MASS SPECTROMETRY-BASED HOLISTIC ANALYTICAL APPROACHES FOR METABOLITE PROFILING IN SYSTEMS BIOLOGY STUDIES

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Metabonomics and metabolomics represent one of the three major platforms in systems biology. To perform metabolomics it is necessary to generate comprehensive "global" metabolite profiles from complex samples, for example, biological fluids or tissue extracts. Analytical technologies based on mass spectrometry (MS), and in particular on liquid chromatography–MS (LC–MS), have become a major tool providing a significant source of global metabolite profiling data. In the present review we describe and compare the utility of the different analytical strategies and technologies used for MS-based metabolomics with a particular focus on LC–MS. Both the advantages offered by the technology and also the challenges and limitations that need to be addressed for the successful application of LC–MS in metabolite analysis are described. Data treatment and approaches resulting in the detection and identification of biomarkers are considered. Special emphasis is given to validation issues, instrument stability, and QA/quality control (QC) procedures. © 2011 Wiley Periodicals, Inc., Mass Spec Rev 30:884–906, 2011
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I. INTRODUCTION

A. Metabolomics and Metabonomics in Systems Biology

Metabonomics is defined as the "the quantitative measurement of the dynamic multiparametric response of a living system to pathophysiological stimuli or genetic modification" (Nicholson, Lindon, & Holmes, 1999; Nicholson et al., 2002). Metabolomics, on the other hand, has a number of definitions ranging from the "systematic study of the unique chemical fingerprints that specific cellular processes leave behind" to "the total biochemical complement of a cell or particular organ." Metabolomics specifically studies small-molecule metabolite profiles (Fiehn, 2002; Daviss, 2005). However, as the field of global metabolite profiling has developed the distinction has become blurred and the use of the terms has tended to become one of personal preference (for a recent discussion, see Lindon & Nicholson, 2008). Irrespective of the term used both approaches apply holistic analytical strategies to collect untargeted analytical data to investigate the patterns and concentrations of low molecular weight organic metabolites in biofluids, cells, or organs within an organism and in the present article both terms will be used. Metabolite profiling is the third "omic" (the other two being genomics and proteomics), an integral and very important part of the systems biology field (see Fig. 1). There is therefore a requirement that the analytical methodologies used produce comprehensive and representative global metabolite profiles from such complex biological samples. As we have noted previously the ultimate metabolite profiling platform for such analyses would provide analysis directly on the samples, without sample preparation or storage, and would provide unbiased results (with respect to different classes of metabolites), rapidly and, at the same time, would also be highly (and equally) sensitive to all the metabolites present in the sample, with a wide dynamic range, and be robust and reproducible (Lenz & Wilson, 2007). These desirable features should also be combined with high information content, which in return would enable straightforward identification of key metabolites. Unfortunately there is no technique currently available that can provide all of the desired properties. Not surprisingly the consensus reached in related scientific meetings, and the common practice of active researchers in the field, is to try to employ multiple analytical platforms, to maximize metabolite coverage of the sample set (see, e.g., Lenz et al., 2004a,b and Wishart's articles and work on the Human Metabolome Project: Wishart et al., 2007, 2008). ^{1}H NMR spectroscopy, liquid chromatography–MS (LC–MS), and gas chromatography–mass spectrometry (GC–MS) are currently the major analytical tools in this effort. These topics have been described in detail in a variety of reviews many of which are referenced below (Villas-Boas et al., 2005; Wilson et al., 2005b; Dettmer, Aronov, & Hammock, 2007; Lenz & Wilson, 2007; Lu et al., 2008b; Theodoridis, Gika, & Wilson, 2008; Wu et al., 2009) and have also recently been the subject of a number of books (Robertson et al., 2005; Griffiths, 2007; Lindon, Nicholson, & Holmes, 2007).

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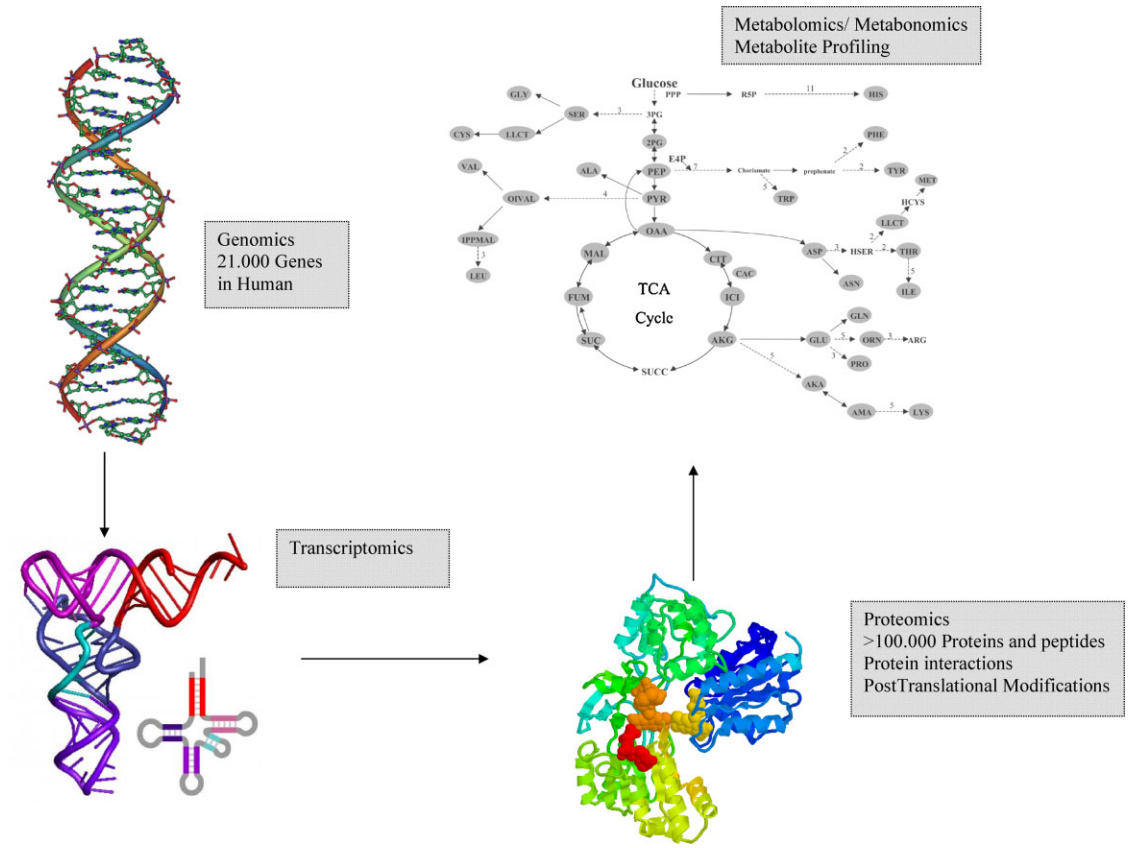

FIGURE 1. A schematic of the systems biology field.

Nuclear magnetic resonance (NMR) spectroscopy represents a powerful technique for metabolite profiling and offers direct identification and quantification of a range of abundant analytes. Typically each research group creates their own library of spectra of compounds (although there is a commercial library of ca. 500 compounds with spectra acquired at seven different pH values). A potential limitation in applying NMR spectroscopy in metabolic profiling is the requirement for a specialist operator for the instrument. In addition, the initial acquisition cost of the spectrometer is high (although subsequent costs per sample are low). As a result the number of NMR facilities available for this type of work is limited. Despite this NMR spectroscopy is currently the most used technology in metabolomics and metabonomics (Wilson, Wade, & Nicholson, 1989; Lenz & Wilson, 2007; Coen et al., 2008).

Gas chromatography–mass spectrometry (GC–MS) is, after NMR and LC–MS, the third pole in the holistic metabolite analysis arena. GC–MS provides a powerful tool with strengths resulting from its sensitivity and metabolite identification potential and weaknesses in its poor ability to analyze large and/or, non-volatile molecules without prior derivatization coupled with the need for extensive, and labor-intensive, sample preparation (Fiehn et al., 2000; Kanani, Chrysanthopoulos, & Klapa, 2008; Strehmel et al., 2008). However, because the majority of the metabolites encountered in biological samples are involatile and, due to the need to obtain the maximum possible coverage of the metabolome, profiling by LC–MS has become a basic part of the analytical toolkit in metabolomic and metabonomic research (Theodoridis, Gika, & Wilson, 2008). Since the first reports of LC–MS-based metabonomics in 2002 (Plumb et al., 2002, 2003a,b) the use of this technology in the field has expanded continuously. There are good reasons to justify this:

1. The large number of available instruments and trained experimental scientists to perform analysis.
2. The wide analyte coverage obtained, giving a more "global" profile. Thus, as a rule LC–MS is much more sensitive than NMR (although this is analyte dependent and there are metabolites such as, e.g., glucose that, because of their poor ionization properties, are much more easily detected by NMR spectroscopy). LC–MS can also analyze both semi-volatiles and non-volatiles in the same run in contrast to GC–MS.
3. The strong interest of both the manufacturers (who have invested in the technology) and the life sciences research community (who are already familiar with the technology for drug bioanalysis, therapeutic drug monitoring, and proteomics).
4. The versatility of the technology, where the same instrument platform can function for bioanalytical work for, for example, therapeutic drug monitoring or environmental analysis or proteomic analysis and with minimal, or no, change can switch to metabolite profiling (targeted and non-targeted).

Here we describe the current state of the art for generating global metabolite profiles, based on the use of MS techniques, with particular emphasis on LC–MS, whilst providing a

commentary on the relative strengths and weaknesses of the individual technologies and strategies for their use. Key issues addressed are the validation of the method, quality control (QC) approaches to evaluate system stability and general performance, ways to perform data extraction with the generated multidimensional data, and methods for the identification of markers found.

II. MASS SPECTROMETRIC TECHNIQUES FOR "HOLISTIC" ANALYSIS

Typically two strategies can be followed in MS-based metabolite profiling analysis. With the first strategy a limited number of known/or unknown metabolites or compound classes are measured. This is often called "targeted" metabolomics, where the peaks are first designated/annotated to metabolites (either identified metabolites or non-identified/non-fully structure elucidated metabolites known to be present in the samples), and subsequent multivariate statistical analysis uses only this "thinned" data set.

The second strategy promotes an unbiased holistic approach towards the profiling of as many features as possible in the metabolome without prior knowledge of the identity of these features. Multivariate statistical analysis then deals with a much bulkier data set than the targeted approach, and the data guides the analyst to recognize the important metabolites that contribute to the differentiation (if any) of the samples. Next effort is put towards the identification of these discriminating molecules (peak annotation or structure elucidation). The following sections describe in more detail the various instrumental configurations used for the development/application of MS-based metabolite profiling research. including DI/FIA–MS, LC–MS, GC–MS, and capillary electrophoresis (CE)–MS.

A. Flow Injection (FI) or Direct Infusion (DI) Analysis-MS

The direct infusion (DI) or flow injection (FI) analysis of biological samples (fluids or tissue extracts) is of interest because of its potential to provide high-throughput profiling. Such an approach could be ideal for application in population studies for mapping the large numbers of biological specimens that are generated. The method so far has utilized very high-resolution mass spectrometers (FT-ICR) and has been mainly used for plant and microbial samples in metabolomic studies. Such fingerprinting generates data on large numbers of masses and these data sets exhibit high variance, thus providing a challenge for data mining. The Drapper group (Aberystwyth University, UK) advocates the utilization of DI or FIA–MS as a "first pass" screen in the search for compositional differences. To achieve unbiased discrimination between sample groups, powerful data analysis algorithms are used along with FIA–MS/MS^n metabolite databases. Three relevant protocols have been reported so far, also expanding to biofluids. Although these are designed for use by biologists/analytical chemists, collaboration with data-mining experts is generally advised by the developers (Beckmann et al., 2008; Enot et al., 2008; Overy et al., 2008).

As a whole DI/FIA–MS provides an interesting approach because if LC is not used the variations that this analytical dimension brings are eliminated. However, the application of DI/FIA–MS to complex and highly variable samples such as urine and plasma/serum where matrix effects and more specifically ion suppression/ion enhancement, and differences in salt concentrations, etc., have the potential to adversely affect the result needs further investigation to prove the validity of the approach. The technique also has obvious limitations in the necessity for high-resolution MS instruments and also in the analysis of isobaric substances.

B. GC–MS

Gas chromatography–mass spectrometry (GC–MS) technology, and applications in analytical chemistry, were developed long before the introduction of LC–MS, and GC–MS remains the platform of choice for the analysis of volatile and semi-volatile analytes. A major advantage for the application of GC–MS in metabolomics and metabonomics is the availability of GC–MS electron impact (EI) spectral libraries. These libraries offer very good possibilities for the identification of unknown biomarkers. In fact the typical process in GC–MS-based metabonomics is first to identify the peaks in the chromatogram, quantify them and then find differences in their concentrations or find trends for these analytes in the sample sets.

Major limitations of the technology are found in the analysis of polar, non-volatile analytes (in the absence of prior derivatization), large molecules and thermolabile compounds. Hence GC–MS cannot provide a global metabolite profile but only a profile of apolar, volatile to semi-volatile molecules (or those that can be made volatile) with MW lower than 600–700 amu. As indicated above, another obstacle is the need for extensive sample preparation to make involatile substances suitable for GC: typically a dual derivatization scheme is applied to samples prior to analysis. First methoxymation is applied to cover the carbonyl groups and next silylation to mask active hydrogen atoms (OH, or amino groups) (for details, see Kanani, Chrysanthopoulos, & Klapa, 2008; Pasikanti, Ho, & Chan, 2008). Kanani, Chrysanthopoulos, and Klapa (2008) report that the length of the derivatization step is critical as it is imperative that all metabolites are derivatized, otherwise there is a risk of accepting differences due to derivatization kinetics as variations of biological significance. Derivatization, extensive sample preparation, and long chromatographic analysis time make GC–MS a rather low-throughput technique for metabolomic/metabonomic applications.

However, a current advantage of GC–MS in this field is that the identification of peaks (peak annotation or, more difficult, the structure elucidation of unknowns) is more straightforward than in LC–MS but is by no means a trivial exercise. This process is often assisted by (1) using retention time or retention index comparison with the values of pure standard compounds and (2) using retention indices of mass spectral library databases. The retention index of a metabolite is calculated by relating the retention time of the compound to retention times of standard *n*-alkanes or other standards (e.g., fatty acid methyl esters (FAMES)) analyzed with the same conditions. Certain analytical software platforms calculate these automatically. In contrast to ESI or APCI LC–MS chromatograms, a GC–MS chromatogram for metabolomics studies does not usually contain molecular ion peaks (due to fragmentation in the EI ion source). A fact that makes peak annotation/identification even more complex is that analytes may appear in several multiderivatized forms. This means that, for example, alanine may be present in the sample either as a molecule with one or two TMS moieties, or a mixture

of both forms. Kanani, Chrysanthopoulos, and Klapa (2008) reported that even after up to 30 hr of silylation time the derivatization of many metabolites is not expected to have been completed and suggest special attention with regard to applying the appropriate derivatization time to avoid biases in GC–MS-based metabolomics. Even worse, all these molecules will undergo extensive fragmentation to several ions in the ion source. Typically only one of these signals can be used for the quantification of the analyte. Alternatively deconvolution of these signals to one peak is performed manually or automatically using special software such as Automated Mass Spectral Deconvolution and Identification System (AMDIS; National Institute of Standards and Technology (NIST), USA). These software can perform spectral deconvolution and library searching against the NIST database. AMDIS has been applied in metabolic profiling with success (Halket et al., 1999); however, automatic deconvolution should be used with caution and should always be followed by manual inspection of the results. A recent review covering the field of GC–MS library searching for compound identification has been produced (Halket et al., 2005). Peak-picking and alignment algorithms used in LC–MS have also found use in GC–MS analysis. Lin et al. (2009) used the XCMS program to detect, normalize, and align features from GC–MS data from the analysis of urine from a Crohn's disease model. A problem encountered in this approach is the multitude of signals per metabolite and the artifacts originating from the derivatizing agents. The authors analyzed blank samples and subtracted these from the test runs before normalization of the signals (in R software). Markers of interest detected by this approach were next identified by searching in mass libraries. This approach takes the reverse route for data handling compared to the one described above for GC–MS metabolomics experiments. As a result the rigorous process of identifying all peaks in the sample before the application of advanced informatics is circumvented and data processing should proceed faster: of the 2,990 features detected in XCMS, 546 were removed as artifacts reducing the number to 2,444. These were then divided into 592 groups based on the finding that the features were sharing the same retention time, thus giving a rough estimate of the number of metabolites detected. Finally, 23 features of interest were found ($P < 0.05$, ANOVA) of which 13 were identified, 8 were unknowns, and 2 corresponded to different derivatives of identified metabolites. Further studies in the application of metabolomics software in GC–MS are needed to evaluate the utility of this approach.

Despite these disadvantages GC–MS is the method of choice for the analysis of a large number of analyte groups that share the necessary characteristics described above. For example, GC–MS is still unchallenged in the analysis of organic acids in urine in the search for (inborn and other forms of) metabolic syndromes (although Bruker has recently produced an NMR platform for this). In these aspects, it cannot still be surpassed by LC–MS or other MS-based analytical platforms as it offers excellent sensitivity, wide dynamic range and quantification, very good identification capabilities, directly searchable databases, very satisfactory robustness, and even possibilities for platform comparison using retention time indices (for a review of the use of GC–MS in metabolome analysis to study mutations of inborn errors of metabolism, see Kuhara, 2005). The combination of these features renders GC–MS a very powerful analytical tool for metabolite profiling.

Mass analyzers used in GC–MS metabolomics include single quadrupoles, ion traps, and TOF-MS machines. Combination with TOF instruments is the first choice (see discussion below in LC–MS), but if this is not available quadrupoles and ion-traps will also suffice. Recently, developments in comprehensive two-dimensional GC (GC × GC) coupled to time-of-flight mass spectrometry (TOF-MS) offer additional perspective to the application of the technology (see Shellie et al., 2005; Welthagen et al., 2005; Almstetter et al., 2009). Analysis time can be reduced and at the same time a larger number of metabolites can be detected in a single analysis (due to the increased peak capacity). In GC × GC components that elute from the first column are subsequently introduced to a second column. The first column is usually non-selective, whereas the second column, which is much shorter, separates compounds on an orthogonal mechanism (e.g., if the first separation is based on molecular weight, or boiling point the second separation is based on polarity). The technology renders it a powerful tool for the analysis of complex mixtures but requires expensive instrumentation for which robustness is not a strong point. Data interpretation and manipulation is not straightforward since the outcome of such instruments is not a typical mass chromatogram but resembles a heat map. A recent article deals with the comparative evaluation of software for the deconvolution of metabolomics GC–TOF-MS data (Lu et al., 2008a).

C. Capillary Electrophoresis-Mass Spectrometry

As a technology CE can provide interesting advantages in terms of (1) high resolution, (2) the ability to analyze a diverse range of chemical compounds, and (3) need for low sample volume. Furthermore, CE, is well suited to the types of samples encountered in metabolic profiling, requires minimal sample preparation (e.g., for urine samples), and has good potential for the analysis of charged or polar molecules (which can represent troublesome molecules for both GC and LC). However, the fact is that, so far, CE has not found widespread application in global metabolite profiling. Major reasons for this are issues with analytical system stability, mainly having to do with the modification of the capillary wall due to sorption of macromolecules following injection of biological samples. As a result reproducibility of migration times and peak areas of the analytes is poor (Ullsten et al., 2006). In targeted analyses (e.g., pharmaceutical analysis) these issues are easily overcome with the introduction of internal standard(s). This is not feasible in non-targeted analysis where peak alignment and quantification would instantly fail. Hence, there have been efforts towards these ends via the development of coated capillaries (Ullsten et al., 2006; Ramautar et al., 2008).

Capillary electrophoresis (CE)-UV and CE–MS studies have been reported mostly in the area of bacterial metabolomics in particular studies on *Bacillus subtilis* (Soga et al., 2003; Jia and Terabe, 2005), but there are also works on plant metabolomics (Sato et al., 2004; Levandi et al., 2008). A recent review covers the field of CE–MS application in bacterial extracts, plant extracts, urine, plasma, and CSF metabolomics (Ramautar, Somsen, & deJong, 2008). Microchip CE and microfluidics application (including micro-LC) have also been applied in metabolomics, and the field was recently reviewed by Kraly et al. (2009). The majority of the reports deal with targeted analysis of bacterial or plant metabolomes in photometric or

electrochemical detection. CE–MS has as well been used mostly in targeted metabolite analysis rather than non-targeted analysis. In an application of CE–TOF-MS (using a fused silica capillary), Soga et al. (2006) identified ophthalmic acid as an oxidative stress biomarker (in liver extracts) associated with the oral administration of acetaminophen (paracetamol, 150 mg/kg) to mice using a metabolomics approach. Recently, CE–ESI-MS was applied for the study of single cell metabolomics and subcellular structures (Lapainis, Rubakhin, & Sweedler, 2009). However, a larger number of applications of CE–MS are still required for it to be considered an alternative metabolite profiling platform.

D. LC–MS, the Tool for Global Metabolite Profiling

Generally metabonomics analyses by LC–MS are performed using solvent gradients, on reversed-phase (RP) packing materials. Columns of between 2.0 and 4.6 mm i.d. and 5–25 cm length are typically used containing 3–5 μm packing materials (Theodoridis, Gika, & Wilson, 2008), although lately the trend is towards sub-2 μm particles (ultra performance LC (UPLC) or ultra high-performance LC (UHPLC) columns). U/HPLC–MS is increasingly used as it provides much higher separation power and peak capacity (Wilson et al., 2005a; Lenz & Wilson, 2007). In fact it can now be claimed that ca. 20% of UPLC applications deal with metabolomics/metabonomics applications. Validation of UPLC–TOF-MS for the metabonomic analysis of human urine has been reported (Gika et al., 2008b) and more recently for blood serum (Zelena et al., 2009) and plasma (Michopoulos et al., 2009).

Analysis times for LC and U/HPLC-MS metabolite profiling studies have ranged from ca. 2 min up to 1 or 2 hr per sample depending upon the application. Such gradient RP separations are well suited for the analysis of compounds of medium and low polarity (which represent a huge number of metabolites); however, they are less suited for the analysis of polar or ionic compounds which elute essentially unretained. Such analytes include important biomolecules such as some amino acids and sugars. A solution to this could be to resort to CE–MS or GC–MS. Another solution is to use hydrophilic interaction chromatography (HILIC), which works essentially as a type of reversed-RP chromatography. HILIC phases are now available from several manufacturers, also in sub-2 μm particles. In HILIC the chromatogram is virtually turned around: thus, the late elutants in RP-LC elute first and the early elutants in RP elute late (metabonomics/metabolomics applications of HILIC–MS include Idborg et al., 2005; Cubbon et al., 2007; Tolstikov, Fiehn, & Tanaka, 2007; Gika, Theodoridis, & Wilson, 2008a).

Another alternative separation mechanism in liquid phase is the application of water-rich (or even water only) mobile phases in combination with high operating temperatures. In one example, Plumb et al. (2006) used a temperature of 90°C for gradient elution in UPLC for the analysis of urine. Others have used even higher temperatures for separations (in excess of 100°C and up to 180°C in some applications) and temperature gradients (Gika et al., 2008a). This system worked very well for the metabonomic analysis of urine, but not as well for blood plasma, where the eluotropic strength of even superheated water did not suffice to elute apolar sample components.

As stated earlier, in non-targeted metabonomic LC–MS analysis, the aim is for an unbiased analysis where the data will drive and guide the bioinformatics analysis. In such a case, UPLC proves superior to LC due to (1) the higher stability of the chromatographic system and hence, (2) the enhanced repeatability, (3) the higher separation power (UPLC should provide more features compared to HPLC), and (4) the higher throughput. A typical UPLC peak width is close to 3–5 sec at the base and hence UPLC must be combined with fast acquisition/ "scanning" mass spectrometers; therefore, the combination of UPLC with TOF or qTOF machines is very common for such applications.

Often in metabolomics the analysis of the samples requires the mass spectrometer to acquire data in both +ve and −ve ESI modes to maximize metabolome coverage by ionizing molecules of different classes that ionize more efficiently in positive mode (e.g., nitrogen containing molecules) or in negative mode (e.g., acids). Currently, the utilization of ion sources, capable of fast switching between ionization modes over the lifetime of the eluting chromatographic peak, is not common. This is due to the loss in sensitivity that is incurred as a result of the switching process and also because polarity switching in older TOF machines cannot be performed "on the fly." In such cases the whole sample set in analyzed in +ve ESI and again in −ve ESI in a second run. Newer TOF machines, or triple quadrupoles, can provide automatic polarity switching hence reducing analysis time (but with the sacrifice of some sensitivity).

A typical UPLC-QTOF total ion current (TIC) trace for a human plasma sample (−ve ESI) along with its corresponding contour plot is shown in Figure 2A. Figure 2B shows the profile of rat urine, also obtained using UPLC-QTOF (+ve ESI). There are many ions present in these TICs, thus advanced tools are needed to study data sets comprising tens to hundreds of such samples. As described below, via the use of a variety of multivariate statistical approaches (e.g., principal components analysis (PCA)) the discovery of differences in the metabolic profiles becomes both possible and practical. It is clear however that urine contains more polar constituents, whereas serum contains apolar compounds of higher molecular weight.

An objective and pragmatic problem is that no matter how good the LC separation is before the introduction of metabolites into the MS, and no matter how sensitive and accurate the detection in MS, it is still likely that an unknown number of sample components will still remain undetected. This unwanted effect is the cumulative result of a number of issues such as the varying polarities of the analytes (leading to non-retention or non-elution under the LC conditions used), poor ionization properties, wide range of molecular mass of the metabolites, low concentrations, and ion suppression by other components present in the sample. Therefore, although the metabolome coverage that LC–MS offers is perhaps the most comprehensive provided by any of the current metabolite profiling technologies it is still not complete. Ion suppression is a well-known problem in MS that can be described as a matrix effect phenomenon resulting from the coelution of matrix components that will affect the detection capability, precision, or accuracy for the analyte(s) of interest. In the case of ion suppression a loss of signal intensity is observed. If the opposite happens (the signal increases) the term ion enhancement is used. Again, in targeted analyses where a number of internal standards (usually stable isotope labeled analogues of the analyte) can be used, these issues can be controlled (i.e., ion suppression may still occur but it is monitored). In untargeted analysis the utilization of tens or even

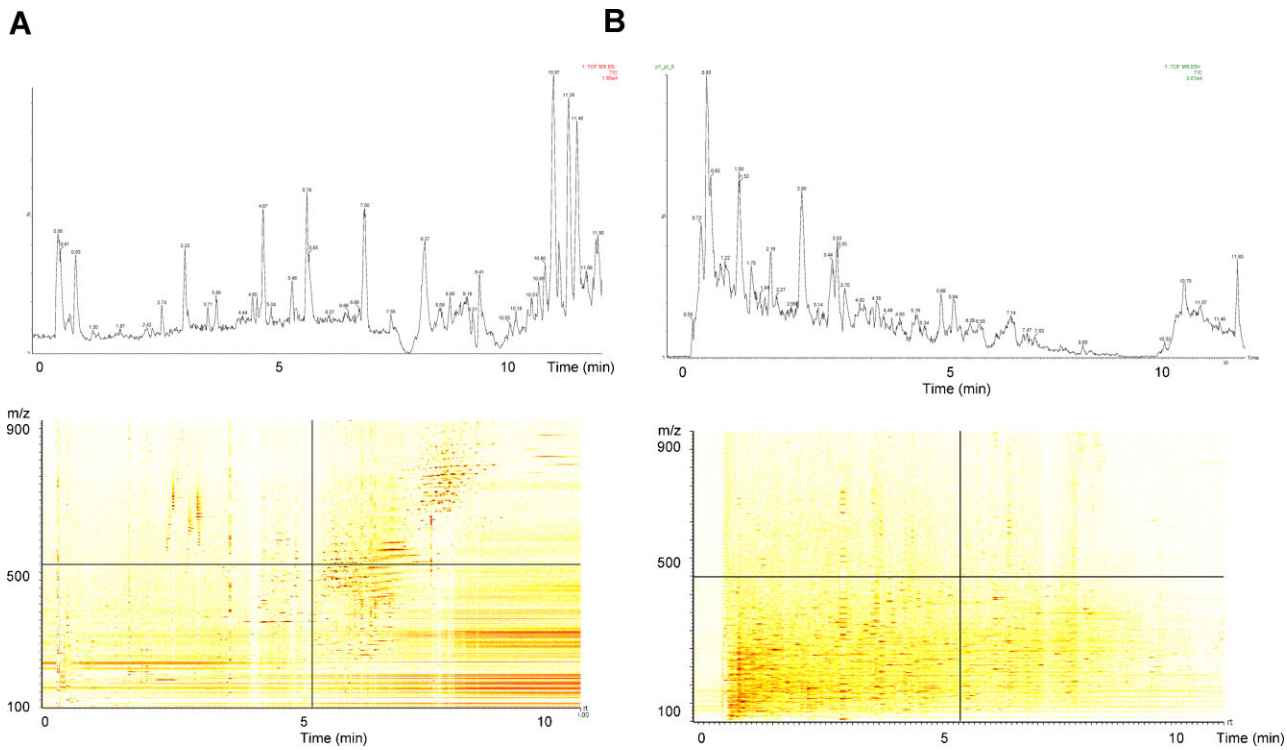

FIGURE 2. TIC and contour plot from human serum analysis in UPLC-QTOF (−ve ESI) (**A**) and from rat urine UPLC-QTOF (+ve ESI) (**B**). The figure shows that compared to urine, serum contains more apolar components that elute late in the chromatogram. In urine a big portion of the peaks and the overall signal is collected in the first part of the mass chromatogram thus corresponding to polar/medium polar analytes. Conditions: UPLC–QTOF-MS analysis of 10 μL of serum (following protein crash); voltages: capillary 3,000 V in (A) and 3,500 kV in (B); sample cone: 35 V; desolvation temperature: 300°C; source temperature: 120°C. LC on acquity HLB columns with gradient elution at 0.4 mL/min. For human serum solvent A is water and solvent B is methanol: Program: 100% A at time 0 and then linear change to 100% B at 16 min and isocratic 100% B for 4 min. For rat urine solvent A is water and solvent B is acetonitrile (both acidified with formic acid 1%, v:v). Program: 95% A at time 0 and then linear change to 80% A at 5 min, then to 55% A at 8 min and 5% A at 9.8 min and then isocratic 5% A for 2 min.

hundreds of deuterated internal standards may correct only for the corresponding non-deuterated analytes and hence cannot provide a practical and easy solution to the problem. Hence high-quality and very stable chromatographic separations are essential if reliable data are to be produced.

E. Mass Spectrometry Platforms

In addition to the obvious role of the chromatographic separation the ionization mechanism employed in LC–MS plays a major role in the profile measured. So, just as with the LC separation it is also the case with respect to MS that it is not prudent to take for granted that one methodology can cover all types of molecules: polar, neutral, ionic, non-polar, and so forth. Obviously, some analytes are ionized more efficiently in one ionization mode or one polarity and some in another mode. Currently, ESI is by far the method of choice in LC–MS metabolomic studies because it produces large numbers of ions via charge exchange in solution (Sana, Waddell, & Fischer, 2008). Atmospheric pressure ionization chemical ionization (APCI) on the other hand has been used in only a few cases. The APCI technique produces ions by charge exchange in the gas phase and may provide wider dynamic range (Want, Cravatt, & Siuzdak, 2005), much lower signal intensities, background noise and in-source fragmentation than ESI as shown in a study of the plant metabolome using FT-ICR-MS (Aharoni et al., 2002). Typically, an APCI full scan TIC will be much less populated and of much lower total signal that the respective ESI TIC. A dual ionization metabolomics approach has recently been described increasing the coverage of the metabolome of human serum (Nordstrom et al., 2008). In another study a group from Agilent Technologies (Sana, Waddell, & Fischer, 2008) found that employing APCI in addition to ESI resulted in a 34% increase in the coverage (number of features detected) of the metabolome of human erythrocytes. Such effects are illustrated in Figure 3 (Sana, Waddell, & Fischer, 2008), which provides an interesting perspective on the differences between the ionization modes. It is well known that two mechanisms of ionization have some ion formation overlap (as also with APPI). Figure 3 shows that the contribution of APCI especially in positive mode is significant and can be considered as a complementary technique.

Our findings concur with the above (Fig. 4). This result is from the LC–MS analysis (QTRAP 4000) of blood plasma from statin-dosed and control rats. It can be seen that certain ions are only seen in ESI (Fig. 4A) and some ions appear only in APCI

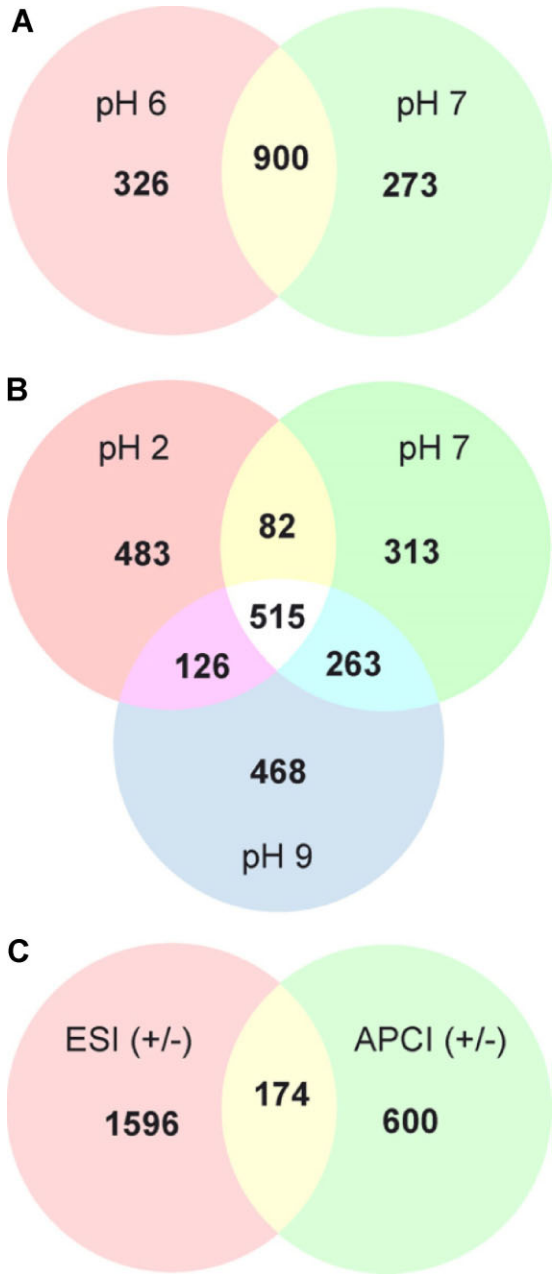

FIGURE 3. ESI versus APCI, pH 6 versus pH 7 versus pH 9. Venn diagrams showing the number of overlapping features between different pH conditions and ionization modes. Comparison of the total number of detected features for (**A**) pH 6 and 7 extracted samples, and (**B**) pH 2, 7, and 9 extracted samples, by combining the results of ESI (+), ESI (−), APCI (+), and APCI (−) modes. **C**: Comparison of the total number of detected features for ESI and APCI modes in both polarities (+/−), where the results for each condition: pH 2, 6, 7, and 9 were combined together in each ionization mode. Reprinted from Sana, Waddell, and Fischer (2008) with permission from Elsevier.

(Fig. 4B). Clearly the combination of both data sets results in a more comprehensive coverage of the metabolome.

Lately interest in newly developments in the ionization technologies such as desorption electrospray ionization (DESI) or extractive electrospray ionization (EESI) and direct analysis real time (DART) has lead to their application in holistic metabolite profiling applications. We used DART for the MS analysis of urine and detected a much smaller number of ions compared to LC–MS runs (data not shown). Zhou, McDonald, and Fernández (2009) reported the application of DART for the TOF-MS profiling of serum applying a dual analysis scheme: non-derivatized serum and next derivatized serum (silylation with MSTFA and TMCS). Although the authors claim sample throughput as major advantage of DART usage versus LC–MS or GC–MS, the need for sample derivatization significantly compromises this, whereas at the same time matrix effects remain, in our opinion, an issue. The group of G. Cooks have reported on the use of DESI and EESI on targeted metabolomics studies on *Escherichia coli* (Jackson et al., 2008); however, the focus was on a very small number of metabolites: the presence of 13 out of 17 selected central carbon metabolites was confirmed in real samples, thus hardly justifying the term metabolomics. Obviously, further evidence is necessary to substantiate the potential of such technologies in holistic metabolite profiling.

Liquid chromatography–mass spectrometry (LC–MS)-based metabolomics analysis is usually performed using high mass accuracy instruments to gain from the higher mass resolution and to assist in marker identification. The platform that is currently emerging as the combination of choice is a UHPLC type of separation combined with a TOF-MS instrument. This combination benefits from the high separation power of the UHPLC and the increased peak capacity on the chromatography side and the fast acquisition and high resolution of the TOF-MS. Metabolomics analysis using triple quadrupole instruments is not common, especially in full scan mode in non-targeted analysis, due to the high background noise (there are however, good examples of the use of triple quadrupole instruments, see Bijlsma et al., 2006; Ceglarek et al., 2009). Other instruments such as linear ion traps and the high-end MS machines such as FT-MS and Orbitrap have also found use. From the linear ion-trap systems the configuration of MDS Sciex of a triple quadrupole where the third quadrupole is replaced by linear ion trap (QTRAP) is the system that has found wide utilization in metabolomics studies. QTRAP systems, introduced in 2002 (QTRAP 4000) and 2008 (QTRAP 5500), provide utilities such as enhanced mass scanning (ion trap scanning in the axial direction towards the detector resulting in higher sensitivity compared to the corresponding triple quadrupoles) and several additional MS and MS–MS functionalities. Fragmentation up to MS^n level is theoretically achievable although in small molecule analysis this feature is not as useful as in proteome analysis. Ion traps, however, do not provide high mass accuracy. Ultra high-resolution MS machines (FT-MS, Orbitrap) provide resolution higher than 100,000 and superior mass accuracy (sub-ppm) compared to other types of mass spectrometer. Figure 5 illustrates the advantages of this ultra-high resolving power which facilitates analysis of complex metabolic extracts without mass spectrum overlap from other species. The figure shows the range of metabolites of nominal mass 279 amu from the spectrum obtained from the analysis of hepatocyte extracts. Five ions were detected in the 279 amu region: one is the common plasticizer 2-ethylhexylphthalate $[M+H]^+$ at 279.1583 amu. The peak of the sodium adduct of palmitic acid $[M+Na]^+$ 279.2288 amu revealed concentration changes related to drug concentration irrespective of the other neighboring peaks (including that of the plasticizer which is only 70 mDA apart). In this case the high

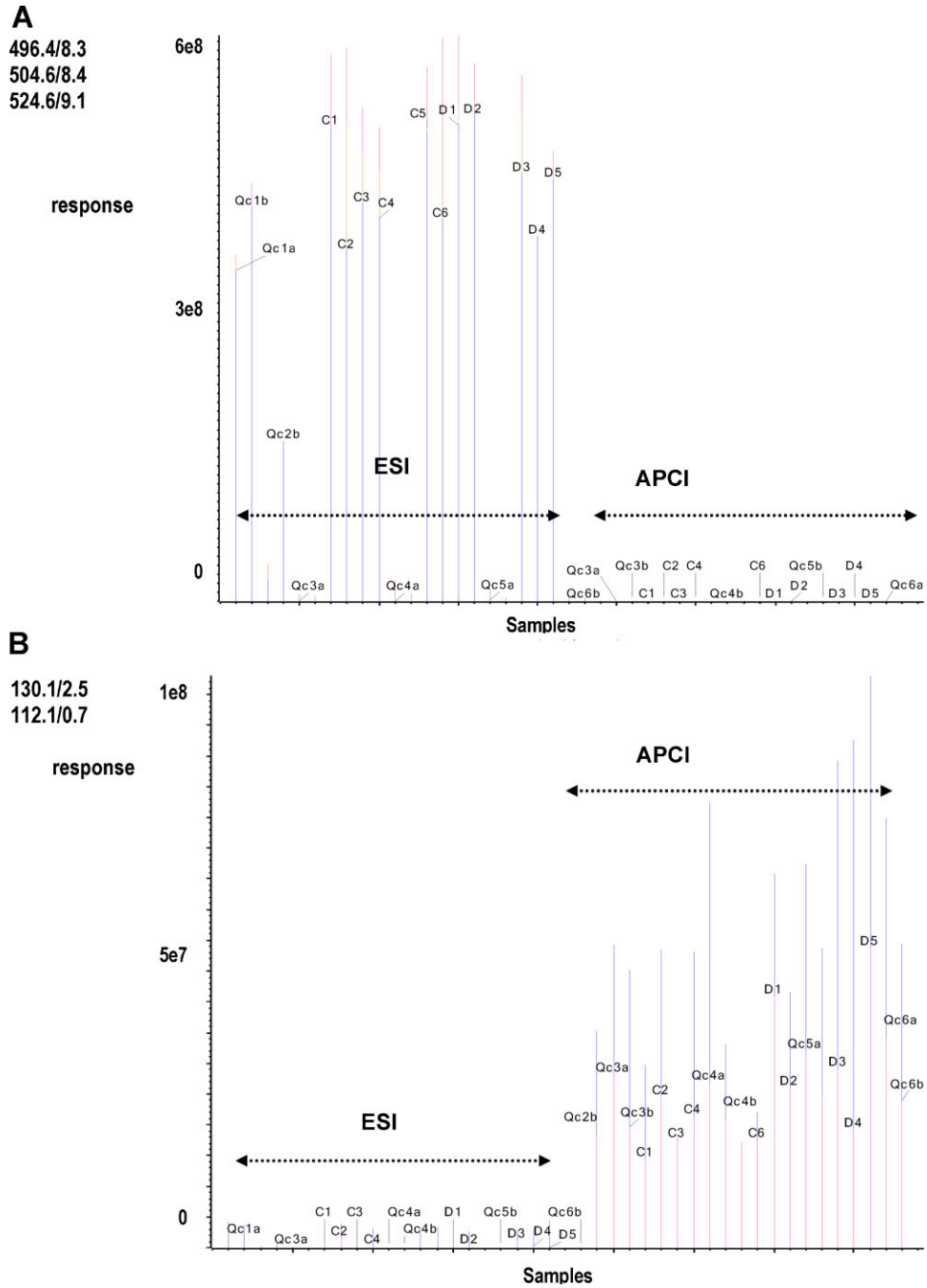

FIGURE 4. Contribution plots for ions that ionize only in ESI (**A**) and only in APCI (**B**). A: Ions 496.4/8.3, 504.6/8.4, and 524.6/9.1 (mass/retention time pairs) appear practically only in ESI mode and not in APCI. B: 130.1/2.5 and 112.1/0.7 appear practically only in APCI mode and not in ESI.

mass accuracy facilitated the unambiguous assignment of the molecular formulae (Brown, Kruppa, & Dasseux, 2005). High mass accuracy is not dependent on the analyte concentration and is also achievable over wide concentration ranges. However, high mass resolution typically requires longer scanning time compared to, for example, TOF instruments, which is not always suitable for the untargeted analysis of unknown samples. The original Orbitrap instrument was rather slow in scanning but newer versions provide faster scanning rates (e.g., Exactive™) and can thus better be combined with UPLC for untargeted holistic analysis.

Of course the use of so many different types of mass spectrometer does beg the question of the comparability of the data obtained on different instruments. MS instruments operate in very different ways, differing in several operational and instrumental configuration parameters (e.g., the ionization probe

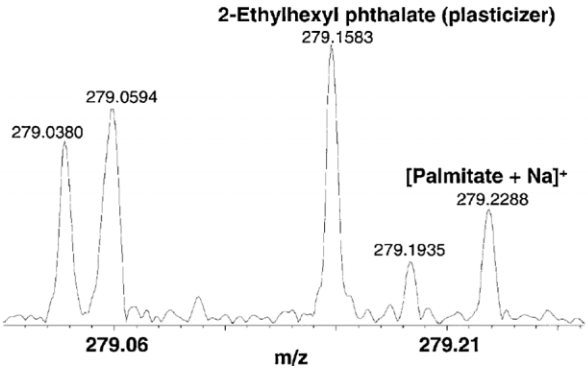

FIGURE 5. Partial FT-ICR-MS spectrum of a hepatocyte cell extract. The 279 *m/z* nominal mass range is shown: five ions are detected of which the sodium adduct of palmitic acid (ion of interest) is clearly resolved from a plasticizer. From Brown, Kruppa, and Dasseux (2005) with permission from Wiley.

design, the ion optics, the operation of the mass analyzer, even the detector and the signal obtained), not to mention the processing software. As a result it is widely understood that single-MS spectra are not reproducible on different types of instruments (Weinmann et al., 2001).

To the best of our knowledge, only limited work has been so far performed comparing analytical platforms in performing metabolomics analyses. Pandher et al. (2009) have recently reported the cross-validation of an Agilent 6510 hybrid quadrupole-TOF and a Waters QTOF Premier used for the analysis of tumor cells. In a study conducted by us a set of 120 urine samples were analyzed using UPLC–ESI-MS with three different mass spectrometers: a Waters QTOF Micro, an API/MDS Sciex QTRAP 4000 (triple quadrupole-linear ion trap operating in EMS mode), and an API/MDS Sciex 365 triple quadrupole. The aim of the study was (1) to compare the profiles obtained from the analysis of 120 human urine samples on three different mass spectrometers, (2) to assess the stability of the three systems, (3) to identify markers separately by each of the three analytical systems, (4) to compare the markers and the patterns revealed by the different instrumental set-ups and software tools. The whole sample set consisted of 120 test samples, 16 QC samples, and 4 reference test mixtures—(in agreement with the sample configuration proposed in Gika et al., 2007) and the analyses lasted for ca. 36 hr in single uninterrupted runs. RP gradient UPLC was used and full scan mass spectrometric data were collected (*m/z*: 80–850) in positive ESI at either 3,500 V (Q-Trap), 5,000 V (3 Q 365), or 3,000 V (Q-TOF). The QC sample was injected in quadruplicate before the commencement of the analysis of the unknown samples, to condition the system, and then every 10 injections run to validate the quality of the profiles obtained. Retention time and intensity variations were assessed for selected peaks of the pooled urine control sample. Multivariate statistical analysis was applied to project the data and extract information. Principal component analysis (PCA) was performed using Marker View (for data obtained in the Q-Trap 4000 and 365 instruments) and Marker Lynx (for data obtained in the Q-TOF). Whilst the TICs obtained on Q-TOF, Q-Trap, and 3-Q instruments for the same samples appeared very different, the PCA results however (Fig. 6 showing PC1 vs. PC2 scores plot in the three MS machines) were strikingly similar. This similarity was despite the fact that: (1) the instruments were made by two different manufacturers, (2) the mass analyzers operated under different principles, and (3) two different data extraction software were used. Comparing the three scores plots it can be seen that the QCs lie closely clustered, in the center of the scores plot, indicating a repeatable method and a trustworthy data set. All three MS systems identified the same samples as "different from the herd," and the same ions were identified in all three systems as being responsible for this separation. Robustness of the results was evaluated by applying different PCA scaling methods (pareto, centered, and UV) in SIMCA P and was found very satisfactory (plots were alike in all three data sets).

This finding may be valid for sample sets where great biological variability is observed: that is, in this case urine samples from humans. However, for sample sets where the biological variability is not as great (e.g., blood samples from inbred, genetically similar, animal models), multivariate statistics do reveal differences between the profiles due to instrumentation differentiation. Thus, in a similar study on rodent plasma UPLC was connected simultaneously, via flow splitting to QTOF and a QTRAP mass spectrometers operating in ESI mode. Data from both instruments were extracted with the same algorithm (XCMS) and were further treated in SIMCA P11 and SIMCA P12. The resulting PCA scores plots again looked very similar (data not shown), clearly separating the two classes of animal. Advanced statistical tools such as O2PLS and hierarchical PCA were used to compare findings and evaluate the complementarity of the two data sets and from this it was seen that the QTRAP mass spectrometer detected a number of unique ions (accounting for ca. 10% of the total), whereas the common ions (picked by both QTOF and QTRAP) account for ca. 80%. The remainder of the ions (ca. 10%) were essentially due to "noise" that can be ignored. Further statistical work is on progress on these data sets and will be reported elsewhere (Gika et al., in preparation).

F. Maximizing Metabolome Coverage in LC–MS-Based Analysis

Clearly there is a tension between the high sample through-put required to profile large numbers of samples (in both positive and negative ionization modes) and obtaining the most comprehensive coverage of the metabolome. Thus, if rapid gradients are used the sample throughput is maximized, but at the expense of the number of features detected. Similarly, the use of certain designs of mass spectrometer results in very accurate mass determination, of great value for identification purposes, but may require chromatographic peak widths that are not consistent with highly efficient separations. As described above separation systems capable of delivering high peak capacities, using microbore separations or U/HPLC-based separations, have been described, but it is not yet clear what proportion of the metabolome these cover. Further to this, both the length and shape of the gradient can affect the number of features detected and these also need careful optimization to maximize coverage. In addition, whilst the bulk of the LC–MS work that has been described for determining global metabolite profiles have used RP gradient chromatography it is obvious that such separations are not ideal for highly polar/ionic species. The addition of HILIC, or ion-exchange LC separations will likely increase metabolome coverage but does not eliminate the need for the RP

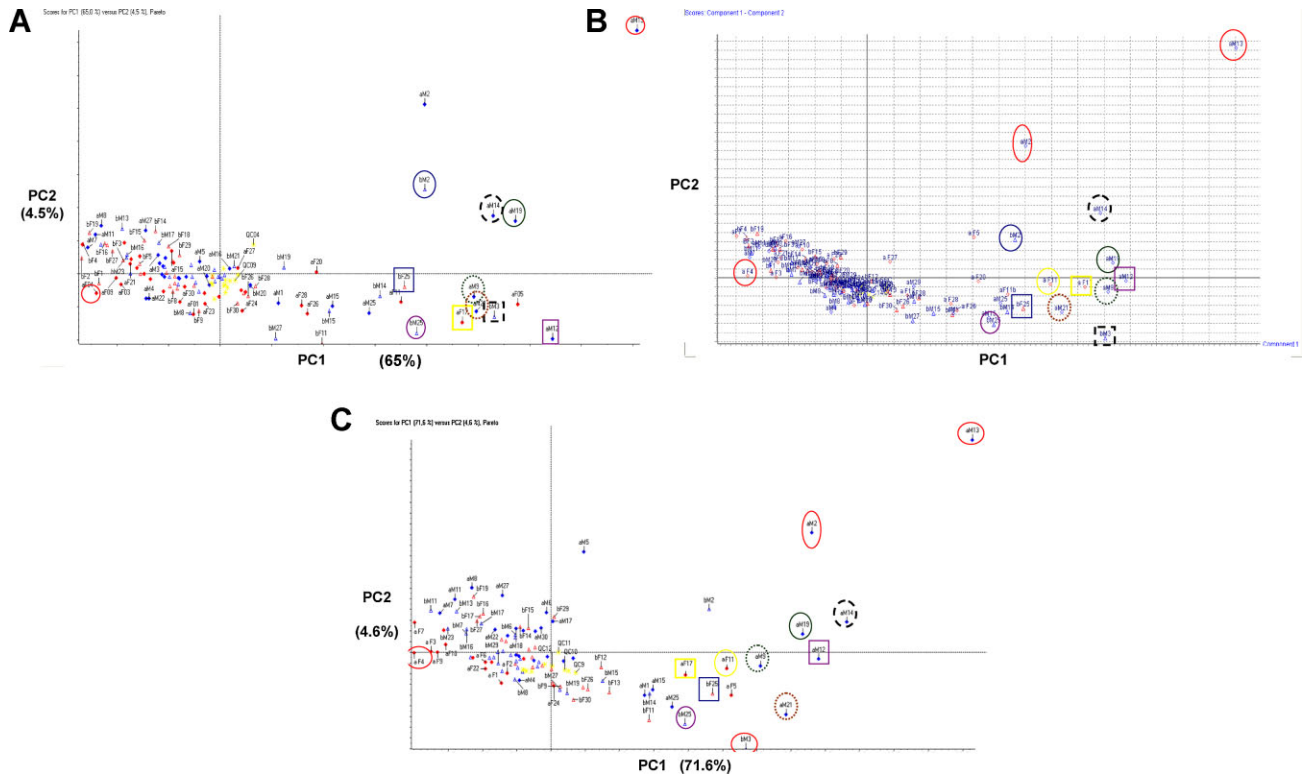

FIGURE 6. PCA scores plot generated from UPLC–MS analysis of 120 urine samples from humans in a QTRAP (**A**), QTOF (**B**), and a triple quadrupole (**C**). Data were extracted and PCA was performed in MarkerView (A,C) and MarkerLynx (B) data treatment software. Despite instrumentation differentiation and the utilization of different peak picking and alignment software, PCA scores plots are very similar with the same samples projected on the same spot in all three graphs. Loading plots (not shown) show the same ions dominating the variability in all three data sets.

profile as well. Faced with these opportunities there are essentially two options. First, the samples can be profiled twice, using two analyses with RP and HILIC undertaken independently (Gika, Theodoridis, & Wilson, 2008a). This option is easy to implement and generates relatively uncomplicated data sets. The alternative is to attempt some type of two-dimensional analysis as reported by, for example, Wang et al. (2008) in an ''orthogonal'' combination of the selectivity of HILIC and RP-LC using a column switching approach. Two-dimensional chromatography requires excellent repeatability and suitable software for subsequent data alignment analysis, if multiple samples are to be analyzed with confidence. Such methods have been used for GC analysis (GC × GC) (Shellie et al., 2005) but the technical problems encountered are by no means trivial. One promising area for introducing a two-dimensional, orthogonal, separation is via the hyphenation of the LC separation to an ion-mobility-based separation immediately prior to mass spectrometry, and such an instrumental set-up has recently been described (Harry et al., 2008).

In addition to the complexity afforded by factors such as the length of the chromatographic gradient and the mode of chromatography, there is also the choice of ionization mechanism to consider as, in addition to positive and negative ESI, there is also APCI to consider given that, as described above, this mode of ionization also give access to a slightly different set of molecules. Self-evidently, there is also the sensitivity of the mass spectrometer to consider as well as, with increasing sensitivity, comes a further increase in the number of features detected.

Of course, to a very great extent, the level of detail to which the metabolome needs to be determined does depends on the purpose of the study. Arguably, if the aim of the study is to detect and identify a few ''diagnostic'' molecules that could be used in, for example, a clinical setting to monitor a disease or its treatment, then complete coverage of the metabolic profile of the sample is less important than finding an appropriate number of suitable markers. If on the other hand, the aim is to provide an in-depth characterization of the sample then clearly the combination of the highest resolution separations systems and most sensitive mass spectrometers are needed.

G. Validation Issues: Is the ''Marker'' Real or Just an Artifact?

As described above (UP)LC–MS is a very powerful tool for global metabolite profiling; however, potential limitations must be kept in mind when developing and applying such a methodology. Such limitations are the potential for drift in both chromatographic and mass spectrometer performance and the repeatability and reproducibility of the method. These limitations should be recognized and efforts should be made to control them. This would mean that to eliminate bias due to a gradual change in the performance of the system, the samples should be analyzed in

a random order, and QC samples should be used to monitor the performance of the system (Sangster et al., 2006; Gika et al., 2007; Gika et al., 2008b; Michopoulos et al., 2009; Zelena et al., 2009). QC samples (generally a pool made from the biological test samples, or a representative bulk control sample) should be assessed against predefined criteria to enable acceptance or rejection of the analytical batch. It is also strongly advisable to implement a number of injections (most conveniently the QC sample) at the beginning of the analytical run to "condition" the system. Such conditioning injections, together with those of standards, can also be used to assess system suitability prior to beginning the main run of test samples. The QC sample should be then analyzed repeatedly at regular intervals throughout the run. Following the analysis the QC data and selected ions should be examined first to test for gross changes; these would denote a large change in the system. This would subsequently mean that the system is not stable along the run and the quality and integrity of the data set are suspect.

If the run passes these initial tests, then selected compounds/ions are monitored and evaluated with criteria having to do with parameters such as peak asymmetry and peak shape, stability of signal intensity, mass accuracy, and retention time. A positive outcome would not indicate that the run is successful, but that the data are worth working further. Thus, Figure 7A shows a plot of the retention times measured for the peaks of hippuric acid within an UPLC-TOF run analyzing 60 human urines over a period of 17 hr. Excellent retention time stability was seen. A similar observation is seen in Figure 7B that illustrates extracted ion chromatograms (XIC) for a randomly selected ion, along a UPLC–TOF-MS run of 114 human urine test samples. In the figure XICs from seven consecutive QC samples (pooled sample from the test samples) show very satisfactory analytical repeatability and precision for both retention time and signal intensity. It is important that signals are analytically examined this way as XICs, not only in multivariate statistics which project the major trends in the data set.

A useful approach is to calculate the CV of the intensities of all of the ions found in the QC samples and apply quantitative criteria for the evaluation of the ions stability. We have adapted the FDA proposed criteria for bioanalytical methods validation (Viswanathan et al., 2007), which correspond to 20% signal variability for LC–MS analysis and 30% tolerance for bioanalytical technologies such as ELISA. For untargeted metabolomic analysis by LC–MS (full scan analysis with no use of internal standards or MRM transitions) values close to 30% CV could be allowed (clearly highly variable ions, with CVs in excess of 30%, would not be good candidates as biomarkers). We typically categorize the ions in groups that pass criteria of CV < 15%, CV < 20%, and CV < 30%. We then try to calculate the percentage of these three categories in the total number of ions. Obviously, the aim of this type of analysis is to have 100% of ions with CV < 30%, but a data set containing >80% of ions with CV < 30% would indicate a good quality data set. Given that many journals now allow the provision of supplementary electronic data to complement the main publication we now advocate the value of providing the QC data as supplementary tables as a means of demonstrating acceptable repeatability (e.g., see Lai et al., 2009; Michopoulos et al., 2009). Of importance is also the relation of an ion mass, RT, and intensity with its repeatability. This is exemplified in Figure 7 which shows the distribution of peaks according to the CV value for the analysis of blood plasma. It is seen (Fig. 7C) that a small number of peaks are rather variable, showing a CV > 30%. A number of these unstable peaks are typically seen eluting early in the separation (near the void volume of the LC system) but others are of high mass eluting towards the end of the analysis. Similar findings were also observed in the analysis of urine or other biosamples such as tissue extracts (unpublished observations). Typically peaks which are less repeatable than others are characterized by low signal intensity, which is sometimes close to the cut-off level of the peak picking algorithm. Hence, in certain samples the peak is picked and in other samples the peak is below the cut-off level and a zero value appears. Multivariate analyses can cope with a large number of zero values in the data matrix as described in the literature (Trygg, Holmes, & Lundstedt, 2007) and also seen by the authors even with data sets with up to 40% zero values. However, certain software incorporate a "gap-filling" utility: a script that re-examines raw data to integrate such peaks that appear in those samples where the signal is below the cut-off (noise threshold). This is a useful utility but it is advisable that researchers use it with caution and investigate the quality of the results with and without the application of the script. Poorly repeatable peaks eluting in the solvent front are also common in water-rich specimens such as urine, which comprise large numbers of polar components that would elute (unretained) in the chromatographic void volume. Ion suppression is most likely the major source of error for these ions. Varying concentrations of abundant polar metabolites may also disturb the ionization, and thus the detection, of other co-eluting metabolites (that have stable concentrations in the samples) and produce false markers. Late eluting poorly repeatable peaks, presumably including lipids and related molecules, are also seen as shown in Figure 7. In the case of these late eluting components the contamination of the LC column and retention time drifts are most likely the cause of the variability in the data, presumably due to poor peak alignment. Application of strict retention time tolerance where there is retention time drift may result in the reporting of several variables for one metabolite (these would have the same mass but several slightly different retention times). Thorough investigation of the data set by application of different statistical univariate and multivariate methods and advanced visualization methodologies helps in identifying trends in the data sets but also in better understanding the biological content of samples (e.g., content of polar molecules, content of higher molecular mass metabolites, and so forth).

If the QC samples pass these preliminary screens then advanced statistical analysis is applied: Multivariate statistical analysis can be performed to test whether the QCs cluster closely together in PCA, or if they show time related or other types of trend. Highly variable QC data would mean that the run had failed, whilst close QC data do not automatically mean that the run was acceptable, but do justify further data analysis. If, following the statistical analysis of the sample data, potential biomarkers have been identified it is possible, and advisable, to re-examine the QC data specifically with regard to the variability of the results obtained for those ions specifically highlighted as biomarkers. Figure 8 shows examples of this approach: Figure 8A shows a PCA scores plot (PC1 vs. PC2 for urine analysis) where a tight cluster of QCs indicates acceptable level of data quality. Figure 8B shows the trend plot of a selected ion illustrating stable performance (repeatable signal intensity in the QCs and variable in the test samples). Such a repeatable peak

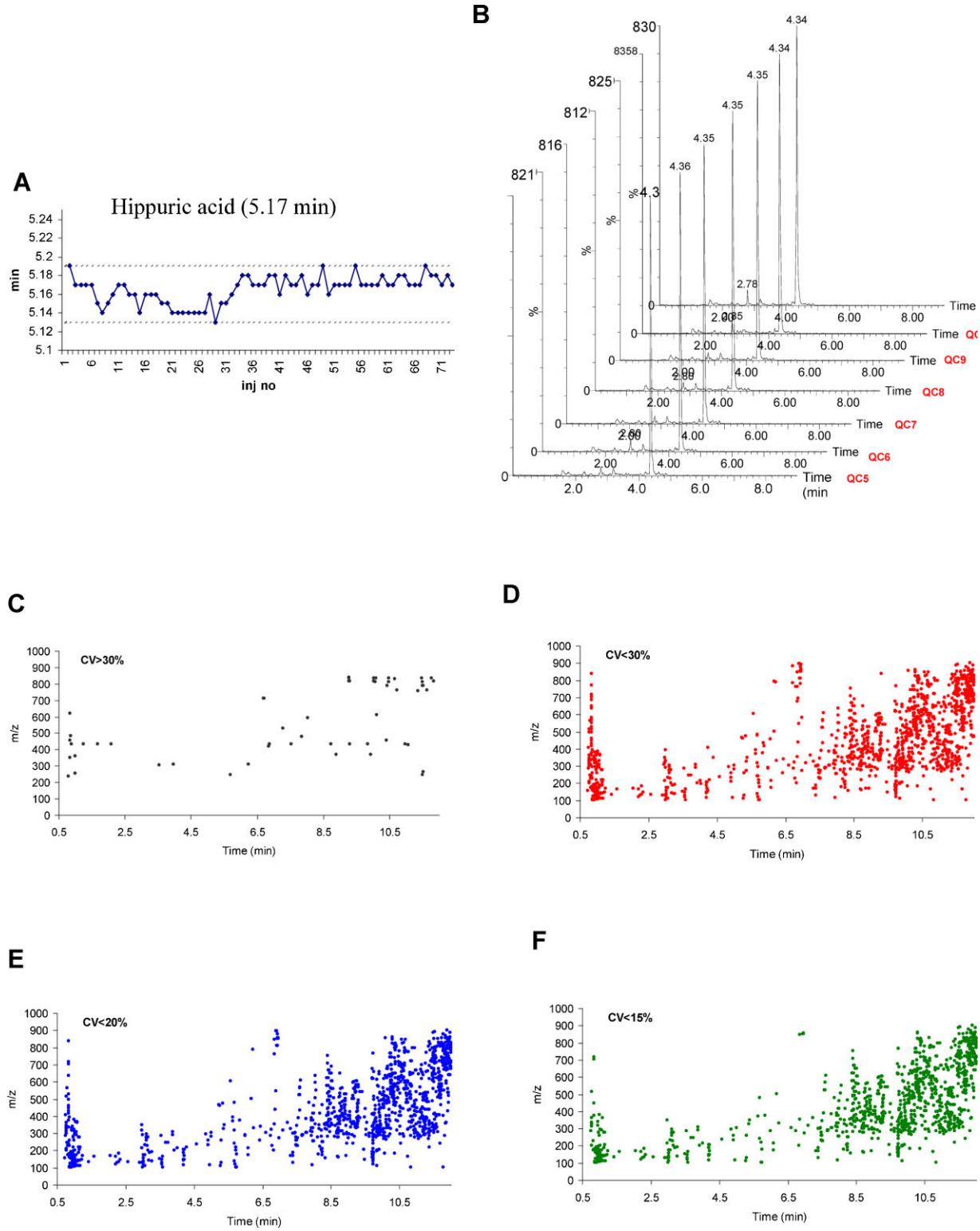

FIGURE 7. Peak repeatability in non-targeted LC–MS metabonomics analysis. **A**: Retention time variation within one data set (duration 17 hr); (**B**) example of excellent stability in UPLC–MS profiling, XIC from seven QC injections spread along a run of ca. 130 urine samples. Distribution of ions according to the signal CV value from the analysis of rodent blood plasma. **C**: Ions with signal's CV > 30% (unstable ions). **D**: Ions with CV < 30%. **E**: Ions with CV < 20% and (**F**) ions with CV < 15%. (B) reprinted from Gika et al. (2008b) with permission from Elsevier.

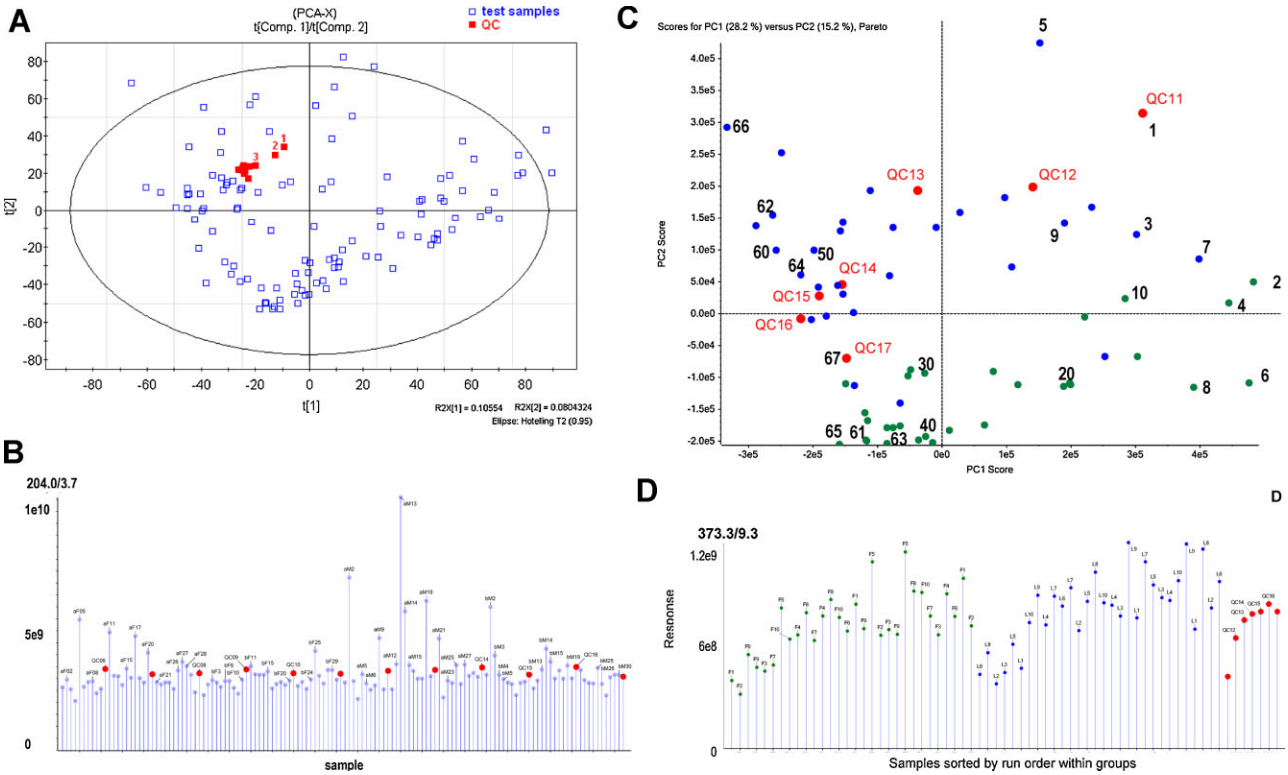

FIGURE 8. Utility of the incorporation of QC samples in the sample set. **A**: PCA scores plot (PC1 vs. PC2) for urine analysis; tight cluster of QCs indicates acceptable level of data quality. **B**: Trend plot of a selected peak (*m/z*: 204.0/rt 3.7 min) illustrating stable performance: repeatable signal intensity in the QCs (red dots) and variable in the test samples ($n = 120$) along a 48 hr LC–MS run of ca. 140 injections. **C**: PCA scores plot (PC1 vs. PC2) from the analysis of Zucker rat plasma samples. Run order effect is evident: see QCs trajectory and sample numbering. **D**: Increasing trend plot for an unstable peak. **C** adapted from Lai et al. (2010), with permission from Royal Society of Chemistry.

could then be investigated further as a potential biomarker. Figure 8C shows a PCA scores plot (PC1 vs. PC2) on the data acquired from the analysis of Zucker rat plasma samples. A run order effect is evident: see the trajectory of the QC samples and also the sample numbering representing the order of injection. Nevertheless separation of the normal Zucker (−/−) wild-type rats (blue dots) from the (fa/fa) strain (green dots) is seen. In such cases deeper investigation of the data may reveal trends as the increasing trend shown in Figure 8D for the peak 373.3/9.3. Peak response is plotted in *y*-axis and run order within group is in *x*-axis. The lean group (−/−) is identified as L (blue dot) and Fat group (fa/fa) is F (green dot). A useful strategy to evaluate such run-order effects is to perform *t*-tests between the first and last samples of the sample set and then investigate the trends of the peaks with the highest *t*-test value (this would indicate ions with great differentiation in intensity along the run).

Metabolite profiling data sets should be thoroughly tested to search for such ions/features. In the ideal case features should show plots such as the one in Figure 8B. However, it is commonly the case that poorly repeatable ions are encountered in which case the researcher has two options: either to fail the run or to fail the ion. Removal of ions (features) from data sets may be considered not to be prudent or useful by hard core informaticians. However, in the experience of the authors (Lai et al., 2009) and others (Burton et al., 2008; Ivosev, Burton, & Bonner, 2008) thinning the data set by removing features that are highly variable, and that do not show a contribution in group separation, is considered to be a "safe" option. One way or the other, before digging deeper into the data to find biomarkers, and then determining their real concentrations via specific targeted methods and unambiguously confirming them as true biomarkers, it is of utmost importance to make sure that the systems performance is at its best by thorough examination of the repeatability of mass signal intensity and retention time data for the majority of the detected ions.

Whilst such factors are a major consideration in metabolic profiling studies of all kinds they become particularly problematic when large-scale epidemiological studies on populations to investigate health or nutrition related issues (specimen mapping, diagnostic biomarkers discovery, drug toxicity studies, etc.) are undertaken. These studies generate large numbers of samples, often collected over a period of years, from many participating subjects. In such cases, whilst it would be desirable to analyze the complete sample set in one long run on a single instrument, however, this is obviously wholly impractical. Either such analyses have to be undertaken on a single instrument using a large number of analytical runs or they must be distributed to a number of instruments, possibly in different facilities. Whichever approach was adopted at some point all of the data from the analyses of these samples would need to be combined for data mining. In an ideal case the analytical systems would be stable and well documented so that issues such as instrument variability, platform variability, or

system stability would not pose problems for metabonomics studies. Data extraction could occur from the whole data set or it would be possible to perform data extraction from the smaller data sets, fuse the data matrices in one, and work further to identify important biomarkers. Some examples of the successful application of this approach for urine analysis by NMR spectroscopy, which is inherently stable and repeatable from laboratory to laboratory and instrument to instrument, have been reported (Dumas et al., 2006; Bertram et al., 2007). However, for LC–MS and GC–MS such approaches are still being developed. Bearing in mind that typical metabolomics runs range in length from 10 to 15 min for LC and 20–35 min or longer for GC and CE, the throughput per day does not seem impressive (30–80 samples and QCs per day). In large epidemiological studies where several hundreds/thousands of samples need to be analyzed it is obvious that this process would require months or years of instrument time. Problems then start to appear from several points: (1) how long are the samples stable? (2) Is the analytical system stable and how can I express this in quantitative manner? (3) How can I minimize batch to batch variation? (4) How can one know when to clean the ion-source or replace a column? (and can one compare the results before and after such an intervention?) (5) How can one compare or correlate the data obtained in one laboratory with those obtained, even on the same type of instrument, in another laboratory? (6) What about joining or comparing results obtained on different analytical platforms (e.g., Orbitrap and QTOF-MS)? (7) Are the biomarkers highlighted the same in both platforms or laboratories? Such questions have been expressed also by other groups and strategies to address these problems have been proposed. Bijlsma et al. (2006) report methodological considerations for experiment planning and data processing for large-scale human metabolomics studies (600 samples). Such considerations and ways to treat unwanted effects and time trends in the analysis of blood plasma from rats and mice have also been recently reported by our group (Lai et al., 2009).

All these issues become important as the metabonomic search for biomarkers can in fact represent a search for items that are sometimes "hidden in the grass." In targeted analyses such issues may be better addressed using internal standards or correlation to other parameters. In targeted analysis clear cut criteria have been established following years of development and debate; for example, FDA or EC directives and guidelines for the validation of bioanalytical methodologies (EC Directive 86/4691986,36 and 657/2002/EC Commission Decision 2002, 8). However, for holistic analytical approaches there is no official guide to evaluate these issues. Finally, one should not forget that the route to valid biomarkers would necessitate a second study and application of independent analytical methods, and these steps unfortunately are rarely taken (Koulman et al., 2009).

Another ongoing debate within MS-based metabolomics circles is the fact that too many variables are recorded (sometimes more than 4,000) from a small number of observations (sample sets of less than 50 samples). This unbalanced data matrix is not optimal for many multivaratiate statistical approaches which were designed to deal with relatively few variables from a large number of samples.

Bearing in mind that this is a genuinely multidisciplinary field, it is obvious that analytical chemists (chromatographers or spectrometrists) with little knowledge of statistics often come to "play" with user-friendly software for data treatment and statistical analysis. It is thus very possible that researchers (unaware of such important critical issues) may be tempted towards over-fitting and be lead to the wrong conclusions.

Debate on the above questions is necessary before planning appropriate experimental protocols to avoid unnecessary variation. Thus, the appropriate length of an analytical run needs to be determined to decide the maximum number of injections that can be performed before data quality is irretrievably affected. Problems of this kind are documented in both GC–MS (e.g., see Fig. 1 in Strehmel et al., 2008) and LC–MS. At present it seems that (UP)LC–MS can handle several hundreds of consecutive injections (of volumes of ca. 10 μL) of diluted urine. However, for plasma or serum much shorter runs (close to 60 injections of samples not including QCs, Lai et al., 2009; Zelena et al., 2009) has been seen to be the practical limit before unacceptable deterioration in performance. In such a case cleaning of the source and the column or even the replacement of precolumn and analytical column are necessary. But then how do the results compare when using different columns? A study conducted by the HUSERMET consortium (Zelena et al., 2009) reported that there was no visual separation in PCA when using two different UPLC acquity columns for the repeat UPLC–TOF-MS analysis of a set of 60 samples of human serum (see Fig. 9A). Furthermore, they have also demonstrated (from the analysis of the same set for four consecutive days) that PCA shows that the results of the two first days lay closer together than the subsequent two, and that there was a clear drift in PC1 corresponding to the run order (see Fig. 9B). This is in agreement with our own experiences (Fig. 9C) for a set of 60 urine samples, where separation of blocks of samples is seen in PC2 (explaining 6.5% of the variation in this study). In this case the 60 urine samples (30 from female and 30 male human subjects) were analyzed using an LC–QTRAP-MS for five consecutive days. As Figure 9C shows, for the first 3 days the result for the samples lie together and cannot be separated, whereas the data from the last 2 days are clearly differentiated. Note that PC1 accounts for 63.4% of the explained variation (having to do mainly with the biological variation of the individual samples). It is also interesting to see how the system becomes stable in this experiment where five QC injections were run first before any test sample to "condition" the system. In Figure 9D the trend plot of the QC injections only is shown for the 5 days of analysis. It is seen that the first injection is always a clear outlier. We have observed that, for urine, five injections of the QC sample usually suffices to condition the system and that from the second injection on the samples fall within the two SD limit. The variation of the initial conditioning QC samples on the first day of analysis was much larger than for the subsequent days; this indicates that once the system is conditioned it can go into the operating conditions much faster. Nevertheless, it is strongly advised that investigators thoroughly examine the trends of the QCs and implement such a conditioning step before the commencement of the analysis. For other specimens such as blood-derived samples (serum or plasma) a more complex process is required for system conditioning and monitoring (see Lai et al., 2009; Michopoulos et al., 2009; Zelena et al., 2009).

Finally, another source of variability comes from the samples themselves. Sample stability has been assessed by both NMR (Lauridsen et al., 2007; Maher et al., 2007) and LC–MS

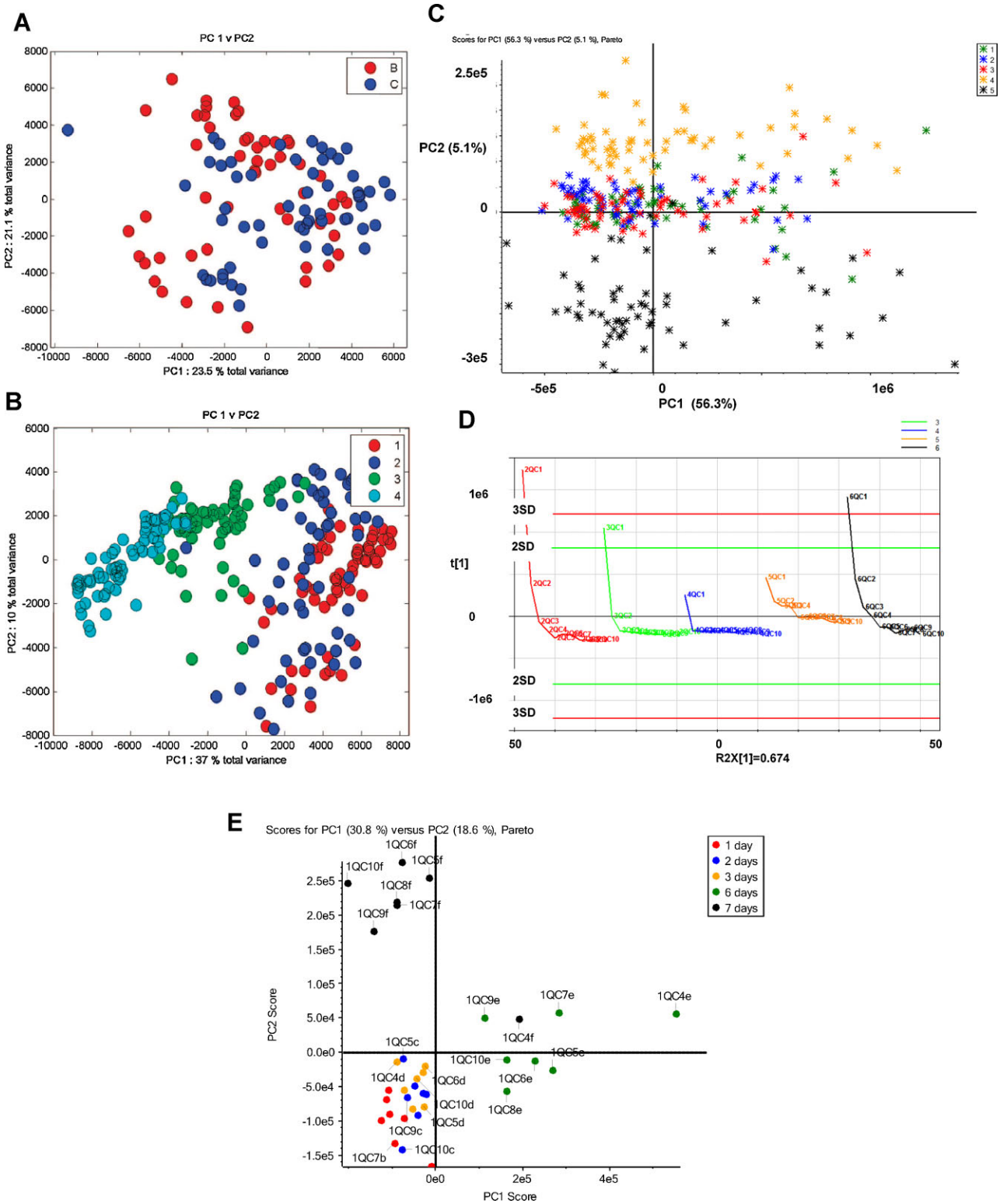

FIGURE 9. **A**: PCA scores plot for data acquired using two UPLC columns from different manufacturing batches. The scores for all the samples analyzed on both columns are overlaid and there is no visual separation between the results from both columns. **B**: PCA scores plot for four identical blocks of human serum samples ($n = 60$) analyzed over a period of 5 days. The first two runs lay together, but there is a significant drift for runs 3 and 4. PC1 contributes to 37% of the total variance. **C**: PCA plot for the LC–MS analysis of 60 human urine samples in five consecutive days (samples colored according to day of analysis). **D**: Plot of $t1$ versus time shows how QC samples settle down with increasing run number. Extract from the data set described in (C). **E**: PCA plot for QCs (human urine) over 7 days storage in the LC–MS autosampler (~4°C). Forty-eight hours seem to be the maximum safe length of time for these samples. (A) and (B) adapted from Zelena et al. (2009), with permission from American Chemical Society.

(Gika, Theodoridis, & Wilson, 2008b). It seems that urine samples are stable for up to 6 months in the freezer (−20 or −80°C) and that 48 hr is the limit for urine samples lying in the autosampler (at 4°C) (see Fig. 9E). After that period samples kept in the autosampler tend to show significant drift. However, more data are needed to evaluate the stability of other specimens and also to evaluate the stability for very-long storage periods (e.g., 3 years or longer, especially bearing in mind the development of biobanks).

H. Data Extraction: How to Treat the Generated Data?

In this type of holistic metabolite analysis the LC–MS derived data can be considered as a cube of data points in three dimensions comprising retention time, *m/z* value, and signal intensity. Typically, the raw data obtained in the mass spectrometer for a metabolomic study represents a series of full scan mass spectra acquired at a given time point. Data treatment prior to multivariate statistical analysis involves a number of processes: noise filtering, baseline correction, centering, normalization, peak picking, peak integration, and alignment. The overall task of these tools is to detect all peaks and collapse the three-dimensional data into a two-dimensional data matrix (a peak-table). A simplified schematic of the process is given in Figure 10 (from Katajamaa and Orešič, 2007). A more detailed scheme of data processing that covers also further statistical and marker evaluation work can be found in Figure 1 in Bijlsma et al. (2006).

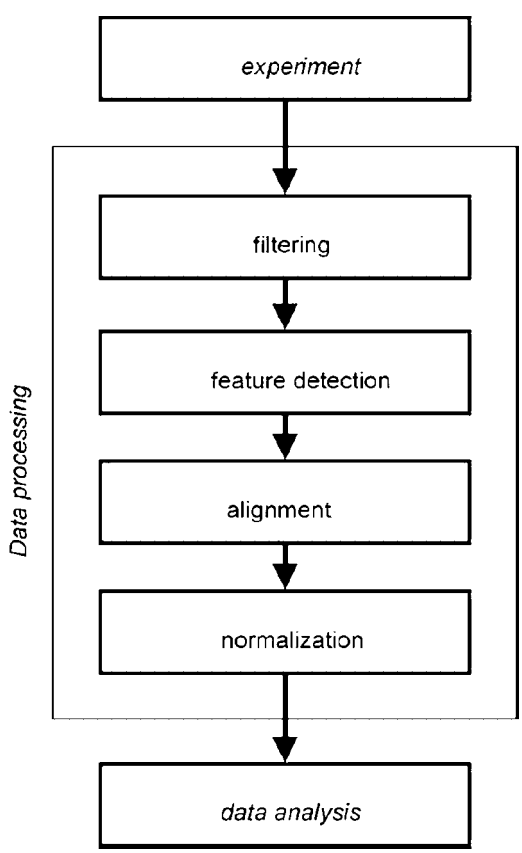

FIGURE 10. Scheme for data extraction: normalization–centering–alignment–peak picking–integration. From Katajamaa and Orešič (2007), with permission from Elsevier.

There are several peak-picking software packages available. Some are freely available, open-source software, which can be found on the Internet. MS manufacturers have also developed their own bespoke metabolomic software, which in some cases operates more like a "black-box" and does not allow modifications or alterations in the way the algorithm operates. In most cases the software also performs multivariate statistics, such as PCA, and provides scores plots. Typically, these software packages can process data originating only from the corresponding mass spectrometer (e.g., MarkerLynx from Waters (Manchester, UK), can analyze data from Waters Instruments, MarkerView similarly works on data from Sciex instruments). Alternatively, the freely available software programs such as MZmine, MetAlign, and XCMS can analyze data in universal formats such as netCDF that originate from any type of MS instrument and often provide more information on the processes involved in data treatment (see, for example, Lommen, 2009).

Some software combine peak picking and alignment with advanced visualization tools, multivariate statistics (both supervised and non-supervised), univariate statistics, and marker identification functionalities (such as automated database search and formulae prediction). For example, the XCMS program (Smith et al., 2006) uses the R platform to perform peak picking, nonlinear retention time alignment, and relative quantification. Data extraction can also be performed with in-house developed, proprietary, software. A list of data processing software is given in Table 1, and Katajamaa and Orešič (2007) have produced a comprehensive review on the field. Some of these programs are directly linked to public on-line databases; web base resources or data depositories for mass spectrometry metabolomics are not as well established as for proteomic or genomic research (Arita, 2009) but developments in that direction are being made (see Tohge & Fernie, 2009 for a review of the topic).

It is accepted that the data processing step is of crucial importance in metabolomics studies. Hence, it is sometimes stated that, starting from the same data set, 10 different researchers will reach 10 different results after data extraction and multivariate analysis. This may happen in sample sets of reduced biological variation (e.g., inbred animals living in the same environment and diet). When applying such advanced statistical tools, one should remember that these operate basically as projection methods that highlight the important trends. So these trends should be viewed with caution and from various "angle points." Hence, it is advisable to apply different scaling methods. These could make huge impact in the resulting scores and loadings plots. However, as we have indicated (see also Fig. 6), we have seen that for sample sets of human urine, analyzed on three different mass spectrometers and subject to data analysis using two different data treatment software we nevertheless obtained very similar results (even when altering the scaling from Pareto to UV and mean centered). Clearly, as LC–MS becomes even more widespread in its use for global metabolite profiling there is a need for a focus on interlaboratory, and cross platform, comparisons to determine data comparability. The truth is that, until hard comparison data are obtained via, for example, "round robin" tests organized within and between different analytical facilities, using a variety of software analysis options, the scale of this potential problem remains unresovled.

TABLE 1. Data extraction software

Software Tool	Developer/Availability	Reference/Information
AnalyserPro	SpectralWorks	http://www.spectralworks.com/analyzerpro.asp
ChromaTOF	LECO	http://www.leco.com/index3.htm
COMSPARI	Freely available	http://www.biomechanic.org/comspari/
Expressionist/ Refiner	MS Genedata	http://www.genedata.com/products/expressionist/index_eng.html
Genespring* Mass Hunter/Mass Profiler	Agilent Technologies	http://www.chem.agilent.com
MarkerLynx™ *	Waters	http://www.waters.com
Markerview™	MDS/Applied Biosystems	http://www.mdssciex.com/
MathDAMP	Freely available	http://mathdamp.iab.keio.ac.jp/
MetAlign	Freely available	http://www.metalign.wur.nl
MET-IDEA	Freely available	http://www.noble.org/PlantBio/MS/MET-IDEA/index.html
MSFACTS	Freely available	http://www.noble.org/PlantBio/MS/MSFACTs/MSFACTs.html
Mzmine *	Freely available	http://mzmine.sourceforge.net/
Profiler M *	Phenomenome Informatics	http://www.phenomenomeinformatics.com/pro_M_main.htm
SIEVE	ThermoFisher Scientific	http://www.thermofisher.com/global/en/home.asp
XCMS / METLIN *	Freely available	http://metlin.scripps.edu/download/
MS Resolver,	Pattern Recognition Systems	http://www.prs.no/MS%20Resolver/MS%20Resolver.html

Marked with an asterisk (*) are denoted the software with database searching for marker identification.

I. Identification of the Markers

Last but not least comes the most difficult part of a metabolic profiling exercise: The identification of those metabolites highlighted as potential markers. The overall aim of any metabonomics study is to come-up with key molecules that play an important role in a biochemical process and characterize certain physiological states. To do this we have to be able to place a chemical identity against the feature that has been identified by the multivariate statistical analysis as a pair of numbers: mass and retention time. This task currently represents the main bottleneck in contemporary metabolomics. Even in the case of GC–MS, with its well-established spectral databases, considerable effort is required, partly because the bulk of the compounds in these databases are of synthetic rather than biological origin. However, by comparison, in LC–MS such databases are still under development and are also often not interchangeable between different instruments or laboratories. Hence, the effort for full identification is much greater and may demand a series of additional experiments. Marker identification can be divided into two different cases: (1) the identification of known compounds (also called peak annotation), for example, the assignment of a feature to a known amino acid and its confirmation against an authentic standard and (2) the identification of unknown chemical entities in unknown samples where structure elucidation is also required.

To achieve these ends a number of desired analytical characteristics are sometimes required: (1) accurate mass measurements, (2) the study of fragmentation patterns, (3) isotope ratio determination, (4) neutral loss experiments, (5) H/D exchange experiments, (6) ring double bond equivalent (RDBE) calculations, (7) application of the nitrogen rule, (8) interrogation of existing spectral or metabolic databases, (9) isolation for additional NMR, TLC, SPE, or GC–MS experiments.

An excellent review article describes in detail the ways to treat MS signal data to reach the identification of metabolites (Werner et al., 2008). A brief description is given here: calculation of the elemental composition is the first step and the resulting formulae can be used in searching in chemical and metabolic databases. Accurate mass measurements are needed instead of nominal mass measurements because the former are much more informative and helpful. Therefore, mass accuracy at a level of 5 ppm or better is strongly recommended. High mass accuracy may reduce the number of candidates to, for example, a number smaller than 10 molecules, but this alone may not suffice (Kind & Fiehn, 2007) as it has been reported that even at 0.5 ppm accuracy, marker identification may fail. In such a case data from fragmentation experiments and isotope ratios are the first additional resources. Exploitation of high collision and low collision energy experiments may provide useful diagnostic ions and has been proposed as a means of highlighting specific classes of metabolite (e.g., glucosides or sulfates; Plumb et al., 2009). Isotope ratios are very useful for elemental composition determination but they can be affected by interferences and saturation effects. Ding et al. (2008) used high-end mass spectrometers (Orbitrap and FT-ICR-MS) for the identification of lipids. Their strategy was to obtain data-dependent MS/MS information of human plasma, erythrocyte, and lymphocyte

lipids in both positive and negative ESI modes to assign lipid class and fatty acid composition. Identification of lipid molecular species was based on manual interpretation and comparison with Lipid Maps data. The methodology managed to identify co-eluting glycerophospholipids because these species displayed characteristic MS/MS fragmentation patterns. Results of this identification strategy can be seen in Table 2.

Loftus et al. (2008) described the use of a special algorithm that incorporates MS–MS, ion ratio, and accurate mass data to automatically provide candidate formulas. These were compared with known databases to reach identification of phospholipids in the blood plasma of Zucker rats. If this process finds no (or a large number of) candidates after searching in the databases the nitrogen rule and/or RDBE can be used to rule out some of the candidates. Caution is needed as the nitrogen rule is not reliable for masses higher than 500 amu and RDBE becomes blurred when dealing with sulfur or phosphorus-containing molecules. In those cases that the identity is still not reached, preparative LC or TLC and subsequent NMR or GC–MS analysis may be needed to solve the problem. Obviously, this process may be extremely labor intensive (and time consuming), so it should be undertaken only for the identification of well-validated and worthy biomarkers. A schematic of the process is shown in Figure 11 (reprinted from Werner et al., 2008) and related figures can be found elsewhere in the literature (e.g., Lu et al., 2008b). Effort at identification may be greatly assisted by the special software tools developed by the mass spectrometer manufacturers (marked with an asterisk in Table 1). In Table 3 existing public databases that contain biological and mass spectral data are listed. Finally, we should not forget that this type of data is multidimensional incorporating LC retention time data. If the latter in some cases may be an additional source of error (due, e.g., to poor alignment), this is because the LC dimension is also a source of information. Retention time in LC (and in GC as discussed in the corresponding paragraph) is governed by the metabolite molecular properties. In RPLC retention time can be correlated to octanol/water distribution (log *P*) although this is fraught with difficulty in practice. However, the use of log *P* data

TABLE 2. MS and MS/MS information for individual classes of lipids under both positive and negative ion modes

Class	Head group	Positive mode		Negative mode	
		Observed *m/z* in MS	Observed *m/z* in MS/MS	Observed *m/z* in MS	Observed *m/z* in MS/MS
Monoacylphosphocholine (LPC), diacylphosphocholine (PC), alkenyl-acyl phosphocholine (*p*-PC), alkyl-acyl phosphocholine (*o*-PC), sphingomyelin (SM)	Phosphocholine	$[M+H]^+$	184 ($C_5H_{15}NO_4P$), $[M+H-59]^+$, $[M+H-183]^+$, $[M+H-R'_2CHCO]^+$, $[M+H-R'_1CHCO]^+$, $[M+H-R_2COOH]^+$, $[M+H-R_1COOH]^+$	$[M+CH_3COO]^-$	$[M-CH_3]^-$, $[M-H]^-$, carboxylate anion
		$[M+Na]^+$	184, $[M+Na-N(CH_3)_3]^+$		N/D
Monoacylphosphoethanolamine (LPE), diacylphosphoethanolamine (PE), alkenyl-acyl phosphoethanolamine (*p*-PE), alkyl-acyl phosphoethanolamine (*o*-PE)	Phosphoethanolamine	$[M+H]^+$, $[M+Na]^+$	$[M+H-141]^+$, neutral loss of 141 ($C_2H_8NO_4P$)	$[M-H]^-$	Carboxylate anion
Oxidized phosphocholine	Phosphocholine	$[M+H]^+$	Loss of water, oxygen atom, methanol	$[M+CH_3COO]^-$	$[M-CH_3]^-$, $[M-H]^-$, $[M-CH_3OH]^-$, carboxylate anion, etc.
Oxidized phosphoethanolamine	Phosphoethanolamine			$[M-H]^-$	$[M-H-H_2O]^-$, $[M-H-CH_3OH]^-$, carboxylate anion, etc.
Phosphoserine (PS)	Phosphoserine	$[M+H]^+$	Neutral loss of 185 ($C_3H_8NO_6P$)	$[M-H]^-$	Neutral loss of 87 ($C_3H_5NO_2$)
Phosphoinositol (PI)	Phosphoinositol	$[M+NH_4]^+$	Neutral loss of 277 ($C_6H_{16}NO_9P$)	$[M-H]^-$	241 ($C_6H_{10}O_8P^-$)
Triacylglycerol (TAG)	N/A	$[M+NH_4]^+$	Neutral loss of fatty acid		N/D
Cholesterol, cholesterol esters	N/A	$[M+NH_4]^+$	369 ($C_{27}H_{45}^+$)		N/D

Reprinted from Ding et al. (2008) with permission from Elsevier.

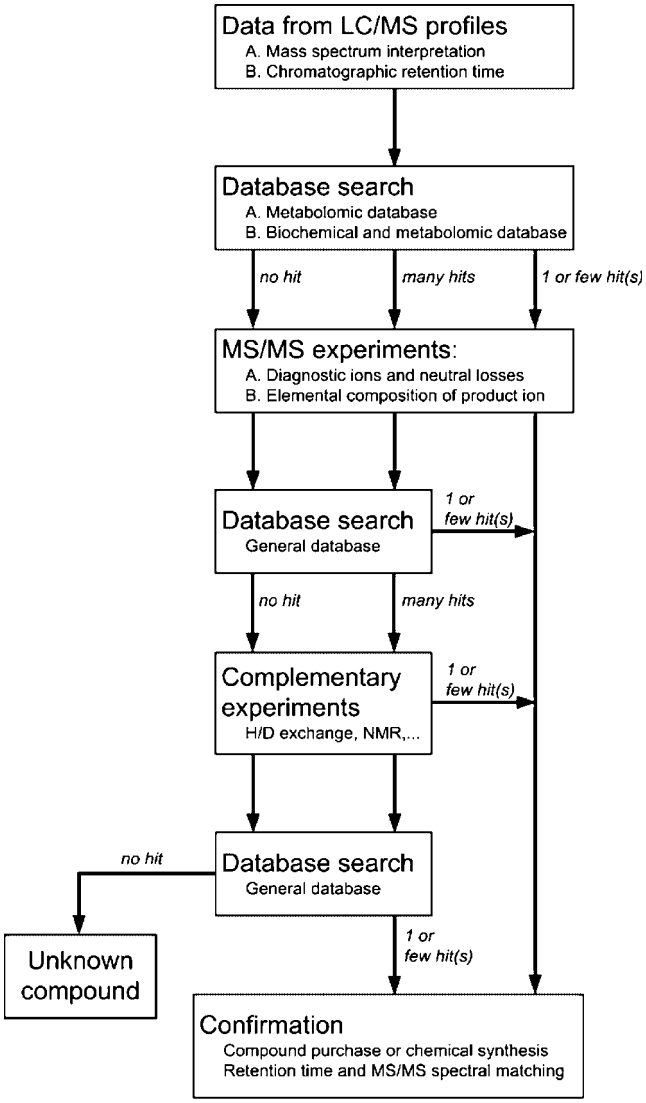

FIGURE 11. Flowchart of a proposed strategy for the identification of potential biomarkers found in LC–MS-based metabolomic/metabonomic analysis. Reprinted from Werner et al. (2008), with permission from Elsevier.

(already in progress in the Human Metabolome Database) may assist in identification of unknowns or even in partial identification of unknown peaks often detected in test samples. Good practice in metabonomic studies is, of course, always to confirm any identification by comparison with an authentic standard where these are available (using two orthogonal separations if possible) and until this is done any identification must be considered provisional. Further validation is advisable: second-stage experiments for sample collection and analysis again by both untargeted analysis and also by specific methods to confirm the findings (Koulman et al., 2009).

III. CONCLUSIONS

Metabolomics and metabonomics represent a major research area in the systems biology arena to complement the information provided by genomics and proteomics (Blow, 2008; Arita, 2009). Although NMR is still the technology with the widest utilization in the field, mass spectrometry is increasingly being applied, coupled to either gas or liquid chromatography. The application of LC–MS in global metabolite profiling has certain desirable features, including the flexibility of the technology arising from the availability of a range of different separation mechanisms and media combined with high separation efficiency, MS-based metabolite characterization and identification, generally good sensitivity, widespread availability, ease of sample preparation, and data extraction. This means that the metabolome coverage provided by LC–MS is probably the most comprehensive currently available from any metabolite profiling platform. However, it must be kept in mind that these holistic metabolic profiling studies may have different end results and aims. If the scope of a study is comprehensive mapping of certain samples, all platforms (NMR, GC–MS, LC–MS) should be applied and we could expect a higher number of metabolites to be found in LC–MS. If the scope of the study is biomarker discovery, the collection of larger numbers of variables does not guarantee by any means that the analysis is more powerful or that it will reveal better markers. Again application of all three platforms should be the best and safest resort for more comprehensive metabolome coverage, better validation of results, or unambiguous marker identification. Efforts are needed to work in parallel or to combine these complex data sets (see, for example, GC–MS and LC–MS data combination in Sreekumar et al., 2009; t'Kindt et al., 2009; Gao et al., 2008; NMR and LC–MS data combinations in Lenz et al., 2004b; Law et al., 2009) and to develop tools to assist data mining.

As more researchers come into the field and as new more powerful MS machines are introduced (e.g., latest generation TOF-MS that reach resolution of more than 40,000), the application of LC–MS in metabolomics will continue to increase. With the current state of the art, UHPLC coupled to QTOF or Orbitrap MS seems to be the best choice for this type of work. Important remaining issues include robustness, instrument variability, cross-platform comparison (and in fact the lack of data in that aspect), application in large sample sets, and data treatment. All these issues are interconnected and related to each other. Before LC–MS metabolomics and metabonomics can be considered to have become routine in clinical, toxicity or food quality applications, solutions (or at least standard operating procedures), need to be assembled and accepted. Obviously, this would require common efforts from the research community, instrument manufacturers, informaticians, and last but not least the end users. Since this is multidisciplinary research, scientists from different starting points and disciplines need to co-operate closely to address current issues and promote the field. This process may change the way that people are working: for example, informaticians should understand the reasons why instrument performance is changing to find ways to overcome this. Further, clinicians should adapt to the requirements of metabonomics experiments when collecting/handling samples. Analytical chemists should learn more biochemistry and/or informatics and so forth.

Global metabolic profiling studies will continue to increase as an important area of "omics" research because it provides answers about what is happening now in the organism (or the organ) of interest. For this reason metabolomics and metabonomics should address the analysis of target organs (Veenstra &

TABLE 3. Databases useful in metabolomics research

DATABASE	Thematic	Web page/Link
The Human Metabolome Database (HMDB)	Human	http://www.hmdb.ca/
The BiGG database	Human	http://bigg.ucsd.edu/
KEGG (Kyoto Encyclopedia of Genes and Genomes)	General	http://www.genome.jp/kegg/
BioCyc/MetaCyc/HumanCyc	Pathways	http://biocyc.org/ http://metacyc.org/ http:/humancyc.org/
ChemFinder	General	www.chemfinder.CambridgeSoft.com
MoTo: Metabolome database for tomato, Wageningen University	Tomato	www.appliedbioinformatics.wur.nl
Golm: Plant Max Planck Institute for Molecular Plant Physiology (Germany)	Plant	www.csbdb.mpimp-golm.mpg.de
Madison Metabolomics Consortium Database	General	http://mmcd.nmrfam.wisc.edu/
CSLS, NIH (USA)	General	http://129.43.27.140/cgi-bin/lookup/search
ChEBI: European Bioinformatics Inst.		www.ebi.ac.uk/chebi/
The DrugBank	Pharma-ceuticals	http://www.drugbank.ca/
Therapeutic Target Database	Pharma-ceuticals	http://xin.cz3.nus.edu.sg/group/ttd/ttd.asp
STITCH		http://stitch.embl.de/
LipidBank	Lipids	http://lipidbankjp/
LipidMaps	Lipids	http://lipidmaps.org/
CHEMnetBase	General	www.chemnetbase.com
KNApSAcK	Natural products	http://kanaya.naist.jp/ KNApSAcK/ KNApSAcK.php
MassBank	Mass Sp	http://www.massbank.jp/
METLIN	Mass Sp	http://metlin.scripps.edu/index.php
Fiehn GC-MS database,	Mass Sp	http://fiehnlab.ucdavis.edu/Metabolite-Library-2007/
BioMagResBank		http://www.bmrb.wisc.edu/metabolomics/
ChemSpider	Mass Sp	http://www.chemspider.com/
SciFinder	General	http://www.acs.org
PubChem	Mass Sp	http://www.ncbi.nlm.nih.gov/

Databases which are mainly biological are listed in the upper part and spectral databases are listed in the lower part (shadowed cells).

Zhou, 2009) and also investigate the correlation of organ profiles with the metabolite profile of other, more accessible and less invasive, samples such as saliva or urine, and MS imaging technologies seem very promising in these aspects. Great prospects and important discoveries lie ahead in this exciting research topic.

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