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Simultaneous determination of hydroxycinnamates and catechins in human urine samples by column switching liquid chromatography coupled to atmospheric pressure chemical ionization mass spectrometry

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Abstract

A quantitative liquid chromatography mass spectrometry (LC–MS) methodology with online sample clean up by column switching is described for the simultaneous determination of the hydroxycinnamates, caffeic acid and chlorogenic acid, and of the catechins, epicatechin and catechin in human urine samples. Enzymatically treated urine samples were directly injected onto the LC–MS system, where sample clean up was performed by a reversed-phase Zorbax 300SB C_3 column and selective elution of the target compounds onto a Zorbax SB C_{18} column resulted in final separation prior to detection by atmospheric pressure chemical ionization (APCI) MS using single ion monitoring (SIM) in negative mode. Linear calibration graphs were achieved in the dynamic range of 10–1000 ng/ml urine. The inter- and intraassay coefficients of variation (C.V.%) for the analysis of the four compounds in quality control urine samples were between 7.8 and 10.9, $n=17$ (reproducibility), and the repeatability of the assay was between 2.5 and 5.0% ($n=12$). Analyses of urine samples from a human dietary intervention study with intake of 600 g of fruits and vegetables were demonstrated. To our knowledge, this is the first method described that allows simultaneous determination of both hydroxycinnamates and catechins in biological samples.

Keywords: Column switching; Hydroxycinnamates; Catechins

1. Introduction

Phenolic compounds form a substantial part of foods of plant origin [1]. Catechins and hydroxycinnamates are two groups of very hydrophilic phenols with antioxidative properties, that have been shown to have antiatherogenic and anticarcinogenic properties [2–6]. Catechins are the major polyphenols found in tea and normally comprise more than 75% of the tea polyphenols, which is about 30% of the dry weight of green tea and 9% of black tea [7,8]. Also other foods and beverages contain catechins, such as red wine, and to a lesser extent, white

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wine, chocolate, fruits, especially apples and pears, whereas coffee and beer contain no catechins. The major catechins in foods are epicatechin and catechin, whereas tea additionally contains epigallocatechin, epigallocatechin gallate and epicatechin gallate [9].

Hydroxycinnamic acids are among the most widely distributed phenylpropanoids in the plant kingdom, with fruits, vegetables, beverages, and cereals being abundant dietary sources [10]. The major representative of hydroxycinnamic acids is caffeic acid, which occurs in foods mainly as an ester with quinic acid called chlorogenic acid (5-caffeoylquinic acid) (Fig. 1). Coffee is a major source of chlorogenic acid in the human diet; daily intake in coffee drinkers is 0.5–1 g; coffee abstainers will usually ingest 100 mg/d. Other dietary sources of chlorogenic acid include apples, pears, berries, artichoke and aubergines [11]. Caffeic acid has been identified as one of the active antioxidant constituents of red wine showing a dose-dependent ability to protect human low-density lipoproteins against oxidation in vitro [12]. Absorption of both chlorogenic acid and of caffeic acid has been shown to be high (33% and 95%, respectively) [13], and these compounds therefore have a high potential to act as antioxidants within our body.

Further knowledge on the absorption, bioavailability, and metabolism of catechins and hydroxycinnamates after normal dietary intakes is essential for our understanding of their mechanisms of action. Methods using high-performance liquid chromatography (HPLC) with ultraviolet absorption detection (UV) have mainly been used in studying the pharmacokinetics of these compounds [14–16], although a few liquid chromatography–mass spectrometry (LC–MS) methods for the analyses of subgroups of either catechins or hydroxycinnamates recently have been developed [10,17]. HPLC–UV is sufficient to analyze samples from human subjects given large

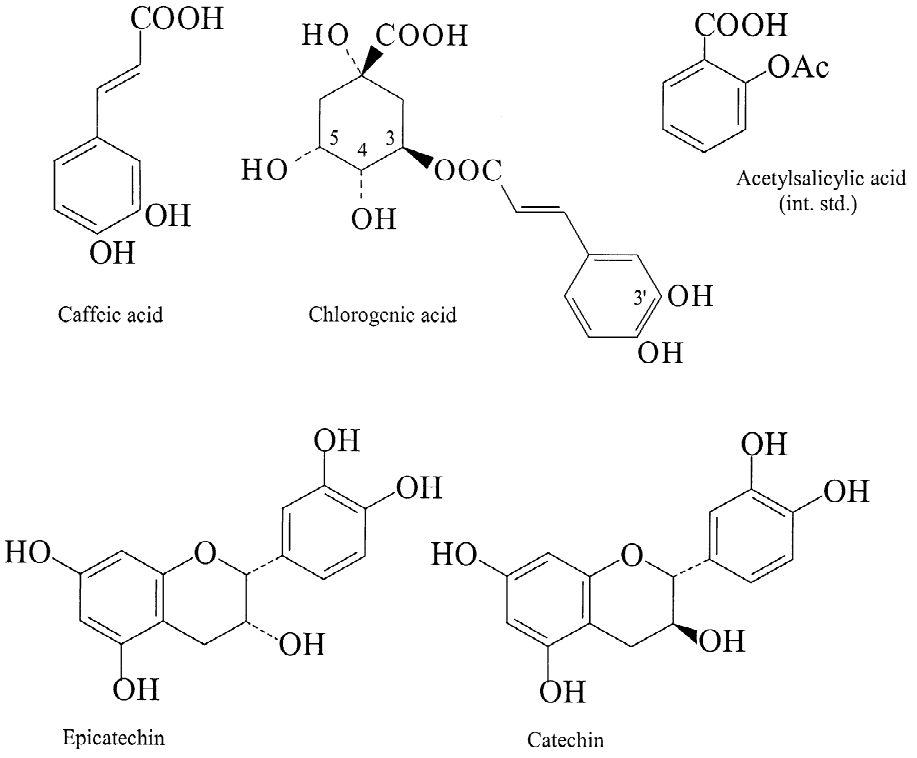

Fig. 1. Chemical structures of the four analytes employed in the HPLC–APCI–MS assay, caffeic acid, chlorogenic acid, epicatechin and catechin and the internal standard, acetylsalicylic acid.

doses of these compounds, but more sensitive and specific methodologies are needed for the analyses of biological samples from un-supplemented subjects or subjects supplemented with doses within the range of normal dietary intake. Multi-methods, where different classes of dietary compounds are determined simultaneously in human biological samples, can provide important insight in the health protective effects of both the individual compounds and of the combination of several potential health protective agents. We have previously demonstrated the successful use of 24 h human urine samples as a medium for the determination of other dietary antioxidants as reliable intake markers [18]. The aim of the present study was to develop and apply a sensitive method that utilizes column switching and HPLC–MS with atmospheric pressure chemical ionization (APCI) in selected ion monitoring (SIM) mode for the simultaneous determination of the catechins, epicatechin and catechin, and of the hydroxycinnamates, chlorogenic acid and caffeic acid in human urine samples.

2. Experimental

2.1. Apparatus

The HPLC–MS system was from Hewlett-Packard (Waldbronn, Germany) and consisted of a Hewlett-Packard 1100 HPLC system equipped with a thermostatically controlled column compartment, an autosampler with a 400 μl loop, an automatic six-port column switching valve, and an ultraviolet (UV) and visual (VIS) diode array detector. The system used an 1100 binary pump connected to eluent A: 0.5% aqueous formic acid, and B: methanol (pump 1), and an 1100 isocratic pump (pump 2) connected to eluent C: acetonitrile. Both pumps were connected to a 1100 vacuum degasser, and the outlet of the isocratic pump was connected to the binary pump via a T-connection prior to the damping module. Thus the two pumps functioned as a single unit with three pump heads. The column temperature was maintained constantly at 40 °C. UV detection was carried out simultaneously at 260 and 350 nm, with peak scanning between 210 and 600 nm (2 nm step). The outlet of the UV–VIS detector was directly connected to a Hewlett-Packard mass spectrometer (1100 MSD module version B) allowing the mobile phase to enter the APCI ion source without splitting.

2.2. Reagents and standards

Acetonitrile and methanol were of HPLC grade and obtained from Rathburne Ltd. (Walkerburn, UK). Catechin, epicatechin, chlorogenic acid, and the internal standard acetylsalicylic acid (see Fig. 1), were all obtained from Sigma Chemicals Co. (St Louis, MO, USA). Caffeic acid (3,4 dihydroxycinnamic acid) was from Aldrich (Steinheim, Germany). All standards were HPLC grade. A stock solution of 1 mg/ml of a mixture of all four target compounds, catechin, epicatechin, caffeic acid and chlorogenic acid was prepared in DMSO. Dilution of the stock solution with DMSO yielded the working solution at concentrations of 1 μg/ml and 10 μg/ml. The internal standard acetyl salicylic acid was also dissolved in DMSO giving a stock solution containing 1 mg/ml. All stock and working solutions were stored at −20 °C and were stable for at least 3 months.

The enzymes used for enzymatic hydrolysis of the urine samples were β-glucuronidase (*E. coli*, >200 standard units/ml) obtained from Boehringer Mannheim (Mannheim, Germany) and arylsulfatase (*Aerobacter aerogenes*, 16.8 standard units/ml) from Sigma Chemicals Co. All other chemicals used were of HPLC grade or reagent grade.

2.3. Samples

Urine samples were obtained from ten subjects (6 males and 4 females) participating in a 24 day long parallel intervention study with strictly controlled intake of a basic diet free from fruits, vegetables, coffee and tea. No medication or vitamin supplements were allowed during the study period. Half of the subjects consumed additionally 600 g vegetables and fruits per day (''6 a day''), consisting of apples (90 g), pears (90 g), orange juice (120 g), broccoli (100 g), carrots (75 g), onion (25 g), and canned tomatoes (100 g) (details will be published elsewhere) [19]. The subjects volunteered for the study and gave their written consent after receiving careful information about the study. The study was approved

by the Scientific Ethics Committee of the municipalities of Copenhagen and Frederiksberg (01-234/99).

The subjects collected a 24 h urine sample at the end of the 24 day dietary intervention period. The urine samples were collected in 2.5 l containers to which were added 50 ml 1 *M* HCl and 10 ml 10% aqueous ascorbic acid prior to collection. The samples were immediately refrigerated after collection and after measurement of the sample volume and pH, the pH of each sample was adjusted to pH<4 by the addition of 10 ml portions of 1 *M* HCl. Aliquots of 25 ml were stored at −80 °C until analysis.

2.4. Calibration samples

Samples for the calibration curves were prepared by spiking blank urine samples (1 ml) from a previous intervention study [20]. The urine samples were centrifuged 5 min at 4000 rpm and the supernatants were then filtered through a 0.2 μm filter. Aliquots of 1 ml urine were then added to either 0, 10, 50, or 100 μl of the working solutions containing catechin, epicatechin, chlorogenic acid and caffeic acid, resulting in concentrations of 0, 10, 50, 100, 500 and 1000 ng/ml. In addition, 10 μl (0.1 mg/ml DMSO) acetyl salicylic acid was added as internal standard. The calibration samples were enzymatically hydrolyzed and otherwise treated similarly to the samples from the intervention study prior to HPLC–APCI–MS.

2.5. Enzymatic hydrolysis

After centrifugation and filtration of the urine samples (as for the calibration samples) the pH of 1 ml aliquots was adjusted to pH 5 (4.9–5.1) by addition of about 100 μl 2 *M* sodium acetate buffer containing 10 mg/ml ascorbic acid. By addition of 10 μl acetylsalicylic acid in DMSO, 1 μg of internal standard was added to each sample. Then 10 μl of an enzyme mixture (consisting of 60 μl β-glucuronidase and 300 μl arylsulfatase preparation) was added and the total volume of each sample was adjusted to 1.190 ml by addition of water, so all samples, including the calibration samples described above, had the same volume. Then argon (g) was blown over the sample, and it was incubated in a sealed vial for 1 h at 37 °C under continuous shaking. Immediately after the enzymatic hydrolysis, aliquots of 250 μl of each sample were injected onto the HPLC–APCI–MS system.

2.6. Chromatography

The columns used were a Zorbax 300SB-C_3 (4.6×50 mm, 3.5 μm) column with guard cartridge (4×4 mm, 5 μm) as column 1 and a Zorbax SB-C_{18} (4.6×150 mm, 5 μm) column as column 2 (all from Agilent technologies). The mobile phases used were: A: 0.5% aqueous formic acid, B: methanol, and C: acetonitrile.

Table 1 shows in detail the chromatographic conditions for the column-switch HPLC methodology developed. An illustration of the column switching system can be found in Ref. [18]. Briefly, due to the very hydrophilic nature of the hydroxycinnamic acids and the catechins, a very aqueous elution profile was applied on column 1 to elute the more hydrophilic urinary impurities to waste prior to the elution of the target compounds. The profile started with pump 1 alone giving 5 min of isocratic elution

Table 1
Chromatographic conditions for the HPLC–APCI–MS system

Time (min)	% Mobile phase B (v/v)	% Mobile phase C (v/v)	Flow (ml/min)	Column switch (min/position of valve)[a]
0.0	0.0	0.0	0.800	0.0/1
5.0	0.0	0.0	0.800	4.5/2
10.0	5.0	0.0	0.800	17.5/1
15.0	5.0	0.0	0.800	22.0/2
18.0	17.0	0.0	1.220	
18.1	100.0	0.0	1.500	
19.0	100.0	0.0	1.500	
19.1	0.0	0.0	1.500	
22.0	0.0	0.0	0.800	
32.0	0.0	20.0	0.800	
35.0	0.0	20.0	0.800	
36.0	0.0	100.0	0.800	
39.0	0.0	100.0	0.800	
39.1	0.0	0.0	0.800	
43.0	0.0	0.0	0.800	

[a] Valve position 1 leads the flow through column 1 (Zorbax 300 SB-C3) and the UV-detector alone (meanwhile, the APCI–MS is switched to ''waste position'' by its own internal valve), position 2 leads the flow through both columns and to the UV- and APCI–MS-detector.

with formic acid buffer alone (solvent A), then a slow gradient between 5 and 10 min to 5% MeOH (B) in A (v/v) followed by additionally 5 min of isocratic elution with 5% B in A (v/v). A steep gradient of 5–17% B in A (v/v) between 15 and 18 min concentrated the four target compounds in a narrow fraction that were eluted onto column 2 between 4.5 and 17.5 min by switching the automatic six-port column switching valve to position 2. The more non-polar impurities remaining on column 1 after elution of the target compounds were removed by a quick column wash with 100% MeOH, followed by reequilibration with pure formic acid buffer (solvent A), with the six-port switching valve in position 1. Finally, by using pump 2 in combination with pump 1, the target compound was eluted through the UV and APCI–MS detector by 20% acetonitrile (C) in aqueous formic acid (A) after switching the flow back to column 2. Both columns were then washed by 100% C from pump 2 and then returned to the initial conditions after 39 min, still with the solvent flow eluting through both columns, thus preparing the system for the next injection.

2.7. APCI–MS

APCI was performed by switching between negative (neg.) and positive (pos.) mode using selected ion monitoring (SIM). The ions selected for SIM are seen in Table 2. The capillary voltage, vaporizer temperature, drying gas temperature, corona current, and fragmentor voltage were all optimized with regard to maximum signal intensity of molecular ions and fragment ions by consecutive injections of 250 μl samples (2 ng/μl) containing all the analytes including the internal standard. The optimal conditions for both positive and negative mode were the following: APCI capillary voltage: 2500 V, vaporizer temperature: 500 °C, nebulizer pressure: 60 p.s.i., drying gas temperature: 350 °C, drying gas flow: 10 l/min, fragmentor voltage: 70 V. Corona current: neg. mode: 20 μA, pos. mode: 4 μA.

2.8. Stability of hydroxycinnamates and catechins in urine

The stability of the analytes, the hydroxycinnamates and the catechins was investigated by keeping a set of spiked urine samples ($n=3$ at each concentration level) prepared as for the calibration curve (see above) at −80 °C for 7 months or at room temperature for 10 days. These sets of samples were then analyzed together with a freshly prepared set of samples, and the concentrations of the analytes were compared (see Table 3).

2.9. Linearity, limit of detection and limit of quantification

Standard curves for each of the 4 catechin and hydroxycinnamate standards were prepared over a concentration range of 0–1000 ng/ml with six different concentration levels and more than triplicate injections at each level. Peak areas were plotted against the corresponding standard concentration using weighed linear regression to generate

Table 2
Retention time, limit of detection and quantification, and fragment ions of the monitored catechins and hydroxycinnamic acids determined by SIM using HPLC–APCI–MS

Compound	Retention time (min)	LOD[b] (ng/ml)	LOQ[c] (ng/ml)	$M-1^{-}$ [d] m/z (intensity)	Qualifier ions[d,e] m/z (intensity)
Catechin	35.8	4	10	289 (100)	291 (100)
Chlorogenic acid	36.1	3	10	353 (100)	191 (70)
Caffeic acid	36.7	6	20	179 (100)	–
Epicatechin	37.2	3	10	289 (100)	291 (100)
Acetylsalicylic acid[a]	40.1	12	40	137 (100)	–

[a] Internal standard.
[b] LOD = limit of detection, determined as $S/N=3$
[c] LOQ = limit of quantification, determined as lowest concentration level employed in the calibration curve (10 ng/ml) or as $S/N=10$.
[d] The intensity is determined relative to the maximum mass peak.
[e] For catechin and epicatechin, these ions are determined in positive mode giving $M+1^{+}$.

Table 3
Stability of hydroxycinnamic acids and catechins in human urine

Storage conditions[a]	Catechin (%)	Chlorogenic acid (%)	Caffeic acid (%)	Epicatechin (%)	Acetylsalicylic acid (%)
7 months at −80 °C	94.8±2.97	96.9±0.7	99.9±2.7	89.8±0.9	84.8±2.7
10 days at room temp.[b]	83.9±15.3	105.9±5.8	89.0±24.4	78.3±9.7	21.2±0.8

[a] Urine samples were spiked with 100, 500 or 1000 ng of each compound ($n=3$) and compared with freshly prepared samples after end of storage time. Data are calculated as % remaining after storage. Mean and SD of all conc. levels are given.
[b] The samples were stored in the darkness.

the standard curves. Conclusions about the limit of detection (LOD) and the limit of quantification (LOQ) were drawn by inspecting the chromatograms obtained at the lowest calibration level (see Table 2).

2.10. Performance verification, precision and specificity

An aliquot of 10 μl acetylsalicylic acid dissolved in DMSO (0.10 mg/ml) was added to the urine samples prior to enzymatic hydrolysis as an internal standard correcting for analytical loss and variations in the performance of the mass spectrometer. Prior to, and after each series of analyses it was controlled so that the retention times of the target compounds on column 1 were within the time frames of column switching. This was done by injection of an aliquot of 500 ng of the catechin and hydroxycinnamate standards, including the internal standard dissolved in 250 μl 1% aqueous methanol containing 1% formic acid on column 1 alone with the flow eluting through the UV- and MS-detector. Additionally, another 250 μl of this mixture was injected to assess the performance of the entire HPLC–APCI–MS method.

As seen in Table 4, the inter-day variation of the assay was evaluated by analyzing blank urine samples spiked with the catechin and hydroxycinnamate standards at concentrations of 50, 100, 500 and 1000 ng/ml in triplicates on the same day (repeatability). Reproducibility was evaluated by analyzing 2–3 blank urine samples spiked with catechin and hydroxycinnamate standards at a concentration of 500 ng/ml within each series of samples over a period of 5 months ($n=17$).

2.11. Statistics

Since the data obtained from the intervention study were not normally distributed, a non-parametric Mann–Whitney *U* test (Statview, Abacus Concepts Inc., version 4.53) was performed to analyze the urinary excretion of catechins and hydroxycinnamates in the group receiving ''6 a day'' of fruits and vegetables versus the group on basic diet.

2.12. Safety considerations

General guidelines for work with organic solvents were respected, and urine samples were handled as potentially infectious.

Table 4
Precision and accuracy evaluation for the analysis of catechins and hydroxycinnamtes in human urine using HPLC–APCI–MS[a]

Concn. added (ng/ml)	Concn. found (ng/ml)	Repeatability (C.V.%, $n=3$)	Reproducibility[b] (C.V.%, $n=17$)	Accuracy (%)
50	53.2 (50.0–55.6)	4.3 (4.0–6.9)	–	6.6 (0.1–11.1)
100	100.6 (93.8–107.7)	5.0 (1.5–8.2)	–	4.2 (1.1–7.7)
500	494.4 (472.2–530.1)	4.3 (1.6–10.1)	–	4.1 (2.4–6.0)
500	507.2 (502.8–515.8)	–	9.3 (7.8–10.9)	1.4 (0.6–3.2)
1000	1023.7 (994.2–1060.2)	2.5 (0.5–4.4)	–	2.7 (0.6–6.0)

[a] Mean of determinations for all 4 standards, range given in parenthesis.
[b] Determined over a period of 5 months.

3. Results and discussion

3.1. Enzymatic hydrolysis

Enzymatic hydrolysis of the glucuronic and sulfate conjugates of the hydroxycinnamates and the catechins was employed instead of acidic hydrolyses as used by Maini and coworkers, avoiding the risk of degrading the catechins [21]. The optimal incubation time for the enzymatic hydrolysis has been investigated previously [18,22], and also a different type of β-glucuronidase has been evaluated for hydrolysis [23].

3.2. Chromatography and mass spectrometry

The future for chromatographic analyses of biological samples is multi-methods, where several different analytes are determined simultaneously in a single run. This is feasible by the use of LC–MS, where quantitative determination is possible of low levels of an analyte even without chromatographic separation from the other components in the sample. The operation in SIM mode furthermore allies excellent sensitivity with high specificity, as only ions with a specific mass are monitored.

Previous methodologies developed for detection of the very hydrophilic compounds, hydroxycinnamates and catechins, in biological fluids have been limited to only one of these compound classes and include sample treatment such as solid-phase extraction or extraction with e.g. ethyl acetate followed by evaporation to dryness and redissolution prior to analyses [10,21,24].

The use of column switching diminished the sample treatment, since the online sample purification on the first column renders the traditional pre-HPLC sample treatment unnecessary. It also increases the reproducibility of the assay, since the manual sample treatment and the number of steps in the procedure is limited. This approach thus makes it realistic to apply the developed methodology on large numbers of samples from e.g. human intervention or epidemiological studies.

We have previously developed a similar assay for the determination of seven different flavonoids in human urine [18]. In a later study, we demonstrated the use of this methodology on a large sample set from a human intervention study including subjects on their own habitual diet [25]. The flavonoids have the advantage of being slightly more non-polar than the hydroxycinnamic acids and the catechins, and they furthermore have higher molecular masses, making the detection easier on the background of urinary impurities. The detection of more polar analytes with low molecular mass such as the hydroxycinnamic acids and the catechins in biological samples, especially urine, is however somewhat more challenging, since these compounds elute in the smear of polar components contained in urine, many also with low molecular masses. This is probably the reason for the very limited number of previous reports on HPLC or LC–MS analyses of hydroxycinnamic acids or catechins in biological samples. MS of these low molecular mass compounds rarely result in useful fragmentation for qualification purposes, such as was the case for the flavonoids [18]. Only the rather high molecular mass compound chlorogenic acid showed a fragment ion at m/z 191 useful for identification and verification originating from the loss of the caffeoyl residue (m/z 163) by cleavage of the ester bond. However, the use of both positive and negative APCI–MS allowed the positive identification of both of the catechins at m/z 289 $(M-1)^-$, and at m/z 291 $(M+1)^+$. Caffeic acid and chlorogenic acid showed low responses in positive mode that were not useful for identification.

To gain sufficient separation of the four analytes, the very polar Zorbax 300SB-C_3 column was selected as the first column for sample clean-up instead of C_8 as previously used [22,23], and a more non-polar Zorbax SB-C_{18} column was selected as the second column, to increase the chromatographic differences between the first and the second column. Furthermore, the MeOH gradient applied to column 1 was developed in order to elute the analytes in the smallest possible heart-cut fraction still having sufficient removal of impurities, preventing contamination of the APCI chamber. By the application of methanol as the organic solvent on column 1 and of acetonitrile on column 2, sufficient separation of the target compounds from interfering compounds in urine was achieved prior to APCI detection. It was necessary to use acetonitrile as the organic solvent on column 2 to achieve sufficient separation of caffeic acid from hippuric acid, also found in urine.

The two compounds have very similar molecular ions, $(M-1)^- = m/z$ 179 and 178, respectively, and since hippuric acid was eluting just prior to caffeic acid (data not shown) the interference between these two compounds had to be avoided. Typical SIM chromatograms of a urine sample from the intervention study in comparison with a standard sample are shown in Fig. 2.

3.3. Stability of hydroxycinnamic acids and catechins in human urine

The stability of the four analytes and of the internal standard, acetylsalicylic acid, was investigated by spiking blank urine samples with different levels of concentration, where after the recovery was investigated subsequent to different storage condi-

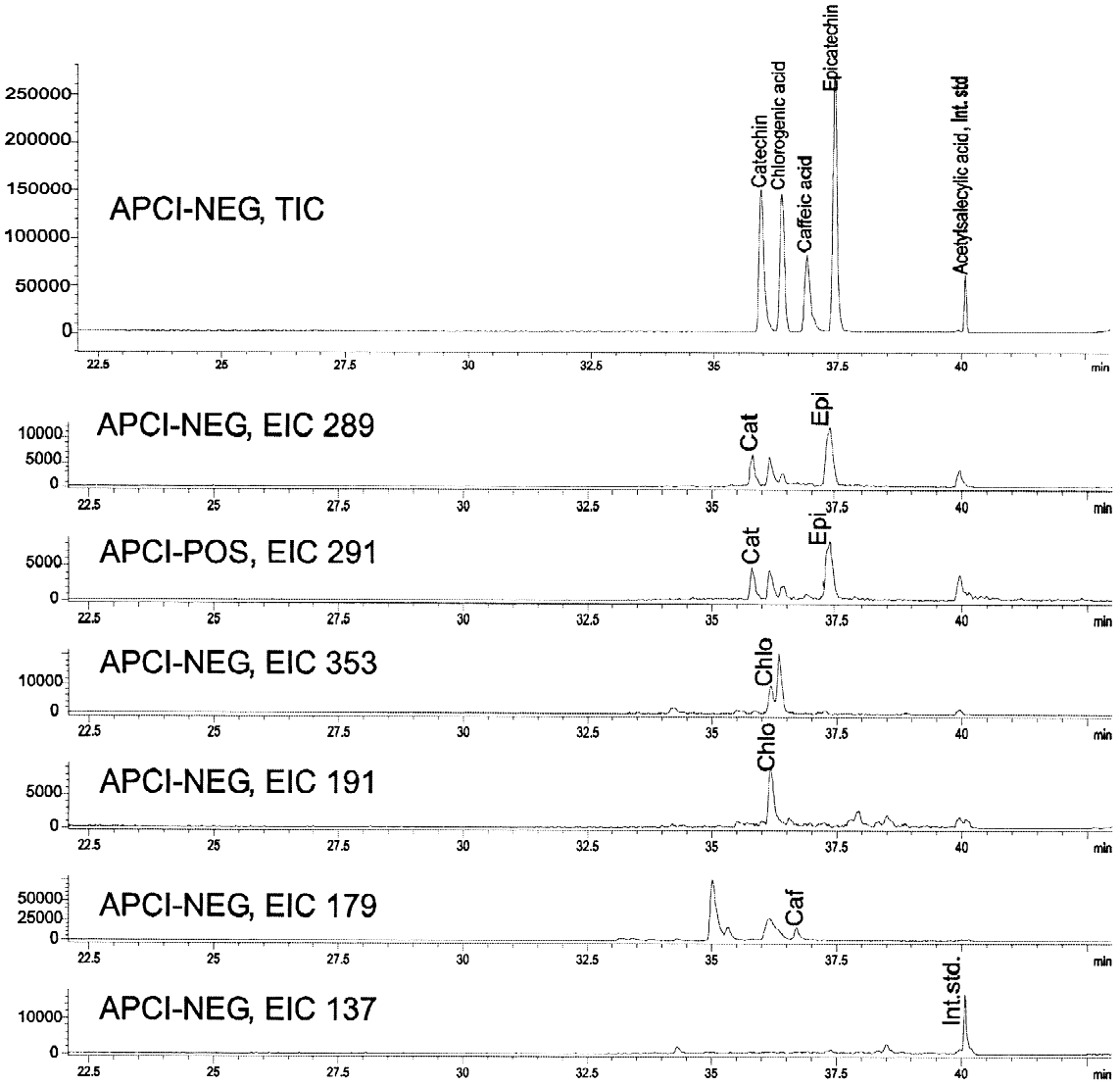

Fig. 2. Upper chromatogram: Total ion track (TIC) of a standard mixture of catechin (cat), epicatechin (epi), chlorogenic acid (chlo), and caffeic acid (caf). The remaining chromatograms are from a urine sample collected after 24 days of intervention with 600 g/day of fruits and vegetables, showing the individual extracted ion tracks (EIC) of each of the target compounds, at m/z for the molecular ion or at the respective qualifier ions, generated by negative or positive APCI in SIM mode after column-switching chromatography of the sample.

tions. As seen in Table 4, the employed hydroxycinnamates and catechins are highly stable in urine when stored at −80 °C. Even after 7 months, more than 90% of the spiked catechin, chlorogenic acid, and caffeic acid still remained intact, and about 90% and 85% of epicatechin and acetylsalicylic acid, respectively, remained at this stage. On the contrary, prolonged storage at room temperature almost totally degraded the internal standard, acetylsalicylic acid, whereas more than 75% of the other compounds remained intact.

3.4. Linearity, limit of detection, and precision

The calibration graphs were linear with correlation coefficients of 0.995 or higher in the range of 10–1000 ng/ml urine for all four target compounds, except for caffeic acid, where quantification at 10 ng/ml was not possible. The LOQs were determined as the lowest level employed in the calibration curves (10 ng/ml) or as for caffeic acid and acetyl salicylic acid as the signal-to-noise ratio = 10 (S/N = 10), and the LODs as S/N ratios >3 (Table 2).

Only a few other studies have analyzed catechins or hydroxycinnamates in human urine, and the employed methodologies have not been thoroughly validated with regard to limit of detection and quantification in urine samples [10,24]. A few studies used HPLC with UV-detection for the determination of hydroxycinnamates and catechins in foods and beverages or in human plasma after ingestion of green tea, and they all report on higher limits of detection in the μg/ml range [16,21,26]. These methodologies would thus not have the sufficient sensitivity for analyses of biological samples from subjects not being supplemented with high doses of catechins or hydroxycinnamates. Cremin and coworkers report on a LC–MS methodology for the analyses of hydroxycinnamates in human urine and plasma [10], but the methodology involves ethyl acetate extraction, which results in poor recoveries of chlorogenic acid (below 30%) in both plasma and urine when the concentration is down at the level measured in the present study. Furthermore, detection limits were only determined using external standards.

The precision and accuracy of the present LC–MS assay were thoroughly investigated for the analysis of urine samples. Table 4 shows the repeatability, accuracy, and reproducibility data obtained for all the compounds determined in the assay at the different concentrations tested. Most of the coefficients of variation (C.V.%) were below 5% with caffeic acid having the highest C.V.%. A slightly higher C.V.% was seen for the reproducibility of the assay when this was evaluated over a period of 5 months (n = 17), but it was still acceptable and meets with the objectives of a routinely applicable analysis for this type of compounds in human urine samples.

3.5. Analysis of samples from dietary intervention

To evaluate the applicability of the method, a subset of urine samples from an intervention study using diets with or without 600 g of fruits and vegetables [19] (details will be published elsewhere) was analyzed. The results are presented in Table 5. As can be seen, there are large differences in the

Table 5
Excretion of hydroxycinnamic acids and catechins in human urine samples determined by HPLC–APCI–MS[a]

Diet	Urinary excretion (μg/24 h)			
	Caffeic acid	Chlorogenic acid	Catechin	Epicatechin
"6 a day"				
Mean	275	67.8*	25.7	114*
(SD)	(115)	(42.4)	(51.4)	(29.0)
Basic				
Mean	102	7.97	n.d.	23.6
(SD)	(142)	(15.9)	(–)	(47.3)

[a] Determined in 24 h urine samples collected from healthy subjects on the last day of 24 days of intervention with either a basic diet (Basic) or supplemented with 600 g fruits and vegetables ("6 a day"). * $P<0.05$ using non parametric test (Mann–Whitney U test), n = 5 in each group, n.d. = not detected.

urinary excretion of all four analytes between the two groups. However, only the differences in excretion of chlorogenic acid and epicatechin reached statistical significance using a non-parametric test ($P < 0.05$).

Studies on excretion of hydroxycinnamates and of catechins in humans after intake of fruits and vegetables are very limited and there are no previous reports on the simultaneous detection of both classes of compounds. Dupont and coworkers [27], however, attempted to determine both catechins and hydroxycinnamates in human urine and plasma after consumption of apple cider using either HPLC with UV or fluorescence detection or HPLC–MS, but neither catechin, epicatechin, caffeic acid or chlorogenic acid was detectable in urine using the employed methodology. Caffeic acid was found in plasma within the first 90 min after consumption, but the catechins and chlorogenic acid were undetectable in plasma by this assay.

The urinary excretion of especially chlorogenic acid and of epicatechin after intake of fruits and vegetables show that the contribution from fruits and vegetables of these two antioxidants is substantial, and that not only tea and coffee are important sources of these compounds. Furthermore, we have demonstrated that the developed methodology has sufficient sensitivity and selectivity to be applied on human urine samples collected from subjects given doses of fruits and vegetables within the range of normal dietary intake.

4. Conclusion

We have successfully developed a very rapid and sensitive HPLC–MS assay for the analysis of dietary catechins and hydroxycinnamates in human urine. The use of column switching and APCI–MS in SIM mode allowed limited sample preparation and gave specific, quantitative and simultaneous determination of 4 different dietary components in human urine samples. Application of small changes to the assay should allow the developed methodology to be used for the determination of other catechins or hydroxycinnamates in human urine samples. The method was validated with regard to linearity, precision, and accuracy and its suitability for analysis of real samples was successfully demonstrated.

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