Journal of Chromatography B, 878 (2010) 2199–2208

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Microscale analysis of amino acids using gas chromatography–mass spectrometry after methyl chloroformate derivatization

Wen-Ping Chen[a,c,*], Xiao-Yuan Yang[b,c], Adrian D. Hegeman[a,c], William M. Gray[b,c], Jerry D. Cohen[a,c]

[a] Department of Horticultural Science, University of Minnesota, Saint Paul, MN 55108, United States
[b] Department of Plant Biology, University of Minnesota, Saint Paul, MN 55108, United States
[c] Microbial and Plant Genomics Institute, University of Minnesota, Saint Paul, MN 55108, United States

ARTICLE INFO

Article history:
Received 8 March 2010
Received in revised form 18 June 2010
Accepted 25 June 2010
Available online 1 July 2010

Keywords:
Arabidopsis
GC–MS
SPE column
Stable isotope
Metabolite profiling
Alkyl chloroformate

ABSTRACT

To conduct studies of stable isotope incorporation and dilution in growing plants, a rapid microscale method for determination of amino acid profiles from minute amounts of plant samples was developed. The method involves solid-phase ion exchange followed by derivatization and analysis by gas chromatography–mass spectrometry (GC–MS). The procedure allowed the eluent to be derivatized directly with methyl chloroformate without sample lyophilization or other evaporation procedures. Sample extraction and derivatization required only ca. 30 min and quantification of the 19 amino acids eluted from the cation exchange solid-phase extraction step from a single cotyledon (0.4 mg fresh weight) or three etiolated 7-day-old *Arabidopsis* seedlings (0.1 mg fresh weight) was easily accomplished in the selected ion monitoring mode. This method was especially useful for monitoring mass isotopic distribution of amino acids as illustrated by *Arabidopsis* seedlings that had been labeled with deuterium oxide and ^{15}N salts. Sample preparation was facile, rapid, economical, and the method is easily modified for integration into robotic systems for analysis with large numbers of samples.

1. Introduction

Two significant categories of current biological research in the post-genomic era have been the comprehensive analysis of metabolites (metabolomics) [1–3] and expressed proteins (proteomics) [4–6] in any given biological system in order to make connections between gene expression and the mechanisms of metabolic regulation in response to internal stimulation and external perturbation. Due to the important functions of amino acids, they have been important targets for metabolic profiling. Not only serving as the monomeric units of proteins, amino acids play central roles as intermediates in many important metabolic pathways, such as the biosynthesis of nucleotides, vitamins and secondary metabolites. Thus, amino acids have been analyzed frequently by many methods, including high performance liquid chromatography [7,8], gas chromatography (GC) [1,2], liquid chromatography (LC) [9], and capillary electrophoresis (CE) [10] coupled to a mass spectrometer (MS). Mass spectrometry remains the primary analytical and detection system for metabolic and peptide profiling due to the accuracy and the information content of such analyses, thus facilitating the identification and measurement of large numbers of biochemical molecules. In addition, MS methods add the capability of monitoring isotope distributions of molecules for metabolic flux analysis [1–5].

GC–MS technology has been demonstrated as a facile and sensitive method for metabolite profiling. However, fast and efficient derivatization techniques are needed to obtain the full potential of GC–MS for high throughput analysis. Various trimethylsilylation methods have been documented and are used often for derivatization of amino and organic acids in preparation for GC–MS analysis [1,10–16]. The trimethylsilyl derivative has many advantages, including good fragmentation during electron impact GC–MS analysis and a significantly high detection response across a range of compounds. However, the derivatization procedure can be relatively time consuming (30–60 min), often requires heating, must be carried out under anhydrous conditions, and the resulting derivatives may not be stable. The latter issue being important should additional post-derivatization purification be required as the combination of procedures can create artifacts [17]. Moreover, the procedure results in the addition of a large and bulky group to the amino acids, which creates problems for precise measurement of isotopic abundance of the target structures [11–16]. In addition, strong isotope and concentration effects have also been observed [14,15].

Recently, derivatization methods for profiling amino and organic acids in biological fluids using alkyl chloroformates have

* Corresponding author at: University of Minnesota, Department of Horticultural Science, 305 Alderman Hall, 1970 Folwell Ave., Saint Paul, MN 55108, United States. Tel.: +1 612 624 6735; fax: +1 612 624 4941.
E-mail address: chenx071@umn.edu (W.-P. Chen).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.06.027

received considerable attention and are gaining popularity. Husek [18] first introduced the basic procedure, which was then investigated by many laboratories for its potential for quantitative and reproducible derivatization of amino acids in different matrices [2,19–32]. Using alkyl chloroformate as a derivatizing reagent has several advantages (1) a rapid one-step reaction can be carried out directly in aqueous solution without the need for sample heating, (2) the reagent costs are very low, and (3) it is easy to separate the resulting derivatives from the reaction mixture using an organic solvent, resulting in less chemical contamination. In addition to these advantages, the method can be easily automated with commercially available analytical robotics [26]. Importantly, this dervitization method adds only a relatively low molecular weight group to the derivatized analytes, which makes the calculation of isotopic abundance simple and maintains accuracy. A commercial kit based on propyl chloroformate derivatization is available [31] and has been applied for analysis of amino acids in biological samples [26,32]. However, with a reported LOQ of 2.5 nmoles per injection, a significantly higher cost per sample, and added manipulations required at the SPE step, it does not appear to be suitable for microscale GC–MS analyses at the levels reported here and would also be difficult to adapt for automation.

The standard trimethylsilylation or alkyl chloroformate protocols typically involve sample sizes of a hundred milligrams fresh weight or more in order to obtain reliable MS peaks for compound identification and quantification. Incorporation of solid-phase extraction (SPE) columns to the standard procedures for amino acid purification and pre-concentration dramatically reduces sample processing time, increases bench to bench consistency and it allows the procedure to be applied to analyze smaller samples [32–35]. In this report, a rapid microscale method for determination of amino acid profiles from minute amounts of plant samples was developed. That is especially suitable for both quantification as well as analysis of amino acid isotopic distributions. Amino acids were first purified using microtips packed with 5 mg DOWEX 50-WX2-200 (H^+) strong cation exchange (SCX) resin and using methyl chloroformate as the amino acid derivatization reagent for GC–MS analysis. We show that following elution of the amino acids with ammonium hydroxide in methanol it is possible to derivatize directly with methyl chloroformate, without the requirement of sample lyophilization or other solvent removal steps. Using this SPE microtip and single solution elution/derivatization protocol, it is possible to monitor amino acid profiles, as well as amino acid isotopomer distributions, from less than 5 mg fresh weight of most plant tissues. The average processing time from sample extraction to GC–MS analysis was under 30 min. Where large sample numbers need to be processed, this method has a potential to be integrated into an automated robotics system for amino acid isolation employing either commercial or self-packed SPE/SCX formats.

2. Experimental

2.1. Chemicals

The amino acid standard mixture was purchased from Thermo Scientific-Pierce (Rockford, IL, USA). Individual amino acids (free base) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Stock standards (2 mg/mL) were prepared in either 10 mM hydrochloric acid or 1:1 (v/v) 8 M NH_4OH:methanol and stored at −20 °C. The derivatizing agent, methyl chloroformate, was obtained from Sigma–Aldrich (Saint Louis, MO, USA) and repackaged into 25 mL bottles under nitrogen gas in a glove bag. [^{13}C]-Labeled algal amino acid mixture, 1,2-[^{13}C]-glycine, [^{13}C]-methanol, [^{15}N]-salts (KNO_3 and $Ca(NO_3)_2$), and deuterium oxide (^2H_2O) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). All other chemicals were analytical-reagent grade.

2.2. Plant material

Arabidopsis thaliana ecotype Columbia (Col-0) seedlings grown on agar in Petri plates were used as a source of plant tissue. Seeds were surface sterilized with 10% (v/v) bleach containing 0.1% (v/v) Triton X-100 for 10 min then rinsed with distilled water three times. For stable isotope labeling, seeds were sown onto *Arabidopsis thaliana* salts (ATS) medium [36] solidified with 0.8% agar (A7049, Sigma–Aldrich, St. Louis, MO, USA) containing either 30% ^2H_2O (99 atom%) or [^{15}N]-salts (98.5 atom%) and grown under continuous illumination (100 μmol m^{-2} s^{-1} supplied by cool white fluorescent bulbs). To obtain etiolated *Arabidopsis* seedlings, seeds were germinated in complete darkness at room temperature in Petri dishes containing half-strength Murashige and Skoog (MS) medium and 100 μM gibberellin A_4. The individual cotyledons or whole etiolated seedlings were each harvested 7 days after germination, weighed, frozen in liquid nitrogen and stored at −80 °C before analysis.

2.3. Preparation of pipette tips filled with strong cation exchange resin

During method development, the Dowex 50W-X2 strong cation exchange (SCX) resin with mesh size at 200–400 was chosen because of its high capacity, easy availability, handling ease and, because of a low degree of divinylbenzene cross-linking, it has higher recoveries for the aromatic amino acids [29,31]. When preparing the SCX tips, a small amount of dimethyldichlorosilane (DMCS)-treated glass wool (Alltech, Deerfield, IL, USA) was place as a bed support at the dispensing end of pipette tips (200 μL, RT-250, Rainin, Oakland, CA, USA). Approximately 5 mg (~7 mm measured from the glass wool support) of deionized water-rinsed Dowex 50WX2-200 (H^+ form) strongly acidic cation exchange (SCX) resin (Sigma–Aldrich, St. Louis, MO, USA) in a water slurry was then transferred into the pipette tip using a clean glass pipette. After rinsing with 100 μL of methanol 5 times, followed by 100 μL of glass distilled water 5 times, the tips were ready to be used. Alternatively, commercial SCX tips (TT2-TWSCX.96, Glygen Corp, Columbia, MD, USA), based on their modified Top-tip format, proved successful and are more convenient for routine use.

2.4. Amino acid extraction

Tissues excised from *Arabidopsis* seedlings were transferred to microcentrifuge tubes, weighed, and then ground using disposable pellet pestles (Kimble Chase Kontes, Vineland, NJ, USA) in 120 μL of 10 mM HCl in the presence of 5 μL of methionine sulfone (100 μg/mL) or a [^{13}C]-labeled amino acid mixture as the internal standard. After vortexing for 15 min at room temperature, samples were centrifuged at 14,000 × *g* for 3 min. The supernatant was removed and subsequently the amino acids bound to the SPE/SCX tips by aspirating and dispensing 10 times using a 200 μL pipettor. The SPE/SCX tips were then washed with 100 μL of 80% methanol by aspirating and dispensing to waste at least 3 times before the amino acids were eluted from the resin with 25 μL of freshly prepared 1:1 (v/v) 8 M NH_4OH:methanol by aspirating and dispensing 5 times into 250 μL conical glass vials (sold as GC autosampler inserts). Alternatively, to increase the speed of analysis when processing a significant number of samples, the pipetting steps were performed using a multichannel pipettor.

If 20 mg or more of tissues were extracted, samples in 1 mL of 10 mM HCl were ground using a grinding mill (MM300, Retsch, Newtown, PA, USA) then SCX solid-phase extraction columns

Table 1
Mass fragment ions of *N*-methoxycarbonyl amino acid methyl esters generated by 70 eV electron impact GC–MS analysis.

Amino acid[a]	Retention time (min)	Molecular ion (*m*/*z*)	Major fragment ions (*m*/*z*)	Detected fragment[b] (*m*/*z*)
Glycine	6.01	147	88	88
Alanine	6.04	161	102, 88	102
Valine	7.00	189	146, 130, 115, 98	130
Leucine	7.52	203	144, 115, 102, 88	144
Iso-leucine	7.61	203	144, 115, 101, 88	144
Threonine (Thr-OMe)	7.68	205	147, 115, 100, 88	147
Proline	7.86	187	128, 84	128
Asparagine	7.93	262	146, 127, 95	127
Aspatic acid	8.28	219	160, 128, 118, 101	160
Serine (Ser-OMe)	8.65	191	176, 144, 114, 100, 88	100
Glutamine	8.75	276	141, 109, 82	141
Glutamic acid	8.95	233	201, 174, 142, 114	174
Methionion	9.06	221	147, 128, 115	221
Cysteine	9.54	192	192, 176, 158, 146, 132	192
Phenalalanine	9.69	237	178, 162, 146, 131, 103, 91	162
Lysine	11.05	276	244, 212, 142, 88	142
Histidine	11.37	285	254, 226, 210, 194, 140, 81	210
Tyrosine	11.90	296	252, 236, 220, 192, 165, 146, 121	236
Tryptophan	13.17	276	130	130

(a) Arginine was not detected following derivatization by this method, and threonine and serine were detected as N-MOC methyl esters with methyl hydroxylether side chains (Thr-OMe and Ser-OMe) and (b) major ion used for detection of the specific amino acid.

(Extract-Clean, 100 mg, Alltech [these columns contain a 50 μm Dowex 50-type sulfonated polystyrene-divinylbenzene copolymer resin]) were used for amino acid purification with elution aided by using a vacuum manifold. In this case, the column was washed with 2 mL of 80% methanol twice after sample loading, then amino acids were eluted with 250 μL of 1:1 (v/v) 8 M NH_4OH:methanol (yielding pH > 9.0).

2.5. Amino acid derivatization

The analyte in 1:1 (v/v) 8 M NH_4OH:methanol was directly derivatized by addition, with mixing, of 2.5 μL of pyridine and 2.5 μL of methyl chloroformate into the 250 μL glass vial insert. To separate the MCF derivatives from the reaction mixture, 50 μL of chloroform and 50 μL of a 50 mM sodium bicarbonate solution were added sequentially and mixed well until a clear bottom phase was obtained. The bottom (chloroform) layer was transferred to a new 250 μL GC insert vial containing a few crystals of sodium sulfate, followed by brief vortex mixing. The dry sample was then transferred to a new 50 μL GC insert vial for GC–MS analysis. These procedures were all conducted in a fume hood and only positive displacement pipettes (Microman; Gilson, Middleton, WI, USA) were used. If the amino acids were extracted by using a Extract-Clean, 100 mg SPE column, 50 μL of analyte was then used for derivatization in a 6× 50 mm disposable glass tube (Fisher Scientific, Waltham, MA, USA) with 5 μL of methyl chloroformate in the presence of 5 μL of pyridine, followed by 90 μL of chloroform and 90 μL of 50 mM sodium bicarbonate solution for partitioning. Derivatized samples could be stored at −20 °C and analyzed within 2 days, as necessary.

2.6. GC–MS analysis

All GC–MS analyses were performed using a Hewlett-Packard 5890 (GC)/5970 mass selective detector (MSD) in electron impact (EI) mode (70 eV) with a system equipped with a fused silica capillary column (HP-5MS, 30 m × 25 mm ID, 0.25 μm film thickness; Agilent J&W Scientific, Folsom, CA, USA). The 2 μL sample was injected using the splitless mode. The oven temperature was initially held at 70 °C for 3 min. Thereafter the temperature was raised at 25 °C/min until 280 °C and held for 5 min. Helium was used as carrier gas and delivered at a constant flow rate at 1 mL/min. The injector temperature was set at 240 °C and the interface temperature was 290 °C. Mass spectra of the MCF-derivatized amino acids and internal standards were obtained in either the full-scan (50–350 *m*/*z*) or selected ion monitoring (SIM) acquisition modes. The retention times, molecular ions and major fragment ions of the 19 derivatized amino acids that could be monitored are shown in Table 1. The ions selected to detect specific amino acids in SIM mode are also indicated.

2.7. Quantification

Absolute quantification of amino acids (except arginine) was performed by analyzing standard solutions containing equimolar amounts of all amino acids. The amino acid standard mixture contain all amino acids except glutamine, asparagine and tryptophan, which are unstable in acidic solution. Those three amino acids were thus prepared separately as the other mixture. For calibration, the two mixtures were mixed in equal amounts to yield a final concentration of 500 μM for each compound except for Cys at 250 μM. The mixture was further diluted to final concentrations of 50 and 5 and 0.5 μM, respectively. Fifty microliters of each diluted sample was pipetted into a 250 μL GC insert vial followed by the addition of the [^{13}C]-labeled amino acid internal standard mixture before being derivatized as described above. The amino acids were normalized by the peak area of the [^{13}C]-labeled amino acids for the generation of calibration curves in the range of 0.5–500 μM. Limit-of-detection (LOD) is defined as the concentration of analyte required to give a signal equal to the background (blank) plus three times the standard deviation of the blank [IUPAC Gold Book, http://goldbook.iupac.org/L03540.html and 37]. Limit-of-quantification (or LOQ) was defined by the lower and upper limits of quantification where the lower limit is ten times standard deviation above the mean blank value [37]. A total of five replicates were used to determine LOD and LOQ for each amino acid using the method presented in this study. The sample recovery values represent the peak area differences in percentage from five independent analyses of 25 nmole/mL of amino acid standard mixture following the whole procedure with and without SPE clean-up with the commercial SCX tips.

2.8. Mass isotopomer distribution analysis

IDCalc software shared by Dr. Michael MacCoss from the University of Washington was used to generate the theoretical isotope distributions for the amino acids measured by

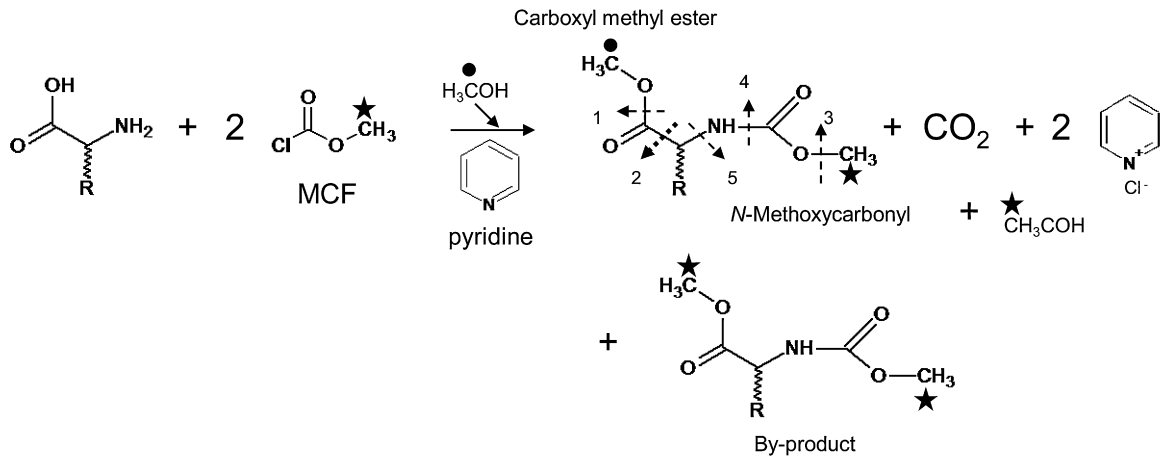

Fig. 1. Reaction mechanism of amino acid derivatization with methyl chloroformate in the presence of methanol and pyridine. The methyl residue (round dot) at the carboxyl group of an amino acid is from methanol via an intermediate exchange reaction after the reaction of methyl chloroformate with the carboxyl group and the methoxycarbonyl residue is formed by the direct reaction with methyl chloroformate. The methyl group (star) in methyl chloroformate is recycled to form methanol. The carboxyl methyl ester residue often is lost during fragmentation in the mass spectrometer (thick arrow 2) resulting in a major fragment ion with mass $(M-59)^+$.

mass spectrometry employing the method reported by Kubinyi [38].

3. Results and discussion

3.1. Derivatization reaction

The proposed reaction of amino acids with MCF in the presence of methanol and pyridine is shown in Fig. 1. It has been proposed [23–25] that both the amino and the carboxyl groups of the amino acids readily react with MCF to form an intermediate, i.e., a mixed anhydride containing 2 *R″* groups of the chloroformate before the stable products, *N*(*O*,*S*)-methyl methoxycarbonyl esters of amino acids. The main product containing the alkyl group *R′* from the alcohol is likely formed by an exchange reaction with the alcohol R′OH; a by-product is, however, formed by decarboxylation of the mixed anhydride, induced by pyridine, to yield the alkyl ester containing the *R″* chain from the chloroformate (by-product, Fig. 1). To further characterize these reactions, we carried out a series of experiments using mixtures of 1,2-[^{13}C]-glycine and [^{13}C]-methanol combined with either MCF or ethyl chloroformate (ECF). The mass spectral fragmentation pattern for derivatized glycine is shown in Fig. 2. The most abundant fragment was at $m/z = 147 - 59 = 88$ (Fig. 2A), which should be due to the loss of the acylium ion $[CO_2CH_3]^+$ derived from the cleavage of the C–C bond in the α-position to the carbonylic function of the esterified carboxyl group. It is unlikely that the loss of the $[CO_2CH_3]^+$ ions from the cleavage of the C–N bond of the amidic bond formed by methoxycarbonylation of the aminic group is occurring, because the major fragment of the ECF- and MCF-derivatized glycine would, in that case, have the same *m*/*z*, and as shown in Fig. 2D, this is not the case. The other characteristic ion at *m*/*z* 59, however, could arise from both the etherified carboxyl group and the methoxycarbonyl aminic group, because both ions *m*/*z* 59 and *m*/*z* 60 (59 + 1a; a, α-carbon from glycine) were observed when [^{13}C]-labeled glycine and unlabeled methanol were used (Fig. 2B) and both ions *m*/*z* 59 and $m/z = 59 + 1a + 1b$ (b, carbon from methanol) were detected when both [^{13}C]-glycine and [^{13}C]-methanol were used (Fig. 2C). Also, an extra ion *m*/*z* 59 + 1 was detected when both labeled glycine and labeled methanol were used in the reaction (Fig. 2C). This is likely to be the acylium ion $[CO_2CH_3]^+$ with a labeled carbon from glycine and the methyl group from the chloroformate rather than from methanol. This result suggests that the by-product was also formed in the reaction, but as only a minor product. These results concerning the reaction mechanism are consistent with previous models [23–25]. In those models, the R′ group in the esterificated carboxyl group of the derivatized amino acid is from the R′ group of the alcohol via an exchange reaction between the anhydride intermediate and the alcohol to yield the main product. As a result, the methyl group of the chloroformate would be recycled to form methanol and a CO_2 released. We also established that the major fragment ion frequently seen in the spectrum results from the loss of the acylium ions $[CO_2CH_3]^+$ at the carboxylic ester group of derivatized amino acids, instead of the loss of methoxycarbonyl aminic group.

3.2. Amino acid extraction and derivatization in the presence of NH_4OH

Amino acid extraction using SPE often involves sample binding to a strong cation exchange resin followed by methanol/water washing then elution with ammonium hydroxide (NH_4OH) [33–35]. Before amino acid derivatization, the eluent, NH_4OH, present in the samples is typically removed by either SpeedVac [33] or by a stream of nitrogen gas [35]. Because evaporation can take from 30 min to several hours to complete, appropriate equipment for evaporation may not always be available, and drying steps frequently result in sample losses, it is desirable to eliminate evaporation steps. We found that the methanol required for the derivatization reaction could be added simultaneously with MCF to the sample in the elution buffer (1:1, v/v; 8 M NH_4OH:methanol), allowing samples to be eluted and derivatized in the same small volume. Using this protocol, one or more samples can be ready for GC–MS analysis in less than 30 min.

As a consequence of elimination of a drying step, NH_4OH is derivatized in the reaction which is seen as an additional peak (5.5 min) that has a retention time shorter than Gly and Ala (Fig. 3) and does not interfere with the analysis. Previously, it was reported that ammonia could lead to diminished derivatization yields of amino acids [30]; however, this appears to be easily compensated for by adding a larger amount of alcohol and RCF [28]. Our results confirm that NH_4OH in the reaction did not significantly reduce the yields of derivatized amino acids over that obtained with standards prepared with NaOH (data not shown) as long as there was an excess of methanol and MCF present. In fact, this procedure resulted in improved yields as a consequence of the fewer manipulation and evaporation steps involved.

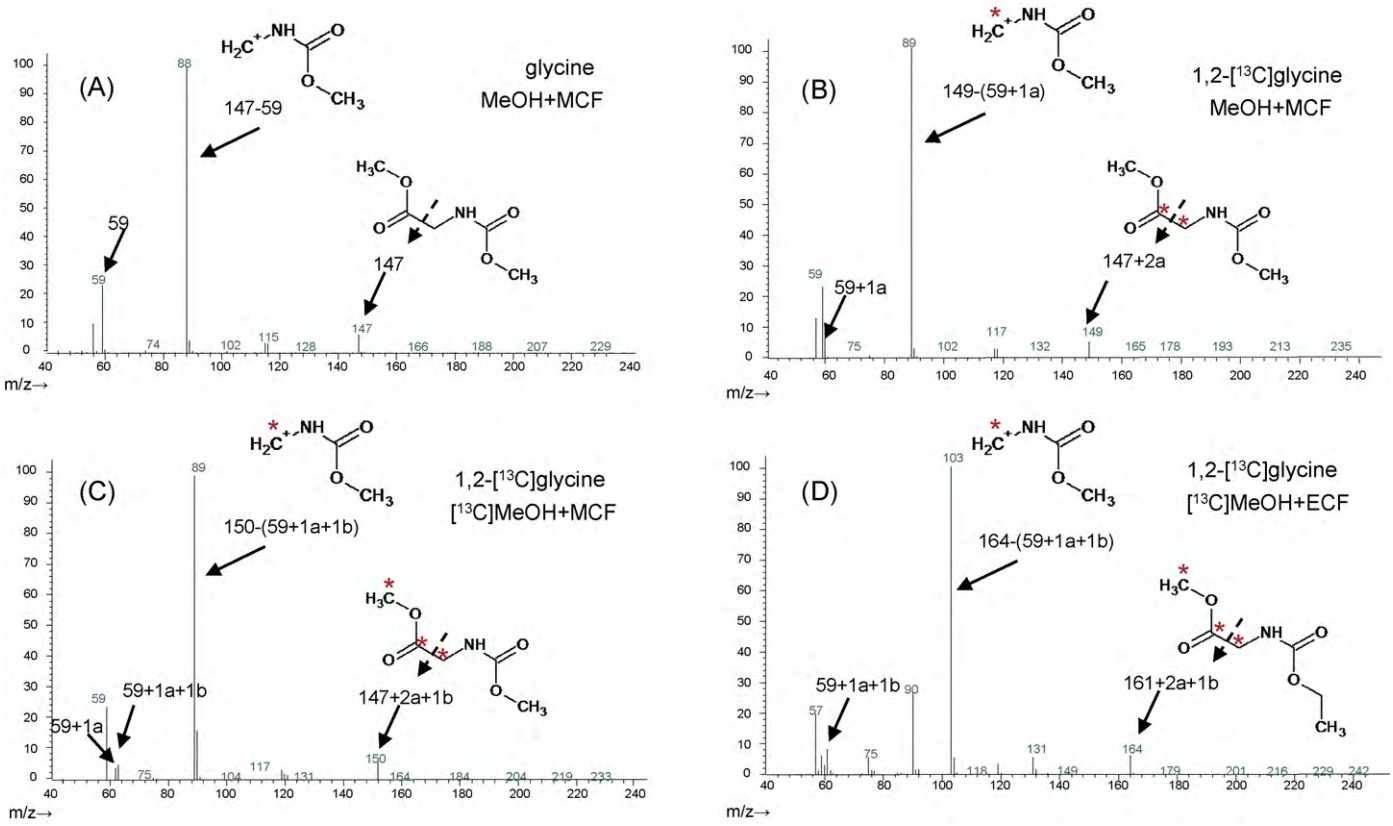

Fig. 2. Verification of derivatization and fragmentation mechanisms with chloroformate using [^{13}C]-labeled glycine and methanol. The molecular ion of N-ethoxycarbonyl glycine methyl ester has *m*/*z* 147. The major fragment ion *m*/*z* 88 is the result of the loss of carboxyl methyl ester residue of the molecular ion confirmed by the correct mass shift of the *m*/*z* 88 and *m*/*z* 59 ions due to the contribution from [^{13}C]-atoms. Note: a, b indicate carbon from the glycine backbone and methanol, respectively. Red stars indicate [^{13}C]-carbon atoms.

3.3. GC–MS analysis of amino acids

The method for derivatization and subsequent separation of amino acids was tested on a mixture containing all 20 amino acids found in proteins. The analytes eluted from the GC column between 6.01 and 13.17 min in the following order: Gly (6.01), Ala (6.04), Val (7.00), Leu (7.52), Ile (7.61), Thr (7.68 with tailing as Thr-OMe), Pro (7.86), Asn (7.93), Asp (8.28), Ser (8.65, as Ser-OMe), Gln (8.75), Glu (8.95), Met (9.06), Cys (9.54), Phe (9.69), Lys (11.05), His (11.37), Tyr (11.90) and Trp (13.17) (Fig. 3). The elution order was determined by injection of derivatized analytical standards of each amino acid individually onto the GC–MS and the identity of the product peak confirmed by examining the resulting spectrum. Using this method, the only amino acid not detected was arginine. The lack of detectable arginine has been accounted for either by the possible low reactivity of the guanidine group under these derivatization conditions [19,25] or by the thermal instability of the derivative that carries a free guanidine group [26]. The detection of both Gln and Asn using this method is in agreement with the result of Kaspar et al. [26] but in contrast with the result

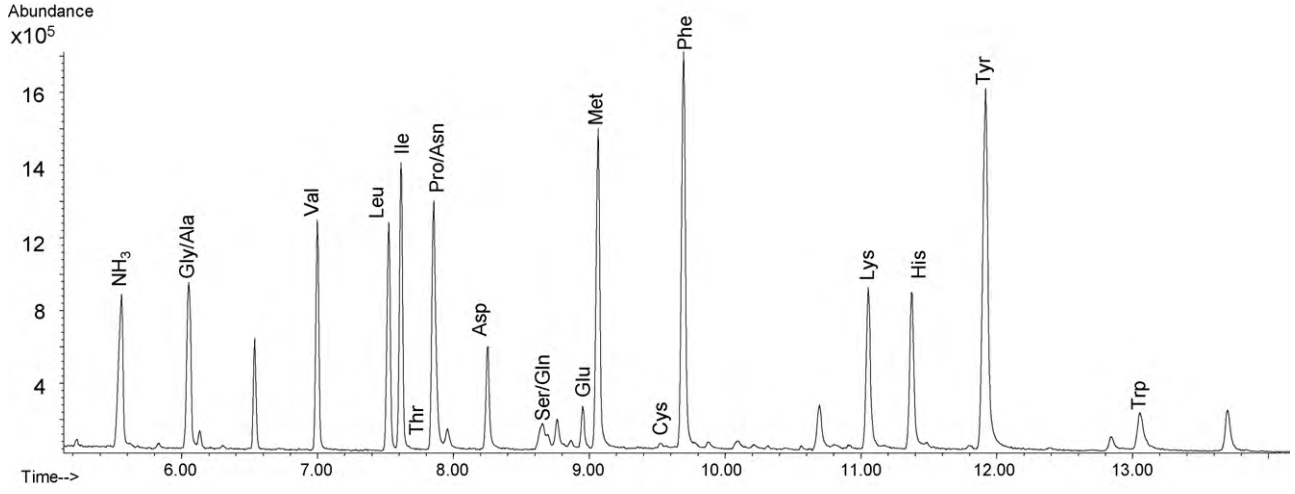

Fig. 3. Typical GC–MS chromatogram from the analysis of an amino acid standard mixture after derivatization with methyl chloroformate in the presence of NH_4OH.

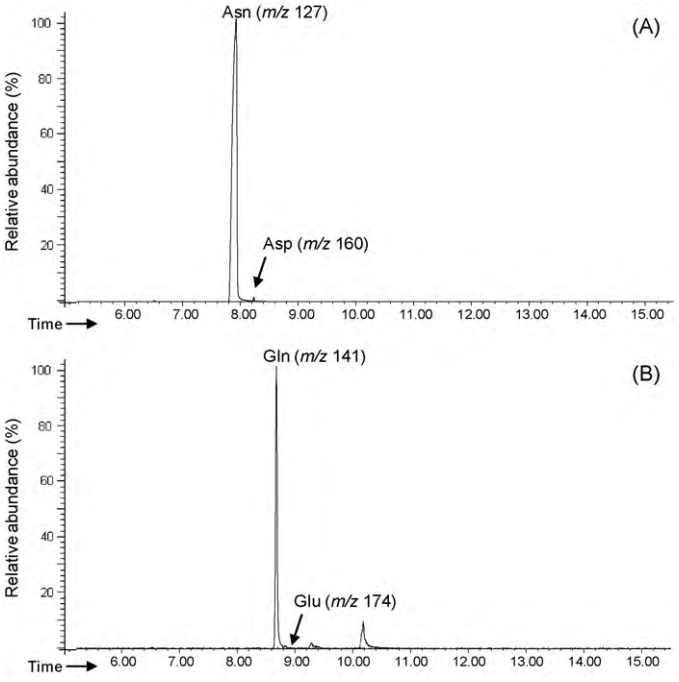

Fig. 4. Analysis of the possible conversion of asparagine to aspartic acid and glutamine to glutamic acid during purification and derivatization. Extracted ion chromatograms were performed to screen for the signature ions *m*/*z* 127 (asparagine) and *m*/*z* 160 (aspartic acid) following full-scan GC–MS analysis for asparagine (A), and signature ions *m*/*z* 141 (glutamine) and *m*/*z* 174 (glutamic acid) in the analysis for glutamine (B). A clear aspartic acid peak was seen in the asparagine sample (A, arrow). However, no clear glutamic acid peak was seen in the glutamine sample run (B, arrow indicates the expected retention time for glutamic acid). The two unlabeled peaks in the chromatogram in panel B were contaminates that also had a *m*/*z* 141 ion.

of Casal et al. [19], in which both amino acids were not detected. Casal et al. [19] hypothesized that there might be a conversion of Asn and Gln to Asp and Glu, respectively, during derivatization with alkyl chloroformate, resulting in the lack of both amino acids on GC chromatograms. To investigate this notion, we looked for Asp and Glu peaks in the Asn and Gln chromatograms by monitoring signature ions for each of these amino acids (Fig. 4). We found that only ~1.5% of Asn is converted to Asp (Fig. 4A) and less than 0.1% of Gln is converted to Glu (Fig. 4B). Likely because of the use of NH_4OH in the elution and derivatization process, the conversion of Asp and Glu to Asn (*m*/*z* 127) and Gln (*m*/*z* 141) was also a concern as ~17% of Asp converted to Asn and ~7% Glu converted to Gln. For turnover analysis, this did not introduce a significant error due to the similarity in metabolic rates. However, if necessary either (1) the use of ^{15}N-labeled NH_4OH to allow a correction for the conversion, or (2) the substitution of another base can be considered. We have found that triethylamine is a suitable substitute for NH_4OH and eliminates the Glu to Gln and Asp to Asn conversion, although it requires a rather high concentration (2.7 M) for elution of amino acids from the SCX resins (data not shown).

Under the GC conditions used in this work, most of the MCF-derivatized amino acids are baseline resolved. There are two issues with overlapping peaks, Gly/Ala and Pro/Asn. Leu and Ile, Ser and Gln as well as Glu and Met peaks are also close to each other but resolved. Since the major fragment ion of the derivatized Gly at *m*/*z* 88 also appears in the spectrum of the derivatized Ala, this could potentially present a problem if a protocol were used where this ion was selected for the quantification of glycine. This complication is easily avoided by use of the molecular ion at *m*/*z* 147, however, it has a somewhat lower abundance (Fig. 2A). The major fragment ion of derivatized Asn at *m*/*z* 127 is typically mixed with the major fragment ion from proline at the same nominal mass. Therefore, under these conditions that ion could not be used for mass isotope distribution analysis. The overlap for Gly/Ala and Pro/Asn, could be solved by a slower temperature ramp at 5 °C/min for the first 15 min GC–MS run, although the peak shapes of many amino acids broadened unacceptably under these conditions (data not shown). Thus, the careful selection of the monitored ions proved a more practical solution.

3.4. Method evaluation

3.4.1. Linearity of response, accuracy, precisions and sample recovery

The calibration responses were obtained by plotting the peak area ratio between the derivatives of amino acids in a concentration range of 0.5 and 500 μM and that of corresponding [^{13}C]-labeled amino acids at a fixed concentration. As shown in Table 2, linearity was obtained for all the amino acids in this range, with all *R*-values greater than 0.98. The lowest *R*-values were for Thr-OMe (0.984), Ser-OMe (0.988) and Gln (0.987).

Accuracy and precision, as given by relative error (RE%) and relative standard deviation (RSD%), respectively, were evaluated (Table 2) by analyzing five replicates of an amino acid standard at 25 nmol/mL (except for Cys at 12.5 nmole/mL). Most of the variations in precision were less than 10% with the lowest variation found for Ala and the highest variation was for Cys. The variations in accuracy were all within 15%, with the highest variation found for Thr-OMe and the lowest for Ala. Higher variations for both accuracy and precision were found for Thr-OMe, Ser-OMe, Gln and Cys which might, in part, be due to the lower MS response to these amino acids as indicated by their higher LODs and LOQ (Table 2). The recovery values are the results from five independent analyses of 25 nmole/mL of the amino acid standard mixture following the entire procedure, from SPE using Glygen SCX tips to amino acid derivatization. Overall, the recovery percentages were >90% for all amino acids, with essentially quantitative recovery for most amino acids and only Thr (90.2), Asn (91.5), Gln (91), Phe (90.1) and Trp (90.6) being slightly lower.

3.4.2. Limits of detection (LOD) and quantitation (LOQ)

The LODs and LOQs by GC–MS in the SIM mode are shown in Table 2. The lowest LODs were 0.1 pmol per injection (2 μL) for Gly, Ala, Val, Leu, Ile, Pro, Tyr and Trp. The LODs higher than 1 pmole were obtained for Thr, Ser, Asn, Gln, and Cys, which yielded the highest LOD at 10 pmole. The lower limit of LOQ was ten times standard deviation above the mean blank value. The LOQs with the current method ranged from 1 to 30 pmole per injection with the highest value determined for Cys.

3.4.3. Stability

The stability of amino acids in plant extracts was assessed for short-term storage by pooling and grinding three 4-week-old *Arabidopsis* plants in 10 mM HCl spiked with the [^{13}C]-labeled algal amino acid mixture, then equally dividing the sample into three microcentrifuge tubes. Samples were then either processed on the same day or stored at −80 °C for 1 and 2 weeks before processing. Repetitive analysis showed essentially equivalent data (RE% < 10%) for all three samples (data not shown). The MCF derivatives of amino acid standards were analyzed by repeated injections at room temperature over a 12 h GC–MS analysis period and derivatized samples was analyzed again after being stored at −20 °C for either 1 and 2 days. The MCF-derivatized amino acids showed no significant change (RE% < 1%) after 12 h at room temperature nor after 1 or 2 days storage at −20 °C (data not shown).

Table 2
Calibration parameters and sample recovery.

Amino acid	R^2[a]	LOD [pmole/pg per injection][b]	LOQ [pmole per injection][c]	RE%[d]	RSD%[d]	Recovery%[e]
Glycine	0.993	0.1/8	1–1000	−2.1	2.8	102.1
Alanine	0.999	0.1/9	1–1000	−0.6	2.1	100.7
Valine	0.999	0.1/12	1–1000	−1.3	2.5	103.2
Leucine	0.999	0.1/13	1–1000	−2.7	3.5	94.6
Iso-leucine	0.996	0.1/13	1–1000	−2.8	3.2	101.5
Threonine	0.987	5/600	20–1000	−13.6	8.3	90.2
Proline	0.998	0.1/12	1–1000	5.4	3.8	101.3
Asparagine	0.991	0.5/66	5–1000	3.2	4.5	91.5
Aspartic acid	0.999	0.2/27	2–1000	−5.6	3.6	96.2
Serine	0.988	5/525	20–1000	−7.2	6.6	97.8
Glutamine	0.987	5/740	20–1000	−8.7	8.4	91.0
Glutamic acid	0.997	0.5/74	5–1000	−3.1	3.2	99.8
Methionine	0.992	0.5/75	5–1000	−2.2	6.1	93.1
Cysteine	0.994	10/1210	30–1000	−12.3	9.3	104.8
Phenylalanine	0.999	0.1/17	1–1000	1.8	2.3	90.1
Lysine	0.997	0.2/29	2–1000	−1.7	2.7	92.6
Histidine	0.992	1/156	5–1000	−3.4	3.2	107.4
Tyrosine	0.995	0.1/18	1–1000	−2.5	2.2	99.7
Tryptophan	0.998	0.1/21	1–1000	−1.8	2.6	90.6

The calibration for the amino acid analyses was obtained using [^{13}C]-labeled amino acids as internal standards.
[a] Coefficient of determination (square of the correlation coefficient R of the regression analysis).
[b] Limit-of-detection (S/N ≥ 3). LODs were determined according to the statistical methods recommended by IUPAC http://goldbook.iupac.org/L03540.html).
[c] Limit-of-quantification (or LOQ) was defined by the lower and upper limits of quantification where the lower limit is ten times standard deviation above the mean blank value.
[d] The RE and RSD represent the accuracy and precision of amino acid standard (25 nmol/mL) levels, respectively, for 5 repeated injections.
[e] The recovery values represents the results from five independent analysis of 25 nmole/mL of amino acid standard mix following the whole procedure from SPE clean-up using Glygen SCX tips to amino acid derivatization.

3.5. Amino acid profiling of Arabidopsis seedlings

In order to do amino acid profiling of minute amounts of plant tissues, selected ion monitoring was used to obtain higher sensitivity. To maximize sensitivity, we divided data acquisition into three retention windows so that only four to seven ions were monitored simultaneously. Group (1) consisted of m/z 102 (Ala), m/z 130 (Val), m/z 144 (Leu, Ile), and m/z 128 (Pro); group (2) consisted of m/z 160 (Asp), m/z 100 (Ser), m/z 174 (Glu), m/z 221 (Met), m/z 192 (Cys) and m/z 162 (Phe); group (3) consisted of m/z 212 (Lys), m/z 210 (His), m/z 236 (Tyr) and m/z 130 (Trp). These ions were chosen on the basis of ion abundance and the absence of interfering material in biological matrices. Gly, Thr, Asn and Gln were excluded in the analysis.

To demonstrate the feasibility of this protocol to analyze minute amounts of plant samples, amino acids were extracted from one cotyledon (~0.4 mg) of a 2-week-old *Arabidopsis* seedling as well as from three 1-week-old etiolated *Arabidopsis* seedlings (~0.1 mg). The derivatized amino acids were then analyzed by GC–SIM-MS (Fig. 5). Clearly, in both samples only Cys was absent and the other 15 were detected including tryptophan that is usually in low abundance. The signal-to-noise ratios were all large enough to suggest that even lower levels of detection might be expected with this method.

3.6. Method evaluation for mass isotopomer distribution analysis

To investigate the potential of our method as a reliable GC–SIM-MS protocol for the determination of amino acid mass isotopomers from minute plant tissues, amino acids extracted from a single cotyledon (~0.4 mg) of a *Arabidopsis* seedling grown on regular medium or 30 atom% deuterium oxide for 2 weeks were analyzed in SIM scan modes. Fig. 6 shows the mass isotopomer distribution (MID) of the major fragment ion (m/z 174) of Glu extracted from either unlabeled plants or 30 atom% ^2H_2O labeled plants. Six isotopomers in the cluster from m0 thru m5 were monitored. This fragment (m/z $[M-59]^+$, $C_7H_{12}NO_4$) contains five hydrogen atoms, derived from Glu in vivo that can be labeled with ^2H_2O. When generating theoretical distributions using the IDCal program for natural abundance distribution, the chemical formula $C_7H_{12}NO_4$ was applied for the calculation. The theoretical relative abundances for the first six isotopomers are: m0 = 100, m1 = 8.9619, m2 = 1.1268, m3 = 0.0747, m4 = 0.0051 and m5 = 0.0003. A Chi-square statistical test of the observed distribution and the theoretical distribution suggested the observed distribution obtained by our GC–SIM-MS method did not differ from the theoretical value (p = 0.05, Table 3). This observation was also true for Pro as shown in Table 3 and several other amino acids analyzed (data not shown). To calculate theoretical distribution for 30 atom% ^2H_2O labeled fragment of Glu,

Table 3
Fitness χ^2 of the observed natural abundance distributions and theoretical distributions of two amino acids extracted from 1-week-old etiolated *Arabidopsis* seedlings. Mass isotopomer distributions were monitored by GC–SIM-MS as described in Fig. 3.

Amino acid	No. of seedlings	Fitness (χ^2)	No. of carbon atoms in the monitored ion	Degrees of freedom	Critical χ^2 value if two distributions are statistically different (p = 0.05)
Proline	1	0.63	6	5	11.07
	3	0.47			
	5	0.36			
Glutamic acid	1	0.22	7	6	12.59
	3	0.08			
	5	0.02			

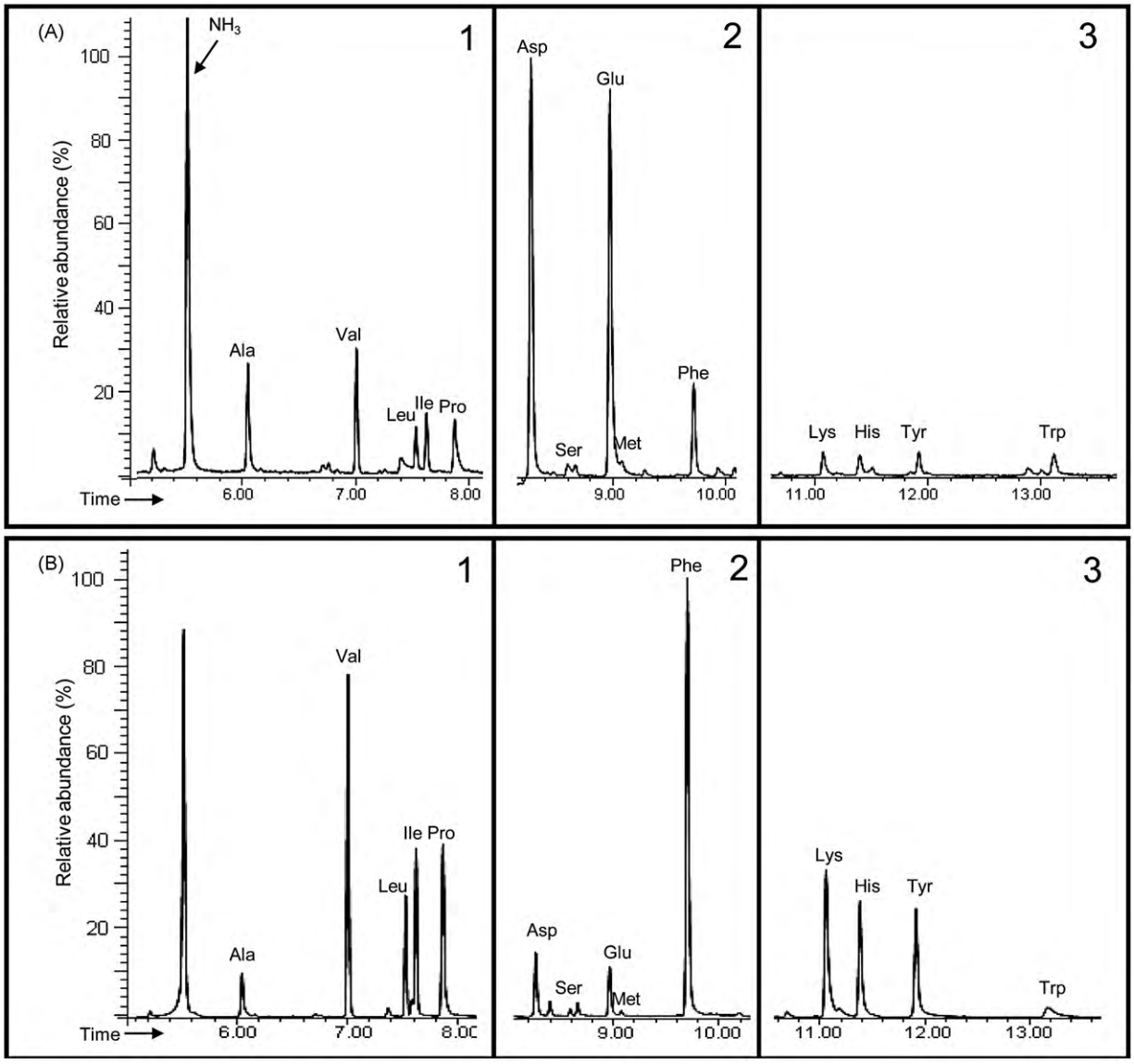

Fig. 5. Amino acids extracted from (A) one single cotyledon (~0.4 mg) of a 2-week-old *Arabidopsis* seedling and from (B) three 1-week-old etiolated *Arabidopsis* seedlings (~0.1 mg), as detected by GC–SIM-MS. A total of 14 amino acids, including tryptophan, were detected. Fragment ions, representing 15 detectable amino acids, were arranged into three SIM groups based on the retention time of the amino acids on the GC column. Group (1) consisted of *m/z* 102 (alanine), *m/z* 130 (valine), *m/z* 144 (leucine, iso-leucine) and *m/z* 128 (proline); group (2) consisted of *m/z* 160 (aspartic acid, *m/z* 100 (serine), *m/z* 174 (glutamic acid), *m/z* 221 (methionine), *m/z* 192 (cysteine) and *m/z* 162 (phenylalanine); group (3) consisted of *m/z* 212 (lysine), *m/z* 210 (histidine), *m/z* 236 (tyrosine) and *m/z* 130 (tryptophan). Gly, Thr, Asn and Gln were excluded from this analysis.

five hydrogen atoms were used as the labeled atoms and the rest of other elements ($C_7H_7NO_4$) in the structure was considered unlabeled and natural isotope abundance was used in the calculation. The calculated isotopomer abundances are: m0 = 44.914, m1 = 100, m2 = 91.0354, m3 = 43.3467, m4 = 11.5191 and m5 = 1.7421. The observed relative abundances were: m0 = 50, m1 = 100, m2 = 92, m3 = 44, m4 = 11.5 and m5 = 1.5. Chi-square test showed no significant difference between observed and theoretical distributions. These results confirm that amino acid derivatization with MCF followed by GC–MS is suitable for high sensitivity mass isotopomer distribution analysis of labeled and unlabeled amino acids.

3.7. Concentration independency of measured MID

Measured mass isotopomer distribution (MID) of t-butyldimethylsilyl (TBDMS)-derivatized amino acid fragments have been reported to be varied over different concentrations, although this was more pronounced using specific GC–MS instrumentation, including an HP/Agilent 5971 [16]. To accurately measure stable isotope enrichments of amino acids in plants, there was a need to confirm that there is no such variation due to sample concentrations. Although we did not have access to the Agilent 5971 system, we were able to analyze an amino acid standard mixture on both HP 5970 and Agilent 5973 systems. Using the HP 5970, the mixture was analyzed repetatively 51 times at varying concentrations and then the MID of each amino acid determined. Fig. 7 shows the mass isotopomer abundances for both unlabeled Ala-102 and Val-130 plotted as a function of total ion counts. The m0 abundances of both amino acid fragments were consistent over a wide range of concentrations. Similar results were also observed for other fragments of MCF-derivatized amino acids (data now shown). When analyzed with the Agilent 5973, which has a similar ion source design as was used in the Agilent 5971, consistency in MID values was again found (data not shown). These results suggest that amino acid derivatization with MCF is reliable for MID analysis using these tested instruments.

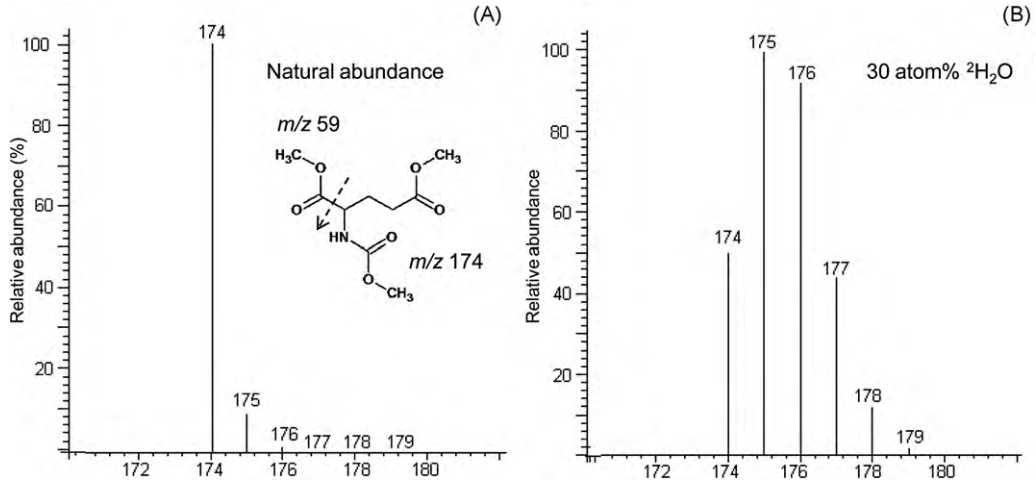

Fig. 6. Mass isotopomer analysis of the major fragment ion from glutamic acid extracted from a single cotyledon (~0.4 mg) of a 2-week-old *Arabidopsis* seedling as determined by GC–SIM-MS. (A) The mass isotopomer distribution of the major fragment ion (174 *m*/*z*) and its associated ion cluster pattern from unlabeled glutamic acid extracted from an unlabeled seedling. (B) Mass isotopomer distribution of the same fragment ion and its associated ion cluster pattern from glutamic acid extracted from a seedling grown on 30 atom% deuterium oxide for 2 weeks. This fragment contains five hydrogen atoms that can be labeled from ^2H_2O.

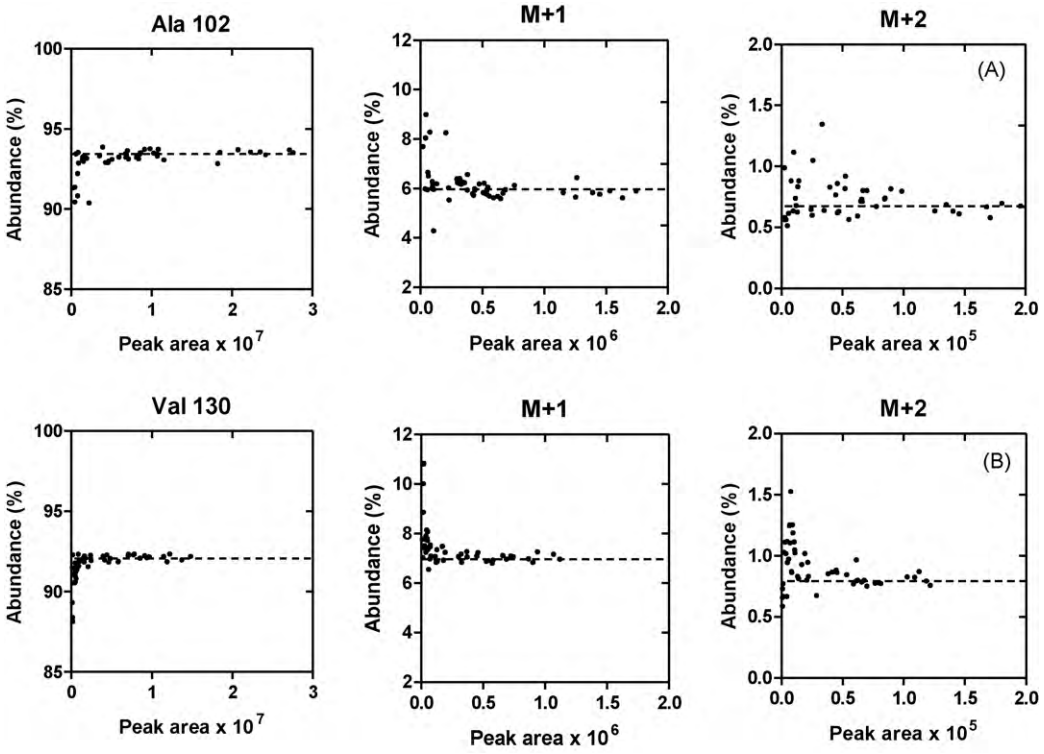

Fig. 7. Concentration independence of mass isotopomer abundances for MCF-derivatized amino acids. Samples were analyzed 51 times at varying concentrations. Each dot represents a separate GC/MS run. Peak area corresponds to the sum of integrated intensities at *m*/*z* 102–104 for natural alanine (A) and *m*/*z* 130–132 for natural valine (B). The dashed horizontal lines represent theoretical mass isotopomer abundances.

4. Conclusions

In this study, the approach using solid-phase extraction for amino acid purification and methyl chloroformate for sample derivatization has been shown to provide an effective platform of rapid profiling of minute amounts of plant samples. The use of SCX SPE minitips allowed microscale analysis when coupled to the elimination of lyophilization/evaporation steps. The reaction's tolerance of ammonium ions dramatically shortens and simplifies the sample preparation and derivatization protocol. The yield and retention times of *N*(*O*,*S*)-alkyl alkoxy carbonyl esters of amino acids using the present method are found comparable to those of previously reported large-scale methods. This protocol was especially suitable for monitoring mass isotope distributions in amino acids in *Arabidopsis* seedlings that had been labeled with stable isotopes. This method can be easily modified for integration into robotic systems for analysis of large numbers of samples and has potential for use in a number of areas in the life sciences where small sample size and/or rapid analyses are important. For example, this procedure was recently shown to be particularly useful for determining amino acid levels in tomato [39] and amino acid turnover in *Arabidopsis* [40].

Acknowledgements

We thank Dr. Michael MacCoss at the University of Washington for sharing IDCal for calculation of theoretical amino acid isotopomer distributions. This work was supported by the U.S. National Science Foundation, Plant Genome Program, grants DBI-0606666 and IOS-0923960, and by funds from the Gordon and Margaret Bailey Endowment for Environmental Horticulture.

References

[1] U. Roessner, A. Luedemann, D. Brust, O. Fiehn, T. Linke, L. Willmitzer, A. Fernie, Plant Cell 13 (2001) 11.
[2] S.G. Villas-Bôas, J.F. Moxley, M. Akesson, G. Stephanopoulos, J. Nielsen, Biochem. J. 388 (2005) 669.
[3] M.K. Hellerstein, Annu. Rev. Nutr. 23 (2003) 379.
[4] J.M. Pratt, J. Petty, I. Riba-Garcia, D.H. Robertson, S.J. Gaskell, S.G. Oliver, R.J. Beynon, Mol. Cell Proteomics 1 (2002) 579.
[5] M.K. Doherty, C. Whitehead, H. McCormack, S.J. Gaskell, R.J. Beynon, Proteomics 5 (2005) 522.
[6] A. Motoyama 3rd, J.R. Yates, Anal. Chem. 80 (2008) 7187.
[7] A.S. Feste, J. Chromatogr. 574 (1992) 23.
[8] H.G. Biggs, L.J. Gentilcore, Clin. Chem. 30 (1984) 851.
[9] B. Thiele, K. Füllner, N. Stein, M. Oldiges, A.J. Kuhn, D. Hofmann, Anal. Bioanal. Chem. 391 (2008) 2663.
[10] K. Harada, E. Fukusaki, T. Bamba, F. Sato, A. Kobayashi, Biotechnol. Prog. 22 (2006) 1003.
[11] W.A. van Winden, C. Wittmann, E. Heinzle, J.J. Heijnen, Biotechnol. Bioeng. 80 (2002) 477.
[12] M.I. Klapa, J.C. Aon, G. Stephanopoulos, Eur. J. Biochem. 270 (2003) 3525.
[13] M. Dauner, U. Sauer, Biotechnol. Prog. 16 (2000) 642.
[14] B.W. Patterson, F. Carraro, R.R. Wolfe, Biol. Mass Spectrom. 22 (1993) 518.
[15] C.K. Fagerquist, R.A. Neese, M.K. Hellerstein, J. Am. Soc. Mass Spectrom. 10 (1999) 430.
[16] M.R. Antoniewicz, J.K. Kelleher, G. Stephanopoulos, Anal. Chem. 79 (2007) 7554.
[17] J.L. Little, J. Chromatogr. A 844 (1999) 1.
[18] P. Husek, J. Chromatogr. 552 (1991) 289.
[19] S. Casal, M.D. Oliveira, M.A. Ferreira, J. Chromatogr. A 866 (2000) 221.
[20] P. Husek, P. Matucha, A. Vrankova, P. Simek, J. Chromatogr. B 789 (2003) 311.
[21] P. Husek, J. Chromatogr. B 669 (1995) 352.
[22] S.G. Villas-Boas, D.G. Delicado, M. Akesson, J. Nielsen, J. Anal. Biochem. 322 (2003) 134.
[23] I. Abe, N. Fujimoto, T. Nishiyama, K. Terada, T. Nakahara, J. Chromatogr. A 722 (1996) 221.
[24] J. Wang, Z.-H. Huang, D.A. Gage, J.T. Watson, J. Chromatogr. A 663 (1994) 71.
[25] M.G. Zampolli, G. Basaglia, F. Dondi, R. Sternberg, C. Szopa, M.C. Pietrogrande, J. Chromatogr. A 1150 (2007) 162.
[26] H. Kaspar, K. Dettmer, W. Gronwald, P.J. Oefner, J. Chromatogr. B 870 (2008) 222.
[27] P. Husek, P. Simek, P. Hartvich, H. Zahradnícková, J. Chromatogr. A 1186 (2008) 391.
[28] L. Yang, Z. Mester, R.E. Sturgeon, Anal. Chem. 76 (2004) 5149.
[29] P. Husek, P. Simek, LC–GC North America 19 (2001) 986.
[30] P. Husek, J. Chromatogr. Libr. 70 (2005) 2.
[31] P. Husek, Phenomenex, Eur. Pat. 00301791.0-2204, priority CZ/04.03.99/CZ 769994, 3 March (1999).
[32] M.J. Nozal, J.L. Bernal, M.L. Toribio, J.C. Diego, A. Ruiz, J. Chromatogr. A 1047 (2004) 137.
[33] J. Persson, T. Näsholm, Physiol. Plant. 113 (2001) 352.
[34] I. Spanik, G. Horvathova, A. Janacova, J. Krupcik, J. Chromatogr. A 1150 (2007) 145.
[35] P.K. Kanaujia, V. Tak, D. Pardasani, A.K. Gupta, D.K. Dubey, J. Chromatogr. A 1185 (2008) 167.
[36] G. Parry, S. Ward, A. Cernac, S. Dharmasiri, M. Estelle, Plant Cell 18 (2006) 1590.
[37] J. Corley, in: P.W. Lee (Ed.), Handbook of Residue Analytical Methods for Agrochemicals, vol. 1, John Wiley & Sons, New Jersey, 2003, p. 59.
[38] H. Kubinyi, Anal. Chim. Acta 247 (1991) 107.
[39] G.S. Maloney, A. Kochevenko, D. Tieman, T. Tohge, U. Krieger, D. Zamir, M.G. Taylor, A.R. Fernie, H.J. Klee, Plant Physiol., doi:10.1104/pp.110.154922.
[40] X.-Y. Yang, W.-P. Chen, A.K. Rendahl, A.D. Hegeman, W.M. Gray, J.D. Cohen, Plant J., doi:10.1111/j.1365-313X.2010.04266.x.