Journal of Chromatography B, 912 (2013) 8–15

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

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

A fast liquid chromatography–mass spectrometry (LC–MS) method for quantification of major polar metabolites in plants

Zhiqian Liu[a,b], Simone Rochfort[a,b,c,*]

[a] Department of Primary Industries, Biosciences Research Division, Bundoora, Victoria, Australia
[b] Dairy Futures Co-operative Research Centre, 1 Park Drive, Bundoora, Victoria, Australia
[c] La Trobe University, Bundoora, Victoria, Australia

ARTICLE INFO

Article history:
Received 12 July 2012
Accepted 30 October 2012
Available online 3 November 2012

Keywords:
Polar compounds
LC–MS
Amino acids
Organic acids
Sugars
Plants

ABSTRACT

Current liquid chromatography (LC) based methods for the analysis of polar plant metabolites require multiple runs using complex mobile phases and a combination of different columns. Here we describe a fast liquid chromatography–mass spectrometry (LC–MS) method for the determination of major polar metabolites in plants that requires only a single run using a single column. The method takes advantage of the ability to acquire both positive and negative data in an ion trap mass spectrometer (MS) and also the accurate mass capability of the orbitrap MS. The separation of polar compounds is achieved with a polar, reversed-phase column (Synergi Hydro-RP). A single analysis with a 25 min runtime is able to reliably determine the level of nearly all essential amino acids, several major organic acids and several major sugars in plant materials, as exemplified by analysis of a perennial ryegrass extract. The level of detection on column was below 0.1 ng (average 0.03 ng) for most amino acids, below 5 ng (average 2.3 ng) for organics acids and below 1 ng (average 0.64 ng) for sugars. The levels of quantified metabolites in ryegrass varied from 22 μg/g dry weight for histidine to 41 mg/g dry weight for sucrose.

1. Introduction

Most plant primary metabolites are small, polar compounds with a wide range of chemistries. Gas chromatography–mass spectrometry (GC–MS) is a powerful technique, able to determine simultaneously a great number of polar metabolites, including sugars, amino acids and organic acids and has been extensively utilized in plant metabolomics for this reason [1,2]. However, there are drawbacks with the technique. Firstly, a relatively complex derivatization process is required to make the polar compounds more volatile and amenable for GC–MS analysis. Secondly, large and thermolabile compounds such as oligosaccharides cannot be detected by GC–MS due to their limited volatility [3]. Furthermore, the formation of isomers from certain compounds such as sugars during derivatization adds a level of complexity to data analysis.

Although numerous methods are available for polar compound analysis using high performance liquid chromatography (HPLC) or LC–MS techniques, most of the earlier methods deal with only one class of metabolites. For example, high performance anion exchange chromatography coupled with electrochemical detection is a powerful technique for carbohydrate analysis [4,5], whereas organic acid quantification can be easily achieved with reverse-phase separation and UV detection [6,7]. In the case of amino acids, various derivatization and detection techniques have been reported [8,9]. The fact that plant primary metabolites comprise several chemical classes implies that multiple HPLC runs would be required to quantify even the few most important classes of polar compounds [5,10,11]. A universal LC based method that can reliably quantify major polar metabolites in a single run would save both time and cost in analysis.

As standard reversed-phase columns have poor retention property for polar analytes, alternative approaches, such as porous graphitic carbon stationary phase chromatography, hydrophilic interaction liquid chromatography (HILIC) and aqueous normal phase chromatography (ANP), have seen a rapid increase in use for the analysis of polar compounds in recent years [12–18]. Some attempts were also made to use HILIC and ANP based LC–MS techniques for simultaneous quantification of a large number of polar metabolites [14–18]. These methods are generally able to quantify amino acids and organic acids, but often involve relatively complex mobile phase systems (such as pH adjusted buffers), long runtime (45–60 min) and sometimes multiple runs. In addition, all comprehensive methods reported were developed for analysis of non-plant samples and thus the most abundant polar metabolites in plants, the sugars, were not measured. Here we report on the method development and validation for the quantitative analysis of major polar metabolites of plants based on the use of a polar,

* Corresponding author at: Department of Primary Industries, Biosciences Research Division, Bundoora, Victoria, Australia.
E-mail address: simone.rochfort@dpi.vic.gov.au (S. Rochfort).

1570-0232/$ – see front matter © 2012 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jchromb.2012.10.040

reversed-phase column (Synergi Hydro-RP) coupled to an ion-trap MS. We show that this method is able to quantify all major amino acids, several major organic acids and several major sugars in plant tissue within a single short run.

2. Materials and methods

2.1. Chemicals

All non-labeled amino acid, organic acid and sugar standards were purchased from Sigma. The stable isotope analogs of alanine (DL-alanine, 3,3,3-D3, 98%), tyrosine (ring-3,5-D2, 98%), leucine (15N, 98%), serine (15N, 98%), phenylalanine (15N, 98%), glutamine (amide-15N, 98%) and valine (DL-valine, D8, 98%) were purchased from Cambridge Isotope Laboratories, Inc. Solvents were of HPLC grade and obtained from Sigma–Aldrich (water with 0.1% formic acid and methanol with 0.1% formic acid) and Merck (for methanol).

2.2. Plant materials

All validation experiments were conducted with whole tillers of perennial ryegrass (*Lolium perenne*) plants grown in the field (Hamilton, Victoria, Australia). Samples were collected from plants at the vegetative growth stage, immediately frozen in liquid nitrogen and stored at −80 °C before freeze drying (Labconco, USA) under vacuum for 48 h. Samples were ground into a fine powder using a mill-mixer (MM400, Retch Germany). Polar compounds were extracted as described by Roessner et al. [19] with some modifications. Briefly, 20 mg of dry powder was extracted twice with 80% methanol (1 mL) by sonication for 15 min. Following centrifugation (10 min, 13,000 × *g*), the combined supernatant was transferred to a HPLC vial and 5 or 10 μL injected directly or after appropriate dilution for LC–MS analysis.

2.3. LC–MS conditions

HPLC separation was achieved using a Synergi Hydro-RP column (250 × 4.6 mm, 4 μm, Phenomenex) on an Agilent 1290 Infinity system (Agilent, Walbronn), including degasser, binary pump, temperature-controlled autosampler (maintained at 4 °C) and column compartment (maintained at 30 °C). The mobile phase was composed of water containing 0.1% formic acid (A) and methanol containing 0.1% formic acid (B). The flow rate was 0.5 mL/min with a gradient elution of 0–100% B over 16 min and then increased to 1 mL/min for post-acquisition column equilibration. Analyte detection was by mass spectrometry LTQ Orbitrap Velos (Thermo Scientific, Waltham, MA, USA; Bremen, Germany) with a heated electrospray ionization (HESI) source. The mass spectrometer was operated with full scan (50–1000 *m*/*z*) in positive FT mode (at a resolution of 30,000) for amino acid analysis and in negative ion trap (IT, 50–1000 *m*/*z*) mode for organic acid and sugar analysis.

The heated capillary was maintained at 270 °C with a source heater temperature of 350 °C and the sheath, auxiliary and sweep gases were at 40, 15 and 8 units respectively. Source voltage was set to 3.2 kV for negative mode and 4.2 kV for positive mode. Prior to data acquisition the system was tuned using an infusion of the extract. The extract was infused *via* syringe pump through a T-piece at a rate of 5 μL/min with a HPLC flow rate of 0.2 mL/min with a solvent composition of 50% A and 50% B.

Molecular ions ($[M+H]^+$ for amino acids and $[M-H]^-$ for organic acids) or formate adduct ions ($[M+HCOO]^-$ for sugars) were extracted from the full scan chromatograms and peak areas integrated using Xcalibur software (Thermo Scientific).

2.4. Method validation

Determination of the limit of detection (LOD) and limit of quantitation (LOQ) as well as the linear range for individual sugars, organic acids and amino acids was carried out using a mixed standard. A series of 10 dilutions (ranging from 0.0001 to 0.1 mg/mL) were prepared for seven major plant sugars/sugar alcohols (sucrose, glucose, fructose, mannitol, inositol, trehalose and raffinose) and seven major organic acids (succinate, citrate, malate, tartrate, maleate, fumarate and *trans*-aconitate), and 12 dilutions (ranging from 0.0004 to 10 μg/mL) for 19 essential amino acids. The concentrations were chosen through preliminary tests to encompass the linear range and also enable quantification in the plant material of interest.

To determine method precision three mass levels (one close to LOQ, one intermediate and one close to the upper limit of linear range) of standard sugars (2.5, 25 and 100 ng), organic acids (5, 100 and 500 ng) and amino acids (0.1, 1 and 10 ng) were injected seven times. The repeatability of peak area and retention time for each compound was estimated by calculation of the respective relative standard deviation (RSD) values.

Measurement accuracy with plant extract was evaluated using the spike recovery test. A mix of seven stable isotope-labeled amino acids (alanine-D3, tyrosine-D2, leucine-15N, serine-15N, phenylalanine-15N, glutamine-15N, and valine-D8), a mix of seven non-labeled organic acid standards and a mix of seven non-labeled sugar standards were spiked respectively to three dilutions of a ryegrass extract (non-diluted extract, 2- and 5-fold diluted extract). The concentration of standard compounds spiked was of intermediate level (0.2, 20 and 5 μg/mL for amino acids, organic acids and sugars, respectively). The potential ion suppression effect of the matrix on the detection of target compounds was estimated by the recovery rate of the spiked standard compounds. For labeled amino acids, the recovery rate was calculated as the percentage of peak area of spiked plant extract relative to that of matrix-free standards. In the case of non-labeled organic acids and sugars, the recovery rate was calculated after correction for the endogenous compound concentrations in the matrix using the following formula:

$$\text{Recovery}\ \ (\%) = \left[\frac{\text{total mass in the spiked sample} - \text{mass in the non-spiked sample}}{\text{mass spiked}}\right] \times 100$$

2.5. Method application

Having confirmed the accuracy in analyzing plant samples, we applied the method to quantification of major polar compounds in ryegrass. Polar compounds were extracted from a ryegrass sample with four replicates and injected into the LC–MS system without prior purification. The reliability of the method was further verified by the intra-sample precision in peak area and retention time of all detected compounds and also by the inter-sample variation in polar compound content. The former was estimated by the RSD of seven repeated injections from the same extract (replicate 1), whereas the latter was judged by the RSD values of the four replicates.

3. Results

3.1. Method performance for standard compounds

The extracted ion chromatogram (EIC) after injection of the amino acid standard mix is shown in Fig. 1. The commercial standard mix contains two types of alanine (β-alanine and L-alanine), hence giving two peaks with identical accurate mass. This

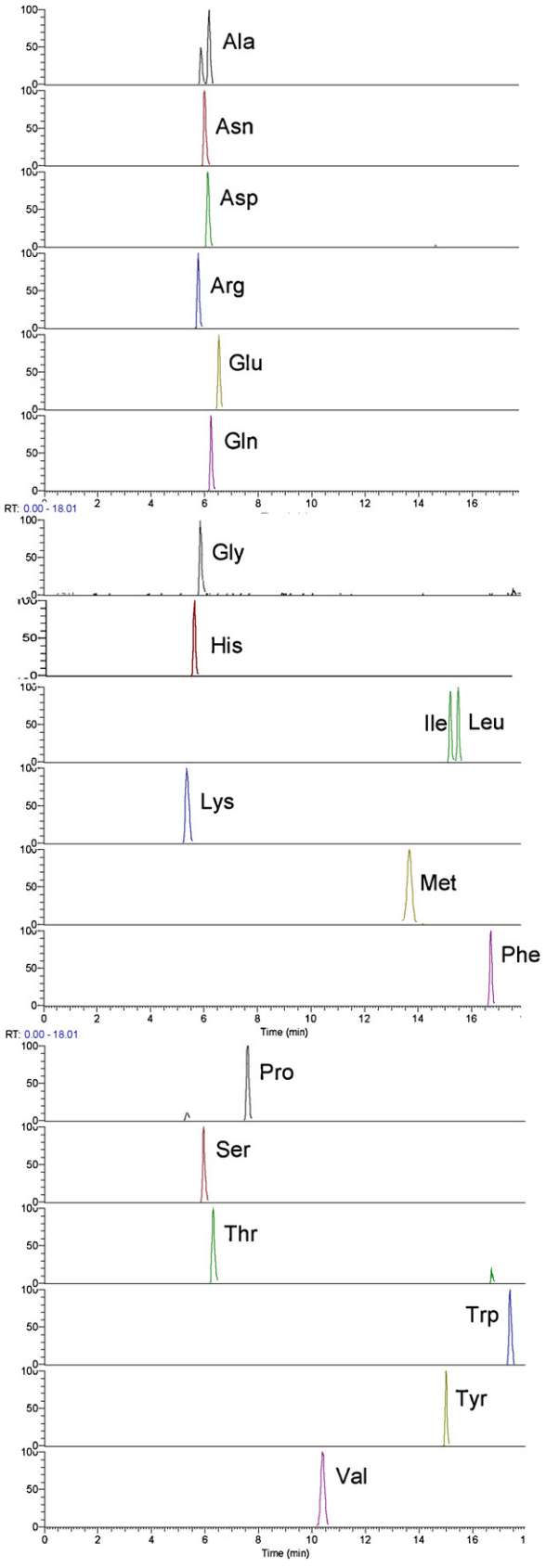

Fig. 1. EIC of 19 amino acid tested (standards).

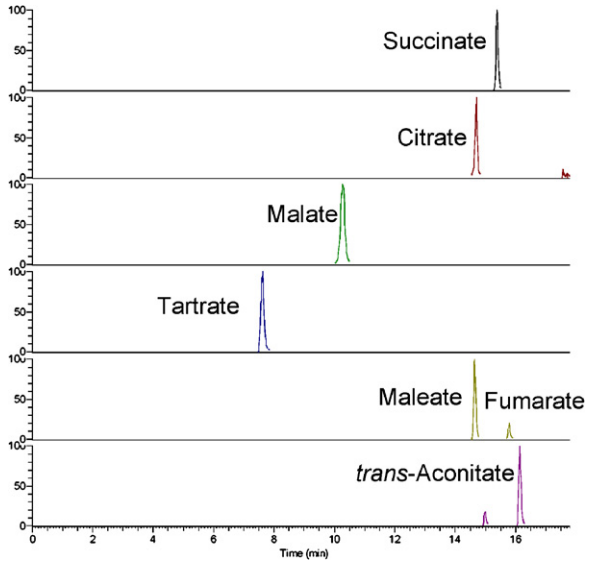

Fig. 2. EIC of the seven organic acids tested (standards).

analytical method is able to resolve amino acids with the same nominal mass (lysine *vs.* glutamine) and the isomers leucine and isoleucine. Extraction of accurate mass (within a narrow window of 0.01 *m*/*z*) also eliminates the potential interference of ^{13}C isotopes when two amino acids differing by a single mass unit are incompletely resolved, as in the case between asparagine and aspartic acid.

The EIC of the seven organic acids is shown in Fig. 2. Five out of the seven compounds including structural isomers (fumaric acid and maleic acid) are chromatographically resolved, whereas co-eluting citric acid and maleic acid can be differentiated by their mass difference.

We have found that sugars/sugar alcohols can be detected in both positive (as sodium adducts) and negative (as deprotonated ions and formate adducts) mode. However, overall the dynamic range for these compounds is larger in negative mode and higher signal intensity was detected for formate adduct ions as compared to deprotonated molecular ions. As a result, formate adducts acquired in negative mode were used for sugar quantification.

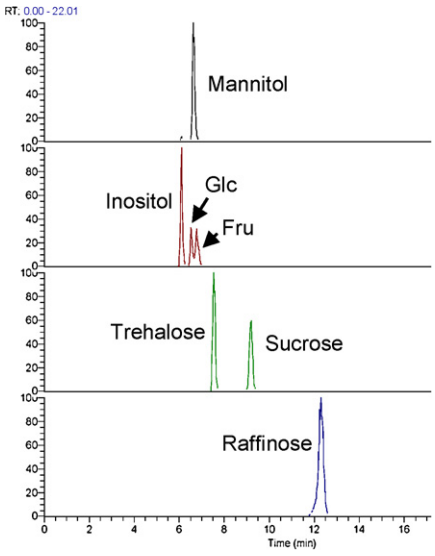

Fig. 3. EIC of the seven sugars tested (standards).

Table 1
LOD, LOQ, linearity range and retention time of polar metabolites tested.

Compounds	Ions quantified	Ion extraction window (*m*/*z*)	LOD (ng)	LOQ (ng)	Linearity range (ng)	R^2	RT (min)
Amino acids							
Alanine	$[M+H]^+$	90.05–90.06	0.02	0.05	0.05–5	0.9929	6.22
Asparagine	$[M+H]^+$	133.06–133.07	0.075	0.15	0.15–35	0.9994	6.07
Aspartic acid	$[M+H]^+$	134.04–134.05	0.015	0.07	0.07–7	0.9974	6.17
Arginine	$[M+H]^+$	175.11–175.12	0.004	0.009	0.009–5	0.9968	5.81
Glutamic acid	$[M+H]^+$	148.06–148.07	0.004	0.04	0.04–15	0.9913	6.57
Glutamine	$[M+H]^+$	147.07–147.08	0.05	0.15	0.15–30	0.9991	6.30
Glycine	$[M+H]^+$	76.03–76.05	0.2	0.4	0.4–8	0.9999	5.93
Histine	$[M+H]^+$	156.07–156.08	0.008	0.02	0.02–4	0.9910	5.62
Isoleucine	$[M+H]^+$	132.10–132.11	0.003	0.007	0.007–13	0.9994	15.18
Leucine	$[M+H]^+$	132.10–132.11	0.003	0.017	0.017–13	0.9996	15.47
Lysine	$[M+H]^+$	147.11–147.12	0.02	0.07	0.07–15	0.9991	5.40
Methionine	$[M+H]^+$	150.05–150.06	0.02	0.04	0.04–15	0.9978	13.42
Phenylalanine	$[M+H]^+$	166.08–166.09	0.004	0.02	0.02–17	0.9999	16.67
Proline	$[M+H]^+$	116.07–116.08	0.015	0.06	0.06–12	0.9998	7.66
Serine	$[M+H]^+$	106.05–106.06	0.015	0.05	0.05–11	0.9916	6.01
Threonine	$[M+H]^+$	120.06–120.07	0.015	0.06	0.06–3	0.9899	6.35
Trptophane	$[M+H]^+$	205.09–205.10	0.01	0.03	0.03–21	0.9994	17.37
Tyrosine	$[M+H]^+$	182.08–182.09	0.009	0.045	0.045–18	0.9990	15.01
Valine	$[M+H]^+$	118.08–118.09	0.03	0.06	0.06–12	0.9994	10.27
Organic acids							
Succinic acid	$[M-H]^-$	116.5–117.5	2	5	5–1000	0.9948	15.35
Citric acid	$[M-H]^-$	190.5–191.5	2	5	5–500	0.9940	14.68
Malic acid	$[M-H]^-$	132.5–133.5	5	10	10–1000	0.9984	10.13
Tartaric acid	$[M-H]^-$	148.5–149.5	2	2	2–1000	0.9953	7.62
Maleic acid	$[M-H]^-$	114.5–115.5	1	2	2–1000	0.9920	14.65
Fumaric acid	$[M-H]^-$	114.5–115.5	2	2	2–500	0.9951	15.72
trans-Aconitic acid	$[M-H]^-$	172.5–173.5	2	5	5–500	0.9931	16.08
Sugars							
Mannitol	$[M+HCOO]^-$	226.5–227.5	0.5	1	1–100	0.9922	6.64
Inositol	$[M+HCOO]^-$	224.5–225.5	0.5	1	1–100	0.9937	6.10
Glucose	$[M+HCOO]^-$	224.5–225.5	1	2.5	2.5–100	0.9910	6.55
Fructose	$[M+HCOO]^-$	224.5–225.5	1	2.5	2.5–100	0.9917	6.77
Sucrose	$[M+HCOO]^-$	386.5–387.5	0.5	1	1–250	0.9872	9.18
Trehalose	$[M+HCOO]^-$	386.5–387.5	0.5	0.5	0.5–100	0.9971	7.53
Raffinose	$[M+HCOO]^-$	548.5–549.5	0.5	1	1–250	0.9997	12.35

RT, retention time.

The EIC of the seven sugars tested is shown in Fig. 3. This method is able to resolve chromatographically or by mass difference five out of the seven sugar compounds. While inositol is well separated from its isobaric compounds, glucose and fructose, the two hexoses are not fully resolved.

3.2. Method validation

3.2.1. LOD, LOQ and linearity

Here the LOD is defined as the smallest amount of a compound that can be reliably distinguished, usually showing a signal to noise (S/N) value above 3 in our system, whereas the LOQ is the smallest amount of a compound that can be reliably quantified (signal intensity variation below 15% between three repeated injections). The linear range is the two points between the LOQ and the highest concentration of a compound, which still maintains a good linearity (with a threshold R^2 value equal to or above 0.99).

This method is very sensitive for amino acid detection with a LOD for most amino acids well below 0.1 ng (Table 1). The only exception was glycine, with a LOD of 0.2 ng. The LOQ ranged from 0.007 to 0.4 ng and as expected is 2–5 times higher than the LOD. The linear range varies from 20 to 2000 times LOQ depending on the amino acid species (Table 1).

The LOD for the organic acids is between 1 and 5 ng, and the LOQ between 2 and 10 ng (Table 1). Signal intensity increases in a linear manner from LOQ to 500–1000 ng as the upper limit.

Compared to amino acids, this method is not as sensitive for sugars with a LOD value ranging from 0.5 to 1 ng, and LOQ from 0.5 to 2.5 ng (Table 1). The linear range was between 0.5 and 250 ng depending on the sugar species.

3.2.2. Precision

This method shows excellent reproducibility in retention time and peak area for all standard amino acids and all injection levels. The overall RSD of seven injections is below 0.3% for retention time and below 9% for peak area (Supplementary materials: Table S1).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.10.040.

The precision in retention time and peak area for standard organic acids is comparable to that of amino acids. The RSD of seven injections is below 0.4% for retention time and below 7% for peak area at lowest injection level (5 ng) and much smaller RSD values for peak area are obtained with intermediate and high injection levels (100 and 500 ng) (Supplementary materials: Table S2).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.10.040.

As observed for amino acid and organic acid compounds, good reproducibility in retention time and peak area was also obtained for all sugars and all injection levels. The overall RSD of repeated injections is below 0.4% for retention time and below 8% for peak area (Supplementary materials: Table S3), despite the partial resolution of glucose and fructose.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.10.040.

Table 2
Recovery rates (% ±SD) of standard compounds spiked to three dilutions of a ryegrass extract (matrix).

Compounds	5-Fold diluted extract	2-Fold diluted extract	Non-diluted extract
Amino acids			
DL-valine (D8)	107.6 ± 6.9	106.2 ± 4.2	107.7 ± 6.2
Phenylalanine (15N)	105.1 ± 1.8	105.4 ± 0.8	105.8 ± 0.6
Leucine (15N)	106.3 ± 0.4	103.3 ± 0.3	102.7 ± 0.8
DL-alanine (D3)	102.6 ± 6.7	101.9 ± 7.2	99.2 ± 3.3
Tyrosine (D2)	105.9 ± 1.0	101.5 ± 2.8	99.0 ± 1.6
Serine (15N)	102.4 ± 3.3	100.0 ± 3.4	95.2 ± 0.3
Glutamine (15N)	101.3 ± 4.1	101.4 ± 5.1	101.5 ± 0.4
Organic acids			
Succinic acid	101.5 ± 2.2	101.4 ± 2.7	101.7 ± 0.2
Citric acid	97.9 ± 1.3	97.9 ± 2.0	nc
Malic acid	104.7 ± 0.7	100.5 ± 1.4	99.6 ± 0.1
Tartaric acid	98.5 ± 7.1	105.5 ± 0.9	100.6 ± 0.7
Maleic acid	103.3 ± 1.9	100.2 ± 0.6	101.0 ± 2.8
Fumaric acid	102.2 ± 5.2	98.7 ± 0.4	99.6 ± 1.4
trans-Aconitic acid	104.2 ± 2.6	99.1 ± 0.2	100.7 ± 3.1
Sugars			
Mannitol	96.6 ± 1.1	93.0 ± 1.3	79.2 ± 1.1
Inositol	104.2 ± 3.3	103.3 ± 1.5	93.2 ± 4.5
Glucose	84.0 ± 4.6	76.4 ± 1.1	73.4 ± 3.5
Fructose	95.9 ± 4.6	99.2 ± 0.4	92.6 ± 5.9
Sucrose	99.7 ± 1.7	92.6 ± 0.4	nc
Trehalose	103.4 ± 0.3	109.8 ± 3.5	116.5 ± 4.3
Raffinose	102.9 ± 4.0	104.8 ± 0.6	104.9 ± 0.6

nc: not calculated. Recovery rates (%) in the table are mean values of three replicates. Concentrations of standard compounds spiked to ryegrass extract are 0.2, 20 and 5 μg/mL for amino acids, organic acids and sugars, respectively.

3.2.3. Matrix effects

To determine the method accuracy for quantitative analysis of polar metabolites in matrix, a spike recovery experiment was conducted for seven representative amino acids and all organic acids and sugars tested in this work. An example of a ryegrass extract spiked with alanine-D3 is shown in Fig. 4. As expected, alanine-D3 co-elutes with endogenous alanine present in the matrix (Fig. 4A and B). The mass spectrum at the apex of alanine peak shows the complexity of matrix. Besides endogenous alanine (accurate mass: 90.055), many other species in the matrix co-elute with alanine-D3 (accurate mass: 93.074) (Fig. 4C).

No ion suppression was observed for the seven amino acids tested, with recovery rates ranging between 95 and 108%, regardless of matrix concentrations (Table 2). For organic acids, which were detected in the negative mode, the presence of matrix had no significant influence on the detection of any of the seven compounds. Their recovery rates were between 98 and 106% for all three dilutions of matrix (Table 2). The recovery rate of citric acid spiked to non-diluted extract was not calculated as a large amount of endogenous citrate was present in the matrix and the total citrate level was over the linear range. In the case of sugars, except for glucose, which showed lower recovery (below 90%), especially in the presence of high concentrations of matrix, ion intensity of other six compounds was not significantly affected by the matrix. Again, recovery rate of sucrose after spiking to non-diluted matrix could not be accurately determined due to its high abundance in the matrix (over the linear range).

3.3. Method application to ryegrass samples

To assess the method for application in plant polar metabolite analysis, field grown ryegrass samples were collected and analyzed with this new methodology. Ryegrass extract is a complex matrix (Supplementary materials: Fig. S1). However, a distinct peak at the expected retention time was extracted from the full scan spectrum (positive mode) for all amino acids, except methionine (Supplementary materials: Fig. S2). This method affords excellent precision in peak area (RSD below 2.5%) and retention time (RSD below 0.2%) for repeated injections for all detected amino acids (Table 3). As expected, the abundance of amino acids differs greatly. Methionine was present in very low level in the ryegrass sample and was not quantifiable due to interfering peaks. All 18 other amino acids could be quantified with an inter-sample RSD (of four replicate extractions) below 7% (Table 3).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.10.040.

The EIC of the seven organic acids in the ryegrass extract is shown in Fig. S3 (Supplementary materials). Due to the lower resolution of the IT mass analyzer and the consequently larger ion extraction window (1 m/z) used, isobaric peaks were detected for nearly all organic acid compounds. As a result, retention time was essential to assign the peak of interest. Four organic acids (succinate, citrate, malate and fumarate) were identified in the ryegrass sample. The precisions of the retention time (RSD below 0.3%) and the peak area (RSD below 7%) for repeated injections (of the same extract) were satisfactory for all the detected organic acids (Table 3). Despite their variable levels in the sample (large amount of malate and citrate compared to very small amount of fumarate), all these four compounds could be quantified with an inter-sample RSD below 10% (Table 3). Tartaric acid, maleic acid and *trans*-aconitic acid were not detected in the sample.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.10.040.

Six out of the seven sugars/sugar alcohols were detected in the ryegrass sample (Supplementary materials: Fig. S4). Overall, the intra-sample variation in peak area was below 4% and the inter-sample variation in metabolite content was below 10% (Table 3). This suggests that the level of the six sugar compounds can be reliably determined by this method. As expected, sucrose, glucose and fructose are present in high concentration in the sample. By contrast, the level of mannitol, inositol and raffinose was much lower, whereas trehalose was not found in this ryegrass sample (Table 3).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.10.040.

4. Discussion

Determination of plant primary metabolites is important for conventional plant breeding and metabolite-engineering projects as well as for the study of plant metabolism under stress conditions and under the influence of a changing climate. Classic analytical techniques have existed for several decades for total sugars and total amino acids and accurate and sensitive HPLC methods are also available for each class of polar compounds. However, a comprehensive analysis of all major primary metabolites using one single method has been a challenge.

The ready access of mass spectrometry detectors in many laboratories simplifies the simultaneous detection and concentration determination of multiple metabolites. However, this needs to be coupled with effective separation technology. Various HILIC and ANP columns have shown promise in the separation of polar metabolites [3,13,20]. HILIC based LC–MS methods for the simultaneous determination of a large number of hydrophilic metabolites in non-plant samples have also been reported [14,18]. However, the performance of these methods for the analysis of sugars was not tested and another drawback of these methods is that mobile phases need to be carefully optimized to achieve adequate separation and peak shape of amino acids and organic acids. To our

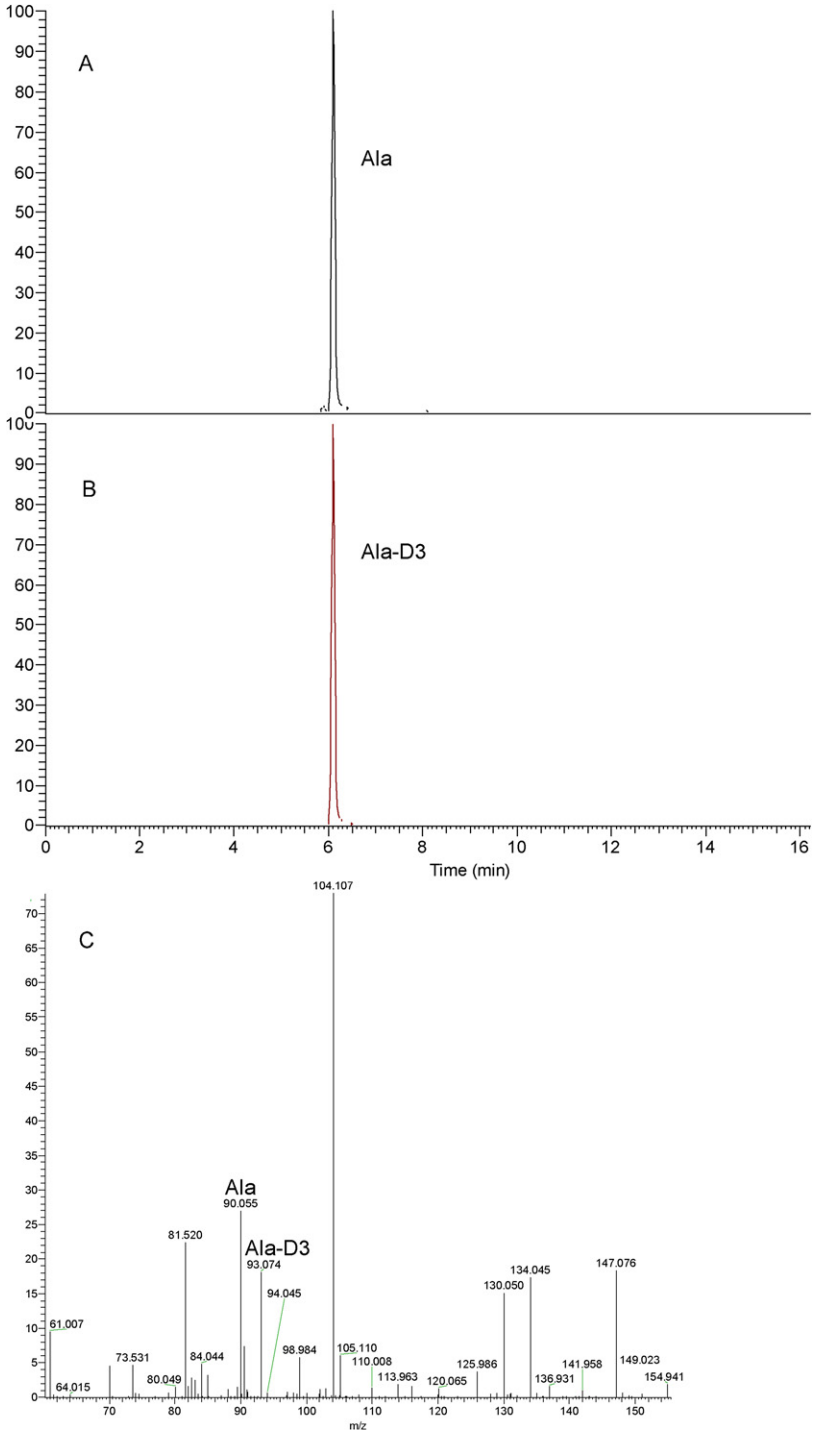

Fig. 4. EIC of endogenous alanine (A) and alanine-D3 (B) from a sample containing alanine-D3 spiked to 2-fold diluted ryegrass extract. (C) Mass spectrum of all ions co-eluted with alanine-D3 (*m*/*z*: 93.074).

knowledge, HILIC based methods that enable the quantification of all three major types of polar compounds of plants have not been reported.

The Synergi Hydro-RP column contains polar-endcapped C18 stationary phase. It provides hydrophobic selectivity and at the same time is able to improve the retention of polar compounds. This column allows adequate separation of the three major classes of polar metabolites, including the isobaric species Leu and Ile, difficult to achieve with HILIC and ANP columns. While most researchers focus on the use of HILIC columns in the separation of polar metabolites, this work demonstrates that modified reversed-phase columns have equal potential in this field.

Although sugars can be detected in either mode, amino acids produce strong signal only in positive ionization mode, whereas most organic acids can only be detected in negative mode. This implies a polarity switching method is required if one wants to determine all of these compounds in a single run. Ideally high resolution data (accurate mass) would be acquired for both positive and negative ionization modes simultaneously. With the model of LTQ-orbitrap MS instrument used for this work, this is not possible and so this led to the use of a combination of low and high

Table 3
Polar compound content in ryegrass and inter- and intra-sample variation of measurements.

Compounds	Intra-sample RSD (%) of RT	Intra-sample RSD (%) of PA	Inter-sample average content (mg/g DW)	Inter-sample SD (mg/g DW)	Inter-sample RSD (%)
Amino acids					
Alanine	0.13	0.86	0.43	0.015	3.49
Asparagine	0	0.95	0.74	0.017	2.30
Aspartic acid	0.06	1.15	0.92	0.023	2.50
Arginine	0.18	0.81	0.064	0.0017	2.66
Glutamic acid	0.12	0.48	0.97	0.067	6.91
Glutamine	0	0.74	3.35	0.13	3.88
Glycine	0.06	1.27	0.055	0.0012	2.18
Histine	0.17	2.37	0.022	0.00028	1.27
Isoleucine	0	0.93	0.11	0.0025	2.27
Leucine	0.03	1.07	0.089	0.0026	2.92
Lysine	0.13	0.91	0.044	0.001	2.27
Phenylalanine	0.04	1.37	0.08	0.0019	2.38
Proline	0.05	0.67	0.32	0.009	2.81
Serine	0.13	0.87	0.94	0.018	1.91
Threonine	0.12	0.95	0.57	0.021	3.68
Trptophane	0.03	0.93	0.058	0.0015	2.59
Tyrosine	0.03	0.52	0.061	0.0013	2.13
Valine	0.17	2.34	0.023	0.0011	4.78
Organic acids					
Malic acid	0.26	3.24	6.87	0.63	9.17
Citric acid	0.09	1.49	2.87	0.041	1.43
Succinic acid	0.05	5.38	0.42	0.011	2.62
Fumaric acid	0.10	6.98	0.084	0.007	8.33
Sugars					
Mannitol	0.18	2.48	0.31	0.02	7.54
Inositol	0.12	3.11	0.50	0.02	4.11
Glucose	0.19	2.72	7.13	0.34	4.73
Fructose	0.15	1.75	6.39	0.61	9.53
Sucrose	0.09	1.94	41.47	1.10	2.66
Raffinose	0.26	3.45	0.29	0.01	4.37

RT, retention time; PA, peak area; SD, standard deviation. Intra-sample RSD (%) of RT and PA was calculated from seven repeated injections of the same extract (replicate 1). Inter-sample average content, SD and RSD (%) were calculated from four replicate extractions of the same sample.

resolution scans in a single method. The choice of combining IT negative with FT positive modes allows us to take advantage of the high mass accuracy capability to differentiate the isobaric species of amino acids. New accurate mass ion trap instruments such as the Q-Exactive are able to rapidly switch between negative and positive modes with high resolution and so use of such instruments may further improve the selectivity of the method described here.

In the current method extraction of polar metabolites from plant tissues was performed using aqueous methanol, which extracts readily polar compounds from plant materials and is compatible with the HPLC mobile phase, facilitating direct injection of the extract.

It is widely known that co-elution of analytes from the matrix and consequently ion suppression on target compounds is a major source for inaccuracies in metabolite quantification using ESI-MS [21–23]. The most common strategies to investigate matrix effects are postextraction addition and postcolumn infusion [18,22,23]. We adopted the postextraction addition method to assess the effect of matrix on measurement accuracy.

This allowed us to confirm that with the typical extraction protocol (20 mg of dry material in 2 mL of solvent) and low injection volume, signal reduction (ion suppression) caused by the matrix is negligible for amino acids, organic acids and most sugars tested. This suggests that most of the major polar metabolites can be reliably quantified even in a complex matrix. Although some ion suppression was found for glucose, owing to its high abundance in plant material, glucose can be measured with highly diluted extract to minimize the ion suppression effect of matrix.

Although not all polar compounds could be tested in this work, the major polar compounds of plant primary metabolism are covered. Consequently, this quick method is particularly suited to research projects where a large number of samples need to be screened for major polar compounds, where a comprehensive quantification of all polar metabolites is not required. Moreover, this method, utilizing the full scan capacity and continuous polarity switching of IT mass spectrometry, provides the possibility of conducting relative quantification (fold change determination) in the same run of both positively and negatively charged polar compounds for which standards are not available.

5. Conclusions

A novel LC–MS method has been developed, which, using a single run of 25 min, is able to quantify most amino acids, several major organic acids and several major sugars. The applicability of this method is demonstrated for ryegrass where quantification of 18 amino acids, four organic acids and six major sugars was achieved with a RSD below 10% (average RSD 3.7%). In addition to the analysis of the above three classes of polar compounds, this method may be equally useful for the analysis of other polar metabolites.

Acknowledgments

The authors wish to thank Dr. Craige Trenerry for thorough critiquing of this manuscript and two anonymous reviewers for valuable suggestions. The financial support of the Dairy Futures Co-operative Research Centre is also acknowledged.

References

[1] O. Fiehn, J. Kopka, P. Dormann, T. Altmann, R.N. Trethewey, L. Willmitzer, Nat. Biotechnol. 18 (2000) 1157.
[2] U. Roessner, C. Wagner, J. Kopka, R.N. Tretheway, L. Willmitzer, Plant J. 23 (2000) 131.
[3] V.V. Tolstikov, O. Fiehn, Anal. Biochem. 301 (2002) 298.

[4] T.R.I. Cataldi, G. Margiotta, L. Lasi, B. Di Chio, C. Xiloyannis, S.A. Bufo, Anal. Chem. 72 (2000) 3902.
[5] S. Schiebold, H. Tschiersch, L. Borisjuk, N. Heinzel, R. Radchuk, H. Rollectschek, Plant Methods 7 (2011) 19.
[6] G.R. Cawthray, J. Chromatogr. A 1011 (2003) 233.
[7] M. Arnetoli, G. Montegrossi, A. Bucciant, C. Gonnelli, J. Agric. Food Chem. 56 (2008) 789.
[8] S.S. Yang, I. Smetena, Chromatographia 37 (1993) 593.
[9] R. Minocha, S. Long, J. Chromatogr. A 1035 (2004) 63.
[10] A.F. Lopez-Millan, F. Morales, A. Abadia, J. Abadia, Plant Physiol. 124 (2000) 873.
[11] P. Armengaud, R. Sulpice, A.J. Miller, M. Stitt, A. Amtmann, Y. Gibon, Plant Physiol. 150 (2009) 772.
[12] C. Antonio, C. Pinheiro, M.M. Chaves, C.P. Ricardo, M.F. Ortuno, J. Thomas-Oates, J. Chromatogr. A 1187 (2008) 111.
[13] T. Langrock, P. Czihal, R. Hoffmann, Amino Acids 30 (2006) 291.
[14] S.U. Bajad, W. Lu, E.H. Kimball, J. Yuan, C. Peterson, J.D. Rabinowitz, J. Chromatogr. A 1125 (2006) 76.
[15] H. Yoshida, T. Mizukoshi, K. Hirayama, H. Miyano, J. Agric. Food Chem. 55 (2007) 551.
[16] J.J. Pesek, M.T. Matyska, S.M. Fischer, T.R. Sana, J. Chromatogr. A 1294 (2008) 48.
[17] B. Preinerstorfer, S. Schiesel, M. Lammerhofer, W. Lindner, J. Chromatogr. A 1217 (2010) 312.
[18] S. Schiesel, M. Lammerhofer, W. Lindner, Anal. Bioanal. Chem. 396 (2010) 1655.
[19] U. Roessner, C. Wagner, J. Kopka, R.N. Trethewey, L. Willmitzer, Plant J. 23 (2000) 131.
[20] D. Callahan, D. De Souza, A. Bacic, U. Roessner, J. Sep. Sci. 32 (2009) 2273.
[21] H.P. Nguyen, K.A. Schug, J. Sep. Sci. 31 (2008) 1465.
[22] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[23] P.J. Taylor, Clin. Biochem. 38 (2005) 328.