

I.1 Gas Chromatography Mass Spectrometry

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1 Introduction

GC-MS technology has been used for decades in studies which aim at the exact quantification of metabolite pool size and metabolite flux. Exact quantification has traditionally been focused on a single or small set of predefined target metabolites. Today GC-MS is one of the most widely applied technology platforms in modern metabolomic studies. Since early applications in unravelling the mode of action of herbicides (Sauter et al. 1988) it has experienced a renaissance (Fig. 1) in post-genomic, high-throughput fingerprinting and metabolite profiling of genetically modified (e. g. Roessner et al. 2001a,b, 2002; Fernie et al. 2004) or experimentally challenged plant samples (e. g. Cook et al. 2004; Kaplan et al. 2004; Urbanczyk-Wochniak and Fernie 2005). Metabolic phenotyping and analysis of respective phenocopies by metabolite profiling has become an integral part of plant functional genomics (Fiehn et al. 2000b; Roessner et al. 2002; Fernie et al. 2004). The essence of metabolite profiling, namely the non-biased screening of biological samples for changes of metabolite levels relative to control samples, has been thoroughly discussed earlier and is clearly distinguished from fingerprinting approaches and the concept of exact quantification (Fiehn et al. 2000b; Sumner et al. 2003; Birkemeyer et al. 2005).

GC-MS-based metabolome profiling analysis is on the verge of becoming a routine technology. This fact substantially contributes to the development of metabolomics as a fourth integral part of the Rosetta stone for functional genomics and molecular physiology (Trethewey et al. 1999; Fiehn et al. 2000b; Trethewey 2004). Nevertheless, GC-MS technology is already challenged again by new bottlenecks and demands for improved data sets which are optimised for the mathematical modelling tools currently developed in the fields of bioinformatics and biological systems analysis.

The challenges of modern, multi-parallel, GC-MS based metabolite analysis are manifold: (i) automation of sample preparation, wet chemistry and data processing after acquisition for increased throughput and reproducibility, (ii) extension of the analytical scope of metabolomics studies, for example by combined analysis of single samples using multiple analytical technology platforms, and combined analysis with the proteome and transcriptome

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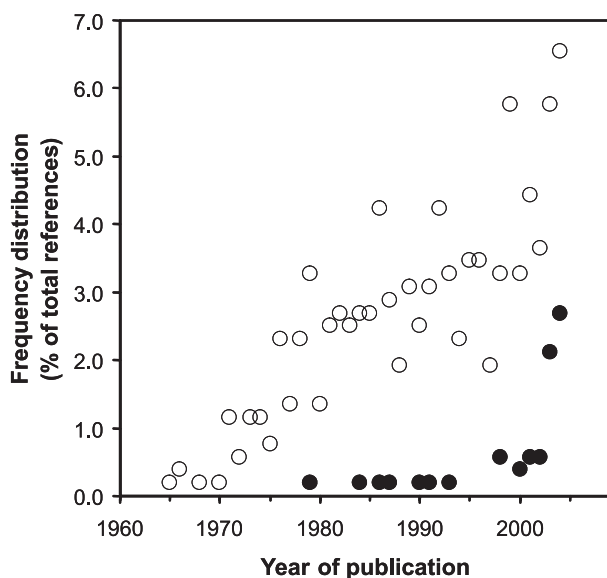


Fig. 1. Literature survey of publications which associate the concepts, “metabolite”, “profiling”, and “gas chromatography” performed on 1/2005. A total of ~500 citations without conference proceedings, abstracts and book chapters were found. The frequency of publications in all biological sciences (*open circles*) is compared to the contribution by plant metabolomics community (*closed circle*)

(Weckwerth et al. 2004b), (iii) profiling of trace compounds, or signalling molecules in the presence of bulk metabolites (Mueller et al. 2002; Birkemeyer et al. 2003; Schmelz et al. 2003, 2004), (iv) increasing accuracy in multi-parallel metabolite quantification (Birkemeyer et al. 2005), (v) combining profiling and flux analyses (Roessner-Tunali et al. 2004), (vi) establishment of quantitative repeatability, unambiguous nomenclature and comparability between analyses performed in different laboratories or using different analytical technology platforms (Schauer et al. 2005), and (vii) finally – perhaps the most important challenge of all metabolomic investigations – the identification of the unidentified majority of metabolic components from metabolite profiling experiments (Fiehn et al. 2000a; Schauer et al. 2005).

In agreement with the focus of this chapter the above challenges have predominantly analytic or technical motivation. The breakthrough of metabolomic investigations, however, will depend on the access to hitherto unavailable fundamental insights into metabolic and systems interactions. Increasingly integrative studies which consider the metabolome, proteome, transcriptome, and genome evolution of an organism have been initiated and are to be expected. Promising steps have been made – using GC-MS technology – towards network analysis (Fiehn 2003; Weckwerth et al. 2004a) and correlation studies between or within metabolome and transcriptome

constituents (Urbanczyk-Wochniak et al. 2003; Steinhäuser et al. 2004; Kopka et al. 2005). A detailed discussion of these general aspects including GC-MS studies and beyond can be found in the applications section of this book.

2 GC-MS Profiling Technology in a Nutshell

Metabolite profiling with GC-MS involves six general steps:

1. *Extraction* of metabolites from the biological sample, which should be as comprehensive as possible, and at the same time avoid degradation or modification of metabolites (e. g. Kopka et al. 2004).
2. *Derivatisation* of metabolites making them amenable to gas chromatography. Metabolites which are not volatile per se require chemical modification prior to GC analysis.
3. *Separation* by GC. High resolution GC can also be highly reproducible as it involves automated sample injection robotics, highly standardised conditions of gas-flow, temperature programming, and standardised capillary column material.
4. *Ionisation* of compounds as they are eluted from the GC. Electron impact (EI) ionisation is most widely used, as it is the technology which is least susceptible to suppression effects and produces reproducible fragmentation patterns.
5. Time resolved *detection* of molecular and fragment ions. Mass separation and detection can be achieved with different mass-detection devices, including sector field detectors, quadrupole detectors (QUAD), ion trap technology, and time-of-flight detectors (TOF). The choice of detectors depends on the targeted analytical niche. GC-MS systems with QUAD detection are most widely spread for routine analysis. Ion trap technology allows MS \times MS (two-dimensional MS) analysis for structural elucidation and targeted quantification of trace compounds (e. g. Mueller et al. 2002). TOF detection can either be tuned to fast scanning rates (van Deursen et al. 2000) or to high mass precision comparable to sector field systems. Fast scanning GC-TOF-MS enables the, today, most advanced technology in the GC-MS field, namely two dimensional GC \times GC-TOF-MS (two-dimensional GC-TOF-MS) (Ryan et al. 2004; Sinha et al. 2004a–c).
6. *Acquisition and evaluation* of GC-MS data files. All GC-MS system manufacturers provide software which is tuned for targeted, quantitative metabolite analysis. The targeted approach involves unequivocal identification of predefined metabolites by expected chromatographic retention times and mass-spectral fragmentation patterns and quantitative calibration by authentic standard concentrations. Recent software developments support the non-targeted analysis of GC-MS patterns, and the full evaluation of all resolved compounds. This feature of GC-MS allows discovery of novel hitherto

unknown metabolites. As we are far from knowing all possible metabolites of a given organism, non-biased, truly comprehensive data evaluation is the most essential requirement of metabolite profiling.

2.1 Chemical Derivatisation and Chromatography

The principles of fast metabolic sample inactivation and nondestructive extraction are common to all metabolome analyses. In contrast to all other technologies GC-MS is inherently restricted to volatile and temperature-stable compounds. The scope of GC-MS for metabolite analysis is limited by the typical temperature range of commercial capillary columns, for example up to 320–350 °C. The lower temperature range is determined by ambient temperature, but cold trapping devices and isothermal GC allow analysis of low molecular weight gases and highly volatile metabolites. GC received a considerable extension of applications through the development of a highly versatile tool box of derivatisation reagents, which chemically transform non-volatile metabolites into volatile analytes for GC-MS analysis (e. g. Knapp 1979; Blau and Halket 1993; Toyo'oka 1999). To date, GC-MS profiling of metabolites in plants has largely been confined to compounds, recovered in the methanol-water phase after methanol-water/chloroform extraction of tissues (Fiehn et al. 2000a; Roessner et al. 2000; Duran et al. 2003; Barsch et al. 2004; Gullberg et al. 2004; Strelkov et al. 2004; Broeckling et al. 2005). Although not all hydrophilic compounds can be volatilised by derivatisation, the following classes of compounds are detected routinely: amino-, organic-, and aromatic-acids, amines, sugars up to trisaccharides, alcohols and polyols, and some mono-phosphorylated metabolites.

The current limitations of metabolite preparation and derivatisation strategy, namely methoxyamination with subsequent direct trimethylsilylation of predominantly polar metabolites, call for extension. Application of other technology platforms is an obvious route and will be discussed in the following chapters. Here a short appraisal of the potential of chemical derivatisation is attempted. Four main types of reaction schemes will be discussed.

1. *Alkoxyamination* by reagents, such as methoxyamine $\text{CH}_3\text{--O--NH}_2$, stabilises carbonyl moieties in native metabolite structures, but forms E- and Z-isomers of the --N=C< double-bond substituents. Keto-enol tautomerism is suppressed, as is the decarboxylation of unstable β -carbonyl-carboxylic acids. In addition, the formation of acetal- or ketal-structures in aqueous solution is inhibited. These equilibrium reactions generate multiple intramolecular and water adducts, for example the typical α - and β -conformers of reducing sugars. Ether- and ester-conjugates are mostly stable when exposed to methoxyamine reagent and maintain conformation. So far other alkoxy-reagents – for example hydroxylamine, ethyloxyamine, or benzyloxyamine – have not been exploited for systematic discovery of metabolites with carbonyl moieties:

2. *Silylation* reagents classify into those which introduce either a trimethylsilyl (TMS) moiety, $-\text{Si}(\text{CH}_3)_3$, or a dimethyl-*(tert-butyl)*-silyl (TBS) moiety, $-\text{Si}(\text{CH}_3)_2-\text{C}(\text{CH}_3)_3$. TMS reagents have been well investigated and are known to have the widest derivatisation spectrum (Little 1999; Halket et al. 2005). TMS has the potential to substitute all exchangeable, “acidic” protons of a metabolite. Steric hindrance of TMS substitution is rare but common with the bulkier TBS reagent. The benefit of the TBS reagent is higher tolerance for the presence of water and clear mass spectral fragmentation. However, vicinal diols, which typically occur in sugars, are only partially derivatised.
3. *Alkylation* reactions, mostly methylation, are widely used to derivatise carboxylic acids and alcohols. The enormous reactivity of available reagents – some allow for flash derivatisation during hot GC injection – leads to transalkylation of ester-bonds and consequently breaks down complex metabolites, such as glycerol- and phospholipids. Alkylation of sugars leads to derivatives which are more volatile than the TMS derivatives and therefore allow analysis of higher sugar oligomers.
4. *Acylation* reactions, mostly acetylation or trifluoro-acetylation, are less reactive than transalkylation. Reagents usually form stable ester and amide bonds and break down only activated metabolic intermediates, e.g. thioesters.

In conclusion further developments of alternate GC-MS profiling techniques need to employ more selective combinations of metabolite fractionation and derivatisation schemes. Solid phase extraction can be explored to partition and concentrate metabolites amenable to alternate subsequent derivatisation. On the other hand, vapour phase extraction (VPE) for the separation and concentration of volatile derivatisation products prior to GC injection may prove promising (Schmelz et al. 2003, 2004). VPE has the potential to be a robust technique and was shown to operate with a range of commonly used reagents.

2.2 Mass Detection and Quantitative Calibration Techniques

One of the major criticisms and pitfalls of metabolome analyses is best explained by so-called matrix effects. This well-known effect describes unexpected losses or increased recovery of metabolites in complex extracts compared to pure authentic preparations. Matrix effects on one hand are caused by the presence of compounds which either specifically inhibit extraction or chemical analysis of metabolites. Positive matrix effects can stabilise otherwise labile compounds in the presence of suitable chemicals. Typical examples are suppression effects of soft ionization techniques, for example electrospray ionization (ESI) or matrix assisted laser desorption ionization (MALDI). Electron-impact ionization (EI) typically used in GC-MS profiling is not susceptible to suppression. Instead GC injection is the crucial step which may

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