Metabolomics, Vol. 3, No. 2, June 2007 (© 2007)
DOI: 10.1007/s11306-007-0057-3

A comparative study of amino acid measurement in leaf extracts by gas chromatography-time of flight-mass spectrometry and high performance liquid chromatography with fluorescence detection

Graham Noctor*, Gaëlle Bergot, Caroline Mauve, Dorothée Thominet, Caroline Lelarge-Trouverie, and Jean-Louis Prioul

Plateforme Métabolisme-Métabolome, IFR87, Institut de Biotechnologie des Plantes, UMR CNRS 8618, Université Paris XI, Orsay cedex, 91405, France

Received 7 November 2006; Accepted 30 March 2007

Gas chromatography coupled to time-of-flight mass spectrometry (GC-TOF-MS) has become a promising technique for simultaneous and rapid analysis of small metabolites in complex mixtures. The aim of this work was to establish the quantitative nature of the information generated by amino acid analysis of crude leaf extracts using GC-TOF-MS. Dried aliquots of methanol/water extracts of *Arabidopsis* leaves were analysed in parallel by GC-TOF-MS following trimethylsilylation or high performance liquid chromatography and fluorescence detection of *o*-phthaldialdehyde derivatives (OPA-HPLC). Twenty amino acids could be routinely detected in leaf extracts by both methods. Because of instability of some trimethylsilylated derivatives, all GC-TOF-MS analyses were performed within a window of 2 h 30 min following derivatization. Repeatability studies showed that relative standard deviations for multiple injections of a single extract were below 20% for both techniques, though significantly smaller for OPA-HPLC. Similar between-extract variability and condition-independent biological variation were detected by OPA-HPLC and GC-TOF-MS, and both techniques detected similar environmentally induced changes in four major amino acids. Recovery of standard compounds through the extraction procedure was between 80% and 120% for OPA-HPLC but more variable when analysed by GC-TOF-MS. When quantified on the basis of tissue fresh weight according to response factors of mixed standards, the two techniques gave consistent values for a number of amino acids but divergent values for others. Taken together, the results suggest GC-TOF-MS analysis of *Arabidopsis* leaves with the present protocol can be used for absolute quantification of 4–7 amino acids, accurate relative quantification of 8–11 amino acids, and more limited quantification for five compounds of this class.

KEY WORDS: metabolite; metabolomics; *Arabidopsis*; quantification; metabolite profiling.

1. Introduction

The metabolome has been defined as 'native small molecules (definable non-polymeric compounds) that are participants in general metabolic reactions and that are required for the maintenance, growth and normal function of a cell' (Goodacre *et al.*, 2004). The study of the metabolome – metabolomics – provides essential information on biological function that is complementary to approaches such as transcriptomics and proteomics (Hall, 2006). Though no one method can provide an exhaustive inventory of all small molecules present in a given sample, metabolomics aims to profile as many metabolites as possible within a single analysis (Weckwerth and Fiehn, 2002; Sumner *et al.*, 2002; Bino *et al.*, 2004; Fernie *et al.*, 2004). Gas chromatography coupled to mass spectrometry (GC-MS) has become one of the most widely used methods to meet this objective. The separative power of GC allows a high degree of chromatographic resolution, detection by MS is relatively unlimited by the physicochemical nature of specific metabolites or their derivatives, and compounds can be identified by reference to available MS databases (Wagner *et al.*, 2003; Hall, 2006). Several hundred polar compounds can be detected during a single analysis, and assigning identity to all these compounds, many of which remain unknown, is a major future challenge (Bino *et al.*, 2004).

Metabolite profiling techniques are providing valuable information in the study of plant metabolism (Roessner-Tunali *et al.*, 2003; Stitt and Fernie, 2003; Nikiforova *et al.*, 2005; Dutilleul *et al.*, 2005; Broekling *et al.*, 2005; Hirai *et al.*, 2005). Pioneering studies of plant tissue analysis by GC-MS used quadrupole detectors (Roessner *et al.*, 2000, 2001). Subsequently, time-of-flight (TOF)-MS has been adapted to the analysis of complex mixtures such as plant extracts (Weckwerth *et al.*, 2004a). The principal advantages of TOF-MS in a metabolomics context are high rates of data acquisition and constant spectral composition throughout peak elution (Wagner *et al.*, 2003). These properties facilitate accurate deconvolution of overlapping or closely eluting compounds and improve detection of minor or narrow peaks, increasing the number of metabolites that can be resolved within the gas chromatogram.

* To whom correspondence should be addressed.
E-mail: graham.noctor@u-psud.fr.

Despite the undoubted interest of GC-TOF-MS, questions remain concerning the type of information that is generated by this technique. In general, an inverse correlation is acknowledged between different techniques concerning their quantitative precision and the number of metabolites that can be profiled (Fernie *et al*., 2004). An important point is therefore to establish to what extent data generated by GC-TOF-MS are quantitative. Applications of GC-MS to profile plant tissues have involved correlation analysis to identify 'metabolic networks' (Weckwerth and Fiehn, 2002; Fiehn, 2003; Weckwerth *et al*., 2004a). For these purposes, relative quantification may be sufficient. Absolute quantification is likewise not the primary requirement in other applications such as identification of diagnostic marker metabolites or rapid screening programmes. However, absolute quantification is necessary or desirable for other current or potential applications of metabolite analysis. Examples are studies seeking to relate changes in metabolite concentrations to properties of proteins (e.g., enzymes or receptors) in the analysis of metabolic regulation or signalling, an application likely to become increasingly important as techniques develop that allow improved spatial resolution of samples (Stitt and Fernie, 2003). Even with relatively heterogenous samples (i.e., whole tissues or cells), quantification is important for studies of metabolite enrichment as a result of genetic manipulation. Investigations of resource allocation within plant tissues, with or without metabolic flux analysis, also call for appropriate quantification of metabolites.

The primary aim of this study was to establish whether the GC-TOF-MS data generated for the analysis of amino acids, an important class of small polar metabolites, are quantitative or not. Because of its sensitive and quantitative nature, we chose as a reference method fluorimetric detection of *o*-phthaldialdehyde (OPA) derivatives after separation by high performance liquid chromatography (HPLC). The results show that the type of information generated by GC-TOF-MS with commonly used conditions of extraction and analysis is metabolite-specific. The method yields absolutely quantitative information for some amino acids, relatively quantitative information for others, and semi-quantitative information for the remainder.

2. Materials and methods

2.1. Chemicals

Amino acid standards, methoxyamine, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), and *o*-phthaldialdehyde (OPA), were from Sigma, Saint Quentin Fallavier, France. Methanol was HPLC grade. Water was millipore-filtered.

2.2. Equipment

GC-TOF-MS was performed on a LECO Pegasus III with an Agilent 6890N GC system with Agilent 7683 automatic liquid sampler. The column was an RTX-5 w/integra-Guard (30 m × 0.25 mm i.d. + 10 m integrated guard column) (Restek, Evry, France).

OPA-HPLC was performed using a Waters Alliance instrument with a Waters 2475 multi-wavelength fluorescence detector set at λ_{exc} = 340, λ_{em} = 455. The reverse-phase column was a Waters Symmetry C_{18} 3.5 μm (150 mm × 4.6 mm i.d.) with a Sentry guard column (10 mm × 2.1 mm i.d.).

2.3. Plant material and extraction

Arabidopsis thaliana, ecotype Columbia, was grown in soil in individual 5 cm pots in controlled environment growth chambers at 8 h light (20 °C), 16 h dark (18 °C), and 60% relative humidity. The irradiance during the light period was 200 μmol.quanta m^{-2} s^{-1} at the leaf surface. Plants were watered once every 2 days with nutrient solution. Ambient CO_2 was maintained at either 0.04 ('air') or 0.45% ('high CO_2'). Leaf samples (100 mg fresh weight) were rapidly frozen in liquid N_2 and stored at −196 °C until extraction. To compare amino acid measurement by the two methods as directly as possible, the procedure depicted in figure 1 was adopted. The protocol was designed to give the same equivalent of leaf extract injected onto the column for both techniques. Samples were ground in a mortar in liquid N_2 then in 2 × 1 mL extraction medium consisting of 80% methanol containing 100 μM α-aminobutyrate as internal standard. For recovery experiments, known concentrations of amino acid standards were pre-added to the extraction medium. Extracts were transferred to 2 mL Eppendorf tubes, incubated on ice for 20 min, then centrifuged at 10,000 × *g* and 4 °C for 15 min. Supernatants were transferred

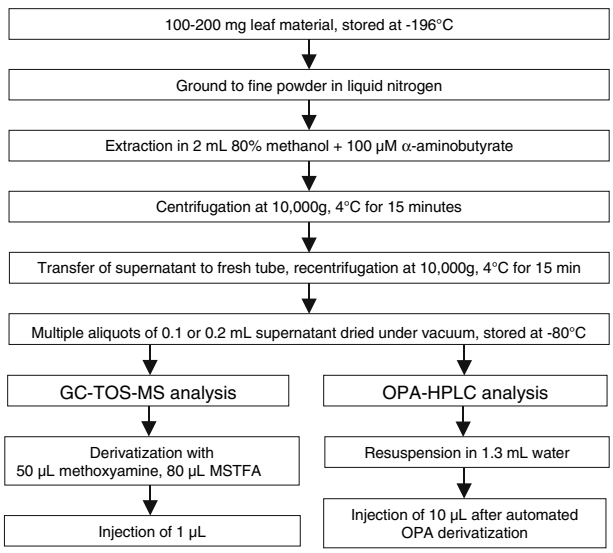

Figure 1. Scheme showing the protocol for analysis of amino acids in parallel by OPA-HPLC and GC-TOF-MS. For further details, see text.

to fresh tubes and centrifuged again. Several aliquots of each extract (usually 0.1 or 0.2 mL, for some experiments also 0.3 mL) were spin-dried under vacuum and stored at −80 °C until analysis. Equivalent dried aliquots of all leaf extracts were directly compared by GC-TOF-MS and OPA-HPLC. For OPA-HPLC, the dried aliquot was resuspended in 1.3 mL water, centrifuged, filtered into autosampler vials, and aliquots were derivatized as described below. For GC-TOF-MS, dried aliquots were derivatized directly as described below. Additional extractions for GC-TOF-MS analysis were also performed according to a phase-partition method adapted from Weckwerth *et al*. (2004b), as follows. Ground leaf powder was extracted into 2 mL of methanol:chloroform:water (2.5:1:1 v/v). The mix was shaken for 30 min at 4 °C then centrifuged for 2 min at 10,000 × *g* and 4 °C. The supernatant was set aside (supernatant 1) and 1 mL of chloroform:methanol (1:1 v/v) was added to the pellet and mixed vigorously. Following centrifugation as above, the supernatant was recovered (supernatant II) and 0.5 mL of water was added to both supernatants I and II. After centrifugation, 2 × 1 mL aliquots were recovered from the polar phase.

2.4. Derivatization and analysis

OPA-HPLC. OPA reagent was made 24 h before first use by dissolving OPA at 54 mg mL^{-1} in methanol and adding 200 μL to 1.8 mL 0.5 M sodium borate (pH 9.5) and 40 μL 2-mercaptoethanol. The reagent was filtered into an autosampler vial and used for up to 3 days. Precolumn derivatization was performed in the injection loop by automated mixing of 10 μL sample and 15 μL OPA reagent, followed by a delay of 2 min prior to injection. The chromatographic separation was performed by gradient elution at 40 °C using buffer A (20% methanol, 80% sodium acetate, 1% tetrahydrofuran, pH 5.9) and buffer B (80% methanol, 20% sodium acetate, pH 5.9). Buffer flow rate was 0.8 mL min^{-1} throughout and total run time per injection was 42 min. Peak identity was confirmed by co-elution with authentic standards.

GC-TOF-MS. Methoxyamine was dissolved in pyridine at 20 mg mL^{-1} and 50 μL added to dried aliquots of standards or extracts. Following vigorous mixing, tubes were incubated for 90 min at 30 °C with shaking. Eighty microlitre of MSTFA was then added, the mix was vortexed, and incubated for 30 min at 37 °C with shaking. The derivatization mix was then incubated for 60 min at room temperature, before loading into the GC autosampler. Unless otherwise stated, injections of 1 μL in splitless mode were made up to 2 h 30 min after loading. Separation was performed in a helium gas-stream at 1 mL min^{-1} using a temperature ramp from 80 to 330 °C between 2 and 18 min followed by 6 min at 330 °C. Total run time per injection was 30 min. Ionization was by electron impact at 70 eV and the MS acquisition rate was 20 spectra s^{-1} over the m/z range 80–500, as in Weckwerth *et al*. (2004a). Peak identity was initially established by reference to mass spectra of derivatives in the US National Institute of Standards and Technology database, a copy of which is supplied with the LECO software. All mass spectra were confirmed by individual injection of authentic standards derivatized as described above.

2.5. Standards

Common standard solutions were prepared for OPA-HPLC and GC-TOF-MS analysis. To minimize possible discrepancies between amino acid responses in extracts and standards, abundant leaf amino acids were present in the standard solutions at higher concentrations than less abundant amino acids. Aliquots of standard solutions were vacuum-dried to give six levels of standards with injected amounts of 10–500 pmoles (Ala, Asn, Asp, Gln, Glu, Gly, Ser, Thr), 2–100 pmoles (β-Ala, homo-Ser, Ile, Leu, Phe, Val), and 1–50 pmoles (γ-ABA, Arg, Lys, Met, Orn, Trp). Dried aliquots were stored at −80 °C until use. For OPA-HPLC, dried aliquots were redissolved in 1.3 mL water, filtered and 10 μL each solution was derivatized prior to injection as described above for leaf extracts. For GC-TOF-MS, dried aliquots were derivatized as described above for leaf extracts.

2.6. Data processing and quantification

Integration of HPLC peaks was done using the Waters Millenium ApexTrack algorithms and verified manually. Quantification was performed relative to standard curves generated using quadratic equations. For GC-TOF-MS, integration of peaks was performed using LECO Pegasus software. Because automated peak integration was occasionally erroneous, integration was verified manually for each analysis. Verified peak areas obtained for mixed standards were copied into Microsoft Excel to generate standard curves, and unknowns were quantified using quadratic equations obtained by curve fitting.

3. Results

Typical chromatograms of mixed standards and *Arabidopsis* leaf extracts of amino acids are shown in figure 2. Twenty amino acids (plus the internal standard) could be detected in leaf extracts by both methods. Other amino acids could be detected or quantified only by one method. These included Tyr and *O*-acetylserine, which were detected by GC-MS but not adequately separated by HPLC, and His, which was separated by HPLC, but which on GC-MS was closely co-eluted with a more abundant glucose peak, preventing adequate

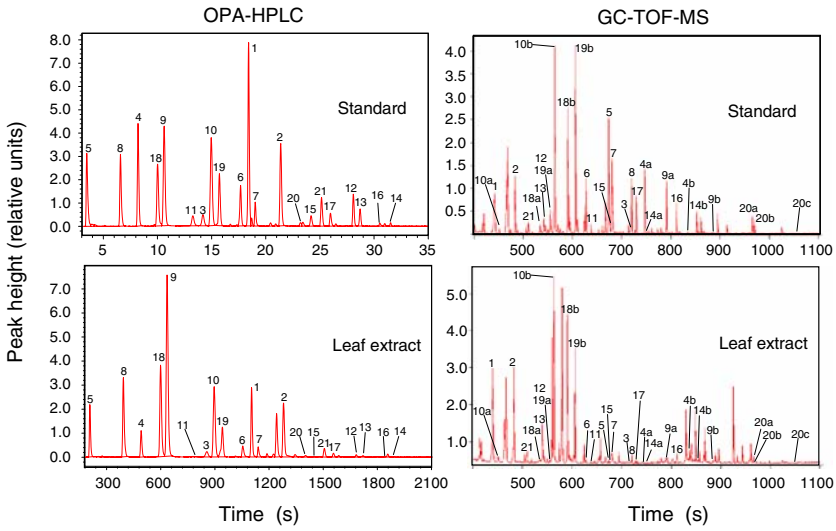

Figure 2. Separation of amino acids by OPA-HPLC (left) and GC-TOF-MS (right). Typical chromatograms are shown for standards and leaf extracts of *Arabidopsis*. Key to peaks (where more than one TMS derivative was detected by GC-MS, these are in brackets): 1. Ala. 2. α-ABA. 3. Arg. 4. Asn (4a. Asn3TMS; 4b. Asn4TMS). 5. Asp. 6. β-Ala. 7. γ-ABA. 8. Glu. 9. Gln (9a. Gln3TMS; 9b. Gln4TMS). 10. Gly (10a. Gly2TMS; 10b. Gly3TMS). 11. homoSer. 12. Ile. 13. Leu. 14. Lys (14a. Lys3TMS; 14b. Lys4TMS). 15. Met. 16. Orn. 17. Phe2TMS. 18. Ser (18a. Ser2TMS; 18b. Ser3TMS). 19. Thr (19a. Thr2TMS; 19b. Thr3TMS). 20. Trp (20a. Trp2TMS. 20b. Trp3TMS. 20c. Trp4TMS). 21. Val.

resolution, even with the deconvolution software. Of the 20 amino acids that could be detected by both techniques, eight gave more than one trimethylsilyl (TMS) derivative when analysed by GC-MS (figure 2 and legend). Preliminary experiments showed that several amino acids were detected in insufficient quantity for analysis by GC-TOF-MS using split injection, and so splitless injection mode was used. The ions monitored for each TMS derivative were chosen on the basis of specificity and, where possible, abundance, and are summarized in table 1.

3.1. *Stability of trimethylsilyl(TMS)-amino acid derivatives*

To avoid problems with certain unstable OPA derivatives (Lindroth and Mopper, 1979), HPLC analysis of amino acids was performed by programmed pre-column derivatization. Because an analogous automated derivatization facility was not available on our GC-TOF-MS system, we first investigated derivative stability by repeated injection of standards over a period of three days following the derivatization procedure. Several patterns were observed (figure 3 and legend). The response factors of seven amino acids declined steadily over the period of analysis, while eight amino acids showed a more stable response, with a pattern similar to those of Gly3TMS and GlyTOT (figure 3), and % relative standard deviation (RSD) values over the 15 injections ranging from 15.7 (Ile3TMS) to 25.1 (Leu2TMS). Most of the amino acids that gave more than one TMS derivative showed changes in derivative response factors, and these were often inverse (e.g., for Ser in figure 3), suggesting slow conversion between derivatives over the course of the experiment. Because of the significant changes in derivative stability, a window of 2 h 30 min following derivatization was defined within which all further analyses were performed. This window allowed analysis of five standards or extracts within a single series. Within this window, % RSD of five injections of a single standard was below 10% for all compounds apart from Arg, though variability was in general somewhat higher than that obtained by OPA-HPLC (table 2).

3.2. *Standard curves for amino acids analysed by GC-TOF-MS*

Using mixed solutions of authentic amino acids, standard curves obtained by OPA-HPLC were reproducible and generally close to linear (data not shown). Representative standard curves are shown for GC-TOF-MS in figure 4. Where an amino acid gave more than one TMS derivative, one was always reproducibly predominant within the analysis window described above (e.g., 3TMS for Gly, 3TMS for Trp). However, preliminary experiments showed that for certain amino acids, the ratio between the abundance of TMS derivatives was different in extracts and standards, and that the most reliable data could be obtained by taking the sum of the different derivatives and quantifying them relative to standard curves produced in a likewise manner (see 'Materials and methods').

Table 1
Characteristic m/z fragments used for quantification of amino acids by GC-TOF-MS

	GC-TOF-MS		
Amino acid	Derivative	Fragment (m/z)	Intensity (%)
Ala	2TMS	116	55.8
Arg	?TMS	142	23.9
Asn	3TMS	188	4.3
	4TMS	190	15.7
Asp	3TMS	232	19.7
β-Ala	3TMS	248	9.4
α-ABA	2TMS	130	23.4
γ-ABA	3TMS	304	3.0
Gln	3TMS	245	3.6
	4TMS	227	9.3
Glu	3TMS	246	14.8
Gly	2TMS	102	
Gly	3TMS	174	28.3
homoSer	3TMS	128	14.4
Ile	2TMS	160	2.0
Leu	2TMS	158	44.0
Lys	3TMS	230	2.0
	4TMS	230	2.8
Met	2TMS	176	24.7
Orn	4TMS	174	9.5
Phe	1TMS	146	24.1
	2TMS	192	14.1
Ser	2TMS	132	11.8
	3TMS	218	11.1
Thr	2TMS	130	65.9
	3TMS	219	8.9
Trp	2TMS	202	35.2
	3TMS	202	51.2
	4TMS	202	28.1
Val	2TMS	144	44.5

Intensity of the monitored ion is expressed relative to the total ion current over the range 80–500 Da detected for the corresponding peak obtained by injection of authentic standards. The number of TMS substituents is indicated for each amino acid derivative, including for those that yielded more than one species. For the single Arg peak, the number of TMS substituents could not be established. Gly2TMS was undetected when Gly was derivatized and injected singly but was routinely detected as a minor peak in mixed standards (see figure 4).

3.3. Between-injection and between-extract variability for amino acids

Variability for the same extract injected and quantified five times was 10% or less for all compounds by OPA-HPLC and 20% or less for GC-TOF-MS (table 2). Variability between single injections of different extracts was fairly similar using both techniques, though generally slightly lower for HPLC (table 3). For GC-MS, relative standard deviation (RSD) values were generally between 10% and 20% (table 3), i.e., of the same order as variability between injections of a single derivatized extract (table 2). Poor extract–extract repeatability was observed for γ-ABA using both methods, and for Orn, particularly by GC-MS (table 3).

3.4. Recovery of authentic standards added at the initial stage of the extraction procedure

Stability of compounds throughout the extraction procedure is an important criterion in quantitative metabolite analysis. Analysis by OPA-HPLC gave recovery quotients through the extraction between 80 and 120% for all amino acids analysed (figure 5), showing that the extraction procedure itself did not cause appreciable loss or transformation of amino acids. Though values between 60% and 120% were obtained for most amino acids quantified by GC-TOF-MS, poor recoveries were observed for some compounds, notably Asn, γ-ABA, Gln, and Trp (figure 5).

3.5. Condition-independent variability between plants

Analysis of condition-independent biological variability was performed by single injection of independent extracts of different plants growing in identical controlled conditions. Variability was significantly higher than that observed between injections or extracts, and for many compounds was of the same order for the two techniques (table 4). Exceptions were Glu and Lys, where more variability was detected by GC-TOF-MS, and Gly, Met, and Orn, for which HPLC analysis yielded higher RSD values (table 4). To assess the ability of GC-TOF-MS to detect changes in leaf amino acid quantities, we tested the GC-MS response to injections of dried aliquots of different volumes of extract (figure 6). The analysis showed an increasing reponse with increasing extract volume for most amino acids, though the highest dried volume (0.3 mL extract) produced aberrant results for certain compounds, in particular Glu, Gln, Asp, Asn, Lys, and Trp (figure 6). All other experiments described here used either 0.1 or 0.2 mL extract.

3.6. Absolute and relative quantification by GC-TOF-MS and HPLC

To compare data produced by the two analytical methods, 12 independent leaf extracts were performed and amino acids measured in parallel in each extract by the protocol shown in figure 1. Data were quantified according to standard curves and leaf fresh weight, and the mean ratios between values obtained by the two techniques are presented in table 5. Very similar values were obtained for Ala, Gly, Leu, and Val, and data for β-Ala, γ-ABA, homoSer, Ile, Ser, and Thr were also reasonably close (less than 2-fold variation). Other amino acids gave more divergent values, notably Arg, Asn, Asp, Gln, Glu, and Trp. For these amino acids, leaf contents measured by GC-TOF-MS were of the order of 10% or less than those obtained by OPA-HPLC (table 5).

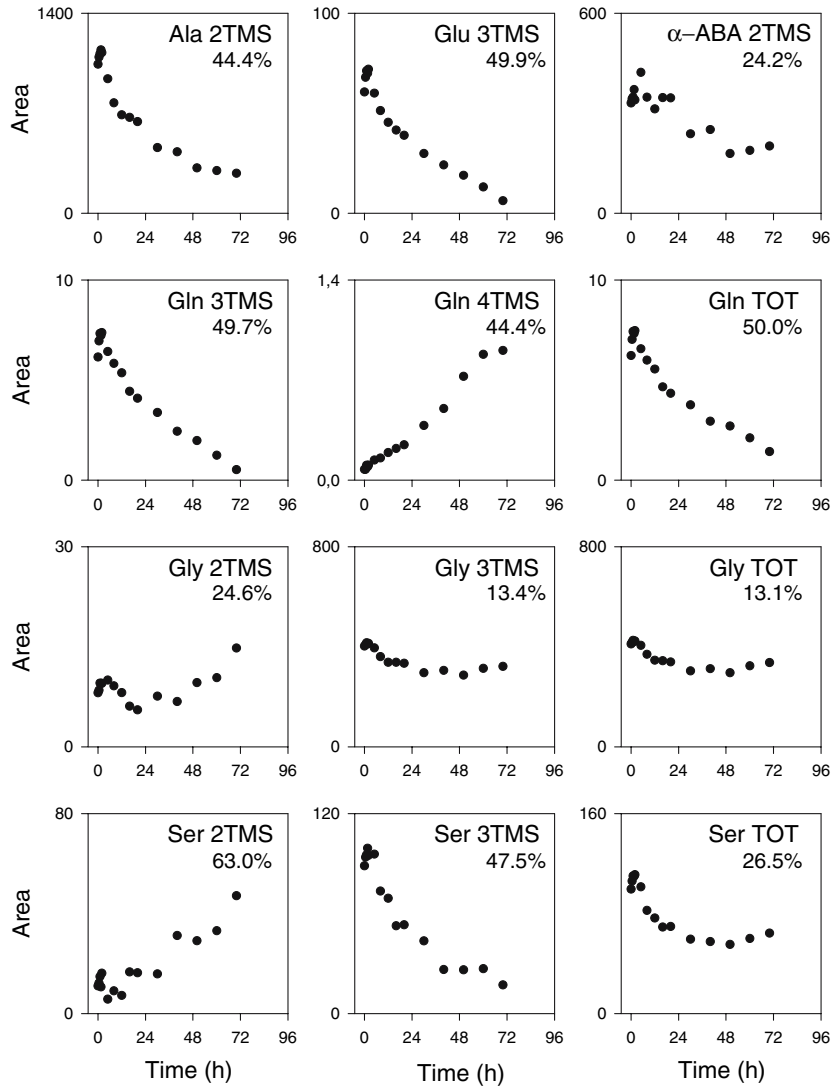

Figure 3. Stability of trimethylsilylated derivatives of amino acids over several days. A single derivatized mixed standard was injected repeatedly over the time shown, and the curves shown are typical examples of the different patterns observed. The *y*-axis is a linear scale from 0 to the maximum value shown (note the large differences in scaling between different derivatives). The numbers indicate % relative standard deviation of the 15 injections. Similar kinetics to those shown for Ala, Glu and the internal standard, α-ABA, were also observed for Arg, Asp, homoSer, and to a lesser extent, for Met. Relatively stable curves, as shown for the major Gly derivative (Gly3TMS) and the sum of the two Gly derivatives were also found for β-Ala, γ-ABA, Ile, Leu, Lys, Orn, and Val. For most amino acids with more than one derivative, distinct kinetics were observed for the different derivatives. Examples are shown for Gln and Ser; other amino acids that showed similar patterns were Asn, Thr, Trp, and Phe.

To examine whether the two techniques produce a similar relative quantification, GC-TOF-MS data were plotted against HPLC data for each extract (figure 7). Within the dataset of 12 extracts, many amino acids showed limited variation when measured by quantitative HPLC, and so there is significant scope for experimental error relative to absolute variation. Despite this, and the large differences in absolute values obtained for some amino acids (table 5), many showed a fairly good relative correspondence between the two techniques (figure 7). Only four amino acids, namely, β-Ala, Ile, Orn, and Trp, showed no or negligible relative correspondence ($r < 0.5$) between GC-TOF-MS and OPA-HPLC (figure 7).

3.7. *Comparison of different extraction protocols and the influence of other compounds abundant in plant extracts*

Because GC-TOF-MS produced much lower values for leaf contents of some amino acids than HPLC, we checked whether this might be linked to the nature of the extraction procedure. Amino acids were measured in *Arabidopsis* leaf extracts by the methanol/water protocol shown in figure 1 and by a methanol/chloroform/water phase-partition method (Weckwerth *et al.*, 2004b). The two methods gave similar between-extract RSD values (between 6% and 30%, see also table 3), and quantities detected were not dramatically

Table 2
Between-injection variability for standards and extracts measured by GC-TOF-MS and OPA-HPLC

	GC-TOF-MS		OPA-HPLC	
Amino acid	Standard	Extract	Standard	Extract
Ala	3.5	9.4	2.4	1.6
Arg	12.1	–	2.0	1.3
Asn	5.6	15.4	1.9	1.4
Asp	4.0	12.1	2.8	1.4
β-Ala	2.3	12.6	2.9	1.7
α-ABA	4.3	–	2.3	–
γ-ABA	3.1	8.8	2.4	2.8
Gln	7.3	20.8	1.9	1.4
Glu	6.7	13.7	1.6	1.2
Gly	1.4	11.6	2.6	1.4
homoSer	3.6	9.4	1.4	1.8
Ile	3.6	17.9	1.8	1.5
Leu	3.9	–	2.2	2.2
Lys	5.3	16.2	3.6	3.3
Met	3.3	12.0	1.8	10.2
Orn	4.7	16.4	4.2	6.3
Phe	3.8	12.0	2.4	1.8
Ser	4.4	9.9	2.1	1.3
Thr	3.5	9.5	1.8	1.8
Trp	7.7	19.8	3.2	2.8
Val	3.6	13.7	2.1	1.2

Values are % relative standard deviation of five injections of a single standard or leaf extract. Data for standards refer to variability in peak area. For leaf extracts, peak areas were converted to pmols by reference to external standard curves, then corrected relative to the internal standard, α-ABA. Arg and Leu were not detected by GC-MS in the leaf extract analysed in this experiment. For GC-TOF-MS, a single extract aliquot was derivatized, injected successively five times, then quantified. For OPA-HPLC, another aliquot of the same leaf extract was injected successively five times following automated pre-column derivatization, then quantified.

different (table 6). In general, values were about 20–50% higher when metabolites were extracted using the methanol/water method.

Discrepancies in absolute leaf contents measured by HPLC and GC-MS could also be linked to interference from other compounds that are not present in standards and that influence the response of amino acids in extracts specifically during the GC-MS derivatization and analysis. We therefore analysed the response on GC-MS of mixed amino acid standards after addition of excess concentrations of five other major metabolites present in leaf extracts (sugars, organic acids). Little or no effect of the presence of these compounds was observed (figure 8).

3.8. *Detection of condition-dependent variations in amino acids by GC-TOF-MS and HPLC*

To evaluate whether the two techniques were able to detect environmentally induced changes in leaf amino acid contents, we examined the response of photorespiratory amino acids to conditions that cause differences in the rate of photorespiration. Photorespiration is an important process in plants such as *Arabidopsis* that impacts on numerous leaf functions, including amino acid metabolism (Leegood *et al*., 1995; Stitt *et al*., 2002; Foyer *et al*., 2003). Four amino acids are directly involved in photorespiratory carbon and nitrogen recycling, the rate of which can be readily manipulated by light intensity or ambient CO_2 concentration. When these four amino acids were compared under conditions favouring or inhibiting photorespiration, significant changes were observed (table 7). The most dramatic changes were in Gly, for which contents were much higher in illuminated leaves kept in air than in darkened leaves or leaves surrounded by enriched CO_2. Both GC-TOF-MS and OPA-HPLC detected these changes, and also less dramatic changes in Ser (table 7). The data also confirmed the marked differences between absolute values of Glu and Gln measured by the two techniques. Despite these large differences in absolute values, both techniques detected condition-dependent changes in Gln and stability of Glu (table 7). Similar trends in Gln/Glu and Gly/Ser ratios were also observed by the two techniques. Both analytical methods revealed that light, with or without photorespiration, increased Gln/Glu compared to dark (though this trend was more apparent for HPLC analysis), and that photorespiration increased Gly/Ser (table 7).

4. Discussion

Amino acids are the organic products of nitrogen assimilation by plants, and are precursors of a wide range of cell components including proteins, nucleotides, chlorophyll, phenylpropanoids, lignin, and other nitrogen-containing compounds such as alkaloids (Morot-Gaudry *et al*., 2001; Stitt *et al*., 2002). Amino acid analysis is an essential part of studies of carbon–nitrogen interactions in plants and provides information on plant nutritional status and metabolic coordination (Foyer *et al*., 1994, 2003; Morcuende *et al*., 1998; Dutilleul *et al*., 2005). Data mining of amino acid profiles generated by HPLC has revealed interesting patterns pointing to possible metabolic and signalling crosstalk between pathways (Noctor *et al*., 2002; Fritz *et al*., 2006) and GC-MS analysis has shown that specific classes of amino acids can be induced by certain stresses (Broekling *et al*., 2005).

While GC-MS has become a method of choice for profiling small metabolites, it is useful to establish the type of information generated by this method. In the present study, we have taken a first step toward this aim by direct comparison of GC-MS data with values obtained by an established quantititative method for amino acid analysis. OPA derivatization of primary amines was introduced by Roth (1971) and has become widely used for quantitative analysis of amines in biological material, including plant tissues (e.g., Noctor and

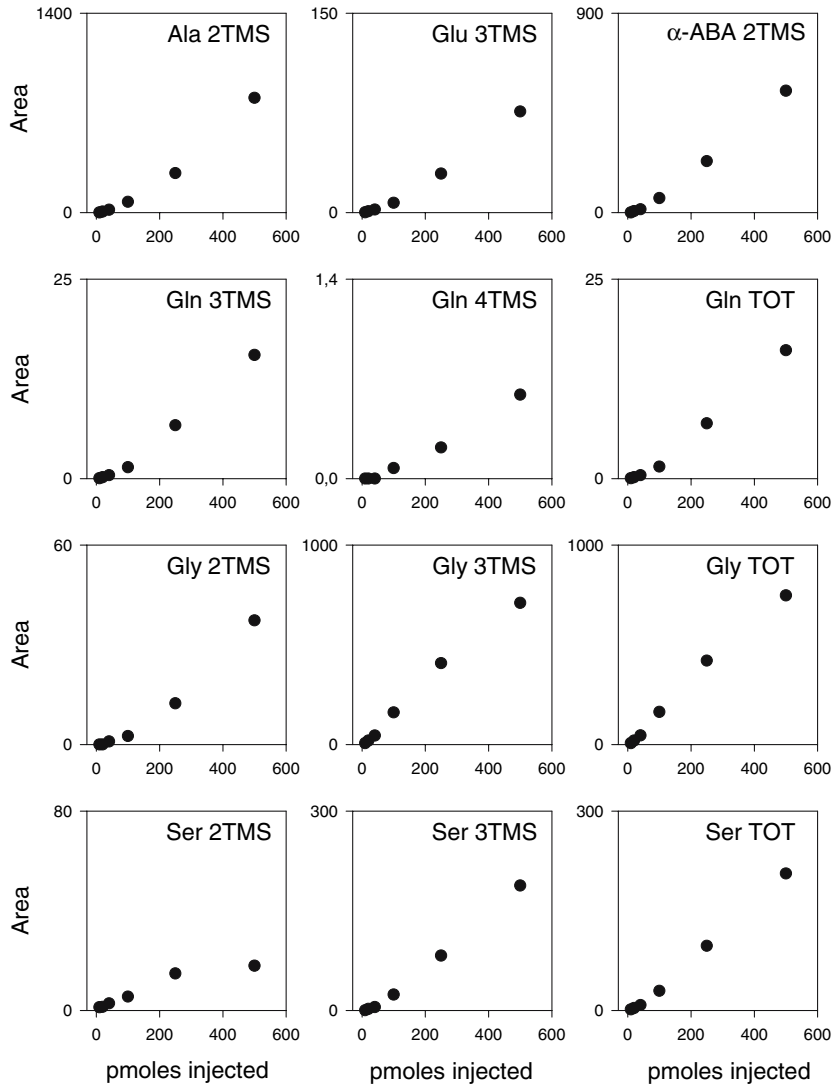

Figure 4. Typical standard curves for selected amino acids measured by GC-TOF-MS. Curves shown are representative and similar to those obtained for other amino acids. TOT, sum of different TMS derivatives for compound indicated.

Foyer, 1998; Noctor *et al.*, 2002; Fritz *et al.*, 2006). Fluorescence detection of OPA derivatives is sensitive down to sub-picomole quantities, though one factor that has to be taken into account is the instability of the derivatives of certain amino acids (Lindroth and Mopper, 1979). This problem can be readily overcome by automated pre-column derivatization. Using this approach, OPA-HPLC generated reproducible standard curves that were linear or close to linear over a 100-fold difference in concentration. To minimize possible matrix effects during the derivatization and potential differences in derivatization efficiency between injections, standards were prepared at relative concentrations that were similar to those found in leaf extracts and an amino acid internal standard was systematically included in extracts. Low between-injection and between-extract variabilities (tables 2 and 3), and high recoveries of metabolites through the extraction procedure (figure 5), support the robustness of the extraction and OPA-HPLC methods for absolute quantification of amino acids. Although minor differences in data between the two methods may be partly or wholly due to imprecisions in OPA-HPLC analysis, consistent marked discrepancies are very likely caused by inaccuracies in GC-TOF-MS.

The most abundant leaf amino acids in many plant species are Glu, Gln, Asp, Ser, Ala, and, depending on conditions, Asn and Gly; this has been found to the case in the present and in numerous previous studies using HPLC with OPA or other derivatizing reagent such as ninhydrin (e.g., Foyer *et al.*, 1994; Noctor and Foyer, 1998; Fritz *et al.*, 2006). Ser and Ala were also found to be amongst the most abundant amino acids in *Arabidopsis* leaves analysed by GC-TOF-MS. However, Glu, Gln, Asp, and Asn were significantly underestimated by this method. These compounds are central to

Table 3
Between-extract variability for amino acids measured by GC-TOF-MS and OPA-HPLC

Amino acid	GC-TOF-MS	OPA-HPLC
Ala	13.6	12.4
Arg	–	10.7
Asn	6.0	11.5
Asp	15.7	11.3
β-Ala	15.4	16.1
γ-ABA	44.7	34.2
Gln	11.6	4.6
Glu	20.1	8.9
Gly	18.3	13.5
homoSer	–	21.7
Ile	18.7	12.5
Leu	14.2	12.5
Lys	17.8	15.6
Met	–	12.4
Orn	61.6	17.0
Phe	19.2	13.5
Ser	11.8	7.0
Thr	17.9	11.7
Trp	23.5	12.4
Val	10.7	8.5

Values are % relative standard deviation of single injections of four independent extracts of a pre-homogenized leaf powder. Each injection was corrected relative to the internal standard, α-ABA. Arg, homoSer, and Met were not detected by GC-MS in all extracts.

Table 4
Estimation of biological variation in amino acids analysed by GC-TOF-MS and OPA-HPLC

Amino acid	GC-TOF-MS	OPA-HPLC
Ala	14.1	16.2
Arg	33.4	33.7
Asn	24.2	19.8
Asp	26.8	16.3
β-Ala	20.4	17.6
γ-ABA	20.7	17.9
Gln	41.5	30.8
Glu	45.3	13.8
Gly	39.3	76.0
homoSer	29.4	26.3
Ile	14.0	11.1
Leu	13.1	13.3
Lys	34.1	10.6
Met	26.4	63.9
Orn	29.1	73.9
Phe	23.7	15.5
Ser	24.1	20.1
Thr	24.2	21.9
Trp	23.5	17.6
Val	18.2	14.9

Values are mean % relative standard deviation of three experiments, each consisting of single injections of extracts of three different plants. Each injection was corrected relative to the internal standard, α-ABA

carbon–nitrogen interactions in plants. Gln and Glu are the major entry points of ammonia into organic compounds, and the amino groups of Glu and Asp and the amido group of Gln are the nitrogen source for most plant compounds containing this element (Morot-Gaudry *et al.*, 2001).

Absolute quantitation depends on efficient and stable extraction, and similar responses of metabolites in extracts and standards. The chemical heterogeneity of metabolites dictates that the choice of extraction medium is a crucial point (Gullberg *et al.*, 2004). Minimal sample preparation is a significant aim of high-throughput metabolomics and so we chose a simple methanol/water extraction with minimal sample clean-up in order to maximize the number of polar metabolites that can be analysed by the method. The high recoveries obtained by HPLC validate the suitability of the extraction method for amino acids. Other approaches seeking simultaneously to extract hydrophilic and lipophilic molecules use phase partitioning (Weckwerth *et al.*, 2004b; Broekling *et al.*, 2005). In this case, lipophilic molecules are eliminated from the methanol/water

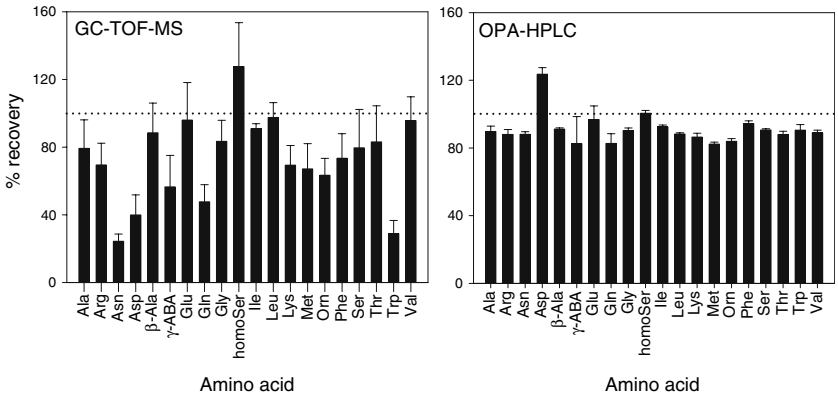

Figure 5. Recovery quotients for amino acids analysed by OPA-HPLC and GC-TOF-MS. Approximately 1 g of *Arabidopsis* leaf material was sliced into small pieces and mixed to produce 8 × 100 mg samples which were promptly frozen. Four samples were extracted into methanol/water (samples A) as in 'Materials and methods', while four were extracted into the same medium containing mixed standards at known concentrations (samples B). Recoveries for each sample B was calculated after subtraction of the mean leaf value obtained by analysis of samples A. The dotted line indicates 100% recovery.

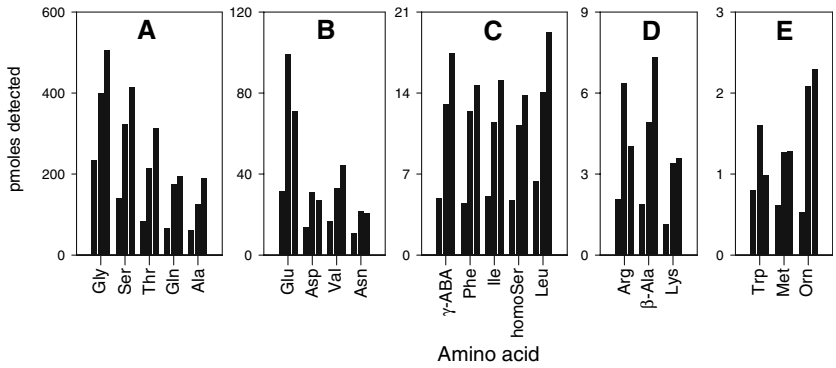

Figure 6. Detector response of amino acids analysed by GC-TOF-MS as a function of extract volume dried for derivatization. The three columns for each amino acid show 1 μL injections of a derivatized dried aliquot of (from left to right) 0.1, 0.2, and 0.3 mL 80% methanol extract. Amino acids are separated into five groups according to differences in abundance (note the difference in y-axis scaling between the graphs).

Table 5
Ratios between leaf contents measured by GC-TOF-MS and OPA-HPLC

Amino acid	GC-TOF-MS:OPA-HPLC
Ala	1.26 ± 0.09
Arg	0.12 ± 0.03
Asn	0.11 ± 0.01
Asp	0.07 ± 0.00
β-Ala	0.63 ± 0.09
γ-ABA	0.56 ± 0.07
Gln	0.07 ± 0.01
Glu	0.08 ± 0.01
Gly	1.10 ± 0.19
homoSer	0.78 ± 0.19
Ile	1.71 ± 0.28
Leu	1.17 ± 0.07
Lys	0.27 ± 0.04
Met	2.05 ± 0.53
Orn	0.53 ± 0.09
Phe	0.24 ± 0.01
Ser	0.61 ± 0.08
Thr	0.62 ± 0.09
Trp	0.08 ± 0.01
Val	1.13 ± 0.04

Values are means ± SE of ratios from 12 independent extracts.

fraction by prior partitioning into chloroform. When *Arabidopsis* leaf samples were extracted by this method, responses were similar (though slightly lower) than those obtained with the simpler methanol/water method (table 6). Underestimation of amino acids such as Glu, Gln, Asp, and Asn by GC-TOF-MS was therefore not specific to the methanol/water extraction method.

Despite the discrepancies in absolute quantities between GC-TOF-MS and HPLC, GC-MS yielded relatively reproducible data for most compounds. Although injection–injection variability was greater than for HPLC, extract–extract variation was similar and the two techniques detected similar condition- and genotype-independent biological variability. Equally, for photorespiratory amino acids, trends associated with light or CO_2 concentration were detected by both techniques. This suggests that even though many amino acids were not absolutely quantifiable by GC-TOF-MS, the technique nevertheless produces biologically useful data for the majority of these compounds. Table 8 summarizes the type of information that GC-TOF-MS analysis under our conditions produces for each amino acid. Where data are defined as relatively quantitative, the most important criteria were considered to be the results of figure 7 and, for photorespiratory amino acids, those shown in table 7.

Analysis by GC-TOF-MS involves solubilization in the derivatizing reagent, volatilization in the injector, and efficient ionization in the source. Incomplete solubilization and/or incomplete derivatization seems unable to explain why some compounds gave disparate results for extracts and standards. First, when extracts were enriched by pre-added standards, some compounds such as Glu that gave poor absolute quantification showed very good recovery quotients. Second, the response of standards was not significantly affected by adding high concentrations of sugars and organic acids found in leaf extracts (figure 8). Ion suppression by co-eluting compounds, recognized as a major problem in LC-MS (Annesley, 2003; Fernie *et al*., 2004), is not expected to be a significant problem during electron impact ionization under strong vacuum. Of the 20 amino acids analysed eight gave more than one TMS derivative. Of these, several (Asn, Gln, Ser, Thr, Phe) showed significant differences in relative abundance between standards and leaf extracts whereas others (Gly, Lys, Trp) showed similar relative abundance between extracts and standards. To overcome this problem we used the sum of all detected TMS derivatives for quantification, and this gave good results for Ser, Thr, and Gly. Moreover, poor quantification was also observed for some amino acids yielding only one TMS derivative such as Asp and Glu. Therefore, multiple TMS derivatives for a given amino acid, and the related problem of different relative abundance in extracts or standards, do not in themselves explain problems with quantification, though they do

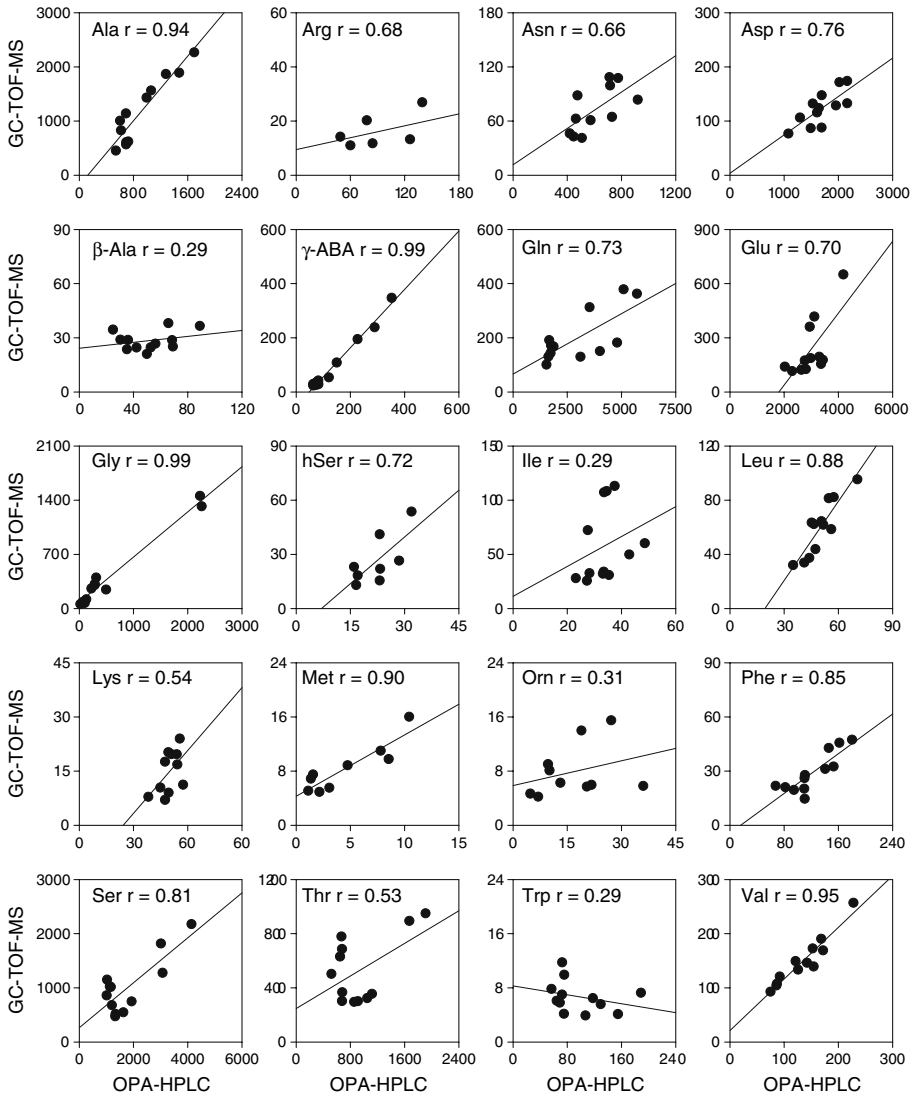

Figure 7. Relationship between leaf values (nmol g^{-1} fresh weight) measured by OPA-HPLC and GC-TOF-MS. Data are compared for up to 12 extracts. Where fewer than 12 points are shown, missing points correspond to extracts where the amino acid could not be measured by both techniques.

illustrate the potential complexity of matrix effects during the TMS derivation.

The choice of ion fragment for quantification was made on the dual basis of specificity and abundance, criteria that were in some cases in conflict. Comparison of tables 1 and 8 does not reveal any consistent correlation between fragment abundance and absolute quantifiability. For instance, the monitored ions for Arg and Trp, two of the amino acids that gave the poorest quantification, were relatively abundant components of the total ion current of the respective derivatives (table 1). Moreover, poor quantification was also observed for some amino acids that were amongst the most abundant (Glu, Gln) when measured by HPLC. The most quantitative data included both abundant amino acids (Ala, Ser) and less abundant ones (homoSer, Val), as well as Gly, whose contents vary considerably with the rate of photorespiration. It seems from our data that quantifiability by GC-TOF-MS may be at least partly related to amino acid structure. Compounds that gave the best quantification (Ala, Gly, homoSer, Leu, Ser, Val) all have simple aliphatic or hydroxyl-containing side chains. Amino acids that gave the most disparate results relative to OPA-HPLC have either complex aromatic or nitrogen-containing side chains (Arg, Lys, Phe, Trp) or additional carboxyl groups (Glu). Other amino acids with side-chain carboxyl or amide groups (Asn, Asp, Gln) also produced much lower values for GC-TOF-MS than OPA-HPLC. We tentatively suggest that the most important factor contributing to underestimation of absolute contents of certain amino acids by GC-MS is extract-specific problems in volatilization/injection.

Table 6
Comparison of GC-TOF-MS response for amino acids following leaf extraction into methanol/water and methanol/chloroform/water

Amino acid	Peak area ratio (methanol/water: $CHCl_3$/methanol/water)
Ala	1.46
Asn	1.69
Asp	1.40
γ-ABA	1.26
Gln	1.52
Glu	1.56
Gly	1.46
Ile	1.21
Leu	1.20
Phe	1.31
Ser	1.50
Thr	1.42
Val	1.21

Three independent leaf samples were extracted into methanol/water or methanol/chloroform/water (Weckwerth *et al.*, 2004a, b; see methods). Mean peak areas are for the major TMS derivatives of each compound (table 1) and were corrected for differences in total methanol/water volumes between the two methods.

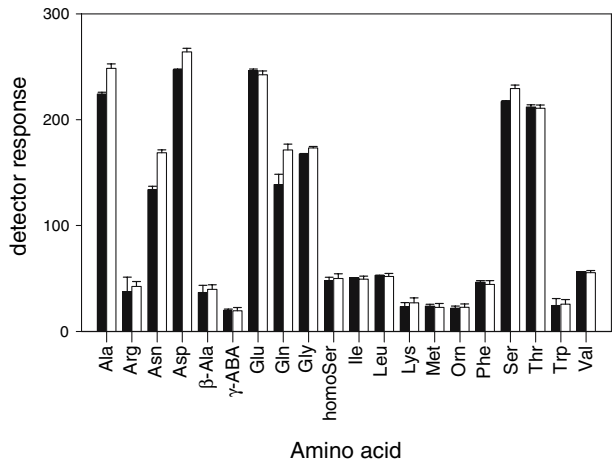

Figure 8. Effect of the presence of major sugars and organic acids during the derivatization on response factors of amino acids analysed by GC-TOF-MS. Black bars, mixed standards of amino acids were dissolved in water, spin-dried under vacuum, then derivatized. White bars, mixed standards of amino acids were dissolved in sucrose, glucose, fructose, malate, and citrate (all at 2 mM), spin-dried under vacuum, then derivatized. The concentrations of sugars and organic acids during the derivatization correspond to an extract of leaf material containing approximately 26 μmol g^{-1} fresh weight of each compound. Data are means ± SE of three derivatizations.

5. Concluding remarks

Our data identify several amino acids that can be quantified absolutely by GC-TOF-MS analysis of leaf extracts, a larger group for which GC-TOF-MS provides relative quantification, and a small group for which the information generated by GC-TOF-MS is at best semi-quantitative. It must be emphasized that the

Table 7
Effect of light and high CO_2 on leaf amino acids involved in photorespiration measured by GC-TOF-MS and OPA-HPLC

	Light, air		Dark, air		Light, 0.45% CO_2	
	Photorespiration		No photorespiration		No photorespiration	
Amino Acid	GC-MS	HPLC	GC-MS	HPLC	GC-MS	HPLC
Gln	0.29 ± 0.14	4.64 ± 1.36	0.18 ± 0.12	2.23 ± 1.12	0.31 ± 0.25	4.40 ± 0.56
Glu	0.31 ± 0.15	2.69 ± 0.58	0.33 ± 0.28	3.66 ± 0.46	0.38 ± 0.33	3.14 ± 0.22
Gly	1.01 ± 0.66	1.66 ± 1.01	0.08 ± 0.03	0.05 ± 0.07	0.12 ± 0.07	0.08 ± 0.03
Ser	1.76 ± 0.45	3.41 ± 0.64	0.58 ± 0.09	1.39 ± 0.21	0.94 ± 0.58	1.63 ± 0.43
Gln/Glu	0.95 ± 0.03	1.71 ± 0.12	0.62 ± 0.07	0.59 ± 0.13	0.84 ± 0.07	1.35 ± 0.13
Gly/Ser	0.53 ± 0.18	0.48 ± 0.17	0.14 ± 0.02	0.04 ± 0.03	0.13 ± 0.01	0.05 ± 0.01
	Ratios between mean values in different conditions					
	Light:Dark		Air:High CO_2			
	GC-MS	HPLC	GC-MS	HPLC		
Gln	1.61	2.08	0.94	1.05		
Glu	0.94	0.73	0.82	0.86		
Gly	12.6	33.2	8.4	20.8		
Ser	3.0	2.5	1.9	2.1		

Values are means ± SD of single injections of three extracts from different plants. Each injection was corrected relative to the internal standard, α-ABA. Data other than ratios are in μmol g^{-1} FW.

results are specific to our conditions of GC-TOF-MS analysis, but that these were based on or similar to previously published protocols (Wagner *et al*., 2003; Weckwerth *et al*., 2004b). Perhaps the most likely explanation of underestimation of amino acids by GC-TOF-MS is related to difficulties in volatilization and injection that are observed in extracts but not in mixed standards. As noted above, this appears to be at least partly related to the chemical structures of the amino acids analysed, which may promote interactions with the sample matrix that are not observed either in the simpler matrix of the mixed amino acid standards or standards supplemented with major sugars and organic acids (figure 8). Such problems could potentially be overcome by isotopically labelled internal standards for each amino acid or more onerous sample clean-up. However, as well as increasing cost and/or analysis time, these refinements would complicate one of the principal objects of non-specific metabolite profiling, which is to produce data on as many metabolites as possible with minimal sample preparation (Gullberg *et al*., 2004). While our data call attention to potential problems associated with quantification of metabolites in complex mixtures by GC-TOF-MS, amino acid analysis by this method simultaneously provides information on many other identifiable and as yet unidentified compounds that are undetectable by more specific techniques such as OPA-HPLC. Nevertheless, it is important to establish potential limitations concerning the information generated.

Lastly, it should be noted that the present study was performed on a single plant species, and that analysis of other biological samples may give better or worse agreement between the two techniques. We have also used the protocol shown in figure 1 to analyse leaf samples of tobacco grown at limiting or non-limiting soil nitrogen concentrations (samples kindly provided by Till Pellny, Rothamsted Research, UK, and Christine Foyer, University of Newcastle, UK). Very similar trends in amino acid abundance between the two conditions were detected by HPLC and GC-TOF-MS, including for amino acids such as Arg and Trp, which gave poor agreement in this study with *Arabidopsis*. Together with the data presented for photorespiratory amino acids analysed in *Arabidopsis* (table 7), these results suggest that GC-TOF-MS provides at least relative quantification for most amino acids. Nevertheless, some of the discrepancies that we report here indicate

Table 8
Summary of quantitative criteria of plant amino acid measurements by GC-TOF-MS

			Agreement with OPA-HPLC		
Amino acid	Response linearity in extracts	Recovery of added metabolite	Absolute	Relative	Type of quantification
Ala	Good	Good	Good	Good	Absolute
Arg	Poor	Good	Poor	Mediocre	Semi
Asn	Mediocre	Poor	Poor	Mediocre	Semi
Asp	Mediocre	Poor	Poor	Good	Relative
β-Ala	Good	Good	Mediocre	Poor	Relative/semi
γ-ABA	Good	Mediocre	Mediocre	Good	Relative
Gln	Mediocre	Mediocre	Poor	Good	Relative
Glu	Poor	Good	Poor	Good	Relative
Gly	Mediocre	Good	Good	Good	Absolute/relative
homoSer	Good	Good	Good	Good	Absolute
Ile	Good	Good	Mediocre	Poor	Relative/semi
Leu	Good	Good	Good	Good	Absolute
Lys	Mediocre	Mediocre	Poor	Mediocre	Semi
Met	Mediocre	Mediocre	Poor	Good	Relative
Orn	Mediocre	Mediocre	Mediocre	Poor	Semi
Phe	Mediocre	Mediocre	Poor	Good	Relative
Ser	Good	Good	Mediocre	Good	Absolute/relative
Thr	Good	Good	Mediocre	Mediocre	Relative
Trp	Poor	Poor	Poor	Poor	Semi
Val	Good	Good	Good	Good	Absolute

'Absolute' means that data can be accurately expressed relative to some physiological parameter such as tissue mass. 'Relative' means the data gives accurate information on changes in contents. 'Semi' means that changes can be detected, but the extent of change cannot be accurately determined. The type of quantification is assigned based on a combination of several criteria. Response linearity is summarized from figure 6. Recoveries are summarized from data of figure 5 by defining values over 70% as 'good', values from 50–70% as 'mediocre' and others as poor. For agreement with HPLC analysis, it is considered that some part of the variation may be due to inaccuracies in HPLC analysis as well as GC-TOF-MS. For absolute agreement, the mean values of table 5 are used, by defining GC-MS value: HPLC value 0.7–1.3 as 'good', 0.5–0.7 and 1.3–2.0 as 'mediocre', and others as 'poor'. Relative agreement with HPLC is estimated from figure 7 by defining r values over 0.7 as 'good', values between 0.5 and 0.7 as 'mediocre', and values below 0.5 as 'poor'. For Gln, Glu, Gly, and Ser, the data of table 7 are also taken into account in assigning the type of quantification.

that for certain compounds, an advisable approach would be to confirm observed changes by other techniques, where these are available.

Acknowledgements

We are grateful to Jean Bleton and Alain Tchapla (LETIAM, IUT de Chimie, Orsay) and Oliver Fiehn (UC Davis) for advice during the initial development of GC-MS analysis, and to Jean Bleton for careful reading of the manuscript in preparation. This work was partly financed by the French Genoplante programme, project GNP036 'MétaboP'.

References

Annesley, T.M. (2003). Ion suppression in mass spectrometry. *Clin. Chem.* **49**, 1041–1044.

Bino, R.J., Hall, R.D., Fiehn, O., Kopka, J., Saito, K., Draper, J., Nikolau, B.J., Mendes, P., Roessner-Tunali, U., Beale, M.H., Trethewey, R.N, Lange, B.M., Wurtele, E.S. and Sumner, L.W. (2004). Potential of metabolomics as a functional genomics tool. *Trends Plant Sci.* **9**, 418–425.

Broeckling, C.D., Huhman, D.V., Farag, M.A., Smith, J.T., May, G.D., Mendes, P., Dixon, R.A. and Sumner, L.W. (2005). Metabolic profiling of *Medicago truncatula* cell cultures reveals the effects of biotic and abiotic elicitors on metabolism. *J. Exp. Bot.* **56**, 323–336.

Dutilleul, C., Lelarge, C., Prioul, J.-L., De Paepe, R., Foyer, C.H. and Noctor, G. (2005). Mitochondria-driven changes in leaf NAD status exert a crucial influence on the control of nitrate assimilation and the integration of carbon and nitrogen metabolism. *Plant Physiol.* **139**, 64–78.

Fernie, A.R., Trethewey, R.N., Krotzky, A.J. and Willmitzer, L. (2004). Metabolite profiling: from diagnostics to systems biology. *Nat. Rev. Mol. Cell Biol.* **5**, 763–769.

Fiehn, O. (2003). Metabolic networks of *Cucurbita maxima* phloem. *Phytochemistry* **62**, 875–886.

Foyer, C.H., Noctor, G., Lelandais, M., Lescure, J.C., Valadier, M.H., Boutin, J.-P. and Horton, P. (1994). Short-term effects of nitrate, nitrite and ammonium assimilation on photosynthesis, carbon partitioning and protein phosphorylation in maize. *Planta* **192**, 211–220.

Foyer, C.H., Parry, M. and Noctor, G. (2003). Markers and signals associated with nitrogen assimilation in higher plants. *J. Exp. Bot.* **54**, 585–593.

Fritz, C., Müller, C., Matt, P., Feil, R. and Stitt, M. (2006). Impact of the C–N status on the amino acid profile in tobacco source leaves. *Plant Cell Environ.* **29**, 2055–2076.

Goodacre, R., Vaidyanathan, S., Dunn, W.B., Harrigan, G.G. and Kell, D.B. (2004). Metabolomics by numbers: acquiring and understanding global metabolite data. *Trends Biotechnol.* **22**, 245–252.

Gullberg, J., Jonsson, P., Nordström, A., Sjöström, M. and Moritz, T. (2004). Design of experiments: an efficient strategy to identify factors influencing extraction and derivatization of *Arabidopsis thaliana* samples in metabolomic studies with gas chromatography/mass spectrometry. *Anal. Biochem.* **331**, 283–295.

Hall, R.D. (2006). Plant metabolomics: from holistic hope, to hype, to hot topic. *New Phytol.* **169**, 453–468.

Hirai, M.Y., Klein, M., Fujikawa, Y., Yano, M., Goodenowe, D.B., Yamazaki, Y., Kanaya, S., Nakamura, Y., Kitayama, M., Suzuki, H., Sakurai, N., Shibata, D., Tokuhisa, J., Reichelt, M., Gershenzon, J., Papenbrock, J. and Saito, K. (2005). Elucidation of gene-to-gene and metabolite-to-gene networks in *Arabidopsis* by integration of metabolomics and transcriptomics. *J. Biol. Chem.* **280**, 25590–25595.

Leegood, R.C., Lea, P.J., Adcock, M.D. and Häusler, R.E. (1995). The regulation and control of photorespiration. *J. Exp. Bot.* **46**, 1397–1414.

Lindroth, P. and Mopper, K. (1979). High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with *o*-phthaldialdehyde. *Anal. Chem.* **51**, 1667–1674.

Morcuende, R., Krapp, A., Hurry, V. and Stitt, M. (1998). Sucrose feeding leads to increased rates of nitrate assimilation, increased rates of α-oxoglutarate synthesis, and increased synthesis of a wide spectrum of amino acids in tobacco leaves. *Planta* **206**, 394–409.

Morot-Gaudry, J.F., Job, D. and Lea, P.J. (2001). Amino acid metabolism in Lea, P.J. and Morot-Gaudry, J.F. (Eds), *Plant Nitrogen*. Springer Verlag, Berlin, pp. 167–211.

Nikiforova, V.J., Kopka, J., Tolstikov, V., Fiehn, O., Hopkins, L., Hawkesford, M.J., Hesse, H. and Höfgen, R. (2005). Systems rebalancing of metabolism in response to sulfur deprivation, as revealed by metabolome analysis of Arabidopsis plants. *Plant Physiol.* **138**, 304–318.

Noctor, G. and Foyer, C.H. (1998). Simultaneous measurement of foliar glutathione, γ-glutamylcystéine, and amino acids by high-performance liquid chromatography: comparison with two other assay methods for glutathione. *Anal. Biochem.* **264**, 98–110.

Noctor, G., Novitskaya, L., Lea, P.J. and Foyer, C.H. (2002). Co-ordination of leaf minor amino acid contents in crop species: significance and interpretation. *J. Exp. Bot.* **53**, 939–945.

Roessner, U., Wagner, C., Kopka, J., Trethewey, R.N. and 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. and Fernie, A.R. (2001). Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified plant systems. *Plant Cell* **13**, 11–29.

Roessner-Tunali, U., Hegemann, B., Lytovchenko, A., Carrari, F., Bruedigam, C., Granot, D. and Fernie, A.R. (2003). Metabolic profiling of transgenic tomato plants overexpressing hexokinase reveals that the influence of hexose phosphorylation diminishes during fruit development. *Plant Physiol.* **133**, 84–99.

Roth, M. (1971). Fluorescence reaction for amino acids. *Anal. Chem.* **43**, 880–882.

Stitt, M. and Fernie, A.R. (2003). From measurements of metabolites to metabolomics: an 'on the fly' perspective illustrated by recent studies of carbon–nitrogen interactions. *Curr. Opin. Biotechnol.* **14**, 136–144.

Stitt, M., Müller, C., Matt, P., Gibon, Y., Carillo, P., Morcuende, R., Scheible, W.R. and Krapp, A. (2002). Steps towards an integrated view of nitrogen metabolism. *J. Exp. Bot.* **53**, 959–970.

Sumner, L.W., Mendes, P. and Dixon, R.A. (2002). Plant metabolomics: large-scale phytochemistry in the functional genomics era. *Phytochemistry* **62**, 817–836.

Wagner, C., Sefkow, M. and Kopka, J. (2003). Construction and application of a mass spectral and retention time index database generated from GC/EI-TOF-MS metabolite profiles. *Phytochemistry* **62**, 887–900.

Weckwerth, W. and Fiehn, O. (2002). Can we discover novel pathways using metabolomic analysis?. *Curr. Opin. Biotechnol.* **13**, 156–160.

Weckwerth, W., Loureiro, M.E., Wenzel, K. and Fiehn, O. (2004a). Differential metabolic networks unravel the effects of silent plant phenotypes. *Proc. Natl. Acad. Sci. USA* **101**, 7809–7814.

Weckwerth, W., Wenzel, K. and Fiehn, O. (2004b). Process for the integrated extraction, identification, and quantification of metabolites, proteins, and RNA to reveal their co-regulation in biochemical networks. *Proteomics* **4**, 78–83.