JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Production of Rare Phyto-Ceramides from Abundant Food Plant Residues

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Supporting Information

ABSTRACT: Ceramides (Cers) are major components of the outermost layer of the skin, the stratum corneum, and play a crucial role in permeability barrier functions. Alterations in Cer composition causing skin diseases are compensated with semisynthetic skin-identical Cers. Plants constitute new resources for Cer production as they contain glucosylceramides (GluCers) as major components. GluCers were purified from industrial waste plant materials, apple pomace (*Malus domestica*), wheat germs (*Triticum* sp.), and coffee grounds (*Coffea* sp.), with GluCer contents of 28.9 mg, 33.7 mg, and 4.4 mg per 100 g of plant material. Forty-five species of GluCers (**1**−**45**) were identified with different sphingoid bases, saturated or monounsaturated α-hydroxy fatty acids (C15−28), and β-glucose as polar headgroup. Three main GluCers were hydrolyzed by a recombinant human glucocerebrosidase to produce phyto-Cers (**46**−**48**). These studies showed that rare and expensive phyto-Cers can be obtained from industrial food plant residues.

KEYWORDS: *ceramide, glucosylceramide, glucocerebrosidase, apple pomace, wheat germs, coffee grounds, Malus domestica, Triticum sp., Coffea sp.*

INTRODUCTION

Ceramides (Cers) and glucosylceramides (GluCers) belong to the class of sphingolipids and consist of a sphingoid base (long-chain base; LCB) and an amide-linked fatty acid (Figure 1).

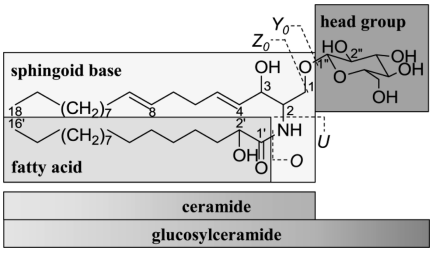

Figure 1. General structure of typical plant GluCers, e.g., Glc-d18:2 h16:0 (**6**) and Cers and major ESI-MS/MS fragmentation patterns. Modifications within the sphingoid base and fatty acid (chain lengths, hydroxylation, and/or desaturation) occur in plant Cers. Positive (Y_0, Z_0, O) and negative (Y_0, Z_0, U) ion fragments are shown according to nomenclature from Ann and Adams.[31]

Cers play a crucial role in the skin by providing a major lipid class (approximately 50%) of the outermost layer of the skin, the stratum corneum.[1] Up to now 18 different Cer classes in human skin were already reported.[2,3] They possess unique functions in membrane stabilization and in barrier formation against water loss.[4] Psoriasis[5] and atopic dermatitis[6] coincide with decreased Cer levels and alterations in Cer composition. Evidence for decreased Cers as cause for skin diseases arises from experimental mice with impaired Cer formation showing psoriasis.[4] The substitution of skin-identical Cers in the form of semisolid dosage forms is expected to improve this balance. Various pharmaceuticals and cosmetics are on the market that contain Cers. It is suggested that phytosphingosine-based Cers have good stabilizing and water-binding properties because of up to four hydroxyl groups in the head core group.[7−10]

Cers are commercially available, but production is semi-synthetic and very expensive. The extraction from animal tissues, such as bovine brain, porcine skin, or hen eggs, is possible but comprises certain risks and constraints. Nowadays, the sphingoid bases are often isolated from fermentation broth of yeasts,[11] followed by an acylation step to provide Cer structures similar to those found in human skin. Therefore, there is a need for cheaper alternatives in production of Cers. In plants, free Cers occur only as a minor fraction of sphingolipids.[12] They are partial constituents of GluCer and (glycosyl-) phosphoryl inositol ceramides that represent the major sphingolipid classes in plant plasma membranes and tonoplasts.[12] Cers as parts of sphingolipids occur ubiquitously in the plant kingdom; they influence membrane integrity and permeability.[13]

Therefore, plants may provide an economical alternative source for natural GluCers. Contents of 1−100 mg/100 g dry mass were reported from different plant materials.[14−16]

Received: September 25, 2016
Revised: December 30, 2016
Accepted: January 24, 2017
Published: January 24, 2017

DOI: 10.1021/acs.jafc.6b04275
J. Agric. Food Chem. 2017, 65, 1507−1517

Specifically, some common edible plants, e.g., soy and rice, were identified as rich sources.[14,17] Huge amounts of industrial plant waste accumulate every day. The value chain often ends as animal feed, e.g., for apple pomace and wheat germs, or in household refuse, e.g., for coffee grounds. Approximately 2.7 million tons of apple pomace (as 25% leftover[18] of apple juice production), 14.7 million tons of wheat germs (as 2% of the whole grain after milling), and 9.2 million tons of coffee were produced for consumption in 2015 worldwide (FAS, 2016). GluCers were already reported as constituents in apple[14,19] and wheat.[20,21] We aim to investigate whether these industrial food byproducts contain sufficient amounts of GluCers to serve as sources for phyto-Cer production.

The nomenclature proposed by Karlsson[22] for individual sphingolipids is in accordance with IUPAC description of lipids. A typical plant GluCer is Glc-d18:2$^{\Delta 4E,8E/Z}$ h16:0, where "d" stands for the dihydroxy sphingoid base ("t" for a trihydroxy base). The carbon chain length and the degree of desaturations of the sphingoid and acyl chains are given as numbers (18:2). Double bond positions and configuration are given as $^{\Delta 8E/Z}$. The α-hydroxylation within the fatty acid is declared by "h", and the sugar moiety is denoted as "Glc" for glucose.

Differences in the chemical structures of human and plant Cers comprise prevalence of sphinganine (d18:0), sphing-4-enine (sphingosine, d18:1$^{\Delta 4}$), and phytosphingosine (t18:0) in human skin, whereas plants appear to contain sphing-8-enine (sphingosine, d18:1$^{\Delta 8}$), sphinga-4,8-dienine (d18:2$^{\Delta 4,8}$), and phytosphing-8-enine (t18:1$^{\Delta 8}$) as major components.[23] Plant sphingoid bases exhibit typical chain lengths of C18, and fatty acid chains range from C14−26, of which 90−95% are α-hydroxylated.[23,24] However, detailed structure differences still need a comprehensive comparison.

Purification of phyto-Cers is necessary for the investigation of functional equivalence. The industrial purification of compounds out of complex matrices requires exhaustive, efficient, and economical methods. For that purpose, an efficient extraction process, isolation (liquid−liquid extraction, column chromatography (CC), pHPLC-MS), and structure elucidation (TLC, HPLC-MS, MS/MS, HR-MS, NMR) for phyto-Cers are described here.

MATERIALS AND METHODS

Materials. Apple pomace (A, *Malus domestica*, apple cultivars) was kindly provided by Becker Eislebener Fruchtsaft (Eisleben, Germany). Wheat germs (W, *Triticum* sp., wheat cultivars) were obtained from Dr. Grandel (Augsburg, Germany) and ground coffee beans (C, *Coffea* sp., coffee varieties) from Jacobs Douwe Egberts (Bremen, Germany). Coffee grounds were obtained after percolation. Analytical TLC was carried out on precoated silica gel F_{254} aluminum plates from Merck (Darmstadt, Germany). For adsorption chromatography, silica gel 60 (0.063−0.200 mm, AppliChem, Darmstadt, Germany) was used. Size exclusion column chromatography was performed using sephadex LH-20 (GE Healthcare, Solingen, Germany).

Methanol used for preparative HPLC was HPLC-grade (99.9%, Roth, Karlsruhe, Germany) and for mass spectrometry analyses ULC/MS-grade (99.98%, Biosolve BV, Valkenswaard, Netherlands). Water was demineralized (σ = 0.055 μS/cm, TKA GenPure, Niederelbert, Germany). Pyridine-d_5 (99.5%) was purchased from Deutero (Kastellaun, Germany). Cerezyme containing the recombinant DNA-produced analogue of human β-glucocerebrosidase imglucerase (EC 3.2.1.45) originated from Genzyme (Cambridge, MA). Sodium taurocholate hydrate (≥97%) was bought from Sigma (St. Louis, MO). A GluCer standard (from soy beans, Glc-d18:2 h16:0, ≥98%) was obtained from Avanti Polar Lipids (Alabaster, AL). TLC reference compounds were cholesteryl oleate (≥98%, Sigma, St. Louis, MO), linoleic acid (≥98%, AppliChem, Darmstadt, Germany), β-sitosterol (≥80%, Roth, Karlsruhe, Germany), β-sitosterol glucoside (≥98%, available from in-house library), squalene (≥97%, Fluka, Neu-Ulm, Germany), and triolein (glycerol trioleate, ≥99%, Sigma, St. Louis, MO).

TLC Analysis. TLC analysis war carried out using chloroform:methanol 85:15 (v/v) as the solvent system.[25] For visualization, developed TLC plates were sprayed with copper(II) sulfate (10%), phosphoric acid (8%), and methanol (5%) in water and heated at 150 °C for 10 min. TLC reference mix was composed of 100 μg/mL of each squalene, triolein, cholesteryl oleate, linoleic acid, β-sitosterol, β-sitosterol glucoside, and GluCer Glc-d18:2 h16:0, in chloroform−methanol 2:1 (v/v). Samples (10 μL) were applied as 6 mm bands using Linomat IV (Camag, Muttenz, Switzerland). The distance from the lower edge was 8 mm, that from the left side was 12 mm, and the space between bands was 4 mm.

Extraction and Purification. Dried plant material (A, 750.0 g; W, 300.0 g; C, 300.0 g) was extracted by isopropanol−*n*-hexane−water (55:20:25 (v/v/v);[12] A, 3.0 L; W and C, 1.2 L) three times using an ultrasonic bath for 15 min. The extracts were combined and evaporated to dryness (A, 99.2 g; W, 110.4 g; C, 34.4 g). This total lipid extract was separated into polar and nonpolar compounds by liquid−liquid partitioning with chloroform−methanol−water (1:1:1, v/v/v; A and W, 0.6 L; C, 0.3 L). Additional 0.5−1.0% (w/v water phase) sodium chloride improved separation. The resulting two phases were additionally partitioned using a mixture of chloroform:methanol 1:1 (v/v) for the aqueous phase and a mixture of methanol:water 1:1 (v/v) for the organic phase (A and W, 0.2 L; C, 0.1 L). The organic phases were combined and evaporated to dryness (A, 30.42 g; W, 30.37 g; C, 30.44 g). For comparison purposes, 20.0 g of each extract were applied to CC on silica gel 60 (250.0 g, 3.8 × 60 cm). Chloroform−methanol (100:0 (v/v), 1 L; 90:10 (v/v), 1 L; 80:20 (v/v), 1 L, flow 1.5−2.5 mL/min) was used for gradient elution. Fractions of 10 or 20 mL were collected with a Cygnet fraction collector (Teledyne Isco, Lincoln, NE). After TLC analysis by comparison with the reference mix, fractions of each plant source containing GluCers and β-sitosterol glucoside (A, 0.666 g; W, 0.278 g; C, 0.279 g) were further separated by size exclusion chromatography on sephadex LH-20 (100.0 g, 1.5 × 60 cm; solvent, dichloromethane−methanol 1:1 (v/v), flow 0.12 mL/min). Fractions of 2.5 mL were collected after TLC and GluCer enriched fractions were obtained (A, 0.222 g; W, 0.084 g; C, 0.050 g). These fractions were further separated by preparative HPLC-MS (flow 25 mL/min, column temperature 25 °C) using a Zorbax Extend-C18 column (5 μm, 21.2 × 150 mm, 80 Å, Agilent, Santa Clara, CA). The split ratio of fraction collector to mass spectrometer was set to 500:1. The injection volume was 300−900 μL, and fractions were pooled according to their retention time and m/z value. Finally, GluCer enriched fractions were obtained from apple pomace (fraction A1 (10.2 mg): **1**−**3**; A2 (10.1 mg): **4**-**5**; A3 (55.6 mg): **6**; A4 (1.1 mg): **9**; A5 (1.1 mg): **12**; A6 (2.0 mg): **16**−**17**; A7 (3.1 mg): **21**−**22**, **24**; A8 (19.5 mg): **27**; A9 (10.7 mg): **30**−**31**; A10 (24.8 mg): **34**−**35**; A11 (3.4 mg): **38**, **40**; A12 (0.9 mg): **42**−**43**), wheat germs (fraction W1 (6.6 mg): **6**−**7**; W2 (4.0 mg): **8**; W3 (3.7 mg): **10**−**11**; W4 (3.8 mg): **14**−**16**, **19**−**20**; W5 (16.7 mg): **20**−**21**, **23**; W6 (2.5 mg): **25**; W7 (13.8 mg): **26**−**28**; W8 (6.3 mg): **29**−**32**; W9 (5.1 mg): **33**−**37**; W10 (2.3 mg): **38**−**40**; W11 (1.0 mg): **41**−**44**; W12 (0.8 mg): **45**) and coffee grounds (fraction C1 (1.0 mg): **6**; C2 (3.0 mg): **27**; C3 (1.8 mg): **30**−**31**; C4 (2.0 mg): **35**; C5 (0.8 mg): **38**, **40**).

Analytical Enzymatic Assay for Cers. A total of 10 μg from each GluCer fraction (A1−12, W1−12, C1−5) were hydrolyzed by imiglucerase. The enzyme was suspended in 50 mM sodium citrate buffer (pH 4.5, final volume 1 mL), and 413.4 μL of a 5 mg/mL sodium taurocholate solution, 2 mM dithiothreitol, and 1 mM ascorbic acid were added as protection against enzyme oxidation. A total of 5 μg of imiglucerase was added, and the reaction mixture was incubated at 37 °C for 14 h. The reaction was quenched with 0.4 mL of chloroform−methanol 2:1 (v/v), and the water phases were extracted two more times (0.4 mL of chloroform−methanol 2:1 (v/v)). The organic phases were combined and evaporated to dryness. Resulting

DOI: 10.1021/acs.jafc.6b04275
J. Agric. Food Chem. 2017, 65, 1507−1517

Table 1. Stepwise Concentration of GluCers

plant material	initial mass [g]	total lipid extract [g]	organic phase [g]	silica gel fraction[a] [mg]	sephadex fraction [mg]	prep. HPLC fraction [mg]	GluCers/100g [mg]
apple pomace	750.0	99.2	30.42	1.012	0.337	216.6	28.9
GluCers w/w	0.029%	0.218%	0.712%	-	-	-	-
wheat germs	300.0	110.4	30.37	0.423	0.128	101.2	33.7
GluCers w/w	0.034%	0.092%	0.333%	-	-	-	-
coffee grounds	300.0	34.4	30.44	0.424	0.076	13,1	4.4
GluCers w/w	0.004%	0.038%	0.043%	-	-	-	-

[a]Applied 20.0 g; the following fractions are calculated for the total organic phase; prep. HPLC − preparative HPLC.

Cers were recognized by comparison with authentic reference standards by TLC.

Enzymatic Hydrolysis and Isolation of Ceramides. Fractions containing suitable amounts of GluCer (A3, **6**, 25.0 mg; A8, **27**, 10.0 mg; A10, **35**, 10.0 mg) were enzymatically hydrolyzed by scaling up the analytical assay to 50 mL (37 °C, 14 h, final volume 50 mL, 20.65 mL of a 5 mg/mL sodium taurocholate solution, 100 μg of imiglucerase, 20 mL of chloroform−methanol 2:1 (v/v) for separation). The organic phase was separated by CC (30.0 g silica gel 60, 1.5 × 38 cm, chloroform−methanol 9:1 (v/v) isocratic, flow 1 mL/min) and afforded Cers **46** (11.7 mg), **47** (5.9 mg), and **48** (4.1 mg).

LC-MS Analysis. For analytical LC-MS analysis, an Agilent 1260 Infinity LC System coupled to an Agilent 6120 B Quadrupole MS (Agilent Technologies, Santa Clara, CA) was used. Separation was achieved on a Zorbax Extend-C18 column (5 μm, 4.6 × 150 mm, 80 Å, Agilent Technologies, Santa Clara, CA) with a precolumn Zorbax Extend-C18 Analytical Guard Column (5 μm, 4.6 × 12.5 mm, Agilent Technologies, Santa Clara, CA). The injection volume was 10 μL, and column temperature was set to 30 °C. The elution solvent was composed of methanol (A) and water (B), each with 0.1% formic acid. The gradient elution program (flow rate 1.2 mL/min) was as follows: linear gradient from 0 min A−B 97:3 (v/v) to 20 min A−B 100:0 (v/v), followed by isocratic elution for 10 min A−B 100:0 (v/v) and an equilibration step (5 min A−B 97:3 (v/v)) to the next run. The APCI/MS parameters were set as follows: discharge voltage +4.5 kV, discharge current 5 μA, gas temperature 200 °C, vaporizer temperature 450 °C, nebulizer gas (nitrogen) 35 psig, and drying gas (nitrogen) 12 L/min. Additionally, a collision-induced dissociation (CID) of 150 V was used. Spectra were recorded in positive and full scan mode (TIC, 150.0−1000.0 m/z). Chromatograms and spectra were evaluated with the Agilent ChemStation (B.04.03) software (Agilent, Santa Clara, CA).

ESI-HRMS and ESI-MS/MS Analyses. Positive ion high resolution ESI mass spectra were obtained from an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) which was equipped with a heated electrospray ion source (spray voltage +4.0 kV, capillary temperature 275 °C, source heater temperature 45 °C). Nitrogen was used as sheath gas. The sample solutions (1 μg/mL in methanol) were injected continuously with a 500 μL of Hamilton syringe pump (flow rate 5 μL/min). Fourier transform MS resolution was 30 000. The instrument was externally calibrated by the Pierce LTQ Velos ESI positive ion calibration solution from Thermo Fisher Scientific (Bremen, Germany). The data were evaluated with the Xcalibur (2.7 SP1) software (Thermo Fisher Scientific, Bremen, Germany).

ESI tandem mass spectra were measured with a Thermo Finnigan LCQ classic ion trap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). It was equipped with an electrospray ion source that was used in positive and negative mode. The MS conditions were optimized with a GluCer standard substance to give the following parameters (positive/negative mode): spray voltage ±4.5 kV, capillary voltage −20/+8.5 V, sheath gas 80/80 AU (nitrogen), capillary temperature 220/220 °C, tube lens offset −20/+35 V. Samples (1 μg/mL in methanol) were injected with a 250 μL Hamilton syringe pump (flow rate 5 μL/min). MS/MS scans were recorded with CID between 35 and 40% (helium, isolation width 1.0−2.0). Spectra were recorded in positive and negative ion mode (m/z 200.0−1000.0) with the Xcalibur (V 2.0) software (Thermo Fisher Scientific, Bremen, Germany).

NMR Analysis. Samples were dried before use in a desiccator with KOH for at least 48 h. All samples were dissolved in 0.65 mL of pyridine-d_5 with TMS as internal standard. 1D- (^{1}H, ^{13}C, 1D-TOCSY) and 2D- (COSY, TOCSY, HSQC, HMBC) NMR spectra were measured at 25 °C with a Varian VNMRS 600 MHz NMR spectrometer (Agilent Technologies, Santa Clara, CA). Proton and carbon NMR frequencies were 599.828 and 150.840 MHz. Chemical shifts δ [ppm] were referenced to the internal tetramethylsilane (δ ^{1}H = 0.000 ppm) and internal pyridine-d_5 (δ ^{13}C = 123.5 ppm), respectively. Spectra were processed with MestReNova (8.0.0) software (Mestrelab Research, Escondido, CA).

RESULTS AND DISCUSSION

Extraction of GluCers. Three industrial waste materials (A, apple pomace; W, wheat germs; and C, coffee grounds) were investigated. The procedure of GluCer enrichment could be monitored by TLC (see Supporting Information). Different lipid classes were assigned according to the reference mixture. GluCer bands of low intensity were detected in the total lipid extract and in the organic phase. Fractionation by the first column chromatography on silica gel removed excessive amounts of triglycerides, sterols, and free fatty acids. The second column chromatography on Sephadex LH20 separated sterol glycosides from GluCer. Total lipid extract of wheat germs (36.8% w/w) was higher than that of apple pomace (13.2% w/w) and coffee grounds (11.5% w/w) (Table 1). However, all organic phases showed similar amounts (approximately 30.4 g) whereas apple pomace had a higher initial mass (750.0 g) in contrast to wheat germs and coffee grounds (300.0 g). The liquid−liquid extraction could remove nearly 2/3 of the total lipid extract of apple pomace and wheat germs in the form of polar compounds. In contrast, this extraction procedure could only eliminate 1/10 of the coffee grounds total lipid extract, and polar compounds were already extracted by percolation beforehand. The lipid contents after extraction and liquid−liquid extraction were similar for apple pomace (4.1% w/w), wheat germs (10.1% w/w), and coffee grounds (10.2% w/w) (Table 1).

Quality of Separation Techniques. In the first separation CC on silica gel 60, GluCers and sterol glucosides eluted together. Only a coarse separation was possible but necessary for an efficient and economical method. The ratio of sample and stationary phase was only 8:100 (20.0:250.0 g, mobile phase 3 L) and not suitable for a complete purification of GluCers. Higher volumes of mobile phase and stationary phase (2000 g of silica gel and 10 L of mobile phase for 20 g of extract) should be used in a single CC step following standard procedures. Size exclusion chromatography on sephadex LH-20 eventually separated sterol glucosides from GluCers (ratio:

DOI: 10.1021/acs.jafc.6b04275
J. Agric. Food Chem. 2017, 65, 1507−1517

sample/stationary phase 0.3−0.7:100) and resulted in highly GluCer enriched fractions. Application of reusable sephadex LH-20 has economical advantages. The combination of two chromatographic separation types is efficient to reduce production costs for GluCer enriched fractions. Subsequent preparative HPLC-MS afforded GluCers fractions A1-12, W1-12, and C1-5. GluCers peaks were not baseline-separated. LC-peaks with shoulders indicate more than one GluCer within one fraction, but compounds could be distinguished individually by m/z as $[M + H]^+$ ions (Figure 2). Each fraction

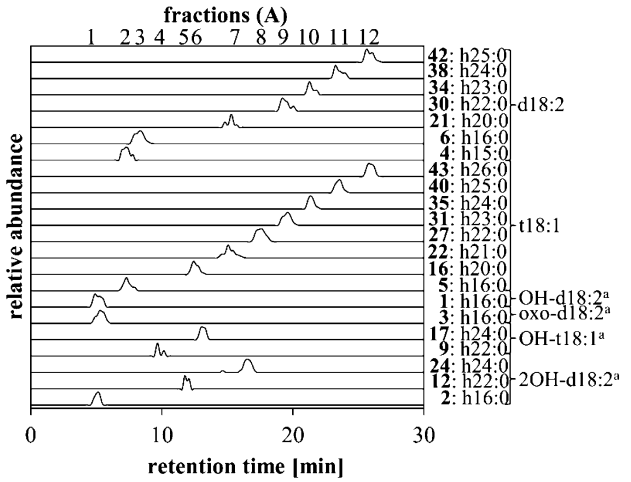

Figure 2. Apple pomace GluCer fractions obtained by HPLC-APCI/MS as extracted ions $[M + H]^+$; compound numbers as given in Figure 3; a, assumed compounds.

contained up to 6 GluCers. Lipids are usually extracted[26] and isolated[27] using chloroform and methanol as solvents. Chloroform is potentially carcinogenic and reprotoxic, and it is therefore necessary to avoid this solvent during large scale production.

GluCer Contents. Apple pomace provided a GluCer content of 28.9 mg, wheat germs of 33.7 mg, and coffee grounds of 4.4 mg per 100 g of plant material. GluCer contents between 17 and 100 mg per 100 g of dry weight were reported in different apple cultivars.[15,16] Therefore, pomace of specific apple varieties should be further studied to enhance the GluCer yield. GluCers between 19.3 and 25.9 mg per 100 g of dry weight (calc. as Glc-d18:2 h16:0, 714 g/mol) were recognized from different wheat sources (whole grain,[28] bran,[21] and flour[14]). The highest lipid content is located in wheat germs. Thus, wheat germs exceeded these amounts with 33.7 mg of GluCers per 100 g of plant material. Maximum GluCer amounts of 28.4 to 35.1 mg per 100 g of dry weight (calc. as Glc-d18:2 h16:0, 714 g/mol) are reported for soy beans used for plant GluCer production.[29,30] Thus, apple pomace and wheat germs may serve as new GluCer resources, while coffee grounds are no suitable source (4.4 mg per 100 g of dry weight).

MS Analyses. In analytical HPLC-APCI/MS, GluCers were detected as $[M + H]^+$ ions or $[M - 18 + H]^+$ in the case of one GluCer class due to fragmentation conditions (CID 150 V) (Table 2). The extracted ion chromatograms of apple pomace GluCers are shown in Figure 2. Phytosphingosine (t18:1) and sphingadienine (d18:2) represented the two major GluCer sphingoid bases. The GluCers were separated according to their fatty acid chain lengths as well as to their sphingoid bases. Thus, Glc-d18:2 h22:0 (**30**) and Glc-t18:1 h23:0 (**31**) eluted together within one fraction (Figure 2, A9). Wheat germs and coffee ground fractions showed similar separation results (Supporting Information).

Structural information was obtained from ESI-HRMS measurements. All compounds were measured as $[M + Na]^+$ ions (Table 2). Errors were between 0.0000 and 4.6510 ppm with most errors ≤2 ppm and greater errors for trace compounds. ESI-MS/MS experiments showed $[M + Na]^+$ ions in positive and $[M - H]^-$ ions in negative mode. Major MS/MS fragments are presented in Figure 1 (all detected fragments in Supporting Information) and were assigned according to Ann and Adams.[31] MS/MS fragmentation revealed sugar cleavage as well as intact Cer structures (Y_0 $[M - C_6H_{10}O_5 + Na]^+$, Z_0 $[M - C_6H_{10}O_5 - H_2O + Na]^+$) and fragments of sphingoid bases (O) and fatty acids (U) for every compound (Table 2). Acid chain lengths were assigned by fragment U (e.g., h16:0 → m/z 271.5, h22:0 → m/z 355.4, h24:0 → m/z 383.5). Desaturations within the fatty acids were indicated by fragment U (e.g., h24:0 → m/z 383.5, h24:1 → 381.5, h24:2 → m/z 379.5) and only detected in wheat germs. Sphingoid bases were identified according to the emerging fragment O (e.g., d18:1 → m/z 484.2, d18:2 → m/z 482.2, d18:3 → m/z 480.2, t18:1 → m/z 500.3).

Unknown derivatives of sphingadienine (d18:2) and phytosphingenine (t18:1) type LCB were identified in trace amounts in apple pomace (Figure 2). ESI-HRMS measurements led to the assumption of additional oxygen groups because of calculated total formulas containing 10 and 11 oxygens (Table 2). MS/MS data indicated an additional +14 amu in the fragment O (m/z 496.2) and suggested an oxo group within the LCB. This LCB was attached to a h16:0 fatty acid (U, m/z 271.4). An additional water elimination in negative mode (Z_0', m/z 528.4) reinforced the structure assignment to give Glc-oxo-d18:2 h16:0 (**3**). Another derivative with an additional hydroxyl group within the LCB was determined (O, m/z 498.3) as OH-d18:2. It was associated with the same fatty acid (h16:0, U m/z 271.4). Two more unknown GluCer classes were identified as 2OH-d18:2 and OH-t18:1. 2OH-d18:2 showed +32 amu (O, m/z 514.3) in contrast to d18:2. In addition to fragment Z_0' (e.g., m/z 658.6 for Glc-2OH-d18:2 h24:0 (**24**)) this class also showed an additional ion Z_0'' (e.g., m/z 640.5 for Glc-2OH-d18:2 h24:0 (**24**)) due to a water elimination. The second class was determined as OH-t18:1 because of a + 16 amu difference of the LCB (O, m/z 516.4) in contrast to t18:1. Fatty acids were h16:0 (m/z 271.4), h22:0 (m/z 355.6) and h24:0 (m/z 383.5). Due insufficient amounts of compounds, the position of the functional groups could not be assigned. Similar compounds have not been reported from plants. Recent studies announced a Cer with unknown location of a hydroxyl group in human skin (Cer NT, tetra18:0 18:0).[2] Their complete structures need to be fully elucidated in future studies.

^{1}H NMR Fingerprinting. As most fractions contained more than one GluCer, NMR measurements were accomplished as ^{1}H NMR fingerprinting. The core structures sphingosine (d18:1 as Glc-d18:1 h16:0, **8**), sphingadienine (d18:2 as Glc-d18:2 h16:0, **6**), and phytosphingenine (t18:1 as Glc-t18:1 h22:0, **27**) were investigated for relevant chemical shifts after extensive structure elucidation by 1D (^{1}H, ^{13}C) and 2D (HSQC, HMBC, COSY, TOCSY) NMR experiments. The configuration of double bonds was determined according to the size of vicinal ^{1}H,^{1}H coupling constants or ^{13}C chemical shifts

DOI: 10.1021/acs.jafc.6b04275
J. Agric. Food Chem. 2017, 65, 1507−1517

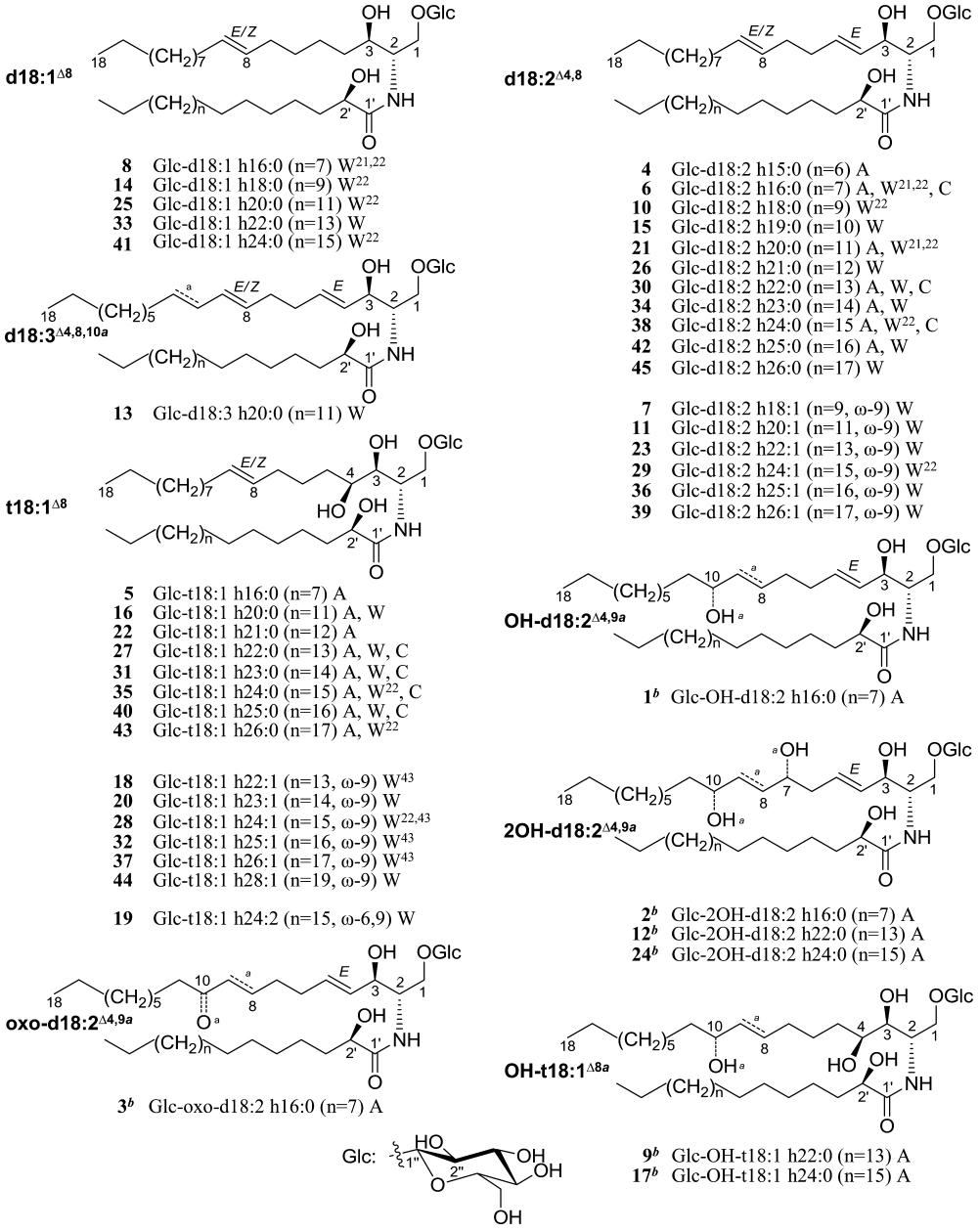

Figure 3. Individual GluCers (**1**–**45**) isolated from apple pomace (A), wheat germs (W), and coffee grounds (C). Intact GluCers were not reported for apple pomace and coffee grounds yet; intact GluCer species identified before in wheat are denoted. Glc - *β*-glucose; *a*, position not proven; *b*, suggested structure.

of adjacent carbons. *E*-double bonds have vicinal coupling constants of 12–16 Hz, *Z*-configuration rather 8–10 Hz.[32] In long acyl chains adjacent carbons show chemical shifts of 27–28 ppm for a *Z*-olefinic double bond and 32–33 ppm for an *E*-olefinic double bond.[33] The configurations were determined by comparison with published ^{13}C chemical shifts of the GluCers as 2*S*, 3*R*, 2′*R* for sphingadienine (d18:2)[34] and sphingosine (d18:1)[21] and 2*S*, 3*S*, 4*R*, 2′*R* for phytosphingenine (t18:1).[35] LCB (NH, Δ4,5, Δ8,9), fatty acid (2′), and sugar (1″ and 5″) proton signals could be assigned (Figure 4). All fractions obtained from apple pomace, wheat germs, and coffee grounds by HPLC (Figure 2; Supporting Information Figures 4, 5) were monitored by NMR fingerprinting. Figure 4 contains a wheat germ fraction with 6 GluCers (W4) as an example (see Supporting Information Figures 19–21 for ^{1}H NMR fingerprinting of all fractions). Three different LCBs were assigned due to NH and sugar signals (d18:1, d18:2, t18:1). An additional NH signal was assumed to belong to d18:3, but this assumption could not be proven due to the missing pure reference substance. Double bonds at positions C-4,5 and C-8,9 were also detected. The attached sugar moiety was glucose due to chemical shifts, and the *β*-glucosidic linkage was indicated by the coupling constants (7.8 Hz).[34] Fatty acids were always *α*-hydroxylated (C-2′). The unsaturation of fatty acids could not be clearly distinguished as these signals fell together with other olefinic signals from C-8,9. In summary, ^{1}H NMR fingerprinting as a simple and fast method supported identification by mass spectrometry.

GluCer in Different Plant Materials. Together, 45 GluCer were elucidated (A, 22; W, 35; C, 7; Figure 3 and Table 2). Glc-d18:2 h16:0 (**6**), Glc-t18:1 h22:0 (**27**), and Glc-t18:1 h24:0 (**35**) were the main compounds of all three plant

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Table 2. MS Identification of GluCers and Cers[a]

		ESI-HRMS		LC-APCI/MS		ESI-MS/MS			
		m/z	formula	RT	*m/z*	*m/z*			
source	compound	$[M + Na]^+$	$[M + Na]^+$	[min]	$[M + H]^+$	$[M + Na]^+$	$[M - acyl + Na]^+$ O	$[M - H^+]^-$	$[M - sug - acyl - H]^-$ U
W	Glc-d18:1 h16:0 (**8**)	738.5496	$C_{40}H_{77}NO_9Na$	9.2	716.5	738.6	484.2	714.5	271.5
W	Glc-d18:1 h18:0 (**14**)	766.5805	$C_{42}H_{81}NO_9Na$	12.3	744.6	766.6	484.3	742.6	299.5
W	Glc-d18:1 h20:0 (**25**)	794.6127	$C_{44}H_{85}NO_9Na$	16.9	772.6	794.6	484.2	770.6	327.4
W	Glc-d18:1 h22:0 (**33**)	822.6459	$C_{46}H_{89}NO_9Na$	21.0	800.7	822.6	484.2	798.6	355.4
W	Glc-d18:1 h24:0 (**41**)	850.6772	$C_{48}H_{93}NO_9Na$	25.4	828.7	850.7	484.2	826.6	383.5
A	Glc-d18:2 h15:0 (**4**)	722.5198	$C_{39}H_{73}NO_9Na$	7.3	700.5	722.5	482.2	698.5[d]	257.4
A, W, C	Glc-d18:2 h16:0 (**6**)	736.5346	$C_{40}H_{75}NO_9Na$	8.4	714.5	736.6	482.2	712.6	271.3
W	Glc-d18:2 h18:0 (**10**)	764.5661	$C_{42}H_{79}NO_9Na$	11.1	742.6	764.6	482.2	740.6	299.4
W	Glc-d18:2 h19:0 (**15**)	778.5836	$C_{43}H_{81}NO_9Na$	12.4	756.5	778.6	482.2	754.6	313.5
A, W	Glc-d18:2 h20:0 (**21**)	792.5967	$C_{44}H_{83}NO_9Na$	15.1	770.5	792.6	482.2	768.6	327.5
W	Glc-d18:2 h21:0 (**26**)	806.6135	$C_{45}H_{85}NO_9Na$	17.4	784.7	806.6	482.3	782.6	341.4
A, W, C	Glc-d18:2 h22:0 (**30**)	820.6277	$C_{46}H_{87}NO_9Na$	19.5	798.7	820.6	482.2	796.6	355.5
A, W	Glc-d18:2 h23:0 (**34**)	834.6432	$C_{47}H_{89}NO_9Na$	21.3	812.6	834.7	482.3	810.3	369.6
A, W, C	Glc-d18:2 h24:0 (**38**)	848.6604	$C_{48}H_{91}NO_9Na$	23.3	826.7	848.6	482.2	824.6	383.5
A, W	Glc-d18:2 h25:0 (**42**)	862.6759	$C_{49}H_{93}NO_9Na$	25.6	840.7	862.7	482.2	838.7	397.5
W	Glc-d18:2 h26:0 (**45**)	876.6927	$C_{50}H_{95}NO_9Na$	28.8	854.6	876.6	482.2	852.7	411.5
W	Glc-d18:2 h18:1 (**7**)	762.5526	$C_{42}H_{77}NO_9Na$	8.5	740.6	762.7	482.2	738.5[d]	297.4
W	Glc-d18:2 h20:1 (**11**)	790.5822	$C_{44}H_{81}NO_9Na$	11.2	768.5	790.6	482.3	766.6	325.3
W	Glc-d18:2 h22:1 (**23**)	818.6113	$C_{46}H_{85}NO_9Na$	15.2	796.7	818.5	482.2	794.5	353.4
W	Glc-d18:2 h24:1 (**29**)	846.6441	$C_{48}H_{89}NO_9Na$	19.4	824.7	846.6	482.3	822.6	381.5
W	Glc-d18:2 h25:1 (**36**)	860.6609	$C_{49}H_{91}NO_9Na$	21.6	838.6	860.7	482.2	836.6	395.6
W	Glc-d18:2 h26:1 (**39**)	874.6749	$C_{50}H_{93}NO_9Na$	23.4	852.6	874.5	482.2	850.6	409.6
W	Glc-d18:3 h20:0 (**13**)	790.5811	$C_{44}H_{81}NO_9Na$	12.2	768.5	790.6	480.2	766.6	327.5
A	Glc-t18:1 h16:0 (**5**)	754.5463	$C_{40}H_{77}NO_{10}Na$	7.3	732.6	754.6	500.3	730.6	271.4
A, W	Glc-t18:1 h20:0 (**16**)	810.6072	$C_{44}H_{85}NO_{10}Na$	12.6	788.5	810.6	500.3	786.6	327.6
A	Glc-t18:1 h21:0 (**22**)	824.6237	$C_{45}H_{87}NO_{10}Na$	15.1	802.6	824.5	500.3	800.6	341.6
A, W, C	Glc-t18:1 h22:0 (**27**)	838.6401	$C_{46}H_{89}NO_{10}Na$	17.6	816.6	838.7	500.3	814.7	355.5
A, W, C	Glc-t18:1 h23:0 (**31**)	852.6540	$C_{47}H_{91}NO_{10}Na$	19.5	830.8	852.7	500.2	828.6	369.7
A, W, C	Glc-t18:1 h24:0 (**35**)	866.6706	$C_{48}H_{93}NO_{10}Na$	21.4	844.7	866.7	500.3	842.6	383.5
A, W, C	Glc-t18:1 h25:0 (**40**)	880.6863	$C_{49}H_{95}NO_{10}Na$	23.8	858.6	880.6	500.3	856.6	397.6
A, W	Glc-t18:1 h26:0 (**43**)	894.7024	$C_{50}H_{97}NO_{10}Na$	25.8	872.7	894.7	500.2	870.7	411.6
W	Glc-t18:1 h22:1 (**18**)	836.6228	$C_{46}H_{87}NO_{10}Na$	12.7	814.5	836.6	500.3	812.6	353.5
W	Glc-t18:1 h23:1 (**20**)	850.6364	$C_{47}H_{89}NO_{10}Na$	15.0	828.7	850.5	500.3	826.5	367.5
W	Glc-t18:1 h24:1 (**28**)	864.6549	$C_{48}H_{91}NO_{10}Na$	17.7	842.7	864.6	500.2	840.6	381.5
W	Glc-t18:1 h25:1 (**32**)	878.6714	$C_{49}H_{93}NO_{10}Na$	19.6	856.6	878.6	500.3	854.8	395.5
W	Glc-t18:1 h26:1 (**37**)	892.6862	$C_{50}H_{95}NO_{10}Na$	21.6	870.7	892.6	500.2	868.6	409.5
W	Glc-t18:1 h28:1 (**44**)	920.7188	$C_{52}H_{99}NO_{10}Na$	25.8	898.6	920.7	500.3	896.4	437.7
W	Glc-t18:1 h24:2 (**19**)	862.6389	$C_{48}H_{89}NO_{10}Na$	13.5	840.6	862.6	500.3	838.6	379.5
A	Glc-OH-d18:2 h16:0[b] (**1**)	752.5254	$C_{40}H_{75}NO_{10}Na$	4.9	730.5	752.5	498.3	728.5	271.4
A	Glc-oxo-d18:2 h16:0[b] (**3**)	750.5123	$C_{40}H_{73}NO_{10}Na$	5.3	728.5	750.5	496.2	726.5	271.4
A	Glc-2OH-d18:2 h16:0[b] (**2**)	768.5236	$C_{40}H_{75}NO_{11}Na$	5.1	746.5	768.4	514.3	744.4	271.4
A	Glc-2OH-d18:2 h22:0[b] (**12**)	852.6189	$C_{46}H_{87}NO_{11}Na$	11.8	830.6	852.6	514.3	828.6	355.5
A	Glc-2OH-d18:2 h24:0[b] (**24**)	880.6498	$C_{48}H_{91}NO_{11}Na$	16.5	858.7	880.6	514.3	856.6	383.5
A	Glc-OH-t18:1 h22:0[b] (**9**)	854.6351	$C_{46}H_{89}NO_{11}Na$	9.7	814.5[c]	854.7	516.3	830.7	355.6
A	Glc-OH-t18:1 h24:0[b] (**17**)	882.6660	$C_{48}H_{93}NO_{11}Na$	11.8	842.5[c]	882.5[d]	516.4	858.6	383.5
A	d18:2 h16:0 (**46**)	574.4810	$C_{34}H_{65}NO_4Na$	9.9	552.5	574.5	320.2	550.6	271.5
A	t18:1 h22:0 (**47**)	676.5853	$C_{40}H_{79}NO_5Na$	21.1	654.6	676.6	338.1	652.6	355.5
A	t18:1 h24:0 (**48**)	704.6165	$C_{42}H_{83}NO_5Na$	24.6	682.7	704.6	338.1	680.6	383.5

[a]Compound numbers (**bold**) as given in Figure 3. RT, retention time; acyl, acyl moiety; sug, sugar moiety. [b]Proposed structure (location and stereochemistry of double bonds and functional groups not known). [c]Detected as $[M - H_2O + H]^+$ with CID 150 V. [d]Ions not detected in MS1 but indirectly by MS/MS measurements

materials. The GluCers consisted mainly of three LCB (d18:1, d18:2, t18:1, all C18), of α-hydroxylated fatty acids (C15−28, saturated, mono- and polyunsaturated) and of β-glucose. Dihydroxy (d18:1, d18:2) LCB were rather bound to short chain fatty acids (≤20) and trihydroxy (t18:1) to long-chain fatty acids (≥22).[36] In mammals sphingosine ($d18:1^{\Delta 4}$) is also mostly attached to short chain and phytosphingosine (t18:0) to long chain fatty acids.[37] All Δ4,5 double bonds were determined as *E*, Δ8,9 as *E* and *Z* configurations. Fatty acid desaturations were only *Z*-configured. Sphingosine (d18:1) and

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J. Agric. Food Chem. 2017, 65, 1507−1517

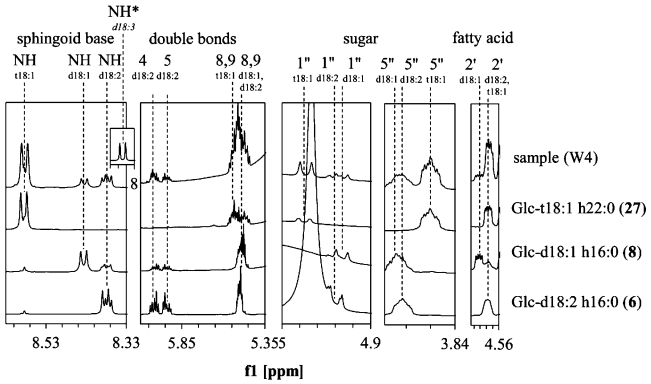

Figure 4. Major ^{1}H NMR signals for structure identification (600 MHz, pyridine-d_5). Hydrogens bound to sphingoid nitrogen assigned as NH, hydrogens bound to carbon indicated by carbon number (′ for fatty acid carbon, ″ for sugar carbon) as in Figure 1; sample W4: Glc-*d18:3** h20:0 (**13**), Glc-d18:1 h18:0 (**14**), Glc-d18:2 h19:0 (**15**), Glc-t18:1 h20:0 (**16**), Glc-t18:1 h22:1 (**18**), Glc-t18:1 h24:2 (**19**); *, signal not proven (no reference available).

unsaturated fatty acids were only detected in wheat germs. Some new LCB derivatives were identified in apple pomace by mass spectrometry but need further proof due to their trace levels.

In apple pomace, Glc-d18:2$^{\Delta4,8}$ h16:0 (**6**) and Glc-t18:1$^{\Delta8}$ h22/23/24:0 (**27**, **31**, **35**) were the main compounds, which is consistent with previous studies.[15,19] Wheat germs showed a more complex range of GluCers. Sphingadienine (d18:2$^{\Delta4,8}$), sphingosine (d18:1$^{\Delta8}$), and phytosphingenine (t18:1$^{\Delta8}$) were found to be the major LCBs and h16:0, h18:0, and h20:0 the main fatty acids. This was also reported before in wheat grains.[28] Glc-d18:2$^{\Delta4,8}$ h16:0 (**6**), Glc-d18:2$^{\Delta4,8}$ h20:0 (**21**), and Glc-d18:1$^{\Delta8}$ h16:0 (**8**) were also reported to be the main compounds in wheat bran[21] and germs.[20] Trace levels of sphingatrienine (d18:3, **13**) were identified in wheat germs. This sphingoid base was detected before in tobacco leaves,[38] rice, and maize[39] as sphinga-4,8,10-trienine (d18:3$^{\Delta4E,8E,10E}$) . The position of desaturation could not be identified here. Unsaturated fatty α-hydroxylated acids were defined as *Z*-type and ω-9 series, which was formerly reported in chilling-resistant plants like wheat.[40] GluCers with fatty acids h28:1 (**44**) and h24:2 (**19**) were identified in wheat for the the first time. Intact GluCers were detected in coffee grounds after roasting and brewing, which gives evidence for the stability of GluCer species. However, their content was low; thus, coffee grounds do not appear suitable for future phyto-Cer production.

Enzymatic Hydrolysis with Imiglucerase. A crucial step of the phyto-Cer production is the sugar hydrolysis. Acidic hydrolyses were not satisfying for GluCer sugar cleavage because of an almost complete loss of trihydroxy ceramides based on phytosphingosine and phytosphingenine. This was stated before, when a two-step oxidation−reduction−hydrolysis was investigated GluCers.[41] Thus, enzymatic hydrolysis was tested. A specific human recombinant glucocebrosidase (imiglucerase, Cerezyme) was used for hydrolysis. All GluCer fractions (A1−12, W1−12, C1−5) were hydrolyzed. TLC was used for survey (Figure 5). All compounds could be cleaved with an active enzyme; the GluCer bands disappeared and Cer bands appeared with the same intensity. GluCers were not hydrolyzed by the inactivated enzyme. There are no reports on enzymatic hydrolysis of plant GluCers with human recombinant glucocebrosidase so far. Glycosylceramidase activity was analyzed in plants before.[23] However, no glucocerebrosidase was assigned in plants yet.

Three GluCers from apple pomace (Glc-d18:2 h16:0 (**7**); Glc-t18:1 h22:0 (**27**); and Glc-t18:1 h24:0 (**29**)) were individually hydrolyzed by the enzyme. The hydrolysis did not proceed quantitatively (yield 51−74%) and was substrate-dependent. Higher substrate concentrations in the higher μM range (200−700 μM) were not hydrolyzed completely. On the other hand, GluCers concentrations in the lower μM range (12−14 μM) were totally converted. The same was observed using an endo glucosylceramidase (EC 3.2.1.123) from leech.[42] The enzyme could only transform approximately 20% of the

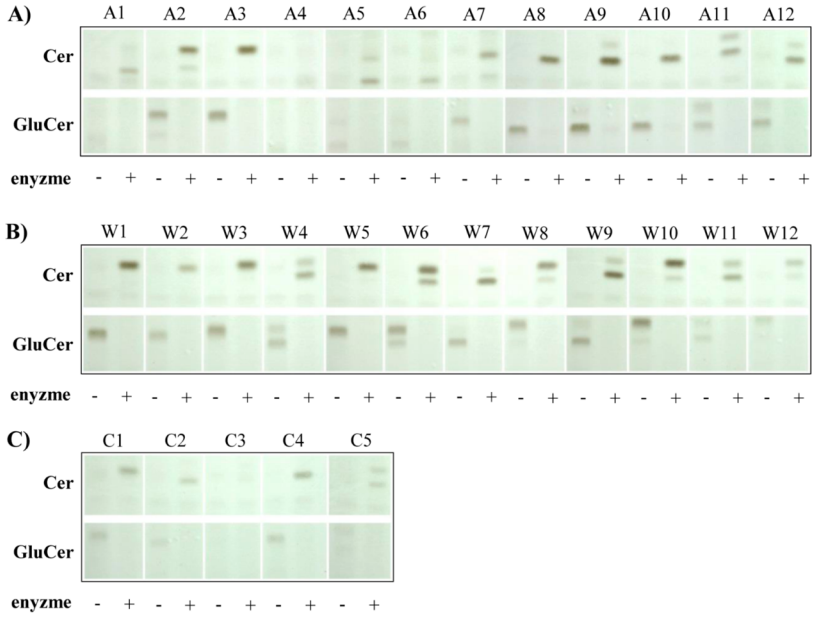

Figure 5. Enzymatic hydrolysis with glucocerebrosidase. Enzymatic hydrolysis of fractions from apple pomace (A; A), wheat germs (W; B), and coffee grounds (C; C) with active (+) and inactive (−) enzyme.

DOI: 10.1021/acs.jafc.6b04275
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substrate (150 μM). Lower substrate concentrations (37.5 μM) and a different detergent led to hydrolysis up to 34%.[42]

The cleavage of the sugar moiety was demonstrated by NMR experiments (see Supporting Information). Sugar-bound hydrogens (H-1″ to H-6″) were not detectable after hydrolysis. The structures of these Cers were also elucidated by HPLC-MS, ESI-MS/MS, ESI-HRMS, and NMR experiments and were consistent with their GluCer precursors. Cers could be analyzed under the same conditions as GluCers. All relevant m/z are shown in Table 2. ^{1}H- and ^{13}C-chemical shifts are presented in Table 3 and Table 4 and were assigned by COSY, TOCSY, HSQC, and HMBC correlations. Structure elucidation confirmed (2*S*,3*R*,4*E*,8*E*/*Z*)-2*N*-[(2′*R*)-2′-hydroxyhexadecanoylamino]-4(*E*),-8(*E*/*Z*)-octadecadiene-1,3-diol (d18:2 h16:0, **46**), (2*S*,3*S*,4*R*,8*E*/*Z*)-2*N*-[(2′*R*)-2′-hydroxydocosanoylamino]-8(*E*/*Z*)-octadecadiene-1,3,4-triol (t18:1 h22:0, **47**), and (2*S*,3*S*,4*R*,8*E*/*Z*)-2*N*-[(2′*R*)-2′-hydroxytetracosanoylamino]-8(*E*/*Z*)-octadecadiene-1,3,4-triol (t18:1 h24:0, **48**) as the hydrolyzed phyto-Cers (Figure 6). These compounds were isolated before from plants as trace substances.[43−45] The enzymatic hydrolysis of these phyto-Cers from GluCers was not reported so far. As Cers contents are essentially lower than GluCers amounts in plants,[12] hydrolysis by glucocerebrosidase is a novel procedure for phyto-Cer production. Availability of the imiglucerase is ensured by heterologous production; however, costs for the human-identical enzyme used for infusion therapy are high. Recombinant glucocerebrosidase is produced in plants, plant cells, and mammalian cells. The heterologous proteins require, in addition to rigorous purification, enzymatic tailoring of glycosylation to ensure nonimmunogenicity and macrophage targeting.[46] For biotechnological GluCer hydrolysis, glucocerebrosidase should have optimal activity, but the protein structure does not need tailoring after expression; thus, heterologous enzyme production can be performed in a convenient microorganism, providing cheap enzyme protein.

Table 3. ^{1}H-, ^{13}C-Chemical Shifts (pyridine-d_5) of d18:2$^{\Delta 4E,\Delta 8E/Z}$ h16:0 (46)

	δ_H [ppm] (M, J [Hz])		δ_C [ppm]	
position	Δ8*Z*	Δ8*E*	Δ8*Z*	Δ8*E*
Sphingoid Base				
1a	4.521 (m)		61.91	61.93
1b	4.264 (m)		-	
2	4.729 (m)		56.13	56.12
3	4.888 (m)		73.09	73.07
4	6.109 (m, 15.4)		132.55	132.43
5	6.040 (m, 15.4)		131.84[d]	131.84[d]
6	2.219[a] (m)		32.96	32.97
7	2.221[a] (m)	2.175[a] (m)	27.41	32.80
8	5.497[c] (m)	5.513[c] (m)	129.43	129.94
9	5.497[c] (m)	5.513[c] (m)	130.65	131.15
10	2.086[c] (m)	2.022[a] (m)	27.61	32.95
11	1.384[b] (m)		29.53−30.08	
12−16	1.260 (m)		29.53−30.08	
16	-		32.14−32.15	
17	1.260 (m)		22.96−22.97	
18	0.873 (t, 6.9)		14.31	
NH	8.392 (d, 8.9)	8.386 (d, 8.8)	-	
Fatty Acid				
1′	-		175.38	175.37
2′	4.635 (dt, 8.0; 3.9)		72.55	72.57
3′a	2.262[a] (m)		35.84	35.85
3′b	2.077[a] (m)		-	
4′a	1.842 (m)		25.92	25.90
4′b	1.764(m)		-	
5′	1.384[b] (m)		29.53−30.08	
6′−14′	1.260 (m)		29.53−30.08	
14′	-		32.14−32.15	
15′	1.260 (m)		22.96−22.97	
16′	0.873 (t, 6.9)		14.31	

[a] ^{1}H chemical shifts from HSQC correlations. [b] ^{1}H chemical shifts from HMBC correlations. [c] ^{1}H chemical shifts from TOCSY correlations. [d] Signals lay upon each other.

Phyto-Cers and Their Possible Role in Dermatotherapy. Phyto-Cers are similar but only partially identical to human skin Cers (Figure 6). Phyto-Cers may differ in chain lengths and degree of desaturation. Skin Cers typically show sphingoid base chain lengths of C18 to 22 and fatty acids of C16 to 30, of which C22 to 26 are most abundant.[37,47] C16, C20, C22, and C24 are the major fatty acid chain lengths in plants. Sphingosine (d18:1) type Cers (e.g., Cer [AS, d18:1 h18:0] and [NS, d18:1 18:0]) represent approximately 17% and phytosphingosine (t18:0) type Cers (e.g., Cers [AP, t18:0 h18:0] and [NP, t18:0 18:0]) approximately 31% of the stratum corneum Cers. Other major Cer groups derive from 6-hydroxysphingosine and from sphinganine (for human skin Cer nomenclature see Figure 6 and Supporting Information).[2]

Cers contribute to the skin barrier function against water loss. The hydroxylated head core groups of the Cers influence the packing of stratum corneum lipids, and phyto-Cers [NP] and [AP] have a high number of hydroxyl groups building hydrogen bonds to increase the stability of the lipid phases.[7,8] Plant Cers with three or four hydroxyl groups may serve as stabilizing and water binding agents in therapy. Investigation of the Cer composition of diseased skin, e.g., of psoriasis skin patches or of skin from atopic dermatitis, revealed a decrease specifically of phyto-Cers and other highly hydroxylated Cer species.[5,6] It is therefore obvious to attempt treatment with phyto-Cers in particular. For this purpose, phyto-Cers need to be available, and the structures and the composition of Cers from plant sources need to be elucidated.

On the other hand, an α-hydroxy group (e.g., Cer [AP]) can lead to decreasing hydrogen bonding[48] and to a less stabilized lamellar order, at least in artificial skin membranes.[49] GluCers with five hydroxyl groups described here (e.g., Glc-2OH-d18:2 h24:0 (**24**) and Glc-OH-t18:1 h24:0 (**17**)) could provide still different features. Their function in plant cells and their possible application in skin therapy must be further elucidated. Further, benefits or disadvantages of desaturations in plant sphingolipids, e.g., at position Δ8 or within the fatty acid (ω-9), need investigation. It was shown that lipids with a *Z*-configuration can stretch the lipid layers and embed water.[50] However, monounsaturated free fatty acids led to a disturbed lipid packing[51] which impairs the integrity of the stratum corneum. Therefore, comparative studies with phyto-Cers and Cers established skin treatment are necessary. For such studies, phyto-Cers need to be isolated. If they show beneficial effects and are similar to each other in activity, complete separation of individual phyto-Cer species will not be necessary. Enriched fractions containing a defined spectrum of phyto-Cers may be applied and can be economically produced.

An upscaling process for phyto-ceramides as rare and expensive natural products appears possible now and should

DOI: 10.1021/acs.jafc.6b04275
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Table 4. ^{1}H-, ^{13}C-Chemical Shifts (pyridine-d_5) of t18:1$^{\Delta 8E/Z}$ h22:0 (47) and t18:1$^{\Delta 8E/Z}$ h24:0 (48)

	t18:1$^{\Delta 8E/Z}$ h22:0				t18:1$^{\Delta 8E/Z}$ h24:0			
	δ_H [ppm] (M, J [Hz])		δ_C [ppm]		δ_H [ppm] (M, J [Hz])		δ_C [ppm]	
position	Δ8Z	Δ8E	Δ8Z	Δ8E	Δ8Z	Δ8E	Δ8Z	Δ8E
Sphingoid Base								
1a	4.531 (dd, 4.5; 10.8)		62.01	62.03	4.533 (dd, 4.6; 10.8)		62.00	62.03
1b	4.444 (dd, 5.0; 10.8)		-		4.446 (dd, 5.0; 10.8)		-	
2	5.139 (m)		52.97	52.99	5.143 (m)		52.97	52.98
3	4.364 (dd, 4.5; 6.8)		76.89	76.87	4.367 (dd, 4.4; 6.8)		76.88	76.86
4	4.307 (ddd, 2.6; 6.8; 9.2)		72.97	72.92	4.310 (ddd, 2.6; 6,8; 9.3)		72.97	72.92
5a	2.335[a] (m)		33.98	33.86	2.335[a] (m)		33.98	33.86
5b	1.977[a] (m)		-		1.977[a] (m)		-	
6a	2.052[a] (m)		26.92	26.76	2.054[a] (m)		26.91	26.76
6b	1.812[a] (m)		-		1.809[a] (m)		-	
7	2.261[a] (m)	2.196[a] (m)	27.94	33.33	2.261[a] (m)	2.196[a] (m)	27.94	33.33
8	5.558[c] (m)	5.559[c] (m)	130.38	130.83	5.558[a] (m)	5.556[a] (m)	130.38	130.83
9	5.475[c] (m)	5.505[c] (m)	130.25	130.72	5.480[a] (m)	5.518[a] (m)	130.25	130.72
10	2.091[a] (m)	2.015[a] (m)	27.60	33.01	2.089[a] (m)	2.013[a] (m)	27.60	33.01
11	1.371[b] (m)		29.54−30.12		1.356[b] (m)		29.54−30.12	
12−16	1.283 (m)		29.54−30.12		1.283 (m)		29.54−30.12	
16	-		32.13−32.15		-		32.13−32.14	
17	1.283 (m)		22.96		1.283 (m)		22.96	
18	0.874 (t, 7.0)		14.31		0.875 (t, 6.8)		14.31	
NH	8.603[d] (d, 9.0)		-		8.607[d] (d, 8.9)		-	
Fatty Acid								
1′	-		175.23		-		175.23	
2′	4.639 (m)		72.47		4.641 (dd, 3.7; 8.0)		72.47	
3′a	2.246[a] (m)		35.74		2.246[a] (m)		35.74	
3′b	2.061[a] (m)		-		2.053[a] (m)		-	
4′a	1.802[a] (m)		25.86	25.84	1.804[a] (m)		25.86	25.84
4′b	1.740[a] (m)		-		1.737[a] (m)		-	
5′	1.371[b] (m)		29.54−30.12		1.381[b] (m)		29.54−30.12	
6′−20′/22′	1.283 (m)		29.54−30.12		1.283 (m)		29.54−30.12	
20′/22′	-		32.13−32.15		-		32.13−32.14	
21′/23′	1.283 (m)		22.96		1.283 (m)		22.96	
22′/24′	0.874 (t, 7.0)		14.31		0.875 (t, 6.8)		14.31	

[a] ^{1}H chemical shifts from HSQC correlations. [b] ^{1}H chemical shifts from HMBC correlations. [c] ^{1}H chemical shifts from TOCSY correlations. [d] Signals overlapping.

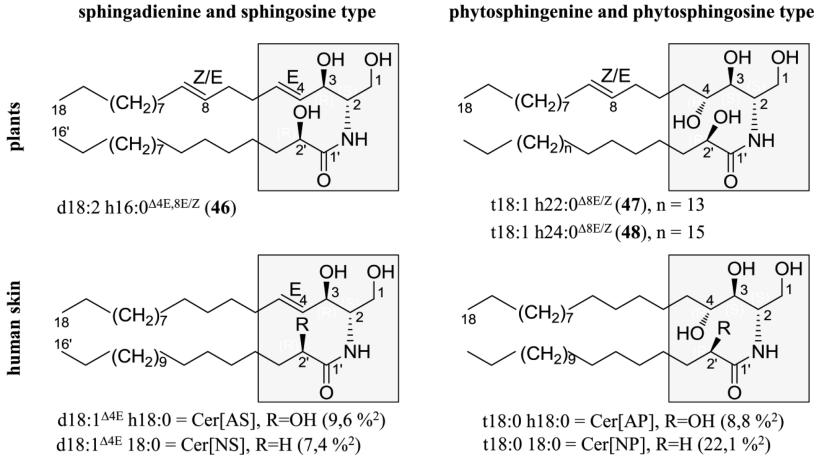

Figure 6. Isolated phyto-Cers and human skin Cers. Cer, ceramide; S, sphingosine; P, phytosphingosine; A, α-hydroxylated fatty acid; N, nonhydroxylated fatty acid.

be pursued. With the methods described here, phyto-Cers can

be obtained from plant material.

DOI: 10.1021/acs.jafc.6b04275
J. Agric. Food Chem. 2017, 65, 1507−1517

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b04275.

GluCer purification of apple pomace, wheat germs, and coffee grounds (TLC); detected GluCer species of wheat germs and coffee grounds (HPLC-MS); detected MS/MS fragments of GluCers and Cers; 1D and 2D NMR spectra and chemical shifts of GluCers and Cers; main and trace compound differentiation (HPLC-MS); isolated Cer compounds; nomenclature system of skin Cers (PDF)

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Funding

M.R. kindly acknowledges the support of the German Academic Exchange Service (DAAD) and of the German Federal Ministry for Education and Research (BMBF) via Project "Welcome to Africa" as well as the support of the Biosolutions GmbH, Halle.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

M. Reisberg is grateful for helpful advice and the enzyme assay conditions from Konrad Sandhoff (Life and Medical Sciences Institute (LIMES), Membrane Biology & Lipid Biochemistry Unit, c/o Kekulé-Institute of Chemistry and Biochemistry, Bonn, Germany) and provision of Cerezyme® by Christoph Baerwald (University Hospital Leipzig, Department for Gastroenterology and Rheumatology, Rheumatology Unit, Leipzig, Germany). The technical assistance from Anja Ehrlich, Manuela Woigk and Annegret Laub is kindly appreciated.

■ ABBREVIATIONS USED

A, apple pomace; APCI, atmospheric pressure chemical ionization; C, coffee grounds; CC, column chromatography; Cer, ceramide; CID, collision-induced dissociation; ESI, electrospray ionization; FAS, Foreign Agricultural Service (United States Department of Agriculture); GluCer, glucosylceramide; LCB, long-chain base; MS, mass spectrometry; NMR, nuclear magnetic resonance; RP, reversed phase; TIC, total ion count; TLC, thin layer chromatography; TMS, tetramethylsilane; W, wheat germs

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DOI: 10.1021/acs.jafc.6b04275
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