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Novel liquid chromatography–electrospray ionization mass spectrometry method for the quantification in human urine of microbial aromatic acid metabolites derived from dietary polyphenols

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Abstract

An HPLC–ESI-MS–MS method was developed to quantify in human urine fourteen aromatic acids known as metabolites of dietary polyphenols. These metabolites were determined simultaneously in a single 20-min chromatographic analysis with multiple reaction monitoring detection. The inter- and intra-day precisions, calculated from quality control samples were 8.8 and 5.3%, respectively, and the mean accuracy was 2.3%. The method was tested on urine samples collected from one healthy volunteer who consumed a polyphenol-rich diet for 3 days. Increased levels of several aromatic acid metabolites were observed, demonstrating that the method can be used to detect changes in the excretion of microbial metabolites induced by the consumption of polyphenol-containing foods in humans.

Keywords: Polyphenols

1. Introduction

Polyphenols are naturally occurring compounds present in many fruits, vegetables and beverages [1–3] and are thus part of our daily diet. Recently there has been a growing interest in polyphenols due to their potential beneficial properties on human health. Polyphenols have been implicated as protective against coronary heart diseases as well as certain cancers [4–7]. Among the various mechanisms of action proposed, their strong antioxidant activity has attracted much attention [8–10].

Biological properties of polyphenols closely depend on their bioavailability. Therefore it is essential to understand how polyphenols are absorbed, metabolized and eliminated from the body. So far, attention has been focused on metabolites produced after polyphenol absorption through the small intestine. However, little is known about the metabolic fate of polyphenols which are either not absorbed, or are excreted in bile and thus reach the colon. In vitro studies with human fecal microflora have shown that they can be metabolized into various benzoic, phenylacetic, phenylpropionic and phenylvaleric

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acids with different hydroxylation profiles in the colon [11–14]. Some of these metabolites once absorbed through the colonic barrier can give glycine conjugates such as hippuric acid formed from benzoic acid in the liver and kidney [15]. Many of these microbial metabolites still bear a free phenolic group and have antioxidant properties [8,16]. To evaluate their potential contribution to the health effects of polyphenols, it is essential to compare the relative abundance of these compounds in the body to that of their precursors, which have until now been the main focus of recent publications [3].

Methodologies developed to date for the analysis of microbial aromatic acid metabolites utilize gas chromatography with mass spectrometry detection (GC–MS) or liquid chromatography coupled with diode-array detection (HPLC–DAD) [13,17,18]. Although the GC–MS method provides useful information about the nature of target compounds by reference to a spectra library, it requires derivatization of the aromatic acids prior to their analysis. The use of HPLC–DAD is limited by its sensitivity and it is thus only applicable to compounds present in high concentrations, such as hippuric acid [18]. These techniques are therefore not suitable for a rapid and sensitive quantification of polyphenol microbial metabolites in tissue or urine samples collected in human dietary intervention and epidemiological studies. The recent development of liquid chromatography–electrospray ionization mass spectrometry (HPLC–ESI-MS and HPLC–ESI-MS–MS) instruments with high sensitivity and selectivity, their user-friendly software and decreasing cost have boosted the use of such analytical techniques in polyphenol metabolism studies [19–23].

We report here the development of an HPLC–ESI-MS–MS method suitable for the rapid and simultaneous estimation of fourteen aromatic acids, which are known as major metabolites of dietary polyphenols.

2. Experimental

2.1. Chemicals

3-Hydroxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid, homovanillic acid, phenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, caffeic acid, ferulic acid, *p*-coumaric acid and syringic acid were purchased from Sigma (St. Louis, MO, USA); 3-hydroxyphenylpropionic acid and 3,4-dihydroxyphenylpropionic acid from Apin (Abingdon, UK). 3-Hydroxyhippuric acid and 4-hydroxyhippuric acid were kindly provided by P.C.H. Hollman (Rikilt, Wageningen University, The Netherlands) and R. Scheline (University of Bergen, Norway), respectively.

2.2. Sampling procedure

Blank urine samples were obtained from twelve healthy volunteers (six females and six males) aged 24 ± 3 years old who participated in an intervention study with highly controlled diets free of polyphenols (to be published elsewhere). The volunteers gave their written consent and the clinical study was approved by the Ethical Committee of the University Hospital in Clermont-Ferrand, France. Volunteers collected 24-h urine after a 3-day polyphenol-free diet containing no fruit, vegetable or polyphenol-rich beverage (coffee, tea, chocolate, fruit juice, red wine). Urine samples were stored at 4 °C and immediately acidified to pH 4 upon reception in the laboratory after completion of the collection, by adding 200 μl of 6 *M* HCl to a 30-ml aliquot. Aliquots collected from each subject were pooled according to the daily volumes. The pooled samples were then stored at −20 °C until analysis.

To evaluate the applicability of the method, a 24-h urine collection was obtained from one volunteer who did not consume polyphenol-containing food or beverage for 3 consecutive days and then followed this with a 3-day diet containing fruits (grape, apple) vegetables (broccoli, lettuce, celery, bean, carrot, salsify, onion, potato, spinach, pumpkin, tomato) orange juice and coffee. Urine samples (24 h), collected over the 3 consecutive days were pooled after acidification as described above and stored at −20 °C prior to analysis.

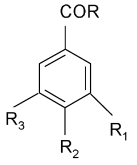
2.3. HPLC–ESI-MS–MS analysis

Urine samples (diluted 1:10, v/v, in 0.1 *M* sodium acetate buffer pH 5, 175 μl) containing syringic acid (3 μ*M*) as internal standard, were acidified to pH 4.9

with 20 μl of 0.58 *M* acetic acid and incubated at 37 °C for 45 min in the presence of an *Helix pomatia* extract containing 1100 U β-glucuronidase and 42 U sulfatase (Sigma). After acidification to pH 2 with 2 μl of 6 *M* HCl, the urine was extracted twice with ethyl acetate (350 μl) and centrifuged at 2400 *g* for 10 min. The supernatant fluid was dried under nitrogen, redissolved in 500 μl of 25% aqueous methanol and filtered (PTFE membrane, 0.45 μm, Millipore, Bedford, USA). A 40-μl aliquot of the filtrate was then injected directly into the HPLC–ESI-MS–MS system.

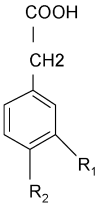
HPLC–ESI-MS–MS analyses were performed on a Hewlett-Packard HPLC system with triple quadrupole MS detection (API 2000, Applied Biosystem, Canada). The column was an Hypersil BDS C_{18} (150×2.1 mm, 5 μm, Touzart and Matignon, Les Ulis, France) and the mobile phases consisted of 5% acetonitrile in 0.1% aqueous formic acid (solvent A) and 40% acetonitrile in 0.1% aqueous formic acid (solvent B). The following gradient was applied: 0–15 min, linear gradient from 0% B to 100% B; 15–20 min: 100% B. The flow-rate was 0.2 ml/min. Detection was carried out using electrospray ionization in negative mode at 450 °C with a nebulizer pressure of 90 p.s.i. (1 p.s.i.=6894.76 Pa), a drying nitrogen gas flow of 11 l/min, a fragmentor voltage of 20 V and a capillary voltage of 4000 V. The MS data were collected in the multiple reaction monitoring (MRM) mode, monitoring the transition of parent and product ions specific for each compound with a dwell time of 500 ms. Fourteen aromatic acids including benzoic, phenylacetic, phenylpropionic and cinnamic acid derivatives, previously reported as

A. Benzoic acids



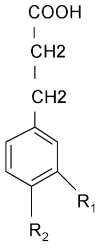
R=OH, R_1=OH, R_2=H, R_3=H: 3-Hydroxybenzoic acid
R=OH, R_1=H, R_2=OH, R_3=H: 4-Hydroxybenzoic acid
R=OH, R_1=OCH3, R_2=OH, R_3=H: Vanillic acid
R=OH, R_1=OCH3, R_2=OH, R_3=OCH3: Syringic acid
R=O-NH-CH2-COOH, R_1=OH, R_2=H, R_3=H: 3-Hydroxyhippuric acid
R=O-NH-CH2-COOH, R_1=H, R_2=OH, R_3=H: 4-Hydroxyhippuric acid

B. Phenylacetic acids



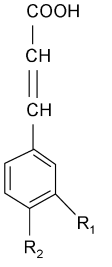
R_1=H, R_2=H: Phenylacetic acid
R_1=OH, R_2=H: 3-Hydroxyphenylacetic acid
R_1=OH, R_2=OH: 3,4-Dihydroxyphenylacetic acid
R_1=OCH3, R_2=OH: Homovanillic acid

C. Phenylpropionic acids



R_1=OH, R_2=H: 3-Hydroxyphenylpropionic acid
R_1=OH, R_2=OH: 3,4-Dihydroxyphenylpropionic acid

D. Cinnamic acids



R_1=OH, R_2=OH: Caffeic acid
R_1=OCH3, R_2=OH: Ferulic acid
R_1=H, R_2=OH: *p*-Coumaric acid

Fig. 1. Chemical structures of the 14 aromatic acids and internal standard syringic acid detected by HPLC–ESI-MS–MS assay.

metabolites of polyphenols [12,13], and the internal standard (syringic acid) were simultaneously detected (Fig. 1).

2.4. Calibration curves, limit of quantification

Calibration curves for HPLC–ESI-MS–MS quantification were prepared by spiking blank urine samples with aliquots of a mixture of standard solutions in the range 0.05–6 μM at six different concentrations with duplicate injections at each level. These samples were treated as described above. Peak areas were plotted against the corresponding standard concentrations to generate the calibration curves. The response factor for each aromatic acid was calculated from the slopes of these linear curves.

2.5. Precision, accuracy and analyte recovery

The precision and accuracy of the assay were evaluated by analyzing quality control samples prepared by spiking blank urine samples with a mixture of standard solutions at concentrations of 0.5, 1.5, 3 and 6 μM over 3 days (six replicates for each concentration) for the determination of inter-day relative standard deviation (RSD) (reproducibility) and in six independent analyses on the same day for the evaluation of intra-day RSD (repeatability).

Analyte recovery was calculated by comparing the response of blank urine spiked with standard solutions at four concentration levels (0.5, 1.5, 3 and 6 μM) prior to extraction with the same urine to which standard solutions had been added after extraction (control). The recovery of the internal standard was similarly determined.

3. Results and discussion

Fourteen aromatic acids, known as metabolites of dietary polyphenols, were selected for analysis in urine (Table 1). They were also identified as major metabolites of polyphenols in a preliminary study using GC–MS analysis [24].

3.1. ESI-MS–MS behavior of pure aromatic acids

Prior to the HPLC–ESI-MS–MS experiments using urine samples, direct injection of a standard solution of each aromatic acid (10–50 μM) with a syringe pump was investigated. The negative ionization mode was found to be more sensitive than positive ionization mode as has been similarly described for other classes of phenolic compounds [21,22,25]. Because the sensitivity of the electrospray ionization is affected by the ability of the mobile phase to support preformed ion in solution, both methanol or acetonitrile containing formic acid

Table1
Parent ions and product ions of aromatic acids monitored in the MRM mode using HPLC–ESI-MS–MS

Compounds	Molecular mass	Parent ions ($[M-H]^-$, m/z)	Product ions (m/z)
3-Hydroxybenzoic acid	138	137	93
4-Hydroxybenzoic acid	138	137	93
3-Hydroxybenzoic acid	195	194	150
4-Hydroxyhippuric acid	195	194	100
Vanillic acid	168	167	123
Homovanillic acid	182	181	137
Phenylacetic acid	136	135	91
3-Hydroxyphenylacetic acid	152	151	107
3,4-Dihydroxyphenylacetic acid	168	167	123
3-Hydroxyphenylpropionic acid	166	165	121
3,4-Dihydroxyphenylpropionic acid	182	181	59
Caffeic acid	180	179	135
Ferulic acid	194	193	134
p-Coumaric acid	164	163	119
Syringic acid[a]	198	197	123

[a] Internal standard.

were investigated as mobile phases. Maximum sensitivity was achieved with a mobile phase of water–acetonitrile–formic acid (59.9:40:0.1, v/v/v). Other parameters affecting the ionization, namely drying gas flow and temperature, nebulizer pressure, capillary voltage and fragmentor voltage were optimized by flow injection analysis of standard solutions at concentrations varying from 1 to 5 μ*M*. These conditions enabled a relatively soft ionization with the major peak for each compound being the $[M-H]^-$ parent ion undergoing a characteristic loss of (COO) in the collision chamber (Table 1). However, three compounds behaved differently. 4-Hydroxyhippuric acid produced a fragment ion at *m*/*z* 100 corresponding to the loss of the glycine moiety. 3,4-Dihydroxyphenylpropionic acid lost the (CH_2–COO) group of the side chain to give a product ion at *m*/*z* 59. Ferulic acid product ion at *m*/*z* 134 was attributed to the simultaneous loss of (COO) and the methyl group.

3.2. Chromatography

The HPLC gradient conditions, together with a preliminary ethyl acetate extraction of the aromatic acids, resulted in sufficient separation of the 14 selected metabolites from interfering compounds present in urine over a short run time of 20 min. A typical set of chromatograms from a human urine sample collected after consumption of a polyphenol-rich diet is shown in Fig. 2. Both microbial aromatic acid metabolites and intact polyphenols like caffeic acid and its methylated metabolite ferulic acid were detected. Peak identity was established by both the characteristic parent and product ion pair and the retention time compared to that of authentic standards. The high selectivity, high sensitivity and low background signal were obtained by the use of MRM which constitutes an extremely powerful detection mode, especially with complex matrices such as biological samples [26].

3.3. Linearity, limit of quantification, precision and analyte recovery

Calibration curves were linear in the range of 0.125–6 μ*M* for all aromatic acids. The limits of quantification (signal-to-noise ratio of more than 3:1) were determined as 0.125 μ*M* for all the aromatic acid metabolites except for vanillic acid (0.5 μ*M*) due to its relatively low ionization efficiency. The limits of quantification defined here were found sufficient to make the present method applicable to the simultaneous analysis of the 14 aromatic acids in human urine even when individuals consumed a diet low in polyphenols (see Section 3.4). For all aromatic acids, calibration curves were characterized by correlation coefficients higher than 0.97 (range 0.9798–0.9987) when plotting peak area against the corresponding analyte concentration.

The precision and accuracy of the HPLC–ESI-MS–MS method developed were also investigated. Table 2 reports the reproducibility, repeatability and accuracy data calculated from quality control samples at four different concentrations. Relative standard deviations (RSDs) for the inter- and intra-assays were 8.8% (range 1.1–14.5%) and 5.3% (range 0.1–14.7%), respectively. The mean accuracy was 2.3% (range −12.1–14.8%). Thus, validation of this method for estimation of such aromatic acids in human urine, was achieved. Extraction efficiency of aromatic acids ranged from 55 to 86% according to the nature of the metabolites (Table 3).

3.4. Quantification of aromatic acid metabolites in human urine after polyphenol consumption

To determine whether this novel method was capable of detecting and quantifying aromatic acid metabolites derived from a normal dietary polyphenol consumption, it was applied to the analysis of samples from one healthy volunteer who followed a diet rich in fruits, vegetables, and coffee over 3 consecutive days after a 3-day diet low in polyphenols. The HPLC–ESI-MS–MS assay reported here facilitated the detection of all of the derivatives of benzoic, phenylacetic, phenylpropionic and cinnamic acids described above (Fig. 2). Urinary levels of these compounds are reported in Table 4. 3-Hydroxyphenylpropionic acid and 3,4-dihydroxyphenylacetic acid were the compounds most affected by the polyphenol-rich diet. Their urinary excretion level increased by a factor of 15- and 12-fold, respectively. 3-Hydroxybenzoic acid, 3-hydroxyhippuric acid, 4-hydroxyhippuric acid, vanillic acid, *p*-coumaric acid, homovanillic acid, phenylacetic

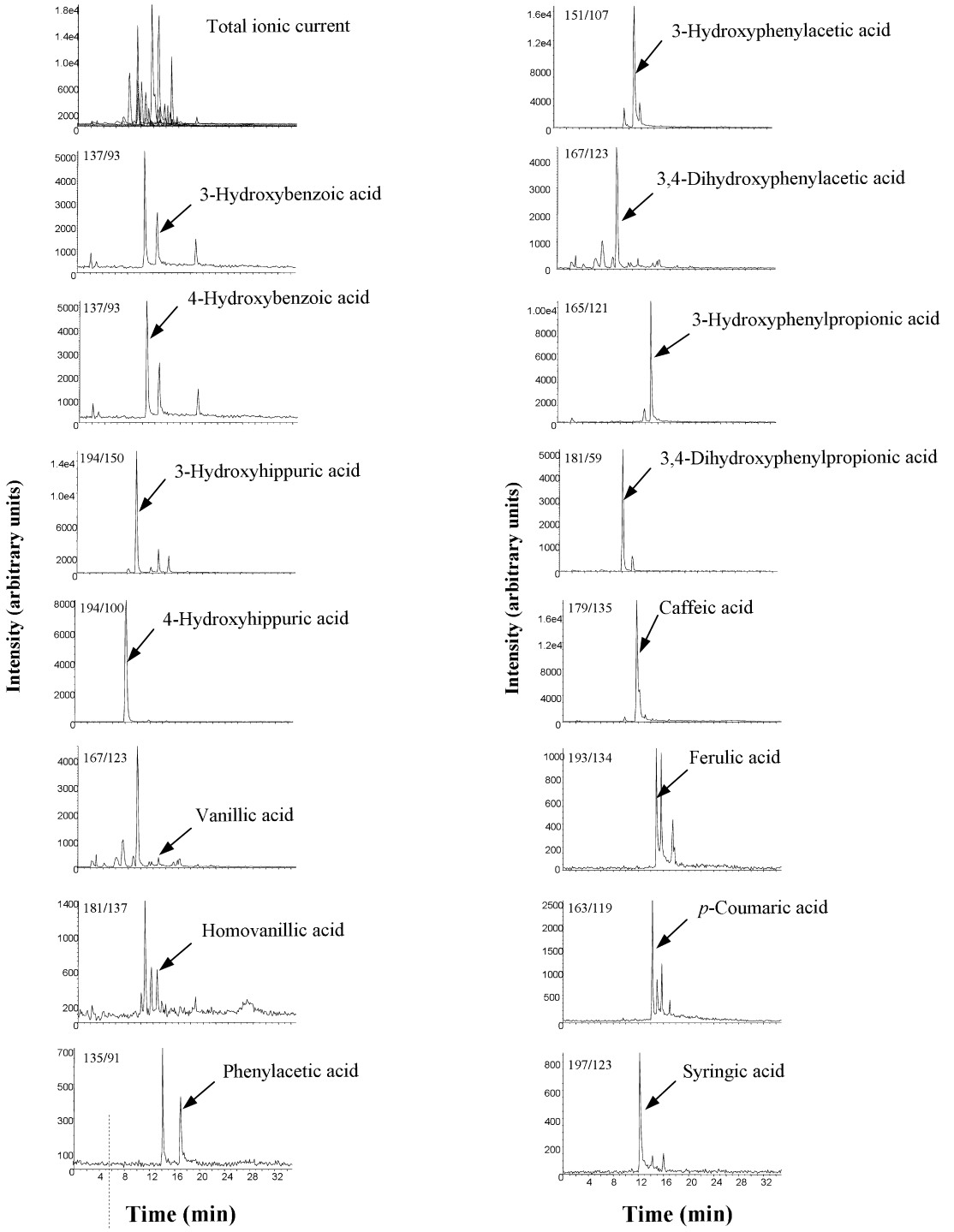


Fig. 2. Typical chromatograms generated by HPLC–ESI-MS–MS in MRM mode of human urine following consumption of a polyphenol-rich diet.

Table 2
Evaluation of precision and accuracy in the analysis of aromatic acids in human urine by HPLC–ESI-MS–MS; inter-, intra-day RSDs and accuracy data were obtained from quality control samples prepared as described in the Experimental section

Concentration (μ*M*)		Inter-day RSD[b] (%, *n* = 6)	Intra-day RSD[b] (%, *n* = 6)	Accuracy[c] (%)
Added	Found[a]			
0.5	0.53 (0.44–0.57)	7.8 (1.1–11.8)	7.9 (1.6–14.7)	6.1 (12.1–14.8)
1.5	1.56 (1.35–1.67)	8.6 (4.2–11.2)	6.3 (2.8–14.6)	4.3 (−9.7–11.8)
3.0	2.89 (2.65–3.14)	8.5 (4.7–12.3)	5.0 (0.3–13.1)	−3.6 (11.7–4.6)
6.0	6.15(5.58–6.56)	10.4 (7.1–14.5)	1.8 (0.1–7.1)	2.5 (−6.9–9.3)

[a] Means for all 14 aromatic acid standards; range given in parentheses. Concentrations were calculated after correction for the recovery of the internal standard.
[b] RSD = (standard deviation / mean) × 100.
[c] Accuracy = [(concentration found − concentration added) / concentration added] × 100.

acid, 3,4-dihydroxyphenylpropionic acid, caffeic acid and ferulic acid also showed an increase in urinary excretion varying between 2- and 8-fold. Some of these metabolites have been reported as major degradation products of polyphenols in previous in vivo and in vitro studies [13,14,27,28]. Hippuric acid, a known metabolite of polyphenols [28] was not included in this study due to its high concentration which would require a high sample dilution for HPLC–ESI-MS analysis not compatible with the lower concentrations of the other aromatic acids. An HPLC method with UV detection was previously developed for its analysis in urine and plasma [18].

Table 3
Recoveries of aromatic acid metabolites in human urine analyzed by HPLC–ESI-MS–MS; a blank urine sample was supplemented with 0.5,1.5,3 or 6 μ*M* of a mixture of standards (*n* = 6) and recoveries calculated as described in the Experimental section

Metabolites	Recoveries (%)
3-Hydroxybenzoic acid	79±11
4-Hydroxybenzoic acid	70±5
3-Hydroxyhippuric acid	66±6
4-Hydroxyhippuric acid	56±8
Vanillic acid	75±13
Homovallic acid	65±9
Phenylacetic acid	55±11
3-Hydroxyphenalacetic acid	74±7
3,4-Dihydroxyphenylacetic acid	68±6
3-Hydroxphenylpropionic acid	72±5
3,4-Dihydroxyphenylpropionic acid	65±6
Caffeic acid	86±9
Ferulic acid	85±10
p-Coumaric acid	72±4

The present results clearly indicate the applicability of this HPLC–ESI-MS–MS method to measure changes in urinary excretion of major aromatic acid metabolites associated with the consumption of polyphenol-containing foods in humans. We have recently applied this method to estimate the aromatic acid metabolites excreted by human volunteers after chocolate consumption [24] and in rats fed a diet enriched in different polyphenols [18]. In these two studies, the aromatic acid metabolites excreted in urine were equally or more abundant than the parent polyphenols which are in most studies the only ones considered.

HPLC–MS and HPLC–MS–MS are well-established techniques for the study of the metabolism of drugs and other xenobiotics [26]. The HPLC–ESI-MS–MS method described here will allow a much more rigorous investigation of the biotransformation of dietary polyphenols whereas our previous attempts to use HPLC–DAD or HPLC coupled to electrochemical detection (HPLC–ECD) were unsuccessful due to a lack of sensitivity or insufficient selectivity of detection. In contrast to GC–MS, which has also been recently used to estimate aromatic acid metabolites in urine [17], liquid chromatography coupled to mass spectrometry can meet the demand for rapid metabolite profiling by simplifying sample preparation, as no derivatization is needed.

Table 4
Urinary excretion of aromatic acid metabolites after consumption of a polyphenol-rich diet by one healthy volunteer; 24-h urine were collected after a 3-day diet low or rich in polyphenols; aromatic acid metabolites were quantified by HPLC–ESI-MS–MS

Metabolites	Urinary excretion (μmol/day)	
	Polyphenol-free diet	Polyphenol-rich diet
3-Hydroxybenzoic acid	0.8	4.7
4-Hydroxybenzoic acid	8.7	10.6
3-Hydroxyhippuric acid	6.5	53.8
4-Hydroxyhippuric acid	7.1	39.1
Vanillic acid	22.1	68.5
Homovallic acid	Nd	3.4
Phenylacetic acid	6.3	10.2
3-Hydroxyphenalacetic acid	7.9	94.1
3,4-Dihydroxyphenylacetic acid	4.4	9.9
3-Hydroxphenylpropionic acid	0.9	13.6
3,4-Dihydroxyphenylpropionic acid	1.0	2.8
Caffeic acid	7.0	17.5
Ferulic acid	5.6	12.6
p-Coumaric acid	0.3	1.4

Nd, not detected.

Acknowledgements

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