Analysis of sphingolipids in potatoes (*Solanum tuberosum* L.) and sweet potatoes (*Ipomoea batatas* (L.) Lam.) by reversed phase high-performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS)

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Ceramides and glucocerebrosides of potatoes (*Solanum tuberosum* L.) and sweet potatoes (*Ipomoea batatas* (L.) Lam.) were analyzed using RP-HPLC-ESI-MS/MS. Ceramides and glucocerebrosides containing the three different long-chain bases 4,8-sphingadienine (d18:2$^{\Delta4,\Delta8}$), 4-hydroxy-8-sphingenine (t18:1$^{\Delta8}$), and 8-sphingenine (d18:1$^{\Delta8}$) acylated to saturated and unsaturated hydroxy- and nonhydroxy fatty acids with 16–26 carbon atoms were detected. For ceramides and glucocerebrosides 4,8-sphingadienine (d18:2$^{\Delta4,\Delta8}$) was found as the major long-chain base, with lesser amounts of 4-hydroxy-8-sphingenine (t18:1$^{\Delta8}$) and 8-sphingenine (d18:1$^{\Delta8}$). 2-(α-)Hydroxypalmitic acid (C16:0h) was the major fatty acid, which was found to be acylated to the long-chain bases. For quantification of these compounds, an RP-HPLC-ESI-MS/MS method with an "echo-peak"-technique simulating internal standard injection was developed. The analyzed samples of potatoes and sweet potatoes showed amounts of ~0.1–8 µg/kg single ceramides and amounts up to 500 µg/kg glucocerebrosides, with C16:0h-glucosyl-4,8-sphingadienine as the major component.

Keywords: Ceramides / Cerebrosides / HPLC-MS/MS / Potatoes / Sphingolipids

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1 Introduction

Sphingolipids, first discovered by Thudichum in 1884 [1], are the most diverse as well as complex class of lipids, which are ubiquitous constituents of all cellular membranes, lipoproteins, and other lipid-rich tissues. They are highly bioactive compounds that serve not only as components of biological structures, but due to their occurrence in cellular membranes, they also participate in the regulation of cell proliferation, cell signaling, cell growth, differentiation, cell–cell and cell–matrix interactions, cell migration, membrane trafficking, and apoptosis [2–7].

Appreciable amounts of sphingolipids are present in both plant and animal foods, but little is known about their nutritional significance. Recent studies have shown that complex dietary sphingolipids such as sphingomyelin and soy glucosphingolipids suppress colon carcinogenesis [8–12]. Animal experiments clearly show that feeding sphingolipids reduces serum LDL cholesterol and elevates HDL, suggesting that sphingolipids represent "functional" constituents of food [13–15]. This protective role may be the result of a turnover to bioactive metabolites, including sphingoid bases (sphingosine and sphinganine) and ceramides, which inhibit proliferation and stimulate apoptosis.

The majority of sphingolipids consist of a sphingoid backbone (such as sphingosine) usually amide-linked to long-chain fatty acids to produce ceramides (Fig. 1). More complex sphingolipids contain polar headgroups such as hexoses or inositolphosphate linked to the C-1 of the *N*-acyl long-chain base, as shown by a few examples in Fig. 1. Variations in the sphingoid backbone, differences in the fatty acid chain length, degree of saturation, and degree of hydroxylation of the fatty acid as well as variations of the headgroup are leading to a large number of different sphingolipids in plant or mammal organisms.

While sphingolipids of mammalian organisms contain mainly sphingosine (*trans*-4-sphingenine, d18:1$^{\Delta4}$) and smaller amounts of sphinganine (d18:0) and 4-hydroxy-

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Abbreviations: EPI, enhanced product ion mode; **MRM,** multiple reaction monitoring

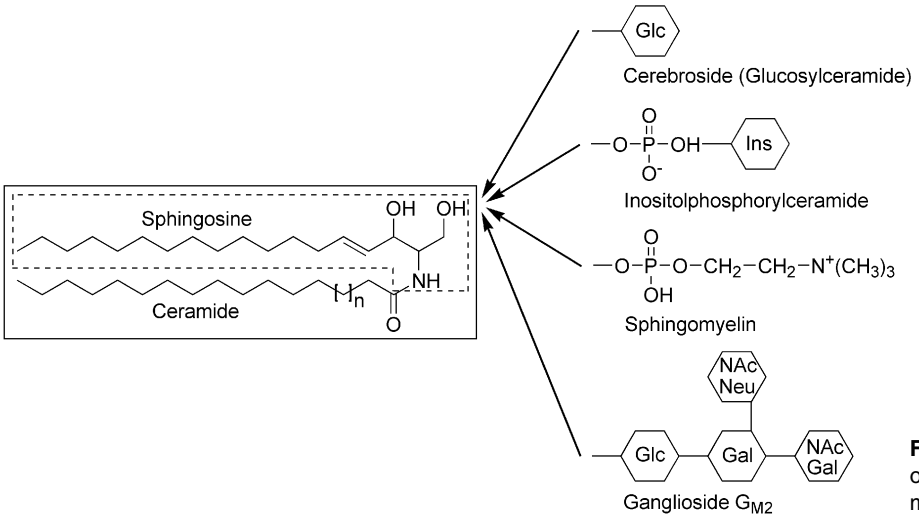

Figure 1. Representative structures of sphingolipids found in plants and mammals.

sphinganine (t18:0; phytosphingosine) as ceramide backbones, plants contain primarily sphinganine (d18:0), 4-hydroxysphinganine (t18:0; phytosphingosine), *cis* and *trans* isomers of 8-sphingenine (d18:1$^{\Delta 8}$), 4,8-sphingadienine (d18:2$^{\Delta 4,\Delta 8}$), and 4-hydroxy-8-sphingenine (t18:1$^{\Delta 8}$) as long-chain bases [16, 17]. Sphingoid bases are abbreviated similar to fatty acids; the chain length and number of double bonds are denoted in the same manner with the prefix "d" or "t" to designate di- and trihydroxy bases, respectively. The described sphingoid bases share the core structure 2-amino-1,3-dihydroxy octadecane. Thus, sphinganine is represented by d18:0, phytosphingosine by t18:0 and sphingosine with an additional double bond at position 4 by d18:1$^{\Delta 4}$ (Fig. 2).

Fatty acids amide-linked to the sphingoid base of complex plant ceramides and glucocerebrosides may be long-chain odd- and even-numbered saturated or unsaturated. Predominantly 2- (or α-) hydroxy fatty acids containing 16 to 26 carbon atoms are found [18–25]. Fatty acids containing no other substituents are denoted as C16:0. Those which contain a 2- (or α-) hydroxy group are referred to as C16:0h. Cerebrosides, chemically belonging to the group of glycosphingolipids, are composed of a polar headgroup like hexose or another carbohydrate moiety bound to a ceramide by a glycosidic linkage in position 1 (Fig. 1). Considering the variation in the sphingoid long-chain bases, the fatty acids and the polar headgroups, the individual molecular species of sphingolipids number in the thousands, which makes them the most structurally diverse as well as complex category of lipids [13, 26].

The structural variability of the sphingolipid classes and lack of reference compounds pose special problems for the analysis of sphingolipids. Little is known about their occurrence in food or in natural sources. Systematic quantitative studies of the sphingolipid content and composition of plant

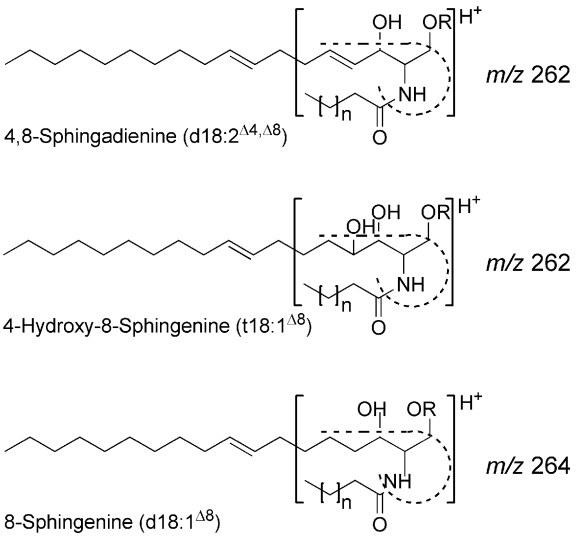

Figure 2. Structures and characteristic fragmentations (positive ionization MS mode) of sphingolipids with the three long-chain bases 4,8-sphingadienine (d18:2$^{\Delta 4,\Delta 8}$), 4-hydroxy-8-sphingenine (t18:1$^{\Delta 8}$), and 8-sphingenine (d18:1$^{\Delta 8}$) found in potatoes and sweet potatoes; ceramide (R = H), cerebroside (R = carbohydrate).

tissues have not been performed yet [16]. There is just a limited number of previous studies concerning primarily the structural analysis of glucocerebrosides, ceramides, and inositolphosphorylceramides in some plant tissues [18–25, 27, 28]. The structures of the long-chain bases and fatty acids of sphingolipid molecules described in those studies were mainly analyzed by TLC, HPLC, GC, or GC-MS following acid or alkaline hydrolysis of the extracts and derivatization, losing structural information by hydrolysis of the linkage positions. Also, the quantification of sphingolipid contents in tissues using these methods can only be an estimation [13].

A powerful tool for the structural elucidation and analysis of complex sphingolipids is the combination of RP-HPLC-ESI-MS/MS, which provides structure specific data for the characterization of known or unknown molecular species as well as the possibility to quantify already characterized molecules. HPLC-MS has been applied more frequently to the analysis of ceramides, sphingomyelins, and various glycocerebrosides in the last few years [29–33]. It has been proven to be an efficient method to characterize intact sphingolipid species and to analyze entire sphingolipid profiles even in complex matrices like plant tissues. However, no applicable methods for the quantification of ceramides and cerebrosides in complex plant matrices have been published yet.

In the course of this study we developed an RP-HPLC-ESI-MS/MS method for the separation, identification, and quantification of ceramides and cerebrosides in plant materials. Using this method numerous new ceramides and cerebrosides could be identified for the first time in potatoes and sweet potatoes. The lack of reference compounds that may be used as internal standards complicated the quantification. Therefore, we developed an "echo-peak"-technique for standard injection, which simulates an internal standard. Thus, interfering matrix effects will be eliminated. The echo-peak-technique for internal standard calibration was applied for the quantification of ceramides and glucocerebrosides for the first time and yielded reproducible results.

2 Materials and methods

2.1 Materials

The plant material, potatoes (*Solanum tuberosum* L., species: Annabelle, Berber, Deutsche Princess, Nicola), and sweet potatoes (*Ipomoea batatas* (L.) Lam.), was purchased from local markets.

N-Stearoylsphingosine was obtained from Sigma-Aldrich (Deisenhofen, Germany). Glucocerebrosides (plant), containing mainly 2-(α-)hydroxypalmitic acid-4,8-sphingadienine-glucocerebroside (C16(OH) β-D-glucosyl cerebroside) for mass spectrometric structural studies was purchased from Matreya LLC (Pleasant Gap, USA).

The echo-peak-standards *N*-lauroyl-D-*erythro*-sphingosine (C12 ceramide) and D-glucosyl-β1-1′-*N*-octanoyl-D-*erythro*-sphingosine (C8 β-D-glucosyl ceramide) were obtained from Avanti Polar Lipids (Alabaster, USA).

All solvents used for sample extraction, chromatography, and MS were purchased from Merck (Darmstadt, Germany) and Carl Roth (Karlsruhe, Germany) in gradient grade quality. Water for HPLC separation was purified with a Milli-Q Gradient A10 system (Millipore, Schwalbach, Germany). Ammonium acetate solution (5 M in water) for the mass spectrometric experiments was obtained from Sigma-Aldrich (Steinheim, Germany).

2.2 Sample preparation

Ceramides and cerebrosides were extracted from potato tubers and sweet potatoes according to a slight modification of the method for lipid extraction of Bligh and Dyer [34]. One hundred grams of freeze–dried sliced potatoes were homogenized in a Waring Blender homogenizer (Waring, USA) for 2 min with 500 mL of boiling ethanol (the ethanol fraction has been analyzed by HPLC-MS/MS and none of the sphingolipids shown in Table 1 were detectable in remarkable amounts). The homogenate was vacuum filtered through a Büchner funnel until dryness. The dry residue was extracted successively with 500 mL of chloroform/methanol (2:1 v/v) and chloroform/methanol (1:2 v/v), respectively, for 30 min at room temperature. The extracts were combined and evaporated to dryness. The lipid extract was dissolved in chloroform/methanol (2:1 v/v) and washed with three equal portions of water. The organic lower layer containing the lipids was evaporated, freeze–dried, taken up in chloroform/methanol (2:1 v/v), and filtered. The filtrate was evaporated to dryness and dissolved in 1 mL of THF/methanol (3:2 v/v).

2.3 ESI-MS/MS

Mass spectrometric experiments for structure analysis of ceramides and cerebrosides were performed using an API 4000 QTrap mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a syringe pump for direct flow infusion. For ESI, the ion spray voltage was set at −4500 V in the negative mode and at 5500 V in the positive mode. Nitrogen served as curtain gas (20 psi), the declustering potential, being the accelerating current from atmospheric pressure into high vacuum, was set at −55 V in the negative mode and at 55 V in the positive mode. For the identification of unknown ceramides or cerebrosides, the mass spectrometer was operated in the full-scan mode, detecting positive or negative ions. For further characterization of the detected substances, the mass spectrometer was operated in the enhanced product ion mode (EPI), detecting the fragmentation of the $[M-H]^-$ or $[M+H]^+$ molecular ions into specific product ions after collision with nitrogen as collision gas (3.9×10^{-5} torr). For the EPI-experiments of *N*-stearoylsphingosine, the collision energy was set to 30 eV in the positive ionization mode and to −20 eV in the negative ionization mode. For the EPI-experiments of C16(OH) β-D-glucosyl cerebroside, a collision energy of

Table 1. HPLC-MS/MS precursor/product ion *m/z*-ratios for +MRM detection of individual molecular species of ceramides and glucocerebrosides

	Ceramides			Glucocerebrosides		
Long-chain base	t18:1	d18:2	d18:1	t18:1	d18:2	d18:1
Product ion	*m/z* = 262,3	*m/z* = 262,3	*m/z* = 264,3	*m/z* = 262,3	*m/z* = 262,3	*m/z* = 264,3
Fatty acid	Precursor $[M+H]^+$	Precursor $[M+H]^+$	Precursor $[M+H]^+$	Precursor $[M+H]^+$	Precursor $[M+H]^+$	Precursor $[M+H]^+$
C16:0	554.5	536.5	538.5	716.6	698.6	700.6
C18:0	582.5	564.5	566.5	744.6	726.6	728.6
C20:0	610.6	592.6	594.6	772.6	754.6	756.6
C21:0	624.6	606.6	608.6	786.6	768.6	770.6
C22:0	638.6	620.6	622.6	800.7	782.6	784.7
C23:0	652.6	634.6	636.6	814.7	796.7	798.7
C24:0	666.6	648.6	650.6	828.7	810.7	812.7
C25:0	680.7	662.6	664.7	842.7	824.7	826.7
C26:0	694.7	676.7	678.7	856.7	838.7	840.7
C16:0h	570.5	552.5	554.5	732.6	714.5	716.6
C18:0h	598.5	580.5	582.5	760.6	742.6	744.6
C20:0h	626.6	608.6	610.6	788.6	770.6	772.6
C21:0h	640.6	622.6	624.6	802.6	784.6	786.6
C22:0h	654.6	636.6	638.6	816.7	798.7	800.7
C23:0h	668.6	650.6	652.6	830.7	812.7	814.7
C24:0h	682.6	664.6	666.6	844.7	826.7	828.7
C25:0h	696.6	678.6	680.7	858.7	840.7	842.7
C26:0h	710.7	692.7	694.7	872.7	854.7	856.7
C22:1	636.6	618.6	620.6	798.7	780.6	782.7
C16:1h	568.5	550.5	552.5	730.6	712.5	714.6
C22:1h	652.6	634.6	636.6	814.7	796.7	798.7

30 eV in the positive ionization mode and −40 eV in the negative ionization mode was used.

2.4 HPLC-ESI-MS/MS analysis

For HPLC-MS/MS analysis, an Agilent 1100 series HPLC was coupled to the mass spectrometer. Data acquisition was carried out with the Analyst 1.4 software (Applied Biosystems). Chromatographic separations were performed on a 150 mm × 2.1 mm id, 4 µm Phenomenex SynergiFusion column (Phenomenex, Aschaffenburg, Germany) with a Phenomenex SynergiFusion precolumn (4.0 mm × 2.0 mm id). A binary gradient consisting of methanol/THF 80:20 as solvent A and water as solvent B, both with addition of 5 mM ammonium acetate was used. The flow rate was 200 µL/min. The following gradient was used for the analysis of ceramides: 0 min, 90% solvent A; 10 min, 100% solvent A; 20 min, 100% solvent A; 21 min, 90% solvent A; 30 min, 90% solvent A. For glucocerebrosides the gradient was: 0 min, 80% solvent A; 5 min, 100% solvent A; 20 min, 100% solvent A; 21 min, 80% solvent A; 30 min, 80% solvent A. After each run, the column was washed with solvent A for 15 min and then equilibrated at the starting conditions for 15 min. The analytes were solved in THF/methanol 3:2. Five microliters of each sample were injected.

For the quantification of the ceramides and glucocerebrosides an echo-peak-technique was used by injecting the internal echo-peak-standards (for details see Section 3). The echo-peak-standards were injected 10 min (ceramides), or 5 min (glucocerebrosides) time-delayed to the injection of the sample. As internal echo-peak-standards, a solution of *N*-lauroyl-D-*erythro*-sphingosine (C12 ceramide) with a concentration of 100 ng/mL (∼1.0 pmol) and a solution of D-glucosyl-β1-1′-*N*-octanoyl-D-*erythro*-sphingosine (C8 β-D-glycosyl ceramide) with a concentration of 200 ng/mL (∼1.7 pmol) were used. The standards were solved in THF/methanol 3:2. Five microliters of the standard solutions were injected, respectively. The calibration curves of the internal echo-peak-standards were proven to be linear in concentrations of 1 ng/mL (10.4 fmol C12 ceramide and 8.5 fmol C8 β-D-glycosyl ceramide) up to 1 µg/mL (10.4 pmol C12 ceramide and 8.5 pmol C8 β-D-glycosyl ceramide) (R = 0.9999).

The quantification of the ceramides and glucocerebrosides by HPLC-MS/MS was performed with the mass spectrometer operating in the positive ionization multiple reaction monitoring (+MRM) mode. Zero-grade air served as nebulizer gas (35 psi) and as turbo gas for solvent drying (45 psi). The temperature of the heated capillary was set at 250°C.

For quantification of the ceramides and glucocerebrosides in the +MRM mode the transitions of the precursor ions $[M + H]^+$ to the main product ions of the long-chain bases were used as precursor/product ion pairs. For the ceramides and cerebrosides with 4-hydroxy-8-sphingenine and 4,8-sphingadienine as long-chain bases, the transitions of $[M + H]^+$ to 262 and for those with 8-sphingenine as long-chain base, the transitions of $[M + H]^+$ to 264 were used as precursor/product ion pairs. The precursor/product ion *m/z*'s of the ceramides and glucocerebrosides considered for quantification are given in Table 1. Using different collision energies (both with standards and extracts) only slight differences regarding the signal intensity were observed. For this reason the collision energy for all transition reactions was set at 35 eV. Up to 15 transitions were monitored in parallel and each transition reaction was monitored for a duration of 150 ms.

3 Results and discussion

3.1 Identification and characterization of ceramides and glucocerebrosides

In order to determine the fragmentation patterns of the different ceramide and cerebroside species, mass spectrometric experiments were first performed with commercially available standard substances. The mass spectrometric parameters were first optimized for the ceramide *N*-stearoylsphingosine (C18:0-d18:1) and for the glucocerebroside C16(OH)-β-D-glucosyl cerebroside (C16:0h-d18:2-Glc), and then applied for the identification of unknown ceramides and cerebrosides in the potato extracts.

In the positive full-scan mode, $[M + H]^+$ was the predominant signal besides small amounts of $[M + Na]^+$ and $[M + NH_4]^+$ and signals showing a loss of water ($[M - H_2O + H]^+$, $[M - 2H_2O + H]^+$). In the negative mode $[M - H]^-$-signals as well as $[M + Cl]^-$ and $[M + FA - H]^-$ adducts, the latter with higher intensity, were found.

For structural analysis of ceramides and glucocerebrosides, $[M + H]^+$ and $[M - H]^-$ ions were used for MS/MS experiments to obtain product ion spectra. In the positive MS/MS mode the fragmentation of the $[M + H]^+$ resulted in a predominant loss of water $[M - H_2O + H]^+$ as shown for *N*-stearoylsphingosine in Fig. 3A. Besides a $[M - 2H_2O + H]^+$ signal, two other signals (*m/z* 264 and 282) which are characteristic for the sphingosine moiety were observed. Ceramides and glucocerebrosides with a sphinganine moiety yielded fragments at *m/z* 266 and 284. Those with 4-hydroxy-8-sphingenine and 4,8-sphingadienine yielded fragments at *m/z* 262 and 280 as shown for C16(OH) β-D-glucosyl cerebroside in Fig. 4A. Furthermore, the positive MS/MS spectrum of the glucocerebroside clearly showed the loss of glucose $[M + H - 162]^+$ (Fig. 4A).

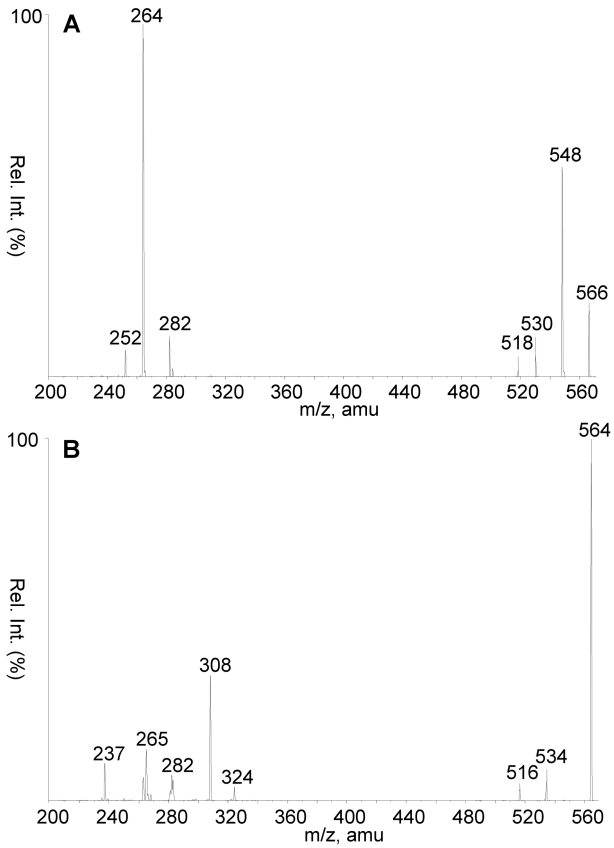

Figure 3. Positive (A) and negative (B) MS/MS spectra of *N*-stearoylsphingosine in the EPI (CE: 30 eV positive mode, –20 eV negative mode).

Besides a loss of water, the negative mode product ion spectra yielded fingerprint spectra providing an optimum of structural information about the fatty acids as well as the long-chain base moieties. As example, Fig. 3B shows the negative MS/MS product ion spectrum of *N*-stearoylsphingosine. The obtained fragments can be classified into three groups (i) fragments formed by the loss of small neutrals ($[M–H_2O–H]^-$ *m/z* = 546, (ii) $[M–CH_2OH]^-$ *m/z* = 534, $[M–CH_2OH–H_2O]^-$ *m/z* = 516), (iii) fragments referring to the long-chain base (*m/z* = 265 and 237), and (iv) fragments referring to the acyl component (*m/z* = 324, 308, and 282). A scheme of the proposed fragmentation is shown in Fig. 5.

The negative product ion spectrum of the glucocerebroside (Fig. 4B) shows a similar fragmentation pattern as obtained for the ceramide (Fig. 3B) yielding fragments referring to the acyl component (*m/z* = 324, 306, 296, 278, 271, 253, and 225). In addition, it shows the loss of glucose ($[M–H–Glc]^-$ *m/z* = 550) as shown for C16(OH) β-D-glucosyl cerebroside in Fig. 4B. Due to the additional hydroxy group

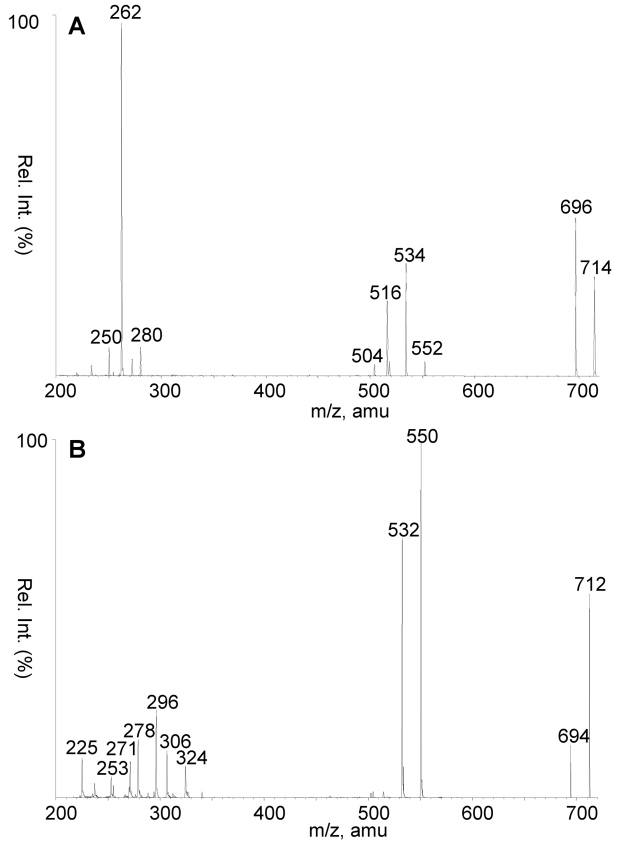

Figure 4. Positive (A) and negative (B) MS/MS spectra of C16(OH) β-D-glucosyl cerebroside in the EPI (CE: 30 eV positive mode, −40 eV negative mode).

attached to the fatty acid, a wider variety of the acyl fragments is observed, providing additional information for the precise characterization of ceramide and cerebroside species. Based on the knowledge of the fragmentation of the standard substances, the ceramides and cerebrosides given in Table 1 were identified in the potato extracts by their product ion spectra of the $[M + H]^+$ and $[M - H]^-$ ions in the positive and negative MS/MS mode and were applied for quantification.

3.2 Quantification with the echo-peak-technique

HPLC-MS has become a widely used analytical technique in many fields of pharmaceutical, environmental, biochemical, and food analysis. The excellent sensitivity and selectivity of modern HPLC-MS instruments employing ESI enable the determination of target analytes at trace levels. A general problem of HPLC-MS analysis is the influence of matrix compounds on the signal intensity. Although they are not visible in the chromatogram, they may result in poor accuracy of the results. Especially in the quantitative analysis of complex biological or food samples, one of the

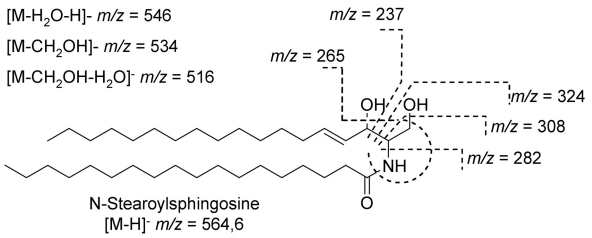

Figure 5. Proposed cleavages of *N*-stearoylsphingosine in the negative MS/MS ionization mode.

major problems is the suppression/enhancement of the analyte signal in the presence of matrix components. As a result of this matrix effects the response of an analyte in pure solvent can differ significantly from that in the matrix sample. To obtain accurate results, matrix effects have to be eliminated or compensated [35].

Concerning the accurate and precise quantification of sphingolipids, the use of internal standards is necessary to control sample losses during the extraction, differences in the chromatographic retention, ionization efficiency, and fragmentation. Ideally, stable isotope labeled standards should be used, having identical physical and chemical properties as the sphingolipids of interest. Because of the multiplicity of individual sphingolipid species in the analyzed plant samples, the use of stable isotope labeled internal standards is not practicable. Additionally, the synthesis of these compounds is difficult and only a few are commercially available. Instead of a stable isotope labeled standard, a panel of internal standards that represent each subclass of sphingolipids to be analyzed (such as ceramides or cerebrosides) is useful. The standard has to approximate the behavior of the analytes during extraction, HPLC elution, and MS ionization and fragmentation [33].

The internal standards and the compounds of interest should ideally coelute from the HPLC columns. This ensures that any factor altering the ionization efficiency will be shared by the standard and analyte. Synthetic homologous which are nearly identical in chemical and physical properties to the analytes, as 17-carbon homologous of sphingosine, are very expensive. Alternatively, internal standards with uncommon fatty acids in the ceramide backbone like the C12 ceramide and the C8 β-D-glucosyl ceramide can be used. Unfortunately, they elute much earlier, because of their shorter chain length compared to the analytes. Regarding this problem, an interesting calibration technique potentially compensating for matrix effects is the so-called echo-peak-technique. The echo-peak-technique is an internal standard method, originally applied to the quantitative analysis of pesticide residues in plant materials [35]. However, no isotopically labeled standards of the target analytes are necessary.

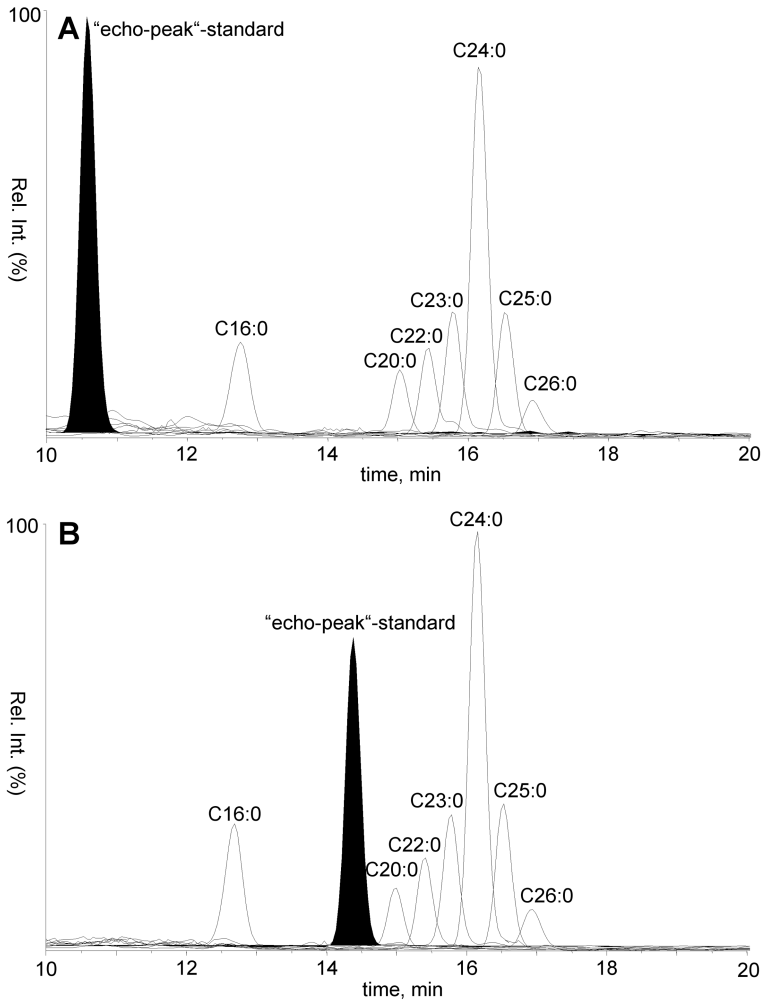

Figure 6. HPLC-ESI-MS/MS analysis of nonhydroxy fatty acid-4-hydroxy-8-sphingenine-ceramides (t18:1) in a potato sample. (A) Shows a chromatogram without using the echo-peak technique for the injection of the C12-ceramide. The standard is injected simultaneously with the sample. (B) Shows a chromatogram with using the echo-peak technique for the injection of the C12-ceramide, simulating an internal standard. The standard is time-delayed injected to the sample (10 min).

The principle of the echo-peak-technique is demonstrated in Fig. 6, showing the HPLC-MS/MS analysis of nonhydroxy fatty acid ceramides of a potato sample. In the chromatogram shown in Fig. 6A no echo-peak-technique was used. The sample and the standard were both injected at the same time. The echo-peak-standard is eluting at around 11 min, while the majority of the ceramide signals elute at 15 min and later. In the chromatogram shown in Fig. 6B the echo-peak-technique was used and the standard was time-delayed injected compared to the sample. With the echo-peak-technique, each analysis comprises of two injections into the HPLC-MS system. The sample and the standard solution are injected consecutively within a short time period, under the specific experimental conditions described in Section 2.3. As a result, the signal of the standard is shifted close to the majority of the ceramide signals, thus forming an echo-peak simulating an internal standard as shown in Fig. 6B. Provided that the retention times of these peaks are close enough to be affected by the coeluting sample components in the same manner, matrix effects should be compensated. However, the echo-peak-technique does not control sample losses during extraction.

Using the echo-peak-technique, all detected sphingolipids, including the echo-peak-standards C12 ceramide and the C8 β-D-glucosyl ceramide elute nearly simultaneously. Therefore, the analytes as well as the echo-peak-standard will be ionized under identical conditions, which avoids most ionization efficiency issues.

3.3 Quantification of ceramides and glucocerebrosides

The described RP-HPLC-ESI-MS/MS method with the echo-peak-technique simulating an internal standard enabled a reliable quantification of the analyzed ceramides and cerebrosides.

As shown by the MS experiments of the standard substances *N*-stearoylsphingosine and C16(OH)-β-D-glucosyl

cerebroside described in Section 3.1, the positive ionization mode was less influenced by the formation of adducts than the negative ionization mode, and was therefore used for quantification. Due to the higher specificity and the increased S/N ratio, quantification was performed in the MRM mode. In MRM, the first mass analyzer is set to pass a specific precursor ion *m/z* ratio, and the second mass analyzer is set to pass a specific product ion *m/z* ratio. Only ions that meet both precursor and product ion *m/z* conditions simultaneously will be detected. The precursor/product ion *m/z*'s of the ceramides and glucocerebrosides considered for quantification are given in Table 1. It is possible to monitor numerous transitions corresponding to different analytes, but the sensitivity is a limiting factor. Therefore, the number of detected precursor/product ion pairs was limited to 15 in one run. By using the positive MRM ionization mode an LOD of approximately 1 ng/mL (S/N 10:1) for ceramides as well as cerebrosides in plant extracts was reached.

The structural similarity of the detected ceramides and cerebrosides made a complete baseline separation by HPLC impossible. However, this is not necessary due to the specificity of the MRM mass spectrometric detection. With respect to the applicability of this method to complex matrices like plant extracts, a minimum of separation is helpful to avoid matrix effects. Besides, sphingolipids might have the same M_r but different structures (for example, both of the two ceramides C22:1h-t18:1 and C23:0-t18:1 have the same precursor/product ion pair of *m/z* 652.6/262.3 in the positive mode). Those ceramides and cerebrosides could be differentiated by their product ion spectra in the negative MS/MS mode (see Section 3.1). Due to the differences in chain length, grade of hydroxylation and saturation of the fatty acid moiety, ceramides and cerebrosides with the same M_r will have different polarities. Therefore, RP-HPLC has been used to separate samples for analysis by MS and to differentiate those compounds by their retention times.

The use of an RP-HPLC column with polar embedded groups yielded a better polar selectivity and retention than a regular RP 18 phase. So, a sufficient separation of the detected ceramides and especially of the cerebrosides was possible. Because of the high affinity of ceramides to the polar embedded RP 18 phase, the mobile phase had to meet several requirements, like best sensitivity for ESI, short retention times, and effective separation. Therefore, the strong eluent THF was added to methanol as mobile phase, to obtain fast and acceptable separations. The addition of water to the gradient increased the retention times without affecting the separation. The differing polarities of the ceramides and the cerebrosides forced the use of two different gradients. Both methods were optimized for peak intensity and acceptable peak shape.

The described echo-peak-HPLC-MS/MS method was applied to ceramides and cerebrosides with the three different long-chain bases 4-hydroxy-8-sphingenine, 4,8-sphingadienine, and 8-sphingenine (Fig. 2). Saturated and unsaturated as well as nonhydroxylated and 2-hydroxylated fatty acids with 16–26 carbon atoms were considered. Since glucose was described as the major carbohydrate head-group of cerebrosides in plants [13], only glucocerebrosides were analyzed in the plant extracts. For example, Fig. 7 shows HPLC-MS/MS chromatograms of a potato sample in the positive MRM mode of (A) 2-hydroxy fatty acid ceramides with 4-hydroxy-8-sphingenine as the long-chain base and of (B) 2-hydroxy fatty acid glucocerebrosides with 4,8-sphingadienine as the long-chain base.

As can be seen from Fig. 7, the separation of the different ceramides as well as cerebrosides is more than sufficient using the HPLC-conditions described above. Even very small differences in chain length or simple saturation of the fatty acid moiety resulted in different retention times (data not shown).

The described RP-HPLC-ESI-MS/MS method with the echo-peak-technique was used to identify, characterize, and quantify ceramides and cerebrosides in potatoes and sweet potatoes. Table 2 gives an overview of the types and amounts of ceramides and cerebrosides in potatoes and sweet potatoes in micrograms *per* kilograms fresh weight. Partly due to very small amounts of individual compounds, the single ceramides and cerebrosides were summed up to the three considered groups of long-chain bases, divided in nonhydroxy fatty acid- and hydroxy fatty acid ceramides and cerebrosides. A single marker long-chain base for ceramides does not exist in potatoes and sweet potatoes, as shown in Table 2A. The determined amounts of individual ceramides vary from 100 ng/kg to 8 µg/kg.

The major ceramides detected in potatoes were those with 4,8-sphingadienine as the long-chain base paired with 2-hydroxypalmitic acid (C16:0h-d18:2) and palmitic acid (C16:0-d18:2), and ceramides with 4-hydroxy-8-sphingenine as the long-chain base paired with 2-hydroxylignoceric acid (C24:0h-t18:1), 2-hydroxypalmitic acid (C16:0h-t18:1), and lignoceric acid (C24:0-t18:1). Table 3 shows the proportional distribution of the containing fatty acid moieties.

In sweet potatoes, 2-hydroxypalmitic acid-4,8-sphingadienine (C16:0h-d18:2), 2-hydroxylignoceric acid-4-hydroxy-8-sphingenine (C24:0h-t18:1), and lignoceric acid-4-hydroxy-8-sphingenine (C24:0-t18:1) were found to be the predominant ceramides. Other pairs of the three considered long-chain bases and the other fatty acids listed in Table 3 were also found in lesser but appreciable amounts.

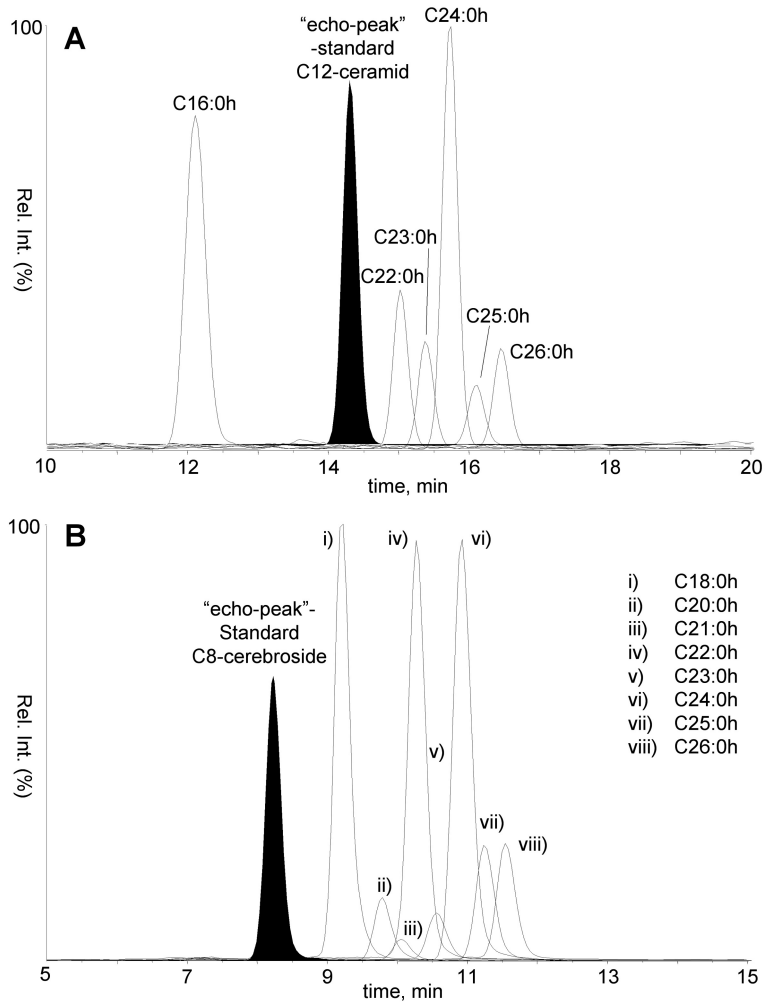

Figure 7. HPLC-ESI-MS/MS in the +MRM mode of a potato sample (species: Berber) with the extracted ion chromatograms of (A) 2-hydroxy fatty acid-4-hydroxy-8-sphingenine-ceramides (t18:1), and (B) 2-hydroxy fatty acid-4,8-sphingadienine (d18:2)-glucocerebrosides measuring the transitions of the $[M + H]^+$ signal to the fragment of the long-chain base (*m/z* 262).

Table 2. Amounts of (A) ceramides and (B) glucocerebrosides found in potatoes and sweet potatoes. The amounts of individual molecular species are summed up to the three considered groups of long-chain bases 4-hydroxy-8-sphingenine (t18:1), 4,8-sphingadienine (d18:2), and 8-sphingenine (d18:1), divided in nonhydroxy fatty acid (NHFA)- and hydroxy fatty acid (HFA)-ceramides and cerebrosides

A	Potato[a)] (μg/kg)	Sweet potato (μg/kg)	B	Potato[a)] (μg/kg)	Sweet potato (μg/kg)
t18:1 NHFA	0.5–1.5	1.5	t18:1 NHFA	1–11	31
t18:1 HFA	0.5–8	1.5	t18:1 HFA	0.2–1	25
d18:2 NHFA	0.2–8	0.2	d18:2 NHFA	5–12	18
d18:2 HFA	1–8	2	d18:2 HFA	33–197	490
d18:1 NHFA	0.1–1	0.3	d18:1 NHFA	0.1–1	10
d18:1 HFA	0.1–1	0.3	d18:1 HFA	1–8	16

a) The range represents the results of the different potato species.

The results of the quantification of cerebrosides in potatoes and sweet potatoes are shown in Table 2B. The amounts of the individual glucocerebrosides vary from 100 ng/kg to 190 μg/kg fresh weight for the potatoes and 100 ng/kg to 440 μg/kg fresh weight for the sweet potatoes. As shown in Table 2B, the long-chain base profile of cerebrosides isolated from potatoes and also from sweet potatoes is dominated by 4,8-sphingadienine (d18:2) which represents ~80–85% of the long-chain bases of all detected cerebrosides. The predominant fatty acid of potato and sweet potato ceramides as well as of cerebrosides is 2-hydroxypalmitic acid (C16:0h) as shown in Table 3.

Table 3. Summary of ceramides and cerebrosides found in potatoes and sweet potatoes by their total amount in % of the containing fatty acids

	Ceramides		Cerebrosides	
	Potato[a)] (%)	Sweet potato (%)	Potato[a)] (%)	Sweet potato (%)
C16:0	4–25	4	6–10	6
C18:0	0.5–12	0.5	0.1–0.2	0.2
C20:0	1–8	4	0.1–0.2	0.3
C21:0	0–1	1	–	–
C22:0	0.5–2	4	0–0.2	0.3
C23:0	0.5–3	4	0–0.1	–
C24:0	2–7	6	0.1–0.2	0.2
C25:0	0.5–4	5	–	–
C26:0	0.5–1	1.5	–	0.1
C16:0h	34–52	32	76–86	78
C18:0h	0.5–1	3	2–2.5	2
C20:0h	0.5–3	6	0.1	1
C21:0h	0–1	1	–	0.2
C22:0h	3–5	5	0.2–1	4
C23:0h	2–3	3	0.1–0.5	0.6
C24:0h	7–11	7	1–2	3
C25:0h	2–4	4	0.1–0.3	1
C26:0h	2–4	3	0.2–0.5	1
C16:1	0–8	–	0.1–9	1.5
C22:1	0–0.5	7	0–1	2
C16:1h	0–0.5	–	0.5	0.1
C22:1h	0.5–8	–	0.2–1.5	1

a) The range represents the results of the different potato species.

The major cerebroside detected in potatoes as well as in sweet potatoes was 2-hydroxypalmitic acid-4,8-sphingadienin-glucocerebroside (C16:0h-d18:2-Glc), which contributed approximately 80% of all the detected cerebrosides. The smaller part of the cerebroside fraction mainly consisted of the long-chain bases 4-hydroxy-8-sphingenine (t18:1) and 4,8-sphingadienine (d18:2) paired with palmitic acid (C16:0), 2-hydroxypalmitic acid (C16:0h), 2-hydroxybehenic acid (C22:0h), and 2-hydroxylignoceric acid (C24:0h) as well as 8-sphingenine (d18:1) acylated to 2-hydroxypalmitic acid (C16:0h).

In the course of this study, several different species of potatoes (species: Annabelle, Berber, Deutsche Princess, and Nicola) were analyzed and compared. Due to the differences in the amounts of ceramides and cerebrosides among the individual varieties, Table 2 shows the variation in the amounts of potato ceramides and cerebrosides.

4 Concluding remarks

Sphingolipids are bioactive membrane components of plant tissues, but due to their great variety and difficult analysis, little is known about their occurrence in food. Systematic quantitative studies of the sphingolipid content and composition of plant tissues have not been performed yet.

In this study, a method for the extraction, identification, and quantification of ceramides and cerebrosides in plant tissues like potatoes and sweet potatoes by RP-HPLC-ESI-MS/MS is described.

The use of MS/MS has provided unambiguous structural assignments for the molecular species of ceramides and cerebrosides from potatoes and sweet potatoes, confirming the presence of the 4,8-sphingadienine ($d18:2^{\Delta4,\Delta8}$), 4-hydroxy-8-sphingenine ($t18:1^{\Delta8}$), and 8-sphingenine ($d18:1^{\Delta8}$) as the major long-chain bases.

Individual ceramides and glucocerebrosides in potatoes and sweet potatoes were analyzed and quantified for the first time. The three described long-chain bases were found to be paired with 22 different fatty acids, to form the individual ceramides and glucocerebrosides. The resolution of such a complex mixture of molecules thus required a powerful chromatographic separation. Using the developed RP-HPLC-MS/MS method, we were able to adequately separate ceramides and glucocerebrosides containing even critical fatty acid pairs like saturated and unsaturated fatty acids.

In quantitative HPLC-MS analysis, the presence of matrix components may cause problems due to the suppression/enhancement of the analyte signal. These problems were solved by using an appropriate calibration method. The echo-peak-technique simulates the use of an internal standard, without the demand for isotopically labeled analogs.

The developed RP-HPLC-MS/MS method provides highly structure specific data for the determination of the exact pairing of fatty acids with long-chain bases and carbohydrate headgroups of ceramides and cerebrosides in plant and other tissues. The use of the echo-techniques allows the quantification of ceramides and cerebrosides in the nanomolar range.

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