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Development of a Stable Isotope Dilution Analysis with Liquid Chromatography−Tandem Mass Spectrometry Detection for the Quantitative Analysis of Di- and Trihydroxybenzenes in Foods and Model Systems

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A straightforward stable isotope dilution analysis (SIDA) for the quantitative determination of the di- and trihydroxybenzenes catechol (**1**), pyrogallol (**2**), 3-methylcatechol (**3**), 4-methylcatechol (**4**), and 4-ethylcatechol (**5**) in foods by means of liquid chromatography−tandem mass spectrometry was developed. With or without sample preparation involving phenylboronyl solid phase extraction, the method allowed the quantification of the target compounds in complex matrices such as coffee beverages with quantification limits of 9 nmol/L for 4-ethylcatechol, 24 nmol/L for catechol, 3-methyl-, and 4-methylcatechol, and 31 nmol/L for pyrogallol. Recovery rates for the analytes ranged from 97 to 103%. Application of the developed SIDA to various commercial food samples showed that quantitative analysis of the target compounds is possible within 30 min and gave first quantitative data on the amounts of di- and trihydroxybenzenes in coffee beverage, coffee powder, coffee surrogate, beer, malt, roasted cocoa powder, bread crust, potato crisps, fruits, and cigarette smoke and human urine. Model precursor studies revealed the carbohydrate/amino acid systems as well as the plant polyphenols catechin and epicatechin as precursors of catechol and 5-*O*-caffeoylquinic acid, caffeic acid as a precursor of catechol and 4-ethylcatechol, and gallocatechin, epigallocatechin, and gallic acid as precursors of pyrogallol.

KEYWORDS: Catechol; pyrogallol; 3-methylcatechol; 4-methylcatechol; 4-ethylcatechol; coffee; stable isotope dilution analysis; Maillard reaction; chlorogenic acid; catechin

INTRODUCTION

Various animal feeding studies showed evidence that 1,2-dihydroxybenzene, named catechol (**1**) (**Figure 1**), initiated and promoted stomach carcinogenesis in rodents in a dose-dependent manner (*1*−*3*). In addition, in the presence of Cu(II) ions and NADH, catechol was found to induce oxidative DNA damage (*4*). As an ingredient in olive mill wastewater, recent studies revealed catechol to exhibit phytotoxic activity showing EC_{50} values ranging from 0.40 mmol/L for the monocotyledon *Sorghum bicolor* to 1.09 mmol/L for the dicotyledonous species *Cucumis sativus* (*5*).

Not only **1** but also pyrogallol (**2**), 3-methylcatechol (**3**), 4-methylcatechol (**4**), and 4-ethylcatechol (**5**) are well-known thermally generated di- and trihydroxybenzenes present in roasted coffee (*6*, *7*) and mainstream cigarette smoke (*8*). First systematic investigations revealed that such di- and trihydroxybenzenes are generated upon thermal degradation of chlorogenic acid and caffeic acid (*6*, *7*). Recent findings demonstrated for the first time that pyrogallol and catechol are also produced upon pyrolysis of mixtures of glucose and glycine by means of Maillard type reaction chemistry (*9*). Beside its thermal generation, 4-ethylcatechol is reported to be formed also as an off-flavor component during wine making as a fermentation product of caffeic acid, which is decarboxylated to 4-vinylcatechol followed by reduction by *Brettanomyces bruxellensis* and *Brettanomyces anomalus* (*10*, *11*).

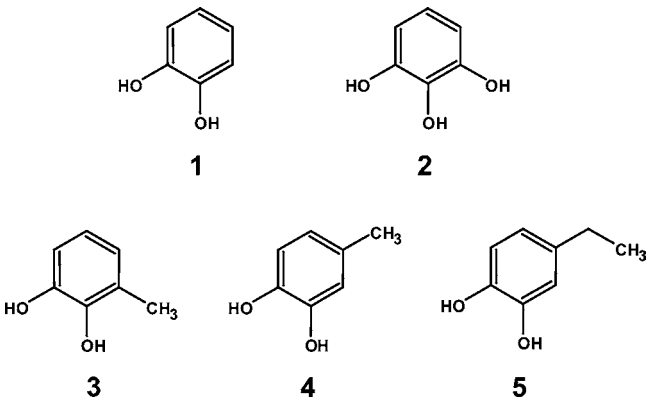

Figure 1. Chemical structures of di- and trihydroxybenzenes: catechol (**1**), pyrogallol (**2**), 3-methylcatechol (**3**), 4-methylcatechol (**4**), and 4-ethylcatechol (**5**).

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Furthermore, catechol, methylcatechols, and ethylcatechols are long-known to be enzymatically formed as metabolites upon biotransformation of the xenobiotics benzene, toluene, and ethylbenzene. It is well-accepted in the literature that the *o*-quinone, formed from the 1,2-dihydroxybenzene moiety by oxidation, is able to generate stable adducts with DNA bases, thus possibly playing a major role in tumor initiation (*12*, *13*).

Because di- and trihydoxybenzenes are discussed as potential carcinogens, various methods have been developed for the quantification of such compounds in foods and other biological samples. Aimed at quantifying catechol, hydroquinone, and phenol as metabolites of the carcinogen benzene, these phenols were analyzed in urine samples by means of time-programmed fluorescence detection using external calibration (*14*). During workup, catechol conjugates were cleaved with hot concentrated hydrochloric acid and the target compounds were separated by liquid−liquid extraction. The lack of suitable internal standards makes this quantification method vulnerable to severe losses and discrimination of the analytes when applied to more complex matrices. Very recently, high-performance capillary electrophoresis with chemiluminescence detection was used for the quantitative analysis of pyrogallol and catechol in river water (*15*). However, this methodology lacks suitable internal standards to overcome the discrimination of catechol and pyrogallol during sample extraction, cleanup, and analysis.

For catechol analysis in mainstream cigarette smoke, a high-performance liquid chromatography (HPLC) method with automated precolumn sample preparation was developed using the trihydroxybenzene pyrogallol as an internal standard. Effective separation from the matrix was achieved by first enriching catechol by means of a dihydroxyboryl-silica stationary phase, followed by elution and analysis by reversed phase (RP)-HPLC with UV detection (*16*). Because pyrogallol is also known to be thermally generated (*6*, *7*, *9*), its use as an internal standard limits the application of this method to all samples of interest. In roasted coffee, various dihydroxybenzenes have been identified by means of high-resolution gas chromatography/mass spectrometry (HR-GC/MS) and quantified using 4-*n*-propylphenol as the internal standard (*17*). The laborious and time-consuming cleanup procedure, followed by the silylation of the analytes, does, however, not allow the rapid quantification of hydroxybenzenes on a high-throughput scale. In addition, the silylation of the analytes takes place at a very late step of the workup; therefore, the recovery of the dihydroxybenzenes is expected to be rather low due to their oxidative instability (*6*). A far more rapid sample preparation was developed for the analysis of phenolic compounds in seawater (*18*). After the addition of the internal standard 2,7-dihydroxynaphthalene as well as acetic anhydride to the buffered aqueous sample, the acetates formed were enriched by means of solid phase extraction (SPE) and then analyzed by means of HR-GC/MS. As large sample volumes of more than 500 mL are needed to quantify small amounts of di- and triphenols, this laborious methodology is not suitable for high-throughput routine analysis.

Because the application of stable isotopomers of analytes is known to enable the correction of compound discrimination during extraction, cleanup, chromatographic separation, and mass spectrometry, deuterated dihydroxybenzenes are the best choice as internal standards for quantitative analysis. As a procedure for the reliable and rapid quantification of the instable, highly polar di- and trihydroxybenzenes in complex samples is not available so far, the purpose of the present investigation was to develop a versatile and reliable stable isotope dilution assay (SIDA) for the quantitative determination of catechol, pyrogallol, 3-methylcatechol, 4-methylcatechol, and 4-ethylcatechol in foods and other samples. Subsequently, this method could be applied to different food samples as well as to model systems in order to evaluate the efficiency of different natural precursors in thermal di- and trihydroxybenzene generation.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: 4-methylcatechol (98%), 3-methylcatechol (99%) (Acros Organics, Geel, Belgium), Amberlyst 15 (dry), catechol (99%), deuteriumoxide (99.9%), pyrogallol (98%), gallocatechin, catechin trihydrate, epicatechin, epigallocatechin, gallic acid, caffeic acid, 5-*O*-caffeoylquinic acid (Sigma-Aldrich, Steinheim, Germany), trifluoroacetic acid, diethyl ether, glucose, glycine, proline, methanol, $Na_2HPO_4 \times 2H_2O$, formic acid, concentrated hydrochloric acid, trichloromethane (Merck KGA, Darmstadt, Germany), 4-ethylcatechol (98%) (Lancaster, Eastgate, England), myricetin, and quercetin dihydrate (Riedel-de Haen, Seelze, Germany). Methanol was of HPLC grade, and water was Millipore grade. DMSO-d_6 was supplied by Euriso-Top (Gif-sûr-Yvette, France). Cocoa powder, malt, and roasted coffee were provided by the food industry, and the other food samples as well as the cigarettes were purchased from local retailers. Urine was pooled from three healthy male individuals.

Synthesis of Deuterium-Labeled Di- and Trihydroxybenzenes d_4-1, d_3-2, d_3-3, d_3-4, and d_3-5. Following the method described in the literature (*19*) with slight modifications, dry Amberlyst-15 catalyst resin (100 mg per 100 mg diphenol, dried 24 h in a desiccator above sulfuric acid in high vacuum) was added to a solution of the phenolic compound (2 mmol) in deuterium oxide (12 mL) in a centrifugation tube (Schott AG) under an atmosphere of nitrogen. After tightly closing the reaction vessel, the mixture was heated for 24 h in an oil bath at 110 °C with exclusion of light. The suspension was cooled to room temperature and extracted 10 times with diethyl ether (10 mL each), and the combined organic layers were then freed from solvent in a vacuum, taken up in water (10 mL), and, finally, freeze-dried. The C−H/C−D exchange was checked by MS-electrospray ionization (ESI^-) as well as ^1H NMR spectroscopy. For quantification of di- and trihydroxybenzenes, the compounds were individually dissolved in water/methanol (1/1, v/v) in concentrations of 25 mg/100 mL. The solutions were kept in the freezer (−20 °C) until use.

[3,4,5,6-$^2H_4$]-1,2-Dihydroxybenzene, d_4-Catechol, d_4-1. MS (ESI^-): *m/z* 113 (100, $[M - 1]^-$), 227 (93, $[2M - 1]^-$), 159 (90, $[M + HCOO]^-$). MS/MS *m/z* (%): 94 (17), 85 (11), 69 (6). ^1H NMR (400 MHz, DMSO-d_6, DQF-COSY): δ/ppm 8.77 (s, 2 × H, HO−$C_{1,2}$). Two residual CH signals resonating at 6.61 and 6.73 ppm, respectively, were detectable with an intensity of less than 8% each.

[4,5,6-$^2H_3$]-1,2,3-Trihydroxybenzene, d_3-Pyrogallol, d_3-2. MS (ESI^-): *m/z* 128 (100, $[M - 1]^-$), 174 (96, $[M + HCOO]^-$). MS/MS *m/z* (%): 111 (12), 109 (11), 100 (20), 84 (20), 81 (38), 72 (32), 54 (12). ^1H NMR (400 MHz, DMSO-d_6, DQF-COSY): δ/ppm 7.96 (s, 1 × H, HO−C_2), 8.69 (s, 2 × H, HO−$C_{1,3}$). Two residual signals resonating at 6.24 and 6.41 ppm, respectively, were detectable with an intensity of less that 6% each.

[4,5,6-$^2H_3$]-1,2-Dihydroxy-3-methylbenzene, d_3-3-Methylcatechol, d_3-3. MS (ESI^-): *m/z* 126 (100, $[M - 1]^-$), 172 (92, $[M + HCOO]^-$). MS/MS *m/z* (%): 111 (24), 107 (7). ^1H NMR (400 MHz, DMSO-d_6, DQF-COSY): δ/ppm 2.11 (s, 3 × H, H−C_7), 8.59 (s, 2 × H, HO−$C_{1,2}$). Three residual signals resonating at 6.40, 6.56, and 6.61, respectively, were detectable with an intensity of less than 5% each.

[3,5,6-$^2H_3$]-1,2-Dihydroxy-4-methylbenzene, d_3-4-Methylcatechol, d_3-4. MS (ESI^-): *m/z* 172 (100, $[M + HCOO]^-$), 126 (98, $[M - 1]^-$). MS/MS *m/z* (%): 111 (24), 107 (9). ^1H NMR (400 MHz, DMSO-d_6, DQF-COSY): δ/ppm 2.10 (s, 3 × H, H−C_7), 8.57 (s, 2 × H, HO−$C_{1,2}$). One residual signal resonating at 6.57 ppm was detectable with an intensity of 35%.

[3,5,6-$^2H_3$]-1,2-Dihydroxy-4-ethylbenzene, d_3-4-Ethylcatechol, d_3-5. MS (ESI^-): *m/z* 186 (100, $[M + HCOO]^-$), 140 (93, $[M - 1]^-$). MS/MS *m/z* (%): 125 (67). ^1H NMR (400 MHz, DMSO-d_6, DQF-COSY): δ/ppm 1.09 (t, 3 × H, J = 7.58 Hz, H−C_8), 2.40 (q, 2 × H, J = 7.58 Hz, H−C_7), 8.581 (s, 2 × H, HO−$C_{1,2}$). Three signals

resonating at 6.43, 6.57, and 6.62 ppm, respectively, were detectable with an intensity of less than 1% each.

Sample Preparation for Quantification of Di- and Trihydroxybenzenes. Beer samples and powdered materials were used for analysis without further preparation. Roasted cocoa nibs and malt samples were frozen in liquid nitrogen and ground with a kitchen mill. Potato crisps were powdered in a mortar. Bread crust was separated from the bread with a kitchen knife, frozen in liquid nitrogen, and ground in a kitchen mill. Apples, peaches, and mangos were washed, freed from the kernel, and cut to pieces. Coffee beverages were prepared by percolating coffee powder (54 g/L) placed in a size 4 cellulose filter (Melitta, Germany) with boiling water (1.1 L), collecting the coffee brew and cooling to room temperature in an ice bath.

Quantification of Di- and Trihydroxybenzenes in Foods. *Liquid Samples. Method A.* The liquid sample (25 mL) was transferred into a 80 mL centrifugation tube with a screw cap (Schott AG, Mainz, Germany), defined amounts of the internal standards d_4-**1**, d_3-**2**, d_3-**3**, d_3-**4**, and d_3-**5** were added, and after closing the tube, the sample was equilibrated for 20 min at room temperature under an atmosphere of nitrogen while stirring. The solution was extracted with diethyl ether (25 mL) and centrifuged (5 min, 3000 rpm), and the clear supernatant was freed from solvent in a vacuum, and the residue was taken up in a mixture (2/8, v/v; 1 mL) of methanol and phosphate buffer (10 mM; pH 8.5). This solution was applied onto a 500 mg Bond Elut-PBA phenylboronyl SPE cartridge (Varian, Darmstadt, Germany), which had been pretreated with phosphate buffer (10 mM; pH 8.5; 5 mL). After the cartridge was rinsed with a mixture (1/1, v/v; 5 mL) of methanol and phosphate buffer (10 mM; pH 8.5), the analytes were eluted with a methanol/water mixture (2/8, v/v; 5 mL) containing 0.25% trifluoroacetic acid. The eluate was extracted with diethyl ether (5 mL), and after the solvent was removed in a vacuum, the residue was taken up in methanol/water (1/1, v/v; 500 μL) and membrane filtered (0.45 μm), and aliquots (5 μL) were analyzed by means of liquid chromatography−tandem mass spectrometry (LC-MS/MS).

Method B. Following closely the procedure of method A, the equilibrated sample spiked with the internal standards was extracted with diethyl ether (25 mL) and centrifuged (3000 rpm, 5 min). The organic supernatant was freed from solvent in a vacuum, taken up in methanol/water (1/1, v/v; 1 mL), and membrane filtered (0.45 μm), and aliquots (5 μL) were analyzed by LC-MS/MS.

Fruits. Fresh-cut fruit samples (100 g) were crushed in an aqueous saturated calcium chloride solution (40 mL) spiked with defined amounts of the internal standards d_4-**1**, d_3-**2**, d_3-**3**, d_3-**4**, and d_3-**5**, by means of a kitchen mixer. After equilibration for 20 min at room temperature, the mixture was extracted with diethyl ether (150 mL) and centrifuged (20 min, 5000 rpm, 4 °C) in a 500 mL centrifugation cup (Kendro Laboratory Products, Langenselbold, Germany). The organic supernatant was freed from solvent in a vacuum, the residue was taken up in methanol/water (1/1, v/v; 2 mL) and membrane filtered (0.45 μm), and aliquots (5 μL) were analyzed by LC-MS/MS.

Coffee Beans, Bread Crust, Cocoa, Potato Crisps, Malt, and Coffee Surrogate. The ground sample (2 g) was maintained in a centrifugation tube, a mixture of methanol and water (70/30, v/v; 25 mL), and the internal standards d_4-**1**, d_3-**2**, d_3-**3**, d_3-**4**, and d_3-**5** were added. After the tube was closed, the mixture was stirred for 20 min at room temperature and then heated for 10 min at 70 °C. After it was cooled to room temperature, the mixture was diluted with water (150 mL), filtered, and then extracted with diethyl ether (180 mL). The organic layer was freed from solvent in a vacuum, the residue was taken up in methanol/water (1/1, v/v; 1 mL) and membrane filtered (0.45 μm), and aliquots (5 μL) were analyzed by LC-MS/MS.

Quantification of Di- and Trihydroxybenzene in Cigarette Smoke. Under reduced pressure, the smoke produced by a lighted cigarette was sucked through a round-bottom flask (500 mL) containing trichloromethane (200 mL) until 0.5 cm of the cigarette was left. The solution was removed from the trap, the solvent was evaporated, and the residue was taken up in methanol (10 mL). An aliquot of the solution (1 mL) was spiked with the internal standards d_4-**1**, d_3-**2**, d_3-**3**, d_3-**4**, and d_3-**5**, diluted with water (100 mL), and, after equilibration (20 min), was extracted with diethyl ether (100 mL). The organic layer was freed from solvent in a vacuum, and the residue was taken up in methanol/water (1/1, v/v; 1 mL) prior to analysis by LC-MS/MS.

Table 1. Monitored Mass Transitions, MS/MS Parameters, and Retention Times of Di- and Trihydroxybenzenes and Deuterated Internal Standards

compound	MW (Da)	mass transition (*m*/*z*)	DP	CE	CXP	RT[a] (min)
catechol (**1**)	110	109 → 81	−76	−30	−13	13.03
		109 → 91[b]				
d_4-catechol (d_4-**1**)	114	113 → 85	−76	−30	−13	12.62
		113 → 94[b]				
pyrogallol (**2**)	126	125 → 69[b]	−76	−20	−15	5.52
		125 → 97				
d_3-pyrogallol (d_3-**2**)	129	128 → 72[b]	−76	−20	−15	5.34
		128 → 100				
3-methylcatechol (**3**)	124	123 → 108[b]	−78	−28	−15	20.08
d_3-3-methylcatechol (d_3-**3**)	127	126 → 111[b]	−78	−28	−15	19.98
4-methylcatechol (**4**)	124	123 → 108[b]	−78	−28	−15	19.50
d_3-4-methylcatechol (d_3-**4**)	127	126 → 111[b]	−78	−28	−15	19.42
4-ethylcatechol (**5**)	138	137 → 122[b]	−78	−26	−11	21.33
d_3-4-ethylcatechol (d_3-**5**)	141	140 → 125[b]	−78	−26	−11	21.29

[a] Retention time. [b] Mass transition used for quantitative analysis.

Quantification of Di- and Trihydroxybenzene in Human Urine. Following a workup procedure reported in the literature (*14*), concentrated hydrochloric acid (100 μL) was added to an aliquot (500 μL) of pooled human urine and was then heated in a boiling water bath for 90 min. After the mixture was cooled to room temperature, the internal standards d_4-**1**, d_3-**2**, d_3-**3**, d_3-**4**, and d_3-**5** and water (2 mL) were added, and after equilibration for 20 min, the solution was extracted with diethyl ether (5 mL). The organic layer was freed from solvent, and the residue was taken up in methanol (200 μL) and then analyzed by LC-MS/MS.

Quantification of Di- and Trihydroxybenzenes in Model Mixtures. *Carbohydrate/Amino Acid Mixtures.* Carbohydrate (10 mmoL) and the amino acid (10 mmoL) were intimately mixed in a mortar, placed into a glass beaker (400 mL), and thermally treated in a lab oven at 200 °C for 10 and 30 min, respectively. After the mixture was cooled to room temperature, water (200 mL, 40 °C) and the internal standards d_4-**1**, d_3-**2**, d_3-**3**, d_3-**4**, and d_3-**5** were added, and the mixture was stirred for equilibration (20 min) and then filtered through a plug of glass wool. After extraction with diethyl ether (200 mL), the organic layer was freed from solvent, the residue was taken up in methanol/water (1/1, v/v; 1 mL) and membrane filtered, and an aliquot (5 μL) was analyzed by LC-MS/MS.

Polyphenols. Quercetin, myricetin, catechin, gallocatechin, epicatechin, epigallocatechin, gallic acid, 5-*O*-caffeoylquinic acid, and caffeic acid (0.01−0.1 mmol each), respectively, were placed in a glass vial and heated in a lab oven at 200 °C for 30 min. After it was cooled to room temperature, the mixture was taken up in methanol/water (1/1, v/v; 2 mL), diluted with water (10 mL), and spiked with the internal standards d_4-**1**, d_3-**2**, d_3-**3**, d_3-**4**, and d_3-**5**. After equilibration for 20 min with stirring, the solution was extracted with diethyl ether (10 mL), the organic layer was separated and freed from solvent in a vacuum, the residue was taken up in methanol (200 μL) and membrane filtered, and an aliquot (5 μL) was analyzed by LC-MS/MS.

HPLC-MS/MS. The Agilent 1100 Series HPLC-system consisted of a pump, a degasser, and an autosampler (Agilent, Waldbronn, Germany) and was connected to a 4000 Q Trap triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems/MDS Sciex, Darmstadt) with an ESI device running in negative ionization mode. The nebulizer gas was zero-grade air, and it was set at 45 psi, while the curtain gas was nitrogen, which was set at 35 psi. Detection was performed in multiple reaction monitoring (MRM) mode, recording the transition from the negative pseudo-molecular ion $[M - H]^-$ to the fragment after collision-induced dissociation. The declustering potential (DP), the cell exit potential (CXP), and the collision energy (CE) were set as detailed in **Table 1**. The dwell time for each mass

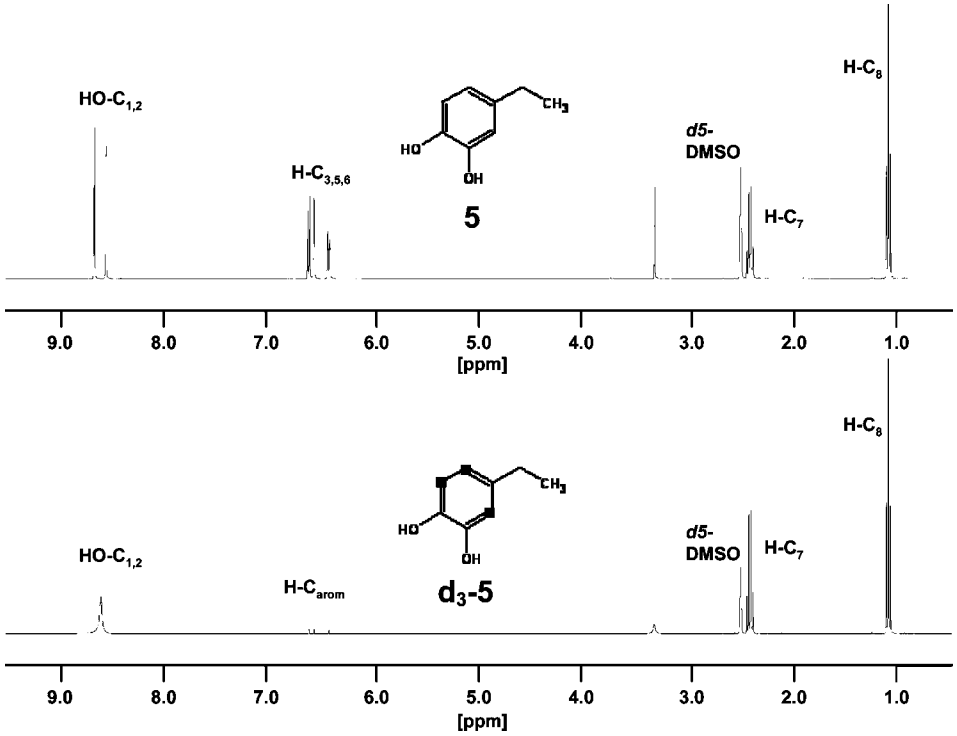

Figure 2. ^{1}H NMR spectra (400 MHz; DMSO-d_6) of (**A**) 4-ethylcatechol (**5**) and (**B**) 3,5,6-$^2H_3$-4-ethylcatechol (d_3-**5**).

transition was 80 ms. The quadrupoles operated at unit mass resolution. For instrumentation control and data collection, Sciex Analyst software (v1.4) was used. Only the mass transitions of the fully labeled internal standards and the nonlabeled analytes were monitored. After sample injection (5 μL), chromatographic separation was carried out on a 150 mm × 2.5 mm i.d., 4 μm, Luna Phenyl-Hexyl column (Phenomenex, Aschaffenburg, Germany) with gradient elution at a flow rate of 0.25 mL/min. Eluent A was 0.5% formic acid in methanol, and eluent B was 0.5% formic acid in water. For chromatography, eluent A was held at 2% for 3 min, then increased linearly to 15% within 7 min, then to 100% within additional 10 min, followed by isocratic elution with 100% for 9 min.

Calibration. Solutions of the deuterated standards and the analytes were prepared in five mass ratios from 0.2 to 5, and analysis was performed. Calibration curves were prepared by plotting peak area ratios of analyte to internal standard against concentration ratios of each analyte to the internal standard using linear regression.

Recovery. Experiments were performed using coffee sample B. Freshly prepared coffee beverage (25 mL) was spiked with known amounts of catechol, pyrogallol, 3-methylcatechol, 4-methylcatechol, and 5-ethylcatechol dissolved in methanol. The mixture was stirred for 20 min for equilibration followed by diethyl ether extraction and quantitative analysis of the di- and trihydroxybenzenes as detailed above. As the basis for the calculation of the recovery rate, the initial concentration of the analytes in unspiked coffee beverage (control) was employed ($n = 5$).

Limits of Quantification. Experiments were performed using coffee sample B. Freshly prepared coffee beverage (25 mL) was spiked with increasing amounts of d_4-**1**, d_3-**2**, d_3-**3**, d_3-**4**, and d_3-**5** and analyzed in triplicate as detailed above. The limit of quantification is the concentration calculated from the maximum height of the 95% confidence interval at the zero addition level and was calculated according to the literature (*20*).

NMR Spectrometry. ^{1}H and COSY experiments were performed on a Bruker AMX-400 instrument (Bruker, Rheinstetten, Germany). Chemical shifts are related to d_5-DMSO (quintet at 2.49 ppm relative to tetramethylsilane).

RESULTS AND DISCUSSION

To accurately determine the amounts of di- and trihydroxybenzenes in foods and model systems, a SIDA with LC-MS/MS detection should be developed. To achieve this, first, corresponding labeled internal standards needed to be synthesized.

Synthesis of Deuterated Di- and Trihydroxybenzenes. For the synthesis of stable isotope-labeled di- and trihydroxybenzenes by means of protium/deuterium exchange, solutions of catechol, pyrogallol, 3-methylcatechol, 4-methylcatechol, and 4-ethylcatechol, respectively, in deuterium oxide were heated in the presence of dry Amberlyst 15 as a catalyst for 24 h under an atmosphere of nitrogen. After cooling to room temperature, the mixtures were extracted with diethyl ether, and after evaporation of the solvent in a vacuum, the residues were taken up in water to selectively reexchange the oxygen-bound deuterium atoms in the deuterated target molecules. After freeze drying, mass spectrometry and ^{1}H NMR spectroscopy confirmed the incorporation of deuterium into the molecule yielding d_4-catechol (d_4-**1**), d_3-pyrogallol (d_3-**2**), d_3-3-methylcatechol (d_3-**3**), d_3-4-methylcatechol (d_3-**4**), and d_3-4-ethylcatechol (d_3-**5**) fully labeled at the aromatic core. The ^{1}H NMR spectra of d_4-**1**, d_3-**2**, d_3-**3**, and d_3-**5**, respectively, obtained in d_6-DMSO, showed just minor amounts of residual carbon-bound protons resonating as singlets, thus demonstrating that neither nonlabeled nor singly deuterated phenol was present. As exemplified in **Figure 2** for d_3-**5**, the ^{1}H NMR spectrum obtained for 4-ethylcatechol after H/D exchange exhibited the proton signals resonating as a triplet at 1.09 ppm and as a quartet as 2.40 ppm, respectively. The three proton signals detected between 6.43 and 6.62 ppm showed strongly decreased intensity, and the resonances of both of the hydroxyl groups showed coalescence as a broad signal at 8.58 ppm. For d_3-**4**, a residual singlet with an intensity of 35% indicated that beside 65% of trideuterated 4-methylcatechol about 35% of dideuterated 4-methylcatechol was present. Because neither nonlabeled nor singly labeled 4-methylcatechol was detectable, the calibration of 4-methylcatechol for quantitative LC-MS/MS was achieved using the masses of nonlabeled and fully labeled 4-methylcatechol.

To check the stability of the deuterated compounds under aqueous acidic conditions, the labeled standards were individu-

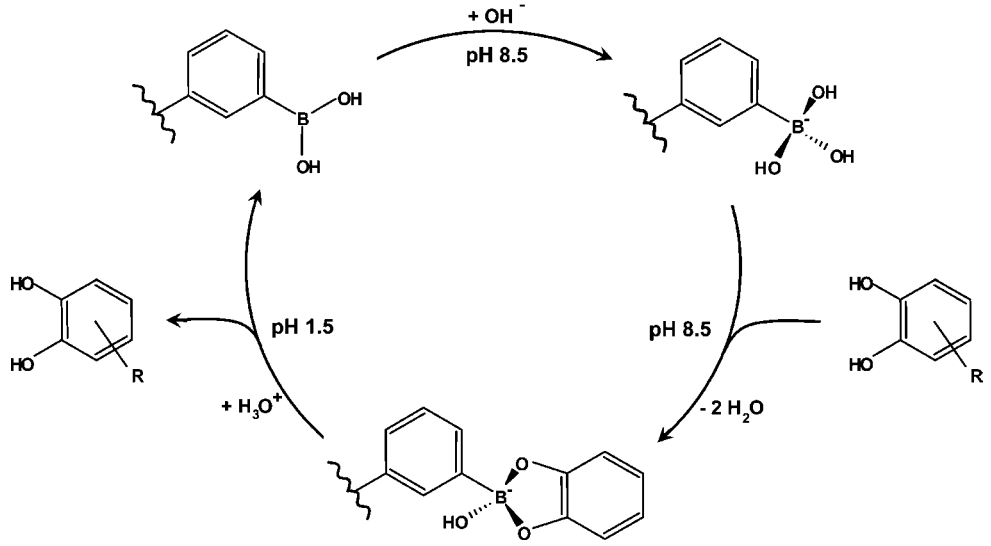

Figure 3. Enrichment of *cis*-diol functionalized benzenes by immobilized phenylboronic acid.

ally incubated in H_2O acidified with HCl to pH 4.0 for 4 h at room temperature and, after lyophilization, were then analyzed by ^{1}H NMR spectroscopy. An increase of the aromatic protons was still not observable for the labeled compound (data not shown), thus demonstrating the lack of any significant C−D/C−H exchange under cleanup conditions and confirming its suitability as internal standards for the SIDA.

Development of a SIDA and Quantitation of Di- and Trihydroxybenzenes in Coffee. To analyze the target compounds with high selectivity by using the MRM mode, repeated manual sample injections were done to determine the pseudomolecular precursor and daughter ions in full scan mode or in product ion scan mode in the range of 50−150 amu, respectively. Using flow injection and the automatic tuning option of the software, the settings were optimized between −76 and −78 for the DP, between −26 and −30 for the collision potential, and between −11 and −15 for the CXP, thus enabling the maximization of the product ion intensity (**Table 1**).

To convert the measured ion intensities into the mass ratios of labeled and nonlabeled di/trihydroxybenzenes, a graph was calculated from calibration mixtures of known mass ratios and the corresponding peak area ratios in HPLC-MS/MS. Good linearity was found for mass ratios ranging from 0.2 to 5.0.

For the quantitative analysis of the di- and trihydroxybenzenes by stable isotope dilution analysis, coffee beverage was spiked with defined amounts of d_4-**1**, d_3-**2**, d_3-**3**, d_3-**4**, and d_3-**5** as the internal standards, followed by equilibration at room temperature and diethyl ether extraction. After solvent removal, di- and trihydroxybenzenes were isolated from the residue by means of SPE using a phenylboronyl cartridge conditioned with phosphate buffer at pH 8.5 (**Figure 3**). After flushing the cartridge with trifluoroacetic acid in aqueous methanol, the effluent was extracted with diethyl ether, and the organic extract was freed from solvent in a vacuum and, finally, analyzed by HPLC-MS/MS on a phenylhexyl phase. The results, given in **Table 2**, show that the highest concentrations of 5485 and 4169 μg/L were found for catechol and pyrogallol, followed by 3-methylcatechol and 4-ethylcatechol present in concentrations of 1069 and 1299 μg/L, respectively. As compared to catechol, 4-methylcatechol was present in 12-fold lower concentrations in coffee beverage.

To shorten the procedure of sample workup and to speed up the overall analysis time, the same coffee sample was analyzed with the stable isotope dilution analysis omitting the SPE.

Table 2. Influence of the SPE Cleanup on the Quantification of Di- and Trihydroxybenzenes in Coffee Beverages[a]

	concentration[d] (μg/L) of				
method	**1**	**2**	**3**	**4**	**5**
with SPE[b]	5485 (±73)	4169 (±72)	1069 (±19)	402 (±11)	1299 (±8)
w/o SPE[c]	5480 (±42)	3999 (±109)	1138 (±13)	407 (±19)	1317 (±46)

[a] The coffee beverage (54 g/L) was prepared from a 100% Arabica Brazil Santos. [b] HPLC-MS/MS analysis was performed after cleanup by means of phenylboronyl SPE (cf. **Figure 3**). [c] HPLC-MS/MS analysis was performed without SPE cleanup. [d] Concentrations are given as the means of five replicates (standard deviation).

HPLC-MS/MS analysis showed that even without the cleanup on phenylboronyl SPE, the mass chromatograms obtained for the individual analytes and internal standards looked rather clean (**Figure 4**). Also, the results given in **Table 2** show that the quantitative data obtained from this analysis did not differ strongly from the data obtained after phenylboronyl cleanup. Therefore, the developed SIDA was used for the analysis of various food samples without any further SPE cleanup.

To check the accuracy of the analytical method without SPE cleanup, recovery experiments were performed in the following. Reference material of the di- and trihydroxycatechols **1**−**5** was added to a freshly prepared coffee beverage in four different concentrations each prior to quantitative analysis, and the amounts determined after workup were compared with those found in the blank coffee sample (control). The recovery rates, calculated on the basis of the content of each di- and trihydroxybenzene added to the coffee beverage prior to workup, were found to be 97.3% for compound **1**, 97.5% for compound **2**, 100.2% for compound **3**, 101.3% for compound **4**, and 103.0% for compound **5**. These data clearly demonstrate the developed SIDA as a reliable tool enabling a rapid and accurate quantitative determination of di- and trihydroxybenzenes in foods.

In addition, the limits of quantification were determined in coffee beverages as the concentration at which the peak height of the internal standard was at least three times higher than the underground noise. To achieve this, the deuterium-labeled internal standards were added to the coffee beverages in different concentrations. With the exception of **5**, which showed a very low quantification limit of 9 nmol/L, the quantification limits of the compounds **1**−**4** were rather comparable, 24 nmol/L for compounds **1**, **3**, and **4** and 31 nmol/L for **2**.

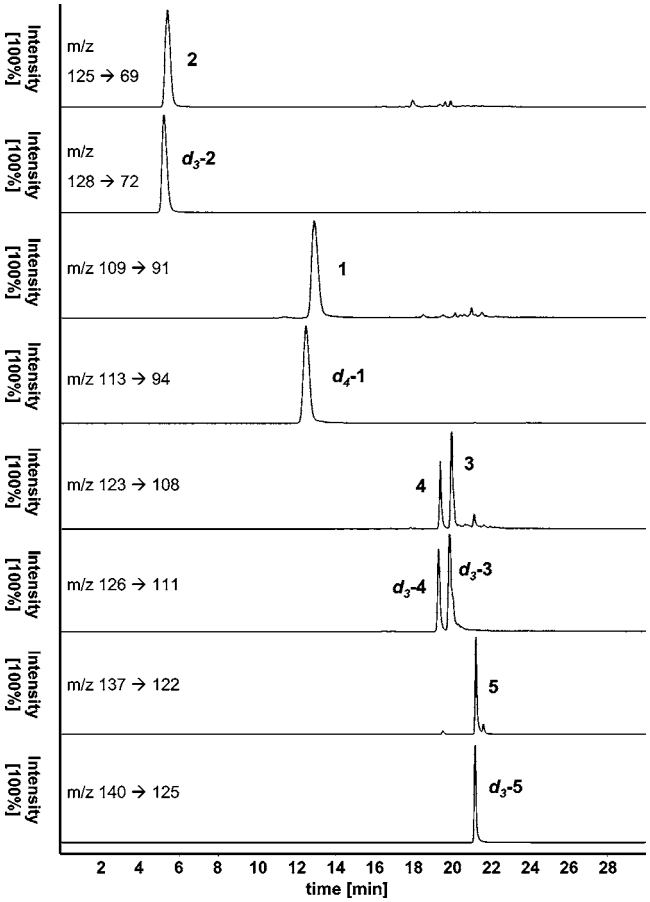

Figure 4. MS/MS chromatograms for the quantitative analysis of the di- and trihydroxybenzenes **1**–**5** in coffee beverage via the internal standards d_4-**1**, d_3-**2**, d_3-**3**, d_3-**4**, and d_3-**5** by using the MRM mode.

Applications of the SIDA for the Quantitative Analysis of Di- and Trihydroxybenzenes. We used the developed SIDA for the quantification of compounds **1**−**5** in various liquid and solid foods as well as fruits, cigarette smoke, and human urine. The workup needed to be slightly adjusted depending on the samples. Solid samples, spiked with the internal standards, were homogenized in aqueous methanol, and after filtration, diethyl ether extraction, and solvent evaporation, the obtained extracts were analyzed by means of HPLC-MS/MS. For the quantification of compounds **1**−**5** in fruits, the edible parts of the fruit were homogenized in saturated calcium chloride prior to extraction in order to inhibit polyphenoloxidase activity. According to the literature (*14*), human urine samples were thermally pretreated with hydrochloric acid to release di- and trihydroxybenzenes from conjugates prior to analysis. Cigarette smoke was trapped in trichloromethane, and after the solvent was removed in a vacuum, the residue was used for analysis.

Analysis of di- and trihydroxybenzenes in three different coffee beverages, a dark beer, and a pale beer revealed the highest concentrations in the coffee samples (**Table 3**). Independent of the coffee sample analyzed, catechol and pyrogallol were found as the predominant compounds with concentrations ranging from 3490 to 7091 and from 4543 to 7811 μg/L, respectively, whereas 4-methylcatechol was detected as the minor component in concentrations between 272 and 503 μg/L. In comparison, both beer samples contained just minor amounts of di- and trihydroxybenzenes (**Table 3**). In contrast to the coffee samples, the concentrations of pyrogallol exceeded those of catechol by a factor of more than three, thus suggesting different precursors for their generation in coffee and beer.

Table 3. Concentrations of Di- and Trihydroxybenzenes in Samples

	concentrations[a] (μg/kg) of				
sample	**1**	**2**	**3**	**4**	**5**
		liquid foods[b]			
coffee A	7091	7811	1189	503	1691
coffee B	5480	3999	1138	407	1317
	(±42)	(±109)	(±13)	(±19)	(±46)
coffee C	3490	4543	802	272	910
dark beer	140	314	29	22	10
pale beer	11	47	1	ND	6
		solid foods[c]			
coffee B (powder)	119351	84023	20657	7766	27954
coffee surrogate	18186	1760	5039	3111	1489
malt, roasted[d]	5649	21512	1073	614	50
malt, unroasted	153	887	48	11	ND
bread crust	1373	5832	277	112	33
cocoa powder	1271	1857	127	98	18
potato crisps	42	126	ND	ND	4
		fruits[e]			
peach	5	ND	ND	ND	615
apple	16	ND	ND	ND	ND
mango	13	ND	ND	ND	5
		miscellaneous			
cigarette smoke[f]	165	3	22	3	35
human urine[g]	16	72	<1	1	1

[a] Concentrations are given as the means of duplicates. [b] Concentrations are given on a liquid basis. [c] Concentration is given on the basis of fresh weight. [d] Malt was roasted for 20 min at 200 °C. [e] Concentration is related to dry matter of edible parts. [f] Concentration in smoke is given in μg per cigarette. [g] Concentration is given in μg/mL pooled human urine; ND, not detectable.

Comparison of both beer samples showed that catechol and pyrogallol were present in 13- and 7-fold higher concentrations than in pale beer. In contrast to dark beer, 3-methylcatechol was only present in trace amounts and 4-methylcatechol was not detectable at all in pale beer (**Table 3**).

Quantitative determination of compounds **1**−**5** in various thermally processed solid foods revealed by far the highest concentrations in roasted coffee beans (coffee sample B). Concentrations of catechol and pyrogallol were 119.4 and 84.0 mg/kg, respectively. In comparison, 3-methylcatechol and 4-ethylcatechol were present in lower concentrations of 20.7 and 27.9 mg/kg, respectively, whereas just 7.8 mg/kg was found for 4-methylcatechol. These data showed that when preparing the beverage by percolating coffee powder B with hot water, the di- and trihydroxybenzenes are close to quantitatively extracted into the beverage, e.g., the contents of compounds **1**, **2**, and **5** in the beverage ranged between 85 and 88% and were quantitative (102 and 97%) for the methylcatechols **3** and **4**. The coffee surrogate made from a mixture of roasted barley, roasted chicory, and roasted wheat contained catechol in concentrations of only 18.2 mg/kg but showed higher concentrations for 3-methylcatechol (5.0 mg/kg) than for pyrogallol (1.7 mg/kg). In contrast to coffee surrogate but similar to coffee, malt roasted for 20 min at 200 °C showed predominant amounts of pyrogallol (21.5 mg/kg). These data clearly indicate the existence of different precursors for the di- and trihydroxybenzenes in these foods. Significantly lower levels of di- and trihydroxybenzenes were found in cocoa powder and dark bread crust, e.g., the concentration of catechol and pyrogallol was 100 and 20 times below those found in roasted coffee (**Table 3**). Comparatively low amounts of catechol type compounds were present in potato crisps.

As compared to the contents of di- and trihydroxybenzenes found in the thermally processed foods above, fruits such as peach, apple, and mango contained only low amounts of catechol

Table 4. Model Experiments on the Thermal Generation of Di- and Trihydroxybenzenes

	amount (μmol/mol) formed				
precursor	**1**	**2**	**3**	**4**	**5**
caffeic acid[a]	563	ND	ND	ND	1831
chlorogenic acid[a]	46	ND	ND	ND	26
gallic acid[a]	ND	109	ND	ND	ND
catechin[a]	2291	ND	ND	ND	ND
epicatechin[a]	86	ND	ND	ND	ND
gallocatechin[a]	ND	2040	ND	ND	ND
epigallocatechin[a]	ND	127	ND	ND	81
quercetin[a]	ND	ND	ND	ND	ND
myricetin[a]	ND	ND	ND	ND	ND
glucose[a]	ND	ND	ND	ND	ND
glucose/glycine[b]	10	7	5	ND	ND
glucose/proline[b]	42	5	7	2	1

[a] Precursor (0.1–0.01 mmol) was heated at 200 °C for 30 min in a glass vial. [b] Glucose and amino acid (10 mmol each) were intimately mixed and then heated at 200 °C for 30 min; ND, not detectable.

and 4-ethylcatechol whereas pyrogallol and 3- and 4-methylcatechol could not be detected (**Table 3**). It is interesting to note that only peaches, with regard to the other analyzed fruit samples, showed a relatively high concentration in 4-ethylcatechol with 615 μg/kg dry matter.

In addition, the amounts of di- and trihydroxybenzenes were determined in cigarette mainstream smoke. The data given in **Table 3** show that the overall concentration of compounds **1**−**5** in the smoke trapped from a single cigarette is in the same range as found for 1 L of dark beer. In contrast to beer, catechol, 3-methylcatechol, and 4-ethylcatechol were found to be the quantitatively predominating compounds in cigarette smoke.

Finally, the suitability of the SIDA for the quantitative analysis of catechols in urine samples should be demonstrated. As an example, compounds **1**−**5** were quantified in a pooled human urine sample (**Table 3**). While the alkylcatechols were detected in concentrations from 0.47 up to 1.05 μg/mL, catechol and pyrogallol were present in significantly higher amounts of 15.59 and 72.14 μg/mL.

Because the absolute amounts as well as the ratios between the individual di- and trihydroxybenzenes in the different samples investigated are rather different, different precursors leading to these catechols and pyrogallol upon thermal processing have to be taken into account. To investigate the potential of thermal polyphenol degradation (*6*, *7*) as well as Maillard type formation from carbohydrates and amino acids (*9*) as precursor systems in generating di- and trihydroxybenzenes, model precursor experiments have been done as follows.

Model Precursor Studies. To gain first insight into the generation of di- and trihydroxybenzenes from natural precursor systems, caffeic acid, 5-*O*-caffeoylquinic acid (chlorogenic acid), the flavan-3-ols catechin, epicatechin, gallocatechin, and epigallocatechin, the flavon-3-ols quercetin and myricetin, and the Maillard mixtures D-glucose/glycine and D-glucose/L-proline were dry-heated for 30 min at 200 °C, and the concentrations of the compounds **1**−**5** were determined by means of the SIDA as given in **Table 4**.

In accordance with literature data (*6*), caffeic acid was found to generate exclusively **5** and **1** with high yields of 1831 and 563 μmol/mol, respectively. 5-*O*-Caffeoylquinic acid produced the diphenols **1** and **5** as well but in drastically lower yields of just 46 and 26 μmol/mol, respectively. In contrast, gallic acid was identified to produce exclusively pyrogallol in amounts of 109 μmol/mol.

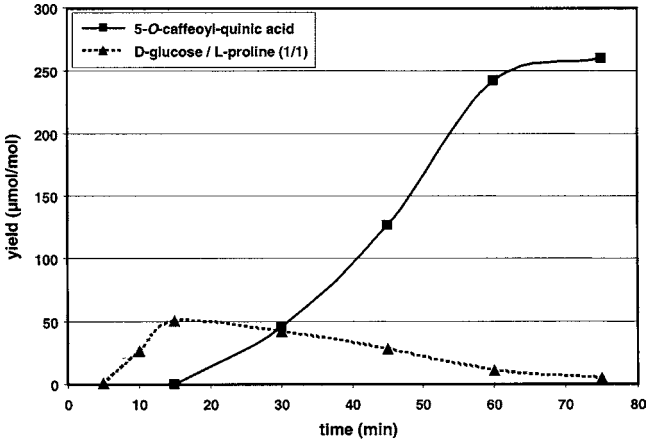

Figure 5. Influence of the reaction time on the generation of catechol (**1**) from 5-*O*-caffeoylquinic acid and D-glucose/L-proline, respectively, at 200 °C.

Thermal treatment of the flavan-3-ols catechin and gallocatechin containing a catechol and a pyrogallol substructure, respectively, resulted in the release of dihydroxybenzene **1** and trihydroxybenzene **2** in rather high yields of 2291 and 2040 μmol/mol (**Table 4**). Interestingly, their diastereomers epicatechin and epigallocatechin with 86 and 127 μmol/mol generated, by far, smaller amounts of **1** and **2**, respectively, and epigallocatechin was found to produce also **5** in yields of 81 μmol/mol. As compared to the flavan-3-ols, the flavon-3-ols, quercetin and myricetin, proved to be far more stable and did not generate any of the di- and trihydroxybenzenes.

In addition, the precursor potential of carbohydrate and amino acids were investigated employing the SIDA (**Table 4**). While roasting glucose alone did not result in the generation of any di- and trihydroxybenzenes, heating equimolar mixtures of D-glucose in the presence of the primary amino acid glycine or the secondary amino acid L-proline induced the production of the target phenols. Independent from the amino acid used, catechol was formed as the predominant diphenol, but the combination glucose/proline was about four times more efficient in generating compound **1** than glucose/glycine. In contrast to catechol, the other phenols were formed in far lower yields. Comparing the phenols produced from the various precursors investigated revealed that 3-methylcatechol seems to be exclusively formed by Maillard type reactions from carbohydrates and amino acids.

Finally, the time-dependent generation of catechol from D-glucose/L-proline mixtures and 5-*O*-caffeoylquinic acid, respectively, was investigated quantitatively. The precursors were heated at 200 °C from 5 up to 75 min, and the concentrations of catechol were determined by means of the SIDA (**Figure 5**). Whereas catechol production from the Maillard system went through a maximum after 15 min, the generation of catechol from 5-*O*-caffeoylquinic acid was favored with increasing the reaction time up to 75 min. These data give evidence that polyphenols might be the key precursors in di- and trihydroxyphenol production in intensely thermally processed food products, whereas Maillard type reactions between reducing carbohydrates and amino acids might play a more important role in phenol generation in short time-heated food products.

In summary, a versatile analytical method enabling an accurate quantitative analysis of di- and trihydroxybenzenes in foods as well as biological samples by means of stable isotope dilution analysis using HPLC-MS/MS was developed, thus

opening the possibility to study the influence of processing technologies on the formation of these compounds in foods.

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