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Identification of polyphenols and their metabolites in human urine after cranberry-syrup consumption

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ABSTRACT

As the beneficial effects of American cranberry (*Vaccinium macrocarpon*) can be partly attributed to its phenolic composition, the evaluation of the physiological behaviour of this fraction is crucial. A rapid and sensitive method by ultra-performance liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry (UPLC-Q-TOF-MS) has been used to identify phenolic metabolites in human urine after a single dose of cranberry syrup. Prior to the analysis, metabolites were extracted using an optimised solid-phase extraction procedure. All possible metabolites were investigated based on retention time, accurate mass data and isotope and fragmentation patterns. Free coumaroyl hexose (isomer 1 and 2), dihydroxybenzoic acid, caffeoyl glucose, dihydroferulic acid 4-*O*-β-D-glucuronide, methoxyquercetin 3-*O*-galactoside, scopoletin, myricetin and quercetin, together with other 23 phase-I and phase-II metabolites, including various isomers, could be tentatively identified in the urine. Afterwards, the metabolites were simultaneously screened in the urine of different subjects at 0, 2, 4, and 6 h after the ingestion of cranberry syrup by Target Analysis™ software.

1. Introduction

American cranberry (*Vaccinium macrocarpon*) is a rich source of bioactive phenolic compounds with antiproliferative, antioxidant (He and Rui, 2006), antimicrobial (Foo et al., 2000), anti-inflammatory and anticarcinogenic activities (Neto et al., 2008). It contains large amounts of sugars, proanthocyanidins, flavonoids, organic acids, and also anthocyanins. Previously, it has been demonstrated that cranberry changes the surface hydrophobicity and biofilm formation of P-fimbriated *Escherichia coli*, which is related to urinary-tract infection (UTI) (Uberos et al., 2011).

Due to the properties of this fruit, research should be directed not only towards the characterization and study of its bioactivity, but also towards revealing the bioavailability of the phenolic components in order to gain new insight into their *in vivo* physiological behaviour. In this sense, six anthocyanins have been identified in human urine by HPLC-MS/MS in multiple-reaction mode (MRM) in which peonidin 3-*O*-galactoside was the most plentiful compound found after cranberry-juice ingestion (Ohnishi et al., 2006). The recovery of total anthocyanins in the urine over 24 h was estimated to be 5.0% of the amount consumed. In the same manner, quercetin and anthocyanins such as peonidin 3-*O*-galactoside and cyanidin 3-*O*-galactoside were detected in the rat urine after cranberry administration (Rajbhandari et al., 2011). The absorption and excretion of 20 cranberry-derived phenolics in plasma and urine have been studied by using GC–MS in selective ion monitoring (SIM) mode after the consumption of cranberry juice, sauces, and fruits (Wang et al., 2012). These studies have performed a targeted analysis to detect cranberry metabolites using MRM or SIM detection mode that represent only a selected part of the urinary spectra of cranberry. Using microLC-MS, the effect of cranberry consumption to prevent oxidative stress has been evaluated, and the main metabolites identified in human urine were isomers of salicyluric and dihydroxybenzoic acids, hippuric acid, and quercetin glucuronide (Valentová et al., 2007). None of these studies examined the phase-I metabolism of polyphenols. Phase-I metabolism involves oxidation, reduction, and hydrolysis, among other less frequent reactions, resulting in minor chemical changes that make a compound more hydrophilic and thus more effectively eliminated by the kidneys. These reactions expose or introduce a reactive functional group (–OH, –SH, $–NH_2$, –NH–, –COOH) in the molecule and usually preceed to phase-II metabolism (Holčapek et al., 2008).

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Liquid chromatography (LC) coupled to mass spectrometry has become a useful tool for separating and identifying metabolites. For improved speed, resolution and sensitivity, HPLC methods can be successfully transferred to UPLC, RRLC, and UHPLC, which operate with smaller particle sizes (<2 μm) of the stationary phase. Q-TOF analysers can provide excellent mass accuracy over a wide dynamic range and enable measurements of the isotope pattern, providing important additional information for determining the elemental composition. Furthermore, this instrument permits the rapid and efficient confirmation of the structure of metabolites in fragmentation studies, and also provides high selectivity using the extracted-ion chromatogram (EIC) mode when there are overlapping peaks. In our previous study, the phenolic fraction of cranberry syrup with beneficial properties against UTI by HPLC-TOF-MS was determined (Iswaldi et al., 2012). For the continuation of the earlier study, the aim of this work was to identify polyphenols from cranberry syrup and their metabolites that appear in human urine after a single dose of cranberry. Urinary metabolites were previously extracted by solid-phase extraction (SPE) with several sorbents and analysed by UPLC-ESI-Q-TOF-MS. Furthermore, a rapid multi-screening method was applied to determine the putative metabolites identified in urine 0, 2, 4 and 6 h after consumption of cranberry syrup.

2. Materials and methods

2.1. Chemicals

All chemicals were analytical HPLC reagent grade and used as received. Formic acid and acetonitrile used for preparing mobile phases were from Fluka (Sigma–Aldrich, Steinheim, Germany) and Lab-Scan (Gliwice, Sowinskiego, Poland), respectively. A G560E Vortex-Genie 2 (Scientific Industries, Bohemia, NY, USA) was used for mixing. Distilled water was deionized by using a Milli-Q system (Millipore, Bedford, MA, USA). The resisitivity of the deionized water was 18.2 MΩ.

2.2. Cranberry syrup

A cranberry syrup was purchased from a local pharmacy. It contained 1.10% (w/v) total phenolics, 0.71% (w/v) proanthocyanidins, and 0.10% (w/v) anthocyanins. The estimation of these amounts and the characterization of the phenolic compounds were consistent with our previous work (Iswaldi et al., 2012).

2.3. Subjects and study design

Four healthy volunteers (2 male and 2 female), who were non-smokers and not taking any medication, participated in the study. They were aged between 25 and 40 years and with a weight between 60 and 80 kg. Subjects were required to follow a diet low on polyphenols, which excluded fruits and vegetables, high-fiber products, and beverages such as tea, coffee, fruit juice, and wine, for 2 days before the study. After overnight fasting, early-morning urine samples were collected before (0 h) the ingestion of cranberry syrup and at time points, 2, 4, 6 h after the consumption of 0.6 mL/kg of cranberry syrup. An aliquot of each urine sample was acidified with 5% aqueous TFA solution (1:10, v/v). The urine samples were stored at −80 °C before analysis. The study protocol was approved by the ethics committee of the University of Granada, Spain.

2.4. Extraction of phenolic-derived metabolites from urine samples

Urine samples (4 mL) were diluted with 4 mL of 10 mM of oxalic acid and applied to Supelclean LC-18 (500 mg) (Supelco, Bellefonte, PA, USA), Evolute ABN (200 mg) (Biotage, Sweden) and Isolute ENV+ (500 mg) (Biotage, Sweden) SPE columns. The columns were previously conditioned with 10 mL of methanol and 30 mL of 10 mM of oxalic acid. Impurities were washed out with 20 mL of 10 mM of oxalic acid and the retained phenolic-derived metabolites were eluted with 25 mL methanol acidified with 0.1% HCl. The flow rate through the column was controlled by means of a vacuum manifold. The eluate obtained was carefully evaporated to dryness under vacuum (<40 °C), redissolved in 200 μL methanol:acetic acid (95:5, v/v), and filtered through 0.22 μm tetrafluoroethylene (PTFE) syringe filters prior to UPLC-Q-TOF-MS analysis.

2.5. Chromatographic and MS conditions

LC analyses were made with a Waters Acquity UPLC™ system (Waters Corporation, Milford, MA, USA). Separation was performed with a Zorbax Eclipse Plus C_{18} analytical column (4.6 × 150 mm, 1.8 μm particle size). Gradient program 1, used for the MS negative ion mode, consisted of 1% formic acid in water/acetonitrile (90:10, v/v) (phase A) and acetonitrile (phase B) at a constant flow rate of 0.5 mL/min using the following multi-step linear gradient: 0–20 min, from 5% B to 20% B; 20–25 min, from 20% B to 40% B; 25–30 min, from 40% B to 5% B; and 30–35 min, isocratic of 5% B. Subsequently, a different chromatographic method (gradient program 2) was used for the MS positive ion mode and consisted of 10% (v/v) aqueous formic acid (phase A) and acetonitrile (phase B) at a constant flow rate of 0.5 mL/min using the following gradient multi-step linear: 0–13 min, from 0% B to 20% B; 13–20 min, from 20% B to 30% B; 20–25 min, from 30% B to 80% B; and 25–30 min, from 80% B to 0% B; and 30–35 min, isocratic of 0% B. The column was set to room temperature and the injection volume was 1 μL for both gradient-elution programs (Iswaldi et al., 2012).

The UPLC instrument was coupled to a micrOTOF-Q™ (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionisation (ESI) interface. Parameters for analysis were set using negative and positive ion modes with spectra acquired over a mass range of 50–1000 *m/z*. The other optimum values of the ESI-MS parameters were: capillary voltage, +4000 V; dry gas temperature, 190 °C; dry gas flow, 9.0 L/min; nebulizer pressure, 2.0 bar; and spectra rate 1 Hz. The calibrant was a sodium formate cluster containing 5 mM sodium hydroxide and 0.2% formic acid in water:isopropanol (1:1, v/v), injected at the beginning of each run with a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA). All the spectra were calibrated prior to the compound identification. All operations were controlled by the newest DataAnalysis 4.0 software (Bruker Daltonik), which provided a list of possible elemental formulas by using the SmartFormula™ editor. The Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula. TargetAnalysis™ 1.2 software (Bruker Daltonik) was used for multi-target screening of metabolites in urine samples.

3. Results and discussion

3.1. Selection of the extraction and analytical conditions for the analysis of phenolic metabolites in urine

In the present study, because of the large number of compounds contained in each human urine sample, the aim was focused on metabolites of polyphenols. Thus, samples were extracted by SPE in order to concentrate metabolites of interest, which are generally

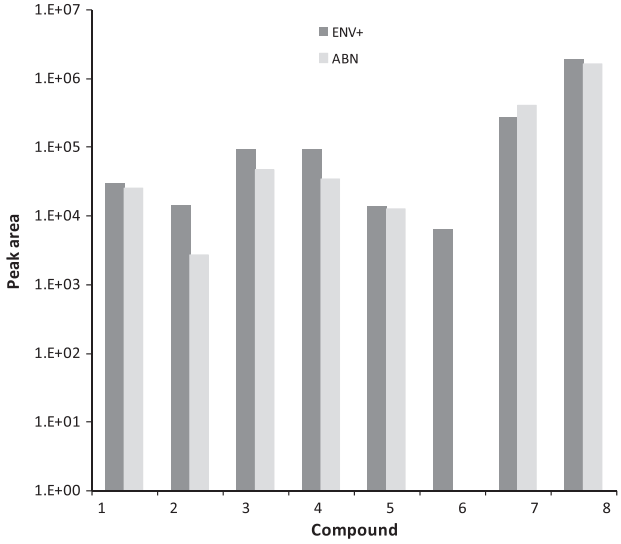

Fig. 1. Comparison of ENV+ and ABN SPE columns. Compound (1) coumaroylhexose isomer 1, (2) dihydroxybenzoic acid, (3) caffeoyl glucose, (4) coumaroylhexose isomer 2, (5) dihydroferulic acid-4-*O*-β-D-glucuronide, (6) methoxyquercetin-3-*O*-galactoside, (7) myricetin, and (8) quercetin. Peak area was represented in logarithmic scale.

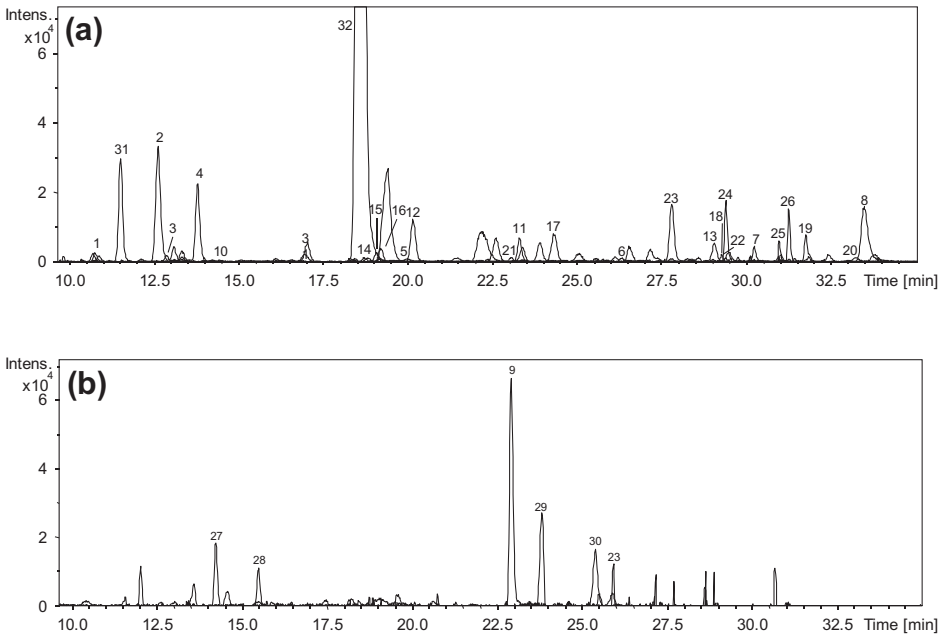

Fig. 2. Chromatographic profiles of urinary metabolites collected 4 h after cranberry-syrup ingestion: (a) extracted ion chromatograms (EICs) for the gradient program 1 and negative-ion mode, and (b) EICs for the gradient program 2 and positive-ion mode. Intensity scale was magnified to show the less-abundant compounds.

found in trace levels, and to avoid the contamination of the instrument. Three types of stationary phases, LC-18 (octadecylsilane phase), Evolute ABN (a water-wettable polymer-based sorbent) and Isolute ENV+ (a hydroxylated polystyrene divinylbenzene copolymer) columns, were tested by using the same extraction method. LC-18 column (500 mg) got saturated during the extraction process. Therefore, the use of this kind of SPE column was discarded for subsequent analysis. Evolute ABN (200 mg) and Isolute ENV+ (500 mg) columns gave cause to better results and the comparison of them to extract the compounds of interest is depicted in Fig. 1. As a preliminary result, larger numbers of compounds were found using Isolute ENV+ and, therefore, this cartridge was selected. This type of sorbent was also found to be appropriate for extracting relatively polar drug metabolites from urine and plasma (Altun et al., 2006). Then, a wide range of polar and semi-polar metabolite compounds were successfully separated with the previously optimised gradients by UPLC and analysed by coupling with Q-TOF-MS in both negative and positive ionisation modes. As an example of the chromatographic profiles, the extracted ion chromatograms (EICs) of the metabolites in human urine 4 h after the intake of cranberry syrup and obtained using negative and positive ion modes are presented in Fig. 2a and b, respectively. The proposed compounds, peak numbers, and retention times according to Fig. 2, molecular formula, *m/z* experimental, error, sigma value, and MS/MS major fragments are listed in Table 1.

3.2. Identification of native polyphenols in urine

The native phenolic compounds were studied in depth based on the matching of molecular weight (parent and product ions), isotope pattern, and retention time with those polyphenols previously characterized in cranberry syrup, and taking into account the polarity of the compounds. As shown in Fig. 3, the retention properties of compounds corresponding to various free polyphenols in cranberry syrup and human urine samples were very similar. Thus, it was possible to identify 10 native forms of polyphenols in urine samples using the negative-ion mode, and one native form using the positive-ion mode.

The structure of the metabolites was confirmed by MS^2 analysis. For instance, Fig. 4 shows the MS^2 spectra of some metabolites identified. For glycosylated compounds, it was taken into account that breakage occurred at the most labile linkage of the molecular ion, corresponding to the glycosidic bond, in agreement with results observed by ion-trap tandem MS and in-source fragmentation by TOF (Iswaldi et al., 2012; Gómez-Romero et al., 2010). In this manner, the neutral loss of monosaccharide residues was established by the mass difference between the glycoside and the aglycone form: a difference of 162 Da for hexose and 176 Da for glucuronide.

Compounds corresponding to peak **1** (RT 10.80 min) and **4** (RT 13.85 min) produced the same deprotonated molecular ion $[M-H]^-$ at *m/z* 325.0939 and 325.0927, respectively, and one prominent product ion $[M-H-162]^-$ at *m/z* 163 by loss of a sugar moiety (Fig. 4a). The minor fragment (*m/z* 119) was the subsequent loss of CO_2 (44 Da), suggesting the presence of carboxylic acid in the parent ion. Therefore, these compounds were proposed as coumaroyl hexose (isomers), this being consistent with a previous study (Iswaldi et al., 2012). In this way, caffeoyl glucose was also tentatively identified, corresponding to peak **3** (RT 12.92 min) with a parent ion at *m/z* 341.0864 and dihydroferulic acid 4-*O*-*β*-D-glucuronide (peak **5**, RT 20.02 min, *m/z* 371.0972). Moreover, the proposed analytical methodology made it possible to detect dihydroxybenzoic acid at RT 12.68 min (peak **2**) and *m/z* 153.0189. The urinary excretion of dihydroxybenzoic acid after ingestion of cranberry products agrees with the previous results (Valentová et al., 2007; Prior et al., 2010).

Three flavonols were tentatively identified in the urine samples: methoxyquercetin 3-*O*-galactoside (peak **6** RT 26.30 min, *m/z* 477.1036), myricetin (peak **7**, RT 30.20 min, *m/z* 317.0306), and quercetin (peak **8,** RT 33.43 min, *m/z* 301.0349). Dihydroferulic acid 4-*O*-*β*-D-glucuronide showed a daughter ion at *m/z* 175, corresponding to the loss of glucuronide moiety ($[M-H-176]^-$). Quercetin (Fig. 4b) presented a characteristic fragmentation pattern, with two major fragments at *m/z* 151 and 179, corresponding to an A^- ring fragment released after retro Diels–Alder (RDA) fission and retrocyclization after fission on bonds 1 and 2 (Dueñas et al.,

Table 1
Free polyphenols, phase I and II metabolites identified in human urine 4 h after intake of cranberry syrup and classified by the type of metabolic reaction.

Conjugation	Peak	Selected ion	RT (min)	Molecular formula	Experimental *m/z*	Error (ppm)	mSigma value	MS/MS fragments	Identification of putative metabolites
Negative ion mode									
Free polyphenols	1	$[M-H]^-$	10.80	$C_{15}H_{17}O_8$	325.0939	3.1	0.0163	163	Coumaroyl hexose (isomer 1)
	2	$[M-H]^-$	12.68	$C_7H_5O_4$	153.0189	2.8	0.0021		Dihydroxybenzoic acid
	3	$[M-H]^-$	12.92	$C_{15}H_{17}O_9$	341.0864	4.2	0.0038	179	Caffeoyl glucose
	4	$[M-H]^-$	13.85	$C_{15}H_{17}O_8$	325.0927	−0.6	0.0048	163	Coumaroyl hexose (isomer 2)
	5	$[M-H]^-$	20.02	$C_{16}H_{19}O_{10}$	371.0972	−3.2	0.0118	175	Dihydroferulic acid 4-*O*-*β*-D-glucuronide
	6	$[M-H]^-$	26.30	$C_{22}H_{21}O_{12}$	477.1036	−0.6	0.0433		Methoxyquercetin 3-*O*-galactoside
	7	$[M-H]^-$	30.20	$C_{15}H_9O_8$	317.0306	1.0	0.0206		Myricetin
	8	$[M-H]^-$	33.43	$C_{15}H_9O_7$	301.0349	−1.5	0.0028	179, 151	Quercetin
Phase I metabolites									
Hydrogenation ($+H_2$)	10	$[M-H]^-$	14.50	$C_{19}H_{27}O_{12}$	447.1463	−3.0	0.0304	445	Canthoside A + H_2
	11	$[M-H]^-$	23.29	$C_{25}H_{29}O_{13}$	537.1584	−5.5	0.0044	373	Caviunin glucoside + H_2
Dehydrogenation ($-H_2$)	12	$[M-H]^-$	20.16	$C_{15}H_{11}O_6$	287.0549	−4.3	0.0030		(+)-Catechin − H_2
	13	$[M-H]^-$	29.03	$C_{15}H_{15}O_9$	339.0709	−3.6	0.0029		Caffeoyl glucose − H_2
Hydroxylation (+OH)	14	$[M-H]^-$	18.74	$C_{16}H_{17}O_{10}$	369.0827	0.0	0.0156		Chlorogenic acid + OH
	15	$[M-H]^-$	19.09	$C_{22}H_{21}O_{13}$	493.0971	−3.3	0.0278		Methoxyquercetin-3-*O*-galactoside + OH
	16	$[M-H]^-$	19.22	$C_{16}H_{19}O_{11}$	387.0934	0.3	0.0085	175	Dihydroferulic acid 4-*O*-*β*-D-glucuronide + OH
Hydration ($+H_2O$)	17	$[M-H]^-$	24.29	$C_{16}H_{19}O_{10}$	371.0981	−0.8	0.0050	353	Chlorogenic acid + H_2O
Phase II metabolites									
Glucuronidation ($+C_6H_8O_6$)	18	$[M-H]^-$	29.20	$C_{21}H_{17}O_{13}$	477.0717	8.9	0.0397	301	Quercetin − glucuronide
Methylation ($+CH_2$)	19	$[M-H]^-$	31.71	$C_{16}H_{11}O_7$	315.0506	−1.4	0.0044	301	Quercetin + CH_3
	20	$[M-H]^-$	33.17	$C_{16}H_{11}O_8$	331.0439	−6.3	0.0129	317	Myricetin + CH_3
Dehydrogenation + glucuronidation	21	$[M-H]^-$	23.04	$C_{21}H_{19}O_{12}$	463.0882	0.1	0.0047		(+)-Catechin − H_2-glucuronide
Methylation + glucuronidation	22	$[M-H]^-$	29.23	$C_{22}H_{19}O_{14}$	507.0766	−2.7	0.0245	317	Myricetin + CH_3-glucuronide
	23	$[M-H]^-$	27.76	$C_{22}H_{19}O_{13}$	491.0830	0.3	0.0069	301	Quercetin + CH_3-glucuronide (isomer 1)
	24	[M−H]–	29.37	$C_{22}H_{19}O_{13}$	491.0824	−1.5	0.0019	301	Quercetin + CH_3-glucuronide (isomer 2)
	25	$[M-H]^-$	30.92	$C_{22}H_{19}O_{13}$	491.0847	−3.3	0.0158	301	Quercetin + CH_3-glucuronide (isomer 3)
	26	$[M-H]^-$	31.21	$C_{22}H_{19}O_{13}$	491.0820	2.2	0.0075	301	Quercetin + CH_3-glucuronide (isomer 4)
Positive ion mode									
Free polyphenols	9	$[M+H]^+$	22.92	$C_{10}H_9O_4$	193.0502	3.7	0.0320		Scopoletin
Dehydrogenation	27	$[M+H]^+$	14.23	$C_7H_5O_3$	137.0233	0.0	0.0124		2-Hydroxybenzoic acid − H_2
Hydroxylation + glucuronidation	28	$[M+H]^+$	15.50	$C_{15}H_{15}O_{10}$	355.0641	−5.4	0.0464		7-Hydroxycoumarin + OH-glucuronide
Methylation + glucuronidation	29	$[M+H]^+$	23.82	$C_{16}H_{17}O_9$	353.0865	−0.5	0.0495		7-Hydroxycoumarin + CH_3-glucuronide
Methylation	30	$[M+H]^+$	25.38	$C_{11}H_{11}O_4$	207.0664	5.7	0.0184		Scopoletin + CH_3
Methylation + glucuronidation	23	$[M+H]^+$	25.92	$C_{22}H_{21}O_{13}$	493.0982	1.1	0.0132		Quercetin + CH_3-glucuronide[a]
Other metabolites									
Glycine conjugation ($+C_2H_3ON$)	31	$[M-H]^-$	11.57	$C_9H_9NO_4$	194.0459	1.9	0.0047		Salicyluric acid (2-hydroxybenzoic acid-glycine)
	32	$[M-H]^-$	18.62	$C_9H_8NO_3$	178.0512	1.5	0.0009	134	Hippuric acid

The same peak number corresponds to the same compound in Fig. 1.
RT: retention time.
[a] This compound has also been detected in negative ion mode.

2008). The presence of myricetin and quercetin have been previously detected in rat urine by HPLC-MS/MS (triple quadrupole) (Rajbhandari et al., 2011). Quercetin was also detected in plasma and human 24 h urine using HPLC with electrochemical detection, showing that this compound is bioavailable from a diet containing moderate amounts of blue and red berries (Koli et al., 2010). In general, flavonoid glycosides are cleaved prior to absorption in different parts of the intestines (Del Rio et al., 2010) and, in fact, although quercetin and myricetin exists in cranberries as aglycone and glycosidic forms (Iswaldi et al., 2012), only the aglycone forms were excreted. In contrast, dihydroferulic acid-4-*O*-*β*-glucuronide and methoxyquercetin 3-*O*-galactoside were detected in human urine.

Using positive ionization mode, scopoletin (peak **9**, RT 22.92 min, *m/z* 193.0502) was the only native polyphenol tentatively identified in urine and it was found as one of the main urinary metabolites (Fig. 2b).

Cranberry procyanidins and anthocyanidins were not found in human urine by the proposed methodology. Similar results were found in rat urine after 5 days of repeated treatment with procyanidin dimer B3 (Gonthier et al., 2003), and very low amounts were found after a single treatment of grape seed procyanidins B1, B2 and B3 to rats (Tsang et al., 2005). The metabolism of procyanidins is controversial (Monagas et al., 2010) and a recent study suggests further degradation by the gut microflora before absorption, and therefore subsequent microbial metabolites could be active forms (Stoupi et al., 2010). Notably, human urine treated *ex vivo* with dried cranberry juice had an inhibitory effect on the adhesion of bacteria related to UTI, although procyanidins were not detected (Valentová et al., 2007). In our previous work, petunidin was the

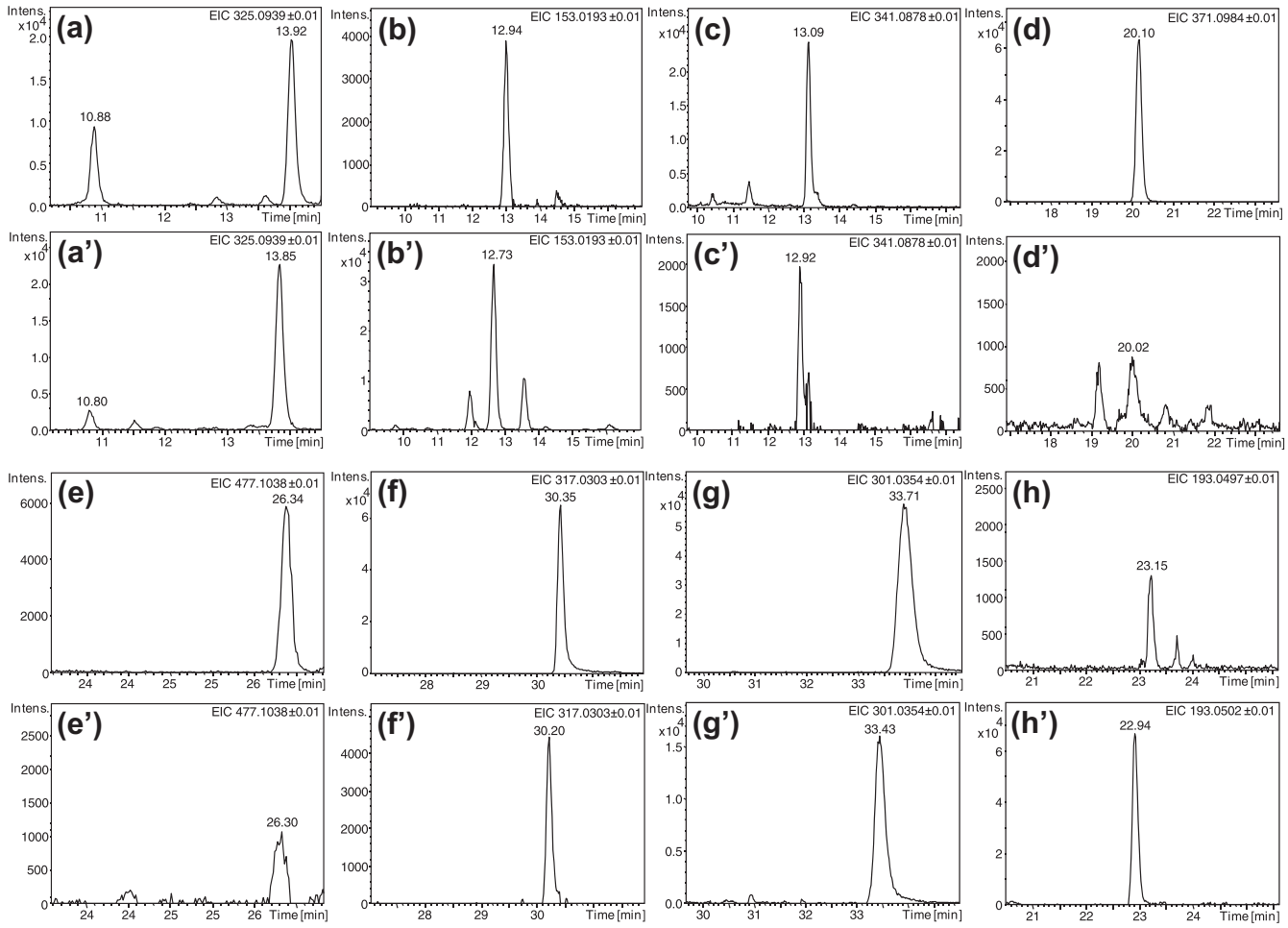

Fig. 3. Representative extracted ion chromatograms (EICs) of native polyphenols identified in cranberry syrup (a–h) and compared with those detected in urine at 4 h (a′–h′). Negative-ion mode: (a) coumaroyl-hexose isomer 1 and 2, (b) dihydroxybenzoic acid, (c) caffeoyl glucose, (d) dihydroferulic acid-4-*O*-*β*-D-glucuronide, (e) methoxyquercetin-3-*O*-galactoside, (f) myricetin, and (g) quercetin. Positive-ion mode: (h) scopoletin.

only anthocyanidin found in cranberry syrup and at a low concentration (Iswaldi et al., 2012). This fact together with the instability of this class of compounds and their metabolites (Manach et al., 2004) hamper their detection in biological samples, such as in urine.

3.3. Identification of phenolic-derived metabolites

As for native polyphenols, a similar procedure was followed for tentatively identified phenolic-derived metabolites in urine while taking into account that all the data provided by the literature concerning metabolic reactions was gained *in vivo*. As mentioned above, high-resolution ESI-Q-TOF measurements provide accurate mass and isotope-pattern data, which enable reductions in the number of possible molecular formulae for presumed novel compounds to be matched against available databases. Table 1 summarizes all the putative metabolites found in urine and classified by the type of metabolism reaction. There were three types of phenolic-derived metabolites: (i) compounds found in their native form, which were described above; (ii) phase-I metabolites formed by chemical modifications, such as hydrogenation ($M+H_2$), dehydrogenation ($M-H_2$), hydroxylation (M+OH), and hydration ($M+H_2O$); and (iii) phase-II metabolites formed by conjugation, such as methylation ($M+CH_3$), glucuronidation ($M+C_6H_8O_6$), and other conjugation reactions.

Nine phase-I metabolites were successfully characterized, the most abundant being phenolic acids (canthoside A + H_2; caffeoyl glucose − H_2; chlorogenic acid + OH and + H_2O; dihydroferulic acid 4-*O*-*β*-D-glucuronide + OH; and 2-hydroxybenzoic − H_2) and flavonols (caviunin glucoside + H_2 and methoxyquercetin 3-*O*-galactoside + OH) that were presumably absorbed and metabolized. One monomeric flavanol, (+)-catechin − H_2, was also detected.

Phase-I reactions usually preceded phase-II metabolism, though not necessarily, since, in our case, parent compounds presented one or more suitable functional groups, concerning OH and COOH. Hence, it was possible to identify mainly glucuronoconjugated and methylated forms of polyphenols and phase-I metabolites from cranberry.

To confirm the structure of phase-II metabolites, tandem MS analysis was automatically performed by Q-TOF. The MS^2 spectrum of the compound with *m/z* 477.0717 (peak **18**, RT 29.20 min) gave an abundant daughter ion at *m/z* 301 by neutral loss of 176 Da. Based on the metabolism rule of phase II *in vivo* and the above MS/MS spectrum data, the latter metabolite was identified as the monoglucuronide product of quercetin, which has been previously identified in human urine as well (Valentová et al., 2007; Watson and Oliveira, 1999; Mullen et al., 2004; Mullen et al., 2006). Peak **19** (RT 31.71 min) was observed as a deprotonated ion $[M-H]^-$ at *m/z* 315.0506. A product ion at *m/z* 301 suggested the loss of a methyl group ($[M-H-14]^-$) to form quercetin

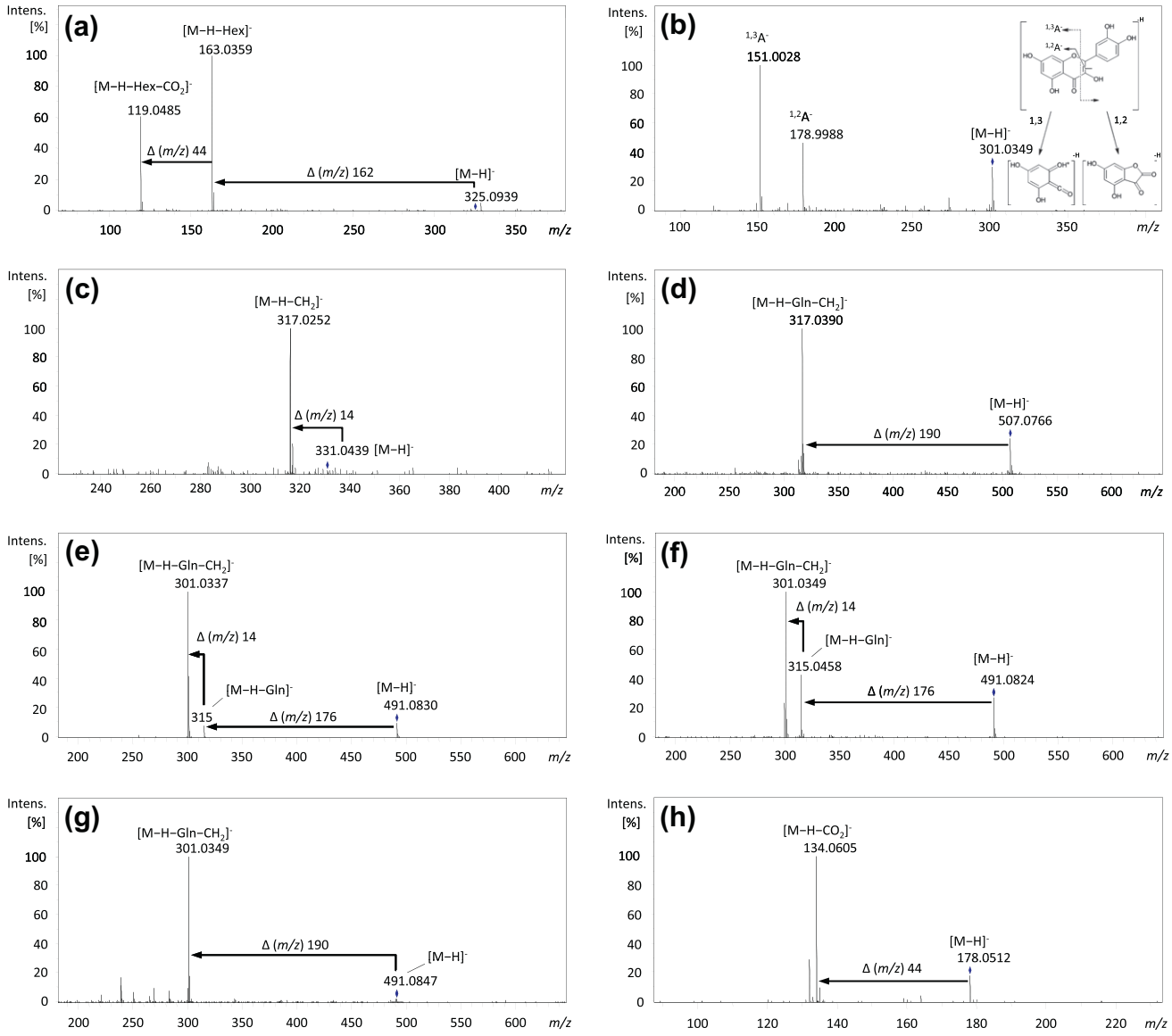

Fig. 4. Negative ESI-MS^2 spectra of (a) coumaroyl-hexose isomer 1, (b) quercetin, (c) myricetin + CH_3, (d) myricetin + CH_3-glucuronide, (e–g) quercetin + CH_3-glucuronide isomers 1–3, and (h) hippuric acid. Hex, hexose; Gln, glucuronide.

aglycone. Therefore, this peak was tentatively identified as quercetin-CH_3. Similarly, the methylation product of myricetin (myricetin-CH_3) (peak **20**, RT 33.17 min) was observed as an $[M-H]^-$ ion at *m/z* 331.0439 and the major MS/MS fragment was at *m/z* 317 (Fig. 4b). According to the metabolism rule of phase II *in vivo*, the methylation reactions may occur at the myricetin skeleton since six free OH groups were found.

Myricetin + CH_3-glucuronide with a *m/z* of 507.0766 (peak **22**, RT 29.23 min) was also detected and it presented a prominent product ion at *m/z* 317, which corresponded to the neutral loss of both the methyl and glucuronide groups (a total of 190 Da). This is consistent with previous studies on rats in which glucuronidated and methylated forms were the main conjugated compounds detected (Lin et al., 2012). Other phase-II metabolites, (+)-catechin − H_2-glucuronide, and several isomers of quercetin + CH_3-glucuronide (for example, Fig. 4e–g) were found in the urine samples. Similarly, eight *O*-methylated and glucuronidated forms of quercetin were identified after incubation of apple-derived polyphenols with hepatocytes cells, which mimic the metabolism occurring in liver (Kahle et al., 2011).

Other metabolites were also identified in urine. The presence of hippuric acid (peak **32**) was detected as major compound in the reconstructed chromatogram at *m/z* 178.0512 (Fig. 2a). The loss of CO_2 resulting in the formation of the ion *m/z* 134 was clearly seen in the related collision spectrum collected over the chromatographic peak (Fig. 4h). The metabolite corresponding to peak **31** (RT 11.57 min) and at *m/z* 194.0450 was tentatively identified as salicyluric acid (also known as 2-hydroxyhippuric acid). This compound is formed by conjugation of salicylic acid (2-hydroxybenzoic acid) with glycine mediated by glycine N-acyltransferase. Urinary excretion of the latter compounds has been previously reported in rats fed concentrated cranberry powder (Koli et al., 2010), and in humans after cranberry-juice consumption (Valentová et al., 2007).

These results suggest that at least one fraction of the ingested compounds was absorbed intact and acted *in vivo* in this form prior to excretion, whereas another fraction was absorbed, metabolised, and excreted in urine as phase-I and phase-II metabolites, glucuronidation and methylation being the most frequent reactions. Further studies of the biological properties of these metabolites would be helpful to understand the action mechanism of cranberry syrup.

Table 2
Metabolites detected in urine from four human subjects at different times after consumption of cranberry syrup.

Peak	Putative metabolites identified	Time points (hours after ingestion of cranberry syrup)			
		Subject-1	Subject-2	Subject-3	Subject-4
Negative ion mode					
Free polyphenols					
1	Coumaroyl-hexose (isomer 1)	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
2	Dihydroxybenzoic acid	0, 2, 4, 6	0, 2, 4, 6	2, 4, 6	2, 4, 6
3	Caffeoyl-glucose	2	2, 4	2, 4	2, 4, 6
4	Coumaroyl-hexose (isomer 2)	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
5	Dihydroferulic acid 4-*O*-*β*-glucuronide	2, 4	2, 4, 6	nd[a]	2, 4
6	Methoxyquercetin-3-*O*-galactoside	2	6	nd	4
7	Myricetin	2, 4, 6	nd	2, 4, 6	2, 4, 6
8	Quercetin	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
Phase I metabolites					
10	Canthoside A + H_2	2, 4, 6	4, 6	4, 6	4, 6
11	Caviunin glucoside + H_2	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
12	(+)-Catechin − H_2	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
13	Caffeoyl glucose – H_2	6	nd	4, 6	4, 6
14	Chlorogenic acid + OH	2, 4	2, 4	nd	4
15	Methoxyquercetin-3-*O*-galactoside + OH	2, 4	2, 4	2	2, 4, 6
16	Dihydroferulic acid 4-*O*-*β*-glucuronide + OH	2, 4	2, 4, 6	2, 4, 6	2, 4, 6
17	Chlorogenic acid + H_2O	4, 6	6	2, 4, 6	2, 4, 6
Phase II metabolites					
18	Quercetin glucuronide	2	nd	nd	2, 4
19	Quercetin + CH_3	2, 4, 6	2, 4, 6	2	2, 4, 6
20	Myricetin + CH_3	2, 4, 6	2, 4	2, 4, 6	2, 4, 6
21	(+)-catechin − H_2-glucuronidation	2, 4, 6	2, 4	2, 4, 6	4, 6
22	Myricetin + CH_3-glucuronide	nd	nd	2, 4	2, 4
23	Quercetin + CH_3-glucuronide (isomer 1)	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
24	Quercetin + CH_3-glucuronide (isomer 2)	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
25	Quercetin + CH_3-glucuronide (isomer 3)	2, 4, 6	2, 4, 6	2	4, 6
26	Quercetin + CH_3-glucuronide (isomer 4)	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
Positive ion mode					
9	Scopoletin	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
23	Quercetin + CH_3-glucuronide	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
27	2-Hydroxybenzoic acid – H_2	2, 4	nd	2, 4, 6	2, 4, 6
28	7-Hydroxycoumarin + OH-glucuronide	nd	4	nd	4
29	7-Hydroxycoumarin + CH_3-glucuronide	2, 4, 6	6	nd	4, 6
30	Scopoletin + CH_3	2, 4, 6	2, 4, 6	2, 4, 6	2, 4, 6
Other metabolites					
31	Salicyluric acid	0, 2, 4, 6	0, 2, 4, 6	0, 2, 4, 6	0, 2, 4, 6
32	Hippuric acid	0, 2, 4, 6	0, 2, 4, 6	0, 2, 4, 6	0, 2, 4, 6

[a] nd: Not detectable.

3.4. Screening of phenolic-derived metabolites during urinary excretion

The urinary-metabolite spectra from four subjects after cranberry-syrup consumption were compared before (wash-out period, 0 time point) and after 2, 4, and 6 h using the novel software TargetAnalysis™ 1.2. This tool enabled an automatic multi-screening target analysis of the metabolites based on the information provided by the UPLC-ESI-Q-TOF analysis, regarding the retention time, accurate *m/z*, and isotope pattern, which were matched using stored data run through our in-house database. Table 2 shows free polyphenols, phase-I and phase-II metabolites detected in human urine at the study times 0, 2, 4 and 6 h. Most of the metabolites appeared between 2 and 4 h after cranberry-syrup consumption. The urinary excretion of several of the metabolites studied varied between subjects. Such variability in absorption or post-absorptive metabolism is attributable partly to variability in the expression of metabolizing enzymes and transporters due to genetic variability within the population (Spencer et al., 2008) and in colonic microbiota (Van Duynhoven et al., 2011).

Presumably, the metabolites studied should not be present at time point 0 and should appear at later points. However, hippuric acid, salicyluric acid, and dihydroxybenzoic acid were detected at time point 0. The origin of such phenolic acids is diverse, as some can be produced by colonic microflora from a number of different phenolic compounds and also endogenously from amino acids such as tyrosine and phenylalanine (Prior et al., 2010). In this sense, hippuric acid is one of the main urinary endogenous metabolites and an important compound from the pathway of benzoate elimination. Previous studies have suggested that dihydroxybenzoic acids may be metabolites of cranberry polyphenols (Zhang and Zuo, 2004) and may be formed by ring fission of quercetin and derived from anthocyanins (Koli et al., 2010). As commented above, our results suggest that dihydroxybenzoic acid may also be excreted as a free native form. In this respect, Fig. 5 shows the time course of this compound and other free polyphenols represented for subject 4 after cranberry-syrup consumption. The estimated amounts of these compounds, expressed as the peak area, reached maximum concentrations in human urine 4 h after administration.

4. Concluding remarks

In the present study, human urinary metabolites of polyphenols from cranberry syrup were firstly extracted by ENV+ SPE and then succesfully profiled using UPLC-Q-TOF-MS. In total, 32 metabolites were tentatively identified, including phase I metabolites and phase II metabolites, such as methylated and glucuronide

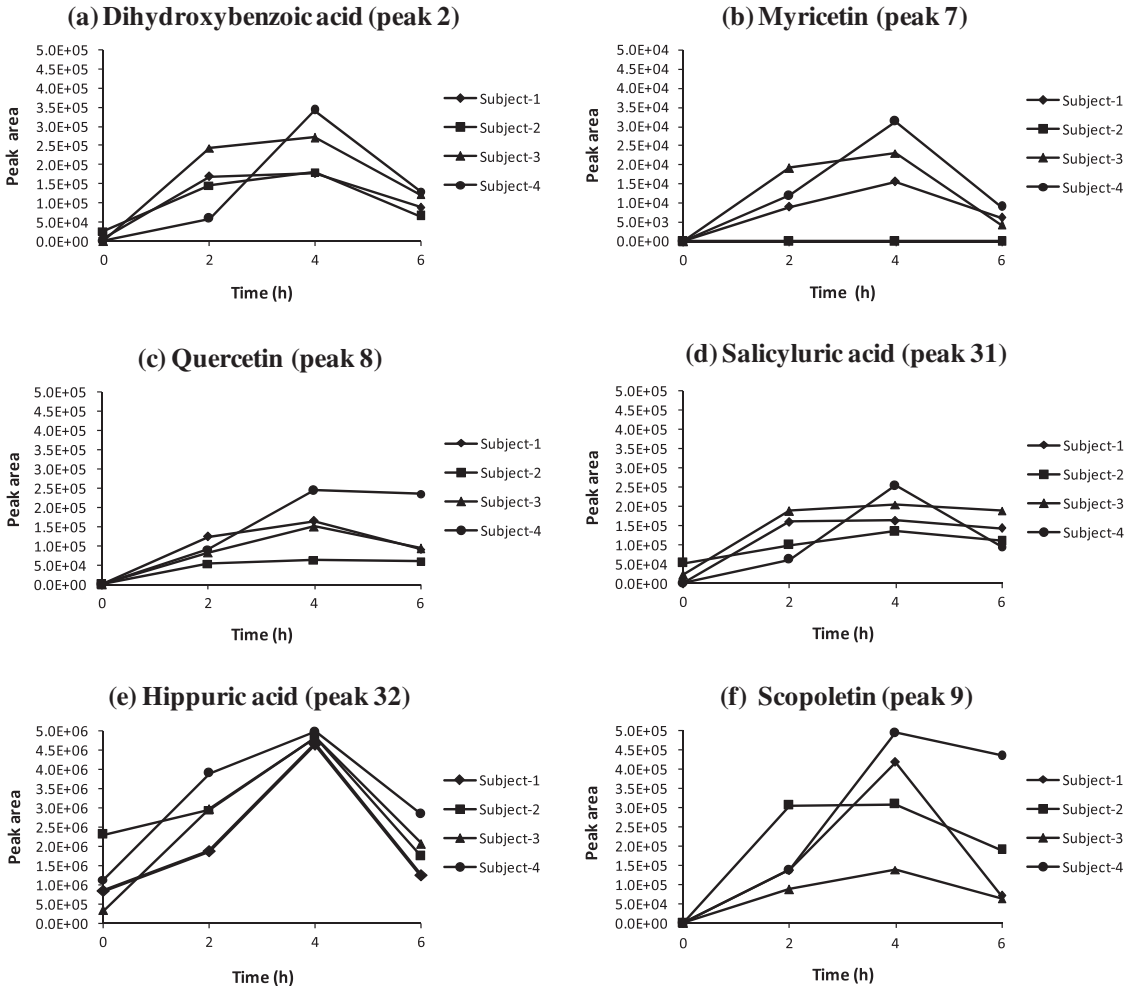

Fig. 5. Changes in major urinary metabolites over time after cranberry-syrup consumption: (a) dihydroxybenzoic acid, (b) myricetin, (c) quercetin, (d) salicyluric acid, (e) hippuric acid, and (f) scopoletin.

conjugated forms. Among them, free phenolic acid derivatives (coumaroyl hexose, dihydroxybenzoic acid, caffeoyl glucose, and dihydroferulic acid 4-*O*-β-D-glucuronide), flavonols (methoxyquercetin 3-*O*-galactoside, myricetin and quercetin) and one coumarin (scopoletin) were detected in the human urine. Most of these urinary metabolites have been identified for the first time after cranberry consumption. The proposed analytical technique has proven to be an useful tool to study biological matrices such as urine. In this manner, further studies are in progress for complementing our knowledge of the bioavailability of cranberry-derived polyphenols, and getting new insights into the active metabolites.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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