The Handbook of Metabonomics and Metabolomics
John C. Lindon, Jeremy K. Nicholson and Elaine Holmes (Editors)

Chapter 5

Chromatographic and Electrophoretic Separations Combined with Mass Spectrometry for Metabonomics

Ian D. Wilson

Department of Drug Metabolism and Pharmacokinetics
AstraZeneca, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK

Abstract

The current use of mass spectrometry, in combination with separation techniques such as gas and liquid chromatography and capillary electrophoresis, for metabonomics research is reviewed. Capillary gas chromatography (GC) is a very high resolution separation technique for this type of "global metabolite profiling", especially when GC × GC is performed, but requires extensive sample pre-treatment and derivatisation prior to analysis. High performance liquid chromatography (HPLC) and capillary LC provide high throughput, and generally require minimal sample preparation other than protein precipitation. The recently introduced ultra-performance liquid chromatography (UPLC), which is based on smaller particles and higher pressures than used in conventional HPLC, provides a significant improvement in terms of resolution and sensitivity. Capillary electrophoresis (CE) is another valuable liquid phase technique because of the alternative separation mechanism involved. The attributes of the various combinations are examined and the advantages and limitations of these techniques relative to each other and to NMR spectroscopy are briefly examined.

5.1. Introduction

The use of increasingly powerful and sophisticated instrumental techniques has resulted in enormous changes in the analytical landscape with respect to metabolite profiling. These advanced techniques have enabled the development of the non-targeted methods for "global" metabolite profiling that are essential for metabonomics. Whilst the bulk of the published applications in metabonomics have relied on the use of high field NMR spectroscopy, there is no doubt that other methodologies, particularly those based on mass spectrometric (MS) techniques, especially when linked to separations (often referred to as "hyphenation"), have the ability to contribute to metabonomics studies. Clearly to be useful for metabonomic studies an analytical technique would ideally provide as comprehensive a metabolic profile as possible and would not preferentially select particular classes of metabolites but be equally sensitive to all the compounds in the samples.

Moderate to high throughput is a real advantage for metabonomics as relatively large sample sets are a feature of this type of work and therefore a short analysis time, with minimal sample workup, is to be aimed for. In addition, a method should be sensitive and have a sufficiently broad dynamic range to be able to cope with the wide range of concentrations of metabolites present in the samples. The analytical techniques used should also provide both quantitative results and sufficient structural data to enable rapid and unambiguous biomarker identification.

Such requirements are very demanding and current MS-based methods can achieve some, but not all of them. However, it is arguable that there is no ideal method for obtaining these global metabolite profiles and that MS covers at least some of the bases. Here the current practice of high performance liquid chromatography coupled to mass spectrometry (HPLC-MS) [1] for metabonomics is considered together with gas chromatography-mass spectrometry (GC-MS) [2, 3] and capillary electrophoresis-mass spectrometry (CE-MS) [4, 5].

5.2. Gas Chromatography-Mass Spectrometry

GC-MS represents one of the oldest and most successful hyphenations of separation techniques to a mass spectrometer. Capillary GC provides an efficient and high resolution separation method and there are few practical problems in coupling to the interface of the mass spectrometer. Separations of complex mixtures of the type encountered in metabonomics are usually based on a programmed temperature gradient, with the most volatile components eluting first. In GC-MS there are essentially two modes of ionisation: electron impact ionisation (EI) and chemical ionisation (CI).

The EI technique provides mass spectra where the molecular ion may be weak but the spectra often contain a number of diagnostic fragments that can enable much structural identification to be carried out (especially when combined with searchable databases). CI, being a rather more "gentle" means of ionisation, provides mostly molecular ion information which can be very valuable as it enables the detection/confirmation of the molecular mass of the unknown.

GC provides a highly developed, stable, selective, sensitive and high resolution separation system. This capability is continually being enhanced and, with the introduction of GC-GC separations, combined with ever more powerful MS detectors, including ToF (time of flight) and ToF-ToF instruments, the comprehensive analysis of very complex samples should be possible. The use of ToF enables accurate masses to be obtained with the benefit that atomic compositions can be deduced providing further useful information for structure determination. Capillary GC-MS is in widespread use for metabolomics in areas such as micro-organisms and plants [2] but published applications of GC-MS to mammalian systems are harder to find.

The most obvious disadvantage of GC-based techniques is that the bulk of the components in biofluid or tissue samples are relatively involatile. There is therefore a requirement for a reasonable amount of pre-processing before the sample can actually be analysed, in order to convert the analytes into volatile derivatives before GC analysis is attempted [6]. In the case of a sample such as plasma it would be usual practice to remove interfering plasma proteins by precipitation with three volumes of acetonitrile [3, 7]. An aliquot of the supernatant would then be evaporated to dryness, followed by derivatisation with first methoxylamine hydrochloride (40 mg/mL in pyridine) at 28 °C for 90 min and then *N*-methyl-*N*-(trimethylsilyl)-trifluoracetamide (MSTFA) at 37 °C for 30 min. For aqueous samples such as urine, even though proteins are generally absent, it is still necessary to perform an extraction to enable the analytes to be dissolved in pyridine, as the derivatisation reactions cannot be performed in water. Extraction on to a suitable solid phase extraction (SPE) cartridge is a possible way of obtaining extracts (and can also be used to concentrate samples). However, with such an SPE step there is always the attendant risk of losing important metabolites that are not retained on the phase.

For such a multi-step process it is clearly necessary to institute good quality control (QC) procedures to ensure the validity of the final result. The usual way to do this where single analytes are being analysed is to use internal standards, however, it is difficult to control the extraction, derivatisation, chromatography and detection of hundreds of different analytes from disparate chemical classes, even using a number of internal standards. For this reason, in our studies we have adopted the pragmatic approach of using a pooled sample as a QC. This sample is distributed at random amongst the study samples and taken through the analytical process with them. Following analysis of all of the samples the data are processed via, for example,

principal components analysis (PCA) and the closeness of the clustering of the QC samples in the scores plot examined. Providing that all of the QC samples map close together, it can be assumed that the method has performed in a sufficiently reproducible fashion for the data from the study samples to be valid. If, on the other hand, there is a widespread distribution of the QC samples, it is reasonable to assume that there is a problem with the analytical method.

This extensive sample preparation required for GC-MS, together with the relatively long run-times currently associated with GC, means that the technique is relatively low throughput compared to some other technologies. However, this is compensated for to some extent by the very high separation efficiency of the system and the availability of large databases that greatly aid identification of unknowns.

An application of the potential of GC-MS for metabonomic analysis is shown by the example of the analysis of plasma from Zucker (fa/fa) obese and normal Wistar-derived animals given in Figures 5.1 and 5.2. The total-ion-current (TIC) traces shown are for GC-MS with EI (Figure 5.1a and b) and CI (Figure 5.2a and b). The excellent separation of these two classes using PCA on the GC-EI-MS data is shown in Figure 5.3. This figure also includes the data obtained for the QC samples (made by pooling together an aliquot of all of the study samples) which cluster

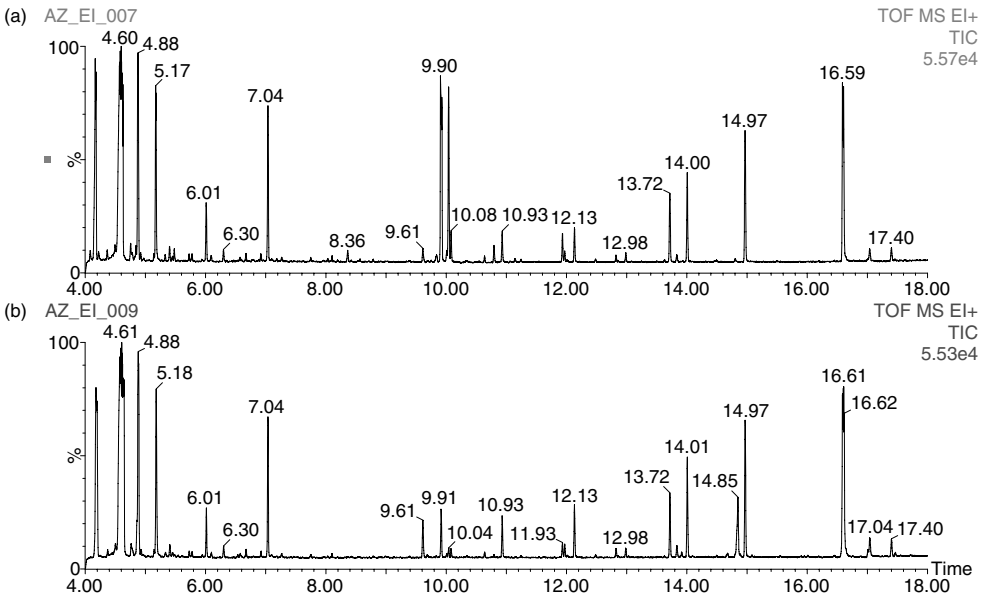

Figure 5.1. Typical TIC traces obtained from GC-EI-MS analysis of plasma obtained from (a) Wistar-derived and (b) Zucker (fa/fa) obese rats.

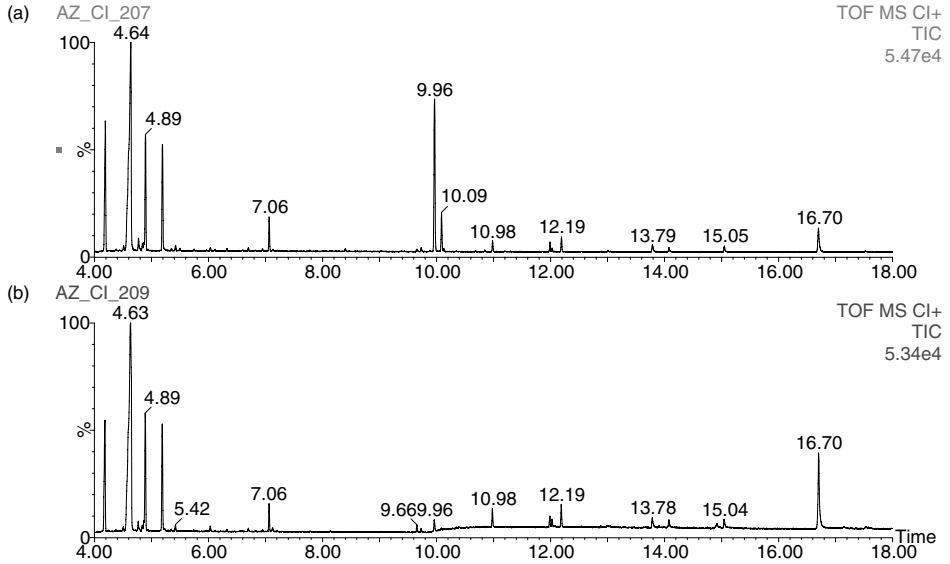

Figure 5.2. Typical TICs obtained from GC-CI-MS analysis of plasma obtained from (a) Wistar-derived and (b) Zucker (fa/fa) obese rats.

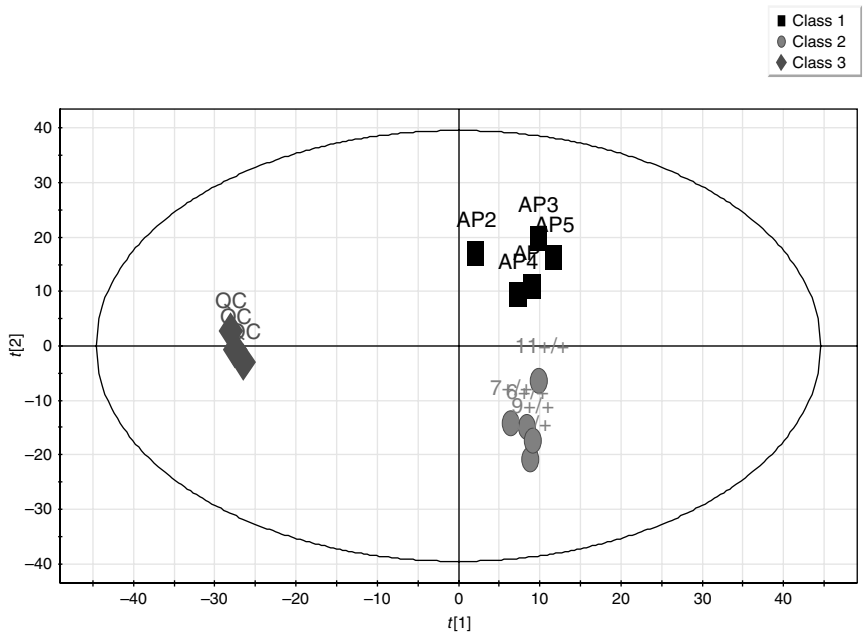

Figure 5.3. **S**cores plot (component 1 versus component 2) obtained following partial least squares discriminant analysis (PLS-DA) of data derived from GC-EI-MS analysis of plasma samples obtained from Wistar-derived (AP) and Zucker (fa/fa) obese (+/+) rats and QC samples analysed in the same run.

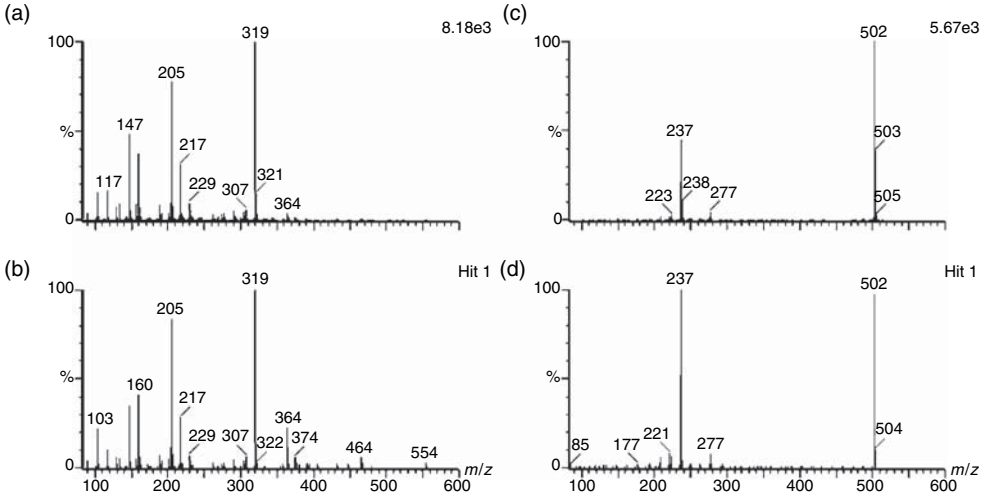

Figure 5.4. Identification of perturbed metabolites from GC-EI-MS analysis: (a) EI-MS for peak eluting at 9.91 min and (b) its library match D-glucose 2,3,4,5,6-pentakis-*O*-(trimethylsilyl-,*O*-methyloxime, (c) EI-MS for peak eluting at 16.58 min and (d) its library match tocopherol (vitamin E), trimethylsilyl derivative.

closely together providing confidence in the overall quality of the data. Figure 5.4 illustrates a typical set of spectra derived from these data for tocopherol.

The capabilities of GC-ToF-MS for obtaining global metabolite profiles of human plasma have recently been demonstrated where, following an automated (closed loop) optimisation, over 950 individual components were detected [3]. This procedure, which was also applied to yeast fermentation broths, involved optimisation of a number of instrumental parameters via a genetic algorithm and provided an almost threefold increase in the number of observable peaks. An example of the type of result possible following such an optimisation is illustrated in Figure 5.5.

A further, major, enhancement in the capabilities of GC-MS to metabolite profiling is the application of multi-dimensional GC × GC (sometimes referred to as "comprehensive GC). In GC × GC, two GC columns are used, the first generally long (typically 30 m) and the second much shorter (e.g. 1.5 m). Compounds eluting from the first column are trapped and focussed cryogenically on the second for a short time followed by a rapid separation on the 2nd column. By using different column chemistries the selectivity of the separation can be modulated, and improved separations can be obtained. Currently applications in metabolite profiling are sparse but in an example GC × GC-ToF-MS was used to examine extracts of spleen from obese NZO and lean C57BL/6 mice [8, 9]. Separations in the first dimension were performed on a 30 m × 250 μm capillary GC column coated with dimethyl polysiloxane (0.25 μm thickness) which separated compounds, using a thermal gradient,

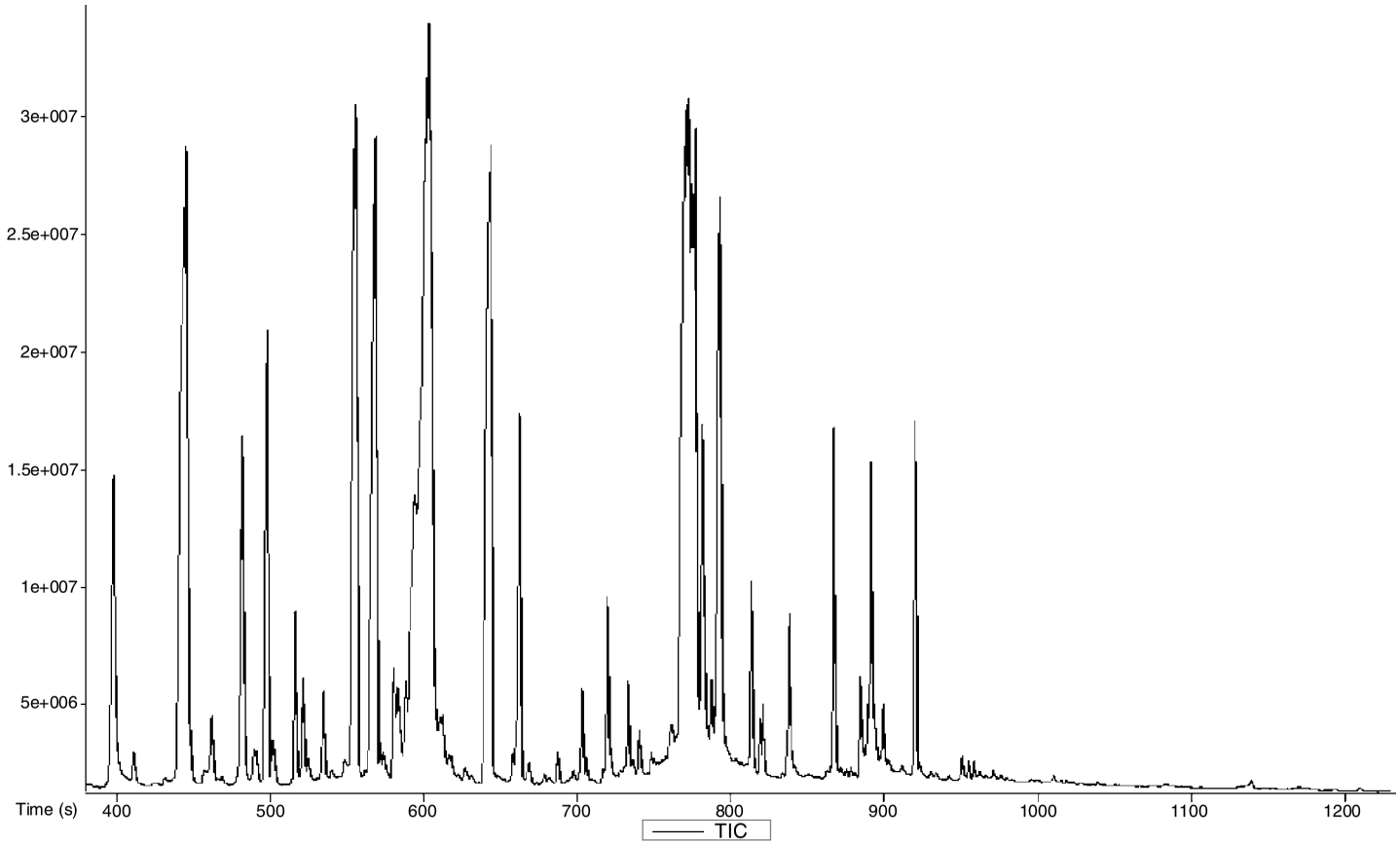

Figure 5.5 A typical total ion chromatogram of human serum using the method optimised as described in Reference 3.

based on volatility. Chromatography in the 2nd dimension was undertaken using a 1.5 m × 100 μm capillary coated with 50% polysilphenylene-siloxane (0.10 μm film thickness), a moderately polar phase, that separated compounds on relative polarity. Compared to conventional GC-ToF-MS (on a 30 m × 250 μm dimethyl-polysiloxane column (0.25 μm film thickness), which enabled the detection of 500 compounds, GC × GC-ToF-MS revealed the presence of some 1200 compounds with a run time of some 65 min.

Despite the current paucity of examples of the use of GC-MS and GC × GC-ToF-MS in metabonomics, there is clearly considerable potential for this powerful analytical technology to make a valuable contribution to the generation of global metabolite profiles and undoubtedly many more examples will be published in the future.

5.3. Liquid Chromatography-Mass Spectrometry

Unlike GC-MS, where the coupling of the separation technique and the spectrometer has proven to be relatively straightforward, the hyphenation of liquid chromatographic separations with mass spectrometers was technically much more demanding. As a result, HPLC-MS as a routine technique is a more recent addition to the bioanalytical tool box and for this reason HPLC-MS has only begun to be used for metabonomics relatively recently [1]. However, HPLC-MS is widely available in bioanalytical laboratories and for this reason, if no other, is destined to become increasingly important in metabonomics studies.

In general, reversed-phase gradient chromatography has been adopted for metabolite profiling work of the sort required for metabonomics studies. This type of separation is compatible with aqueous biological samples such as urine, making analysis possible with minimal sample preparation. More complex samples such as plasma do, however, require more extensive sample preparation. Protein precipitation using about two or three volumes of acetonitrile followed by centrifugation is generally required to prevent irreversible degradation of the HPLC column. Detection to date has usually been performed by electrospray ionisation (ESI), although CI is also an option. Self-evidently, detection in mass spectrometric methods depends upon the ionisation of the molecules in the sample. Because some analytes ionise better in positive ESI and others in negative ESI, it is good practice to analyse the samples using both ionisation modes (usually in separate analytical runs).

Something that has to be borne in mind with all liquid chromatography-mass spectrometry (LC-MS) analyses is that not all molecules ionise equally well leading to differences in sensitivity even when compounds are present in equal molar concentrations. In addition, HPLC-MS studies are complicated by the phenomenon known as "ion suppression" (and also enhancement) whereby the presence of co-eluting substances adversely affects the ionisation of a particular analyte causing its signal

to be reduced [10, 11]. In drug analysis in biological fluids, where the identity of the analyte is known, these effects can be studied, minimised through careful sample preparation and chromatographic optimisation and compensated for to some extent by the addition of an internal standard (usually a deuterated version of the analyte itself). However, in a complex mixture of unknown composition, such as a urine or plasma sample, where all of the components may be important, the strategies used for single known analytes are not easily applied. It seems clear therefore that, for a biomarker to be shown to be valid, once the analytes have been identified there is then a need to develop specific and comprehensively validated analytical methods. These methods can then be applied to the samples to confirm that the changes observed, for example, in plasma concentrations of particular substances really do correlate with an observed physiological change.

The very characteristics that make reversed-phase liquid chromatography so well suited to the direct injection of biological fluids with minimal sample preparation also make the technique vulnerable to failure as a result of column degradation and source contamination. Column degradation can reveal itself by changes in column performance (loss of peak shape, changes in retention time) whilst contamination of the ion source usually results in loss of sensitivity. The need for careful monitoring and QC of the methodology in order to ensure the validity of the conclusions is therefore essential if valid data are to be produced. The regular injection of test mixtures through the run can clearly help in determining whether or not system performance is maintained, and internal standards may also have a role to play. However, given the complexity and structural diversity of the molecules present in biological samples then the difficulty of choosing an internal standard(s) is obvious. Once again we have adopted the pragmatic approach of using a pooled sample that is run at regular intervals throughout the analysis in order to monitor the behaviour of the LC-MS analyses undertaken on biofluid samples.

5.4. HPLC-MS for metabonomics

Conventional HPLC separations for metabonomic analysis are undertaken using 4.6 or 3.0 mm i.d. columns, of between 5 and 25 cm in length, packed with 3–5 μm solid materials. These analyses are usually performed using reversed-phase gradients in order to separate the widest range of molecules in a single run. In our own work, the typical chromatographic conditions that we have employed involved separation on a 2.1 mm × 10 cm Symmetry® C18, 3.5 μm column held at 40 °C in a column oven and solvents composed of 0.1% formic acid and acetonitrile. Elution was performed using a two part gradient from 0 to 20% acetonitrile over 0.5–4 min. and then to 95% acetonitrile over the next 4 min. The eluent composition is then held at 95% acetonitrile for 1 min. before returning to 100% aqueous formic acid at 9.1 min. For a

column of these dimensions a flow rate of 600 μl/min was used (with 100 μl directed into the ion source of the mass spectrometer), and typically 5–10 μl of rat urine would be injected. This protocol results in an analysis time of ca. 10 min per sample.

The recently published applications of HPLC-MS for metabonomics studies include examples in the investigation of toxicity [12–18], metabotyping, where, for example, strain, gender, aging and diurnal variation in rodents have been studied [19–21] and for disease models [20]. Examples of the results obtained for a typical HPLC-MS analysis of mouse urine (in both +ve and −ve ESI) are shown in Figure 5.6. The TIC traces themselves are not particularly informative, though it is possible to discern differences in the profiles obtained depending upon the ionisation techniques used. However, PCA of the data obtained in this way to compare, for example, different strains of mouse, as shown in Figure 5.7, reveal the power of HPLC-MS as a means of distinguishing animal strains.

Studies on the urine of male and female Zucker (fa/fa) obese rats [20], using both ^{1}H-NMR spectroscopy and HPLC-MS, allowed both diurnal and gender-based differences to be determined. Whilst diurnal effects were marked by increases in taurine, creatinine, allantoin and α-ketoglutarate in the ^{1}H NMR spectra of the Zucker rats for the evening samples, HPLC-MS indicated that (unidentified) ions at *m/z* 285.0753, 291.0536 and 297.1492 in positive ESI, and 461.1939 in negative ESI were elevated in these samples. Discrimination between male and female Zucker rats was also obtained using both analytical approaches, with HPLC-MS affording the clearest distinction. Thus, in the ^{1}H NMR spectra hippuric acid, succinate, α-ketoglutarate and dimethylglycine were higher in the urine of females compared to males, together with ions at m/z 431.1047, 325.0655, 271.0635 and 447.0946 in positive ESI, and m/z 815.5495 and 459.0985 in negative ESI by HPLC-MS. In addition, a comparison was also performed against samples of urine obtained from a Wistar-derived rat strain with ^{1}H NMR spectroscopic analysis pinpointing higher concentrations of taurine, hippurate and formate and decreased betaine, α-ketoglutarate, succinate and acetate in Zucker versus Wistar-derived rats. The HPLC-MS detected increased hippurate and unidentified ions at m/z 255.0640 and 285.0770 in positive, and 245.0122 and 261.0065 in negative ESI respectively.

More recently, we have used a similar approach of combining both ^{1}H NMR spectroscopy and HPLC-ToF/MS using ESI to study the effect of aging and development [21] in Wistar-derived rats on the profile of endogenous urinary metabolites. Samples were collected from male rats every 2 weeks, from just after weaning at 4 weeks up to 20 weeks of age, and the resulting spectroscopic data were analysed using multivariate data analysis. This enabled age-related metabolite changes to be detected, with urine samples collected at 4 and 6 weeks showing the greatest differences by both analytical techniques. Markers detected by ^{1}H NMR spectroscopy included creatinine, taurine, hippurate and resonances associated with amino acids/fatty acids, which increased with age, whilst citrate and resonances

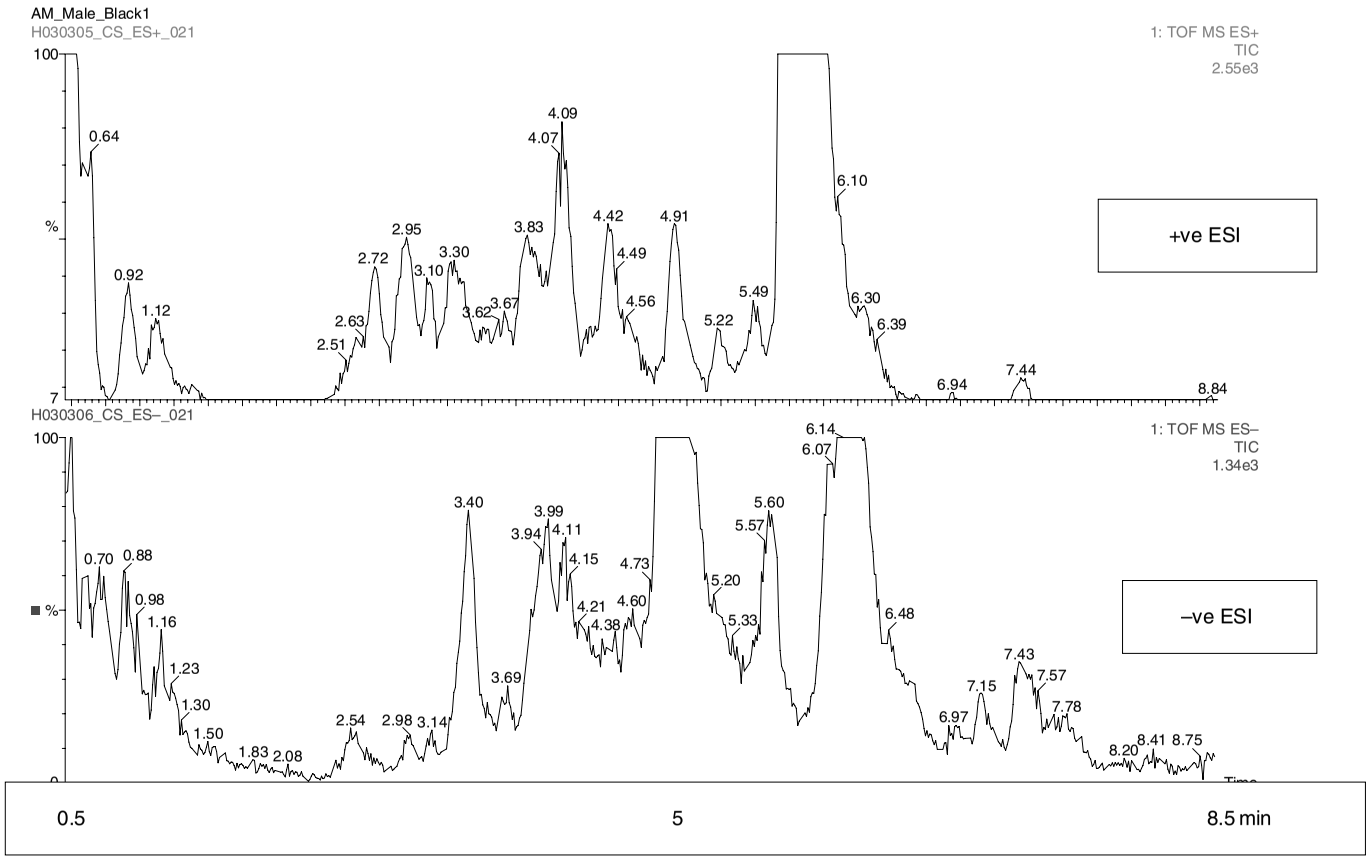

Figure 5.6. Gradient HPLC-oa-ToF-MS of mouse urine. A) total ion current for +ve and B) −ve ion electrospray. The column was a Waters Symmetry C18, 2.1 mm × 10 cm, 3.5 μm packing. Elution was via a linear gradient from 0 to 20% acetonitrile versus 0.1% formic acid in water from 0.5 to 4 min and then 95% acetonitrile at 8 min, followed by a return to 100% aqueous formic acid at 9 min. The column eluent was monitored by ESI-oa-TOF-MS from 100–1400 *m/z*.

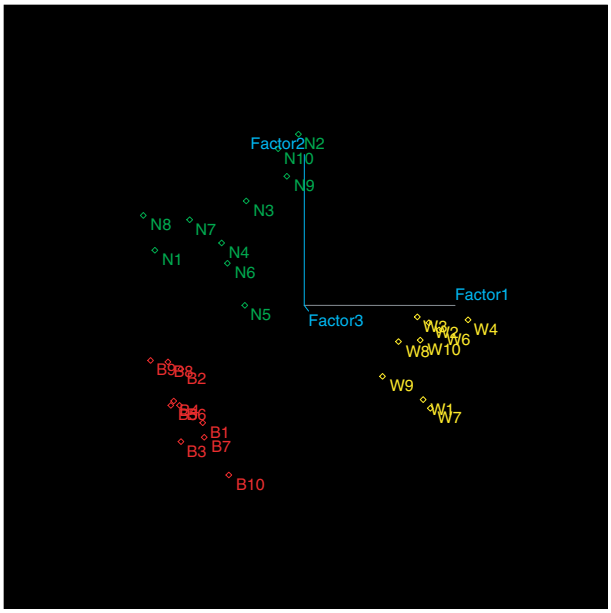

Figure 5.7. Scores plot for the PCA of LC-MS data obtained from urine of male animals from three mouse strains (key: green = nude, yellow = white and red = black). The samples were collected in the morning.

resulting from glucose/myoinositol declined. Interestingly a number of ions were detected with HPLC-MS that were only present in the 4 week urine samples, in both positive and negative ESI. The HPLC-MS analysis also showed age-related increases for a number of compounds, including, for example, carnitine which increased in samples from the older animals. A conclusion from both of these studies in the rat where ^{1}H NMR spectroscopy and HPLC-MS were used was that their use together provided a powerful and complementary approach to sample analysis, and this is also a conclusion of studies in the area of toxicology discussed below.

In addition to the metabotyping experiments described above, there have also been a significant number of applications concerning the investigation of study of toxicity in rats. The first application of HPLC-MS in this area involved an undisclosed candidate drug administered to both male and female rats at two dose levels over a 3-month period [11]. One of the ions detected in the urine of these animals, that appeared to be associated with toxicity in this study, was identified as indican (a metabolite of tryptophan). Identification was made using a combination of accurate mass, MS/MS and comparison with an authentic standard. Shortly afterwards a study of the effects of the drug citalopram was described as part of investigations of phospholipidosis [12]. Rather than use direct injection of the samples, these were subjected to solid phase extraction on a polymeric phase.

The investigation of the nephrotoxicity of heavy metal salts such as uranium nitrate or cadmium chloride using HPLC-MS, with both +ve and −ve ESI, was described [14] using reversed-phase chromatography on an Xterra MS C18 bonded material with a long gradient (ca. 2 h). Markers of toxicity were determined by a manual examination of the data, with compounds such as riboflavin, phenol sulfate and ferrulic acid, amongst others ions identified.

In a series of studies, we have investigated the application of gradient reversed-phase HPLC-ToF-MS, with both + and −ve ESI, for the analysis of urine obtained from rats exposed to a number of nephrotoxins [15–18]. In the first of these studies, an investigation was performed of the effects of the administration of a single 2.0 mg/kg s.c. dose of mercuric chloride to male Wistar-derived rats on the urinary metabolite profiles [15]. In this study, urine was collected for 9 days and then analysed using both HPLC-oa-ToF-MS and ^{1}H NMR spectroscopy. Unsurprisingly both methods of analysis identified marked changes in the pattern of endogenous metabolites with the most pronounced disturbances observed at 3 days post-dose. Thereafter, the metabolite profile gradually returned to a more normal composition. The HPLC-MS-detected markers of nephrotoxicity seen using positive ESI included decreased amounts of kynurenic, xanthurenic and pantothenic acids as well as 7-methylguanine. In addition, an ion at m/z 188, possibly 3-amino-2-naphthoic acid, was observed to increase whilst several unidentified ions at, for example, m/z 297 and 267 decreased after $HgCl_2$ administration. Analysis with negative ESI revealed a number of sulfated compounds such as phenol sulfate and benzene diol sulfate, both of which appeared to decrease in concentration in response to dosing, together with an unidentified glucuronide (m/z 326).

Similar studies on the nephrotoxicity of the immunosuppressant, cyclosporin A[16], involved 9 daily doses of 45 mg/kg/day of the drug. Disturbances of the urinary metabolite profile were only observed after 7-days dosing. The HPLC-MS interpretation of the data was complicated in this instance as a result of the presence of ions derived from cyclosporin, its metabolites and the dosing vehicle which had to be removed prior to PCA. However, as noted with the mercuric chloride example above, an excellent concordance was seen between HPLC-MS and NMR spectroscopy for the time course of toxicity.

A third example of the study of nephrotoxicity using HPLC-MS involved the twice daily administration 60 mg/kg of the antibiotic gentamicin for 7 days [17]. Changes in the pattern of endogenous metabolites were readily detected shortly after the start of dosing using both positive and negative ESI, with the major changes observed beginning on days 5/6 of the study. The MS data showed, as observed for mercuric chloride, reduced xanthurenic acid and kynurenic acid whilst neutral loss experiments also revealed a changed pattern of sulfate conjugation on gentamicin administration. A similar time course was noted when the same samples were analysed using ^{1}H NMR spectroscopy followed by PCA. However, such data require

careful interpretation and it is likely that some of the observed changes were due not to the toxicity of the drug on the kidney but to the toxicity of the compound to the gut microflora resulting in changes in the urinary profile of microflora-derived metabolites.

In the same way that differences in patterns of a subset of metabolites were examined in the above study, a recent report described a metabonomic study where constant neutral loss experiments were performed in order to detect only mercapturates present in the urines of human volunteers who were administered 50–500 mg of acetaminophen (paracetamol) [22].

HPLC-ToF-MS was also used to investigate urinary metabolic perturbations associated with D-serine-induced nephrotoxicity [18]. D-Serine is known to cause selective necrosis of the proximal straight tubules in the rat kidney accompanied by aminoaciduria, proteinuria and glucosuria. In these studies, Wistar-derived rats received either D-serine (250 mg/kg i.p.) or vehicle (deionised water), and urine was collected up to 48 h post-dose. Samples were then analysed using reversed-phase gradient HPLC with ToF-MS in both positive or negative ion mode. Changes to the urinary profile were detected at all time points after administration. For example, in negative ESI-MS increased concentrations of serine, an ion at m/z 104.0376 (possibly hydroxypyruvate) and glycerate were detected (the latter being a metabolite of D-serine). Also observed were increased amounts of tryptophan, phenylalanine and lactate, together with decreased methylsuccinic and sebacic acids. In the case of positive ESI, decreased concentrations of xanthurenic acid were noted, together with a general aminoaciduria, including proline, methionine, leucine, tyrosine and valine and an increase in acetylcarnitine.

As well as applications in animal models, HPLC-MS has also been used for applications on human samples for diseases such as type-2 diabetes [23], liver cancer, hepatitis and liver cirrhosis [24]. Although described as metabonomics applications by the authors in fact, in both cases, only a subset of the total metabolic profile was studied (either phospholipids or *cis*-diols) making these studies more akin to conventional class-specific metabolic profiling. However, as the separation methodology employed, including the use of multivariate statistical analysis, is relevant, both applications are briefly described here.

In the application to type-2 diabetes [23] normal phase chromatography was performed on a silica-based "diol" stationary phase using gradient elution. The solvents used for chromatography were mixtures of hexane: propan-1-ol:formic acid:aqueous ammonia (79:20:0.6:0.07 v/v) and propan-1-ol:water:formic acid:aqueous ammonia (88:10:0.6:0.07 v/v) at a flow rate of $0.4\,\mathrm{ml}\cdot\mathrm{min}^{-1}$. With re-equilibration of the column following the gradient the analysis took ca. 60 min per sample. Plasma samples were analysed for phospholipids by ESI-MS/MS, with negative ESI used for profiling and both positive and negative ESI for characterisation and identification.

Multivariate statistical analysis of these data enabled the authors to distinguish between normal and diabetic subjects.

For the analysis of urine from subjects suffering from a variety of liver diseases [24], reversed-phase gradient chromatography was performed using 5 mm ammonium acetate and methanol as solvents on a 25 cm × 4.6 mm i.d. column packed with a 5 μm ODS phase with ESI-MS/MS. Run times, including re-equilibration, were of the order of 70 min. Samples were prepared using extraction onto phenylboronic acid solid-phase extraction cartridges to extract the required *cis*-diols (mainly metabolites of nucleic acids). The data were then analysed by multivariate statistical methods following peak alignment. Identification of the biomarkers found by this approach was using a combination of MS/MS and authentic standards. Some eight such compounds were detected which enabled patients with liver cancer to be distinguished from subjects with other types of liver disease.

5.5. Capillary LC-MS

Alternatives to conventional HPLC such as narrow bore (ca. 2 mm i.d.), micro bore (0.5, 1.0 mm i.d.) and capillary HPLC column formats have also been employed for metabolite profiling [25, 26]. Capillary HPLC offers similar benefits for the analysis of complex mixtures to capillary separations in gas chromatography and, by providing an increased number of theoretical plates via a combination of smaller particles and increased column length, results in greater peak capacity. In addition, sample requirements are modest. However, the use of very long capillaries also requires the use of high operating pressures to force the solvent through the column, and this can prove a limiting factor.

There have been a number of applications of capillary LC to metabonomic and metabolomic assays. In one of these, C18-bonded silica monolithic columns (0.2 mm i.d.), of between 30 and 90 cm in length, were used in combination with MS detection for the analysis of extracts of the plant *Aradopsis thaliana* [25]. In another example capillary LC-MS with 10 cm × 320 μm columns filled with a 3.5 μm C18-bonded packing material was employed for the analysis of urine obtained from Zucker rats [26]. A gradient separation was used in this application (from 0 to 95% acetonitrile with 0.1% aqueous formic acid). In this example approximately twice as many ions were detected for the same samples with the capillary separation compared to conventional HPLC-MS with columns of the same length packed with the same stationary phase whilst consuming a fraction of the sample. Despite the much reduced amount of sample introduced into the system, a much better sensitivity was noted (ca. 100-fold for some metabolites) for the capillary method compared to HPLC-MS on the same samples. The increase in the number of components observed using the capillary separation reflects reduced ion suppression compared to the conventional

separation. Interestingly, in this study it was possible to observe diurnal variation in sample composition for both male and female animals using data derived from either HPLC-MS or capillary LC-MS analysis, however, there was little overlap in the ions detected that enabled this classification to be made.

As indicated above, the use of long capillaries requires high operating pressures. Recently the use of such high pressures (20 kpsi), in combination with gradient reversed-phase capillary chromatography, has been reported for the separation of cell lysates of the microorganism *Shewanella oneidnedensis* [27]. The separation was performed at 20 kpsi using a 50 μm i.d. fused silica capillary, 200 cm in length, packed with a stationary phase of 3 μm porous C18-bonded particles. The gradient separation resulted in the detection of more than 5000 metabolites. However, whilst the separation was impressive, the analysis time was long with some 2000 min required for completion.

5.6. Ultra performance Liquid Chromatography-Mass Spectrometry

One of the problems associated with biological samples is their complexity, which places a considerable strain on the ability of the separation system employed to resolve all of the components. Recently chromatography on 1.7 μm stationary phases has been introduced in the form of UPLC (Ultra performance LC)-MS offering a substantial improvement in performance for complex mixture analysis compared to conventional HPLC. The use of such packing materials generally requires the use of much higher operating pressures than are normally encountered in HPLC to achieve high flow rates. However, this combination of high pressure and small particle size results in separations of much greater efficiency than can be obtained using conventional technology. A typical example of the UPLC-MS of mouse urine is shown in the TIC shown in Figure 5.8. The improved resolution and increased number of peaks detected in the UPLC-MS run compared to conventional HPLC-MS is clear when these results are compared to those shown in Figure 5.6.

The first metabonomics application of UPLC-MS was a "functional genomic" investigation involving obtaining metabolic profiling of urines from males and females of two groups of phenotypically normal mouse strains (C57BL19J and Alpk:ApfCD) and a "nude mouse" strain [28]. When compared with conventional HPLC-MS under similar analytical conditions an improved phenotypic classification was seen by the use of UPLC-MS.

As well as the use of UPLC to increase the number of peaks detected in the same run time as a normal HPLC analysis, it can also be used to drastically reduce analysis time for high throughput analyses. An example of this is provided by the use of UPLC with a rapid (1.5 min) reversed-phase gradient applied to the analysis of urine samples from rodents, including normal and Zucker (fa/fa) obese rats and three

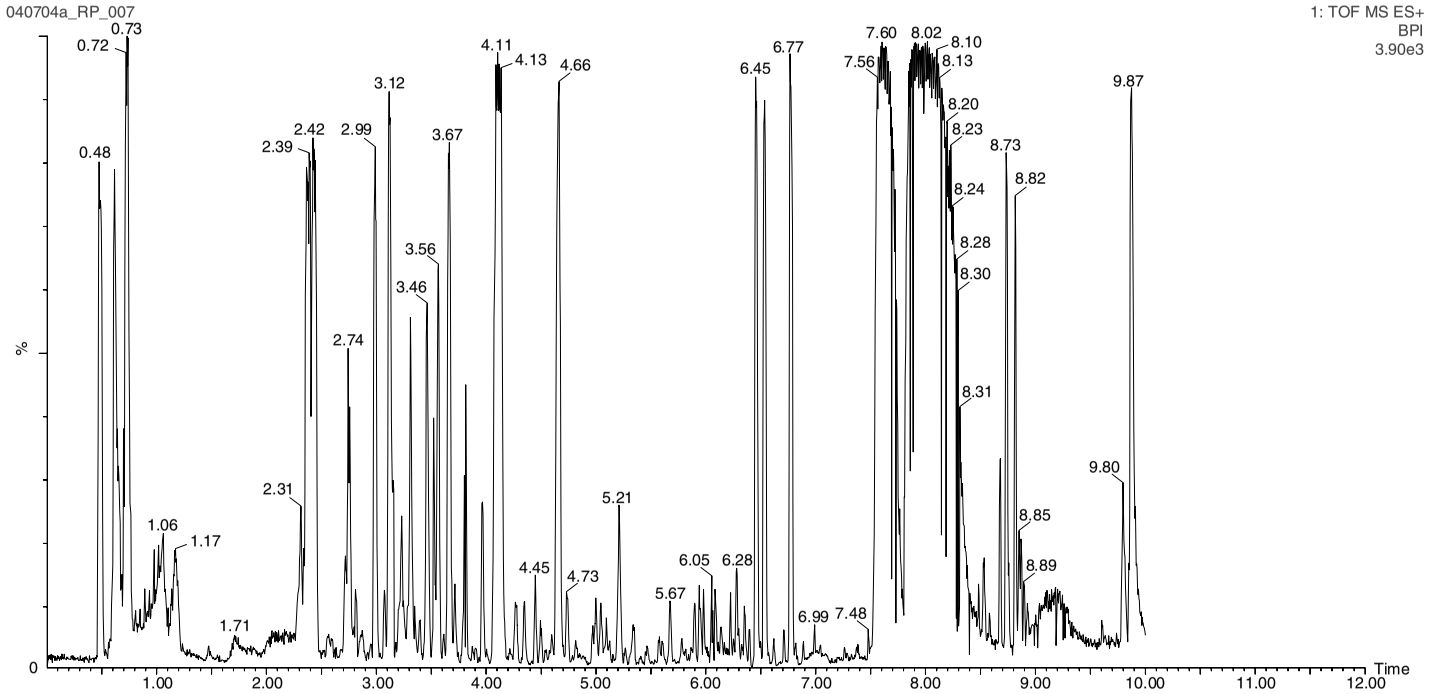

Figure 5.8. UPLC separation of white female mouse urine on a 2.1 mm × 5 cm Waters ACQUITY™ 1.7 μm C18 column. The column was eluted with 0–95% aqueous formic acid: acetonitrile (0.1% formic acid) gradient (10 min) at 500 μL/min. The column eluent was monitored by ESI oa-TOF-MS from 50 to 850 *m/z* in positive ion mode. The sample was collected in the morning.

strains of mice (of both sexes) [29]. This methodology enabled rapid discrimination between age, strain, gender and diurnal variation. The peak capacity and the number of marker ions detected using these fast UPLC separations and oa-TOF MS was found to be similar to that generated by conventional HPLC-MS methods with a 10 min separation.

5.7. Capillary zone electrophoresis-Mass Spectrometry

Another separation technique with considerable potential that can provide metabolite profiles is capillary zone electrophoresis-MS (CZE-MS, CE-MS). To date there have been relatively few published applications of CE-MS, and these have been concentrated in the area of bacterial metabolomics. In these studies, samples have been investigated using "targeted" analyses against a panel of up to ca. 1700 standards [4, 5]. Like capillary HPLC an advantage of CE methods is that they require only very small samples which need little or no preparation for samples such as urine. Because the separation is based on electrophoresis and therefore on charge, a different separation mechanism is utilised compared to HPLC or GC with potential benefits to selectivity and many further applications of the techniques may be anticipated in the future.

5.8. GC-, LC- and CE-MS for metabonomics: A perspective

From the examples provided above it is clear that the combination of MS with any of the three types of separation techniques provides a very powerful addition to the metabolite profiling techniques available for metabonomics. However, all MS-based techniques suffer from the disadvantage that the response of the detector is compound dependent, and can vary widely. Thus, for detection in MS it is necessary to form ions and, if a compound ionises poorly it will not be detected with great sensitivity. Conversely, compounds that ionise well compared to others of similar concentration will dominate the TIC. The widely touted advantage of the great sensitivity of MS compared with many other analytical techniques (in particular, NMR spectroscopy) must therefore be viewed in this context. Because of the compound-dependent response of MS, absolute, rather than relative, quantification is impossible in the absence of an authentic standard, and if the biomarkers are unknown, or not commercially available this can be problematic. In addition, ESI and, to a lesser extent APCI, the most popular ionisation techniques used in liquid-based separations such as HPLC and CE are subject to the phenomenon of ion suppression/ion enhancement. This phenomenon can result in apparent changes in the relative amounts of a component where none have occurred and reinforces

the need to identify potential biomarkers and then develop specific and validated bioanalytical methods to confirm their value.

It is also worth noting that GC, LC and CE are not equivalent to each other and each has advantages and disadvantages. Thus the tremendous resolving power and sensitivity of GC and GC × GC-based MS come at the price of a need for extensive sample preparation. The high resolution of GC and GC × GC also requires fairly lengthy analysis times. A useful feature of GC-MS-based metabonomics is that there are already in existence extensive databases of structures to aid in biomarker identification (although these are not yet comprehensive). For liquid chromatography, the advantages include the potential for minimal sample preparation and high throughput. There are currently no equivalents in LC of the extensive databases available for GC making the identification of unknowns more difficult. However, it is fairly easy to scale up HPLC separations to a preparative level and this allows for the isolation and identification of unknowns using a battery of spectroscopic techniques. The widespread availability of HPLC-MS, combined with its relative ease of use, suggests that it will become very widely applied in metabonomics/metabolomic research. However, our own, limited, studies comparing the information derived from the analysis of plasma samples with GC, UPLC and ^{1}H NMR spectroscopy have illustrated the complementary nature of these techniques [7]. We would therefore, like others, advocate a multianalytical-platform approach to global metabolite profiling rather than an over-reliance on a single technology.

Similarly CE-MS, whilst currently less widely available, currently combines the advantages of LC in terms of minimal sample preparation and ease of use with a complementary separation mechanism (based on charge). CE therefore provides a different selectivity which may be advantageous in the analysis of complex mixtures as biofluids.

Clearly, in the case of all of these methods a complex 3D dataset results comprising of retention time, signal intensity and mass. Factors that can complicate the analysis of these data include the potential for drift in retention times and mass accuracy, together with time-dependent changes in detector response. Whilst the discussion of data processing is outside the scope of this article, the use of appropriate quality control measures at all stages of the analytical process is clearly essential if valid and meaningful biomarkers are to be determined.

5.9. Conclusions

The application of hyphenated-MS systems to metabonomics is still at a relatively early stage of development, but there is absolutely no doubt that these techniques will make a substantial contribution to the future development of the field. The ease of use and widespread availability of LC-MS systems will probably ensure that the

bulk of the applications will be performed using these instruments. However, the power of GC, especially GC × GC, will ensure an important and continuing role for the technique in this area. Similarly CE-MS, with its high resolution, will also develop into an important tool for metabolite profiling.

References

[1] Wilson, I.D., Plumb, R., Granger, J., Major, H., Williams, R., Lenz, E.M. *J.Chrom. B*. **2005**, *817*, 67–76.

[2] Fiehn, O., Kopka, J., Trethewey, R.N., Willmitzer, L. *Anal. Chem.* **2000**, *72*, 3573–3580.

[3] O'Hagan, S., Dunn, W.B., Brown, M., Knowles, J.D., Kell, D.B. *Anal.Chem.*, **2005**, *77*, 290–303.

[4] Soga, T., Ohashi, Y., Ueno, Y., Naraoka, H., Tomita, M., Nishioka, T. *J. Proteome Res.* **2003**, *2*, 488–494.

[5] Jia, L., Terabe, S. in *Metabolome Analysis*, eds Vaidyanathan, S., Harrigan, G.G., Goodacre, R, Springer, New York, **2005**, pp. 83–101.

[6] Halket, J.M., Waterman, D., Przyborowska, A.M., Patel, R.K.P., Fraser, P.D., Bramley, P.M. *J.Exp. Bot*. **2005**, *56*, 219–243.

[7] Williams, R., Lenz, E.M., Wilson, A.J., Granger, J., Wilson, I.D., Major, H., Stumpf, C., Plumb, R. *Mol. Biosystems,* **2006**, *2*, 174–183.

[8] Welthagen, W., Shellie, R.A., Spranger, J., Ristow, M., Zimmermann, R., Fiehn, O., *Metabolomics*, **2005**, *1*, 65–73.

[9] Shellie, R.A., Welthagen, W., Zrostlikova, J., Spranger, J., Ristow, M. Fiehn, O., Zimmermann, R. *J. Chrom. A*. **2005,** *1086*, 83–90.

[10] Matuszewski, B.K., Constanzer, M.L., Chavez-Eng, C.M *Anal. Chem.*, **1998**, *70*, 882–889.

[11] Law, B., Temesi, D, *J.Chromatogr., B*, **2000**, *748*, 21.

[12] Plumb, R.S., Stumpf, C.L., Gorenstein, M.V., Castro-Perez, J.M., Dear, G.J., Anthony, M., Sweatman, B.C., Connor, S.C., Haselden, J.N. *Rapid Commun. Mass Spectrom*, **2002**, *16*, 1991–1996.

[13] Idborg-Bjorkman, H., Edlund, P.-O., Kvalheim, O.M., Schuppe-Koistinen, I. Jacobsson S.P. *Anal. Chem*. **2003**, *75*, 4784–4792.

[14] Lafaye, A., Junot, C., Ramounet-le Gall, B., Fritsch, P., Tabet, J.-C., Ezan, E., *Rapid Commun. Mass Spectrom*., **2003**, *17*, 2541–2549.

[15] Lenz, E.M., Bright, J., Knight, R., Wilson, I.D., Major, H. *The Analyst.* **2004**, *129*, 535–541.

[16] Lenz, E.M., Bright, J., Knight, R., Wilson, I.D., Major, H. *J. Pharm. Biomed. Anal*. **2004**, *35*, 599–608.

[17] Lenz, E.M., Bright, J., Knight, R., Westwood, F.R., Davies, D., Major, H. Wilson, I.D. *Biomarkers.* **2005**, *10*, 173–187.

[18] Williams, R.E., Major, H., Lock, E.A., Lenz, E.M: Wilson, I.D. *Toxicology.* **2005**, *207*, 179–190.

[19] Plumb, R.S., Granger, J., Stumpf, C., Wilson, I.D., Evans, J.A., Lenz, E.M. *The Analyst*, **2005**, *128*, 819–823.

[20] Williams, R.E., Lenz, E.M., Evans, J.A, Wilson, I.D., Granger, J.H., Plumb, R., Stumpf, C. *J. Pharm. Biomed. Anal*. **2005**, *38*, 465–471.

[21] Williams, R.E., Lenz, E.M., Lowden, J., Rantalainen, M., Wilson, I.D., *Mol. Biosystems*, **2005**, *1*, 166–180.

[22] Wagner, S., Scholz, K., Donegan M., Burton, L., Wingate, J., Volkel, W., *Anal Chem.* **2005**.

[23] Wang, C., Kong, H., Guan, Y., Yang, J., Gu, J., Yang, S., Xu, G., *Anal. Chem*., **2005**, *77*, 4108–4116.
[24] Yang, J., Xu, G., Zheng, Y., Kong, Y., Wang, C., Zhao, X., Pang, T., *J. Chromatogr., A*, **2005**, *1084*, 214–221.
[25] Tolstikov, V.T., Lommen, A., Nakanishi, K., Tanaka, A., Fiehn, O. *Anal. Chem*., **2003**, *75*, 6737–6740.
[26] Granger, J., Plumb, R., Castro-Perez, J., Wilson, I.D., *Chromatographia*, **2005**, *61*, 375–380.
[27] Shen, Y., Zhang, Y., Moore, R.J., Kim, J., Metz, T.O., Hixon, K.K., Zhao, R., Livesay, E.A., Udseth, H.R and Smith, R.D., *Anal. Chem*., **2005**, *77*, 3090–3100.
[28] Wilson, I.D., Nicholson, J.K., Castro-Perez, J., Granger, J.H., Johnson, K., Smith, B.W., Plumb, R.S. *J. Proteome Res*. **2005**, *4*, 591–598.
[29] Plumb, R.S., Granger, J.H., Stumpf, C.L., Johnson, K., Smith, B.W., Gaulitz, S., Wilson, I.D., Castro-Perez, J. *The Analyst*, **2005**, *130*, 844–849.