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Development of a QuEChERS-Based Stable-Isotope Dilution LC-MS/MS Method To Quantitate Ferulic Acid and Its Main Microbial and Hepatic Metabolites in Milk

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ABSTRACT: Forage plants of the Poaceae family are grown as pasturage or used for the production of hay, straw, corn stover, etc. Although ferulic acid contents of grasses are generally high, the amount of ingested ferulic acid differs depending on the type of forage, resulting in varying contents of ferulic acid and its microbial and hepatic metabolites in milk. Concentrations and patterns of these metabolites may be used as markers to track different forages in livestock feeding. Therefore, we developed a stable isotope dilution assay to quantitate ferulic acid, 12 ferulic acid-based metabolites, *p*-coumaric acid, and cinnamic acid in milk. Because most analytes were not commercially available as stable isotope labeled standard compounds, they were synthesized as ^{13}C- or deuterium-labeled standard compounds. A modification of the QuEChERS method, a Quick, Easy, Cheap, Effective, Rugged, and Safe approach usually applied to analyze pesticides in plant-based products, was used to extract the phenolic acids from milk. Determination was carried out by LC-ESI-MS/MS in scheduled multiple reaction monitoring modus. By using three different milk samples, the applicability of the validated approach was demonstrated.

KEYWORDS: *phenolic acids, hydroxycinnamic acids, ferulic acid, metabolism, milk, QuEChERS, isotopic labeling, stable isotope dilution assay, mass spectrometry*

INTRODUCTION

Ferulic acid (4-hydroxy-3-methoxycinammic acid, FA) largely contributes to the stability of plant cell walls of grasses and other plants. As a potential cell wall cross-link it is also a main factor in cell wall recalcitrance against chemical or enzymatic degradation. Larger amounts of FA can be found in grasses (members of the family Poaceae), especially in maize (*Zea mays*), whereas quantities in dicotyledonous plants of the order of Caryophyllales are generally lower.[1−3] Only small amounts of FA occur in its free form, but most FA is linked to cell wall polymers, i.e. arabinoxylans, pectins, and/or lignin.[4−6] These polymers can be cross-linked by ferulic acid and its derivatives, such as ferulate oligomers, limiting cell growth and increasing the robustness of the cellulose−hemicellulose network.[7,8]

With many ruminant forages being based on grasses, dairy cows ingest comparably large amounts of FA. Microbial esterases in the rumen can liberate FA; other enzymes are able to reduce the unsaturated aliphatic side-chain of FA and to demethylate and dehydroxylate its phenolic unit (Figure 1, solid arrows).[9−11] Microbial decarboxylation or acetate elimination can transform FA to 4-vinylguaiacol or vanillin, respectively.[12,13] Also, phenylacetic acid (PAA), which is potentially formed from phenylpropionic acid (PPA) by microbial α-oxidation, was found in milk.[13−15] FA and its microbial metabolites are partially absorbed and further metabolized, for example through hepatic β-oxidation (Figure 1, dotted arrows).[16,17] Through phase-II metabolism, benzoic acids are conjugated with glycine to form hippuric acids.[13,18] Additionally, conjugates with sulfuric or glucuronic acids are potential phase-II metabolites.[18,19] Most of these compounds were detected in cow's milk earlier.[20,21] It has to be emphasized that the analyzed metabolites are not exclusively formed from FA, but may also originate from, for example, *p*-coumaric (*p*-CA) and cinnamic acid (CinnA, Figure 1), as well as from polyphenols such as flavonoids and from the aromatic amino acids phenylalanine and tyrosine. These phenolic compounds also naturally occur in plant materials, and their microbial and/or hepatic metabolism partially results in the same metabolites as FA.[22,23]

The amounts of FA in different forages vary considerably. Consequently, the concentrations of FA and its metabolites in milk potentially differ largely, depending on the cow's feeding type. For example, feeding maize silage should result in a higher FA intake if compared to many other grasses or products thereof. Also, feeding pure roughages compared to roughages with the addition of concentrates might result in differences of the phenolic acid profiles of milk. Thus, specific phenolic acids may be used as markers to trace the forage types of dairy cows. Increased consumer expectations on the potential health effects of organic milk or milk with particular labels as well as the demand of ethical husbandry have become important social issues. However, currently, cattle forages are not routinely traced back. In the past, fatty acid profiles were proposed as potential indicators for different forage sources.[24]

Here, we report the development of a stable isotope dilution assay to quantitate phenolic acids in milk as potential markers for different cattle forages. QuEChERS-based sample extraction[25,26] in combination with LC-MS/MS analysis provides an accurate, precise, selective, and highly sensitive method. Whereas the QuEChERS-based sample preparation simplifies and speeds up

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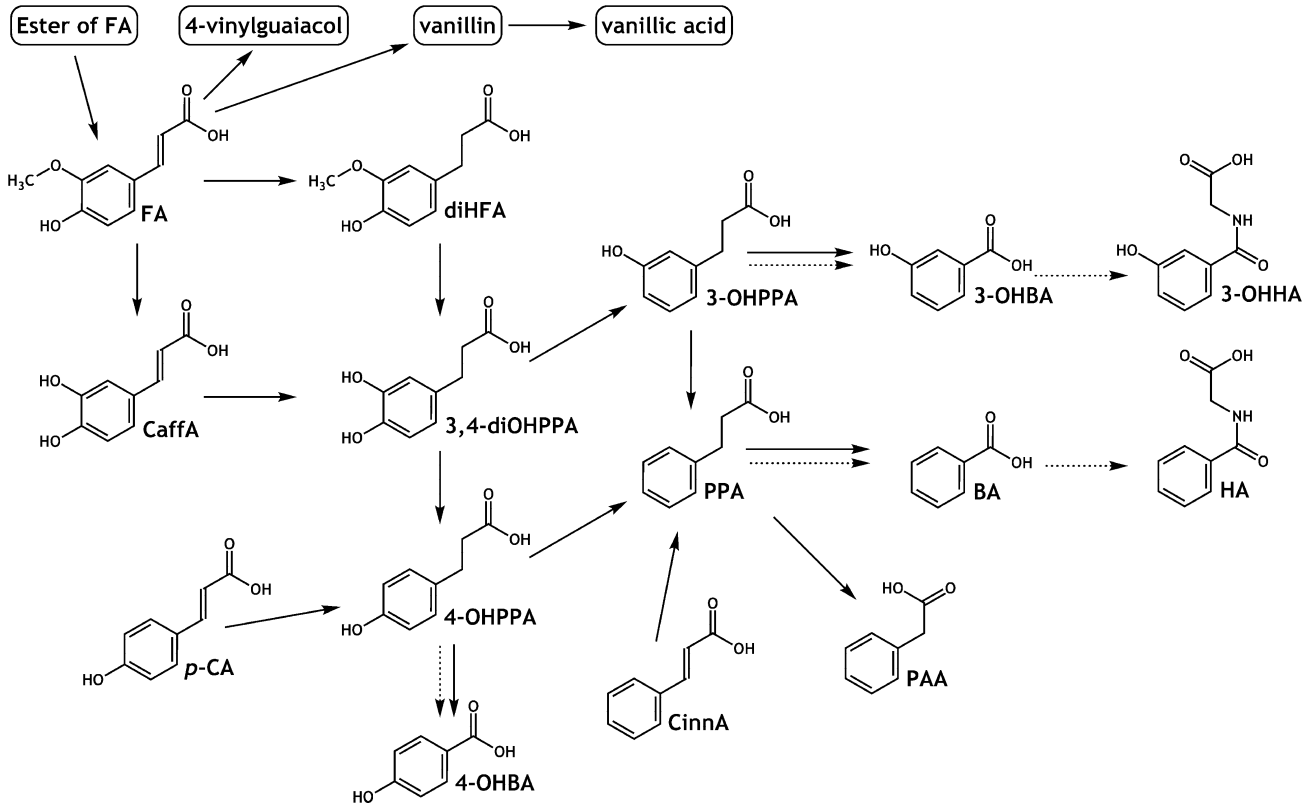

Figure 1. Potential microbial (solid arrows) and hepatic (dotted arrows) metabolic pathways of ferulic acid (FA), *p*-coumaric acid (*p*-CA), and cinnamic acid (CinnA) in ruminants. BA, benzoic acid; CaffA, caffeic acid; diHFA, dihydroferulic acid; 3,4-diOHPPA, 3-(3,4-dihydroxyphenyl)propionic acid; HA, hippuric acid; 3-OHBA, 3-hydroxybenzoic acid; 4-OHBA, 4-hydroxybenzoic acid; 3-OHHA, 3-hydroxyhippuric acid; 3-OHPPA, 3-(3-hydroxyphenyl)propionic acid; 4-OHPPA, 3-(4-hydroxyphenyl)propionic acid; PAA, phenylacetic acid; PPA, 3-phenylpropionic acid.

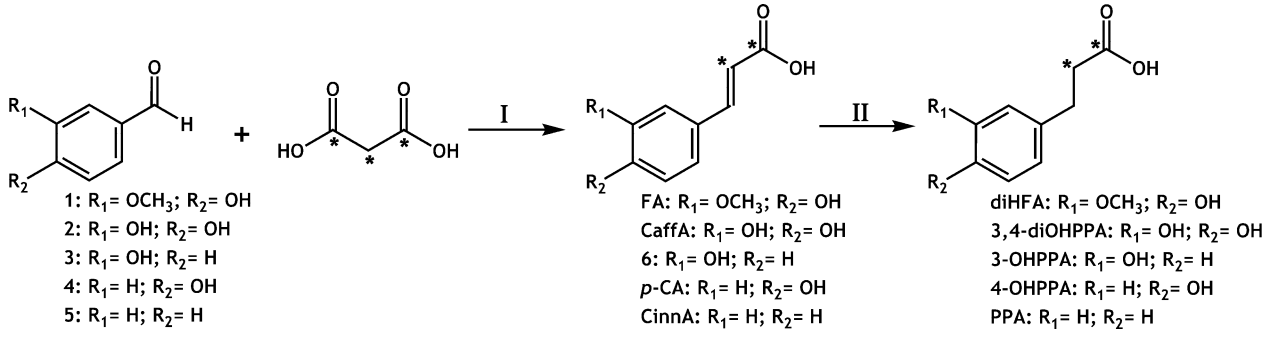

Figure 2. Scheme for the syntheses of ^{13}C-labeled standard compounds. Benzaldehydes were converted to cinnamic acids by using the Knoevenagel reaction (**I**; pyridine, aniline). Hydrogenation of the propenylic side-chains (**II**; methanol, palladium (10%) on carbon, H_2) resulted in 3-phenylpropionic acids. Asterisks indicate ^{13}C-labels. **1**, vanillin; **2**, 3,4-dihydroxybenzaldehyde; **3**, 3-hydroxybenzaldehyde; **4**, 4-hydroxybenzaldehyde; **5**, benzaldehyde; **6**, 3-hydroxycinnamic acid; CaffA, caffeic acid; CinnA, cinnamic acid; diHFA, dihydroferulic acid; 3,4-diOHPPA, 3-(3,4-dihydroxyphenyl)propionic acid; FA, ferulic acid; 3-OHPPA, 3-(3-hydroxyphenyl)propionic acid; 4-OHPPA, 3-(4-hydroxyphenyl)propionic acid; PPA, 3-phenylpropionic acid; *p*-CA, *p*-coumaric acid.

the procedure, using isotopically labeled standard compounds improves both the accuracy and precision of this method by compensating for, for example, analyte losses during sample cleanup procedures and matrix induced suppression of ionization.

MATERIALS AND METHODS

Chemicals. [$^{13}C_2$]Glycine (99%), [$^{13}C_3$]malonic acid (99%), acetone-d_6 (99.9%), deuterium (99.8%), deuterium chloride 35 wt % (99%), 3,4-dihydroxybenzaldehyde (97%), ethyl acetate, *trans*-FA (99%), PAA (99%), and sodium hydroxide 40 wt % (99.5%) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Aniline, benzaldehyde (99%), dihydroferulic acid (diHFA, 97%), 3-(3,4-dihydroxyphenyl)propionic acid (3,4-diOHPPA, 98%), 3-hydroxybenzoic acid (3-OHBA, 99%), 4-hydroxybenzoic acid (4-OHBA, 99%), 3-(3-hydroxyphenyl)propionic acid (3-OHPPA, 98%), 3-(4-hydroxyphenyl)propionic acid (4-OHPPA, 99%), and phenylpropionic acid (PPA, 99%) were from Alfa Aesar (Haverhill, Massachusetts, USA). Benzoic acid (BA, 99.5%), *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, 99%), caffeic acid (CaffA, 97%), CinnA (99%), 3-hydroxybenzaldehyde (95%), glycine (99%), *p*-CA (98%), and vanillin (99%) were from Fluka (Buchs, Switzerland), and benzoyl chloride (99%), hippuric acid (HA, 99%), acetonitrile, methanol, magnesium sulfate, pyridine, and toluol were from VWR International (Radnor, Pennsylvania, USA). 4-Hydroxybenzaldehyde (96%), formic acid, and

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palladium (10%) on activated carbon were from Merck KGaA (Darmstadt, Germany), [3,5-D_2]BA (98.9%) was from CDN isotopes (Québec, Canada), D_2O was from Deutero GmbH (Kastellaun, Germany), thionyl chloride (98%) was from Riedel-de Haën AG (Seelze, Germany), and hydrogen was produced in-house by using a hydrogen generator.

Milk Samples. Conventional milk samples with 1.5% and 3.5% fat contents (UHT, homogenized) were purchased in April 2016 from a local grocery store (Rheinland-Pfalz, Germany). An organic milk sample with a fat content of 3.8% (pasteurized, nonhomogenized) was also purchased in April 2016 from a local dairy farm (Baden-Württemberg, Germany), which produces exclusively organic milk-products following demeter-principles.

Nuclear Magnetic Resonance Spectroscopy. ^{1}H NMR experiments were performed at 298 K on a Bruker (Rheinstetten, Germany) Ascend 500 MHz NMR spectrometer equipped with a Prodigy cryoprobe using standard Bruker implementations. Samples were dissolved in acetone-d_6 (carboxylic and phenolic protons were partially exchanged for deuterium), and the acetone residual peak was used for spectrum calibration (methyl proton, δ_H 2.05 ppm).[27]

Preparative Liquid Chromatography. Preparative separations were performed on a Shimadzu (Kyoto, Japan) HPLC equipped with two pumps (LC-8A), an UV-detector (SPD-20A), and a communication bus module (CBM-20A). Injection was accomplished manually via a six valve port with a 2 mL sample loop.

LC-DAD-MS/MS. The LC-DAD-MS/MS consisted of a 2690 separations module, pumps, degasser, autosampler, and a 996 diode array detector (Waters Corporation (Milford, Massachusetts, USA)) coupled to a Micromass Quattro Micro mass spectrometer (Waters Corporation) and a column oven (Jetstream Plus, Beckman Coulter, Krefeld, Germany).

Synthesis of Cinnamic Acids. Syntheses of the ^{13}C-labeled cinnamic acids [$^{13}C_2$]FA, [$^{13}C_2$]CaffA, [$^{13}C_2$]*p*-CA, [$^{13}C_2$]3-hydroxycinnamic acid, and [$^{13}C_2$]CinnA were performed by using the Knoevenagel reaction with Doebner modification (Figure 2), similar to a formerly published approach.[28] In brief, the respective benzaldehyde was added to [$^{13}C_3$]malonic acid, pyridine, and aniline (amounts see below) in a 2 mL glass vial. Prevented from light, the sealed vial was heated to 55 °C for 16 h. Cold water (1 mL) was slowly added, and the solution was acidified by adding dropwise 0.5 mL of concentrated hydrochloric acid. The reaction product was transferred to a larger vial and extracted into ethyl acetate (5 × 2 mL). The combined extracts were evaporated to dryness using a rotary evaporator.

Purification of the reaction products by preparative HPLC using a phenyl-hexyl column (Phenomenex, Aschaffenburg, Germany, Luna, 250 × 10 mm, 5 μm) yielded the standard compounds in sufficient purity (>98%): Methanolic solutions of the raw products were fractionated at room temperature with a flow rate of 3.5 mL min^{-1} and a detection wavelength of 280 nm; water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B) were used as eluents. Starting conditions for the separation of [$^{13}C_2$]FA, [$^{13}C_2$]CaffA, [$^{13}C_2$]*p*-CA, and [$^{13}C_2$]3-hydroxycinnamic acid used 20% B; the eluent was ramped to 45% B in 25 min and ramped to 100% B within 10 min, following an equilibration step. To purify [$^{13}C_2$]CinnA, 35% B was initially used, followed by a linear increase to 100% B within 35 min and an equilibration step. The purified, dried compounds were analyzed with LC-DAD-MS to determine their purity. In addition, 5 mg of the compounds were dissolved in acetone-d_6 to acquire ^{1}H NMR spectra.

[8,9-$^{13}C_2$]*trans*-Ferulic Acid. starting material, 65 mg (0.61 mmol) of [$^{13}C_3$]malonic acid, 142.8 mg (0.92 mmol) of vanillin, 300 μL of pyridine, 30 μL of aniline; product, 85.2 mg (0.43 mmol, yield 71%) of [$^{13}C_2$]FA. δ_H [ppm] 3.92 (s, 3H, OCH_3); 6.37 (ddd, J = 160.8; 15.9; 2.7 Hz, 1H, 8); 6.87 (d, J = 8.2 Hz, 1H, 5); 7.14 (dd, J = 8.2; 2.0 Hz, 1H, 6); 7.33 (d, J = 2.0 Hz, 1H, 2); 7.59 (ddd, J = 15.9; 6.8; 2.9 Hz, 1H, 7).

[8,9-$^{13}C_2$]*trans*-Caffeic Acid. starting material, 65 mg (0.61 mmol) of [$^{13}C_3$]malonic acid, 127 mg (0.92 mmol) of 3,4-dihydroxybenzaldehyde, 300 μL of pyridine, 30 μL of aniline; product, 79.1 mg (0.43 mmol, yield 71%) of [$^{13}C_2$]CaffA. δ_H [ppm] 6.26 (ddd, J = 160.4; 15.9; 2.8 Hz, 1H, 8); 6.87 (d, J = 8.1 Hz, 1H, 5); 7.04 (dd, J = 8.1; 2.1 Hz, 1H, 6); 7.16 (d, J = 2.1 Hz, 1H, 2); 7.54 (ddd, J = 15.9; 6.8; 2.8 Hz, 1H, 7).

[8,9-$^{13}C_2$]*trans*-*p*-Coumaric Acid. starting material, 60 mg (0.56 mmol) of [$^{13}C_3$]malonic acid, 112.4 mg (0.92 mmol) of 4-hydroxybenzaldehyde, 300 μL of pyridine, 30 μL of aniline; product, 72.6 mg (0.44 mmol, yield 78%) of [$^{13}C_2$]*p*-CA. δ_H [ppm] 6.33 (ddd, J = 160.6; 15.9; 2.7 Hz, 1H, 8); 6.90 (m, 2H, 3;5); 7.55 (m, 2H, 2;6); 7.61 (ddd, J = 15.9; 6.8; 2.9 Hz, 1H, 7).

[8,9-$^{13}C_2$]*trans*-3-Hydroxycinnamic Acid. starting material, 30 mg (0.28 mmol) of [$^{13}C_3$]malonic acid, 56.2 mg (0.46 mmol) of [$^{13}C_2$]3-hydroxybenzaldehyde, 150 μL of pyridine, 15 μL of aniline; product, 40.2 mg (0.24 mmol, yield 86%) of [$^{13}C_2$]3-hydroxycinnamic acid.

[8,9-$^{13}C_2$]*trans*-Cinnamic Acid. starting material, 60 mg (0.28 mmol) of [$^{13}C_3$]malonic acid, 97.6 mg (0.92 mmol) of benzaldehyde, 300 μL of pyridine, 30 μL of aniline; product, 44.8 mg (0.3 mmol, yield 54%) of [$^{13}C_2$]CinnA. δ_H [ppm] 6.53 (ddd, J = 161.3; 16.0; 2.7 Hz, 1H, 8); 7.44 (m, 3H, 3;4;5); 7.68 (m, 3H, 2;6;7).

Synthesis of Phenylpropionic Acids. Syntheses of ^{13}C-labeled 3-phenylpropionic acids [$^{13}C_2$]diHFA, [$^{13}C_2$]3,4-diOHPPA, [$^{13}C_2$]4-OHPPA, [$^{13}C_2$]3-OHPPA, and [$^{13}C_2$]PPA were carried out by hydrogenation of the corresponding carbon-labeled cinnamic acids (Figure 2). The cinnamic acids (amounts see below) were reduced in 1.5 mL of methanol using palladium (10%) on carbon (approximately 5 mg) as catalyst under H_2 atmosphere. After 16 h, the catalyst was removed by centrifugation, and the solvent was evaporated. Structure and purity of the products were evaluated by LC-DAD-MS and NMR as described above.

[8,9-$^{13}C_2$]Dihydroferulic Acid. 49.1 mg (0.25 mmol) of [$^{13}C_2$]FA was reduced to 47.3 mg (0.24 mmol, yield 96%) of [$^{13}C_2$]diHFA. δ_H [ppm] 2.54 (ddt, J = 128.2; 7.3; 7.3 Hz, 2H, 8); 3.80 (s, 3H, OCH_3); 6.66 (dd, J = 8.0; 1.9 Hz, 1H, 6); 6.72 (d, J = 8.0 Hz, 1H, 5); 6.84 (d, J = 1.9 Hz, 1H, 2).

[8,9-$^{13}C_2$]3-(3,4-Dihydroxyphenyl)propionic Acid. 43.7 mg (0.24 mmol) of [$^{13}C_2$]CaffA was reduced to 42.6 mg (0.23 mmol, yield 96%) of [$^{13}C_2$]3,4-diOHPPA. δ_H [ppm] 2.53 (ddt, J = 128.1; 7.2; 7.2 Hz, 2H, 8); 2.76 (m, 2H, 7); 6.57 (dd, J = 8.0; 2.0 Hz, 1H, 6); 6.72 (d, J = 8.0 Hz; 1H, 5); 6.73 (bs, 1H, 2).

[8,9-$^{13}C_2$]3-(4-Hydroxyphenyl)propionic Acid. 31.0 mg (0.19 mmol) of [$^{13}C_2$]*p*-CA was reduced to 30.0 mg (0.18 mmol, yield 95%) of [$^{13}C_2$]4-OHPPA. δ_H [ppm] 2.54 (ddt, J = 128.1; 7.4; 7.4 Hz, 2H 8); 2.81 (m, 2H, 7); 6.74 (m, 2H, 3;5); 7.07 (m, 2H, 2;6).

[8,9-$^{13}C_2$]3-(3-Hydroxyphenyl)propionic Acid. 40.2 mg (0.24 mmol) of [$^{13}C_2$]3-hydroxycinnamic acid was reduced to 39.6 mg (0.23 mmol, yield 96%) of [$^{13}C_2$]3-OHPPA. δ_H [ppm] 2.58 (ddt, J = 128.3; 7.3; 7.3 Hz, 2H, 8); 2.84 (m, 2H, 7); 6.70 (m, 3H, 2;4;6); 7.09 (t, J = 7.8 Hz, 1H; 5).

[8,9-$^{13}C_2$]3-Phenylpropionic Acid. 22.4 mg (0.15 mmol) of [$^{13}C_2$]CinnA was reduced to 20.6 mg (0.14 mmol, yield 91%) of [$^{13}C_2$]PPA. δ_H [ppm] 2.61 (ddt, J = 128.3, 7.4, 7.4 Hz, 2H, 8); 2.91 (m, 2H, 7); 7.18 (m, 1H, 4); 7.27 (m, 4H, 2;3;5;6).

[7,7-D_2]Phenylacetic Acid. PPA (400 mg, 2.34 mmol), D_2O (884 μL), and NaOD-solution (294 μL, 40 wt %) were placed in a high-pressure bottle and air was exchanged for N_2. After stirring overnight at 100 °C, the solution was cooled down to room temperature, acidified with concentrated HCl (130 μL), and extracted into ethyl acetate. The combined extracts were dried under reduced pressure. This procedure was repeated twice to yield 303 mg of product (yield 75%) with 96% 2-fold deuterium incorporation in benzylic position.[29] δ_H [ppm] 7.25 (m, 1H, 4); 7.32 (m, 4H, 2;3;5;6).

[3,5-D_2]4-Hydroxybenzoic Acid and [2,4,6-D_3]3-Hydroxybenzoic Acid. 4-OHBA (100 mg, 0.725 mmol) and 3-OHBA (100 mg, 0.725 mmol), respectively, were placed in high-pressure bottles. D_2O (745 μL) and DCl-solution (124 μL, 35 wt %) were added, and the mixtures were stirred for 48 h at 100 °C. Then, the solution was cooled to 0 °C, and the crystallization product was collected by centrifugation, washed with chilled water and lyophilized overnight. This procedure was repeated once for [D_2]4-OHBA and three times for [D_3]3-OHBA. Yields were 88.9 mg (89%) with 99% 2-fold deuterium incorporation for 4-OHBA and 84.8 mg (85%) with 98% 3-fold deuterium incorporation for 3-OHBA, respectively. [D_2]4-OHBA, δ_H [ppm] 7.92 (s, 2H, 2;6); 9.13 (bs, 1H, 4-OH); 3-OHBA, δ_H [ppm] 7.32 (s, 1H, 5).

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Table 1. Parameters of the Multiple Reaction Monitoring Using Specific Time Frames for the Monitored Transitions[a]

analyte	mass transition [m/z]	cone voltage [V]	collision energy voltage [V]	time frame [min]
3-OHHA	194 → 150	24	11	5−16
[$^{13}C_2$]3-OHHA	196 → 151			
4-OHBA	137 → 93	23	12	16−18.9
[D_2]4-OHBA	139 → 95			
HA	178 → 134	24	11	18.3−21
[$^{13}C_2$]HA	180 → 135			
3,4-diOHPPA	181 → 137[b]	24	12	18.9−22
	181 → 109[c]			
[$^{13}C_2$]3,4-diOHPPA	183 → 138[b]			
	183 → 109[c]			
3-OHBA	137 → 93	20	13	22−25
[D_3]3-OHBA	140 → 96			
CaffA	179 → 135	20	15	25−28
[$^{13}C_2$]CaffA	181 → 136			
4-OHPPA	165 → 93[b]	27	13	27−31
	165 → 121[c]			
[$^{13}C_2$]4-OHPPA	167 → 93[b]			
	167 → 122[c]			
diHFA	195 → 136[b]	25	17	31−34.5
	195 → 121[c]			
[$^{13}C_2$]diHFA	197 → 137[b]			
	197 → 121[c]			
3-OHPPA	165 → 121[b]	24	12	31−34.5
	165 → 119[c]			
[$^{13}C_2$]3-OHPPA	167 → 122[b]			
	167 → 120[c]			
p-CA	163 → 119	20	14	33.7−36.5
[$^{13}C_2$]*p*-CA	165 → 120			
PAA	135 → 91	15	7	36.5−39.5
[D_2]PAA	137 → 93			
BA	121 → 77	20	11	36.5−39.5
[D_2]BA	123 → 79			
FA	193 → 134[b]	22	15	39−50
	193 → 178[c]			
[$^{13}C_2$]FA	195 → 135[b]			
	195 → 180[c]			
PPA	149 → 105	24	11	47−54.4
[$^{13}C_2$]PPA	151 → 106			
CinnA	147 → 103	22	11	54.2−65
[$^{13}C_2$]CinnA	149 → 104			

[a]BA, benzoic acid; CaffA, caffeic acid; CinnA, cinnamic acid; diHFA, dihydroferulic acid; 3,4-diOHPPA, 3-(3,4-dihydroxyphenyl)propionic acid; FA, ferulic acid; HA, hippuric acid; 3-OHBA, 3-hydroxybenzoic acid; 4-OHBA, 4-hydroxybenzoic acid; 3-OHHA, 3-hydroxyhippuric acid; 3-OHPPA, 3-(3-hydroxyphenyl)propionic acid; 4-OHPPA, 3-(4-hydroxyphenyl)propionic acid; PAA, phenylacetic acid; PPA, 3-phenylpropionic acid; *p*-CA, *p*-coumaric acid. [b]Quantifier mass transition. [c]Qualifier mass transition.

[1,2-$^{13}C_2$]Hippuric Acid. [$^{13}C_2$]Glycine (100 mg, 1.27 mmol) was dissolved in NaOH-solution (2 mL, 2 M). The solution was cooled to 0 °C, and benzoyl chloride (186 μL, 1.62 mmol) was added dropwise while stirring vigorously. After stirring for another 2 h at 0 °C, the solution was acidified to pH 1 and the crystallization product was filtered under vacuum and dried afterward. The resulting byproduct BA was dissolved in toluene (20 mL) at room temperature and removed by vacuum filtration. Recrystallization in H_2O yielded 195.5 mg (yield 85%) of the desired product. δ_H [ppm] 4.09 (dd, J = 139.2; 5.9 Hz, 2H, CH_2); 7.45 (m, 2H, 3;5); 7.52 (m, 1H, 4); 7.91 (m, 2H, 2;6).

3-Hydroxyhippuric Acid and [1,2-$^{13}C_2$]3-Hydroxyhippuric Acid. 3-OHBA (100 mg, 0.725 mmol), BSTFA (1 mL, 0.96 g, 3.73 mmol), and triethylamine (100 μL, 73 mg, 0.72 mmol) were allowed to react in a 1.5 mL glass vial at 60 °C for 16 h. The solvent was removed under reduced pressure at 40 °C, and the residue was lyophilized afterward. Acetone (500 μL) was added to the oily product, the solution was cooled to 0 °C, and thionyl chloride (52.6 μL, 86.28 mg, 0.725 mmol) was added slowly. The reaction mixture was stirred for 2 h, before the solvent was evaporated under N_2. The brown, solid residue was suspended in toluene (400 μL) by ultrasonication and added dropwise to a solution of glycine (54.4 mg, 0.725 mmol) in aqueous NaOH (500 μL, 2 M) at 0 °C. After stirring for 1 h, the reaction mixture was warmed to room temperature and stirred for another 4 h. Toluene was evaporated under N_2, the aqueous layer was acidified with concentrated HCl and extracted with ethyl acetate three times. The combined extracts were dried under reduced pressure to give an oily residue. To remove remaining trimethylsilyl groups, the crude product was dissolved in 2.5 mL of acetic acid/H_2O/acetonitrile (3:1:1, v/v/v) and kept at 60 °C for 16 h. After drying under reduced pressure, the residue was dissolved in acetonitrile/H_2O (1:1, v/v) and purified by preparative HPLC, using a Luna C18(2) reversed phase column (Phenomenex, 250 × 15 mm, 5 μm, 100 Å). Eluent A was 0.1% formic acid in H_2O, and eluent B was acetonitrile. The linear gradient (flow rate of 8 mL min^{-1}) increased from 10% B to 20% B within 15 min, followed

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by rinsing and equilibration steps. Detection was carried out at 288 nm, and collection of the desired compound was performed manually. Evaporation of the eluent resulted in pure 3-hydroxyhippuric acid (3-OHHA, 21.1 mg, 0,108 mmol, yield 15%). δ_H [ppm] 4.12 (d, J = 5.5 Hz, 2H, CH_2); 7.00 (m, 1H, 4); 7.28 (t, J = 7.8 Hz, 1H, 5); 7.39 (m, 2H, 1;6). The analogous synthesis using [$^{13}C_2$]glycine (55.8 mg, 0.725 mmol) resulted in [$^{13}C_2$]3-OHHA (40.5 mg, 0,205 mmol, yield 28%). δ_H [ppm] 4.13 (ddd, J = 139.2; 5.8; 5.0 Hz, 1H, CH_2); 7.00 (m, 1H, 4); 7.28 (t, J = 7.8 Hz, 1H, 5); 7.39 (m, 2H, 1;6).

Sample Analysis. *Sample Preparation.* A 15 g aliquot of vigorously shaken milk was weighed into a 50 mL polypropylene vial. After adding 100 μL of a mixture of isotopically labeled standard compounds in methanol/H_2O (1:1, v/v) the sample was equilibrated for a minimum of 20 min. To deconjugate sulfonated and glucuronated analytes, the pH of the sample was lowered with HCl (600 μL, 1 M) to pH 4.9, and sulfatase (containing glucuronidase activity) from *Helix pomatia* (Sigma-Aldrich, type H-1, 100 U) in acetate buffer (500 μL, 1 M, pH 4.9) was added. The vial was incubated for 18 h at 38 °C with gentle agitation. Afterward, the solution was acidified with HCl (2.1 mL, 1 M) to pH 1.5. To determine the nonconjugated analytes only, the sample was directly acidified with HCl (2.7 mL, 1 M) without prior addition of and incubation with the enzymes. Then, acetonitrile (10 mL) was added followed by rigorous shaking. Addition of $MgSO_4$ (7.5 g) and NaCl (2 g) induced phase separation. Samples were mixed rigorously for 3 × 30 s. After centrifugation at 4000 rcf for 5 min, about 8 mL of the organic layer were transferred into a 15 mL polypropylene vial, and $MgSO_4$ (2 g) was added. The sample was shaken rigorously for 3 × 30 s and centrifuged at 4000 rcf for 5 min. About 5 mL of the organic layer were transferred into a 15 mL polypropylene vial. The residue after vacuum evaporation at 45 °C for 3.5 h was dissolved in methanol:H_2O:concentrated HCl (250:50:1, v/v/v) by ultrasonication for 5 min. This solution was centrifuged during which phase separation partially occurred. Thus, depending on the behavior of the individual sample, either the solution or the upper layer (if phase separation occurred) was analyzed with LC-MS/MS, directly and following 1:100 dilution with methanol:H_2O (50:50, v/v).

LC-MS/MS Analysis. HPLC was carried out using a phenyl hexyl column (Phenomenex, Kinetex, 150 × 4.6 mm, 2.6 μm, 100 Å) with the eluents 0.01% formic acid in H_2O (A), 0.01% formic acid in methanol (B), and 0.01% formic acid in acetonitrile (C). Elution at 24 °C was carried out at a flow rate of 0.5 mL min^{-1} using a linear gradient: initial conditions 95% A, 5% B, linear increase to 83% A, 17% B within 10 min, held for 10 min, linear change to 87% A, 13% C within 1 min, held for 11 min, linear change to 75% A, 25% B within 1 min, held for 12 min, linear increase to 100% B within 10 min, rinsing and equilibration steps. The following MS parameters were used: ionization, ESI negative mode; capillary, 3.4 kV; cone, analyte-dependent (Table 1); extractor, 3 V; RF lens, 0.1 V; source temp, 120 °C; desolvation temperature, 350 °C; desolvation gas flow, 750 L/h; cone gas flow, 50 L/h; LM resolution 1, 15; HM resolution 1, 15; ion energy 1, 0.8; entrance, −1; collision energy, analyte-dependent (Table 1), exit, 1; LM resolution 2, 15; HM resolution 2, 15; ion energy 2, 1.0; multiplier, 650; cell gas pirani, 3.2e^{-3}. Mass transitions and time frames for the multiple reaction monitoring-modus are given in Table 1.

RESULTS AND DISCUSSION

Synthesis of Stable Isotope Labeled Standard Compounds. Because isotopologues of most of the analytes (Figure 1) were either not commercially available or available at exceptionally high prices only, synthetic routes were established in our laboratory. Deuterium labeling is often the favored pathway, because deuteration reagents are more affordable than ^{13}C-labeled educts. On the other hand, ^{13}C-labels are generally more stable, and isotopic shifts during chromatographic separation procedures (different retention times for isotopologues) are less distinct for ^{13}C-labeled compounds compared to deuterium labeled compounds.

Electrophilic aromatic substitution has been used for the replacement of aromatic hydrogen atoms earlier. Using protocols comparable to the method described by Kirby and Ogunkoya,[30] H/D-exchange reactions were performed for 3,4-dihydroxybenzaldehyde, 4-hydroxybenzaldehyde, CaffA, *p*-CA, and 3,4-diOHPPA. Although benzaldehydes were not required as standard compounds in this project, they can be converted into cinnamic acids or phenylpropionic acids as shown in Figure 2. However, electrophilic aromatic substitution under the conditions tested did not result in the required 2-fold labeled products. Either the replacement rate was marginal, or more than two deuterium labels were incorporated into the molecule, which, however, was not complete, resulting in a mixture of differently labeled compounds.

Reduction of the particular cinnamic acids with D_2 using palladium (10%) on carbon as catalyst was another approach to obtain 2-fold deuterium-labeled 3-phenylpropionic acids.[31−33] Different from our expectations, it was, however, not possible to exclusively incorporate two deuterium labels into the products. Depending on the solvent used (methanol or methanol-d_4), less or more than two labels, respectively, were detected in the products. This indicates that additional H/D-exchange mechanisms between the gas atmosphere, solvent, and reactants were triggered by the catalyst. Several mechanisms are described in the literature aiming to explain similar observations,[34−37] but these were not further investigated for our compounds. Instead, cinnamic acids were synthesized with two ^{13}C-labels and partly reduced to phenylpropionic acids.

Generally, cinnamic acids are obtained from benzaldehydes by three different reaction types, all of which can be used to incorporate two ^{13}C-labels. In the Perkin-reaction, acetic anhydride is used as solvent and reactant, and pyridine serves as catalyst.[38−40] The Horner−Wadsworth−Emmons-modification of the Wittig-reaction uses trialkyl phosphonoacetates in combination with anhydrous bases,[41,42] and the Knoevenagel-reaction with Doebner-modification uses malonic acid in pyridine as reagent/solvent and piperidine or aniline as catalyst to obtain cinnamic acids.[43−45] Here, the Knoevenagel-reaction with Doebner-modification was used, as it turned out to be the least expensive and most practical reaction pathway. 2-Fold ^{13}C-labeled cinnamic acids were obtained, and hydrogenation with H_2 and palladium (10%) on carbon as catalyst resulted in 2-fold ^{13}C-labeled 3-phenylpropionic acids (Figure 2).

HA can be obtained in a one-pot Schotten−Baumann reaction[46,47] using benzoyl chloride and glycine. Analogous synthesis of 3-OHHA requires, however, protection of the hydroxyl group of 3-OHBA; otherwise, intermolecular coupling occurs after conversion to 3-hydroxybenzoyl chloride. Here, trimethylsilylation was a suitable protection strategy, because the carboxylic group still reacts to form benzoyl chloride, and the protected hydroxyl group is stable enough to endure alkaline Schotten−Baumann conditions. Deprotection is easily achieved under acidic conditions (Figure 3).

Synthetic routes to incorporate ^{13}C-labels into benzoic acids and PAA are not as trivial as for cinnamic acids or hippuric acids. Therefore, H/D-exchanges were accomplished using harsher conditions. For PAA, alkaline conditions facilitate keto−enol-tautomerism, leading to a substitution of α-protons. For 3-OHBA and 4-OHBA, electrophilic aromatic substitution in aqueous deuterium chloride is facilitated by the hydroxyl groups, which accelerate the reaction and direct substitution to both *ortho* and *para* positions. Hence, 3-OHBA was the only analyte which was not obtained as 2-fold labeled standard compound but

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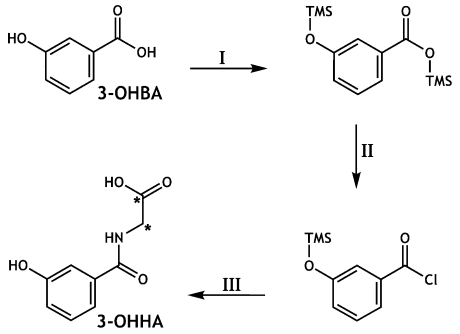

Figure 3. Synthesis of 3-hydroxyhippuric acid (3-OHHA) and [$^{13}C_2$]3-OHHA starting with 3-hydroxybenzaldehyde (3-OHBA). **I**; *N,O*-bis(trimethylsilyl)trifluoroacetamide, triethylamine, 60 °C, 16 h. **II**; thionyl chloride. **III**; toluol, glycine, or [$^{13}C_2$]glycine in aqueous NaOH. Asterisks indicate ^{13}C-labels. TMS, trimethylsilyl.

contains three labels. Different from the benzaldehydes and the cinnamic acids described above, 3-fold substitution was, however, complete. Lack of an aromatic hydroxyl group prevents a selective exchange in case of BA, but an appropriately labeled isotopologue was commercially available.

Sample Preparation. The QuEChERS method, first published in 2003,[25] describes a frequently used procedure to analyze pesticides in low-fat food products. Since its first publication, multiple variations of the procedure expanded the use of the QuEChERS method to a wide range of applications.[48] Here, the principles of a previously described QuEChERS-based method to extract pesticides from milk samples were used.[26] Some modifications were, however, necessary to adjust the procedure to the analytes of interest. First, the pH of the milk samples was adjusted to lower values in order to optimize extraction of our acidic analytes into acetonitrile. Second, dispersive solid phase extraction with primary and secondary amines was omitted, because this cleanup step eliminates carboxylic acids. Third, a larger aliquot of the extraction solution was sampled and concentrated before injection to increase method sensitivity by a factor of about 25. Although the concentration step is most useful to analyze the minor metabolites, it takes an additional 3.5 h. Fourth, enzyme (sulfatase, glucuronidase) treatment enables the determination of sulfate- and glucuronide-conjugated metabolites next to the nonconjugated analytes. Because these conjugates can be formed during hepatic phase II metabolism, it is advised to include them into the method. Quantitative release of bound analytes was verified by incubation of milk samples with different enzyme activities (50 U, 100 U, and 300 U) in triplicate. For 10 out of 15 analytes, including the more dominant HA, BA, 3-OHHA, PAA, and FA, no significant differences ($p < 0.05$) were found for the higher enzyme concentrations (100 U and 300 U). Differences were only found for *p*-CA and CinnA; however, this is more likely due to their low concentrations close to the LOD. CaffA, 3,4-diOHPPA, and 4-OHPPA were not quantifiable in these samples (see below).

In some extracts, phase separation occurred following the addition of methanol:H_2O:concentrated HCl (250:50:1, v/v/v) to the residue after concentrating an aliquot of the acetonitrile extraction solution. Because of the acidic conditions, it was assumed that the analytes are located in the organic fraction after centrifugation. Analysis of the methanol fraction by LC-MS/MS analysis confirmed this assumption.

LC-MS/MS Analysis. Suitable MS/MS mass transitions were determined for all analytes (Table 1). If possible, a qualifier mass transition was chosen in addition to the quantifier mass transition. Chromatographic separation was achieved on a core−shell phenyl−hexyl column using a ternary gradient. In

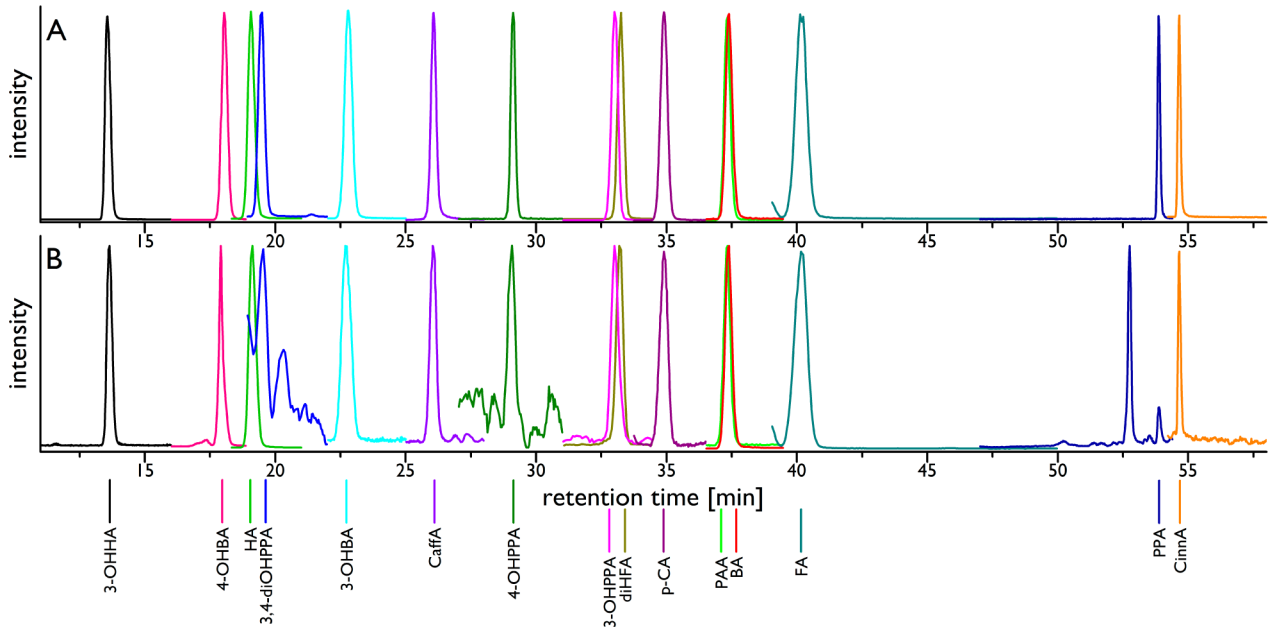

Figure 4. HPLC-MS/MS standard (A) and sample (B, organic cow milk, 3.8% fat) chromatograms combining chromatograms from different mass transitions in single chromatograms. 3-Hydroxyhippuric acid (3-OHHA) and hippuric acid (HA) were analyzed after 1:100 dilution of the evaporated and redissolved extract. BA, benzoic acid; CaffA, caffeic acid; CinnA, cinnamic acid; diHFA, dihydroferulic acid; 3,4-diOHPPA, 3-(3,4-dihydroxyphenyl)propionic acid; FA, ferulic acid; 3-OHBA, 3-hydroxybenzoic acid; 4-OHBA, 4-hydroxybenzoic acid; 3-OHPPA, 3-(3-hydroxyphenyl)propionic acid; 4-OHPPA, 3-(4-hydroxyphenyl)propionic acid; PAA, phenylacetic acid; PPA, 3-phenylpropionic acid; *p*-CA, *p*-coumaric acid.

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Table 2. Lowest Calibration Levels (LCL), Calibration Equations,[b] and Coefficients of Determination (R^2) for the Analytes and Their Corresponding Isotopically Labeled Standard Compounds[a]

	LCL [μg/mL]	calibration equation (LCL − 1 μg/mL)	R^2	calibration equation (1 μg/mL − 11 μg/mL)	R^2
3-OHHA	0.005	$y = -6163.66x^2 + 54610.42x + 3.20$	0.99999	$y = -1240.58x^2 + 40879.31x + 8794.48$	0.99969
[$^{13}C_2$]3-OHHA	0.005	$y = -5129.54x^2 + 48533.708x - 1.86$	0.99999	$y = -1024.93x^2 + 35527.96x + 8935.23$	0.99983
4-OHBA	0.025	$y = -3652.18x^2 + 64510.75x + 29.31$	0.99995	$y = -1560.89x^2 + 56804.96x + 6549.16$	0.99993
[D_2]4-OHBA	0.025	$y = -7741.37x^2 + 69114.40x - 68.40$	0.99939	$y = -1533.40x^2 + 57405.16x + 7849.06$	0.99995
HA	0.005	$y = -4796.57x^2 + 41409.25x + 5.96$	0.99996	$y = -704.16x^2 + 32078.89x + 4934.99$	0.99974
[$^{13}C_2$]HA	0.005	$y = -5355.09x^2 + 41557.83x + 0.88$	0.99985	$y = -619.48x^2 + 31072.73x + 6224.93$	0.99951
3,4-diHOPPA	0.005	$y = -453.56x^2 + 27213.196x - 17.70$	0.99992	$y = -613.59x^2 + 24708.4546x + 2737.89$	0.99967
[$^{13}C_2$]3,4-diHOPPA	0.005	$y = -464.67x^2 + 24370.40x - 11.35$	0.99985	$y = -434.04x^2 + 20663.44x + 3842.84$	0.99948
3-OHBA	0.005	$y = -4517.41x^2 + 56068.35x - 0.81$	0.99982	$y = -930.82x^2 + 45440.91x + 7283.44$	0.99958
[D_3]3-OHBA	0.005	$y = -6873.74x^2 + 65166.19x + 13.61$	0.99989	$y = -1415.59x^2 + 55038.14x + 5370.07$	0.99994
CaffA	0.005	$y = -1527.42x^2 + 77767.75x - 64.29$	0.99997	$y = -1791.70x^2 + 67860.64x + 13119.72$	0.99914
[$^{13}C_2$]CaffA	0.005	$y = -2702.00x^2 + 91859.04x - 42.57$	0.99986	$y = -2008.78x^2 + 78871.14x + 15054.20$	0.99932
4-OHPPA	0.025	$y = 68.39x^2 + 1943.31x - 1.36$	0.99941	$y = -35.83x^2 + 2106.87x + 12.93$	0.99988
[$^{13}C_2$]4-OHPPA	0.025	$y = 108.86x^2 + 1834.36x - 0.74$	0.99982	$y = -30.76x^2 + 1947.76x + 75.65$	0.99972
diHFA	0.005	$y = -7749.06x^2 + 52160.46x - 16.82$	0.99962	$y = -683.80x^2 + 30437.26x + 17065.50$	0.99854
[$^{13}C_2$]diHFA	0.005	$y = -7915.49x^2 + 51371.02x - 20.6$	0.99974	$y = -698.08x^2 + 29930.07x + 15733.76$	0.99863
3-OHPPA	0.005	$y = -7187.23x^2 + 87843.14x - 13.11$	0.99997	$y = -1453.87x^2 + 66017.93x + 19445.92$	0.99925
[$^{13}C_2$]3-OHPPA	0.005	$y = -7048.66x^2 + 95509.69x - 11.98$	0.99998	$y = -1709.90x^2 + 73005.74x + 18985.64$	0.99945
p-CA	0.005	$y = -1688.11x^2 + 128469.96x - 14.23$	0.99971	$y = -2185.78x^2 + 103535.68x + 25990.06$	0.99988
[$^{13}C_2$]*p*-CA	0.005	$y = -2681.35x^2 + 140329.39x - 32.94$	0.99988	$y = -2291.10x^2 + 112070.76x + 28014.01$	0.99993
PAA	0.005	$y = 109.72x^2 + 14030.99x + 0.06$	0.99982	$y = -59.73x^2 + 16607.21 \times -2219.39$	0.99982
[D_2]PAA	0.005	$y = -178.86x^2 + 13732.97x - 7.06$	0.99959	$y = -26.59x^2 + 15537.15x - 1653.99$	0.99999
BA	0.025	$y = -92.43x^2 + 4744.17x + 86.33$	0.99992	$y = -37.87x^2 + 4654.97x + 175.15$	0.99984
[D_2]BA	0.025	$y = -262.17x^2 + 4375.13x - 3.39$	0.99984	$y = -27.58x^2 + 4126.50x + 83.88$	0.99989
FA	0.005	$y = 1284.21x^2 + 46816.70x + 5.14$	0.99909	$y = -536.75x^2 + 46297.26x + 2725.98$	0.99987
[$^{13}C_2$]FA	0.005	$y = 2273.74x^2 + 45554.03x + 0.97$	0.99870	$y = -459.34x^2 + 44581.16x + 4263.07$	0.99993
PPA	0.025	$y = 291.68x^2 + 2477.92x + 0.21$	0.99997	$y = 11.68x^2 + 2961.81x - 210.83$	0.99999
[$^{13}C_2$]PPA	0.025	$y = 271.99x^2 + 2736.43x - 2.20$	0.99994	$y = 17.21x^2 + 3182.75x - 194.23$	1.00000
CinnA	0.005	$y = 404.37x^2 + 14060.01x + 8.96$	0.99998	$y = -87.45x^2 + 15405.87x - 865.67$	0.99999
[$^{13}C_2$]CinnA	0.005	$y = 439.62x^2 + 15382.77x + 4.81$	0.99989	$y = -68.05x^2 + 16382.18x - 424.01$	0.99999

[a]BA, benzoic acid; CaffA, caffeic acid; CinnA, cinnamic acid; diHFA, dihydroferulic acid; 3,4-diOHPPA, 3-(3,4-dihydroxyphenyl)propionic acid; FA, ferulic acid; HA, hippuric acid; 3-OHBA, 3-hydroxybenzoic acid; 4-OHBA, 4-hydroxybenzoic acid; 3-OHHA, 3-hydroxyhippuric acid; 3-OHPPA, 3-(3-hydroxyphenyl)propionic acid; 4-OHPPA, 3-(4-hydroxyphenyl)propionic acid; PAA, phenylacetic acid; PPA, 3-phenylpropionic acid; *p*-CA, *p*-coumaric acid. [b]y = peak area, x = analyte concentration [μg/mL].

Figure 4, the chromatograms from all quantifier mass transitions of all analytes of a milk sample (organic cow milk containing 3.8% fat) are combined in a single chromatogram (data from 3-OHHA and HA are taken from the analysis of the diluted sample; see below) and compared to a chromatogram of standard compounds. The chromatographic separation of 4-OHBA and 3,4-diOHPPA is not affected by the high concentration of coeluting HA, which shows a 2 min broad, tailing peak in the concentrated sample. A maximum of eight mass transitions is recorded within a given time frame (Table 1), resulting in sufficient (at least 12) data points per peak to ensure correct peak integration. For some quantifier mass transitions, more than one peak appears in the chromatogram. In these cases, retention times of analytes and isotopically labeled standard compounds need to be compared, and, where defined, qualifier mass transitions have to be considered for an unambiguous identification.

The first quantitative experiments were performed to roughly estimate the contents of all analytes in different milk samples in order to add comparable amounts of isotopically labeled standard compounds to the milk samples. Due to large differences among the analyte concentrations in milk samples, two measurements (concentrated and in 1:100 dilution) of each sample are advised. Without dilution, HA concentrations exceed the calibration range; also, the 3-OHHA levels are high and should therefore be assessed from measuring the diluted sample extract.

Method Validation and Application. The lowest calibration levels (LCL) were determined by diluting mixtures of all standard compounds until signal-to-noise ratios of at least 9:1 (LCL, Table 2) were reached. Calibration ranges were chosen from the respective LCL up to concentrations of 11 μg mL^{-1}, divided into two sections: section 1, LCL to 1 μg mL^{-1}; and section 2, 1 μg mL^{-1} to 11 μg mL^{-1}. Both sections covered six equidistant calibration points. Each calibration point was measured in triplicate, and, because homogeneity of variances was not given, weighted regression (with reduced chi^2, weighting factor 1/standard $deviation^2$) was used. The obtained data fit second order polynomial regression better than linear regression, which was also confirmed by residual analysis. Calibration equations and the corresponding coefficients of determination (R^2) are given in Table 2. Assessing both limit of detection (LOD) and limit of quantitation (LOQ) is not a straightforward process, because empty matrices (milk samples which do not contain our analytes) are not available and mimicking a milk matrix is problematic because both caseins and whey protein may adsorb phenolic compounds, thus potentially carrying analytes into our empty matrix. Spiking milk samples with our isotopically

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Table 3. Limits of Detection (LOD), Limits of Quantitation (LOQ), and Recovery Rates of Isotopically Labeled Analytes Added to Conventional or Organic Milk Samples with Different Fat Contents (1.5; 3.5; or 3.8%)[a]

analyte	LOD in conventional milk 3.5% fat [μg/kg]	LOQ in conventional milk 3.5% fat [μg/kg]	recovery rate in conventional milk 1.5% fat [%]	recovery rate in conventional milk 3.5% fat [%]	recovery rate in organic milk 3.8% fat [%]
[$^{13}C_2$]3-OHHA	0.15	0.4	67	69	68
[D_2]4-OHBA	0.2	0.7	52	56	55
[$^{13}C_2$]HA	N/A[b]	N/A[b]	61	64	63
[$^{13}C_2$]3,4-diOHPPA	0.7	2	41	37	38
[D_3]3-OHBA	0.15	0.4	60	63	59
[$^{13}C_2$]CaffA	0.15	0.4	76	74	84
[$^{13}C_2$]4-OHPPA	3	9	43	41	30
[$^{13}C_2$]diHFA	0.2	0.7	22	21	47
[$^{13}C_2$]3-OHPPA	0.1	0.3	29	25	45
[$^{13}C_2$]*p*-CA	0.03	0.1	60	64	64
[D_2]PAA	0.7	2	31	30	29
[D_2]BA	2	7	39	44	42
[$^{13}C_2$]FA	0.1	0.3	90	94	90
[$^{13}C_2$]PPA	0.2	0.6	59	61	47
[$^{13}C_2$]CinnA	0.07	0.2	60	62	56

[a]BA, benzoic acid; CaffA, caffeic acid; CinnA, cinnamic acid; diHFA, dihydroferulic acid; 3,4-diOHPPA, 3-(3,4-dihydroxyphenyl)propionic acid; FA, ferulic acid; HA, hippuric acid; 3-OHBA, 3-hydroxybenzoic acid; 4-OHBA, 4-hydroxybenzoic acid; 3-OHHA, 3-hydroxyhippuric acid; 3-OHPPA, 3-(3-hydroxyphenyl)propionic acid; 4-OHPPA, 3-(4-hydroxyphenyl)propionic acid; PAA, phenylacetic acid; PPA, 3-phenylpropionic acid; *p*-CA, *p*-coumaric acid. [b]See text.

labeled compounds is an option; however, due to the small but existing spectral overlap between the labeled and unlabeled analytes, this strategy does not work for analytes with large concentrations in the milk samples. Nevertheless, this approach was used to determine approximate LODs and LOQs, and mixtures of all isotopically labeled standard compounds were added in decreasing concentrations to samples of 15 g of conventional milk with 3.5% fat. Samples were processed as described with enzyme treatment. Concentrations, which resulted in a signal-to-noise-ratio of 3:1 and 9:1, were defined as LOD and LOQ, respectively (Table 3). However, the LOD and LOQ of [$^{13}C_2$]HA are not assessable because the high levels of naturally occurring HA result in a severe overloading of the HPLC column and, therefore, in a broad signal with shifted retention time. Additionally, 0.1% of the natural HA are recorded with the same mass transition as for [$^{13}C_2$]HA because of the natural abundance of ^{13}C as described above.

The applicability of the method was tested by analyzing three different milk samples (conventional cow milk, 1.5% fat; conventional cow milk, 3.5% fat; organic cow milk, 3.8% fat) in triplicate. As shown in Figure 5, it was possible to quantitate all analytes with the exception of 3,4-diOHPPA, 4-OHPPA, and nonconjugated CaffA in these samples. Incubation with sulfatase and glucuronidase before extraction resulted in similar or higher concentrations of all analytes compared to nonenzyme treatment data. Also, metabolites that can only be ester-conjugated (BA, PPA, PAA, and CinnA) were determined in higher concentrations after enzyme incubation.

Standard deviations of the total concentrations (free and conjugated) of the metabolites for the analyzed milk samples representing the precision of the method range between <1% and 8.9%. The data were also used to calculate recovery rates of the added isotopically labeled standard compounds. Considering that after QuEChERS extraction only about 5 out of 10 mL of the organic fraction was used for concentration and LC-MS/MS analysis, recovery rates are between 21% for [$^{13}C_2$]diHFA and 94% for [$^{13}C_2$]FA (Table 3). However, although some recovery rates are apparently quite low, it needs to be mentioned that these values only influence the method sensitivity, but should not affect the accuracy of the method. To verify this assumption, spiking procedures were performed in triplicate by adding a mixture of nonlabeled standard compounds (together with the labeled standards) to conventional milk samples containing 3.5% fat. Recovery rates of 90−110% were determined for most of the analytes. However, recovery rates of 4-OHBA, 3-PPA, and CinnA were only around 50−70%. Therefore, data for these analytes should be labeled as semiquantitative only.

Comparing the phenolic acid contents in the three analyzed milk samples, promising differences in the metabolite patterns can be observed. The concentrations of 3-OHPPA, 3-OHBA, 3-OHHA, 4-OHBA, and BA are higher in the analyzed organic milk sample compared to the milk samples from conventional production, whereas diHFA and PAA seem to be more abundant in conventional milk samples. However, whether or not these differences are only due to the amounts of ingested FA or whether and how other ingested phenolic compounds influence these patterns cannot be answered at this point. Larger studies with milk samples of known history will be necessary to estimate the potential of differentiating organic and conventional milk based on ferulic acid metabolites. In comparison to the results from other studies,[20] some analyte concentrations differ widely. However, Besle et al. showed that well-defined diets result in variations of the phenolic acid contents in milk.[20]

In conclusion, a stable isotope dilution analysis approach based on HPLC-MS/MS was developed for sensitive, accurate, and precise determination of 15 phenolic acids (nonconjugated and conjugated) in cow's milk. One essential requirement, however, is the appropriate addition of labeled standard compounds to the samples, particularly when second-order calibration curves are used. Although only three milk samples were analyzed by using this method so far, differences among the milk samples profiles suggest that the application of the developed method on a large number of samples of known origin in combination with multivariate data analysis may help to trace back cattle forages.

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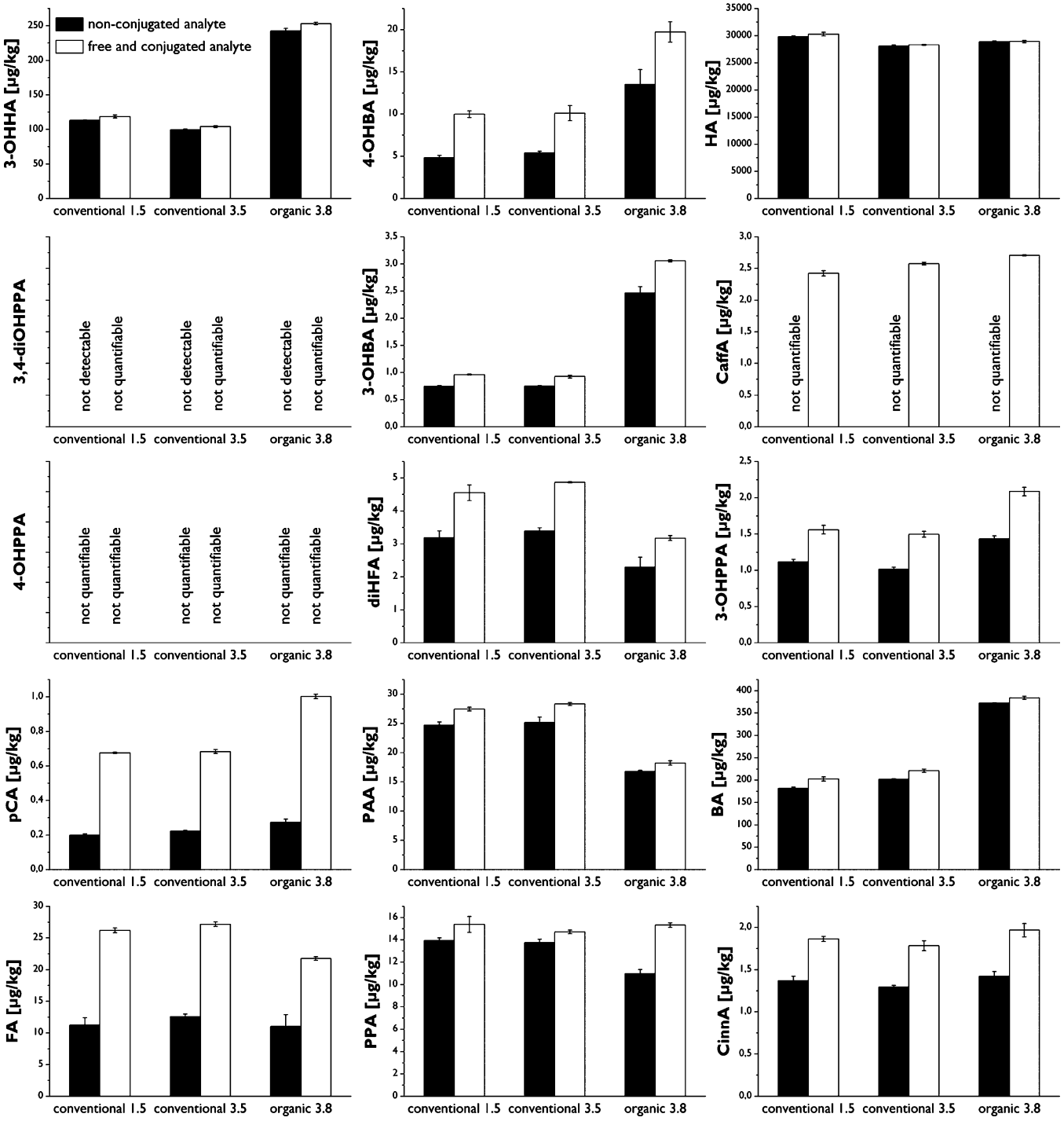

Figure 5. Concentrations of phenolic acids in conventional and organic cow milk samples with different fat contents (1.5, 3.5, or 3.8%) by LC-MS/MS. The sums of nonconjugated and conjugated analytes were measured after incubation with sulfatase and glucuronidase. Data are shown as mean ± standard deviation, $n = 3$. BA, benzoic acid; CaffA, caffeic acid; CinnA, cinnamic acid; diHFA, dihydroferulic acid; 3,4-diOHPPA, 3-(3,4-dihydroxyphenyl)propionic acid; FA, ferulic acid; HA, hippuric acid; 3-OHBA, 3-hydroxybenzoic acid; 4-OHBA, 4-hydroxybenzoic acid; 3-OHHA, 3-hydroxyhippuric acid; 3-OHPPA, 3-(3-hydroxyphenyl)propionic acid; 4-OHPPA, 3-(4-hydroxyphenyl)propionic acid; PAA, phenylacetic acid; PPA, 3-phenylpropionic acid; *p*-CA, *p*-coumaric acid.

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Notes

The authors declare no competing financial interest.

ABBREVATIONS

BA, benzoic acid; BSTFA, *N,O*-bis(trimethylsilyl)-trifluoroacetamide; CaffA, caffeic acid; CinnA, cinnamic acid; diHFA, dihydroferulic acid; 3,4-diOHPPA, 3-(3,4-

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dihydroxyphenyl)propionic acid; ESI, electrospray ionization; FA, ferulic acid; HA, hippuric acid; MS, mass spectrometry; NMR, nuclear magnetic resonance; 3-OHBA, 3-hydroxybenzoic acid; 4-OHBA, 4-hydroxybenzoic acid; 3-OHHA, 3-hydroxyhippuric acid; 3-OHPPA, 3-(3-hydroxyphenyl)propionic acid; 4-OHPPA, 3-(4-hydroxyphenyl)propionic acid; PAA, phenylacetic acid; PPA, 3-phenylpropionic acid; *p*-CA, *p*-coumaric acid

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