Mass spectrometry in metabolome analysis. Mass Spectrom Rev

ARTICLE *in* MASS SPECTROMETRY REVIEWS · SEPTEMBER 2005

Impact Factor: 7.71 · DOI: 10.1002/mas.20032 · Source: PubMed

5 AUTHORS, INCLUDING:

Silas G Villas-Bôas
University of Auckland
92 PUBLICATIONS **2,113** CITATIONS
SEE PROFILE

Mats Akesson
Novo Nordisk
24 PUBLICATIONS **1,504** CITATIONS
SEE PROFILE

MASS SPECTROMETRY IN METABOLOME ANALYSIS

Silas G. Villas-Bôas, Sandrine Mas, Mats Åkesson, Jørn Smedsgaard and, Jens Nielsen*

Center for Microbial Biotechnology, BioCentrum-DTU, Technical University of Denmark, Building 223, DK-2800 Kgs. Lyngby, Denmark

Received 1 March 2004; received (revised) 19 May 2004; accepted 7 June 2004

Published online 19 August 2004 in Wiley InterScience (www.interscience.wiley.com) DOI 10.1002/mas.20032

In the post-genomic era, increasing efforts have been made to describe the relationship between the genome and the phenotype in cells and organisms. It has become clear that even a complete understanding of the state of the genes, messages, and proteins in a living system does not reveal its phenotype. Therefore, researchers have started to study the metabolome (or the metabolic complement of functional genomics). Within this context, mass spectrometry (MS) has increasingly occupied a central position in the methodologies developed for determination of the metabolic state. This review is mainly focused on the status of MS in the metabolome field, trying to direct the reader to the main approaches for analysis of metabolites, reviewing basic methodologies in sample preparation, and the most recent MS techniques introduced. Apart from the description of the different methods, this review will try to state a general comparison between the several different techniques that involve MS and metabolite analysis, and will highlight their limitations and preferred applicability. © 2004 Wiley Periodicals, Inc., Mass Spec Rev 24:613–646, 2005

Keywords: *metabolite profiling; functional genomics; metabolic fingerprinting; metabolic footprinting; phenotypic characterization; metabolite analysis*

I. INTRODUCTION

Being the intermediates of biochemical reactions, metabolites play a very important role in connecting the many different pathways that operate within a living cell. The level of the metabolites are determined by the concentration and the properties of the enzymes, and their level is, therefore, a complex function of many different regulatory processes inside the cell; i.e., regulation of transcription and translation, regulation of protein–protein interactions, and allosteric regulation of enzymes through their interaction with metabolites. Thus, the level of metabolites represents integrative information of the cellular function, and, hence, defines the phenotype of a cell or tissue in response to genetic or environmental changes. Adding further to the complexity is the fact that many intracellular metabolites participate in a large number of different biochemical reactions, and, hereby, tie many different parts of the cellular metabolism together as a tightly controlled metabolic network (Nielsen, 2003). Analysis of cellular function at the molecular level requires recruitment of several different analytical techniques. Whereas comprehensive methods for analysis at the transcriptional level (transcriptome) and at the translational level (proteome) are currently in a rapid state of development, and high throughput analytical methods are already in use (Godovac-Zimmermann & Brown, 2001; Baldi & Hatfield, 2002; Cristoni & Bernardi, 2003), methods for analysis of the metabolomic approaches are, however, so far less common.

Although metabolite profiling has long been applied for medical and diagnostic purposes (Horning & Horning, 1971; Gates & Sweeley, 1978) as well as for phenotypic characterization (Frisvad & Filtenborg, 1983), it is not until recently that increasing efforts have been undertaken to develop methods to screen of a high number of intracellular metabolites in the context of functional genomics (Fiehn, 2001; Trethewey, 2001). However, increases in mRNA levels do not always correlate with increases in protein levels (Gygi et al., 1999), and once translated a protein may or may not be enzymatically active (Sumner et al., 2003). Therefore, changes observed in the transcriptome or in the proteome do not always correspond to phenotypic alterations. Thus, measurement of the metabolites synthesized by a biological system, "the metabolome," constitutes an important complement to assess genetic function (Oliver, 1997; Oliver et al., 1998; Hellerstein, 2004).

In common with the transcriptome and the proteome, the metabolome is context-dependent, and the levels of each metabolite depend on the physiological, developmental, and pathological state of a cell, tissue, or organism. However, an important difference is that, unlike mRNA and proteins, it is difficult or impossible to establish a direct link between genes and metabolites. The convoluted nature of cell metabolism, where the same metabolite can participate in many different pathways, complicates the interpretation of metabolite data. In addition, Schwab (2003) showed that, for complex organisms such as plants, there are more metabolites than genes in a biological system, whereas for microorganisms there are generally fewer metabolites than genes; i.e., for the yeast *Saccharomyces cerevisiae* there are fewer than 600 metabolites (Förster et al., 2003). Multiple mRNAs could be formed from one gene, multiple proteins from one mRNA, and multiple metabolites may be formed from one enzyme, because many enzymes may accept more than one substrate, although enzymes generally have high selectivity.

Metabolome analysis covers the identification and quantification of all intracellular and extracellular metabolites with molecular mass lower than 1000 Da, using different analytical techniques. In addition to applications in functional genomics, quantification of metabolite concentrations enables identification of the kinetics that underlies specific intracellular reactions

**Correspondence to:* Jens Nielsen, Center for Microbial Biotechnology, BioCentrum-DTU, Technical University of Denmark, Building 223, DK-2800 Kgs. Lyngby, Denmark. E-mail: jn@biocentrum.dtu.dk

(Buchholz et al., 2002; Hellerstein, 2004). Together with the structure of a metabolic network, the levels of the intracellular metabolites represent key information to gaining understand about how the fluxes through different intracellular metabolites are regulated; information is valuable not only in functional genomics but also in the development of efficient cell factories through metabolic engineering (Nielsen, 2001, 2003; Hellerstein, 2004).

The genome, transcriptome, and proteome elucidations are based on target chemical analyses of biopolymers composed of 4 different nucleotides (genome and transcriptome) or 22 amino acids (proteome). Those compounds are highly similar chemically, and facilitate high-throughput analytical approaches. Within the metabolome, there is, however, a large variance in chemical structures and properties. Thus, the metabolome consists of extremely diverse chemical compounds from ionic inorganic species to hydrophilic carbohydrates, volatile alcohols and ketones, amino and non-amino organic acids, hydrophobic lipids, and complex natural products. That complexity makes it virtually impossible to simultaneously determine the complete metabolome. Therefore, the metabolome has been studied with efficient sample preparation and with selective extractions coupled to a combination of different analytical techniques to achieve as much information as possible.

Figure 1 summarizes the different strategies to study the metabolome. Fiehn (2002) defined different approaches for analysis of the metabolome; e.g., target analysis, metabolite profiling, metabolomics, and metabolic fingerprinting. However, we argue that, from the methodological point of view, there are basically only two different strategies: (i) target analysis and (ii) metabolite profiling. In our opinion, target analysis is restricted to quantitative analysis of a class of compounds that are related to a specific pathway or to intersecting pathways. Targeted analysis is very useful for the study of the primary effect of a genetic alteration, and the analytical procedures must include identification and absolute quantification of the selected metabolites in the sample. Metabolite profiling (or, sometimes, metabolic profiling), on the other hand, involves rapid analysis, often not quantitative, of a large number of different metabolites with the objective to identify a specific metabolite profile that characterizes a given sample. This approach can be sub-divided into (a) metabolic fingerprinting and (b) metabolic footprinting. Fingerprinting covers the scanning of a large number of intracellular metabolites detected by a selected analytical technique or by a combination of different techniques in a defined situation. Not all metabolites must be identified and quantified, and ''raw'' data can be used; i.e., one may use the information content from mass spectrometry (MS) profiles or nuclear magnetic resonance

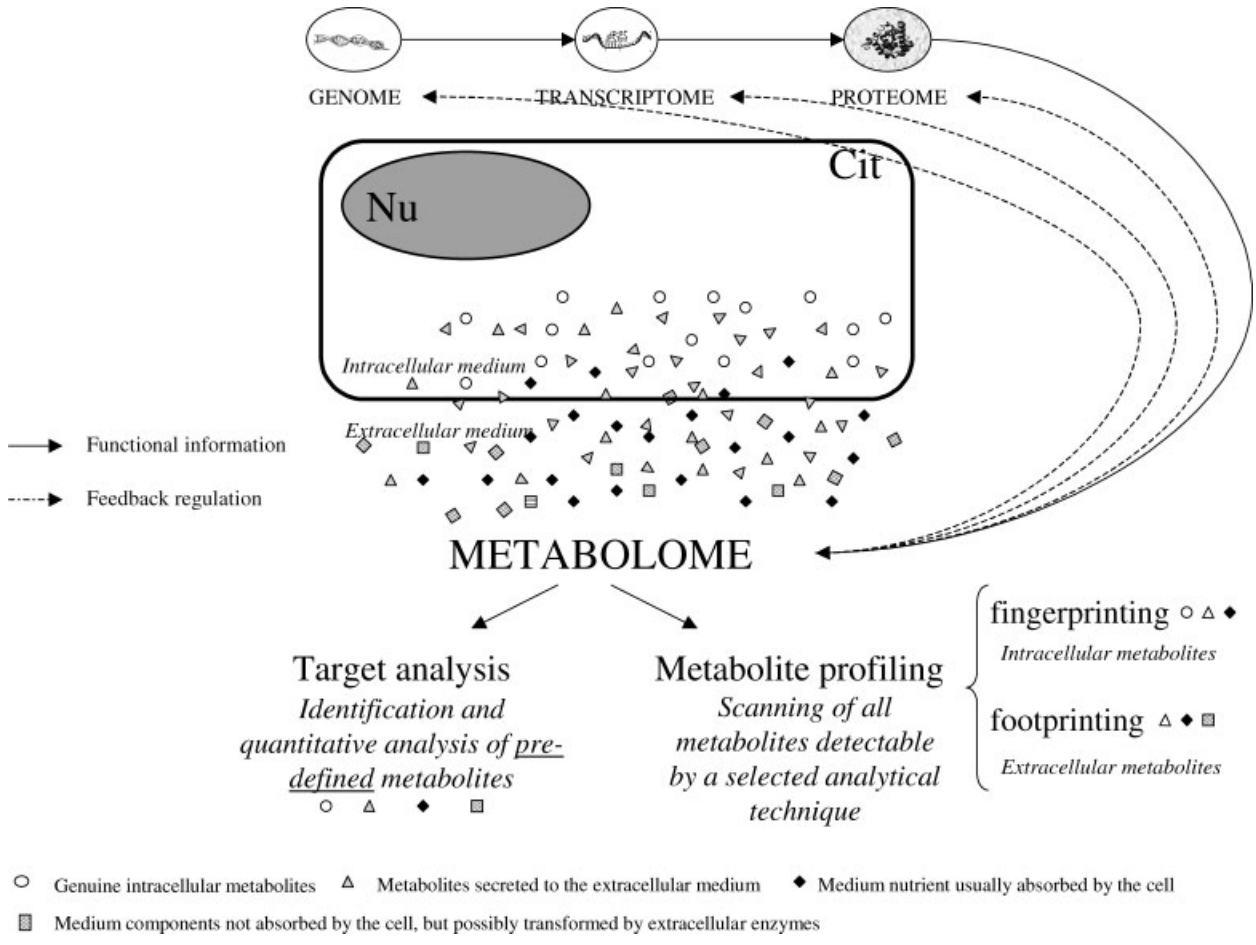

FIGURE 1. Metabolome analysis in the context of functional genomics. Nu, nucleus; Cit, cytoplasm.

(NMR) spectra directly, but the method must give a reproducible profile of the sample. Metabolic footprinting is a more recently proposed approach (Allen et al., 2003), which is technically similar to fingerprinting, but is focused on the measurement of all extracellular metabolites present in a spent culture medium. The compounds determined are metabolites secreted by the cells into the medium and the medium components biochemically transformed by the organism. Both approaches of metabolite profiling can be used to distinguish between different physiological states of wild-type strains, and between single-gene deletion mutants from even nominally closely related areas of metabolism. Important physiological information can be extracted using these approaches, but it requires identification of the individual metabolites analyzed, and it is often laborious and difficult. Our definitions of key terms used in the field of metabolome analysis are given in Table 1.

Mass spectrometry (MS) and NMR are the most frequently employed methods of detection in the analysis of the metabolome. NMR in particular, is very useful for structure characterization of unknown compounds, and has been applied to the analysis of metabolites in biological fluids and cells extracts (Shockcor et al., 1996). However, in certain circumstances, the ^{1}H NMR spectrum is insufficient on its own to provide information that will fully characterize a metabolite. This limitation is obviously the case where analytes contain functional groups that are deficient in protons or where the protons can readily chemically exchange with the solvent; the signals are broadened beyond detection. Alternatively, other nuclei can also be used; such as ^{13}C NMR for labeled metabolites (Shockcor et al., 1996; Des Rosiers et al., 2004). However, ^{13}C NMR spectroscopy presents relatively low sensitivity; i.e., in the range of μmol to mmol. In addition, ^{13}C NMR analysis may take several hours for

TABLE 1. Definitions of terms used in this review*

Metabolome	The complete set of small molecules (metabolites) produced by a cell as a result of its metabolism; time-resolved, and substrate-dependent.
Metabolome analysis	Determination of the metabolome. In practice, this goal is not possible because there is no single method (or limited set of methods) that enables that analysis of the complete metabolome. However, this term is often used as a broad term to describe the general experimental approach to analyze groups of metabolites that are produced by a cell as a function of its metabolism; i.e., either though a targeted analysis or a metabolite profiling (screening).
Targeted analysis	Quantitative analysis of a single or selected metabolites produced by a cell
Metabolite profiling	A large number of metabolites produced by a cell as seen by specific methodologies (combined sampling-extraction-analyses); often a rapid, and not necessarily quantitative, approach.
Metabolic fingerprinting	A large number of intracellular metabolites as seen by specific methodologies (combined sampling-extraction-analyses); often a rapid, and not necessarily quantitative, approach
Metabolic footprinting	Profile of metabolites that remain in the media; i.e., extracellular metabolites or the "exome", determined by a specific methodology (combined sampling-extraction-analysis); often a rapid, and not necessarily quantitative approach. Biotransformation of substrate components is a part of the footprint.

*Molecules with a mass below approximately 1000 Da is considered as a part of the metabolome.

a single sample, as a consequence of its low sensitivity (Des Rosiers et al., 2004), and the equipment costs are much higher compared to MS based techniques.

The most important advantages of MS are its high sensitivity, and high-throughput in combination with the possibility to confirm the identity of the components present in the complex biological samples as well as the detection and, in most of the cases, the identification of unknown and unexpected compounds. Furthermore, the combination of separation techniques (e.g., chromatography) with MS tremendously expands the capability of the chemical analysis of highly complex biological samples. The basic information of mass spectra is characterized by its simplicity. The spectrum displays masses of the ionized molecule and its fragments, and those masses are simply the sums of the masses of the component atoms. In some cases, a mass spectrum contains a wealth of specific analytical and structural information, much more information than the expert in the field currently can utilize; unfortunately, that abundance of information can discourage the novice who turns to compendia of MS information for help. Nevertheless, it is comparatively simple to handle the mass spectra and there are several available software applications that make the interpretation of MS data relatively easy.

This review will focus on applications of MS for metabolome analysis, and will highlight recent developments with a focus on the advantages and disadvantages in the use of MS as a stand-alone technique or combined with a separation technique. Because sample preparation has a major impact on the results obtainable, a discussion of quenching techniques, metabolite extraction, and concentration methodologies is also included. Finally, we address the challenges in data analysis combined with some examples of applications on functional genomics.

II. SAMPLE PREPARATION

In metabolome analysis, adequate methods for sample preparation are of outmost importance. Figure 2 illustrates the key steps in sample preparation. Because the metabolite levels reflect the final response of a biological system to a genetic or an environmental change, rapid quenching of all biochemical processes is the first step. That step is very important for metabolome analysis because the metabolite concentrations very rapidly reflect changes induced by any (unnoticed) variation in the environment of the cell (Koning & van Dam, 1992). For *S. cerevisiae* growing aerobically on glucose, the typical half-life for an intracellular metabolite is on the order of a second or less; i.e., cytosolic glucose is converted at a rate of approximately 1 mM/s and ATP at approx 1.5 mM/s (Koning & van Dam, 1992). Also, for an analysis of extracellular metabolites it is important to quench cellular activity, however, generally the half-life for extracellular metabolites is longer than for intracellular metabolites.

Following the quenching step, it is necessary to separate the cellular phase from the extracellular medium, and if intracellular metabolites are to be measured, then those metabolites must be

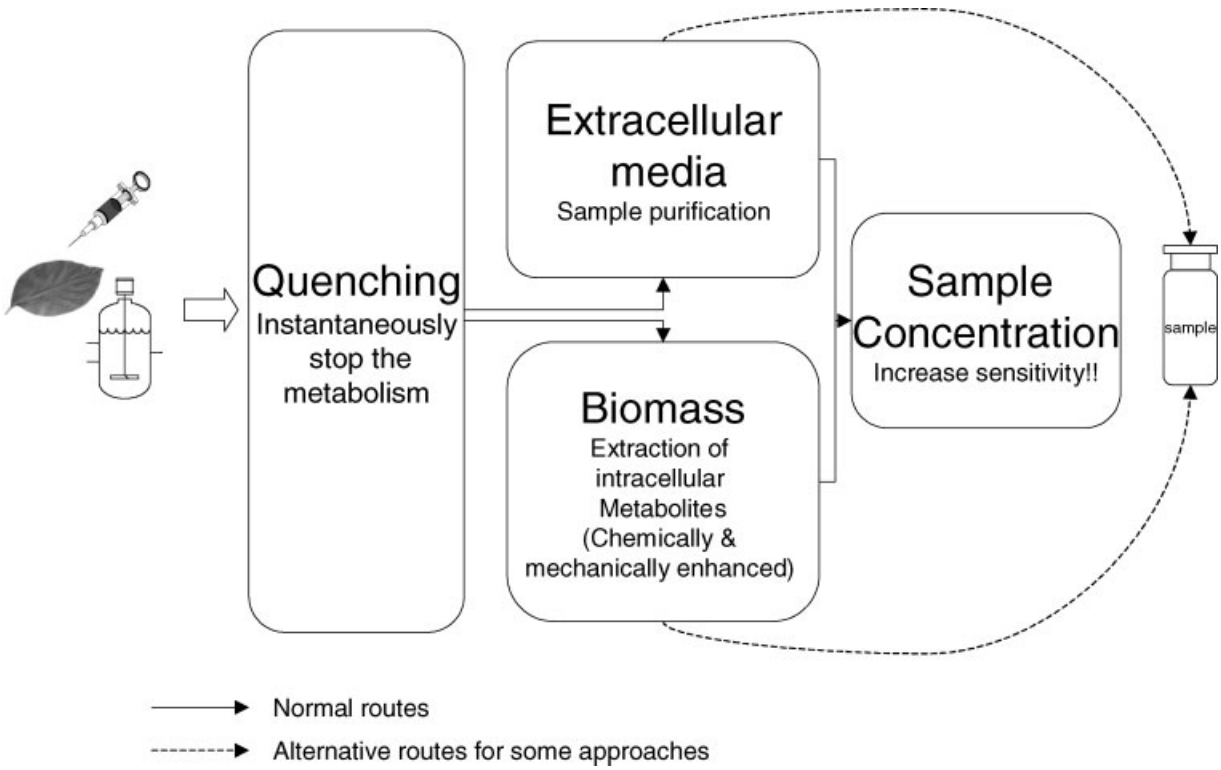

FIGURE 2. The main steps involved in sample preparation during metabolome analysis. All steps should be evaluated for any losses of metabolites by degradation, further biochemical reactions, or sample mishandling. The different options applied in each step are discussed in the text.

extracted and archiving minimal losses because of degradation or further biochemical conversions. Extraction of intracellular metabolites makes them accessible to many different analytical methods. However, the extraction procedures are often the most time-consuming step in metabolome analysis, and it is virtually impossible to avoid losses because of many different factors, particularly because of the large chemical variability of the different metabolites. During the extraction of intracellular metabolites, inevitable extra variability results in a comparatively low reproducibility of any analytical method. In addition, most of the available extraction methods produce high sample dilutions that result in even lower concentrations of metabolites, thus a concentration step is required.

A. Quenching

A rapid inactivation of metabolism is usually achieved through rapid changes in temperature or pH. There are two general strategies that depend on the objective: (1) quenching and extraction are combined, typically when the quenching procedure results in partial extraction of the intracellular metabolites because of disruption of the cell wall. In that case the extracellular and intracellular metabolites will be analyzed together. (2) Quenching followed by a separation of the biomass from the extracellular medium, and thereafter a washing step and extraction of intracellular metabolites. Obviously, separation of the biomass from the extracellular medium eliminates the interference of extracellular compounds on analysis of intracellular metabolites; however, a strict requirement is a conserved quenching method that avoids cell leakage.

Table 2 summarizes the main features of the different quenching methods described in the literature, taking in account their different applications. Freeze clamping, immediate freezing in liquid nitrogen, or acidic treatments with perchloric or nitric acid are the main quenching methods used for plant and animal tissues (Bundy et al., 2002; Fiehn, 2002). According to Fiehn (2002), freezing in liquid nitrogen is the most reasonable way to stop enzymatic activity for plant tissue. On the other hand, quenching a microbial cell culture is limited by the high dilution ratio between biomass and the extracellular medium. The most commonly used method was originally proposed by Koning & van Dam (1992), who analyzed intracellular metabolites in the yeast *S. cerevisiae*. That method enabled the inactivation of the metabolism within 100 ms by spraying 10–15 mL samples of incubated yeast suspension in a stirred solution of 60% methanol kept at −40°C. A major advantage is that the cells can be separated from the extracellular medium though centrifugation; however, it requires sample handling at low temperatures to eliminate or at least minimize further biochemical conversions within the cells before extraction, and it does not work for quenching of bacterial cells (Wittmann et al., 2004).

Microbial cells can also be quenched with perchloric acid (Cordeiro & Freire, 1996; Larsson & Törnkvist, 1996; Theobald et al., 1997; Weuster-Botz, 1997). That procedure is sufficiently rapid to inactivate the metabolism within a few seconds (Larsson & Törnkvist, 1996). However, not all metabolites are stable at the low pH employed (Shryock, Rubio, & Berne, 1986; Maharjan & Ferenci, 2003). Liquid nitrogen can also be used to quench the cellular metabolism by rapidly freezing the sample (Buziol et al., 2002). However, Mashego et al. (2003) reported low reproducibility in measurements of residual glucose in fermentation media after quenching cells of *S. cerevisiae* in liquid nitrogen.

The physiology and the morphology of filamentous fungi are quite different from those of yeast, and, therefore, different quenching methods must be considered. The cultures of filamentous fungi are usually highly viscous and heterogeneous, and it is, therefore, difficult to obtain a representative sample from a fermentation process. The easiest methods for quenching this kind of samples are either with liquid nitrogen or cold methanol solution (Hajjaj et al., 1998). Quenching by liquid nitrogen allows rapid and repeated sampling under short periods of time, but it does not allow separation between intra- and extracellular metabolites. On the contrary, quenching in cold methanol allows separation of intra- and extracellular metabolites, but technical adaptations of the method developed for yeasts are needed to use the method on short time-scales (Ruijter & Visser, 1996; Hajjaj et al., 1998).

B. Extraction

For analysis of intracellular metabolites, it is important to have a good extraction method that will extract all or the maximum number of metabolites in their original state and in a quantitative manner (Maharjan & Ferenci, 2003). There is no unique extraction procedure for metabolome analysis that will cover all intracellular metabolites. The extraction step aims at dissolving a maximum number of metabolites from the cells. The extraction solvent should be able to prevent any further physical and chemical alterations of the molecules, and the whole entire extraction process should ensure a minimal loss of the metabolites to be extracted. Furthermore, the extraction solvent should be chosen such that it is compatible with the subsequent analytical procedures. Tables 3 and 4 summarize the most frequently used extraction methods described in the literature and typical applications of the methods.

In metabolome studies, the intracellular metabolites are usually extracted with organic solvents. Frequently, more than one solvent is used in the extraction procedure: polar solvents like methanol, methanol–water mixtures, or ethanol to extract polar metabolites, and non-polar solvents like chloroform, ethyl acetate, or hexane to extract lipophilic components (Koning & van Dam, 1992; Fiehn, 2002; Maharjan & Ferenci, 2003). Koning & van Dam (1992) adapted a methodology originally designed for extraction of total lipids from animal tissues (Folch, Lees, & Stanley, 1957), based on buffered methanol–water mixture and chloroform at low temperatures (−40 to −20°C), to extract polar metabolites in a yeast-cell suspension. That method is well described for the analysis of intracellular metabolites from microorganisms (Smits et al., 1998; Jensen, Jokumsen, & Villadsen, 1999; Zaldivar et al., 2002; Maharjan & Ferenci, 2003), filamentous fungi (Cremin et al., 1995; Ruijter & Visser, 1996), and even from animal tissues (Le Belle et al., 2002).

Extraction at elevated temperatures with boiling solvents is another very popular extraction method (Gonzalez, François, & Renaud, 1997; Tweeddale, Notley-Mcrobb, & Ferenci, 1998; Hans, Heinzle, & Wittmann, 2001; Lange et al., 2001; Visser

TABLE 2. Summary of the main different quenching methods

METHOD	APPLICATIONS	ADVANTAGES	DISADVANTAGES	REFERENCES
Cold Methanol	1. Microbial cells (mainly yeasts) 2. Filamentous fungi	Consistent and fast drop of temperature; Separation between extra- and intracellular metabolites; Automatic devices to enhance quench reproducibility available; Applicable for different microorganisms.	Significant disturbance on the fermenter operation; Hard to reproduce sample size; Methanol: water ration must be seriously controlled to avoid cell leakage; Not indicated for bacterial cells.	Koning and Van Dam, 1992 Gonzalez et al., 1997 Hajjaj et al., 1998 Schaefer et al., 1999 Letisse and Lindley, 2000 Hans et al., 2001 Lange et al., 2001 Buchholz et al., 2002 Wittmann et al., 2004
Liquid Nitrogen	1. Plant/animal tissues 2. Filamentous fungi 3. Microbial cells	Fast stop of cell metabolism; Extremely low temperature (-196 °C); Easily removed from the sample.	Not homogeneous freezing; Considered slow process for plant/animal tissues; No separation between extra- and intracellular metabolites.	Hajjaj et al., 1998 Bundy et al., 2002 Buziol et al., 2002 Fiehn, 2002
Perchloric acid (PCA)	1. Microbial cells	Inactivation of metabolism in fraction of seconds; Analysis of nucleotides and amides; Automatic devices to enhance quench reproducibility available compounds.	Very oxidative medium; Several metabolites are not stable at low pH; No separation between extra- and intracellular compounds; Big losses of analytes during sample preparation. literature; No evidence of efficiency on other biological matrixes.	Cole et al., 1967 Shryock et al., 1986 Cordeiro and Freire, 1996 Larsson and Törnkvist, 1996 Theobald, 1997 Weuster-Botz, 1997 Maharjan and Ferenci, 2003
Acid/alkali	1. Plant/animal tissues 2. Microbial cells 3. Filamentous fungi	Very efficient for extraction of amines, amides.	Several metabolites are not stable on extreme pHs and oxidative/reducing media; Bad recovery.	Shryock et al., 1986 Kopka et al., 1995 Cordeiro and Freire, 1996 Larsson and Törnkvist, 1996 Chen et al., 1998 Hajjaj et al., 1998 Adams et al., 1999 Bouchereau et al., 2000 Buchholz et al., 2002 Buziol et al., 2002 Conneely et al., 2002 And many others.

et al., 2002; Castrillo et al., 2003). According to Gonzalez, FranÓois, & Renaud (1997), the method is simple, fast, accurate, and reliable. Glucose 6-phosphate, ATP, pyruvate, NAD, and NADH seem to be very stable in buffered boiling ethanol, and present an excellent recovery after extraction. However, the method is not suitable for analysis of many intracellular metabolites because several metabolites are not stable at the extraction temperature; e.g., trehalose, glutamate, glutamine, glutathione, and succinate showed poor recovery compared with the methanol:chloroform method (Maharjan & Ferenci, 2003).

Acidic and alkaline extractions are classical methods for the extraction of intracellular metabolites, and are commonly used

TABLE 3. Summary of the main different chemical extraction methods

METHOD	APPLICATIONS	ADVANTAGES	DISADVANTAGES	REFERENCES
Exhaustive extraction with organic solvents	1. Plant tissues	100% efficiency in extraction of plant secondary metabolites; Fractionation of polar and non-polar compounds.	Slow and laborious; Several primary metabolites are not stable during the process; Requires large sample; Requires large volumes of organic solvent.	Starmans and Nijhuis, 1996 Roessner et al., 2000 Fiehn, 2002
Cold methanol: chloroform: buffer	1. Microbial cells 2. Filamentous fungi 3. Animal tissues	Efficient and reproducible extraction; Denaturation of enzymes by chloroform avoiding further reactions; Good recovery for phosphorylated compounds; Possibility to separate polar from non-polar metabolites.	Work at very low temperatures (-20 to –40 °C); Tedious and time-consuming; Chloroform toxicity; Difficult automation.	Koning and van Dam, 1992 Cremin et al., 1995 Ruijter and Vesse, 1996 Smits et al., 1998 Jensen et al., 1999 Le Belle et al., 2002 Zaldivar et al., 2002 Maharjan and Ferenci, 2003
Boiling ethanol	1. Microbial cells	Simple and fast; Accurate and reliable; Works finely for high diluted cell density culture; Excellent recovery for selected group of compounds (glucose 6-phosphate, ATP, pyruvate, NAD and NADH).	A number of metabolites are not stable at the high temperature applied for extraction (trehalose, glutamate, glutamine, glutathione, succinate, pentose-phosphates, 2-oxoglutarate, PEP, and others).	Entian et al., 1977 Gonzalez et al., 1997 Tweeddale et al., 1998 Hans et al., 2001 Lange et al., 2001 Visser et al., 2002 Castrillo et al., 2003 Maharjan and Ferenci, 2003
Cold methanol	1. Bacterial cells	All advantages of boiling ethanol extraction; Non-degradation of thermo-labile	Reliability of the method is discussible; Scarce information	Maharjan and Ferenci, 2003

for the extraction of acid- and alkaline-stable compounds, respectively. Those methods have been widely used for extraction of metabolites from animal and plant tissues (Shryock, Rubio, & Berne, 1986; Kopka, Ohlrogge, & Jaworski, 1995; Chen et al., 1998; Adams et al., 1999; Bouchereau, Guénot, & Larher, 2000; Conneely, Nugent, & O'Keeffe, 2002), microorganisms (Cordeiro & Freire, 1996; Weuster-Botz, 1997; Schaefer et al., 1999; Vaseghi et al., 1999; Letisse & Lindley, 2000; Martins, Cordeiro, & Freire, 2001; Buchholz et al., 2002; Buziol et al., 2002; Maharjan & Ferenci, 2003), and filamentous fungi (Hajjaj et al., 1998). That procedure is well-suited for extraction of amines from biological samples (Bouchereau, Guénot, & Larher, 2000). Acidic extraction is also commonly used for extraction of nucleotides and water-soluble metabolites (Vaseghi et al., 1999; Martins, Cordeiro, & Freire, 2001; Buchholz et al., 2002); however in metabolome studies, it is evident that not all metabolites are stable at the very low pH, and even destruction of some nucleotides has been reported (Hajjaj et al., 1998; Maharjan & Ferenci, 2003).

The extraction process can be enhanced by using conventional organic solvents at temperatures above their atmospheric boiling points [pressurized liquid extraction (PLE)] or by using microwaves or sonic waves (Smith, 2003). Microwave- and ultrasound-assisted extraction methods have been shown to be very efficient to extract metabolites from biological samples when compared with the classical solvent extraction methods, and they require smaller samples and reagent volumes (Stout et al., 1996; Sargenti & Vichnewski, 2000; Smith, 2003). Supercritical fluid extraction (SFE) also presents a great potential to enhance the extraction of intracellular metabolites. SFE is increasingly being used for extraction of metabolites from plants (Reverchon, 1997; Sargenti & Lanças, 1997; Murga et al., 2000; Maraschin et al., 2001; Beek, 2002; Vaher & Koel, 2003), whereas there are only few examples of applying SFE to extract metabolites from animal tissues (Brooks & Uden, 1995; Jacobson et al., 1997; Stolker et al., 2002), filamentous fungi (Young & Games, 1993; Abdullah, Young, & Games, 1994), and microorganisms (Gharaibeh & Voorhees, 1996; Lim et al., 2002).

C. Sample Concentration

After extraction, intracellular metabolites are often present in diluted solutions, because the low amount of metabolites extr-

TABLE 4. Summary of the main different mechanically enhanced extraction methods

METHODS	APPLICATIONS	ADVANTAGES	DESADVANTAGES	REFERENCES
Pressurized liquid extraction (PLE)	1. Plant tissue Potentially useful for other matrixes	Fast; Small sample sizes; Excellent reproducibility; Suitable for high-throughput screening programs.	Possible thermal decomposition of thermo-labile compounds; Scarce information on literature.	Bethin et al., 1999 Smith, 2003
Microwave and sonic wave	1. Filamentous fungi 2. Plant tissue	Quick and easy method; Multiple samples can be simultaneously extracted; Small sample sizes; Reduced solvent volume; Possibility to be combined with PLE.	Sample vessel have to be cooled before the extraction; Difficult to automate; Some primary metabolites can be potentially degraded.	Young, 1995 Stout et al., 1996 Smedsgaard, 1997 Castro et al., 1999 Sargenti and Vichnewski, 2000 Smith, 2003
Supercritical fluid extraction (SFE)	1. Plant/animal tissues 2. Filamentous fungi 3. Microbial cells	Short extraction time; Reduced amounts of solvents; Small sample sizes; Easy automation; Possibility of on-line coupling to GC/LC-MS; High purity and small volume of the final extract (easy sample concentration); High selectivity.	Optimization is strictly related to sample source; Difficult to extract polar compounds; Decomposition under high pressure may be observed for some labile metabolites.	Young and Games, 1993 Abdullah et al., 1994 Gharaibeh and Voorhees, 1996 Reverchon, 1997 Murga et al., 2000 Namieśnik and Górecki, 2000 Beek, 2002 Lim et al., 2002 Stolker et al., 2002 Smith, 2003 And many others.

acted from the cells and the large volume of extraction solvents normally used. Often, there is the same problem with analysis of extracellular metabolites. Thus, prior to sample analysis, the solvent(s) must be partially or totally removed from the samples.

Solvent evaporation under vacuum is one alternative, using commercially available devices such as a Rotavapor. However, that procedure is relatively time-consuming and the numbers of samples that can be concentrated simultaneously is limited. In addition, many metabolites are not stable at the temperatures required for solvent evaporation (Ferreira et al., 1995).

Freeze-drying, or lyophilization, is commonly used to remove water from aqueous samples to avoid any thermal degradation. That method combines the advantage of deep-freezing and dehydration. The metabolites are stabilized by a non-aggressive technology as they are dried from the frozen solution, thus avoiding concentration effects and thermal degradation. Finally, being free of water, many metabolites usually show good stability, which allows storage almost indefinitely if kept in a cold, dry, and neutral atmosphere, and for some metabolites in the dark.

Solid-phase extraction (SPE) and solid-phase micro-extraction (SPME) can also be employed for sample concentration. SPE and SPME concentrate the analytes from a diluted sample by passing the sample through an SPE cartridge or by incubating under a SPME fiber. Here, the metabolites of interest are trapped, and they can subsequently be eluted into a small volume of eluting solvent (SPE) or by heating the fiber into a GC injection port (SPME). The limitations of those methods are that different trapping materials are needed to trap different classes of metabolites, and that not all classes of metabolites can be effectively concentrated by with SPE or SPME methods.

III. ANALYSIS

The application of MS for analysis of cellular metabolites has grown dramatically over the last two decades, and today MS is the single most important detector method in biotechnology. Especially the introduction of ESI and APCI LC-MS techniques in the late 80s has revolutionized bioanalysis of small molecules. It is a consensus that MS plays an important role in the chemical analysis of metabolites mainly because of its accessibility, versatility, and powerful technology that are best-suited to solve research and analytical problems in the metabolome field.

Figure 3 illustrates the main different options the analyst has to approach the metabolome, with MS as a single detector. MS combined with a separation technique offers tremendous opportunities for analysis of complex biological samples because it

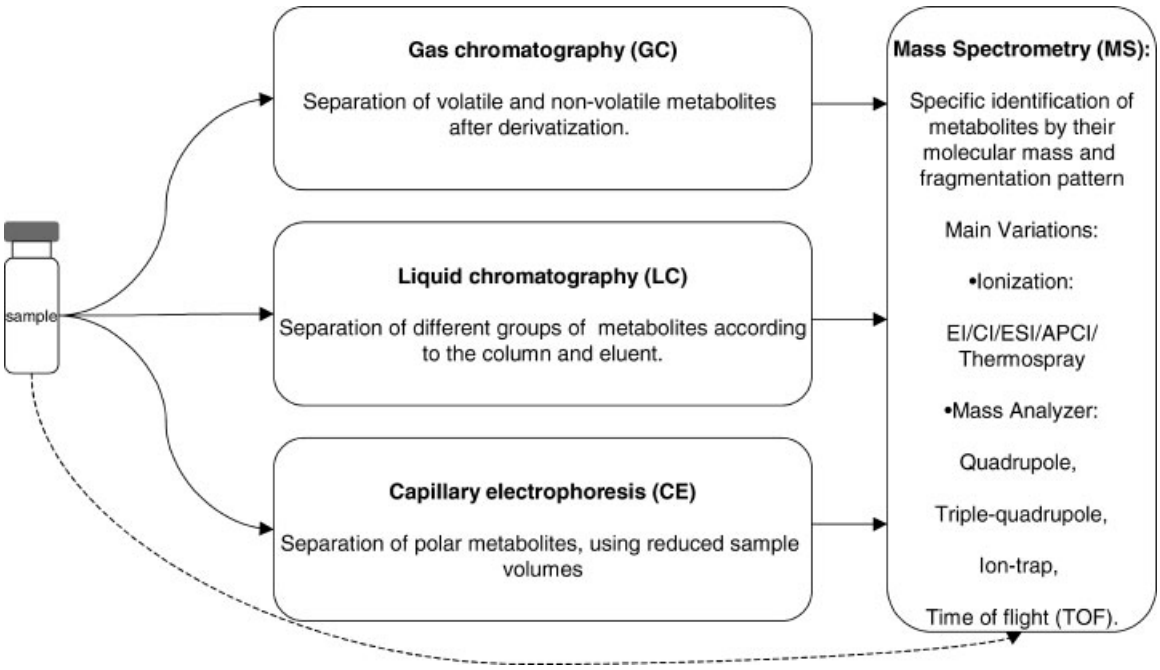

FIGURE 3. Alternative ways for the most used MS analysis of metabolites. The sample can be directly analyzed by MS (dashed arrow), or it can be first resolved with different on-line separation techniques (full arrows).

enables the determination and identification of a large number of metabolites in a single analysis. Furthermore, it is often possible to choose between different methods for analysis of a certain group of metabolites. However, for metabolome analysis, two issues must be considered in connection with choosing an appropriate analysis method: (a) scope/resolution, because the metabolome comprises different classes of metabolites with distinct physicochemical properties, and (b) sensitivity, referring mainly to intracellular analysis, where metabolite concentrations are relatively low.

In the following, we review different MS methods described in the literature, and we start with methods that combine on-line separations with MS, because of their broader applicability in metabolome analyses. The technical aspects of GC-MS techniques will be discussed in more detail because this method is considered a mature technique for analysis of metabolites. The use of direct infusion into mass spectrometer is discussed at the end of this section, covering mainly fingerprinting and footprinting analysis with no identification of the analyzed compounds.

A. GC-MS

Gas chromatography (GC) coupled to MS has been extensively used in metabolome analysis because of its high separation efficiency that can resolve very complex biological mixtures. In addition, the easy and complete interfacing of GC with MS permits the reliable identification of compounds, which is further enhanced by the ability to deconvolute overlapping chromatographic peaks using an automated mass spectral deconvolution and identification system (AMDIS) (Halket et al., 1999; Stein, 1999). GC-MS is a relatively low-cost method for analysis of metabolites and modern systems are easy to operate. It can be used to analyze a wide range of volatile compounds and through chemical derivatization it is also possible to analyze many semi-volatile metabolites.

Usually, GC-MS analysis is performed on single-quadrupole mass spectrometers, which provide nominal-mass information. More recently, the introduction of GC-TOFMS systems offered an attractive supplement to quadrupole instruments and provided greater mass accuracy. TOF instruments also provide high scan speeds that are compatible with ultrafast GC-MS (Davis, Makarov, & Hughes, 1999), and the potential to profile complex mixtures in less time. Similarly, GC coupled to multistage MS-MS [e.g., MS-MS or MS^n (triple-quadrupole and ion-traps)] instruments enabled the acquisition of very detailed fragmentation information, a higher level of molecular specificity, and higher selectivity (Nielsen et al., 2003). Nevertheless, quadrupole mass analyzers still present some extra advantages in metabolite analysis such as higher repeatability and large dynamic range for quantitative analysis. Quadrupole MS spectra can be easily compared with commercial or inter-laboratories databases, and according to Nielsen et al. (2003), it is possible to improve detection sensitivity from the ng-level to the pg-level through the scanning for a selected number of characteristic ions [selected ion monitoring (SIM)], which is very useful for quantitative target analysis.

However, the limitation of GC-MS is that the samples must be volatile to be separated on a GC column. Most naturally occurring metabolites are not sufficiently volatile to be analyzed directly on a GC system. Derivatization of the metabolites is, therefore, required, and this step adds time to the analysis as well

as causes more complex sample-handling and an increased variance in the analysis. Furthermore, heat-labile compounds can not be analyzed, and identification of unknown derivatized compounds can be difficult because they are chemically modified.

1. Scope and Resolution of GC-MS

Historically, GC-MS has proven to be the most valuable bioanalytical tool to resolve complex mixtures of metabolites because of its outstanding high-resolution capillary GC assisted by identification of compounds with MS. The capillary columns used in GC enable the separation of more than 100 compounds in a single analysis (Roessner et al., 2000; Förster, 2002). Those compounds are usually metabolites that belong to different chemical classes and present completely different physicochemical characteristics; i.e., alcohols, sugars, lipids, amino and non-amino organic acids, phosphorylated compounds, peptides, and others (Katona, Sass, & Molnár-Perl, 1999; Roessner et al., 2000). To obtain the highest resolution in GC-MS analysis, the capillary column should be carefully chosen. The choice of stationary phases and film thickness depends on the polarity and volatility of the compounds to be separated, and there is not a single ideal column for metabolome analysis. As a rule of thumb, more-volatile compounds require a thicker film column, because high separation power is better obtained by a thick-film column (Grob, 1993).

The application of GC-MS in metabolome analysis can be divided into two groups: (1) naturally occurring volatile metabolites, and (2) metabolites that can be made volatile through derivatization. Volatile metabolites are compounds that are usually secreted by the cell/organism and that have a boiling point lower than 300°C. Those volatiles can be analyzed by GC-MS without any derivatization. The volatiles include ketones, aldehydes, alcohols, esters, furan and pyrrole derivatives, other heterocyclic compounds, isocyanates, isothiocyanates, sulfides, some lipids, and hydrocarbons with 1–12 carbons. The semi-volatile and non-volatile metabolites are often unstable at the high temperature required for evaporation into the injector. Those compounds must be chemically modified to be analyzed by GC-MS, and that step involves a decrease of their boiling points by derivatization, which, also, increases their stability at high temperatures. Most intracellular metabolites and several extracellular metabolites such as sugars, sugar-phosphates, sugar-alcohols, organic acids, amino acids, lipids, peptides, long-chain alcohols, alkaloids, amines, amides, etc. are semi-volatile metabolites.

2. Analysis of Volatile Metabolites

The profile of volatile metabolites has been extensively used to screen microorganisms in food science, in connection with toxicological studies (e.g., Nilsson et al., 1996; Vergnais et al., 1998; Gao et al., 2002; Demyttenaere, Moriña, & Sandra, 2003), and in the clinical diagnostic of diseases (Niwa, 1986). It has, however, also been applied in functional genomic studies for phenotypic characterization of mutants (Alonzo et al., 2001; Förster, 2002) as well as in chemotaxonomic studies (Savard et al., 1994; Larsen & Frisvad, 1995). Target analyses of some important volatiles have been used in several applications; for example, physiological studies of human (Pietrogrande, Rossi, & Paganetto, 2003), plants (Chiang, Pusateri, & Leitz, 1998), microorganisms (Masson et al., 1999; Beck, Hansen, & Lauritsen, 2003), and insects (Southwell et al., 2003).

In that kind of analysis, the volatile compounds are either extracted from the sample with an organic solvent (Savard et al., 1994; Sargenti & Lanças, 1997; Chiang, Pusateri, & Leitz, 1998; Pietrogrande, Rossi, & Paganetto, 2003; Southwell et al., 2003), by trapping on an absorbent (Nilsson et al., 1996; Vergnais et al., 1998; Masson et al., 1999; Alonzo et al., 2001; Förster, 2002; Beck, Hansen, & Lauritsen, 2003; Demyttenaere, Moriña, & Sandra, 2003), or collected directly from the sample headspace and injected into the GC (Nilsson et al., 1996; Masson et al., 1999). They are usually separated with long capillary columns (30–60 m) with thick films (0.25–0.05 μm ID), and the samples are injected at relatively high temperatures (200–280°C).

SPME techniques are the most-used sampling procedure for analysis of volatile metabolites. Lord & Pawliszyn (2000) have extensively reviewed the SPME technology. According to Lord & Pawliszyn (2000), a split/splitless GC injector can be used for SPME with a narrow liner with an inside diameter close to the outside diameter of the needle. Those narrow liners are required to increase the linear flow around the fiber as well as to increase heat transfer, resulting in an efficient removal of desorbed analytes. SPME injection should be performed in the splitless mode to ensure a complete transfer of trapped metabolites. The speed of desorption is usually limited by the relatively long time required to introduce the fiber into the heated zone with a standard split/splitless injector. It is, therefore, suggested to use rapid-injection autosampling devices; e.g., programmed temperature-vaporizing (PTV) injection. Those devices allow the introduction of the fiber into a cold injector, which is heated very rapidly to release the metabolites into the carrier-gas stream.

SPME technology has definitely enhanced the scope of GC-MS analysis of volatile metabolites, and has enabled higher sensitivity and reproducibility. For instance, Nilsson et al. (1996) determined more than 50 different volatile metabolites from the headspace of *Penicillium* sp. cultures cultivated on petri dishes. Förster (2002) detected and identified ca. 100 volatile compounds in the bioreactor headspace of three different *S. cerevisiae* strains with an SPME technique. Those results reflected the effect of single-gene deletions on the profile of volatile metabolites. The three strains were clearly differentiated from each other with the metabolite profiles.

3. Analysis of Semi-Volatile and Non-Volatile Metabolites

The analysis of semi-volatile and non-volatile metabolites by GC-MS relies on efficient derivatization techniques. The derivatization methods expanded tremendously the use of GC-MS, but derivatization adds an additional step to the sample-preparation procedure. Great care must be taken to ensure that the reaction worked properly, and an ideal derivatization method should be (i) simple (few steps, low reagent costs, and mild reaction conditions), (ii) efficient (broad use, reproducible yields,

TABLE 5. Types of derivatizations commonly used in the GC-MS analysis of polar metabolites

Type of Reaction	Type of Reagent	Typical reaction conditions	Classes of metabolites derivatized
Silylation	TMS, HMDS, TBS, MTBSTFA, BSTFA, MBDSTFA, QSM, MSHFBA, TSIM, MSTFA, TMCS, TMSDEA, BSA, etc.	Reaction in pyridine under anhydrous conditions and heating	Sugars and derivatives, amino acids, organic acids, terpenoids, fatty acids, flavonoids, amides, and phytohormones.
Alkylation/esterification	Chloroformates (MCF, ECF, PCF, CECF, TCECF)	Reaction in pyridine in aqueous solution, room temperature, with or without a second reactant	Amino acids and derivatives, mono- and polycarboxylic acids, keto acids, hydroxy acids, fatty acids, aliphatic and alicyclic amines, and amine-alcohols.
	Diazomethane	Non-polar solvent in the absence of water	
	Acidic esterification	H_2SO_4, Na_2SO_4, methanol/ethanol	

and stability of the derivatives), and (iii) fast (fast reaction course, possibility of automation). A very large number of derivatization methods for analysis of cellular metabolites have been reported, but only a few are used for routine analysis. Table 5 presents an overview of the principal derivatization methods applied for metabolome analysis by GC-MS.

Silylation of organic compounds is the classical and most widely used derivatization procedure for metabolome analysis by GC-MS. By silylation, a silyl group [-Si$(CH_3)_3$] is introduced into the molecule, in most cases through the substitution of active hydrogens. The reagents that have been used for that propose are derivatives of trimethylsilane, $(CH_3)_3SiH$. The active hydrogen of a hydroxyl, thiol, amine, or carboxylic acid group can be replaced by an alkyl-silyl group such as trimethylsilyl (TMS). The reactivity of the functional groups towards silylation is alcohols > phenols > carboxylic acids = amines > amides. Silyl derivatives are generally more volatile, less polar, and thermally more stable than their parent compounds. The several distinct classes of metabolites that can be derivatized by trimethylsilane derivatives are summarized in Table 5. Sugars and their derivatives (sugar alcohols, phosphorylated sugars, amino sugars, and others) are the most important class of metabolites derivatized by silylation. Silylation is characterized as being efficient with a broad range of applications, and results in stable derivatives with good reproducibility. However, silylation is not simple, fast, or easy to automate. Silylation reactions are performed under anhydrous conditions, and they, therefore, require the complete removal of water from the samples. Furthermore, the samples must be heated for more than 1 hr, resulting in the loss of thermo-labile metabolites. Finally, the residual reagents in the samples after derivatization are not removed, and after injected into the chromatographic column, produces a decreased column lifetime.

Alkylation or esterification is another derivatization technique that is often used in metabolite analysis by GC-MS. The applications of the alkylation/esterification methods are listed in Table 5. Those methods are primarily used for derivatization of polyfunctional amines and organic acids. The use of esterification reactions based on chloroformate derivatives (CFs) has recently become popular. Those derivatives represent several advantages compared to silylation: (i) reactions are fast (10–60 s), (ii) reaction takes place at room temperature, (iii) reactions occur in aqueous media, (iv) there is negligible reagent costs, and (v) the reactions are easy to automate. CF derivatization is reproducible, and most of the derivatives are sufficiently stable for quantitative analysis (Hušek, 1992; Villas-Bôas et al., 2003). The only limitation of those methods is their application range. Compared to silylation, the rouse of applicability of CF derivatizations is smaller. However, similar reaction conditions can be used to profile a variety of compounds. Recently, we reported a new protocol for CF derivatization with MCF as reagent to enable simultaneous separation, detection, and quantitation of more than 36 different amino and non-amino organic acids in a single GC-MS analysis (Villas-Bôas et al., 2003). These compounds include important intermediates in the cellular metabolism. The action of CF in aqueous solution allows many substituted carboxylic acids, amines, amino alcohols, and amino acids to be converted to volatile derivatives to thereby extend the application of CFs for the derivatization of cellular metabolites for metabolome analysis by GC-MS (Hušek, 1992, 1998).

Alternatively, alkylation can also be performed with diazomethane as reagent. That method has been used for methylation of carboxylic and fatty acids (Abdullah, Young, & Games, 1994). The reaction is simple and rapid, and produces minimum by-products (Wells, 1999). The resulting methyl esters provide more characteristic mass spectra compared to other derivatives, which is an advantage in metabolic-profiling studies. However, diazomethane is highly toxic, has a limited storage time (Wells, 1999), and is normally produced *in situ*. Hence, that method is employed less in metabolite analysis. Classical esterifications under strong acidic conditions have been applied to analyze fatty acids in microorganisms (Gharaibeh & Voorhees, 1996), but are rarely used in metabolite profiling because of their high oxidative power.

4. Sensitivity

Many intracellular metabolites are found at very low concentrations, and a high sensitivity is, therefore, an important parameter to consider. Table 6 presents a list of some metabolites and their concentrations detected by GC-MS in different organisms. The choice of the mass analyzer, the data-acquisition mode (full-scan/SIM), and the chromatographic setup determine the GC-MS sensitivity. As mentioned before, TOFMS and ion traps can give a very high sensitivity, mainly in the SIM mode. However, the dynamic range of those instruments is lower than for quadrupoles operated in SIM mode. In full-scan analysis, a continuous series

TABLE 6. Concentrations and detection limits of different GC-MS analyzed metabolites

ORGANISM	METABOLITE	CONCENTRATION (μg/ml)	DETECTION LIMIT (ppm)	INJECTION TECHNIQUE & DATA ACQUISITION MODE	REFERENCE
Eucalyptus sp. (leaves)	Lactate	3.2	1.8	Splitless/SIM	Chen et al., 1998
	Malate	13.3	n.d.[a]	Splitless/SIM	
	Alanine	d.n.s.[b]	1.5	Splitless/SIM	
	Glycine	d.n.s.	1.2	Splitless/SIM	
	Valine	d.n.s.	1.8	Splitless/SIM	
	Leucine	d.n.s.	2.1	Splitless/SIM	
	Isoleucine	d.n.s.	2.0	Splitless/SIM	
	Proline	37.3	1.5	Splitless/SIM	
	Methionine	21.9	2.4	Splitless/SIM	
	Serine	d.n.s.	1.5	Splitless/SIM	
	Threonine	d.n.s.	1.5	Splitless/SIM	
	Phenylalanine	35.6	2.0	Splitless/SIM	
	Asparagine	d.n.s.	2.0	Splitless/SIM	
	OH-proline	48.6	1.5	Splitless/SIM	
	Cysteine	22.3	15.0	Splitless/SIM	
	Ornithine	d.n.s.	5.0	Splitless/SIM	
	Lysine	7.4	3.1	Splitless/SIM	
	Glutamine	d.n.s.	4.0	Splitless/SIM	
	Arginine	d.n.s.	16.0	Splitless/SIM	
	Histidine	d.n.s.	18.0	Splitless/SIM	
Human plasma	L-hydroxypyrene*	0.02	0.1	Splitless/scan	Tienpont et al., 2002
			0.0001	Splitless/SIM	
Garlic cloves (*Allium sativum* L.)	S-methylcysteine sulfoxide	$57x10^3$	1.0	Split/?	Kubec et al., 1999
Arabidopsis thaliana	Indole-3-acetic acid	1.75×10^{-5} - 1.75×10^{-4}	0.05	Splitless/SIM	Birkemeyer et al., 2003
	Jasmonic acid	2.12×10^{-5} - 2.12×10^{-4}	5.0	Splitless/SIM	
	Salicylic acid	1.38×10^{-6} - 1.38×10^{-5}	0.05	Splitless/SIM	
	Abscisic acid	2.6×10^{-6} - 2.6×10^{-5}	2.0	Splitless/SIM	
	meta-Topolin	n.d.	2.0	Splitless/SIM	
	trans-Zeatin	n.d.	5.0	Splitless/SIM	
	1-aminocyclopropane-1-carboxylic acid	10^{-5} - 10^{-4}	0.5	Splitless/SIM	

SIM, selected-ion monitoring mode.
*Not a natural metabolite.
[a]Not determined.
[b]Data not shown.

of mass spectra is acquired during the chromatographic analysis to produce a large set of data files. Sufficient fast-scanning is required to acquire a sufficient number of data points (typically 10–20) to adequately describe the chromatographic elution profile. On the other hand, in SIM mode analysis, the intensity of only a number of pre-selected ions that is monitored in the mass analyzer increases substantially the selectivity, and in the case of scanning instruments, the sensitivity (Tienpont et al., 2002; Birkemeyer, Kolasa, & Kopka, 2003). That technique will, however, impede detection of unexpected compounds; however in most modern instruments, a full-scan analysis can be combined with SIM mode, and if the equipment does not allow that combination, then the sample may have to be injected twice (Nielsen & Thrane, 2001; Birkemeyer, Kolasa, & Kopka, 2003). The SIM mode is the most sensitive method for quantitative analysis, and is the method of choice for the quantitative analysis of isotopes (Christensen & Nielsen, 1999). However, it is not suited for metabolite profiling.

Several parameters can be optimized to improve the sensitivity in chromatographic separation. Although often neglected, the most important step in GC analysis is the injection (Grob, 1993). Classical injection techniques used for metabolite analysis are split, splitless, or in a few cases, on-column injection. However, ca. 30% of the publications related to metabolite analysis by GC-MS do not provide any details on the injection method used. Randomly checked in a group of 70 scientific papers related to metabolome analysis by GC, 30% employed split, 37% splitless, and 3% on-column injection. In the remaining papers, the injection technique was not specified.

Splitless injection is the most frequently applied technique for the quantitative determination of low-concentration samples, and is the preferred choice for quantitative approaches in analysis of metabolites (Kopka, Ohlrogge, & Jaworski, 1995; Chen et al., 1998; Nielsen & Thrane, 2001; Birkemeyer, Kolasa, & Kopka, 2003, and many others). Splitless injection is suitable for use with solute concentrations between 0.1 and 50 ppm (ng/μL). Table 6 presents some detection limits of metabolites published by different authors that used splitless injection and collected the ions either in the scan or SIM mode. Sensitivities down to the ppm level are usually only obtained with splitless injection. The sensitivity makes the methods well-suited for metabolome analysis, where samples usually are very diluted. However, splitless injection has some adverse effects; e.g., discrimination of low-volatility compounds, sorption, and thermodegradation (Zroslíková et al., 2001). A very comprehensive and practical description of split/splitless injection in GC was published by Grob (1993, 2001).

For metabolome analysis, most of the GC-MS split-injection methods are non-quantitative, and have primarily been used for target analysis or to profile a selected group of metabolites; e.g., toxins produced by filamentous fungi (Demyttenaere, Moriña, & Sandra, 2003), or volatile metabolites (Nilsson et al., 1996). The detection limits were rarely determined or given. However, some split-injection methods are highly sensitive. For example, Roessner et al. (2000) described a robust method to detect more than 150 intracellular metabolites of plants in a single GC-MS analysis. After derivatization, the samples were injected with a split ratio of 25:1, using a hot-needle technique. Using a quadrupole mass analyzer, the sensitivity of the method was very high. Kubec, Svobodová, & Velíšek (1999) developed a method to determine *S*-alk(en)ylcysteine sulfoxides, which are important aroma precursors of *Allium* and *Brassica* species that are generally difficult to quantify because of their instability. The derivatized samples were injected with a split ratio of 1:10, and the mass spectra were collected with a quadrupole MS. The method presented an outstanding sensitivity estimated to be ca. 1 ppm.

5. Repeatability/Reproducibility of GC-MS for Metabolome Analysis

Reproducibility is crucial for metabolome analysis, particularly because even small changes in metabolite levels reflect substantial changes in the metabolism. In many cases, it is not necessary to measure absolute concentrations, as it is for a comparison of different growth conditions or different genotypes. Three components contribute to the variability in GC-MS analyses: (1) the analytical methodology (chromatography, detection, and stability of derivatized samples), (2) the sample preparation (quenching, extraction, and concentration), and (3) sampling and other biological variations.

At the data-acquisition step, the larger source of variability is the stability of the compounds to be analyzed (derivatized or not). A good derivatization technique must produce considerably stable derivatives because derivatized samples normally reside in the autosampler for some time before their injection. If the derivatives are not sufficiently stable; (i.e., stable within 24 hr) then large standard deviations may be observed for analyzing the same sample more than once. Losses by evaporation are another cause of variability for samples held in the autosampler. Adapting a cold tray at the GC injector can reduce that problem. Leakages at the injection port, at the connections of the capillary column, or at the ion source are common causes of instrumental variability that can easily be checked prior to each analysis.

Typically, GC-MS instruments present satisfactory reproducibility and repeatability with a variability below 10% (Sass & Endres, 1997; Baldizsár et al., 1998; Chiang, Pusateri, & Leitz, 1998; Dasgupta, 1998; Molnár-Perl, Horváth, & Bartha, 1998; Katona, Sass, & Molnár-Perl, 1999; Kubec, Svobodová, & Velíšek, 1999; Peláez et al., 2000; Tienpont et al., 2002). Nilsson et al. (1996) and Roessner et al. (2000) suggested that the variability in the determination of metabolite concentrations by GC-MS originated primarily from a variability of the samples themselves. Roessner et al. (2000) analyzed 33 representative metabolites by GC-MS, and the data showed that the deviations introduced by data acquisition and by sample preparation were, as a rule, below 6% of the mean. However, the variability of the same metabolites between the biological samples (potato tubers) was significantly higher—ca. 20% for most metabolites. Sample preparation usually provides an acceptable reproducibility for the determination of metabolites for metabolome analysis, but is highly dependent on the biological matrix as well as on the procedures used for quenching, extraction, and concentration. Nonetheless, internal standardization is preferred to minimize the sample-to-sample variance. In general, the internal standard should be added as early as possible in the process; i.e., if derivatization of the sample is needed, then the internal standard should also be derivatized, it should not exhibit a significantly higher or lower boiling points than the analytes, and should not

interfere with the metabolites found in the sample. Typical internal standards used in GC-MS are synthetic compounds (Dasgupta, 1998; Birkemeyer, Kolasa, & Kopka, 2003; Villas-Bôas et al., 2003), isotopically labeled metabolites (Tienpont et al., 2002), or metabolites that are not expected to be found in sample (Sass & Endres, 1997; Roessner et al., 2000).

B. LC-MS

By combining MS with liquid chromatography, molecular identification and quantification of polar, less-polar, and neutral metabolites can be achieved, even when they are present at relatively low concentrations levels and in a complex matrix. There have been major difficulties in coupling LC with MS; e.g., the incompatibility caused by the liquid flow rate into the high vacuum of the mass spectrometer, the ionization of non-volatile and thermally labile analytes, and the incompatibility caused by the non-volatile mobile phase used in the chromatographic separation. Technical solutions have been provided by the recent introduction of the powerful soft-ionization techniques thermospray, APCI, and especially electrospray (Abian, 1999; Niessen, 1999), and by the modification of the liquid-chromatography methods. Thermospray allows the ionization of moderately polar compounds (polyphenol, terpenoids) in the mass range 200–800 Da, whereas ESI and APCI are suitable for a broader range of metabolites (Wolfender et al., 1995). Electrospray is the most widely method used for sensitive analysis of polar and ionic compounds. APCI can be used for the analysis of less-polar and neutral chemical species. Generally APCI and ESI produce only molecular ions, but the multicharge ionization mechanism of electrospray can extend the mass range of the instrument to provide a mass range of greater than 80,000 Da to permit the accurate mass determination of macromolecules such as intact proteins. The impact of those innovations has been felt notably in pharmaceutical research, and more recently in the area of metabolome analysis.

1. LC-MS for Target Analysis

By providing a unique combination of resolving power, sensitivity, and specificity, LC-MS and particularly LC-MS^n offer the possibility to identify target metabolites in a very complex mixture. Actually, LC-MS^n is a tool used more frequently for the identification of metabolites in crude plant extracts to provide not only molecular mass information, but also structure information that is deduced from fragmentation patterns obtained by collision-induced dissociation. The advantage of LC-MS is the ability to determine selected metabolites in a few minutes from only a small amount of material and with a simple sample preparation. For LC-MS analysis, the sample preparation, chromatographic separation and MS methods are developed simultaneously. Because of its compatibility with ESI, the most frequently used LC method is reversed-phase HPLC separations with a volatile mobile phase.

Special strategies have been developed for the analysis of plant metabolites. For example, an LC-APCI-MS method was developed and optimized for the analysis of chlorophylls (Airs & Keely, 2000), and enhanced sensitivity was achieved by one-line demetallation of the metabolites through the post-column introduction of formic acid into the eluent flow. In the report of Mellon et al. (2002), glucosinolates in crude plant extracts were analyzed by LC-MS in the negative-ion mode. The sensitivity and the selectivity of a programmed cone-voltage electrospray LC-MS method were found to be better than a LC-MS-MS (triple quadrupole) method. The detection limits were in the ng range. In another report, the differentiation of C-glycosidic flavonoid isomers was evaluated by quadrupole-TOF tandem-MS and ion-trap multiple-stage MS at different collision-induced dissociation energy values (Waridel et al., 2001). Isomers were discriminated by the two approaches, but the specificity of multiple-stage experiments may be an advantage for the analysis of a mixture that contained co-eluting metabolites. LC-ESI-MS^n has been applied to analyze metabolites in several different matrices: identification and determination of nucleosides in rat brain microdialysates (Zhu et al., 2001); semi-quantification of monophosphate nucleotides in wine (Aussenac et al., 2001); quantification of metabolites involved in purine and pyrimidine metabolism defects in urine (Ito et al., 2000); and quantification of underivatized amino acids in blood (Qu et al., 2002). Finally, there are several examples on targeted analysis for medical diagnosis, using LC-MS (Yu, Cui, & Davis, 1999; Ito et al., 2000).

2. LC-MS for Metabolite Profiling

LC-MS can provide information on analytes that: display chemical diversities, are labile, or are difficult to separate at the preparative scale. By using LC-MS, known and unknown compounds that are present in a biological matrix can be detected and identified without any prior knowledge of their exact chemical structure. LC-MS is, therefore, an important tool for metabolite profiling.

The use of LC-MS and LC-NMR to screen bioactive compounds in plant extracts has been reviewed by Wolfender et al. (Wolfender, Rodriguez, & Hostettmann, 1998; Wolfender, Ndjoko, & Hostettmann, 2003). The combination of several analytical tools may be necessary to overcome the limitations of each technique and to provide the means to rapidly screen for many metabolites in crude plant extracts (avoiding the isolation of compounds and the eventual degradation of unstable compounds), and even for the identification of metabolites of potential interest. Queiroz et al. (2002) analyzed crude plant extracts with high-resolution LC-APCI-Q-TOF-MS to obtain an overview of the composition of the crude extract; subsequently, the molecular masses of interesting compounds were determined accurately with Q-TOF. The mass of the compounds and the mass of lost fragment indicated the presence of prenylated isoflavanones or isoflavones; LC-NMR and MS-MS finally confirmed that result. In the publications of Schaller et al. (2001) and Cogne et al. (2003), crude plant extracts were analyzed by LC/thermospray-MS and LC-UV-ESI/MS to determine the presence of certain classes of compounds such as diterpenic compounds and unstable cinnamic ester derivatives, respectively, whereas NMR experiments established the structure of isomeric pairs. Generally, it was observed that MS detection was more sensitive

than NMR to detect the metabolites, but was less efficient for compound identification.

The capabilities of LC-MS are expanded further with MS^n, especially the structure elucidation through the formation of characteristic fragments. As an example, multistage MS-MS experiments were performed to determine the sugar sequence of saponins (glycosides) by Wolfender, Rodriguez, & Hostettmann (1998). The isolation of the ion of interest and its cleavage, one sugar at a time, were possible, and simplified dramatically the spectrum interpretation and, therefore, the structure elucidation of the compounds. Saponins were also studied by Huhman & Sumner (2002), using reversed-phase HPLC/ photodiode array detection/ESI/MS/MS. Negative-ion ESI yielded a maximum MS response for saponins, whereas an acidic environment was preferred because of enhanced HPLC chromatographic resolution. The post-column addition of a base to the acidic HPLC eluent has been attempted, but higher ion abundances were recorded for 0.1% acetic acid. MS spectra and HPLC retention times were used to profile and identify 27 saponins in *Medicago truncatula*. Other classes of compounds, such as oligosaccharides, glycosides, amino sugars, amino acids, and sugar nucleotides in plants, have been quantified with hydrophilic-interaction liquid chromatography-ESI-MS^n (Tolstikov & Fiehn, 2002). The detection limit was in the range of 0.5 ng.

Identification, but also quantification, of plant metabolites has been achieved in many studies. Jensen et al. (2002) have quantified within 14 min the terpene constituents of *Ginkgo biloba* in the pg range by LC-APCI-MS in the negative mode. MS was found to be more sensitive than UV dectection because of the lack of UV chromophores in the metabolites and because flavonoids interfered with their UV detection. LC-MS has been preferred to GC-MS, LC with evaporative light-scattering detection, and refractive index because those techniques require a long process of sample preparation and a long analysis time. The APCI interface was chosen to ionize metabolites because APCI produced more-intense and more stable ions than electrospray, the thermospray interface showed variability and poor stability (Camponovo, Wolfender, & Hostettmann, 1995; Jensen et al., 2002). Prinsen et al. (1998) analyzed indoles and cytokinins with micro- and capillary- LC ESI-MS-MS in the positive-ion mode. The detection limits and linearity ranges were, respectively, 0.1 fmol and between 1 fmol and 5 pmol for the capillary LC-MS (0.3 mm column), and 5 fmol and between 5 fmol and 1 nmol for the micro LC-MS (1 mm column). LC-MS sensitivity is improved by the miniaturization of the LC dimensions, and by the large and extra-large pre-volume injections. Capillary LC-MS has also been used by Witters et al. (1999) for the quantification of isoprenoids and cyclic nucleotides at the femtomol level. Online Capillary HPLC (0.3 mm column) ESI-MS-MS was combined with a column-switching technique, using a micro-precolumn and an analytical column. The positive-ion mode was chosen for isoprenoids, and the negative-ion mode for cyclic nucleotides. Detection limits were, respectively, 2.5–25 and 25 fmol. The determination of endogenous levels of all natural isoprenoids by reversed-phase LC-ESI-MS in the positive-ion mode (Novák et al., 2003) led to limits of detection between 10 and 50 fmol. Van Rhijn et al. (2001) quantified plant metabolites with micro LC-ESI-MS-MS. The detection limits were between 50 and 100 fmol, and the linearity range was between 0.5 and 10 pmol. That sensitivity was sufficient to detect a clear increase in the isoprenoid content of plant tissue extracts for a hemizygote plant in comparison the wild-type *Nicotiana tabacum*. Moreover, *cis*- and *trans*- isomers have been identified on the basis of their LC retention time. The effect of genetic modification of plants on the cytokinin content was determined with LC-MS capabilities. Bednarek et al. (2001) have studied with LC-ESI-MS the changes in metabolism of isoflavonoids and their conjugates in *Lupinus albus* that was treated with biotic elicitor by. In elicited lupine seedlings, an increased amount of prenylated isoflavone aglycones was identified. In those examples, plants extracts were analyzed by LC-MS after immunoaffinity purification (Prinsen et al., 1998; Witters et al., 1999; Novák et al., 2003) or SPE (Bednarek et al., 2001). That step was necessary because the metabolites were present at low concentrations (van Rhijn et al., 2001).

Generally, it was observed that combined LC-ESI-MS could be used to analyze of plant metabolites with a comparable or better sensitivity than GC-MS without any prior derivatization that is required for GC-MS. ESI, but also APCI, thermospray, and fast atom bombardment, have been used as the ionization technique for the MS analysis of plant metabolites in combination with reversed-phase liquid chromatography. Today, ESI is the most-used technique for the study of cellular metabolites.

LC combined with ESI-MS has also been successfully used for metabolite profiling of microorganisms. As an example, isoprenoid quinones have been profiled by LC-FAB-MS for the chemotaxonomy of microorganisms such as bacteria and yeasts (Nishijima, Araki-Sakai, & Sano, 1997). The isoprenoid quinines were identified by their HPLC retention times, and by UV and mass spectra. The compounds were quantified from the HPLC chromatogram.

Metabolic pathways have also been studied with LC-MS. For instance, the biosynthetic pathway that leads to the plant hormone indole-3-acetic acid (IAA), which is present in bacteria that live in close association with plants, has been studied by reversed-phase LC-MS (Prinsen et al., 1997). Before analysis, the samples were methylated to improve up to 1000-fold the detection limit for tryptophane, IAA and other intermediates. Detection limits were from 10 fmol to 10 pmol. Recently, Buchholz, Takors, & Wandrey (2001) have carried out a quantitative study on bacteria metabolites under "*in vivo*" conditions to obtain information on the kinetics of metabolic reactions. The intracellular concentrations of glycolytic intermediates and nucleotides in *Escherichia coli* K12 were measured under defined growth conditions. Special attention was given to the quenching because the turnover of most metabolites is at the subsecond scale (Koning & van Dam, 1992). After extraction, metabolites were analyzed with an LC-ESI-MS (ion trap) in the negative-ion mode. Using retention time, m/z, specific MS-MS fragmentation patterns, and spiking with known compounds achieved metabolite identification. Two chromatographic systems, compatible with the MS analysis, were used: (i) two Nucleodex α-OH columns connected in series, and a mobile phase based on aqueous ammonium acetate/methanol, and (ii) a porous-graphite carbon HyperCard column, and a mobile phase based on aqueous ammonium acetate/2-propanol. The first system had many advantages: hydrophilic and hydrophobic interactions provided by

cyclodextrin-bonded phases, size-dependent and enantio-specific inclusion complexes can be formed, and an anion-exchange mechanism accounts for the chromatographic differentiation of negatively charged compounds. The second system, using a porous-graphite carbon column, was employed to resolve isobaric substances such as G6P/F6P and 3PG/2PG, but it had the disadvantage of a slow column re-equilibration. That method was validated by a comparison with enzymatic assays and UV detection for many of the metabolites that were analyzed. The detection limits (intracellular concentration) were around 0.02–0.50 mM. Intracellular concentrations were estimated to be in the range 0.01–4 mM; e.g., 0.22 and 0.25 mM for G6P and F6P, respectively. However, for some compounds the estimated concentrations were quite close to the detection limit. Using the method of Buchholz, Takors, & Wandrey (2001), it was possible to quantify metabolites present in a complex matrix at low concentrations, even though they had a chemical similarity and a minimal UV adsorption, and using only a small amount of sample compared to what is required for enzymatic determinations. Another interesting example is the report of van Dam et al. (2002), where glycolytic intermediates were quantified in the yeast *S. cerevisiae* samples from a carbon-limited aerobic chemostat culture. The carbohydrate analysis by LC-MS has already been investigated (Simpson et al., 1990; Wunschel et al., 1997; Fox et al., 1998; Torto et al., 1998). In the publication of van Dam et al. (2002), the cellular metabolism was quenched, and metabolites were extracted and separated by high-performance anion-exchange chromatography, using a sodium hydroxide gradient. On-line desalting (Simpson et al., 1990) was performed before the MS analysis to avoid ESI interface fouling and ionization suppression that would result in the loss of sensitivity (Niessen, 1999). Glycolytic intermediates were identified by ESI-MS (negative-ion mode) in the MRM (multiple-reaction monitoring) mode at optimized conditions. Quantification used the standard addition of the individual metabolites. Concentrations of intracellular G6P and F6P were estimated to be 1282 and 182 nmol/g dry weight, respectively, and the method was confirmed by enzymatic G6P analysis. In an earlier study, Feurle et al. (1998) had also reported the analysis of various sugar phosphates by LC-ESI-MS-MS, but by using a β-cyclodextrin-bonded phase column, which avoided any anion-exchange column and micromembrane desalting system because of the loss of aminosugars and sensitivity problems discussed by Conboy & Henion (1992). Nevertheless, in the study of Feurle et al. (1998), the metabolite quantification has not been attempted in spite of the successful separation and structural characterization of many phosphorylated carbohydrates. However, the method was particularly useful to demonstrate the enzymatic formation of D-1-deoxyxylulose 5-phosphate by yeast transketolase. Fungal metabolites have also been analyzed by LC-MS (Larsen, Svendsen, & Smedsgaard, 2001), where 48 *Penicillium verrucosum* isolates were analyzed with HPLC coupled with diode-array detector (DAD) or MS for their ability to produce secondary metabolites in particular, the mycotoxin ochratoxin A. Twenty-two known and unknown metabolites were detected. Using cluster analysis of qualitative secondary metabolite production for 13 metabolite groups (containing metabolites with very similar UV spectra), the 48 isolates were grouped into two separate clusters.

C. CE-MS

Capillary electrophoresis (CE) can perform fast and efficient separation of a wide variety of charged and uncharged species, and the combination of CE with MS appears to be very promising and takes advantage of the high levels of speed, selectivity, and sensitivity of each of the two analytical techniques. The CE-MS coupling requires a specific interface, which usually includes a coaxial liquid sheath flow. The CE separation quality is improved by high ionic strength, whereas the presence of non-volatile ionic species in an ESI spray is deleterious (Cech & Enke, 2001). The buffer system for the CE analysis and the sheath liquid composition should be chosen to be compatible with CE and MS. For instance, the electrolyte should have a mobility that is similar to that of the analytes and should interfere as little as possible with the ES-MS detection. Most research in the field of metabolome analysis with CE-MS has focused on the optimization of the operational parameters; the results of that optimization are summarized in Table 7.

1. CE-MS for Target Analysis

In medical laboratories, the metabolite analysis routinely performed by GC-MS analysis takes several hours. However, CE-MS offers the possibility to overcome that drawback. Many efforts have been done to develop a fast and reliable method for screening patients, because these disorders, resulting of genetic defects, cause often the accumulation of a specific metabolite in serum or urine and ultimately organ damage. For instance, CE-MS has been successfully applied for the diagnosis of various human metabolic disorders, with an analysis of only fifteen minutes (He et al., 1999; Vuorensola et al., 2001). Vuorensola et al. (2001) used CE-MS to analyze catecholamines that differ only by one amino group. Compared to LC-MS analysis, one major drawback was the high concentration limit of detection because of the very low injection volume used in CE. In that study, the limits of detection obtained after optimization (0.19–0.63 mg/L) were sufficient to analyze catecholamines in concentrated urine samples. He et al. (1999) also analyzed ''diagnostic metabolites'' in urine by CE-MS. Adenylosuccinate-glutathione and -ornithine were detected in the positive-ion mode, whereas the negative-ion mode was preferred for organic acids like homogentisic acid, histidine, and pyroglutamate. The conditions applied in those methods are given in Table 7. It should be noted that a good MS response could be obtained even with the use of a nonvolatile buffer. To increase the specificity of the MS analysis, authors have performed MS-MS on glutathione, and confirmed that the fragmentation pattern corresponded to its structure. The detection concentration limits were in the range of 0.01–0.1 mg/mL. Elgstoen et al. (2001) have also used direct CE-MS-MS (triple quadrupole) to analyze the urine of patients with different metabolic disorders. Specific transitions that were characteristic for each metabolite have been determined by MRM, and were used to identify diagnostic metabolites that were not sufficiently separated. Compared to CE-DAD (Elgstoen & Jellum, 1997), compounds that absorb light in the UV range and that do not absorb could both be detected by CE-MS.

The CE-MS analysis of classes of compounds such as secondary metabolites, amino acids, organic acids, and carbohy-

TABLE 7. Optimized parameters of CE-MS analysis (amm, ammonium; Me, methanol)

		diagnostic metabolites (amines, nucleotides, amino acids, etc)				alkaloids	
		Vuorensola et al. (2001)	He et al. (1999)	He et al. (1999)	Elgstoen et al. (2001)	Unger et al. (1997)	Bianco et al. (2003)
CE	*capillary type*	50 μm i.d., 80 cm	75 μm i.d., 111cm fused-silica	75 μm i.d., 111cm fused-silica	50 μm i.d., 98cm fused-silica	50 μm i.d., 55cm fused-silica	uncoated, 50 μm i.d., 80cm fused-silica
	voltage	25 kV	30 kV	20 kV	25 kV	15 kV	25.5 kV
	buffer	50 mM amm acetate pH 4.0 with acetic acid	borate buffer (300 mM) pH 8.5 by amm hydroxide	amm bicarbonate (100 mM) pH 8.5 by amm hydroxide	amm acetate 20 mM pH 8.5	amm acetate 100 mM -Acetonitrile 50:50 (v/v) pH 3.1 with acetic acid	MeCN–MeOH 90:10 (v/v) with 50 mM amm acetate and 1.2 M acetic acid
	temperature	23°C				20°C	
Sample	*volume*		7.2 nl	7.2 nl			5.5 nl
	pressure	0.5 psi	0.5 psi	0.5 psi	100 mbars	345 mbars	0.5 psi
	duration	30 s	3 s	3 s	12 s		5 s
Liquid sheath		Me-water 80:20 (v/v) with 0.5% acetic acid	acetic acid–Me–water (10:45:45, v/v/v)	Me–water (50:50, v/v)	Me–water (1:1, v/v) amm acetate 2 mM	Me–water or acetonitrile/water with 1% acetic acid	Me–water (1:1, v/v) with 1% acetic acid
		6 μL min −1	5 μL min −1	5 μL min −1	5 μL min −1	1-2 μL min −1	2.5 μL min −1
ESI	*mode*	positive	positive	negative	negative	positive	positive
	voltage	4.0 kV				5 kV	4.5 kV
MS		ESI - triple quadrupole	ESI -quadrupole ion trap	ESI -quadrupole ion trap	ESI - triple quadrupole	ESI - sector field MS	ESI - quadrupole ion trap
	capillary temp.	70°C				100-250°C	180°C
	capillary voltage	20V					32 V

		amino acids		organic acids	
		Soga and Heiger (2000)	Schultz and Moini (2003)	Hagberg (2003)	Johnson et al. (1999)
CE	*capillary type*	50 μm i.d., 100cm fused-silica	20 μm i.d., 130cm or 115cm (for blood), fused-silica	50 μm i.d., 103cm fused-silica	100 μm i.d., 100cm fused-silica
	voltage	30 kV	30 kV	- 17 kV	- 25 kV
	buffer	Formic acid 1M	Formic acid 1M or 30 mM of (+)-(18-crown-6)-2,3,11,12 -tetracarboxylic acid	2 mM TMA, 5 mM Tris, pH 8.5 by amm hydroxide	pyromellitic acid 4 mM, NDS 4mM, DETA 2 mM / Me (80:20, v/v)
	Temperature	20°C		25°C	
Sample	*volume*	3 nl	0.4 nl		60 nl
	pressure	50 mbar		30 mbar	
	duration	3 s		10 s	
Liquid sheath		Me–water (1:1, v/v) 5mM amm acetate	any	0.25% (v/v) formic acid in 2-propanol–water (50:50, v/v)	pyromellitic acid 4 mM, NDS 4mM, DETA 2 mM / Me (80:20, v/v)
		10 μL/min		4 μL/min	10 μL/min
ESI	*mode*	positive	positive	negative	negative
	voltage	4 kV		3.4 kV	3 kV
MS		ESI - quadrupole	ESI -quadrupole	ESI - quadrupole	ESI - quadrupole

(*Continued*)

TABLE 7. (*Continued*)

		carbohydrates		different metabolite classes	
		Che et al. (1999)	Larsson et al. (2001)	Soga et al. (2002a)	Soga et al. (2002b)
CE	*capillary type*	75 mm i.d., 57cm fused-silica	50 mm i.d., 70-100cm coated with polyacrilamide or not	50 mm i.d., 100cm cationic capillary	51 mm i.d., 100cm, non-charged (poly-dimethylsiloxane)
	voltage	- 20 kV	- 30 kV	- 30 kV	- 30 kV
	buffer	1% HAc-NH_4OH pH 3.4	background electrolyte	50mM amm acetate pH 9.0	50mM amm acetate pH 7.6
	temperature	25°C		20°C	30°C
Sample	*volume*	17 nl		30 nl	30 nl
	pressure		50 mbar	50 mbar	50 mbar
	duration	15 s	6 s	30 s	30 s
Liquid sheath		ethanol-water (60:40) with formic acid 1%	isopropanol - 0.5% BGE acetic acid– formic acid (1:1, v/v) - low pH	Me-water (50:50, v/v) with 5 mM amm acetate	Me-water (50:50, v/v) with 5 mM amm acetate
		1 µL/min	5 µL/min	10 µL/min	10 µL/min
ESI	*mode*	negative	negative	negative	negative
	voltage	4.5 kV	3.8 kV	3.5 kV	3.5 kV
MS		ESI - quadrupole ion trap	ESI - single quadrupole	ESI - quadrupole	ESI - quadrupole
	capillary temp.	200°C			

drates is recent. The specific analysis of related chemical species is probably technically easier than analyzing all cell metabolites, and can be a comprehensive approach to characterize the whole pool of cellular metabolites.

In the pharmaceutical area, attention is focused on plant secondary metabolites, which have potentially a therapeutic value. Unger et al. (1997) have presented different CE-MS methods to analyze more than 30 alkaloids without any pre-treatment with the advantage to overcome the problems that are normally associated with the derivatization procedures that are required for GC-MS analysis. Even if qualitative and quantitative analysis of alkaloids can be performed with HPLC, CE provides, in a shorter time, higher resolution and efficiency than LC (Tomás-Barberán, 1995). Accordingly, CE-MS has been used to analyze four different classes of alkaloids; i.e., monoterpenoid indole alkaloids, protoberberines/ benzophenanthridines, beta-carboline alkaloids and isoquinolines with only minor changes of the instrumental conditions. Almost all alkaloids within one class were separated by CE, and were identified with electrophoretic mobility and m/z. Bianco et al. (2003) evaluated the glycoalkaloids (GA) content of wild-type potato plants and genetically modified virus Y-resistant potato plants with non-aqueous CE coupled with ESI-MS (Bianco et al., 2002). The improvement of potato plants resistance to virus infections can trigger changes in the levels of natural toxicants, like GA, those changes can be problematic in plants used as food. Wild-type potato plants were transformed, and subsequently their GA content was characterized and quantified (Driedger et al., 2000). The GA levels were, in both cases, inferior to the limit value recommended for food safety.

Analysis of amino acids is valuable for many different applications such as biochemical studies, medical diagnosis, food technology, and biotechnology. Many methods have been developed for analysis of amino acids by GC, GC-MS, HPLC, and CE, but those methods usually require the chemical derivatization of the amino acids. That step can be avoided with CE-MS. Soga & Heiger (2000) have described a CE-ESI-MS method for the determination of 19 standard underivatized amino acids in 17 min. All of the amino acids were not electrophoretically separated, but were selectively detected by MS in the positive-ion mode. The limits of detection (concentration) for alkaline amino acids such as Arg, His, and Lys were between 0.3 and 1.1 µmol/L, whereas it was 6 and 11 µmol/L for the acidic amino acids glutamate and aspartate, respectively. The sensitivity of the CE-ESI-MS method is dramatically increased compared to a CE method with indirect UV detection (Soga & Ross, 1999), but is inferior to an HPLC method that used derivatization. Schultz & Moini (2003) have analyzed in 35 min 20 underivatized amino acids and their D/L enantiomers by CE-ESI-MS. With a limit of detection in the femtomol range for amino acids, the method had more sensitivity than those proposed by Soga & Heiger (2000). It is probably because of the utilization of a sheathless CE-MS interface that delivered a few nL/min (Moini, 2001) instead of the sheath-flow interface that delivered a few µL/min. The method has been applied for the analysis of blood with minimal sample preparation, and thereby used for the diagnosis of metabolic diseases like phenylketonurea and tyrosinemia by identifying increased levels of phenylalanine and tyrosine, respectively, in blood from sick infants compared to healthy infants.

Low molecular mass organic acids also play an important role in biochemistry and food science, as well as in environmental science. Those compounds have also been studied by CE-MS to avoid any metabolite derivatization (contrary to GC analysis) and to improve the metabolite detection in comparison to CE-UV analysis. Johnson et al. (1999) have analyzed by CE-MS within 35 min a mixture of succinic, maleic, malonic, and glutaric acids. An electroosmotic flow modifier (2 mM diethylene triamine) was introduced into the CE mobile phase to cause the electroosmotic flow and the anions to move in the same direction. The optimal separation and ES-MS detection were achieved with the addition of pyromellitic acid and naphthalene disulfonate. Detection limits were between 1 and 10 mg/L. The authors suggested the use of micro- or nano-ESI to improve the limit of detection. In another report, Hagberg (2003) analyzed with CE-MS 11 organic acids in 21 min (oxalate, malate, malonate, citrate, tartrate, succinate, glutarate, adipate, lactate, iso-saccharinate, and gluconate). Limits of detection (concentration) were between 0.05 and 0.10 mg/L. Those values are inferior to those obtained for a similar CE-UV analysis. The method was applied to filtered samples to quantify organic acids.

CE can separate oligosaccharides with high resolution (El Rassi, 1999), whereas MS can give information on the complex structure and the molecular weight of carbohydrates (Papac, Wong, & Jones, 1996; Suzuki et al., 1997). Those two techniques have been coupled recently for carbohydrates analysis. Che et al. (1999) described the first use of ESI-MS as on-line detector for the analysis of CE-separated 8-aminonaphthalene-1,3,6 trisulfonic acid-derivatized oligosaccharides (ANTSDOs). The advantage of that derivatization (Jackson, 1990) is to add a negative charge to the analytes, and to enhance the CE separation and MS ionization. Using CE-UV (223 nm), the analysis of a mixture of derivatized dextran hydrolysates resulted in 13 peaks, which can be theoretically attributed to ANTSDOs with degrees of polymerization (DPs) from 1 to 13. Using CE-MS, the mass of ANTSDOs has been determined with an accuracy between 0.01 and 0.07%. The oligosaccharides with a DP higher than 8, present at low level, were not detected by the ion-trap mass spectrometer used. Micro- or nano-ESI could be used to ionize molecules without any sheath liquid dilution to thus improve the detection limit. Larsson, Sundberg, & Folestad (2001) have also analyzed ANTSDOs by CE-MS. However, all compounds were not completely separated, and the MS background levels were high. Thus, the authors suggested an improvement of the detection limits by implementing mathematical methods to extract relevant data from a spectral background. In some publications, oligosaccharides were not derivatized prior to analyses. For example, a complex mixture of naturally charged hyaluronic acid fragments was analyzed directly by CE-MS (Kühn et al., 2003). Another example is the CE-MS analysis of heparin, a complex mixture of sulfated polysaccharides extracted from mammalian tissues (Duteil et al., 1999).

2. CE-MS for Metabolite Profiling

The feasibility of the CE analysis of organic acids, amino acids, carbohydrates, and nucleotides has been demonstrated by Soga & Ross (1999) and Soga & Imaizumi (2001). However, UV detection is often insufficient to determine many metabolites in complex mixtures. By coupling CE and MS, Soga et al. (2002a) have achieved a simultaneous and quantitative analysis in only 22 min of multivalent anions, such as carboxylic acids, phosphorylated saccharides, and nucleotides. A cationic polymer-coated capillary was employed together with reverse electroosmotic flow, and CE-ESI-MS was performed in the negative-ion mode. The detection limits (concentration) were between 0.3 and 6.7 μmol/L, and the correlation coefficients for the calibration curves were superior to 0.993 in the range 5–100 μmol/L. All of the components were not electrophoretically separated, but were identified according to their mass. That method was used to analyze the metabolic intermediates extracted from *Bacillus subtilis* at two cell sporulation stages (cells in the initial sporulation-phase t0 and sporulating cells t1) to elucidate the metabolic and signaling role of the TCA cycle in the sporulation process. Metabolites were extracted at different times (t). Twenty-seven anionic metabolites were analyzed directly and were quantified in 25 minutes: 2-oxoglutarate and citrate concentrations were high at t1, but were not detected at t0. Those observations were in agreement with the fact that 2-oxoglutarate and citrate are believed to be involved in the induction of *B. subtilis* sporulation. The same authors (Soga et al., 2002b) have changed slightly that method to analyze in 20 min more specifically nucleotides and CoA esters. A capillary coated with a non-charged polymer was used to avoid metabolite adsorption on the column. Because a current drop often occurred in this system, constant liquid flow towards the mass spectrometer was driven by air pressure (50 mbar). The detection limits (concentration) were between 0.5 and 3.7 μmol/L, and the correlation coefficients were superior to 0.991 in the range 10–500 μmol/L. That method was used to analyze *B. subtilis* extracts, but the compounds were not well-separated by CE. Citrate and CoA exhibited poor peak shape or split peak. The intracellular concentrations of 14 metabolites were determined with an RSD ($n = 6$) inferior to 7.2%. Those reports have shown that CE-MS is a suitable tool for the quantification of metabolites, and for the study of biological events and bacterial differentiation.

D. Direct-Infusion Mass Spectrometry

Direct-infusion MS is compatible with the quest to analyze all metabolites in connection with metabolome analysis. With developments in interfacing techniques, especially atmospheric pressure ionization (API) interfaces, a metabolites mass spectrum can be obtained in a few seconds by the direct infusion of a biological sample into a mass spectrometer without any chromatography separation, and in some cases without any metabolite derivatization. Direct-infusion MS can be used for target analysis and metabolite profiling in several different ways: metabolite quantification, structure elucidation of metabolites by tandem MS or MS^n, or screening of metabolites in a complex biological sample.

1. Direct-Infusion MS for Targeted Analysis

The analysis of metabolites by direct-infusion MS can be performed within a few seconds. Direct-infusion MS is, there-

fore, an interesting methodological tool for high-throughput approaches like the screening of 500 samples/day/instrument (Rashed et al., 1997). The assignment of the mass peaks is promoted by soft ionization techniques that produce little or no fragmentation of fragile, volatile or not, thermolabile molecules. A myriad of recent publications are devoted to the direct-infusion MS study of non-endogenously synthesized metabolites used as drugs, but clinical laboratories have also investigated products and substrates of endogenous metabolism by MS (Rashed, 2001). One of the most important high-throughput applications of MS in medicine concerns the screening of inborn errors of metabolism. An early diagnosis of metabolic disorders is necessary to provide a proper therapeutical treatment to prevent later disability. Those diseases are usually diagnosed by the analysis of biological fluids, using chemical, spectrophotometric, radiochemical, and chromatographic methods. However, those methods cover only a narrow spectrum of diseases, have a insufficient level of specificity or sensitivity (or both), need a laborious sample preparation, and a long analysis time, and are, therefore, not well-suited to screen large populations. In the last decade, numerous reports have described robust, accurate, and automated methods based on MS or multistage MS for the screening of metabolic diseases.

A rapid method with the potential to screen for many of the peroxisomal disorders has been developed by Johnson (2000). Analysis of very long-chain fatty acids (VLCFA) in plasma and in a blood spot has been performed by ESI-MS-MS in 2 hr, including sample preparation, whereas such analyses by GC and GC-MS take significantly longer times (Moser & Moser, 1991; Vallance & Applegarth, 1994; Inoue et al., 1997; Vreken et al., 1998). The strategy was to esterify all of the VLCFAs with an alcohol that contained a protonatable functional group to assist the electrospray ionization, and to quantify them with MRM. Despite the overestimation of the content of fatty acids compared to GC analysis, the ESI-MS results showed that the VLCFAs ratios are significantly increased in the plasma of patients with peroxisomal disorders compared to controls. Valianpour et al. (2003) have analyzed non-esterified VLCFAs in plasma samples by ESI-MS-MS and by GC-MS according to the method of Moser & Moser (1991). Differences between results from both analytical methods were explained by non-identified errors in both methods. The ESI-MS method is a good alternative to the current methods, and enabled the high-throughput analysis of VLCFAs. Another interesting example recently published by Nagy et al. (2003) is the method for the analysis of 19 amino acids in dry blood spots. Compared to the well-established method that includes a butyl-esterification of the free carboxyl groups of amino acids to enhance the electrospray ionization efficiency (Rashed et al., 1995; Chace et al., 1996, 1998; Shigematsu et al., 1999; Zytkovicz et al., 2001), the new method does not require any derivatization of the amino acids before ESI-MS-MS analysis. That method is, therefore, not limited to the analysis of compounds that have a butyl-formate loss in connection with derivatization. Moreover, MRM was used to optimize the individual sensitivity and selectivity for each analyzed compound, instead of constant neutral-loss scans. Twenty-two underivatized amino acids instead of ten were identified. That method was based on an extraction in an aqueous phase instead of a methanol-based extraction, followed by ultrafiltration to remove macromolecules and desalting with SPE to avoid an ionization suppression in the analysis. Accordingly, the sensitivity of MS detection was improved 2- to 5-fold compared with the conventional method. Johnson (2001) has also proposed another alternative to the current method. A superior derivatizing agent, dimethylformamide dimethylacetal, has been used for amino-acid derivatization to increase the signal intensity from the butyl esters of small amino acids such as glycine, alanine, and valine. In that way, the signal intensity could be increased up to 20-fold. Although more amino acids can be characterized by this method, the suitability of the method for quantitative analysis has not been evaluated, and the method involves a derivatization step. Acylcarinitines have also been the target in the screening of newborns because the accumulation of these compounds in blood or urine indicates organic acidaemias and fatty-acid oxidation defects (Rashed et al., 1995, 1997; Chace et al., 1997; Shigematsu et al., 1999; Vreken et al., 1999; Mueller et al., 2003). Acylcarinitines, as well as amino acids, from dried blood spots were analyzed by ESI-MS after butyl-esterification. Those metabolites were identified according to their specific MS fragmentation from C2 to C18:1 (Chace et al., 1997), from C3 to C14 and derivatives (Rashed et al., 1997), and from C3 to C16 (Zytkovicz et al., 2001). In the same way, more than 10 different acylglycines associated with 14 diseases were identified in the study of Bonafé et al. (2000). Chace et al. (1997) analyzed all acylcarnitines and 12 amino acids from dried blood spot in less than 3 min with a tandem MS. Medium-chain acyl-CoA dehydrogenase deficiency (MCAD) has been diagnosed on the basis of the increase of medium-chain length acylcarnitines. MS-MS acylcarnitine profiles exhibited very high octanoylcarnitine peak intensity in the case of MCAD newborns, later confirmed by DNA analysis. Application of that technology to newborn screening in a set of ca. 300,000 samples revealed a disease frequency of 1 in approximately 17,000.

In the medical area, even if high-throughput is required, key diagnostic metabolites were quantified to provide decision criteria for the presence of metabolic diseases. Direct-infusion MS without any chromatographic separation can achieve metabolite quantification; the levels of each metabolite or peak ratios of metabolite pairs were determined. For instance, the quantitative analysis of tyrosine in blood is required to detect tyrosinemia (Rashed et al., 1997), and the ratio between phenylalanine and tyrosine is required to determine phenylketonurea (Chace et al., 1998). Quantification was achieved by comparing the peak height of the amino acid of interest (ion product) to an internal standard, usually an isotopically labeled analog. Excellent calibration curves generated with standard isotope-dilution techniques could be obtained. Quantitative analyses of metabolites by direct-infusion MS have been validated, and were used in large newborn-screening programs (Zytkovicz et al., 2001; Schulze et al., 2003; Wilcken et al., 2003).

With the aid of automated sample preparation, and of computers to assist in the recognition of a specific disease in a patient, rapid MS-MS analysis can now be used to perform real-time newborn screening (Rashed et al., 1997; Shigematsu et al., 1999). In the medical area, very high and reproducible sample throughput has been achieved with tandem MS for the analysis of an array of metabolic disorders, and already in 2000, more than 1 million blood and plasma samples had been tested by MS in laboratories throughout the world (Chace, DiPerna, & Naylor,

1999). Zytkovicz et al. (2001) screened 160,000 newborns by MS/MS for 23 metabolic disorders in a 2-year screening program in New England. Schulze et al. (2003) screened 250,000 neonates in a German population with ESI-MS-MS for 23 metabolic disorders. The MS/MS screening has ca. doubled the detection rate compared to that achieved by the conventional methods used in Germany. In the period 1998–2002, 362,000 newborns in Australia were screened for 31 metabolic disorders by MS-MS (Wilcken et al., 2003). That study has shown that more cases of inborn errors of metabolism can be diagnosed with tandem MS for screening compared with classical diagnosis.

Direct-infusion MS has also been used for the quantitative analysis of metabolites in plants and microorganisms. For example, the semi-quantitative determination of bis-indole alkaloids from *Catharanthus roseus* has been done by ESI-MS (Favretto et al., 2001). Using the selected ion-monitoring mode, peak area was determined and was plotted versus alkaloid concentration to allow the estimation of alkaloids in natural extracts. In the publication of Wittmann & Heinzle (2001a), MALDI-TOF MS has been used for the quantification of lysine, arginine, and glucose, using stable isotopes as standards. The presence of non-labeled molecules contained in the internal standard and the presence of natural isotopes of the analyte were considered in the calculation of concentrations from peak ratios. Excellent agreement has been found between those results and those obtained by HPLC (lysine, alanine) and enzyme assay (glucose). The method has been applied to the study of the production of lysine in cultivations of *Corynebacterium glutamicum*. Wittmann & Heinzle (2001b) have also used MALDI-TOF MS to quantify the flux distribution in the central metabolism of *C. glutamicum*. To achieve that goal, ^{13}C-tracer studies with labeling measurements were performed with MALDI-TOF MS, and metabolite balancing was developed. An advantage of the developed method is that it only requires the analysis of a few metabolites, which are identified by computer-based experimental design. The labeling of those metabolites provided the missing information to obtain the entire flux-distribution map; 37 metabolic fluxes were determined, and provided with a low experimental effort interesting insights into the functioning of the metabolism of the strain.

2. Direct-Infusion MS for Metabolite Profiling

The rapid screening of natural metabolites produced by plants and fungi, primarily secondary metabolites, is an efficient way to find new biologically bioactive molecules. The presence of secondary metabolites in microbial extracts can be estimated in ca. 1 min by direct infusion ESI-MS (Higgs et al., 2001). In a continuous research, Zahn, Higgs, & Hilton (2001) have used that method to guide culture-conditions improvement to enhance the expression of secondary metabolites. The expression of 10 secondary metabolites in 44 actinomycetes strains under different fermentation conditions was analyzed. The results showed that significant differences in the production of secondary metabolites occurred in response to the growth conditions (fermentation growth medium), and, therefore, the analysis could be used to classify the cultivation conditions based on the metabolite profile. The m/z ratio of ions, combined with information on the nature/origin of the sample and knowledge on the metabolism of the species studied, can be sufficient to identify metabolites by MS (Higgs et al., 2001). The MS^n capability can facilitate greatly the interpretation of the spectra and the structure elucidation of unknown secondary metabolites produced by plants and microorganisms, and also avoid time-consuming isolation of pure constituents and the loss of low-concentration compounds. In the study of Cui et al. (1999), three unknown triterpenoid saponins in crude extracts were investigated by ESI-MS^5. The characteristic fragmentation pattern of each metabolite was proposed and discussed in terms of ion adducts, rearrangement, and ion stability. The mass spectra in the positive- and negative-ion modes suggested the presence of the same glycosyl moiety in three metabolites, whereas differences existed in the aglycone unit. The same approach was used to characterize anthocyanins (Piovan, Filippini, & Favretto, 1998). Petunidin, malvidin, and hirsutidin were MS-identified as aglycone moieties in hydrolyzed extracts of *C. roseus*. To identify anthocyanin glycosides, the ions detected in the full scan of non-hydrolyzed extracts were isolated and were subjected to collision-induced dissociation to check among their fragmentation products for the presence of one of the aglycones identified. In this manner, six glycosides were detected. With MS/MS experiments in presence of D_2O, which was added to indicate the presence of exchangeable protons, the presence of a coumaroyl residue could be confirmed. A deuteration experiment combined with ESI-MS^n was also performed to identify some steps in the ring fragmentation of polyketides (Gates et al., 1999). It was demonstrated that the only difference in the structure of erythromycin analogs was the starter acid used in the biosynthesis. In conjunction with specific labeling and derivatization, ESI-MS^n has provided pertinent information on product-ion structure and allowed the distinction of isomeric species (Viseux, de Hoffmann, & Domon, 1998). New sulfated molecules in the pathogenic bacteria *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* were discovered (Mougous et al., 2002). Sulfur-containing molecules were identified by mass spectral analysis of cell extracts from mycobacteria that were labeled metabolically with a stable isotope of sulfur ($^{34}SO_4^{2-}$). Sulfated molecules were distinguished from those possessing reduced sulfur (i.e., compound with thiol) by performing the experiment with mutants that lacked the reductive branch of the sulfate-assimilation pathway. That approach may be very useful in studies of metabolic flux and in the identification of new biosynthetic pathways.

Direct-infusion MS has also been applied for the high-throughput classification and identification of microorganisms (Smedsgaard & Frisvad, 1996, 1997; Goodacre et al., 2003), bioproduct classification (Goodacre et al., 2002), and analysis of large mutant collections for functional genomic studies (Allen et al., 2003; Castrillo et al., 2003). The ''profiles of masses'' obtained for different samples were compared with the aid of specific data-analysis and bioinformatics methods.

Metabolite profiles can be used to discriminate microorganisms, and Smedsgaard & Frisvad (1996, 1997) applied MS to the chemotaxonomic segregation of 36 *Penicillium* species that were associated with stored cereals. Mass-profile spectra were obtained within few minutes by the direct-injection ESI-MS of a fungal crude extract, and were correlated with the production of

specific metabolites considered as taxonomic keys. A database of metabolite profiles was built that could be used for the rapid identification of species, even though not all the peaks or fragmentation patterns were identified. The data-processing approach, based on cluster analysis, showed that ESI-MS analysis could produce chemotaxonomically significant mass profiles. Another work that focuses on the pattern-recognition of metabolic phenotypes rather than on the inventory of a specific compound has been described (Goodacre et al., 2003), where ESI-MS was used in combination with various post-analysis chemometric methods to discriminate among the metabolic profiles of plants that had been subjected to different photoperiod treatments. Unfractionated *Pharbitis nil* leaf-sap samples were analyzed by ESI-MS in less than 30 s, and with an extremely simple sample preparation. The mass spectra showed peaks of organic acids and sugars. Discrimination was not possible by a visual comparison, but rather with chemometric methods including discriminant function analysis and the machine-learning methods of artificial neural networks and genetic programming. Those chemometric methods were also used in a previous report (Goodacre et al., 2002) to discriminate plant oils. Recently, non-targeted metabolic analysis has been performed on strawberry fruit and tobacco flower extracts with direct-injection Fourier Transform Ion Cyclotron Mass Spectrometry (Aharoni et al., 2002). The identification of the metabolites was achieved by determining their elemental compositions deduced from accurate mass determinations. Relative quantification was obtained by internal calibration. The developed method could simultaneously and rapidly detect masses that corresponded to various types of compounds (flavor compounds, terpenes, carbohydrates, anthocyanins) that were known to accumulate during the ripening of the strawberry fruit. In another set of experiments, the method was used to study changes in the metabolic profiles of transgenic tobacco plants that overexpressed a regulatory gene.

Direct-infusion ESI-MS has been investigated for the global analysis of intracellular metabolites of different strains of the yeast *S. cerevisiae* (Castrillo et al., 2003). More than 25 intracellular metabolites were simultaneously detected. Mass spectra showed clear differences between the metabolic profiles of different strains. The high-throughput classification of yeast mutants for functional genomics has been achieved by so-called metabolic ''footprinting,'' a term that has been introduced by Allen et al. (2003). That approach was defined as ''the recognition of the significance of overflow metabolism in appropriate media.'' The method consisted of the direct and non-invasive MS monitoring of extracellular metabolites. The advantage was to avoid the technical difficulties associated with metabolite extraction from microbial cells, and, therefore, could perform high-throughput analysis (2 min/sample). Results have shown that metabolic footprinting can distinguish between different physiological states of wild-type yeast and mutants, and is a tool for the classification of mutants, through different types of cluster analysis.

E. Comparison of the Different Analytical Techniques

Neither on-line separation combined to MS nor direct-infusion MS can cover the analysis of the complete metabolome of an organism. Applications and limitations of the different techniques, therefore, must guide the analyst to choose the most appropriate method for the given application. Table 8 summarizes the applications of the different analytical techniques that use MS as the detector, and highlight the advantages and disadvantages of each method.

GC-MS is a more mature technique in bioanalysis and, as a consequence, has a broader application described in the literature. It has a great potential for targeted analysis and metabolite profiling, and the variation between those two approaches are basically performed through a switching of the detection from the SIM to the scan mode. However, the requirement to derivatize non-volatile metabolites often complicates the identification of unknown compounds, and it also increases the sample preparation variability.

On the other hand, the API-MS can be applied to the analysis of non-volatile and thermally unstable metabolites that displayed a wide variety of polarities and molecular masses, without any derivatization. API-MS generates mass spectra with little or no metabolite fragmentation; ''fingerprint'' of molecular ions is obtained in a few minutes by direct-infusion API-MS. The direct infusion of crude extract into an API-MS instrument is appropriate for the rapid screening of metabolites produced by plants and fungi. The molecular structure of the unknown metabolites, potentially new bioactive molecules, can often be deduced from the specific fragmentation patterns obtained by multistage MS. Furthermore, direct-infusion MS offers the possibility to quantify metabolites directly from complex biological fluids. That approach is faster than any technique that involved a separation step, and it is, therefore, compatible with high-throughput analysis. Daily ion source cleaning is necessary, but the cleaning frequency can be increased to maintain the efficiency of the MS instrument, especially if ionic species or complex samples are injected into the MS.

The analysis of multi-component mixtures by direct-infusion MS can, however, be hindered by the competitive ionization of the analytes that leads to a low sensitivity. Through a combination of liquid/liquid extraction procedures that might ensure the separation of metabolites and thereby reduce any matrix effects, and ESI-Fourier Transform Mass Spectrometry (FTMS), the competitive ionization effects can be minimized. FTMS is currently the only MS technology that provides the resolution and the mass accuracy that are required to reliably assign molecular formulas to detected metabolites. Some of the typical characteristics are a mass resolution greater than 100,000, an attomole sensitivity, and better than 1 ppm mass accuracy. However, such technology is expensive, and requires advanced informatics tools for data analysis.

The combination of LC with MS enables the analysis of many classes of compounds: plant and fungal secondary metabolites, amino acids, nucleotides, oligosaccharides, and glycolytic intermediates. Those metabolites can be identified with data from a UV detector or from MS^n data. The LC-MS methods have proven to be efficient for the identification, but also the quantification, of metabolites that have even similar molecular masses (i.e., glycolytic intermediates) and in complex mixture. One challenge is to convert LC methods to LC-MS methods according to important factors, such as the mobile-phase volatility and the ion-suppression effects to attain a compromise

TABLE 8. Examples of application of different analytical techniques for metabolome analysis

Analysis Technique	Application	Advantages	Disadvantages	Type of Analysis	Reference
GC-MS	Simultaneous separation, identification, and quantification of different classes of metabolites (volatile and non-volatile) in a single analysis. Basically, the type of analysis (targeted or metabolite profiling) is defined by switching the detection between the SIM[1] and SCAN modes.	High chromatographic resolution, ideal to resolve complex biological samples; Enable simultaneous analysis of different classes of metabolites.	Unable to analyze thermo-labile metabolites; Non-volatile metabolites must be derivatized before analysis. Difficult to identify unknown compounds after derivatization	**Targeted analysis** *S-alk(en)ylcysteine, sulforaphane, sulforaphane nitrile* **Metabolite Profiling** *all different classes of metabolites.*	Chiang et al., 1998 Kubec et al., 1999 and others. Nilsson et al. 1996 Baldizsár et al., 1998 Katona et al., 1999 Roessner et al., 2000 Demyttenaere et al., 2003 Villas-Bôas et al., 2003 and others
LC-MS	Separation, identification and quantification of a very broad group of metabolites. Limited potential in identification unless a MS-MS techniques is used.	High sensitivity; Average to high chromatographic resolution; No derivatization required; Enable analysis of thermo-labile metabolites.	A few restrictions on LC eluents, De-salting may be needed. Limited structural information Matrix effects	**Targeted analysis** *glucosinolates* **Metabolite profiling** *glycolytic intermediates, nucleotides, isoprenoids.*	Mellon et al., 2002 Buchholz et al., 2001 van Dam et al., 2002 Cogne et al., 2003 Novák et al., 2003
CE-MS	Separation, identification, and quantification of polar metabolites, using small sample volumes.	Useful for complex biological samples Small volumes High resolution	Complex methodology and quantification, Buffer incompatibility; Difficulty in interfacing; Need further development	**Targeted analysis** *Oligosaccharides, amino acids, glycoalkaloids* **Metabolite profiling** *Carboxylic acids, phosphorylated saccharides, nucleotides.*	Che et al., 1999 Soga & Heiger 2000 Bianco et al., 2003 Schultz & Moini 2003 Soga et al., 2002a
MS	The applications vary according to the ionization method. Generally applied for determination of metabolites by their molecular mass and fragmentation pattern. Usually applied in metabolite profiling without any quantification and identification of the metabolites.	Rapid screen of metabolites; Negligent sample clean-up for metabolite profiling; High-sensitivity Easy database storage Recommended for identification of unknown compounds (MS-MS)	Identification of metabolites require MS-MS; Matrix effects, Incompatibility with higher ionic strength (used in some metabolite extractions)	**Targeted analysis** *Acylcarinitines, amino acids, carbohydrates.* **Metabolite profiling** *No-identification Finger- and foot-Prints.*	Chace et al., 1997 Wittmann & Heinzle 2001b Nagy et al., 2003 Castrillo et al., 2003 Allen et al., 2003

[1]Selected ion monitoring.

between the LC separation and the MS detection. Most of the time, the reversed-phase is preferred to an ion-exchange column to avoid any negative effects on metabolite ionization. Otherwise, the desalting step necessary prior to the ionization process could be problematic for certain salt-like metabolites. To direct all the effluent towards the MS and to maintain a sufficient plate number, the LC column should have a short length with a low particle diameter.

On the other hand, monolithic columns have been used instead of conventional particulate HPLC columns for fast, high-quality separations of peptides, proteins, and pharmaceutical compounds. The monolithic separation medium is made of a continuous, rigid polymeric rod with a porous structure. The lack of any intraparticle void volume improves the mass transfer and separation efficiency. Wu et al. (2001) have measured three pharmaceutical components in 600 plasma extracts within 12 hr with a LC-MS-MS system that used a monolithic column. The use of such columns, therefore, seems to be very promising for high-throughput profiling.

The combination of MS and CE is more complicated. The existing CE methods must be modified to be compatible with the electrospray ionization process. Many efforts have been made to

establish parameters, especially regarding the composition of the CE background electrolyte, which allow a satisfactory separation coupled to an efficient ionization by ESI (Table 7). The small sample volumes required for CE can be an advantage, but the technique can suffer from insufficient sensitivity. The advantage of CE-MS is that it is versatile, flexible, and, therefore, applicable to a wide range of compounds from a wide variety of chemical classes that are present in complex matrices; however, currently only polar compounds can be analyzed by this technique.

Capillary electrochromatography (CEC) combined with MS is one of the newest analytical tools for the pharmaceutical, clinical, forensic, veterinary, and life science fields. For example, Que & Novotny (2003) have separated neutral oligosaccharides by CEC by using polar monolithic columns, whereas an on-line tandem MS has been used to elucidate the structure of the isomeric forms. CEC is a hybrid of CE and capillary HPLC, and uses an electric field rather than hydraulic pressure to propel the mobile phase through a standard packed bed HPLC. The absence of backpressure means that particles as small as sub-μm and columns considerably longer than in HPLC can be used. A second factor responsible for enhancing separation efficiency in CEC, as compared with HPLC, is the uniformity of flow across the capillary. Plug flow has the advantage of moving the analyte molecules along the column more uniformly, resulting in narrow analyte bands, and therefore improved resolution.

IV. APPLICATION ON FUNCTIONAL GENOMICS

The high-throughput nature of MS makes it well-suited for functional genomics, because it is hereby possible to analyze a large number of mutants and/or growth conditions of cellular system under study. At the interface between the environment and DNA-encoded processes, metabolite levels are quantitative phenotypic indicators that provide an important complement to the measurements of mRNA and proteins when studying cellular function. In common with the transcriptome and the proteome, the metabolome is context-dependent, and, thereby, reflects the actual environmental conditions. On the other hand, there is no one-to-one correspondence between genes and metabolites. The same metabolite may, for instance interact with several different gene products in different metabolic pathways, or one gene product may catalyze several different reactions that involve different metabolites. In that respect, measurements of metabolite levels represent truly integrative information. It has been stated as an advantage that the size of the metabolome is ''smaller'' than the genome and the genome-related entities; e.g., the estimated number of small metabolites in the yeast *S. cerevisiae* is one order of magnitude less than the number of genes (Raamsdonk et al., 2001). That property is clearly organism-dependent because the number of metabolites in many plants and fungi, with more diversified secondary metabolism, well matches or even exceeds that of the genes (Schwab, 2003). A potential advantage for large-scale studies is that the cost per sample is moderate compared to the analysis of the transcriptome and proteome; however, for a comprehensive analysis of the metabolome, the great physicochemical variation implies that several different analytical and sample processing methods will be required.

The most prominent applications thus far have been demonstrated within the field of plant science. For *Arabidopsis thaliana* (Fiehn et al., 2000) and potato tuber (Roessner et al., 2000), GC-MS protocols enabled the simultaneous detection of more than 300, and the identification of 150 intracellular metabolites, respectively, including sugars, sugar phosphates, amino acids, and organic acids. A following study that assessed the impact from genetic and environmental modifications on the metabolite profiles in wild-type and transgenic potato lines demonstrated the potential of metabolome analysis for functional studies (Roessner et al., 2001). Outside the plant realm, a ''guilt-by-association'' concept for functional genomics based on analysis of intracellular metabolite profiles was demonstrated in the yeast *S. cerevisiae* (Raamsdonk et al., 2001). The underlying idea was that single-gene deletion mutants with similar metabolite profiles also should have a similar function, or at least be involved in the same biological process. Thus, the function of orphan genes could be deduced by comparing with the metabolite profiles that correspondng to deletions of well-characterized genes. Using a similar approach for the data analysis, but avoiding the need for quenching, separation, and extraction, Allen et al. (2003) suggested a strategy based on the direct-infusion MS-analysis of culture media, thereby fingerprinting the consumption and production of extracellular metabolites. Their footprinting approach with a sample throughput of 2 min may be particularly attractive for large-scale studies, although the restriction to extracellular metabolites may limit the discriminatory power for certain phenotypes.

The matter of determining gene function from similarities in metabolite profiles is closely related to the classification problem in taxonomy, where metabolite profiling has become an important tool. Standardized protocols based on direct-infusion ESI-MS or LC-ESI-MS have been applied for the classification, identification, and characterization of fungal species based on their secondary metabolite levels (Smedsgaard & Frisvad, 1996; Nielsen & Smedsgaard, 2003). Within the context of metabolome analysis and MS, one can also mention metabolic network analysis, which is a powerful approach for phenotypic characterization based on ^{13}C and $^{2}H_2O$ tracer-experiments (Christensen & Nielsen, 2000; Gombert et al., 2001; Wittmann, 2002; Hellerstein, 2004). The labeling patterns of selected metabolites were analyzed by MS, which allows an estimation of intracellular fluxes as well as the identification of active pathways. Finally, it should be appreciated that considerable metabolomics efforts are made in industry with large research programs in several companies (e.g., Metanomics, Paradigm Genetics, Phenomenome Discoveries, Plant Research International). Even though the scientific publications from that sector are scarce, one can note the application of FT-MS (Aharoni et al., 2002) as well as a study that integrated the transcriptional and metabolite profiles for metabolic engineering of lovastatin-producing *Aspergillus* strains (Askenazi et al., 2003).

Figure 4 shows the key steps in data handling and analysis. Following data acquisition, there are still many steps before the acquired data are converted into some kind of useful information, and we will here briefly comment upon topics that relate to the pre-processing of raw data, data analysis and interpretation, and

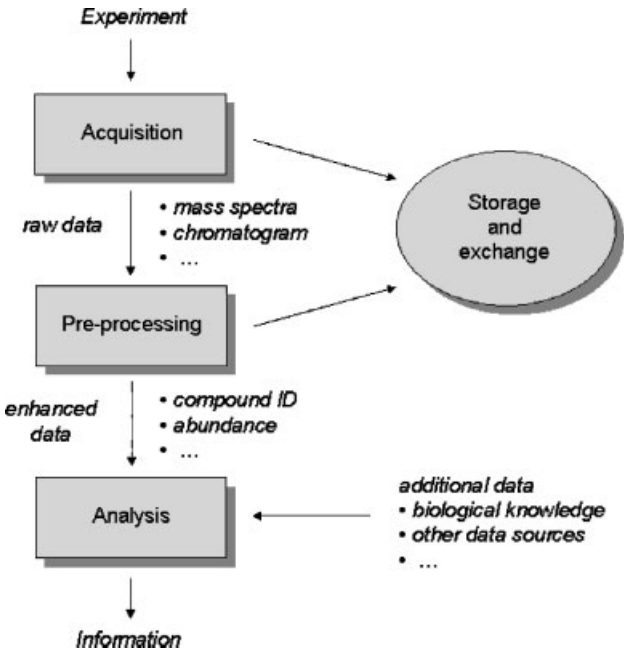

FIGURE 4. Key steps in data-handling and analysis to convert the experimental data to useful information.

the storage and exchange of data. Several of those issues have been extensively reviewed (Mendes, 2002; Sumner et al., 2003).

An important topic, especially for large-scale parallel studies, is the management of the generated data files because the analysis of a single sample may range in size from a few MB to several hundred MB, depending on the type of analysis, the type of detector, and the mode of acquisition. To facilitate the exchange and interpretation of data, it is also important to define what information should be stored. With the development of public repositories for transcriptome and proteome data, there are already initiatives for standardized data descriptions that are supplemented with experimental conditions, sample processing, measurement technique, instrument settings, normalization and controls, etc. (Brazma et al., 2001; Taylor et al., 2003). Similar efforts for metabolomics will for several reasons require more efforts; one reason is the requirement for different analytical methods for a comprehensive coverage of the metabolome. Another issue is the fact that it is not known *a priori* what metabolites are present in a sample, suggesting that some kind of ''raw'' data must be stored.

Regardless of the application, the raw instrumental data requires some processing to extract information for further analysis and interpretation. That pre-processing would at least involve instrument-specific normalization and conversion to standard data formats that are appropriate for the subsequent analysis. In many applications, it is of interest to quantify the abundance of targeted compounds, or to identify what compounds are present in the sample. Automated methods for peak deconvolution and interpretation of spectra, together with public or proprietary databases are invaluable tools to obtain an efficient analysis flow (Stein, 1999; Nielsen & Smedsgaard, 2003). The pre-processing may also include procedures for the identification of unknown compounds.

Once converted to a quantitative description, one can, in principle, apply any technique for the analysis of multivariate data sets; for instance, those techniques used for other types of '-omics' data. Visualization is a key aspect because the data may contain hundreds of variables. Often, singular-value decomposition (SVD) or principal-component analysis (PCA) (Jolliffe, 1986) was used prior to the analysis to reduce the dimensionality of the data while retaining most of the information. If the experiments have been replicated, then one may use statistical tests to confine the analysis to those variables or metabolites that change significantly between different sample groups (Zhao & Pan, 2003).

It is often of interest to compare the experimental responses from different conditions, strains, or mutants, and many of the applications mentioned above exploit multivariate tools for the classification and discrimination of the samples, or for the exploration and visualization of any patterns in the data. That type of analysis, where a distinction is made between unsupervised and supervised methods, can be applied for metabolic fingerprints. Unsupervised methods, where one may include PCA, groups the samples solely on the information in the measurement data. In that category we find clustering methods, such as hierarchical clustering (HCA), K-means clustering, and self-organizing maps (see e.g., Roessner et al., 2001; Beckonert et al., 2003a,b). Supervised methods require a calibration or reference data set, where the user has associated samples with qualitative or quantitative traits such as sample class or concentration of a metabolite in the sample. The inclusion of prior knowledge typically leads to an improved performance (i.e., improved discrimination between sample groups), but the outcome is highly dependent on the quality and the diversity of the reference data. Examples of supervised methods applied within the metabolome field are discriminant analysis, artificial neural networks, and evolutionary algorithms (Kell, Darby, & Draper, 2001; Allen et al., 2003). For supervised and unsupervised methods, the choice of normalization and distance measure may impact the result, and it is, therefore, wise to investigate several different options.

To a varying degree, the above methods provide some information about what characterized a particular sample class on the metabolite/variable level. In other approaches, the metabolomic data are used more directly to generate hypotheses and to infer any underlying biological mechanisms. Correlation between metabolite levels may also contain important information (Steuer et al., 2003), and schemes that reconstruct metabolic networks from time-series data have been suggested (Arkin, Shen, & Ross, 1997). One may instead exploit available knowledge on metabolic networks for visualization (Mendes, 2002) as well as directly in the analysis (Förster, Gombert, & Nielsen, 2002). For instance, an approach that identified subnetworks with significantly changed expression in networks of protein–protein and protein–DNA interactions (Ideker et al., 2002) may straightforwardly be extended with metabolic networks and inclusion of measured metabolite levels. The integrative nature of the metabolome suggest that the full benefit of metabolomic data will only be exploited in combination with other data sources, and at the same time makes the metabolome

an important piece of the puzzle in functional genomics and systems biology.

V. FUTURE OUTLOOK

The sample preparation required for metabolome analysis must be standardized to minimize the variability between laboratories and analysts. Standard quenching methods for the different matrices and improved extraction procedures of intracellular metabolites to achieve minimal losses, are required. In that way, mechanically enhanced extraction techniques present great potential to be explored to obtain improved recoveries and reproducibilities simultaneously with automation. Similarly, sample-concentration methods that present low-discrimination pattern (i.e., lyophilization) must also be optimized and standardized. What is currently seen in the analysis of metabolites is a miscellany of sample-preparation methods that are better-suited for target analysis than for a metabolite-profiling approach. As far as metabolite profiling is concerned, standard rules on sample preparation must be established.

It is also expected that the combination of metabolome data with data from the other ''-omes'' will have a significant impact on systems biology and functional genomics. In particular, the complementary and integrative nature of the metabolome will be instrumental for the analysis and understanding of complex physiological phenomena.

Concerning data acquisition techniques, there has been much progress during the last decades, and those techniques will certainly improve continuously with the availability of more sophisticated analytical devices that, each time, will be more accurate, sensitive, and faster. There is no ideal acquisition method for metabolome analysis today, and there will probably not be one in the future. Metabolome studies are likely to always rely on a combination of several different analytical techniques to cover the analysis of the majority of metabolites that are expressed by a living organism.

Although GC-MS is often considered a matured analytical technique, there are continuing developments, especially with respect to an increased speed of analysis. The typical analysis times of GC-MS in metabolite profiling are in the range of 30–100 min/sample, which is generally considered to be too slow for high-throughput analysis. High-speed GC analysis, with peak widths that may be less than 100 ms, presents problems for MS detection similar to that presented by fast LC and by CE (Tomer, 2001). The increase in the data-acquisition speed resulted in skewed mass spectra, mainly in the scan mode, which made difficult the computerized identification of the compounds by comparison with standard spectra.

GC-TOF-MS seems to be promising for fast GC-MS analysis, because the current commercially available instruments present a spectral-acquisition rate of 500 spectra/s over 5–1000 Da, whereas a quadrupole-based mass spectrometer typically acquires data in less than 40 scans/s over a 100 Da mass range (Tomer, 2001). GC-TOF-MS instruments present high sensitivity, but their resolution in scanning complex biological mixtures remains to be optimized.

In-vial derivatizations and micro-derivatizations will also be the tendency in the future of metabolite GC-MS analysis. Although derivatization protocols are generally regarded as time-consuming and result in poor reproducibility, there is a trend to introduce automatization that will employ robotic devices. Linked to automatic in-vial derivatization, derivatization of very low sample volumes (2–20 μL) will facilitate the high-throughput analysis of very small samples, and result in improvements in sensitivity (the possibility to resuspend the samples in a smaller volume) and reproducibility (divide the samples in 3, 4, or 5 portions).

Compared with GC-MS, LC-MS is a younger technique with great possibilities for improvements. The knowledge of the ionization mechanism in the LC/MS interface is scarce, and is the most-needed area for improvement to facilitate the development of new methods. The difficulty with exchanging a spectra library from LC-MS analysis is a major drawback of that technology, particularly for metabolite profiling, where the identification of a large number of compounds is desirable. Increasing our knowledge in ionization mechanisms in LC-MS may lead to the possibility to exchange spectra libraries between laboratories and research groups, that exchange will have a substantial impact on the entire field of metabolome analysis.

On the other hand, the recent developments in separation technique and MS coupling CEC-MS, LC-MALDI-MS tends to improve the use of MS for metabolite analysis. Over the past few years, interest has grown rapidly in electroseparation techniques, because they show higher efficiency and resolution than liquid chromatography. The wide variety of stationary phases available for CEC (i.e., monolithic capillaries) makes this technique convenient for neutral, acidic, and basic compounds and, therefore, for a wide range of metabolites. The use of nano-electrospray ionization interface, for the coupling of such miniaturized separation techniques and a mass spectrometer, affords enhanced mass and concentration sensitivity over other ESI interfaces. The coupling of LC-MALDI is more delicate because MALDI, based on desorption of molecules from a solid surface layer, is *a priori* not compatible with LC or CE. A simple alternative to circumvent this limitation is the automatic deposition of fractions from a chromatographic separation on a MALDI-TOF target. More advanced techniques have been developed recently: electrospray deposition, electrically mediated deposition, rotating ball inlet, continuous vacuum deposition, continuous off-line atmospheric-pressure deposition (Wehr, 2003). The current interfacing improvements will surely expand the use of LC-MALDI in the metabolomic area.

In parallel with the development of more efficient analytical techniques, it is also important to develop novel theoretical techniques for the treatment of MS data. In particular, the issue to link the metabolome to the genome is central for a more widespread use of metabolome analysis in the field of functional genomics. However, because of the ease of analysis and the many different analytical techniques already developed for metabolite analysis, metabolome analysis by MS will surely play an increasingly important role in medicine, food technology, biotechnology, and functional genomics.

ACKNOWLEDGMENTS

We thank our colleagues, past and present, who contributed to the study described here.

REFERENCES

Abdullah MI, Young JC, Games DE. 1994. Supercritical fluid extraction of carboxylic and fatty acids from *Agaricus* Spp. mushrooms. J Agric Food Chem 42:718–722.

Abian J. 1999. The coupling of gas and liquid chromatography with mass spectrometry. J Mass Spectrom 34:157–168.

Adams MA, Chen Z, Landman P, Colmer TD. 1999. Simultaneous determination by capillary gas Chromatography of organic acids, sugars, and sugar alcohols in plant tissue extracts as their trimethylsilyl derivatives. Anal Biochem 266:77–84.

Aharoni A, Ric De Vos CH, Verhoeven HA, Maliepaard CA, Kruppa G, Bino R, Goodenowe DB. 2002. Nontargeted metabolome analysis by use of Fourier Transform Ion Cyclotron Mass Spectrometry. OMICS 6:217–234.

Airs RL, Keely BJ. 2000. A novel approach for sensitivity enhancement in atmospheric pressure chemical ionization liquid chromatography/mass spectrometry of chlorophylls. Rapid Commun Mass Spectrom 14:125–128.

Allen J, Davey HM, Broadhurst D, Heald JK, Rowland JJ, Oliver SG, Kell DB. 2003. High-throughput classification of yeast mutants for functional genomics using metabolic footprinting. Nat Biotechnol 21:692–696.

Alonzo G, Saiano F, Tusa N, Del Bosco SF. 2001. Analysis of volatile compounds released from embryogenic cultures and somatic embryos of sweet oranges by headspace SPME. Plant Cell Tiss Org Cult 66: 31–34.

Arkin A, Shen PD, Ross J. 1997. A test case of correlation metric construction of a reaction pathway from measurements. Science 277:1275–1279.

Askenazi M, Driggers EM, Holtzman DA, Norman TC, Iverson S, Zimmer DP, Boers ME, Blomquist PR, Martinez EJ, Monreal AW, Feibelmanb TP, Mayorga ME, Maxon ME, Sykes K, Tobin JV, Cordero E, Salama SR, Trueheart J, Royer JC, Madden KT. 2003. Integrating transcriptional and metabolite profiles to direct the engineering of lovastatin-producing fungal strains. Nat Biotechnol 21:150–156.

Aussenac J, Chassagne D, Claparols C, Charpentier M, Duteurtre B, Feuillat M, Charpentier C. 2001. Purification method for the isolation of monophosphate nucleotides from Champagne wine and their identification by mass spectrometry. J Chromatogr 907:155–164.

Baldi P, Hatfield GH. 2002. DNA microarrays and gene expression. Cambridge: Cambridge University Press. 213p.

Baldizsár I, Horváth K, Szedlay Gy, Molnár-Perl I. 1998. Simultaneous GC-MS quantitation of acids and sugars in the hydrolyzates of immunostimulant, water-soluble polysaccharides of basidiomycetes. Chromatogr 47:413–419.

Beck HC, Hansen AM, Lauritsen FR. 2003. Novel pyrazine metabolites found in polymyxin biosynthesis by *Paenibacillus polymyxa*. FEMS Microbiol Let 220:67–73.

Beckonert O, Bollard ME, Ebbels TMD, Keun HC, Antti H, Holmes E, Lindon JC, Nicholson JK. 2003a. NMR-based metabonomic toxicity classification: Hierarchical cluster analysis and k-nearest-neighbour approaches. Anal Chim Acta 490:3–15.

Beckonert O, Monnerjahn K, Bonk U, Leibfritz D. 2003b. Visualizing metabolic changes in breast-cancer tissue using H-1-NMR spectroscopy and self-organizing maps. NMR Biomed 16:1–11.

Bednarek P, Franski R, Kerhoas L, Einhorn J, Wojtaszek P, Stobiecki M. 2001. Profiling changes in metabolism of isoflavonoids and their conjugates in *Lupinus albus* treated with biotic elicitor. Phytochem 56:77–85.

Beek TA. 2002. Chemical analysis of *Ginkgo biloba* leaves and extracts. J Chromatogr A 967:21–55.

Bethin B, Danz H, Hamburger M. 1999. Pressurized liquid extraction of medicinal plants. J Chromatogr A 837:211–219.

Bianco G, Schmitt-Kopplin P, De Benedetto G, Kettrup A, Cataldi TRI. 2002. Determination of glycoalkaloids and relative aglycones by nonaqueous capillary electrophoresis coupled with electrospray ionization-ion trap mass spectrometry. Electrophoresis 23:2904–2912.

Bianco G, Schmitt-Kopplin P, Crescenzi A, Comes S, Kettrup A, Cataldi TRI. 2003. Evaluation of glycoalkaloids in tubers of genetically modified virus Y-resistant potato plants (var. Désirée) by non-aqueous capillary electrophoresis coupled with electrospray ionization mass spectrometry. Anal Bioanal Chem 375:799–804.

Birkemeyer C, Kolasa A, Kopka J. 2003. Comprehensive chemical derivatization for gas chromatography-mass spectrometry-based multi-targeted profiling of the major phytohormones. J Chromatogr A 993:89–102.

Bonafé L, Troxler H, Kuster T, Heizmann CW, Chamoles NA, Burlina AB, Blau N. 2000. Evaluation of urinary acylglycines by electrospray tandem mass spectrometry in mitochondrial energy metabolism defects and organic acidurias. Mol Genet Metab 9:302–311.

Bouchereau A, Guénot P, Larher F. 2000. Analysis of amines in plant materials. J Chromatogr B 747:49–67.

Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, Gaasterland T, Glenisson P, Holstege FC, Kim IF, Markowitz V, Matese JC, Parkinson H, Robinson A, Sarkans U, Schulze-Kremer S, Stewart J, Taylor R, Vilo J, Vingron M. 2001. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet 29:365–371.

Brooks MW, Uden PC. 1995. The determination of abamectin from soil and animal tissue by supercritical fluid extraction and fluorescence detection. Pesticide Sci 43:141–146.

Buchholz A, Takors R, Wandrey C. 2001. Quantification of intracellular metabolites in *Escherichia coli* K12 using liquid chromatographic-electrospray ionization tandem mass spectrometric techniques. Anal Biochem 295:129–137.

Buchholz A, Hurlebaus J, Wandrey C, Takors R. 2002. Metabolomics: Quantification of intracellular metabolite dynamics. Biomol Eng 19: 5–15.

Bundy JG, Spurgeon DJ, Svendsen C, Hankard PK, Osborn D, Lindon JC, Nicholson JK. 2002. Earthworm species of the genus *Eisenia* can be phenotypically differentiated by metabolic profiling. FEBS Let 521: 115–120.

Buziol S, Bashir I, Baumeister A, Claaßen W, Noisommit-Rizzi N, Mailinger W, Reuss M. 2002. New bioreactor-coupled rapid stopped-flow sampling technique for measurements of metabolite dynamics on a subsecond time scale. Biotechnol Bioeng 80:632–636.

Camponovo FF, Wolfender JL, Hostettmann K. 1995. Evaporative light scattering and thermospray mass spectrometry: Two alternative methods for detection and quantitative liquid chromatographic determination of ginkgolides and bilobalide in *Ginkgo biloba* leaf extracts and phytopharmaceuticals. Phytochem Anal 6:141–148.

Castrillo JI, Hayes A, Mohammed S, Gaskell SJ, Oliver SG. 2003. An optimized protocol for metabolome analysis in yeast using direct infusion electrospray mass spectrometry. Phytochem 62:929–937.

Castro MDL, Jiménez-Carmona MM, Fernández-Pérez V. 1999. Towards more rational techniques for the isolation of valuable essential oils from plants. Trends Anal Chem 18:708–716.

Cech NB, Enke CG. 2001. Practical implications of some recent studies in electrospray ionization fundamentals. Mass Spectrom Rev 20:362–387.

Chace DH, DiPerna J, Naylor E. 1999. Laboratory integration and utilization of tandem mass spectrometry in neonatal screening: A model for clinical mass spectrometry in the next millennium. Acta Paediatrica 88:45–47.

Chace DH, Hillman SL, Millington DS, Kahler SG, Adam BW, Levy HL. 1996. Rapid diagnosis of homocystinuria and other hypermethionemias from newborns' blood spots by tandem mass spectrometry. Clin Chem 42:349–355.

Chace DH, Hillman SL, Van Hove JLK, Naylor EW. 1997. Rapid diagnosis of MCAD deficiency: Quantitative analysis of octanoylcarnitine and other acylcarnitines in newborn blood spots by tandem mass spectrometry. Clin Chem 43:2106–2113.

Chace DH, Sherwin JE, Hillman SL, Lorey F, Cunningham GC. 1998. Use of phenylalanine-to-tyrosine ratio determined by tandem mass spectrometry to improve newborn screening for phenylketonurea of early discharge specimens collected in the first 24 h. Clin Chem 44:2405–2409.

Che FY, Song JF, Zeng R, Wang KY, Xia QC. 1999. Analysis of 8-aminonaphthalene-1,3,6-trisulfonate-derivatized oligosaccharides by capillary electrophoresis—Electrospray ionization quadrupole ion trap mass spectrometry. J Chromatogr A 858:229–238.

Chen Z, Landman P, Colmer TD, Adams MA. 1998. Simultaneous analysis of amino and organic acids in extracts of plant leaves as *tert*-butyldimethylsilyl derivatives by capillary gas Chromatography. Anal Biochem 259:203–211.

Chiang WCK, Pusateri DJ, Leitz REA. 1998. Gas chromatography/mass spectrometry method for the determination of Sulforaphane and Sulforaphane nitrile in broccoli. J Agric Food Chem 46:1018–1021.

Christensen B, Nielsen J. 1999. Isotopomer analysis using GC-MS. Metab Eng 1:282–283.

Christensen B, Nielsen J. 2000. Metabolic network analysis: A powerful tool in metabolic engineering. Adv Biochem Eng Biotechnol 66:209–231.

Cogne AL, Queiroz EF, Wolfender JL, Marston A, Mavi S, Hostettmann K. 2003. On-line identification of unstable catalpol derivatives from *Jamesbrittenia fodina* by LC-MS and LC-NMR. Phytochem Anal 14:67–73.

Conboy JJ, Henion J. 1992. High-performance anion-exchange chromatography coupled with mass spectrometry for the determination of carbohydrates. Biol Mass Spectrom 21:397–407.

Conneely A, Nugent A, O'Keeffe M. 2002. Use of solid phase extraction for the isolation and clean up of a derivatized furazolidone metabolite from animal tissues. Analyst 127:705–709.

Cordeiro C, Freire AP. 1996. Methylglyoxal assay in cells as 2-methylquinoxaline using 1,2-diaminobenzene as derivatizing reagent. Anal Biochem 234:221–224.

Cremin P, Donnelly DMX, Wolfender JL, Hostettmann K. 1995. Liquid chromatography-thermospray mass spectrometric analysis of sesquiterpenes of *Armillaria* (Eumycota: Basidiomycotina) species. J Chromatogr A 710:273–285.

Cristoni S, Bernardi LR. 2003. Development of new methodologies for the mass spectrometry study of bioorganic macromolecules. Mass Spectrom Rev 22:369–406.

Cui M, Sun W, Song F, Liu Z, Liu S. 1999. Multi-stage mass spectrometric studies of triterpenoid saponins in crude extracts from *Acanthopanax senticosus* harms. Rapid Commun Mass Spectrom 13:873–879.

Dasgupta A. 1998. Gas chromatography-mass spectrometric identification and quantification of aniline after extraction from serum and derivatization with 2,2,2-trichloroethyl chloroformate, a novel derivative. J Chromatogr B 716:354–358.

Davis SC, Makarov AA, Hughes JD. 1999. Ultrafast gas chromatography using time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 13:237–241.

Demyttenaere JCR, Moriña RM, Sandra P. 2003. Monitoring and fast detection of mycotoxins-producing fungi based on headspace solid-phase microextraction and headspace sorptive extraction of the volatile metabolites. J Chromatogr A 985:127–135.

Des Rosiers C, Lloyd S, Comte B, Chatham JC. 2004. A critical perspective of the use of ^{13}C-isotopomer analysis by GCMS and NMR as applied to cardiac metabolism. Metab Eng 6:44–58.

Driedger DR, LeBlanc RJ, LeBlanc E, Sporns P. 2000. A capillary electrophoresis laser-induced fluorescence method for analysis of potato glycoalkaloids based on a solution-phase immunoassay. 1. Separation and quantification of immunoassay products. J Agr Food Chem 48:1135–1139.

Duteil S, Gareil P, Girault S, Mallet A, Feve C, Siret L. 1999. Identification of heparin oligosaccharides by direct coupling of capillary electrophoresis/ion spray-mass spectrometry. Rapid Commun Mass Spectrom 13:1889–1898.

El Rassi Z. 1999. Recent developments in capillary electrophoresis and capillary electrochromatography of carbohydrate species. Electrophoresis 20:3134–3144.

Elgstoen KBP, Jellum E. 1997. Capillary electrophoresis for diagnosis of metabolic disease. Electrophoresis 18:1857–1860.

Elgstoen KBP, Zhao JY, Anacleto JF, Jellum E. 2001. Potential of capillary electrophoresis, tandem mass spectrometry and coupled capillary electrophoresis—Tandem mass spectrometry as diagnostic tools. J Chromatogr A 914:265–275.

Entian KD, Zimmermann FK, Scheel I. 1977. A partial defect in carbon catabolite repression mutants of *Saccharomyces cerevisiae* with reduced hexose phosphorylation. Mol Gen Genet 156:99–105.

Favretto D, Piovan A, Filippini R, Caniato R. 2001. Monitoring the production yields of vincristine and vinblastine in *Catharanthus roseus* from somatic embryogenesis. Semiquantitative determination by flow-injection electrospray ionization mass spectrometry. Rapid Commun Mass Spectrom 15:364–369.

Ferreira V, Fernández P, Meléndez J, Cacho J. 1995. Analytical characteristics of sample evaporation with the micro-Kuderna-Danish concentrator. J Chromatogr A 695:41–55.

Feurle J, Jomaa H, Wilhelm M, Gutsche B, Herderich M. 1998. Analysis of phosphorylated carbohydrates by high-performance liquid chromatography-electrospray ionization tandem mass spectrometry utilizing a β-cyclodextrin bonded stationary phase. J Chromatogr 803:111–119.

Fiehn O. 2001. Combining genomics, metabolome analysis, and biochemical modeling to understand metabolic networks. Com Funct Genom 2: 155–168.

Fiehn O. 2002. Metabolomics—The link between genotypes and phenotypes. Plant Mol Biol 48:155–171.

Fiehn O, Kopka J, Dormann P, Altmann T, Trethewey RN, Willmitzer L. 2000. Metabolite profiling for plant functional genomics. Nat Biotechnol 18:1157–1161.

Folch J, Lees M, Stanley GH. 1957. A simple method for the isolation and purification of total lipids from animal tissue. Biol Chem 226:497–509.

Förster J. 2002. Metabolite profiling of single deletion mutants of *Saccharomyces cerevisiae*. In: Förster J, editor. Pathway analysis of the metabolic network of *Saccharomyces cerevisiae*. Kongens Lyngby: CPB-BioCentrum, Technical University of Denmark (Ph.D. thesis). pp 51–62.

Förster J, Gombert AK, Nielsen J. 2002. A functional genomics approach using metabolomics and in silico pathway analysis. Biotechnol Bioeng 79:703–712.

Förster J, Famili I, Fu P, Palsson BØ, Nielsen J. 2003. Genome-scale reconstruction of the *Saccharomyces cerevisiae* metabolic network. Genome Res 13:244–253.

Fox KF, Wunschel DS, Fox A, Stewart GC. 1998. Complementarities of GC-MS and LC-MS analyses for determination of carbohydrate profiles of vegetative cells and spores of bacilli. J Microbiol Met 33:1–11.

Frisvad JC, Filtenborg O. 1983. Classification of terverticillate penicillia based on profiles of mycotoxins and other secondary metabolites. App Environ Microbiol 46:1301–1310.

Gao P, Korley F, Martin J, Chen BT. 2002. Determination of unique microbial volatile organic compounds produced by five *Aspergillus* species commonly found in problem buildings. AIHA J 63:135–140.

Gates SC, Sweeley CC. 1978. Quantitative metabolic profiling based on gas chromatography. Clin Chem 24:1663–1673.

Gates PJ, Kearney GC, Jones R, Leadlay PF, Staunton J. 1999. Structural elucidation studies of erythromycins by electrospray tandem mass spectrometry. Rapid Commun Mass Spectrom 13:242–246.

Gharaibeh AA, Voorhees KJ. 1996. Characterization of lipid fatty acids in whole-cell microorganisms using *in situ* supercritical fluid derivatization/extraction and gas Chromatography/mass spectrometry. Anal Chem 68:2805–2810.

Godovac-Zimmermann J, Brown LR. 2001. Perspectives for mass spectrometry and functional proteomics. Mass Spectrom Rev 20: 1–57.

Gombert AK, Moreira SM, Christensen B, Nielsen J. 2001. Network identification and flux quantification in the central metabolism of *Saccharomyces cerevisiae* under different conditions of glucose repression. J Bacteriol 183:1441–1451.

Gonzalez B, François J, Renaud M. 1997. A rapid and reliable method for metabolite extraction in yeast using boiling buffered ethanol. Yeast 13: 1347–1356.

Goodacre R, Vaidyanathan S, Bianchi G, Kell DB. 2002. Metabolic profiling using direct infusion electrospray ionization mass spectrometry for the characterization of olive oils. Analyst 127:1457–1462.

Goodacre R, York EV, Heald JK, Scott IM. 2003. Chemometric discrimination of unfractionated plant extracts analyzed by electrospray mass spectrometry. Phytochem 62:859–863.

Grob K. 1993. Split and splitless injection in capillary GC. Heidelberg: Hüthig. 571p.

Grob K. 2001. Split and splitless injection for quantitative gas chromatography: Concepts, processes, practical guidelines, and sources of error. Wiley-VCH. 480p.

Gygi SP, Rochon Y, Franza BR, Aebersold R. 1999. Gene expression: Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 19:1720–1730.

Hagberg J. 2003. Analysis of low-molecular-mass organic acids using capillary zone electrophoresis–electrospray ionization mass spectrometry. J Chromatogr A 988:127–133.

Hajjaj H, Blanc PJ, Goma G, François J. 1998. Sampling techniques and comparative extraction procedures for quantitative determination of intra- and extracellular metabolites in filamentous fungi. FEMS Microbiol Let 164:195–200.

Halket JM, Przyborowska A, Stein SE, Mallard WG, Down S, Chalmers RA. 1999. Deconvolution gas chromatography/mass spectrometry of urinary organic acids—Potential pattern recognition and automated identification of metabolic disorders. Rapid Commun Mass Spectrom 13:279–284.

Hans MA, Heinzle E, Wittmann C. 2001. Quantification of intracellular amino acids in batch cultures of *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 56:776–779.

He T, Quinn D, Fu E, Wang YK. 1999. Analysis of diagnostic metabolites by capillary electrophoresis–mass spectrometry. J Chromatogr B 727: 43–52.

Hellerstein MK. 2004. New stable isotope-mass spectrometric techniques for measuring fluxes through intact metabolic pathways in mammalian systems: Introduction of moving pictures into functional genomics and biochemical phenotyping. Metab Eng 6:85–100.

Higgs RE, Zahn JA, Gygi JD, Hilton MD. 2001. Rapid method to estimate the presence of secondary metabolites in microbial extracts. Appl Environ Microbiol 67:371–376.

Horning EC, Horning MG. 1971. Human metabolic profiles obtained by GC and GC/MS. J Chromatogr Sci 9:129–140.

Huhman DV, Sumner LW. 2002. Metabolic profiling of saponins in *Medicago sativa* and *Medicago truncatula* using HPLC coupled to an electrospray ion-trap mass spectrometer. Phytochem 59:347–360.

Hušek P. 1992. Fast derivatization with chloroformates for gas chromatographic analysis. LC GC Intl 5:43–49.

Hušek P. 1998. Chloroformates in gas chromatography as general purpose derivatizing agents. J Chromatogr B 717:57–91.

Ideker T, Ozier O, Schwikowski B, Siegel AF. 2002. Discovering regulatory and signaling circuits in molecular interaction networks. Bioinform 18(Suppl 1):S233–S240.

Inoue K, Suzuki Y, Yajima S, Shimozawa N, Orii T, Kondo N. 1997. Very long chain fatty acid analysis of dried blood spots on filter paper to screen for adrenoleukodystrophy. Clin Chem 43:2197–2198.

Ito T, van Kuilenburg ABP, Bootsma AH, Haasnoot AJ, van Cruchten A, Wada Y, van Gennip AH. 2000. Rapid screening of high-risk patients for disorders of purine and pyrimidine metabolism using HPLC-electrospray tandem mass spectrometry of liquid urine or urine-soaked filter paper strips. Clin Chem 46:445–452.

Jackson P. 1990. The use of polyacrylamide-gel electrophoresis for the high-resolution separation of reducing saccharides labeled with the fluorophore 8-aminonaphthalene-1,3,6-trisulphonic acid. Detection of picomolar quantities by an imaging system based on a cooled charge-coupled device. Biochem J 270(3):705–713.

Jacobson GB, Moulder R, Lu L, Bergström M, Markides KE, Långström B. 1997. Supercritical fluid extraction of ^{11}C-labeled metabolites in tissue using supercritical ammonia. Anal Chem 69:275–280.

Jensen NBS, Jokumsen KV, Villadsen J. 1999. Determination of the phosporylated sugars of the Embden-Meyerhoff-Parnas pathway in *Lactococcus lactis* using a fast sampling technique and solid phase extraction. Biotechnol Bioeng 63:357–362.

Jensen AG, Ndjoko K, Wolfender JL, Hostettmann K, Camponovo F, Soldati F. 2002. Liquid chromatography-atmospheric pressure chemical ionization/mass spectrometry: A rapid and selective method for the quantitative determination of ginkgolides and bilobalide in Ginkgo leaf extracts and phytopharmaceuticals. Phytochem Anal 13:31–38.

Johnson DW. 2000. A rapid screening procedure for the diagnosis of peroxisomal disorders: Quantification of very long-chain fatty acids, as dimethylaminoethyl esters, in plasma and blood spots, by electrospray tandem mass spectrometry. J Inherit Metab Dis 23:475–486.

Johnson DW. 2001. Analysis of amino acids as formamidene butyl esters by electrospray ionization tandem mass spectrometry. Rapid Commun Mass Spectrom 15:2198–2205.

Johnson SK, Houk LL, Johnson DC, Houk RS. 1999. Determination of small carboxylic acids by capillary electrophoresis with electrospray-mass spectrometry. Anal Chim Acta 389:1–8.

Jolliffe IT. 1986. Principal component analysis. Springer-Verlag. 271p.

Katona ZsF, Sass P, Molnár-Perl I. 1999. Simultaneous determination of sugars, sugar alcohols, acids and amino acids in apricots by gas chromatography-mass spectrometry. J Chromatogr A 847:91–102.

Kell DB, Darby RM, Draper J. 2001. Genomic computing: Explanatory analysis of plant expression profiling data using machine learning. Plant Physiol 126:943–951.

Koning W, van Dam K. 1992. A method for the determination of changes of glycolytic metabolites in yeast on a subsecond time scale using extraction at neutral pH. Anal Biochem 204:118–123.

Kopka J, Ohlrogge JB, Jaworski JG. 1995. Analysis of *in vivo* levels of acyl-thioesters with gas Chromatography/mass spectrometry of the butylamide derivative. Anal Biochem 224:51–60.

Kubec R, Svobodová M, Velíšek J. 1999. Gas chromatographic determination of *S*-alk(en)ylcysteine sulfoxides. J Chromatogr A 862:85–94.

Kühn AV, Rüttinger HH, Neubert RHH, Raith K. 2003. Identification of hyaluronic acid oligosaccharides by direct coupling of capillary electrophoresis with electrospray ion trap mass spectrometry. Rapid Commun Mass Spectrom 17:576–582.

Lange HC, Eman M, van Zuijlen G, Visser D, van Dam JC, Frank J, Teixeira de Mattos MJ, Heijnen JJ. 2001. Improved rapid sampling for in vivo kinetics of intracellular metabolites in *Saccharomyces cerevisiae*. Biotechnol Bioeng 75:406–415.

Larsen TO, Svendsen A, Smedsgaard J. 2001. Biochemical characterization of ochratoxin A-producing strains of the genus *Penicillium*. Appl Environ Microbiol 67:3630–3635.

Larsen TO, Frisvad JC. 1995. Characterization of volatile metabolites from 47 *Penicillium* taxa. Mycol Res 99:1153–1166.

Larsson M, Sundberg R, Folestad S. 2001. On-line capillary electrophoresis with mass spectrometry detection for the analysis of carbohydrates after derivatization with 8-aminonaphthalene-1,3,6-trisulfonic acid. J Chromatogr A 934:75–85.

Larsson G, Törnkvist M. 1996. Rapid sampling, cell inactivation and evaluation of low extracellular glucose concentrations during fed-batch cultivation. J Biotechnol 49:69–82.

Le Belle JE, Harris NG, Williams SR, Bhakoo KK. 2002. A comparison of cell and tissue extraction techniques using high-resolution ^{1}H-NMR spectrometry. NMR Biomed 15:37–44.

Letisse F, Lindley ND. 2000. An intracellular metabolite quantification technique applicable to polysaccharide-producing bacteria. Biotechnol Let 22:1673–1677.

Lim GB, Lee SY, Lee EK, Haam SJ, Kim WS. 2002. Separation of astaxanthin from red yeast *Phaffia rhodozyma* by supercritical carbon dioxide extraction. Biochem Eng J 11:181–187.

Lord H, Pawliszyn J. 2000. Evolution of solid-phase microextraction technology. J Chromatogr A 885:153–193.

Maharjan RP, Ferenci T. 2003. Global metabolite analysis: The influence of extraction methodology on metabolome profiles of *Escherichia coli*. Anal Biochem 313:145–154.

Maraschin M, Sugui JA, Wood KV, Bonham C, Lanças FM, Araujo PS, Yunes RA, Verpoorte R, Fontana JD. 2001. Supercritical CO_2 extraction of velunitol *A* from *Mandevilla velutina*(*Apocynaceae*) cultured cells and MALDI-TOF MS analysis. Biotech Let 23:77–82.

Martins AMTBS, Cordeiro CAA, Freire AMJP. 2001. *In situ* analysis of Methylglyoxal metabolism in *Saccharomyces cerevisiae*. FEBS Let 499:41–44.

Mashego MR, van Gulik WM, Vinke JL, Heijnen JJ. 2003. Critical evaluation of sampling techniques for residual glucose determination in carbon-limited chemostat culture of *Saccharomyces cerevisiae*. Biotechnol Bioeng 83:395–399.

Masson F, Hinrichsen L, Talon R, Montel MC. 1999. Factors influencing leucine catabolism by a strain of *Staphylococcus carnosus*. Int J Food Microbiol 49:173–178.

Mellon FA, Bennett RN, Holst B, Williamson G. 2002. Intact glucosinolate analysis in plant extracts by programmed cone voltage electrospray LC/MS: Performance and comparison with LC/MS/MS methods. Anal Biochem 306:83–91.

Mendes P. 2002. Emerging bioinformatics for the metabolome. Brief Bioinform 3:134–145.

Moini M. 2001. Design and performance of a universal sheathless capillary electrophoresis to mass spectrometry interface using a split-flow technique. Anal Chem 73:3497–3501.

Molnár-Perl I, Horváth K, Bartha R. 1998. GC-MS quantitation of benzoic and aralkyl carboxylic acids as their trimethylsilyl derivatives: In model solutions I. Chromatogr 48:101–110.

Moser HW, Moser AB. 1991. Measurement of saturated very long chain fatty acids in plasma. In: Hommes FA, editor. Techniques in diagnostic human biochemical genetics. New York: Wiley-Liss. pp 177–191.

Mougous JD, Leavell MD, Senaratne RH, Leigh CD, Williams SJ, Riley LW, Leary JA, Bertozzi CR. 2002. Discovery of sulfated metabolites in mycobacteria with a genetic and mass spectrometric approach. Proc Natl Acad Sci USA 99:17037–17042.

Mueller P, Schulze A, Schindler I, Ethofer T, Buehrdel P, Ceglarek U. 2003. Validation of an ESI-MS/MS screening method for acylcarnitine profiling in urine specimens of neonates, children, adolescents and adults. Clin Chim Acta 327:47–57.

Murga R, Ruiz R, Beltrán S, Cabezas JL. 2000. Extraction of natural complex phenols and tannins from grape seeds by using supercritical mixtures of carbon dioxide and alcohol. J Agric Food Chem 48:3408–3412.

Nagy K, Takats Z, Pollreisz F, Szabo T, Vekey K. 2003. Direct tandem mass spectrometric analysis of amino acids in dried blood spots without chemical derivatization for neonatal screening. Rapid Commun Mass Spectrom 17:983–990.

Namieśnik J, Górecki T. 2000. Sample preparation for Chromatographic analysis of plant material. J Planar Chromatogr 13:404–413.

Nielsen J. 2001. Metabolic Engineering. Appl Microbiol Biotechnol 55:883–887.

Nielsen J. 2003. It is all about metabolic fluxes. J Bacteriol 185:7031–7035.

Nielsen KF, Smedsgaard J. 2003. Fungal metabolite screening: Database of 474 mycotoxins and fungal metabolites for dereplication by standardized liquid chromatography-UV-mass spectrometry methodology. J Chromatogr A 1002:111–136.

Nielsen KF, Thrane U. 2001. Fast methods for screening of trichothecenes in fungal cultures using gas chromatography-tandem mass spectrometry. J Chromatogr A 929:75–87.

Nielsen KF, Smedsgaard J, Larsen TO, Lund F, Thrane U, Frisvad JC. 2003. Chemical identification of fungi: metabolite profiling and metabolomics. In: Arora DK, editor. Fungal Biotechnology in Agricultural, Food, and Environmental Applications. New York: Marcel Dekker, p. 19–35.

Niessen WM. 1999. State-of-the-art in liquid chromatography-mass spectrometry. J Chromatogr A 856:179–197.

Nilsson T, Larsen TO, Montanarella L, Madsen JØ. 1996. Application of headspace solid-phase microextraction for the analysis of volatile metabolites emitted by *Penicillium* species. J Microbiol Methods 25: 245–255.

Nishijima M, Araki-Sakai M, Sano H. 1997. Identification of isoprenoid quinones by frit-FAB liquid chromatography-mass spectrometry for the chemotaxonomy of microorganisms. J Microbiol Met 28:113–122.

Niwa T. 1986. Metabolic profiling with gas chromatography-mass spectrometry and its application to clinical medicine. J Chromatogr 379:313–345.

Novák O, Tarkowski P, Tarkowská D, Doležal K, Lenobel R, Strnad M. 2003. Quantitative analysis of cytokinins in plants by liquid chromatography–single-quadrupole mass spectrometry. Anal Chim Acta 480:207–218.

Oliver SG. 1997. Yeast as a navigational aid in genome analysis. Microbiol 7:405–409.

Oliver SG, Winson MK, Kell DB, Baganz F. 1998. Systematic functional analysis of the yeast genome. TIBTECH 16:373–378.

Papac DI, Wong A, Jones AJS. 1996. Analysis of acidic oligosaccharides and glycopeptides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal Chem 68:3215–3223.

Peláez MV, Bayón MM, Alonso JIG, Sanz-Medel A. 2000. A comparison of different derivatization approaches for the determination of selenomethionine by GC-ICP-MS. J Anal At Spectrom 15:1217–1222.

Pietrogrande MC, Rossi D, Paganetto G. 2003. Gas chromatography-mass spectrometry analysis of di(2-ethylhexyl) phthalate and its metabolites in hepatic microsomal incubations. Analytica Chimica Acta 480:1–10.

Piovan A, Filippini R, Favretto D. 1998. Characterization of the anthocyanins of *Catharanthus roseus* (L.) G. Don in vivo and in vitro by electrospray ionization ion trap mass spectrometry. Rapid Commun Mass Spectrom 12:361–367.

Prinsen E, van Dongen W, Esmans EL, van Onckelen HA. 1997. HPLC linked electrospray tandem mass spectrometry: A rapid and reliable method to analyze indole-3-acetic acid metabolism in bacteria. J Mass Spectrom 32:12–22.

Prinsen E, van Dongen W, Esmans EL, van Onckelen HA. 1998. Micro and capillary liquid chromatography-tandem mass spectrometry: A new dimension in phytohormone research. J Chromatogr 826:25–37.

Qu J, Wang Y, Luo G, Wu Z, Yang C. 2002. Validated quantitation of underivatized amino acids in human blood samples by volatile ion-pair reversed-phase liquid chromatography coupled to isotope dilution tandem mass spectrometry. Anal Chem 74:2034–2040.

Que AH, Novotny MV. 2003. Structural characterization of neutral oligosaccharide mixtures through a combination of capillary electrochromatography and ion trap tandem mass spectrometry. Anal Bioanal Chem 375:599–608.

Queiroz EF, Wolfender JL, Atindehou KK, Traore D, Hostettmann K. 2002. On-line identification of the antifungal constituents of *Erythrina vogelii* by liquid chromatography with tandem mass spectrometry, ultraviolet absorbance detection and nuclear magnetic resonance spectrometry combined with liquid chromatographic micro-fractionation. J Chromatogr A 974:123–134.

Raamsdonk LM, Teusink B, Broadhurst D, Zhang NS, Hayes A, Walsh MC, Berden JA, Brindle KM, Kell DB, Rowland JJ, Westerhoff HV, van Dam K, Oliver SG. 2001. A functional genomics strategy that uses metabolome data to reveal the phenotype of silent mutations. Nat Biotechnol 19:45–50.

Rashed MS. 2001. Clinical applications of tandem mass spectrometry: Ten years of diagnosis and screening for inherited metabolic diseases. J Chromatogr B 758:27–48.

Rashed MS, Ozand PT, Bucknall MP, Little D. 1995. Diagnosis of inborn errors of metabolism from blood spots by acylcarnitines and amino acids profiling using automated electrospray tandem mass spectrometry. Pediatr Res 38:324–331.

Rashed MS, Bucknall MP, Little D, Awad A, Jacob M, Alamoudi M, Alwattar M, Ozand PT. 1997. Screening blood spots for inborn errors of metabolism by electrospray tandem mass spectrometry with a microbatch process and a computer algorithm for automated flagging of abnormal profiles. Clin Chem 43:1129–1141.

Reverchon E. 1997. Supercritical fluid extraction and fractionation of essential oils and related products. J Supercritical Fluids 10:1–37.

Roessner U, Wagner C, Kopka J, Trethewey RN, Willmitzer L. 2000. Simultaneous analysis of metabolites in potato tuber by gas Chromatography-mass spectrometry. Plant J 23:131–142.

Roessner U, Luedemann A, Brust D, Fiehn O, Linke T, Willmitzer L, Fernie AR. 2001. Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified plant systems. Plant Cell 13: 11–29.

Ruijter GJG, Visser J. 1996. Determination of intermediary metabolites in *Aspergillus niger*. J Microbiol Methods 25:295–302.

Sargenti SR, Lanças FM. 1997. Supercritical fluid extraction of *Cymbopogon citrates*(DC) Stapf. Chromatogr 46:285–290.

Sargenti SR, Vichnewski W. 2000. Sonication and liquid Chromatography as a rapid technique for extraction and fractionation of plant material. Phytochem Anal 11:69–73.

Sass JO, Endres W. 1997. Quantitation of total homocysteine in human plasma by derivatization to its N(O,S)-propoxycarbonyl propyl ester and gas chromatography-mass spectrometry analysis. J Chromatogr A 776:342–347.

Savard ME, Miller JD, Blais LA, Seifert KA, Samson RA. 1994. Secondary metabolites of *Penicillium bilaii*strain PB-50. Mycopathol 127: 19–27.

Schaefer U, Boos W, Takors R, Weuster-Botz D. 1999. Automated sampling device for monitoring intracellular metabolite dynamics. Anal Biochem 270:88–96.

Schaller F, Wolfender JL, Hostettmann K, Mavi S. 2001. New antifungal 'quinone methide' diterpenes from *Bobgunnia madagascariensis* and study of their interconversion by LC/NMR. Helv Chim Acta 84:222–229.

Schultz CL, Moini M. 2003. Analysis of underivatized amino acids and their D/L-enantiomers by sheathless capillary electrophoresis/electrospray ionization-mass spectrometry. Anal Chem 75:1508–1513.

Schulze A, Lindner M, Kohlmuller D, Olgemoller K, Mayatepek E, Hoffmann GF. 2003. Expanded newborn screening for inborn errors of metabolism by electrospray ionization-tandem mass spectrometry: Results, outcome, and implications. Pediatrics 111:1399–1406.

Schwab W. 2003. Metabolome diversity: Too few genes, too many metabolites? Phytochem 62:837–849.

Shigematsu Y, Hata I, Kikawa Y, Mayumi M, Tanaka Y, Sudo M, Kado N. 1999. Modifications in electrospray tandem mass spectrometry for a neonatal-screening pilot study in Japan. J Chromatogr B 731:97–103.

Shockcor JP, Unger SE, Wilson ID, Foxall PJD, Nicholson JK, Lindon JC. 1996. Combined HPLC, NMR spectroscopy, and ion-trap mass spectrometry with application to the detection and characterization of xenobiotic and endogenous metabolites in human urine. Anal Chem 68:4431–4435.

Shryock JC, Rubio R, Berne RM. 1986. Extraction of adenine nucleotides from cultured endothelial cells. Anal Biochem 159:73–81.

Simpson RC, Fenselau CC, Hardy MR, Townsend RR, Lee YC, Cotter RJ. 1990. Adaptation of a thermospray liquid chromatography/mass spectrometry interface for use with alkaline anion exchange liquid chromatography of carbohydrates. Anal Chem 62:248–252.

Smedsgaard J. 1997. Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. J Chromatogr A 760:264–270.

Smedsgaard J, Frisvad JC. 1996. Using direct electrospray mass spectrometry in taxonomy and secondary metabolite profiling of crude fungal extracts. J Microbiol Met 25:5–17.

Smedsgaard J, Frisvad JC. 1997. Terverticillate penicillia studied by direct electrospray mass spectrometric profiling of crude extracts. I. Chemosystematics. Biochem Syst Ecol 25:51–64.

Smith RM. 2003 . Before the injection—Modern methods of sample preparation for separation techniques. J Chromatogr A 1000:3–27.

Smits HP, Cohen A, Buttler T, Nielsen J, Olsson L. 1998. Cleanup and analysis of sugar phosphates in biological extracts by using solid-phase extraction and anion-exchange Chromatography with pulsed amperometric detection. Anal Biochem 261:36–42.

Soga T, Heiger DN. 2000. Amino acid analysis by capillary electrophoresis electrospray ionization mass spectrometry. Anal Chem 72:1236–1241.

Soga T, Imaizumi M. 2001. Capillary electrophoresis method for the analysis of inorganic anions, organic acids, amino acids, nucleotides, carbohydrates and other anionic compounds. Electrophoresis 22:3418–3425.

Soga T, Ross GA. 1999. Simultaneous determination of inorganic anions, organic acids, amino acids and carbohydrates by capillary electrophoresis. J Chromatogr A 837:231–239.

Soga T, Ueno Y, Naraoka H, Ohashi Y, Tomita M, Nishioka T. 2002a. Simultaneous determination of anionic intermediates for *Bacillus subtilis* metabolic pathways by capillary electrophoresis electrospray ionization mass spectrometry. Anal Chem 74:2233–2239.

Soga T, Ueno Y, Naraoka H, Matsuda K, Tomita M, Nishioka T. 2002b. Pressure-assisted capillary electrophoresis electrospray ionization mass spectrometry for analysis of multivalent anions. Anal Chem 74:6224–6229.

Southwell IA, Russell MF, Maddox CDA, Wheeler GS. 2003. Differential metabolism of 1,8-cineole in insects. J Chem Ecol 29:83–94.

Starmans DAJ, Nijhuis HH. 1996. Extraction of secondary metabolites from plant material: A review. Trends Food Sci Technol 7:191–197.

Stein SE. 1999. An integrated method for spectrum extraction and compound identification from gas chromatography/mass spectrometry. J Am Soc Mass Spectrom 10:770–781.

Steuer R, Kurths J, Fiehn O, Weckwerth W. 2003. Observing and interpreting correlations in metabolomic networks. Bioinform 19:1019–1026.

Stolker AAM, Zoontjes PW, Schwillens PLWJ, Kootstra PR, van Ginkel LA, Stephany RW, Brinkman UATh. 2002. Determination of acetyl gestagenic steroids in kidney fat by automated supercritical fluid extr-

action and liquid Chromatography ion-trap mass spectrometry. Analyst 127:748–754.

Stout SJ, daCunha AR, Picard GL, Safarpour MM. 1996. Microwave-assisted extraction coupled with liquid Chromatography/electrospray ionization mass spectrometry for the simplified determination of imidazolinone herbicides and their metabolites in plant tissue. J Agric Food Chem 44:3548–3553.

Sumner LW, Mendes P, Dixon RA. 2003. Plant metabolomics: Large-scale phytochemistry in the functional genomics era. Phytochem 62:817–836.

Suzuki H, Muller O, Guttman A, Karger BL. 1997. Analysis of 1-aminopyrene-3,6,8-trisulfonate-derivatized oligosaccharides by capillary electrophoresis with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal Chem 69:4554–4559.

Taylor CF, Paton NW, Garwood KL, Kirby PD, Stead DA, Yin Z, Deutsch EW, Selway L, Walker J, Riba-Garcia I, Mohammed S, Deery MJ, Howard JA, Dunkley T, Aebersold R, Kell DB, Lilley KS, Roepstorff P, Yates JR, Brass A, Brown AJ, Cash P, Gaskell SJ, Hubbard SJ, Oliver SG. 2003. A systematic approach to modeling, capturing, and disseminating proteomics experimental data. Nat Biotechnol 21:247–254.

Theobald U, Mailinger W, Baltes M, Rizzi M, Reuss M. 1997 . *In vivo* analysis of metabolic dynamics in *Saccharomyces cerevisiae*: I. Experimental observations. Biotechnol Bioeng 55:305–316.

Tienpont B, David F, Desmet K, Sandra P. 2002. Stir bar sorptive extraction-thermal desorption-capillary GC-MS applied to biological fluids. Anal Bioanal Chem 373:46–55.

Tolstikov VV, Fiehn O. 2002 . Analysis of highly polar compounds of plant origin: Combination of hydrophilic interaction chromatography and electrospray ion trap mass spectrometry. Anal Biochem 301:298–307.

Tomás-Barberán FA. 1995. Capillary electrophoresis: A new technique in the analysis of plant secondary metabolites. Phytochem Anal 6:177–192.

Tomer KB. 2001 . Separations combined with mass spectrometry. Chem Rev 101:297–328.

Torto N, Hofte A, van der Hoeven R, Tjaden U, Gorton L, Marko-Varga G, Bruggink C, van der Greef J. 1998. Microdialysis introduction high-performance anion-exchange chromatography/ion-spray mass spectrometry for monitoring of on-line desalted carbohydrate hydrolysates. J Mass Spectrom 33:334–341.

Trethewey RN. 2001. Gene discovery via metabolic profiling. Cur Opinion Biotechnol 12:135–138.

Tweeddale H, Notley-Mcrobb L, Ferenci T. 1998. Effect of slow growth on metabolism of *Escherichia coli*, as revealed by global metabolite pool (''metabolome'') analysis. J Bacteriol 180:5109–5116.

Unger M, Stöckigt D, Belder D, Stöckigt J. 1997. General approach for the analysis of various alkaloid classes using capillary electrophoresis and capillary electrophoresis-mass spectrometry. J Chromatogr A 767:273–276.

Vaher M, Koel M. 2003. Separation of polyphenolic compounds extracted from plant matrices using capillary electrophoresis. J Chromatogr A 990:225–230.

Valianpour F, Selhorst JJM, van Lint LEM, van Gennip AH, Wanders RJA, Kemp S. 2003. Analysis of very long-chain fatty acids using electrospray ionization mass spectrometry. Mol Genet Metab 79:189–196.

Vallance H, Applegarth D. 1994. An improved method for quantification of very long chain fatty acids in plasma. Clin Biochem 27:183–186.

van Dam JC, Eman MR, Frank J, Lange HC, van Dedem GWK, Heijnen SJ. 2002. Analysis of glycolytic intermediates in *Saccharomyces cerevisiae* using anion exchange chromatography and electrospray ionization with tandem mass spectrometric detection. Anal Chim Acta 460:209–218.

van Rhijn JA, Heskamp HH, Davelaar E, Jordi W, Leloux MS, Brinkman UAT. 2001. Quantitative determination of glycosylated and aglycon isoprenoid cytokinins at sub-picomolar levels by microcolumn liquid chromatography combined with electrospray tandem mass spectrometry. J Chromatogr 929:31–42.

Vaseghi S, Baumeister A, Rizzi M, Reuss M. 1999. *In vivo* dynamics of pentose phosphate pathway in *Saccharomyces cerevisiae*. Metabolic Eng 1:128–140.

Vergnais L, Masson F, Montel MC, Berdagué JL, Talon R. 1998. Evaluation of solid-phase microextraction for analysis of volatile metabolites produced by Staphylococci. J Agric Food Chem 46:228–234.

Villas-Bôas SG, Delicado DG, Åkesson M, Nielsen J. 2003. Simultaneous analysis of amino and nonamino organic acids as methyl chloroformate derivatives using gas Chromatography-mass spectrometry. Anal Biochem 322:134–138.

Viseux N, de Hoffmann E, Domon B. 1998. Structural assignment of permethylated oligosaccharide subunits using sequential tandem mass spectrometry. Anal Chem 70:4951–4959.

Visser D, van Zuylen GA, van Dam JC, Oudshoorn A, Eman MR, Ras C, van Gulik WM, Frank J, van Dedem GWK, Heijnen JJ. 2002. Rapid sampling for analysis of *in vivo* kinetics using the BioScope: A system for continuous-pulse experiments. Biotechnol Bioeng 79:674–681.

Vreken P, van Lint AEM, Bootsma AH, Overmars H, Wanders RJA, van Gennip AH. 1998. Rapid stable isotope dilution analysis of very-long-chain fatty acids, pristanic acid and phytanic acid using gas chromatography–electron impact mass spectrometry. J Chromatogr B 713: 281–287.

Vreken P, van Lint AEM, Bootsma AH, Overmars H, Wanders RJA, van Gennip AH. 1999. Quantitative plasma acylcarnitine analysis using electrospray tandem mass spectrometry for the diagnosis of organic acidaemias and fatty acid oxidation defects. J Inherit Metab Dis 22: 302–306.

Vuorensola K, Kokkonen J, Sirén H, Ketola RA. 2001. Optimization of capillary electrophoretic-electrospray ionization-mass spectrometric analysis of catecholamines. Electrophoresis 22:4347–4354.

Waridel P, Wolfender JL, Ndjoko K, Hobby KR, Major HJ, Hostettmann K. 2001. Evaluation of quadrupole time-of-flight tandem mass spectrometry and ion trap multiple-stage mass spectrometry for the differentiation of C-glycosidic flavonoid isomers. J Chromatogr 926:29–41.

Wehr T. 2003. Coupling liquid-phase separations and MALDI-MS. LC GC N AM 10:974–982.

Wells RJ. 1999. Recent advances in non-silylation derivatization techniques for gas chromatography. J Chromatogr A 843:1–18.

Weuster-Botz D. 1997. Sampling tube device for monitoring intracellular metabolite dynamics. Anal Biochem 246:225–233.

Wilcken B, Wiley V, Hammond J, Carpenter K. 2003. Screening newborns for inborn errors of metabolism by tandem mass spectrometry. N Engl J Med 348:2304–2312.

Witters E, Vanhoutte K, Dewitte W, Machackova I, Benkova E, van Dongen W, Esmans EL, van Onckelen HA. 1999. Analysis of Cyclic Nucleotides and Cytokinins in Minute Plant Samples using Phase-System Switching Capillary Electrospray-Liquid Chromatography-Tandem Mass Spectrometry. Phytochem Anal 10:143–151.

Wittmann C. 2002. Metabolic flux analysis using mass spectrometry. Adv Biochem Eng Biotechnol 74:39–64.

Wittmann C, Heinzle E. 2001a. MALDI-TOF MS for quantification of substrates and products in cultivations of *Corynebacterium glutamicum*. Biotechnol Bioeng 72:642–647.

Wittmann C, Heinzle E. 2001b. Application of MALDI-TOF MS to lysine-producing *Corynebacterium glutamicum*: A novel approach for metabolic flux analysis. Eur J Biochem 268:2441–2455.

Wittmann C, Krömer JO, Kiefer P, Binz T, Heinzle E. 2004. Impact of the cold shock phenomenon on quantification of intracellular metabolites in bacteria. Anal Biochem 327:135–139.

Wolfender JL, Ndjoko K, Hostettmann K. 2003. Liquid chromatography with ultraviolet absorbance—Mass spectrometric detection and with nuclear magnetic resonance spectroscopy: A powerful combination for the on-line structural investigation of plant metabolites. J Chromatogr A 1000: 437–455.

Wolfender JL, Rodriguez S, Hostettmann K. 1998. Liquid chromatography coupled to mass spectrometry and nuclear magnetic resonance spectroscopy for the screening of plant constituents. J Chromatogr A 794:299–316.

Wolfender JL, Rodriguez S, Hostettmann K, Wagner-Redeker W. 1995. Comparison of Liquid chromatography/electrospray, atmospheric pressure chemical ionization, thermospray and continuous-flow fast atom bombardment mass spectrometry for the determination of secondary metabolites in crude plant extracts. J Mass Spectrom 30: S35–S36.

Wu JT, Zeng H, Deng Y, Unger SE. 2001. High-speed liquid chromatography/tandem mass spectrometry using a monolithic column for high-throughput bioanalysis. Rapid Commun Mass Spectrom 13:1113–1119.

Wunschel DS, Fox KF, Fox A, Nagpal ML, Kim K, Stewart GC, Shahgholi M. 1997. Quantitative analysis of neutral and acidic sugars in whole bacterial cell hydrolysates using high-performance anion-exchange liquid chromatography-electrospray ionization tandem mass spectrometry. J Chromatogr A 776:205–219.

Young JC. 1995. Microwave-assisted extraction of the fungal metabolite ergosterol and total fatty acids. J Agric Food Chem 43:2904–2910.

Young JC, Games DE. 1993. Supercritical fluid extraction and supercritical fluid Chromatography of the fungal metabolite ergosterol. J Agric Food Chem 41:577–581.

Yu X, Cui D, Davis MR. 1999 . Identification of in vitro metabolites of indinavir by ''intelligent automated LC-MS/MS'' (INTAMS) utilizing triple quadrupole tandem mass spectrometry. J Am Soc Mass Spectrom 10:175–183.

Zahn JA, Higgs RE, Hilton MD. 2001. Use of direct-infusion electrospray mass spectrometry to guide empirical development of improved conditions for expression of secondary metabolites from actinomycetes. Appl Environ Microbiol 67:377–386.

Zaldivar J, Borges A, Johansson B, Smits HP, Villas-Bôas SG, Nielsen J, Olsson L. 2002. Fermentation performance and intracellular metabolite patterns in laboratory and industrial xylose-fermenting *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 59:436–442.

Zhao YL, Pan W. 2003. Modified nonparametric approaches to detecting differentially expressed genes in replicated microarray experiments. Bioinform 19:1046–1054.

Zhu Y, Wong PSH, Zhou QH, Sotoyama H, Kissinger PT. 2001. Identification and determination of nucleosides in rat brain microdialysates by liquid chromatography/electrospray tandem mass spectrometry. J Pharmaceut Biomed 26:967–973.

Zroslíková J, Hajšlová J, Godula M, Maštovská K. 2001. Performance of programmed temperature vaporizer pulsed splitless and on-column injection techniques in analysis of pesticide residues in plant matrices. J Chromatogr A 937:73–86.

Zytkovicz TH, Fitzgerald EF, Marsden D, Larson CA, Shih VE, Johnson DM, Strauss AW, Comeau AM, Eaton RB, Grady GF. 2001. Tandem mass spectrometric analysis for amino, organic, and fatty acid disorders in newborn dried blood spots: A two-year summary from the New England Newborn Screening Program. Clin Chem 47:1945–1955.

Silas G. Villas-Bôas, a Biologist, graduated in 1998 from Campinas State University in Brazil and received an MSc degree in Biotechnology in 2001, studying the biological conversion of agro-industrial wastes by selected microorganisms at the Department of Microbiology and Parasitology, Federal University of Santa Catarina, Brazil. In 2001 he joined the Center for Microbial Biotechnology at the Technical University of Denmark (DTU) as a research assistant developing methods for analysis of pentose phosphates using HPLC-PAD. Later, in 2001, he started a PhD program in Biotechnology focused on method development for metabolome analysis of yeasts using a GC-MS platform. S. G. Villas-Bôas is currently finishing his PhD studies at DTU and shortly will join the AgResearch Grasslands in New Zealand as a post-doctoral fellow.

Sandrine Mas received an MSc in Microbiology, Molecular and Cellular Biology in 1997 at the University of Montpellier, France. She got a Ph.D. degree in Microbial Engineering (2001) from the University of Montpellier studing the control of high cell density process and developing on-line instrumentations for measurement of gas, biomass, flocculation (GC, capacitance probe, image analysis). She taught diverse courses such as micro-biological systematics and engineering, process engineering, and genetics at the University of Montpellier, ISIM and INSA engineering schools (France). After working two years as a teaching and research assistant in France, she joined the Center for Microbial Biotechnology, DTU, Denmark, in 2002, as a post-doctoral fellow, where her research activity is focused on high-throughput yeast metabolome analysis by mass spectrometry.

Mats Åkesson obtained his PhD in Control Engineering in 1999 from Lund University, Sweden. He thereafter worked with development of fermentation processes for production of biopharmaceuticals at Biotecnol SA, Lisbon, Portugal. From 2001 to 2003 he was assistant professor at the Center for Process Biotechnology, Technical University of Denmark, heading the research group on Systems Biology and Metabolite Profiling. Dr Åkesson is currently with Novo Nordisk A/S, Bagsvaerd, Denmark.

Jørn Smedsgaard received an MSc in engineering from Department of Organic Chemistry, Technical University of Denmark in 1986 studying analytical organic chemistry and enzyme kinetics. After working a short period for a commercial analytical laboratory, he joined Department of Dairy Technology at the Royal Veterinarian and Agricultural University in 1987 to setup a mass spectrometric facility. In 1991 he returned to The Technical University of Denmark to setup a mass spectrometric facility at Department of Biotechnology while doing a PhD study. He received a PhD in 1995 in Chemosystematics of fungi studied by mass spectrometry. J. Smedsgaard is today associated professor at Center for Microbial Biotechnology, Biocentrum-DTU where his core research interest is focused on biological mass spectrometry and data processing for detection and profiling metabolites in microbes, especially filamentous fungi.

Jens Nielsen is professor in fermentation physiology at the Technical University of Denmark. He holds an MSc degree in chemical engineering and a PhD in biotechnology, both from the Technical University of Denmark. He has been at several international universities, including University of Hannover and MIT. He currently serves as director of Center for Microbial Biotechnology, a leading research center in the field of metabolic engineering of yeast and filamentous fungi. Jens Nielsen has authored and co-authored close to 200 research papers in international journals with peer review. His research group focuses on metabolic engineering of yeast and filamentous fungi for improved production of various chemicals, e.g., bioethanol, organic acids, antibiotics, and isoprenoids. Particularly his research involves the development of novel methods for functional characterization of microorganisms, e.g., methods for analysis of intracellular metabolites, flux quantification, and genome-wide transcription analysis.