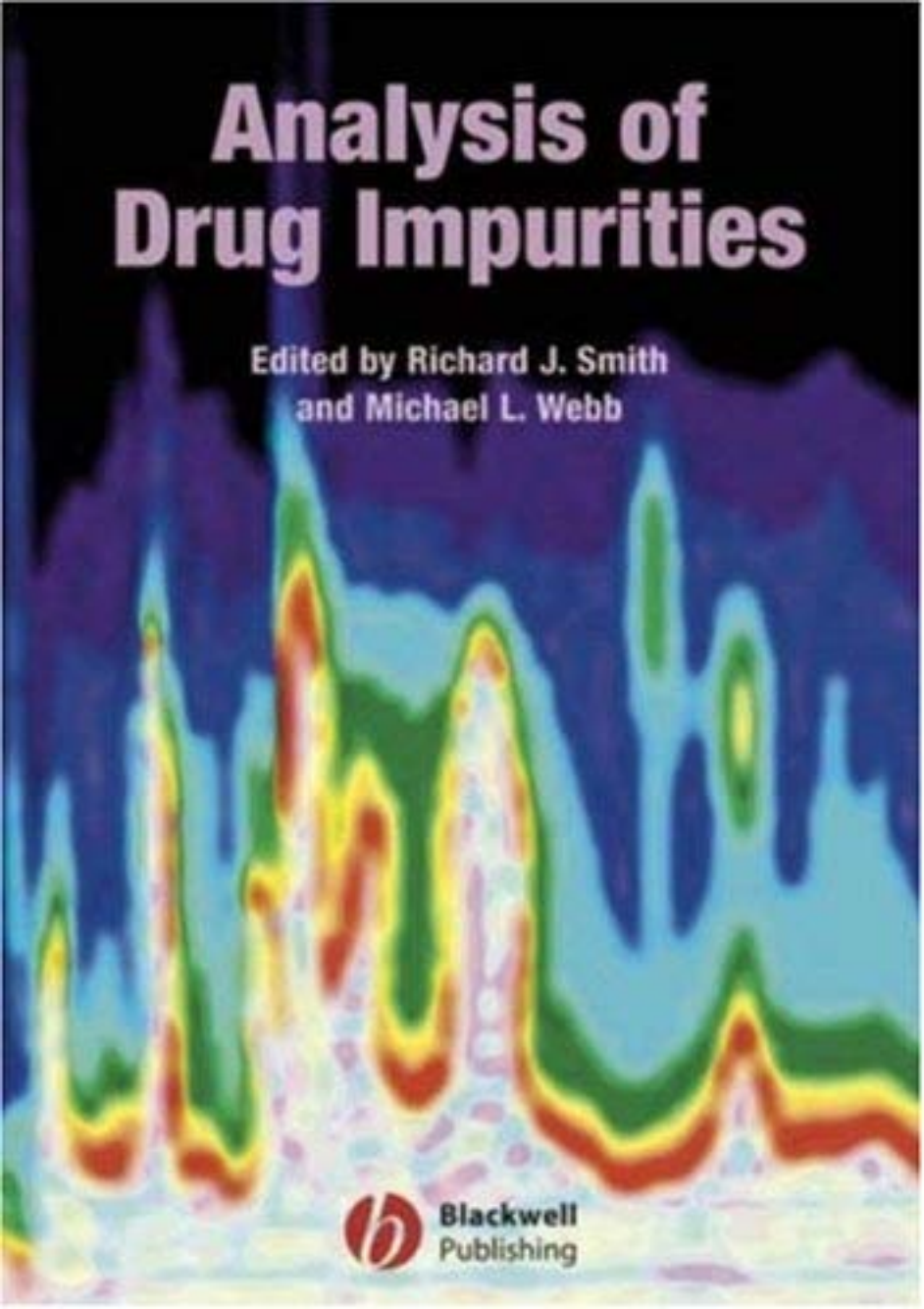
Analysis of Drug Impurities

Edited by Richard J. Smith and Michael L. Webb



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Analysis of Drug Impurities

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Preface

A key component of the quality of pharmaceutical drugs is the control of impurities. It is important to identify and quantify levels of impurities that may be present to provide safe, effective and well-controlled medicines. The measurement and identification of impurities to today's standards presents significant challenges to the analytical chemist. The development of modern quantitative methods is driven by these challenges, and the rapid development of spectrometers has provided increasing opportunity to identify the structure, and therefore the origin and safety potential, of such impurities. In this book, we will provide a source of information for the analytical chemist to understand the challenges and the techniques available to permit accurate identification and quantification of drug impurities.

This book is intended for student and practitioners alike and is structured to bring the relevant disciplines together in one reference book.

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1 Organic impurities in drug substance: origin, control, and measurement

Linda Ng, George Lunn, and Patrick Faustino

1.1 Introduction

The search for medicine to combat diseases began with natural products. Through the years drug substances have been developed from many sources. The earliest drugs were extracts from plant sources. Nowadays, the drug substance could come from many sources, such as wild or farm-grown plants, microbial or marine organisms, animal parts, chemical synthesis, or recombinant technology. Drugs from natural products may include a few or many steps of synthetic modifications and purifications.

During the manufacturing process, whether by chemical synthesis, extraction, cell culture/fermentation, recovery from natural sources, or any combination of these processes, impurities may arise. Impurities are extraneous compounds that are not the drug substance (also known as the active ingredient or the Active Pharmaceutical Ingredient [API]), but arise during the synthesis, extraction, purification, or storage of the drug. Understanding the origin, control, and measurement of impurities is critical to the production of high-quality drug substances.

In addition to guidances from the local authorities of many countries, a series of guidances developed in recent years by the Expert Working Group of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, commonly known as ICH, have been increasingly accepted by the pharmaceutical community. The guidances on quality will be referred to, from time to time, in this chapter. The guidances may be found at http://www.ich.org/cache/compo/276-254-1.html, among other places.

1.2 Origin of impurities

Impurities generally fall into three main categories: process impurities, degradation impurities, and contaminant impurities. Additionally, enantiomers and polymorphs may be considered impurities under some circumstances.

Process impurities arise during the manufacture of the drug substance.
Degradation impurities arise during the storage of the drug substance.
Contaminant impurities are not drug related but are inadvertently introduced during processing or storage, and are not part of the synthesis, extraction, or fermentation process.

Impurities that cause the greatest concern are those that are toxic, defined by the US Pharmacopeia (USP) as impurities that have significant undesirable biological activity [1], and host cell contaminants in biopharmaceuticals that have potential risks of allergic reaction or other immunopathological effects [2].

1.2.1 Process impurities

These include inorganic impurities, organic impurities, and residual solvents.

Organic impurities may be unwanted by-products of a chemical synthesis. They may arise by many different routes, for example, by the reaction of an intermediate with the solvent rather than the desired substrate, by cyclization in the wrong direction. Organic impurities can also arise from impurities in the starting materials, for example, traces of propylamine in butylamine may lead to the propyl analog of the drug substance or they may be reagents used during the reaction. Unreacted starting materials and intermediates may also be present as impurities in the drug substance. In some cases, enzymes are used during a chemical synthesis. These materials may be present in the drug substance as impurities. Very frequently, batches of drug substance made by different synthetic routes will contain different impurities.

In a chemical synthesis, the unwanted compounds that are not removed during the synthetic or purification steps will become impurities. In a similar fashion, the extraction, purification, and later synthetic steps for natural, fermentation, or recombinant products may also give rise to such impurities. Biotechnological processes may give rise to impurities such as media components and host cell proteins. For animal and plant sources, the manufacturing process begins with the organ, fluid, or tissue of the animal or the whole plant or part of the plant. For biotechnology and classical fermentation products, the process begins with the cell bank, master and working cell banks, as appropriate. The further the source step is from the final end product, the less likely the presence of an impurity in the drug substance. Understanding the source of the impurity will make it easier to devise a means of eliminating the impurity, thus resulting in a drug substance of improved quality.

Inorganic impurities include water, salts from buffers, reagents, ligands, catalysts, heavy metals, or other residual metals, and inorganic compounds used in processing, such as filter aids and charcoal. Inorganic impurities can also arise by leaching from equipment as a result of the unit manufacturing process.

Residual solvents are considered a subset of organic impurities. Solvents used to create a solution or suspension during the manufacturing process may not be completely eliminated in the course of manufacture. Solvents used later in the synthesis are more likely to be present in the drug substance, although solvents that have low volatility may persist from earlier steps.

1.2.2 Degradation impurities

Normally, degradants are chemical breakdown compounds of the drug substance formed during storage. In rare cases, degradants are formed when the drug

substance chemically interacts with other compounds or contaminants. In addition, degradants may also be formed by physical degradation, for example, aggregates of proteinaceous material, dimers, trimers, and so forth, of synthetic compounds, polymorphs of synthetic compounds. Degradants may be chemically identical to process impurities. However, the levels of degradants will increase during storage, while the levels of process impurities will remain constant. The rate of increase of degradants resulting from storage is dependant on the chemical nature of the drug substance. An understanding of the potential degradation pathways of the drug substance will lead to optimization of the storage conditions and will result in less impurities.

1.2.3 Contamination impurities

Contamination impurities are unexpected adulterating compounds found in the drug substance.

Current manufacturing technology has reduced many of the contaminant impurities observed in drugs prepared decades ago. For example, heavy metals like lead that leached from pipes or manufacturing/storage tanks gave rise to the commonly used limit test for heavy metals in the drug substance. Current pipes and tanks are primarily stainless steel or glass-lined to reduce this concern, although the type of material is ultimately dependent on the nature of the reactions, the nature of the drug substance, and the nature of the manufacturing unit operations. Other contaminants could be, but not likely agents sprayed to improve the environment in the manufacturing plant or accidental droppings like human hair or paint chips from walls. Disinfectants such as mono-, di-, or trichloroacetic acids or chloramines may be present.

For drug substances from plants, examples of contaminant impurities could be herbicides, for example, diquat and glyphosate, or pesticides, for example, carbofuran and endrin, sprayed in the environment. Additionally, heavy metals or polycyclic aromatic hydrocarbons, if present in the soil, may be absorbed through the root systems of the plants. Polycyclic aromatic hydrocarbons, if present in the air, may be absorbed through the leaves of the plant. These contaminants will be present as residues in the drug substance if the selection, cleaning, extraction, and purification processes do not reduce or eliminate them.

1.2.4 Other impurities

Different polymorphic forms may be categorized as impurities in the drug substance. Different polymorphs may be generated by changes in the manufacturing process, or by seeding effects if a new polymorph arises. In some cases, the presence of different polymorphic forms in various batches of drug substance is not important, for example, if the drug product is a solution and all polymorphs are highly soluble, but in other cases, the polymorphic form of the drug substance is critical to the performance of the drug product. For example, the original formulation of the protease inhibitor ritonavir (Norvir) was that of a capsule containing a hydroalcoholic solution of ritonavir [3]. The appearance of a new and

dramatically less soluble polymorph of ritonavir made it impossible to manufacture this product and necessitated a change in the formulation. Other categories of impurities, not discussed in this chapter, are enantiomers and diastereomers. This is the subject of Chapter 3.

1.3 Control of drug substance impurities

In theory, all impurities should be eliminated. In practice, it is generally not economically feasible to totally eliminate all impurities. However, the levels of all impurities should be controlled to provide a consistent product. In most cases, only low levels of impurities should be allowed, but in rare cases, even quite high levels of impurities are tolerated. In some cases, for example, biotechnology derived products such as macrocyclic antibiotics, or extracts of a botanical source such as some dietary supplements, the drug substance or active component contains multiple compounds, all of which have biological activity. However, only organic impurities, which include residual solvents in the drug substance, are addressed in this chapter.

For each drug substance, the maximum acceptable levels of the various impurities are described in the drug substance monograph or the specification included in the submissions to the regulatory authorities. In this chapter, the ICH Q6A [4] and Q6B [5] definition of specification is used. A specification consists of three parts: the test (e.g. moisture content, impurities), references to the analytical procedure (e.g. high-performance liquid chromatography [HPLC], gas chromatography [GC]), and the acceptance criterion (e.g. not more than 0.50%).

1.3.1 Control of residual solvents

Residual solvents (also known as organic volatile impurities [OVIs]) are discussed in ICH Q3C [6]. The nature of the various residual solvents is discussed in Q3C, and lists of the maximum acceptable amounts, from the toxicological perspective, are provided. For the solvents where toxicological data are available, ICH Q3C classifies the residual solvents as Class 1 solvents that should be avoided, Class 2 solvents that should be limited, and Class 3 solvents to be limited by current good manufacturing practice (cGMP) or other quality-based requirements. The tightest limits are recommended for solvents that are thought to be most toxic, and lower limits are recommended for solvents that have lesser toxicity concerns. However, it may be desirable to control all solvents at levels lower than those recommended by ICH Q3C for process reasons, for example, to improve the flow characteristics of the drug substance. Broadly, ICH Q3C states that 'Since there is no therapeutic benefit from residual solvents, all residual solvents should be removed to the extent possible to meet product specifications, good manufacturing practices, or other quality-based requirements.'

Various techniques are used to reduce the amounts of these solvents left in the drug substance, such as washing with water to remove water-soluble solvents,

distillation, or drying under reduced pressure. Modification of the reaction scheme to use more easily removed solvents may also be a valid approach.

1.3.2 Control of synthetic impurities

Generally, the reaction conditions are adjusted to reduce the amounts of by-products produced during each step of the reaction. The reaction conditions are tightly controlled to prevent varying levels of impurities, or even new impurities, from arising. High-quality starting materials may also lead to lower amounts of impurities in the final product when starting material impurities are carried through to drug substance impurities. Similarly, the use of high-quality reagents may help avoid the generation of unwanted by-products. Other options to reduce these impurities are the introduction of additional intermediate or final purification steps.

Understandably, the impurity profiles of the same drug substance produced by different synthetic routes will differ qualitatively and quantitatively. This is commonly observed when a drug substance is provided by different suppliers. For example, the HPLC chromatograms from samples of fluoxetine hydrochloride obtained from four different suppliers show the differences in the impurities produced by the presumably different synthetic routes (Figure 1.1) [7]. Supplier A is the innovator company, Supplier B is in Italy, and Suppliers C and D are in India.

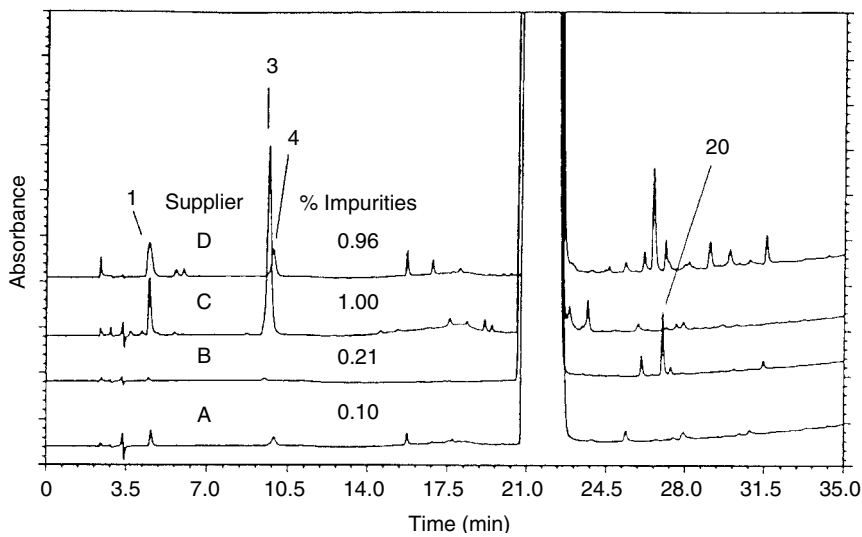

Figure 1.1 HPLC chromatograms of samples of fluoxetine hydrochloride from four different suppliers (A–D). Reproduced from [7]. Reproduced by permission from the publisher and authors. (Column 250 × 4.6 mm i.d. 5 μm Zorbax SB-C8; mobile-phase gradient acetonitrile : water : trifluoroacetic: 20 : 80 : 0.07 for 5 min, to 85 : 15 : 0.07 over 25 min, maintain at 85 : 15 : 0.07 for 5 min, return to initial conditions over 5 min, re-equilibrate for 10 min; flow rate: 1 ml/min; injection: 10 μl of 10 mg/ml solution in the initial mobile phase; detector UV: 260 nm.)

Table 1.1 Limits or thresholds for the reporting, identification, and qualification of impurities

Maximum daily dose[a] (g/day)	Reporting threshold[b,c] (%)	Identification threshold[c]	Qualification threshold[c]
≤2	0.05	0.10% or 1.0 mg/day intake (whichever is lower)	0.15% or 1.0 mg/day intake (whichever is lower)
>2	0.03	0.05%	0.05%

Source: From ICH Q3A(R2).

Notes:

[a] The amount of drug substance administered per day.

[b] Higher reporting thresholds should be scientifically justified.

[c] Lower thresholds can be appropriate if the impurity is unusually toxic.

Examination of the chromatograms shows that some impurities are unique to one particular manufacturer while others are common to more than one manufacturer although the relative amounts may vary widely. Note also that the total impurities values range from 0.10% to 1.00%. The very large peak at retention time of 21.1 min is fluoxetine.

ICH Q3A(R) [8] provides a clear guidance for the control of the organic impurities. Table 1.1 describes the limits or thresholds for the reporting, identification, and qualification of the impurities based on the maximum daily dose of the drug substance administered per day.

Attempts at structural identification of the compound are expected for impurities above the identification threshold. The evaluation of biological safety is expected above the qualification threshold.

Broadly, the upper limits for the various impurities are set on the basis of toxicological considerations and process capabilities. On the basis of animal studies, preferably with drug substance containing enhanced levels of impurities, a dose level that produces no adverse events in the animals is identified. This is converted to an equivalent of the oral dose level in humans. Although high-dose pharmaceuticals are different, for the majority of drugs, impurities above 0.05% are reported as unidentified impurities and included in the total impurity amount, impurities above 0.10% are identified and included in the specification, and impurities above 0.15% are toxicologically qualified.

Genotoxic impurities, which are observed in production batches, or for which there are theoretical grounds for believing that they may be present, should be treated with great caution using a much lower acceptance criterion for control.

Impurities are generally toxicologically qualified when the drug is first approved for marketing, but changes in the synthetic process that lead to an existing or new impurity being routinely produced at a level above the qualification threshold

may require additional preclinical toxicological testing. Similarly, drug substances from a new supplier that have the same impurity profile as the original product will generally be accepted without additional toxicological testing. However, drug substances with existing or new impurities above the qualification threshold may require toxicological testing.

1.3.3 Control of impurities in biological and botanical products

For biological and botanical products, the initial step is frequently an extraction. The use of different extraction solvents and conditions may lead to differences in the impurity profiles. Different batches of the same plants may have different impurity profiles because of seasonal changes, variation in geographic location, or soil differences or variations in the plants parts, for example, root, stem, leaf, utilized in the production process. Additionally, genetic variations in the plant species can result in different impurity profiles. For recombinant or fermentation products, slight differences in the master or working cell banks may result in differences in the levels of impurities. Only the organic impurities that include process and residual solvents in these drug substances are discussed.

Although it may not always be possible to control the sources or extraction conditions to produce only a single component, they should be arranged so as to produce, as far as possible, a consistent mixture. Impurity profiles of botanical products are often monitored by a number of analytical procedures to ensure product quality. Minor components that have pharmacological activity should not necessarily be viewed as impurities. In some cases, the activity of a botanical or fermentation drug substance may be attributed to a number of components.

In many cases, biotechnology-derived products may have many components that have biological activity. The aim should be to devise controls that monitor the various components so as to retain a consistent potency and purity. For example, the USP monograph for erythromycin [9] indicates that the principal component is erythromycin A and that the percentage of erythromycin A, erythromycin B, and erythromycin C is not less than 85.0% and not more than 100.5%. Within these parameters, the relative ratios of erythromycins A, B, and C may change. This is not always the case for biotechnology-derived products, however. For example, the USP monograph for amoxicillin [10] allows for only one active component.

Table 1.2, adapted from Paesen *et al*. [11], shows the varying amounts of some major impurities in the fermentation drug substance erythromycin from seven sources. The major components that make up the drug substance are identified. Note that the level of water also varies quite widely.

Figure 1.2 illustrates the different process impurities present in a sample of erythromycin [11]. A polymeric column was used.

It is not uncommon for facilities to be dedicated for processing only one, or only one type of, drug substance to avoid cross-contamination. For example, some people are allergic to the penicillins, and fatal reactions are known to occur.

Table 1.2 Major impurities in erythromycin from seven sources

Compound	Abbreviation	Sample (%)						
		1	2	3	4	5	6	7
Erythromycin F	EF	<0.1	0.35	<0.1	0.1	<0.1	0.3	1.0
Erythromycin C	EC	<0.1	1.4	<0.1	1.2	0.5	1.9	2.1
Erythromycin E	EE	<0.15	1.3	<0.15	0.9	<0.15	1.5	2.5
Erythromycin A	EA	94.7	92.3	93.8	85.4	93.0	89.0	80.5
Anhydroerythromycin A	AEA	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	2.5
Pseudoerythromycin A enol ether	PsEAEN	0.05	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03
Erythromycin B	EB	<0.4	1.6	<0.4	6.3	<0.4	1.0	3.0
Water		4.7	0.01	NA	5.8	1.3	4.6	4.4

Source: Adapted from [11].

Note: NA – Not available.

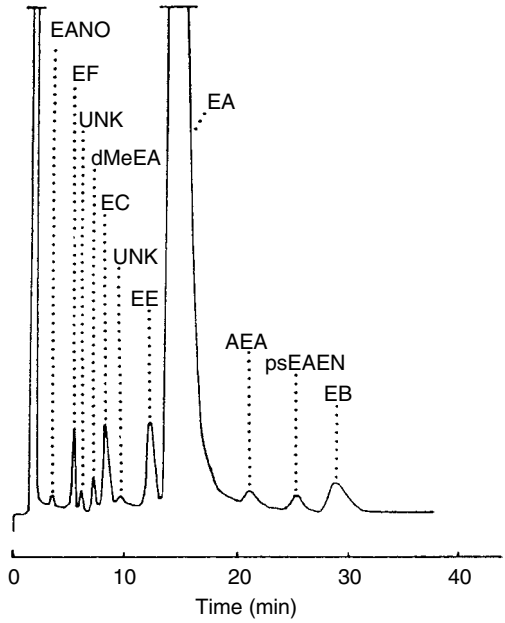

Figure 1.2 Impurities in a sample of erythromycin A. Reproduced from [11]. Reproduced by permission from the publisher and authors. (Column 250 × 4.6 mm i.d. 8 μm 1000 Å; PLRP-S; mobile-phase acetonitrile : *t*-butanol : 200 mM pH 9.0 phosphate buffer : water: 3 : 16.5 : 5 : 75.5; flow rate: 2 mL/min; column temperature: 70°C; injection: 100 μl of a 4 mg/ml solution; detector UV: 215 nm.)

Facilities for the manufacture of penicillins are frequently dedicated so that other drugs will not be contaminated with even small amounts of these potential allergens.

1.3.4 Purification processes

Purification processes, such as crystallization, sieving, filtration, and preparative chromatography, are widely used.

Although the purpose of the purification step is to increase the purity of the drug substance, it is possible to introduce impurities at this step. Equally, a poorly designed purification process may not be as effective as it could be.

For these reasons, an understanding of the purification conditions and materials used is useful. For example, on using one solvent, impurities could crystallize under the same conditions as the compound of interest, but on using another solvent, the impurities may be left in the solution. Use of a relatively nonvolatile solvent may lead to residual amounts, which are difficult to eliminate. If the drug substance is dissolved in a solvent, filtration is an effective method of removing particulate matter.

For synthetic drug substances, purification is most often accomplished through crystallization. Preparative chromatography is currently more widely used for biotechnology-derived products than for synthetic drug substances

because of the expense of the equipment and the complexity of the mixtures. This technique is very selective although impurities that elute close to the drug substance may not be eliminated. Depending on how the column is cleaned or regenerated, it is possible that impurities leached from the column or solvents used in the chromatographic procedure may contaminate the purified material. Figure 1.3 [12] illustrates the chromatographic purification of pravastatin sodium indicating that all impurities except for the two at retention times 7.6 and 8.6 min were removed after a chromatographic purification. These two remaining impurities were greatly reduced.

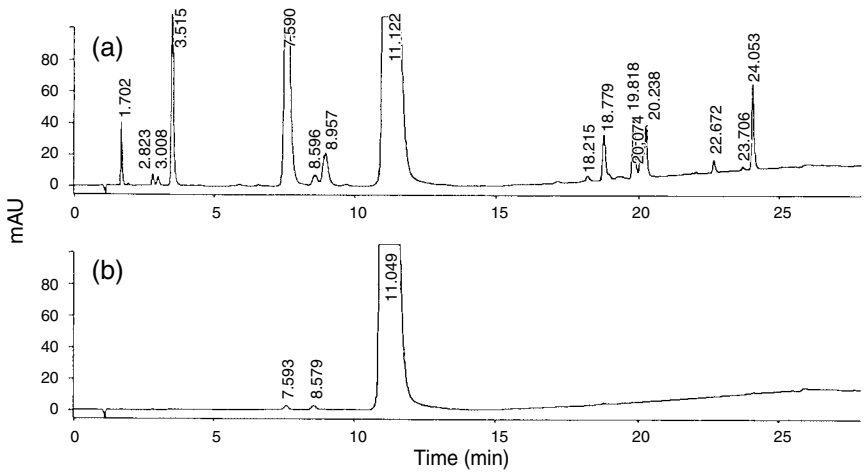

Figure 1.3 Purification of pravastatin sodium by preparative liquid chromatography. Reprinted from [12], copyright 2001, with permission from Elsevier. (Column 125 × 4.6 mm i.d. 3 μm Hypersil ODS; mobile-phase gradient methanol : water : triethylamine : acetic acid: 45 : 54.8 : 0.1 : 0.1 for 13 min, to 99.8 : 0 : 0.1 : 0.1 over 9 min; flow rate: 1.2 ml/min; detector UV: 235 nm.)

1.3.5 Control of impurities from packaging

The majority of drug substances are solids packaged in polyethylene bags and stored at ambient temperature. Some are stored as liquids or lyophilized solids in plastic or glass containers at ambient, refrigerated, or freezer storage temperature, depending on the stability of the drug substance. Impurities such as plasticizers may leach from the product contact material if it is plastic. Small particles of glass could become detached from glass containers. Thus, compatibility between the drug substance and the container should be considered for selection of the storage container.

For sterile drug substances, an understanding of the drug substance and the sterilization process will help in avoiding potential impurities, for example, compounds formed by the appropriate functional groups of the drug substance reacting with residual ethylene oxide from the sterilization process.

1.3.6 Control of contamination impurities

Contaminants may include such items as lubricants and antistatic agents used during manufacturing, leachables from equipment such as chromatographic columns, and particles from the environment. All these contaminants are controlled by manufacturing the drug substance under cGMPs. Such practices have dramatically reduced, if not completely eliminated, such contaminants. ICH Q7A [13] has recommendations for personnel and their sanitation and health habits, design of the facilities, and control of the environment within the facilities.

1.3.7 Control of degradants on stability

It is expected that the potency, purity, and quality of the drug substance be maintained over time. The chemical, physical, enzymatic, and microbiological quality, as appropriate, should be retained through to the claimed life of the drug substance and also through the life of the formulated drug substance, that is, the marketed drug product.

Using an understanding of the drug substance gained from stability studies, an optimum storage condition to maximize the quality of the drug substance can be determined. The types of degradation that a drug substance can undergo are described in Chapter 2. Generally, storage conditions are adjusted to keep chemical or physical degradation to a minimum.

For drug substances, a retest date is generally determined. The period up to the retest date is a period during which the material is known to adhere to its specification, for example, for assay or degradants. The retest date may be assigned on the basis of real-time stability data obtained at the recommended storage conditions or on the basis of accelerated stability data obtained under more stressful conditions.

During the period up to the retest date, the material can be used to manufacture drug product with confidence. At the end of this period, the material should be retested to see if it still meets its specification before being used to manufacture the drug product. It is important to realize that the drug substance retest period may not necessarily be the same as the drug product expiration dating period. Examples are known where the drug substance is less stable than the drug product, and examples are also known where the reverse is the case. The drug substance retest date should be regarded as a completely separate parameter from the drug product expiration date.

1.4 Measurement of drug substance impurities

Currently, organic drug substance impurities are almost exclusively measured using chromatographic procedures. Chromatographic procedures should involve a separation mode that allows for the resolution of impurities from the drug substance and a detection mode that allows for the accurate measurement of impurities.

Particles may be controlled by microscopic examination or by laser diffraction technologies and moisture may be determined by Karl Fischer titration or loss on drying. Loss on drying may also provide a crude measure of the total amount of residual solvent(s) that is present. Note that Karl Fischer titration will determine all the water in a sample, including water of hydration, but will be insensitive to residual solvents, whereas loss on drying will determine only water that can readily be removed under the drying conditions (generally not water of hydration) but will determine most volatile solvents. Therefore, the results obtained by the two methods may not be comparable. In cases such as this, thermal techniques such as thermal gravimetric analysis and differential scanning calorimetry can be used to monitor the amount of water of hydration (bound) and (unbound) to resolve and verify the nature of the water in the drug substance.

Owing to the polar and nonvolatile nature of most compounds used as medicinal drugs, reversed-phase HPLC is the most common technique for monitoring the drug substance and its impurities. GC is also used, particularly for residual solvents, and capillary electrophoresis (CE) has been introduced in more recent times. Some older methods use thin-layer chromatography (TLC), but use of this methodology for the quantitative measurement of impurities is not common.

As a result of advances in detection technology, newer HPLC detection techniques may be utilized. For example, evaporative light-scattering detection and refractive index detection may be used to quantify impurities with poor or no UV chromophore. Conductivity detection may also be used. In some cases, when no chromophore is present, chemical derivatization may be used to add a chromophore. Increasingly, tandem techniques such as liquid chromatography mass spectrometry (LC-MS) and GC-MS are utilized for impurity characterization. The highly selective nature of LC-MS ensures that few impurities are undetected.

Appropriate techniques should be selected depending on the type of impurity. With the large variation in the types of impurities, especially for the natural or biotechnology-sourced products, orthogonal techniques may be necessary for any given drug substance. Orthogonal techniques are complementary techniques that use different separation principles to qualify and quantify the compounds of interest. For example, if an impurity co-elutes with the drug substance in an HPLC C-18 ODS column, a cyano column, as an orthogonal technique, may be used to resolve the two peaks. Another example would be using page gel electrophoresis following a chromatographic technique.

1.4.1 HPLC

The most common technique for monitoring impurities is HPLC with UV detection. Quantification of impurities is achieved by reference standard, when available, or by area percent or height percent relative to the parent compound. Figure 1.4 shows a typical chromatogram enlarged to show the various impurities. One of the impurities is the USP Compound B that is added to the furosemide drug substance.

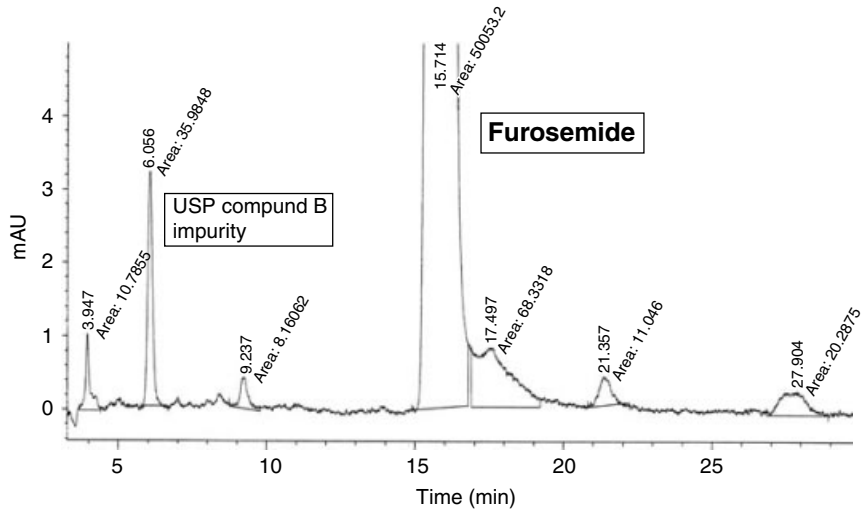

Figure 1.4 Furosemide drug substance showing USP Compound B impurity together with other impurities. (Column 250 × 4.6 mm i.d. Phenomenex Luna (2) C-18; mobile-phase THF : water : acetic acid: 35 : 65 : 0.1; flow rate: 1 ml/min; detector UV: 272 nm.)

HPLC methods should be optimized to resolve all the impurities from the drug substance. For example, Figure 1.5 [14] illustrates the use of different columns, organic components, pHs, and buffers for the separation of impurities A, B, and C from the main peak (*P*). Other parameters such as ion-pairing agents, gradient steepness, or column temperature may also be varied. The varying of column type, length, and diameter as well as particle and pore size can be optimized to ensure that all impurities are detected and accurately measured. Commercial programs may be used to efficiently assist with the optimization of chromatographic parameters to ensure impurities separation.

Although UV detection is most commonly used in the quality control of drug substances, other detectors such as fluorescence, electrochemical, near infrared, refractive index, evaporative light scattering, or mass spectrometry may be used as appropriate.

1.4.2 GC

GC is most commonly used to determine residual solvents since these compounds are volatile. For example, Figure 1.6 [15] illustrates the use of GC to measure ICH Class 2 solvents. The solvents are dissolved in DMF and heated at 80°C for 60 min, and a sample of the headspace is injected into a capillary GC system fitted with a flame ionization detector.

Varying residual solvent profiles can be used to identify drug substance samples obtained from different sources [16] for forensic purposes.

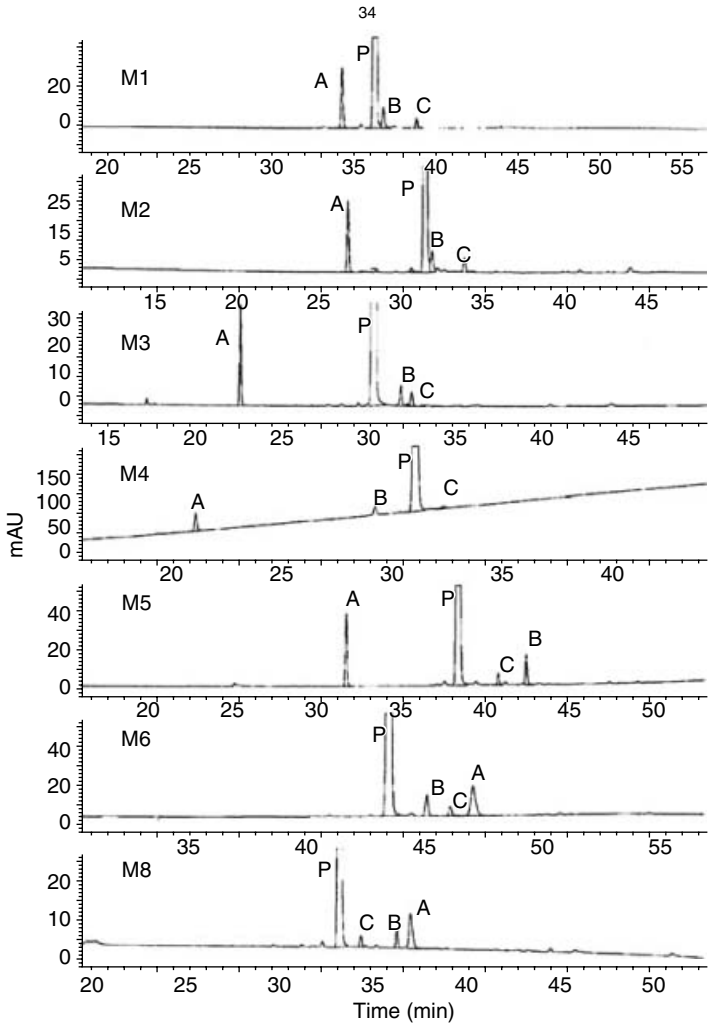

Figure 1.5 Separation of impurities A, B, and C from the peak of interest (*P*) using seven different HPLC systems. Reprinted from [14], copyright 2004, with permission from Elsevier. (For each system the column temperature is 30°C, the detector is UV 254 nm and the gradient is a 60-min gradient from 5% to 95% organic modifier. M1 column 250 × 4.6 mm i.d. 5 μm Kromasil C4, mobile-phase acetonitrile/0.1% trifluoroacetic acid [pH 1.9]; M2 column 100 × 4.6 mm i.d. 5 μm Luna phenyl-hexyl, mobile-phase acetonitrile/0.1% acetic acid adjusted to pH 3.5 with ammonium hydroxide; M3 column 100 × 4.6 mm i.d. 5 μm Luna phenyl-hexyl, mobile-phase acetonitrile/10 mM ammonium acetate [pH 7.0]; M4 column 100 × 4.6 mm i.d. 5 μm Luna phenyl-hexyl, mobile phase THF/10 mM ammonium acetate adjusted to pH 5.0 with glacial acetic acid; M5 column 150 × 4.6 mm i.d. 3 μm Spherisorb ODS1, mobile-phase methanol/10 mM ammonium acetate [pH 7.0]; M6 column 150 × 4.6 mm i.d. 5 μm Monitor C18, mobile-phase methanol/0.1% acetic acid adjusted to pH 3.5 with ammonium acetate; M8 column 100 × 4.6 mm i.d. 4 μm YMC J'Sphere ODS H80, mobile-phase acetonitrile/0.1% formic acid [pH 2.1] [M7 is a variation on the M8 gradient and is not shown].)

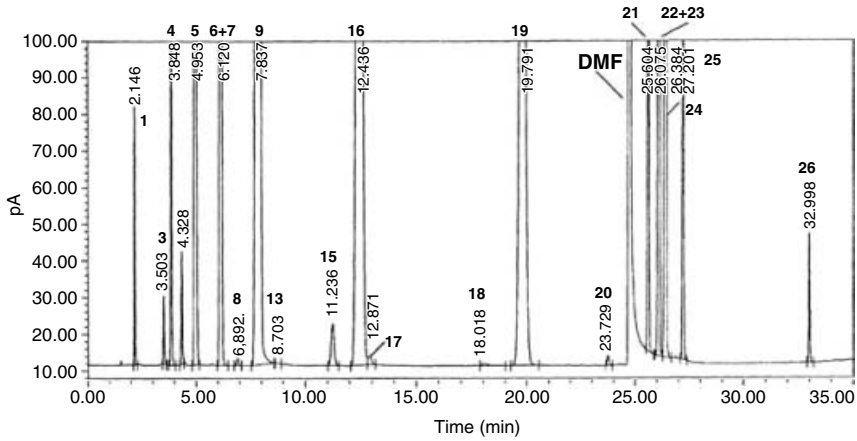

Figure 1.6 ICH Class 2 solvents measured using GC. Purification of pravastatin sodium by preparative liquid chromatography. Reprinted from [15], copyright 2004, with permission from Elsevier. (Column 30 m × 0.53 mm i.d. 3 μm OVI-G43 (Supelco); carrier gas helium at 5 ml/min; injection in split mode; total flow 25 ml/min; injector temperature: 140°C; flame ionization detector temperature: 250°C; and oven temperature: 40°C for 20 min, to 240°C at 10°C/min, maintain at 240°C for 20 min. The components are 1 methanol, 3 acetonitrile, 4 dichloromethane, 5 hexane, 6 *cis*-1,2-dichloroethylene, 7 nitromethane, 8 chloroform, 9 cyclohexane, 13 1,2-dimethoxyethane, 15 1,1,2-trichloroethylene, 16 methylcyclohexane, 17 1,4-dioxane, 18 pyridine, 19 toluene, 20 2-hexanone, 21 chlorobenzene, 22 ethylbenzene, 23 *m*-xylene, 24 *p*-xylene, 25 *o*-xylene, and 26 tetralin. The solvents are dissolved in DMF and heated at 80°C for 60 min, and a sample of the headspace is injected.)

Depending on the nature of the drug substance and impurities, GC may sometimes be an appropriate technique. For example, the USP method for amantadine involves GC. Figure 1.7 [7] illustrates the impurities found in two samples of fluoxetine hydrochloride as determined by GC. Compounds monitored by GC are normally low molecular weight compounds that are not heat sensitive.

1.4.3 CE

Recently, CE has been developed for the analysis of drug substances. It is not employed with great frequency for quality control (owing to inherent sensitivity issues), but on occasion, CE procedures can be employed when HPLC procedures have failed to adequately measure the impurities. CE is particularly useful for the separation of closely related compounds such as the diastereomers and enantiomers of compounds with more than one chiral center. Figure 1.8 illustrates the separation of the diastereomers quinine (QN) and quinidine (QD) and some impurities using CE [17].

Figure 1.9 [18] illustrates the use of CE for the analysis of amoxicillin (AMOX) from various suppliers. Unidentified impurities (1 and 2) and degradant (DG) are seen, and the mixtures are spiked with ampicillin (AMP) and penicillin V (PENV).

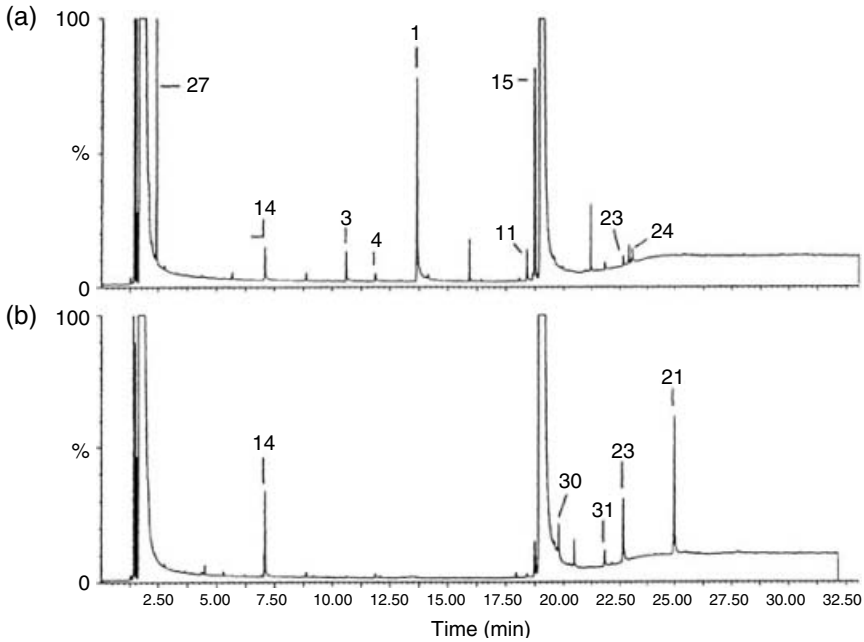

Figure 1.7 GC-MS (total ion current) chromatograms for samples of fluoxetine hydrochloride from two different suppliers: (a) India and (b) Costa Rica. Reproduced from [7]. Reproduced by permission from the publisher and authors. (Column 30 m × 0.25 mm i.d. 1 μm DB-1 (J&W); carrier gas helium; split ratio: 30 : 1; flame-ionization detector; and oven temperature: 100°C for 3 min, to 300°C at 10°C/min, maintain at 300°C for 10 min.)

1.4.4 General considerations

An impurities analytical procedure should be described adequately so that any qualified analyst can readily reproduce the method. The description should include the scientific principle behind the procedure. A list of reagents and equipment, for example, instrument type, detector, column type, and dimensions, should be included. Equipment parameters, for example, flow rate, temperatures, run time, and wavelength settings, should be specified. How the analytical procedure is carried out, including the standard and sample preparations, the calculation formulae, and how to report results, should be described. A representative chromatogram with labeled peak(s) should be included in the procedure.

In the monitoring of impurities by UV detection, impurities that elute at the solvent front and late- or noneluters may not be observed. Impurities that have a chromophore that is significantly different from that of the drug substance may not be accurately quantified unless a correction factor is used. Generally, attempts are made to isolate, characterize, and synthesize the impurity to create analytical impurity standards that can be used to accurately quantify the impurity. In the

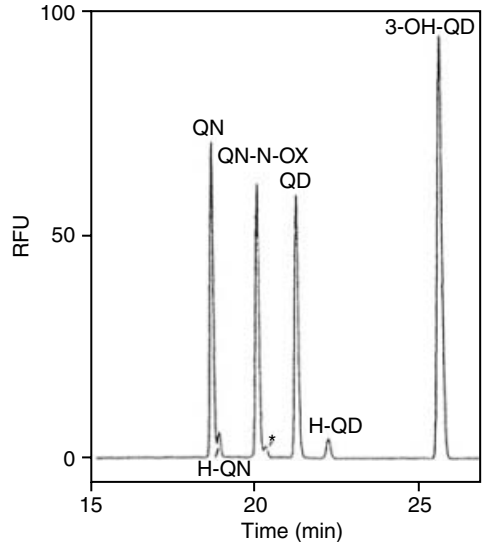

Figure 1.8 Use of capillary electrophoresis for separating the diastereomers quinine (QN) and quinidine (QD) (H-QN is hydroquinine, QD-N-OX is quinidine *N*-oxide, H-QD is hydroquinidine, 3-OH-QD is 3-hydroxyquinidine, and asterisk is an unidentified impurity). Reprinted from [17], copyright 2001, with permission from Elsevier. (Capillary 47 cm × 75 μm i.d. (40 cm to detector) (Polymicro Technologies); running buffer 50 mM phosphoric acid containing 15 mM β-cyclodextrin adjusted to pH 2.5 with NaOH; voltage: 7 kV; current: 21 μA; injection at 0.5 psi for 4 s; detector fluorescence (HeCd laser) excitation: 325 nm, emission: 450 nm.)

extreme, impurities with no chromophore will not be detected. In general, an HPLC method should be stability indicating, that is, a validated quantitative analytical procedure that can detect changes with time in the pertinent properties of the drug substance and drug product. A stability-indicating method accurately measures the active ingredients, without interference from degradation products, process impurities, or other potential impurities. Hence, it is desirable, for stability studies, that a stability-indicating impurities assay, which is capable of qualitatively and quantitatively measuring the impurities, be used. If it becomes obvious that some degradants are not being detected, then a different analytical technique should be considered. For example, if the degradant is volatile and has no UV chromophore, GC may be of use. The general principles on monitoring impurities by UV detection can be extrapolated to other measurement techniques mentioned in this chapter.

System suitability tests for chromatographic impurities procedures, such as precision, resolution factor, calibration standard, and tailing factor, should be considered as appropriate. In the presence of multiple peaks, a resolution factor between the two closest peaks should be proposed. For reliable quantitation, baseline resolution of the impurities will provide accurate measurement of the

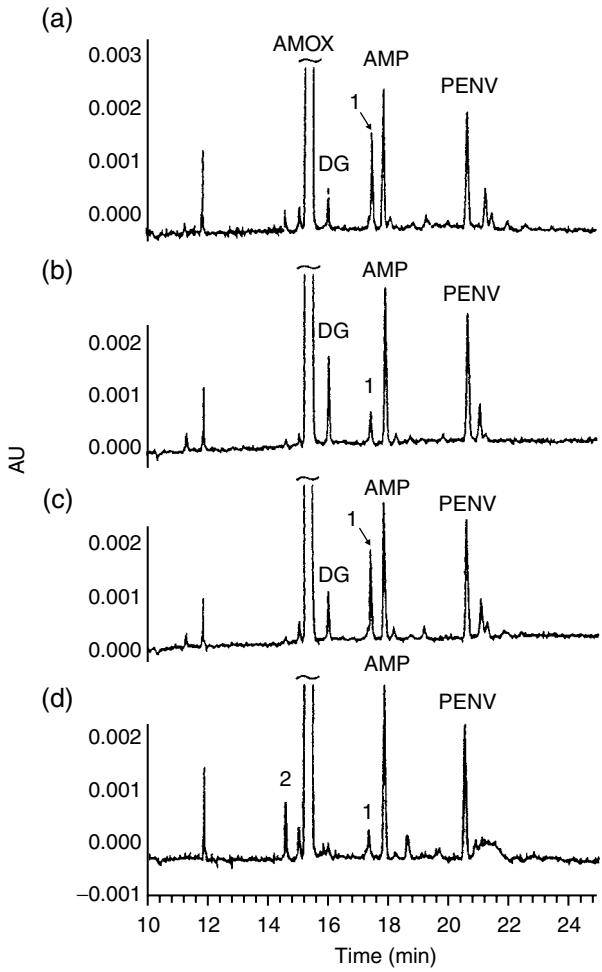

Figure 1.9 Use of capillary electrophoresis for the analysis of amoxicillin from four suppliers (a–d). Reprinted from [18], copyright 1994, with permission from Elsevier. (AMOX is amoxicillin, AMP is ampicillin, PENV is penicillin V, DG is a degradant, and 1 and 2 are unspecified impurities. Capillary 80 cm × 50 μm i.d. [75 cm to detector] [Polymicro Technologies]; running buffer 100 mM pH 8 Na_2HPO_4 containing 50 mM sodium dodecyl sulfate and 50 mM sodium borate; voltage: 18 kV; injection at 100 mm for 15 s; detector UV: 205 nm.)

peak areas [19]. A calibration standard containing either an impurity or drug substance at the limit of quantitation will be a useful tool to ensure that impurities may be quantitatively determined down to the level of the quantitation limit at the time of analysis [20]. Detector sensitivity, especially UV detectors, can vary over an order of magnitude with the model, manufacturer, and age of the source.

1.5 Conclusions

By understanding the manufacturing process and the stability of the drug substance, whether from synthetic, natural, or recombinant sources, the chemist is able to identify, control, and measure the impurities, and so the quality of the drug substance and reproducibility from production batch to batch are maintained.

Disclaimer

The views expressed above are the personal views of the authors. The content of this article does not necessarily reflect the views or policies of the Food and Drug Administration, nor does the mention of trade names, commercial products, or organizations imply endorsement by the U.S. government.

References

1. USPharmacopeia 29/National Formulary 24, 1086, Rockville, MD, 2006, p. 2921.
2. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH S6: Preclinical Safety Evaluation of Biotechnology-derived Pharmaceuticals, 1997.
3. J. Bauer, S. Spanton, R. Henry, *et al*., *Pharm. Res*., 2001, **18**, 859–866.
4. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Q6A: Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances, 1999.
5. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Q6B: Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products, 1999.
6. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Q3C(R3): Impurities: Residual Solvents, 2005.
7. D. D. Wirth, B. A. Olsen, D. K. Hallenbeck, M. E. Lake, S. M. Gregg and F. M. Perry, *Chromatographia*, 1997, **46**, 511–523.
8. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Q3A(R1): Impurities in New Drug Substances, 2002.
9. USPharmacopeia 29/National Formulary 24, Rockville, MD, 2006, p. 828.
10. USPharmacopeia 29/National Formulary 24, Rockville, MD, 2006, p. 157.
11. J. Paesen, E. Roets and J. Hoogmartens, *Chromatographia*, 1991, **32**, 162–166.
12. R. Grahek, D. Milivojevic, A. Bastarda and M. Kracun, *J. Chromatogr. A*, 2001, **918**, 319–324.
13. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Q7A: Good Manufacturing Practice Guidance for Active Pharmaceutical Ingredients, 2000.
14. G. Xue, A. D. Bendick, R. Chen and S. S. Sekulic, *J. Chromatogr. A*, 2004, **1050**, 159–171.
15. R. Otero, G. Carrera, J. F. Dulsat, J. L. Fábregas and J. Claramunt, *J. Chromatogr. A*, 2004, **1057**, 193–201.

16. K. J. Mulligan, T. W. Brueggemeyer, D. F. Crockett and J. B. Schepman, *J. Chromatogr. B*, 1996, **686**, 85–95.
17. S. Zaugg and W. Thormann, *J. Pharm. Biomed. Anal.*, 2001, **24**, 785–799.
18. C. L. Flurer and K. A. Wolnik, *J. Chromatogr. A*, 1994, **674**, 153–163.
19. FDA/CDER Reviewer Guidance: Validation of Chromatographic Methods, November 1994, p. 23 (http://www.fda.gov/cder/guidance/index.htm).
20. FDA/CDER Reviewer Guidance: Validation of Chromatographic Methods, November 1994, p. 9 (http://www.fda.gov/cder/guidance/index.htm).

Analysis of Drug Impurities
Edited by Richard J. Smith, Michael L. Webb

2 Organic impurities in drug products: origin, control and measurement

David P. Elder

2.1 Introduction

Drug products contain both drug substance (sometimes referred to as the Active Pharmaceutical Ingredient [API]) and excipients. The resultant biological, chemical and physical properties of the drug product are directly dependent on the excipients chosen, their concentration and interactions with the API [1].

Excipients are sub-divided into various functional classifications, depending on the role that they are intended to play in the resultant formulation, for example, fillers, disintegrants, binders, lubricants and glidants. An added complexity is the fact that certain excipients can have different functional roles in different formulation types. Thus, lactose is widely used as a filler or diluent in solid oral dosage forms, for example, tablets and capsules [2] and as a carrier for inhalation products [3].

In addition, individual excipients can have different grades, types and sources depending on those different functional roles. For example, various lactose grades that are commercially available have different physical properties, for example, flow characteristics or particle size distribution. In theory, this permits the optimum selection of the most suitable grade for a particular utilisation. Usually, fine grades of lactose are utilised when manufacturing tablets by the wet granulation route, as it utilises the binder more efficiently and permits better mixing. In contrast, spray-dried lactose is used for direct compression tablets, as it flows better and is more compressible.

Whereas excipients are usually biologically inactive, the same cannot be said from a chemical perspective. Excipients, and the impurities present therein, can stabilise and/or destabilise drug products. The allowable level of any given impurity or impurities that are permitted in drug products, without explicit non-clinical safety testing, are defined by ICH Q3B (R) [4]. Synthetic impurities *per se* are covered by separate guidelines, ICH Q3A (R) [5]. The amounts of impurities that are allowable are based on the total daily intake of the drug product. There are separate limits (or thresholds) for reporting, identification and qualification of drug product impurities, sometimes referred to as degradants or degradation products. The reporting threshold is defined as the level that must be reported to regulatory agencies to alert them to the presence of a specified impurity. The identification threshold is defined as the level that requires analytical identification of a specified impurity. Finally, the qualification threshold is defined as the level where the specified impurity must be subjected to

Table 2.1 Guidance on allowable degradants in drug products

Threshold	Maximum daily dose of drug product	Threshold limit based on total daily intake (TDI)
Reporting	≤1 g	0.1% TDI
	>1 g	0.05% TDI
Identification	<1 mg	1.0% TDI or 5 μg
	1–10 mg	0.5% TDI or 20 μg
	10 mg–2 g	0.2% or 2 mg
	>2 g	0.10% TDI
Qualification	<10 mg	1.0% TDI or 50 μg
	10–100 mg	0.5% TDI or 200 μg
	100 mg–2 g	0.2% or 3 mg
	>2 g	0.10% TDI

Source: From ICH Q3B.

non-clinical toxicological testing to demonstrate safety. These threshold limits are defined as a percentage of the total daily intake of the drug product, or in absolute terms as the total allowable amount, whichever is lower (see Table 2.1).

2.2 Analytical methodology

Analytical methodology needs to be stability specific, that is, capable of resolving all potential impurities likely to be formed during the products' entire shelflife at the designated storage conditions. As such, chromatographic high performance liquid chromatography (HPLC) or electrochromatographic (capillary electrophoresis [CE]) are the methods of choice. Bakshi and Singh [6] reported recently on the development and validation of stability-indicating test methodologies. Typically, these methodologies are evaluated for specificity, linearity, sensitivity, precision (within-day and between-day), accuracy and robustness [7]. Method validation should be performed according to internationally agreed protocols for chromatographic validation [8].

A typical example of HPLC method development and validation was provided by Boneschans *et al*. [9]. They developed an HPLC method for piroxicam benzoate and its major hydrolytic degradation products, piroxicam and benzoic acid. The authors utilised a robust stationary phase (Phenomenex Luna, C_{18}), with an optimised mobile phase comprising of acetonitrile/water/acetic acid (45/7/8 v/v), and a flow rate of 1.5 ml/min. The operating pH of the mobile phase (pH 2.45) was selected on the basis that it is ca. 2 pH units from the pKa of the drug, and hence reasonably insensitive to changes in mobile-phase preparation. The injection volume was 20 μl with a detection wavelength of 254 nm. They utilised

standard chromatographic parameters, for example, capacity factors, efficiency and peak asymmetry to evaluate routine system performance.

The authors determined specificity using the known hydrolytic degradation products. The precision of spiked samples of these degradation products were determined and found to be acceptable (99.9 ± 0.4%). Accuracy of the method was determined using spiked recoveries of piroxicam benzoate, and the recoveries were acceptable (99.1–100.5%). Assay precision ($n = 6$, RSD = 0.4%) was in accord with recommended criteria [7]. Within-day precision was performed on two instruments on two separate days, and the overall intermediate precision was 1.0%. The method was linear over the expected analyte concentration range giving a regression line of $R^2 = 0.999$. The detection (DL) and quantification levels (QL) were assessed, and the latter was determined as 0.185 μg/ml (ca. 0.04%).

A major consequence of using regulatory limits based on degradant formation, rather than absolute change of the API level in the drug product, is that it necessitates the application and routine use of very sensitive analytical techniques [10]. In addition, the need to resolve both structurally similar, as well as structurally diverse degradants of the API, mandates the use of analytical separation techniques, for example, HPLC, CE, often coupled with highly sensitive detection modes, for example, ultraviolet (UV) spectroscopy, fluorescence (F) spectroscopy, electrochemical detection (EC), mass spectroscopy (MS), tandem mass spectroscopy (MS-MS) and so forth.

HPLC-MS and HPLC-MS-MS methods have been applied to the determination of impurities and degradants in API and drug product. HPLC-MS methods were utilised for the determination of impurities in the 1 mg/ml oral solution of the angiotensin converting enzyme (ACE) inhibitor, quinapril. The impurities were composed of the principal hydrolysis product, quinaprilat and a cyclic impurity. All of the MS methods gave enhanced sensitivity compared with the standard HPLC-UV or stand-alone MS methods. Both methods gave equivalent results. The authors commented that the very low detection limits for the HPLC-MS-MS method (10 pg/ml) make this technique particularly suited for detecting API, and related impurities lacking chromophores or fluorophores, and that this method could be preferable to alternative detection systems; for example, refractive index (RI), electrochemical (EC), chemiluminescence (CL) or evaporative light scattering (ELS).

CE has been typically utilised as an ‘orthogonal’ analytical technique to HPLC, providing complimentary information. Weinberger and Lurie [11] resolved the acidic and neutral impurities found in illicit heroin drug products, with acceptable precision using a CE method. They showed that the CE method had greater resolving power compared with the reference HPLC method, whereas HPLC gave better precision. A fully validated CE method was recently reported for the assay of Ximelagatran and related substances, in both drug substance and drug product [12]. A low pH phosphate buffer containing both acetonitrile and hydroxypropyl-β-cyclodextrin was employed to achieve the required specificity for related substances.

2.3 Drug–excipient compatibility experimental design

Before initiating drug product development, the formulation scientists must fully consider the chemical structure of the drug substance, the type of delivery system required and the proposed manufacturing process. Initial selection of excipients should be based on expert systems, appropriate delivery characteristics, personal choice and knowledge of potential mechanisms of degradation for the drug in question [13]. Known chemical incompatibilities of common excipients may be obtained from existing published information [1].

The objectives of drug–excipient compatibility studies are to delineate, as quickly as possible, interactions between potential formulation excipients and the API, thus allowing the rapid optimisation of a particular dosage form with respect to patentability, processing, drug release, elegance, and physical and chemical stability. Drug–excipient interactions tend to be studied using two complimentary approaches. The first approach favoured by Monkhouse *et al*. [14] is to conduct short-term stability studies using prototype formulations, under stressed conditions. Both the chemical stability, as measured by chromatographic methods and physical stability as measured by microscopic, particle analysis, *in vitro* dissolution methods and so forth, are performed.

The second approach is to perform traditional pre-formulational studies using full factorial or Plackett Burman experimental designs [15]. Here, the preferred analytical methodology tends to be thermal and spectroscopic, rather than chromatographic, although the latter methodologies are still utilised. Differential scanning calorimetry (DSC), isothermal calorimetry (ITC) or Fourier-transform infrared (FT-IR) spectroscopy have all been utilised successfully.

In the typical drug–excipient compatibility testing program, binary powder mixes are prepared by triturating API with the individual excipients. These powder samples, usually with or without added water and occasionally compacted or prepared as slurries, are stored under accelerated conditions and analysed by stability-indicating methodology, for example, HPLC, CE and so forth. This entire process takes considerable time and resources.

Alternatively, samples are quickly screened by thermal methods, such as DSC or ITC. This alternative approach eliminates the necessity for stability set-downs; hence cycle times and sample consumption are reduced. However, the data obtained are difficult to interpret and may be misleading; false positives and negatives are routinely encountered [14].

Another approach, designed to reduce the overall effort, is through a variety of statistical designs. ‘One-factor-at-a-time’ methods have been supplanted by factorial design or reduced factorial design. The factorial design can be useful for screening purposes or as an aid to identifying effects in complex systems. Excipients that should be included in these designs are fillers, disintegrants, binders, lubricants and granulating liquids. With 5 factors, a 2^5 factorial design results in 32 experiments, which probably involves too much work [16]. Additionally, other factors such as temperature, relative humidity, compaction and particle size, may still require investigation. A typical 2^3 factorial design is given in Table 2.2. This was intended to investigate the effects of temperature,

Table 2.2 Typical 2^3 factorial design for an aspirin suspension

Factor	Low value (−)	High value (+)
Temperature (A)	40°C	60°C
Tablet filler (B)		
Avicel PH 101 (B1)	0%	50%
Emcompress (B2)	0%	50%
Lactose (B3)	0%	50%
Mechanical treatment (C)	Crystalline	Ball milled, 20 h

Source: Derived from [17].

tablet fillers and mechanical treatment on the degradation of aspirin in suspension [17].

However, it is well known that the chemical compatibility of a drug substance in a binary mixture may differ completely from a multi-component prototype formulation. Some investigators in this field have attempted to apply statistical designs to determine the occurrence of chemical interactions in complex formulations, with a view towards establishing which excipients cause incompatibility within a given mixture. Leuenberger and Becher [18] used a 2^5 factorial design, consisting of four excipients (filler, lubricant, disintegrant and binder) and the fifth factor represented humidity, encountered during the wet granulation process.

Serajuddin *et al*. [19] reported on a drug–excipient compatibility screening approach that they claimed was more predictive than previous models. The approach involved storage of 200 mg of drug–excipient blends in closed vials at 50°C with 20% added water, the amount of drug substance in the blend being modified according to the anticipated drug–excipient ratio in the final compression blend. This allowed the evaluation of the role of the chemical nature of the excipient, the ratio of the drug–excipient blend, pH of the drug–excipient blend, role of moisture in the drug–excipient blend, as well as the more classically important roles of temperature, light and humidity of the external environment. The authors disagreed with Monkhouse and Maderich [14] about the utility of drug–excipient testing. They concluded that on the basis of case histories that the stability of drug products could be optimised by selection of excipients according to this approach, and that late-stage development surprises were less likely if this model was adopted.

2.4 Degradation mechanisms

The manner in which drugs degrade in solid oral dosage forms is still rather obscure, despite the best efforts of several eminent investigators in the field [20, 21]. The kinetics of the degradation reactions are difficult to interpret and the orders of the reaction are often complex. The following mechanisms have been proposed by Wells [22] and are summarised in Table 2.3.

Table 2.3 Solid-state reaction mechanisms

Mechanism	Description
i	Degradation by nucleation, via the gaseous phase
ii	A contracting surface (sphere or cylinder) due to nucleation
iii	Degradation mediated by surface moisture or eutectic films
iv	Oxidation
v	Photolysis

Source: Derived from [22].

Probably the most important reaction mechanism is the liquid-mediated process (*iii*). This is because most drugs, even those not particularly susceptible to hydrolysis, become less stable as the surrounding moisture levels increase. It has been speculated that degradation proceeds via a thin film of moisture on the surface of the drug substance [23]. However, studies have indicated that the moisture is concentrated in local regions of molecular disorder, rather than in thin films [24]. These regions that are crystal defects or amorphous areas, equate to the reaction nuclei of mechanisms (*i*) and (*ii*).

Ahlneck and Zografi [20] have postulated that it is not necessary for the drug substance to be dissolved, to induce degradation. They propose that water adsorbed into regions of localised disorder can act as plasticisers, lowering the glass transition temperature of excipients and API, which by permitting increased local molecular movement can increase chemical reactivity. In some cases, there has been shown to be a correlation between the reaction rates and the glass transition temperature, T_g [25]. This hypothesis supports the observation that even relatively low moisture levels can destabilise drug products. Hancock and Zografi [26] indicated that the destabilising affect of small amounts of amorphous material in a crystalline matrix can be hugely amplified, as a result of local areas of greatly increased water content relative to the total water content.

It has been shown in a number of instances that under identical conditions the reaction rates of amorphous forms are greater than in crystalline forms of the same drug [21]. Generally, water has a destabilising effect in the majority of cases, for example, deamidation, hydrolysis or oxidation [27].

In summary, there are at least four ways in which residual moisture in the amorphous state can impact on chemical reactivity. First, as a direct interaction with the drug, for example, in various hydrolytic reactions. Second, water can influence reactivity as a by-product of the reaction, by inhibiting the rate of the forward reaction, for example, in various condensation reactions, such as the Maillard reaction. Third, water acting locally as a solvent or medium facilitating a reaction, without direct participation. Finally, by virtue of its high free volume and low T_g, water can act as a plasticiser, reducing viscosity and enhancing diffusivity [28].

2.5 Excipients' role in drug product destabilisation

Water can be associated with excipients in a number of very different ways, as crystal hydrates, by absorption onto the bulk phase of crystalline or amorphous solids, by adsorption to a surface as a monolayer or multilayers and by capillary condensation into micropores [29]. Callahan *et al*. [30] divided excipients into four classes; non-hygroscopic (I), slightly hygroscopic (II), moderately hygroscopic (III) and very hygroscopic (IV). The authors determined the equilibrium moisture values for 30 common excipients and classified the excipients on this basis. Kontny [31] described how in a closed system water from excipients will re-equilibrate between the individual components of the formulation, via the vapour phase, to attain the most thermodynamically stable state. In practice, as most excipients contain more available moisture than the drug substance, this results in the API being the net recipient of the available moisture, resulting in an increased potential for degradation.

It has been postulated by Carstensen *et al*. [32] that, in the case of hydrophobic excipients, there is the potential for drug to be adsorbed onto the surface of the excipient, resulting in the formation of a drug monolayer, which would be more susceptible to chemical instability. This view was supported by Ahlneck and Alderborn [33], who suggested that increased degradation of acetylsalicylic acid could be the result of diffusion of the dissolved drug onto the microcrystalline cellulose, in binary mixtures of the two compounds. For maximum stability it is critical to optimise the pH of the micro-environment, by judicious selection of excipients; this is especially true for degradation pathways that are pH sensitive.

The degradation rate for many drugs varies as a function of pH of the surrounding environment. This appears to be equally true in the solid state as it is in the solution state [34]. There is evidence that the pH within the micro-environment of a solid oral dosage form can impact on the stability of the formulation. Ahlneck and Lundgren [35] studied the compatibility of acetylsalicylic acid in the presence of three common diluents: lactose, microcrystalline cellulose and dicalcium phosphate. The authors demonstrated that dicalcium phosphate, despite having much lower moisture pick-up levels than microcrystalline cellulose, had a greater destabilising effect on the drug. The authors attributed this to the alkalinity of the dicalcium phosphate in the solid state. The increase in the pH adversely affects the stability of the formulation, despite its very slight solubility in water.

Serajuddin *et al*. [19] reported that the degradation pathway of prevastatin sodium was directly linked to the micro-pH environment within the formulation. Under neutral conditions (pH 6.5), the statin formed two degradation products, a cyclic lactone and an internal hydroxyl rearrangement product. However, as the pH was increased to 9.9 with the incorporation of magnesium oxide into the blend, the only degradation mechanism involved the formation of the cleavage product, 2-methylpropanoic acid. This latter approach of increasing the

formulation pH was adopted to facilitate the long-term stabilisation of the drug product [36].

Thoma and Kerker [37] showed that the photostability of molsidomine solutions were pH dependent, with the drug being more stable under neutral and basic conditions compared with that under acidic conditions. In contrast, in the solid state the pH of the micro-environment appears not to be significant. Aman and Thoma [38] showed that the photostability of molsidomine tablets manufactured using differing lubricants with different acidities or basicities was essentially the same. They utilised stearic acid, glycerylpalmitostearate and magnesium stearate to represent acidic, neutral or basic lubricants. In all cases, the photodegradation was similar, and marked, with drug losses between 35% and 40% after 12 h irradiation.

Despite being marketed for over five decades, levothyroxine tablets, 50 μg, continue to have numerous product recalls due to degradation, and resultant failure to meet approved product specifications [39]. The API is stable at accelerated storage conditions and non-hygroscopic under normal processing and storage conditions [40]. However, it is well known that the lower the active content, and hence the higher the resultant excipient content, the greater can be the potential for instability issues [41]. Levoxythyroxine has a complex stability profile and is sensitive to heat, light, moisture, pH and oxidation [42]. In solution, the degradation follows first-order kinetics, and the rate decreases as the pH increases, with the degradation mechanism involving deiodination. In contrast, Won [42] indicated that the solid-state degradation mechanism was biphasic and involved primarily deamination.

Patel *et al*. [40] found that moisture and the pH of the micro-environment influenced degradation the most. They identified the best diluent for tablet manufacture as being dibasic calcium phosphate, with a basic modifier (sodium carbonate, sodium bicarbonate or magnesium oxide). The authors indicated that the degradation pathways observed were deiodination, deamination and decarboxylation. The data are shown in Table 2.4.

However, as might be anticipated, there is no universal panacea to drug stabilisation. Basic excipients can also destabilise formulations. Serajuddin *et al*. [19] reported on the drug–excipient compatibility of a calcium channel

Table 2.4 Effect of modifiers on stability (percentage label claim) of levothyroxine tablets, 50 μg, 40°C/75% RH for 6 months

Months	Without modifier	Na_2CO_3	$NaHCO_3$	MgO	Tartaric acid	Citric acid
0	101.6	96.6	96.0	100.0	100.2	100.5
3	91.1	98.8	95.1	98.6	83.4	87.6
6	87.2	95.2	84.7	96.8	78.3	74.4

Source: Derived from [40].

blocker (II). The drug, which was a secondary amine, did not have any significant incompatibilities with either lactose or mannitol in binary mixtures. Ternary mixes of the drug, diluent and acidic lubricants (stearic acid) were stable. However, in the presence of basic lubricants (magnesium stearate and sodium stearyl fumarate), hydrolytic degradation was observed, attributed to the hydrolysis of the *O*-acetyl moiety. In addition, for the blends with lactose, there were early eluting peaks observed in the chromatogram that were attributed to the interaction with lactose to form a glycosylamine (Maillard condensation). The authors indicated that under acidic conditions the reactivity would be reduced as a result of protonation of the amine and a consequent decrease in the nucleophilicity of the base. However, in the presence of the more basic lubricants, the pH of the micro-environment would increase, liberating the free base of the drug to react with lactose to form a condensation product. Abdoh *et al*. [43] recently reported the destabilisation of lactose formulations of amlodipine in the presence of aqueous slurries of basic excipients.

In many cases, the excipient-induced instability is greater than can be explained, solely on the basis of an increase in the moisture content. This phenomenon may be illustrated by the stability of binary mixtures of acetylsalicylic acid, with a variety of different cellulose excipients [33]. The authors demonstrated that the drug degradation rate increases proportionally with the amount of excipient present in the mixture. As the study was conducted in open containers at constant relative humidity, the increased drug degradation rates cannot be attributed to equilibration of moisture from the excipients alone. However, these results can be explained in terms of increased surface contact between the excipient and drug, facilitating increased levels of drug degradation. This is illustrated by the fact that homogenous mixtures of drug and excipient, either as powder blends or compacts, give similar degradation rates, whereas two-layer compacts give significantly reduced degradation rates.

Carstensen and Kothari [44] used contact points between alkoxyfuroic acids and microcrystalline cellulose to explain the increased degradation rates. The mechanism for this reaction has not been fully elucidated, but the formation of water bridges between drug and excipient has been postulated. These ‘bridges’ would act as new reaction nuclei, thus increasing the initial degradation rate. Badawy *et al*. [41] presented data in support of this concept. The authors showed that the levels of the two hydrolytic degradation products (I and II) of DMP 754 in a tableted blend were significantly higher than the same blend under identical conditions. They reported levels of the hydrolytic degradation products of 1.5% (I) and 1.9% (II) in the blend vs 6.0% (I) and 2.6% (II) in the compressed product. The authors postulated that compression increases the number of contact points between lactose and DMP754, thereby enhancing their chemical reactivity.

In addition to facilitating the transfer of water, intimate contact between drugs and excipients can lead to the formation of eutectic mixtures and subsequent degradation. Mroso *et al*. [45] reported on mixtures of acetylsalicylic acid and alkali stearates. They demonstrated that there was a linear relationship between

Table 2.5 Numerical summary of lubricant-ibuprofen phase diagrams

System	Eutectic point (expressed as percentage weight of lubricant)	Eutectic melting point (°C)
Ibuprofen : stearic acid	56.0	49
Ibuprofen : stearyl alcohol	64.2	51.5
Ibuprofen : calcium stearate	37.5	55
Ibuprofen : magnesium stearate	32.6	56
Ibuprofen	–	75–77

Source: Derived from [47].

log of the rate and the reciprocal of the melting point of the drug–excipient mixture. The authors demonstrated that the degradation rate increases as the level of alkali stearate increases, which is supportive of the view that from the perspective of stability levels of stearate lubricants should be kept as low as practically possible. Fortunately, this is also a prerequisite from the perspective of dissolution and bioavailability, as owing to its hydrophobic nature, magnesium stearate may retard the dissolution of a drug from a solid oral dosage form [46]. It is likely that other low melting lubricants will behave in a similar manner.

Ibuprofen is known to form eutectics with excipients, resulting in loss of potency due to sublimation. Gordon *et al*. [47] in a DSC investigation of the compatibility of Ibuprofen with stearate lubricants were able to demonstrate that the melting point of Ibuprofen could be reduced by some 20–30°C, via the formation of eutectic mixtures (see Table 2.5).

2.6 Processing as a source of moisture

Crowley and Martini [48] reported on several studies evaluating the impact of unit process operations on hydrates. All showed some level of dehydration liberating freed 'crystalline' water to participate in moisture-mediated reactions. The authors speculated that such energetic processing conditions are likely to have a similar affect on hydrated excipients with a potential deleterious effect on moisture-sensitive APIs. They commented that classical excipient compatibility studies were ill-equipped to predict such moisture-mediated interactions and that compression, attrition and other energy-intensive unit operations were rarely mentioned as requiring investigations.

High-energy processes (milling, lyophilisation, granulating, drying) can introduce certain amounts of amorphicity into otherwise highly crystalline material [20]. As has been previously indicated, enhanced levels of amorphicity lead to increased local levels of moisture, and increased chemical reactivity in these areas. Hancock and Zografi [49] reported on the impact of a roller-compaction process on the water vapour sorption of a sample of aspirin. They speculated that

this was attributed to increased levels of amorphicity in the sample. They estimated the amorphous content of the sample to be of the order of 10%. Buckton *et al*. [50] showed that ball milling causes irregularity, surface faults and imperfections in aspirin crystals. The degree of crystal damage could be directly correlated with the energy of the milling process. Using flow microcalorimetry (ITC), the authors showed that this could also be correlated by increases in enthalpy of water vapour adsorption, consistent with increases in amorphicity.

2.7 Hydrolysis

One of the most common pathways of drug product degradation is hydrolysis. Flutamide, a non-steriodal anti-androgen, is an acetanilide derivative and undergoes both acid- and base-catalysed amide hydrolysis [51], at the extremes of pH to form 4-nitro-3-trifluoromethylaniline (NTMA). Solutions of flutamide stored at pH 1 for 2 weeks at 45°C gave 39% of NTMA, whereas solutions at pH 10 gave 21% of NTMA under the same storage conditions. In contrast, neutral solution of flutamide were stable (<0.4% NTMA).

The novel oral cephalosporin, cefalcor, is administered as both oral solutions and solidoral dosage forms. The stability of this product under aqueous acidic and solid-state conditions was described by Baertschi *et al*. [52, 53]. The degradation of cefalcor in solution is initiated by hydrolysis of the β-lactam ring followed by internal rearrangements, ring contraction of the six-membered cephem ring system to the five-membered thiazol derivatives via an episulphonium ion intermediate and attack of the phenyglycine primary amine to form substituted pyrazines. In all, a total of more than a dozen degradation products via several different degradation pathways were observed.

In contrast, in the solid state, hydrolysis of the β-lactam ring is a minor degradation pathway. Many of the degradation pathways are unique to the solid state, and appear to arise from two separate pathways. An isomeric conversion to Δ^6-cefalcor proceeds via a protonated carbonium ion, which can subsequently decarboxylate. Oxidative attack occurs at the C4 position of the dihydrothiazine ring, which is the predominant degradation pathway at ambient temperature. The other three minor degradation pathways are common to the solution state (hydrolysis of the β-lactam ring, formation of five-membered thiazol derivatives and formation of substituted pyrazines). In total, 17 degradation products were isolated and characterised.

The source of the raw materials can greatly influence hydrolytic reactions. This is exemplified by Gold and Campbell [54] where talc obtained from different sources impacts markedly on the overall stability of the aspirin tablet formulation. This is possibly attributable to the effect of different types and amounts of surface impurities, which are dissolved in the adsorbed moisture layer, where they subsequently react with the API. It could also influence the pH of the micro-environment.

2.8 Oxidation

Oxidation is broadly defined as a loss of electrons in a system, but it can be restated as an increase in oxygen or a decrease in hydrogen content [55]. Oxidation always occurs in tandem with reduction, the so-called redox reaction couple. More generally, it can be defined as the loss of an electron-positive atom, radical or electron, or the addition of an electronegative moiety. Oxidation reactions can be catalysed by oxygen, heavy metals, light, leading to free radical formation (initiation). Free radicals then react with oxygen to form peroxy radicals, which react with the oxidative substrate to yield further complex radicals (propagation), and finally the reaction ceases (termination).

Excipients play a key role in oxidation, either as a primary source of oxidants, trace amounts of metals, or other contaminants. Auto-oxidation of diethylstilbesterol to the peroxide and conjugated quinone degradation products was attributable to the presence of colloidal silicon dioxide, used as a glidant in solid oral dosage forms [48]. This was ascribed to the fact that silicon dioxide can act as a Lewis acid (an electron acceptor or oxidising agent), under anhydrous conditions.

Peroxides are a very common impurity in many excipients, particularly polymeric excipients [56]. They are used as initiators in polymerisation reactions, but are difficult to remove. Ding [57] monitored the peroxide concentrations within polysorbate 80 solutions, and demonstrated the effect of light, heat and concentration on peroxide concentrations. The author showed that the peroxide concentrations increased 9-fold at the lowest polysorbate concentration versus increases of only 1.5-fold at the highest polysorbate concentration. However, the absolute peroxide levels at the higher concentration were much higher.

Hartauer *et al*. [58] reported that peroxide residues in povidone (binder) and crospovidone (disintegrant) were attributable to the formation of the *N*-oxide oxidation product of raloxifene. The authors correlated residual levels of peroxide in the excipients with *N*-oxide formation and thereby gained understanding of the degradation mechanism. A radical-initiated oxidation mechanism would be expected to show a typical 'S'-shaped autocatalytic curve, whereas these curves showed 'fickian' kinetics; that is, rapid initial formation of the *N*-oxide followed by a plateauing of the rate, with consumption of the peroxides, leading to a slowing of the reaction rate.

Huang *et al*. [59] evaluated some common excipients for residual levels of hydrogen peroxide. They found levels of H_2O_2 in the range 0–244 ppm, but perhaps of more concern, they showed quite pronounced differences between different batches of the same vendor and between different vendors of the same excipient. For example, although there is some variability of residual peroxide levels in PEG 400 (polyethylene glycol) from vendor B (5–16 ppm), the differences were small, in contrast with those seen from vendor C (2–59 ppm). Similarly, the differences in residual peroxide in the binder PVP (polyvinyl pyrolidone) from two different vendors were quite marked (73 vs 244 ppm). Formulations optimised for excipients from specific sources could well be sub-optimal if the

vendor source is changed. For this reason, dual sourcing of key excipients is recommended.

Transition metals (iron, copper, nickel and cobalt) catalyse oxidation by shortening the induction period, and by promoting free radical formation [60]. Hong *et al*. [61] reported on the oxidation of a substituted α-hydroxyamine in an intravenous formulation. The kinetic investigations showed that the molecule underwent a one-electron transfer oxidative mechanism, which was catalysed by transition metals. This yielded two oxidative degradants: 4-hydroxybenzaldehyde and 4-hydroxy-4-phenylpiperidine. It has been previously shown that α-hydroxyamines are good metal ion chelators [62], and that this can induce oxidative attack on the α-hydroxy functionality.

Crystalline packing is an important parameter for auto-oxidation in the solid state, as molecular oxygen must be able to access susceptible moieties in the molecule. Lyn *et al*. [63] showed that only one (hexagonal form) of the five different polymorphic forms of prednisolone *tert*-butylacetate were susceptible to oxidation, yielding the 11-ketone product. The authors attributed this oxidative reactivity to channels in the crystal structure allowing access to the labile 11-alcohol position. Bryn *et al*. [64] also identified a similar phenomenon in the photo-mediated oxidation of 21-cortisol *tert*-butylacetate to the corresponding ketone.

Clay *et al*. [65] showed clear correlation between the oxidative reactivity of dialuric acid monohydrate and prevailing moisture contents. Below 93% relative humidity the drug was reasonably stable, oxidation occurring over a 2-month period, whereas the same reaction took less than a day at humidities in excess of 93%.

Losartan, an ACE inhibitor, follows two oxidation-mediated degradation pathways [66]. The first is via the oxidation of the hydroxyl group to the aldehyde derivative. Second, via oxidative dimerisation forming two different degradation products (mediated by the condensation of two losartan molecules and the subsequent elimination of water).

It is well documented that amines oxidise differently in non-aqueous environments to those pathways seen in aqueous systems. In the former systems, hydrogen abstraction of the α-carbon predominates. The reactivity is in the decreasing order: tertiary > secondary > primary amines. Oxidation in non-aqueous systems results in amides, aldehydes and carbon–nitrogen cleavage products [67].

The metal-catalysed oxidation of the anti-emetic, RG-12915, which proceeds via the hydrogen abstraction of the benzylic hydrogen of the fused benzofuran system, and *N*-oxide formation of the bicyclic heterocyclic ring can be controlled using EDTA, citric and tartaric acids. These excipients act as chelators that sequestrate residual metal contaminants, for example, Cu^{2+}, thereby stabilising the formulation. Insights into the overall reaction mechanisms can often be gleaned in this manner. Use of propyl gallate inhibited the benzylic oxidation, but did not prevent *N*-oxide formation, suggesting that it was implicated in disruption of the oxidative propagation steps, but not the initiation steps [68].

Simvastatin, a conjugated alkene, can polymerise as a result of peroxyl radical addition. The peroxide-linked oligomers can be subsequently cleaved to produce epoxides, which in turn degrade to form ketones and alcohols [69]. Inclusion of vitamin E (α-tocopherol) into formulations was found to inhibit chain-oxidation of simvastatin, lovastatin and other structurally related statins.

Hydroquinone oxidation to quinone occurs via two linked one-electron transfer stages. The first step (typically metal catalysed) yields the semiquinone radical intermediate, which is resonance stabilised. The second step involves electron transfer to molecular oxygen to generate superoxide and quinone [70]. This reaction mechanism is common to all hydroquinones, catechols, resorcinols, and so forth.

Powell *et al*. [71] reported on the moisture-mediated oxidation of lonapalene, an ester pro-drug for the treatment of psoriasis. Many ester pro-drugs are formulated in alcoholic and semi-solid vehicles to preclude (or reduce) hydrolysis. The authors investigated the degradation kinetics in these vehicles as a function of pH. Lonapalene is less stable in neutral and basic conditions than at acidic pHs. The authors demonstrated the effect of pH modifiers during the formulation optimisation. When citric acid was added to non-aqueous formulations of lonapalene, it increased the shelflife by 2–5-fold depending on solvent ratio. More significantly, citric acid also stabilised lonapalene in propylene glycol based ointment formulations, by 6-fold, compared with the standard non-acid-stabilised formulation.

2.9 Photolysis

Sunlight (both in the UV and visible regions) may degrade drug products and excipients, and, consequently, photolabile APIs can raise many formulation issues [72].

The need to characterise and define light sources in photodegradation studies was outlined by investigators from the FDA [73]. They reported on UV-A-induced photolytic decomposition products of the anti-diuretics, chlorothiazide and hydrochlorothiazide. Both drugs are reported to be potent photosensitisers; however, their photodegradation had previously been reported only under very specific conditions [74, 75].

Revelle *et al*. [73] showed that the principal degradation products for both thiazides were the dehalogenated products. For hydrochlorothiazide, the authors also showed that a major photodegradation product was the dehydrogenation product. In contrast, Tamat and Moore [75] found that the principal degradation pathway for hydrochlorothiazide was *via* a hydrolytic pathway, and did not report seeing the dehydrogenation product. In addition, Ulvi and Tammiletho [76] reported different photodegradation products for chlorothiazide, and they did not report seeing the dehalogenated product. All of these differences were attributed to variability in the wavelength and energy spectra between the various irradiation sources, which were used in the various different studies.

The understanding of some photodegradation pathways appears to be still in its infancy. Photostability studies on a new ester of methylprednisolone

identified two unknown degradation products, in addition to the well-established oxidation (17-keto and 11-keto) and hydrolytic (methylprednisolone) pathways [77]. Both of the new degradation products appeared to be formed via a novel bicyclo [3.1.0] hex-3-en-2-one intermediate and subsequent rearrangement of the cross-conjugated dienones. Surprisingly, one of the new degradation products (1, 11-epoxy) was formed in high yields.

The ionisation state of molecules in the solution state appears to be an important variable in photodegradation mechanisms. A recent publication on riboflavin oral liquid preparations shows that the formulation is most photostable at pHs between 5 and 6, where the non-ionised form predominates [78]. The rate of photolysis increase 80-fold at pH 10.0, owing to increased redox potential. Conversely, at pH 3.0, the increased photolysis is associated with the excited singlet state, in addition to the triplet state.

There is often little difference between photo-induced oxidation and oxidation, both following similar pathways. Silchenko *et al*. [79] reported on the photo-mediated auto-oxidation of dithiophene, an anti-cancer drug. Dithiophene photodegrades via oxidation of the alcohol function to the 2-aldehyde and subsequently the 2-carboxylic acid degradants. Interestingly, under anaerobic conditions the degradation was more complex, but one of the photodegradants was the same: the 2-aldehyde. It was speculated that the degradation pathway under these conditions was primarily via a disproportionation mechanism.

After clinical treatment with topical products photodecomposition may occur on the skin surface, or indeed during the transit through the dermis, and may subsequently cause adverse skin reactions [80, 81]. Non-steriodal anti-inflammatory agents (NSAID) are often developed as topical products for the treatment of pain and swelling, particularly associated with sporting injuries. Several members of the NSAID family have been reported as having issues with photoinstability [80, 81]. The photochemistry of one of the more popular topical NSAIDs, ketoprofen, is complex, and is influenced by the surrounding environment [72]. In all, nine different photodecomposition products of ketoprofen have been identified, involving molecular rearrangement, decarboxylation, reduction, dimerisation and the formation of hydrogen peroxide [82]. The latter finding is particularly worrying, given the role of peroxide as a dermal sensitiser.

Recently, Lodén *et al*. [83] investigated the use of titanium dioxide as an opacifier in topical formulations of ketoprofen. The results demonstrated qualitative differences in the level of photostabilisation based on the grade of titanium dioxide utilised. Surface-coated particles were shown to induce greater photostabilisation than pharmaceutical grades of the excipient. The authors showed clear superiority of the opacified topical formulation containing 4% titanium dioxide versus the standard clear gel.

An elegant publication from Aman and Thoma [84] outlined the various options open to the stabilisation of photolabile oral products. The addition of light-absorbing agents is a well-established approach to stabilising photolabile products [85]. Aman and Thoma [84] reported that the incorporation of light-absorbers and pigments considerably improved the photostability of the very light sensitive

Table 2.6 Photodegradation of sorivudine

Colouring agent	Direct compression (% Z-isomer formed)	Wet granulation (% Z-isomer formed)
No colouring agent	21.1	9.0
Red iron oxide	3.4	0.7
Yellow iron oxide	2.6	0.7
Black iron oxide	2.6	1.3

Source: Derived from [86].

molsidomine tablets. Pigments were superior to colorants or UV absorbers. However, they warned that the use of titanium dioxide (an opacifier) needs to be considered carefully. Although preblending of titanium dioxide with the drug compression blend was successful, surface-treated material, which according to Lodén *et al*. [83] reduces photocalalytic activity, was inferior to the untreated excipient.

Desai *et al*. [86] reported on the photolytic degradation of the anti-viral, sorivudine, which formed the inactive Z-isomer. On the basis of extensive drug–excipient compatibility studies it was found that the incorporation of iron oxide pigments into the blends (direct compression or wet granulated) stabilised the drug to photodegradation; indeed, so much so that the tablet was found not to require a film coat. The data are summarised in Table 2.6.

Interestingly, titanium dioxide can also act as a photocatalyst [87]. In some investigations into these phenomena a moisture-mediated redox reaction has been postulated.

The 1,4-dihydropyridines, for example, nifedipine, nicardipine and amlodipine, are a well-established class of anti-hypertensive drugs. They are photolabile, some markedly so, for example, nifedipine. In all cases, the major light degradation product is the resonance-stabilised, fully aromatic, pyridine analogue [38].

2.10 Impact of processing on photostability

As well as affecting the stability of hydrolytically unstable products, processing can impact on the photostability of drug products. Interestingly, although the particle size of the API can significantly impact on the photolability, it appears to have a lesser impact on the drug product. This is possibly due to the fact that photodegradation is a surface-mediated phenomenon following topochemical reaction kinetics and the excipients within the formulation will reduce the impact of photodegradation of API at the tablet surface.

Teroka *et al*. [88] investigated the effect of grinding on the photostability of tablets incorporating the α- and β-forms of the photolabile drug, nicardipine.

They found that both crystalline forms of the drug were totally converted to the amorphous form after 2.5 h grinding in a 'mixer' mill. The formation of the pyridine photodegradation product was monitored on the surface of tablets using FT-IR. The authors indicated that as solid-state photodegradation is a surface phenomenon, FT-IR was more appropriate than HPLC. The degradation rate constants of both crystalline polymorphs were similar (α-form = 0.039 per day; β-form = 0.046 per day). Both polymorphs showed an increased photodegradation rate after grinding (α = 1.5 times and β = 2.5 times). However, the thermodynamically less stable polymorph (β) showed a significantly increased photodegradation rate (0.159 per day) compared with the thermodynamically more stable α-polymorph (0.061 per day).

These findings were in accord with those of Qin and Frech [89], who reported that the photodegradation rate of the amorphous form of the development drug, MK-912, was approximately 40 times greater than its crystalline counterpart, under ICH irradiation conditions. Interestingly, unlike the photodegradation of nicardipine, which gave the same photodegradation product from crystalline and amorphous forms, MK-912 gave different degradation products dependant on the physical form. Photodegradation yielded three ketone and one alcohol degradant for the crystalline form. In contrast, the amorphous form yielded three ketone, five alcohol and one *N*-oxide degradant, all structurally identified by HPLC-MS-MS. The authors explained the differences by postulating photodegradation of the crystalline form via the molecule's triplet excited state. In contrast, the amorphous form behaves like a singlet-oxygen sensitiser. The crystalline form has an ordered close-packed structure, whereas the amorphous form does not, and singlet-oxygen has greater reactivity resulting in faster photodegradation and more photodegradants.

Aman and Thoma [38] studied the processing of the photolabile drugs, nifedipine and molsidomine. They found that the greater the compression force, and hence the lower the tablet porosity, the greater the photoinstability (see Table 2.7). The influence of tablet manufacturing process was also investigated. The authors investigated direct compression (DC), aqueous wet granulation (WG) and alcoholic wet granulation (WG); in the latter cases they used polvinylpyrollidone (PVP) as the binder. The product was destabilised to a

Table 2.7 Impact of compression force on photoinstability of nolsidomine tablets

Compression force (kN)	Tablet porosity (%)	Percentage degradation after 3 h photo-irradiation
3.5	27.5	12
9.0	14.0	22
21.0	8.0	24

Source: Derived from [38].

greater extent by the WG processes and showed 4% greater degradation levels than the corresponding DC product. This was not related to the residual moisture levels in the products, as the moisture levels in the DC and the alcoholic WG tablets were approximately the same (4.9% vs 4.8%, respectively), whereas in the aqueous WG product it was only slightly higher (5.7%). They speculated that it was probably attributable to the more amorphous nature of the drug substance in the WG products, after wet massing and drying.

2.11 Miscellaneous reactions

Dextrose is widely used as an isotonic media in parenteral formulations. Sterilisation using autoclaving has been reported to induce the formation of fructose via an isomerisation reaction, with the resultant formation of 5-hydroxymethyl-furfural [90].

Aspartame is relatively unstable in solution, undergoing cyclisation by intramolecular self-aminolysis at pH values in excess of 2.0 [91]. This follows nucleophilic attack of the free base N-terminal amino group on the phenylalanine carboxyl group resulting in the formation of 3-methylenecarboxyl-6-benzyl-2, 5-diketopiperazine (DKP). The DKP further hydrolyses to L-aspartyl-L-phenylalanine and to L-phenylalanine-L-aspartate [92]. Grant and co-workers [93] have extensively investigated the solid-state stability of aspartame. At elevated temperatures, dehydration followed by loss of methanol and the resultant cyclisation to DKP were observed. The solid-state reaction mechanism was described as Prout-Tompkins kinetics (via nucleation control mechanism).

The rarely observed alkyl-nitrogen heterolysis reaction was reported by Raghaven *et al*. [94] for the acid-catalysed degradation of DMP-777, a potent elastase inhibitor. The exceptional stability of one of the by-products, an alkyl-substituted benzylic cation, was postulated as being the driving force behind this unusual reaction mechanism. Under basic conditions, DMP-777 undergoes β-lactam hydrolysis, leading to the formation of parabanic acid piperazineamide (1-methyl-(4′-hydroxy)-4-phenoxypiperazine) and a substituted methylenedioxyphenyl fragment. Interestingly, monocyclic β-lactams are usually considered to be stable to alkaline hydrolysis, whereas in contrast, bicyclic β-lactams are readily hydrolysed under alkaline conditions. Surprisingly, the monocyclic β-lactam ring system in DMP-777 appears to be highly reactive and readily susceptible to hydrolysis, possibly attributable to the reduced basicity of the β-lactam nitrogen, making it a good leaving group.

The Maillard condensation is one of the most extensively studied reactions within the field of degradation chemistry, particularly in the area of food and nutritional science. Louis Mallard reported in 1912 that some amines react with reducing carbohydrates to produce brown pigments. The condensation typically yields a simple glycosylamine, which then readily undergoes the Amadori rearrangement to produce 1-amino-1-deoxy-2-ketoses [95]. Reducing carbohydrates

such as glucose, maltose, lactose and their derivatives are substrates for this reaction as they can tautomerise to produce the reactive aldehydic form. In contrast, non-reducing carbohydrates such as sucrose, mannitol and trehalose are not reactive. Most amines, primary and secondary, aromatic and aliphatic, can undergo this reaction.

Wirth *et al*. [95] reported on the Maillard condensation reaction that occurred between fluoxetine (the active principal in Prozac) and lactose. Whereas Prozac is formulated with starch, several generic fluoextine formulations used lactose as the diluent. The authors demonstrated the formation of the intermediate glycosamine and characterised the Amadori rearrangement product as being primarily *N*-formylfluoxetine. The by-products of the rearrangement reaction were volatile and could be monitored using GC-MS. The authors proposed using the *N*-formyl compound as a specific marker for this drug–excipient incompatability, as it is relatively easy to prepare.

It has been shown in previous sections of this chapter that excipients and the impurities therein can significantly influence drug product degradation. Less well known is that some of these excipient impurities are themselves unstable and can generate additional reactive impurities. Excipients containing polyoxyethelene chains, for example, polyethylene glycol 300 (PEG 300) and polysorbate are known to generate formaldehyde, which is a degradation product of the known peroxide impurity [96]. The authors examined the levels of formaldehyde in PEG 300, polysorbate 80 and ethanol using HPLC with post-column derivatisation. The authors found levels of formaldehyde in the range 0–165 ppm, but perhaps of more concern, they found quite pronounced differences between different batches from the same vendor and between different vendors of the same excipient. Worse still, there were pronounced differences between different containers of the same lot (9–165 ppm). This may be due to inappropriate storage of the excipients, as the levels of formaldehyde, as well as other aldehydes, can increase in the presence of atmospheric oxygen. These findings mirrored the earlier investigations of Huang *et al*. [59], who evaluated residual hydrogen peroxide levels in some common excipients.

BMS-204352, a novel substituted 3-fluorooxindole, is a potassium channel opener being developed for the treatment of stroke. Nassar *et al*. [96] reported on the development of a non-aqueous parenteral formulation of BMS-204352. This formulation was composed of a mixture of PEG 300, polysorbate 80, ethanol and water. The authors reported on the formation of 1-hydroxymethyl adduct of BMS-204352 (formaldehyde adduct), which was linked with residual levels of formaldehyde in the polymeric excipients.

Duloxetine hydrochloride, a novel anti-depressive, is known to be acid labile and, consequently, it has been formulated as an enteric-coated tablet. Interestingly, Jansen *et al*. [97] subsequently found that the drug was destabilised by degradation products within these enteric polymers. The authors found that succinyl and phthalyl residues from the hydroxypropyl methylcellulose acetate succinate (HPMCAS) and hydroxypropyl methylcellulose phthalate (HPMCP) formed

succinamide and phthalamide degradants, respectively. This reaction occurred via the free acid or anhydride reactive intermediates.

2.12 Container–closure systems

The applicability of the container–closure system(s) for its intended role in product protection requires thorough investigation. Hydrolytically labile products can be stabilised via several packaging options; however, there are several variables that need to be carefully considered. In addition to the water activity within the formulation, the water vapour transition rate (WVTR) through the container–closure system and the water levels within the container head-space need to be assessed. Chen and Li [98] reported recently on a novel mathematical model for predicting moisture uptake by packaged drug products during storage. The experimental and modelled data showed very good accord, and allowed the packaging scientist to predict, *a priori*, the moisture uptake by container–closure systems, under varying storage conditions, over the predicted shelflife.

The WVTR through the container is determined by the container wall thickness, the permeability of the material and the difference between the external and internal relative humidity environments. Waterman *et al*. [99] determined the theoretical rate of water permeation through a standard 60-cc bottle when stored at 40°C/75% RH. This equated to an uptake of 1 mg of water per day. They commented that even if the product had been packed under low water vapour conditions the relative humidity conditions within the container would be equate to 50% RH within 1 day. The WVTRs (see Table 2.8) for some common packaging materials were reported by Waterman *et al*. [99].

Table 2.8 WVTRs of common packaging materials at 38°C/90% RH

Container/closure	WVTR (g mm/m^2 day)
Nylon 6	7.5–7.9
PVC	1.8
Polypropylene	0.54
PET	0.39–0.51
HDPE	0.12
Aclar 22A	0.011
Aclar UltRx	0.0006
Cold form foil blister	<0.0005

Source: Derived from [99].

Note: PVC, Polyvinylchloride; PET, polyethyleneteraphthalate; HDPE, high density polyethylene.

The choice of packing material is decided by several variables: degree of protection required by the product, cost, marketing preferences and regulatory considerations. Lusina *et al*. [100] reporting on the packaging optimisation trials they performed on a losartan/hydrochlorothiazide combination tablet. The level of moisture protection could only be evaluated by comparative testing under realistic storage conditions. The authors evaluated two different blister pack types. A PVC blister (polyvinylchloride: 250 μm), which afforded partial protection, and a cold-form Al blister, which afforded total protection against moisture ingress. However, the latter pack was more costly, and a less elegant presentation. The authors found that the latter pack gave adequate protection when stored at 40°C/75% RH for 6 months, whereas the former pack showed unacceptably high levels of the hydrolysis degradation product of hydrochlorothiazide, 4-amino-6-chlorobenzene-1, 3-disulphonamide. The losartin component was stable in both pack presentations. On the basis of these data, the product was assigned a 24-month shelflife.

Desiccants have been utilised to control the exposure of products to the ingress of moisture. Desiccants vary in their capacity and the rate that they adsorb/absorb ingressed moisture. Silica gel is very efficient at absorbing moisture at high relative humidities, but comparatively poor at lower relative humidities, whereas for molecular sieve desiccants the opposite scenario prevails. As a consequence, more molecular sieve is required at higher relative humidities, and the greater the handling precautions that are required during packaging operations. On the basis of the calculated WVTR of known container components and the rate of moisture adsorbed by desiccants, the amount of desiccant that would be required to maintain a specified relative humidity over the product's shelflife can be determined [101].

Blister packs afford greater product protection by restricting the headspace and thereby controlling the water levels within the immediate vicinity of the product [100]. Unfortunately, there are significant compromises that need to be taken. Unfortunately, the better the barrier protection, the greater the cost. Additionally, the better the moisture barrier protection, the worse the barrier to oxygen ingress, and vice versa. Unfortunately, blister packs have a high surface area per unit dose ratio and the WVTRs are problematical under conditions of high temperature and humidities. Therefore, the only meaningful option for retaining low relative humidity conditions when exposed to high external relative humidity environments are cold-form Al foil blisters.

Similar considerations are relevant to protection of products that are labile to oxidative degradation. The permeability of plastic containers to oxygen ingress has also been evaluated, and is summarised in Table 2.9 [102].

Waterman *et al*. [103] determined the theoretical rate of oxygen permeation through a standard 30-cc bottle when stored in a well-sealed container. This equated to an uptake of 0.2 mMol of oxygen per year. In addition to permeation through the container walls, the key vulnerability in any container–closure system is the closure. With screw-topped closures leakage can be significant. Hence for

Table 2.9 Oxygen vapour transmission rates (OVTR) for some common packaging containers

Container/closure	OVTR (ml mm/m^2 day)
LDPE	241
HDPE	102
Polystyrene	127
Polycarbonate	114
Polypropylene	89
PVC	4
PET	2

Source: Derived from [102].

Note: LDPE, low density polyethylene; HDPE, high density polyethylene; PVC, polyvinylchloride; PET, polyethyleneteraphthalate.

oxidatively labile dosage forms an oxygen impermeable seal is required, and induction heat-sealed containers are particularly useful. Levels of oxygen in the headspace of the container–closure can be significant, and packaging under an inert atmosphere although doable is problematical.

Polymeric additives within the container can migrate into aqueous or non-aqueous liquid formulations. Polyolefin polymers used in the packaging of pharmaceuticals and foodstuffs require the addition of stabilisers (particularly antioxidants) to ensure their physical and chemical integrity [104]. A large number of publications [105] have appeared in the literature concerning the migration, or leaching, of antioxidant additives from food, drug and cosmetic plastic packs into the formulations. Jencke [105] provided a comprehensive review on this area, citing 124 references on extractable/leachable substances derived from plastic containers and devices. The identities of over 100 extractable/leachable substances were reported, arising from the various plastic container/closures, as well as their reported levels in actual and simulated products.

One consequence of these large numbers of packaging-related impurities is the potential for abundant chemical interactions of a non-predictable nature. A recent report on the identification of a malodorous impurity in a heat-stressed packaging material exemplifies the nature of these issues. Sides *et al*. [106] reported on the formation of a thioester (ethyl-2-mercaptoacetate) resulting from the unanticipated reaction between small levels of residual ethanol in the tablet disintegrant and low levels of thioglycollic acid. The thioglycollate arises as a reactant or synthetic intermediate of an organotin heat-stabilising plastic additive (di-*n*-octyltin-bis [*iso*-octylthioglycollate]). The residual ethanol was found to arise from the tablet disintegrant, explotab.

Confirmation of the reaction mechanism was performed by heating samples of the suspect lot of the PVC foil with deuterated methanol. The authors selected methanol as it would not interfere and would form the analogous methyl

thioester (methyl-2-mercaptoacetate) and the deuterated analogue would make MS identification unequivocal. The packaging material developed the same unmistakable odour, and GC-MS confirmed the identity of the malodorous impurity.

References

1. R. C. Rowe, P. J. Sheskey and P. J. Weller, eds, *Handbook of Pharmaceutical Excipients*, Joint Publication of A.P.S. and R.S.P.G.B., xvii, 2003.
2. R. C. Hwang and G. R. Peck, *Pharm. Technol*., 2001, **25**, 54.
3. M. P. Timsina, G. P. Martin and C. Marriott, *Int. J. Pharm*., 1994, **101**, 1.
4. International Conference of Harmonization Q3B (R), *Impurities in New Drug Products (Revised Guideline)*, Federal Register 68, 2003.
5. International Conference of Harmonization Q3A (R), *Impurities in New Drug Substances (Revised Guideline)*, Federal Register 68, 2003.
6. M. Bakshi and S. Singh, *J. Pharm. Biomed. Anal*., 2002, **28**, 1011.
7. L. Huber, *LC/GC Magazine*, 1998, 2.
8. International Conference of Harmonization Q2B, *Validation of Analytical Procedures: Methodology*, Geneva, Switzerland, 1996.
9. B. Boneschans, A. Wessels, J. van Staden, M. Zovko, B. Zorc and J. Bergh, *Drug. Dev. Ind. Pharm*., 2003, **29**, 155.
10. K. C. Waterman and R. C. Adami, *Int. J. Pharm*., 2005, **293**, 101.
11. R. Weinberger and I. S. Lurie, *Anal. Chem*., 1991, **63**, 43.
12. P. K. Owens, H. Wilkstrom, S. Nagard and L. Karlsson, *J. Pharm. Biomed. Anal*., 2002, **27**, 587.
13. M. J. Akers, *Can. J. Pharm. Sci*., 1976, **11**, 1.
14. D. C. Monkhouse and A. Maderich, *Drug Dev. Ind. Pharm*., 1989, **15**, 2115.
15. K. S. Paswani and J. K. Lalla, *J. Parenter. Sci. Technol*., 1990, **44**, 336.
16. H. Nyqvist, *Drug Dev. Ind. Pharm*., 1986, **12**, 953.
17. J.-O. Waltersson, *Acta. Pharm. Suec*., 1986, **23**, 129.
18. H. Leuenberger and W. Becher, *Pharm. Acta. Helv*., 1975, **50**, 88.
19. A. T. M. Serajuddin, A. B. Thakur, R. N. Ghoshal *et al*., *J. Pharm. Sci*., 1999, **88**, 696.
20. C. Ahlneck and G. Zografi, *Int. J. Pharm*., 1990, **62**, 87.
21. J. T. Carstensen and T. Morris, *J. Pharm. Sci*., 1993, **82**, 657.
22. J. I. Wells, *Pharmaceutical Preformulation, Excipient Compatibility*, Ellis Horwood, Chichester, Chapter 8, 1988.
23. L. J. Leeson and A. M. Mattocks, *J. Am. Pharm. Assoc. Sci. Ed*., 1958, **47**, 329.
24. M. J. Kontny, G. P. Grandolfi and G. Zografi, *Pharm. Res*., 1987, **4**, 104.
25. M. L. Roy, M. J. Pikal, E. C. Rikard and A. M. Maloney, *Dev. Biol. Stand*., 1991, **74**, 232.
26. B. C. Hancock and G. Zografi, *J. Pharm. Sci*., 1997, **86**, 1.
27. E. Y. Shalav and G. Zografi, *J. Pharm. Sci*., 1996, **85**, 1137.
28. G. Zografi and B. Hancock, in *Topics in Pharmaceutical Sciences*, Editors D. J. A. Crommelin, K. K. Midha and T. Nagai, Medpharm Scientific Publishers, Stuttgart, 1993, 405.
29. G. Zografi, *Drug Dev. Ind. Pharm*., 1988, **14**, 1905.
30. J. C. Callahan, G. W. Cleary, M. Elefant, G. Kaplan, T. Kensler and R. A. Nash, *Drug Dev. Ind. Pharm*., 1982, **8**, 355.

31. M. J. Kontny, *Drug Dev. Ind. Pharm.*, 1988, **14**, 1991.
32. J. T. Carstensen, *Drug Dev. Ind. Pharm.*, 1988, **14**, 1927.
33. C. Ahlneck and G. Alderborn, *Acta. Pharm. Suec.*, 1988, **25**, 41.
34. J. T. Carstensen, in *Drug Stability Principles and Practices*, Marcel Dekker, New York, 1990, 155.
35. C. Ahlneck and P. Lundgren, *Acta. Pharm. Suec.*, 1985, **22**, 305.
36. Y. M. Joshi, P. Chiesa and N. B. Jain, U.S. Patent 5, 180, 589, Jan. 19th (1993).
37. K. Thoma and R. Kerker, *Pharm. Ind.*, 1992, **54**, 630.
38. W. Aman and K. Thoma, *Pharm. Ind.*, 2002, **64**, 1287.
39. C. J. Rhodes, *Clin. Res. Reg. Aff.*, 1998, **15**, 173.
40. H. Patel, A. Stalcup, R. Dansereau and A. Sakr, *Int. J. Pharm.*, 2003, **264**, 35.
41. S. I. F. Badawy, R. C. Williams and D. L. Gilbert, *Pharm. Dev. Technol.*, 1999, **4**, 325.
42. C. M. Won, *Pharm. Res.*, 1992, **9**, 131.
43. A. Abdoh, M. M. Al-Omari, A. A. Badwan and A. M. Y. Jaber, *Pharm. Dev. Technol.*, 2004, **9**, 15.
44. J. T. Carstensen and R. C. Kothari, *J. Pharm. Sci.*, 1983, **72**, 1149.
45. P. V. Mroso, A. Li Wan Po and W. J. Irvin, *J. Pharm. Sci.*, 1982, **71**, 1096.
46. L. V. Allen and P. E. Luner, *Handbook of Pharmaceutical Excipients*, Joint Publication of A.P.S. and R.S.P.G.B., 2003, 354.
47. R. E. Gordon, C. L. VanKoevering and D. J. Reits, *Int. J. Pharm.*, 1984, **21**, 99.
48. P. J. Crowley and L. G. Martini, *Pharm. Technol. Eur.*, March 2001.
49. B. C. Hancock and G. Zografi, *J. Pharm. Sci.*, 1996, **85**, 246.
50. G. Buckton, A. Choularton, A. E. Beezer and S. M. Chatham, *Int. J. Pharm.*, 1988, **47**, 121–128.
51. R Sternal and N Nigura, *Anal. Prof. Drug Subs. Excip.*, 2001, **27**, 115.
52. S. W. Baertschi, D. E. Dorman, J. L. Occolowitz, M. W. Collins, L. A. Spangle, G. A. Stephenson and L. J. Lorenz, *J. Pharm. Sci.*, 1997, **86**, 526.
53. D. E. Dorman, L. J. Lorenz, J. L. Occolowitz *et al.*, *J. Pharm. Sci.*, 1997, **86**, 526.
54. G. Gold and J. Campbell, *J. Pharm. Sci.*, 1964, **53**, 52.
55. S. W. Hovoska and C. Shoenich, *J. Pharm. Sci.*, 2001, **90**, 253.
56. K. C. Waterman, R. C. Adami, K. M. Alsante *et al.*, *Pharm. Dev. Technol.*, 2002, **7**, 1113.
57. S. Ding, *J. Pharm. Biomed. Anal.*, 1993, **11**, 95–101.
58. K. J. Hartauer, G. N. Arbuthnot, S. W. Baertschi *et al.*, *Pharm. Dev. Technol.*, 2000, **5**, 303.
59. T. Huang, M. E. Garceau and P. Gao, *J. Pharm. Biomed. Anal.*, 2003, **31**, 1203.
60. J. A. Mollica, S. Ahuja and J. Cohen, *J. Pharm. Sci.*, 1978, **67**, 443.
61. J. Hong, E. Lee, J. C. Carter, J. A. Masse and D. A. Oksanen, *Pharm. Dev. Technol.*, 2004, **9**, 171.
62. Z. Foldi, T. Foldi and A. Foldi, *Chem. Ind.*, 1955, 1297.
63. C-T. Lyn, P. Perrier, G. G. Clay, P. A. Sutton and S. R. Bryn, *J. Org. Chem.*, 1982, **47**, 2978.
64. S. R. Bryn, P. A. Sutton, B. Tobias, J. Frye and P. Main, *J. Am. Chem. Soc.*, 1988, **110**, 1609.
65. R. J. Clay, A. M. Knevel and S. R. Bryn, *J. Pharm. Sci.*, 1982, **71**, 1289.
66. Z. Zhao, Q. Wang, E. W. Tsai, X. Z. Quin and D. Ip, *J. Pharm. Biomed. Anal.*, 1999, **20**, 129.
67. A. L. J. Beckwith, P. H. Eichinger, B. A. Mooney and R. H. Prager, *Aus. J. Chem.*, 1983, **36**, 719–739.

68. C. M. Won, S-Y. Tang and C. L. Strohbeck, *Int. J. Pharm.*, 1995, **121**, 95.
69. G. B. Smith, L. DiMichele, L. F. Colwell Jr., G. C. Dezeny, A. W. Douglas, R. A. Reamer and T. R. Verhoeven, *Tetrahedron*, 1993, **49**, 4447.
70. Y. Li and M. A. Thrush, *Arch. Biochem. Biophys.*, 1993, **300**, 346.
71. M. F. Powell, A. Magill, A. R. Becker, R. A. Kenley, S. Chen and G. C. Visor, *Int. J. Pharm.*, 1988, **44**, 225.
72. H. H. Tonnesen, *Int. J. Pharm.*, 2001, **225**, 1.
73. L. K. Revelle, S. R. Musser, B. J. Rowe and I. C. Feldman, *J. Pharm. Sci.*, 1997, **86**, 631.
74. D. E. Moore, *J. Pharm. Sci.*, 1977, **66**, 1282–1284.
75. S. R. Tamat and D. E. Moore, *J. Pharm. Sci.*, 1983, **72**, 1282.
76. V. Ulvi and S. Tammiletho, *Acta. Pharm. Nord.,* 1989, **1**, 195.
77. M. Ogata, Y. Noro, M. Yamada, T. Tahara and T. Nishimura, *J. Pharm. Sci.*, 1998, **87**, 91.
78. I. Ahmad, Q. Fasihullah, A. Noor, I. A. Ansari and Q. M. N. Ali, *Int. J. Pharm.*, 2004, **280**, 199.
79. S. Silchenko, C. Schöneich, B. J. Carlson and V. J. Stella, *Int. J. Pharm.*, 2003, **264**, 97–105.
80. J. W. Gould, M. G. Mercurio and C. A. Elmets, *J. Am. Acad. Dermotol.*, 1995, **33**, 574.
81. H. Bagheri, V. Liaubet, J. L. Montastruc and N. Chouini-Laianne, *Drug Saf.*, 2000, **22**, 339.
82. F. Bosca, M. A. Miranda, G. Carganico and D. Mauleön, *J. Photochem. Photobiol.*, 1994, **60**, 96.
83. M. Lodén, U. Åkerström, K. Lindahl and B. Berne, *J. Pharm. Sci.*, 2005, **94**, 781–787.
84. W. Aman and K. Thoma, *J. Pharm. Sci.*, 2004, **93**, 1860.
85. K. Thoma and R. Klimek, *Int. J. Pharm.*, 1991, **67**, 169.
86. D. S. Desai, M. A. Abdelnasser, B. A. Rubitski and S. A. Varia, *Int. J. Pharm.*, 1994, **103**, 69–76.
87. J. Fujishima, T. N. Rao and D. A. Tryc, *J. Photochem. Photobiol.* C, 2000, **1**, 1.
88. R. Teroka, M. Otsuka and Y. Matsuda, *Int. J. Pharm.*, 1999, **184**, 35.
89. X. Qin and P. Frech, *J. Pharm. Sci.*, 2001, **90**, 833.
90. P. C. Buxton, R. Jahnke and S. Keady, *Eur. J. Phar. Biopharm.*, 1994, **40**, 172.
91. S. M. Gaines and J. L. Bada, *J. Org. Chem.*, 1988, **53**, 2757.
92. R. J. Prankerd, *Anal. Prof. Drug Subs. Excip.*, 2002, **29**, 7.
93. S. S. Leung, B. E. Padden, E. J. Munsen and D. J. W. Grant, *J. Pharm. Sci.*, 1998, **87**, 501.
94. K. S. Raghaven, D. B. Gray, T. H. Scolz, G. A. Nemeth and M. A. Hussain, *Pharm. Res.*, 1996, **13**, 1815.
95. D. D. Wirth, S. W. Baertschi, R. A. Johnson *et al.*, *J. Pharm. Sci.*, 1998, **87**, 31.
96. M. N. Nassar, V. N. Nesarikur, R. Lozano, W. L. Parker, Y. Huang, V. Paliniswamy, W. Xu and N. Khaselev, *Pharm. Dev. Technol.*, 2004, **9**, 189–195.
97. P. J. Jansen, P. L. Oren, C. A. Kemp, S. R. Maple and S. W. Baertschi, *J. Pharm. Sci.*, 1998, **87**, 81.
98. Y. Chen and Y. Li, *Int. J. Pharm.*, 2003, **255**, 217–225.
99. K. C. Waterman, R. C. Adami, K. M. Alsante *et al.*, *Pharm. Dev. Technol.*, 2002, **7**, 113.
100. M. Lusina, T. Cindric, J. Tomaic, M. Pekp, L. Pozaic and N. Musulin, *Int. J. Pharm.*, 2005, **291**, 127.

101. R. L. Dobson, *J. Packag. Technol.*, 1987, **1**, 127–131.
102. Y. Wang, A. J. Easteal and X. D. Chen, *Packag. Technol. Sci.*, 1998, **11**, 169.
103. K. C. Waterman, R. C. Adami, K. M. Alsante *et al.*, *Pharm. Dev. Technol.*, 2002, **7**, 1.
104. G. Ferrara, M. Bertoldo, M. Scoponio and F. Ciardelli, *Polym. Degrad. Stabil.*, 2001, **73**, 411.
105. D. Jencke, *J. Pharm. Sci. Technol.*, 2002, **56**, 332–371.
106. S. L. Sides, K. B. Polowy, A. D. Thornquest and D. J. Burinsky, *J. Pharm. Biomed. Anal.*, 2001, **25**, 379–386.

Analysis of Drug Impurities
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3 Stereochemical impurities

Mark R. Hadley

3.1 Introduction

The chiral nature of living organisms has obvious implications for the interaction of chiral drug molecules with such systems. The stereoselectivity of drug–receptor interactions stems from the chirality of the constituent amino acid building blocks of proteins. Protein function in biological systems is inextricably linked to their three-dimensional structure, which is governed by the amino acid sequence present. Only homochiral amino acids are known to form proteins with the required alpha helical structure necessary for biological activity, with proteins in terrestrial biological systems consisting entirely of L-amino acid residues [1]. In addition, many other endogenous biochemicals, for example, sugars are also present as homochiral molecules in living organisms. Chirality is not a requirement for drug activity, but when present can lead to marked differences in the pharmacokinetic, pharmacodynamic and toxicological properties of the resulting drug stereoisomers [2]. The overall *in vivo* effect of a drug is dependant on these properties, so it is unsurprising that stereoisomers often cause different responses in such systems. Different combinations of these properties can potentially lead to dramatically different biological consequences. For example, most commonly prescribed β-blockers are sold as racemates, but the anti-hypertensive effects of this drug class reside essentially with the *S*-enantiomer. The *R*-enantiomer in each case has considerably lower anti-hypertensive potency but, however, does not cause any major side effects [3]. The enantiomer with the highest potency for a particular pharmacological action has been termed the eutomer, the enantiomer that is less potent has been termed the distomer and the ratio between the two has been termed the eudismic ratio [4]. In cases where the distomer has considerably lower potency for a particular pharmacological action, Ariëns referred to this as 'stereochemical ballast' if it is present in pharmaceutical products [4]. Another situation encountered *in vivo* is where the distomer exhibits undesirable side effects. For example, the anti-arthritic drug penicillamine is marketed as the pure *S*-enantiomer, as the distomer (*R*-enantiomer) is a potent toxin [3].

There are also known cases of drug enantiomers that possess completely different therapeutic properties. The (+)-2*R*,3*S*-stereoisomer of propoxyphene (dextropropoxyphene) is marketed as an analgesic agent, whereas its enantiomer (−)-(2*S*,3*R*)-propoxyphene (levopropoxyphene) is available as an effective antitussive agent [3]. The enantiomers of some chiral drugs are known to possess essentially identical qualitative and quantitative pharmacological activities, for example, the antihistamine promethazine, which is marketed as a racemate [5].

Drugs in the latter category are, however, in the minority and it is rare that living systems are not able to distinguish between the stereoisomeric forms of a chiral pharmaceutical. A large amount of literature is available on the subject of stereoselectivity of drugs *in vivo* but further discussion is outside the scope of the chapter, and the reader is encouraged to seek more specific references if required. A major factor contributing to the *in vivo* study of stereoselective drug processes is the recent, rapid advance in stereoselective analytical techniques available over the last 25 years. Such advances have enabled an examination of both new and existing chiral drugs that has also contributed to the increased interest in this area from the various drug regulatory authorities throughout the world. For regulating the development of new chiral drugs, a common approach has essentially been adopted by the authorities whereby the decision to develop a single stereoisomer or a racemic mixture must be scientifically justified [6]. Clinical studies must now be performed with individual stereoisomers whether developing a single isomer or the racemate. Development of new racemic drugs is not prohibited but the majority of new chiral drugs tend to be developed as single isomers. Recent developments in stereoselective synthesis have also contributed significantly to this trend. Sensitive stereoselective analytical methodology is an obvious prerequisite for monitoring clinical studies and for controlling the quality of the drug substance and drug product used.

In cases where a single stereoisomer is selected for development as a new drug substance, the International Conference on Harmonisation (ICH) Topic Q6A provides guidance on test procedures and acceptance criteria [7]. Control of stereoisomeric impurities in new drug substances and products should be considered in the same manner as for other achiral impurities. The guidance does, however, recognise that technical limitations of some stereospecific analytical methodologies may preclude the same limits from being applied. Where no suitable methods are available for the determination of stereoisomeric impurities, appropriate testing of starting materials or intermediates may be used as an assurance of control if justified. Stereoisomeric impurity levels registered in specifications should be qualified by clinical trials and safety studies using drug substance batches containing the impurities at levels such that appropriate exposure occurs [8]. With established single isomer drugs, specifications detailing limits for stereoisomeric impurities and test methods are recorded in relevant pharmacopoeias, some examples of which are discussed in later sections. The key to ensuring the stereoisomeric quality of chiral pharmaceuticals in development and on the market is the establishment of appropriate specifications supported by validated methodology. ICH topic Q2B outlines the validation requirements for analytical methods used to test new drug substances and products during registration for human use [9]. No differentiation is made between validation requirements for analytical techniques used to determine chiral or achiral drug quality.

Stereochemical impurities present in drug substances or drug products can arise from a number of different sources. Armstrong *et al*. performed an extensive study in the 1990s focusing on enantiomeric impurities in chiral synthons

used in stereoselective synthesis such as amino acids and terpenes, an example of which is presented in a later section [10–17]. The presence of unwanted stereoisomers in such materials does not, however, necessarily mean that the resulting drug substance/product will be contaminated and will depend on the downstream chemistry and purification processes used during manufacture. The establishment of suitable specifications for chiral raw materials is a requirement during the registration process for all new drug applications [18]. In addition, other chemicals used in stereoselective synthetic processes such as chiral auxiliaries and catalyst ligands may not be stereochemically pure and will potentially contribute to the overall stereochemical integrity of the resulting drug substance/product [10, 11, 19]. Stereochemical purity may also be compromised by isomerisation of raw materials or intermediates during chemical processing. Chiral drugs or their intermediates may be deliberately manufactured as racemates or stereoisomeric mixtures and subsequently resolved using crystallisation (preferential or diastereomeric), kinetic resolution (chemical or enzymatic) or chiral chromatography [3]. Such purifications may not provide total stereochemical purity, and processes obviously need to be monitored to ensure the required product quality. Isomerisation of stereoisomerically pure drug substance or drug product may also occur during storage depending on the chemical structure and storage conditions [20]. ICH Topic Q1A discusses requirements for stability testing of drugs during development [21].

The main emphasis of this chapter is on the various analytical tools now available to the analyst to determine trace-level stereoisomeric impurities in raw materials, intermediates and drug substance. In particular, it will focus on the measurement of enantiomeric impurities as opposed to diastereomeric impurities (a category that, by definition, includes *cis–trans* isomers), as the latter differ in terms of their physico-chemical properties and can often be measured using conventional achiral analytical techniques as described in Chapters 1 and 2. The main advantages and disadvantages of the various enantiospecific techniques are discussed and illustrated with examples from our laboratories and from published literature. The majority of the chapter is devoted to a discussion on separation techniques, in particular high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE), owing to their relative importance in this area.

3.2 Separation techniques: direct resolution

Although the majority of current pharmacopoeial methods describe the use of specific optical rotation ($[\alpha]$) for the determination of stereoisomer identity and/or content, separation techniques such as HPLC and CE are now the most frequently applied technologies during drug development and for new pharmaceutical products entering the market. Indeed, Supplementary Chapter K in Volume IV of the 2004 British Pharmacopoeia (BP) deals with this specific issue [22]. It states that in future when a monograph describes an enantiomer it will include both a test for identity ($[\alpha]$) and a test to control stereoisomeric purity

such as chiral chromatography. The last 25 years has seen a rapid rise in the availability of chiral stationary phases (CSPs) and chiral additives such that the development of direct separation methods for stereoisomeric purity determination is now often a relatively straightforward process. The direct approach refers to the use of CSPs or chiral additives to form transient diastereomeric complexes with analyte stereoisomers, thus facilitating physical separation [2]. One interesting point to note is the fact that there is actually no requirement for CSPs or chiral additives to be totally enantiomerically pure when using such approaches. The resolving power of the phase/additive is obviously not optimal under such conditions but the resolution achieved may still be useful.

3.2.1 HPLC using CSPs

HPLC CSPs are undoubtedly the most widely used separation media for stereoisomeric pharmaceutical molecules at the present time [23]. Pioneering work by Pirkle *et al*. in the late 1970s and early 1980s led to the introduction of the brush-type donor–acceptor phases [24–27]. Pirkle's initial CSP design was based on his successful use of chiral solvating agents in nuclear magnetic resonance (NMR) spectroscopy, in particular (−)-(*R*)-2,2,2-trifluoro-1-phenylethanol [28–32]. During the 1980s, a number of other CSPs were designed and commercialised, for example, the derivatised polysaccharide phases, protein-based CSPs and the bonded cyclodextrin (CD) phases. Wainer originally classified HPLC CSPs into five different types in 1987 [33], and for each class prediction of their suitability for separation was sometimes possible according to analyte structure. Since then, a plethora of additional CSPs have been commercialised, and to date more than 100 different phases are now available [34]. As yet, however, the search for a universal CSP for the separation of all different types of stereoisomers has been unsuccessful. Indeed, the current trend for HPLC chiral method development appears to be in the use of 'scatter-gun' chiral column screening, using automated column and solvent switching, with a number of publications proposing strategies for this type of approach [35–39]. An alternative approach is the use of a database such as the commercially available package ChirBase™ [40]. Such systems allow analysts to locate stereoisomeric separation methods for chiral compounds that have previously been examined. Chiral applications databases from ChirBase™ are available for HPLC, gas chromatography (GC), CE and thin-layer chromatography (TLC).

Irrespective of the mechanism of resolution, HPLC CSPs work by providing a chiral environment for analyte stereoisomers to interact with. Resolution relies upon the formation of reversible, transient diastereomers on the CSP that have different free energies of interaction and therefore stability. The stereoisomer forming the most stable diastereomer with the CSP will be the most retained and vice versa. Free energy differences are typically small in such systems but may be large enough to produce useable resolutions provided the column efficiency is sufficient [41]. If column efficiency is insufficient to allow complete separation of the stereoisomers, inaccurate integrations can result in erroneous

data especially when dealing with trace-level impurities [42]. Another potential problem with HPLC CSPs is the presence of additional chemical contaminants that may not be resolved from the stereoisomers of interest and interfere with their accurate integration. This section will examine some of the more popular CSPs commercially available to provide examples where such systems are used to measure and control trace-level stereoisomeric impurities in pharmaceuticals.

3.2.1.1 Derivatised polysaccharide CSPs

Derivatised polysaccharide CSPs originally developed in the 1980s [43, 44] and commercialised by Daicel Chemical Industries Ltd have become undoubtedly the most successful media for the separation of stereoisomers. The importance of this class of CSP should not be underestimated; an examination of the aforementioned HPLC ChirBase™ in 2002 indicated that polysaccharide-based CSPs were used in approximately 40% of all stereoisomeric separations listed [45]. The first derivatised polysaccharide CSP produced that possessed practically useful chiral-recognition abilities for a range of stereoisomers was microcrystalline cellulose triacetate [46]. The main drawbacks with this material, however, are its compressibility and relatively large irregular particle size [41]. In the late 1970s, Okamoto *et al*. developed a synthetic helical vinyl polymer that when adsorbed onto macroporous silica gel exhibited good chiral-recognition properties for stereoisomers containing aromatic groups [47]. The coating technique was then subsequently applied to the preparation of derivatised polysaccharide CSPs, with the use of a silica support ensuring resistance to compression and better control of the particle shape and size [43, 44]. This discovery has stimulated considerable research and development in the area of polysaccharide CSPs, leading to the commercialisation of a number of different CSPs.

The phases are derived from either cellulose or amylose derivatised with a number of different chemical groups through ester or carbamate linkages, which are then coated onto a silica support. The most successful of the phases contain phenylcarbamate, benzylcarbamate or benzoate groups. Four CSPs in particular have been shown to be extremely successful for the separation of a wide range of chiral analytes, namely CHIRALPAK® AD (amylose *tris* (3,5-dimethylphenylcarbamate)), CHIRALPAK® AS (amylose *tris* [(*S*)-α-methylbenzylcarbamate]), CHIRALCEL® OD (cellulose *tris* (3,5-dimethylphenylcarbamate)) and CHIRALCEL® OJ (cellulose *tris* (4-methylbenzoate)) (Figure 3.1) [23]. These types of CSPs are generally used with normal-phase solvents such as hexane and an alcohol modifier although they can also be used with purely polar organic solvents such as methanol and acetonitrile. Reversed-phase and, more recently, bonded versions of some of the polysaccharide CSPs have also now become available. The phases can also be used in supercritical fluid chromatography (SFC). There appears to be a vast amount of literature relating to the use of these phases for the separation of stereoisomers, the majority of successful applications being achieved with normal-phase solvents [23]. Chiral recognition on derivatised polysaccharide CSPs is usually attributed to shape selective inclusion into

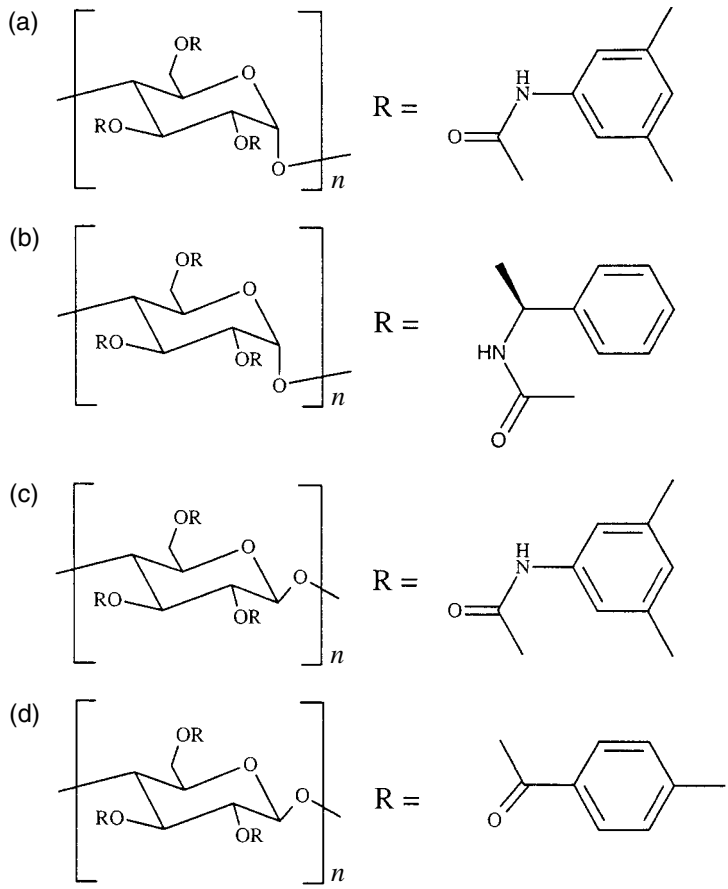

Figure 3.1 Chemical structures of the current most successfully employed derivatised polysaccharide CSPs. (a) CHIRALPAK® AD: Amylose *tris* (3,5-dimethylphenylcarbamate) coated onto a silica support, (b) CHIRALPAK® AS: Amylose *tris* [(*S*)-α-methylbenzylcarbamate] coated onto a silica support, (c) CHIRALCEL® OD: Cellulose *tris* (3,5-dimethylphenylcarbamate) coated onto a silica support, (d) CHIRALCEL® OJ: Cellulose *tris* (4-methylbenzoate) coated onto a silica support.

chiral grooves present in the helical polymers and subsequent interactions of the analytes with the derivative moieties present through hydrogen bonding, dipole interactions, π–π interactions and van der Waals forces, which will be influenced by the mobile phase employed [34]. When used with normal-phase solvents, the alcohol modifier can sometimes have a pronounced effect on resolution. Reversal of chromatographic elution order by simply changing between different alcohols has even been reported [48].

Figure 3.2 shows the separation of the enantiomers of a bicylic thiolactone that is used as a chiral contributory raw material for the production of a drug candidate currently in development in our laboratories. This particular example is

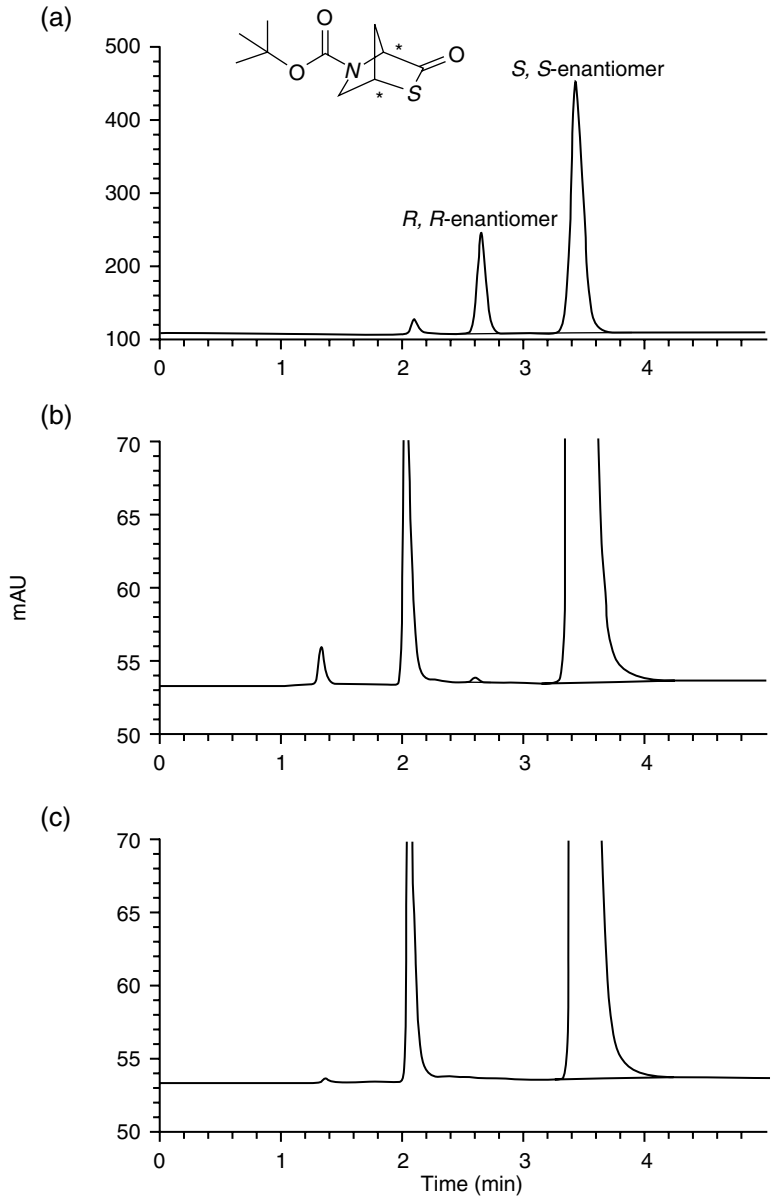

Figure 3.2 Resolution of the enantiomers of *t*-butyl-3-oxo-2-thia-5-azabicyclo[2.2.1]heptane-5-carboxylate using a CHIRALPAK® AD CSP. (a) *S*,*S*-Enantiomer standard artificially enriched with 30%w/w *R*,*R*-enantiomer, (b) *S*,*S*-enantiomer standard artificially enriched with 0.05%w/w *R*,*R*-enantiomer and (c) chirally pure *S*,*S*-enantiomer. (Conditions: CHIRALPAK® AD-H 15 cm × 0.46 cm i.d.; mobile phase: methanol at 1 ml/min; detection: UV at 210 nm; column temperature: 15°C; sample preparation: 0.4 mg/ml in methanol; injection volume: 10 µl.)

interesting for a number of reasons. The method was developed quickly and efficiently using a screening procedure involving automated column and solvent switching as discussed previously. The screen was run overnight and produced a usable chiral resolution without any further method development. CHIRALPAK® AD was chosen as the most suitable CSP, using a polar organic solvent (methanol) as the mobile phase without additional modifiers. Although the presence of aromatic groups in analytes can enhance chiral resolution on derivatised polysaccharide CSPs, they are by no means essential. One very important property of this class of CSPs is their usefulness for chiral resolution of aliphatic compounds [41]. The absence of an aromatic moiety did, however, also reduce the ultraviolet (UV) molar extinction coefficient in comparison with aromatic derivatives, requiring the use of relatively high column loading and UV detection at a relatively low wavelength (210 nm) to improve sensitivity. As an example, chiral-phase chromatograms of authentic standards and a representative batch of the raw material are shown in Figure 3.2. Under the conditions employed, the limit of detection for the undesired enantiomer was less than 0.1% area. The method also resolved some additional chemical impurities present in the batch from the analyte enantiomers, with an overall analysis time of less than 5 min. The method has been used to determine the enantiomeric purity of the raw material before its use in the manufacture of a chiral drug substance for clinical trials, and the batch data generated has been used to establish a provisional buying specification.

Monographs in the 2004 British Pharmacopoeia (BP) that contain HPLC methods employing CSPs to measure and control stereoisomeric drug substance purity also exemplify the relative importance and utility of derivatised polysaccharide CSPs [22]. Although at present there are only seven such monographs, five of the methods listed employ derivatised polysaccharide CSPs for stereoisomer resolution, mainly in the presence of normal-phase solvents. The methods specified to determine enantiomeric impurity levels in levodropropizine, timolol maleate, selegiline hydrochloride, dexchlorpheniramine maleate and oxaliplatin drug substances all employ derivatised polysaccharide CSPs, with pharmacopoeial limits set between 0.1 and 2.0% for the undesired enantiomer, depending on the product [22]. In addition, both the CHIRALCEL® OD and CHIRALPAK® AD CSPs are listed in the current United States Pharmacopoeia (USP) chromatographic reagent section as HPLC packings L40 and L51, respectively [49].

3.2.1.2 Macrocyclic antibiotic CSPs

The use of macrocyclic antibiotics as chiral selectors for HPLC was first proposed by Armstrong *et al*. [50] in 1994. The most successful of the CSPs are based on the glycopeptide antibiotics vancomycin, teicoplanin and ristocetin A and are commercially available through Advanced Separation Technologies Inc. (Astec Inc.) as Chirobiotic V™ , Chirobiotic T™ and Chirobiotic R™ , respectively. More recently, a number of other derivatives of these antibiotics have also been developed offering different stereoselectivities. A comprehensive handbook is now available from Astec Inc. [51] alongside a number of recent review articles

in the literature providing details on method-development strategies and pharmaceutical applications of this important class of CSP [52–55]. Structurally they consist of an aglycone core of three or four fused macrocyclic rings composed of amino acids and substituted phenols, which form a characteristic shallow 'basket' shape for inclusion. Different sugar moieties are attached to the aglycone basket depending on the antibiotic type, which are all free to rotate and therefore assume a number of different orientations [52]. All three glycopeptides contain multiple stereogeneic centres and are bonded to a silica support through multiple ether linkages [52]. Stereoselectivity with the Chirobiotic™ CSPs depends upon a number of potential interactions occurring such as inclusion complexation, π–π interactions, hydrogen bonding, dipole stacking, steric repulsion and ionic interactions. The relative importance of the various potential interactions is dependant on the structure of the glycopeptide and analyte and the chromatographic mode [50]. The shallow 'basket' region, which is assumed to play a role in inclusion complexation, is thought to generate weaker binding energies relative to phases such as the bonded cyclodextrin CSPs under similar mobile-phase conditions, leading to faster kinetics for the formation and breakdown of complexes, facilitating rapid, efficient separations [51].

An important feature of the Chirobiotic CSPs is their ability to be used successfully with a wide range of mobile-phase solvents spanning the entire polarity range including halogenated solvents. The reversed-phase mode tends to yield the highest percentage of successful stereoselective resolutions. They offer broad-spectrum applicability but are particularly useful for the separation of amino acids and carboxylic acids generally [34]. The Chirobiotic CSPs exhibit good mechanical and chemical stability and possess loading capacities sufficient for use in preparative chromatography [51]. The water-soluble parent glycopeptide antibiotics have also been used successfully as chiral selectors in CE [52]. As the Chirobiotic CSPs offer broad-spectrum stereoselectivity and a relatively high success rate and can be used with a variety of mobile-phase solvents, they are now often used in screening systems along with polysaccharide CSPs [38, 39]. One particular application of interest is the use of different, relatively short (10 cm) Chirobiotic columns connected in series for screening purposes, an approach that may be beneficial in terms of the speed of method development [56].

3.2.1.3 Pirkle-type CSPs

The Pirkle-type CSPs consist of low molecular weight amino acid derivatives possessing aromatic moieties that can associate with aromatic analytes through π–π donor–acceptor interactions. The associations may be stabilised through additional interactions such as hydrogen bonding, depending on the structure of the analyte. The phases are covalently bonded to silica supports, are chemically stable with universal mobile-phase compatibility and exhibit fast mass-transfer kinetics [34]. As mentioned previously, the concept for Pirkle-type CSPs arose from the successful use of chiral solvating agents (CSAs) to induce non-equivalence in the NMR spectra of selected chiral analytes for the determination of their enantiomeric composition [28–32]. The success of the studies eventually

led to the design of the first brush-type donor–acceptor phase whereby one of the π-donor CSAs, (−)-(*R*)-2,2,2-trifluoro-1-(9-anthryl)ethanol (TFAE or Pirkle's alcohol) was covalently bound to a silica support and used for the chiral resolution of a number of π-acceptor racemic *N*-(3,5-dinitrobenzoyl) amino acid derivatives [24]. The reciprocity concept was then applied to the design of the next CSP whereby (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine was employed as the covalently bonded π-acceptor phase and used for the enantiomeric resolution of racemic 2,2,2-trifluoro-1-(9-anthryl)ethanol and other related π-donor chiral fluoroalcohols [25].

A number of *N*-(3,5-dinitrobenzoyl) amino acid CSPs were subsequently developed, with phenylglycine and leucine derivatives being commercialised by Regis Technologies Inc. A series of phases have since been developed by Pirkle's group and commercialised by Regis Technologies Inc., including a naphthylleucine π-donor phase and combined π-donor/π-acceptor phases such as the Whelk-O® CSPs. A comprehensive applications and method-development handbook is now available from Regis Technologies Inc. [57]. The Pirkle-type class of CSPs is, however, not patent protected to the same degree as some other classes of CSPs, and a number of other research groups have developed alternative brush-type donor–acceptor phases and successfully commercialised them in conjunction with HPLC phase manufacturers [23, 58]. Bonded *N*-(3,5-dinitrobenzoyl)phenylglycine CSPs are listed in the current USP chromatographic reagent section as HPLC packing L36 [49].

The CSPs are usually used in conjunction with normal-phase solvents such as hexane with an aliphatic alcohol modifier in order to maximise any potential π–π and hydrogen bond interactions although a wide polarity range of solvents can be used on all the bonded Pirkle-type CSPs. The enantiomeric resolution of a large number of chiral aromatic pharmaceutical compounds has been achieved using this class of CSP including β-blockers, non-steroidal anti-inflammatory drugs (NSAIDs) and benzodiazepines. Analytes without aromatic groups can be derivatised such that π–π interactions are possible. Prediction of enantiomeric elution order is also sometimes possible with the Pirkle-type CSPs, as the mechanisms are relatively well understood and the selector acts independently allowing interactions to be modelled [34]. Also of interest is the fact that with the exception of the naphthylleucine π-donor phase, all the Pirkle-type CSPs commercially available through Regis Technologies Inc. are manufactured in both enantiomeric forms [57]. The ability to invert the chromatographic elution when measuring trace-level stereoisomeric impurities, to ensure that the minor component present elutes before the major component, can be beneficial in ensuring accurate integration of the resulting chromatograms [42]. The Whelk-O® 1 CSP has the broadest applicability of the Pirkle-type phases in part due to the presence of both π-acceptor dinitrobenzoyl and π-donor naphthyl moieties. Although originally developed for the resolution of the enantiomers of naproxen, it has been found to be useful for the enantiomeric resolution of many chiral pharmaceutical compounds containing an aromatic system and an H-bond acceptor group in close proximity to the chiral centre [2]. The CSP is available in both *R,R* and

S,*S*-versions to allow reversal of chromatographic elution order if required. The basic structure of the Whelk-O® 1 CSP is shown in Figure 3.3.

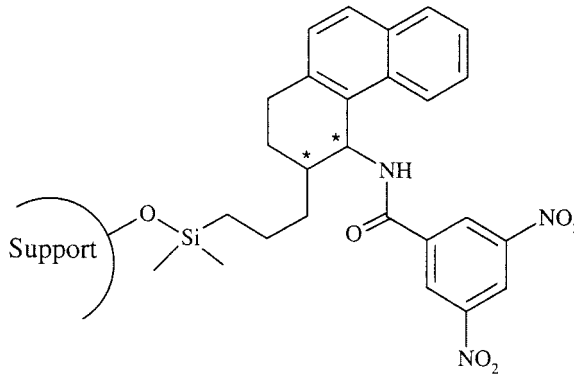

Figure 3.3 Chemical structure of the Whelk-O® 1 CSP.

3.2.1.4 Cyclodextrin phases

Cyclodextrins (CDs) are natural macrocyclic polymers of D-glucose units connected by α-(1,4) glycosidic linkages. The most widely used CDs contain six, seven or eight glucose units and are referred to as α-CD, β-CD and γ-CD, respectively, and contain multiple stereogenic centres (Figure 3.4). The most stable configuration for such cyclic structures is that of a hollow conical frustum (truncated cone with the top sliced off parallel to the base) [59]. The smaller opening of the cone presents primary 6-position hydroxyl groups to the local environment, whereas the larger opening presents secondary 2- and 3-position hydroxyl groups. The interior of a CD molecule is relatively hydrophobic owing to the electron-rich environment, whereas the exterior is relatively hydrophilic owing to the presence of the hydroxyl groups, thus making native CDs water soluble. The CD with the broadest applicability for the separation of stereoisomers of pharmaceutical interest is undoubtedly β-CD owing to its cavity diameter and general availability. In terms of their use as chiral selectors for analytical scale chromatography, CDs were initially employed as chiral mobile-phase additives for TLC and HPLC [41]. The first commercially viable bonded phases were produced in 1984 by Armstrong and DeMond [60], which were subsequently developed by Astec Inc. into the CYCLOBOND™ series of CSPs [61]. As with the Pirkle-type phases, other HPLC phase manufacturers have also developed bonded Cyclodextrin-based CSPs for stereoisomeric separations [58]. The 6-position primary hydroxyls are used to link the CDs to the surface of the support [61]. A number of native and derivatised bonded CD phases are now commercially available with a range of physical and chemical properties designed to extend the separation capability of this class of CSP. Selective derivatisation of CD molecules is possible at the 2- and 3-position secondary hydroxyl groups around the

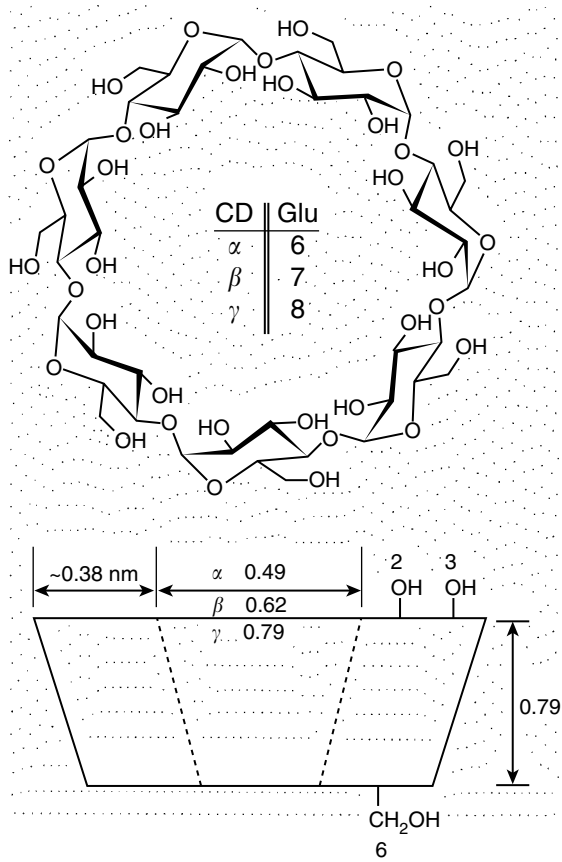

Figure 3.4 Chemical structures of native α-, β- and γ-cyclodextrins. Reproduced with permission from Dr J. Jindrich, Department of Organic Chemistry, Charles University, Prague, Czech Republic.

larger opening. The most useful of the CYCLOBOND™ series of CSPs to date are the hydroxypropyl- and naphthylethyl carbamate-β-CD derivatives [61]. Bonded β-CD CSPs are listed in the current USP chromatographic reagent section as HPLC packing L45 [49].

Bonded CD-type CSPs can be used in conjunction with a wide polarity range of mobile-phase solvents, although they were initially used almost exclusively in the reversed-phase mode where inclusion complexation is the main retention mechanism. Kinetics of inclusion complexation are relatively fast in this mode, generally leading to good chromatographic efficiency [23]. Any additional electrostatic interactions such as hydrogen bonding and the steric character of both the host and guest molecules can also affect the stability of the CD–analyte complexes. Buffer ionic strength and pH may also be effective parameters in controlling the strength of interactions [34]. The presence of an aromatic ring is

generally required for inclusion complexation, with stereoselective interactions often occurring where molecules possess both aromatic and hydrogen bonding moieties in close proximity to the stereogenic centre [34]. Owing to the mechanism of separation, minor differences in analyte structure can sometimes lead to major differences in chromatographic stereoseparation [23]. A number of chiral molecules of pharmaceutical interest have been resolved using bonded CD CSPs in reversed-phase mode including β-blockers, anti-depressants and NSAIDs [62, 63].

More recently, another approach that has been used very successfully with this class of CSP is the use of polar organic solvents such as methanol and/or acetonitrile in combination with small quantities of acetic acid and triethylamine [64]. The mobile phase contains no water and the CD–analyte complexes are thought to occur mainly through interactions with the secondary hydroxyl groups present on native CDs or with functional group residues on derivatised CDs. Inclusion complexation is not thought to contribute significantly under such conditions [64]. Indeed, this type of approach has been used successfully for stereoisomeric separations of non-aromatic molecules on bonded CD CSPs [61]. It is also claimed that the polar organic mode often results in more efficient chromatography that is quicker and more reproducible, with higher sample capacity when used as a preparative medium [61]. Chiral analytes possessing multiple hydrogen bonding moieties and bulky groups near the stereogenic centre can often be resolved in this mode [34].

An interesting example of the use of this type of CSP in the polar organic mode is presented in Figure 3.5. Zukowski used a CYCLOBOND™ I *2000*RSP phase (bonded *R,S*-hydroxypropyl-β-CD) to determine the stereoisomeric purity of commercially available Jacobsen's catalyst used for the chiral epoxidation of an olefin-containing pharmaceutical intermediate [19]. Such reagents have been used successfully to generate pharmaceutical products of high enantiomeric purity. However, when Jacobsen's catalyst is used to generate a chiral epoxide of a chiral olefin, kinetic differences in the rates of epoxidation may occur, which may magnify (or diminish) the amount of unwanted stereoisomeric product resulting from the chiral catalyst impurity [19]. Figure 3.5 shows the determination of the enantiomeric purity of Jacobsen's catalyst purchased from one particular supplier. Optical rotation (OR) was used by the manufacturer to confirm the stereochemical identity of the catalyst, but this technique is generally unsuitable for the measurement of trace-level stereochemical impurities. The chiral chromatography method developed enabled the measurement of the stereochemical purity of Jacobsen's catalyst down to low levels (<0.1% by area). Such methodology can be extremely useful for controlling the stereoisomeric quality of asymmetric reagents used in the manufacture of chiral pharmaceuticals.

3.2.1.5 Protein phases

Proteins are polymers of L-amino acids containing numerous chiral centres, each possessing a characteristic three-dimensional shape, or conformation. Most globular proteins such as albumins undergo extensive folding of the chains into

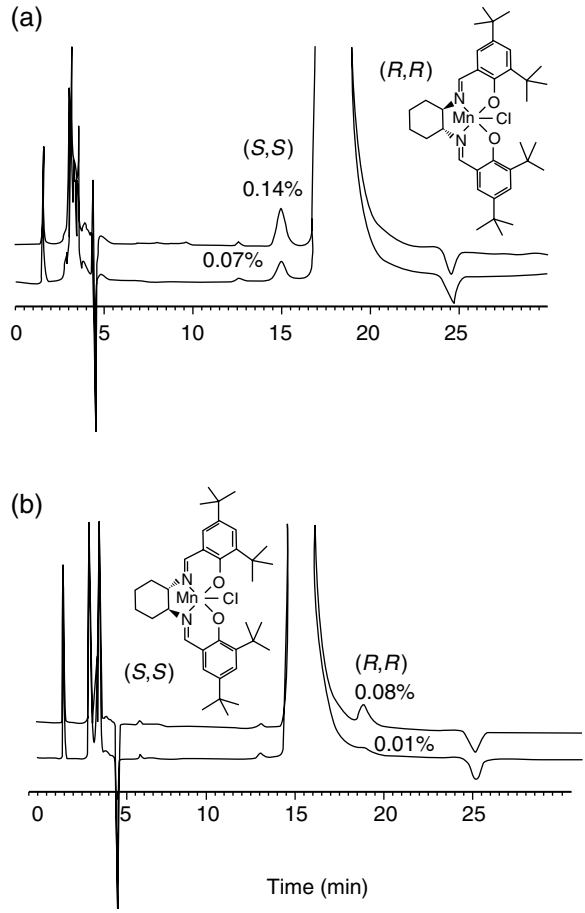

Figure 3.5 Measurement of the chiral purity of commercially available Jacobson's catalyst using a cyclodextrin-based CSP. (a) Lower trace: *R*,*R*-enantiomer product; upper trace: *R*,*R*-enantiomer product artificially enriched with *S*,*S*-enantiomer and (b) lower trace: *S*,*S*-enantiomer product; upper trace: *S*,*S*-enantiomer product artificially enriched with *R*,*R*-enantiomer. (Conditions: CYCLOBOND™ I *2000*RSP 25 cm × 0.46 cm i.d.; mobile phase: acetonitrile: triethylamine: glacial acetic acid [1000: 0.5: 2.5, v/v]; flow rate: 1 ml/min; temperature: ambient; detection: UV at 240 nm; sample preparation: 1 mg/ml in acetonitrile; injection volume: 10 μl). Reprinted from [19], copyright 1998, with permission of Wiley-Liss, Inc., a subsidiary of John Wiley and Sons, Inc.

complex three-dimensional shapes that are referred to as tertiary structures. Such proteins are generally water soluble and assume spherical or elliptical shapes in aqueous media. Certain proteins are known to stereoselectively bind small chiral molecules in solution and, as such, are obvious candidates for use as chiral selectors in HPLC [65, 66]. Stewart and Doherty recognised this potential in 1973 and developed the first protein-based CSP by bonding bovine serum albumin (BSA) to agarose beads, which they used in a packed column to successfully separate

the enantiomers of tryptophan [67]. Since then, a number of protein-based HPLC CSPs have been developed that are mostly derived from serum proteins bonded to silica supports. The range includes α_1-acid glycoprotein (α_1-AGP) (CHIRAL-AGP, ChromTech Ltd.), BSA (Resolvosil BSA-7, Macherey-Nagel; ULTRON ES-BSA, Shinwa Chemical Industries Ltd.), human serum albumin (CHIRAL-HSA, ChromTec Ltd.), ovomucoid (ULTRON ES-OVM, Shinwa Chemical Industries Ltd.) and cellobiohydrolase (CHIRAL-CBH, ChromTech Ltd.). Both the α_1-AGP and ovomucoid-based CSPs are listed in the USP chromatographic reagent section as HPLC packings L41 and L57, respectively [49].

Protein-based CSPs are used exclusively in the reversed-phase mode, with retention being influenced through variation of the ionic composition of the buffer (type, pH and molarity), temperature and organic modifier [34]. The mechanism of retention with this class of CSP is multi-modal owing to the complex nature of the selectors, and thus stereoselectivity is difficult to predict [58]. Complexation is thought to be primarily due to hydrophobic interactions in a lipophilic region inside the protein. Additional electrostatic and steric interactions will also be involved depending on the protein type and analyte structure [34]. They offer broad-spectrum applicability and have been used to resolve the stereoisomers of large number of neutral, acidic and basic analytes of pharmaceutical interest. α_1-AGP and BSA phases were the first to be introduced and are currently the most commonly used of the bonded protein CSPs [23]. Capacities do, however, tend to be relatively low with this class of CSP owing to the low surface coverage of proteins on the silica support, limiting their use to analytical scale separations [58]. Chromatographic efficiency is also generally lower with protein-based CSPs owing to slow mass-transfer kinetics, which is related in part to the size of the selectors [23].

Figure 3.6 shows the application of an α_1-AGP CSP for the determination of the enantiomeric purity of the *S*-enantiomer of omeprazole (esomeprazole), the Active Pharmaceutical Ingredient (API) present in AstraZeneca's Nexium®. This product was the first PPI to show a significant clinical advantage over Losec®, AstraZeneca's original racemic product and the previous 'gold standard' of treatment in the management of acid-related disorders. The single isomer API is manufactured using an innovative asymmetric sulphide oxidation generating material of high enantiomeric purity [68, 69]. The chromatogram illustrates the high stereoselectivity of this CSP towards the enantiomers of omeprazole, facilitating the development and validation of a fast, sensitive direct method to monitor and control the enantiomeric purity of the single isomer API [70].

3.2.2 HPLC using chiral mobile-phase additives

The development of a plethora of HPLC CSPs in the 1980s and 1990s has, to a large extent, made the use of chiral mobile-phase additives (CMPAs) redundant in most modern pharmaceutical analytical laboratories [23]. Before this period, chiral selectors were used routinely as additives in HPLC, but are now only used for a small number of specific applications [23]. CMPAs are used to form

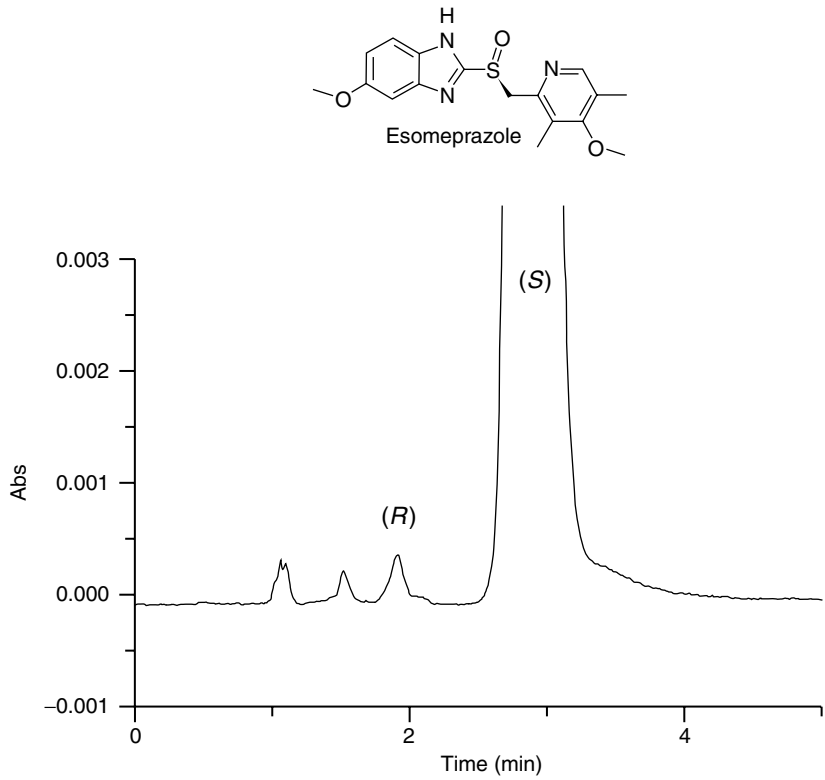

Figure 3.6 Resolution of the enantiomers of omeprazole using a protein-derived CSP. The chromatogram shows the analysis of esomeprazole API artificially enriched with 0.1% w/w of the *R*-enantiomer. (Conditions: α_1-AGP 10 cm × 0.4 cm i.d.; mobile phase: sodium phosphate [pH 6.0, 60 mM] : acetonitrile [85 : 15, v/v]; flow rate: 1 ml/min; detection: UV at 302 nm; column temperature: ambient; sample preparation: 0.02 mg/ml in sodium phosphate [pH 11.0, 18 mM] : methanol [98 : 2, v/v]; injection volume: 20 μl.)

transient diastereomeric complexes in solution that subsequently interact differentially with an achiral HPLC stationary phase to afford stereoisomeric resolutions [71]. Examples of CMPAs include chiral host molecules such as CDs [72–74], ion-pairing reagents such as (+)-10-camphorsulphonic acid [75, 76] and ligand exchange systems such as *N*-alkyl-L-hydroxyproline in combination with copper (II) acetate [77]. The main advantages of this approach to stereoisomeric resolution by HPLC include the fact that relatively inexpensive achiral HPLC phases may be used and a wide range of CMPAs are available [78]. The main disadvantages, however, probably outweigh the advantages and are related to the high consumption of potentially costly chiral selectors (if recycling is not an option) [23], high UV background noise if the selector is UV absorbing [23] and that the analyte character may be modified such that retention may be difficult to predict. Of interest to note at this point is that many of the CMPAs

originally used in HPLC are now used routinely and very successfully as chiral selectors in CE and are discussed in the next section.

3.2.3 Capillary electrophoresis using chiral selectors

One of the most important advances in stereoisomer analysis since the introduction of CSPs for HPLC was the development of CE as an analytical technique in the early 1980s by Jorgenson and Lukacs [79–83]. CE is a microanalytical technique and is an instrumental approach to electrophoresis [83] whereby high voltages (up to 30 kV) are applied across buffer-filled fused-silica capillaries, causing ions present to migrate depending on their size, charge and shape. The use of high voltages facilitates rapid analysis, thus minimising band broadening due to diffusion but does however generate heat within the system. The capillary format is required to ensure effective heat dissipation and prevent joule heating, which can lead to loss of efficiency [79]. An additional driving force that may also be present in CE is electroosmotic flow (EOF), which is a consequence of the surface charge on the interior wall of the capillary. With a charged capillary surface, a solution double layer of oppositely charged ions will accumulate and, on application of an electric field, the more diffuse outer ions will migrate towards the oppositely charged electrode and draw the bulk solution with them. As most CE is performed in fused-silica capillaries, the ionisation state of the silanol groups present on the inner wall will have a major influence on the strength of the resulting EOF. At high pH values (>7), silanol groups are generally de-protonated, resulting in high cathodic EOF. However, at low pH values (<4), silanol protonation occurs, neutralising the surface and suppressing the EOF. The force and direction of EOF will influence the amount of time analytes remain in the capillary by superposition onto analyte mobility. One major advantage of EOF over pressure-driven systems such as HPLC is the resulting plug-like flow with a flat velocity profile across the capillary diameter, producing less distortion of the analyte zones. However, it is worth noting that where robust CE methods are required, EOF may need to be carefully controlled as it is dependent on a number of different factors and can be highly variable.

Analytes in solution are introduced onto capillaries using either pressure or voltage differences applied across the capillary. Detection is usually performed on-capillary using UV-visible absorption detectors although a number of alternative detector types are now available, depending on the application [84]. Neutral species can also be separated by CE using charged selectors to complex the analyte molecules and induce electrophoretic mobility. This important sub-technique of CE was introduced by Terabe *et al*. [85] in 1984 and is referred to as capillary electrokinetic chromatography (CEKC). The main advantage of CE over other separation techniques is the extremely high peak efficiency achievable, often producing useable resolutions from small selectivity differences. Other advantages include the fact that method development and optimisation is often a very straightforward process and the relatively low running costs of CE due, in part, to the microanalytical nature of the technique [78]. The main limitation of CE

with respect to trace analysis is sensitivity when using on-capillary UV-visible absorption detection due to the relatively short light pathlength available [23]. In situations where sensitivity is a limiting factor, a number of approaches can be used to reduce limits of detection such that practically useful methods can be developed and include the use of low UV wavelengths (<220 nm), larger diameter capillaries, extended light path capillaries, increased loading, use of on-line sample pre-concentration techniques such as stacking or alternative detectors [23]. Since its introduction, CE has found its niche in certain areas of analysis and is used routinely in many pharmaceutical laboratories for stereoisomer analysis. The success of CE as a separation technique in such applications is reflected in the sheer number of books, reviews and research papers dedicated to this subject [84, 86]. Although there are some examples of chiral CE methods in recent drug regulatory submissions and pharmacopoeial monographs [87–90], as a technique it is more likely to be used in the development phase of pharmaceutical R&D as opposed to a quality control tool in pharmaceutical manufacturing [23]. However, chiral CE can be used very effectively as an alternative to chiral HPLC if method development is unsuccessful or if validation procedures highlight any method deficiencies [87, 88].

As enantiomers have identical electrophoretic mobilities in free solution (capillary zone electrophoresis [CZE]), the use of a chiral selector is required for separation [84]. The first reported enantiomeric separations in CE employed ligand exchange systems for the resolution of racemic dansylated amino acids [91] and, as stated in the previous section, many chiral selectors originally used in HPLC are now used to much greater effect in CE including CDs, crown ethers, polysaccharides and proteins. The correct name for CE in the presence of chiral selectors (both neutral and charged) is chiral CEKC, as separation depends upon both kinetic and distributive (chromatographic) mechanisms [84]. For a chiral selector to be of use in CE it must be able to interact stereoselectively to form transient diastereomeric complexes with analyte molecules, the complexes formed must possess different mobilities to the uncomplexed analyte molecules and the exchange between free and complexed forms must be sufficiently rapid to minimise band broadening [84]. Stereoisomeric resolutions of neutral analytes are therefore only achievable in the presence of charged chiral selectors.

Without doubt the most important class of chiral selectors in CE are the CDs, mainly owing to their water solubility, stability, UV transparency, commercial availability and low cost [84]. They are available in a range of sizes and chemistries and generally produce efficient separations in CE owing to fast kinetics of complexation [86]. The mechanisms of interaction of CDs in solution with analyte molecules are described in Section 3.2.3. A number of chiral CE screening strategies are now available in the literature, the majority of which suggest the use of an array of different CDs using an automated sequence, with generally high success rates reported [92–101]. Figure 3.7 shows the application of CE in the presence of dimethyl-β-CD for the determination of the enantiomeric purity of SB-214857-A, a single isomer API previously in development as an antithrombotic agent [87]. In this particular example, chiral CE was investigated

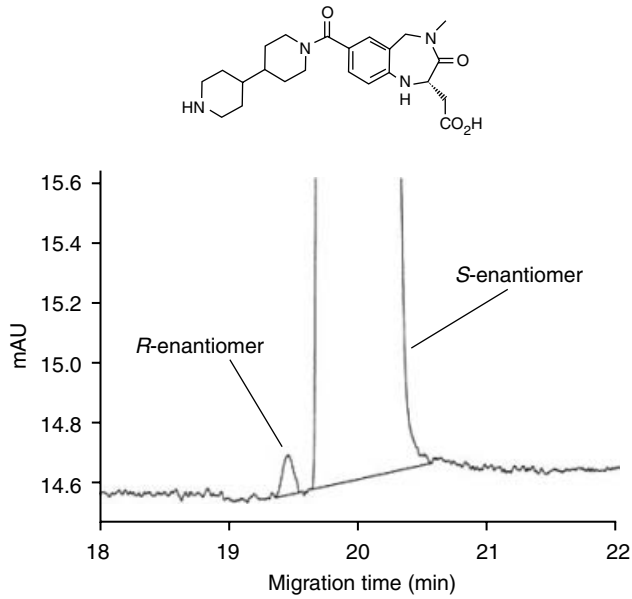

Figure 3.7 Determination of the enantiomeric purity of SB-214857 API using CD-modified CE. Distomer content measured at 0.06% by area. (Conditions: plain fused silica capillary, 50 cm effective length, 57 cm total length, 75 μm i.d.; buffer: lithium phosphate [pH 3.0, 100 mM] containing 0.05% (w/v) hydroxyethylcellulose and 1.5 mM dimethyl-β-CD; voltage: 30 kV; temperature: 20°C; detection: UV at 200 nm; sample preparation: 0.2 mg/ml in water; sample introduction: 5 s at 35 mbar, capillary inlet at anode.)

as an alternative to chiral HPLC owing to significant column batch-to-batch variability encountered during validation. An automated screen was performed using an array of different β-CD derivatives, with dimethyl-β-CD producing the most promising separation. Further optimisation and validation lead to a sensitive, robust CE method capable of detecting and quantifying the distomer down to low levels (Figure 3.7). Detection sensitivity was improved during method development by use of a low UV wavelength for detection (200 nm) and a relatively wide bore capillary (75 μm i.d.). Chiral CE was chosen as the primary analytical technique for the determination of the enantiomeric purity of SB-214857-A API and was included in regulatory submissions, and the technology was successfully transferred to a primary manufacturing facility. Figure 3.8 shows the analytical effect of using dimethyl-β-CD purchased from three different suppliers, resulting in only minor differences in migration times and resolutions. There are, however, literature examples where derivatised CD batch variations have resulted in significant analytical differences, and such effects should always be considered and investigated during validation exercises [102].

Figure 3.9 shows the application of chiral CE for the determination of the enantiomeric purity of SB-240093, a pharmaceutical intermediate used in the

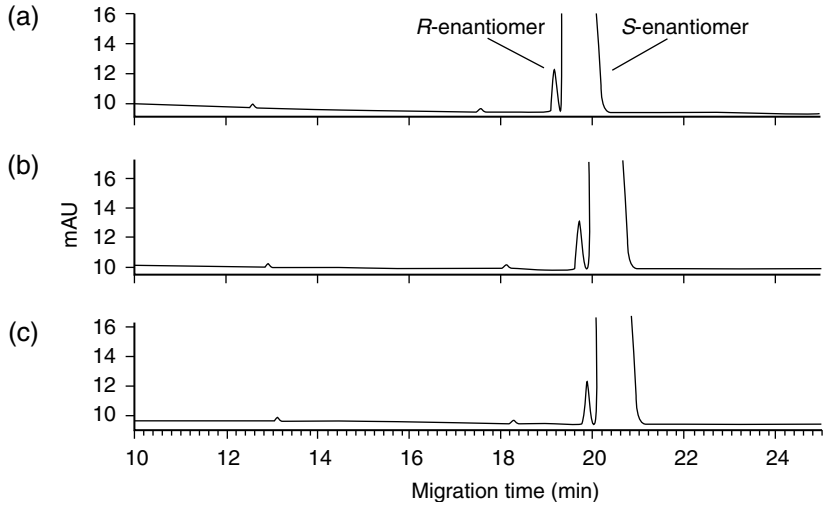

Figure 3.8 Example electropherograms of the SB-214857 chiral system suitability standard resolved using dimethyl-β-CD from different suppliers: (a) Aldrich product, (b) Wacker product and (c) Technicol product. (Conditions: as in Figure 3.7.) Reprinted from [87], copyright 2000, with permission from Elsevier.

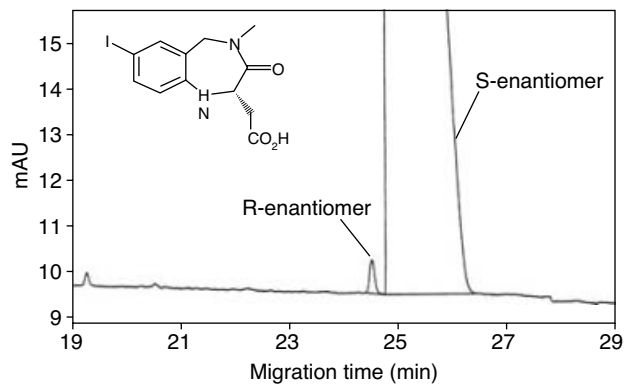

Figure 3.9 Measurement of the enantiomeric purity of the pharmaceutical intermediate SB-240093 using CD-modified CE. The electropherogram shows the analysis of the chiral system suitability standard containing 0.5% w/w of the *R*-enantiomer. (Conditions: PVA-coated fused silica capillary, 50 cm effective length, 57 cm total length, 50 μm i.d.; buffer: sodium phosphate [pH 7.0, 100 mM] containing 1.75 mM dimethyl-β-CD; voltage: −30 kV [reversed polarity]; temperature: 20°C; detection: UV at 200 nm; sample preparation: 0.5 mg/ml in water : DMSO (95 : 5, v/v); sample introduction: 6 s at 35 mbar, capillary inlet at cathode.)

synthesis of SB-214857-A. The material was produced to high enantiomeric purity by kinetic resolution using a commercially available immobilised lipase enzyme. With this particular example, extensive chiral HPLC screening failed to produce a method for the determination of the enantiomeric purity of SB-240093; thus chiral CE was investigated as an alternative separation technique. As with

the previous example, an automated screen was performed using an array of different β-CD derivatives, but employing a different background electrolyte with a higher pH to deprotonate the carboxylic acid moiety (Figure 3.9). Under these conditions, the molecules were negatively charged and could be analysed using CE in the reversed polarity mode (capillary inlet at cathode) using a coated capillary to suppress the resulting EOF such that separation was not performed against the flow, which would have resulted in longer, less reproducible migration times. Dimethyl-β-CD proved again to be the selector of choice, and after optimisation and validation, a robust method with similar sensitivity to that achieved for the API was transferred to the same production facility as a quality control tool for stereoisomeric purity. With both methods, the distomer ((+)-*R*-enantiomer in each case) migrated before the eutomer, enabling accurate integration of trace-level amounts.

One particularly interesting development in CE over the last 15 years has been the introduction of charged CDs for chiral resolutions. Terabe *et al*. first advocated the use of charged CDs for achiral electrokinetic chromatography in 1985 [103]. Chiral separations using charged CDs were initially reported in 1989 [104], and since then a number of derivatives have become commercially available [105]. Sulphated CDs in particular are now used extensively in CE mainly owing to their anionic nature over the entire pH range accessible to CE [106] and their UV transparency, facilitating low wavelength UV detection to improve sensitivity for trace-level analysis [107]. Although charged selectors are an obvious requirement for the separation of neutral analytes in CE, the application of anionic CDs for the enantioresolution of cationic analytes has also proved to be extremely successful [86]. In situations where analyte molecules carry the opposite charge to that of the selector, mobility differences between bound and free analytes can be maximised owing to a counter migration mechanism, which does, however, require the use of multiply charged selectors [86]. In addition, complexes can form without the need for inclusion through ion-pairing interactions, potentially allowing resolution of non-aromatic stereoisomers. Furthermore, ion-pairing interactions can also occur in the presence of non-aqueous media, enabling the use of a wider polarity range of solvents to be employed for such applications [105].

3.2.4 Supercritical fluid chromatography using chiral stationary phases

A supercritical fluid is defined as gas with liquid-like densities in which the pressure and temperature are above the critical point. Such fluids have unique properties, possessing significant solvation strengths, whilst retaining many of the advantageous qualities of gases [108]. SFC can be performed using both open tubular capillary and packed-column formats, with the latter technique now predominating in pharmaceutical environments owing to mobile-phase limitations, low sample capacity and a lack of commercially available CSPs with the open tubular format [109]. With packed-column formats, supercritical fluids have several advantages over liquid mobile phases including reduced viscosity and higher diffusivity, permitting the use of higher linear velocities, thereby reducing

analysis times without compromising the separation efficiency [109]. In addition, the possibility of using higher linear velocities can reduce column equilibration times, facilitating rapid column screening for method development [109]. The most common fluid used in SFC is carbon dioxide mainly because of its low cost, low toxicity, available purity, ease of use and moderate critical point [110]. It is often used in combination with a more polar modifier such as methanol to increase the elution strength for polar analytes. Both isocratic and gradient elution modes are used routinely with SFC and, in addition, miscible acid and base modifiers can also be used where necessary to improve peak shape. However, as modifiers increase the temperature and pressure necessary to maintain supercritical conditions, it is thought that many such analyses are actually performed under subcritical conditions [108]. Modified (subcritical) carbon dioxide eluents possess higher diffusivity than pure solvent eluents and thus there are still considerable benefits to be gained when using such systems [108].

With packed-column chiral SFC, many phases originally designed for HPLC applications are employed as selectors because of their wide availability [111]. However, as supercritical carbon dioxide is comparable with pentane in terms of polarity and solvent strength [110], only certain HPLC phases are suitable as SFC CSPs, such as derivatised polysaccharides, bonded CDs, macrocyclic antibiotics and Pirkle-type phases [109]. The first reported application of packed-column SFC to enantiomeric separations was by Mourier *et al*. [112] in 1985, who employed a Pirkle-type phase for the resolution of the enantiomers of a series of chiral phosphine oxides. Since this initial application, the interest in chiral SFC has increased steadily with many reports suggesting improved separation efficiency with supercritical fluids in comparison with normal-phase eluents on a variety of different CSPs [108]. It is, however, worth noting that analyte–CSP interactions are not necessarily equivalent between supercritical fluids and normal-phase liquids and analyte-dependant selectivity differences have been reported when comparing the two modes [109]. Despite its many potential advantages over chiral HPLC, chiral SFC has not yet established itself as a major analytical technique in the pharmaceutical industry, which may in part be due to higher cost of SFC instrumentation and the fact that HPLC enjoys such a dominant position with respect to chiral analysis [108]. Recent technical advances in SFC instrument design have, however, improved overall performance and reliability such that there is likely to be considerable renewed interest in its use for stereoisomer separations in the near future.

Figure 3.10 exemplifies the application of packed-column SFC for the rapid determination of the enantiomeric purity of pharmaceutical materials. The method employed a CHIRALCEL OD® CSP with a carbon dioxide/methanol/isopropylamine mobile phase under isocratic conditions. A flow rate of 4 ml/min was used to generate a high linear velocity whilst maintaining an outlet pressure of 200 bar by use of a back-pressure regulator to ensure reproducible chromatography. Baseline resolution of the enantiomers of propanolol was achieved in approximately 3 min with an overall cycle time of 4 min. Detection was by UV

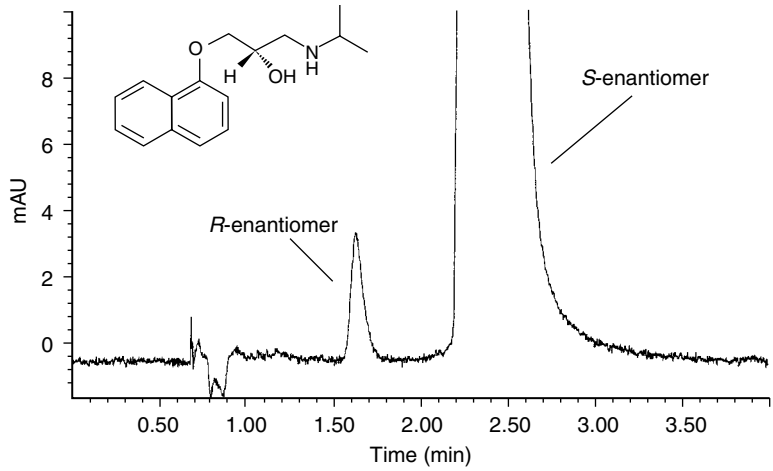

Figure 3.10 Determination of the enantiomeric purity of propranolol API using packed-column chiral SFC. Distomer content measured at 0.04% by area. (Conditions: CHIRALCEL® OD 25 cm × 0.46 cm i.d.; mobile phase: carbon dioxide: methanol containing 0.5% isopropylamine [70 : 30, v/v isocratic]; flow rate: 4 ml/min constant; outlet back-pressure regulator: 200 bar; detection: UV at 220 nm; column temperature: 30°C; injection volume: 10 µl.)

at 220 nm, which allowed the trace determination of the distomer (*R*-enantiomer) down to levels significantly below 0.1% by area. Nowadays, reproducible, fast and efficient performance is not uncommon with modern instrumentation such that it is worthwhile to seriously consider SFC as a viable alternative to HPLC for stereoisomeric resolutions.

3.2.5 Gas chromatography using chiral stationary phases

Gas chromatography (GC) was originally developed and commercialised in the 1950s, with the first reported use for enantiomeric separations by Gil-Av *et al*. in 1966 [113]. They demonstrated the possibility of direct stereoisomeric resolution of derivatised amino acids in GC using glass capillaries coated with a *N*-TFA-L-isoleucine lauryl ester, at a time when it was generally considered to be unlikely that such an approach would be successful. After almost 40 years of development, a series of capillary GC CSPs are now commercially available, which have been classified into three basic types depending on the mode of interaction [114]. As with all chromatographic techniques, separation depends upon partitioning of analyte molecules between a mobile and stationary phase. With GC, the mobile phase consists of a gas and the stationary phase consists of a selector either bonded or coated onto a support or directly onto the surface of a column. Most separations in GC are now performed using fused-silica capillary columns, although packed columns can still be purchased for specific applications.

In common with all capillary-based techniques, modern GC offers high-efficiency separations, allowing analyte resolution even with relatively low selectivity differences. Flame ionisation is now the most common form of detection used for organic analytes in GC and is universal for hydrocarbon-containing species. Although achiral GC is used widely in the pharmaceutical industry for the analysis of residual solvents and volatile analytes, its application to chiral analysis tends to be limited to chiral raw materials and smaller intermediates. As the mobile phase is a gas, only volatile analytes are applicable to analysis by GC, often precluding its use for the analysis of relatively large, complex API molecules. Moreover, analyte molecules also need to be thermally stable to the temperatures required to ensure volatilisation [114].

The first class of GC CSPs are based on the original concept from Gil-Av *et al*. and use amino acid derivatives as chiral selectors to form transient diastereomeric complexes with analyte molecules through hydrogen-bonding interactions. The commercially available CSP of this type, Chirasil-Val™ (Alltech Associates), is derived from valine chemically bonded to polysiloxanes and immobilised onto the inner wall of the fused-silica capillary. Both D- and L-forms of the CSP are available to allow peak switching to aid trace-level analysis if required [114]. Chirasil-Val™ CSPs are used routinely for the stereoisomeric resolutions of amino acids, although derivatisation is usually required to increase volatility and improve chiral recognition through additional hydrogen-bonding possibilities. A second class of GC CSPs was introduced in 1977 by Schurig *et al*. [115] on the basis of enantioselective interactions with chiral transition metal complexes. The technique is referred to as complexation GC and uses selectors consisting of chiral β-dicarbonyl ligands derived from natural products such as (+)-(1*R*)-camphor complexed to transition metals such as nickel or rhodium [116]. The mechanism of stereoselective retention is based on the formation of transient diastereomeric complexes through electron donation to the complexed metal, making this type of CSP suitable for the resolution of non-polar compounds with electron-rich centres such as cyclic olefins, ethers and ketones [41]. The biggest advance in chiral GC undoubtedly came with the introduction of CDs as chiral selectors in 1983 by Kościelski *et al*. [117]. Originally, packed columns were employed for enantiomeric resolutions, which were eventually superseded by the use of more efficient fused-silica capillaries coated with derivatised CDs dissolved in moderately polar siloxanes [114]. A number of CD-based GC CSPs are commercially available using this coating technique and, in addition, chemically bonded phases have also now been developed. Both the coated and bonded CD CSPs have good temperature stability and are applicable to a wide range of chiral analytes including non-aromatic apolar species [114]. Often, little functionality is required for chiral separation on this class of CSP, with no logical dependence on analyte structure [114]. Of interest to note is the fact that molecular inclusion may not be a prerequisite for chiral recognition/resolution in this mode. Many of the diverse range of chiral catalysts, auxiliaries, synthons and resolving agents investigated by Armstrong *et al*. [10, 11] in the late 1990s were analysed using CD-based GC CSPs.

3.3 Separation techniques: indirect resolution

The indirect approach for separation-based enantiospecific analysis involves the formation of stable diastereomeric derivatives by reaction with chiral derivatising agents (CDAs) [118]. The resulting diastereomers may then be resolved using appropriate achiral separation techniques such as HPLC, CE, GC or SFC, depending on the resulting structure [118]. CDAs suitable for use in NMR spectroscopy are discussed separately in Section 3.4.2. This technique has been termed the 'indirect approach' as enantiomers are not analysed directly in their original form [2]. Prerequisites include the need for a suitable functional group to derivatise, fast reproducible reactions that progress to completion and the need for enantiomerically pure CDAs (also referred to as uni-CDAs) [118]. The latter point is important to emphasise because of the fact that in the presence of chirally impure CDAs additional stereoisomeric products will be produced upon reaction with analyte stereoisomers, the enantiomeric pairs of which will not be resolved using achiral separation techniques, thus producing a false picture of the true stereoisomeric content of the sample [118]. In addition, partial racemisation of the CDA and/or analyte during derivatisation, kinetic resolution (stereoselective derivatisation) and differences in detection responses for the diastereomeric products may also have an influence on the measured enantiomeric purity of the sample [2]. It is therefore essential to investigate all potential sources of inaccuracy with this approach during analytical validation. The presence of competing derivatisable functionalities in analyte molecules may also lead to the formation of additional products that may complicate or hinder method development [2]. The potential limitations associated with the indirect approach have, to a large extent, contributed to the recent emphasis on direct determination. Indirect determination of stereoisomeric content does, however, have several advantages over the direct approach and is still used routinely for certain applications, in particular for bioanalysis [2, 119]. Many different CDAs are commercially available that are both chirally pure and relatively inexpensive, the majority of which are based on acylating agents, amines, alcohols or isocyanates. Separation of the resulting diastereoisomers may be achieved using conventional achiral chromatography or electrophoresis, and a judicious choice of the CDA may enable the use of certain favoured analytical techniques and/or enhance detection sensitivity [2]. For example, a number of CDAs are commercially available, which can be used for the preparation of diastereomeric derivatives that possess a fluorophore, facilitating the development of highly sensitive, specific analytical methods using fluorescence detection [2, 119].

3.4 Non-separation techniques

3.4.1 Chiroptical spectroscopy

Chiral molecules possess the unique property of interacting differentially with left and right circularly polarised light. A differential absorption is known as

circular dichroism (CD); a differential speed (refractive index) is the basis of the optical rotation (OR) of the plane of linearly polarised radiation. Collectively, these two phenomena as a function of wavelength are known as chiroptical spectroscopy. In the main, chiroptical phenomena are measured in the UV/Vis region of the electromagnetic spectrum associated with electronic transitions as in ordinary UV/Vis absorption spectroscopy [120]. OR has sign (+/−, clockwise/anticlockwise), magnitude and a wavelength dependency [120]. The optical rotatory dispersion (ORD) spectrum is the wavelength dependence of OR and is measured using a polarimeter. Specific OR ($[\alpha]$) at a particular wavelength normalises measurements with respect to sample pathlength and analyte concentration (g 100 ml^{-1}). The wavelength most commonly used for OR measurement is 589 nm (often referred to as the sodium D line) in the visible region of the spectrum. As mentioned previously, $[\alpha]$ remains the specified method for labelling chiral identity in all pharmacopoeial monographs and is also frequently used for the determination of enantiomeric purity [6]. As a stand-alone technique, OR is not specific as measurements relate to net effects; the presence of many electronic transitions in a molecule and other optically active impurities can obviously influence the measurement of OR. In addition, the analytical variability associated with the use of current polarimeters can sometimes be too great for compliance with regulatory guidelines, especially in cases where $[\alpha]$ is relatively small [6]. Specific online OR detectors are commercially available for use with enantioselective and ordinary HPLC such that OR measurements can be performed on separated components in a mixture. Both fixed and variable wavelength systems are available; the choice of wavelength will depend on the chiroptical characteristics of the analyte molecules. OR-based detectors are classed as universal by analogy with ordinary refractive index detection, as they do not require the presence of a chromophore [121]. However, such systems are generally considered to be relatively insensitive for most small organic molecules of pharmaceutical interest and are affected by solvent changes, making detection difficult with gradient elution chromatography [121]. However, recent improvements in polarimetric detection technology may address some of the shortcomings of this approach for enantiomeric purity determination [6].

CD is defined as the differential absorption of left and right circularly polarised light passing through an optically active substance and is measured using a CD spectrometer [120]. In modern instruments, the polarisation of light passing through the sample modulates between left and right circular with the detector and signal 'frequency-locked' into this modulation cycle to record directly the differential transmittance and absorbance. In this way, the true measure of CD is achieved as the differential absorbance $(A_L - A_R) = \Delta A = \Delta\varepsilon \cdot c \cdot l$, where $\Delta\varepsilon$ is the differential extinction coefficient, c is the concentration in moles per litre and l is the pathlength in centimetres. ΔA is very small (10^{-3}–10^{-5}) and could not be measured directly until the advent of 'modern' technology in the 1960s. Before 1960, CD was often measured as the change in the ellipticity (θ) of linearly polarised light as it passed through a chiral sample. In this case, θ of the transmitted light beam was detected as a measure of CD. Unfortunately, the older units, although redundant, are often reported. In both units, CD is normally

reported as a molar quantity, either the molar $\Delta\varepsilon$ or the molar ellipticity [Θ]. CD is dependant on the nature of the sample ($\Delta\varepsilon$ being +ve or −ve), wavelength, pathlength and analyte concentration. The CD spectrum is simply the wavelength dependence of ΔA or θ and resembles the ordinary absorption spectrum albeit some features are positive and others negative in sign [120]. The signed character of CD means that the maximum in a CD spectrum will not always be coincident with the absorption λ_{max} in the ordinary UV-visible spectrum. Both $\Delta\varepsilon$ and [Θ] are analogous to [α] in that measurements are normalised with respect to sample pathlength and analyte concentration (molar rather than g 100 mL^{-1}). CD is an absorption-based phenomenon, and a chromophore is therefore necessary for detection [121].

Online CD detectors are now commercially available for use with HPLC that are inherently more sensitive than corresponding OR detectors and not affected by solvent changes to the same extent and are thus more gradient compatible [121]. Provided $\Delta\varepsilon$ and the concentration of an analyte are known with good precision/accuracy, the measurement of CD will allow the determination of enantiomeric purity. In addition, with CD-based detection systems, both chiroptical and ordinary absorbance can be determined simultaneously allowing the measurement of the *g*-factor (or dissymmetry factor), which is defined as the ratio of the CD to the absorbance ($\Delta A/A$) [122]. The *g*-factor is concentration independent and its measurement allows a more reliable determination of enantiomeric purity (without using a CSP) with reference to standards of known enantiomeric composition irrespective of their concentration [123]. A small number of recent literature examples have suggested the potential use of achiral HPLC with online CD detection for the determination of extreme enantiomeric ratios [121, 124–126]; however, chiral separation techniques currently provide a more reliable measurement of enantiomeric purity.

3.4.2 Nuclear magnetic resonance spectroscopy

The only other non-separation technique that is used for the determination of the stereochemical purity of materials of pharmaceutical interest is NMR spectroscopy, which can often be a very straightforward approach. Various studies have investigated the use of NMR spectroscopy as a viable tool for assessing the stereoisomeric purity of APIs and, in all cases, the technique was found to be suitable for such purposes and in some cases superior to the use of [α]. However, the one obvious disadvantage of using NMR spectroscopy is instrument availability, and, as with CE, it is more likely to be used by pharmaceutical companies in the areas of drug discovery or development to monitor stereoisomeric resolutions or to measure the stereoisomeric purity of intermediates [23]. Spectral nonequivalences may be induced indirectly by the formation of permanent diastereomeric species using CDAs or directly by the formation of transient diastereomeric species using CSAs [127]. The indirect approach was pioneered by Mosher *et al*. [128] in the late 1960s, who introduced the use of α-methoxy-α-trifluoromethylphenylacetic acid (MTPA or Mosher's acid) as a versatile reagent for the determination of the enantiomeric composition of chiral alcohols

and amines by formation of diastereomeric esters and amides. This reagent has since been used extensively for the stereochemical analysis of such materials mainly because of the extent of spectral non-equivalence seen with proton and fluorine NMR signals, its stability to racemisation even under severe conditions, its availability as a chirally pure reagent and its general versatility [128]. In addition, the use of fluorine NMR can sometimes be of great benefit for trace analysis, as any signal non-equivalence will occur in uncongested spectral regions, potentially making integration easier and more accurate [128]. Mosher's acid has also been used for the assignment of the absolute configuration of chiral alcohols and amines. A number of CDAs are now commercially available for use in NMR for the determination of enantiomeric purity and absolute configuration of a wide range of chiral molecules, with many of the new reagents incorporating the same general attributes of Mosher's acid [127]. The main disadvantages of this technique are the need to covalently bond the CDA and analyte stereoisomers and the requirement of a chirally pure CDA, as with indirect resolution using separation techniques (Section 3.3).

The direct approach of chiral discrimination in NMR uses CSAs to form transient diastereomeric complexes to induce chemical shift differences in NMR signals [127]. CSAs are typically chirally pure compounds that interact with analyte molecules through non-covalent intermolecular forces [127]. Several approaches have been described including the use of chiral lanthanide shift reagents (LSRs), chiral donor–acceptor reagents and chiral host molecules such as CDs [127]. For spectral resolution to be possible, the association constants between the analyte isomers and the CSA must be different, resulting in different time-averaged solvation environments [127]. Such differences may result in signal non-equivalences potentially allowing the direct measurement of stereoisomeric content by integration [41]. The fact that a time-averaged picture of the transient diastereomeric complexes is produced is an important feature of this type of system, the consequence of which is that CSAs do not need to be chirally pure for direct resolution by NMR spectroscopy, which is analogous to the situation with direct separation techniques. The chiral purity of the CSA only affects the magnitude of the chemical shift difference, not the measured integrals [41].

Chiral LSRs were first introduced in the late 1960s/early 1970s as CSAs suitable for the determination of enantiomeric purity by NMR spectroscopy [129, 130]. They consist of chiral β-diketones complexed with lanthanide metal ions such as europium III^+ and praseodymium III^+, forming stable paramagnetic species [41]. The lanthanide metal cations can coordinate to sites on analyte molecules containing electron-rich centres such as nitrogen and oxygen atoms [41]. The reagents are readily soluble in many typical NMR solvents such as deuterochloroform and can produce large pseudocontact downfield shifts due to the paramagnetic nature of the lanthanide ions [41]. More recently, binuclear lanthanide–silver reagents have been introduced as shift reagents for soft Lewis bases such as alkenes and aromatics [127]. Although LSRs are still an important class of CSAs, their use has diminished in recent years mainly due to the

paramagnetic line broadening they cause, which is exacerbated at the higher field strengths now commonly used [127].

Chiral host reagents such as CDs represent another important class of CSAs [127]. Their use in NMR spectroscopy as chiral resolving agents was first established in the late 1980s and since then a multitude of applications have been reported using both native and derivatised versions. The mechanisms of interaction of CDs in solution with analyte molecules are described in Section 3.2.3. Solvent choice with CDs is generally limited to D_2O, although some derivatives are soluble in polar organic solvents such as deuteromethanol, thus increasing their range of application. The main advantages of CDs as chiral host reagents are their widespread availability, their versatility, the narrow chemical shift range of their proton signals and the lack of signal broadening in comparison to LSRs [131]. The main disadvantages relate to their solubility in a limited range of polar solvents and relatively small chemical shift differences induced in comparison to LSRs. However, where such differences are insufficient to allow accurate integration, a number of chemometric resolution enhancement techniques are now available to aid data interpretation. Of the commercially available CDs, acetylated-β-CD in particular has been shown to be a very useful CSA when used with certain analyte classes [131]. Chiral crown ethers such as (18-crown-6)-2,3,11,12-tetracarboxylic acid have been shown to be particularly useful as host reagents for the stereochemical resolution of chiral analytes containing primary amine groups, although this class of CSA has limited applicability due to the specific mechanism of interaction.

Chiral donor–acceptor type CSAs were first introduced by Pirkle and coworkers in the late 1970s as previously described in Section 3.2.1 and are still routinely used as chiral discriminating reagents in NMR spectroscopy [28–32]. The most widely used CSAs of this type are undoubtedly the 2,2,2-trifluoromethyl-1-arylethanol derivatives such as TFAE (Figure 3.11), although many different reagents have now been shown to be of use for this type of analysis, depending on the functionality of the analyte [127]. TFAE has the broadest applicability and generally induces the largest stereochemical shift differences of its class due to the extent of diamagnetic anisotropy exerted by the anthracene moiety [127]. It is commercially available as an enantiomerically pure reagent and is generally used in conjunction with non-solvating solvents such as deuterochloroform to maximise any potential π–π, dipole–dipole or hydrogen bonding interactions. The main advantages of TFAE as a CSA are its versatility and the lack of signal broadening in comparison to LSRs. As with Mosher's acid, TFAE can also be used in fluorine NMR spectroscopy when performing trace level stereoisomer analysis. The main disadvantages of TFAE as a CSA are the relatively small chemical shift differences induced in comparison to LSRs and the limited range of solvents it can be used in conjunction with. Figure 3.11 shows the application of TFAE for the determination of the enantiomeric purity of ZD6169 ((*S*)-*N*-(4-benzoylphenyl)-3,3,3-trifluoro-2-hydroxy-2-methylpropionamide), an API previously in development as a potassium channel opener for the treatment of urinary incontinence. The application exemplifies the use of TFAE

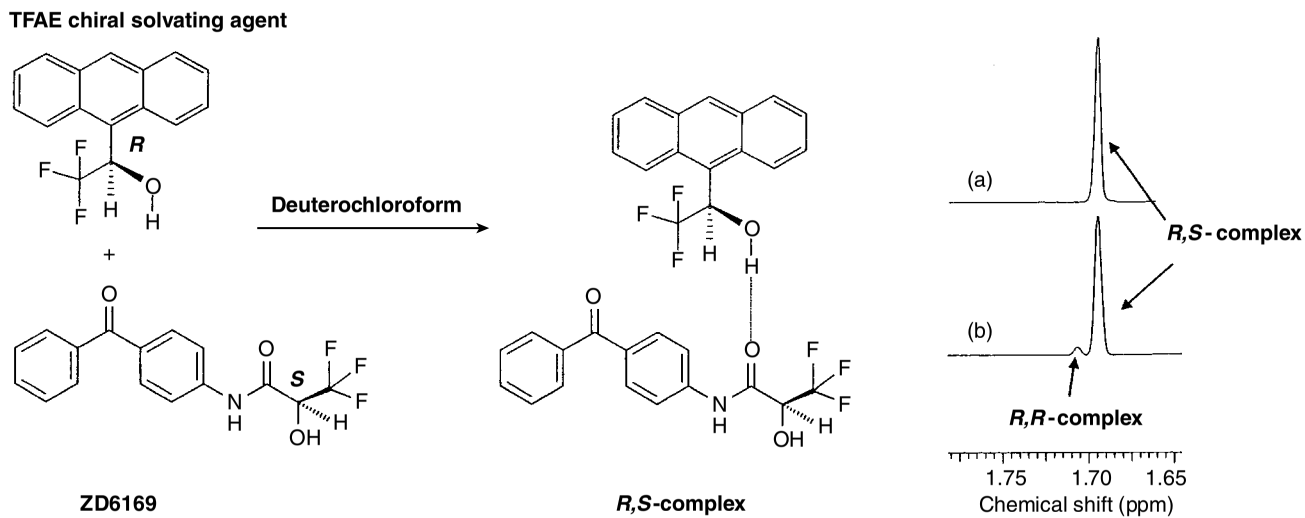

Figure 3.11 Application of ^{1}H-NMR for the determination of the enantiomeric purity of ZD6169 API. (a) Signal associated with the methyl proton of enantiomerically pure ZD6169 API. (b) Signals associated with the methyl protons of ZD6169 API artificially enriched with approximately 5%w/w (*R*)-enantiomer (distomer). (Conditions: field strength: 500 MHz; temperature: 300 K; number of transients: 256; pulse: 90°; pulse repetition time: 6.5 s; Gaussian multiplication; Gaussian broadening factor: 0.1; line-broadening factor: −0.5; sample preparation: 20 mg/ml in deuterochloroform containing 10 mol equivalents TFAE.)

as a CSA in that it was used in deuterochloroform solution to induce a relatively small chemical shift difference (ca. 0.1 ppm) in the signals corresponding to the methyl protons of the two enantiomers in the NMR spectrum without causing significant line broadening. The transient diastereomeric complexes formed through hydrogen bonding between the donor alcohol group on the CSA and the acceptor carbonyl group on the analyte molecule. Sufficient resolution was achieved with minimal method development to facilitate accurate integration and measurement of enantiomeric purity at levels down to approximately 1% w/w of the unwanted *R*-enantiomer.

3.5 Conclusions

This chapter has presented a general overview of the current most common analytical techniques for the measurement of chiral impurities. From just a cursory glance at recent scientific literature, it is evident that the modern analytical chemist has a plethora of reliable technologies at their disposal for such determinations ranging from frequently used separation techniques such as HPLC and CE to less-frequent (but equally as valid), non-separation techniques such as chiroptical and NMR spectroscopy. Often, more than one enantiospecific analytical technique will be suitable for chiral purity determinations, with the choice being related to equipment availability and analyst familiarity. It is fair to say that progress in this field during the last 25 years has been remarkable such that development of enantiospecific methodologies for pharmaceutical applications is often now a fairly routine task. Improvements to existing technologies continue to facilitate faster, more reliable method development and also help reduce limits of detection to cope with increasingly stringent regulatory requirements. An increasingly cost-conscious pharmaceutical industry is likely to ensure that stereospecific analytical approaches continue to evolve such that in future the most efficient methods are automatically applied even in the absence of specialist knowledge.

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References

1. L. E. Orgel, *The Origins of Life. Molecules and Natural Selection*, John Wiley and Sons, New York, 1973, p. 166.

2. A. J. Hutt and B. K. Patel, in *Drug Metabolism: Towards the Next Millenium*, ed. N. J. Gooderham, IOS Press, Amsterdam, 1998, p. 196.
3. R. A. Sheldon, *Chirotechnology. Industrial Synthesis of Optically Active Compounds*, Marcel Dekker, New York, 1993.
4. E. J. Ariëns, *TIPS*, 1986, **7**, 200.
5. I. Toldy, L. Wargha, I. Tolh and J. Borsy, *Acta Chem. Acad. Sci. Hung.*, **19**, 273.
6. U. Schmitt, S. K. Branch and U. Holzgrabe, *J. Sep. Sci.*, 2002, **25**, 959.
7. ICH Topic Q6A. *Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances*, 1999.
8. ICH Topic Q3A (R). *Impurities in New Drug Substances*, 2002.
9. ICH Topic Q2B. *Validation of Analytical Procedures: Methodology*, 1996.
10. D. W. Armstrong, J. T. Lee and L. W. Chang, *Tetrahedron: Asymmetry*, 1998, **9**, 2043.
11. D. W. Armstrong, L. He, T. Yu, J. T. Lee and Y.-S. Liu, *Tetrahedron: Asymmetry*, 1999, **10**, 37.
12. D. W. Armstrong, J. D. Duncan and S. H. Lee, *Amino Acids*, 1991, **1**, 97.
13. J. Zukowski, M. Pawlowska and D. W. Armstrong, *J. Chromatogr.*, 1992, **623**, 33.
14. S. C. Chang, L. R. Wang and D. W. Armstrong, *J. Liq. Chromatogr.*, 1992, **15**, 1411.
15. M. Pawlowska, S. Chen and D. W. Armstrong, *J. Chromatogr.*, 1993, **641**, 257.
16. D. W. Armstrong, M. P. Gasper, S. H. Lee, N. Ercal and J. Zukowski, *Amino Acids*, 1993, **5**, 299.
17. D. W. Armstrong and J. Zukowski, *J. Chromatogr. A*, 1994, **666**, 445.
18. ICH Topic Q7A. *Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients*, 2000.
19. J. Zukowski, *Chirality*, 1998, **10**, 362.
20. M. Reist, B. Testa, P.-A. Carrupt, M. Jung and V. Schurig, *Chirality*, 1995, **7**, 396.
21. ICH Topic Q1A. *Stability Testing of New Drug Substances and Products*, 2000.
22. https://www.the-stationery-office.co.uk/bp2004/bpfrls.htm
23. W. J. Lough, in *Pharmaceutical Analysis*, eds. D. C. Lee and M. Webb, Blackwell Publishing, Oxford, 2003, p. 74.
24. W. H. Pirkle and D. W. House, *J. Org. Chem.*, 1979, **44**, 1957.
25. W. H. Pirkle, D. W. House and J. M. Finn, *J. Chromatogr.*, 1980, **192**, 143.
26. W. H. Pirkle and J. M. Finn, *J. Org. Chem.*, 1981, **46**, 2935.
27. W. H. Pirkle, J. M. Finn, J. L. Schreiner, B. C. Hamper, *J. Am. Chem. Soc.*, 1981, **103**, 3964.
28. W. H. Pirkle, *J. Am. Chem. Soc.*, 1966, **88**, 1837.
29. T. G. Burlingame and W. H. Pirkle, *J. Am. Chem. Soc.*, 1966, **88**, 4294.
30. W. H. Pirkle and S. D. Beare, *J. Am. Chem. Soc.*, 1967, **89**, 5485.
31. W. H. Pirkle and S. D. Beare, *J. Am. Chem. Soc.*, 1968, **90**, 6250.
32. W. H. Pirkle and S. D. Beare, *J. Am. Chem. Soc.*, 1969, **91**, 5150.
33. I. W. Wainer, *Trends Anal. Chem.*, 1987, **6**, 125.
34. R. Thompson, *J. Liq. Chromatogr. Rel. Technol.*, 2005, **28**, 1215.
35. J. A. Whatley, *J. Chromatogr. A*, 1995, **697**, 257.
36. C. Perrin, V. A. Vu, N. Matthijs, M. Maftouh, D. L. Massart and Y. Vander Heyden, *J. Chromatogr. A*, 2002, **947**, 69.
37. C. Perrin, N. Matthijs, D. Mangelings *et al.*, *J. Chromatogr. A*, 2002, **966**, 119.
38. P. Borman, B. Boughtflower, K. Cattanach *et al.*, *Chirality*, 2003, **15**, S1.
39. M. E. Andersson, D. Aslan, A. Clarke, J. Roeraade and G. Hagman, *J. Chromatogr. A,* 2003, **1005**, 83.
40. http://chirbase.u-3mrs.fr.
41. S. G. Allenmark, *Chromatographic Enantioseparation: Methods and Applications*, Ellis Horwood, Chichester, 1988.

42. V. Mayer, *Chirality*, 1995, **7**, 567.
43. Y. Okamoto, M. Kawashima, K. Yamamoto and K. Hatada, *Chem. Lett*., 1984, 739.
44. A. Ichida, T. Shibata, I. Okamoto, Y. Yuki, H. Namikashi and Y. Toga, *Chromatographia*, 1984, **19**, 280.
45. Z. Juvancz and J. Szetjtli, *Trends Anal. Chem*., 2002, **21**, 379.
46. G. Hesse and R. Hagel, *Chromatographia*, 1973, **6**, 277.
47. Y. Okamoto, S, Honda, I. Okamoto *et al*., *J. Am. Chem. Soc*., 1981, **103**, 6971.
48. T. Wang and Y. W. Chen, *J. Chromatogr. A*, 1999, **855**, 411.
49. http://www.usp.org/USPNF/
50. D. W. Armstrong, Y. Tang, S. Chen, Y. Zhou, C. Bagwill and J. -R. Chen, *Anal. Chem*., 1994, **66**, 1473.
51. http://www.astecusa.com/publications/CHIROBIOTIC_HANDBOOK_2004.pdf
52. T. J. Ward and A. B. Farris III, *J. Chromatogr. A*, 2001, **906**, 73.
53. T. E. Beesley, J. T. Lee and A. X. Wang, in *Chiral Separation Techniques: A Practical Approach (2nd edition)*, ed. G. Subramanian, Wiley-VCH, Weinheim, 2001, p. 25.
54. J. Ďungelová, J. Lehotay and T. Rojkovičová, *Chem. Anal. (Warsaw)*, 2004, **41**, 1.
55. T. L. Xiao and D. W. Armstrong, in *Chiral Separations: Methods and Protocols*, ed. G. Gübitz and M. G. Schmid, Humana Press, Totowa, NJ, 2003, p. 113.
56. A. X. Wang, J. T. Lee and T. E. Beesley, *LCGC*, 2000, **18**, 626.
57. http://www.registech.com/chiral/ChiralGuideV.pdf
58. S. Rudaz and J.-L. Veuthey, in *Handbook of Experimental Pharmacology (Stereochemical Aspects of Drug Action and Disposition) vol. 153*, ed. M. Eichelbaum, B. Testa and A. Somogyi, Springer-Verlag, Berlin, 2003, p. 45.
59. http://mathworld.wolfram.com/ConicalFrustum.html
60. D. W. Armstrong and W. DeMond, *J. Chromatogr. Sci*., 1984, **22**, 411.
61. http://www.astecusa.com/publications/cyclobond-handbook-01.pdf
62. A. Berthod, H. L. Jin, T. E. Beesley, J. D. Duncan and D. W. Armstrong, *J. Pharm. Biomed. Anal*., 1990, **8**, 123.
63. D. W. Armstrong, C. D. Chang and S. H. Lee, *J. Chromatogr*., 1991, **539**, 83.
64. S. C. Chang, G. L. Reid III, S. Chen, C. D. Chang and D. W. Armstrong, *Trends Anal. Chem*., 1993, **12**, 144.
65. R. H. McMenamy and J. L. Oncley, *J. Biol. Chem*., 1958, **233**, 1436.
66. N. Varsano-Aharon and S. Ulick, *J. Biol. Chem*., 1972, **247**, 4939.
67. K. K. Stewart and R. F. Doherty, *Proc. Natl. Acad. Sci. U.S.A*., 1973, **70**, 2850.
68. H.-J. Federsel, *Chirality*, 2003, **15**, S128.
69. H.-J. Federsel and M. Larsson, in *Asymmetric Catalysis on Industiral Scale Challenges, Approaches and Solutions*, ed. H. U. Blaser and E. Schmidt, Wiley-VCH, Weinheim, 2003, p. 413.
70. A. A. S. Karlsson, personal communication.
71. W. H. Porter, *Pure Appl. Chem*. 1991, **63**, 1122.
72. J. Dobashi, D. Sybilska and J. Jurczak, *J. Chromatogr*., 1982, **237**, 303.
73. J. Dobashi, D. Sybilska and J. Jurczak, *Chromatographia*, 1982, **16**, 198.
74. D. Sybilska and J. Zukowski, in *Chiral Separations by HPLC. Applications to Pharmaceutical Compounds*, ed. A. M. Krstulovic, Ellis Horwood, Chichester, 1989, p. 147.
75. C. Pettersson and G. Schill, *J. Chromatogr*., 1981, **204**, 179.
76. C. Pettersson and G. Schill, *Chromatographia*, 1982, **16**, 192.
77. V. A. Davankov, A. S. Bochkov, A. A. Kurganov, P. Roumeliotis and K. K. Unger, *Chromatographia*, 1980, **13**, 677.
78. W. J. Lough, in *Chirality in Natural and Applied Science*, ed. W. J. Lough and I. W. Wainer, Blackwell Publishing, Oxford, 2002, p. 179.

79. J. W. Jorgenson and K. D. Lukacs, *Anal. Chem.*, 1981, **53**, 1298.
80. J. W. Jorgenson and K. D. Lukacs, *J. Chromatogr.*, 1981, **218**, 209.
81. J. W. Jorgenson and K. D. Lukacs, *Clin. Chem.*, 1981, **27**, 1551.
82. J. W. Jorgenson and K. D. Lukacs, *J. H. R. C. & C. C.*, 1981, **4**, 230.
83. J. W. Jorgenson and K. D. Lukacs, *Science*, 1983, **222**, 266.
84. B. Chankvetadze, *Capillary Electrophoresis in Chiral Analysis*, John Wiley and Sons, Chichester, 1997.
85. S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, *Anal. Chem.*, 1984, **56**, 111.
86. S. A. C. Wren, *Chromatographia*, 2001, **54 (Suppl.)**, S1.
87. M. Hadley, M. Gilges, J. Senior, A. Shah and P. Camilleri, *J. Chromatogr. B*, 2000, **745**, 177.
88. H. Wikström and P. K. Owens, *J. Sep. Sci.*, 2002, **25**, 1167.
89. M. Jimidar, W. Van Ael, M. De Smet and P. Cockaerts, *LC-GC Eur.*, 2002, **15**, 230.
90. H. Fabre and K. D. Altria, *LC-GC Eur.*, 2001, **14**, 302.
91. E. Gassman, J. E. Kuo and R. N. Zare, *Science*, 1985, **230**, 813.
92. A. Guttman, *Electrophoresis*, 1995, **16**, 1900.
93. N. Roos, K. Ganzler, J. Szemán and S. Fanali, *J. Chromatogr. A*, 1997, **782**, 257.
94. M. Fillet, P. Hubert and J. Crommen, *Electrophoresis*, 1998, **19**, 2834.
95. L. Liu and M. A. Nussbaum, *J. Pharm. Biomed. Anal.*, 1999, **19**, 679.
96. C. Perrin, Y. Vander Heyden, M. Maftouh and D. Luc Massart, *Electrophoresis*, 2001, **22**, 3203.
97. N. Matthijs, C. Perrin, M. Maftouh, D. Luc Massart and Y. Vander Heyden, *J. Pharm. Biomed. Anal.*, 2002, **27**, 515.
98. L. Zhou, R. Thompson, S. Song, D. Ellison and J. M. Wyvratt, *J. Pharm. Biomed. Anal.*, 2002, **27**, 541.
99. W. Zhong and E. S. Yeung, *Electrophoresis*, 2002, **23**, 2996.
100. N. Matthijs, S. Van Hemelryck, M. Maftouh, D. Luc Massart and Y. Vander Heyden, *Anal. Chim. Acta*, 2004, **525**, 247.
101. M. Jimidar, W. Van Ael, P. Van Nyen, M. Peeters, D. Redlich and M. De Smet, *Electrophoresis*, 2004, **25**, 2772.
102. C. E. Sänger-van de Griend and K. Gröningsson, *J. Pharm. Biomed. Anal.*, 1996, **14**, 295.
103. S. Terabe, H. Ozaki and T. Ando, *J. Chromatogr.*, 1985, **332**, 211.
104. S. Terabe, *Trends Anal. Chem.*, 1989, **8**, 129.
105. T. de Boer, R. A. de Zeeuw, G. J. de Jong and K. Ensing, *Electrophoresis*, 2000, **21**, 3220.
106. G. M. Janini, G. M. Muschik and H. J. Issaq, *Electrophoresis*, 1996, **17**, 1575.
107. M. R. Hadley, M. Decrette, G. Guilloré *et al.*, *J. Sep. Sci.*, 2001, **24**, 766.
108. K. W. Phinney, *Anal. Chem.*, 2000, **72**, 204A.
109. Y. Liu, A. W. Lantz and D. W. Armstrong, *J. Liq. Chromatogr. Relat. Technol.*, 2004, **27**, 1121.
110. T. A. Berger, *J. Chromatogr. A*, 1997, **785**, 3.
111. G. Terfloth, *J. Chromatogr. A*, 2001, **906**, 301.
112. P. A. Mourier, E. Eliot, M. H. Caude and R. H. Rosset, *Anal. Chem.*, 1985, **57**, 2819.
113. E. Gil-Av, B. Feibush and R. Charles-Sigler, *Tetrahedron Lett.*, 1966, 1009.
114. V. Schurig, *J. Chromatogr. A*, 2001, **906**, 275.
115. V. Schurig, *Angew. Chem. Int. Ed. Engl.*, 1977, **16**, 110.
116. V. Schurig, *J. Chromatogr. A*, 2002, **965**, 315.
117. T. Kościelski, D. Sybilska and J. Jurczak, *J. Chromatogr.*, 1983, **280**, 131.
118. M. Sculte, in *Chiral Separation Techniques: A Practical Approach (2nd edition)*, ed. G. Subramanian, Wiley-VCH, Weinheim, 2001, p. 185.
119. N. R. Srinivas, *Biomed. Chromatogr.*, 2004, **18**, 207.

120. P. Salvatori and L. Di Bari, in *Chirality in Natural and Applied Science*, ed. W. J. Lough and I. W. Wainer, Blackwell Publishing, Oxford, 2002, p. 203.
121. A. L. Jenkins and W. A. Hedgepeth, *Chirality*, 2005, **17**, S24.
122. W. Kuhn, *Annu. Rev. Phys. Chem.*, 1958, **9**, 417.
123. A. F. Drake, J. M. Gould and S. F. Mason, *J. Chromatogr.*, 1980, **202**, 239.
124. C. Bertucci, V. Andrisano, V. Cavrini and E. Castiglioni, *Chirality*, 2000, **12**, 84.
125. M. R. Hadley and G. D. Jonas, *Enantiomer*, 2000, **5**, 357.
126. R. D. Shah and L. A. Nafie, *Curr. Opin. Drug Discov. Devel.*, 2001, **4**, 764.
127. T. J. Wenzel and J. D. Wilcox, *Chirality*, 2003, **15**, 256.
128. J. A. Dale, D. L. Dull and H. S. Mosher, *J. Org. Chem.*, 1969, **9**, 2543.
129. C. C. Hinckley, *J. Am. Chem. Soc.*, 1969, **91**, 5160.
130. G. M. Whitesides and D. W. Lewis, *J. Am. Chem. Soc.*, 1970, **92**, 6979.
131. M. Thunhorst and U. Holzgrabe, *Magn. Reson. Chem.*, 1998, **36**, 211.

Analysis of Drug Impurities
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4 Low-level measurement of potent toxins

Peter W. Skett

4.1 Introduction

In addition to solvents, inorganic and drug-related impurities, the pharmaceutical industry must pay close attention to the presence of compounds whose biological effects are known or likely to be seriously detrimental to the health of the patient. These are highly toxic impurities, and of particular concern are genetic toxins. These compounds are implicated in one way or another in increasing the risk of cancer.

Genetic toxicity is a complex field of toxicology and will not be discussed in detail here. For more information the reader is directed to references [1–3]. DNA damage is common to all cancers. Carcinogens can be classified into two types: those that cause damage to DNA directly (or through their metabolites) and those that act indirectly by increasing susceptibility to genetic changes (e.g. through effects on cell proliferation or inflammation). The former are genotoxic and the latter are non-genotoxic carcinogens. Alkylating agents are often genotoxins, since they can, if appropriately absorbed into tissues, act by alkylating DNA. 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) and chloroform are examples of compounds whose carcinogenicity is believed to be through non-genotoxic mechanisms.

A simplistic theory holds that non-genotoxic carcinogens give rise to a threshold dose effect: below a threshold dose no significant increase in tumour incidence occurs, but above this a definite dose response occurs [2]. In this view, genotoxins give rise to a linear dose response even at very low concentrations. However, whilst initial DNA damage may be proportional to concentration of genotoxin at very low doses, other mechanisms can ameliorate or compound this effect [4]. For example, metabolic activation or detoxification (DNA repair) can occur. Either of these mechanisms can be saturated at higher dose levels, causing reduced or increased rate of damage, respectively. The rate of cell division may decrease at low concentrations of genotoxin but increase as the concentration increases, giving rise to a 'J-shaped' curve for the dose response [2, 5].

For these reasons analytical chemists are highly dependent on advice from toxicologists on what levels of particular genetic toxins may be deemed safe. Where novel compounds are under consideration, particularly during early phases of drug development, no toxicological data may be available for the analyte in question. In these circumstances, the toxicologist can resort to a substantial body of work on structure–activity relationships (SARs), which can provide predictive information on genetic toxicity.

What is common to toxins causing damage to DNA is that they contain electrophilic groups. The electrophilic group may be inherent in the drug impurity (such as in alkylating agents) or in some species generated indirectly by its biological effects (e.g. oxygen radicals generated during an inflammatory response). Table 4.1 summarises some of the main electrophilic species associated with DNA damage, together with examples of relevant carcinogens.

Table 4.1 Electrophilic species of some chemical carcinogens

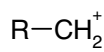
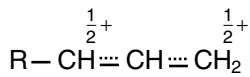
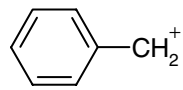
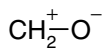
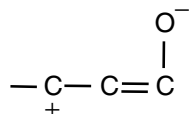
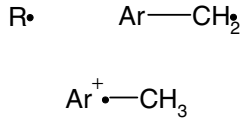
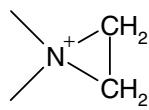
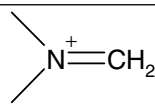
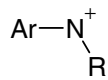
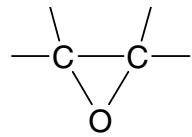
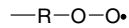
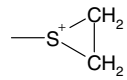
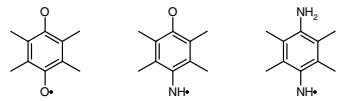
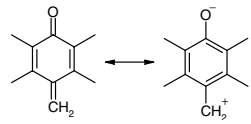
Electrophilic species	Structures	Examples of carcinogens
Alkylcarbonium ions		Diethylnitrosamine; dimethylsulfate; 1,2-dimethylhydrazine
Arylcarbonium ions		benzidine; 1-(4-methoxyphenyl)-1, 3-dimethyltriazene
Allylic carbonium ions		Allyl methanesulfonate; lasiocarpine; safrole
Benzylic carbonium ions		Benzyl chloride; 7,12-dimethylbenz(a)anthracene
Formaldehyde		Formaldehyde; hexamethylphosphoramide
α,β-Unsaturated carbonyls or carboxylates		acrylates; arecoline; cyclophosphamide; diallate; ptaquiloside
Acylating moieties		Benzoyl chloride
Carbon-centred free radicals or radical cations		Carbon tetrachloride; 7,12-dimethylbenz(a)anthracene
Carbene safrole		Carbon tetrachloride;
Aziridinium ions		Aziridine; cyclophosphamide; nitrogen mustard

Table 4.1 *Continued*

Electrophilic species	Structures	Examples of carcinogens
Iminium ions		Hexamethylmelamine; bis-(morpholino)-methane; hycanthone
Arylnitrenium ions		2-acetylaminofluuorine; benzidine; *N*,*N*-dimethyl-4-aminoazobenzene
Nitrogen-centred free radicals or radical cations		benzidine; 2-naphthylamine
Epoxides		Aflotoxin B_1; capsaicin; ethylene oxide; vinyl chloride
Oxonium ions (α-haloethers)		Bis(chloromethyl) ether
Peroxy free radicals		Di-*t*-butylperoxide
Episulfonium ions		1,2 Dichloroethane; sulfur mustard
Sulfonium ions (α-halothioethers)		Dichloromethane
Semiquinone and its quinoneimine and diamine/diimine analogues		1,4-Benzoquinone; adriamycon; benzidine; 2-naphthylamine; phenacetin
Quinonemethide		Daunomycin; quercetin

Source: Reproduced with permission from [6]

4.2 Classes of genotoxic impurity

Computer programmes are available for predicting the potential toxicity of drug impurities on the basis of chemical structure [7]. Whilst these may not be foolproof, they give an indication of which one would be ill advised to ignore without data from specific toxicity studies. There is always a balance between the resource required to carry out animal genetic toxicity studies and that required to determine trace levels of a predicted genotoxin in batches of drug substance earmarked for the clinic.

Nevertheless, chemical structures are a currency with which synthetic and analytical chemists are used to dealing, so it is useful to categorise compounds into types that will alert them to potential issues.

4.2.1 Alkylating agents

There are, of course, a number of structural motifs that give rise to alkylating ability and these are further sub-categorised below. The main concern with alkylators is the possibility of alkylating DNA bases (on N-7 of guanine and N-3 of adenine) [8], although other biomolecules may also be affected. Alkylation provides an effective means of forming bonds to aliphatic carbon atoms. Often short carbon chains or similar moieties can be added by this means during synthesis, but more complex intermediates with alkylating functionality may also be used. What must also be considered is the possibility of generating an unwanted alkylating agent during synthesis. This might happen if, for example, a salt counterion of a basic intermediate or drug molecule (e.g. HCl or similar salt) reacts with an alcohol used as a solvent to form, say, an alkyl halide.

4.2.1.1 Alkyl halides

These include alkyl iodides, bromides and chlorides (the carbon–fluorine bond normally being too strong for general use in alkylation reactions). Nucleophilic substitution, typically at an sp^3 carbon, results in formation of the bond to this carbon with loss of the halide anion as the leaving group. Reactivities are in the order I > Br > Cl. Hence iodide salts are sometimes used to catalyse a reaction with alkyl chlorides, since I^- is a good nucleophile that displaces the chloride. The resulting alkyl iodide is a more powerful alkylating agent because iodide is a much better leaving group than chloride. In these circumstances, the analytical chemist must consider whether to analyse for residual alkyl iodide as well as the original chloride.

4.2.1.2 Alkyl sulfonates

Commonly used alkyl sulfonates are esters of methanesulfonic acid (mesylates), benzenesulfonic acid (besylates) and *p*-toluenesulfonic acid (tosylates). The sulfonate anion is another extremely good leaving group, making the esters particularly good alkylating agents. In addition, sulfonic acids are often used to form salts of bases for purification of API or intermediates or as the preferred salt form of the drug for reasons of bioavailability. Interactions of these with residual alcohols may be a cause for concern [9], and this has led to significant pressures on the industry from some reviewers from regulatory agencies in recent times.

4.2.1.3 Dialkyl sulfates

Dimethyl sulfate and diethyl sulfate are commonly used as methylating and ethylating agents, respectively. Again, the possibility of sulfate salts giving rise to these compounds in the presence of methanol or ethanol must be considered.

4.2.1.4 Epoxides

Ring opening of an epoxide results in a reactive ion that can alkylate DNA. Quinolines can be metabolised to form an epoxide on the nitrogen ring, although substituents in the 2, 3 and 4 positions reduce the likelihood of this.

4.2.1.5 Aziridines

The strained ring in aziridines makes them reactive, with ring opening under attack from any nucleophile to create a new nucleophile–carbon bond. Aziridine rings can be formed from intramolecular substitution of a halogen atom by the nitrogen in nitrogen mustards.

4.2.2 Reactive amines

Aromatic amines (anilines) may become activated *in vivo* to form reactive amines. These are nucleophiles and may attack DNA, forming covalent modifications. Aromatic nitro compounds can be metabolised to also form reactive amines. *N*-nitroso compounds result in the alkylation of oxygen sites in DNA bases (O-6 in guanine and O-4 in thymidine) [8, 10].

4.2.3 Fused tricyclics

These compounds are able to intercalate between the coils of the DNA double helix.

4.2.4 Substituted purines and pyrimidines

Substituted purines and pyrimidines can themselves be erroneously incorporated into the DNA sequence, leading to mutations.

4.2.5 Hydroperoxides

Hydroperoxides result in oxidative damage to DNA.

4.3 The analytical challenge of genetic toxins

From the above classification it is possible to at least have an indication of when a compound is *potentially* genotoxic or might result in cancer risk through an indirect, non-genotoxic mechanism. Whenever it is not possible to rule out the risk of potentially toxic levels being present in a drug on purely synthetic grounds, the onus rests with the analytical chemist to determine the offending analyte at trace levels. The regulatory pressures on this issue are presently severe and unlikely to relent.

This poses a significant challenge. Conventional analytical procedures for measuring drug impurities are rarely sufficient to deliver the limits of detection (LOD) required for these potentially toxic impurities or known carcinogens (typically low- or sub-ppm relative to drug). Hence one fact that cannot be ignored in any analytical methodology is that the drug itself may be present at five, six or more orders of magnitude higher than the compound being measured. In favourable cases LC-UV impurities methods may give sufficient chromatographic selectivity and sensitivity, but the probability of a plethora of other, non-toxic

drug-related impurities being present at these levels is high, and these could easily cloud the issue.

Hence, in most cases the selectivity, specificity and sensitivity will only be achieved by using more selective or orthogonal separation and/or detection techniques. The best approach depends upon the physico-chemical properties of both the analyte and the drug Active Pharmaceutical Ingredient (API). In most cases the latter will be non-volatile, so volatile impurities may be amenable to measurement at trace levels by gas chromatography without significant sample pre-treatment or pre-concentration. However, where the properties of the toxic analyte are similar to the drug (such as alkylating intermediates or toxic by-products), typically a condensed-phase separation method will be required and selectivity for the analyte may require a more selective detection method, such as mass spectrometry. Alternatively, a sample pre-concentration method may be necessary. Various chromatographic techniques with appropriate detection methods are described in the following sections. More general applicability of these techniques for measuring drug impurities has been described by Okafo and Roberts in a previous monograph [11]. Hence the present discussion focuses on their relevance to trace analysis of toxic impurities.

4.4 Gas chromatography

4.4.1 Sample introduction techniques

4.4.1.1 Split/splitless injectors

Direct injection is the most commonly used technique for sample introduction in GC, typically using combined split/splitless injectors. In split mode, a portion of the sample passes onto the column and the rest is directed to waste. After sufficient split time to completely flush the injector the split vent may be closed to save gas, although this is optional. The injector is set to a sufficiently high temperature to eliminate discrimination between analytes. The sensitivity of the technique is inversely proportional to the split ratio.

In splitless injection, the split vent is closed for a period of typically 0.5–1 min, during which time the column oven is set to a temperature at least 10°C lower than the boiling point of the injection solvent. This allows the analytes to condense onto the head of the column in a tight band. After the splitless period, the split valve is opened to flush the injector of any remaining sample. The oven is then programmed to elute the sample peaks. Sensitivity is much greater than in split mode, although the robustness of the technique is much more affected by high-level components or involatiles such as the drug substance.

Thermal stability and/or involatility of the main component can be a serious issue with direct injection techniques. At the very least, thermal degradants or involatiles can contaminate the injector and the head of the column, affecting the inertness of the system and causing deterioration in performance (both in recoveries due to adsorption and sensitivity and selectivity caused by peak tailing).

In other situations, degradation of the main component can lead to volatile components chromatographing and interfering with the analysis. In exceptional circumstances, the drug substance may thermally degrade to yield the analyte itself, thus making the analysis untenable.

4.4.1.2 Cold on-column injectors

Cold on-column injection (COCI) provides an alternative means of splitless injection. Here, the injector has a low thermal mass and so can be heated and cooled relatively quickly. An injection gap is required (a short section of fused silica of wide enough bore for the syringe needle to pass inside) between the injector and the head of the column to take up the volume of the injection. The injector remains 'cold' (lower temperature than for conventional split/splitless injections) until the sample is loaded, after which it is temperature programmed to ensure that vaporisation of the analyte is achieved and the oven is also programmed to effect chromatography. This injection mode is particularly suited to thermally unstable analytes or matrices, since degradation is minimised. However, the large excess of drug substance in pharmaceutical samples can still pose significant problems for this technique. Nevertheless, COCI has been demonstrated to provide a method to analyse an epoxide impurity in a drug where conventional split/splitless injection resulted in degradation of the drug to form the analyte. Liquid–liquid extraction (see below) was used to minimise interference from the drug [12].

4.4.1.3 Headspace injection

For the above reasons it is desirable to minimise contamination of the injector and column by avoiding the introduction of a large excess of drug substance present in samples where toxic impurities are being analysed. One way of achieving this for volatile analytes is to use headspace injection. Here, the sample is dissolved in a high-boiling solvent such as water or DMSO and several hundred microlitres or up to a few millilitres of the solution is enclosed in a headspace vial. The vial is incubated at a temperature below the boiling point of the solvent. Volatiles partition into the headspace, which is sampled using a gas syringe (typically 0.5–1 ml injection) and introduced into a conventional split injector.

The advantage of headspace mode is that only volatile components that will not contaminate the GC are injected. Involatiles do not partition into the headspace and so never enter the injector. Effectively, the analyte is decoupled from the influence of the drug (but see the discussion on validation below). However, many analytes that are amenable to GC by direct injection are not sufficiently volatile to give a high-enough vapour pressure to be detected by conventional headspace injection. These semi-volatile components can sometimes be successfully analysed using a variant of the headspace technique known as total vaporisation headspace injection. In this instance, a few microlitres of the sample solution are injected into the headspace vial, which is then incubated at a temperature that vaporises the solvent completely into the headspace.

Figure 4.1 depicts the difference between these two modes. Since there is no longer any partitioning of the analyte between the condensed phase and the vapour phase, compounds of limited volatility may yield a higher vapour pressure in this mode than by the conventional headspace approach, where they predominantly remain in solution. Involatile materials (such as API usually) do not vaporise, but instead condense onto the inside of the vial. The headspace vial effectively becomes a disposable injector liner. Care must be taken to operate at an incubation temperature that will not cause degradation of API into volatile components, which might interfere with the analysis.

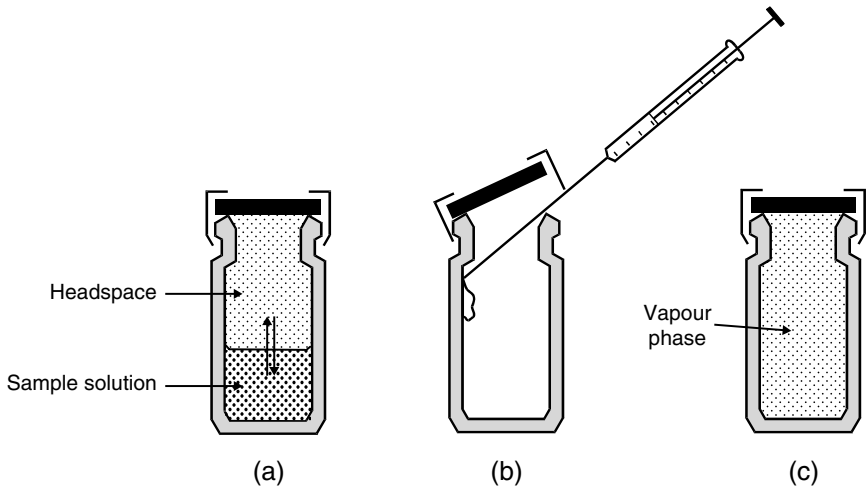


Figure 4.1 Comparison of conventional headspace and total vaporisation headspace modes. (a) Conventional headspace mode during equilibration phase, (b) total vaporisation mode during sample loading and (c) total vaporisation mode during equilibration phase.

4.4.1.4 Programmed temperature vaporiser

Some analytes are volatile enough to be analysed by GC, but too involatile to give sufficient sensitivity by headspace injection modes. In these cases, all may not be lost. The programmed temperature vaporiser (PTV) can provide a means of selectively injecting the desired analyte onto the column whilst excluding undesired components such as solvents and involatiles. This is variously known as selective extraction or selective exclusion. It permits large volume injections (LVI) and difficult matrix introduction (DMI).

Like cold on-column injection the PTV has a low thermal mass, so can be heated and cooled quickly. However, unlike cold on-column the sample is injected into an injector liner and not direct onto the column. Unlike conventional split/splitless injection, the aim is to make use of volatility selectivity of the system rather than to inhibit it. By selecting an appropriate starting temperature, the analytes condense in the liner; if desired the solvent may be flushed away through

the split line (e.g. in LVI mode) and then the injector is temperature programmed up to an appropriate isothermal temperature with the split vent closed. During this extraction period, desired components are vaporised and flushed onto the column. Once efficient extraction has taken place the split vent may be opened with a high flow and the injector programmed up to a high temperature to flush out any undesired matrix components.

A number of different temperature and split-vent programmes allow the PTV to be used in a number of different modes, including as a conventional split/splitless injector. What may seem surprising is the isothermal temperatures required to exclude components from or extract them onto the column. Hundred per cent extraction can be achieved at isothermal temperatures very much lower than the boiling point of the analyte. Figure 4.2 shows the complete exclusion and complete extraction isothermal temperatures for some high-boiling alkanes plotted against their boiling points at atmospheric pressure. Below the lower line all components are completely excluded from the column, while above the upper line they are extracted with 100% efficiency. By choosing a suitable temperature it is possible to discriminate between components of a mixture. As a rule of thumb, a difference in boiling points of ~70°C is sufficient to allow complete discrimination between two compounds in a sample. Where a smaller difference applies, a trade-off is required where extraction efficiency may have to be sacrificed in order to exclude another component sufficiently for the analysis.

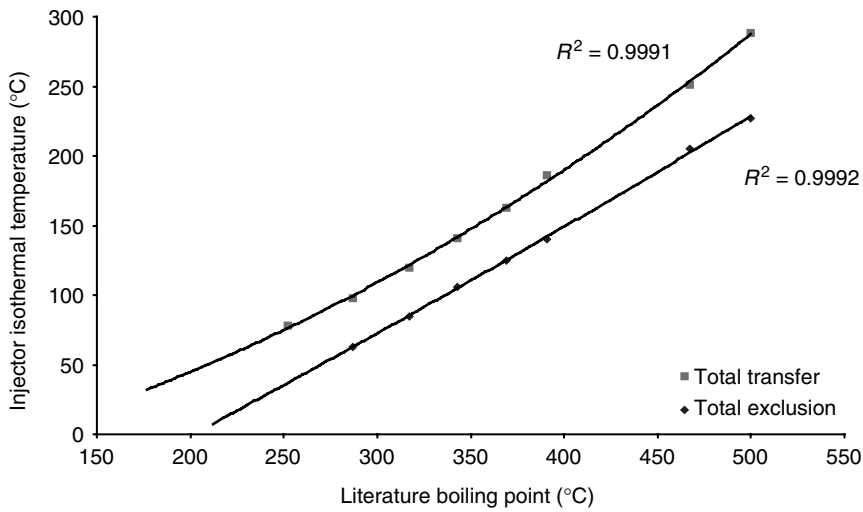


Figure 4.2 Selective exclusion and selective transfer temperatures for long-chain alkanes plotted against boiling points. Data supplied by and reproduced with permission of Anatune Ltd.

Despite the many capabilities of the PTV, there is often still a problem with the vast excess of drug substance when one is carrying out what is still a modified form of direct injection. Where this is volatile it can be excluded or

chromatographed. Indeed, one of the properties of this system is that, given an appropriate column, everything transferred from the injector onto the column is capable of being chromatographed. Hence contamination of the column is not a concern. However, in many cases pharmaceutical actives are polar and involatile, so the injector can suffer serious contamination due to condensed or degraded drug, with consequent deterioration in chromatographic performance. This may be less of an issue than for a conventional split/splitless injector, but problematical it remains.

4.4.1.5 Automatic injection liner exchange

Replacement or cleaning of injection liners can restore system performance, but if this must be done too frequently it can seriously impact on laboratory efficiency. Instrument manufacturers have now introduced systems that permit automatic liner exchange. Where necessary, after every few injections or, in extreme circumstances, after every injection, the liner can be replaced by a clean one without operator intervention. At present, commercially available liners are too expensive to be considered disposable, but some systems permit the inclusion of an insert into the liner that acts a liner within a liner. One disadvantage of automated liner exchange is that air is inevitably allowed into the injector. Since oxygen is a good electron-capture agent, this would lead to an enormous background signal if one were to attempt to combine this with electron-capture detection.

These liner exchange systems make feasible yet another analysis mode: direct thermal desorption (DTD). Here the liner or an insert is packed with the solid sample. The liner exchange system can then be used in place of a conventional autosampler. The liner is automatically inserted into the PTV and the volatiles thermally desorbed onto the column. Some analysts may feel uneasy about such desorption from the solid phase: how does one know that all of the volatile analytes have been released from the sample crystal lattice? However, where applicable, this approach may not be as difficult to validate as one might imagine. For instance, the PTV can be cooled after the analyte transfer, and then, at the end of the chromatographic temperature programme, reheated to repeat the process. Ideally all of the analyte should transfer in the first cycle and none in the second, demonstrating that complete desorption occurs in the method.

4.4.2 Detectors

4.4.2.1 Flame-ionisation detector

Despite its longevity, flame-ionisation detection (FID) remains the most universal and widely applicable detector available for GC. Any compound that is combustible (which includes almost all organic molecules) gives a signal. The response depends largely on the number of carbon atoms, so sensitivity will increase among homologous series for instance. Nevertheless, its simplicity of use and near universality makes it extremely versatile. Its lack of specificity can be both an advantage and, at some times, a disadvantage. Where one is measuring trace levels of toxic impurities in a large excess of drug, the response of the

main component can, if it chromatographs, give rise to significant interference. Nevertheless, capillary GC is an extremely high-resolution technique, so selectivity is not usually an issue. The FID is not as sensitive as some of the more selective detectors *for specific analytes*, so limitations in sample loading can impact on detectability of some trace components. In such instances, some of the more selective detectors may prove valuable. Li used a direct GC-FID method with large, splitless injections (5 μl) of high concentrations of drug (40 mg/ml) to measure methyl, ethyl and isopropyl methanesulfonates down to detection limits of 1 ppm relative to drug substance. (A chromatogram from standard solutions from this work is shown in Figure 4.3.) However, at these loadings it was necessary to replace the injection liner every 15–20 injections to avoid contamination impacting on recoveries [13].

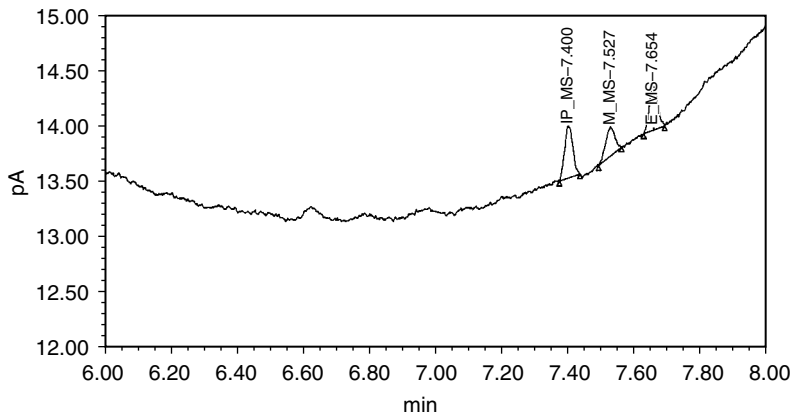


Figure 4.3 Chromatogram of a standard solution containing 0.04 μg/ml each of isopropylmethane sulfonate (IP_MS), methylmethane sulfonate (M_MS) and ethylmethane sulfonate (E_MS). Reproduced from [13] with permission from Elsevier.

4.4.2.2 Electron-capture detector

One of the next most commonly used detectors in GC is electron-capture detection (ECD). This is of particular relevance to the present consideration, because a significant proportion of the genotoxic analytes often encountered in the pharmaceutical industry are alkyl halides and halogens give a strong response to ECD. Sensitivity increases in the order Cl < Br < I. Figures 4.4–4.6 show the responses of a number of short-chain alkyl halide standards analysed by headspace GC-ECD, all analysed at the same concentration (selected to mimic 10 ppm relative to a 10 mg/ml solution of API). The alkyl chlorides give a very weak response, the bromides a stronger signal and the iodides all saturate the detector. Hence alkyl iodides give a very strong response, but molecules with a bromine or multiple chlorines can also yield very good sensitivity. If the drug molecule contains a strong electron-capturing atom then it will obviously give

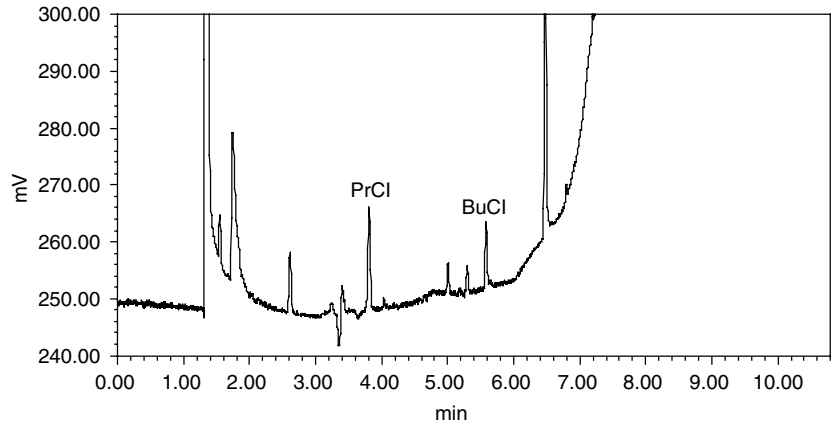


Figure 4.4 Headspace GC-ECD of some short-chain alkyl chlorides.

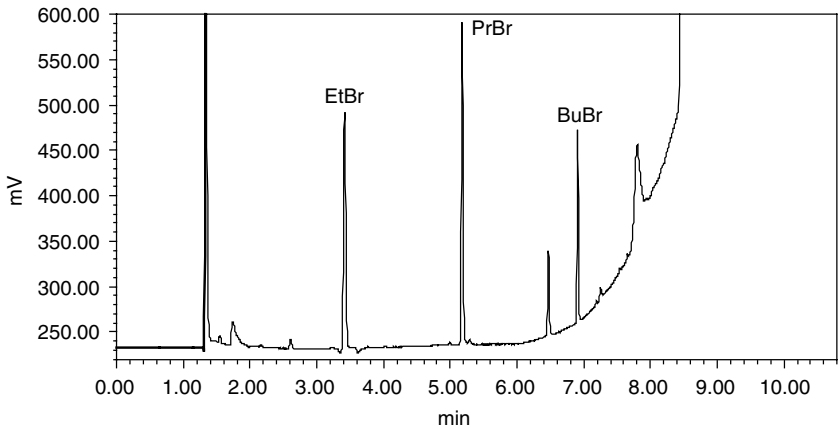


Figure 4.5 Headspace GC-ECD of some short-chain alkyl bromides.

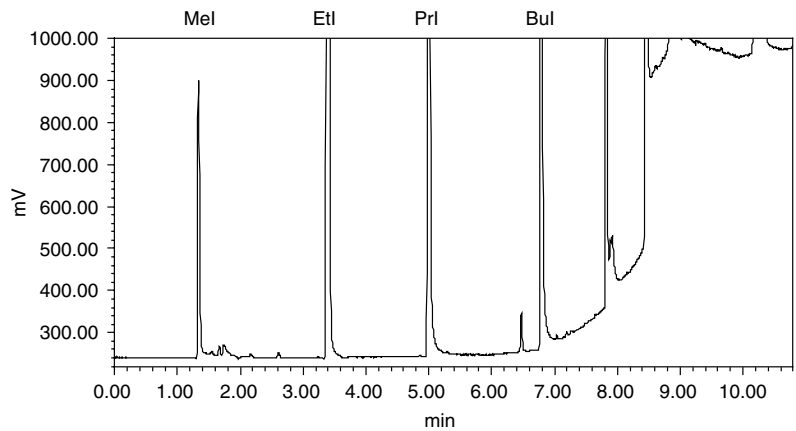


Figure 4.6 Headspace GC-ECD of some short-chain alkyl iodides. Reproduced with permission from S. Hamilton [27].

a response far in excess of that of the analyte. In these circumstances, the value of the ECD as a selective detector is lost, although its sensitivity remains, provided the drug does not interfere chromatographically.

4.4.2.3 Atom-selective detectors

Where the target analyte contains heteroatoms such as nitrogen, phosphorus and sulfur, atom-selective detectors can provide an ideal detection method. A number of examples appear in the literature of the use of a detector called a thermal energy analyser (TEA) for the measurement of *N*-nitroso compounds [14–17] and aromatic nitro compounds [18]. This has also been used as an HPLC detector [19, 20], and a modified TEA has been reported to be useful for analysis of amines and other nitrogen-containing compounds [17]. Unfortunately, this technique appears not to have gained in popularity, since no reports have appeared in the literature for over two decades.

4.4.2.4 Mass spectrometry

Mass spectrometry (MS) has many distinct advantages as a detector that counterbalances its cost. First, it gives excellent sensitivity for many organic molecules, especially if used in selected ion monitoring (SIM) mode. Ramjit, Singh and Coddington [9] used GC/MS in SIM mode to show detection of low ppm quantities of alkylsulfonates generated in the formation of a sulfonate salt of a pharmaceutical. Second, it can provide qualitative identification of a compound in scanning mode. The specificity of MS is unparalleled amongst GC detectors. With most instrument configurations, by focusing on a specific m/z ratio (or a sequence of ratios) only the ions of interest are transmitted to the detector. Hence, even if much higher levels of other components are present in the sample, their responses can be effectively switched off if they do not give ions of the appropriate mass. Of course, one must take care to avoid the main peak in the sample exactly co-eluting with the compound of interest; otherwise ion suppression might take place owing to space-charge effects in the ion source. This could seriously inhibit the response of minor components under the main peak, leading to their underestimation.

Where 'chemical noise' (background signal at the desired m/z ratio and chromatographic region from the main peak, other impurities or components in the blank) interferes with the measurement of an analyte, MS/MS can provide a means of removing this. Instead of SIM, triple quadrupole instruments can be used in selected reaction-monitoring (SRM) mode. Here the first mass analysis segment of the instrument (Q1 in triple quadrupoles) selectively transmits a single precursor ion (typically the molecular ion). This is then fragmented in a collision region and the second mass analysis segment (Q3) is set to transmit a specific product ion. The instrument can be switched between different precursor–product ion combinations to investigate multiple analytes or obtain multiple responses for identity confirmation. Lower overall signal may result from MS/MS, but by removing the response from unrelated background interferences, better signal-to-noise may be obtained.

The most common ionisation mode used for GC/MS is electron ionisation (EI), sometimes alternatively described as electron impact ionisation. Here, the compound is vaporised into the ion source. Electrons are emitted from a heated filament and accelerated to a kinetic energy of normally 70 eV through the sample vapour. This is much higher than the ionisation potential of organic compounds, so interaction of the sample molecules with electrons results in ionisation by loss of an electron.

$$M \xrightarrow{e^- \ 70\ eV} M^{+\cdot} + e^-$$

The result is a radical cation, M^+, the molecular ion. Depending upon the magnitude of the interaction of the molecules with the high-energy electron, additional internal energy may be imparted into them. In some cases, this may be sufficient to cause fragmentation

$$M^{+\cdot} \longrightarrow F^{+\cdot} + n$$

$$M^{+\cdot} \searrow F^{+} + n\cdot$$

where F is a fragment ion and n is the neutral lost. In the upper scenario, the fragment is a radical cation. The lower case involves an even-electron ion and radical neutral loss. Even-electron ions tend to be more stable than radical ions, so they are less prone to further fragmentation than the radical cations. Furthermore, the even-electron rule states that an even-electron ion is unlikely to fragment by loss of a neutral radical to form a radical fragment ion (since both are likely to be less stable than the precursor). Hence, even-electron ions tend to fragment by rearrangement rather than homolytic cleavages.

The resulting spectrum usually contains the molecular ion plus a number of fragment ions, since a spread of internal energies results from different degrees of interaction of sample molecules with the electron beam. However, labile compounds may fragment readily and give no observable molecular ion. When this is the case, chemical ionisation (CI) may be preferred, since molecular weight information is often more analyte specific than fragment ion m/z ratios.

In CI, a reagent gas is present in the ion source, which is ionised by the electron beam (usually somewhat lower energy than that for EI)

$$R \xrightarrow{e^-} R^{+\cdot} + e^-$$

where R is the reagent gas molecule. Ion–molecule reactions lead to the formation of even-electron ion species such as CH_5^+ for methane or NH_4^+ for ammonia. These then are able to protonate the sample molecule by gas-phase acid–base chemistry, for example,

$$NH_4^+ + M \longrightarrow [M + H]^+ + NH_3$$

In order for the molecule M to be ionised by protonation, it must have a higher gas-phase proton affinity than the reagent gas molecule R. When ammonia is the reagent gas, then this only applies to basic molecules. If methane or other less basic gas is used then more compounds are amenable to ionisation by protonation. However, adduct formation may also occur

$$M + Cat^+ \rightarrow [M + Cat]^+$$

where Cat^+ is a cation, provided M is polar enough to form a non-covalent adduct with a cation (e.g. NH_4^+). The ionisation process is much less energetic than in EI, so resulting ions tend to retain lower internal energies and thus fragment less readily. One is therefore much more likely to obtain molecular weight information from CI.

It should be noted that CI invariably generates even-electron ions, so fragmentation channels involving radical fragments and neutrals are unlikely. At times these might give useful structural information. One disadvantage of CI is that the spectra obtained are highly dependent upon the ion source conditions (source temperature or reagent gas pressure) and so can be much more variable than EI spectra, both between instruments and even in day-to-day operation on the same instrument.

The cheapest bench-top GC/MS systems consist of EI-only ion sources with small single quadrupole mass filters. These are appropriate to many applications, but some molecules fragment excessively under EI conditions, yielding less-specific low-mass fragment ions. In these cases CI may produce less fragmentation and thus better specificity for the desired compound (such as by yielding intact $[M + H]^+$ or $[M - H]^-$ ions). Instruments capable of CI require higher specification pumping systems than EI-only spectrometers, so inevitably cost more.

As indicated above, the effect of the main peak on the ionisation of co-eluting components must not be ignored. One must not imagine that MS/MS is a cure-all for this situation. Suppression in the ion source will reduce the number of analyte ions produced and thus the sensitivity of any MS method.

4.5 High-performance liquid chromatography

4.5.1 Separation modes

4.5.1.1 Normal phase

Normal phase (NP) was the original mode of high-performance liquid chromatography (HPLC) reported. This uses a polar stationary phase (e.g. silica, alumina) and non-polar mobile phase. It can also be referred to as adsorption

chromatography, since analytes are involved in adsorption/desorption interactions with the surface of the stationary phase. Because retention is based on polarity, it is well suited to non-polar to moderately polar molecules. Highly polar compounds can undergo very strong or effectively irreversible binding with the stationary phase. When using silica columns the degree of water saturation of the mobile phase can make a large impact on the chromatography achieved. Bonded polar phases available (such as cyanopropyl-derivatised silica) are more reliable in this respect, but typically with less retention than bare silica.

4.5.1.2 Reversed phase

Given that most pharmaceutical compounds are quite polar, often with acidic or basic groups, normal phase has become less popular in the industry. Instead, reversed-phase (RP) HPLC is much more widely used. This uses a stationary phase that is less polar than the mobile phase, which always contains water as a component. The stationary phase is usually a non-polar functionality (such as an alkyl group) chemically bonded to the surface of silica particles, although many other formats are in use. Sample components are injected into the mobile phase, and these partitions between the mobile phase, and the non-polar bonded phase. Hence this is a form of partition chromatography. It is much better suited to polar compounds than normal phase, since these readily partition into the polar mobile phase. Retention is largely determined by hydrophobicity, although other effects have an influence, such as ion-exchange interactions of basic molecules with ionisable, underivatised silanols ('residual silanols') on the surface of the silica.

4.5.1.3 Hydrophilic interaction chromatography

Where RP-HPLC tends to be less successful is when analytes are very polar and non-hydrophobic. These tend to be completely unretained, even with weakly eluting, highly aqueous mobile phases. Indeed, such highly aqueous mobile phases can give rise to wettability problems with the stationary phase, which can result in retention variability. In recent years, column manufacturers have made great strides in producing columns that are less susceptible to these problems, but retention of these analytes remains an issue in many cases. In these situations, an alternative separation method is hydrophilic interaction chromatography (HILIC). In HILIC, a polar stationary phase (such as silica, aminopropyl silica or ion-exchange phases) is used with an organic mobile phase (usually acetonitrile) with a small amount of water, typically 1–30%v/v. The water is believed to form a layer on the surface of the stationary phase, into which polar analytes can partition. Extremely good retention can be achieved for very polar compounds with this mode of chromatography. Indeed, some compounds that are used as void markers in RP-HPLC, such as uracil, can give good retention in HILIC. It has been described as 'reversed reversed-phase', since greater elution strength is obtained with higher water contents. What must be borne in mind with HILIC is that the sample diluent must not be too aqueous, or its water content can overwhelm that of the eluent, resulting in lack of retention [21]. With many polar

molecules this poses something of a problem, since often high water content may be necessary to fully dissolve the polar analytes to which the separation mechanism is suited. This is probably the main factor reducing the scope of HILIC. Where analysis of trace toxic impurities is concerned, solubility in organic, typically acetonitrile-based diluents, may be sufficient at the levels at which they are present, provided strategies can be used to fully dissolve the drug molecule to ensure good recoveries. In the current context, analytes like purine and pyrimidine bases and aromatic amines might be suitable for the HILIC approach.

4.5.1.4 Ion chromatography

Another useful chromatographic technique is ion-exchange chromatography. The stationary phase uses ionic groups such as sulfonates (strong cation exchange) or quaternary ammonium species (strong anion exchange) to form ion pairs with ionic analytes (such as ionised bases or acids, respectively). The eluent contains an ionic species that forms a stronger ion pair with the stationary phase, thus displacing the analyte and causing elution. Where an analyte is ionic this can provide a useful means of obtaining retention, selectivity and separation where RP-HPLC might fail. One might consider using this approach in conjunction with a derivatisation scheme that generates ionic species (see Section 4.9.2).

4.5.1.5 Flow injection analysis

Sometimes it is not necessary to use the selectivity of a chromatographic technique. Sensitive analysis can sometimes be achieved with selective detection in flow injection analysis (FIA). Whilst some of the detectors described below may be appropriate in themselves in favourable cases, in most cases more sophisticated detection regimes are necessary, such as post-injection derivatisation of the analyte. Strategies involving some of the derivatisation methods outlined in Section 4.9.2 may be considered.

4.5.2 Detection techniques

4.5.2.1 UV/visible detection

The most commonly used detector in HPLC is UV/visible absorption spectroscopy. This is not due to it being an ideal detector, but simply because it is currently the most generally applicable. Its prominence is due to the lack of a more universal and sensitive HPLC detector rather than any great merit in its own right. In its favour it is accepted by regulatory agencies for quantitative analyses, and most main peak assays and impurity profiles submitted in dossiers entail LC-UV data. It is reasonably sensitive, although this of course depends upon the presence and nature of any chromophore in the molecules being analysed. In favourable cases, LC-UV may be sufficiently sensitive to measure trace impurities. However, generally more sensitive detection modes are required, unless some form of analyte pre-concentration is used.

4.5.2.2 Fluorescence detection

Fluorescence detectors can be more sensitive, but have a much narrower applicability. Only a small proportion of organic molecules exhibit natural fluorescence. One may choose to derivatise samples with a fluorescent or fluorogenic agent, but this adds to the complexity of the analysis and the validation required.

4.5.2.3 Electrochemical detection

Electrochemically active functional groups are more common in organic molecules. Amines, alcohols and other functionalities may be oxidised to give a response using coulometric or amperometric detection. Thiols will oxidise, but at a voltage that tends to give a significant response from oxidation of the eluent. Here, integrating amperometry can be used.

Whilst many functionalities may be reduced, reductive HPLC detectors are less commonly used. Another form of electrochemical detection is conductivity, which is useful when an analyte is ionic. Hence, it is often used in conjunction with ion-exchange chromatography (see Section 4.5.1.4).

4.5.2.4 Chemiluminescence detection

Another option that might be considered is the detectors based on chemiluminescence. Chemiluminescence nitrogen and sulfur detectors (CLND and CLSD, respectively) have shown some distinct advantages. These are that the compound does not need to have a chromophore and that the response is proportional to the number of nitrogens (or sulfurs) in the molecule. Hence, where the structure is known (which is inevitable in target compound analysis) quantification can be done without standards of the specific analyte. Sensitivity of 0.6 ng nitrogen is quoted by the manufacturers. Under achievable drug injection parameters for HPLC (typically 20 ul of a 10 mg/ml solution for trace analysis), for an analyte molecule of molecular weight 300 containing a single nitrogen this would correspond to a limit of detection (LOD) of several tens of parts per million (ppm) relative to the drug. Whilst this might be sufficient in some cases, in general it would not. One must also consider the stipulation that the complete HPLC system must be free of nitrogen-containing compounds, including acetonitrile, one of the most commonly used eluent components. In practical terms, this may mean an instrument that has never been used with acetonitrile. It would appear that this approach would only be applicable to genetic toxin analysis in very limited, highly favourable cases.

4.5.2.5 Evaporative light scattering detection

The evaporative light scattering detector (ELSD) may have a role in this field. The response is highly dependent upon the size of analyte particles formed during evaporation of the mobile phase in the interface with the HPLC. Hence, it is hard to predict how sensitive it will be for a specific compound. Furthermore, volatile

compounds may be lost in the interface along with the eluent. Another important consideration is that concentration response is non-linear. Hence, one must construct a calibration curve for quantitative purposes. This ought not to impact on its use for limit tests, however. The author is unaware of any literature applications of ELSD for genetic toxins in drugs.

4.5.2.6 Mass spectrometry

Undoubtedly, mass spectrometric detection has a substantial role to play in condensed-phase chromatographic analyses of toxic impurities. As in GC/MS, it can be highly sensitive, although this is probably more analyte-specific than in GC/MS. Selectivity can be gained by SIM on single quadrupoles or, if necessary, SRM on MS/MS instruments. What must be considered is the appropriate ionisation mode to be used in LC/MS. Most modern instruments use atmospheric pressure ionisation sources, including electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI) and more recently atmospheric pressure photoionisation (APPI).

ESI is a very 'soft' ionisation technique, meaning that very little excess energy is imparted into the molecule during the ionisation process and therefore producing little fragmentation. The prerequisite is that the analyte molecule M has to be basic enough to be protonated

$$M + B - H \rightarrow [M + H]^+ + B^-,$$

and acidic enough to be de-protonated

$$M + B \rightarrow [M - H]^- + BH^+$$

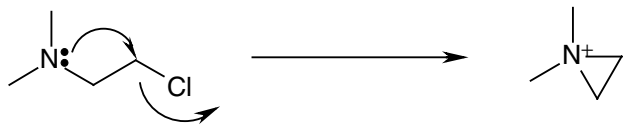
or have sufficiently polar functionality to form a stable adduct with either a cation or an anion

$$M + Cat^+ \rightarrow [M + Cat]^+$$

$$M + An^- \rightarrow [M + An]^-$$

where B is a basic component in the eluent, Cat^+ is a cation and An^- is an anion. Whilst a number of mechanisms occur, molecules that are ionised in one of these ways *in solution* tend to give good sensitivity in ESI, whereas those that are not likely to give rather poor response, if any. Compounds that are already ionic (e.g. quaternary ammonium ions) give excellent responses on ESI.

ESI LC/MS has been shown to be effective in measuring *N,N*-dimethylaminoethyl chloride as its related dimethylaziridinium species in drug samples [22].



N, N-dimethylaminoethyl chloride Dimethylaziridinium

The nitrogen on the *N,N*-dimethylaminoethyl chloride is readily protonated to form a detectable species and the dimethylaziridinium is ionic, so it gives an extremely good response (see Figure 4.7). Clarke demonstrated LC/MS methods for analysing some synthetic intermediate alkyl bromides [23]. Other trace impurities in pharmaceuticals have been detected using electrospray LC/MS [24, 25]. A column-switching approach has been shown to effectively remove the suppression effects of the API in measuring trace components in pharmaceuticals [26].

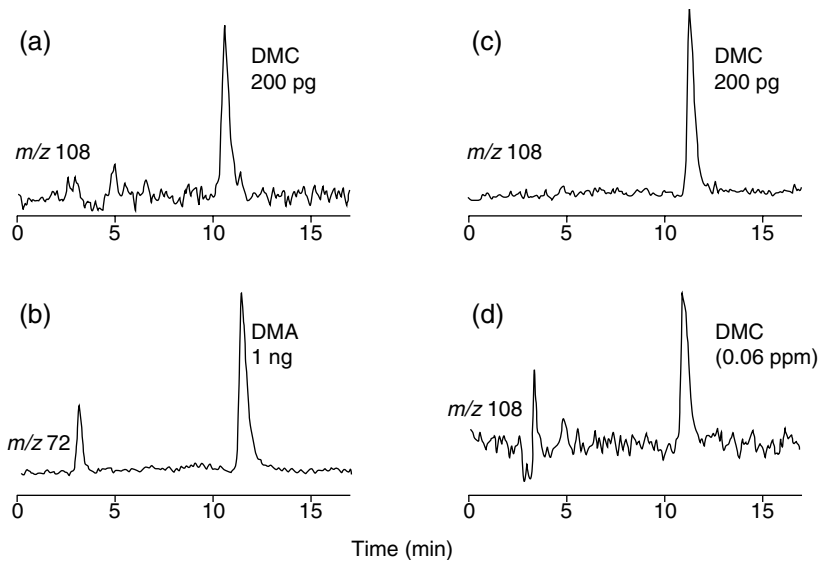


Figure 4.7 Chromatograms from selected ion monitoring: (a) *N,N*-dimethylaminoethyl chloride (DMC), 200 pg corresponding to 0.2 ppm, operator 1; (b) dimethylaziridinium (DMA), 1 ng corresponding to 1 ppm, operator 1; (c) DMC, 200 pg, operator 2; (d) batch of diltiazem hydrochloride estimated to contain 0.06 ppm DMC (operator 1). Normal day-to-day variation in retention times is illustrated; DMA elutes later than DMC (Rs = 1.6). Reproduced from [22] by permission of the Royal Society of Chemistry.

APCI also relies on protonation, deprotonation or adduct formation, but these occur in the gas phase. Water and/or alcohols form the protic solvents that mediate the ionisation process. Thermal energy is imparted into the eluent to desolvate the solute molecules, and fewer collisions occur in the gas phase to dissipate internal energy than in solution. For this reason it tends to be less suitable than

ESI for labile compounds and a higher degree of fragmentation tends to occur. However, in both ESI and APCI, invariably the more stable even-electron ions are formed by ionised species. An interesting approach to alkyl halide analysis is the use of negative ion APCI. In this ionisation mode, a major fragment ion is the halide anion, I^-, Br^- or Cl^-. Selected ion monitoring of *m*/*z* 127, 79 and 35, respectively, gives rise to a response for compounds containing the appropriate halogens. Of course, this is not specific to individual compounds, so chromatographic speciation must be used. Figure 4.8 shows LC/MS using negative ion APCI of some simple alkyl iodide standards with good sensitivity [27]. Hence for alkyl iodides that are stable enough to chromatograph under RP-HPLC conditions, this might form a suitable technique for their analysis. Bromides and chlorides give successively lower response, so this approach is probably not suitable for trace analysis of these compounds in API.

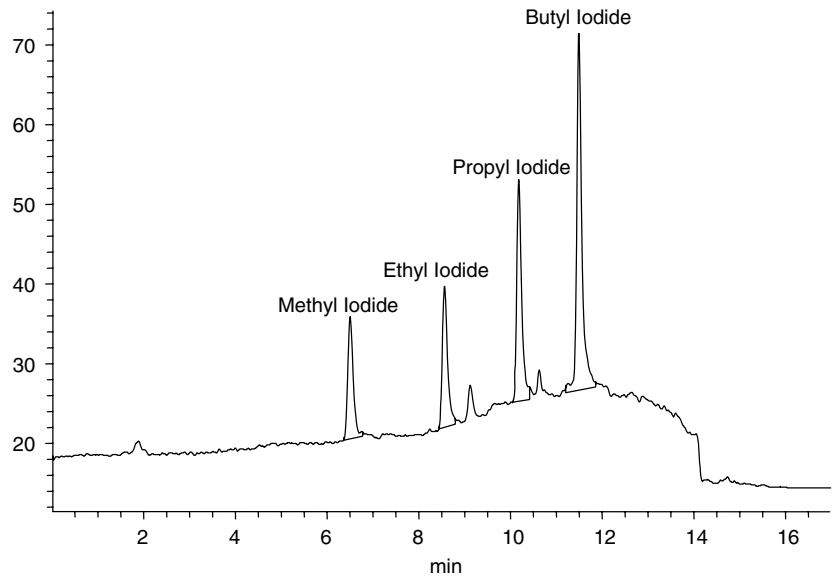


Figure 4.8 LC/MS using negative ion APCI of alkyl iodide standards. Sample concentrations of 50 ng/ml were used. Reproduced from [27] with permission.

APPI is a relatively recent development compared with the other techniques. Here, ionisation is achieved photochemically, either directly or mediated by a dopant such as acetone added to the eluent. Both even- and the less stable odd-electron ions (e.g. $M^{+\cdot}$) may be formed. At the time of writing, the mechanisms involved and scope of the technique are still not fully understood. What is apparent is that it provides a complementary technique to ESI and APCI.

4.6 Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) is an intermediate chromatographic technique between GC and HPLC. It depends upon the fact that when a fluid becomes supercritical (both the temperature and pressure are at or above its critical point) it develops some of the solvating properties of a liquid whilst retaining the low viscosity of a gas. Hence, mass transfer (essential to efficient chromatography) is more akin to that of GC than HPLC, but many compounds can be chromatographed at temperatures much lower than what would be required by GC, so some thermally labile compounds are amenable to SFC where they would degrade under GC conditions [28].

Typically, supercritical carbon dioxide is used as the basic eluent constituent. Methanol is often added to increase the solubility of more polar molecules. Other solvents such as water or ammonia can be used, but their critical points are at significantly higher temperatures and pressures than CO_2.

GC-type capillary columns and conventional packed HPLC columns may be used. Modified GC and HPLC instrumentation is used respectively, such that the eluent can be maintained above the critical point throughout the chromatographic system. One of the advantages of SFC is that many of the detectors from both GC and HPLC are compatible. Hence SFC-FID is common, giving the near universality of FID when analysing compounds that are not amenable to GC.

SFC has been used as a technique to analyse nitroaromatics in aqueous environmental samples [29]. It was necessary to use sample pre-concentration methods (see next section) to obtain suitable detection limits.

4.7 Thin-layer chromatography

A few publications cite the use of thin-layer chromatography (TLC) for analysis of alkylating agents [30–32]. There are, of course, advantages in principle in applying TLC to trace analyses: the whole sample is observed, disposable layers allow minimal sample clean-up for dirty samples, and a plethora of derivatisation techniques are available that can be tailored to visualisation of the target analytes. However, in the references cited, which report use of NBP as a derivatisation agent (see Section 4.9.2 for more information), either these were for qualitative purposes or detection limits in the region of 1 μg were quoted. Clearly, at these levels very large sample amounts would be required to determine low ppm levels of analyte relative to API and significant sample pre-concentration would be required to enable realistic loadings onto TLC plates. Preliminary investigations by the present author did not give grounds for optimism that TLC using this derivatisation approach at least would provide a practical approach for trace impurities in pharmaceuticals [33].

4.8 Sample pre-concentration

Where none of the above techniques is capable of providing a sufficiently low limit of detection (LOD), it may be necessary to pre-concentrate the analyte relative to the drug substance as part of the method. Alternatively, a cheaper, less powerful detector may be suitable for the analysis given this enrichment. This section describes various approaches.

4.8.1 Liquid–liquid extraction

Liquid–liquid extraction (LLE) is widely used in many aspects of organic chemistry to purify materials. It relies on the compounds to be separated having different partition coefficients between two immiscible solvents, such that the compound of interest preferentially partitions into one layer relative to the other component. When separating a very polar compound from a very non-polar one, this can yield extremely efficient separation. In less favourable circumstances, only a partial pre-concentration is achieved. Sometimes repetitive extractions can provide the desired pre-concentration, but with compounded losses and thus reduced sensitivity.

Table 4.2 summarises a selection of solvent pairs that are sufficiently immiscible to form biphasic mixtures suitable for LLE. Klick used LLE as one step in a method to measure an epoxide degradant of a pharmaceutical [12]. In an application of SFC to environmental analysis, LLE was found to give higher extraction efficiencies than solid-phase extraction (SPE – see below) [29].

Table 4.2 Polar and non-polar solvent combinations suitable for liquid–liquid extraction

Polar solvent	Non-polar solvent
Water, aqueous buffers	Alkanes: pentane, hexane, heptane, cyclohexane
Water, aqueous buffers	Chlorinated solvents: chloroform, dichloromethane, 1,2-dichloroethane
Water, aqueous buffers	Ethers: diethyl ether, methyl-*t*-butyl ether, diisopropyl ether
Water, aqueous buffers	Esters: ethyl acetate, butyl acetate
Water, aqueous buffers	Long-chain alcohols: butanol, octanol
Water, aqueous buffers	Aromatics: benzene, toluene, xylene
Dimethylsulfoxide	Alkanes: pentane, hexane, heptane, cyclohexane
Dimethylsulfoxide	Diethyl ether
Dimethylformamide	Alkanes: pentane, hexane, heptane, cyclohexane
Dimethylformamide	Diisopropyl ether
Methanol	Alkanes: pentane, hexane, heptane, cyclohexane
Acetonitrile	Alkanes: pentane, hexane, heptane, cyclohexane

4.8.2 Solid-phase extraction

Solid-phase extraction (SPE) is widely used in environmental and biomedical analysis. A wide range of stationary phases are available, including a variety of reversed-phase, normal-phase, ion-exchange and mixed modes. These are packed into plastic or glass cartridges that are normally disposable. The technique can be seen as a low efficiency equivalent of HPLC, since the solvent flows are generated by gravity, moderate over-pressure or moderate vacuum. Unlike HPLC, gradients are generated step-wise rather than continuously. Also, the solvents permissible in the mobile phase in SPE are not as restricted as those with the compressibility, volatility and UV absorption characteristics required for HPLC.

SPE generally consists of the following operations:

1. Rinse the SPE cartridge with a strong solvent to remove adsorbed impurities.
2. Wash the cartridge with a solvent suitable for initial conditions.
3. Load the sample.
4. Wash off any unwanted fast-eluting impurities.
5. Dry the cartridge.
6. Elute the required analyte and collect fractions.
7. Analyse the fractions as required.

Depending upon the application, steps 4 and/or 5 may not be required. By careful selection of eluting solvents the compound to be measured may not only be enriched relative to the drug, but may also be concentrated relative to the original loading solution, thus enhancing sensitivity.

4.8.3 Solid-phase microextraction

Solid-phase microextraction (SPME) is effectively a miniaturised version of SPE. Instead of using a packed cartridge, a rod is typically used, which is coated with the stationary phase. This is dipped into a solution of the analyte and allowed to extract for a pre-determined period of time. After this incubation period, the rod is removed from the solution and may be inserted directly into the injection system of the GC or HPLC. All of these operations can be automated on an autosampler. Clearly, the success of this technique depends intimately on the affinity of the analyte for the stationary phase. Frost, Hussain and Raghani [34] used SPME with GC-FID to measure benzyl chloride and chloroethylmethyl ether (amongst other process impurities) in pharmaceutical preparations.

4.8.4 Liquid-phase microextraction

More recently, a miniaturised version of liquid–liquid extraction has been developed, known as liquid-phase microextraction (LPME). Two main forms of this

technique have been reported. In the first of these, a single drop of extracting solvent is suspended from an injection syringe into the sample solution and allowed to extract the analyte of interest. This is then withdrawn back into the syringe and injected onto the chromatographic system. Stirring may be used during the extraction phase, which may take tens of minutes.

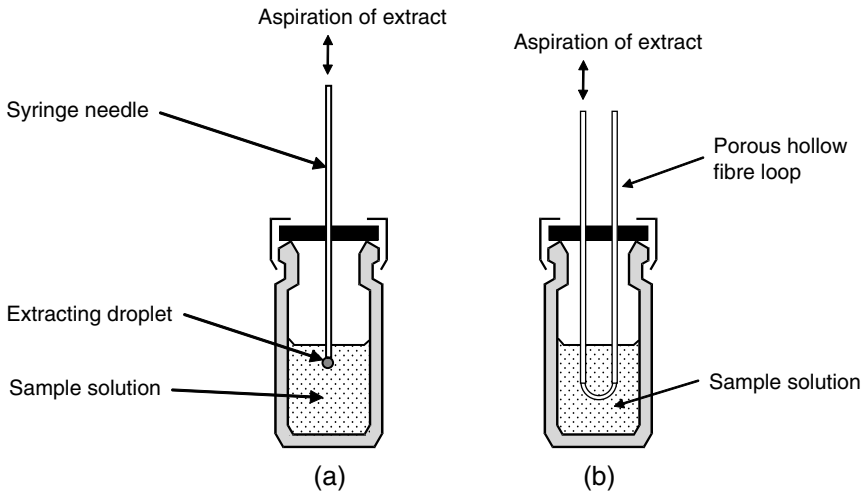


Figure 4.9 Two modes of liquid phase microextraction. (a) Suspended drop method and (b) membrane loop method.

The second variant of LPME uses a disposable, porous, hollow microfibre. In this case, the sample solution is placed in a vial equipped with a modified lid. A loop of microfibre passes in and out of the lid, such that the loop dips into the sample solution in the vial. The extraction solvent is injected into the capillary fibre from the outside. Again, stirring may be used. After an equilibration period, where solutes are able to traverse the fibre wall into the extraction solvent, the contents of the capillary are withdrawn and injected onto the chromatographic system. The two forms of this technique are illustrated in Figure 4.9.

The majority of the applications of LPME have been analysis of drugs in biofluids [35–40] or of pollutants in water samples [41–45]. However, it has been successfully applied to the analysis of triphenylphosphine oxide (TPPO) as an impurity in pharmaceuticals [46].

It is now possible to purchase commercially available systems for achieving something akin to LPME. In this instance, instead of a capillary microfibre, the extraction solvent is contained in a porous insert that sits inside the sample vial. This can be sampled automatically using the autosampler syringe after an appropriate incubation time.

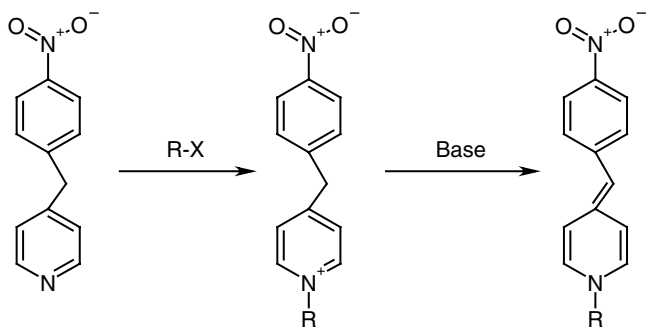
4.9 Other techniques

4.9.1 Electrochemical measurements

Barek *et al*. have reported on the determination of *N*-nitroso compounds, azo compounds, heterocyclics, aromatic nitro compounds, heterocyclic amines and even benzyl chloride using electrochemical methods such as voltammetry and polarography. The nitro and *N*-nitroso compounds work particularly well in reductive mode [47, 48]. For appropriate analytes, adsorptive stripping voltammetry and anodic stripping voltammetry can give orders of magnitude lower detection limits than are available from HPLC with electrochemical detection [48].

4.9.2 Derivatisation methods

By far the most commonly used reagent for detecting and measuring alkylating agents is 4-(4′-nitrobenzyl)pyridine (NBP) [49–53]. This reacts in the following way:



where R is the alkyl group and X the leaving group of the alkylating agent. Initially the pyridine ring is alkylated. Addition of base removes a benzylic proton and results in conjugation of both ring systems with one another. The product is highly coloured and can be detected spectrophotometrically. This technique has been applied to measuring alkylating compounds in environmental and biomedical research [54, 55], in the latter case the measurement of antineoplastic anti-cancer drugs *in vivo* in particular. It has also featured in patents for field test kits for measuring mustard gas in military applications [50, 56–58]. Whilst this has typically been applied in non-chromatographic, spectrophotometric measurements, there are some references to using NBP as a derivatisation reagent in TLC [30, 32, 59]. In another example, the authors allude to its use as a derivatisation approach in combination with HPLC for the measurement of impurities in pharmaceuticals, although no details are given [60].

There are some issues with using this reagent. Some authors have commented on problems with this method, principally due to the instability of the product [55].

Hence the response can be short-lived. Since the rate of the alkylation step is highly dependent on the reactivity of the alkylating agent, weak alkylators only reach a very low concentration of the quaternary intermediate in the timescale of the reaction, so sensitivity is not good in these cases. Indeed, one is not measuring the concentration of the analyte, but a combination of its concentration and reactivity or *alkylating potential* [49, 61]. What is not clear from the literature is whether NBP derivatisation provides a sensitive-enough approach to attain the very low detection limits required in trace toxic impurity determinations in pharmaceuticals. Attempts by this author to investigate NBP as a post-chromatographic TLC derivatisation approach for a number of alkylating agents showed the responses to be variable (largely dependent upon reactivity of the analyte) and falling far short of that required for a practical trace analytical method [33].

A number of other spectrophotometric reagents and combinations thereof have been used and compared with NBP: 4-pyridinecarboxaldehyde 4-nitrophenylhydrazone; 4-pyridinecarboxaldehyde 2-benzothiazolylhydrazone; 4-acetylpyridine 4-nitrophenylhydrazone; 4-acetylpyridine 2-benzothiazolylhydrazone [62]. *S*-Dodecylisothiouronium bromide has been used with primary and secondary alkyl halides [63]. 4-Nitrothiophenol has been claimed to be better than NBP, but only when used in combination with HPLC and not as a stand-alone reagent [64].

An alternative approach to getting around the difficulties with NBP might be to avoid using base completely, but instead measure the quaternary ammonium species using mass spectrometry. In this strategy, any quaternary ammonium ion would be expected to be highly sensitive in positive ion electrospray mode. Quirke, Adams, and Van Berkel refer to using *N,N*-dimethyldocecylamine to derivatise alkyl halides for use in this strategy. Tertiary phosphines and triethylamine were also examined [63].

Ehrsson and Hassan [65] used sodium iodide to derivatise the antineoplastic alkylating agent busulfan for biofluid analysis by GC-ECD. Other GC- and GC/MS-compatible derivatisations include tetrafluorothiophenol (TFTP), BSTFA, trifluoroacetic anhydride. Alternatively, a number of reagents have been used for LC-UV and LC-fluorescence applications: diethyldithiocarbamate, *p*-thiocresol, 8-mercaptoquinoline, *o*-phthalaldehyde (OPA), 4-(2-phthalimidyl) benzoyl chloride (for *N*-nitrosamines). A comprehensive review of separation, derivatisation and detection methods for antineoplastic agents was given by Paci *et al*. in 2001 [66] and this provides a useful reference for those looking for derivatisation approaches for alkylating agents.

What must be borne in mind with the majority of these examples is that these have invariably been measurement of alkylating agents in biomedical samples (biofluid analysis), food research or environmental analysis. There have been relatively few instances in the open literature of derivatisation of alkylators (or other genetic toxins) for determining quality of drugs. In one case, Lee *et al*. used sodium thiocyanate to react with methyl and ethyl methanesulfonates in headspace vials to generate, respectively, methane and ethane thiocyanates and isothiocyanates (Figure 4.10). These species are much more volatile than the

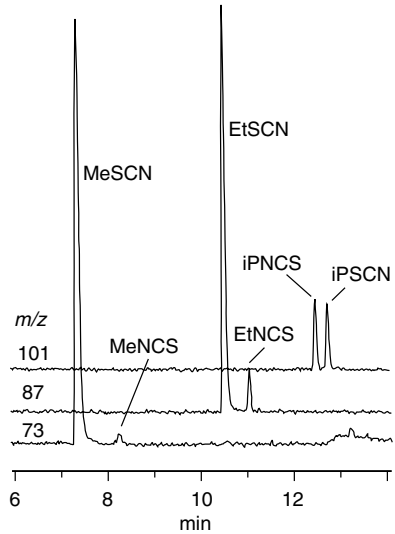


Figure 4.10 Selected ion-monitoring chromatograms of the thiocyanate and isothiocyanate derivatives of methyl, ethyl and isopropyl mesylates (final concentrations: 0.25 μg/ml). Reproduced from [60] by permission of the Royal Society of Chemistry.

sulfonate esters, so partition into the headspace much more readily. Low ppm levels of these alkyl sulfonates could be detected in drugs using this technique [60]. Of course, these sulfonates are fairly reactive alkylating agents. It is not clear if the same technique could be used for other, less-reactive methylating or ethylating agents.

4.10 Adapting analytical methods from fields beyond pharmaceuticals impurities analysis

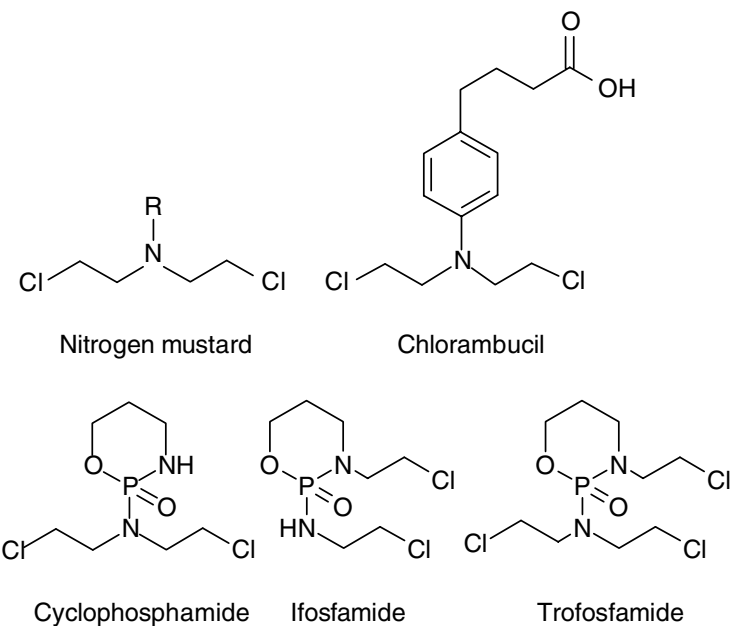
At the time of writing, there exists a relative scarcity of publications on the analysis of genetic toxins in pharmaceuticals, with only a few examples appearing in the recent literature. This is probably for a number of reasons. First, regulatory pressures, whilst having always been significant in this area, have grown considerably in the last few years. For example, by-products of salt formation, such as esters of sulfonic acids generated at trace levels by interaction of residual alcohols during the formation of sulfonate salts of drugs, have come under intense scrutiny of late. This has had the effect of focusing the industry into committing ever more resources into resolving such issues, often through the development of trace analytical methods.

The breadth of science that is undoubtedly taking place but has not yet been published does not aid a review of the field. Instead, one must look more widely. The analytical problems faced in doing such trace analysis are not unique to pharmaceutical analysis. The same analytes are often found in other areas, such

as environmental and biomedical analysis. Hence, one can learn from the extensive literature in these fields. The matrices in which the compounds are found are quite different from those relevant to the present study. In many cases these are more complex or the analyte is present at even greater dilution than is the case in drug analysis (or at least the perceived safe testing limits). Therefore, inevitably methodologies often employ extensive sample preparation techniques to clean up and/or concentrate the analyte. Such methods are generally of low resolution and depend upon the analyte having significantly different physico-chemical properties from the matrix. It must be recognised that whilst this is sometimes the case in pharmaceutical impurities testing, often it is not. Even when the analyte differs significantly from the drug substance, sample clean-up will inevitably differ from that used in the environmental or biomedical fields. Hence one is unlikely to find off-the-shelf methods from these disciplines for the present subject without adaptation. However, these may form a good starting point from which to develop a more suitable method.

4.10.1 Antineoplastic agents

One of the fields in which a considerable amount of analysis has been carried out is in the determination of antineoplastic agents in biofluids. These compounds are used as anti-cancer drugs, since they are highly cytotoxic and target cancer cells with some degree of selectivity. They vary in structure, but many contain the nitrogen mustard moiety, such as in the cyclophosphamide and its analogues, ifosfmide and trofosfamide. Such bifunctional alkylating moieties are able to cross-link DNA, causing irreparable damage to the genome.



Other compounds in the nitrogen mustard class are mechlorethamine, chlorambucil, phenylacetic acid mustard, melphalan, prednimustine, estramustine and oxazaphosphorine drugs. Alkyl sulfonates busulfan and treosulfan are members of another group of alkylating antineoplastic agents. Reference has already been made to the review by Paci *et al*. of separation methods for antineoplastic agents, which contains much information on the analysis of these compounds. It covers detection methods, including derivatisation strategies. The application was to analysis of biological fluids [66].

Another review covered metabolic studies on cylophosphamide, ifosfamide and trofosfamide. The analytical techniques covered were GC/MS, LC/MS and LC/MS/MS [67]. The same class of drugs has been determined in urine by GC/MS/MS with on-column injection and LC/MS/MS with LLE [68, 69]. Chiral derivatisation has been used in conjunction with HPLC to measure the ratio of cyclophosphamide enantiomers [70].

Alkyl halides, alkyl sulfonates and other alkylating agents have also been subject to scrutiny in spheres other than pharmaceuticals, such as in environmental analysis. Various approaches have included two-step SPE, derivatisation with trifluoroacetic anhydride followed by GC/MS (for cyclophosphamide and its analogues in sewage water); SPE on surface water to isolate the antineoplastic agents carmustine, chlorambucil, cyclophosphamide and melphalan for LC-UV and LC-fluorescence measurements; and derivatisation of alkyl halides and epoxides with 4-nitrothiophenol followed by HPLC-UV detection (claimed to be better than NBP derivatisation). A patent exists for a field test kit for mustard gases in military use based on NBP derivatisation.

4.10.2 Other fields

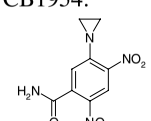
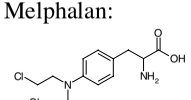
Space does not permit a comprehensive review of all potentially relevant fields of biomedical and environmental analysis. Instead, a selection of published applications is presented in Table 4.3.

4.11 Validation of trace analytical methods

Validation of analytical methods is important both from the standpoint of good science and to satisfy regulators of the reliability of results reported in dossiers. When approaching validation of any analytical method, one must always ensure that the method is fit for purpose. The amount of resource should be appropriate to the phase of development and the degree to which the process is defined and the methods finalised.

The first decision that must be taken when considering validation is whether the analyte should be quantified, or whether a limit test should be used. The latter involves significantly less validation and is recommended wherever possible. ICH Q2A requires only specificity and sensitivity for limit tests of impurities [81]. However, when one is considering trace impurities, scientific judgement

Table 4.3 Literature analytical approaches for genetic toxins in non-pharmaceutical applications

Compound	Matrix/ application	Sample preparation	Separation mode	Detection method	Comments	Reference
Iodoform; iodoacetic acid; 1-iodo-octadecane; 1-bromo-octadecane; 1-chloro-octadecane; ethyl methanesulfonate; ethyl *p*-toluenesulfonate; many other alkylating pollutants	Atmospheric particulate fractions	Derivatisation with NBP; 4-pyridinecarboxaldehyde 4-nitrophenylhydrazone; 4-pyridinecarboxaldehyde 2-benzothiazolylhydrazone; 4-acetylpyridine 4-nitrophenylhydrazone; 4-acetylpyridine 2-benzothiazolylhydrazone		Photometric		[62]
CB1954: 	Human plasma samples	Centrifugation	HPLC	UV		[71]
Bis-(2-chloroethyl)amine; bis-(2-chloroethyl)methylamine	Tissue homogenates	LLE, heat denaturation for biological samples NBP derivatisation		Photometric		[54]
Melphalan: 	Plasma samples	Column switching	HPLC	UV	Column switching to remove proteins	[72]

Alkyl sulfonates busulfan & treosulfan; aziridines ThioTEPA and TEPA; methylmelamine altretamine; N-mustards mechlorethamine, chlorambucil, phenylacetic acid mustard, melphalan, prednimustine, estramustine; nitrosoureas tallimustine, tauromustine, lomustine/carmustine; dacarbazine, temozolomide and mitozolomide	Biological fluids	Protein precipitation; LLE; SPE derivatisation methods: NaI; TFTP; DDTC; TFAA; *N*-acetylcysteine, 3-aminophenol; Bratton–Marshall reagent	GC, HPLC, LC, CE, TLC	Variety of detection methods	Review	[66]
Alkyl halides		Derivatisation methods including: *S*-dodecylisothiouronium bromide; *N*,*N*-dimethyldocecylamine; *t*-phosphines; thiourea; triethylamine; Mg/CO_2	LC	ESI MS		[63]
Chlorambucil	Plasma	LLE Na_2S derivatisation to form a thiazane	GC	MS		[73]

Continued

Table 4.3 *Continued*

Compound	Matrix/ application	Sample preparation	Separation mode	Detection method	Comments	Reference
N-mustards, aziridines		NBP derivatisation		Photometric	Comparison of chemical and biological activities	[74]
Cyclophosphamide, bis(2-chloroethyl)amine hydrochloride	Mouse blood	NBP derivatisation		Photometric	Derivatisation method modified to produce a stable product	[55]
Cyclophosphamide, ifosfamide, trofosfamide	Sewage water	Two-step SPE TFAA derivatisation	GC	GC/MS		[75]
Cyclophosphamide, ifosfamide, trofosfamide and metabolites	Metabolism		GC, LC	MS and MS/MS	Review	[67]
Cyclophosphamide, ifosfamide	Urine	LLE derivatisation with HFBA	GC (on-column inj)	MS/MS		[68]
Cyclophosphamide	Urine	LLE	LC	MS/MS		[69]
Carmustine, chlorambucil, cyclophosphamide, melphalan	Surface water	SPE	LC	UV, fluorescence		[76]

Cyclophosphamide enantiomers	Plasma	Chiral derivatisation	LC	UV		[70]
α-Bromobenzyl cyanide, *o*-chlorobenzalmalononitrile, α-chloroacetophenone, diphenylaminechloroarsine	Military and law enforcement irritant agents	Various derivatisation reagents, including NBP	TLC	Visual	For qualitative analysis	[77]
N- and *S*-mustards	Soil, vegetation, water or tissues	NBP derivatisation	TLC	Visual	Detection limits 'slightly less than a microgram'	[30]
1,3-Bis(2-chloroethyl)-1-nitrosourea and related mustard alkylating agents		NBP derivatisation		Photometric	Measuring alkylating activity	[78]
Bis(2-chloroethyl)sulphide, Bis[2-(2-ethylthio)ethyl]ether and other mustards	Warfare agents	Various derivatisation reagents		Spot test	Patent for field test kit	[56]
Benzyl bromide, 2,4-dinitrobenzyl bromide, chlorinated pyrimidines and other labile Cl-cntng cpds		NBP derivatisation	TLC	Visual		[32]
Thermally labile alkyl sulfonates			SFC	FID	Compounds difficult to analyse by GC or HPLC	[28]

Continued

Table 4.3 *Continued*

Compound	Matrix/ application	Sample preparation	Separation mode	Detection method	Comments	Reference
N-nitroso cpds, azo cpds, heterocyclics, aromatic nitro cpds, heterocyclic amines	Environment analysis	SPE		Electrochemical methods		[47]
Amino-PAHs, nitro-PAHs, *N*-nitroso cpds, azo cpds, benzyl chloride	Metabolism, environmental analysis			Polarography and voltammetry	Review	[48]
N-nitrosamines		HBr-AcOH with 4-(2-phthalimidyl)benzoyl chloride (pre-column derivatisation)	LC	Fluorescence	0.4–1.6 pmol per injection limits of detection	[79]
N-nitroso cpds	Biological matrices and foodstuffs		GC	Thermal energy analyser		[16]
Nitrobenzenes	Water samples	LPME	GC	ECD		[80]
N-nitrosomethylurea and other carcinogens	Food products incubated with nitrite under simulated gastric conditions	Column chromatography NBP derivatisation		Photometric	Measuring alkylating activity	[10]

NBP, 4-(4-nitrobenzyl)pyridine; TFTP, 2,3,5,6-tetrafluorothiophenol; DDTC, Diethyldithiocarbamate; BSTFA, bis (trimethylsilyl) trifluoroacetamide TFAA, trifluoroacetic anhydride; *o*-PA, *o*-phthalaldehyde; HFBA, heptafluorobutyric anhydride.

must be used. Low-level components may be subject to losses due to adsorption. Also, the more complex methods often required for trace analysis compared with conventional impurities methods may give rise to lower recoveries. Hence these issues must also be considered to confirm the accuracy of the method. If one chooses to quantify trace impurities, then linearity and precision must also be considered.

4.11.1 Sensitivity

The first consideration is the limit of detection (LOD) and/or limit of quantification (LOQ) required of the method. These will need to be low enough to be below a specification limit for the analyte to ensure that the level in the drug product is lower than that which would give rise to safety concerns for the patient. This will depend upon genetic toxicology data for the toxic analyte in question. In early development studies, such toxicology information may be limited, so advice from experienced toxicologists is crucial. In general, low- or sub-ppm concentrations of toxic analytes relative to drug substance will be necessary. However, it may be possible to base the limit on absolute dose to patient of the toxic component, especially when low-dose or high-dose pharmaceuticals are involved. In general, the LOD and/or LOQ will be one of the first parameters of the analytical method that will be tested to ensure suitability. If appropriate LOD or LOQ cannot be achieved, then an alternative method will need to be sought.

4.11.2 Specificity

It is important to ensure that the method will be specific for the analyte in question and not affected by responses from other impurities or artefacts of the method. Analysis of suitable blanks from solvents used and matrix components will determine this.

Specificity may be achieved through sample preparation, chromatographic selectivity, the selectivity of the detection method or combinations of these. It is often tempting to utilise the most selective detection method available (such as MS or MS/MS), since this can reduce the effort required in optimising the sample preparation and chromatography. However, whilst this is often the most expedient approach in early development, it may not always be suitable to transfer expensive, highly technical methods and instrumentation into a manufacturing environment if this is required.

4.11.3 Accuracy

One of the crucial factors that must not be ignored is the accuracy, or recovery of the method. It is not sufficient to show sensitivity and selectivity using just standard solutions. Recovery of the analyte from API or drug product (where appropriate) during the method must be sufficient to allow confidence in the method. This is particularly important when reactive compounds such as alkylating agents

are concerned. These may react with API, excipients, solvents or other components in the sample matrix or those used in the method, thus significantly reducing recovery. This may be of particular concern when sample heating is involved, such as in GC injectors or headspace systems. Also, extraction methods in sample preparation are invariably not 100% efficient.

The normal way of determining recoveries is to spike analyte standards into a blank sample matrix (one not containing the analyte) and compare these with standard solutions. The best way to obtain such a blank sample is to synthesise the API using a different route that cannot give rise to the impurity to be measured. Alternatively, a sample can be highly purified to eliminate the analyte. Such a blank sample may not be available, in which case standard addition may be required. Here, different levels of the analyte are spiked into the sample and the response can be measured from the slope of the resulting line. This can be compared with the results from standard solutions. Ideally, recoveries will be more than 80%, although in difficult analyses this may not always be achievable.

4.11.4 Solution stability

The stability of sample and standard solutions over the course of the experiments must be demonstrated to ensure that the results are not erroneous owing to degradation. Bracketing of standard and sample injections can also help in this respect.

4.11.5 Linearity and precision

Compared with conventional impurities measurements, trace analyses cannot be expected to achieve the same linearity and precision values. This is due to the lower signal-to-noise ratios inevitable at low levels. Hence, while the same approaches can be used, greater latitude will be necessary in the acceptance criteria. What must be demonstrated is that the data is statistically valid to show that the levels of toxic analytes are below their specification limits.

4.12 Conclusions

It will be observed from the above discussion that this field remains a challenging one, with difficult analytical methodologies facing pressure on resources within the industry and externally from regulatory bodies demanding ever more stringent testing regimes. There remains no simple solution to the many and varied problems analytical chemists face in dealing with toxic impurities. This is a challenging environment that demands creativity and resilience, but also offers many opportunities to seek new ways of working to bring greater efficiencies into this area.

The industry also has an important need to provide good science to regulatory agencies to support its viewpoint. It is only in cooperation that a reasoned and realistic position can be achieved.

Another sector that may be able to contribute to this field is the analytical instrument manufacturing industry. Many of the developments in analytical instrumentation addressed towards pharmaceuticals over the last several years have been focussed on supporting high-throughput chemistry and proteomics. However, very few developments have addressed by design the challenging issues discussed in this chapter. Again, pharmaceutical companies need to be influencing this industry sector to understand the problems faced and to see potential new markets for their technologies.

References

1. W. N. Choy, *Genetic Toxicology and Cancer Risk Assessment*, Marcel Dekker, New York, 2001.
2. W. K. Lutz and A. Kopp-Schneider, Threshold dose response for tumor induction by genotoxic carcinogens modeled via cell-cycle delay. *Toxicological Sciences,* 1999, **49**(1), 110–115.
3. W. K. Lutz, S. Vamvakas, A. Kopp-Schneider, J. Schlatter and H. Stopper, Deviation from additivity in mixture toxicity: relevance of nonlinear dose-response relationships and cell line differences in genotoxicity assays with combinations of chemical mutagens and g-radiation. *Environmental Health Perspectives Supplements,* 2002, **110**(6), 915–918.
4. W. Slikker Jr., M. E. Andersen, M. S. Bogdanffy *et al*. Dose-dependent transitions in mechanisms of toxicity. *Toxicology and Applied Pharmacology,* 2004, **201**(3), 203–225.
5. J. G. Hengstler, M. S. Bogdanffy, H. M. Bolt and F. Oesch, Challenging dogma: thresholds for genotoxic carcinogens? The case of vinyl acetate. *Annual Review of Pharmacology and Toxicology,* 2003, **43**, 485–520.
6. D. Y. Lai and L.-T. Woo, Structure-activity relationships (SAR) and its roles in cancer risk assessment, in *Genetic Toxicology and Cancer Risk Assessment*, ed. W. N. Choy, Marcel Dekker, New York, 2001, pp. 73–91.
7. R. D. Snyder, G. S. Pearl, G. Mandakas, W. N. Choy, F. Goodsaid, I. Y. Rosenblum, Assessment of the sensitivity of the computational programs DEREK, TOPKAT, and MCASE in the prediction of the genotoxicity of pharmaceutical molecules. *Environmental and Molecular Mutagenesis,* 2004, **43**(3), 143–158.
8. W. N. Choy, Genotoxic and nongenotoxic mechanisms of carcinogenesis, in *Genetic Toxicology and Cancer Risk Assessment*, ed. W. N. Choy, Marcel Dekker, New York, 2001, pp. 47–71.
9. H. G. Ramjit, M. M. Singh and A. B. Coddington, Gas chromatographic/mass spectrometric analysis of methyl methanesulfonate and ethyl methanesulfonate in the bismesylate salt of DPI 201–106, a positive inotropic agent for the treatment of heart failure. *Journal of Mass Spectrometry*, **31**(8), 867–872. 1996.
10. P. J. Groenen and E. Busink, Alkylating activity in food products – especially sauerkraut and sour fermented dairy products – after incubation with nitrite under quasi-gastric conditions. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, **26**(3), 215–225. 1988.
11. G. N. Okafo and J. K. Roberts, Development of achiral separation methods in pharmaceutical analysis. *Pharmaceutical Analysis*, 2003, 31–73.
12. S. Klick, Evaluation of different injection techniques in the gas chromatographic determination of thermolabile trace impurities in a drug substance. *Journal of Chromatography A*, 1995, **689**(1), 69–76.

13. W. Li, Trace analysis of residual methyl methanesulfonate, ethyl methanesulfonate and isopropyl methanesulfonate in pharmaceuticals by capillary gas chromatography with flame ionization detection. *Journal of Chromatography A*, 2004, **104**6(1–2), 297–301.
14. D. H. Fine and D. P. Rounbehler, Trace analysis of volatile N-nitroso compounds by combined gas chromatography and thermal energy analysis. *Journal of Chromatography*, 1975, **109**(2), 271–279.
15. D. H. Fine, D. Lieb and F. Rufeh, Principle of operation of the thermal energy analyzer for the trace analysis of volatile and nonvolatile N-nitroso compounds. *Journal of Chromatography,* 1975, **107**(2), 351–357.
16. D. H. Fine, F. Rufeh, D. Lieb and D. P. Rounbehler, Description of the thermal energy analyzer (TEA) for trace determination of volatile and nonvolatile N-nitroso compounds. *Analytical Chemistry,* 1975, **47**(7), 1188–1191.
17. D. P. Rounbehler, S. J. Bradley, B. C. Challis, D. H. Fine and E.A. Walker, Trace determination of amines and other nitrogen containing compounds with a modified thermal energy analyzer (TEA). *Chromatographia,* 1982, **16**, 354–358.
18. A. L. Lafleur and K. M. Mills, Trace level determination of selected nitroaromatic compounds by gas chromatography with pyrolysis/chemiluminescent detection. *Analytical Chemistry,* 1981, **53**, 1202–1205.
19. D. H. Fine, D. P. Rounbehler, A. Silvergleid and R. Ross, Trace analysis of polar and apolar N-nitroso compounds by combined high-performance liquid chromatography and thermal energy analysis. *Proceedings of the 2nd International Symposium on Nitrite Meat Production*, 1977, pp. 191–198.
20. P. E. Oettinger, F. Huffman, D. H. Fine and D. Lieb, Liquid chromatograph detector for trace analysis of nonvolatile N-nitroso compounds. *Analytical Letters,* 1975, **8**(6), 411–414.
21. M. McAlpine and P. W. Skett, Unpublished Work. 2001.
22. C. R. Lee, M. Hubert, C. Nguyen Van Dau, D. Peter, and A. M. Krstulovic, Determination of N,N-dimethylaminoethyl chloride and the dimethylaziridinium ion at sub-ppm levels in diltiazem hydrochloride by LC-MS with electrospray ionization. *Analyst*, 2000, **125**(7), 1255–1259.
23. C. Clarke, Development and validation of LC-MS methods that demonstrates the control of toxic impurities to low ppm levels in drug substance. *Proceedings of the International Separations Conference,* 2004.
24. C. Eckers, N. Haskins and J. Langridge, The use of liquid chromatography combined with a quadrupole time-of-flight analyzer for the identification of trace impurities in drug substance. *Rapid Communications in Mass Spectrometry,* 1997, **11**(17), 1916–1922.
25. N. J. Haskins, C. Eckers, A. J. Organ, M. F. Dunk and B. E. Winger, The use of electrospray ionization with Fourier transform ion cyclotron resonance mass spectrometry in the analysis of trace impurities in a drug substance. *Rapid Communications in Mass Spectrometry,* 1995, **9**(11), 1027–1030.
26. J. C. Wolff, L. Barr and P. Moss, New ultraviolet signal actuated switching valve for the measurement of low level impurities by liquid chromatography/mass spectrometry. *Rapid Communications in Mass Spectrometry,* 1999, **13**(23), 2376–2381.
27. S. Hamilton, A. Ray and K. Dennis, LC/MS of alkyl iodides using HPLC with negative ion atmospheric pressure chemical ionisation. Unpublished Work. 2003.
28. T. Netscher, Sulfonate leaving groups for nucleophilic substitution reactions-improved structures and procedures. *Recent Research Developments in Organic Chemistry,* 2003, **7**, 71–83.
29. P. O. Chye, P. C. Kiat, K. L. Hian and F. Y. L. Sam, Analysis of nitroaromatics in aqueous samples by capillary supercritical fluid chromatography. *International Journal of Environmental Studies,* 1992, **41**, 17–25.

30. S. Sass and M. H. Stutz, Thin-layer chromatography of some sulfur and nitrogen mustards. *Journal of Chromatography,* 1981, **213**(1), 173–176.
31. W. D. Ludemann, M. H. Stutz and S. Sass, Qualitative thin-layer chromatography of some irritants. *Analytical Chemistry,* 1969, **41**(4), 679–681.
32. A. Cee New detection method for compounds with a labile halogen on thin-layer chromatograms. *Journal of Chromatography,* 1978, **150**(1), 290–299.
33. P. W. Skett Unpublished Work. 2005.
34. R. P. Frost, M. S. Hussain and A. R. Raghani, Determination of pharmaceutical process impurities by solid phase microextraction gas chromatography. *Journal of Separation Science,* 2003, **26**(12–13), 1097–1103.
35. H. T. Gronhaug, Pedersen-Bjergaard S, Rasmussen KE. Reduction of extraction times in liquid-phase microextraction. *Journal of Chromatography B, Biomedical Sciences and Applications,* 2001, **760**(2), 219–226.
36. T. G. Halvorsen, S. Pedersen-Bjergaard and K. E. Rasmussen, Liquid-phase microextraction and capillary electrophoresis of citalopram, an antidepressant drug. *Journal of Chromatography A,* 2001, **909**(1), 87–93.
37. T. S. Ho, S. Pedersen-Bjergaard and K. E. Rasmussen, Recovery, enrichment and selectivity in liquid-phase microextraction comparison with conventional liquid-liquid extraction. *Journal of Chromatography A,* 2002, **963**(1–2), 3–17.
38. S. Myung, S. Yoon and M. Kim, Analysis of benzene ethylamine derivatives in urine using the programmable dynamic liquid-phase microextraction (LPME) device. *Analyst,* 2003, **128**(12), 1443–1446.
39. S. Pedersen-Bjergaard, T. S. Ho and K. E. Rasmussen, Fundamental studies on selectivity in 3-phase liquid-phase microextraction (LPME) of basic drugs. *Journal of Separation Science,* 2002, **25**(3), 141–146.
40. K. E. Rasmussen, S. Pedersen-Bjergaard, M. Krogh, H. G. Ugland and T. Gronhaug, Development of a simple in-vial liquid-phase microextraction device for drug analysis compatible with capillary gas chromatography, capillary electrophoresis and high-performance liquid chromatography. *Journal of Chromatography, A* **873**(1), 3–11. 2000.
41. L. Hou, G. Shen and H. K. Lee, Automated hollow fiber-protected dynamic liquid-phase microextraction of pesticides for gas chromatography-mass spectrometric analysis. *Journal of Chromatography A,* 2003, **985**(1–2), 107–116.
42. P. Kotianova and E. Matisova, Liquid-phase microextraction and its utilization for trace analysis of organic compounds in water matrix. *Chemicke Listy,* 2000, **94**(4), 220–225.
43. Liu Jf, Chi Yg, Jiang Gb, C. Tai, Peng Jf and JT. Hu, Ionic liquid-based liquid-phase microextraction, a new sample enrichment procedure for liquid chromatography. *Journal of Chromatography A,* 2004, **1026**(1–2), 143–147.
44. Liu Jf, Chi Yg and Jiang Gb, Screening the extractability of some typical environmental pollutants by ionic liquids in liquid-phase microextraction. *Journal of Separation Science,* 2005, **28**(1), 87–91.
45. E. Psillakis and N. Kalogerakis, Hollow-fibre liquid-phase microextraction of phthalate esters from water. *Journal of Chromatography A,* 2003, **999**(1–2), 145–153.
46 S. M. Richoll and I. Colon, Liquid-phase Microextraction (LPME): A Novel Approach to the Isolation and Quantitation of Low Level Impurities in Pharmaceutical Applications. Abstracts of Pittcon 2004.
47 J. Barek, J. Cvacka, A. Muck, V. Quaiserova and J. Zima, Electrochemical methods for monitoring of environmental carcinogens. *Fresenius' Journal of Analytical Chemistry,* 2001, **369**(7–8), 556–562.
48. J. Barek, V. Mejstrik, A. Muck and J. Zima, Polarographic and voltammetric determination of chemical carcinogens. *Critical Reviews in Analytical Chemistry,* 2000, **30**(1), 37–57.

49 J. H. Kim and J. J. Thomas, Use of 4-(nitrobenzyl)pyridine (4-NBP) to test mutagenic potential of slow-reacting epoxides, their corresponding olefins, and other alkylating agents. *Bulletin of Environmental Contamination and Toxicology,* 1992, **49**(6), 879–885.
50. T. J. Novak and K. A. Quinn, Catalytic NBP spot test for the detection of trichothecene mycotoxin T-2. *Analytical Letters,* 1986, **19**(19–20), 2001–2009.
51. W. Preibsch, K. D. Hofmann and W. Krafft, Method for determination of alkylating activity in aqueous solutions by reaction with NBP [4-(p-nitrobenzyl)pyridine]. *Archiv fuer Geschwulstforschung,* 1972, **40**(3), 259–262.
52. R. Preussmann, H. Schneider and F. Epple, Detection of alkylating agents. II. Detection of different classes of alkylating agents by a modification of the color reaction with 4-(4-nitrobenzyl)pyridine(NBP). *Arzneimittel-Forschung,* 1969, **19**(7), 1059–1073.
53. R. Sohr, G. Schmeck, R. Preiss and M. Matthias, Methodology of the colorimetric determination of alkylating cytostatics with 4-(4´-nitrobenzyl)pyridine (NBP) in plasma. *Pharmazie,* 1982, **37**(11), 777–779.
54. O. M. Friedman and E. Boger, Colorimetric estimation of nitrogen mustards in aqueous media. Hydrolytic behavior of bis(2-chloroethyl)amine, nor-HN2. *Analytical Chemistry,* 1961, **33**, 906–910.
55. R. A. Christian, S. K. Chaffee, C. J. Hovick and W. J. Steele, A stable colorimetric assay for cyclophosphamide and its alkylating metabolites based on the alkylation of 4-(4'-nitrobenzyl)-pyridine. *Life sciences,* 1980, **27**(25–26), 2595–2599.
56. T. J. Novak (United States Dept. of the Army U, ed.), Microspot test methods and field test kit for on-site inspections of chemical agents. US patent 5935862. 19990810.
57. T. J. Novak (United States Dept. of the Army U, ed.), Microspot test kit and method for chemical testing. US patent 6787366. 20040907.
58. T. J. Novak (United States Dept. of the Army U, ed.), Field microspot test method for on-site chemical testing. US patent 6420181. 20020716.
59. J. A. Guth and L. P. Manner, 4-(p-Nitrobenzyl)pyridine as a reagent for thin-layer chromatographic determination of various aza heterocycles. *Journal of Chromatography,* 1967, **30**(2), 514–521.
60. C. R. Lee, F. Guivarch, C. Nguyen Van Dau, D. Tessier and A. M. Krstulovic, Determination of polar alkylating agents as thiocyanate/isothiocyanate derivatives by reaction headspace gas chromatography. *Analyst,* 2003, **128**(7), 857–863.
61. G. Bellucci, A. Lippi and F. Marioni, A simple conductimetric method as an alternative to the colorimetric p-nitrobenzylpyridine test for the measurement of the reactivity of potentially mutagenic alkylating compounds. *Chemico-Biological Interactions,* 1986, **59**(3), 255–263.
62. E. Sawicki, D. F. Bender, T. R. Hauser, R. M. Wilson Jr. and J. E. Meeker, Five new methods for the spectrophotometric determination of alkylating agents including some extremely sensitive autocatalytic methods. *Analytical Chemistry,* 1963, **35**(10), 1479–1486.
63. J. M. Quirke, C. L. Adams and G. J. Van Berkel, Chemical Derivatization for Electrospray Ionization Mass Spectrometry. 1. Alkyl Halides, Alcohols, Phenols, Thiols, and Amines. *Analytical Chemistry,* 1994, **66**(8), 1302–1315.
64. A. M. Cheh and R. E. Carlson, Determination of potentially mutagenic and carcinogenic electrophiles in environmental samples. 1981, *Analytical Chemistry,* **53**(7), 1001–1006.
65. M. Hassan and H. Ehrsson, Gas chromatographic determination of busulfan in plasma with electron-capture detection. *Journal of Chromatography,* 1983, **277**, 374–380.
66 A. Paci, A. Rieutord, F. Brion and P. Prognon, Separation methods for alkylating antineoplastic compounds. *Journal of Chromatography.B, Biomedical Sciences and Applications,* 2001, **764**(1–2), 255–287.

67. F. Baumann and R. Preiss, Application of liquid chromatography-mass spectrometry in the determination of oxazaphosphorines and their metabolites. *Analytica Chimica Acta,* 2003, **492**(1–2), 147–156.
68. N. Sannolo, N. Miraglia, M. Biglietto, A. Acampora and A. Malorni, Determination of cyclophosphamide and ifosphamide in urine at trace levels by gas chromatography/tandem mass spectrometry. *Journal of Mass Spectrometry,* 1999, **34**(8), 845–849.
69. C. Sottani, R. Turci, L. Perbellini and C. Minoia, Liquid–liquid extraction procedure for trace determination of cyclophosphamide in human urine by high-performance liquid chromatography tandem mass spectrometry. *Rapid Communications in Mass Spectrometry:* RCM **12**(16), 1063–1068. 1998.
70. J. M. Reid, J. F. Stobaugh and L. A. Sternson, Liquid chromatographic determination of cyclophosphamide enantiomers in plasma by precolumn chiral derivatization. *Analytical Chemistry,* **61**(5), 441–446. 1989.
71. D. Anderson, D. R. Ferry, R. J. Knox *et al*. High-performance liquid chromatographic method for sensitive determination of the alkylating agent CB1954 in human plasma. *Journal of Chromatography. B, Biomedical Sciences and Applications,* 1999, **731**(2), 293–298.
72. Y. Kato, H. Kaneko, T. Matsushita *et al*. Diect injection analysis of melphalan in plasma using column-switching high-performance liquid chromatography. *Therapeutic Drug Monitoring,* 1992, **14**:66–71.
73. H. Ehrsson, S. Eksborg, I. Wallin, Y. Marde and Joansson Bo, Detemination of chlorambucil in plasma by GLC with selected-ion monitoring. *Journal of Pharmaceutical Sciences,* **69**(6), 710–712. 1980.
74. T. J. Bardos, N. Datta-Gupta, P. Hebborn and D. J. Triggle, A study of comparative chemical and biological activities of alkylating agents. *Journal of Medicinal Chemistry,* 1965, **8**, 167–174.
75. T. Steger-Hartmann, K. Kuemmerer and J. Schecker, Trace analysis of the antineoplastics ifosfamide and cyclophosphamide in wastewater by two-step solid-phase extraction and gas chromatography – mass spectrometry. *Journal of Chromatography A*, 1996, **726**(1 + 2), 179–184.
76. T. Kiffmeyer, H. J. Goetze, M. Jursch and U. Lueders, Trace enrichment, chromatographic separation, and biodegradation of cytostatic compounds in surface water. *Fresenius' Journal of Analytical Chemistry,* 1998, **361**(2), 185–191.
77. W. D. Ludemann, M. H. Stutz and S. Sass, Thin-layer chromatography of some irritants. *Analytical Chemistry* 1969, **41**(4), 679–681.
78. G. P. Wheeler and S. Chumley, Alkylating activity of 1,3-bis(2-chloroethyl)-1-nitrosourea and related compounds. *Journal of Medicinal Chemistry,* 1967, **10**, 259–261.
79. M. Zheng, C. Fu and H. Xu, High-performance liquid chromatographic detection of trace N-nitrosoamines by precolumn derivatization with 4-(2-phthalimidyl)benzoyl chloride. *Analyst*, 1993, **118**(3), 269–271.
80. R. Zhao, S. Chu and X. Xu, Optimization of nonequilibrium liquid-phase microextraction for the determination of nitrobenzenes in aqueous samples by gas chromatography-electron capture detection. *Analytical Sciences: International Journal of the Japan Society for Analytical Chemistry*, 2004, **20**(4), 663–666.
81. Text on Validation of Analytical Procedures, Adopted by CPMP, November 94, issued as CPMP/ICH/381/95. CPMP/ICH/381/95, 11260. 1995. Published in the Federal Register, Vol. 60.

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5 A systematic approach to impurity identification

Gary E. Martin

5.1 Introduction

The observation and subsequent structure characterization of an impurity or degradant in the pharmaceutical industry is a frequent, highly specialized, and labor-intensive undertaking. The identification of impurities and/or degradants in pharmaceuticals is critically important for reasons of both product efficacy and patient safety. The importance of this issue is underscored by several monographs [1, 2], a chapter [3–5], and numerous general reviews [6–12], as well as a review on impurities contained in excipients used in pharmaceutical formulations [13–15]. In terms of efficacy and patient safety, it is important to isolate and identify impurities and/or degradants to ensure that their presence will not evoke any form of adverse response, either pharmacologic or toxicologic, in a patient taking the medication.

Regulatory bodies such as the U.S. Food and Drug Administration (FDA) and the International Congress on Harmonization (ICH) have established rigorous guidelines for the identification of extraneous compounds in pharmaceutical agents. Generally, for drugs dosed at <2 g/day, impurities and/or degradation products present at >0.1% require isolation and structural characterization. For drugs dosed at >2 g/day, the threshold for isolation and identification is lower at 0.05% according to ICH guidelines [16–18]. An exception to these thresholds may arise when there is a highly colored material formed during the synthetic preparation of a pharmaceutical agent. In such cases, it may become necessary to isolate and attempt to identify the causative agent, which may be present at much lower levels because of a chromophore in the structure of the molecule. Colored contaminants are among the most challenging impurity and degradant characterization problems since they often involve only trace quantities of highly conjugated molecules with intense chromophores. Finally, insofar as regulatory issues surrounding impurity and degradant characterization, it is also worth noting that in a newsletter published in 2003, the FDA noted that structure characterization is effectively a form of functional group analysis, and as such, does not constitute an undertaking that falls under GMP guidelines [19].

In terms of drug efficacy, it is important to determine whether the presence of an impurity or degradant can have an impact on the intended pharmacologic response of a drug entity [20]. Generally, one can only evaluate the pharmacologic activity of impurity or degradant and hence the potential impact on the efficacy of a pharmaceutical agent by synthesizing the molecule in question and

then subjecting that discrete chemical entity to pharmacologic and toxicologic screening. In some cases, this screening will be based on testing; in others, *in silico* screening may be sufficient after the structure of the material has been definitively established. Knowledge of impurities that may be formed during large-scale synthesis can also be used to refine the synthetic process, thereby engineering these impurities out of the finished drug molecule in question.

In the simplest terms, the time line of events leading to a request to identify an impurity or degradant begins with the initial detection of the spurious entity by whatever means are being employed for standard assays of the drug. Next, a dialogue between the group detecting the presence of the new impurity and the structure characterization group(s) must be initiated. Quite often, it will be a stability or quality assurance group that will first detect the presence of a new impurity or degradant in either a batch of Active Pharmaceutical Ingredient or a finished dosage form. Integral to the communication between whichever group is responsible for the initial observation of a new impurity or degradant and the structure characterization group(s) will be details on how the new impurity or degradant was detected including chromatographic methods employed, information pertaining to the synthetic route being utilized if the spurious entity is a process impurity, or stability conditions that the drug molecule was subjected to in the case of a degradant. During the course of that discussion, information and whatever initial spectral data, for example, a molecular weight if an LC/MS system was used for the initial detection, should be conveyed to the structure group followed by an appropriate feedstock from which the impurity can be isolated. It is also important to factor into the ensuing discussions any input from colleagues with a first-hand knowledge of the chemical process used to prepare the drug moiety in question. Their intimate knowledge of the synthetic route, solvents utilized, and handling can prove invaluable in speeding the isolation and subsequent identification of an impurity. Along with preliminary spectroscopic information, it is also important for priority and time constraints regarding the isolation and identification of the impurity or degradant to be communicated to facilitate the prioritization of the structure characterization request relative to competing requests that the structure characterization group may already have queued.

Following the receipt of all available information including any spectral data, how the request for assistance is handled by the group(s) responsible for providing structural characterization will depend, in part, on how structure elucidation/characterization function is organized, which can vary from company to company and even between sites within a single company. The most prominent models for the organization of structure characterization units within the pharmaceutical industry are very briefly considered in Section 5.1.1.

5.1.1 Structure elucidation group organizational models

Structure elucidation functions within the pharmaceutical industry fall principally into three organizational constructs. These range from each discipline

functioning as a discrete unit to partially integrated organizations, where typically NMR and mass spectrometry (MS) are grouped together with preparative chromatography and perhaps vibrational spectroscopy provided by an organizationally separate group or groups, and finally, fully integrated operations where all of the necessary spectroscopic expertise and chromatographic support are found in a self-contained chemical structure group. Depending on the nature of the parent organization, and the specific tasking of the individual spectroscopic groups, each of the aforementioned organizational constructs may have some inherent advantages. From the vantage point of the author, full integration of the necessary spectroscopic and chromatographic expertise provides the most powerful and efficient approach to the rapid isolation and characterization of impurity and degradant structures, but this is by no means the only workable mechanism for handling the problem of impurity identification within the pharmaceutical industry.

5.1.2 Whether or not to isolate and identify an impurity or degradant

The isolation and identification of an impurity or degradation product in a drug is a very labor-intensive and expensive undertaking. Groups tasked with this responsibility typically represent a capital investment of millions of dollars. Work directed to such a group always prompts the question of the prioritization of one project vs others that the group is already working on or has in their queue. Generally, it is desirable to use regulatory guidelines as the trigger points for the isolation and identification of an impurity or degradation product (see Figure 5.1). In some cases during stability studies, the rate at which levels of a given degradant are increasing may be such that for a given set of conditions, for example, 40°C/75% relative humidity, it will be evident that the levels of the degradant in question will exceed allowable threshold limits before the completion of the stability study. In such cases, if the quantities of drug on stability are sufficient or if it is possible to produce the same degradant under accelerated conditions, then the isolation and identification of that degradant may be warranted earlier.

Another facet of the question of when to isolate and characterize a degradant arises when forced degradation studies of a drug are contemplated. It may be attractive to isolate and identify potential degradants from a forced degradation study, but again, this is a time-consuming and labor-intensive undertaking. An effort should be made to ascertain, most commonly using LC/MS methods, that a degradant being formed in a forced degradation study is a legitimate degradant of the drug molecule being formed in standard, regulatory stability studies of the drug. In such cases, the isolation and identification of that specific degradant may be warranted. Other degradants formed in the forced degradation study can be isolated during the preparative chromatography of the targeted degradant and it may be desirable to hold those samples in abeyance should it later become evident that one or more of those components is also a legitimate degradant of the drug. Ideally though, degradants whose formation in a regulatory stability study cannot be confirmed should not be characterized as a cost and

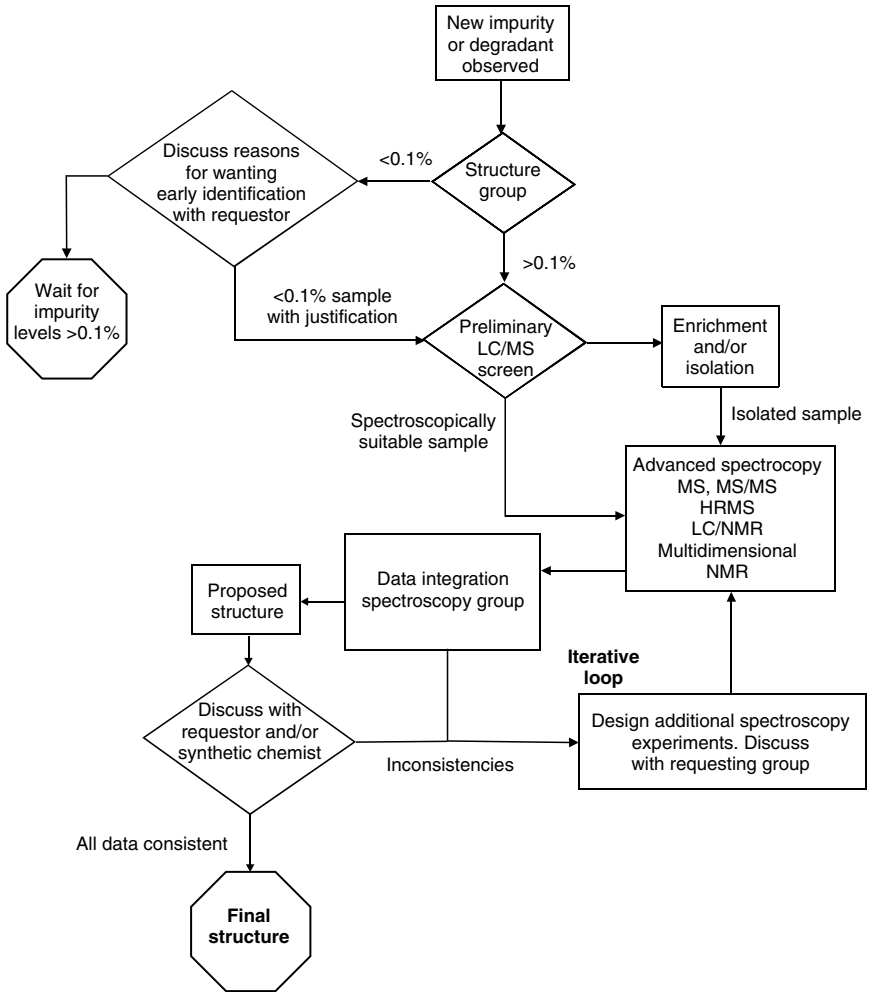


Figure 5.1 Interactive strategy for impurity and degradant characterization as it might be implemented in a contemporary structure characterization group charged with this responsibility within the pharmaceutical industry. The workflow for impurity/degradant isolation and characterization is governed, in part, by regulatory thresholds that specify the requirements for the identification of impurities and/or degradants. As is apparent from the flow chart, heavy reliance is placed on LC/MS methods for initial screening, followed either by chromatography when necessary to obtain an enriched sample or pure isolate or by direct spectroscopy in some cases. Data integration is an important and generally iterative phase of the process. The author has found it highly beneficial to involve groups originating the request for isolation and identification, as well as synthetic chemists familiar with the drug and process chemistry used in the synthetic preparation of the molecule in these discussions. The process continues with more data or new experiments designed as necessary until sufficient data are in hand to establish the structure of the impurity or degradant that is consistent with all of the available spectroscopic data as well as stability and/or process and synthetic chemistry considerations.

time-saving measure until their legitimacy as a degradant formed in a stability study is established.

5.1.3 Time lines for impurity/degradant identification

One of the first tasks of any group undertaking the structural characterization of an impurity or degradant is the necessity of reproducing the chromatography in use when that impurity or degradant peak was first observed. Ideally, the chromatographic method will be LC/MS 'friendly' but that is not always the case. Occasionally, nonvolatile buffers, pairing ions, heated columns, and the like, must be worked around before preparative isolation can be initiated. The primary goal is to establish the molecular weight for the impurity/degradant in question, thereby providing a degree of assurance that the correct small peak is eventually isolated for characterization. Unfortunately, most individuals working in this area of the pharmaceutical industry will eventually encounter a case where small peaks with very similar relative retention times (RRTs) 'trade places' in a chromatogram during the development of preparative chromatographic conditions if isolation is necessary, which has the potential of leading to the embarrassing isolation and characterization of the wrong peak if one is not careful. In the case of small isobaric impurity peaks in the chromatogram, even the availability of LC/MS data does not provide the means of determining that peaks have changed elution order in many cases.

Following chromatography replication to obtain initial molecular weight information for a given impurity or degradant peak, a decision typically must be made to determine whether or not preparative chromatographic isolation will be necessary. Simple chemical modification of an otherwise largely intact drug may be amenable to characterization through LC/MS data or a combination of LC/MS and LC/NMR data. Readers concerned with problems of this type are directed to Chapter 6 for a more detailed discussion of this approach to impurity characterization. More complex chemical modification, that is, those that would normally require the acquisition of direct and long-range heteronuclear shift correlation data for the structure to be characterized, or a total unknown, typically will necessitate preparative chromatographic isolation to provide a sample suitable for heteronuclear 2D NMR data acquisition. It should be noted, however, that this statement has some potential of becoming dated because of gains in sensitivity being made with cryogenic NMR probes with flow capabilities. The size of the sample that must be isolated will largely depend on the NMR instrumentation available and the complement of probe technology available to the structure group. Groups responsible for impurity/degradant structure characterization are typically equipped with 500- or 600-MHz NMR instruments, and in a few cases, with 700-MHz instruments. Laboratories with instruments equipped with either conventional small volume, high-sensitivity NMR probe technology (e.g. 3- or 2.5-mm microprobes, 1.7-mm submicro- or SMIDG™ probes, or Bruker's recently developed 1-mm CapNMR™ probes) or cryogenic NMR probe capabilities will require correspondingly smaller samples than that will be necessary for

laboratories using conventional 5-mm NMR probe technology [21]. In the case of groups limited to 5-mm probe technology, there is still some advantage to acquiring data in a 3-mm tube positioned coaxially in the 5-mm probe despite filling factor losses. There is further discussion on this specific point below with reference to cryogenic probes. The ability to work with smaller samples, in general, corresponds to a more time-efficient approach since less work will be required to isolate a sample suitable for NMR spectroscopy. Conversely, the much higher sensitivity of small volume and/or cryogenic NMR probes places correspondingly higher demands on the staff member responsible for the preparative chromatography to produce a highly pure sample. Access to high-sensitivity small volume probes can also facilitate the successful isolation and characterization of a new impurity in the late stages of a stability study when the amount of drug or dosage units available to use as feedstock may be severely restricted.

Generally, once a sample of an impurity or degradant has been chromatographically purified, it is desirable to split the sample into aliquots for MS, NMR, and vibrational spectroscopy. Because of the high sensitivity of MS and vibrational spectroscopy relative to NMR, which is an inherently insensitive technique, it is relatively easy to provide suitably sized samples for all spectroscopic techniques from a single chromatographic isolate. Repetition of the initial MS on the purified isolate is desirable. First, it is useful to confirm that the molecular weight of the isolated fraction corresponds to the initial LC/MS data, thereby reconfirming that the correct chromatographic specie was isolated in the absence of multiple isobaric impurities or degradants. Second, the determination of fragmentation pathways from MS/MS data is generally much easier with an isolate sample than from initial low-level samples in whatever matrix they are being isolated from, for example, the bulk pharmaceutical agent. Finally, if the capability is available, an empirical formula based on HRMS data can also be quite useful, particularly if the compound in question is a true unknown and unrelated to the drug itself. It is likewise useful to interrogate the sample by vibrational spectroscopy, using FT-IR, FT-Raman, or both, as a means of assessing the type, and under favorable spectroscopic circumstances, the numbers of specific functional groups present. While MS and vibrational spectroscopy are being done, NMR data acquisition is also normally initiated. The complexity of the NMR experiments performed will generally depend on the nature of the problem – simple chemical structure modifications may be confirmed by nothing more than a proton spectrum while a complete unknown will generally require much more extensive data acquisition including a variety of homo- and heteronuclear direct and long-range chemical shift correlation experiments.

Interpretation of the spectroscopic data from the individual spectroscopic techniques is generally done as the data are amassed. When all of the data are available, it is useful for the participating scientists to integrate their respective data, which is discussed in more detail below. The overall elapsed time for the isolation and identification of a new impurity or degradation product is quite variable. The difficulty of the actual isolation and the structural complexity of the molecule both impinge on the process. On the basis of the author's experience,

the average time required for structure group responsible for isolating and identifying impurities and degradation products to have at least a verbal reply back to the requesting scientist is about 2 weeks. Very simple problems may take considerably less time and, conversely, extremely challenging problems may take considerably more than 2 weeks, but 2 weeks does seem to be the average time required in >90% of cases.

5.1.4 Impurities vs degradation products

Although the terms impurity and degradant sound synonymous, they are really not. A degradant is the simplest unwanted constituent in a pharmaceutical agent to describe. As the name implies, it may be formed by the degradation of the pharmaceutical agent itself or through an interaction or reaction of the active ingredient in a formulation with one of the other constituents in a dosage form, and is hence generally closely related structurally to the drug molecule. As such, there are a few commonly encountered types of processes leading to the formation of a degradant.

The most common degradation pathways encountered involve the oxidation or hydrolysis of the drug molecule. The former, as an example, may lead to the conversion of an alcohol to a moiety in a higher oxidation state, such as an aldehyde, ketone, or carboxyl group, or, less commonly, to the oxidation of a nitrogen to the corresponding N-oxide. Hydroxyl groups can also be involved in dehydrations, leading to a degradant with a molecular weight 2 Da lower than the parent drug molecule. Hydrolysis reactions are often manifest in the hydrolytic cleavage of esters or lactone rings, and so on. Some of the less-frequently encountered types of degradants involve rearrangements and isomerization or epimerization. Generally, the level of a degradation product will increase with time; eventually the level of a degradation product present in a pharmaceutical agent may reach a level (typically 0.1% by FDA guidelines for drugs dosed at <2 g/day and 0.05% for drugs dosed at >2 g/day in the ICH guidelines) that triggers the decision to isolate and identify the component.

Impurities, in contrast to degradation products, might or might not have any relation to the drug molecule. In the simplest case, residual quantities of a solvent used at some step in the process chemistry can become an impurity in the drug product. A recent, very extensive publication to which the interested reader is referred, listed the proton and carbon NMR chemical shifts of a large number of potential solvent impurities in a number of commonly employed NMR solvents [22]. There have also been several reviews on the topic of residual solvents in pharmaceuticals [23, 24].

Extraneous constituents present in a synthetic reagent used in the chemical process can also end up as an impurity in the pharmaceutical agent. Such extraneous constituents may or may not react chemically during the synthesis of the desired pharmaceutical agent. Compounds that do not react chemically may be present at low levels in the final product and may be present at higher levels in

the mother liquor from which the drug product was isolated or crystallized. Chromatographic indication of the presence of such an impurity might prompt an investigator to examine the mother liquor to see if larger quantities are available and isolable from that source if it is necessary to identify the material. An impurity contained in a synthetic reagent that is capable of reacting during the synthesis of the target pharmaceutical agent corresponds to a different type of impurity. This type of impurity is most commonly encountered during process chemistry development. One example encountered by the author involved a synthetic process that relied on the reaction of phenyl isocyanate in the final step of the reaction sequence. An impurity was observed in a drug product by LC/MS methods at $> 0.1\%$ that differed from the drug product by being 6 Da heavier than the drug itself. A mass difference of 6 Da between the drug and an impurity is very uncommon. The origin of the impurity was traced back to small quantities of cyclohexyl isocyanate present in the phenyl isocyanate used in the final step of the synthetic route. The former was capable of reacting to form the cyclohexyl analog of the drug rather than the desired phenyl analog. Once the impurity was identified, it was possible to tighten specifications on the phenyl isocyanate being used in the drug synthesis to effectively 'engineer' the cyclohexyl impurity out of the process. Other examples of impurities of this type that can arise include reagents involving an alkyl chain as a part of their structure where the chain differs either in chain length, for example, *n*-propyl-containing reagent contaminated with small quantities of the ethyl or butyl analog, or where the chains differ in branching, for example, an *n*-propyl compound contaminated with a quantity of the isopropyl analog. Impurities formed in the latter case will be isobaric with the drug and may also be chromatographically difficult to distinguish from the drug molecule itself.

Another type of impurity arises when the synthetic process gives rise to low-level impurities formed by what might be considered 'minor' pathways. The author has encountered numerous impurities of this type over the years that made no sense on the basis of the chemical knowledge that would be utilized in designing a synthetic route to a molecule. However, the pathways leading to such low-level impurities are not the sort that would be employed for synthesis, and it is advisable for an investigator to keep a very open mind when working on the structural characterization of an impurity or degradation product – common chemical intuition and knowledge may not be safe criteria to use in discarding a structure hypothesis.

A third type of process impurity can arise when a synthetic route to a molecule involves a cyclization reaction and there are two pathways along which the cyclization can proceed. In this case an isobaric impurity may be formed with a chemical skeleton that differs from the target pharmaceutical agent. A very similar situation arises when a reaction can lead to major and minor epimers. There are numerous other examples that could be cited, but those that have been mentioned at least give the reader a glimpse of the diverse nature of impurities that can be encountered during the drug-development process.

5.2 Isolation and identification of impurities and degradants

5.2.1 Preparative chromatography

Any comprehensive discussion of chromatographic methods is well beyond the scope of both this chapter and the author's area of expertise, but the importance of high-quality isolates nevertheless warrants comment. There are a number of published reviews dealing with various aspects of preparative chromatography in relation to pharmaceutical impurities to which the interested reader is referred [25–30], as well as to Chapter 7.

Present preparative chromatographic methods are heavily reliant on reversed-phase chromatography, which employs a stationary nonpolar phase and a polar mobile phase that may incorporate polarity modifiers, pairing ions, buffers, and the like. Because of the diverse nature of impurity and degradant characterization requests, investigators should keep in mind that reversed-phase chromatography might not be the method best suited for all problems, and that there will be occasions when normal-phase chromatography, supercritical fluid chromatography (SFC), gas chromatography [31], capillary electrophoresis [32–39], or still other methods may be the best choice.

Irrespective of which preparative isolation method is employed, an isolate will likely still contain artefacts from the isolation process, constituents that are probably best referred to as 'chemical noise.' It is highly desirable to take steps to reduce such contaminants in the final isolate insofar as possible before initiating spectroscopic data collection. This step is of particular importance when small volume or high-sensitivity NMR probes (e.g. 1.7 -mm and smaller conventional probes, cryogenic NMR probes) will be employed in the acquisition of the NMR data. In the case of isolates prepared by reversed-phase chromatographic methods, as an example, it is sometimes useful to load the isolate onto a small, prepacked cartridge capable of trapping undesired components in the isolate. Ideally, if the isolate can be washed back off the trapping cartridge with a pure solvent such as acetonitrile, with the undesired components left behind on the trapping column, a much higher quality isolate can be obtained. In addition, the isolate washed back off a column in this manner will also frequently be concentrated relative to when it was applied to the trapping column, which can also be very beneficial.

5.2.2 Spectroscopic data from various techniques

Each of the various types of spectroscopy that can be applied to the determination of an impurity or degradant structure allows an investigator many choices and considerable flexibility. Not all of the various types of experiments that can be performed should be done for any given isolate. Ideally, the team of scientists working on an impurity or degradant structure problem should try to select those experiments for their respective spectroscopic methods that will provide the most useful information. Then, if instrument time permits, additional experiments can

be performed to accumulate data that may be useful if the structure cannot be solved with what might be considered 'front-line' experiments.

5.2.2.1 MS

MS is a very high sensitivity albeit destructive technique. Coupled or hyphenated MS methods and their application to pharmaceutical impurities have been the subject of several prior reviews [40–47]. Coupled with liquid chromatography to separate the peak of interest from others present in the initial sample, mass spectrometric measurements offer the potential for determining the molecular weight of the target molecule as well as affording 'structure-indicating' fragment ions as noted by Lohr, Alsante, and coworkers in a pair for published articles dealing with the isolation and identification of impurities and degradant structures [10, 11].

Simple molecular weight changes relative to the parent molecule may indicate the gain or loss of the equivalent of a methylene group (i.e. the loss of a methyl group replaced by a proton), oxidation, and hydration/dehydration or hydrolysis to list only a few possibilities. The formation of adduct ions, for example, sodium, potassium, or acetonitrile at +23, +39, and +43 Da, respectively, can provide the means for the preliminary identification of the molecular ion of a molecule. Isotope patterns, for example, from $^{35/37}$Cl, $^{79/81}$Br, and even $^{32/34}$S can also be diagnostically beneficial. Finally, high-resolution MS (HRMS) measurements can provide empirical formulae in many cases that can be useful when dealing with something unrelated to the parent molecule.

Beyond the constraints that can be derived from empirical formula-related considerations, the next highly desirable information that can be deduced from the mass spectral data is associated with the fragmentation of the molecule. Generally, when an impurity or degradation product of a pharmaceutical agent is encountered, the parent drug molecule will already be well characterized and the mass spectral fragmentation pathway(s) well understood. Differences in fragmentation and/or the absence of a major fragment ion in the MS/MS data can provide diagnostically useful insight into the locus of chemical modification as well as possibly the nature of that modification. In most cases though, mass spectral information is not diagnostically useful for differentiating impurities that are positional isomers of the parent drug molecule.

5.2.2.2 NMR spectroscopy

The complement of NMR data typically acquired consists of a proton reference spectrum, COSY or TOCSY data, as the user may prefer of other considerations may dictate, to establish the proton homonuclear connectivity network, and a multiplicity-edited HSQC spectrum followed in many if not most cases by an HMBC or some other long-range heteronuclear shift correlation experiment [48]. Typically, heteronuclear shift correlation experiments will be limited to ^{1}H–^{13}C experiments, although the greatly increased sensitivity offered by cryogenic NMR probes is opening the door for the regular acquisition of ^{1}H–^{15}N direct and long-range correlation studies of impurities and degradants [49]. Direct and

long-range heteronuclear shift correlation data can obviate the acquisition of a ^{13}C reference spectrum in most but not all cases. In those cases where a ^{13}C reference spectrum is still needed to account for carbons with no long-range correlations to them or for regulatory purposes, specialized probes such as a heteronuclear Nano-probe™ or ^{13}C cryoprobes can facilitate the acquisition of these data [49].

A further consideration associated with the acquisition of NMR data for scarce samples is the question of whether or not to utilize gradients for coherence pathway selection. Contemporary NMR spectrometers within the pharmaceutical industry routinely have gradient capabilities and probes. When dealing with larger samples, gradient-based direct and long-range heteronuclear shift correlation experiments are greatly facilitated by the use of gradients for coherence pathway selection and to flatten the noise floor of the experiment. In the author's own experience and as also reported by Reynolds and Enriquez [50], as the size of the sample being interrogated by NMR methods diminishes, there is a threshold beneath which it is advisable not to use gradients. As a general rule of thumb, when the approximate sample size is <0.25 μmol or when one is faced with accumulating more transients/t_1 increment than that are employed in the phase-cycled variant of the experiment in question, there is generally a performance advantage to be had by using phase cycling rather than gradient selection. A similar situation prevails in the acquisition of multiplicity-edited HSQC direct correlation spectra. We have generally found that the decision to employ multiplicity-editing reduces the *s*/*n* ratio of the experiment by about 20% relative to that of the conventional HSQC experiment.

The order in which various NMR data are acquired is largely one of user preference. Acquisition of the proton reference spectrum will invariably be undertaken first. Whether a user next seeks to establish homo- or heteronuclear shift correlations is where individual preferences come into play. Many spectroscopists proceed from the proton reference spectrum to either a COSY or a TOCSY spectrum next, while others may prefer to establish direct proton–carbon chemical shift correlations. This author's preference is for the latter approach. From a multiplicity-edited HSQC spectrum you obtain not only the carbon chemical shifts, which give an indication of the location of heteroatoms, the degree of unsaturation and the like, but also the number of directly attached protons, which eliminates the need for the acquisition of a DEPT spectrum [51, 52]. The statement in the prior sentence presupposes, of course, that there the sensitivity losses associated with the acquisition of multiplicity-edited HSQC data are tolerable.

The first three NMR experiments done on any sample, regardless of the order in which the data are acquired, are in most cases relatively short-duration experiments. While an NMR spectroscopist is beginning to interpret these data, the acquisition of an HMBC or other long-range heteronuclear shift correlation experiment can be initiated. Taking advantage of the high sensitivity associated with cryogenic NMR probes, we have also found it useful to queue the acquisition of a ^{1}H–^{15}N long-range correlation experiment while the other data are being interpreted [53–55]. Rather than performing a ^{1}H–^{15}N GHMBC experiment, we

have found that the variability of long-range $^{1}H–^{15}N$ couplings is better accommodated by using the accordion-optimized IMPEACH-MBC experiment [56].

The direct and long-range heteronuclear shift correlation experiments will be sufficient in the determination of most impurity and/or degradant structures. In some cases, such as steroids that have especially congested proton or carbon spectra, hyphenated two-dimensional NMR experiments such as the inverted direct response (IDR)-HSQC-TOCSY experiment have also proved to be beneficial [57, 58]. Unfortunately, the sensitivity of the IDR-HSQC-TOCSY experiment is quite low, roughly on a par with long-range $^{1}H–^{15}N$ correlation experiments in this author's experience, which makes it highly desirable to perform these experiments, when necessary, using either a small volume or cryogenic NMR probe for optimal sensitivity [49, 59, 60].

Hyphenated NMR methods are not considered in the present chapter. The interested reader is referred to Chapter 6 or to one of the numerous reviews that have appeared recently that deal with various hyphenated NMR methods [40, 61–63].

5.2.2.3 Vibrational spectroscopy

Contemporary approaches to chemical structure elucidation are now heavily reliant on mass spectrometry and NMR spectroscopy. Since the advent of 2D NMR methods, in many laboratories vibrational data are either not acquired or not considered, which represents a paradigm shift from approaches to chemical structure elucidation as recently as 20 years ago when vibrational spectroscopic data were an integral part of the structure elucidation data assembled to characterize an unknown structure. In contrast, we have found it useful to continue to acquire and utilize vibrational data for the characterization of impurities and degradation products [64, 65].

Vibrational data, primarily FT-IR and FT-Raman, are both high-sensitivity spectroscopic methods that require only small samples relative to what is normally used in the acquisition of NMR data. It is thus convenient to provide a small aliquot of the isolate sample for interrogation by vibrational methods in parallel with the acquisition of MS and NMR spectroscopic data. One of the strong points of vibrational spectroscopic methods is in the area of functional group analysis/characterization. As an example, consider carbonyl groups in the structure of an impurity or degradant. Carbonyl moieties, other than aldehydes, are transparent in the proton reference, COSY, and multiplicity-edited HSQC experiments. Long-range correlations are generally observed to carbonyl species in HMBC experiments, but these data are usually among the last NMR data to be acquired for reasons of sensitivity. In addition, while the HMBC data allow a given carbonyl carbon to be positioned in the structure being elucidated, ^{13}C chemical shift alone is not a good 'reporter' of the nature of the carbonyl in question, that is, lactone, vs lactam, vs ester, and so forth, nor is the ^{13}C shift particularly sensitive to variations in ring size, for example, γ- vs δ-lactone, which vibrational data can readily provide.

Overall, we have found vibrational spectroscopy data to be highly complementary to other spectral data amassed during the characterization of the structure of a degradant or impurity. As such, given the relative ease of obtaining these data, it seems obvious that they should be acquired and incorporated into the structure elucidation protocol used when impurities and degradants of a pharmaceutical agent are characterized.

5.2.3 Data integration

Following the acquisition of the various fundamental spectroscopic data, it is very beneficial for the various scientists responsible for these data to meet to integrate their results [64]. Data integration meetings are useful in that they often suggest additional experimental data that can be beneficially acquired, or the process of integrating the data may trigger alternative, viable interpretations of various portions of the data leading to the assembly of a working structural hypothesis. Involvement of the synthetic chemist working with the process in question and the colleague responsible for stability studies – if that is when a new impurity or degradation product is first encountered – should also be a participant in these discussions. Synthetic chemistry colleagues in particular, given their intimate experience with the class of molecule in question, may be able to provide further chemical or stability insights that can facilitate the determination of the structure.

In the case of fully integrated structure characterization groups, data-integration sessions are generally a logical next step in the problem-solving process after the completion of data acquisition. With partially integrated groups, or in the case of organizations that utilize separate spectroscopic groups, coordinated data interpretation is a step that probably must be undertaken overtly to ensure that when the full data ensemble are interpreted that all of the data are consistent with the proposed structure of the impurity or degradant.

The last step of the impurity/degradant identification time line is typically the assembly of whatever form of documentation or report is appropriate for the problem at hand. Depending on the individual organization or the type of problem, this could range from a simple e-mail containing the structure to a formal report suitable for incorporation into a regulatory filing package for the drug in question. How this is handled is probably the least important step in the process and will certainly vary from problem to problem and from one organization to another.

5.3 Impurity and degradant characterization studies

Any comprehensive survey of impurities in pharmaceuticals is completely beyond the scope of this contribution. A SciFinder search using the terminology ‘pharmaceutical impurities’ uncovered 1933 citations. Sorting those citations by year gave a rather interesting picture of the importance of this area of investigation.

The earliest report found in that search was published in 1908. For many years thereafter, there was generally only one report published. By the mid-1960s, there were generally ten or fewer reports being published that dealt with impurities and/or degradants in pharmaceuticals annually. By the late 1970s and early 1980s, the number of reports dealing with pharmaceutical impurities was approaching an average of 25 reports per year. By 1990, there were generally about 50 studies being reported in this area on a yearly basis. The number of reports has steadily risen, with 143 reports addressing various aspects of impurity identification and characterization appearing in 2004.

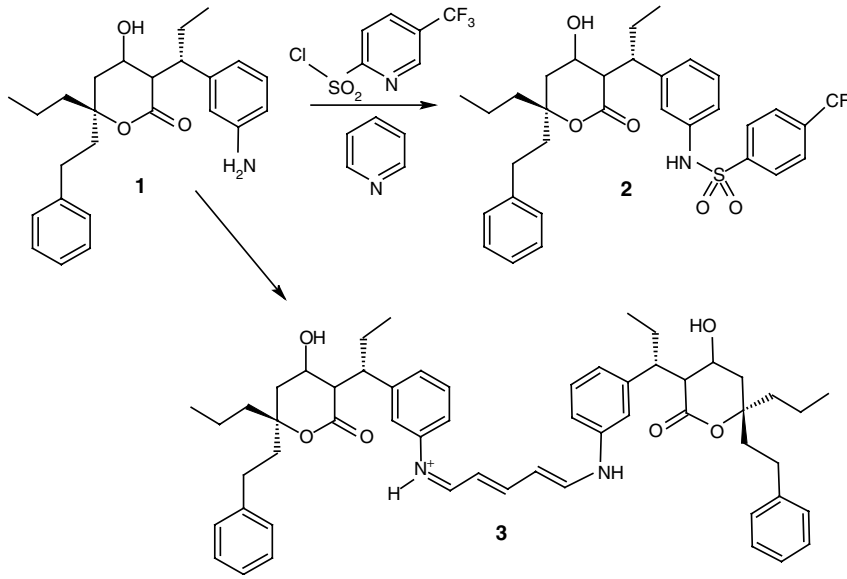
Rather than attempting what would of necessity be a far less than comprehensive survey of impurity characterization, it is more logical to give a few examples to provide the reader with a picture of the range of problems that one can encounter when challenged with the isolation and identification of drug impurities. Examples discussed briefly will include unstable process impurities, an impurity formed by cyclization during thermal stress testing, and finally the challenge of identifying some of the degradants of a severely degraded sample stored in DMSO for a prolonged period of time, which mimics the sort of problem one might face in trying to identify an active fraction from a compound collection sample.

5.3.1 Characterization of an unstable process impurity in the protease inhibitor Tipranavir

While using the 0.1% threshold as a determinant for when an impurity should be isolated to meet regulatory requirements is a good practice, there are other times when it becomes necessary to work with extraneous compounds at still lower levels. During the development of process chemistry for the synthesis of the protease inhibitor Tipranavir™ several synthetic lots of the drug were discolored, appearing pinkish rather than white as they should. It was determined that a low-level (<<0.1%) highly colored material was responsible for the problem with those lots and a request for the isolation and characterization of that contaminant was received [66].

From discussions with the process chemists engaged in the synthesis of Tipranavir, it was determined that the colored contaminant was being formed in the final step of the synthetic process, which involved coupling an aniline-like derivative with a chlorosulfonyl pyridine in the presence of pyridine being used as an acid scavenger as shown in Scheme 5.1. A sample of the colored impurity formed was obtained by methanol stripping of the silica gel chromatography column being used to remove the contaminant from bulk quantities of the drug.

The methanol solution containing the colored impurity was bright red and provided the feedstock used for preparative chromatographic isolation of the unstable, colored impurity, which had a half life of about 18 h in methanol and <10 m in acetone. Using a 1.7-mm 600-MHz SMIDG probe, which was the most sensitive probe technology routinely available when this work was done in 1999 [49, 59], a sample was prepared and a data set consisting of a proton reference



Scheme 5.1

spectrum, TOCSY, GHSQC, and 10-Hz optimized GHMBC spectra was acquired. The proton reference spectrum, a TOCSY spectrum with a 24 ms mixing time (23 m), and a GHSQC spectrum (2 h 9 min) required ~2.5 h to acquire. The 10-Hz optimized GHMBC spectrum was acquired in 12.5 h. The GHSQC and GHMBC spectra are shown in Figures 5.2 and 5.3, respectively.

The aromatic region of the reference spectrum, in addition to the resonances normally observed for the drug, showed three new well-resolved signals, corresponding to a doublet and two triplets at 8.25, 7.82, and 6.27 ppm, respectively. The doublet (8.25) and one of the triplets (6.27 ppm) were twice the intensity of the other triplet (7.82 ppm), which had an integrated intensity corresponding to half of that of a one proton signal in the drug molecule. This observation suggested that the impurity contained two molecules of drug and that the new resonances were present in a ratio of 2 : 2 : 1. The TOCSY spectrum confirmed the coupling of the new resonances in the aromatic region. A HRMS spectrum gave an exact mass of 848.47287 Da (less the ionizing proton) corresponding to the molecular weight of two of the aniline precursor molecules and a C_5H_5 fragment consistent with the integration of the new aromatic resonances in the proton spectrum. The GHSQC data (Figure 5.2) established the following direct proton–carbon correspondence: 8.25/152.7, 7.82/125.8, and 6.27/106.2 ppm as shown by the inset structural fragment in Figure 5.3. The GHMBC correlations linked the 8.25/152.7 ppm resonant pair to the aromatic quaternary carbon bearing what had been the aniline group resonating at 142.6 ppm. The other correlations were internal to the five-carbon fragment.

The deductions drawn from the NMR data were also supported by vibrational data. Prominent vibrational spectral features included an increase in the intensity of the band at 1636 cm^{-1}, suggesting increased double bond character in the

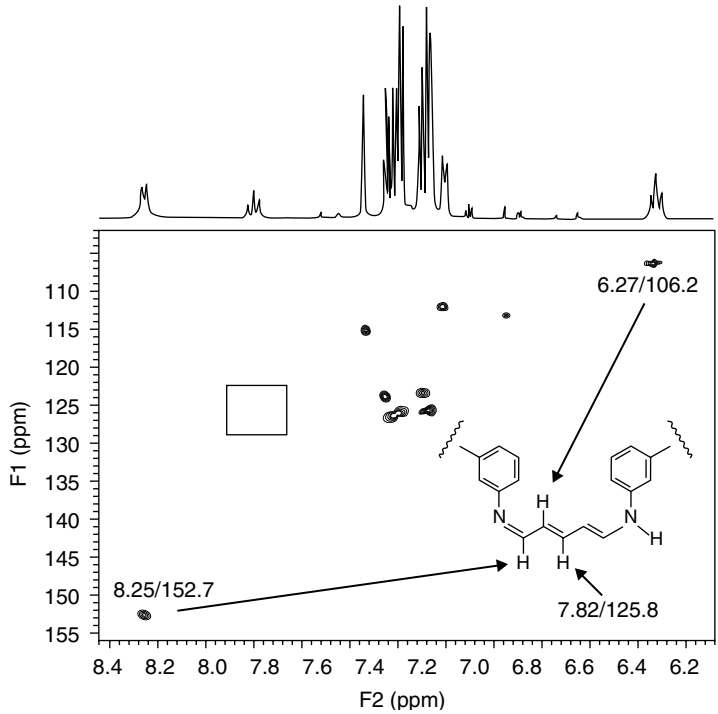


Figure 5.2 GHSQC spectrum of a colored impurity formed during the synthesis of Tipranavir in 2 h 9 m in with a 160 Hz optimization for the one-bond coupling constant. The triplet resonating at ~7.8 ppm gave a very weak response within the boxed region that was below the threshold used to plot this spectrum.

impurity relative to the parent drug and an intense IR band at 1547 cm^{-1} consistent with C = N stretching that is not present in Tipranavir. Changes were also noted in the 1170 cm^{-1} region of the spectrum consistent with an increase in C–O stretching and C–OH bending that can be accounted for by the dimeric nature of the impurity.

Working directly with the synthetic chemist involved in the process chemistry and taking advantage of his knowledge of both the chemistry and chemical literature, it was determined that what had formed in the final step of the process was a Zincke salt [67, 68]. The impurity exhibited maximum UV absorbance at 487 nm, which is consistent with the protonated form of various Zincke salts. The five carbon fragment linking the two aniline moieties, as shown in Scheme 5.1, arose from the pyridine ring coming apart during the reaction, which was first reported in the literature in 1903! Armed with the knowledge that pyridine was an inappropriate base to use as a scavenger in the final step of the synthesis, the process chemists were able to engineer out the formation of the impurity by switching to a more appropriate base.

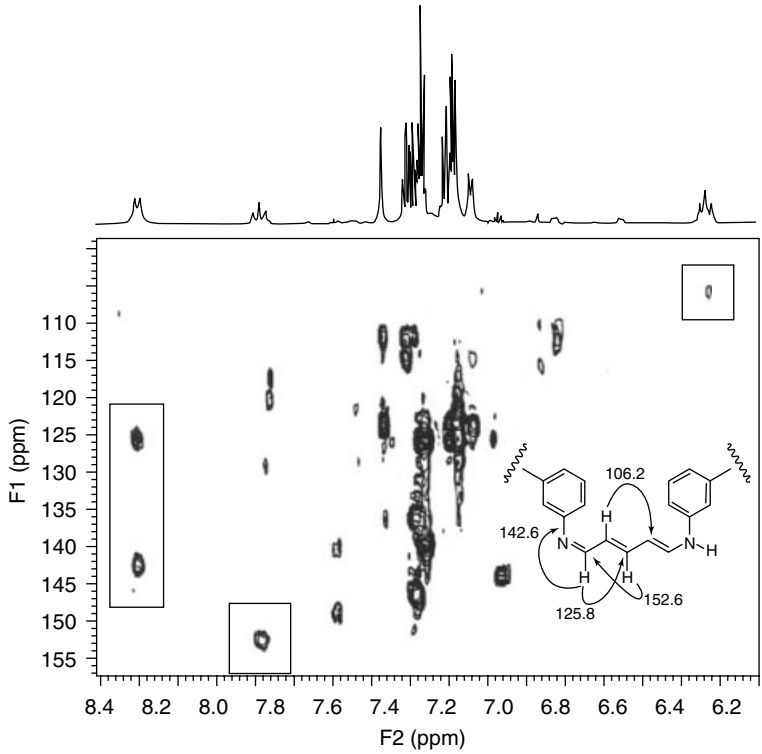
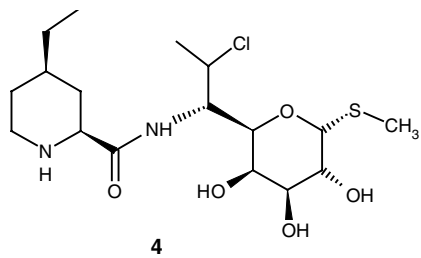


Figure 5.3 GHMBC spectrum of a colored impurity formed during the synthesis of Tipranavir. The long-range delay in the experiment was optimized for 10 Hz; the data were acquired in 12.5 h. Chemical shift labels show the chemical shift of the carbon to which a given proton is long-range coupled. As can be seen by simple inspection, there was considerable degradation of the sample during the course of the data acquisition as there are peaks in the contour plot corresponding to responses that were not observed in the proton spectrum taken at the outset of data acquisition, which is plotted above the contour plot.

5.3.2 Characterization of an impurity of pirlimycin formed by cyclization

During the development of a sterile formulation of pirlimycin, a semisynthetic, veterinary lincosaminide antibiotic derived from lincomycin **4**, three lots of material were subjected to thermal stress at 40°C for a period of 4 months, which led to the observation of a previously unreported degradant present at 0.2% [69].



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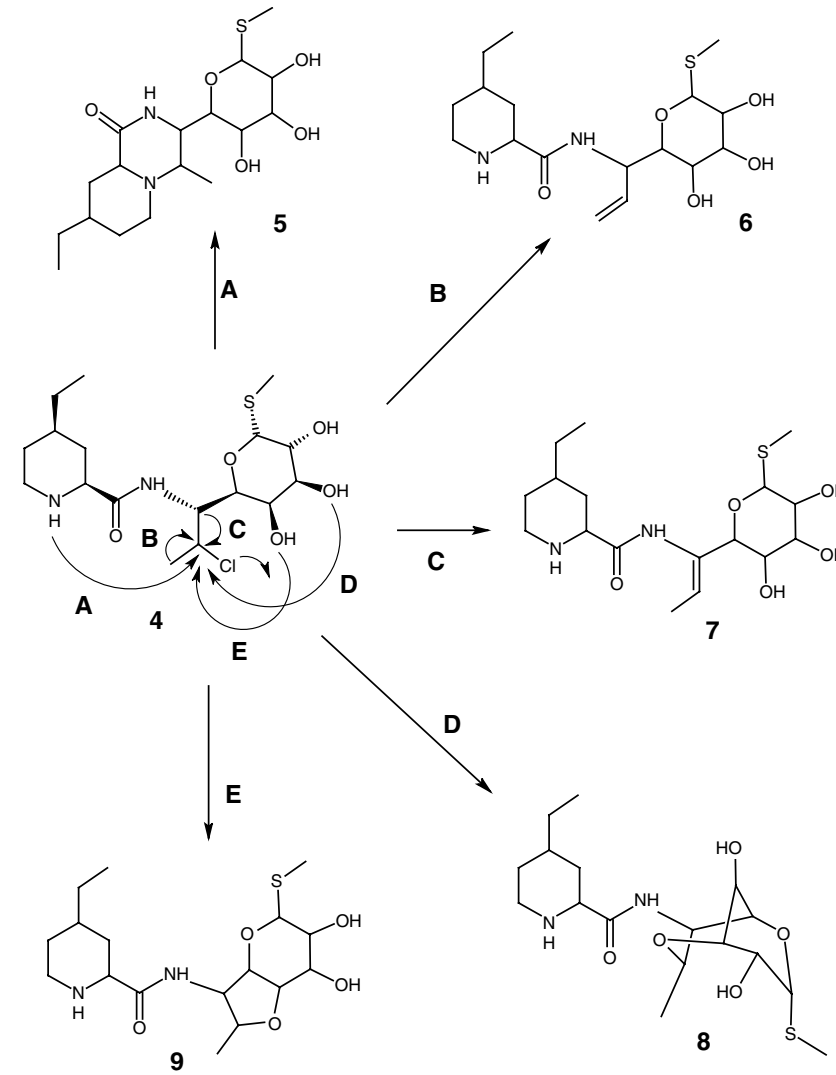
Samples of the degradant were isolated by preparative reversed-phase chromatography for structural characterization. There was sufficient sample available for the NMR measurements to be performed in a conventional 3-mm micro-NMR probe.

Preliminary LC/MS data recorded for the sample of degraded drug showed that the new degradant, which had a RRT = 0.26 relative to the parent molecule, had a molecular weight of 375 Da, which is 36 Da lower than the parent drug. In addition, the ^{37}Cl isotope peak was missing, confirming that the degradant had lost HCl relative to pirlimycin. Examining the structure, the five possibilities for the elimination of HCl are shown in Scheme 5.2; not all of the potential pathways leading to elimination, obviously, are plausible.

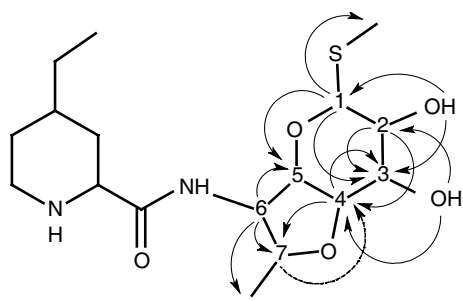


Scheme 5.2

Examining the mass spectral fragmentation pathways exhibited by the isolated degradant did afford some insights into the possible structure of the degradant formed by the dehydrohalogenation process. Fragment ions at 112 amu were observed for the parent drug, **4**, and the isolated degradant. The structural feature giving rise to this fragment ion in the MS/MS data corresponds to an intact 4′-ethyl-2′-piperidine ring, which suggests that the elimination via pathway A in Scheme 5.2, which leads to a piperidone system, **5**, is unlikely. There were no fragment ions observed that allowed any speculation on the side-chain elimination products, **6** and **7**, nor were there any fragment ions observed that would allow any conclusions to be drawn on cyclization products, **8**, and **9**, formed via pathways **D** and **E**, respectively. The exact mass of 374.18759 agreed to within 0.11 ppm of an empirical formula of $C_{17}H_{30}N_2O_5S$, consistent with dehydrohalogenation.

Considering the elimination pathways shown in Scheme 5.2, three pathways, A, D, and E, lead to elimination via cyclizations, while two pathways, B and C, are simple dehydrohalogenations entirely within the chloro-containing side chain. The complete absence of any protonated vinyl resonances in the gradient HSQC experiment performed on the isolated degradant immediately eliminated **6** and **7**, arising via elimination pathways **B** and **C**, respectively, from consideration. Of the three cyclization pathways, **D**, which ultimately leads to the formation of an eight-membered bridged bicyclo structure, was considered much less plausible than pathways **A** and **E**, which lead to a piperidinone and tetrahydrofuranyltetrahydropyran, respectively.

The structure of the degradation product formed by dehydrohalogenation was ultimately assigned as the tetrahydrofuranyltetrahydropyran analog, **9**, on the basis of the long-range correlations observed in a 7.8-Hz optimized GHMBC experiment. Long-range correlations observed for the degradant in the tetrahydrofuranyltetrahydropyran portion of the molecule are shown by **10**. Two key long-range correlations established the formation of the ether bridge of the tetrahydrofuran ring. The H4 methine proton resonating at 4.26 ppm was long-range coupled to the oxygen-bearing C7 methine carbon, which resonated at 75.7 ppm. The H7 methine proton resonating at 3.91 ppm was also weakly long-range coupled in the GHMBC data to the C4 methine carbon resonating at



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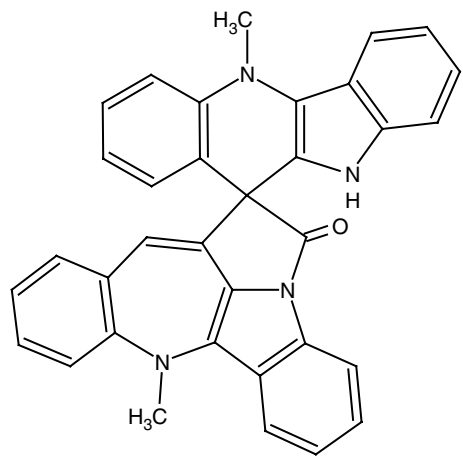
78.3 ppm. Consistent with the formation of the tetrahydrofuran ring, the chemical shifts of both C4 and C7 shifted downfield, 67.4 → 78.3 and 59.4 → 75.7 ppm, respectively, relative to where they were assigned for the parent molecule, **4**.

5.3.3 Structural characterization of degradation products of an alkaloid sample subject to long-term storage in DMSO

The quest for potential therapeutic leads has evolved over the years and is now largely based on high-throughput screening (HTS) methods through which libraries of compounds can be funneled. To facilitate HTS screening, compound libraries are now generally prepared in solution that can be deposited in 96-well plates that can be frozen between uses. Differences in compound solubility have made it attractive, in many cases, to use DMSO as the solvent of choice for this sort of application. Unfortunately, DMSO is a reactive solvent and compounds subjected to long-term storage in DMSO may undergo chemical degradation. To illustrate the sort of scenario that might be encountered in the search for a new therapeutic lead, the author and coworkers examined the fate of a 2.5 mg sample of the complex alkaloid cryptospirolepine (**11**) [70] subjected to storage in a sealed NMR tube in DMSO for a period of 10 years [71]. While the present example represents an extreme case because of the extensive nature of the degradation, in working with groups searching for active therapeutic leads, it is not uncommon to be faced with a sample that has decomposed, necessitating fractionation and subsequent characterization of the active fraction based on testing in an appropriate HTS screen.

When work was undertaken to isolate and identify some of the degradants of cryptospirolepine (**11**) represented by the chromatogram shown in Figure 5.4, we



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had no prior knowledge of the type of degradation processes that might have gone on during storage. The two largest peaks in the chromatogram were targeted for initial isolation and characterization. As the chromatographic method used for achieving the separation shown in Figure 5.4 was developed with preparative isolation in mind, the method was LC/MS-friendly and easily scaled up for preparative isolation. Isolates of the two largest chromatographic components were accomplished using a 21.2-mm × 250-mm Kromasil C18 column with an acetonitrile-aqueous trifluoroacetic acid mobile phase with detection at 270 nm. Collected fractions of the two major components, which were labeled DP-1 and DP-2, were pooled, concentrated, and desalted via trapping on a 10-mm × 250-mm Kromasil C18 column. Eluent from the trapping column containing the degradants was freeze-dried to yield isolates >95% pure.

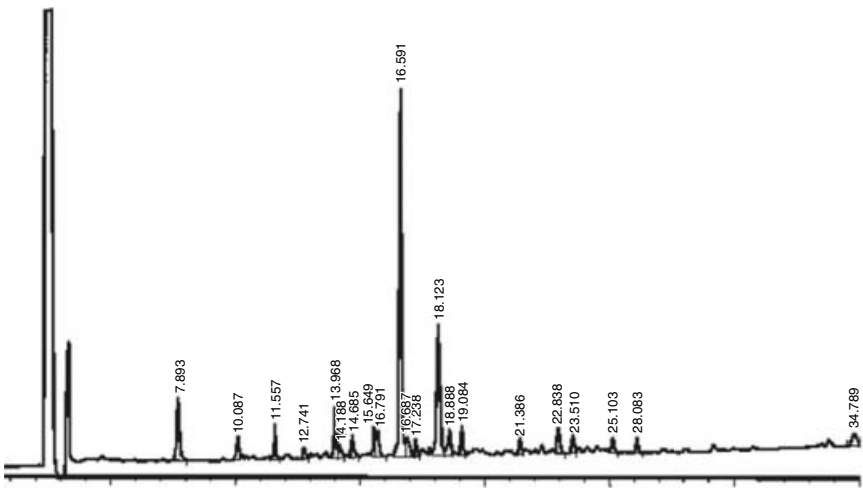
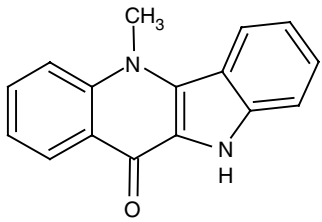


Figure 5.4 Reversed-phase HPLC chromatogram of a sample of the complex alkaloid cryptospirolepine (**11**) that had been stored in d_6-DMSO in a sealed NMR tube for a period of 10 years. None of the starting alkaloid remains; there are a total of 26 components observed in the chromatogram, the two major peaks at 16.591 and 18.123 min retention times constituting >45% of the degraded sample.

By LC/MS, the major peak in the chromatogram was found to give a parent ion, $MH^+ = 249$, which was immediately suggestive of oxidative decomposition of **11** at the spiro center. The mass spectral data suggested that the indolo [3,2-b]quinoline 'top' of the molecule was intact and probably oxidized at the 11-position to afford cryptolepinone (**12**), a known alkaloid of *Cryptolepis sanguinolenta*, the Ghanian plant from which cryptospirolepine (**11**) was isolated. A proton reference spectrum and COSY and GHSQC spectra were recorded, confirming the structure of the molecule as cryptolepinone (**12**) in approximately 20 min total.

The elucidation of the structure of the second, smaller degradant, DP-2, was considerably more challenging than the characterization of cryptolepinone (**12**).



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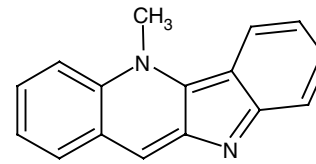

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MS on the DP-2 isolate gave a molecular ion $MH^+ = 479$, which corresponds to an empirical formula of $C_{32}H_{22}N_4O$ and a loss of C_2H_2 relative to cryptospirolepine (**11**) with an empirical formula of $C_{34}H_{24}N_4O$. Prominent fragment ions were observed in an MS/MS experiment at 464, 447, 435, 432, 247, 232, and 217 Da. The 232 Da daughter ion was enlightening and suggestive of the structure of another known alkaloid, cryptolepine (**13**), minus a proton as a possible structural component of the structure of DP-2.

The ~100 μg NMR sample of DP-2 in d_6-DMSO solution was initially reddish-orange and somewhat broad, suggesting the possibility of protonation. A small quantity of ammonia gas was bubbled through the sample, causing the color to shift to deep purple, which is characteristic of cryptolepine (**13**) and also suggestive of an 11-cryptolepinyl fragment incorporated into the structure of the degradant. The proton spectrum after treatment with ammonia gas was considerably sharper, as seen in Figure 5.5.

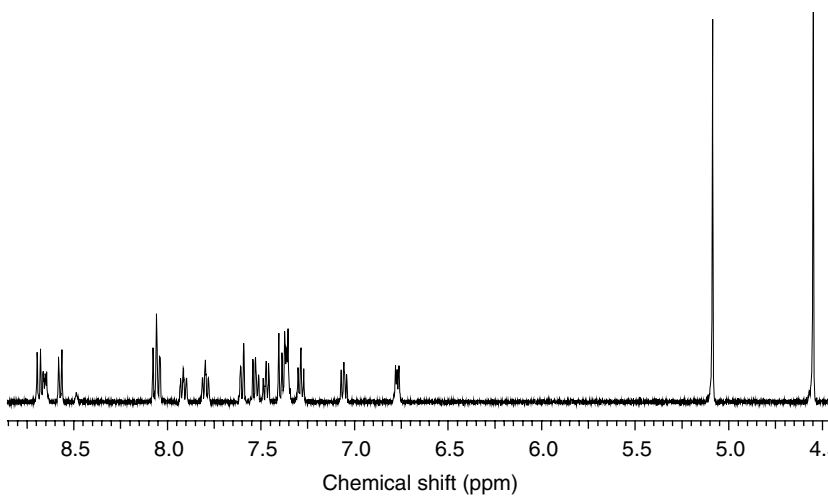


Figure 5.5 Proton spectrum of degradant DP-2 recorded in d_6-DMSO at 500-MHz following treatment with ammonia gas.

COSY and GHSQC spectra were acquired overnight on the sample at 500-MHz using a 3-mm gradient inverse triple resonance NMR probe, followed by a ROESY spectrum with a 500 ms mixing time. These data readily facilitated the identification of an 11-cryptolepinyl moiety as expected on the basis of the MS/MS data. A phase-sensitive 8-Hz optimized HMBC experiment was next performed overnight using a Varian INOVA 500-MHz spectrometer equipped with a 5-mm gradient inverse triple resonance Varian Cold-probe™ to avoid what would otherwise be a minimum of a weekend-long acquisition using a conventional NMR probe. A comparison of the conventional and phase-sensitive HMBC presentations is shown in Figure 5.6 and can be quite useful for molecules with highly congested NMR spectra as in the present case with DP-2.

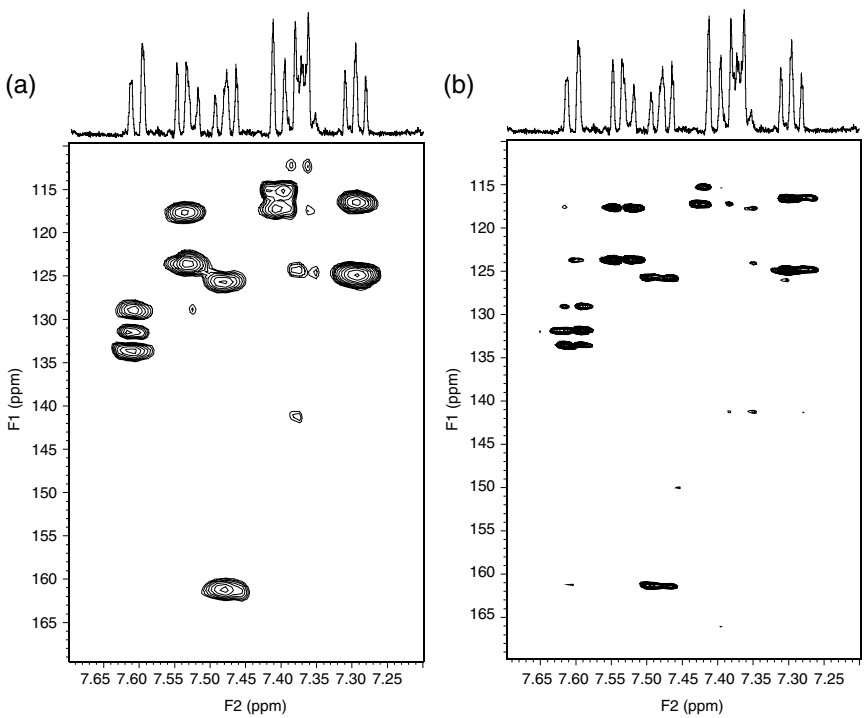


Figure 5.6 Comparative segments from (a) conventional and (b) phase-sensitive 8-Hz optimized HMBC spectra of DP-2 recorded using a 3-mm sample positioned coaxially in a 5-mm gradient inverse triple resonance Varian Cold-probe™ .

Long-range ^{1}H–^{13}C connectivity information in the phase-sensitive HMBC spectrum (Figure 5.7) allowed the assembly of the structural fragment shown by **14**. The wavy lines denote the point beyond which long-range correlations could not reach. Structural fragment **14** and an 11-cryptolepinyl moiety derived from **13** account for 31 of 32 carbons in the empirical formula of the molecule. A sole

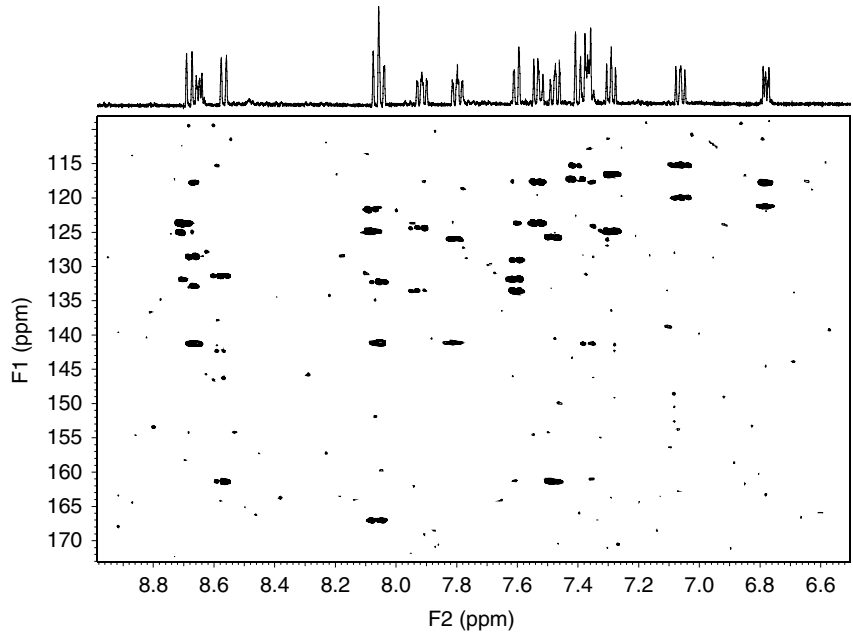
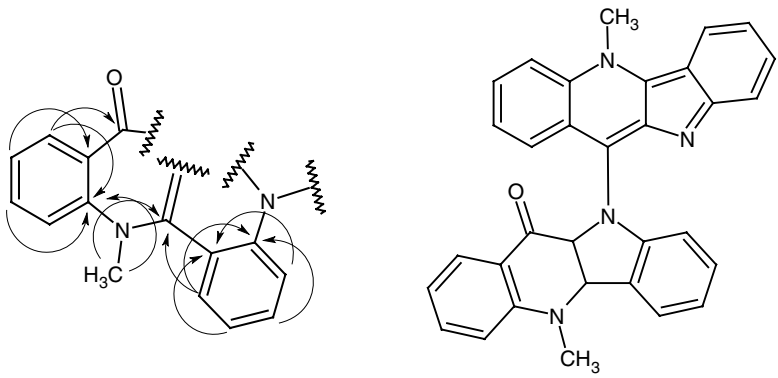


Figure 5.7 Phase-sensitive 8-Hz optimized HMBC spectrum of degradant DP-2 recorded overnight using a 500-MHz Varian Cold-probe™ .



14 **15**

quaternary carbon resonance at 130.0 ppm remains unassigned, and it is logical to assemble the pieces available to afford the bis indolo[3,2-*b*]quinoline, **15**.

As a final piece of data to further support the structure of **15**, a 3–6-Hz optimized ^{1}H–^{15}N CIGAR-HMBC experiment was also acquired over a weekend using a 500-MHz Varian Cold-probe™ , which identified correlations to three of the four nitrogen resonances in the structure of **15** despite the small, ~100 μg,

sample available for analysis. The ^{15}N shifts of the nitrogens to which correlations were observed were consistent with those of other members of the *Cryptolepis* family of indoloquinoline alkaloids that have been previously studied by long-range ^{1}H–^{15}N 2D NMR methods [53, 55].

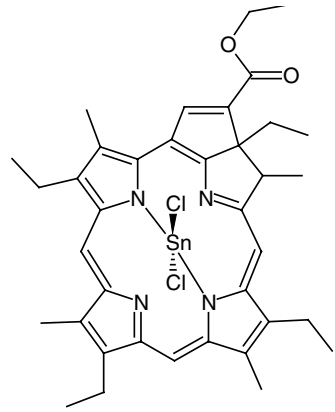
In parallel to the elucidation of the structure of **15** by the author, computer-assisted elucidation of the structure was simultaneously undertaken using Advanced Chemistry Development's Structure Elucidator software package [72–75]. Using the data from the proton, COSY, HSQC, and ^{1}H–^{13}C HMBC spectra as data input, the program generated 4700 structures during a 15 h computation. After removal of duplicates, 334 structures remained. When the remaining 334 structures were ordered on the basis of calculated ^{13}C chemical shift, **15** was in the second position of the output table. The compound in the first position could be readily rejected on the basis of one of the indoloquinoline ring fusions, which was an indolo[2,3-*b*]quinoline rather than the indolo[3,2-*b*]quinoline of the cryptospirolepine (**11**) from which the degradation began.

When the 11-cryptolepinyl fragment and the ^{1}H–^{15}N long-range correlation data were used as an input constraint for Structure Elucidator, the process was dramatically faster. With the modified input, the program generated only 111 structures in 20 min, with the correct structure again in the second position of the ^{13}C sorted output list. The value of the constraints afforded by the availability of user-defined fragments and long-range ^{1}H–^{15}N data are obvious. Although Structure Elucidator is not used for every unknown impurity or degradant problem in the author's laboratory, the program package does serve as a very useful tool when particularly challenging unknown structures have to be identified, especially if those structures are relatively proton deficient in nature.

5.3.4 The impact of experiment selection on the elucidation of the structure of a complex thermal degradant

During the terminal heat sterilization of the lipid emulsion formulation of the photodynamic therapeutic (PDT) agent tin ethyl etiopurpurin (SnET2, **16**), a new degradant was observed at low levels and required isolation and characterization. The problem of characterizing the degradant was made more challenging by the photo-instability of the molecule, coupled with difficulties in the preparative chromatography as well as the acquisition of both mass spectral and NMR data [76].

Chromatographically, the lipid emulsion formulation of the drug and the nature of the degradant, which was very late eluting with RRT $\cong$ 2.5, frustrated the use of normal reversed-phase preparative chromatography. Instead, it was necessary to resort to normal-phase chromatography using a YMC cyano column and a hexane-acetone-*N*,*N*-dimethyl formamide mobile phase. The pooled green fractions of the degradant were concentrated under a stream of dry nitrogen in the dark followed by heated vacuum centrifugation to afford ~250 μg of a photolabile isolate of ~85% purity.



16

Difficulties in the acquisition of the mass spectral data were encountered in the reproducibility of the parent ion cluster for the degradant in positive ion modes. After some trial and error investigation, it was found that normal-phase LC/MS negative ion MS with a 350°C nebulizer temperature gave a reproducible parent ion cluster with little degradation. The nominal molecular weight of the degradant was 814 Da, corresponding to an empirical formula of $C_{37}H_{44}Cl_2N_4O_5Sn$. The experimental and predicted parent ion clusters for the molecule are shown in Figure 5.8. MS/MS studies did not prove fruitful with this degradant, as only trivial, neutral fragment losses were observed. Likewise, the vibrational data did not give any insights into the nature of the chemical modification of the degradant relative to the parent, which suggests that the change may have introduced another functional group of a type already present in the structure.

Despite access to 1.7-mm SMIDG NMR probe capabilities on a 600-MHz spectrometer, the ~250 μg isolate sample dissolved in *d_7-N,N*-dimethylformamide gave a disappointingly low number of long-range correlations in an 8-Hz optimized HMBC experiment acquired in the attempt to characterize the compound. In contrast, when a 2–10-Hz optimized IMPEACH-MBC [48, 77] experiment was acquired (comparison shown in Figure 5.9), there were sufficient long-range correlations observed to allow the characterization of the SnET2 degradant structure shown by **17**. The difference between the results with the HMBC and IMPEACH-MBC experiments underscores the need to consider alternative experiments whenever any experiment fails to provide the anticipated results. Long-range correlations observed in the HMBC and IMPEACH-MBC experiments are superimposed over the degradant structure in **17**. Long-range correlations shown in black were observed in the 8-Hz optimized HMBC experiment. Long-range correlations denoted by both grey and black arrows were observed in the 2–10-Hz accordion-optimized IMPEACH-MBC experiment. While there are

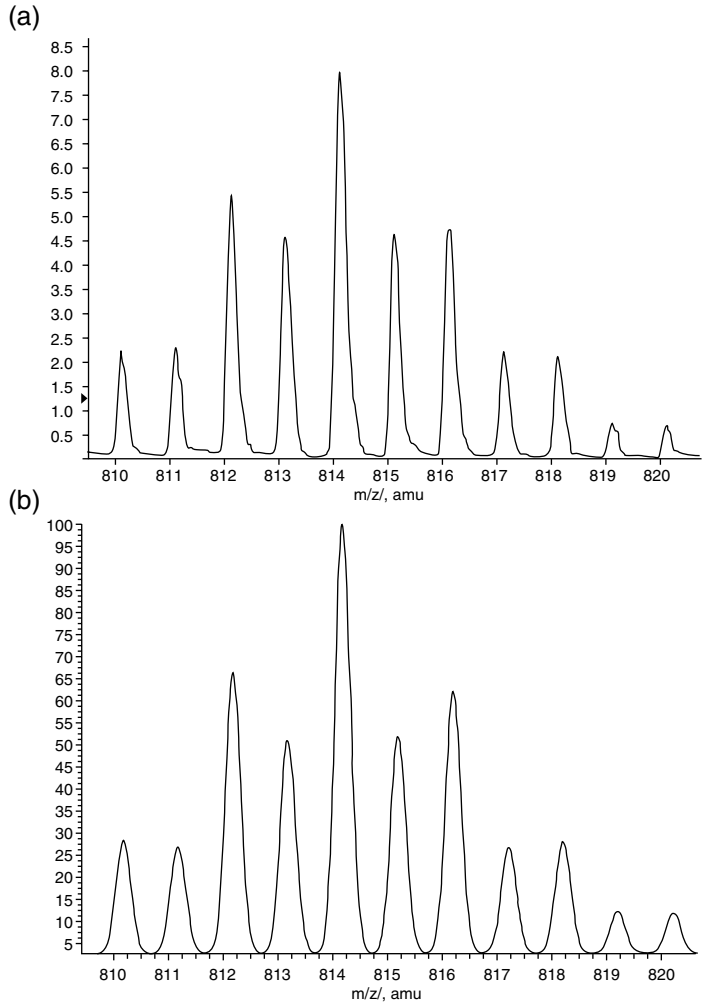


Figure 5.8 (a) Calculated and (b) observed negative ion APCI molecular clusters for an isolated degradant of SnET2 (**16**).

sensitivity losses associated with the use of the accordion-optimized experiments in general, these losses are frequently offset by the greater numbers of correlations, particularly $n > 3$ $^{n}J_{CH}$ correlations.

5.4 Conclusions

The isolation and structural characterization of impurities and degradation products of pharmaceuticals is a necessary and sometimes challenging undertaking.

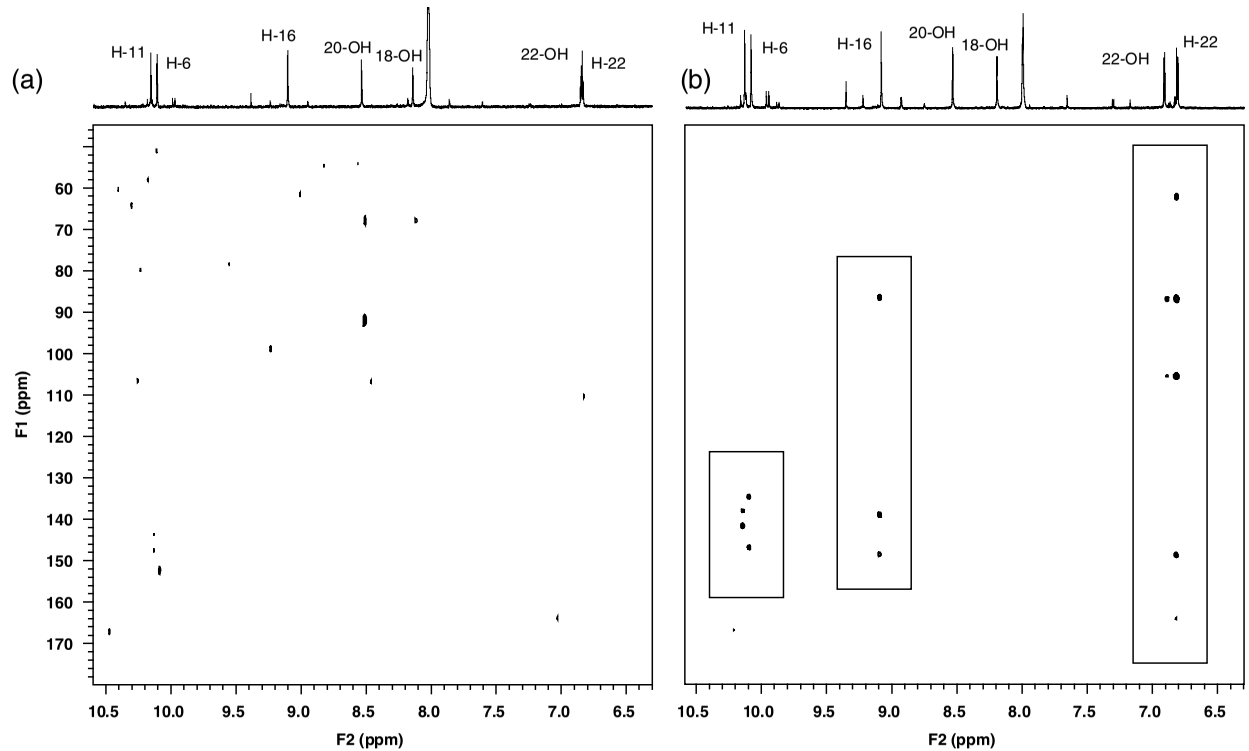
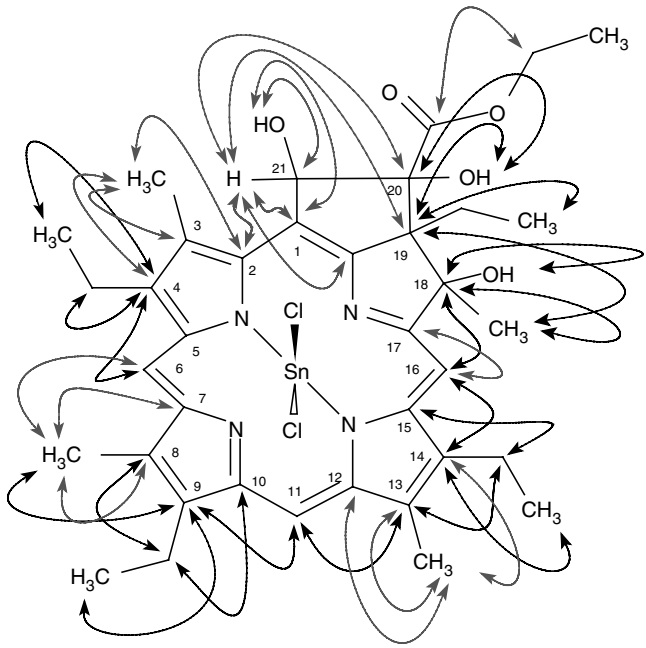


Figure 5.9 Comparison plots of the downfield region of (a) an 8-Hz optimized HMBC and (b) a 2–10-Hz accordion-optimized IMPEACH-MBC experiment. Both spectra were acquired using a 1.7-mm SMIDG NMR probe for identical times at 600-MHz.



17

The overall process is quite labor and capital intensive. Groups tasked with the responsibility of isolating and identifying impurities and degradation products typically require a capital investment of millions of dollars in hardware, as well as individuals highly skilled in the utilization of the equipment and the interpretation of the data it is capable of generating. Development timelines, clinical trial starting dates, and the like, require a high degree of efficiency that is most readily attainable through the integration of the preparative chromatography and spectroscopic disciplines necessary to solve these structures. Beyond the integration of the various scientific disciplines involved, the investigators must individually be able to adapt quickly to the vagaries of a given problem, changing the experiments utilized to obtain the highest quality data possible in the shortest amount of time. After all data are in hand, the scientists in a group tasked with impurity/degradant identification need to be able to divorce themselves from their respective disciplines to facilitate the rigorous evaluation and interpretation of all of the data in hand to be certain that the structure proposed during the characterization process is fully consistent with all of the available data.

References

1. S. Görög, ed. *Identification and Determination of Impurities in Drugs*, Elsevier, Amsterdam, 2000.

2. S. Ahuja and K. Alsante, eds., *Handbook of Isolation and Characterization of Impurities in Pharmaceuticals, 5*, Elsevier, Amsterdam, 2003.
3. T. Cecil and E. Sheinin, in *Handbook of Pharmaceutical Analysis by HPLC*, U.S. Pharmacopeia, Rockville, 2005, pp. 359–377.
4. K. M. Alsante, F. C. Friedmann, T. D. Hatajik *et al*., in *Handbook of Modern Pharmaceutical Analysis* (Seperation Science and Technology), ed. S. Ahuja and S. Scypinski, Elsevier, Academic Press, San Diego, 2001, pp. 85–172.
5. S. Husain and R. N. Rao, *J. Chromatogr. Libr.*, 1998, **60**, 833–888.
6. I. N. Glazkov, N. L. Cochkareva and I. A. Revel'skii, *J. Anal. Chem*., 2005, **60**, 107–118.
7. S. Görög, *Anal. Bioanal. Chem*., 2003, **377**, 852–862.
8. U. Holzgrabe, R. Deubner, N. Novatchem and F. Wienin, *Deut. Apoth. Zeit*., 2003, **143**, 57–64.
9. J. Roy, *AAPS Pharm. Sci. Technol*, **3**(2): article 6: available online *via* the following: http://aapspharmscitech.org/scientificjournals/pharmscitech/vol3issue2/030206/030206.htm
10. L. L. Lohr, T. R. Sharp, K. M. Alsante and T. D. Hatajik, *Am. Pharm. Rev*., 2001, **4**, 104–113.
11. K. M. Alsante, T. D. Hatajik, L. L. Lohr and T. R. Sharp, *Am. Pharm. Rev*., 2001, **4**, 70–78.
12. J. C. Berridge, *J. Pharm. Biomed. Anal*., 1995, **14**, 7–12.
13. D. L. Norwood and F. Qiu, *Am. Pharm. Rev*., 2004, **7**, 92–99.
14. P. Crowley and L. Martini, *Pharm. Technol. Eur*., 2001, **13**, 26–34.
15. D. Jakel and M. Keck, *Drugs Pharm. Sci*., 2000, **103**, 21–58.
16. http://www.ich.org/cache/compo/276–254–1.html
17. J. D. Orr, I. S. Krull and M. E. Schwartz, *LC-GC North America*, 2003, **21**, 1146–1152.
18. J. D. Orr, I. S. Krull and M. E. Schwartz, *LC-GC North America*, 2003, **21**, 626–633.
19. Drug GMP Report, No. 126, 2003, pp. 1,2.
20. S. Görög, *Trends Anal. Chem*., 2003, **22**, 407–415.
21. G. E. Martin, in *Annual Report in NMR Spectroscopy*, ed. G. A. Webb, Elsevier, Amsterdam, 2005, pp. 1–98.
22. I. C. Jones, G. J. Sharman and J. Pidgeon, *Magn. Reson. Chem*., 2005, **43**, 497–509.
23. A. M. Swivedi, *Int. J. Pharm. Excipients*, 2003, 33–37.
24. C. B'Hymer, *Pharm. Res*., 2003, **20**, 37–344.
25. C. Todd and E. Sheinin, in *Handbook of Pharmaceutical Analysis by HPLC* (Seperation Science and Technology), ed. S. Ahuja and M.W. Dong, Elsevier, Academic Press, San Diego, 2005, pp. 359–377.
26. R. Nageswara and V. Nagaraju, *J. Pharm. Biomed. Anal*., 2003, **33**, 335–377.
27. A. Aranyi, *Prog. Pharm. Biomed. Anal*., 2000, **4**, 240–251.
28. M. Gazdg, *Prog. Pharm. Biomed. Anal*., 2000, **4**, 210–239.
29. R. God, H. Gumm, M. Juschka and C. Heuer, *GIT Lab. J*., 1999, **3**,188–191.
30. S. Husain and R. R. Rao, *Proc. Control Quality*, 1997, **10**, 41–57.
31. A. Lauko, *Prog. Pharm. Biomed. Anal*., 2000, 183–209.
32. M. I. R. M. Santoro, M. S. A. Prado, M. Steppe and E. R. M. Kedor-Hackmann, *Rev. Brasileira de Ciencias Farm*., 2000, **36**, 97–110.
33. M. R. Euerby, *Prog. Pharm. Biomed. Anal*., 2000, **4**, 346–358.
34. K. D. Altria, *Prog. Pharm. Biomed. Anal*., 2000, **4**, 323–345.
35. C. L. Flurer, *Electrophoresis*, **20**, 1999, 3269–3279.
36. K. D. Altria, in *High Performance Capillary Electrophoresis*, Chemical Analysis, New York, 1998, pp. 853–877.
37. K. D. Altria, M. A. Kelly and B. J. Clark, *Trends Anal. Chem*., 1998, **17**, 214–226.
38. K. D. Altria, M. A. Kelly and B. J. Clark, *Trends Anal. Chem*., 1998, **17**, 204–214.

39. K. D. Altria, *J. Chromatogr. A.*, 1996, **735**, 43–56.
40. N. D. Gangal, *Chem. Ind. Digest.*, 2004, **17**, 63–70.
41. K. Yamashita, M. Okamoto and K. Nakai, *Environ. Health Sci. Lab.*, 2002, 56–64.
42. A. M. Krstulov, C. R. Lee, S. Firmin, G. Jacquet, C. Nguyen Van Dau and D. Tessier, *LC-GC Eur.*, 2002, **15**, 31–41.
43. J. Ermer and M. Vogel, *Biomed. Chromatogr.*, 2000, **14**, 373–383.
44. S. J. Lane, *Prog. Pharm. Biomed. Anal.*, 2000, **4**, 359–381.
45. L. Tollsten, *Prog. Pharm. Biomed. Anal.*, 2000, **4**, 266–298.
46. M. Mak, G. Czier and J. Brlik, *Prog. Pharm. Biomed. Anal.*, 2000, **4**, 97–109.
47. W. M. A. Niessen, *Chimia*, 1999, **53**, 379–483.
48. G. E. Martin, in *Annual Report in NMR Spectroscopy, vol. 46*, ed. G. A. Webb, Academic Press, San Diego, 2002, pp. 37–100.
49. G. E. Martin, in *Annual Report NMR Spectroscopy, vol. 56*, ed. G. A. Webb, Elsevier, Amsterdam, 2005, pp. 1–119.
50. W. F. Reynolds and R. G. Enriquez, *Magn. Reson. Chem.*, 2001, **39**, 531–538.
51. T. D. W. Claridge, *High-Resolution NMR Techniques in Organic Chemistry*, Pergammon Press, Amsterdam, 1999, pp. 129–143.
52. G. E. Martin, C. E. Hadden and D. J. Russell, in *Handbook of Spectroscopy, vol. 1*, ed. G. Gauglitz and T. Vo-Dinh, Wiley-VCH, New York, 2003, pp. 213–216.
53. G. E. Martin and C. E. Hadden, *J. Nat. Prod.*, 2000, **63**, 543.
54. R. Marek and A. Lycka, *Curr. Org. Chem.*, 2002, **6**, 35.
55. G. E. Martin and A. J. Williams, in *Annual Report in NMR Spectroscopy*, ed. G. A. Webb, Elsevier, Amsterdam, 2005, pp. 1–119.
56. G. E. Martin and C. E. Hadden, *Magn. Reson. Chem.*, 2000, **38**, 251–256.
57. R. C. Crouch, A. O. Davis and G. E. Martin, *Magn. Reson. Chem.*, 1995, **95**, 174.
58. C. E. Hadden, G. E. Martin, J.-K. Luo and R. N. Castle, *J. Heterocyclic Chem.*, 2000, **37**, 821–825.
59. G. E. Martin, in *Encyclopedia of NMR Spectroscopy, vol. 9*, ed. D. M. Grant and R. K. Harris, John Wiley and Sons, Chichester, 2002, pp. 98–112.
60. G. E. Martin, in *Handbook of Modern Magnetic Resonance*, ed. G. A. Webb, Springer-Verlag, Berlin, 2006, in press.
61. H. Kovacs, D. Moskau and M. F. Spraul, *Prog. Nucl. Magn. Reson. Spec.*, 2005, **46**, 131–155.
62. J. C. Lindon, J. K. Nicholson and I. D. Wilson, *J. Chromatogr, B.*, 2000, **748**, 233–258.
63. I. D. Wilson, L. Griffiths, J. C. Lindon and J. K. Nicholson, *Prog. Pharm. Biomed. Anal.*, 2000, 299–322.
64. P. G. Cummings, P. Offen, M. A. Olsen, S. Kennedy-Gabb and G. Zuber, *Mass Spec. Chem. Develop.*, 2003, **6**, 88–92.
65. B. Hegedus, *Prog. Pharm. Biomed. Anal.*, 2000, **4**, 580–591.
66. G. E. Martin, B. A. Pearlman, R. H. Robins *et al.*, *J. Heterocyclic Chem.*, 1999, **36**, 1107–1114.
67. T. Zincke, *Leibigs Ann. Chem.*, 1903, **330**, 361.
68. T. Zincke, *Leibigs Ann. Chem.*, 1904, **333**, 296.
69. F. W. Crow, J. R. Blinn, C. G. Chidester *et al.*, *J. Heterocyclic Chem.*, 1999, **36**, 1049–1055.
70. A. N. Tackie, G. L. Boye, M. H. M. Sharaf *et al.*, *J. Nat. Prod.*, 1991, **56**, 533.
71. G. E. Martin, C. E. Hadden, D. J. Russell *et al.*, *J. Heterocyclic Chem.*, 2002, **39**, 1241–1250.
72. K. A. Blinov, D. Carlson, M. E. Elyashberg *et al.*, *Magn. Reson. Chem.*, 2003, **41**, 359.
73. M. E. Elyashberg, K. A. Blinov, E. R. Martirosian, S. G. Molodtsov, A. J. Williams and G. E. Martin, *J. Heterocyclic Chem.*, 2003, **40**, 1017.

74. M. E. Elyashberg, K. A. Blinov, S. G. Molodtsov, A. J. Williams and G. E. Martin, *J. Chem. Inf. Comput. Sci.*, 2004, **44**, 771.
75. S. G. Molodtsov, M. E. Elyashberg, K. A. Blinov, A. J. Williams and G. E. Martin, *J. Chem. Inf. Comput. Sci.*, 2004, **44**, 1737.
76. C. E. Hadden, W. K. Duholke, B. D. Kaluzny *et al.*, *J. Heterocyclic Chem.*, 2003, **40**, 389–393.
77. C. E. Hadden, G. E. Martin and V. V. Krishnamurthy, *J. Magn. Reson.*, 1999 **140**, 274.

Analysis of Drug Impurities
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6 The use of chromatography and online structure elucidation using spectroscopy

Janet Hammond and Ian Jones

The manufacture of drug substances is regulated by a variety of national authorities with a strong emphasis on the purity of the drug substance. For drugs that are about to be launched on to the market, there is a regulatory requirement to identify impurities in the drug substance at a level of 0.10% or above [1]. The use of LC-MS alone is often sufficient for impurity identification and is a common approach within the early stages of pharmaceutical development where some risk is acceptable. LC-NMR is usually applied in analyses where more certainty is required or when LC-MS is inconclusive, for example in analysis of compounds that ionise poorly or where we need to distinguish between possible isomers.

6.1 Introduction to LC-MS

Structural elucidation and confirmation of impurities is required in all areas of drug development [2]. In process development, impurities during all the synthetic stages need to be identified to control the quality of the drug substance and drug product. Degradation of both drug substance and drug product can be due to simple processes such as oxidation, hydrolysis or dehydration. On the other hand, it can be complex and sometimes unpredictable. This depends on the structure of the active ingredient, composition of its formulation, how it is prepared, the storage conditions and storage containers used, and many other factors. Drug metabolite identification in both discovery and development phases requires extensive structure elucidation. Because of its speed, selectivity and sensitivity and compatibility with reversed-phase chromatography, LC-MS and the tandem techniques of MS-MS have become indispensable analytical techniques for the analysis of pharmaceuticals and have become the method of first choice [3–5]. LC-MS has also provided major support in chromatographic method development and its associated validation, for example in demonstrating peak purity.

The great advantage of the technique is that it provides molecular weight information and on some instruments structural fragments and empirical formulae on line, without the need for time-consuming isolation, in a large number of cases.

In the early days of the technique, from the early 1970s to around 1990, LC-MS was very much in the realm of the dedicated mass spectroscopist: it was a very difficult technique to use, unreliable, insensitive and, to many, of

doubtful value. Fortunately, the early proponents of the technique continued to develop ever more ingenious ways of getting the liquid output from an HPLC column into the gas phase required by the mass spectrometer.

It was not until the introduction of Thermospray Ionisation in the 1980s that a system was available that enabled sufficiently reliable LC-MS instruments to be manufactured [6]. At this point the HPLC community began to realise this was a technique that might just answer their wish for a much needed universal detector. Thermospray ionisation never quite gave the degree of reliability, nor the sensitivity, required to fulfil this dream, but it did show that LC-MS was a very valuable technique to have available. The impetus for further development was therefore present.

Fenn published work in 1989 [7–9] showing ionisation of large molecules by electrospray ionisation (ESI). Fenn built on the early work of Malcolm Dole [10] but Fenn used a counter current gas to assist with desolvation of the droplets and aid the formation of the ions. In the early 1990s, experiments with atmospheric pressure ionisation (API) showed promise and in a short space of time the first commercial systems utilising the new techniques of ESI [11] and Atmospheric Pressure Chemical Ionisation (APCI) began to appear on analysts' benches. The sensitive, reliable and easily operated LC-MS system had arrived.

ESI and APCI [12, 13] are the dominant techniques today for LC-MS in pharmaceutical analysis. These both occur at atmospheric pressure; hence the phrase atmospheric pressure ionisation (API).

In this chapter, we give an overview on how the API techniques work and which factors have an important influence on the performance. Examples are presented mostly from published work to demonstrate how LC-MS, LC-MS-MS, collision-induced dissociations (CIDs), accurate mass measurements and hydrogen/deuterium exchange have been systematically and successfully applied in the structural elucidation of impurities, degradation products and metabolites. In addition, these also illustrate how mass spectrometry has offered a third dimension to chromatographic method development and validation.

6.2 API techniques

6.2.1 Electrospray process

The electrospray process transforms ions in solution into ions in the gas phase [11]. A flow of analyte in solution is passed through a narrow metal capillary. A high potential difference (around 3 kV) is applied to the capillary at the spray tip. This high potential creates a fine mist of highly charged droplets. Nebulising gas flowing co-axially around the capillary aids this process. The charge of the particles can be either positive or negative depending on the ionising voltage polarity. The charged droplets decrease in size by desolvation and this is assisted by a flow of warm nitrogen drying gas. The electrical charge density at the surface

increases as desolvation occurs and reaches a critical point known as the Rayleigh stability limit. At this point the droplets divide into smaller droplets because the electrostatic repulsion is greater than the surface tension. These are known as coulombic explosions. This process repeats itself until analyte ions free from solvent are released from the droplet. These desolvated ions are then passed through the sample cone into the high vacuum mass analyser.

Two mechanisms for electrospray process have been proposed:

1. Charged residue mechanism. This was proposed by Dole and suggests that after multiple coloumbic explosions droplets are formed, which contain only one ion [10]. This mechanism is thought to be important for the ionisation of macromolecules.
2. Iribarne and Thompson proposed the ion-evaporation mechanism. This suggests desorption of the ions from the droplets when they are less than 10 nm in size [14]. This is thought be the dominant mechanism for small molecules.

What is certain about the process is that from the time the initial (neutral) solution is passed through the capillary to the detection of the ions in the mass spectrometer a complex series of reactions has taken place.

The early ESI interfaces were all optimised for flow rates between 1 and 10 μl/min. In trying to achieve direct compatibility with analytical HPLC, much development work has been done to accommodate higher flow rates and increase the efficiency of the nebulisation process. The present pneumatically assisted ESI interface is optimised around flow rates of 50–300 μl/min. It is not the intention here to describe all the different manufacturers' interfaces and source designs for API but the technology has been well documented [15].

6.2.2 APCI

The APCI process is akin to chemical ionisation in that the ionisation takes place in the gas phase. The APCI process requires a flow of the analyte in solution to be passed through a heated capillary in to the source. The capillary tip is held at a high temperature, around 400–500°C, to vaporise the high flow of eluent, which can be anything up to 2 ml/min. The temperature may also be chosen to suit the analyte. The solvent flow from the capillary is converted into an aerosol with the aid of the nebulising gas and begins to evaporate very quickly. Close to the probe is a corona discharge needle held at around 3–8 kV. A corona effect is a partial discharge around the electrode that leads to ionisation and electrical breakdown of the surrounding area. In the case of APCI, the area around the corona electrodes consists of solvent vapour, nitrogen gas and analyte molecules. The solvent vapours are ionised by the corona effect and react with the analyte molecules in the gas phase.

Scheme 6.1 shows a series of reactions that starts with the electron-initiated cation formation with nitrogen gas in the positive ion mode.

Primary ion formation

$N_2 + e^- \longrightarrow N_2^{+\bullet} + 2e^-$

Secondary ion formation

$N_2^{+\bullet} + H_2O \longrightarrow N_2 + H_2O^{+\bullet}$

$H_2O^{+\bullet} + H_2O \longrightarrow H_3O^+ + HO\bullet$

Primary transfer

$H_3O^+ + M \longrightarrow [MH]^+ + H_2O$, where *M* is the analyte

Scheme 6.1

In essence, solvent ions can transfer a proton to the analyte if the analyte's proton affinity (PA) is higher than that of the eluent molecules, by means of gas-phase reactions.

In negative ion mode, $(M–H)^-$ is typically formed by the proton abstraction by OH^-.

APCI sources exhibit mass-dependent sensitivity, that is, the more analyte that enters the source, the greater the signal from that analyte (up to a point), so it is advantageous to allow the full flow into the source.

6.2.3 Atmospheric pressure photo ionisation

The introduction of atmospheric pressure photo ionisation (APPI) technique in 2000 has enabled a wider range of analytes to be examined by LC-MS [16, 17]. These include neutral non-polar compounds that have very little or no sensitivity to ESI and APCI techniques [18]. APPI is analogous to APCI in that the liquid flow is vaporised in the heated nebuliser but the corona discharge needle has been replaced by a krypton discharge lamp. The lamp operates at 10.2 eV, which is above the ionisation potential (IP) of most analytes (7–10 eV) but below the IP of typical HPLC solvents, for example water, acetontrile and methanol, which are 12.61, 12.19 and 10.85 eV, respectively. This allows ionisation of the sample material without the resulting mass spectrum being dominated by mobile-phase solvent ions. Direct ionisation takes place when the photon energy is greater than IP of the analyte.

The process shown in Scheme 6.2 occurs if the PA of M is greater than the PA of the solvent.

$M + hv \longrightarrow M^{+\bullet}$ M is the analyte

$S + M^{+\bullet} \longrightarrow [MH]^+ + [S–H]$ S is the solvent

Scheme 6.2

A second mode of operation is to add a dopant. This is a readily ionisable compound that raises the number of ionised molecules in the source. The dopant absorbs the photon energy and transfers it to the analyte as shown in Scheme 6. 3.

Dopants (D) commonly used include toluene, acetone and, more recently, anisole [19].

$$D + hv \longrightarrow D^{+\bullet}$$

Two different mechanism can now take place

$$D^{+} + M \longrightarrow MH^{+} + D\text{–}H$$ D^{+} ionises analyte M by proton transfer

$$D^{+} + M \longrightarrow M^{+} + D$$ D^{+} ionises analyte M by proton transfer

Scheme 6.3

Proton abstraction from the solvent can also take place if the proton affinities are favourable, to give MH^{+}.

As indicated above, APPI has the ability to produce both $M^{+\cdot}$ and MH^{+} ions, and $M^{-\cdot}$ and $[M\text{-}H]^{-}$ in the negative mode.

Figure 6.1 shows the mass spectrum of an impurity run in the positive APPI mode and shows both $M^{+\cdot}$ at *m/z* 352 and MH^{+} *m/z* 353. This is an example where the mechanisms of proton transfer and electron transfer are both taking place. This can be confusing when dealing with complete unknowns and demonstrates why it is unsuitable for routine use in an open access multi-user environment or by inexperienced users. A better understanding of the processes involved and the role of mobile phase and dopant is required before this can be put to routine use. There are a number of papers published on this topic [20, 21].

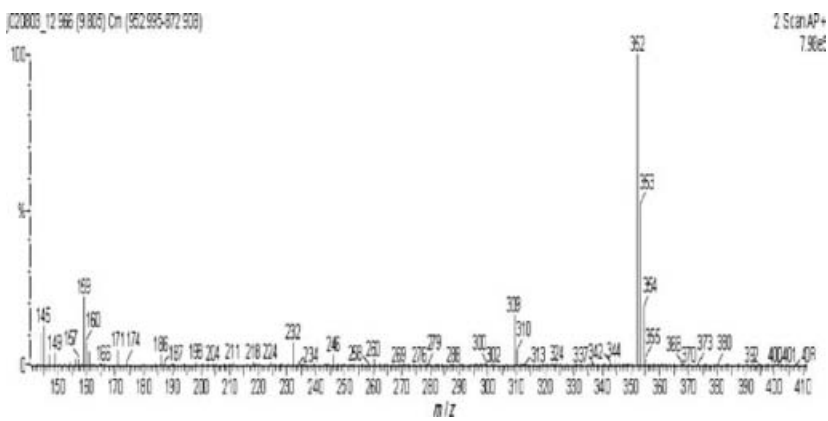


Figure 6.1 Mass spectrum of an impurity run in the positive APPI mode.

6.2.4 ESI, APCI or APPI?

API techniques provide efficient ionisation for a variety of molecules including polar, labile and high mass molecules. They are soft ionisation techniques useful

for ionising large molecules without fragmentation. As most drugs have polar substituents, they are suitable for the API techniques. ESI and APCI both rely on the acidity and basicity of the analyte, so there is some overlap in their applicability. Usually ESI works best for strongly basic and acidic compounds and high-mass compounds such as peptides and proteins. ESI can produce multiply charged species $[M+nH]^{n+}$. Normally, for compounds below mass 1000 multiply charged ions are seldom seen.

APCI has been found to be better for the more weakly acidic and basic compounds up to mass 1500 daltons. It is less susceptible to ion suppression, does not show multiply charged species but is unsuitable for thermally unstable compounds. Although API techniques have reached maturity, it is still crucial for detection that every component is ionised, and sometimes with a complete unknown it is difficult to predict which ionisation technique to use. This is demonstrated in Figure 6.2, which shows the UV and total ion chromatograms (TICs) for three impurities eluting between 27 and 29 minutes, analysed under several different ionisation modes [22]. Using ESI and APCI in the positive ion mode a response for only one of the impurities was seen and no responses for any of the impurities were seen from ESI in the negative mode. APCI in the negative

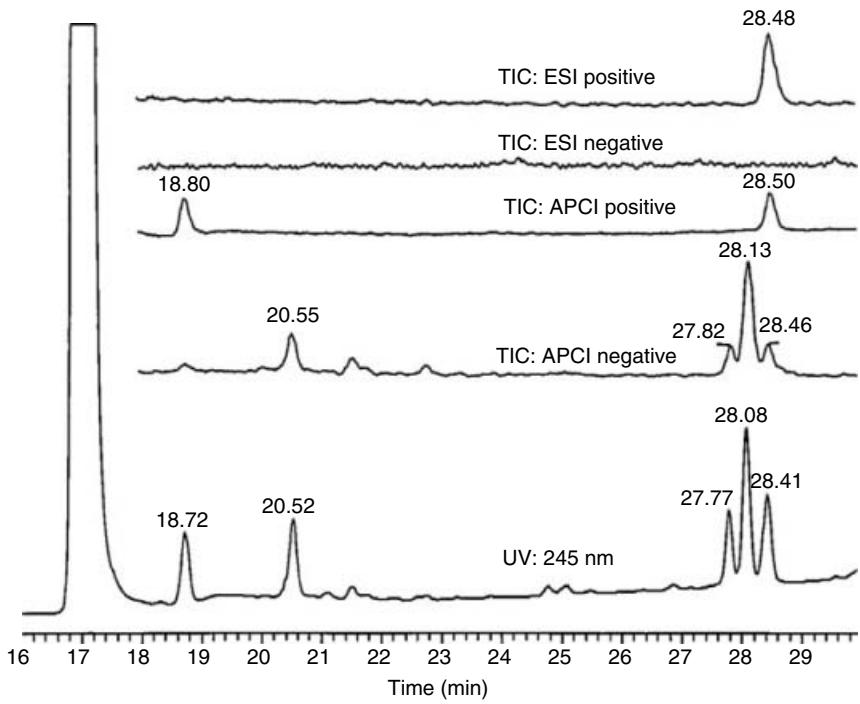


Figure 6.2 UV and TIC chromatograms of three unknowns at retention time 27–29 min. Reproduced from [22] with permission of John Wiley and Sons Ltd.

mode showed responses for all three impurities and therefore was the ionisation method of choice, but this could not have been predicted. In general, negative ion mode is used less than positive ion mode, but negative ion mode can in some cases be more specific. This is due to less chemical noise being generated in the negative mode, so that the signal-to-noise ratio is better when compared with the positive ion mode.

6.3 Practical considerations

It is worth considering the practicalities of LC-MS [23] before we go further, as a knowledge of these is a necessary precursor to structure elucidation.

6.3.1 Eluent considerations

Solvents used with the API techniques should permit formation of ions in solution and should be volatile to permit easy transfer of ions from solution into the gas phase. The ESI process is affected by droplet size, surface charge, liquid surface tensions and ion solvation strength. Large droplets with high surface tension, low volatility, strong ion solvation, low surface charge and high conductivity prevent good ESI.

Modern orthogonal-spraying API sources are remarkably tolerant of the type of solvent system used. Virtually all combinations of water and organic modifier are possible, from 100% water to nearly 100% solvent. At 100% water the surface tension of the droplets is very high and it becomes difficult for them to desolvate and ultimately break into smaller droplets. This can cause very noisy baselines on the total ion current chromatogram owing to incompletely desolvated clusters entering the mass spectrometer. Increasing the flow of desolvation gas usually helps to overcome this. At the other end of the scale, 100% organic modifier sprays easily enough, but sensitivity tends to be very low as ionisation efficiency is low because of the low dielectric constant of pure organic solvents. For most practical purposes, a range of 95% water to 95% organic modifier is fine. Methanol, acetonitrile (ACN), isopropanol and tetrahydofuran (THF) have all been used successfully. THF can cause problems at high levels (>10% v/v), especially when it is the only organic modifier in the eluent, because of its high PA, but in the presence of other organic modifiers such as methanol it is usually well tolerated.

Acids and bases are used to increase ionisation by protonation or deprotonation of the analyte, and it is important to choose the buffer and buffer strength very carefully because both have a noticeable effect on sensitivity. In the positive mode, high PA additives such as triethylamine (TEA) will successfully compete with the analyte for available protons and show an intense ion at m/z 102. Sensitivity can drop substantially when TEA is buffered with trifluroacetic acid (TFA). This is shown in Figure 6.3. The top diagram shows the mass spectrum of a compound analysed using acetonitrile/H_2O/TFA as the eluent and shows MH^+ at m/z 511. The mass spectrum of the same compound when analysed using

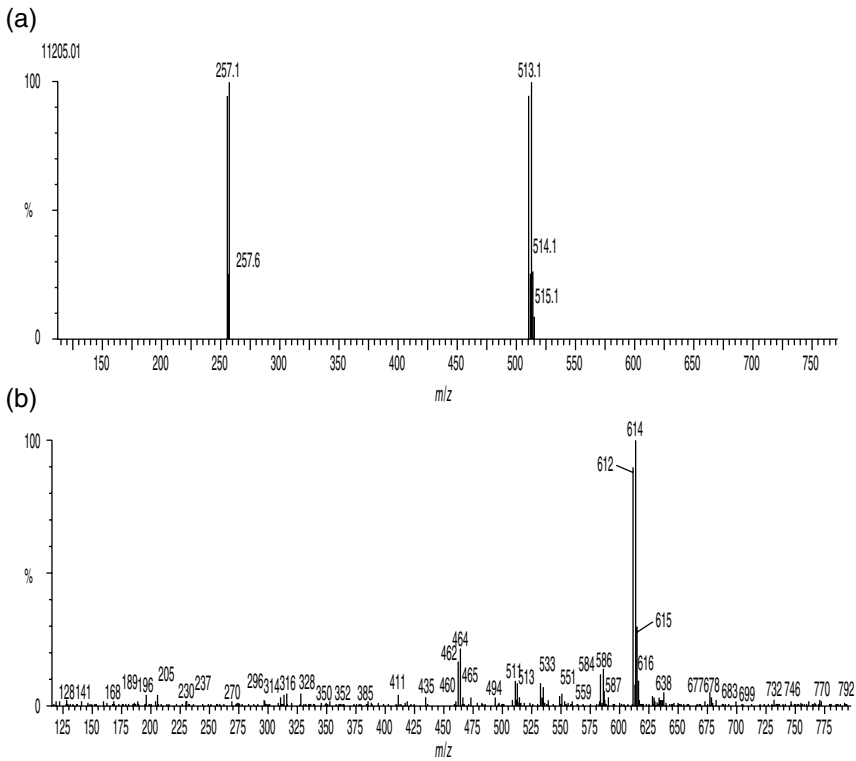


Figure 6.3 Analyte run in eluent: (a) CH_3CN : H_2O : TFA and (b) CH_3CN : H_2O : TFA:TEA.

a TEA/TFA buffered system (Figure 6.3, bottom diagram) shows *m*/*z* 612, which is the $[MHTEA]^+$ adduct, and the spectrum is much weaker. When trying to identify unknowns at low levels this adduct formation can be confusing.

The use of formic acid, acetic acid and ammonium formate rather than trifluoroacetic acid can substantially increase sensitivity because their proton affinities are lower than that of the TFA anion – though TFA is often used in the analysis of peptides. It is always advisable to keep the level of acid additives to less than 0.1% v/v, and preferably 0.03–0.05% v/v, in the final eluent. Triethylamine or ammonium hydroxide can be used successfully in negative mode because they promote deprotonation of acidic species.

Non-volatile buffers such as phosphates, borates, perchlorates and phosphoric acid should be avoided at all costs because of high background ion current, source contamination and blockages, and in the case of perchlorates, explosions. Figure 6.4 shows the mass spectrum of typical background when using phosphoric acid in the eluent. If the solvent system for a particular analysis does not assist the electrospray process, it is possible to enhance ionisation by post-column addition of a suitable volatile buffer.

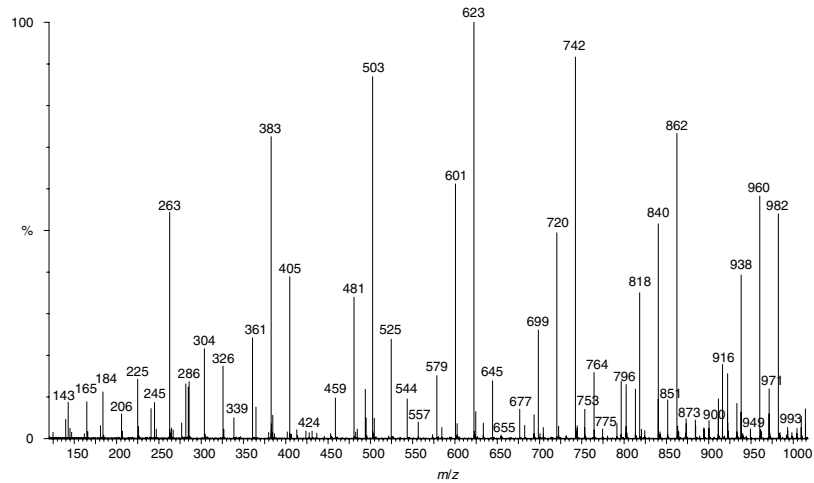


Figure 6.4 Cluster ions in phosphoric-containing eluent.

6.3.2 Flow rate and interfacing considerations

The present pneumatically assisted ESI interface is optimised around flow rates of 50–300 μl/min. The use of analytical columns 3–4.6 mm i.d. with flow rates between 0.5 to 2 ml/min and narrow bore columns of 1–2 mm id with flow rates of 0.2–0.5 ml/min is routine in most pharmaceutical laboratories for HPLC analysis [24]. Capillary LC columns, because of their limited commercial availability and special practical considerations are used more where there is limited sample available or when sensitivity issues are present [25].

Electrospray ion sources display all the characteristics of being concentration dependent. It is therefore normal to split the eluent flow after the UV detector, such that around 300 μl/min goes to the mass spectrometer and the remainder to waste. Care needs to be taken to ensure that there is minimal band broadening caused by the split. It should be possible to minimise band broadening by careful choice of tubing size and positioning of the split point.

Ultra performance liquid chromatography (UPLC) became commercially available in 2004 and has already made a big impact in LC-MS in the pharmaceutical industry [26]. UPLC utilises sub 2 μm particle size column packing with high linear velocity for the mobile phase. The small particle size requires the LC instrument to operate at pressures in the region of 6000–15,000 psi. This results in increased resolution, sensitivity and a shorter analysis time. The increased sensitivity can be clearly demonstrated by comparing the two mass spectra in Figure 6.5a, which shows a mass spectrum obtained using HPLC, and Figure 6.5b, which shows the mass spectrum using UPLC [27].

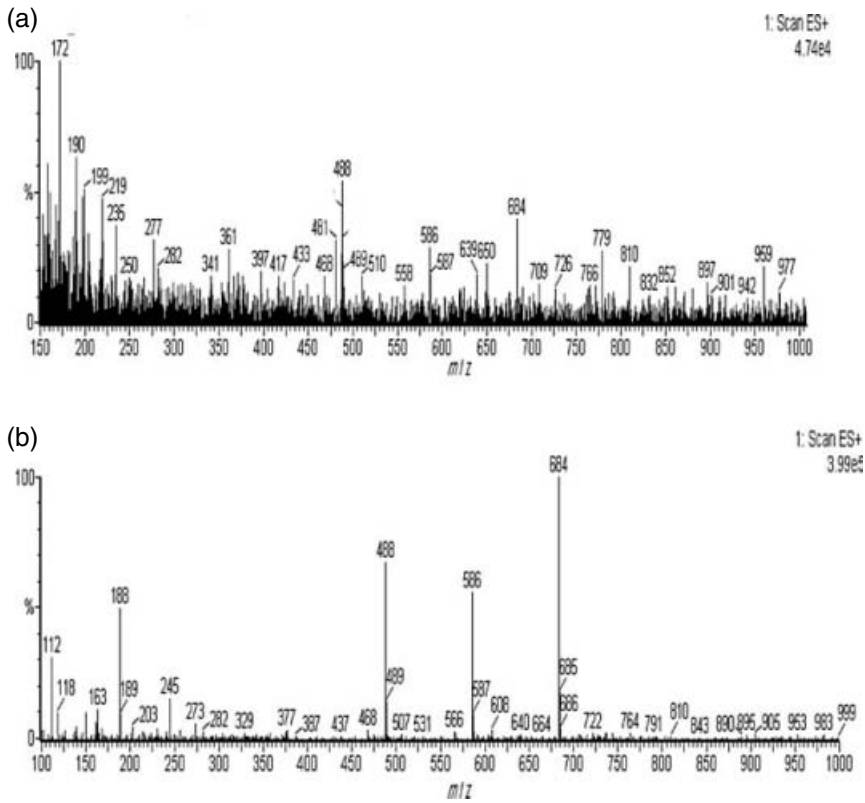


Figure 6.5 MS spectrum from (a) HPLC and (b) UPLC.

6.3.3 Data processing

The combination of liquid chromatography with mass spectrometry especially using ESI can result in the TIC having very high levels of background and chemical noise. It can be time consuming and frustrating to sift manually through data-rich LC-MS TIC traces with the associated risk of losing useful information. Background subtraction can sometimes reduce this problem, but frequently has limited success. Using a spectral/chromatographic search algorithm called component detection algorithm (CODA) considerably improved chromatograms, as seen in Figure 6.6, and can be produced data processing time can be reduced significantly [28]. In the top chromatogram the main component is only just visible at 8.2 min. After CODA was applied the baseline was reset and the other components can clearly be seen, so processing the data set is much easier.

Metabolynx™ is a spectral and chromatographic software program associated with Masslynx™ and developed by Micromass/Waters [29]. Most mass spectrometer manufacturers have similar software. It was specifically designed for

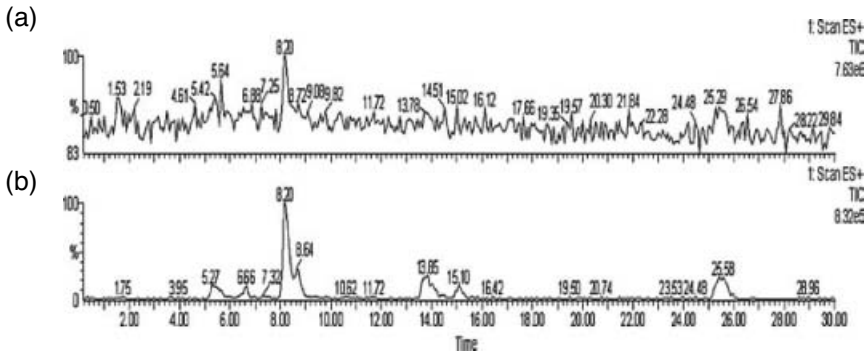


Figure 6.6 TIC (a) before CODA and (b) after CODA.

metabolite identification [30] but can also be applied to impurity and degradant identification. Metabolynx™ compares the LC-MS data for targeted metabolites and degradants by comparison with a control LC-MS data set [31]. The detection and reporting is done automatically but certain processing parameters need to be optimised beforehand. Isotopic clustering analysis can also be done, so chlorine and bromine containing compounds, which have characteristic isotope patterns, can very quickly be recognised.

6.4 Structural elucidation of unknowns

In mass spectrometry, both energetic effects and kinetic effects influence fragmentation behaviour. Therefore, mass spectra, unlike nuclear magnetic resonance (NMR) spectra, cannot be calculated theoretically. With experience the structures of impurities can be inferred from the fragmentation with a high degree of success. In most cases this is enough, especially in the very early stages of drug development where synthetic routes and formulations have not been finalised. When the compound is further into development there may be a need for isolation or synthesis and analysis by NMR for certain impurities, to confirm structures. Taking this phased approach saves valuable time, money and speeds up early drug development.

6.4.1 General interpretation considerations

Both ESI and APCI spectra can look relatively simple in most cases, just showing the pseudo-molecular ion MH^+ or adduct ion in the positive mode, and deprotonation or adduct ions in the negative mode. With API techniques we are dealing with even-electron (non-radical) MH^+ ions as opposed to odd-electron $M^{+\cdot}$ species that result from electron ionisation. Once an ion has achieved an even-electron state, it is unlikely to revert to an odd-electron state, as this is energetically unfavourable. This means that fragmentations from MH^+ should

maintain this even-electron state, losing neutral species such as water, ammonia, alcohols, carbon dioxide and sulphur dioxide. A butyl ester, for example, would lose butene in preference to loss of a butyl radical. Similarly, a chlorinated molecule will not lose 34 Da. Thus the presence of an ion 34 Da less than the MH^+ would indicate the presence of a co-eluting non-chlorinated homologue. Another example is the loss of water (18 Da) which is often seen with acids, but loss of 17 Da should not be interpreted as loss of OH (a radical) from an acid or alcohol, but more probably loss of ammonia, from an amine or similar compound. One mass unit really does make a difference! Also, loss of CN (a radical of 26 mass units) from a heterocyclic ring system is common in EI spectra, but would be seen as loss of 27 Da as HCN (neutral species) in ESI or APCI. Therefore, a loss of 26 Da in ESI or APCI would typically be due to loss of C_2H_2, with completely different implications for structure assignment.

Loss of a radical can, however, take place in certain circumstances, as exemplified by Methoxsalem. Figure 6.7 shows an ion at *m/z* 202, which corresponds to the loss of 15 Da from *m/z* 217 MH^+ [32]. In this case, it is the loss of a methyl radical from the methoxy group attached to the conjugated ring system. The resulting radical ion can effectively distribute the lone electron via several canonical forms, thus making the radical loss energetically more favourable.

6.4.2 Recognising multiply charged species

Multiply charged species can be seen when using ESI. In positive ion mode, the number of charged species normally observed is determined by the number of basic sites on a molecule that can be protonated at low pH. Singly charged species will show isotopic peaks that differ by 1 mass unit; doubly charged ions show isotopic peaks that differ by 0.5 mass units.

The molecular mass can be deduced using the equation below:

$$m/z = \frac{M + n \times 1.0078}{n}$$

where *m/z* is the ion seen in the spectrum, *M* is the actual molecular mass and *n* is the number of multiple charges.

Confusion often arises with genuine covalently bound dimers, where doubly charged ions can appear. The masses of the ions in each case will be the same: consider a compound of mass 500, giving an MH^+ at *m/z* 501 and an $(M + 2H)^{2+}$ ion at *m/z* 251. These ions would be the same as those from a component of mass 250 giving MH^+ at *m/z* 251 and $(2M + H)^+$ at *m/z* 501. The best way to distinguish between these two scenarios is to increase the cone voltage: in the first case, the doubly charged ion will revert to MH^+ by ion–molecule reactions, so *m/z* 501 will predominate, whereas with the second case the real $(MH)^+$ will dominate at *m/z* 251. Doubling the cone voltage is usually sufficient to bring about these changes.

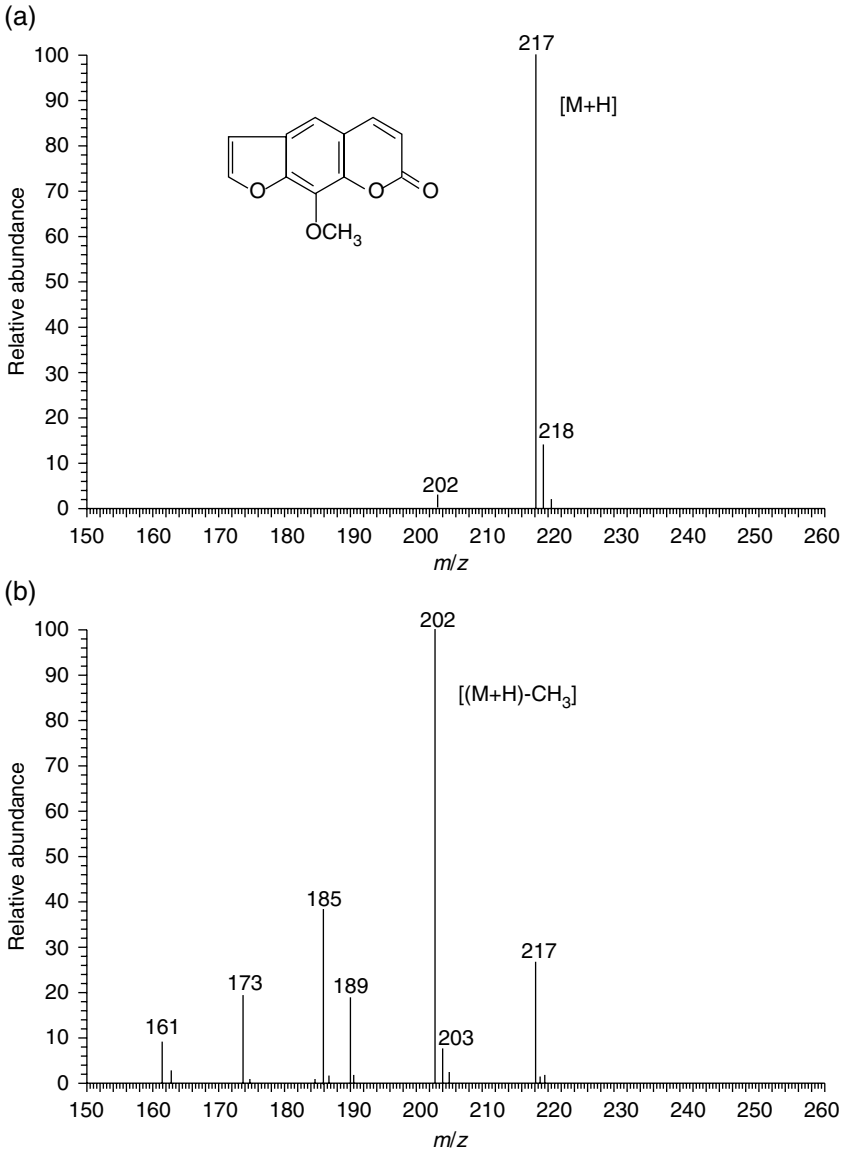


Figure 6.7 APCI mass spectra of methoxsalem: (a) LC-MS (b) LC-MS-MS. Reproduced from [32] with permission of Elsevier.

Another way of distinguishing between the two cases is to look carefully at the separation between the masses of the two ions at, in this case, *m*/*z* 251. As seen in Figure 6.8, the doubly charged ion will have a separation between adjacent masses of only 0.5 Da, whereas it will be 1 Da for an MH^+ ion. This way is simpler in some respects, but does need good instrument resolution.

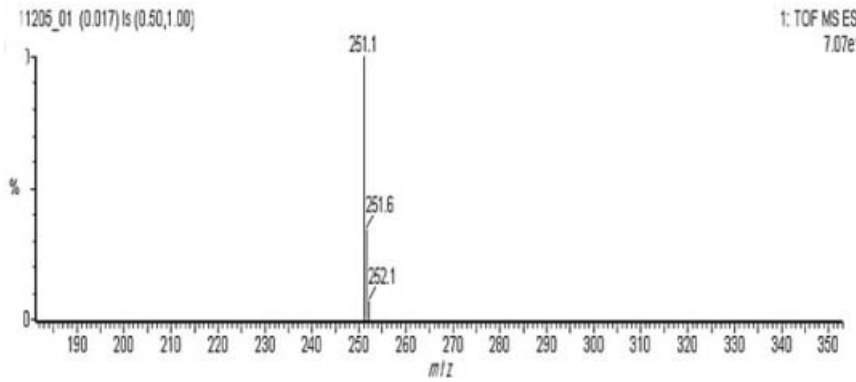


Figure 6.8 Doubly charged ion at *m/z* 251.

In general, a mass separation of 0.5–0.7 Da would be indicative of a doubly charged ion. Similarly, a separation of only 0.3 mass units would indicate a triply charged ion.

6.4.3 Using adduct ions

ESI and APCI produce predominantly pseudo-molecular ions $[M + H]^+$ species in a positive mode and $[M - H]^-$ in negative ion mode, with variable fragmentation depending upon the cone voltage used and, in the case of APCI, on the temperature used. Adduct ions are very often seen in ESI, and it is these that can cause most problems to inexperienced users, but they can be useful. Their formation depends on the coordinating properties, polarity and concentration of the analyte and to some extent on the solvent being used.

It is tempting to assume that an ion corresponding to $(M + 18)^+$ may be $(M + H_2O)^+$ but as both M and H_2O are neutral species this would be incorrect. The ion is $(M + NH_4)^+$ and can arise from two different sources. The first source is from ammonium buffer if present in the eluent. The second source originates from the nitrogen gas used for nebulisation. Inside the source nitrogen, water and a high voltage combine to give a mini-Haber process producing ammonia, which ionises to give NH_4^+.

An ion seen at 22 Da higher than MH^+ is caused by addition of Na^+ to an uncharged molecule resulting in $(M + Na)^+$. This process is known as cationisation and is quite common. The relative amount of $(M + Na)^+$ usually increases when the cone voltage is increased, as the higher collision energies involved make this process more favourable. Cationisation can be very useful to confirm that an ion really is MH^+. Cationisation using silver ions, argentation, is sometimes used, especially where compounds contain pi-bonds to which the Ag^+ ion can coordinate [33]. This technique however is not one that has been widely applied.

Table 6.1 A list of adduct ions

Ion observed	Interpretation
$(MH+ 17)^+$	$(MH + NH_3)^+$
$(MH+ 18)^+$	$(MH + H_2O)^+$
$(MH+ 22)^+$	$(M + Na)^+$
$(MH+ 32)^+$	$(MH + CH_3OH)^+$
$(MH+ 38)^+$	$(M + K)^+$
$(MH+ 41)^+$	$(MH + CH_3CN)^+$
$(MH+ 63)^+$	$(M + Na + CH_3CN)^+$

There are no rules as to which compounds will give this adduct and which will not. Solvent adduct formation usually decreases with increasing cone voltage, owing to the ion–molecule collisions in the cone region breaking up any such clusters still existing at that stage. If you suspect any ion to be an adduct ion of some sort, raising the cone voltage and observing what happens is good practice. A list of adduct ions can be found in Table 6.1.

Ions appearing at approximately twice the mass of the parent ion are known as multimers. These usually appear at $(2M + H)^+$ owing to the formation of a protonated dimer in the MS.

Intensities of adducts can vary depending on different eluents and composition. Using both high and low cone voltages in all the above cases will help in the interpretation of the spectra.

Only looking at the mass spectrum of an impurity that was obtained using a low cone voltage of 25 volts, as in Figure 6.9a, it would be reasonable to assume MH^+ is *m*/*z* 474. Looking at Figure 6.9b, which is the mass spectrum obtained using a higher cone voltage of 50 volts, you can see that MH^+ is in fact *m*/*z* 457 and the ions at *m*/*z* 479 and 495 that are $[M + Na]^+$ and $[M + K]^+$, respectively, are now seen. No $[MNH4]^+$ at *m*/*z* 474 was seen in the spectrum obtained at the higher cone voltage. This demonstrates that it is good practice to use both high and low cone voltage experiments in the identification of unknowns.

A compound of MH^+ 591 shows an impurity that when using both ESI and APPI in the positive ion mode suggests that MH^+ is *m*/*z* 589 (Figure 6.10a). This indicates a mass difference of 2 Da from the parent. The same impurity when run in the negative ESI mode shows *m*/*z* 733 (Figure 6.10b). In the negative ESI mode, it is common to see the TFA adduct ions $[M + CF_3COO]^-$, which gives M + 113. So assuming this is the case the molecular weight of the impurity is 620 Da (733 – 113). From this information it seems that in both APPI and ESI positive mode the impurity was thermally degraded, losing a mass of 32, which was thought to be methanol to give *m*/*z* 589. From later mass measurements in the negative ion mode, the impurity was confirmed as incorporating an OCH_3 group. This problem demonstrates how useful adduct ions are, but also how important it is to employ both positive and negative ion modes in structure elucidation.

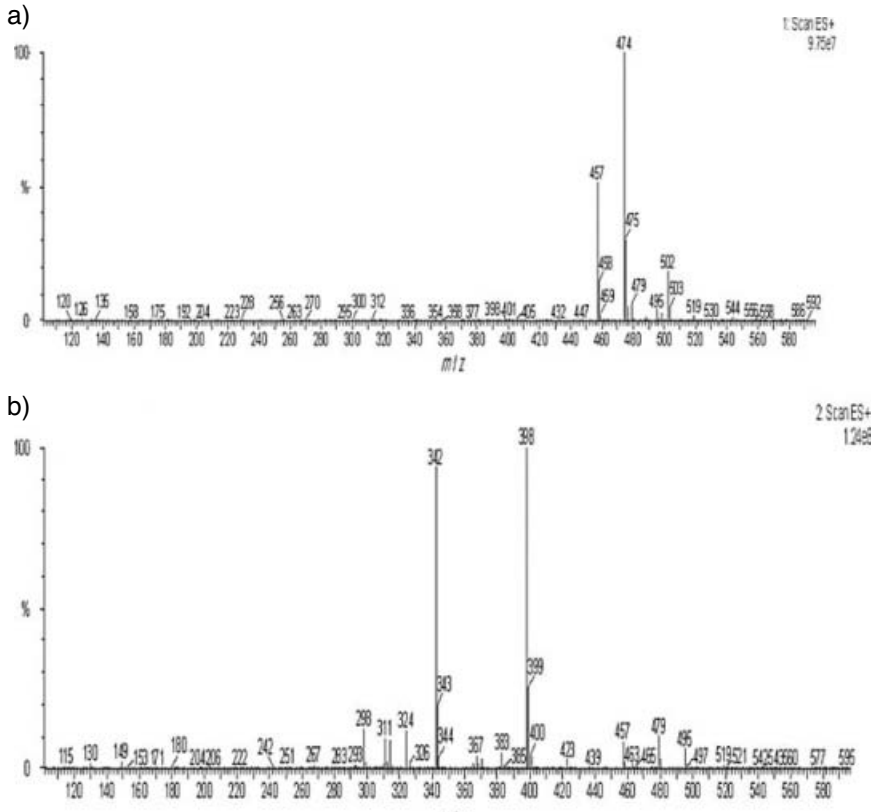


Figure 6.9 Mass spectra of an analyte (a) cone voltage 20 V and (b) cone volts 50 V.

6.4.4 Use of in-source fragmentation

Knowing the molecular weight of an unknown is a prerequisite for its identification, and in some cases no additional information is needed for a reasonable conclusion as to the structure to be drawn. However, having some structurally diagnostic fragmentation information, via either in-source fragmentations or CID, can greatly increase confidence in the structural assignments.

In-source fragmentation has a high success rate in identification of single eluting components and can be achieved by raising the cone voltage. This has the effect of increasing the velocity of the ions between the cone and the skimmer in the source, so that the ions collide with the neutral gas molecules, which are more energetic and therefore cause the molecules to fragment.

Unfortunately, this technique is not selective and all components are affected in the same way so that if exact co-elution of sample components occurs, this may not be detected. Using a single quadrupole instrument limits the user to using in-source fragmentation, but in many cases this can provide enough information to identify unknowns.

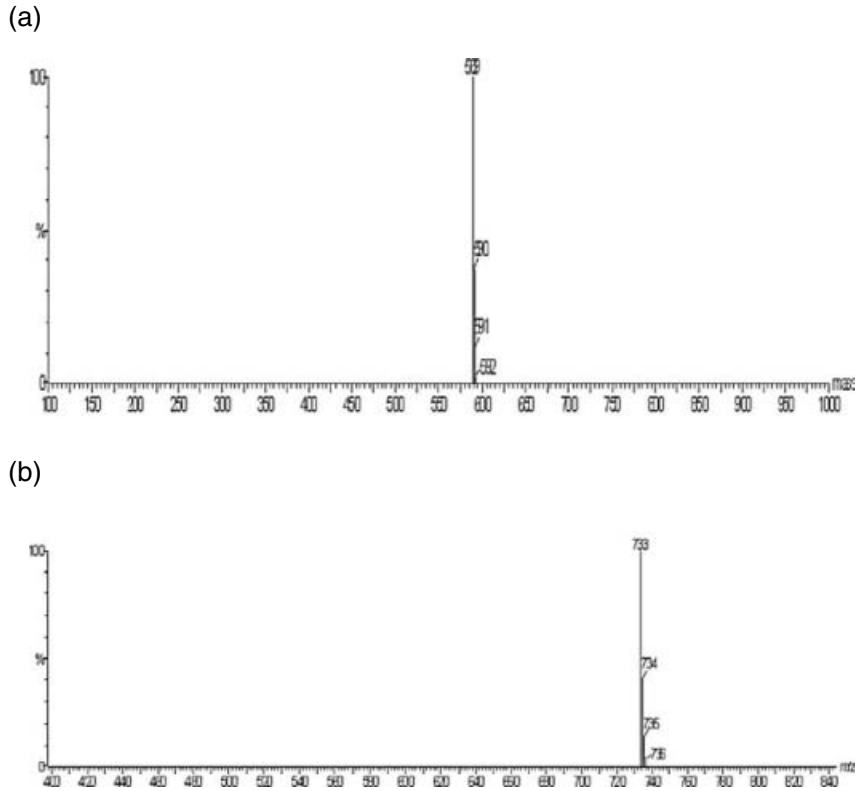


Figure 6.10 Mass spectra of an impurity run in (a) APCI mode and (b) negative ESI mode.

Two impurities I and II were found in a process support sample (SAP). Scanning using a single quadrupole mass spectrometer at cone voltages below 50 V only showed MH^+ at *m*/*z* 382 for SAP and the two impurities I and II both showed MH^+ at *m*/*z* 396. Increasing the cone voltage to 60 V generated many fragment ions, as shown in Figure 6.11. On comparing the mass spectra of both the impurities with each other and with the SAP spectrum, it was seen that the fragments in I all differed by 14 mass units but II showed very similar fragments to SAP. Without any further analysis the fragments and structures were reliably assigned as seen in Figure 6.12, as the ethyl ester (I) and ethyl sulphonamide (II) impurities, respectively.

6.4.5 Using tandem MS–MS techniques

Tandem mass spectrometry, more commonly known as MS-MS, utilises multiple stages of analysis. It is very selective and provides useful structural information. A variety of scan modes can be used to provide specific information for structural

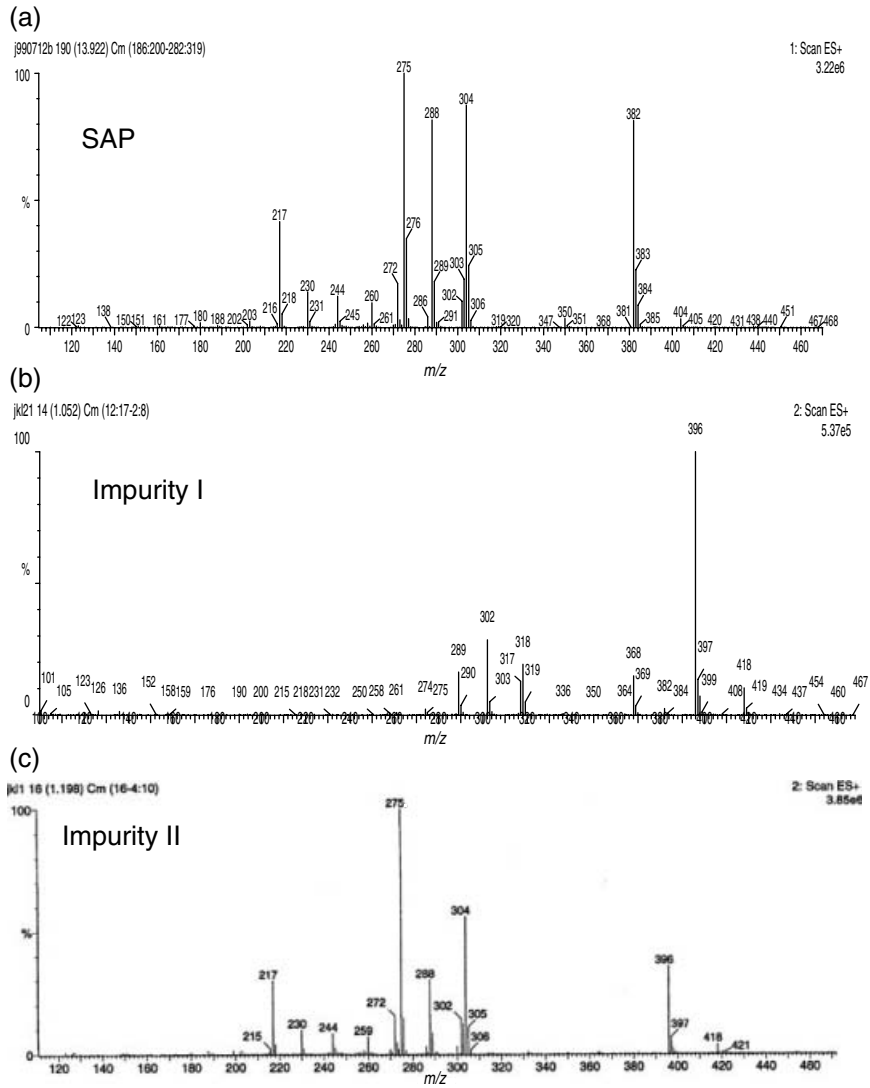


Figure 6.11 Mass spectra of (a) SAP and (b, c) impurities I and II.

elucidation combined with chromatography: these are described in more detail below.

The two most common instruments for these experiments are a triple quadrupole mass analyser [34] and a quadrupole time of flight (Q-TOF) spectrometer [35]. The triple quadrupole instrument comprises two conventional quadrupole analysers usually referred to as Q1 and Q3 separated by a third known as Q2, which acts as an ion-focusing device and a collision cell. All the scan modes below (Figure 6.13) can be carried out on a triple quadrupole instrument.

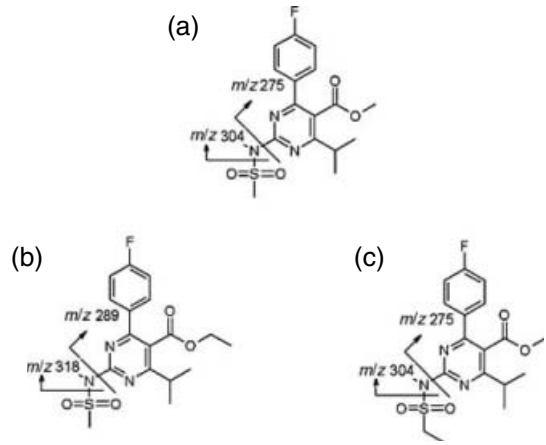


Figure 6.12 Structures and assignments for (a) SAP and (b, c) impurities I and II.

A Q-TOF spectrometer is similar to a triple quadrupole but Q3 is replaced by an orthogonal TOF mass spectrometer. Using a Q-TOF instrument only the product ion scan mode can be collected, but because of its high resolving power, accurate masses for both the precursor ion and product ions can be obtained. (See the section below on accurate mass measurements.)

6.4.5.1 Product ion scan

Figure 6.13a shows a typical product ion scan experiment on a triple quadrupole mass spectrometer. Q1 is used to select a precursor ion of interest; this ion is passed into a collision cell (usually referred to as Q2). Here the ions come in contact with an inert gas – usually helium or argon – where (CID) occurs. The resulting fragments or product ions are detected in Q3. The product ion spectra will depend on the collision energy and the collision gas pressure. Usually, high collision energies cause further decomposition of the original product ions to produces smaller mass product ions. Spectra run on different instruments run under the same conditions can look very different. It is difficult, if not impossible, to reproduce the exact experimental parameters such as collision energy and collision pressure on different types of instruments from different manufacturers.

The product ion scan mode is probably the most used in mass spectrometry for structural elucidation.

That product ion scans can give more information than in-source fragmentation is shown by the next example. Various batches of Mosapride, which is a potent gastroprokinetic agent, showed an impurity with levels varying between 0.05 and 0.1% [36]. To identify this impurity by LC-MS the original LC method was successfully modified, replacing the phosphate buffer with an LC-MS-compatible solvent system, utilising ammonium acetate as the volatile buffer.

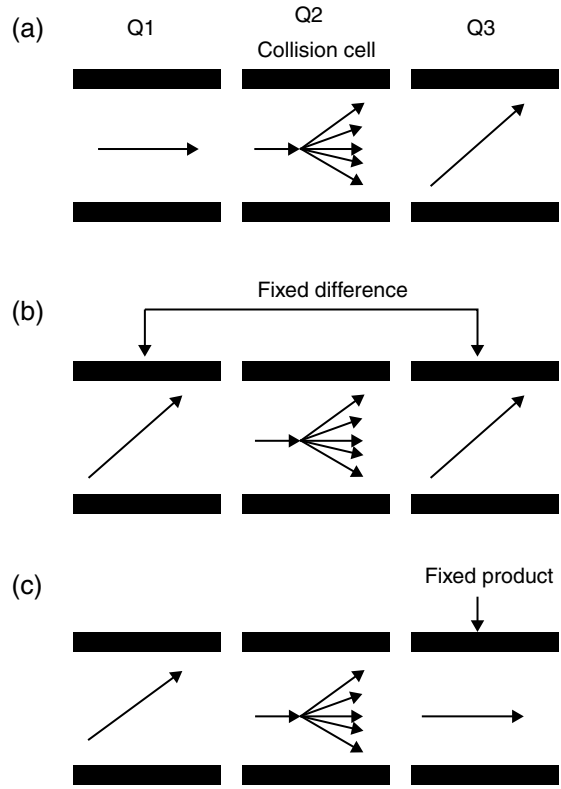


Figure 6.13 Schematic illustrations of (a) product ion scan, (b) neutral loss scan, (c) precursor ion detection on a triple quadrupole mass spectrometer. Reproduced from [37] with permission from Elsevier.

The mass spectra of mosapride and the impurity generated by LC-MS were compared (Figure 6.14). The impurity shows MH^+ at *m*/*z* 404, and comparing this with MH^+ of mosapride at *m*/*z* 422, the mass difference observed is 18 Da. From the single quadrupole experiments, fragments at *m*/*z* 198 and 170 with the characteristic chlorine isotopic abundance can be seen in both mosapride and its impurity. This indicated that the 4-amino-5-choro-2-ethoxy-benzamide moiety was unmodified.

Using MS-MS, the product ion scan of *m*/*z* 422 from mosapride showed *m*/*z* 109 for the fluorotropylium ion, whereas the product ion scan of *m*/*z* 404 for the impurity showed a tropylium ion at *m*/*z* 91 (see Figure 6.15). On the basis of this information the des-fluoro structure was proposed for the impurity, as indicated in Figure 6.16. Its formation was rationalised by the presence of a des-fluoro impurity in the starting material.

In another example, the stability of a solution of a peptide drug shown in Figure 6.17, being developed as an injectable formulation development, was

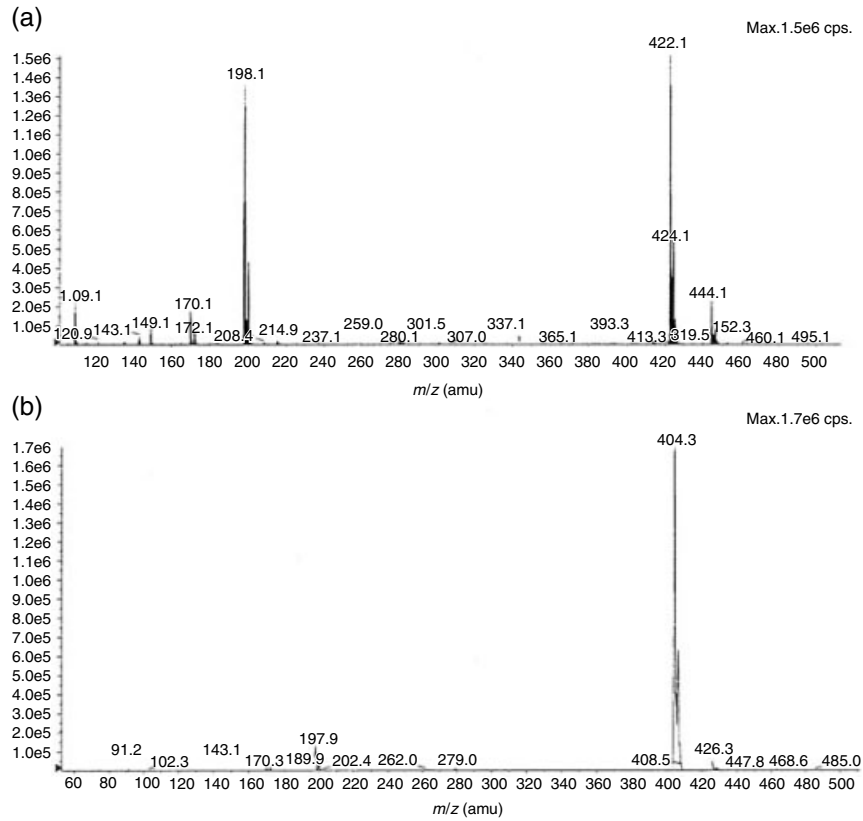


Figure 6.14 Mass spectra of (a) Mosapride and (b) impurity. Reproduced from [36] with permission from Elsevier.

investigated. Figure 6.18 shows two main degradants as the pH of the solution is increased. These were isomeric impurities at MH^+ 551 that were 1 Da higher than their parent. From the nitrogen rule the change in mass indicated the loss of a nitrogen atom and gain of an oxygen atom. The product ion spectra of *m/z* 551 showed two key sets of product ions *m/z* 163/164 and *m/z* 319/320 (see Figure 6.18). The ions at *m/z* 163 and 319 were both present in the parent spectra, which indicates that impurity C was formed by hydrolysis of the N-terminal amidine. The presence of *m/z* 320 and 164 one mass unit higher suggests that impurity B is formed by the hydrolysis of the amide to the corresponding carboxylic acid.

6.4.5.2 Precursor ion scans

As seen from Figure 6.13c, in this mode Q1 is set to scan from low to high mass, fragmentation occurs in Q2 as described previously but Q3 only transmits the

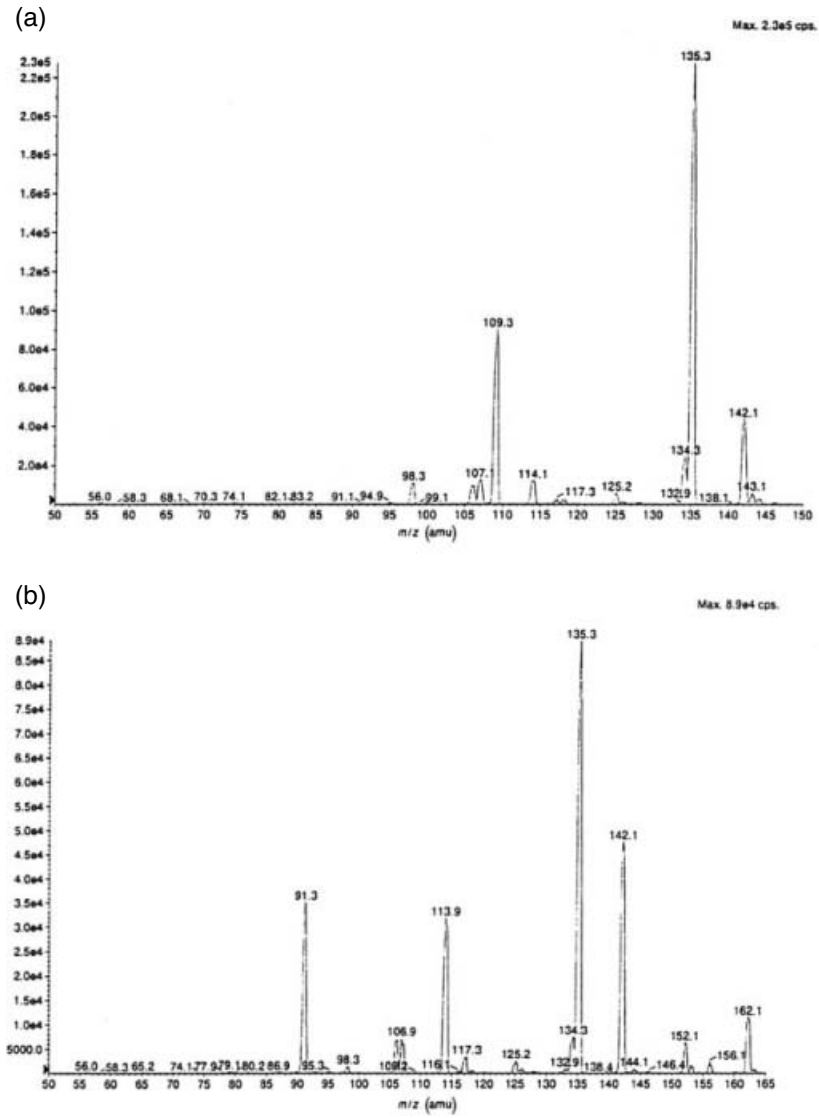


Figure 6.15 Product ion spectra of (a) Mosapride and (b) impurity. Reproduced from [36] with permission from Elsevier.

selected product ion. This produces a TIC that only shows the precursor ions that give rise to the selected product ion. In this mode, mass spectra of all the components that fragment to a common mass owing to a common structural feature are recorded. This can be a rapid and efficient way of screening metabolites, stability samples and degradants that all show a common fragment.

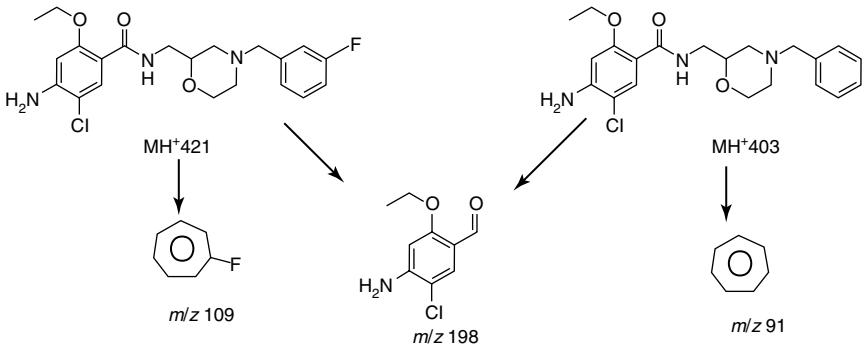


Figure 6.16 The mass fragmentation pathways for Mosapride and an impurity. Reproduced from [36] with permission from Elsevier.

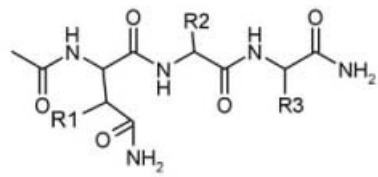


Figure 6.17 Part structure of peptide drug. Reproduced from [22] with permission of John Wiley and Sons Ltd.

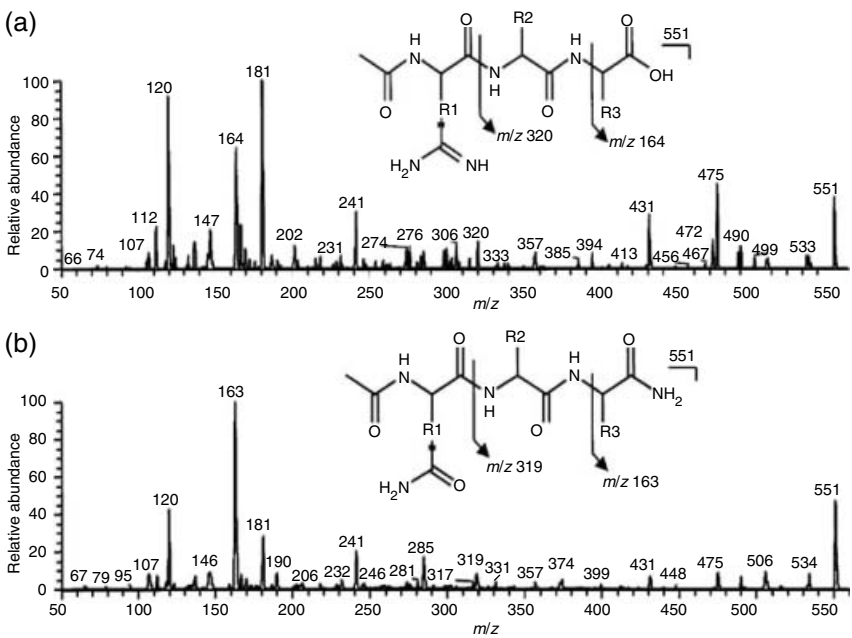


Figure 6.18 Product ion spectra of *m*/*z* 551 of two peptide impurities: (a) peak B and (b) peak C. Reproduced from [22] with permission of John Wiley and Sons Ltd.

An example where this was utilised effectively is seen where compound X showed an intense fragment ion at *m/z* 112. Figure 6.19 shows the TIC produced from precursor ion scanning for *m/z* 112. When compared with the UV chromatogram it can clearly show which peaks contained the partial structure for fragment ion *m/z* 112 and in some cases show components not seen by UV detection.

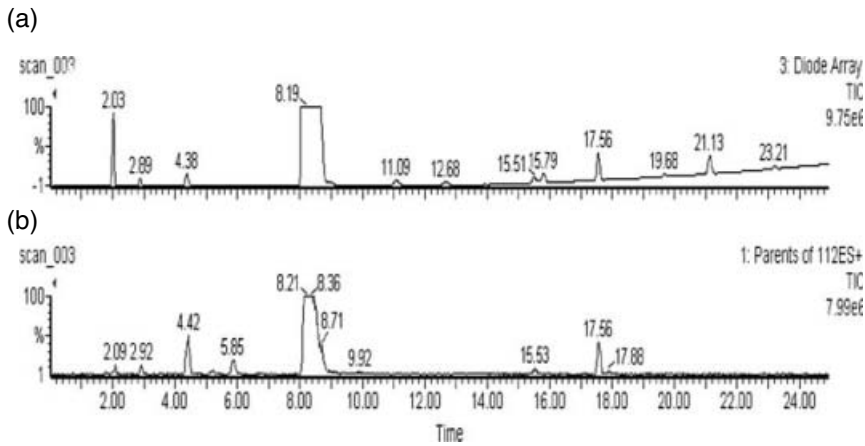


Figure 6.19 (a) Diode array scan and (b) precursor ion scan *m/z* 112.

6.4.5.3 Neutral loss scan

As shown in Figure 6.13b, Q1 and Q3 are both scanning from low to high mass but with a fixed mass difference that corresponds to the mass of the neutral molecule lost during CID. This can be especially useful when analysing the same class of compounds or for group-specific detection.

Compound A produces a predominant fragment at *m/z* 264, which corresponds to a neutral loss of 175, which in this case corresponds to the loss of 4-trifluoromethylbenzylamine [37]. This neutral loss can be used to monitor the presence of other species sharing this common feature. Radioactively labelled compound A was incubated in rat liver microsomes, analysed by LC-MS-MS and the TIC for the neutral loss of a mass of 175 (Figure 6.20a) was obtained. When compared with the radioactivity profile in Figure 6.20b, two extra components were detected in the TIC. This was due to the loss of the ^{14}C radiolabel during metabolism. LC-MS-MS is very useful as a complementary detection method where the radiolabel is lost during metabolism or in situations where a radiolabel is not available.

Precursor ion and neutral loss scan functions are unique to a triple quadrupole mass spectrometer. These are powerful techniques for targeted detection of compounds and their related impurities that produces a characteristic neutral loss or a fragment ion corresponding to a unique structural feature. Both neutral loss and

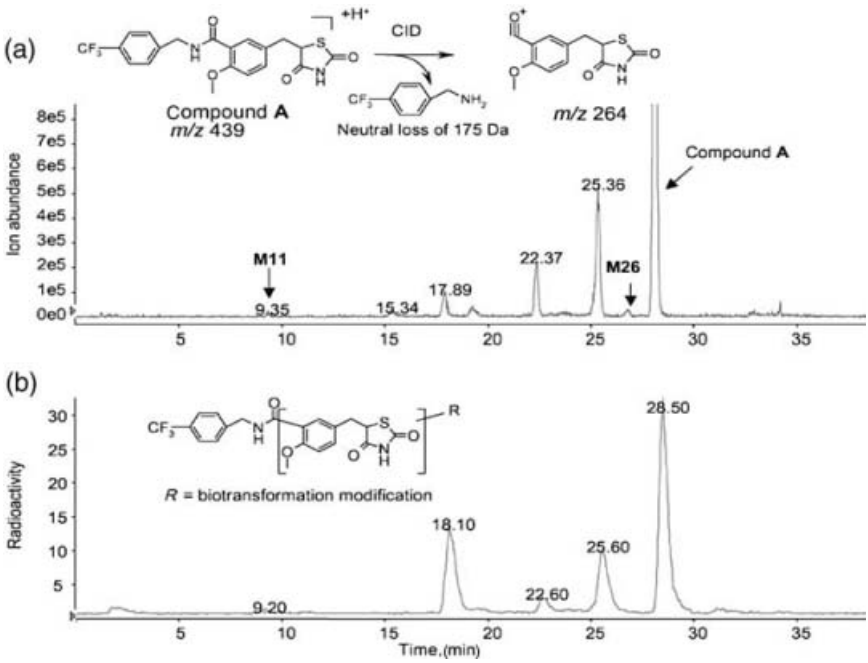


Figure 6.20 (a) LC-MS total ion current of neutral loss 175 and (b) HPLC-radioactivity profile of a rat liver microsomal incubation of radioactive compound A. Reproduced from [37] with permission from Elsevier.

precursor ion modes are highly effective in detecting molecules that closely resemble their parent and gives you a quick visual guide to which part of the molecule has been modified.

6.4.5.4 MS^n on a quadrupole ion trap

Using a triple quadrupole or a Q-TOF only MS^2 data can be generated. This can be taken a step further by using a quadrupole ion trap [38]. This instrument can provide continuous generation of product ions from a fragment ion produced in a previous stage. This multistage MS^n capability provides structurally rich information in a very short time for assigning fragmentation pathways and for structural elucidation of unknowns and metabolites. Usually, for smaller molecules the limit is about five stages of fragmentation before the signal strength becomes too low to be detected.

In some situations, the fragment ion of a metabolite may not necessarily possess the same structure as that of its parent even though they both have the same *m*/*z* value. This is shown by a compound C in Figure 6.21, which gave rise to a prominent fragment at *m*/*z* 268 in its MS^2 spectrum as shown [37]. A metabolite of this compound had a mass of 18 Da higher indicating a

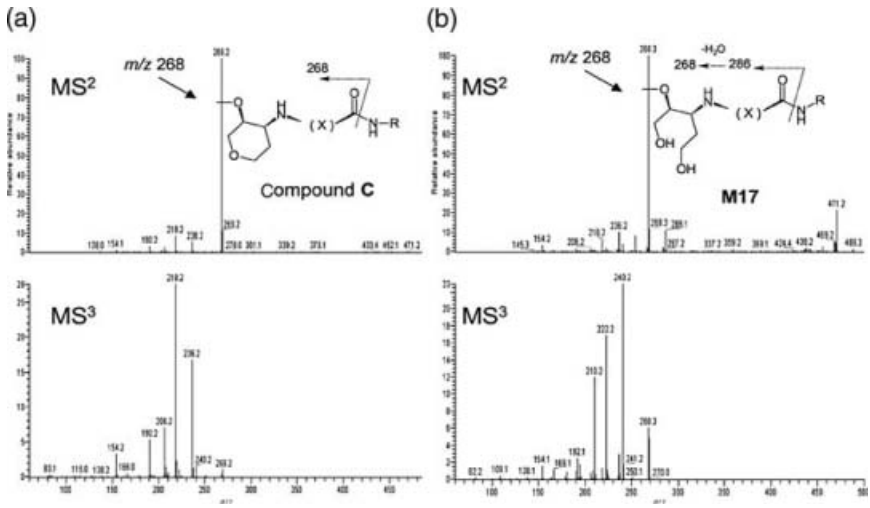


Figure 6.21 MS^2 and MS^3 spectra of (a) compound C and (b) M17, generated on a Finnigan LCQ ion trap mass spectrometer. Reproduced from [37] with permission from Elsevier.

modification by oxidative ring opening. This metabolite also showed a prominent *m/z* 268 in its MS^2 spectrum. On first looking at this information it was not possible to conclude whether the modification had taken place on the R group or on the tetrahydropyran ring. Taking this a step further, the MS^3 of the two *m/z* 268 fragments of the metabolite and the parent are very different. The MS^3 spectrum of the metabolite shows a prominent ion at *m/z* 240 corresponding to loss of the carbonyl group, which then eliminates a molecule of water to form *m/z* 222. The ion at *m/z* 268 from the parent displayed loss of methanol to give *m/z* 236, which further eliminates a molecule of water to give *m/z* 218. Interpretation of this MS^3 data from both parent and metabolite leads to a structure corresponding to the oxidative opening of the tetrahydropyran ring, but relying solely on the MS^2 data would not have made this interpretation possible.

6.4.6 Accurate mass measurements

Most spectra have mass of the individual ion expressed to the nearest whole number, that is, nominal mass. In some cases, as seen previously, this is enough information, along with some fragmentation and knowing the origin of the unknown, to assign a structure successfully. In other cases, however, such data are not sufficient to characterise fully the molecule in question and more information needs to be obtained. Accurately measuring the masses of appropriate ions to four decimal places and obtaining molecular formulae from these masses can provide additional data. To provide unequivocal formulae, the mass needs to

be measured to a high degree of accuracy, usually expressed as an error figure in parts per million or millimass units.

Consider an ion of nominal mass 300 Da. If the mass is measured to within 1 ppm, that is, 300.0000 ± 0.0003, then there is only one combination of, for example, C, H, O and N, which will match this mass. If the mass can only be measured to within 50 ppm, that is, ± 0.015, then 27 combinations of C, H, O and N are theoretically possible. At 100 ppm this becomes 52 combinations.

It is therefore important to acquire data of the highest accuracy if unequivocal molecular compositions are to be determined. As a general rule, any molecular formulae for which the error (i.e. the difference between measured and theoretical masses) is greater than 10 ppm should be discounted.

High-resolution measurement has always played a big role in mass spectrometry [39]. Nowadays accurate mass measurement in LC-MS for structure elucidation plays a major part with the use of API-TOF instrumentation. This provides high mass accuracy of better than 10 ppm, combined with fast scanning and full scan sensitivity at a resolution of 5000–10,000. This also provides most probable elemental formulae for all pseudo-molecular ions. Using a Q-TOF instrument, elemental composition of both product ion and precursor ions are readily obtained. It is important for distinguishing between isobaric molecules and assigning fragment ions for elucidation of fragmentation mechanisms. Knowing the accurate mass can confirm the molecular formula of the chemical entity under investigation. The difference in the accurate mass measurements between two ions can also provide important neutral loss information. In some cases, the specific and structurally related elemental formulae are vital in creating a complete picture of an entire molecule.

Accurate mass measurement and the associated empirical formulae allow routine calculation of the 'double bond equivalent' (DBE) by the spectrometer's computer system. This is a measure of the number of double bonds and/or the number of rings in a molecule, and is derived from a consideration of the valences of the various elements in a given composition. This gives information about the aromaticity or conjugation of the unknown. The values given by the MS data system are based on a simple calculation:

For a compound $C_w H_x N_y O_z$, DBEs $= 0.5\ (2w - x + y + 2)$.

For example, for benzene with six carbons and six hydrogen atoms, the DBE is 4, corresponding to one ring and three double bonds. For acetaldehyde, CH_3CHO, DBE $= 1$, that is, one carbonyl function.

The DBE number is most useful when it comes to deciding which of perhaps two or three possible molecular formulae are correct. If there is a strong possibility that a particular unknown is related to the parent drug, for example, and that drug had a ring system with a DBE of 5, then the unknown should have a value of at least 5, and more if additional double bonds were introduced.

An in-process sample when examined by LC-MS on a single quadrupole showed three impurities, all with the same MH^+ at the nominal mass of m/z 402. This equated to an extra 14 Da higher than the parent mass. From the structure

Table 6.2 Mass measurements, formulae and DBE for *m/z* 402

Sample	Measurement	Formula	PPM difference from theoretical	DBE
Parent	388.0265	$C_{18}H_{12}N_3O_3Cl_2$	2.4	14
Impurity 1	402.0415	$C_{19}H_{14}N_3O_3Cl_2$	0.7	14
Impurity 2	402.0419	$C_{19}H_{142}N_3O_3Cl_2$	1.7	14
Impurity 3	402.0036	$C_{18}H_{10}N_3O_4Cl_2$	−3.1	15

(not shown owing to confidentiality considerations), it was easy for the chemist to account for two different sites for methylation, but a third could not be rationalised. All three impurities were mass measured on a Q-TOF using leucine enkephalin as the lock mass. The measurements and the DBEs are shown in Table 6.2. It was confirmed that there were only two peaks resulting from methylation, and from the measurement and an increase in the DBE the third was shown to be oxidation of a CH_3 group to the aldehyde CHO.

Going a step further, Lui *et al*. [37] showed the unusual migration of a fluorine atom from a trifluromethoxy group to an indole ring using MS-MS, by confirming elemental compositions of the fragment ions from accurate mass measurements on a Quadrupole TOF instrument.

In Figure 6.22, the product ion scan of $(M-H)^-$ *m/z* 352, $C_{17}H_{10}N_1O_2F_3Cl$, showed three major product ions at *m/z* 286 (loss of F_2CO) with the fluorine migrating to the indole ring, *m/z* 283 (loss of CF_3 radical) and *m/z* 266 (loss of CF_3OH). Using the $(M-H)^-$ ion at *m/z* 352 as the reference mass the measured exact masses were all within 5.8 ppm of the theoretical value for the proposed formula.

Although unlikely, a loss of NH_3 from *m/z* 283 could also give rise to *m/z* 266. This mechanism was excluded because the mass measurement of 266.0388 was 96 ppm from the theoretical value expected for $C_{16}H_6O_2Cl$. However, this measurement was within 6 ppm of $C_{16}H_9NOCl$; therefore this fragment could arise from elimination of CF_3OH from the parent molecule or the loss of HF from *m/z* 286. When the product ion scan of *m/z* 286 on a LCQ ion trap did not show *m/z* 266, elimination of CF_3OH from the parent substance was concluded to be the most likely fragmentation mechanism.

6.4.7 Hydrogen/deuterium exchange

The use of deuterium exchange of labile hydrogen atoms such as OH, NH and SH greatly enhances the information to help in structural elucidation and has been used in mass spectrometry for many years. Deuterium has a mass of 2 as opposed to 1 for hydrogen; therefore, when using deuterium oxide in place of water to exchange each labile hydrogen function the mass increases by 1 Da per hydrogen exchanged. Karlsson demonstrated the use of deuterium oxide as a LC

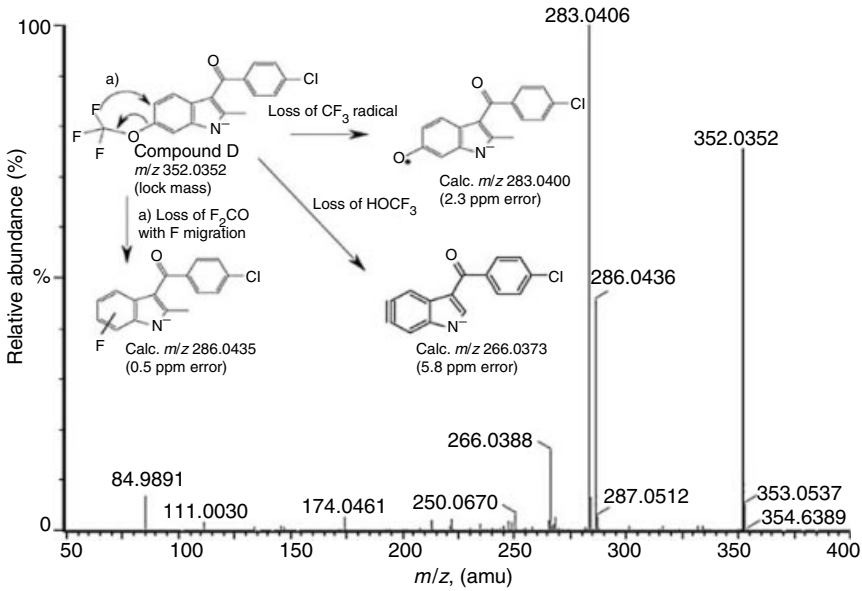


Figure 6.22 Product ion spectra (negative ion mode) of *m/z* 352 with mass measurements for the product ions. Reproduced from [37] with permission from Elsevier.

mobile phase on a micro column [40]. This idea is now being fully utilised in pharmaceutical analysis for the identification of impurities, degradants and metabolites [41]. Replacement of water with deuterium oxide in commonly used mobile phases makes virtually no difference to equilibration time [42]. Replacing water with deuterium oxide in some cases can change the retention time slightly, but experience shows that it does not interfere with the analysis. The cost of deuterium oxide for approximately 50 ml per run is not excessive when compared with the valuable information obtained as demonstrated in the next application.

Liu *et al*. [43] have demonstrated the use of online hydrogen/deuterium exchange, LC-MS and LC-MS-MS for successfully distinguishing between S-oxidation and hydroxylation in metabolite identification for compound I.

Using water in the mobile phase the parent compound I gave an abundant MH^+ at *m/z* 203. The product ion scan of *m/z* 203 showed a prominent ion at *m/z* 86. However, when D_2O was used as the aqueous component in the mobile phase, compound I showed MD^+ at *m/z* 206. This 3 Da increase confirms the presence of two exchangeable hydrogen atoms in the structure of I (see Figure 6.23). The product ion scan of *m/z* 206 showed a prominent ion at *m/z* 88, indicating the presence of two exchangeable hydrogen atoms in the A fragments (Figure 6.24).

M1 gave an MH^+ at *m/z* 219, 16 Da higher than the parent compound (Figure 6.25). Similar to I, M1 gave a prominent ion at *m/z* 86 from the product ion scan of *m/z* 219, indicating that the A fragment was unchanged. However, the B ion was seen at *m/z* 106, 16 Da higher than the B fragment in I, indicating

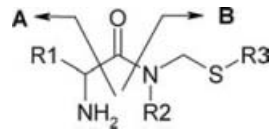


Figure 6.23 Part structure of compound I. Reproduced from Lui *et al*. [43] with permission of John Wiley and Sons Ltd.

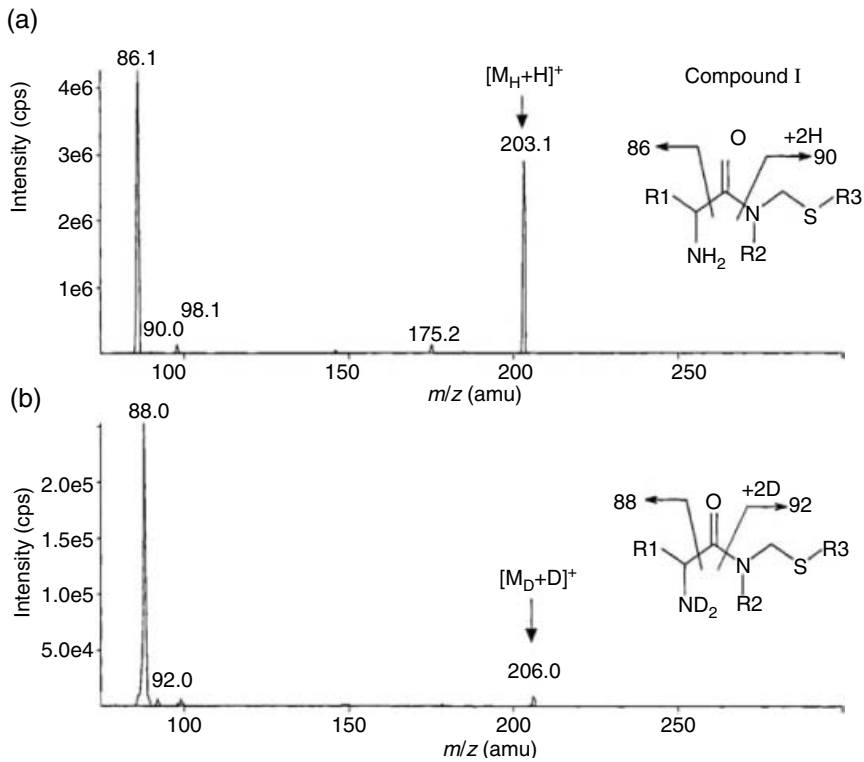


Figure 6.24 MS-MS spectra of compound I: (a) H_2O and (b) D_2O. Reproduced from [43] with permission of John Wiley and Sons Ltd.

oxidative modification of the B moiety. This could correspond either to a hydroxyl group or the sulphoxide and hydrogen/deuterium exchange would increase the molecular weight by 4 or 3 Da, respectively. The online hydrogen/deuterium exchange LC-MS showed an MD^+ ion at *m/z* 222 for M1. This increase of 3 Da revealed that M1 still had two exchangeable hydrogen atoms, which rules out the possibility of a hydroxyl group.

The product ion scan of *m/z* 222 gave an abundant ion for the A fragment at *m/z* 88, which was also observed for the parent molecule I. The B fragment

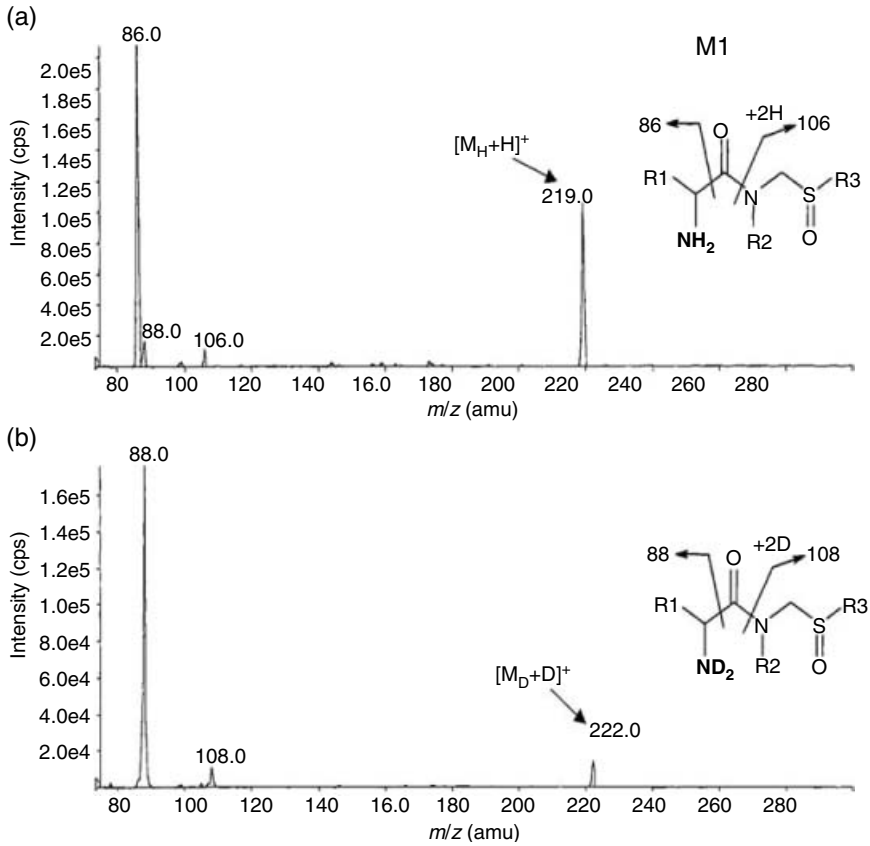


Figure 6.25 MS-MS spectra of M1: (a) H_2O and (b) D_2O. Reproduced from [43] with permission of John Wiley and Sons Ltd.

increased in mass from *m/z* 106 to 108, owing to the formation of the corresponding deuterated ammonium cation. As the oxidation to the B moiety did not add any exchangeable hydrogen atoms to the rest of the structure, this therefore supports the sulphoxide structure for M1.

In a similar manner, M2 gave rise to MH^+ at *m/z* 235, which is 32 Da higher than the parent, I. Similarly to compound I, M2 retained the characteristic A fragment at *m/z* 86 in water. The molecular weight increase suggests the addition of two oxygen atoms. The online hydrogen/deuterium exchange data showed MD^+ at *m/z* 238, indicating that M2 also had two exchangeable hydrogen atoms, which rules out the possibility of a hydroxyl group and confirms the sulphone species.

6.5 A practical example

The next problem shows how using a combination of the mass spectrometry tools available including hydrogen/deuterium exchange, product ion scans and

accurate mass measurements of both the MH^+ and the product ions, it was possible to solve a difficult problem.

In the conversion from A to B (Figure 6.26), an impurity was formed that had a relative retention time of approximately 2.1 with respect to B. The mass spectrum of B is very simple, showing MH^+ at *m/z* 358 and the major product ion of *m/z* 358 is *m/z* 299, representing the loss of acetamide (CH_3CONH_2) from the side chain. The impurity showed MH^+ at *m/z* 741 and the mass measurements gave an empirical formula of $C_{42}H_{49}N_2O_{10}$, as shown in Table 6.3, suggesting a dimer of B plus two carbon and two hydrogen atoms. A deuterium exchange experiment showed that the impurity had four labile hydrogen atoms. Figure 6.27 is the product ion spectrum of *m/z* 741. The loss of water from *m/z* 741 to *m/z* 723 implies the presence of a non-aromatic OH group. The peak at *m/z* 664 represents the loss of CH_3CONH_2 from *m/z* 723 but there is only one loss, indicating that only one acetamide group is present in the impurity. The ion at *m/z* 425 was shown to have two labile hydrogen atoms and an elemental composition of $C_{24}H_{29}N_2O_5$, which equates to a loss of $C_{18}H_{18}O_4$ from *m/z* 723. There is also a reduction of one labile hydrogen atom and the molecule has nine DBEs. This suggests that the ion at *m/z* 425 arises from cleavage between the ring system and

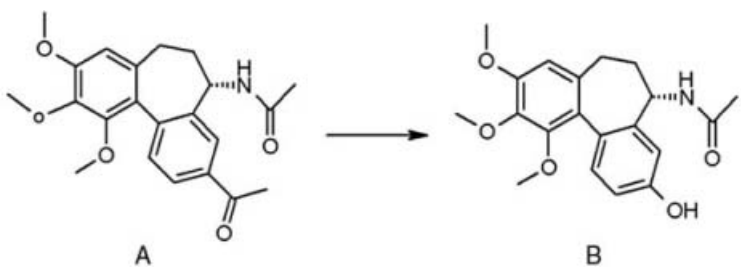


Figure 6.26 Synthetic route for conversion of A to B.

Table 6.3 Molecular formulae, labile hydrogens and DBEs for MH^+ 741

m/z H_2O	*m/z* D_2O	Labile hydrogen atoms	Elemental composition	DBEs
741	746	4	$C_{42}H_{49}N_2O_{10}$	20
723	726	3	$C_{42}H_{47}N_2O_9$	21
425	427	2	$C_{24}H_{29}N_2O_5$	12
384	386	2	$C_{22}H_{26}NO_5$	10
358	360	2	$C_{20}H_{25}NO_5$	10
325	326	1	$C_{20}H_{21}O_4$	9
299	300	1	$C_{18}H_{19}O_4$	9

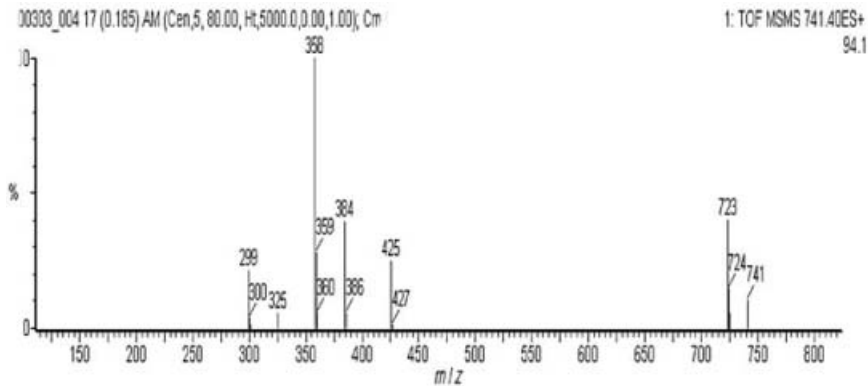


Figure 6.27 Product ion spectrum from *m*/*z* 741 from a dimeric impurity RRT 2.1.

the nitrogen atom (see Figure 6.28). This now confirms that a modification had taken place on the methyl carbon of the acetyl group of the original parent B. The ion at *m*/*z* 384 still has an acetyl group because loss of acetamide to give an ion with *m*/*z* 325 is observed. The ion at *m*/*z* 384 has an elemental composition of $C_{22}H_{26}NO_5$ and ten DBEs, which is one DBE less and two hydrogen atoms more than the starting material A. This supports the conclusion that part of the molecule had undergone reduction from a ketone to an alcohol. It was postulated that the impurity structure shown in Figure 6.28 was formed via an aldol reaction. This was later confirmed by NMR.

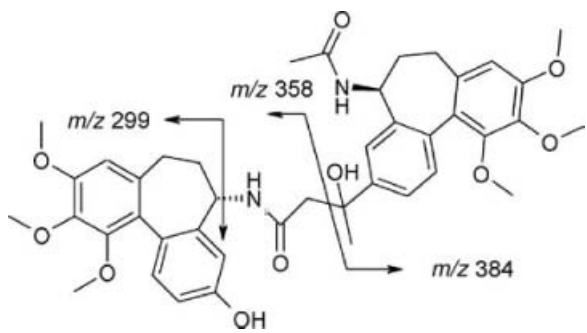


Figure 6.28 Structure of dimer impurity at RRT 2.1.

6.6 LC method development and validation

Investigation of peak homogeneity is an important topic in the validation of the analytical methodology. Method validation requirements are described in the ICH guidelines [1]. Method development involves the separation of drug

substance from all the drug impurities and degradation products. In the past, UV with diode array detection (DAD) was used for investigation of peak homogeneity but it can be limiting because drugs and their related impurities can have very similar UV spectral properties and it can therefore be difficult to detect very low levels of co-eluting impurities by DAD. Mass spectrometry offers a third dimension to check for co-elution of impurities with each other and the drug substance.

Figure 6.29 shows mass spectra recorded during elution reduced to a two-dimensional contour plot. Each point is produced from pseudo-molecular ions, cluster formation or fragmentation. All ions eluting in parallel with respect to time, at c. 29 min are assumed to belong to the main component, but there are some points clearly seen on the front edge of the main peak that indicate the presence of an impurity. This was confirmed by the production of a mass chromatogram of *m*/*z* 486.

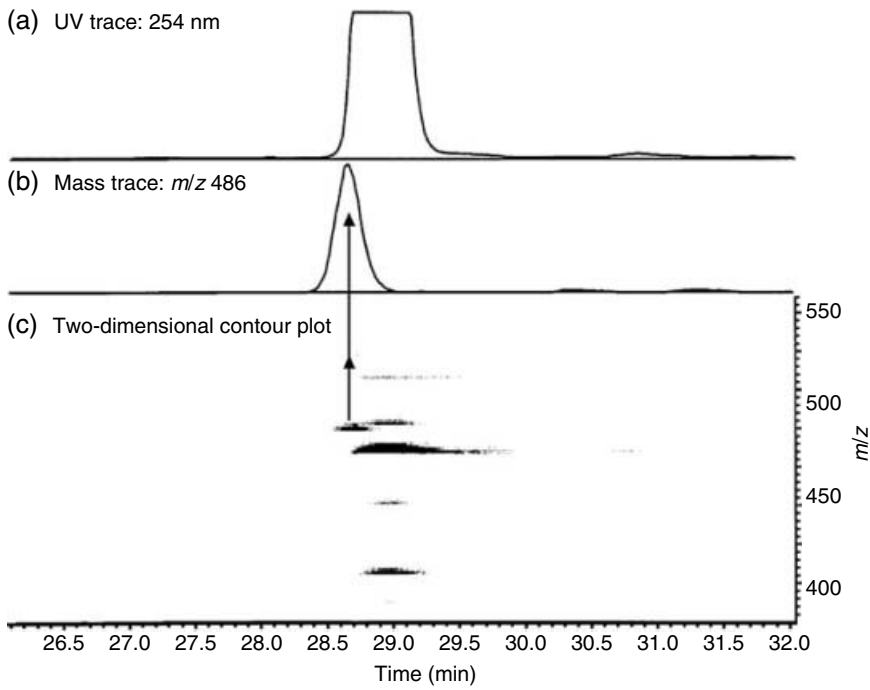


Figure 6.29 (a) UV trace of the drug, (b) mass chromatogram of *m*/*z* 486 of an impurity component and (c) two-dimensional contour plot of the mass spectra from *m*/*z* 400 to *m*/*z* 550. Reproduced from [22] with permission of John Wiley and Sons Ltd.

A case of exactly coincident peak elution is shown in Figure 6.30 [22]. The two ions *m*/*z* 469 and 780 are co-eluting as shown in Figure 6.30. From this information alone it is not clear whether these are two different chemical species with MH^+ 780 and 469, respectively, or if *m*/*z* 469 is a fragment ion from *m*/*z* 780. A product ion spectrum of *m*/*z* 780 was collected and clearly no ion at *m*/*z* 469

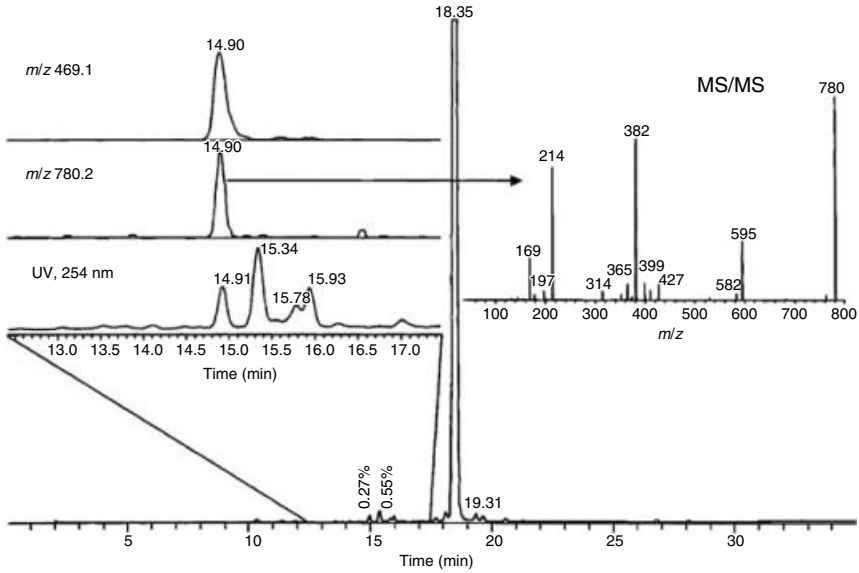


Figure 6.30 UV chromatogram of a drug substance. Expanded section from 13 to 17 min along with two mass traces for the peak at RT 14.9 min and the corresponding product ion spectrum of *m/z* 780. Reproduced from [22] with permission of John Wiley and Sons Ltd.

was seen. This permits confident deduction that *m/z* 780 and 469 are from two different but exactly co-eluting species.

Caution needs to be exercised in confirmation of method specificity, as peak purity cannot be inferred from the absence of any mass spectrometric signals, as an absence could be because of ion suppression, or the response may too low to be detected, or the unknown may have different ionisation properties. Peak purity should be tested by using an orthogonal technique.

6.7 Online NMR spectroscopy

The coupling of high-performance liquid chromatography and NMR (LC-NMR) is a well-established and routine technique for the study of mixtures [44–46]. First pioneered in the 1980s, it has found wide application from the mid-1990s in many areas of pharmaceutical research and development, including drug metabolism [46], impurities in pharmaceutical products [47–52], combinatorial chemistry [53–55] and natural products [46, 56]. Coupling the chromatographic separation and the NMR detection effectively removes a preparative step, which can result in significant gains in efficiency, particularly in situations where there are mixtures with many components. However, the advantages of LC-NMR do not come entirely without a cost, namely the compromise between the optimum conditions for chromatography and those for NMR [57, 58].

If the identities of drug impurities are inconclusive when analysed by LC-MS then LC-NMR can be used. Very often these drug impurities are present at low levels, less than 1% of the main drug compound, making the nature of the problem very different from traditional applications of LC-NMR where higher levels are analysed [57]. Identification of impurities at levels of less than 0.5% has only been successful under conditions of very high sample loading and narrow (low volume) chromatographic peaks [46]. However, recent developments such as cryogenic probes, solid-phase extraction (SPE) units and shielded high-field magnets have made LC-NMR significantly more sensitive, making it possible to routinely analyse low-level impurities [59–62]. Several good reviews on LC-NMR and related techniques are available [44–46, 63], but this chapter will focus on its use in impurity identification within pharmaceutical development.

6.7.1 LC-NMR experimental set-up

A typical experimental arrangement of HPLC-NMR coupling is shown in Figure 6.31. The outlet of the UV detector is connected via a capillary to a peak sampling unit (PSU) or valve that is connected to the NMR flow probe in the magnet. NMR spectra of analytes are acquired when they either pass through (on-flow) or are stopped in (stop-flow) the active volume of the flow cell. The flow probe was originally designed for continuous flow but the need for longer

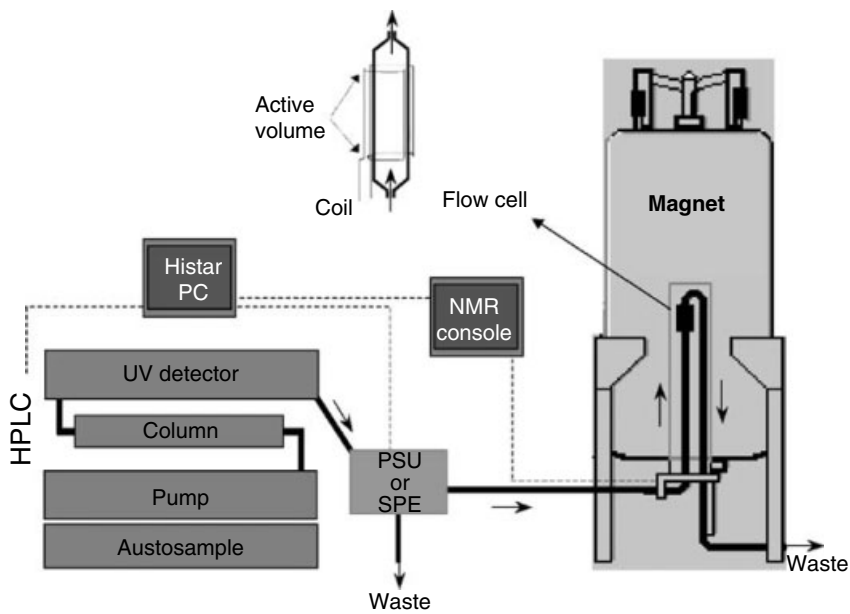


Figure 6.31 Schematic for HPLC-NMR coupling. (→) direction of flow; (---) electronic junctions; (PSU) peak sampling unit; (SPE) solid phase extraction unit.

acquisition times in two-dimensional experiments and for the analysis of low-level analytes led to applications in the stop-flow mode. To acquire the optimum signal-to-noise ratio the transfer time of the analyte 'peak' between the UV detector and the NMR flow cell has to be precisely measured. To prevent back pressure in the flow system the stop-flow mode requires switching valves between the UV detector and the NMR flow probe. One manufacturer sells a software-controlled PSU to do this switching and also a number of capillary loops for the storage of analytes, which can be sent to the NMR flow cell under manual or automatic operation. The switching unit can be substituted by an SPE unit for improved sensitivity.

Flow probes are available for 400–800 MHz spectrometers with cell active volumes ranging from 10 (1 mm internal diameter) to 200 μl (5 mm internal diameter). The selection of the flow-cell size depends on the size of the LC column used, which in turn depends on the sample size to be analysed [58, 64, 65]. When used with unshielded cryomagnets the HPLC and PSU are located at a distance of at least two metres from the magnet. With highly shielded cryomagnets, which have recently become available, the units can be located directly adjacent to the NMR magnet with a transfer line length of 30 cm or less, thus reducing the chromatographic peak broadening during transfer of the analyte to the NMR detection cell [57].

6.7.2 LC-NMR solvents

The increased solvent requirements for LC-NMR over tube NMR make the technique expensive if fully deuterated solvents are used. D_2O is usually substituted for H_2O in the eluent, mainly because of its low cost (~£150 per l), but also because it provides a lock solvent for the NMR. Fully deuterated organic modifiers such as d_3-acetonitrile may not be absolutely required, but in practice they are often also used in many pharmaceutical laboratories as they give reduced solvent signals. This reduces the need for solvent suppression, which lessens the risk that diagnostic resonances are also suppressed in error.

Increased sample loading and replacing non-deuterated solvents with deuterated solvents can give rise to changes in chromatographic retention times. It is therefore good practice to run the chromatography initially with the deuterated solvent and normal sample loading before overloading the conditions for stop-flow LC-NMR. Solvent quality is also very important in LC-NMR as minor impurities in the solvent can complicate the resulting spectra. Most HPLC solvents and deuterated solvents contain small amounts of impurities such as stabilisers, which are often UV inactive. High-quality LC-NMR solvents are available, but it is always advisable to obtain an impurity profile spectrum from each solvent.

6.7.3 Solvent suppression

The implementation of solvent suppression was a major step in the development of LC-NMR [66]. The use of non-deuterated organic solvents gives rise to

dynamic range problems, but application of solvent suppression pulse sequences reduces their signal intensity to that of the analyte resonances. Many pulse sequences are available including NOESY-type presaturation [46], WET [66] and excitation sculpting [67], with the latter two being capable of suppressing several solvent resonances with minimum baseline distortions. One consequence of improved suppression is that the ^{13}C satellites from solvents such as methanol or acetonitrile become a problem as they can be usually larger than low-level impurity signals. However,^{13}C decoupling during acquisition will remove these satellites [66, 67].

The LC solvent system is restricted to the number of signals that can be simultaneously suppressed. For example, two solvent peaks can be suppressed on spectrometers equipped with two radio frequency (RF) channels, so allowing the use of solvent systems such as acetonitrile/water. Acid modifiers such as trifluoroacetic acid (TFA) can be added to such a solvent system without the problem of a third solvent peak requiring suppression, as the acid proton is in rapid exchange with water protons. One disadvantage of suppressing solvent signals is that any nearby analyte signals will also be suppressed, resulting in the possible loss of vital structural information, as shown in Figure 6.32. The use of superheated water alone as an LC solvent has been reported and has the advantage of simplifying the solvent suppression to that of the water peak, which resonates in a relatively clear part of the spectrum [45, 68].

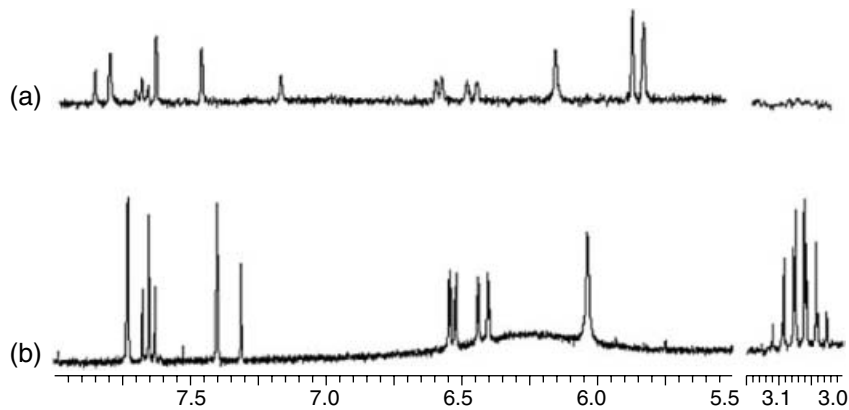


Figure 6.32 500 MHz ^1H NMR spectra of a drug impurity (a) by stopped flow LC-NMR and (b) by a preparative approach. In addition to the improved signal to noise, the peak at 3.06 ppm is missing in the LC-NMR experiment owing to its close proximity to the pre-saturated water signal. Reproduced from [57] with permission of John Wiley and Sons Ltd.

6.7.4 LC-NMR optimisation

For many practitioners of LC-NMR in pharmaceutical development, the LC method is pre-determined by that developed by the analytical chemist.

Conditions that optimize the LC-NMR sensitivity include a very high sample loading (typically 10–20 μl of a concentrated solution) and an analyte LC peak width at half height equal to the active volume in the NMR flow cell [58]. The HPLC parameters that need to be considered and optimised include column dimensions, properties of the stationary phase, mobile-phase composition and flow rate. HPLC columns of 10–25 cm length and 3–5 mm diameter containing 3–5 μm particle size stationary phase have been a popular choice for LC-NMR applications [46]. Under high loading conditions, isocratic methods generally give broad peaks, especially for late-eluting components, but some C_{30} solid phases are available that give minimum broadening with very high sample loadings [69]. Gradient elution is preferred for LC-NMR applications since the LC peak widths are much narrower, giving a much higher concentration of analyte in the active volume of the NMR cell, resulting in enhanced LC-NMR sensitivity. The optimisation of LC and NMR conditions for LC-NMR applications has been extensively discussed previously [58, 64].

The signal-to-noise (S/N) ratio of NMR spectra will be proportional to the concentration of the analyte in the NMR cell active volume and, ultimately, the amount of sample injected. A sample loading for analytical HPLC is typically about 10 μg, which gives, for an impurity present at 0.1%, 10 ng in the active volume of the LC cell, assuming all could be contained. The minimum amount of sample required in the active volume to get good quality data (from which reliable conclusions can be drawn) is a good guide as to whether or not to attempt LC-NMR. The amount will depend on the LC probe design, magnetic field and NMR experiment being conducted. A one-dimensional ^{1}H experiment, for example, will require less analyte than a two-dimensional long-range proton–carbon correlation experiment. One approach to determine the minimum amount of analyte is to fill a flow probe with solutions of known concentration, giving a known amount of analyte in the active volume [57, 70]. Figure 6.33 shows the resulting spectra from a 4-mm LC probe at 500 MHz [57]. In this particular example, a compound with a molecular weight of approximately 500 gives a lower limit of 500 ng after 3 h acquisition time. This figure would be deemed an absolute

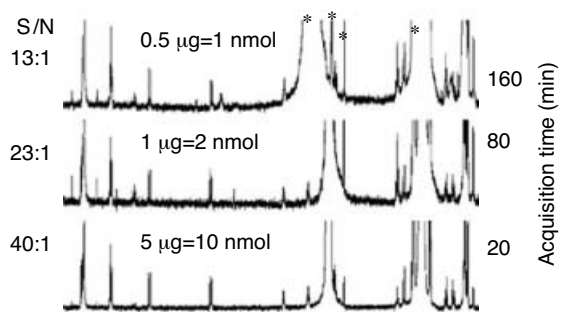


Figure 6.33 Spectra from which Table 6.3 is derived. Starred peaks are due to solvent or impurities therein. Reproduced from [57] with permission of John Wiley and Sons Ltd.

Table 6.4 Minimum weight of compound required to obtain good-quality spectra on a Bruker DRX500 with a 16 h acquisition time

Experiment	4 mm (120 μl) SEI *z*-gradient LC probe (μg)	5 mm (600 μl) BBI probe (μg)	4 mm (120 μl) LC cryogenic probe[a] (μg)
^{1}H	0.25	0.5	0.125
COSY	2.5	5	0.6
TOCSY	1	2	0.25
SE-HMQC	20	40	5
HMBC	40	80	10

[a]Based on a four-fold gain over a conventional LC probe [59].

minimum (even after an acquisition time of 16 h), as it will depend on the quality of the solvents, use of solvent suppression and multiplicity of the resonances. Table 6.4 summarises the minimum amounts of analyte required for various experiments on three different probes.

For an analytical LC injection, there is a shortfall of analyte in the active volume by between 50 and 100-fold, and therefore LC injections loadings must be increased by this order just to obtain a one-dimensional proton spectrum. The sensitivity problem is exacerbated by the fact that LC peaks are generally of larger volume than the active volume (Figure 6.34) and so not all the analyte is detected [57].

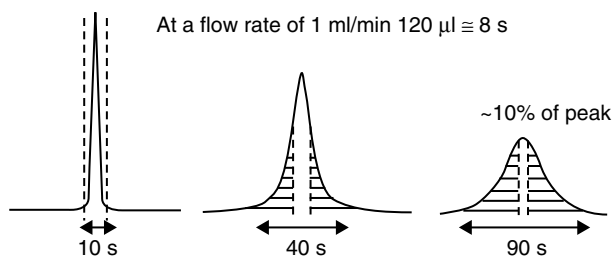


Figure 6.34 Some typical HPLC peak widths compared with LC-NMR cell active volume for a 4-mm probe. For a peak 90 s wide only 10% of the peak is in the active volume of the flow cell. Reproduced from [57] with permission from John Wiley and Sons Ltd.

Broadening of peaks in the flow system present yet another problem for sensitivity and can mean that even less of the analyte peak is used, even if it would appear narrow enough to be totally contained in the flow cell. Broadening of peaks during filling of the cell is much larger for peaks that are narrower than the volume of the cell compared with those that are larger than the cell volume [58]. This can lead to a further deterioration of LC-NMR sensitivity by a factor of 2 for narrow peaks [57].

6.7.5 Modes of LC-NMR and their application

There are four general modes of operation for LC–NMR: on-flow, direct stop-flow, time-sliced and loop collection/transfer. The mode selected will depend on the level and complexity of the analyte and also on the NMR information required. All modes of LC-NMR can be run under full automation for LC peak-picking, LC peak transfer to storage loops or NMR flow cell, and NMR detection [46].

6.7.5.1 On-flow LC-NMR

The NMR experiments are acquired continuously with solvent suppression while the sample is flowing through the detection cell of the NMR probe. The result is a pseudo two-dimensional spectrum (NMR chromatogram), a contour plot of intensity with chemical shift in the horizontal axis and chromatographic retention time on the vertical axis. On-flow experiments require high sample amounts (10 μg or more in the active volume) owing to the short residence time of the component in the active volume (for flow rates of 1 ml/min or more), which limits this approach to one-dimensional proton or fluorine spectra from the main components in a mixture. One advantage of using on-flow is that the NMR spectra of all components, including UV-inactive impurities, can be obtained in a single automated run [46]. Substantially lowering the flow rate for on-flow experiments increases the S/N for each spectrum, making it possible to obtain useful information from lower-level components [71]. This approach has also been used for impurity profiling using fluorine-19 NMR with flow rates as low as 0.1 ml/min [72]. In general, however, on-flow is not ideally suited for the analysis of low-level drug impurities owing to the lack of sensitivity.

6.7.5.2 Direct stop-flow LC-NMR

Direct stop-flow has been extensively used for identifying drug impurities and degradation products at levels of 0.5% or more [47, 49, 51, 73]. In stop-flow LC-NMR, the LC pump is stopped when a peak of interest is in the active volume of the flow cell, back-flow being prevented by valves (e.g. the PSU) controlled by the software. The time taken for the peak (at maximum UV absorbance) to move from the UV detector to the centre of the active volume in the NMR flow cell is precisely calibrated with the software calculating the time thereafter for different flow rates. Shimming, tuning and matching are optimised and then data are acquired in a similar way to conventional tube NMR until sufficient quality one- and two-dimensional spectra are obtained. However, as described above, peak broadening in the flow system reduces the amount of analyte in the active volume, making it difficult for even a one-dimensional proton spectrum of sufficient quality to be obtained from sample analytes present at less than 0.5% of the main analyte unless a cryogenic flow probe is used. Software control allows the whole process to be automated, from injecting the sample to obtaining spectra (with solvent suppression if required) that satisfy a given preset signal-to-noise ratio. The chromatographic peaks of interest can be defined by retention times and peak height. An application to a drug dosage formulation is shown in Figure 6.35.

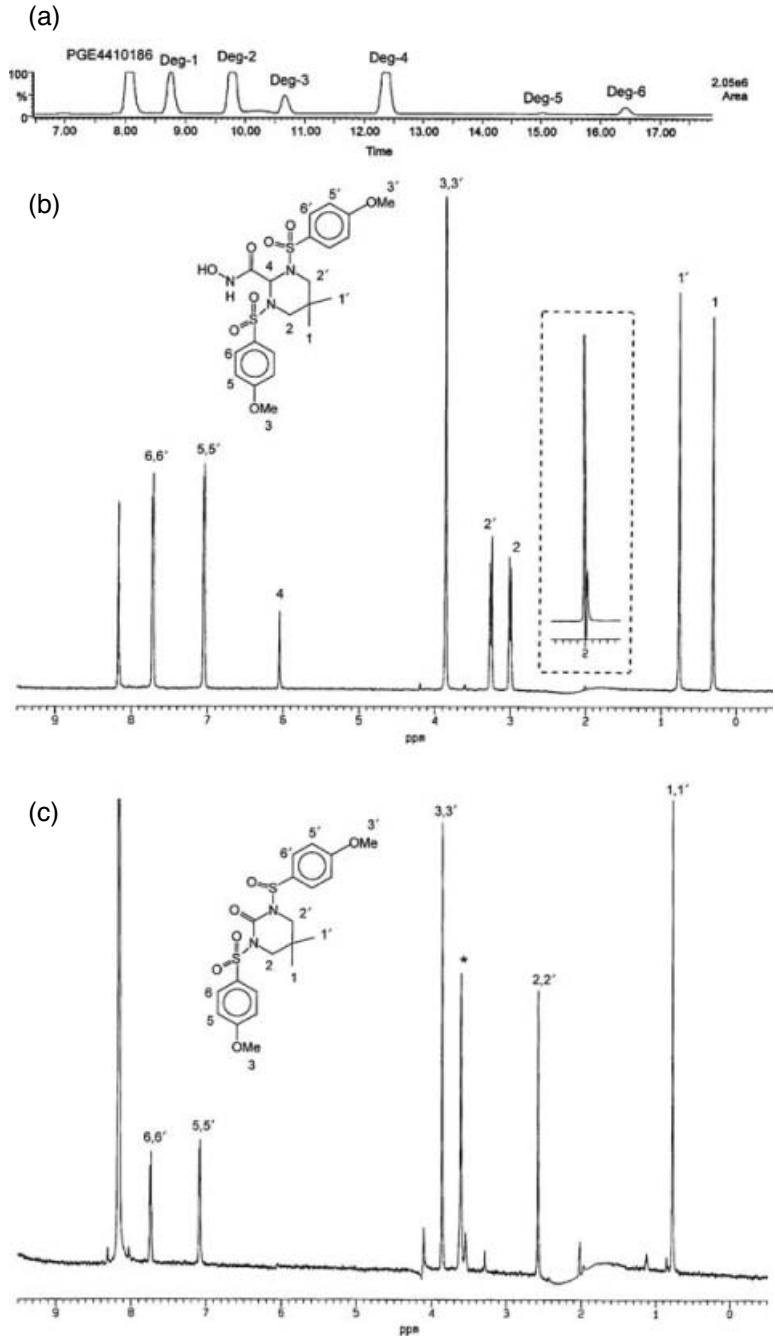


Figure 6.35 600 MHz ^{1}H NMR spectra obtained during a stop-flow LC-NMR experiment on a degradation sample of PGE4410186 in a dosage formulation. (a) LC chromatogram, (b) spectrum corresponding to the parent compound, PGE4410186, and (c) degradant 5. In (b), the inset above 2 ppm shows the suppressed acetonitrile resonance using the WET sequence with ^{13}C decoupling prior to convolution baseline correction. In (c) the peak labelled * is a low-level impurity in the mobile phase. Spectrometer: Bruker Avance DRX600 ^{1}H/^{13}C 3-mm *z*-gradient probe with 60 µl active cell volume. Reproduced from [49] with permission from Elsevier.

In practice, if the NMR acquisition times are longer than 30 min then on-column diffusion dilutes subsequent peaks, thus reducing the sensitivity [63]. Typically, LC peaks will be broadened by 30% and 50% after the flow is stopped for 5 and 10 h, respectively [74], significantly reducing the LC-NMR sensitivity. Such peaks are more efficiently analysed by repeating the chromatography.

Complete resolution of the LC peaks of interest is not always necessary as shown in Figure 6.36. In this case the identity of the minor co-eluting peak was known from LC-MS, which aided in the identification of the unknown impurity.

Carry-over can be problematic for closely eluting peaks in stop-flow mode [46], and these are better analysed using the loop collection mode (see below).

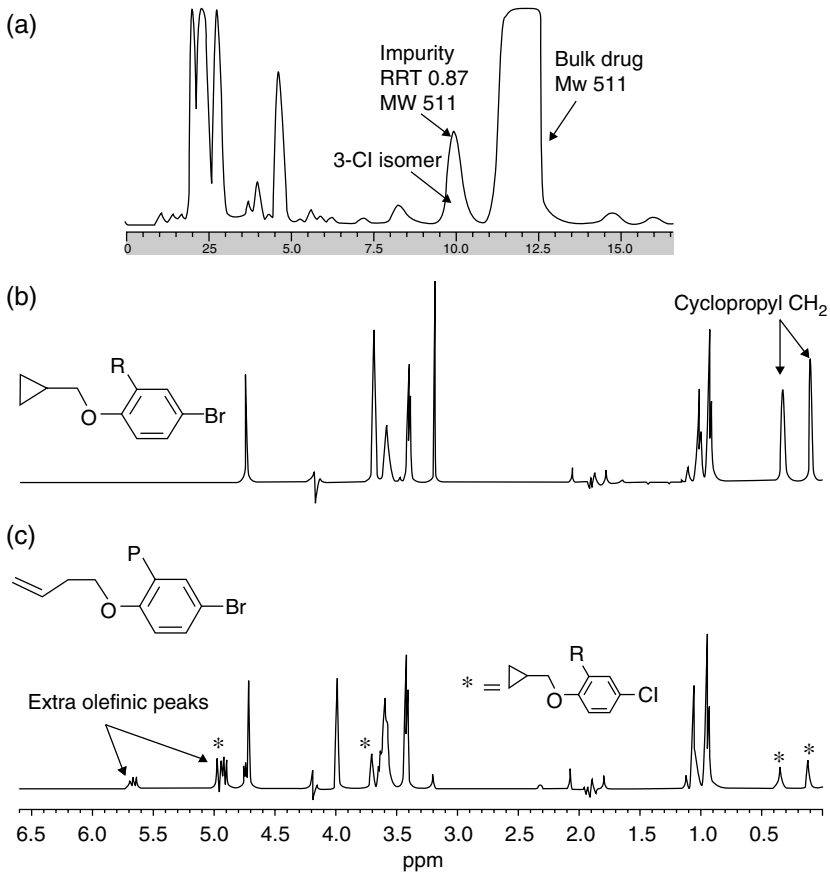


Figure 6.36 500 MHz ^{1}H NMR spectra obtained during a stop-flow LC-NMR experiment on a 1 mg injection of a crude sample of a drug compound. (a) LC chromatogram, (b) spectrum corresponding to the parent bulk drug compound acquired for 64 transients and (c) to the impurity peak RRT 0.87 (~3% by area), acquired for 1024 transients. NOESY-type presaturation was used to suppress the solvent resonances. Bruker DRX500; ^{1}H/ ^{13}C 4-mm *z*-gradient probe with a 120 µl active cell volume.

In addition, carry-over from the main peak is very often problematic for low-level late-running impurities. One way of overcoming this problem is to divert the flow to waste at the PSU for the early-running analytes, bypassing the probe and then switching the flow to the probe to analyse the minor peak(s) of interest.

Many pharmaceutical compounds contain fluorine atoms, and stop-flow LC-NMR using ^{19}F-NMR spectroscopy has proven to be useful for such compounds [70, 75, 76]. The sensitivity of ^{19}F is comparable with ^{1}H, typically requiring 500 ng of analyte to give good quality spectra [75]. However, 40 ng has been detected for compounds containing a trifluoromethyl group on an overnight acquisition [70]. Other advantages of ^{19}F-NMR are that solvent resonances do not dominate the spectrum in the way they dominate ^{1}H spectra, and therefore no solvent suppression is required and also spectra can be acquired with proton decoupling to aid interpretation (Figure 6.37).

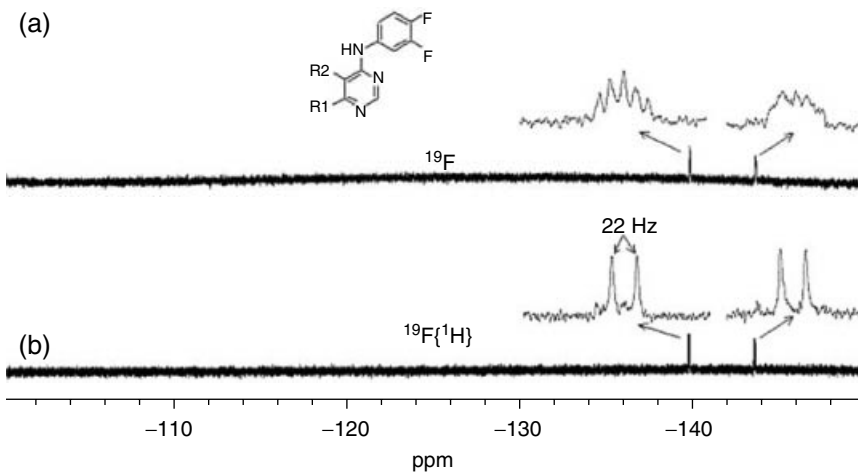


Figure 6.37 Stop flow ^{19}F spectra obtained from a 3,4-difluoro impurity with RRT 1.5 (0.5% by area) in a drug substance containing a 3-fluoro, 4-chloro aniline functional group. Acquisition time was for (a) 2 h and (b) 30 min. Spectra were acquired on a Bruker DRX500 fitted with a 4-mm SEI *z*-gradient LC probe with a 120 µl active cell volume.

6.7.5.3 Time-slice LC-NMR

Time-slicing is a variation of the stop-flow mode where the flow is stopped in a time-dependent manner (e.g. every 15 s) for the whole chromatographic run, in a sense the ultimate approach in slow-flow. When the flow is stopped, sufficient scans can be made to give the desired level of detection before moving on to the next time-slice. The whole process can be automated through software control. There is no requirement for an UV detector, and the approach has been used to identify non-UV-active components in drug substances [48]. The peak purity of

fluticasone propionate has been investigated by stopping the flow every 15 s over the main LC peak and ^{1}H spectra acquired [52]. Low-level impurities (<1%) can be detected if enough scans are taken at each time-slice, assuming there are sufficient chemical shift differences between major and minor component resonances [72].

6.7.5.4 Loop collection (peak-directed stop-flow) LC-NMR

Loop collection is used when there are several chromatographic peaks of interest in the same sample where it has the advantage of there being no interruption in the chromatographic run. The chromatographic peaks are stored in 200 μl capillary storage loops within the PSU (in, say, 12 or 36 loops). The LC detector selects chromatographic peaks for loop storage and the storage loop is isolated after the time delay required for the peak to flow from the detector into the storage loop. Further peaks can be trapped without interruption to the chromatographic run with no carry-over from the previous peak [46]. In this case, the components must be chemically stable inside the loops during the extended period of analysis. The stored chromatographic peaks can be analysed in a different order from the chromatographic run. To maximise sensitivity, the loop volume should ideally be the same volume as the flow cell [46].

Loop collection works better than simple direct stop-flow for closely eluting components, since during stop-flow analytes take longer to leave the NMR flow cell than they do to enter it, giving rise to back-mixing in the flow cell. The flow cell is rinsed clean between each loop analysis, lessening the possibility of mixing. However, in cases where the volume of the chromatographic peak is less than the volume of the loop, the sample concentration in the active volume will be less than that for direct stop-flow, resulting in reduced LC-NMR sensitivity.

Automation of LC-NMR is now at a stage where the operator can inject a sample and leave the HPLC interface to detect and store peaks and the NMR spectrometer to collect one- and two-dimensional data with signal-to-noise-dependent collection. An example of automated loop collection and transfer of closely eluting peaks is shown in Figure 6.38. Structures were deduced from the aromatic peak patterns and LC-MS information. Peaks 1–5 all elute within 5 min with no carry-over present in any of the ^{1}H spectra.

6.7.6 Enhancing the sensitivity of LC-NMR

The relative insensitivity of LC-NMR has driven practitioners and probe designers to develop new techniques to improve overall sensitivity. The development of cryogenic flow probes offer on average a four-fold increase in sensitivity compared with conventional flow probes [77]. The inclusion of an SPE unit after the chromatographic separation results in substantial increases in analyte concentration and significant improvements in sensitivity [60, 62, 78]. The principle and application of these techniques are discussed below. In addition, the SPE technique can be combined with cryogenic flow probes to give even further improvements [59]. Gains in sensitivity have also been obtained through miniaturisation of the NMR detection cell [79].

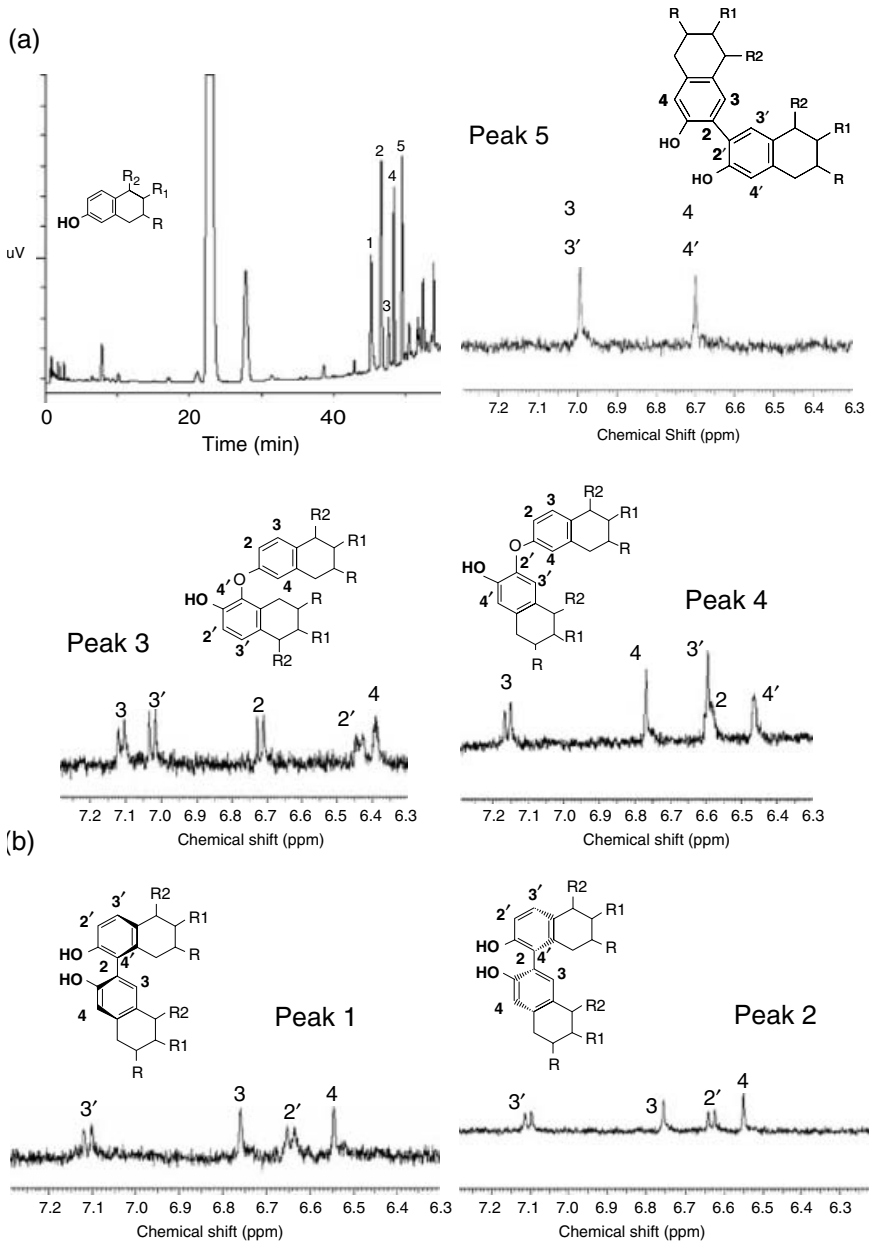


Figure 6.38 (a) The chromatogram from a 0.75 mg injection of an impure steroidal drug substance. Peaks 1–5 are at levels of 2–3% each. The individual peaks were stored in BPSU loops. (b) NMR spectra were acquired with NOESY presaturation with an acquisition time of 10 h on a Bruker DRX500 fitted with a 4-mm SEI z-gradient LC probe with a 120 μl active cell volume.

6.7.6.1 Cryogenic probes

The cryogenic cooling of the RF coils and electronics to give enhanced sensitivity is arguably the most significant recent advance in NMR spectroscopy [77]. In cryogenically cooled probes the RF coils and electronic components of the probe are cooled to 20 K, giving a significant reduction in electronic noise, which results in a three- to four-fold increase in the S/N ratio over that obtained with conventional probes [70, 80]. For a given experiment time the detection limits are up to four times less and for a given amount of sample the experiment time is reduced by a factor of up to 16, which offers significant advantages for the analysis of samples where the amount of the component of interest is limited [77, 81].

Cryogenic flow probes with active volumes ranging from 30 to 200 µl are available for spectrometers operating between 300 and 900 MHz [82]. A cryogenic probe has recently been developed that can operate both in flow and tube modes with conversion between the two modes taking only a few minutes, saving several hours over a standard cryogenic probe change.

6.7.6.2 LC-SPE-NMR

In LC-SPE-NMR, individual components eluting from the HPLC column are trapped on SPE cartridges, as shown in Figure 6.39a, dried with nitrogen to remove water and solvents (Figure 6.38b), and then eluted using a deuterated solvent (Figure 6.39c) to give a narrow, highly concentrated band resulting in an improved S/N ratio compared with conventional HPLC-NMR [61, 62, 83, 84]. Repeat injections of the same solution on to the HPLC column and successive trapping of a particular component on the same SPE cartridge can give further significant increases in the S/N ratio, as illustrated in Figures 6.40 and 6.41. This approach is particularly useful for analysing low-level impurities.

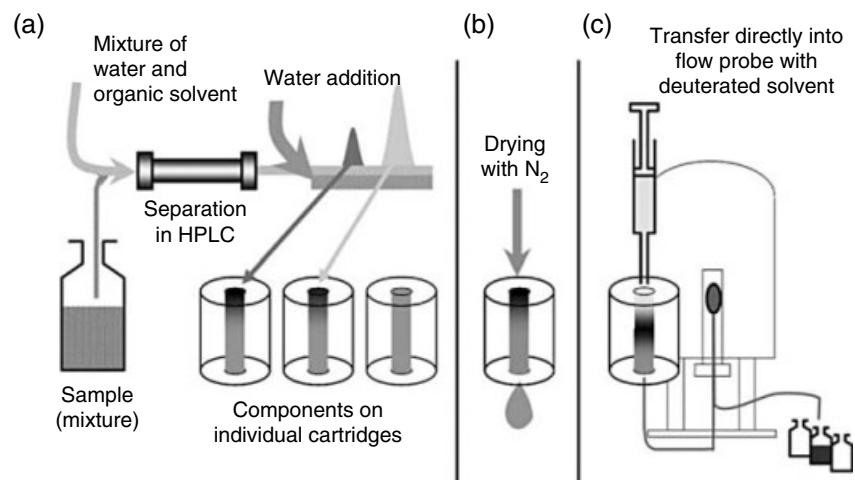


Figure 6.39 The flow path of the post-column SPE that is used in LC-SPE-NMR. Reproduced from [82] with permission from Elsevier.

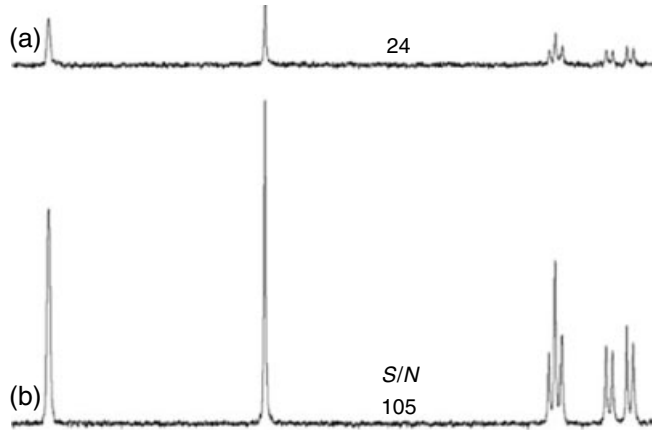


Figure 6.40 Multiple peak trapping on a SPE cartridge: (a) single trap and (b) four times trapping. 1 μg of hydrocortisone per injection, 2 mm C_{18} HD SPE cartridge, 600 MHz, 3-mm *z*-gradient LC-SEI probe with an active volume of 60 μl, 1024 transients.

The advantages of LC-SPE-NMR approach over conventional LC-NMR are:

1. better reproduction of the analytical chromatography by using HPLC conditions closer to the analytical method: use protonated solvents for the HPLC eluents and less LC sample overloading giving fewer regulatory compliance issues, virtually eliminating the need for 'LC peak tracking' by LC-MS;
2. multiple trapping of a single component on the same cartridge followed by elution with a small 'cell matched' volume (~30 μl) of deuterated solvent to optimise the concentration in the active volume of the NMR flow cell;
3. drying cartridges and eluting with small volumes of deuterated solvents (cost-effective) gives very little requirement for solvent suppression, leading to significantly cleaner baselines and no loss of useful resonances in the NMR spectrum;
4. NMR sensitivity is independent of the chromatographic peak volume, allowing more efficient characterisation of late-eluting broad peaks, which were problematic by LC-NMR;
5. good quality two-dimensional spectra (H-H and H-C) can be acquired in reasonable times for impurities at 0.3% or higher levels. For impurities at 0.2% or less cryogenic flow probe with an active volume of 30 μl is required;
6. exchangeable protons can be observed in the NMR spectrum by using an organic, non-protic deuterated solvent.

A recent in-depth study into the applicability of LC-SPE-NMR in pharmaceutical development has shown that trapping efficiency is dependent on compound polarity [61]. The study further concluded that good sensitivity gains can be

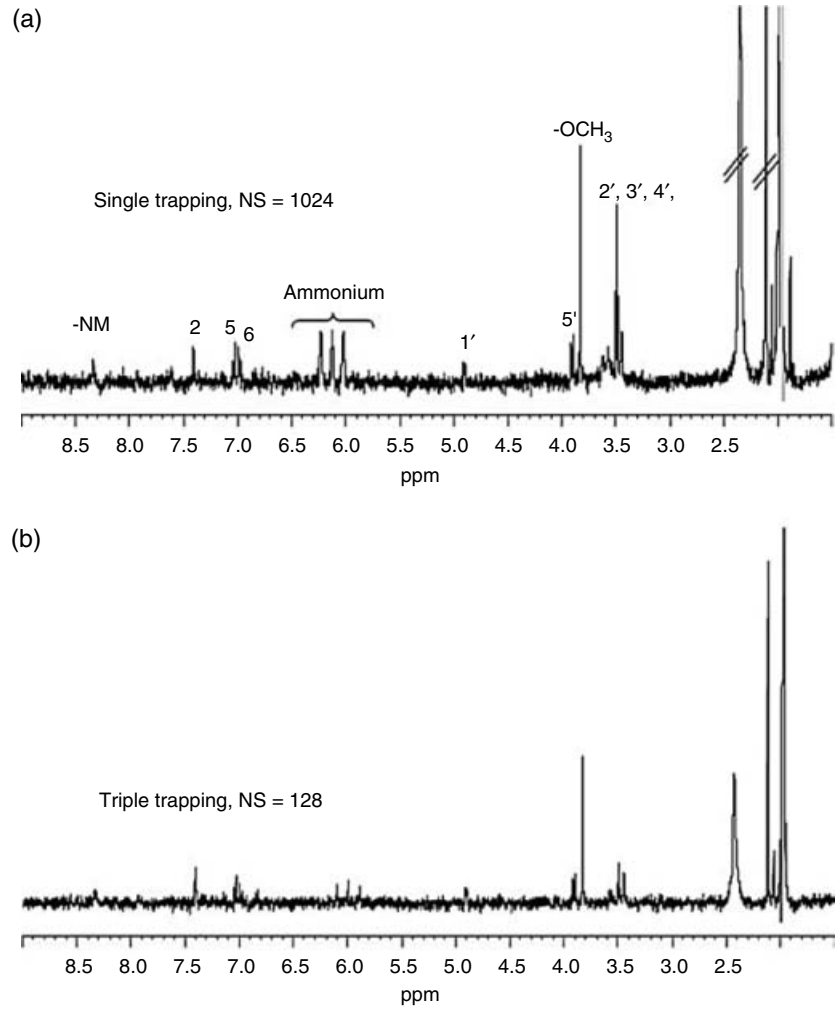


Figure 6.41 50 μl paracetamol in human urine was injected for SPE trapping. (a) Shows the result after single trapping and (b) the result after triple trapping. Urine was obtained from a normal healthy male 4 h after an oral dose of 1 μg of paracetamol. Bruker Avance 500 MHz NMR spectrometer operated at 500.13 MHz, equipped with a 3.0 mm $^{1}H/^{13}C$ inverse LC flow probe (active volume 60 μl). Reproduced from [59] with permission from Elsevier.

made (over conventional LC-NMR) for compounds of medium and low polarity, but there are no sensitivity gains for polar compounds [61]. With further investigation into a range of SPE phases and different drug compounds, it will be possible to select a solid phase for the retention of different classes of compounds (e.g. polar, non-polar, specific functional groups).

The efficiency of trapping of cortisol on different SPE solid phases is shown in Figure 6.42. The large efficiency difference observed highlights the need for

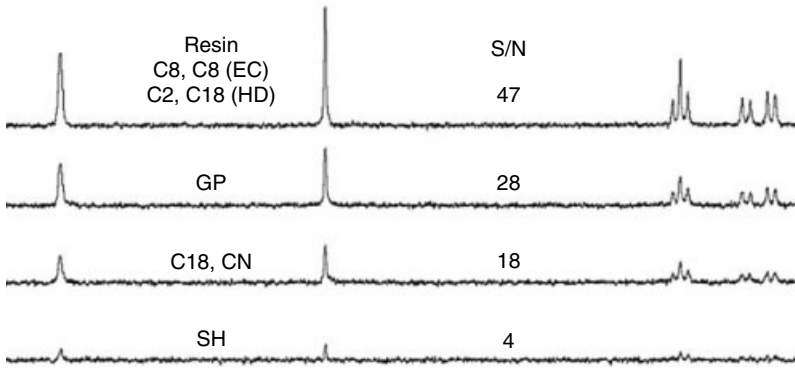


Figure 6.42 Single trap using different SPE phases for a steroid-type drug. 10 μg of hydrocortisone per injection, 2-mm SPE cartridges, 600 MHz, 3-mm *z*-gradient LC-SEI probe with an active volume of 60 μl, 512 transients.

some prior knowledge of how different classes of drug compound will behave, in terms of retention and elution from the SPE cartridges. To make the most efficient use of the SPE technique, the following criteria should ideally be met:

1. No elution of analyte from the SPE cartridge during the trapping process. This requires a solid phase applicable to a wide range of different classes of chemical compounds (drug impurities and degradants).
2. Elution of all the trapped analyte from the SPE cartridge using approximately 30 μl of deuterated solvent with no peak broadening (requires an elution solvent that dissolves a wide range of compound classes).
3. A short transfer line (≤30 cm) from the SPE unit to the flow probe, so minimising broadening of the eluted analyte peak. Such short transfer lines are only practical with shielded magnets since unshielded magnets require a minimum length of 2 m [46].

A recent report using online semi-preparative LC-SPE-NMR shows that even further gains in efficiency can be obtained [84]. The approach uses a five-fold increase in analyte concentration in a single LC-SPE trapping experiment, and so shortening the overall LC-SPE experiment time compared with multi-trapping on an analytical LC-SPE cartridge.

6.7.6.3 LC-SPE-NMR with cryogenic flow probes

The combination of both SPE and cryogenic flow probes currently provides the largest sensitivity gains for hyphenation to HPLC and is optimum if a probe with a matched active volume of 30 μl is used in combination with 2 mm SPE cartridges [59, 77, 78, 82]. Using multiple SPE trapping, a 73-fold gain in sensitivity has been obtained when compared with loop-collection LC-NMR on a conventional flow probe [82]. Further examples of this enhanced sensitivity are demonstrated in Figures 6.43 and 6.44.

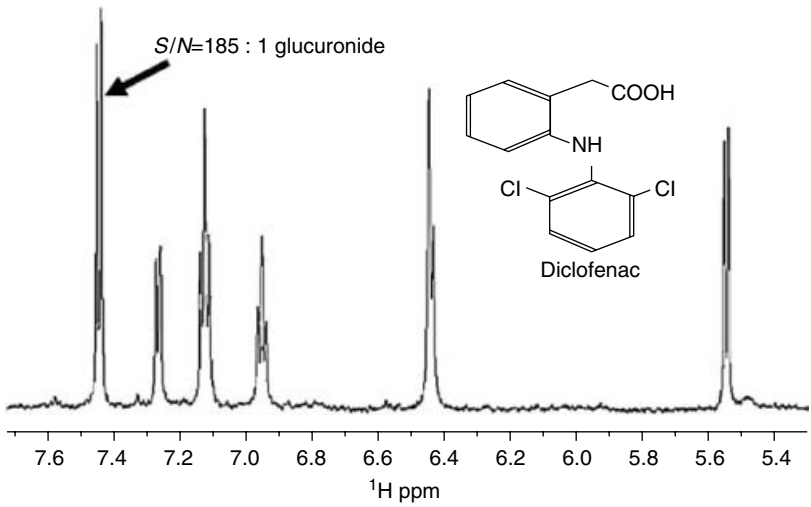


Figure 6.43 ^{1}H spectrum of diclofenac glucuronide obtained after single trapping from a 100 μl injection of female urine 4 h after dosage of 50 mg of diclofenac. The spectrum was recorded using a cryogenic flow probe at 600 MHz. The spectrum indicates that the sensitivity is sufficient to run all two-dimensional experiments needed for structure elucidation. Reproduced from [82] with permission from Elsevier.

6.7.7 Coupling of NMR to other separation techniques

The last 10 years have seen the emergence of hyphenation with several important related techniques such as supercritical fluid chromatography (SFC), gel permeation chromatography (GPC), capillary electrophoresis (CE), capillary electrochromatography (CEC), capillary isotachophoresis (cITP), capillary LC (capLC) and microbore LC [46, 50, 65, 85–87], the latter two being the most useful techniques for analysing drug impurities. The hyphenation to micro-separation techniques (such as capLC and microbore LC) has been made possible through the development of low-volume NMR flow probes [46, 85, 86]. Figure 6.45 shows an experimental arrangement for hyphenation to a microbore LC system.

Low-volume probes with solenoidal or saddle coil diameters of ≤2 mm and active volumes ranging from 5 nl to 1 μl show very high mass sensitivity, a property that is inversely proportional to coil diameter [79, 87]. Reducing the coil diameter from 1 mm to 50 μm shows a 20-fold improvement in mass limits of detection, shortening the acquisition time by a factor of 400 [86]. Additionally, the analyte concentration in the maximum of the chromatographic peak is inversely proportional to the square of the column internal diameter, giving relatively narrow LC peak widths when the diameter is typically ≤1 mm [87]. The combination of very narrow chromatographic peak widths and matched volume NMR detection cells maximises the amount of analyte available for detection, and in the example shown in Figure 6.46 quite impressive detection limits (<1 μg) have been achieved in on-flow experiments [87].

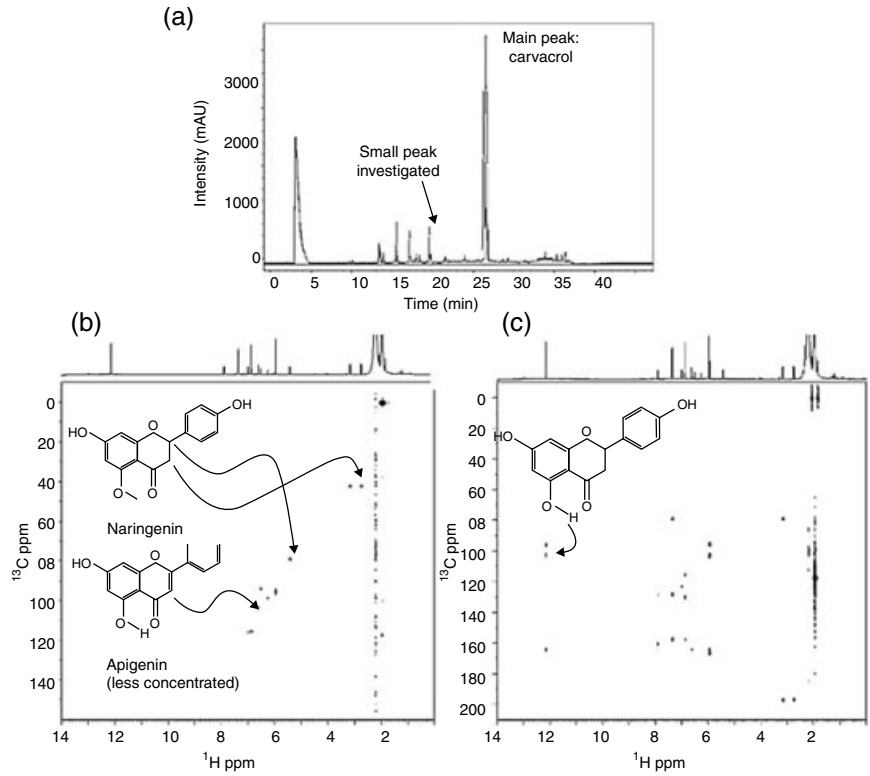


Figure 6.44 (a) Reversed-phase HPLC separation of an oregano extract with UV detection on a 4.6 × 150 mm RP-18 column after injection of 20 µl of acetone extract, (b) ^{1}H–^{13}C HSQC and (c) ^{1}H–^{13}C HMBC NMR spectra recorded on the HPLC peak appearing at 20 min in (a), after triple trapping and transfer with CD_3CN into a cryogenic flow probe at 600 MHz. The active volume was 30 µl, total experiment time c. 14 h for both spectra. Reproduced from [82] with permission from Elsevier.

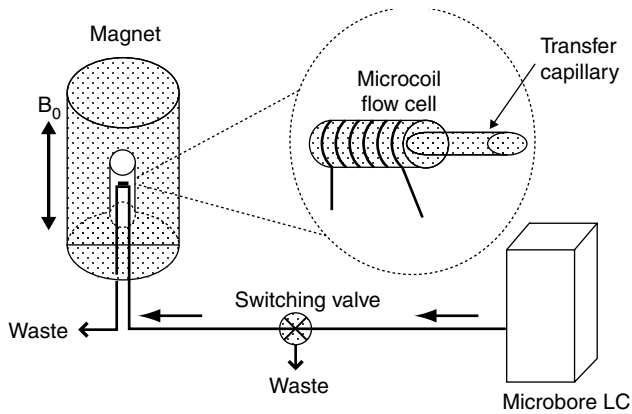


Figure 6.45 Microbore LC-NMR layout. A Microbore HPLC system with a 0.5 mm × 150 mm C18 column is interfaced to a solenoidal microcoil probe. The transfer capillary is connected to the NMR flow cell with a polyamide resin. Reproduced from [85] with permission. Copyright 1999 American Chemical Society.

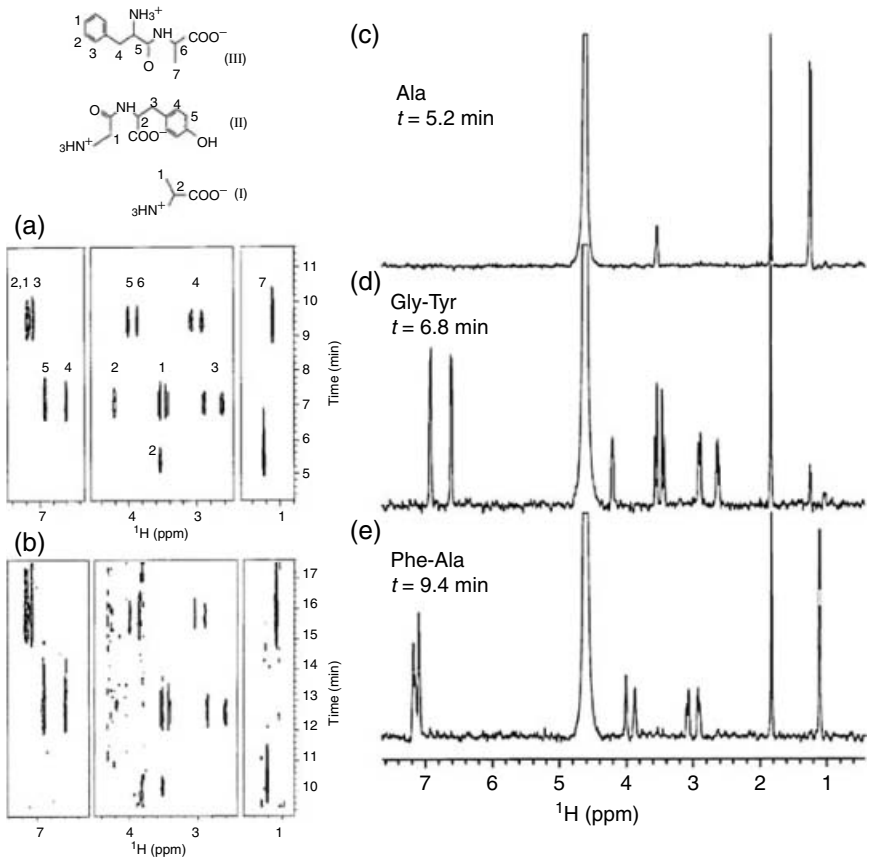


Figure 6.46 LC-NMR chromatogram at 500 MHz from system described in Figure 6.45. Each NMR spectrum represents 12 coadded scans acquired in 12 s. Flow rate, 5 μl/min; active volume, 1.1 l. No solvent suppression scheme was used, and all spectra were acquired with the spectrometer gain set to maximum. (a) Injected amounts: 2.3 (26 nmol) 4.8 (20 nmol), and 4.8 μg (21 nmol) of Ala, Gly-Tyr and Phe-Ala (I, II, III), respectively, (b) injected amounts: 8 nmol of each component. Extracted NMR spectra shown in (c) Ala, (I), (d) Gly-Tyr (II), (e) Phe-Ala (III). Reproduced from [85] with permission. Copyright 1999 American Chemical Society.

Vastly reduced solvent consumption for micro-separation techniques has advantages in that it gives superior solvent suppression when protonated solvents are used [88]. Reduced solvent volumes also make the use of fully deuterated solvents more attractive, eliminating the need for solvent suppression [87]. A low-volume capillary probe with a 7 μl cell volume (1.5 μl active) is commercially available and its application to metabolite identification has been reported [89].

However, issues remain with sample loading capacity, and CapLC–NMR may be best suited to mass-limited samples where the component of interest is present at a sufficient concentration such that column loading does not become an issue [88]. Capillary probes have therefore been used most effectively where

extremely high concentrations have been achieved by effects such as isoelectric focusing in CEC [50].

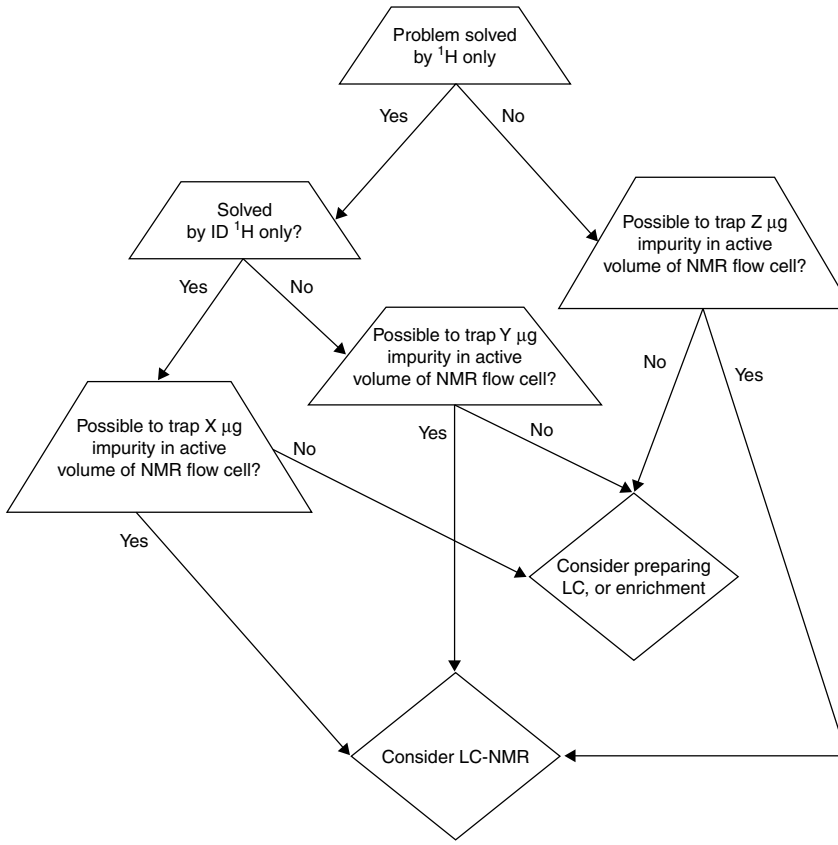
6.7.8 Considerations for online analysis

The speed of analysis provided by LC-NMR can potentially give significant efficiency gains over off-line techniques. However, there are several factors that need to be considered before deciding which approach to take:

1. The number of impurities requiring identification. The automation routines offered by LC-NMR gives large efficiency gains when there are several impurities present in a sample (or multiple samples).
2. The level and structural complexity of the impurities. Complex impurities require higher analyte concentrations to acquire two-dimensional experiments in reasonable time frames. Low-level impurities may not give the desired spectral quality.
3. Prior knowledge of the type of problem to be solved. For example, if aromatic substitution is suspected in an impurity then the coupling pattern of aromatic protons in a one-dimensional ^{1}H spectrum is sufficient to distinguish the possible isomers.
4. Equipment available (HPLC, automated PSU, SPE, flow probe, high-field spectrometer) will determine the overall 'LC-NMR sensitivity' and levels of impurities that can be analysed. For low-level impurities the time required for multiple trapping on SPE cartridges may also need to be considered [84].
5. Availability of the equipment with consideration given to the time taken for probe changes (including GMP/GLP documentation) and also any major disruption to tube NMR work required on the spectrometer.

Figure 6.47 shows a decision scheme for the analysis of impurities by LC-NMR at 500 MHz. The weights indicate the amount of analyte required in the active volume of the flow cell to obtain good quality data from which reliable conclusions can be drawn. For a cryogenic flow probe with an active volume of 120 μl, 0.3 μg of analyte is required to give a one-dimensional proton spectrum in 2 h, approximately 2 μg for two-dimensional homonuclear spectra (COSY, TOCSY) and 25 μg for inverse correlation spectra (HMQC, HMBC). Cryogenic flow probes with lower active volumes (30 or 60 μl) have higher mass sensitivity and therefore require less analyte to acquire spectra of the same quality.

With current commercially available equipment the ideal set-up for online analysis would be an HPLC-SPE system, a cryogenic flow probe (30 μl active volume) that is in permanent use within an actively shielded magnet operating at 500 MHz or higher. The system would offer the optimum LC-NMR sensitivity (no dependency on LC peak volumes), and complex impurities as low as 0.1% could be identified by one- and two-dimensional NMR experiments, provided that the impurities are sufficiently stable to permit isolation on the SPE cartridges,



Sample amount[a] for flow probe (120 µl active volume) on a DRX500

	4-mm SEI z-gradient (µg)	4-mm TXI cryoprobe[b] (µg)
X	1	0.3
Y	10	2.5
Z	100	25

[a]Amount to give good-quality spectra after 2 h acquisition.
[b]Average theoretical gain.

Figure 6.47 Decision tree for analysis by LC-NMR.

with the cartridges showing good trapping and elution efficiencies. The inclusion of a mass spectrometer in this set-up (LC-SPE-NMR-MS) offers even further increases in efficiency [59].

For systems without an SPE unit (or other post-LC column sample concentrating device) the quality of the NMR data will depend on the volume of the chromatographic peak, volume of the NMR flow cell, probe sensitivity and the use of chromatography solvents that can be suppressed. For the analysis of impurities at <1% the overloading required to attempt to obtain sufficient analyte in the active volume tends to broaden peaks significantly. Indeed, many

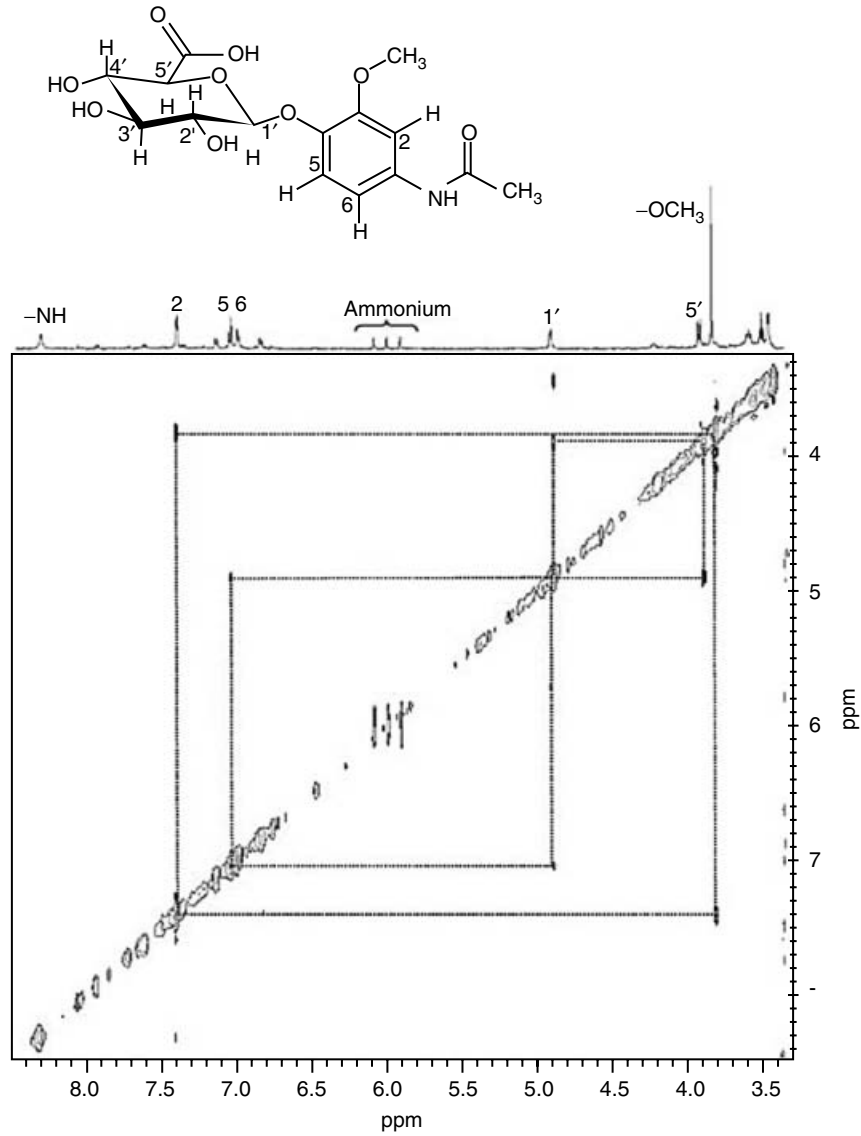


Figure 6.48 Two-dimensional NOESY spectrum (mixing time = 2 s) of the ether glucuronide of 3-methoxy-paracetamol at 600 MHz in a 3-mm cryogenic probe head (total experiment time: 20 h). The sample was recovered from a conventional 3-mm LC probe head after a triple trapping SPE-NMR run (result shown in Figure 6.41). Reproduced from [59] with permission from Elsevier.

chromatographic methods simply cannot take the increase in the required loading, resulting in extremely broad peaks and leading to even further degradation in LC-NMR sensitivity.

One solution to this would be to redevelop a chromatography method to narrow the peaks and simultaneously cope with high loading. Approaches such as gradient LC methods can be useful in this respect, and some chromatographic stationary phases have been shown to cope well with increased loading [69]. However, method redevelopment takes time, during which the analyte(s) could be more quickly isolated by a preparative approach (Chapter 7). This has to be taken into account when thinking of overall efficiency.

Furthermore, in a pharmaceutical development environment (working to GMP), redeveloping a method is not a simple task since peak tracking (by LC-MS) and validation may be required and, in the case of registered methods, results obtained by 'modified' methods may not be acceptable. In such cases, it may be more efficient to use a preparative approach followed by conventional tube NMR where optimum sensitivity can be obtained through the use of cryogenic probes (Figure 6.48) or low-volume 1 mm probes [90].

References

1. ICH Steering Committee 2002, *International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use*, Impurities in new drugs substances ICH Steering Committee, 1997.
2. M. S. Lee and H. S. Kerns, *Mass Spectrom. Rev.*, 1999, **18**, 187–279.
3. R. Kostiainen, T. Kotiaho, T. Kuuranne and S. Auriola, *J. Mass Spectrom.*, 2003, **38**, 357–372.
4. Y. Wu, *Biomed. Chromatogr.* 2000, **14**, 384–396.
5. E. J. Oliveira and D. G. Watson, *Biomed. Chromatogr.*, 2000, **14**, 351–372.
6. C. R. Blakely and M. L. Vestal, *Anal. Chem.*, 1983, **55**, 750–754.
7. M. Yamashita and J. B. Fenn, *J. Chem. Phys.*, 1984, **80**, 4451–4459.
8. R. N. Dreyer, J. B. Fenn, C. M. Whitehouse and M. Yamashita, *Anal. Chem.*, 1985, **57**, 675–679.
9. J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong and C. M. Whitehouse, *Science*, 1989, **246**, 64–71.
10. M. Dole, L. L. Mack, R. L. Hines, R. C. Mobley, L. D. Ferguson and M. B. Alice, *J. Chem. Phys.*, 1968, **49**, 2240–2249.
11. M. Mann and J. B. Fenn, 'Electrospray Mass Spectrometry; Principles and Methods', in *Mass Spectrometry; Clinical and Biomedical,* Vol. 1, ed. D. M. Desiderio, Plenum Press, New York, 1992
12. A. P. Bruins, T. R. Convey and J. D. Henion, *Anal. Chem.*, 1987, **59**, 2642–2646.
13. J. B. Fenn, *J. Am. Soc., Mass Spectrom.*, 1993, **4**, 524–535.
14. J. V. Iribarne and J. Thompson, *Chem. Phys.*, 1974, **64**, 2287–2294.
15. W. M. A. Niessen, *Liquid Chromatography – Mass Spectrometry*, 2nd ed., Chromatographic Science Series, Vol. 79, Marcel Dekker, New York, 1999.
16. D. B. Robb, A. P. Bruins and T. R. Convey, *Anal. Chem.*, 2000, **72**, 3653–3659.
17. J. A. Syage and M. Evans, *Spectroscopy*, 2001, **16**, 14–21.
18. A. Raffaelli and A. Saba, *Mass Spectrom. Rev.*, 2003, **22**, 318–331.
19. A. P. Bruins, T. J. Kauppila and R. Kostiainen, *Rapid Commun. Mass Spectrom.*, 2004, **18**, 808–815.

20. M. Blades and D. B. Robb, *Am. Soc. Mass Spectrom.*, 2005, **16**, 1275–1290.
21. A. P. Bruins, R. Kostiainen and T. P. Kauppila, *Am. Soc. Mass Spectrom.*, 2005, **16**, 1399–1407.
22. J. Emer and M. Vogel, *Biomed. Chromatogr.*, 2000, **14**, 373–383.
23. N. B. Cech and C. G. Enke, *Mass Spectrom. Rev.*, 2001, **20**, 362–387.
24. R. N. Rao and V. Nagaraju, *J. Pharm. Biomed. Anal.*, 2003, **33**, 335–377.
25. J. J. Abian, E. Gelpí and A. J. Oosterkamp, *J. Mass Spectrom.*, 1999, **34**, 244–254.
26. P. Plumb, J. Castro-Perez, J. Granger, I Beattie, K. Joncour and A. Wright, *Rapid Commun. Mass Spectrom.*, 2004, **18**, 2331–2337.
27. P. Tchelitcheff and S. Wren, *Astrazeneca PAR&D*, Alderley Edge, Cheshire.
28. W. Windig, J. M. Phalp and A. Payne, *Anal. Chem.*, 1996, **68**, 3602–3606.
29. Metabolynx HYPERLINK "http://www.waters.com" www.waters.com
30. J. Castro-Perez and J. Kirby, *Am. Biotechnol. Lab.*, 2000, **18**, 42–44.
31. C. A. Kingsmill, A. L. Freed, U. Kale, H. Ando and A. D. T. Rossi, *J. Pharm. Biomed. Anal.*, 2004, **35**, 727–738.
32. T. L. Barry, J. D. Franolic, G. J. Lehr, G. Petzinger and P. Scheiner, *J. Pharm. Biomed. Anal.*, 2003, **33**, 627–637.
33. K. Albert, E. Bayer, C. Rental and S. Strohschein, *Anal. Chem.*, 1998, **70**, 4394–4400.
34. C. G. Enke and R. A. Yost, *J. Am. Chem. Soc.*, 1978, **100**, 2274–2275.
35. H. Morris, T. Paxton, A. Dell *et al.*, *Rapid Comm. Mass Spectrom.*, 1996, **10**, 889–896.
36. M. J. Babu, Y. R. Kumar, M. S. P. Sarma *et al.*, *J. Pharm. Biomed. Anal.*, 2003, **32**, 361–368.
37. Q. Lui and C. E. C. A. Hop, *J. Pharm. Biomed. Anal.*, 2005, **37**, 1–18.
38. G. C. Stafford, P. E .Kelley, J. E. P. Syka, W. E. Reynolds and J. F. Todd, *Int. J. Mass Spectrom. Ion Proc.*, 1984, **60**, 65.
39. K. Biemann, in *Mass Spectrometry and its Organic Applications*, McGraw Hill, New York, 1962.
40. K. E. Karlsson, *J. Chromatogr.*, 1993, **647**, 31–38.
41. R. Ramanathan and W. Lam, *J. Am. Soc. Mass Spectrom.*, 2002, **13**, 345–353.
42. M. Olsen, P. G. Cummings, S. Kennedy-Gabb, B. M. Wagner, G. R. Nicol and B. Munson, *Anal. Chem.*, 2000, **72**, 5070–5078.
43. D. Lui, C. E. C. A. Hop, M. G. Beconi, A. Mao and S. L. Chui, *Rapid Commun. Mass Spectrom.*, 2001, **15**, 1832–1839.
44. K. Albert, *J. Chromatogr. A*, 1999, **856**(1+2), 199–211.
45. J. C. Lindon, J. K. Nicholson and I. D. Wilson, *J. Chromatogr. B*, 2000, **748**(1), 233–258.
46. K. Albert, ed., *On-Line LC–NMR and Related Techniques*, John Wiley and Sons, Chichester, 2002.
47. B. C. M. Potts, K. F. Albizati, M. O. Johnson and J. P. James, *Magn. Reson. Chem.*, 1999, **37**(6), 393–400.
48. S. D. McCrossen, D. K. Bryant, B. R. Cook and J. J. Richards, *J. Pharm. Biomed. Anal.*, 1998, **17**(3), 455–471.
49. S. X. Peng, B. Borah, R. L. M. Dobson, Y. D. Liu and S. Pikul, *J. Pharm. Biomed. Anal.*, 1999, **20**(1–2), 75–89.
50 R. A. Kautz, M. E. Lacey, A. M. Wolters *et al.*, *J. Am. Chem. Soc.*, 2001, **123**(13), 3159–3160.
51. J. K. Roberts and R. J. Smith, *J. Chromatogr. A*, 1994, **677**(2), 385–389.
52. N. Mistry, I. M. Ismail, M. S. Smith, J. K. Nicholson and J. C. Lindon, *J. Pharm. Biomed. Anal.*, 1997, **16**(4), 697–705.
53. J. C. Lindon, R. D. Farrant, P. N. Sanderson *et al.*, *Magn. Reson. Chem.*, 1995, **33**(11), 857–863.
54. J. Chin, J. B. Fell, M. Jarosinski, M. J. Shapiro and J. R. Wareing, *J. Org. Chem.*, 1998, **63**, 386–390.

55. P. A. Keifer, S. H. Smallcombe, E. H. Williams *et al*., *J. Comb. Chem*., 2000, **2**(2), 151–171.
56. J. L. Wolfender, K. Ndjoko and K. Hostettmann, *Curr. Org. Chem*., 1998, **2**(6), 575.
57. G. J. Sharman and I. C. Jones, *Magn. Reson. Chem*., 2003, **41**(6), 448–454.
58. L. Griffiths, *Anal. Chem*., 1995, **67**(22), 4091–4095.
59. M. Godejohann, L. Tseng, U. Braumann, J. Fuchser and M. Spraul, *J. Chromatogr. A*, 2004, **1058**(1–2), 191–196.
60. A. J. Simpson, L. Tseng, M. J. Simpson *et al*., *Analyst*, 2004, **129**(12), 1216–1222.
61. M. Sandvoss, B. Bardsley, T. L. Beck *et al*., *Magn. Reson. Chem*., 2005, **43**(9), 762–770.
62. N. T. Nyberg, H. Baumann and L. Kenne, *Magn. Reson. Chem*., 2001, **39**(5), 236–240.
63. M. V. S. Elipe, *Anal. Chim. Acta*, 2003, **497**, 1–25.
64. L. Griffiths, *Magn. Reson. Chem*., 1997, **35**(4), 257–261.
65. K. Albert, *J. Chromatogr. A*, 1995, **703**(1+2), 123–147.
66. S. H. Smallcombe, S. L. Patt and P. A. Keifer, *J. Magn. Reson. Ser. A*, 1995, **117**(2), 295–303.
67. C. Dalvit, G. Shapiro, J. Böhlen and T. Parella, *Magn. Reson. Chem*., 1999, **37**(1), 7–14.
68. B. J. Stockman, *Curr. Opin. Drug Discov. Devel*., 2000, **3**(3), 269–274.
69. S. Strohschein, M. Pursch, D. Lubda and K. Albert, *Anal. Chem*., 1998, **70**(1), 13–18.
70. P. Styles, N. F. Soffe, C. A. Scott, D. A. Cragg, D. J. White and P. C. J. White, J. *Magn. Reson*., 1984, **60**(3), 397–404.
71. M. Godejohann, A. Preiss, C. Mügge and G. Wünsch, *Anal. Chem*., 1997, **69**(18), 3832–3837.
72. R. J. Smith and A. J. Edwards, in *Pharmaceutical Analysis*, ed. D. C. Lee and M. L. Webb, Blackwell Publishing, Oxford, 2003.
73. N. Wu, W. Feng, E. Lin *et al*., *J. Pharm. Biomed. Anal*., 2002, **30**(4), 1143–1155.
74. S. X. Peng, *Biomed. Chromatogr*., 2000, **14**(6), 430–441.
75. E. A. Crowe, J. K. Roberts and R. J. Smith, *Pharm. Sci*., 1995, **1**(2), 103–105.
76. J. C. Lindon, J. K. Nicholson and I. D. Wilson, *Prog. Nucl. Magn. Reson. Spec*., 1996, **29**(1–2), 1–49.
77. M. Spraul, A. S. Freund, R. E. Nast, R. S. Withers, W. E. Maas and O. Corcoran, *Anal. Chem*., 2003, **75**(6), 1546–1551.
78. V. Exarchou, M. Godejohann, T. A. van Beek, I. P. Erothanassis and J. Vervoort, *Anal. Chem*., 2003, **75**(22), 6288–6294.
79. A. G. Webb, *Magn. Reson. Chem*., 2005, **43**(9), 688–696.
80. Z. Serber, C. Richter, D. Moskau *et al*., *J. Am. Chem. Soc*., 2000, **122**(14), 3554–3555.
81. O. Corcoran and M. Spraul, *Drug Discov. Today*, 2003, **8**(14), 624–631.
82. H. Kovacs, D. Moskau and M. Spraul, *Prog. Nucl. Magn. Reson. Spec*., 2005, **46**(2–3), 131–155.
83. L. Griffiths and R. Horton, *Magn. Reson. Chem*., 1998, **36**(2), 104–109.
84. F. Xu and A. J. Alexander, *Magn. Reson. Chem*., 2005, **43**, 776–782.
85. R. Subramanian, W. P. Kelley, P. D. Floyd, Z. J. Tan, A. G. Webb and J. V. Sweedler, *Anal. Chem.,* 1999, **71**(23), 5335–5339.
86. N. Wu, A. Webb, T. L. Peck and J. V. Sweedler, *Anal. Chem*., 1995, **67**(18), 3101–3107.
87. M. E. Lacey, J. Tan, A. G. Webb and *J. V. Sweedler, J. Chromatogr. A*, 2001, **922**(1–2), 139–149.
88. R. J. Lewis, M. A. Bernstein, S. J. Duncan and C. J. Sleigh, *Magn. Reson. Chem*., 2005, **43**(9), 783–789.
89. M. Sandvoss, A. D. Roberts, I. M. Ismail and S. E. North, *J. Chromatogr. A*, 2004, **1028**(2), 259–266.
90. G. Schlotterbeck, A. Ross, R. Hochstrasser *et al*., *Anal. Chem*., 2002, **74**(17), 4464–4471.

Analysis of Drug Impurities
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7 Preparative isolation of impurities

Gerald Terfloth

7.1 Introduction

In the pharmaceutical development environment, the rapid preparative isolation of impurities is required for many reasons – ranging from use as chromatographic markers, for the purposes of structure elucidation and determination of relative response factors, to the use as reference standards, potentially for the lifetime of the product on the market. This chapter covers a range of chromatographic techniques on a scale from micrograms to multigram amounts, including ultraviolet (UV)- and mass-directed fraction collection, aspects of automation, efficient isolation of impurities, scale-up of chromatography processes, and determination of the most efficient conditions using high-performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC). Figure 7.1 depicts a graphical map to the chapter.

The approach taken is loosely based on the input–process–output meta-model utilized to transform a problem statement into a functional process. The section Scope definition discusses the intended purpose and potential constraints of the isolation effort, followed by an overview of the Toolbox available to the practitioner (input). The section Method development: scouting and scale-up reviews platform-based, highly automated approaches to selectivity scouting, development of the isolation as well as options for scaling up the chromatographic separation depending on purpose and constraints (process). The final section, Performing the task, explores a work breakdown structure approach to the preparative isolation of impurities as a unit operation in the development process (output).

7.2 Scope definition

The preparative isolation of impurities may become necessary when an unambiguous identification cannot be achieved using hyphenated chromatographic–spectroscopic techniques, as discussed in Chapter 6 and in an excellent review [1]. The purpose of the isolation is to provide material

1. in sufficient quantity (submilligrams to several milligram amounts), and
2. of acceptable quality to elucidate the structure by mass spectrometry, nuclear magnetic resonance and other spectroscopic techniques, as required (refer to Chapter 5), or
3. for use as reference material (grams to tens of grams).

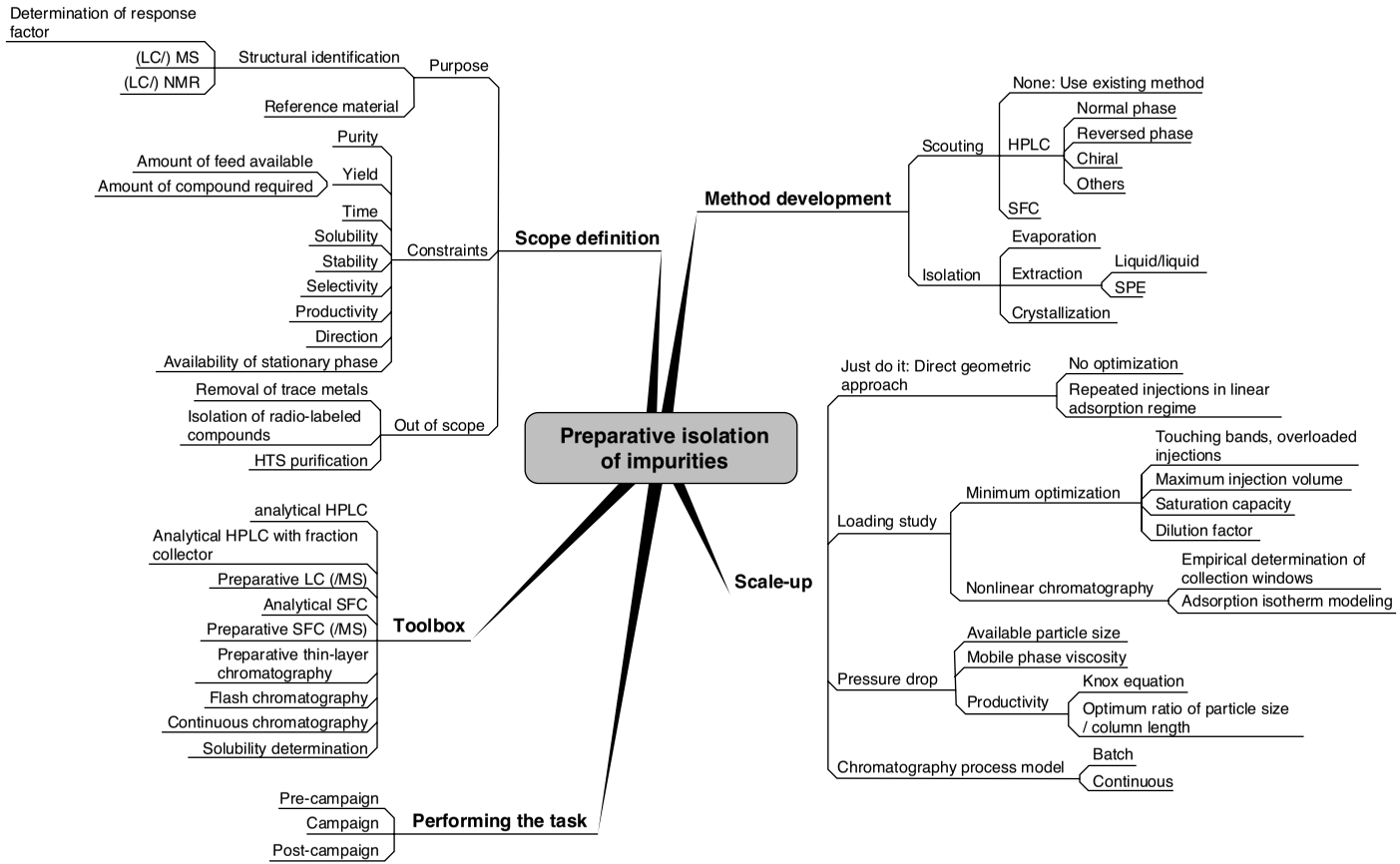

Figure 7.1 Map of 'preparative isolation of impurities.'

The high-throughput purification in a discovery environment and the removal of transition metals using adsorption on or crystallization in the presence of activated carbon, glass-bead sponges, polymeric fibers, or silica-bound scavengers and the preparative isolation of radiolabeled compounds are out of the scope of this contribution.

The chemist attempting to isolate an impurity is faced with a complex multivariable optimization problem and a number of constraints, among which are (in a subjective order of decreasing impact)

- solubility of feed
- selectivity: critical pair separation
- stability of drug and impurities
- amount of material available
- time available to finish task
- purity and yield requirements
- detection
- limited information.

The following statement may be self-evident, but it is worthwhile to point out that to increase the probability of success addressing most of these constraints in an information-gathering exercise before beginning any laboratory work is highly advisable. At the minimum, the synthetic route, solubility and stability in solvents commonly used in chromatography, UV spectra of the drug and known impurities, and information regarding available analytical methods (thin layer chromatography [TLC], HPLC, others) should be evaluated. However, situations may arise where the only way forward is to 'just do it' and to repeatedly inject, separate and collect the peak of interest using the existing analytical method.

Reference materials can accelerate the preparative chromatography development process, for example, in establishing starting material (feed) purity, selecting an appropriate feed, ensuring the desired compound is isolated, and mass balance. However, they may be the direct scope of the effort at hand. Specifically, the yield and purity requirements should be agreed upon at the onset of the isolation, as highly motivated chemists tend to exhibit strong ownership of their compounds. Establishing mass balance, within reason ('what goes in must come out'), for the desired component is prudent, as discrepancies can point to potential issues with the process, for example, buildup on the column or lack of stability. This can be achieved by assessing the content in the feed, the fractions collected and the final amount isolated using first intent assay methodology or a dedicated method.

Solubility of the feed may also vary widely, as the purity of compounds typically increases while progressing through development. This can result in a challenge for the preparative chromatographer because the solubility of an early laboratory batch used in evaluation activities may be significantly higher than that coming from a later synthesis, requiring the redevelopment of the chromatography process. Potential sources – other than mother liquors – for feed

materials are crude intermediates or products, reaction products from experimental designs exploring boundary conditions, samples from bulk drug stress testing, or enriched fractions from a chromatographic purification in the preparation of the compound.

The motivation for choosing the preparative isolation vs chemical synthesis frequently stems from the need for reference materials or impurities that are not easily available through synthesis owing to length, labor intensity, or uncertainty of the outcome, or that they are only available in small amounts with unknown quality. In an early development environment, chromatography often is the fastest and least labor-intensive approach to isolating impurities and to obtaining reference materials. The probability of success using chromatography can be evaluated on the basis of a very limited number of experiments and the help of fundamental equations from chromatography theory.

Equations 1–4 allow the practitioner to quickly get their bearings from one analytical and one overloaded injection on an analytical HPLC and to estimate if the equipment and column(s) at hand will suffice to 'get the job done', while equation 5 relates reduced plate height and reduced velocity, providing an assessment of intrinsic column performance and allowing for a judgment of the maximum operating flow rate possible for a given column efficiency constraint. Equation 6 provides help with answering the question whether or not a separation obtained on a short analytical column packed with small particles can be achieved on a longer preparative column with larger particles of the same stationary phase. The outcome of such an evaluation will help in defining the scope of the effort.

$$V_{\text{inj, max}} = \frac{k_2' - k_1'}{3} V_0$$

Equation 1: Estimation of maximum injection volume, $V_{\text{inj,max}}$ (ml), with capacity factors of the components, k_i', and void volume, V_0 (ml).

$$w_s = \frac{\sqrt{2Nk'}\, k'_0 \text{load}}{(1 + k')(k'_0 - k')}$$

Equation 2: Estimation of saturation capacity, w_s (mg), with plate number of analytical injection, N; capacity factor of overloaded injection, k'; capacity factor of analytical injection, k'_0; and load amount on column (mg).

$$\text{DF} = \frac{V_{\text{inj}}}{V_R} \sqrt{\frac{2\pi}{N}}$$

Equation 3: Dilution factor, DF, with injection volume, V_{inj} (ml); retention volume, V_R [ml]; and plate number, N.

$$\Delta p = \frac{uL\eta\, k_p}{dp^2}$$

Equation 4: Estimation of pressure drop, Δp (10^{-6} bar), with linear velocity (flow/cross section), u (cm/s); column length, L (cm); viscosity, η (cP); characteristic constant (600 for spherical particle), k_p; and particle size, dp (cm).

$$h = \frac{B}{v} + Av^{1/3} + Cv$$

Equation 5: Knox equation, with reduced plate height, h; reduced velocity ($u\ dp/D_m$), ν; coefficient B, describing axial diffusion (typical value 2); coefficient A, describing bed homogeneity (typical value 1–2); and coefficient C, describing mass transfer (typical value: 0.05).

$$\left(\frac{dp^2}{L}\right)\text{opt} = \frac{1}{4}\ \frac{\alpha - 1}{\alpha}\ \frac{k'_{02}}{1 + k'_{02}}\sqrt{\frac{D_m\eta}{3}\ k_0 C\Delta p}$$

Equation 6: Calculation of optimum ratio of particle size and column length, with selectivity factor, α; capacity factor of second component of critical pair under analytical chromatography conditions, k'_{02}; diffusion coefficient, D_m (cm^2/s) (typical value for MW 1000: 10^{-5} cm^2/s); viscosity, η (cP); specific permeability (1.2×10^{-3} for spherical particles), k_0; third term of the Knox equation, C; and maximum safe operating pressure, Δp, (bar).

7.3 Toolbox

This section provides a cursory overview of the equipment and systems commonly used for the isolation of impurities by chromatographic techniques (see Table 7.1).

Table 7.1 Representative preparative chromatography equipment

Equipment, column inner diameter (cm)	Flow rate (l/h)	Amount stationary phase (kg)	Throughput, feed (g/day)
SFC, 2	0.6	0.05	10–50
HPLC, 5	<10	0.3	1–100
HPLC, 10	20–30	1	10–1000
SMB, 5	5–10	1	10–1000
HPLC, 20	100–300	5	500–5000

The workhorses undoubtedly are the preparative HPLC and SFC systems. The equipment should be chosen and set up to reduce the number of variables for scale-up, ideally limited to a purely geometrical scaling factor, with focus on chromatographic efficiency by carefully minimizing variance caused by extra-column volume in the critical flow path (gradient formation, sample introduction, tubing length and diameter, flow-cell volume, and flow-splitter design). This includes standardization of control software and implementation of equipment-operating procedures.

Most modern HPLC systems can be modified to solvent and column-switching capability for scouting purposes or to implement an existing method without the need to reconfigure the system mechanically, resulting in significant efficiency gains. Adding an integrated fraction collector can further help with capitalizing from rapidly being able to carry out separations with 'first intent' methodology. Throughput and quality are obtained by repeated and automated injection, elution and collection on the analytical column, often while working in the analytical (linear, nonoverloaded) regime.

7.3.1 Preparative HPLC(/MS)

Figure 7.2 highlights the highly interactive nature of the isolation development process. The ability to trigger the fraction collection on the basis of the mass selective detector (MSD) signal significantly reduces ambiguity and effort when transferring the method from an analytical liquid chromatography mass spectrometry (LC-MS) instrument. The fraction collection for the preparative system can be based on time, UV response, mass, or some other predefined detector signal, for example, laser light scattering detection. Boolean logic determination of fraction collection allows for detection using signals from two different detectors. The preparative pumps are capable of delivering up to 100 ml/min, and Table 7.2 provides a summary of typical operating parameters.

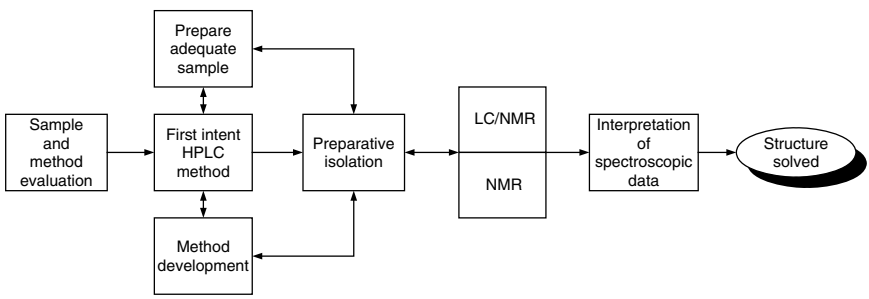

Figure 7.2 Unknown impurity isolation and identification workflow.

The system is used for the isolation of multimilligrams to grams of impurities, intermediates, and reference materials. A high degree of automation enables operation with minimum operator intervention and recoveries of >80% with

Table 7.2 Typical prepLC/MS operating parameters, selectivity >1.5

Column inner diameter (mm)	Flow rate (ml/min)	Loading (mg)
2.1	0.2	0.4
3	0.4	0.8
4.6	1–3	1–30
10	5–14	10–200
21	20–60	100–1000

minimum or no optimization effort, making this the platform of choice for most small-scale isolations. The MSD can be operated in both negative and positive ionization modes utilizing electrospray ionization (ESI), atmospheric pressure chemical ionisation (APCI) and atmospheric pressure photo ionization (APPI) capabilities.

7.3.2 SFC

SFC has demonstrated its advantages, low viscosity and high diffusivity resulting in reduced analysis time and improved efficiency, as described previously [2]. In addition to speed and reduced cost, the use of carbon dioxide as a mobile-phase component greatly simplifies the isolation of the desired compound from the fraction by minimizing or eliminating the need to evaporate solvent. SFC is orthogonal to reversed-phase high-performance liquid chromatography (RP-HPLC) and has been successfully applied to chiral and achiral preparative separations in a high-throughput environment [3, 4]. These features make it a complementary tool and technology of choice when faced with chiral separation problems.

7.3.3 Preparative TLC, flash chromatography

While not considered to be high-technology approach, preparative TLC may provide advantages when faced with the challenge to rapidly develop an orthogonal isolation methodology. The isolation of a hydrochlorothiazide–formaldehyde adduct impurity in drug substance using reversed-phase preparative HPLC followed by preparative TLC and characterization using LC-MS has been described [5], as was the combination of overloaded flash chromatography and preparative HPLC to isolate low-level drug substance impurities for structure elucidation [6]. Challenges one may encounter are the removal of silica from the final sample and limited yield due to lack of efficiency (number of theoretical plates available) of the separation as well as skill required to perform the task. Practitioners with a background in natural products isolation most likely will have significant proficiency in this area.

7.3.4 Continuous chromatography

The technologies described so far can be classified as batch processes – they achieve throughput by repeated injection, elution, and fraction collection. Continuous chromatography processes, such as simulated moving bed (SMB) chromatography and Varicol ([7] and references provided therein for a detailed description), are characterized by a seemingly continuous feed injection and product stream collection. SMB systems are binary separators and therefore ideally positioned to separate racemic mixtures. However, processes can be developed to separate one compound of interest from a complex mixture. Commercial and home-built benchtop systems using 1-cm inner diameter columns have successfully been used for the isolation of small gram amounts. However, given the process development effort and typical time constraint for isolating impurities for structure elucidation, traditional preparative batch chromatography often is the more effective tool, with continuous chromatography having an impressive proven track record in the lab and large-scale production environment. Novel approaches to feedback control of the process, specifically in the start-up phase, may minimize the development required to obtain a stable process in the years to come.

7.3.5 Solubility determination

The thermodynamic solubility determination using shaking or sonication followed by filtration and HPLC analysis is commonly used in the crystallization process development. High-throughput approaches based on 96-well plates and parallel microfluidic HPLC [8] or UV plate readers [9] have been described, while in-silico approaches to predicting aqueous solubility are difficult. IUPAC has recently completed a project to codify and publish reliable methods for solubility determination resulting in a comprehensive monograph with practical guidelines [10].

7.3.6 Stability determination

The stability of the drug and its solution in HPLC sample diluents is assessed during the method development and validation of stability-indicating methodology. Should this information not be available or turn out not to be applicable – owing to the solvent system used for the preparative separation or when trying to isolate a degradant of unknown structure and properties, it is advisable to determine solution stability at different concentrations and ambient and elevated temperature, since evaporation is most likely one of the isolation operations. Aging an autosampler vial containing the feed solution in an oven or more conveniently in a heated autosampler tray allows one to rapidly and, with minimum intervention, assess any area of concern.

7.4 Method development: scouting and scale-up

7.4.1 HPLC

Detailed aspects of analytical and preparative chromatography are discussed in two fundamental monographs [11, 12]. The identification of a chromatographic system providing adequate selectivity (α), that is, sufficient separation of the compound of interest from the closest eluting peak (critical pair), often is the most challenging task given the solubility – often lack thereof – of the feed, as discussed in Section 7.4.3.

It is worthwhile to restate that the target of the method development is to maximize the separation of the critical pair, not the separation of all components from each other, as would be the case for a stability-indicating impurity profile method. The capacity factor (k') should be in the range of 1 to 5 to avoid the potential for coelution with early eluting compounds or impractical run times.

Scouting experiments can be automated and are carried out using analytical chromatography systems with solvent and column switching. This approach may appear to be 'brute force' and not very scientific. However, depending on the outcome of the initial information-gathering exercise, the scouting can be taken on in a biased fashion, eliminating a significant number of experiments based on constraints such as solubility, stability, and pK value of the compound, to name a few.

As RP-HPLC is the method of choice for purity analysis, it provides a natural starting point for the selectivity scouting. The approach described here for RP-HPLC is directly applicable to NP, chiral, and other chromatography modes. Figure 7.3 provides examples for stationary and mobile phases providing a starting point for the screening exercise.

Coupling a screening system with an analytical fraction collector can be helpful when very small amounts (submilligrams to single-digit milligrams) are required. It often also provides the first opportunity to isolate enriched samples for further use in the method development, for example, as retention time markers. The choice of stationary phases in the method development system can be based on

1. the availability of the packing material in bulk or in larger format columns;
2. a chemometric-based evaluation of the selectivity space using a defined or historic sample set.

The scouting exercise is comprised of a screening of the stationary phases with aqueous gradients and organic modifiers such as acetonitrile, methanol, tetrahydrofuran, and others. For the reasons discussed in the Isolation section, the use of additives and buffers is avoided or minimized. Having identified a suitable chromatographic system (e.g. as in Figure 7.4), the practitioner may choose to further

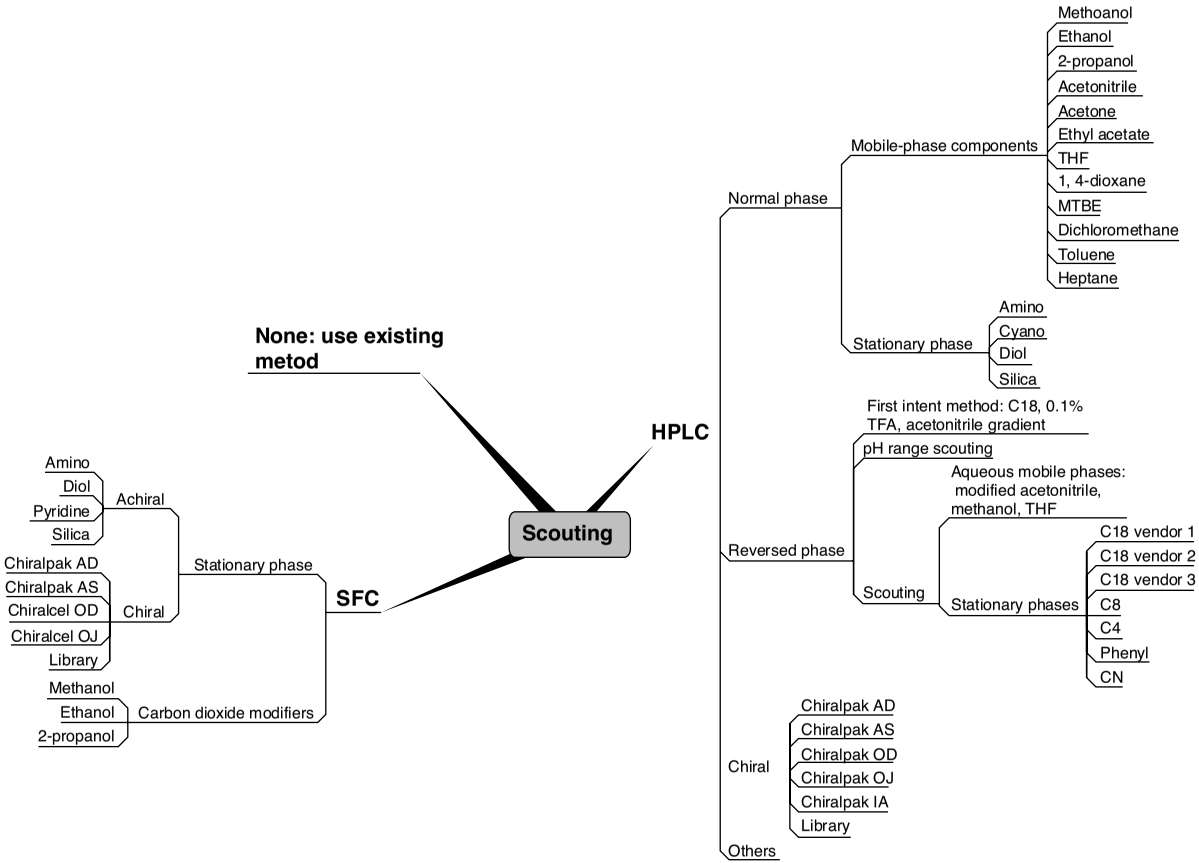

Figure 7.3 Method development scouting.

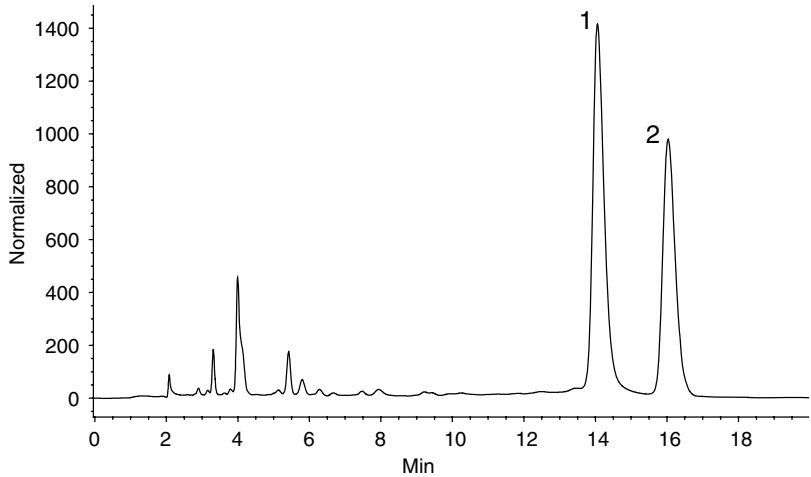

Figure 7.4 Analytical separation of a regioisomer mixture with a resolution of 1.78. The first peak is the desired compound. Conditions: feed concentration: 20 mg/ml; injection volume: 5 µl; Kromasil KR100-10-C18, 250 × 4.6 (i.d.) mm; flow rate: 1.0 ml/min; 80/20 (v/v) acetonitrile/water; UV: 254 nm.

elaborate the gradient using tools such as Drylab or Chromsword, or to develop an isocratic method with a step gradient as a wash, as required.

Stability of the stationary phase is an important factor to take into consideration before commencing, as loss of bonded phase, development of voids in the column, and column contamination are common causes for column failure. Monitoring column performance by evaluating capacity factors, selectivity, tailing factors, number of theoretical plates and back-pressure can identify potential issues. Causes can be operating the column outside of the recommended pH range or excessive pressure owing to malfunctioning equipment or precipitation of contaminants or the feed itself in the system, to name a few. While the use of guard columns is advocated – and is well justified in a high-throughput environment – diligence in solubility determination and feed dissolution including filtration affords a more cost-effective and robust solution in a development environment. This approach has enabled the author's team to using some packing materials and columns over the course of a decade.

7.4.2 SFC

The approach to method development is similar to the one described for HPLC and can be characterized as a rapid stationary phase screen using column and solvent switching with gradient elution followed by development of an isocratic preparative method. SFC has been successfully applied to the analytical and preparative separation of achiral and chiral compounds.

The chiral screen uses columns of 10 cm length, high flow rates, and fast gradients [3, 4, 13]. An initial assessment with methanol as the modifier and the four columns is run serially, followed by screens using ethanol and isopropanol. One racemate can be analyzed in 80 min, making it possible to screen several samples overnight, to isolate rapidly small amounts on the analytical system and to scale up to the preprative system on the next day. The authors report a success rate of >90%.

For achiral separations, the stationary phases screened are amino-, diol-, cyano-, and pyridine-modified silica, and native silica using methanol, isopropanol, and acetonitrile as modifiers. The screening is performed on analytical columns (250 mm × 4.6 mm), maintaining the outlet pressure at 120 bar, with the oven set to a temperature of 35°C and a flow rate of 2.5 ml/min. The modifier concentration is increased from 5% to 55% at a rate of 5% per minute, with the final concentration held for 1 min. Experience shows that if the resolution of the critical pair is >0.5 under these conditions, an effective isocratic preparative separation method can be developed by adjusting the concentration of the organic modifier while keeping all other parameters constant. Typically, this can be done by reducing in half the organic modifier concentration at the elution time of the critical pair observed during the scouting gradient while maintaining or increasing the selectivity in the resulting isocratic method. An isocratic method is often preferred, since it allows for an efficient stacked injection sequence.

Figure 7.5 depicts the preparative separation of a mixture of *cis*/*trans* isomers. A total of 680 injections were made to process 34 g of crude. The system operated unattended for 23 h to produce both isomers with 100% purity.

7.4.3 Scale-up

Depending on the scope and constraints of the isolation effort, the only option may be to 'Just do it', that is, to take the direct geometric scale-up approach by using a larger inner diameter column and carrying out the separation on a semipreparative HPLC system without any further optimization, making repeated injections in the linear adsorption regime and at the linear velocity used for the analytical separation. Analytical and semipreparative autosamplers are limited to a certain maximum injection volume, often requiring the operator to change a loop for adjustment. In addition, the autosampler tray size limits the volume that can be processed in an unattended fashion. The introduction of a feed pump (Figure 7.6) addresses this.

Having the ability to trigger fraction collection by combined UV and MS detection is an advantage when operating with very limited process robustness understanding. The risk associated with this approach is low to moderate, with solvent consumption, dilute fractions and time required to process as the major trade-offs. This scenario can be improved upon with minimum effort by establishing 'touching bands' (critical pair just separated at the baseline) in an overloaded mode (column load >2 mg feed per gram stationary phase). The maximum injection volume, saturation capacity, and dilution factor can be readily estimated

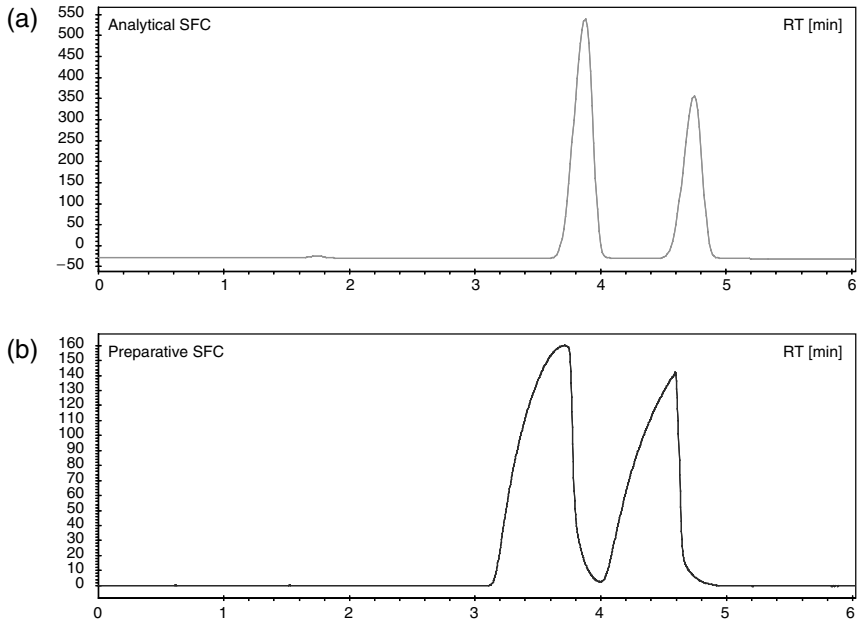

Figure 7.5 Separation of a *cis*/*trans* isomer mixture by SFC using 6% methanol, isocratic elution. (a) Analytical SFC separation. Conditions: column: 250 × 4.6 (i.d.) mm Berger NH_2; flow rate: 2.5 ml/min; oven temperature: 35°C; nozzle temperature: 40°C; outlet pressure: 120 bar; sample concentration: 5 mg/ml in methanol; injection volume: 5 μl; UV: 220 nm. (b) Preparative SFC separation. Conditions: column: 150 × 21.2 (i.d.) mm Berger NH_2; flow rate: 50 ml/min; oven temperature: 35°C; nozzle temperature: 60°C; outlet pressure: 100 bar; sample concentration: 50 mg/ml in methanol; injection volume: 1 ml; UV: 220 nm.

from fundamental characteristics of chromatograms obtained under analytical and overloaded conditions (refer to equations 1–3). Equation 3 points toward the attraction to effective columns – they afford more concentrated fractions. Hence, practitioners tend to utilize small-size particles but may be limited in their choice owing to the lack of availability of commercial columns or bulk stationary phase. Should the same stationary phase with a larger particle size be available for scale-up, the optimum ratio of $(\text{particle size})^2$/bed length can be obtained from equation 6 and applied to identifying a suitable column length for the particle size available.

Owing to the pressure ratings of analytical and semipreparative chromatography systems, most chromatographers do not consider the impact of particle size on pressure drop (equation 4). However, a quick estimation of pressure drop due to the column and comparison with the actual pressure (column plus extra-column contribution, e.g. tubing, detector cell) while maintaining a safety margin to the pressure rating of the system can help in avoiding unwanted system shutdowns. Special attention should be given to water–methanol mobile-phase

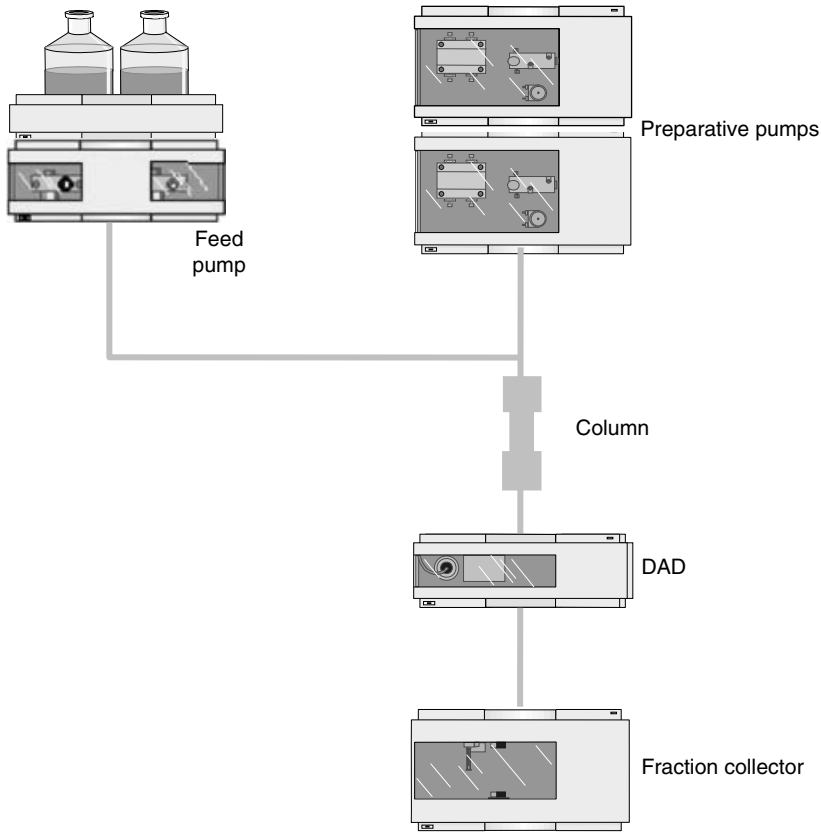

Figure 7.6 Semipreparative HPLC system using feed pump for sample introduction. Icons courtesy of Agilent Technologies Inc., Santa Clara, CA, USA.

systems, as they undergo a significant viscosity change (water: 0.89 cP, methanol: 0.55 cP, 1:1 [v/v] water : methanol: 1.62 cP) as a function of their composition.

As has been discussed before, concentration overloading is superior to volume overloading [12], although limited solubility of the feed in the mobile phase may limit the amount that can be introduced onto the column. This can be addressed by using a solvent in which the sample has higher solubility. However, crystallization leading to changes in permeability and blocking of the column can occur after introduction of the sample into the chromatographic system because of the mismatch in solvent strength of diluent and mobile phase. Deformation of the peak profile and peak splitting can be observed [14].

In situations where larger amounts, low concentration impurities or dilute feed need to be processed, a more detailed assessment and chromatography process development is advisable. Time frequently is the most stringent driver in addition to the constraints mentioned above. The purpose of the process development is to maximize throughput while maintaining acceptable robustness. Two limiting

case scenarios may be considered – obtaining throughput by repeatedly injecting, eluting and collecting on a small (<5 cm column diameter) chromatography system or by carrying out a single or few injections on a pilot or production scale system (>10 cm column diameter). The information provided earlier in Table 7.1 provides some general operating ranges. Capital cost for process chromatography equipment as well as the ability to mitigate risk typically favor obtaining throughput by repetition on a smaller-scale system.

When operating in the nonlinear chromatography mode, displacement of closely eluting components can be observed. Two cases can be discussed. In the preferred case, the impurity elutes before a major component and is displaced by the more concentrated component, resulting in concentrating the band of the impurity. The chromatographer can use this to dramatically improve throughput by overloading the column. However, in the reversed situation, the impurity elutes after a major component and the more concentrated component drags the impurity with it (tag-along effect), often resulting in the loss of what appeared to be a reasonable separation under analytical conditions. Recognizing elution order and elution order reversal in the scouting phase of the method development can pay dividend by choosing a chromatographic system that exhibits the desirable displacement of the compound of interest. Further increase of throughput can be achieved by stacking injections, recycling partially resolved components, and external or closed-loop steady state recycling [15]. The empirical determination of collection windows by evaluating the loading using fraction collection followed by analysis and reconstruction of the chromatogram is illustrated in Figure 7.7.

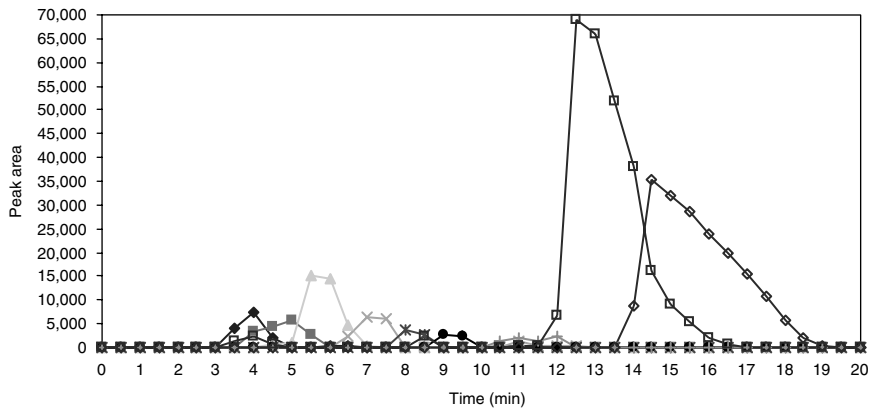

Figure 7.7 Reconstructed chromatogram from a preparative injection of 50 mg of authentic feed at a concentration of 50 mg/ml. Fractions were collected at 30-s intervals.

A simple spreadsheet tool can be used to capture the results of the fraction analyses, provide data visualization, integrate the responses, and thereby establish starting points for the fraction collection window(s), estimation of target purity and amount recovered. This data is useful for extrapolating the number of

injections required to carry out the separation, total processing time and solvent consumption, and helps with mitigating risk in the scale-up by providing a deconvoluted representation of the chromatogram.

So far, the discussion has focused on scaling up a single chromatographic purification step. However, the isolation problem may be addressed more effectively by enriching (repeatedly, if required) the feed material before a final chromatographic purification. As solubility, selectivity and cycle time are the key drivers for increasing throughput, the development of a coupled isolation process typically focuses on reducing cycle time by simplifying the chromatographic separation problem. A possible approach is to remove all components eluting before or after the target compound using orthogonal methodology or unit operations such as crystallization of extraction. The use of an orthogonal technique (e.g. NP vs RP) allows one to influence elution order and to utilize displacement in an advantageous fashion. Similar preparative chromatography examples are described in the literature [16–18]. The development of a detailed predictive process model for batch and/or continuous chromatography by fitting experimental kinetic and thermodynamic data to mathematical chromatography model, determining the order of and which parameters to estimate, identifying dependencies, and carrying out computer simulations is typically not required for a one-time impurity isolation. However, a limited set of experiments can provide a better understanding of the process and improve robustness, should a significant chromatography campaign be required to deliver the impurity. Factors to consider are flow rate/pressure drop dependence (explore at two different linear velocities), variability in the amount on column due to change in feed composition and concentration (explore maximum injection volume and saturation capacity), and temperature (consider conditioning feed solution, solvent and column, as environmental conditions may change as a function of time of the day and year, resulting in changes in chromatography or precipitation of the starting material).

7.4.4 Isolation

Developing an isolation approach is an activity that is frequently overlooked or addressed as an afterthought. However, solubility and stability data may dictate the development of a chromatographic method that requires the elaboration of the isolation, that is, it is more complicated than a simple evaporation of the mobile phase. The development of the chromatographic process should be linked to and interactively codeveloped with the isolation. Ideally, the isolated impurity sample should not contain other compounds or artifacts, such as solvents, mobile-phase additives or particulate matter from the preparative chromatography, as they may interfere with the structure elucidation effort or adversely affect the stability of the impurity during the isolation process. Therefore, it is preferable to avoid or minimize the use of mobile-phase additives. However, should this prove to be impossible, the additive used should be easy to remove. The judicious choice of mobile phase in the HPLC process increases the ability to recover the compound of interest without or with minimum degradation. The most common

processes used for isolation are filtration, evaporation, liquid/liquid extraction, solid-phase extraction, column desalting, (re)crystallization, precipitation, and freeze-drying. Frequently, a combination of these processes is required to isolate and recover the impurity from the mobile phase with a quality appropriate for the subsequent analyses and/or for use as reference material. Expert knowledge in the development of some of these processes may not reside with the chromatography practitioners, mandating a collaborative approach with synthetic chemists and engineers to devise the approach. Evaporation under mild conditions (ambient temperature and high vacuum) followed by filtration of the precipitate often affords material of acceptable quality. Water-soluble additives can also be removed in this way. However, should the impurity be water soluble, evaporation of the organic mobile-phase component followed by (repeated) freeze-drying is a useful approach, as this methodology also removes most commonly used volatile additives (ammonium carbonate, formate, acetate; organic bases such as triethyl and diethyl amine; formic, acetic, and trifluoroacetic acid). Solid-phase extraction can be carried out in a way that may appear unconventional to analytically trained chromatographers. The fraction containing the compound of interest can be introduced to a column packed with a highly retaining stationary phase and the breakthrough can be monitored by following the UV signal. As sample breakthrough is observed, feed introduction is stopped and the compound that is now concentrated in one column volume is eluted using a strong solvent. In an RP-phase operating scenario, this may mean further dilution of the collected fraction with water, followed by pumping it onto a C18 column and elution with a solvent such as acetonitrile. The log k' vs solvent composition data enabling such an approach can be obtained from the initial method development effort.

7.5 Performing the task

It is highly desirable to standardize the workflow when carrying out preparative isolations. The benefits are primarily in the areas of safety, quality, and efficiency. When faced with an isolation problem, a significant number of choices and decisions need to be made. Depending on the scope of the task, the best way to address the impurity isolation may be to ‘just do it’, that is, to implement the existing analytical method on a larger-size column, observe the separation campaign for a short period of time and to rely on automation to finish the job at hand. The maintenance and readiness of the equipment are the major challenges in this scenario. While laboratory systems are designed to operate unattended and for extended periods of time, one cannot expect them to perform like dedicated process equipment. They can be addressed by implementing a scheme that involves scheduled preventive maintenance and calibration as well as automated system performance verification by carrying out test separations as part of a daily, or as may be required more frequent, monitoring routine. The need to operate under overloaded conditions, be it touching band or in the nonlinear regime, carries a higher risk. However, utilizing a work breakdown structure in a blueprint

fashion allows one to rapidly evaluate, scale up, and carry out a separation, while providing a tool for continuous improvement. The impurity isolation by preparative chromatography can be broken down into three phases (see Figure 7.8): precampaign, campaign, and postcampaign.

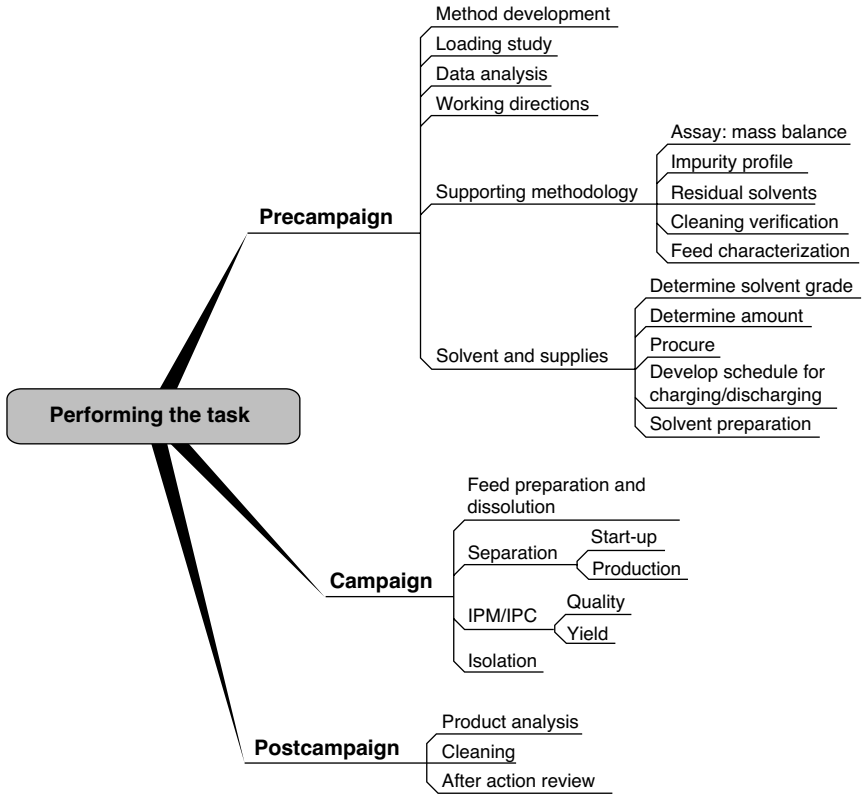

Figure 7.8 Performing the task.

In the precampaign phase, supporting analytical methodology may need to be developed and some effort may be required to address the logistics requirements of solvents and supplies. Following the approach described in this chapter, the method development and scale-up was carried out using stationary phases available in larger amounts. However, procurement can become the major driver of the time line. Depending on the scope of the isolation, the choice of solvent quality may be limited to 'HPLC grade'. However, the requirement to carry out the separation on a significant scale (greater than multikilogram), for example, as part of a multistage isolation strategy, may warrant the exploration of solvent grades more typically used in a pilot plant environment. The safe preparation of mixed mobile phases on a scale beyond the typical analytical solvent reservoirs may require a dedicated environment, which may reside with a different functional group within the organization, requiring planning and coordination.

The charging and discharging of feed, solvents, and waste vessels should be scheduled to ensure seamless processing during the campaign and to minimize safety hazards due to the volume of solvent in the laboratory environment. Capturing the task in working directions provides the benefit of ease of transfer of the process and the ability to revisit and scale up further, when required. The volume to be processed should be taken into consideration when choosing equipment. Having prepared in this way, the campaign can focus on the actual separation and isolation as a routine operation, with occasional checks (IPM/IPC) to ensure quality and yield. The postcampaign phase includes analysis of the product using chromatographic, spectroscopic, and other methodologies to fully characterize the material. An 'after action review' may prove useful to identify learnings and to further improve the operation. Cleaning of the equipment can be a scientific challenge and time-consuming activity. Preferably, a 'cleaning in place' approach is taken, using a known strong solvent (methanol, acetonitrile, acetone) to thoroughly and systematically rinse the chromatographic equipment. However, check-valves, column inlet frits, and feed tubing may be contaminated in a way that requires them to be changed. Cleaning methodology developed for pilot plant equipment can be adopted to help meet cleaning limits.

The approach described reflects an industrial view of the preparative isolation of impurities based on a meta-model developed with the aim of implementing a functional process while maximizing success under a given set of constraints and mitigating risk. This chapter is a snapshot of its current state of evolution.

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References

1. A. Aranyi, *Progress in Pharmaceutical and Biomedical Analysis*, **4** (Identification and Determination of Impurities in Drugs), 2000, 240–251.
2. G. Terfloth, *Journal of Chromatography A*, 2001, **906**(1–2), 301–307.
3. C. White, *Journal of Chromatography A*, 2005, **1074**, 163–173.
4. C. White and J. Burnett, *Journal of Chromatography A*, 2005, **1074**, 175–185.
5. J. D. Franolic, G. J. Lehr, T. L. Barry and G. Petzinger, *Journal of Pharmaceutical and Biomedical Analysis*, 2001, **26**(4), 651–663.
6. R. M. Ladd and A. Taylor, LC-GC, 1989, **7**(7), 584, 586–589.
7. O. Ludemann-Hombourger, G. Pigorini, R. M. Nicoud, D. S. Ross and G. Terfloth, *Journal of Chromatography A*, 2002, **947**(1), 59–68.
8. S. Hobbs, P. Patel, C. Coyne and K. Oldenburg, *Society for Biomolecular Screening, 10th Conference and Exhibition*, 2004, Orlando, FL., USA.

9. T. M. Chen, H. Shen and C. Zhu, *Combinatorial Chemistry & High Throughput Screening*, 2002, **5**(7), 575–581.
10. G. Hefter and R. P. T. Tomkins, *The Experimental Determination of Solubilities*, John Wiley and Sons, New York, 2003.
11. L. R. Snyder, J. J. Kirkland and J. L. Glajch, *Practical HPLC Method Development*, 2nd edn, John Wiley and Sons, New York, 1998.
12. G. Guiochon, S. Golshan-Shirazi and A. Katti, *Fundamentals of Preparative and Non-Linear Chromatography*, Academic Press, New York, 1994.
13. M. Biba, C. J. Welch, J. O. daSilva and D. J. Mathre, *American Pharmaceutical Review*, 2005, **8**(2), 68–72, 84.
14. K. M. Gedicke, D. A. Tomusiak and A. Seidel-Morgenstern, *Journal of Chromatography A*, 2005, **1092**, 142–148.
15. C. M. Grill and L. M. Miller, in *Preparative Enantioselective Chromatography*, ed. G. B. Cox, Blackwell Publishing, Oxford, 2005, pp. 149–175.
16. L. Miller and R. Bergeron, *Journal of Chromatography*, 1994, **658**(2), 489–496.
17. I. Cepanec, H. Mikuldas, M. Litvic and I. Vukusic, *Pharmazie*, 2001, **56**(11), 857–859.
18. R. N. Rao, S. N. Alvi and B. N. Rao, *Journal of Chromatography A*, 2005, **1076**(1–2), 189–192.

Analysis of Drug Impurities
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8 The impact of continuous processing

Frank Cottee

8.1 Context – a brief history of drug impurity analysis

The pharmaceutical industry has traditionally been one of the major drivers for innovation in organic chemistry analysis. There are several reasons for this need for careful characterisation of the products of the industry, which will be explored briefly in this section.

8.1.1 Structural characterisation

First and foremost, Active Pharmaceutical Ingredients (APIs) are relatively complex molecules by the standards of the rest of the chemical industry and the synthetic routes to produce them can themselves be complex. The synthetic organic chemist has therefore sought ways of characterising the products of each stage of his reaction sequence, as well as the final drug substance, in the most efficient manner [1]. This information is necessary to guide the design of an effective synthesis strategy as well as simply to confirm that a given reaction has delivered the desired target molecule.

The period following World War II saw the rapid development of the pharmaceutical industry. It also coincided with a huge growth in instrumental analysis techniques, many of them enabled by military technology developed during the war. The need for *qualitative* analysis was therefore met by an increasing range of molecular spectroscopy techniques of increasing complexity. Table 8.1 summarises the important characteristics of the most common of these techniques as currently available. Table 8.1 also comments briefly on the suitability of these techniques for online analysis, a topic followed up in more detail later in this chapter.

When spectroscopic instruments first became commercially available, they were generally sufficiently complex and capricious that they needed the expertise of a specialist spectroscopist to enable their best use. The interpretation of the resulting spectra was itself a specialist skill, and, furthermore, research grade instruments were often extremely costly. All of these factors combined to ensure that the standard model for pharmaceutical analysis was that of a specialist laboratory, often remote from the synthetic chemistry laboratory, almost certainly remote from the manufacturing plant and usually working to a timescale driven by the instrumentation and not by the needs of the chemist. We will see in later sections how this situation has gradually changed in response to the needs of the industry and the opportunities offered by new technology.

Table 8.1 Common molecular spectroscopy techniques

Technique	Property measured	Comments	Suitability for on-line analysis
Microwave	Molecular rotation	More often used for putting energy into a reaction than for measurement	Sensitive; not diagnostic; requires engineering of waveguides
IR	Bond vibrations (change in dipole moment)	In principle contains wealth of structural information, but difficult to extract in practice	Fairly sensitive and specific; broadly applicable; new fibre optics will increase suitability
Raman	Bond vibrations (change in molecular polarisability)	As IR, but complementary in application Fluorescence can be a problem	As IR, but truly non-invasive
NIR	Vibrational overtones and combination bands	Very high S/N Relatively low sensitivity facilitates minimum sample preparation	Most popular on-line technique, *but* not as sensitive or diagnostic as IR and Raman. Sensitivity to physical as well as chemical status can be a problem or an opportunity, depending on application
UV/vis	Electronic transitions	Commonest detector for HPLC. Not very structurally informative	Sensitive; lack of specificity usually requires deconvolution techniques
NMR	Nuclear (proton) rotation	Most structurally informative technique Costly and requires considerable infrastructure	Only low-field instruments suitable
MS	Mass-to-charge ratio	Highly sensitive as MW detector	Good for gas-phase monitoring

8.1.2 Separations

It is clearly not sufficient merely to know the structures of the components of a reaction mixture. In order to gauge the extent of reaction, for example, or to compare one reaction condition with another it is necessary to have a *quantitative* measure of the components of the reaction mixture. Although this information can sometimes be given to a first approximation by some spectroscopic techniques, the maximum *specificity* and *sensitivity* is usually delivered by some form of separation of the mixture before quantification of its components. It is also worth noting that structural analysis is considerably easier for a single isolated component than for an unseparated mixture.

Several technology leaps have taken place in separation sciences during the lifetime of the pharmaceutical industry. The development of chromatography at the end of the nineteenth century was the first of these revolutions and its transformation into thin-layer chromatography (TLC) provided the mainstay for quantitative analysis well into the second half of the twentieth century. With the development of gas chromatography (GC) after World War II and high-performance liquid chromatography (HPLC) two decades later, the age of fully instrumented separation science had arrived.

The need for specialist separations scientists and the relatively high cost of instrumentation had the same effect on quantitative analysis as it had done in spectroscopy. The analytical laboratory had, by economic necessity, to be separate from the functions for which it was providing the measurements. Even before the advent of online analysis it was recognised that this separation of analytical results from their point of use was inefficient and often had the effect of inhibiting scientific progress in the case of laboratory sample measurements or of producing financial penalties in the case of plant sample measurements. There have been two responses to this dilemma. The first has been to improve the efficiency of laboratory measurements through the use of laboratory automation and by means of organisational tools such as Laboratory Information Management Systems (LIMS). The second, which we will return to later in the chapter, is to move the analysis closer to the point and the time at which the results are required.

8.1.3 The uses of drug impurity data

We have discussed one driver for drug impurity analysis, namely the need for the synthetic organic chemist to monitor his reactions. We will return to this theme later in the chapter. However, there are two other drivers for drug impurity analysis that are relevant to any discussion of change.

The most important of these is the regulatory environment in which the industry works. In a series of judgements resulting from isolated cases of poor laboratory practice the regulatory authorities of many countries, particularly the United States, have defined a strict code of practice for the industry to ensure that the highest standards of development and manufacture are observed by all

pharmaceutical companies. These codes of practice are at once advisory in their detail but mandatory in their principles. Until relatively recently the detail of the regulations, for example for permitted impurity levels, have varied between national agencies. However, thanks to the work of the International Committee on Harmonisation (ICH) there is now a degree of unanimity on such details [2, 3].

The net result of such regulation is that there are well-developed standards for the way in which quantitative and, increasingly, qualitative measurements are carried out. There are also well-defined limits for various categories of impurity, all of which tends to emphasise the importance of measurements on the final API rather than on the process that produces it, for which the requirement is simply that the process remains 'under control'. The importance of this summary is that at present, whatever the means of production, there is a requirement that the API will reach certain standards of purity as demonstrated by *end-point measurement*. Those standards effectively demand quantification of impurities down to about 0.05% for related organic impurities, in the region of ppb to low ppm for known toxins and low ppm levels for inorganics such as catalytic metals.

As we will discuss later, almost the last reason to carry out detailed impurity analysis is to give a detailed understanding of the chemistry selected for API production, or of the production process resulting from it. The one exception to this sweeping generalisation of past practice is in the field of intellectual property protection. One very effective strategy for detecting patent infringement is to have an intimate knowledge of the synthetic route and process in order to be able to demonstrate that certain impurities are 'route- or process-indicating', an additional driver for drug impurity analysis.

8.2 Continuous processing

Unlike most of the rest of the chemical industry, continuous processing is a relatively new concept in API manufacture. It is therefore necessary to understand where the industry has come from in terms of manufacturing methods before considering the factors necessary for change.

8.2.1 Batch processing

The manufacturing model for API since the beginnings of the industry has been almost entirely based on batch processing. There are many good reasons for this. The production volumes for most drugs are relatively small; tens of tonnes per annum rather than thousands of tonnes. In the past, this has inhibited investment in dedicated facilities and has led to manufacturing campaigns in flexible batch equipment that can be switched between products relatively easily. For a similar reason, the control systems associated with such batch plant tend to be generic rather than specific to any particular molecule. A second reason for using generic plant is that by chemical industry standards the number of new products coming

through the development pipeline is high and the lifetime of any particular drug is relatively short, again predicating against the design of dedicated manufacturing plant. It is also the case that batch processing equipment bears a strong similarity to the bench-scale apparatus used by development chemists in designing a manufacturing process. The unit processes such as mixing, isolation and drying are similar to their bench equivalents and this allows the chemist to scale his process through pilot facilities and into manufacturing scale plant with relative ease.

All of this comes at a price – it has been said that development chemists in the past have not scaled up their processes; they have simply run them in larger and larger equipment. In other words, issues of scale-up such as mixing, shear and heat transfer have been dealt with in a pragmatic way at each stage of scale-up rather than being designed into the process. In a competitive industry driven by speed to market, it was inevitable that process design would take second place to throughput of new products. Equally inevitably, this has led to future manufacturing problems essentially being designed into the processes being transferred from development organisations, leading to later batch failures, re-works and, worst of all, product recalls.

Until recently there has been a great resistance to change from this batch processing model, driven by several factors:

1. The wastage and inefficiency of the batch process development model was acceptable at a time when the value of the product produced was extremely high – it was a small price to pay for getting the product to the market fast.
2. There is significant investment in batch equipment and the associated operational expertise and therefore a high hurdle to changing this investment pattern to alternative manufacturing technology.
3. Batch processing is familiar to both the industry and its regulators and therefore any change was seen as carrying a high regulatory risk.

As a result, the manufacturing equipment and methods used by the industry would have been familiar to the founding fathers of the industry – it has been said that we are still using the same ‘pots and pans’ that were used in the nineteenth century.

In the past five to ten years the pharmaceutical industry, has seen a huge change in its fortunes, which have caused it to re-think its whole discovery and development processes. Figure 8.1 illustrates some of these pressures.

In discovery organisations across the industry the response has been to automate, develop high-throughput technologies and to refine disease targets and screens [4]. The response from development organisations has been somewhat slower and has been driven partly by pressures from the pipeline of new drug candidates from discovery organisations and partly by a seismic shift in thinking by the regulatory authorities, particularly the American Food and Drug Administration (FDA). In the next sections, we will consider the moves to continuous processing [5] and how this has been partly facilitated by the FDA’s process analytical technology (PAT) initiative.

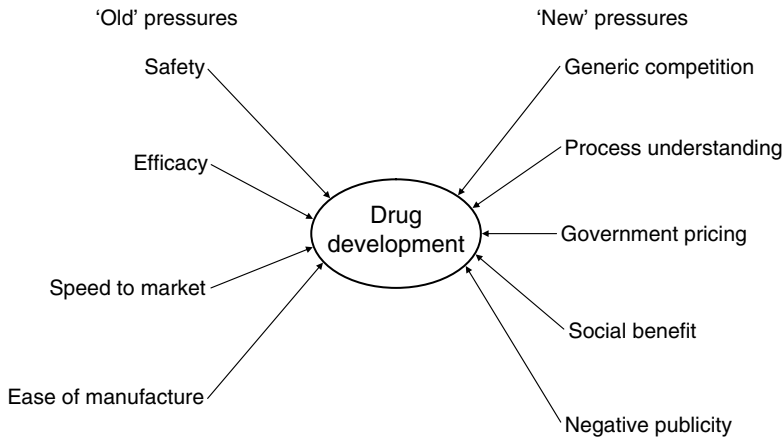

Figure 8.1 The pressures on the pharmaceutical industry.

8.2.2 Continuous processing

It has long been recognised that *process intensification*, of which continuous processing is an example, can lead to significant benefits compared with batch processing [6]. Process intensification aims to produce highly efficient reaction and processing systems through the use of system configurations that significantly reduce reactor sizes and maximise catalytic and mass and heat-transfer efficiencies. The Fine Chemicals industry, which is a good model for pharmaceuticals and indeed a supplier of intermediates, is already a limited user of continuous processing technology. Continuous manufacturing has been demonstrated to provide the following benefits:

1. *Reduced inventory* – as materials are used at a steady rate and API is produced continuously. Supply logistics can therefore be designed around regular time intervals rather than having to store material for a complete manufacturing campaign.
2. *Reduced size of manufacturing equipment*. Supply volume is achieved by producing gram quantities per hour rather than several hundred kilograms in one manufacturing campaign.
3. *Reduced cost of manufacturing equipment*. Cost reductions of 30% compared with the equivalent batch plant have been claimed [7].
4. *Flexibility in location of equipment*. Because of its smaller size, reduced inventory and improved safety the siting of continuous manufacturing equipment requires less infrastructure than the equivalent batch plant. Portacabin-sized equipment is already available, which even makes temporary location of plant a feasibility.
5. *Energy efficiency*. Temperature gradients are steady state and take place in space rather than in time, reducing the need for the expensive heating and cooling cycles common in batch equipment.

6. *Safety*. As well as the reduced inventory of chemicals and solvents, highly exothermic reactions can be safely handled because of the excellent heat flow properties of a flow reactor and the fact that smaller quantities of reactants are present at any time. Similarly, toxic reagents are present in smaller quantities and require minimum handling.
7. *Flexibility*. Although less relevant to pharmaceutical manufacturing at present, continuous processing plant is capable of online changes of product specification. The plastics industry is a good example of the use of this facility [8].

With these advantages, it is therefore something of a surprise that across manufacturing industry as a whole the ratio of batch to continuous processes is about 85 : 15 [9] and in the pharmaceutical industry the bias is even greater towards batch processing. This is why there is currently both industry and government focus on the benefits of process intensification if Western manufacturing industry is to have any chance of competing with the lower cost economies of the developing world.

The scope for continuous reactors does have some limitations and it is unlikely that they will completely replace batch reactors in the foreseeable future. The most important current limitations are as follows:

1. Continuous reactors have traditionally been used for reactions with fast kinetics [10]. Solutions to this limitation are being developed, such as recycling or pulsed flow reactors [11]. However, an alternative solution to this limitation is simply to avoid it by re-thinking the chemical route to the target molecule.
2. Capacity is an inverse problem in that a typical 15-mm diameter tube reactor of 5–6 m length will process in the order of 20 l/h. This therefore sets a lower product volume limit to the usefulness of continuous reactors.
3. Heterogeneous reactions are more difficult to handle in flow reactors, particularly as size is reduced.
4. Some regulatory definitions need to be re-thought, for example, the definition of a 'batch' for release and product genealogy purposes. However, the FDA's current definition of a batch as 'a specific quantity of a drug or other material that is intended to have uniform character and quality, within specified limits, and is produced according to a single manufacturing order during the same cycle of manufacture' [12] is almost certainly adaptable to continuous processing.

A careful analysis of the current portfolio of one major pharmaceutical company indicates that about 60% of the chemistry is suitable for continuous processing. About 50% of this chemistry is homogeneous and therefore readily transferable to existing continuous processing technology. The remaining 50% is heterogeneous and will therefore require implementation of some of the current advances in continuous flow equipment such as oscillatory flow reactors [13]. Technically, the transfer of these processes from batch to continuous could happen within

three years. Even with the infrastructure difficulties noted above, a 10-year timescale should be achievable.

Two important concepts are key to process intensification. First, the chemistry itself must be telescoped as far as possible, that is, isolation of intermediates reduced to a minimum. This is already a goal in batch processing but is more readily achieved in a continuous process because of the process control inherent in the system. Second, the system must be designed for 'right first time' processing, allowing redundancy in the plant to be removed [7]. It is obvious from these two requirements that design of the chemistry and monitoring of the process are two areas where analytical monitoring is a critical requirement. This is a theme that will be returned to later in the chapter. A corollary to the second requirement is that human intervention should be reduced to a minimum by use of automation and closed-loop control, unlike the situation with batch processing. All manual valves, switches and manual overrides are opportunities for error. An interesting observation from ref. [7] is that human beings are good at developing and exploiting new things, which means that they will always want to improve processes to which they have access – the very antithesis of consistency.

A consistent theme in the process intensification literature is that in order to reap the benefits of continuous processing a deeper understanding of the process is required than has been traditional in the pharmaceutical industry [14]. This understanding requires a thorough knowledge of the kinetics of the chemistry, rate constants, mass balance and the like. From this information a process model can be built, and armed with this understanding it is then possible to use process systems engineering design tools to enhance the effectiveness of the process [15]. Again, this requirement fits absolutely with the new regulatory environment embodied by PAT and demonstrates further the need for analytical monitoring at an early stage of chemical route and process design. Building a process model from first principles is always desirable but may not always be possible [16]. Such a model is often referred to as a 'hard' or 'white' model. The other extreme is to build a process model based solely on empirical data, usually gained from an online monitoring technique such as near-infrared (NIR) spectroscopy, using statistical techniques such as regression or neural networks. Such a model is referred to as a 'black box' model and may be a very good fit to the experimental data, but will be unhelpful in deriving chemically meaningful information. Reference [19] shows how 'grey' models combine the advantages of both white models and black box models and, furthermore, are excellent tools for process diagnosis.

The concept of process intensification does not need to apply to the whole of an API production process. There is merit in looking at hybrid reaction schemes, which retain the benefits of, or capital investment in, batch equipment but use continuous processes for the generation of hazardous intermediates [17] or for certain unit processes. Of these, hydrogenation [18], filtration [19], phase separation [20], crystallisation [21] and drying [22] are good examples.

Strategies and factors for success for continuous processing have been widely reported [23]. It is important to select for success by avoiding any of the known risk areas, such as reactions with many side products, and to choose a high-value

product for which the impact will be clearly visible [24]. It is as important to acknowledge human factors in planning for change [25] as it is to plan for infrastructure changes [26].

8.2.3 Comparison with other industries

The adoption of continuous processing technologies as part of the overall pharmaceutical manufacturing strategy will be greatly aided by the long experience of other industries with this technology. For example, the petrochemical industry is virtually fully centred on continuous processing. It is highly efficient and the waste produced is typically less than 1% of total input. This is contrasted with the fine chemicals and pharmaceutical industries, which are largely reliant on batch processing and typically generate 25–100 kg of waste for every kilogram of product [27].

Continuous processing is widely used in manufacturing processes such as textiles [28, 29], ceramics [30], plastics [8] and biotechnology [31]. Of particular interest to the pharmaceutical industry are examples from food processing [32–35], which shares some of the same unit operations such as mixing and drying. A challenge that is shared with all these industries is that of competition from developing countries, which has resulted in a considerable reduction in the manufacturing base in industrialised countries. An unforeseen effect of the resulting downsizing is the loss of engineering expertise that enables process analysers to be coupled to reactors. This has led to a move to standardise such interfaces through a Center for Process Analytical Chemistry (CPAC) (see Section on Process analysis) sponsored initiative called the New Sampling and Sensor Initiative (NeSSI) [36]. NeSSI is intended to be an electronic as well as a physical standard for process interfacing and has been enthusiastically received by parts of industry. At the time of writing, it remains to be seen what impact it will have on interfacing to pharmaceutical continuous processes.

8.3 Process Analytical Technology

As previously noted, the close regulation of the pharmaceutical industry had led to a situation by the end of the last century where the industry was extremely risk averse. Nowhere was this more true than in its manufacturing strategy, which for API production was almost entirely based on batch manufacturing campaigns. Not surprisingly, a perception developed in the regulatory authorities, particularly the FDA, that pharmaceutical manufacturing lagged behind the rest of the chemical manufacturing industry in its adoption of innovative manufacturing technology. Furthermore, the sheer volume of regulatory applications compiled under the risk-averse paradigm was threatening to overwhelm the regulators. With industry unwilling to be the first to take a manufacturing risk and the regulators looking for change, something had to happen to break the deadlock. That something was an initiative forged between the FDA's Center for Drug

Evaluation and Research (CDER), Office of Regulatory Affairs (ORA) and the Center for Veterinary Medicine (CVM), which was titled the PAT initiative.

The initiative was, from the outset, a collaboration with industry, with the first public consultation taking place at an FDA Advisory Committee for Pharmaceutical Science meeting in 2001 [37]. The Draft Guidance for Industry was issued for discussion in 2003 [38] and was the trigger for an upsurge of activity across the industry in all the areas covered by this chapter, namely the online analysis of processes and the move to continuous processing driven by deeper understanding of those processes. The updated guidance has recently been issued [39]. The PAT guidance outlines a scientific, risk-based framework that is founded on process understanding, exactly the requirements for developing a continuous process and the analytical strategy that goes with it.

The Office of New Drug Chemistry (ONDC) is establishing a new risk-based pharmaceutical quality assessment system to replace its current review system for chemistry, manufacturing and controls (CMC) issues in submissions to FDA's CDER. FDA expects that this new system will help speed the drug approval process, reduce the need to submit manufacturing supplements (for changes to already approved drugs), prevent supply shortages or disruptions and encourage manufacturers to implement new technologies. Planned ONDC changes include

1. using small teams of scientists rather than a single chemistry reviewer throughout a product's lifecycle;
2. meeting with drug sponsors more frequently on CMC-only issues during product development;
3. encouraging new drug sponsors to submit more of the development information used to support process validation and to develop control strategies;
4. becoming more directly involved in pre-approval and CGMP inspections.

FDA assessors and inspectors will all receive technical training that has been designed in collaboration with industry and academia.

The FDA's own definition of PAT is a useful starting point for the discussion in later sections of this chapter:

> PAT is a system for designing, analysing and controlling manufacturing through timely measurement (i.e. during processing) of critical quality and performance attributes of raw and in-process materials and processes, with the goal of ensuring final product quality.

Note that 'product' in this context covers both API, which is the subject of this chapter, and the formulated drug product itself. It is likely that the major applications of the PAT approach will be in secondary manufacturing, and indeed successful examples have already been published [40–42]. The FDA is also careful to qualify that the word 'analytical' is meant in its broadest sense and covers chemical, physical, microbiological, mathematical and risk analysis conducted in an integrated manner. Although this chapter is primarily concerned with the first

of these, it is important to note the word 'integrated'. It is also worth noting that the timely measurements referred to may be different in the development, validation and manufacturing phases of the process. Indeed, it should be a goal that the measurements become simpler as the process develops. The FDA goes on to categorise the tools that it anticipates will achieve its goal of an integrated system. These are:

- multivariate data acquisition and analysis tools;
- modern process analysers or process analytical chemistry tools;
- process and end-point monitoring and control tools;
- continuous improvement and knowledge management tools.

The aim of the FDA, and of other regulatory authorities who have taken up this theme, is to move from an industry that relies heavily on validated and fixed processes where quality is ensured by end-point testing to one whose process are well understood and controlled. In other words, quality is to be built into a product by design since it cannot be built in merely by testing. This begs the question of what is meant by a 'well-understood' process. The Agency's own interpretation [43] is that a process is well understood when

1. all critical sources of variability are identified and explained;
2. variability is managed by the process;
3. product quality attributes can be accurately predicted over the design space established.

Note that none of the requirements of PAT exclude the use of batch processes, but they do clearly require the depth of process understanding that makes a continuous process a viable and often preferable alternative.

In its consultation with industry the FDA has been careful to ensure that the PAT initiative is seen as a 'win-win' both by industry, who will have to change their ways of working with the regulators, and by the FDA who will have to make the initiative workable [44, 45]. There are predicted to be gains in quality, safety and efficiency, depending on the nature of the product. These are likely to come from

1. reductions in production cycle times by using on-, in-, and/or at-line measurements and controls;
2. preventing rejects, scrap and re-processing;
3. increasing automation to improve operator safety and reduce human error;
4. facilitating continuous processing to improve efficiency and manage variability, which in turn will allow use of smaller-scale equipment, thus avoiding certain scale-up issues, and improve energy and material use.

Of course, the first point refers to production cycle times, not development cycle times. One of the key challenges to the industry in adopting this new way of

process development is to gain the necessary process knowledge in the most efficient way possible. This will be one of the topics in the Section on Online Analysis.

It would be wrong to give the impression that the PAT initiative is confined to the FDA. The FDA has increased its collaboration with international health and regulatory partners, including the International Conference on Harmonisation for the Technical Requirements for Registration of Pharmaceuticals (ICH) and the International Cooperation on Harmonisation of Technical Requirements for Veterinary Medicinal Products (VICH).

Although not within the scope of this chapter, it is worth noting that the PAT initiative also involves the development of rapid microbiological methods [46], the word 'rapid' in this context being the analogue to 'process analytical' for chemical and physical measurements. The scope of microbial testing within this initiative includes

1. *qualitative methods* – presence or absence of microbes above a specified limit, for example, sterility testing;
2. *quantitative methods* – enumeration of microorganisms present, for example, microbial limit tests;
3. *microbial identification*.

Although regulatory guidance for rapid microbiological testing is less complete than for physical and chemical testing, regulatory submission have already been made in this area.

8.4 Analytical strategies

8.4.1 Batch processes

The role of analysis in support of process development has evolved over the years to become increasingly responsive and timely. However, it is true to say that the role of analysis in the traditional development of batch processes has been essentially laboratory based, univariate and off-line. This is summarised in Figure 8.2, which shows how analysis feeds into the various stages of the development process.

At every stage of the development process, the results of a reaction or process stage will be analysed in one of two ways. The reaction mixture itself will be sampled and analysed to yield information such as extent/completion of reaction, reaction yield or reaction purity. Alternatively, the reaction product will be isolated and dried before sampling and analysis. Typical analytical information in this case would include both chemical and physical characterisation, plus quantitative data to ensure conformance with some pre-defined specification or to provide batch data on which a suitable specification will ultimately be based. Note that however quickly the analytical data are provided, there is a disconnect from the reaction, which means that reaction control is impossible and that

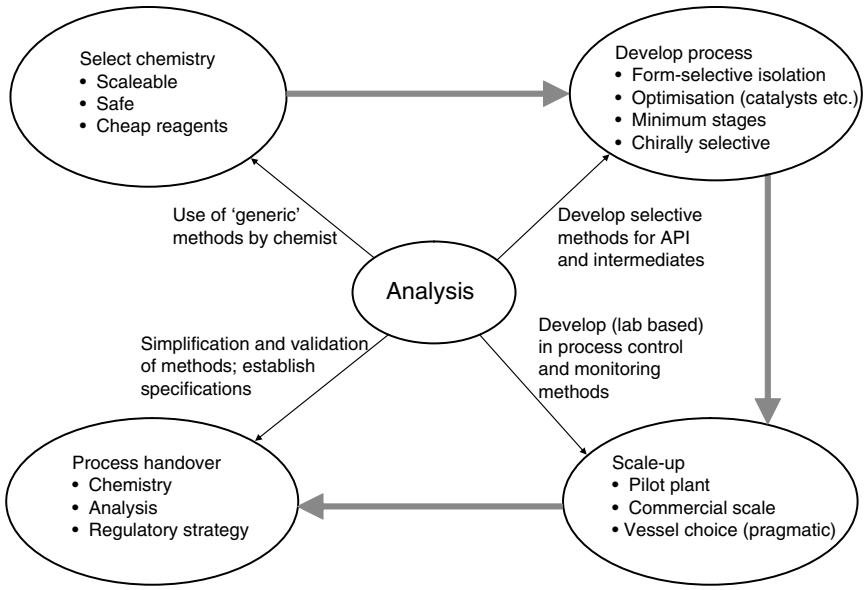

Figure 8.2 The relationship between analysis and the development of a batch process.

even process improvement becomes a tedious stepwise process. Even using sophisticated designed experiments or parallel synthesis regimes the rate of process learning cannot be as fast as when the analytical data are obtained online.

One advantage of laboratory-based analytical data is that the quality of the results can be checked at every stage of their production, from validation of the method, through the use of system suitability checks to ensure fitness for purpose at the time of use to final checking and transcription of the data. Additionally, experimental conditions can be chosen so that the analytical technique is operating under optimal conditions. For example, the appropriate NMR solvent can be employed, column loadings for HPLC can be optimised for maximum sensitivity and a suitable IR sample method can be selected to reduce polymorphic changes. In the context of drug impurity analysis, this means that techniques will be operated at their highest sensitivity or dynamic range, resulting in the lowest detection and quantification limits for impurities.

Reference was made earlier to testing against specifications. It is an obvious statement, but one worth repeating, that testing against a regulatory specification may or may not ensure fitness for purpose for the intended use of the material. For example, the average API specification in a regulatory submission is concerned primarily with drug purity and the absence of toxic impurities. On its own it almost certainly would not guarantee batch-to-batch consistency in any subsequent processing, such as a wet granulation. That requires an interactive knowledge between the wet granulation process parameters and the production process for the API.

8.4.2 Continuous processes

The analytical strategy for a continuous process is necessarily predominantly online, as summarised in Figure 8.3. However, the correlations between continuous process development and online analysis on the one hand and batch process development and off-line analysis on the other are not simple. For example, many aspects of batch process development would benefit from the availability of online analysis and monitoring. Similarly, some of the early development stages of a continuous process will utilise data from batch (i.e. non-continuous) experiments.

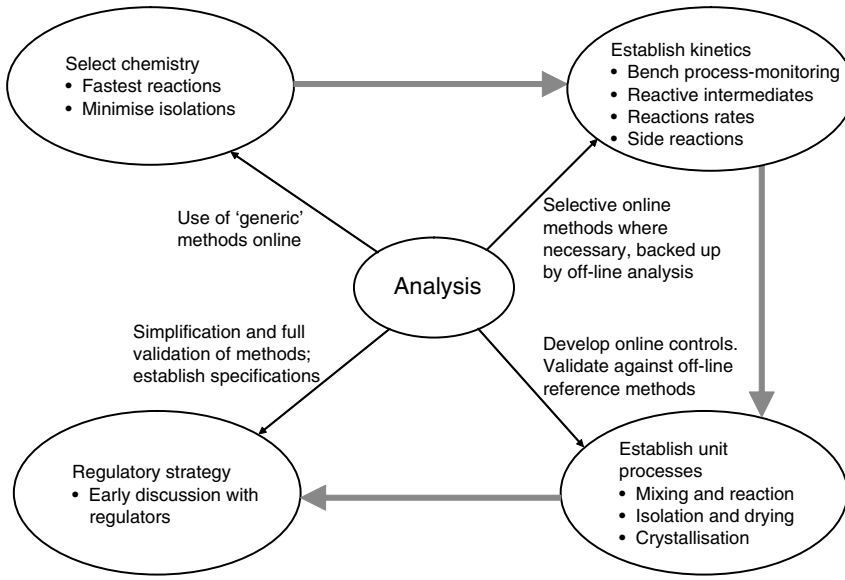

Figure 8.3 The relationship between analysis and the development of a continuous process.

All process development starts with chemistry. The selection criteria for the most suitable chemistry for a continuous process do not suffer from the same constraints as those for a large-scale batch process. For example, highly exothermic reactions are not only possible in a flow reactor, but are in fact preferred [47]. As operator exposure will be low and so will stock levels, different safety considerations come into play that may allow utilisation of otherwise intolerably toxic reagents. Process telescoping is a necessity to minimise the number of intermediate isolations. Examination of all these factors is facilitated by online analysis because of its speed and maintenance of experimental integrity (i.e. no requirement for sampling).

Chemistry route-scouting and optimisation is increasingly carried out using parallel synthesis equipment [48], for which online analysis is a given, simply because the demands on off-line laboratory analyses would be overwhelming.

An even more powerful option is the use of micro flow reactors, which can potentially be automated to provide a complete series of optimisations [49]. For example, a glycosylation reaction was monitored over a series of reaction temperatures. Although the optimum reaction temperature for maximum yield was determined to be −60°C, it was noted that by increasing the temperature of the reaction to −35°C the yield only dropped slightly but the reaction rate increased considerably. It was therefore possible to achieve a ten-fold increase in production rate. The complete optimisation took one afternoon and consumed 2 mg of starting material.

There is as yet no standardisation of the design of microreactors, and numerous other configurations exist [50–52]. All run true continuous processes with all the throughput and versatility that goes with that. Notable achievements that have been published to date included the following:

1. The devices are capable of running single- and multi-phase reactions [53];
2. the processes can be monitored by all the common spectroscopic techniques. Of particular interest is the use of Raman spectroscopy, which can take advantage of the low scatter dispersion of these inherently flat devices in order to produce excellent sensitivities [54, 55];
3. scale-up to produce gram quantities of material has been achieved by 'scaling-out', that is, by running several devices in parallel, thus avoiding any need for changes in reaction conditions;
4. the devices are relatively easy to automate. For example, a system is described which will carry out systematic screening of process parameters and can also be used to produce kilogram quantities of material under automated operation [56]. Its use has been exemplified by the nitration of 2-(4-chlorobenzoyl-)benzoic acid;
5. experiment throughput is high. For a typical acid chloride synthesis the throughput of optimisation experiments increase from batch (typically 48 h) through automated parallel (~0.4 reactions per hour) to microfluidic (0.5 reactions per minute).

We have already noted that the increased process understanding demanded by continuous processes and by PAT requires a thorough investigation of the reaction kinetics [57]. Paradoxically, this is usually provided by a series of (bench-scale) batch reactions because of the flexibility of experimental design possible with such equipment; a typical strategy applied to a continuous pilot plant reactor is shown in ref. [56]. However, the analysis of such experiments is best carried out online for two reasons. First, it is important that the experiment is not disturbed by the need to extract samples, which may also require subsequent quenching. For the same reason, online analysis is frequently able to provide information on reactive or hidden intermediates, which would simply be lost during the sampling process.

Using mid-IR process monitoring, for example, it has been possible to interrogate a catalytic three-stage Pd/C hydrogenation in order to establish reaction

mechanism and kinetics. Enantioselective hydrogenation of prochiral, exocyclic α,β-unsaturated ketones, using (*S*)-proline as a chiral auxiliary in various solvents was previously thought to progress via an intermediate formed by the chemical interaction between an α,β-unsaturated ketone and (*S*)-proline. However, the IR monitoring demonstrated absence of the proposed intermediate down to the detection limit of 0.2 mmol/l, therefore suggesting an alternative pathway and alternative optimum reaction conditions [58]. Similarly, Raman spectroscopy has been used to elucidate the kinetics of the nucleophilic addition of a primary amine to a ketone in chloroform solution [59]. The intensity of the C=O stretching mode at 1684 cm^{-1} was measured to determine the rate constant of the imine formation reaction over a period of 8 h, using the chloroform C–Cl bending peak at 666 cm^{-1} to compensate for spectrometer-induced variation. The second-order rates at three different temperatures were determined. Last, the kinetics of a catalysed vinylation reaction have been measured by FT-Raman spectroscopy, resulting in a greatly improved process model [60].

8.5 Process analysis

As discussed previously, the analytical techniques available to gather real-time process information are not new and have been in use by other manufacturing industries for many years. A variety of techniques is available, producing information on both physical and chemical properties and varying in their degree of invasiveness of the process. What they all have in common is that they produce information essentially in real-time compared with the timescale of the process and that they produce multivariate (e.g. spectral) data rather than single-point (e.g. a temperature measurement) data. The term 'information rich' is often used to describe this type of data, although the information often has to be extracted by some form of data manipulation. This is the subject of the *Chemometrics* section of this chapter. To avoid confusion in terminology, the description of 'online analysis' will be used to include all its possible variants including

1. *non-invasive* – monitoring of the process through some form of window, for example, ultrasound, Raman;
2. *on-line* – using some form of probe into the process, for example, IR;
3. *in-line* – sampling via some form of side arm from the process vessel;
4. *at-line* – near to real-time monitoring involving the in-line removal of sample, possibly with some form of sample conditioning, for example, process LC;
5. *near-line* – a traditional laboratory analytical measurement carried out on the plant.

Several industry/university consortia exist to further the science and technology of this type of measurement. The first consortia were started in the US, with the University of Washington based CPAC in 1984 [61] followed by the

Measurement and Control Engineering Center (MCEC), based in the University of Tennessee (Knoxville, TN). The UK-based Centre for Process Analytics and Control Technology (CPACT, University of Strathclyde) plays a similar role and was initiated in 1995. One of the key roles played by such consortia is to facilitate adaptation of existing online analytical technology to pharmaceutical applications by engaging academia, the instrument manufacturers and the end-users. The field is also served by at least two regular technical conferences. The International Forum on Process Analytical Chemistry (IFPAC) in the United States (IFPAC Committee, InfoScience Services, Inc., Gray slake, IL) and Advances in Process Analytics and Control Technology (APACT) (CPACT, University of Strathclyde, Glasgow) in the United Kingdom.

Reviews of online analysers for the chemical industry date back as far as the mid-1980s [62] and equipment was already widely in use in the 1970s and 1980s [63], when growth was estimated at around 10% per annum. Several reviews of process analytical techniques have appeared in recent years [64–68], which confirm the remarkable growth in range and capability of the instrumentation available currently. It is a sad repetition of history that, just as technology developments in World War II led directly to the upsurge of instrumental analysis technology in the post-war period, so the 'War on Terror' following the events of September 11, 2001 has provided a great impetus to the development of miniature automated instrumentation, which is directly applicable to pharmaceutical process analysis [69]. In the following sections, we will explore these techniques in more depth, and in the process it will become apparent that there is a bewildering array of technologies and technology combinations that can be brought to bear on any given process. An attempt to simplify the choices to be made has recently been published, which introduces the concept of the *measurability factor* [70]. This combines instrument characteristics such as precision, sampling rate, effective sample volume and response rate to enable the choice and location of sensors to be put on a more rational basis [71].

An important point to note in a book on the analysis of drug impurities is that many of the techniques discussed do not necessarily directly measure drug impurities. Their relevance in the context of continuous processing is that they enable production of API of defined quality, and therefore impurity profile, for several reasons that have been discussed previously:

1. The use of chemically informative online analysers in the early stages of route development enable identification of critical processing parameters.
2. This knowledge can be used to select the optimal stages of the process to monitor in order to ensure control of variability.
3. The control can be enabled by multivariate statistical process control (MSPC) using process models, control charts and the like.
4. The variables used to build the necessary models need only to be sensitive to the critical processing parameters, in other words they can be *surrogates* of the actual variables (impurities, pH, etc.) being controlled.

In other words, the assurance of quality by measurement of process impurities in the end product has been replaced by assurance of quality by the removal of variance in the process (by continuous monitoring of a continuous process). Naturally, whether online process analysis is being used as a surrogate for an alternative off-line technique to measure specific analytes or as a monitor to reduce process variance it needs *calibration* and *validation*. These stages require measurement of process analytes by a reference off-line technique, usually HPLC, and subsequent demonstration that the resulting calibration model has reliable predictive power.

8.5.1 UV/visible spectroscopy

Electronic spectra are not the most discriminating spectra and therefore at face value not the first choice for process monitoring. However, a combination of two technologies can often eliminate the shortcomings of either one of them. For example, the high sensitivity of UV/vis detection can be harnessed by eliminating chemical interference, and this interference reduction can be achieved chromatographically by presenting the individual components of a mixture to the UV detector one at a time. This is exactly the procedure employed in one of the most common laboratory analytical measurements, HPLC. The scaled-up continuous process version of HPLC is also monitored by UV/vis and isolation of high-value products or production of drug impurities for further study can be managed by sophisticated UV control systems [72].

When attempting to determine minor impurities in a chemical process the concept of dynamic range of the detection technique is a more useful concept than sensitivity, because sensitivity is usually limited by chemical interference. It remains the case that the dynamic range of the HPLC/UV combination is the highest of all the commonly available analytical techniques, and therefore when it is essential to determine minor impurities online then this is still the preferred combination, despite its interfacing difficulties. The use of online HPLC is further discussed in the chromatography section.

Despite the lack of inherent selectivity, it is still possible to obtain good quantitative data from online UV/vis monitoring by making use of chemometric techniques to resolve the overlapping spectra. The most common application is in dissolution testing [73, 74], where results that are at least as accurate as those of the reference (and much slower and more costly) HPLC method have been demonstrated.

Monitoring of conventional chemical reactions is hampered by the high concentrations involved, where the sensitivity of UV spectroscopy actually becomes a disadvantage. Two approaches can be used to overcome the problem; first by using a short path-length probe or alternatively by the use of fibre optics. A good example is the monitoring of a series of optimisation experiments designed to select the best solvent for scaling up a reaction. Traditionally, this would be done off-line by running samples on a generic HPLC method in order to determine solution yields. Walker *et al*. [75] utilised a fibre-optic probe to monitor multiple

reactions and then used iterative target transformation factor analysis (ITTFA) to derive the component profiles and pure spectra of reactants, intermediates and products without the need for calibration or reference standards. Pairwise analysis of consecutive reactions allows standardless comparisons between reactions in order to establish a rank order of reaction speed.

A further level of sophistication is illustrated by a submersible UV/vis spectrometer, currently being developed for batch process analysis but potentially also applicable to continuous processes. This instrument also overcomes the sensor-fouling problem by using an auto cleaning system based on pressurised air [76].

Lastly, UV/vis monitoring is the commonest method for measuring vessel cleaning by rinse analysis (Spectr Alliance, Inc., St Louis, MO; Carl Zeiss Jena GmbH, Jena, Germany). Although this is primarily a requirement for batch equipment because of its use in manufacturing campaigns for different APIs and intermediates, the same technology is equally applicable to continuous flow equipment in the event of process changes.

8.5.2 Infrared (IR) spectroscopy

Table 8.1 showed infrared spectroscopy to have one of the highest information contents of the spectroscopy techniques. In principle, the molecular vibrations measured by an IR spectrum contain information not only on its primary molecular structure but also on conformational change and interactions with its immediate neighbours, for example, hydrogen bonding and solvent interactions. Furthermore, in principle, the infrared spectrum of a molecule can be calculated *ab initio*. Unfortunately, in practice this is too formidable a task even with present-day computing power for any molecule much larger than hydrogen. In addition, the wealth of information potentially available in an IR spectrum is usually impossible to extract in practice because of band overlap, rotational broadening and chemical noise. Absorbance in the infrared region is strong, which on the one hand makes IR a relatively sensitive technique, but on the other makes for difficulties at the volume efficiencies used in a continuous process. For this reason attenuated total internal reflectance (ATR) is often the technique of choice for process monitoring [77]. Because conventional glasses are strong infrared absorbers, the manufacture of windows or fibre optics for the mid-IR region is challenging. Again, enormous strides are being made in the development of new materials to extend the useful spectral range for mid-IR process monitoring.

Despite these caveats, IR is an excellent tool for API process monitoring because of its chemical information content. This is particularly valuable in early-stage development when it can yield crucial information about unexpected reaction intermediates and side reactions and therefore lead directly to a more robust process. Commercial instrumentation is widely available for this purpose [78] and development of cheaper, smaller and more rugged instrumentation continues apace [79]. For example, a miniaturised mid-infrared spectrometer and

ATR immersion probe have been used to monitor the esterification of crotonic acid by 2-butanol in toluene [80]. Univariate calibration, using the signal at 1188 cm^{-1} in the second derivative spectrum gave accuracy and precision comparable with that of the off-line reference GC method and was superior to that of other in-line process analysers attached to the same reaction (NIR, Raman and UV/vis spectrometers). Similarly, mid-IR and Raman spectroscopy were compared to probe the catalytic hydrogenation of 1-chloro-2-nitrobenzene in a heterogeneous slurry [81]. Using chemometric modelling techniques, both were found to yield good results for this reaction. Although a later section of this chapter will examine the role of chemometrics in extracting quantitative information from online spectral data, it is always preferable to use a signal that is directly and uniquely attributable to the desired analyte. This is where mid-IR can score over NIR, because the latter consists of overtones and combination bands that are less directly relatable to a particular analyte.

An alternative to univariate calibration is to use multivariate techniques to sense when a steady state has been reached in a chemical reaction. This approach has been successfully applied to the detection of reaction end points [82]. A very similar technique can be used to establish deviation from steady state in a continuous process reactor.

Mid-IR is sensitive to polymorphic form and can therefore be useful to monitor crystallisation processes. Although beyond the scope of this chapter, an interesting example is given in ref. [83]. A further area for development is the application of mid-IR fibre-optic sensors [84]. There are two main trends of interest that have relevance to monitoring of continuous processes. The first is the development of broadband sensors that can be used for multi-component analysis. These are likely to have the widest process application and are critically dependent on the development of improved fibre-optic materials. The second is the development of sensing devices by coupling an ATR fibre with a suitably selective membrane, the fibre-optic evanescent wave sensors (FEWSs) [85], that may have application for the monitoring of trace species in a reaction. As well as the development of better IR-transmitting fibres mentioned earlier, other technical developments will enhance the application of mid-IR process-monitoring equipment. Better detectors include microbolometers and improved on-chip integration. However, the most dramatic changes will be in IR sources:

1. *LEDs* – relatively weak sources, but extremely small;
2. *optical parametric oscillators (OPOs)* – a powerful solid-state source of broadly tuneable coherent radiation;
3. *quantum cascade lasers (QCLs)* – a novel type of semiconductor laser [86].

8.5.3 Raman spectroscopy

Both infrared and Raman spectra are concerned with measuring molecular vibration and rotational energy changes. However, the selection rules for Raman spectroscopy are very different from those of infrared – a change of polarisability

of the molecule is required rather than a change in dipole moment. Immediate consequences of these selection rules are that Raman spectroscopy can detect homo-nuclear molecules and that polar solvents are no longer a completely opaque medium. Raman scattering is an extremely weak phenomenon and therefore laser sources are essential to obtaining spectra in process systems. This and a range of other technical challenges have meant that Raman spectroscopy is a relative latecomer to the field of online process monitoring [87]. Technology advances that have helped to increase the utility of Raman spectroscopy outside the laboratory include the development of stable diode lasers [88], fibre-optic sample probes, compact interferometer designs with no moving parts, high quantum efficiency detectors and the availability of increased computer power [89].

An important feature of FT-Raman is that the excitation wavelength can be in the NIR region. This confers two important benefits. First, the problem of fluorescence, the major problem with excitation in the visible region, is overcome. Second, all the window and fibre-optic materials that are transparent in the NIR are available for Raman monitoring, even though the information being obtained is in the infrared region. Indeed, the use of single-ended fibre-optic probes allows the Raman instrument to be placed several hundred meters from the process being monitored [90] and therefore avoids the complication and cost of intrinsically safe instrument housing.

One of the requirements of a continuous process is that quality and/or processing conditions are monitored and controlled throughout the supply chain. This requirement starts right at the beginning of the process, with the control of raw material and reagent quality. Raman spectroscopy, like NIR, requires virtually no sample preparation and is therefore well suited for this purpose. This then begs the obvious question as to whether to use Raman or NIR for raw material monitoring. In the view of the author the final choice depends on the information required. If the purpose of the testing is simply to confirm that the material is correctly labelled and that it lies within a previously determined quality envelope then NIR is probably the technique of choice because it is cheap and robust, can be housed in a warehouse and can give a simple ‘pass/fail’ indication to a non-analyst. However, for starting materials, particularly during the development phase of a process, more chemical information about the material is essential in building up knowledge about the robustness of the process. This information is better provided by the chemically information-rich Raman technique.

Producing accurate quantitative information from Raman process-monitoring data requires a certain amount of care to compensate for variations in excitation intensity and temperature effects [91, 92]. Typically, normalised spectra would be acquired by reference to an internal (e.g. a solvent band) or external standard. Quantitative accuracy of ± 0.5% has been reported in a simulated moving bed application [93]. Multivariate calibration requires careful selection of variables because of the sparse sampling, process dynamics, fluorescence and short accumulation times typical in online monitoring of a continuous process [94]. A strategy based on multiple linear regressions and stepwise variable selection following a Fourier de-noising algorithm has been reported [95].

Discussion so far has concentrated on the chemical characterisation of the API production process. Although strictly outside the scope of this book, one of the greatest challenges to consistency across the whole pharmaceutical product development line is that of solid-state habit and form. Raman spectroscopy is not only able to carry out direct measurements on solids but it is also sensitive to polymorphic form, hydration and to particle size [96, 97]. It is therefore ideally suited for monitoring of continuous crystallisation processes in order to demonstrate consistency of solid-state form. Variable temperature Raman spectroscopy has been used, for example, to study the dynamics of crystallisation of menthol from ethanol under a variety of cooling regimes in order to provide insight into the isolation of different polymorphic forms [98]. As Raman spectroscopy produces information about both the solution phase and the crystallising solid, it is possible to simultaneously measure both de-supersaturation and polymorphic form, as demonstrated for the crystallisation of flufenamic acid systems [99].

8.5.4 NIR spectroscopy

There is probably more experience of NIR spectroscopy in continuous process monitoring than any other spectroscopic technique [100]. The technique has been used for qualitative and quantitative measurements in the agricultural, food, chemical and pharmaceutical industries for several decades [101]. Because of the complexity of correlations within the spectra, the technique has almost driven the specialism of chemometrics, which is essential for extracting useful information. In this section, we shall explore how NIR spectroscopy has achieved this dominant position and how it measures up against alternative techniques as a process-monitoring technique for continuous processes.

The NIR region of the electromagnetic spectrum covers the 800–2500 nm range and is characterised by broad overlapping bands that arise from the combination and overtones of the fundamental molecular vibrations found in the mid-IR region of the spectrum. Partly for this reason NIR absorption bands are one to two orders of magnitude weaker than those in the mid-IR region, which at first sight seems like a disadvantage for sensitivity. However, for the concentrations of organic species in a typical volume-efficient continuous process this is an ideal situation, avoiding the need for online dilutions or the use of very narrow pathlengths, with the associated fouling problems. Silica glass is essentially transparent in the NIR region and therefore NIR optics are relatively inexpensive. Special low OH-silica can be used to fabricate fibre optics that are useable up to many meters and cover the entire NIR spectral region. The dominant absorptions in the NIR arise from OH, NH and CH bonds, which makes it very suitable for monitoring typical API chemistry, although it has to be said that working backwards from the spectral information to structural correlations is fairly unreliable.

In addition to chemical information, NIR spectra also contain information about the physical characteristics of samples and systems that can manifest itself, for example, as a baseline shift caused by scattering differences. On the one hand, this information is extremely useful in characterising particle size [102],

fermentation biomass [103], different polymorphs or the degree of crystallinity [104–106]. However, it also means that in order to achieve quantitative information multivariate calibrations need to include all potential sources of variation, physical as well as chemical, in the calibration set in order to ensure reliable results [107, 108]. For a typical continuous process plant this can be extremely tedious. For this reason, there is an increase in semi-quantitative, or 'calibration-less', applications of NIR in which trends and endpoints are measured rather than quantitative concentration measures [82, 109].

Most literature references to pharmaceutical primary process monitoring are for batch processes, where a model of the process is built from calibration experiments [110, 111]. Many of these examples have led to greater understanding of the process monitored and can therefore be a precursor to design of a continuous process. For example, the acid-catalysed esterification of butan-1-ol by acetic acid was monitored through a factorial designed series of experiments in order to establish reaction kinetics, rate constants, end points, yields, equilibrium constants and the influence of initial water. Statistical analysis demonstrated that high temperatures and an excess of acetic acid were the optimal conditions [112].

The logical extension of online process monitoring is process control. An example is the automatic dosing of ammonia for the production of low methoxylated amidated (LMA) pectin [113]. Fibre optical NIR transmittance spectroscopy was used to monitor the amidated liquid, consisting of ammonia, isopropyl alcohol, water and minor impurities. One of the technical difficulties to be overcome was the high temperature dependence of the measurements in the reaction range, a good example of the physical property sensitivity mentioned earlier. This was overcome by careful selection of the spectral region for monitoring and by the use of first derivative spectra. The robustness of the calibration was demonstrated by over one year's operation in the factory.

Because of the high sensitivity of NIR to changes in OH bond vibrations, it is often possible to use the OH region of the spectrum as surrogate for a bond that cannot be measured directly by NIR. For example, in a feasibility study on the monitoring of the acetic acid process, it was required to monitor acetic acid, methyl acetate, methyl iodide, water and potassium iodide [114]. During the process iodide ion is generated, which cannot be directly measured by NIR. However, by using the resulting wavelength shift and intensity change of the water absorption band a good calibration was obtained. All of these approaches to batch process monitoring and control are equally applicable to continuous processes.

Drug impurities include solvents as well as related organic impurities. Because of its high sensitivity to water, NIR is an excellent technique for monitoring drying processes, whether these are batch or continuous. This use can also be extended to other solvents by using the appropriate calibration models [115]. By placing the NIR monitor in the effluent stream of a drum drier it was possible to determine the percent solvent vapour in real time and therefore establish the end of the drying process. This reference also demonstrates the use of an acousto-optic tuneable filter (AOTF) to select the wavelengths to be monitored, a development that increases the robustness of the technology as it contains no

moving parts. In an extension of this work [116], it was shown that monitoring solvent vapour in the effluent stream or in the material being dried produced equivalent results.

It has already been mentioned that control of a continuous process includes control and monitoring of raw materials used in the process. This is a very successful area of application for NIR because of the minimal sample preparation required, the robustness of the instrumentation and its ability to measure both chemical and physical properties [117]. Libraries of raw material can be compiled, which can then be shared between manufacturing locations given adequate calibration checks between measuring equipment [118].

8.5.5 NMR spectroscopy

NMR spectroscopy is not an obvious candidate for plant-based online monitoring because of its size, cost and infrastructure requirements. In addition, it is not as sensitive as the vibrational spectroscopy techniques that are commonly used for process monitoring. Early attempts at chemical shift NMR at-line or online process analysis simply used high-field laboratory instruments that had been moved into the plant. Not surprisingly, process operators found problems in the calibration and maintenance of these instruments. Despite this inauspicious history there have been several examples of bench-scale online reaction monitoring, and, in addition, flow-NMR technology is available, which makes simulation of continuous flow reactors a feasibility. The reason to persevere with efforts to couple NMR to directly process equipment is that it is by far the most chemically informative of the spectroscopic techniques and therefore offers not only unprecedented specificity but also high diagnostic value in the event of process malfunction. A recent successful example of online coupling in development chemistry probed the equilibria and kinetics of complex formaldehyde-containing mixtures and the kinetics of both homogeneously and heterogeneously catalysed esterification reactions [119].

However, it is not necessary to employ the very high field strengths that we have become accustomed to in the laboratory environment in order to derive useful chemical information from a process. Benchtop NMR instruments have been used for quality control in the food industry for many years, but in this application relaxation times rather than chemical shifts are measured in order to monitor the distribution of fats and water [120]. Chemical shift NMR spectrometry has the potential of being a very useful technique in process environments as it is non-destructive and does not require the measurement probe to be inserted into the process liquors, which avoids fouling problems. Recently, small, dedicated low-field (<60 MHz) NMR systems, based on permanent magnet technology, have been developed, which in turn has led to more reports of at-line and online process applications [121]. The signals from this type of instrument are highly overlapped and therefore the same chemometric techniques that are applied to NIR are necessary in order to extract the information from the spectra. Perhaps the most promising applications at these low field strengths are those

utilising ^{19}F NMR [122]. The intrinsic sensitivity to ^{19}F is only slightly lower than that for the more common proton NMR measurements, but the amount of chemical noise from interfering species is greatly reduced because of the rarity of this species. Although this is a niche application, the occurrence of fluorine-containing molecules in new chemical entities is sufficiently common to make it worth exploring.

8.5.6 Mass spectrometry

In order to understand the strengths and weaknesses of mass spectrometry as a potential process analysis tool, it is helpful to re-visit the principles of the technique. Mass spectrometry is destructive, in that material is consumed in the process of ionisation. However, since the detectors employed are capable of detecting single ions it is the efficiency of the ionisation process and subsequent transmission through the instrument that determines overall sensitivity, and the sensitivity of mass spectrometry tends to be amongst the highest of the common spectroscopic techniques. A variety of ionisation sources exists, but the most interesting for process-monitoring purposes are electron impact ionisation for volatile materials and electrospray/ionspray for organics in solution, which brings us to the first challenge of mass spectrometry – since we wish to mass analyse the ions formed from the sample the subsequent ion manipulation needs to be carried out in a very high vacuum in order to minimise ion–molecule collisions during the analysis process. The technical challenges of interfacing a solution sample with a high vacuum system are understandably huge and occupied the instrument manufacturers for almost a decade during the 1980s and 1990s. It is true to say that interfacing a solution sample is no longer a major problem, but obtaining a representative sample and producing reliable quantitative data from it remains a challenge.

Process mass spectrometers also come with a variety of different mass analysers, each suited to a particular type of application but tending to make the market fragmented and confusing. The simplest and oldest of the analysers is the permanent magnet type, which depends on different ion trajectories in a powerful homogeneous magnetic field to give mass (actually mass-to-charge ratio) separation. This type of instrument probably has the longest pedigree in process applications as it is well suited to gas-phase analysis and requires electronics mainly for the detection and ion source functions, thus simplifying installation requirements. The instrument has been used for decades in the oil industry and is widely used in the pharmaceutical industry to monitor fermentations [123–125]. Not only will the instrument monitor gases and volatiles in the head gas of the fermentation, but by making use of a hydrophobic membrane a sampling device can actually be immersed in the fermentation broth. Thus the instrument can monitor not only the rate of fermentation via CO_2 production, but also volatile metabolites. Development of these applications has been greatly facilitated by the synergy with the oil and environmental monitoring sectors, where the sensitivity and specificity of the technique are so valuable.

For analysis of larger, typically pharmaceutical, molecules different types of mass analyser are better suited in order to give mass range and speed of analysis. Of these the most common for process monitoring are the quadrupole, the ion trap and the time-of-flight instruments. At the current time the majority of pharmaceutical applications are in the biopharmaceutical area where commercial instrumentation is available for monitoring and control of fermentation plant. Monitoring of batch and continuous small molecule reactors is uncommon, but the increasing robustness and miniaturisation of mass spectrometers along with improvements in sampling probe design will undoubtedly lead to further applications [124].

For completeness, it is worth noting the technique of ion mobility spectrometry (IMS). This can be thought of as a form of atmospheric pressure mass spectrometry where, rather than trying to avoid ion–molecule collisions after ionisation of the sample, sample ions are deliberately thermally stabilised by collision with atmospheric pressure gases and then characterised by their drift times across an electrostatic field. The strengths of this technique are its simplicity, as the complexity of high vacuum technology is dispensed with, and its very high sensitivity. Its relevance to a discussion of analysis in continuous processing is that it has been very successfully applied to cleaning validation [126]. It is also finding applications in formulation process development, which are beyond the scope of this chapter.

8.5.7 Ultrasound

Ultrasound can be thought of as the ultimate non-invasive monitoring technique, as in one configuration, passive ultrasonics, it does not even probe the sample with any form of radiation but merely 'listens' to the sound generated by the process itself. This is essentially the definition of acoustic emission (AE), the class of phenomena whereby an elastic wave, in the range of ultrasound usually between 20 kHz and 1 MHz, is generated by the rapid release of energy from the source within a material. The elastic wave propagates through the solid to the surface, where it can be recorded by one or more sensors. The sensor is a transducer that converts the mechanical wave into an electrical signal. The technique has been used for materials testing for about 50 years [127], but it is only recently that its application to pharmaceutical processes has been explored. Its potential advantages for pharmaceutical process monitoring are as follows:

- It is rich in process information.
- It is capable of real-time measurement (and therefore potentially for control).
- It is non-invasive – the sensors are attached to the exterior of the process vessel.
- It is non-destructive – the ultrasound energy originates from within the process.
- It is intrinsically safe – the sensors are essentially passive devices.
- It is applicable to a wide range of processes.

Applications in the pharmaceutical industry included determining the end points of high-shear and fluid bed granulation [128–130], fluid bed drying, powder mixing [131], crystallisation [132] and obtaining information from other processes involving particulates [133].

The application of AE to chemical process monitoring is very much in its infancy, but it has already been investigated to monitor a heterogeneous esterification reaction [133]. The source of AE in the reaction between itaconic acid and 1-butanol is largely from itaconic acid particles interacting and colliding with the vessel walls. By suitable choice of frequency, other sources of ultrasound, such as the oil jacket and stirrer, were minimised. It was possible to detect differences in the rate and extent of reaction under different conditions and also to identify when a combination of the concentration and/or size of itaconic acid particles had reached a steady state. However, it was not possible to differentiate between changes in particle size and concentration using the resonant sensor employed in this study.

Active ultrasound, in which the attenuation of applied ultrasonic energy is monitored [134, 135], has been used to study the crystallisation of (L)-glutamic acid α-polymorph [136, 137]. The study was initiated to investigate ultrasonic spectroscopy as a particle-size monitor. High-precision measurements of ultrasonic attenuation in the frequency range 7–100 MHz were performed, the deconvolution of which enabled process measurements of crystal size distribution and solid concentration throughout the crystallisation process. Real-time measurements of secondary nucleation, growth and crystal breakage were derived, which enabled the kinetic parameters to be extracted.

It is easy to see how this technology might develop in the future to allow better monitoring, and ultimately control, of continuous crystallisation processes.

8.5.8 Chromatography

Gas Chromatography (GC), as the older of the two common instrumental separation techniques, has a long history of process monitoring and control applications. The petroleum industry took up the technique shortly after its development as a laboratory separation technique and commercial instrumentation is well developed. The emphasis on technology development of process GC is focused on increasingly rapid methods [138, 139]. It is not a huge leap from monitoring of petroleum processes to the monitoring of other processes where gas composition is an important factor. There are thus applications in the food, electronics, heavy chemicals [140] and fermentation industries [141]. The last of these is of interest to fermentation production in the pharmaceutical industry, and the technology is well used for this application, usually with non-selective detection such as flame-ionisation, but sometimes coupled to mass spectrometry. It is unlikely that GC will be applied significantly in API production processes because it is strictly limited to gas-phase monitoring and detection.

HPLC has opened up a whole range of analytical capability in the pharmaceutical industry since its development in the 1960s and 1970s and is now the

mainstay of analysis in the analytical laboratory [142]. Its transfer to the plant is more problematical since sampling is a highly invasive procedure and until recently the analysis times were long compared with the time course of a reaction. Applications therefore tended to be largely at-line. Some of the earliest online applications of HPLC were to fermentation processes [143], and at least two manufacturers produced online instruments in the 1980s. However, a number of challenges were encountered that prevented widespread use of the instrumentation. These included sampling and sample conditioning problems, the disposal cost and hazard of flammable solvents, high maintenance costs and sensor fouling [144]. Column and instrument technology developments have overcome many of these problems, producing smaller, simpler instruments and fast separations. However, the major advance has been in reliable sampling systems based on ultrafiltration technology [145].

Applications of HPLC to chemical process monitoring have been published. For example, online HPLC is an integral part of an automated parallel synthesis system [146], where it has been used to monitor alkylation, reduction, oxidation, reductive amination, displacement and cyclisation reactions. A similar system has been used to support a complete experimental design strategy exemplified by the desilylation of a silyl ether [147]. Both these systems, and others like them, are easily adapted to monitoring of a continuous flow reactor. HPLC remains the best way of detecting and characterising (when coupled with MS), low-level impurities in a reaction mixture. As explained earlier, this is not because of a lack of sensitivity on the part of many alternative reaction-monitoring techniques, but rather a lack of specificity. In other words, their full dynamic range is limited by interfering chemical noise, which is precisely the strength of a high-resolution separation technique such as HPLC – it provides the specificity that many spectroscopies lack. Analysis of low-level impurities will remain an essential part of the development and validation of a continuous process, but in principle may be removed from a fully validated process. Whether this will satisfy the requirements of *parametric release* is a call for the regulators, which we will watch with interest.

8.5.9 Other online analytical examples

Preferential crystallisation is one option for optical resolution on a manufacturing scale. Online polarimetry and refractometry have been used to dynamically optimise the process for resolution of DL-threonine in aqueous solution by variation of process parameters such as degree of supersaturation, seed quantity, initial enantiomeric excess and scale [148]. The method is claimed to be suitable for control of 'quasi-continuous' processes.

The application of biosensors for process monitoring has been dogged by problems with fouling, selectivity, degrading bioactivity and long-term stability. Recent developments in molecularly imprinted polymers (MIPs) show some promise as synthetic receptors and have been used for this purpose for assays and as HPLC stationary phases. Recent work has shown that MIPs show increased robustness, storage endurance and lower cost compared with biosensors, which

gives them some potential for online chemical monitors. Examples to date have been in the food and environmental sectors, but this is clearly a technology to watch [149].

8.6 Chemometrics

Analytical scientists have grown used to a multivariate world, where phenomena may be characterised by several variables rather than just one. For this reason it is easy to underestimate the revolution in thinking that this concept represents. Until relatively recently graphical displays were used to confirm a rational pre-conceived model of the phenomena that make up our world, a strategy that dates back to the Greek philosophers and beyond [150]. Think of the determination of linearity of an HPLC method – the responses to injections of various concentrations of analyte are plotted on a two-dimensional display and if all goes well the analyst can draw a straight line through the resulting points and determine slope and linearity, comfortable in the knowledge that his data fit the expected model for his experiment.

Psychologists, economists, epidemiologists, social scientists and the like had rather different needs of their data – they had no pre-conceived models and simply wished to see how their data related. Hence they developed the idea of the graph as an experimental tool – as a way of seeing what structure, if any, was hidden in their data. From this revolutionary change in use of the graph came a whole string of specialist branches of statistics: psychometrics, econometrics and so on. It was hardly a surprise, then, when Svante Wold coined the term 'Chemometrics' in 1972 for that development of statistics, particularly multivariate statistics, which had to do with the display and analysis of chemical data [151]. As well as harnessing the power of statistics to extract meaning from the data, chemometricians also make use of display techniques to enhance the visualisation of the data, and therefore our ability to understand the underlying structure. Without realising it, their use of colour, multilayer plots and plot rotation capitalise on human evolutionary skills that were developed in order to survive in a multivariate (and dangerous) world [152].

There are broadly two uses of chemometrics that interest the process chemist. The first of these is simply *data display*. It is a truism that the human eye is the best analytical tool, and by displaying multivariate data in a way that can be easily assimilated by eye a number of diagnostic assessments can be made of the state of health of a process, or of reasons for its failure [153], a process known as MSPC [154–156]. The key concept in MSPC is the acknowledgement that variability in process quality can arise not just by variation in single process parameters such as temperature, but by subtle combinations of process parameters. This source of product variability would be missed by simple control charts for the individual process parameters. This is also the concept behind the use of experimental design during process development in order to identify such variability in the minimum number of experiments.

An important consequence of MSPC in the context of this chapter is that it is a key tool in the development of process understanding [157]. These references also illustrate the utility of multivariate control charts in identifying abnormal events and how these can then be used to aid understanding of the underlying fault conditions. It has to be said that most literature examples apply to batch processes, but the principles are equally applicable to continuous processes.

One of the keys to multivariate analysis is the ability to reduce the dimensionality of the data so that it can be displayed in two, three or four (time-dependent) dimensional displays. The primary tool for achieving this, principal component analysis (PCA) [158], is the cornerstone of chemometrics as it accomplishes several things:

1. It reduces the dimensionality of the data so that it can be displayed or manipulated further.
2. It links variables into meaningful combinations – for example, particle data including particle dimensions might have these combined into a new variable that simply represents the particle size.
3. It achieves the above by reducing redundancy (or correlation) in the data, so that, for example, related spectral points are displayed as a single data point.
4. It ranks the data in order of its significance to the observed variance, so that noise is isolated in the least significant principal components.
5. It facilitates interpretation of inherent structure, by allowing the variables that contribute towards a particular data structure to be extracted.

There are numerous reviews of chemometric tools, from the basic overview [159] to comprehensive literature reviews [160].

The chemometric tools that enable data display are also the first steps to *data modelling*. The objective here is to construct a model of the process from suitable calibration data and to use that model to test future states of the process. The simplest example of data modelling is the construction of a multivariate calibration, which can be used to predict future concentrations of an analyte (e.g. a component of a process stream) from multivariate data [161]. A key step in constructing the calibration data is a proper experimental design, which will ensure that all the expected variability is covered by the calibration experiments, that sufficient data are acquired to define the model and that the data are obtained in as efficient a way as possible [162]. As described earlier in this chapter, construction of a full quantitative calibration may be unnecessary for many applications such as end-point detection, thus greatly reducing the overheads to implementing multivariate (spectroscopic) process monitoring [82]. Indeed, recent work shows that quantitative data can be obtained from a real-time calibration-free algorithm as long as the spectra (Raman in this case) of the pure components are available [163].

8.7 The future

This chapter has given a high-level summary of analytical capabilities and the interpretation power of chemometrics and multivariate analysis. Exciting possibilities remain. For example, a limiting factor with online spectroscopic process monitoring is the sensitivity, or more correctly the dynamic range, of the measurements. PCA has the potential to extract quantitative information for small amounts of chemical species even when swamped by major components [164, 165], and sensitivities down to the ICH 0.1% qualification limit have been demonstrated. The use of prior knowledge of systems, formalised in Bayesian statistics [166], will allow even more information to be extracted from online measurements. Another tantalising possibility is the elimination of the need for reference samples by extracting quantitative information and spectral responses directly from process mixtures [167]. Indeed, the pharmaceutical industry stands to gain considerably from further application of chemometrics to derive faster, better and cheaper products [168].

The further development of miniaturised spectrometers will reduce cost and infrastructure requirements and increase ruggedness [165]. This trend will become even more critical as chemical processing equipment itself becomes miniaturised towards the 'lab on a chip' extreme. An interesting question is how far this process of miniaturisation will proceed – is there an optimum size for continuous processing equipment and what is the interplay between scale-up (bigger tubes) and scale-out (lots of small tubes in parallel)? A necessary development is further improvement in analytical sensor intelligence. This extends beyond the need for self-calibration and self-diagnosis, although these would go a long way in reducing the cost of ownership of sophisticated monitoring equipment. What is needed is a degree of in-built data processing and decision making that would reduce the electronic traffic to plant control systems and further facilitate wireless networking of plant control equipment – which in turn increases reliability and reduces cost in a continuing virtuous circle.

Whatever the details of future processing and analytical technology development, it is clear that there is no going back to end-point testing and the traditional analytical laboratory of the past. Whether parametric release becomes a reality is not a critical issue. What is important is that all scientists and engineers involved in the development process will have a better understanding of the products and processes that they develop. That has to be a more rewarding future for scientists as well as a more comforting one for patients.

References

1. B. L. Milman, *Trends in Analytical Chemistry*, 2005, **24**(6), 493–508.
2. J. C Berridge, *Journal of Pharmaceutical and Biomedical Analysis*, 1995, **14**, 7–12.
3. P. F. D'Arcy and D. W. G. Harron (eds), *Proceedings of the First International Conference on Harmonisation*, Queens University of Belfast, 1992.

4. T. Chapman, *Nature*, 2003, **421**, 661.
5. A. Behr, V. A. Brehme, Ch. L. J. Ewers *et al*., *Engineering in Life Sciences*, 2004, **4**(1), 15–24.
6. C. Ramshaw, *The Incentive for Process Intensification*, in *Process Intensification for the Chemical Industry*, ed. C. Ramshaw, Mechanical Engineering Publications, London, 1995.
7. J. L. A. Koolen, *Design of Low-Cost Chemical Plants*, in *3rd International Conference on Process Intensification for the Chemical Industry*, ed. A. Green, Professional Engineering Publishing, London, 1999.
8. D. Rosato, *Plastics Processing Data Handbook*, Springer-Verlag Berlin, 1997.
9. C. Ewers, S. Kueppers and H. Weinmann, *The Pharma Supply Chain*, Editio Cantor Verlag, Aulendorf, 2002.
10. C. Brechtelsbauer, N. Lewis, P. Oxley and F. Ricard, *Organic Process Research and Development*, 2001, **5**, 65–68.
11. X. Ni, M.R. Mackley, A. P. Harvey, P. Stonestreet, M. H. I. Baird and N. V. Rama Rao, *Chemical Engineering Research and Design*, 2003, **81**(3), 373–383.
12. US Food and Drug Administration, Center for Drug Evaluation and Research, 21 Code of Federal Regulations, Parts 210 and 211 Current Good Manufacturing Practice for Manufacturing, Processing, Packing, or Holding of Drugs. http://www.fda.gov/cder/dmpq/cgmpregs.htm.
13. X-W. Ni, From maximum to most efficient production using a continuous oscillatory baffled reactor, http://www.nitechsolutions.co.
14. J. D. Perkins, Process Systems Engineering to Support the Development of Future Processing Systems, *in in proceedigs of the 2nd International Conference on Process Intensification in Practice*, ed. J. Semel, Mechanical Engineering Publications, London, 1997.
15. I. K. Kookos and J. D Perkins, *Industrial and Engineering Chemistry Research*, 2001, **40**(19), 4079–4088.
16. E. N. M. van Sprang, H-J. Ramaker, J. A. Westerhuis, A. K. Smilde and D. Wienke, *Applied Spectroscopy*, 2003, **57**(8), 1007–1019.
17. L. D. Proctor and A. J. Warr, *Organic Process Research and Development*, 2002, **6**, 884–892.
18. E. Toukoniitty, P. Maki-Arvela, N. Kumar, T. Salmi and D. Y. Murzin, *Catalysis Letters*, 2004, **95**(3–4), 179–183.
19. P. Mudieller, *Filtration and Separation*, 2003, **40**(6), 34–36.
20. S. A. K. Jeelani and S. Hartland, *Chemical Engineering Science*, 1993, **48**(2), 239–254.
21. J. W. Mullin, *Crystallisation 3rd Edition*, Butterworth-Henemann, Oxford, 1997.
22. A. J. Hickey and D. Ganderton, Unit Processes in Pharmacy Unit Operations*, in Encyclopedia of Pharmaceutical Technology 4th Edition*, ed. J. Swarbrick and J. Boyle, Marcel Dekker, New York, 2002.
23. H. van den Berg, Process Technology Solutions, in *Better Processes for Better Products, Proceedings of the 4th International Conference on Process Intensification for the Chemical Industry, Brugge*, ed. M. Gough, BHR Group Ltd, Cranfield, 2001.
24. J. I. Walpot, *Journal of Chemical Technology and Biotechnology*, 2003, **78**(2–3), 236–240.
25. P. M. Herder, A. L. Turk, E. Subrahmanian and A. W. Westerberg, *Computers and Chemical Engineering*, 2000, **24**(2), 1775–1780.
26. C. E. Miller, *Spectroscopy*, 1997, **12**(1), 24.
27. R. A. Sheldon, *Pure and Applied Chemistry*, 2000, **72**(7), 1233–1248.
28. F. Fourne, *Synthetic Fibres – Machines and equipment Manufacture, Properties*, Hanser Publishers, Munich, 1999.
29. W. S. Simpson and G. H. Crawshaw (eds), *Wool: Science and Technology*, Woodhead Publishing, Cambridge, 2002.

30. J. G. P. Binner (ed.), *Advanced Ceramic Processing and Technology*, William Andrew Publishing, New York, 1990.
31. M. C. Flickinger and S. W. Drew (eds), *Encyclopedia of Bioprocess Technology – Fermentation, Biocatalysis and Bioseparation*, John Wiley and Sons, New Jersey, 1999.
32. P. J. Fellows, *Food Processing Technology – Principles and Practice*, Woodhead Publishing, Cambridge, 2002.
33. A. S. Grandison and M. J. Lewis (eds), *Separation Processes in the Food and Biotechnology Industries*, Woodhead Publishing, Cambridge, 1996.
34. E. Kress-Rogers and C. J. B. Brimelow (eds), *Instrumentation and Sensors for the Food Industry*, Woodhead Publishing, Cambridge, 2001.
35. M. Lewis and N. Heppell, *Continuous Thermal Processing of Foods – Pasteurisation and UHT Sterilisation*, Aspen Publishers, New York, 2000.
36. Center for Process Analytical Chemistry; http//www.cpac.wahington.edu.
37. T. Layl, *Process Analytical Technologies Subcommittee Report*, FDA, 2002
38. *PAT – A Framework for Innovative Pharmaceutical Manufacturing and Quality Assurance*, FDA, 2003
39. *Pharmaceuticals for the 21st Century: A Risk-Based Approach*, FDA, 2004.
40. O. Berntsson, L-G. Danielsson, B. Lagerholm and S. Folestad, *Powder Technology*, 2002, **123**(2–3), 185–193.
41. S. S. Sekulic, H. W. Ward II, D. R. Brannegan *et al*., *Analytical Chemistry*, 1996, **68**, 509–513.
42. R. de Maesschalck, F. Cuesta Sanchez, D. L. Massart, P. Doherty and P. Hailey, *Applied Spectroscopy*, 1998, **52**(5), 725–731.
43. A. M. Afnan, *The Journal of Process Analytical Technology*, 2004, **1**(1), 8–9.
44. R. Miller, *American Pharmaceutical Review*, 2002, **5**(2), 25–29.
45. R. Miller, *American Pharmaceutical Review*, 2003, **6**(1), 52–61.
46. *FDA Advisory Committee for Pharmaceutical Science* transcripts, 2002, 23.
47. J. Antes, D. Boskovic, H. Krause *et al*., *Chemical Engineering Research and Design*, 2003, **81**(7), 760–765.
48. D. G Cork, T. Sugawara, J. S. Linsey, L. A. Corkan and H. Du, *Laboratory Robotics and Automation*, 1999, **11**(4), 217–223.
49. D. M. Ratner, E. R. Murphy, M. Jhunjhunwala, D. A. Snyder, K. F. Jensen and P. H. Seeberger, *Chemical Communications*, 2005, **5**, 578–580.
50. V. Skelton, G. M. Greenway, S. J. Haswell *et al*., *Analyst*, 2001, **126**, 7–10.
51. V. Skelton, G. M. Greenway, S. J. Haswell *et al*., *Analyst*, 2001, **126**, 11–13.
52. T. McCreedy and N. G. Wilson, *Analyst*, 2001, **126**, 21–23.
53. S. P. Fodor, J. L. Read, M. C. Pirrung, L. Stryer, A. T. Lu and D. Solas, *Science*, 1991, **251**, 767–773.
54. P. A. Walker III, M. D. Morris, M. A. Burns and B. N. Johnson, *Analytical Chemistry*, 1998, **70**, 3766.
55. S.-A. Leung, R. F. Winkle, R. C. R. Wootton and A. J. deMello, *Analyst*, 2005, **130**, 46–51.
56. A. Chamayou, H. Delmas and G. Casamatta, *Chemical Engineering and Technology*, 1996, **19**(1), 67–74.
57. V.-M. Taavitsainen, H. Haario and M. Lane, *Journal of Chemometrics*, 2003, **17**(2), 140–150.
58. A. Pintar, R. Malacea, C. Pinel, G. Fogassy and M. Besson, *Applied Catalysis A: General*, 2004, **264**(1), 1–12.
59. M. Lee, H. Kim, H. Rhee and J. Choo, *Bulletin of the Korean Chemical Society*, 2003, **24**(2), 205–208.
60. F. Verpoort, *Applied Spectroscopy*, 2002, **56**(10), 1314–1319.
61. D. Illman, *Trends in Analytical Chemistry*, 1986, **5**(7), 164–172.

62. J. R. P. Clarke, *Analytica Chimica Acta*, 1986, **190**, 1–11.
63. Frost-Sullivan Inc., *On-stream Process Analysers Market in Europe*, Report E397, New York, 1980.
64. K. R. Beebe, W.W. Blaser, R. A. Bredeweg *et al*., *Analytical Chemistry*, 1993, **64**, 199R
65. W. W. Blaser, R. A. Bredeweg, R. S. Harner *et al*., *Analytical Chemistry*, 1995, **67**(12), 47R–70R.
66. J. Workman Jr., D. J. Veltkamp, S. Doherty *et al*., *Analytical Chemistry*, 1999, **71**(12), 121R.
67. J. Workman Jr., K. E. Creasy, S. Doherty *et al*., 2001, **73**(12), 2705–2718.
68. J. Workman Jr., M. Koch and D. J. Veltkamp, *Analytical Chemistry*, 2003, **75**(12), 2859–2876.
69. C. M Harris, *Analytical Chemistry*, 2002, **74**, 127A–133A.
70. F. W. J. van den Berg, H. C. J. Hoefsloot and A. K. Smilde, *Analytical Chemistry*, 2002, **74**, 3105–3111.
71. A. K. Smilde, F. W. J. van den Berg and H. C. J. Hoefsloot, *Analytical Chemistry*, 2002, **74**, 368A–373A.
72. B. Chen, K. Shem and J. Kong, *Journal of Separation Science*, 2005, **28**(3), 286–290.
73. C. Buhlmann, *Spectroscopy Europe*, 2000, **12**(2), 18.
74. V. Fuerte, M. Maldonado and G. D. Rees, *Journal of Pharmaceutical and Biomedical Analysis*, 1999, **21**(2), 267–272.
75. D. S. Walker, K. Purdy, F. J. Tarczynski, Electro-Optic, Integrated Optic and Electronic Technologies for Online Chemical Process Monitoring, in *Proceedings of SPIE*, *International Society of Optical Engineers*, 1999, **26**, 3537.
76. G. Langergraber, J. K. Gupta, A. Pressl *et al*., *Water Science and Technology*, 2004, **50**(10), 73–80.
77. D. Bunimovich, R. Kellner, R. Krska *et al*., *Journal of Molecular Structure*, 1993, **292**, 125–132.
78. M. A. Druy, *Spectroscopy*, 2004, **19**(2), 60–63.
79. P. Wilks, *Spectroscopy*, 2004 **19**(9), 24–34.
80. C. A. McGill, R. H. Ferguson, K. Donoghue, A. Nordon and D. Littlejohn, *Analyst*, 2003, **128**(12), 1467–1470.
81. R. M. Dyson, M. Hacenkamp, K. Kaufmann, M. Maeder, M. Studer and A. Zilian, *Journal of Chemometrics*, 2000, **14**(5–6), 737–750.
82. K. D. Braekeleer, A. D. Juan, F. C. Sanchez *et al*., *Applied Spectroscopy*, 2000, **54**(4), 601–607.
83. K. Pöllänen, A. Häkkinen, S-P. Reinikainen *et al*., *Journal of Pharmaceutical and Biomedical Analysis*, 2005, **38**(2), 275–284.
84. B. Mizaikoff, *Analytical Chemistry*, 2003, **75**(11), 258A–267A.
85. M. Saito and K. Kikuchi, *Optical Reviews*, 1997, **4**(5), 527.
86. J. Faist, F. Capasso, D. L. Sivco, C. Sirtori, A. L. Hutchinson and A. Y. Cho, *Science*, 1994, **264**, 553.
87. S. Farquharson, W. W. Smith, R. M. Carangelo and C. R. Brouillette, in *Proceedings of the International Society of Optical Engineering, SPIE*, 1999, **3850**, 14
88. S. M. Angel, M. Carrabba and T. F. Cooney, *Spectrochimica Acta* Part A, 1995, **51**, 1779–1800.
89. T. Vankeirsbilck, A. Vercauteren, W. Baeyens *et al*., *Trends in Analytical Chemistry*, 2002, **21**(12), 869–877.
90. N. Q. Dao and M. Jouan, *Sensors and Actuators, B: Chemical*, 1993, **B11**(1–3), 147–160.
91. T. J. Vickers, C. A. Rosen and C. K. Mann, *Applied Spectroscopy,* 1996, **50**(8), 1074–1081.
92. S. G. Skoulika and C. A. Georgiou, *Applied Spectroscopy*, 2001, **55**, 1259.

93. P. Marteau, N. Zanier-Szydlowski, A. Aoufi, G. Hotier and F. Cansell, *Vibrational Spectroscopy*, 1995, **9**(1), 101–109.
94. R. J. Ampiah-Bonney and A. D. Walmsley, *Analyst*, 1999, **124**(12), 1817–1821.
95. F. Estienne, D. L. Massart, N. Zanier-Szydlowski and P. Marteau, *Analytical Chimica Acta*, 2000, **424**(2), 185–201.
96. F. W. Langkilde, J. Sjöblom, L. Tekenbergs-Hjelte and J. Mrak, *Journal of Pharmaceutical and Biomedical Analysis*, 1997, **15**, 687.
97. F. W. Langkilde and A. J. Svantesson, *Pharmaceutical and Biomedical Spectroscopy*, 1995, **13**, 409
98. W. P. Findlay and D. E. Bugay, *Journal of Pharmaceutical and Biomedical Analysis*, 1998, **16**, 921.
99. Y. Hu, J. K. Liang, A. S. Myerson and L. S. Taylor, *Industrial and Engineering Chemistry Research*, 2005, **44**(5), 1233–1240.
100. D. A. Burms and E. W. Ciurczak, *Handbook of Near-Infrared Analysis*, 2nd Edition, Marcel Dekker, New York, 2001.
101. P. Williams and K. Norris, *Near-Infrared Technology in the Agricultural and Food Industries*, 2nd Edition, American Association of Cereal Chemists, St. Paul, MN, 2001.
102. P. Frake *et al*., *International Journal of Pharmacy*, 1997, **151**, 75–80.
103. S. A. Arnold, L. M. Harvey and B. McNeil, *BioPharm International*, 2002, **15**(11), 26–34.
104. P. K. Aldridge, C. L. Evans, H. W. Ward, II, S. T. Colgan, N. Boyer and P. J. Gemperline, *Analytical Chemistry*, 1996, **68**(6), 997–1002.
105. A. D. Patel, P. E. Luner and M. S. Kemper, *Journal of Pharmaceutical Sciences*, 2001, **90**(3), 360–370.
106. J. J. Seyer, P. E. Luner and M. S. Kemper, *Journal of Pharmaceutical Sciences*, 2000, **89**(10), 1305–1316.
107. ASTM E1655-00, *Standard Practice for Infrared Quantitative Analysis*, ASTM International, Pennsylvania.
108. ASTM E6122-99, *Standard Practice for Validation of Multivariate Infrared Spectrophotometers*, ASTM International, Pennsylvania.
109. M. S. Sánchez, E. Bertran, L. A. Sarabia, M. C. Ortiz, M. Blanco and J. Coello, *Chemometrics and Intelligent Laboratory Systems*, 2000, **53**(1–2), 69–80.
110. H. W. Ward II, S. S. Sekulic, M. J. Wheeler *et al*., *Applied Spectroscopy*, 1998, **52**(1), 17–21.
111. J. Wiss, M. Länzlinger and M. Wermuth, *Organic Process Research and Development*, 2005, **9**(3), 365–3711.
112. M. Blanco and D. Serrano, *Analyst*, 2000, **125**(11), 2059–2064.
113. C. B. Zachariassen, J. Larsen, F. van den Berg and S. Balling Engelsen, *Chemometrics and Intelligent Laboratory Systems*, 2005, **76**(2), 149–161.
114. H. Chung and M. S. Ku, *Vibrational Spectroscopy*, 2003, **31**(1), 125–131.
115. D. S. Walker *et al*., *Applied Spectroscopy*, 1998, **52**(5), 717–724.
116. D. S. Walker *et al*., *Applied Spectroscopy*, 2001, **55**(8), 1109–1111.
117. M. Blanco and M. A. Romero, *Analyst*, 2001, **126**(12), 2212–2217.
118. R. N. Feudale *et al*., *Chemometrics and Intelligent Laboratory Systems*, 2002, **64**(2), 181–192.
119. M. Maiwald *et al*., *Journal of Magnetic Resonance*, 2004, **166**(2), 135–146.
120. S. Gunasekaran, *Nondestructive Food Evaluation: Techniques to Analyze*, Marcel Dekker, New York, 2004.
121. A. Nordon, C. A. McGill and D. Littlejohn, *Applied Spectroscopy*, 2002, **56**(1), 75–82.

122. A. Nordon, C. Meunier, A. McGill and D. Littlejohn, *Applied Spectroscopy*, 2002, **56**(4), 515–520.
123. K. D. Cook, *Industrial and Engineering Chemistry Research*, 1999, **38**(4), 1192–1204.
124. R. Zimmermann, *Nachrichten aus der Chemie*, 2002, **50**(2), 166–169.
125. C. Cimander and C.F. Mandenius, *Journal of Chemical Technology and Biotechnology*, 2002, **77**(10), 1157–1168.
126. R. DeBono and J. J. Carroll, *LC-GC North America*, 2003, **21**(2 Suppl), 50.
127. J. W. R. Boyd and J. Varley, *Chemical Engineering Science*, 2001, **56**, 1749–1767.
128. M. Whitaker, G. R. Baker, J. Westrup *et al*., *International Journal of Pharmaceutics*, 2000, **205**, 79–92.
129. R. Belchamber, *Spectroscopy Europe*, 2003, **15**(6), 26–27.
130. H. Tsujimoto, T. Yokoyama, C. C. Huang and I. Sekiguchi, *Powder Technology*, 2000, **113**(1–2), 88–96.
131. A. Nordon *et al*., *Spectroscopy Europe*, 2002, 30–32
132. J. G. Bouchard, P.A. Payne and S. Szyszko, *Chemical Engineering Research and Design, Part A: Transactions of the Institute of Chemical Engineers*, 1994, **72**(A1), 20–25.PA67.
133. A. Nordon, R. J. H. Waddell, L. J. Bellamy *et al*., *Analyst*, 2004, **129**(5), 463–467.
134. P. Kippax and D. Higgs, *American Laboratory*, 2001, **33**(23), 8–110.
135. V. Buckin *et al*., *Spectroscopy Europe*, 2003, **15**(1), 20–25.
136. P. Mougin, A. Thomas, D. Wilkinson *et al*., *AIChE Journal*, 2003, **49**(2), 373–378.
137. H. Grön, P. Mougin, A. Thomas *et al*., *Industrial and Engineering Chemistry Research*, 2003, **42**(20), 4888–4898.
138. M. S. Klee and L.M. Blumberg, *Journal of Chromatographic Science*, 2002, **40**(5), 234–247.
139. J. V. Hinshaw, *LC-GC North America*, 2004, **22**(1), 32–40.
140. R. M. Al-Gailani Bashar and T. McCready, *Chemical Communications*, 2003, **9**(1), 120–121.
141. G. A. Eiceman, J. Gardea-Torresdey, E. Overton, K. Carney and F. Dorman, *Analytical Chemistry*, 2002, **74**(12), 2771–2780.
142. R. Nageswara and V. Nagaraju, *Journal of Pharmaceutical and Biomedical Analysis*, 2003, **33**(3), 335–377.
143. S. K. Paliwal, T. K. Nadler, D. I. C. Wang and F. E. Regnier, *Analytical Chemistry*, 1993, **65**, 3363–3367; and Y. Liu, F. S. Wang and W. C. Lee, *Biochemical Engineering Journal*, 2001, **7**(1), 17–25.
144. C. Turner, N. F. Thornhill and N. M. Fish, *Biotechnology Techniques*, 1993, **7**, 19–24.
145. A. Rehorek, K. Urbig, R. Meurer, C. Schäfer, A. Plum and G. Braun, *Journal of Chromatography A*, 2002, **949**(1–2), 263–268.
146. O. W. Gooding, T. Lindberg, W. Miller, E. Munyak and L. Vo, *Organic Process Research and Development*, 2001, **5**(3), 283–293.
147. M. R. Owen, C. Luscombe, L. W. Lai, S. Godbert, D. L. Crookes, D. Emiabata-Smith, *Organic Process Research and Development*, 2001, **5**(3), 308–323.
148. A. A. Rodrigo, H. Lorenz and A. Seidel-Morgenstern, *Chirality*, 2004, **16**(8), 499–508.
149. J. O. Mahony, *Analytica Chimica Acta*, 2005, **534**(1), 31–39.
150. H. Wainer and P. F. Velleman, *Annual Reviews in Psychology*, 2001, **52**, 305–335.
151. S. Wold, Chemometrics: What do we mean by it and what do we want from it?, presented at InCINC '94, Institute of Chemistry, Umea University, 1994.
152. J. Bronowski, *The Origins of Knowledge and Imagination*, Vail-Ballou, Binghampton, NY, 1978.
153. J. Zhang, E. B. Martin and A. J. Morris, *Chemical Engineering Research and Design, Part A: Transactions of the Institute of Chemical Engineers*, 1996, **74**(A1), 89–96.

154. S. Yoon and J. F. MacGregor, *AIChE Journal*, 2004, **50**(11), 2891–2903.
155. J. S. Qin, *Journal of Chemometrics*, 2003, **17**(8–9), 480–502.
156. T. Kourti, *Process Analytical Technology*, 2004, **1**(1), 13–19.
157. C. W. L. Wong *et al*., Realising the power of integration of process and spectroscopic data for batch process monitoring, *Proceedings of IChemE 7th World Congress of Chemical Engineering*, 2005, Glasgow UK.
158. A. M. C. Davies and T. Fearn, *Spectroscopy Europe*, 2005, **1**, 20–23.
159. M. L. Balboni, *Pharmaceutical Technology*, 2003, **10**, 54–67.
160. B. K. Lavine, *Analytical Chemistry*, 2000, **72**(12), 91R–97R.
161. R. G. Brereton, *Analyst*, 2000, **125**(11), 2125–2154.
162. J. Gabrielsson *et al*., *Journal of Chemometrics*, 2002, **16**(3), 141–160.
163. K. S. Setarehdan, *Journal of Chemometrics*, 2004, **18**(9), 414–421.
164. T. Hasegawa, *Trends in Analytical Chemistry*, 2001, **20**(2), 53–64.
165. C. Niedinski and R. Z. Morawski, *Journal of Chemometrics*, 2004, **18**(5), 217–230.
166. T. Leonard and J. S. J. Hsu, *Bayesian Methods*, Cambridge University Press, Cambridge, 1999.
167. M. Kubista *et al*., *Critical Reviews in Analytical Chemistry*, 1999, **29**(1), 1–28.
168. J. Workman J., *Chemometrics and Intelligent Laboratory Systems*, 2002, **60**(1–2), 13–23.

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