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Analysis of Highly Polar Compounds of Plant Origin: Combination of Hydrophilic Interaction Chromatography and Electrospray Ion Trap Mass Spectrometry[1]

Vladimir V. Tolstikov[2] and Oliver Fiehn
Max Planck Institute of Molecular Plant Physiology, Department of Lothar Willmitzer, 14424 Potsdam, Germany

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The primary goal of metabolomic analysis is the unbiased relative quantification of every metabolite in a biological system. A number of different metabolite-profiling techniques must be combined to make this possible. Here we report the separation and analysis of highly polar compounds in a proof of concept study. Compounds were separated and analyzed using hydrophilic interaction liquid chromatography (HILIC) coupled to electrospray ionization (ESI) mass spectrometry. Two types of HILIC microbore columns (Polyhydroxyethyl A and TSK Gel Amide 80) were compared to normal phase silica HPLC columns. The best separations of standards mixtures and plant samples were achieved using the Amide 80 stationary phase. ESI enabled the detection of both positively and negatively charged metabolites, when coupled to a quadrupole ion trap mass spectrometer using continuous polarity switching. By stepwise mass spectrometric fragmentation of the most intense ions, unknown compounds could be identified and then included into a custom mass spectrometric library. This method was used to detect oligosaccharides, glycosides, amino sugars, amino acids, and sugar nucleotides in phloem exudates from petioles of fully expanded *Cucurbita maxima* leaves. Quantitative analysis was performed using external standards. The detection limit for stachyose was 0.5 ng per injection (Amide 80). The concentration of stachyose in investigated phloem samples was in the range of 1–7 mM depending on the plant.

Key Words: **hydrophilic interaction; HILIC; mass spectrometry; quadrupole ion trap; fragmentation; metabolite profiling; metabolomics.**

To understand cellular responses to genetic or environmental perturbations at a new level, much effort is now being directed at the simultaneous detection of all gene products (mRNA transcripts, proteins, and metabolites). Functional genomic methods have been used with considerable success to generate mRNA expression profiles and protein profiles. However, the development and application of a single method for the simultaneous detection of all the metabolites in a biological system (e.g., an organ such as a plant leaf)—the metabolome—is still beyond reach. Due to the large diversity of chemical and physical properties of metabolites, different methods must be combined to determine plant metabolomes. The most universal, sensitive, and versatile detection method applicable to metabolite detection is mass spectrometry. By coupling gas chromatography to mass spectrometry (GC/MS), the relative quantification of 326 metabolites has been demonstrated for single *Arabidopsis thaliana* leaf extracts (1). GC/MS can also be used for *de novo* identification of small plant metabolites (2). However, large and thermolabile compounds such as sugar nucleotides and large oligosaccharides cannot be detected by GC/MS due to their limited volatility. Instead, liquid chromatographic methods must be applied for such plant constituents. Reverse-phase liquid chromatography (RP-HPLC)[3] methods are widely recognized and used for the separation of nonpolar compounds such as flavonoids (3), lipids (4), or phenolics (5). Unfortunately it is hardly applicable for analysis and profiling of carbohydrates and related highly polar compounds. In this case, ion-exchange chromatography is regularly used to enable separation of organic anions and carbohydrates, coupled to amperometric, light scattering, or

[1] This work was supported by the Max Planck Society, Germany.
[2] To whom correspondence should be addressed. Fax: +49-0331-5678250. E-mail: tolstikov@mpimp-golm.mpg.de.

[3] Abbreviations used: HILIC, hydrophilic interaction liquid chromatography; RP-HPLC, reversed-phase high-performance liquid chromatography; DAB, 1,4-dideoxy-1,4-imino-D-arabinitol; CID, collision-induced dissociation.

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conductivity detection (6–8). Whereas such detection methods have proven useful for the detection of high amounts of known compounds in plants (9), coupling to more sensitive mass spectrometers should be advantageous due to the ability to quantify known target analytes and in the same run to identify unknown metabolites. Unfortunately, ion-exchange chromatography involves HPLC solvents with high concentrations of involatile inorganic salts, which makes coupling to mass spectrometry hardly applicable. Alternatively, volatile ion-pairing reagents such as triethylamine can be added to improve separation for inorganic anions like sulfonated aromatics (10); however, neutral polar organics like oligosaccharides do not form ion pairs with such agents. Hydrophilic interaction chromatography is an alternative method of separation of highly polar compounds. Its efficiency has been demonstrated for simple and complex carbohydrates (11), peptides (12), and other natural products (13–16). The retention mechanism has been suggested (11) to be an interaction of polar moieties like hydroxyl groups with the stationary phase as well as with the organic and aqueous liquid eluents. A mechanistic study was not intended in the present report but rather an exploration of its suitability for analyses of different compound classes found in plants, and its coupling to mass spectrometry for quantification and identification purposes.

MATERIALS AND METHODS

Standards and Samples

Using previously garnered knowledge of metabolites occurring in different plant species, a set of reference compounds with diverse physical and chemical properties was put together. *N*-Acetyl-D-glucosamine, 2-amino-2-deoxy-D-glucose, 1,4-dideoxy-1,4-imino-D-arabinitol, L-methionine, D(+)-raffinose, sucrose, uridine-5-diphosphoglucose, uridine-5-diphospho-(*N*-acetyl)galactosamine, L-alanyl-L-alanine, glucosaminic acid, *N*-methyl-1-deoxynojirimycin, L-homoserine, stachyose, and verbascose were purchased from SAF (Taufkirchen and Seelze, Germany). Maltoheptaose was purchased from Supelco (Taufkirchen, Germany). A stock solution of a mixture of references was prepared by water and acetonitrile (1/1, v/v) at a content of 1 μg/μl per compound. Starting from this stock solution, a binary dilution series was prepared in water/acetonitrile (1/1, v/v) for determining calibration curves. Phloem samples were acquired from the fully expanded mature leaves that did not show any signs of senescence of 8-week-old plants. Phloem sampling was done according to (24). In order to preserve water-soluble components intact and simultaneously stop enzymatic activity, phloem exudates were diluted in 300 μL of pure water, and proteins were precipitated by vortexing with 300 μL of chloroform. The water phase was completely desiccated and then dissolved in 50 μL water/acetonitrile (1/1, v/v) without any further derivatization prior to injection (2).

LC/MS$_n$

The LC/MS system consisted of a Finnigan LCQ DECA mass spectrometer (ThermoFinnigan, San Jose, CA), a Rheos 2000 pump (Flux Instruments AB, Karlskoga, Sweden), and an HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland). The system was operated under the Xcalibur software (version 1.2 SP1, ThermoFinnigan). Liquid chromatography was performed using acetonitrile (LiChrosolv, Merck, Darmstadt, Germany) (A) and 6.5 mM ammonium acetate (pH 5.5, adjusted by acetic acid) (B) as the mobile phase at flow rates of 0.15 mL/min at ambient temperature. Ammonium acetate and acetic acid were HPLC grade and purchased from SAF (Taufkirchen and Seelze). Three columns were compared: a normal phase silica column (Nucleosil NH_2, 150 $\times$ 1.0 mm, 5-μm particle size (Phenomenex, Torrance, CA)), and two hydrophilic interaction columns (a 150 $\times$ 1.0 mm, 5-μm particle size Polyhydroxyethyl A column (PolyLC Inc., Columbia, MD)) and a 250 $\times$ 2.0 mm, 5-μm particle size TSK Gel Amide 80 (TosoHaas, Montgomeryville, PA). All stationary phases used have the silica backbone but different modifications and/or coating. Nucleosil NH_2 has propylamine arms attached, Polyhydroxyethyl A includes poly(aspartamide) coating and TSK Gel Amide 80 comprises covalently bonded carbamoyl groups. After a 5-min isocratic run at 0% B, a gradient to 15% B was concluded at 10 min, and then a gradient to 60% B was completed at 60 min for the Nucleosil NH_2 column, to 35% B for the Polyhydroxyethyl A column, and to 55% B for the TSK Amide 80 column. The injection volume was set to 10 μL. HPLC columns were connected to the electrospray interface of the mass spectrometer without splitting. Nitrogen sheath gas pressure was set to 6 bar at a flow rate of 0.8 L/min. Spray voltage was set to 5 kV. The temperature of the heated transfer capillary was maintained at 250°C. The helium collision gas incoming pressure was 2.6 bar, and the ion gauge pressure was 0.89×10^{-5}. Full scan mass spectra were acquired from 150 to 2000 amu at unit mass resolution. For stepwise fragmentation experiments, data-dependent scanning was chosen with the wideband activation turned off. The normalized collision energy was set to 35%, and the activation Q was 0.250 with the source fragmentation turned off.

RESULTS AND DISCUSSION

Separation

In order to develop a method for efficient separation of highly polar components from plant samples,

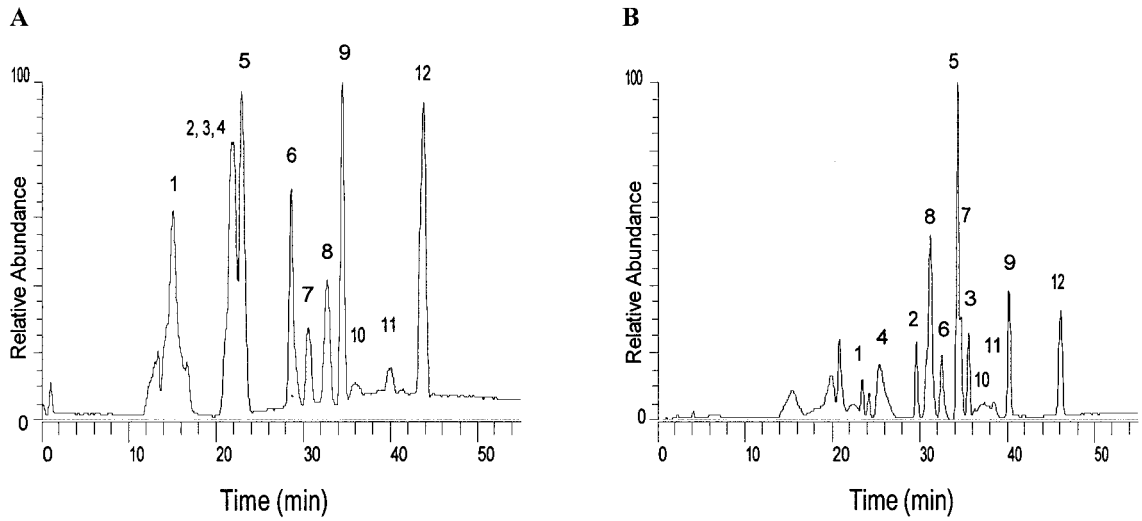

FIG. 1. HILIC/MS base peak chromatograms of a mixture of standards. (A) Polyhydroxyethyl A with a gradient of 15 to 35% B from 10 to 60 min. (B) TSK Gel Amide 80 with a gradient of 15 to 55% B from 10 to 60 min. Identified peaks: (1) *N*-acetyl-D-glucosamine, (2) sucrose, (3) D(+)-raffinose, (4) L-methionine, (5) *N*-methyl-1-deoxynojirimycin, (6) L-alanyl-L-alanine, (7) 1,4-dideoxy-1,4-imino-D-arabinitol (DAB), (8) uridine-5-diphosphoglucose (UDPGlc), (9) stachyose, (10) glucosaminic acid, (11) 2-amino-2-deoxy-D-glucose, (12) maltoheptaose.

we started by separating a mixture of reference compounds that commonly occur in plants. In preceding experiments, we tested the use of a classical normal phase column. Although a reasonable separation could be achieved for most of the reference compounds (data not shown), peak shapes were unsatisfactory and very broad (>2 min), therefore decreasing sensitivity. Furthermore, retention times were not very reproducible, with fluctuations exceeding 4-min time windows, accompanied by changes in retention times relative to the other compounds. As an alternative, we tested the use of HILIC columns (14). For each column, an optimal gradient profile was developed in order to obtain sufficient separation for all reference compounds. The achievable separation is illustrated in Fig. 1, representing the base peak chromatograms recorded in positive and in negative ionization modes. Several conclusions can be drawn from this figure. First, more than 13 peaks were observed, indicating impurities in the mixture of reference compounds. Second, baseline separation could not be achieved for all compounds (see the summary in Table 1). Methionine, sucrose, and raffinose strongly coeluted from the Polyhydroxyethyl HILIC column. Such non-isobaric compounds can still be individually analyzed by extracted ion chromatograms, making use of the superior resolution power of mass spectrometry compared to classical

TABLE 1

Retention Times of Standards on HILIC/MS for Polyhydroxyethyl A and TSK Gel Amide 80 Columns[a]

No.	Standard	Retention time (min)	
		Polyhydroxyethyl A	TSK Gel Amide 80
1.	*N*-Acetyl-D-glucosamine	15.05	23.49
2.	Sucrose	21.91	29.57
3.	D(+)-Raffinose	21.91	35.57
4.	L-Methionine	21.96	25.48
5.	*N*-Methyl-1-deoxynojirimycin	22.86	34.29
6.	L-Alanyl-L-alanine	28.96	32.49
7.	1,4-Dideoxy-1,4-imino-D-arabinitol	30.62	34.74
8.	Uridine-5-diphosphoglucose	32.74	31.11
9.	Stachyose	34.52	40.21
10.	Glucosaminic acid	35.99	37.29
11.	2-Amino-2-deoxy-D-glucose	40.06	38.43
12.	Maltoheptaose	43.73	46.16

[a] Flow rate 0.15 ml/min. Elution time 60 min.

detection methods such as refractory index detectors. However, due to potential ion suppression effects (17), coelution should still be diminished as much as possible in order to allow robust quantification (see below). For both columns, retention times were dependent on compound hydrophilicity. For example, the elution order of oligosaccharides depended on the number of hydroxyl moieties (maltoheptaose > stachyose > raffinose > sucrose), indicating that the retention mechanism of hydrophilic interaction is between the hydroxy groups and the stationary phases. Surprisingly, small molecules such as simple amino acids like methionine also showed strong retention on both phases. Generally, each amino group in the molecular structure added a significant shift in retention time. For example, 2-amino-2-deoxyglucose is eluting 20 min later than glucose (data not shown), although the molecular size as well as the number of hydroxy groups is very similar. This finding suggests the presence of an additional retention mechanism in HILIC chromatography, such as weak cationic interaction, in addition to hydrogen bridges between hydroxy groups of the analyte and the stationary phase. As outlined above, a detailed investigation of the retention mechanisms was beyond the scope of this study. It may be noted, however, that the increased retention of amino groups in HILIC chromatography is complementary to the more difficult chromatographic behavior of polar amino compounds in reverse-phase HPLC (18).

Some mass spectrometers such as time-of-flight instruments do not allow continuous polarity switching for electrospray ionization during LC/MS run. Further, any polarity switching represents a single mass spectrometric experiment, which takes about 1–3 s to be carried out. In metabolomic analyses, trace ions might therefore be overlooked if coeluting with major compounds in data-dependent MS/MS and MS/MS/MS experiments. When comparing the positive to the negative mode base peak chromatogram, each compound can be detected and analyzed in either mode. However, compounds that are prone to get positively ionized such as amino sugars and amino acids are more abundant in the positive mode, whereas neutral oligosaccharides that partly get deprotonized even at pH 5.5 are found to be more abundant in the negative mode than in the corresponding sodiated $[M+Na]^+$ form. Overall separation efficiency was found to be slightly better using the Amide 80 instead of the Polyhydroxyethyl A column for both the mixtures of reference compounds and plant samples (see below). Moreover, peaks shapes were sharper with less peak broadening, less tailing, and reduced variability of absolute retention times for the Amide 80 column. Therefore, this column was chosen for further analysis.

Identification

Many polar compounds have different functional groups in their structures that result in different fragmentation patterns in positive and negative modes (19, 20). Positively charged ions have electron-density distribution quite unlike negative ions. Therefore, collision-induced cleavage of chemical bonds, fragmentation pathways, and fragment stability are compound specific. Moreover, MS/MS and MS_n spectra should be identical under tightly controlled instrument parameters if acquired by quadrupole ion-trap mass spectrometers, independent of instrument type and manufacturer. This offers the possibility of creating custom libraries of reference compounds, and to share these libraries with all other research groups who acquire data by ion trap MS/MS. Therefore, tentative identification of unknown compounds is accomplished not only by similarities of retention times to known compounds, but mostly by interpretation of fragmentation pathways. If possible, structural elucidation is further aided by calculating elemental compositions by exact mass measurements and investigation of additional spectral characteristics, such as interpretation of nuclear magnetic resonance spectra. The characterization of fragmentation pathways is therefore first exemplified for reference compounds. The identification of 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) included in the mixture of references is illustrated in Fig. 2, and the fragmentation of stachyose is presented in Fig. 3. First, the molecular ion needs to be assigned. Since electrospray is a soft ionization technique that often leads to adduct formation in the positive mode, such as $[M+NH_4]^+$, $[M+Na]^+$, $[M+K]^+$, or $[M+M+Na]^+$, the molecular ion $[M+H]^+$ is not in all cases the most abundant ion present in MS spectra. This is highly dependent on the molecular structure, and the true molecular mass may often be revealed only at the MS/MS stage, or if comparing mass spectra gained at negative and positive modes. When analyzing metabolites bearing basic amino groups, $[M+H]^+$ ions are regularly found. This directly helps in identifying unknown compounds, since in cases where masses can be established as the true $[M+H]^+$ ions (e.g., for DAB, m/z 134), the molecular mass must be odd (e.g., for DAB, M = 133 amu). Due to the nitrogen rule, any (unknown) peak revealing an odd molecular mass must bear an odd number of nitrogen atoms. Once a molecular structure is proposed, its probability can be further evaluated by an interpretation of fragments found at the MS/MS or MS_n stage. In the example of DAB (Fig. 2), masses of the fragments generated by Mass Frontier 2.0 software matched the observed ones. Collision-induced dissociation (CID), upon ion collision with He gas inside the ion trap, is a complex process including bond cleavage, bond formation, rearrangements, hydrogen, and charge transfer. Computer-gen-

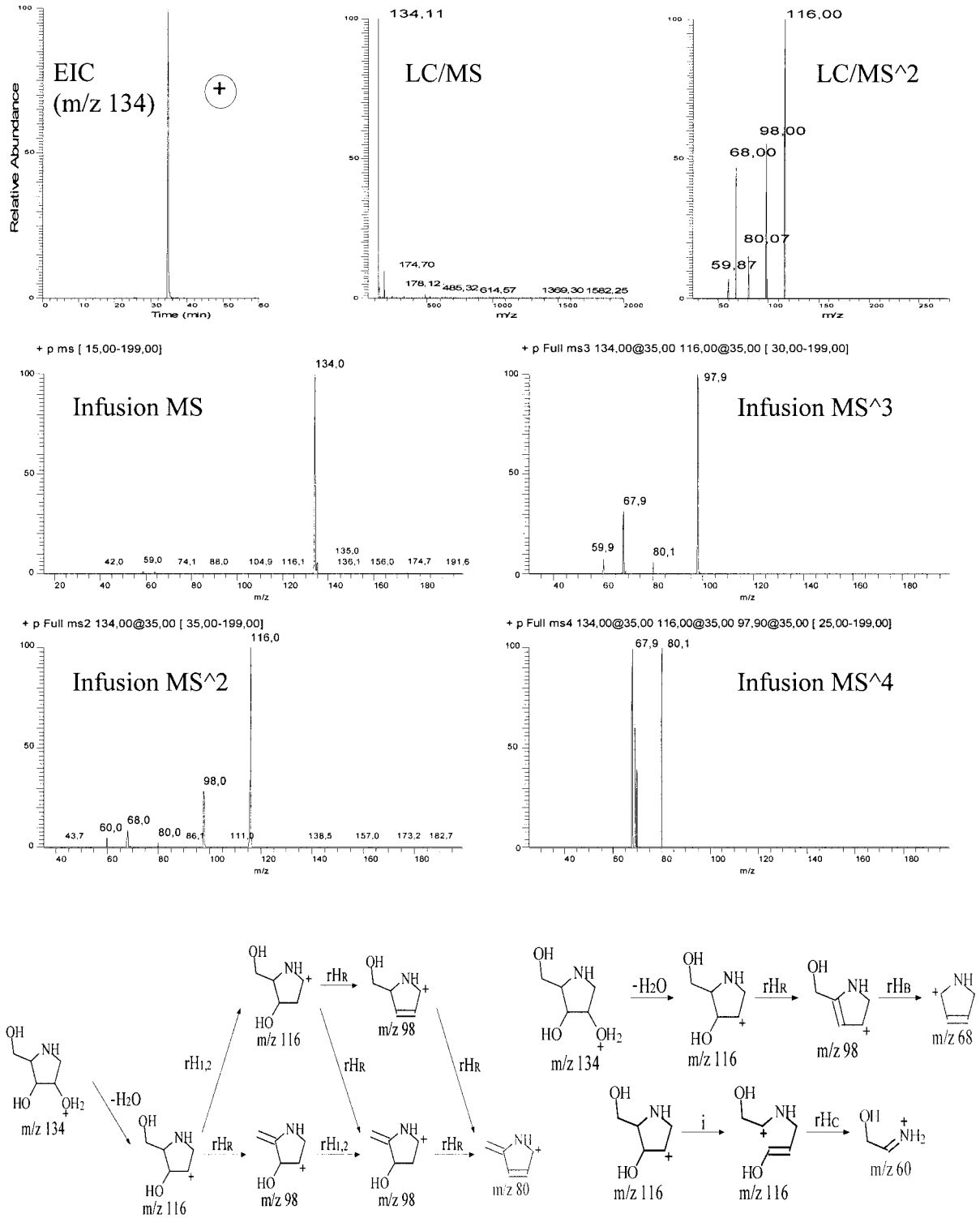

FIG. 2. Extracted ion chromatogram of 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) (TSK Gel Amide 80, elution time is 60 min), in positive mode with the following MS and MS_2 spectra (upper panel); MS fragmentation spectra of DAB obtained by direct infusion of a standard (lower panel); fragmentation scheme generated by Mass Frontier 2.0 software.

erated fragments (Fig. 2, bottom) do not necessarily present the most stable form of cations which were detected for DAB. For example, the ion with *m/z* 116 can have a more favorable form as N-protonated olefin, the ion with *m/z* 98 as an N-protonated diolefin (more likely hydroxymethylpyrrol), the ion with *m/z* 68 as an

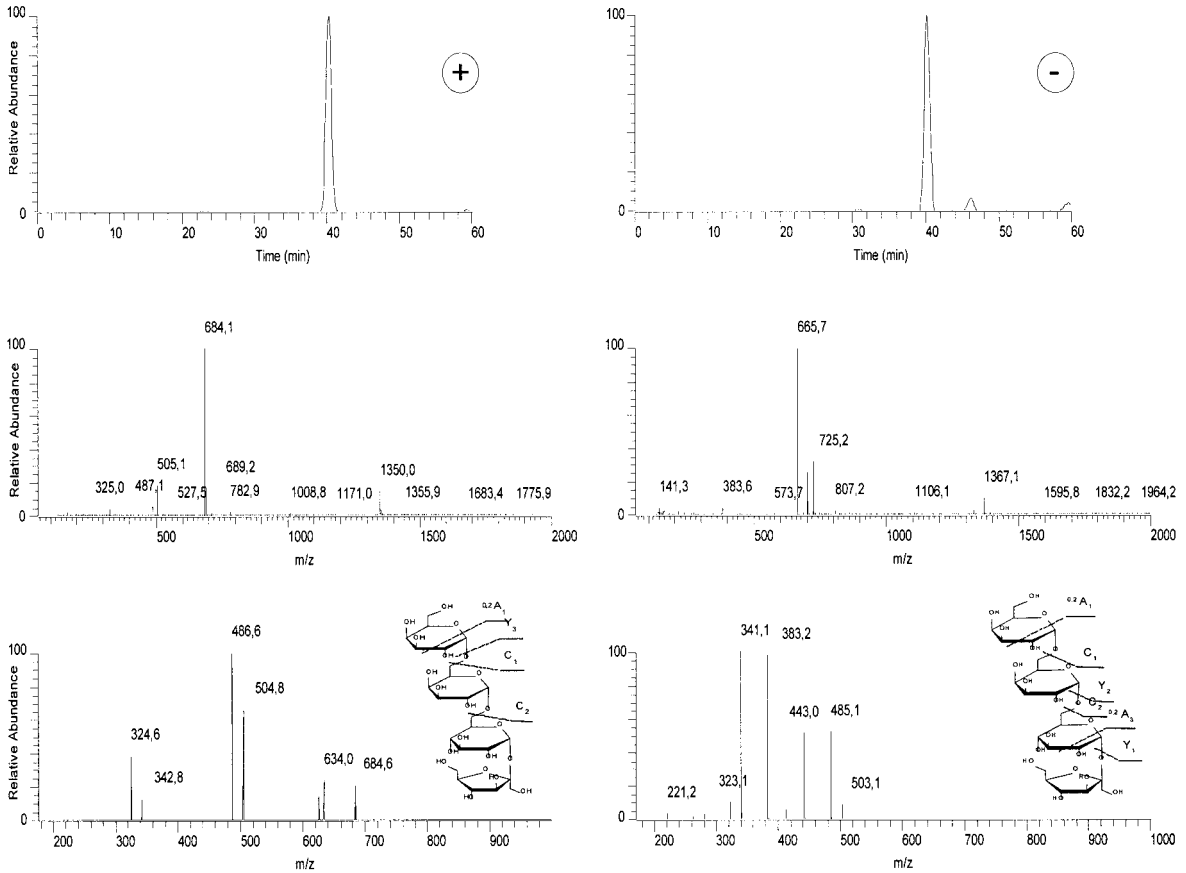

FIG. 3. Extracted ion chromatogram of stachyose (TSK Gel Amide 80, elution time is 60 min), in positive and negative modes with the following MS and MS_2 spectra.

N-protonated pyrrol, and the ion with *m/z* 60 as it is shown in Fig. 2. Therefore, the Mass Frontier software proves to be helpful by quickly suggesting potential fragmentation pathways that can explain the observed signal abundances, such as in the MS/MS spectra of DAB. Furthermore, the software may be even more helpful if used for the interpretation of MS_n spectra obtained from ion-trap mass spectrometers. Regularly peak widths are too narrow in liquid chromatography to allow data-dependent MS_n fragmentation in both positive and negative mode. Therefore, pure DAB was dissolved in elution buffer and directly infused into the mass spectrometer in order to investigate the software usability for the interpretation of DAB MS_4 spectra. The DAB fragmentation pathway is illustrated in Fig. 2 (lower panel). Infusion MS and MS_2 spectra were almost identical to those obtained by LC/MS and MS_2 since the same instrument parameters were chosen as in LC runs. For the MS_3 spectrum with *m/z* 116 as parent ion, the ion at *m/z* 98 might be formed by two different fragmentation pathways leading to two different structures. These structures both give rise to the ion *m/z* 80 which is observed in all DAB spectra (MS_2, MS_3, and MS_4). Furthermore, the Mass Frontier software helps explaining why the ion *m/z* 60 can only be obtained from *m/z* 116 as observed in the MS_3 spectrum, but not from *m/z* 98 as it is missing in the MS_4 spectrum. Such considerations clearly aid in suggesting chemical structures for unknown compounds, if some additional background information is known (such as the elemental composition as derived from exact mass measurements). For compounds that are not commercially available, such investigation could be performed by infusing purified fractions after LC separation and fraction collection, or by stopped-flow techniques during LC runs.

Fragmentation pathways are quite different for compounds that do not contain nitrogen in their molecular structures. The tetrasaccharide stachyose (MW 666.58, monoisotopic mass 666.22), for example, does not form $[M+H]^+$ at *m/z* 667 ions under microscale HPLC ionization conditions (Fig. 3) due to its nonbasic nature. Instead, the base peak is found for $[M+NH_4]^+$ at *m/z* 684, and the second most intense is $[M+Na]^+$ at *m/z* 689. This 5 amu difference already indicates an even molecular mass with M = 666, which is further supported by observing an abundant $[M-H]^-$ at *m/z* 665 in the negative electrospray ionization mode. An ad-

TABLE 2

Retention Times of Identified Compounds from *C. maxima* Phloem Samples by HILIC/MS on a TSK Gel Amide 80 Column[a]

No.	Substance	Retention time (min)
1.	3-*O*-[β-D-Xylopyranosyl-(1-6)-β-glucopyranosyl]-(3*R*)-octen-3-ol	29
2.	Sucrose	32
3.	Uridine-5-diphospho-(*N*-acetyl)galactosamine	41
4.	Uridine-5-diphosphoglucose	42
5.	Raffinose	44
6.	Homoserine	45
8.	Stachyose	56
9.	Verbascose	60

[a] Flow rate 0.15 ml/min. Elution time 90 min.

duct formation of $[M+CH_3COO_2]^-$ is also seen at *m/z* 725 but is far less abundant. At the MS/MS level, hexose cleavages are regularly found for carbohydrates, as is observed for stachyose as $[M-H-Hex]^-$ at *m/z* 485 in the negative mode, and as $[M+H-Hex]^+$ at *m/z* 487 in the positive mode. The latter cleavage can be interpreted as a loss of a hexose moiety and neutral ammonia from the $[M+NH_4]^+$ ion with a simultaneous proton transfer to the carbohydrate backbone. Moreover, a loss of two hexoses is observed in the positive mode, indicated by *m/z* 359 for $[M+NH_4-(Hex)_2]^+$. In both ionization modes, a number of cross-ring cleavages is found at the MS/MS level, that are specific for stachyose. This fragmentation pattern is then included in a custom library to identify stachyose in plant samples. Substances carrying carboxyl and/or phosphate groups are nicely detected in negative mode. Uridine-5-diphosphoglucose, uridine-5-diphospho-(*N*-acetyl)-galactosamine (Fig. 5), and glucosaminic acid were detected and fragmented in negative mode. All the compounds listed in Table 2 were identified by the comparison of retention times, MS, and MS_n spectra with the corresponding data obtained for standards as described for DAB.

Quantification

In metabolomic applications, absolute metabolite levels are rarely determined. Instead, the regulation of metabolite levels is quantitatively studied relative to a control, e.g., in mutant/wild-type comparisons, or under stressed and unstressed environmental conditions. However, in a few cases, the exact metabolite quantities are needed, e.g., for calculation of fluxes along biochemical pathways or for better understanding of long-distance transport. Furthermore, low detection limits and linear calibration curves are intrinsically

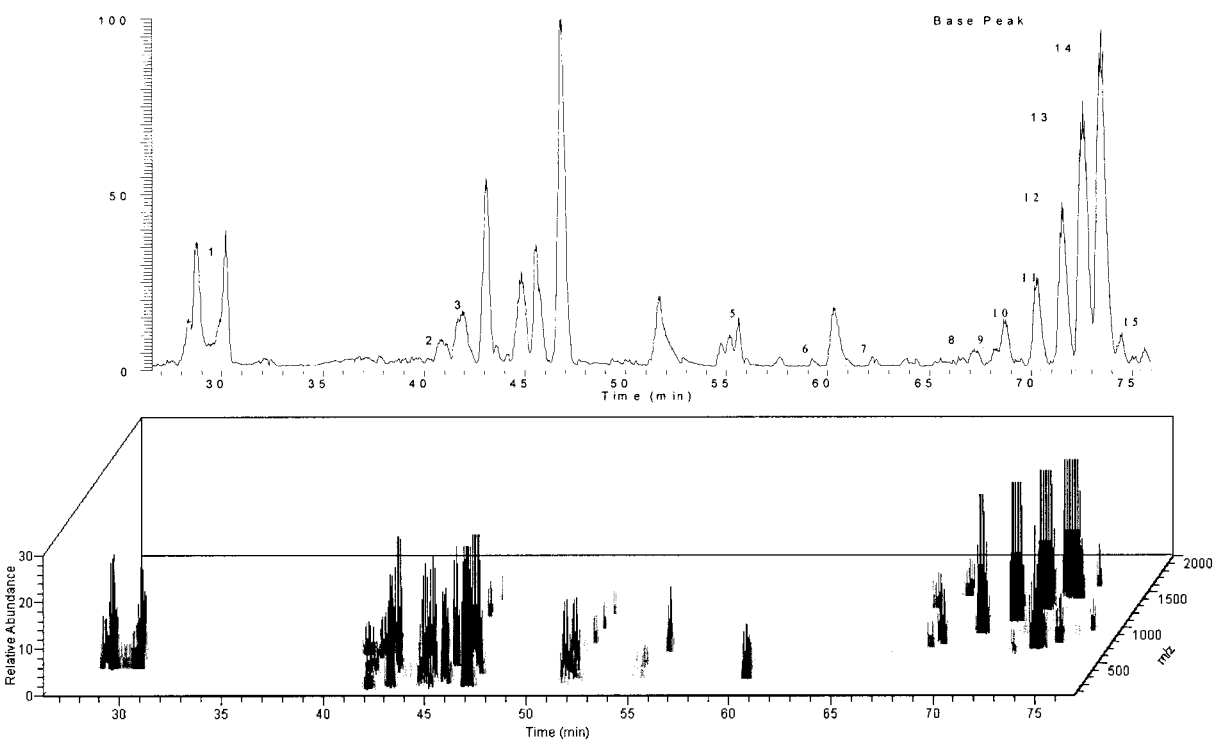

FIG. 4. HILIC/MS base peak chromatogram of *Cucurbita maxima* phloem, presented in a map view (TSK Gel Amide 80, gradient to 60% B was completed at 90 min). Identified peaks are (1) 3-*O*-[β-D-xylopyranosyl-(1-6)-β-glucopyranosyl]-(3*R*)-octen-3-ol, (2) UDP(NAc)Gal, (3) UDPGlc, (4) DAB, (5) stachyose, (6) verbascose, (7–15) *O*-glycans.

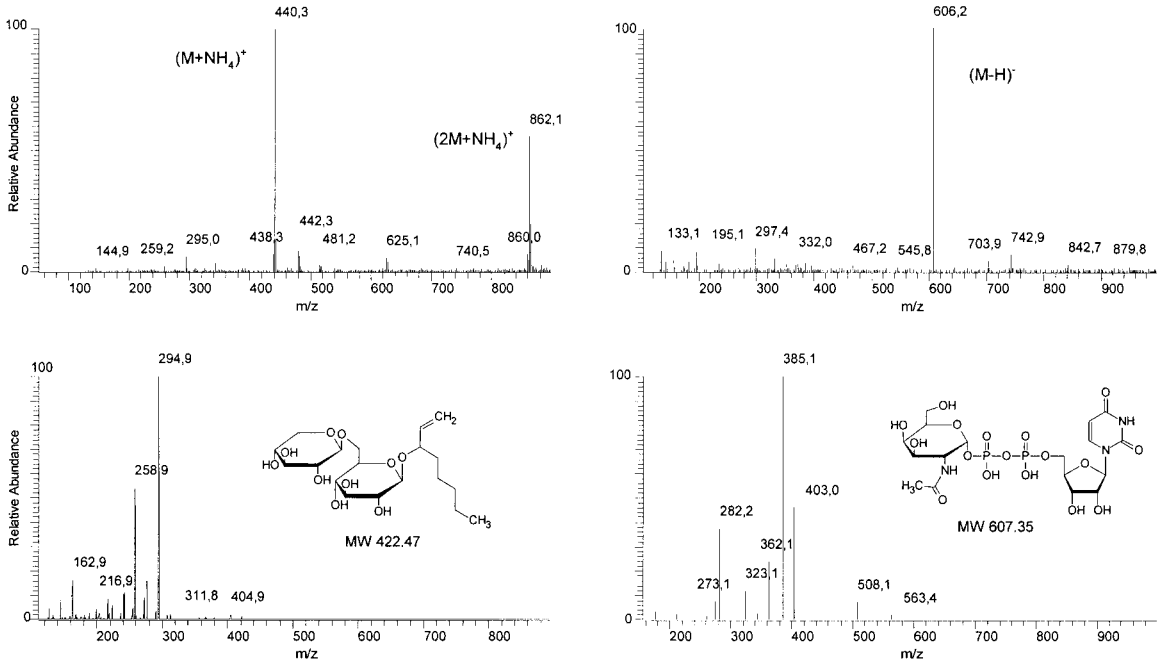

FIG. 5. MS/MS spectra of novel metabolites detected in phloem of *Cucurbita maxima*. (Left) 3-*O*-[β-D-xylopyranosyl-(1-6)-β-glucopyranosyl]-(3*R*)-octen-3-ol. (Right) Uridine-5-diphospho-(*N*-acetyl)galactosamine.

assumed in relative quantifications, even though they might not hold true in all cases. New metabolomic methods must be tested to ensure analytical precision for a range of metabolites, to ensure that the method of metabolite determination itself does not significantly contribute to overall variability. Quantitative analysis has been performed with the LC_{QUAN} program (the instrument's software) by external references. To determine calibration curves, fivefold dilution series were performed using a water/acetonitrile mixture (1/1, v/v) starting from a stock solution of a mixture of standards (1000 ng/μl per compound). Five-point calibration curves were generated for all standards separated on Amide 80 stationary phase. Over the established concentration range, linear regression of concentration versus observed peak areas gave acceptable fits. In the vascular system of symplastic loaders, stachyose is believed to be one of the major forms in which assimilated carbon is transported. Therefore, stachyose was used as an example to test the linearity and sensitivity of HILIC/MS quantification. In the MS mode, the detection limit was 0.5 ng (loaded onto the microbore Amide 80 column). Detector saturation was observed at high concentrations (above 2 μg per injection). In this case, the linearity of calibration curves could be propagated using the MS_2 fragments of stachyose. In order to quantify stachyose in plant samples where concentration of stachyose may significantly vary, the calibration curves were fit using weighted quadratic equation of $1/X$ ($R^2 > 0.9958$). The concentration of stachyose was found in the range of 1–7 mM depending on the sampling position and on the developmental stage of the individual plant.

Application

The metabolite profile of phloem sampled from *Cucurbita maxima* Duch. is presented in Fig. 4. This species is believed to mainly transport raffinose-family oligosaccharides such as raffinose, stachyose, and verbascose. This increase in size is thought to be due to a symplastic phloem-loading mechanism, which involves passive transport across cell walls by open plasmodesmata. In order to inhibit backflow, the transport form of metabolites is believed to be size trapped (21, 22). In fact, raffinose, stachyose, and verbascose can be detected in phloem collected from *C. maxima* petioles. However, quantification of stachyose amounts only revealed 1–7 mM concentrations, which is consistent with earlier reports (23). Instead, a high number of both smaller and larger metabolites can be found in phloem exudates. Among them, the important sucrose catabolite UDP-glucose is present, indicating that sucrose synthase rather than invertase is active here as the sucrose-cleaving enzyme (24). Furthermore, amino acids such as homoserine and methionine could be detected, and larger oligosaccharides that were characterized as nitrogen-containing *O*-glycans (25). In total, 60 peaks were clearly revealed by visual inspection of HILIC/MS phloem chromatograms. However, structural investigation of these oligosaccharides will require linkage determination and investigation of the

monosaccharide compositions. By interpretation of mass spectra and comparison to an external reference (see Acknowledgments), the most abundant peak at 30 min was successfully identified as 3-*O*-[β-D-xylopyranosyl-(1-6)-β-glucopyranosyl]-(3*R*)-octen-3-ol (Fig. 5). In addition, uridine-5-diphospho-(*N*-acetyl)galactosamine was detected in phloem exudates for the first time. To be certain of their absolute configurations, an investigation of NMR spectra of the purified compounds would be needed, either by fraction collection and off-line analysis or by on-line LC-NMR/MS studies. The biological role of these compounds and larger oligosaccharides is as yet unknown. By using HILIC/MS, the function of such metabolites may be studied by determining quantitative and qualitative changes in comparison of different *Cucurbitaceae* species with respect to developmental and environmental alterations.

CONCLUSION

The combination of hydrophilic interaction chromatography and electrospray quadrupole ion-trap mass spectrometry has proven to be a powerful tool for profiling oligosaccharides and sugar nucleotides in metabolomic studies. In addition to a set of metabolites known to be important in phloem-loading mechanisms, novel components of plant phloem exudates have been identified that provide insights into the metabolic processes involved in plant long-distance transport. Such methods have the potential to be further developed to more comprehensive HPLC methods capable of separating hydrophobic and hydrophilic compounds in one chromatographic run by HILIC/reverse-phase HPLC columns coupling prior to MS detection.

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