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RESEARCH ARTICLE

Untargeted Metabolomics Analytical Strategy Based on Liquid Chromatography/Electrospray Ionization Linear Ion Trap Quadrupole/Orbitrap Mass Spectrometry for Discovering New Polyphenol Metabolites in Human Biofluids after Acute Ingestion of *Vaccinium myrtillus* Berry Supplement

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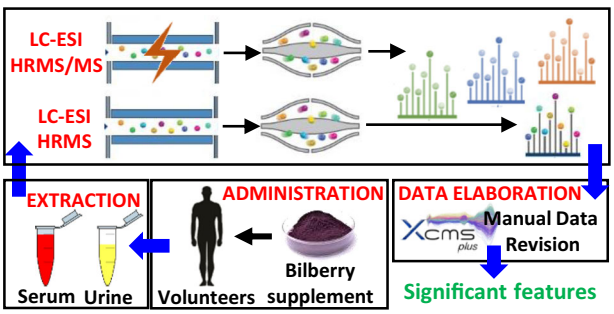

Abstract. In this work, liquid chromatography, coupled with an electrospray ionization hybrid linear ion trap quadrupole/Orbitrap mass spectrometry, has been used to accurately identify polyphenol metabolites in human serum and urine after acute ingestion of a *V. myrtillus* berry supplement. The supplement was obtained by cryo-milling of bilberries, which were freeze-dried within 1 week after their harvesting, to maintain the berry native composition. Thirty-six derivatives of benzoic acids, hydroxyhippuric acids, cinnamic acids, phenylpropionic acids, phenylvaleric acids, phenylpentenoic acids and abscisic acid, together with two berry-native anthocyanins, one flavonol metabolite and two catechol derivatives were putatively identified in the investigated biofluids. The annotated compounds included 13 metabolites, among glucuronides and sulphates of phenylvaleric and phenylpentenoic acids, which have been identified for the first time in human biofluids after ingestion of *V. myrtillus* berries. It should be emphasized that the presence of phenylvaleric and phenylpentenoic acid derivatives is in agreement with their origin from fruit native flavanol monomers and oligomers, which are widely distributed in *Vaccinium* berries, but usually overlooked in metabolomics studies regarding bilberry. The identification of these compounds confirmed the key-role of untargeted metabolomics approach in the discovery of new metabolites which could be biologically active.

Keywords: Untargeted metabolomics, High-resolution mass spectrometry, Human serum, Human urine, *Vaccinium myrtillus*, Polyphenol metabolites

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Introduction

Vaccinium myrtillus berry (bilberry) is widely used for the preparation of transformed products and supplements, owing to its high content of phenolic compounds that have been often related to a number of health-protecting properties [1, 2], thus suggesting bilberry as a functional food in human

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diet [3]. For example, some in vitro studies showed an antiproliferative effect of polyphenol-rich bilberry extracts against different cancer cell lines [4–6], as well as antimicrobial activities towards some important human pathogens [7, 8].

However, digestive processes produce significant modifications of fruit native polyphenols, thus making the in vitro studies not actually informative about the in vivo effects. Nevertheless, it should also be noted that the intake of polyphenol-rich fruits, including bilberry, has been demonstrated to provide beneficial effects against many diseases (e.g. hyperglycaemia, cardiovascular risk, colon cancer) both in animal studies and clinical trials [9–11], making extremely interesting to understand the modifications of native compounds during digestion.

Despite the importance of understanding the final products of digestion of fruit native polyphenols, the absorption, distribution, metabolism and elimination (ADME) of berry phenolic compounds have been investigated in depth only with limited extent [12–15] and, as far as we know, no untargeted studies focusing on the ADME of bilberry polyphenols have been reported in literature up to date. Moreover, in both animals and humans, the fate of anthocyanins–the most abundant bilberry polyphenols–is currently poorly understood, notwithstanding the high number of targeted studies investigating anthocyanin bioavailability in biological fluids after ingestion of various small berries or their transformed products [16–26]. The limited comprehension of the metabolic fate of anthocyanins is probably due to the targeted metabolomics approach adopted in these studies that strongly limited the number and type of potentially identifiable metabolites. More in detail, in these studies, a targeted investigation based on liquid chromatography coupled with diode-array or tandem mass spectrometric (LC-MS/MS) analysis of native anthocyanins and their predicted metabolites was carried out. The latter analytes were methylated derivatives, sulphate and glucuronide conjugates deriving from phase II metabolism [21], as well as benzoic, phenylacetic and hydrocinnamic acids suggested by in vitro studies as the gut microflora metabolites of anthocyanins [27]. LC-MS/MS targeted analysis allowed for quantifying the aforementioned metabolites, even at low concentrations (i.e. pmol/mL-nmol/mL), using relatively easy analytical protocols and instruments. However, the identification of new potentially bioactive metabolites is not possible with this approach. Conversely, the untargeted metabolomics analysis, although it requires more complex instrumentations (typically high-resolution mass spectrometers, HRMS), and analytical protocols, is able to provide the exact mass of the detected ions, thus drastically reducing the number of possible elemental formula ascribable to the parent molecules. Furthermore, untargeted approaches produce tens of thousands signals and requires a long elaboration of the acquired data [28], thus requiring highly trained staff in the field of study.

It is also important to underline that, although some studies focused on anthocyanin metabolites in human biofluids after ingestion of *V. myrtillus* berries, these fruits contain significant concentrations of a number of polyphenols belonging to classes other than anthocyanins [29, 30]. In this regard, the polyphenolic composition of *V. myrtillus* berries was recently investigated by our research group, highlighting the occurrence of a number of flavonols, flavanol oligomers and phenolic acids, in addition to anthocyanins [31]. Accordingly, also the metabolites of non-anthocyanidin phenolic classes could be involved in the biological activity of *V. myrtillus* fruits.

For all these reasons, the untargeted metabolomics analysis of human biological fluids after the ingestion of *V. myrtillus* berries is necessary in order to identify new significant metabolites of bilberry polyphenols in human body. Accordingly, the aim of this research was the LC-HRMS untargeted metabolomics analysis of serum and urine polyphenolic metabolites in healthy volunteers after the administration of a bilberry-based supplement. Figure 1 illustrates an intuitive graph of the workflow adopted in this study for the untargeted serum and urine metabolomics.

Experimental

Reagents and Standards

Methanol and acetonitrile LC-MS Ultra CHROMASOLV™ and formic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). The ultrapure water was obtained by purifying demineralized water in a Milli-Q system from Millipore (Bedford, MA, USA). The internal standard trans-cinnamic-d5 acid (CIN-d5) was purchased from CDN ISOTOPES Inc. (Pointe-Claire, Quebec, Canada). The surrogate standards L-tryptophan-2′,4′,5′,6′,7′-d5 (TRY-d5) and *N*-benzoyl-d5-glycine (hippuric acid-d5, HIP-d5) were obtained from Sigma Aldrich and CDN ISOTOPES Inc., respectively. Reference standards for compound identification were supplied as follows: delphinidin-3-glucoside, delphinidin-3-galactoside, cyanidin-3-galactoside and cyanidin-3-glucoside by Extrasynthese (Genay, France); (4R)-5-(3,4-dihydroxyphenyl)-γ-hydroxyvaleric acid sodium salt by Toronto Research Chemicals (Toronto, Canada); vanillic acid, ferulic acid, abscisic acid, syringic acid, sinapic acid, chlorogenic acid, α-hydroxyhippuric acid (α-HA), *o*-hydroxyhyppuric acid (o-HA), *p*-hydroxyhyppuric acid (p-HA), 3,4-dimethoxycinnamic acid, 3,4,5-trimethoxycinnamic acid, 3-(4-hydroxyphenyl)propionic acid and 3-(3,4-dihydroxyphenyl)propionic acid by Sigma Aldrich.

Study Design

Ten healthy voluntary subjects participated in the study. None of them presented concomitant food allergies or metabolic diseases. The study followed the guidelines set by the Helsinki Declaration (http://www.fda.gov/ohrms/dockets/dockets/06d0331/06D-0331-EC20-Attach-1.pdf) and all subjects provided written informed consent prior to the study.

V. myrtillus-based supplement was obtained as specified in the section S1 of the Supplementary Material. A single dose of 25 g of this berry supplement mixed with 500 mL of drinking water was orally administered to all subjects convened early in the morning after 10 h of fasting. Then, the volunteers remained

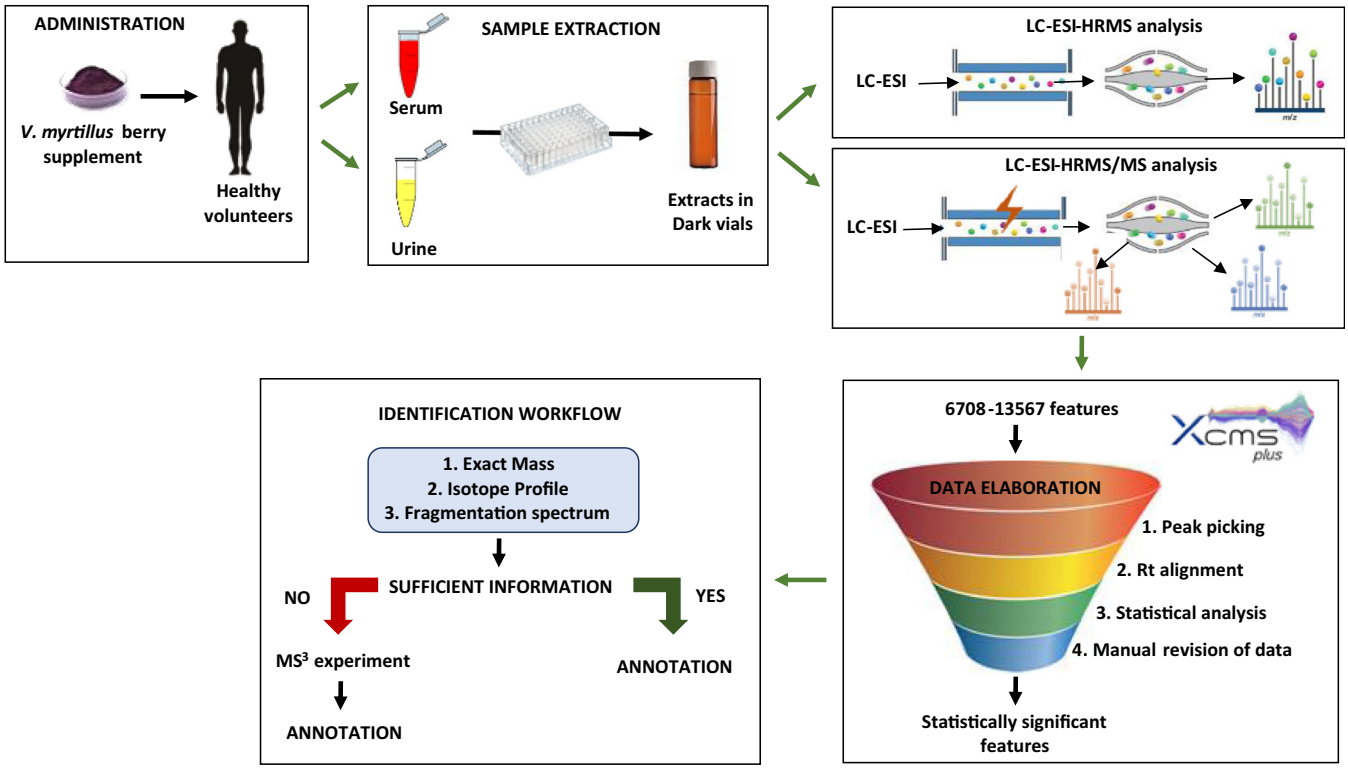


Figure 1. Workflow of the experimental steps followed in this study for human serum and urine metabolomics of bilberry polyphenols

fasting for 6 h, drinking approximately 100 mL of water every 30 min. The supplement dose was chosen in order to simulate real experimental conditions elsewhere adopted within randomized phase II clinical studies. In our study, total polyphenol content of the supplement was 38.8 mg catechin equivalent/gram (see the section S1 of the Supplementary Material), thus each volunteer received a total dose of polyphenols of about 1 g. This amount was an equivalent of the daily dose administered to a group of patients suffering from prostate cancer, resulting effective in slowing the progression of the disease [32].

Ethical approval for the administration was obtained for a phase I-II study (approval n. SPE 14.178 AOUC, 30th May 2016).

Serum and urine of each volunteer were collected at baseline and different sampling times (30, 60, 120, 240 and 360 min) after the supplement consumption. For urine, cumulative samples were also collected after 24 and 48 h from the *V. myrtillus* supplement ingestion. In this regard, it should be remarked that volunteers were asked to keep off fruits and vegetables during the whole study period.

After the collection, both serum and urine samples were divided in aliquots of 500 µL, frozen at −80 °C and stored until LC-HRMS analysis was performed.

Urine and Serum Extraction

Before the LC-MS analysis, urine and plasma aliquots were thawed and extracted according to the methods described in the section S2 of the Supplementary Material. Briefly, urine samples were filtered and diluted according to the preparation procedure for untargeted metabolomics studies using LC-MS analysis [33]. As regards serum samples, the matrix was more complex than urine because of the presence of high molecular weight molecules that must be removed [34]. For this purpose, the extraction of serum samples consisted in the precipitation of proteins and removal of phospholipids adding different mixture of cold organic solvents (see S2 section of the Supplementary Material for details).

For both urine and serum samples, surrogated (TRY-d5 and HIP-d5) and internal (CIN-d5) standards were added in order to evaluate the quality of extraction and LC-MS analysis. In addition, the extraction blanks and the quality control (QC) samples (consisting of a mixture of the same aliquot of all urine or serum samples treated with the same aforementioned extraction conditions) were injected before the LC-MS analysis of the individual samples.

LC-MS and LC-MS/MS Analyses

For both serum and urine samples, LC analysis was performed on a Dionex Ultimate 3000 HPLC system equipped with a Kinetex C18 column (150 mm×2.1 mm I.D., particle size 2.6 µm) and a guard column containing the same stationary phase (Phenomenex, Torrance, CA, USA). The LC system was coupled with a hybrid linear ion trap Fourier Transform (LTQ FT) Orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) by an electrospray ionization (ESI) probe for MS and MS^n analysis both in positive and negative ionization.

According to the general protocols reported in the scientific literature for serum and urine metabolomics [33, 34], the chromatographic gradient was optimized aiming at obtaining the best compromise between throughput and metabolome coverage/peak resolution, since short runs will detect fewer ions than long ones for a variety of reasons, including ion suppression. Moreover, longer batches are more prone to a loss of instrumental stability, such as a decline in sensitivity, retention time shifts or changes in mass accuracy. Empirically, a reasonable compromise between analysis time and metabolome coverage is provided by a 10–12 min LC-MS gradient, which will allow the achievement of good metabolite separation, together with a satisfactory analytical throughput [33]. Furthermore, an isocratic step of 1 min at a very high water percentage (e.g. 95–99%) is usually recommended to allow the salts to be washed away from the column [33]. In our study, the chromatographic conditions below reported were selected on the basis of the results obtained by some experiments aiming at the evaluation of peak area stability, retention time shifts and *m/z* mass accuracy of the aforementioned surrogated and internal standards over 3 days' injections. According to these experiments, for both serum and urine analysis, the column temperature was set at 40 °C, the injection volume was 5 μL and the flow rate was 350 μL/min. Water (eluent A) and acetonitrile (eluent B), both with 0.1% formic acid, were used as mobile phases. For chromatographic analysis of urine sample, the following gradient was adopted: 0–1 min isocratic 5% B, 1–7 min linear gradient 5–45% B, 7–8.5 min linear gradient 45–80% B, 8.5–10.5 min isocratic 80% B, 10.5–11 min linear gradient 80–5% B, 11–12 min 5% B. The elution gradient used for the analysis of serum samples was the following: 0–1 min isocratic 5% B, 1–12.5 min linear gradient 5–100% B, 12.5–14 min isocratic 100% B, 14–14.3 min linear gradient 100–5% B, 14.3–15.3 min 5% B. Full details regarding chromatographic analysis, including a reconstructed chromatogram of the identified metabolites in a representative urine sample, as well as ESI and mass spectrometer conditions, were described in the section S3 of the Supplementary Material. Both the urine and serum QC samples were injected 70 times under the chromatographic conditions specified above, obtaining very good performances for the entire injection queues (e.g. RSD% values of peak area in between 3.1 and 13.2%, see Figures S1–S6 and Tables S1–S4 of the Supplementary Materials for the complete results).

As regards the dataset from nutritional challenge, each sample was analyzed, both under positive and negative ionization, using two different mass acquisition methods for each ionization mode. The first method consisted of a full scan (mass range from 100 to 1000 Da) at a mass resolution of 30,000 FWHM (full width at half maximum for *m/z* 400) in centroid mode. Then, based on the data-dependent acquisition (DDA) mode, the LC-MS/MS analysis of each sample was performed in order to achieve the mass fragmentation spectra. In this method during the chromatographic run, both full scan and MS^2 spectra of the three most intense ions of each full scan were acquired. The resolving power for MS^2 scans was 7500. Product ions were generated in the LTQ trap at collision energy 35 eV using an isolation width of 2 Da. Such double injections with two different mass spectrometry acquisition methods allowed for the collection of high-quality full scan data dedicated to further statistical analysis, while second injection with DDA modality allowed for an automatic collection of high number of MS^2 and pseudo-MS^n spectra.

For both urine (or serum) LC-MS and LC-MS/MS analyses, a quality control (QC) sample consisting of a pool of all urine (or serum) samples, together with a solvent solution (methanol/water 1:1), were injected in every 10 samples for the evaluation of the performance of the analytical system in terms of retention times, mass accuracy, signal drift, and carry over phenomena. For the injection order, samples were randomized within each injection batch.

In addition, whenever necessary MS^3 experiments were carried out for selected ions showing statistically significant differences before and after *V. myrtillus* berry ingestion. These additional experiments were performed in order to obtain the loss of glucuronide and sulphate units in the first mass fragmentation experiment (MS^2) and to achieve the fragmentation of deconjugated metabolites in the second mass fragmentation experiment (MS^3) for the comparison with the reference standard spectrum. Three samples were reinjected for each ion of interest and samples were chosen based on maximal intensity.

Data Processing and Statistical Analysis

All raw data were manually inspected using the Qual browser module of Xcalibur version 2.0.7 (Thermo Fisher Scientific). The LC-MS raw files were converted to mzXML with the MSConvert utility included in ProteoWizard [35]. Then, the mzXML files were processed with the software XCMS *plus* (The Scripps Research Institute, La Jolla, CA, USA) that allows for obtaining data processing (feature detection and retention time alignment) and data analysis through statistics tools [36]. Firstly, feature detection step aimed to the identification of all signals caused by true ions and avoid detection of false positives. During this processing, the maximum mass accuracy was set equal to 5 ppm, the minimum signal to noise ratio was fixed equal to 3 and only peaks with an intensity greater than 10,000 cps for at least three consecutive scans were considered as features. Then, retention time alignment is needed for correcting retention time differences among runs and combining data from different samples. After this step, the maximum retention time deviation across samples to consider two peaks in two different samples as the same compound was set equal to 5 s. The parameters evaluated in order to optimize the extraction of features and the values of such parameters selected after the optimization are given in Table S5 and Table S6 of the Supplementary Material. Among different XCMS parameters the minfrac (i.e. the minimum number of samples per group, expressed as percentage, necessary for a *m/z* feature to be considered significant and to be retained in the following workflow of identification, see Figure 1) was the most critical, as it largely influenced the amount of features obtained during the entire data processing. Several values of

minfrac were tested in this study as shown in the aforementioned supplementary tables. Final decision considered minfrac of 0.8, meaning that, in order to consider a feature as valid, at least 80% of samples in a group must contain that feature. Reason for such value was motivated by the scope to verify the occurrence of common for most of participant's responses to a dietary intervention, rather than identification of individuals or subgroups characterized by particular metabotypes.

As regards urine, the normalization of feature intensities for the volume of each sample was performed in order to remove differences due to the diverse urine volumes [37].

Afterward, statistical analysis of data was performed in order to evidence features with statistically different intensities in urine and/or serum of volunteers before and after *V. myrtillus* berry administration. Both for serum and urine samples, the statistic comparison between different sampling time groups were performed using one-way analysis of variance (ANOVA) and the non-parametric Wilcoxon signed-rank test, as suggested by Gowda and co-workers [36]. Accordingly, the *P* value associated to the comparison among the baseline and the post-ingestion intensities were calculated. Principal component analysis (PCA) was performed by the Compound Discoverer software, version 2.1 (Thermo Fisher Scientific).

Finally, the identification of features with an intensity statistically higher in biofluids collected after bilberry administration in respect to the baseline ones was carried out according to the criteria previously reported by our research group [31]. Briefly, the detected mass of the pseudo-molecular ion must differ from the exact mass of proposed formula in a maximum value of 5 ppm (mass accuracy ≤5 ppm) and the isotope ratio difference compared to the theoretical isotope profile must be below 20%. Then, the structural elucidation of ions of interest was performed through the evaluation of fragment ions present in MS^2 and MS^3 fragmentation spectra.

According to metabolomics guidelines, four levels of identification are distinguished. Briefly, level I is defined when the proposed structure is compared with the reference standard (identified compound); for level II the comparison with reference standards is not necessary and the identification is performed based upon physicochemical properties and similarity with spectral libraries (putatively annotated compound); identification at level III is based on characteristic physicochemical properties of a chemical class of compounds, or by spectral similarity to known compounds of a chemical class (putatively characterized compound classes); level IV includes the unknown compound [38]. Recently, a more detailed level of identification was proposed for unknown compounds distinguishing the cases in which an unequivocal molecular formula can be attributed (level IV) and only the exact mass was available (level V) [39]. In the present study, the absence of commercial standards of glucuronide and sulphate conjugated compounds made impossible the identification with level I. For this reason, the majority of the features considered in the identification workflow corresponded to putatively annotated compounds (level II), namely without chemical reference standards, based upon chromatographic behaviour and similarity with spectral libraries. In this manuscript, we used the terms "putative/putatively" or "tentative/tentatively", meaning a level II identification.

Results and Discussion

Assessment of Data Quality

The QC injected each ten samples during every LC-MS and LC-MS/MS batches assessed the quality of metabolomics data excluding mass calibration problems and retention times drift. Indeed, the mass accuracy and the retention time variations of surrogate and internal standards were found below 5 ppm and 2 s, respectively. Moreover, the variation of integrated area of the surrogate standards (added before the extraction) and internal standard (added before the analysis) in all the QC injections resulted lower than 20%, thus confirming the repeatability of the extraction and excluding the possibility of signal drift and carry over phenomena during the LC-MS and LC-MS/MS analysis. Details regarding analytical performance for internal and external standards in QC samples and entire dataset are given in Table 1.

Figure 2 shows PCA score plots of serum and urine samples under positive and negative ionization modes. It can be easily observed that QC samples (black circles) are tightly grouped in all four PCA plots, showing no batch effect, or trend due to the injection order (i.e. drop in signal intensity). Study samples are distributed homogenously with no visible trends according to post-prandial time point. Interestingly, few outliers can be however identified. More in detail, in case of the serum matrix, samples from volunteers 4, 7 and 8 analyzed in positive ionization mode (Figure 2A) were clearly separated from all the others, whereas in negative ionization (Figure 2B) the separation interested volunteers 3 and 4, even though it was less evident. As regards urine, in positive ionization (Figure 2C) samples from the volunteer 3 tend to be separated from the rest of study samples, while the analysis in negative mode (Figure 2D) gave rise to a more widespread distribution of the analyzed samples, with only the volunteer 10 slightly separated from the others.

Feature Selection

Data processing with XCMS Plus (peak picking and t_R alignment) resulted in a very large number of *m/z* features in both serum and urinary samples (i.e. 6708 and 13,567, respectively). Statistical analysis allowed to reduce ions of interest to a few hundreds, highlighting features that showed in serum and/or urine statistically different signals ($P \leq 0.05$) between the baseline level and the maximum intensity observed after supplement ingestion. The time profile intensities of ions were further manually inspected in order to highlight only features showing an increasing trend in respect to baseline. Finally, the resulting list of features has been reviewed in order to manually exclude *m/z* features, such as in-source fragmentation and other solvent/metal adducts of the same ion. From this procedure of data processing 36 features were selected (Table 2). All the selected features showed a statically different maximum intensity in

Table 1. Mean Values, Standard Deviation (SD) and Relative Standard Deviation (RSD%) of Peak Area Determined for Surrogate/Internal Standards Hippuric Acid-d5 (HIP-d5), Tryptophan-d5 (TRY-d5) and *Trans*-Cinnamic Acid-d5 (CIN-d5) in Quality Control, as well as in Serum and Urine Samples, both in Positive (+) and Negative (−) Ionization

	HIP-d5 *Serum* (+)	TRY-d5	CIN-d5	HIP-d5 *Serum* (−)	TRY-d5	CIN-d5
QC ($n = 27$)						
Mean	1.3E + 08	3.2E + 08	4.2E + 05	2.4E + 08	1.3E + 07	1.1E + 08
SD	5.9E + 06	1.4E + 07	2.2E + 04	1.1E + 07	8.2E + 05	3.5E + 06
RSD%	4.6	4.4	5.3	4.6	6.1	3.3
QC + Samples ($n = 82$)						
Mean	1.3E + 08	3.3E + 08	4.3E + 05	2.4E + 08	1.4E + 07	1.1E + 08
SD	7.9E + 06	2.4E + 07	2.4E + 04	2.0E + 07	1.3E + 06	7.7E + 06
RSD%	5.9	7.2	5.6	8.3	9.7	7.2
	Urine (+)			*Urine* (−)		
QC ($n = 35$)						
Mean	9.4E + 07	1.6E + 07	2.3E + 06	6.4E + 08	2.6E + 07	9.5E + 04
SD	1.3E + 07	1.4E + 06	2.5E + 05	3.1E + 07	1.7E + 06	9.5E + 03
RSD%	13.8	9.1	10.8	4.8	6.7	10.0
QC + Samples ($n = 110$)						
Mean	8.5E + 07	1.6E + 07	2.3E + 06	6.3E + 08	2.7E + 07	9.8E + 04
SD	6.6E + 06	1.5E + 06	3.7E + 05	5.8E + 07	3.7E + 06	9.7E + 03
RSD%	7.7	9.5	15.7	9.2	13.7	9.9

serum and/or urine collected within 6 h after the supplement ingestion, compared to the pre-ingestion level. More in detail, among the 36 features selected, 3 were identified in serum, 24 in urine and 9 in both biofluids.

Table S7 of the Supplementary Material summarises the most probable level I identification and ID reported in Human Metabolome Database (HMD), PubChem (PC) and Chemical Entities for Biological Interest (CHEBI) for the identified

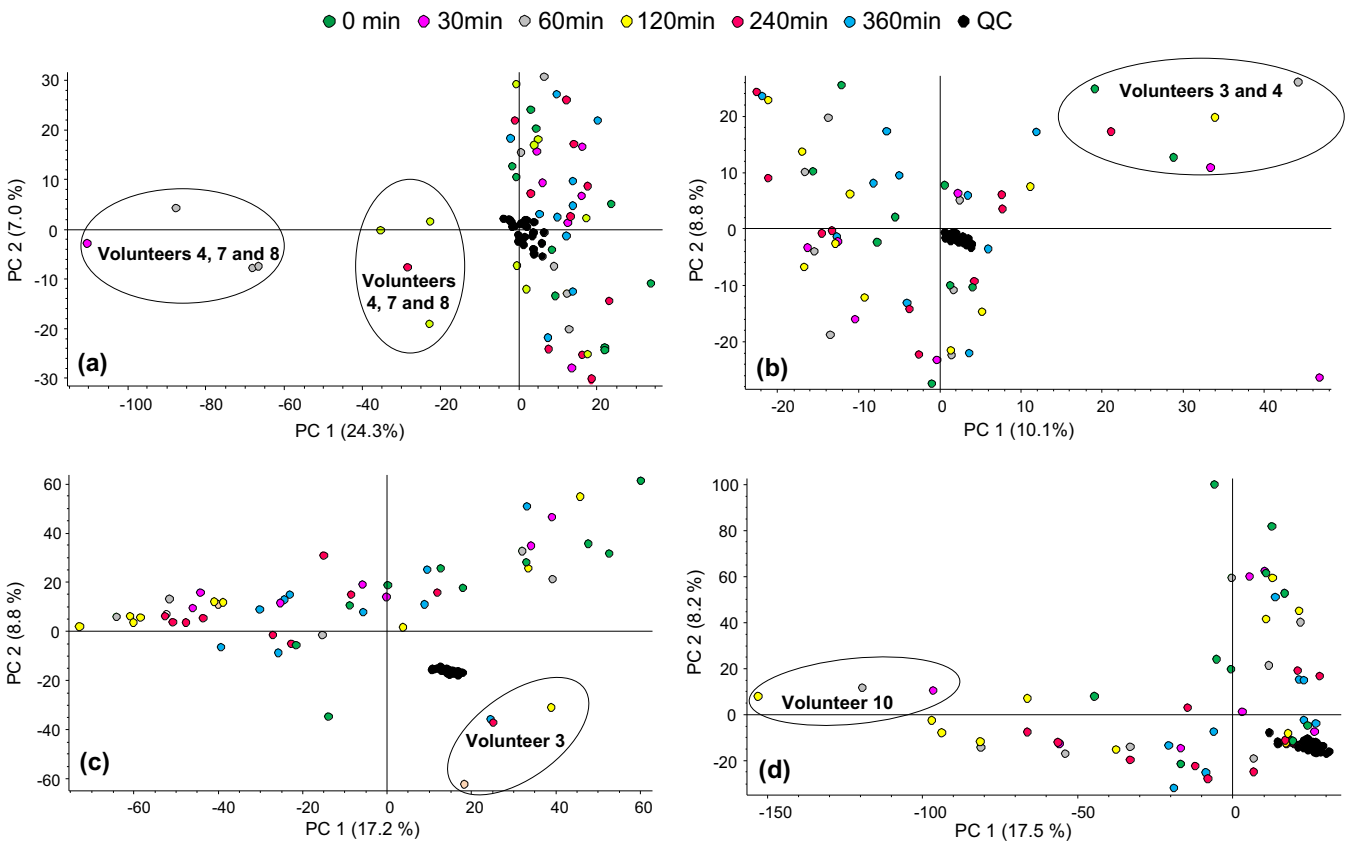


Figure 2. Principal component score plots (PC 1 vs. PC 2) of serum and urine samples analyzed under positive and negative ionization modes. (**a**) Serum samples analyzed under positive ionization; explained variance 31.3%. (**b**) Serum samples analyzed under negative ionization; explained variance 18.9%. (**c**) Urine samples analyzed under positive ionization; explained variance 26.0%. (**d**) Urine samples analyzed under negative ionization; explained variance 25.7%. Note that circles have only the visual function of highlighting outlier samples and are not the result of a cluster analysis

Table 2. Retention Time (t_R, min), Experimental Mass of the Parent Ion (Da), Characteristic Mass Fragments, Proposed Formula, Corresponding Exact Mass (Da), and Accuracy (Δ, ppm) of Peaks Tentatively Identified in Serum (S) and/or Urine (U) Samples after Acute Ingestion of *Vaccinium myrtillus* Supplement. Mass Fragments Can Be Produced during MS^2 and/or MS^3 Experiments, Being the Latter the Result of In-Source Fragmentation or Dedicated Experiment; in both Cases the MS^2 mass fragments reported in italic were the precursor ions of MS^3 fragments, either resulting of in-source fragmentation or dedicated experiment. Fragments reported in bold were the most abundant of each fragmentation spectrum. The Attributions Reported in Bracket in the Identification Column Are the Most Probable Level I Annotations

Peak	t_R	Parent ion	Mass Fragments		Formula	Exact Mass	Δ	Identification	Biological fluid
			MS^2	MS^3					
1	2.4	343.0663[a]	175.0243, *167.0346*, **113.0243**	152.0113, **123.0450**, 108.0218	$C_{14}H_{16}O_{10}$	343.0671	−2.3	Hydroxy-methoxy benzoic acid glucuronide I (Vanillic acid glucuronide I)	U ($P = 0.006$)
2	3.0	274.0019 [a]	**194.0455**	**150.0561**	$C_9H_9NO_7S$	274.0027	−2.9	*o*-Hydroxyhippuric acid sulphate	S ($P = 0.006$); U ($P = 0.006$)
3	3.3	277.0017 [a]	233.0121, **197.0452**	**182.0221**, 153.0554	$C_9H_{10}O_8S$	277.0024	−2.6	Hydroxy-dimethoxy benzoic acid sulphate (Syringic acid sulphate)	U ($P = 0.014$)
4	3.3	373.0768 [a]	***197.0452***, 182.0212, 175.0247, 113.0243	**182.0218**, 153.0554	$C_{15}H_{18}O_{11}$	373.0776	−2.1	Hydroxy-dimethoxy benzoic acid glucuronide I (Syringic acid glucuronide I)	U ($P = 0.006$)
5	3.4	188.9860 [a]	**109.0297**, 79.9576	NA	$C_6H_6O_5S$	188.9863	−1.6	Catechol sulphate	S ($P = 0.011$); U ($P = 0.019$)
6	3.5	329.0873 [a]	167.0347, **123.0451**	NA	$C_{14}H_{18}O_9$	329.0870	0.9	Hydroxy-methoxy benzoic acid hexoside (Vanillic acid glucoside)	U ($P = 0.006$)
7	3.6	343.0663 [a]	175.0246, ***167.0349***, 113.0245	**152.0114**, 123.0452, 108.0218	$C_{14}H_{16}O_{10}$	343.0671	−2.3	Hydroxy-methoxy benzoic acid glucuronide II (Vanillic acid glucuronide II)	U ($P = 0.008$)
8	3.7	399.0924 [a]	***223.0606***, 175.0241	**179.0711**	$C_{17}H_{20}O_{11}$	399.0933	−2.2	Hydroxy-(dihydroxyphenyl) pentenoic acid glucuronide	U ($P = 0.011$)
9	3.8	401.1081 [a]	***225.0768***, 175.0245	207.0658, 163.0767, **101.0246**	$C_{17}H_{22}O_{11}$	401.1089	−2.0	Hydroxy-(dihydroxyphenyl) valeric acid glucuronide I	U ($P = 0.009$)
10	3.9	373.0767 [a]	***197.0455***, 175.0249, 113.0246	182.0221, **153.0557**, 121.0295	$C_{15}H_{18}O_{11}$	373.0776	−2.4	Hydroxy-dimethoxy benzoic acid glucuronide II (Syringic acid glucuronide I)	U ($P = 0.032$)
11	3.9	224.0561 [a]	180.0661, 123.0452, **100.0040**	NA	$C_{10}H_{11}NO_5$	224.0564	−1.3	Hydroxy-methoxy hippuric acid	U ($P = 0.006$)
12	4.0	303.0173 [a]	***223.0608***, 221.0452	**179.0714**	$C_{11}H_{12}O_8S$	303.0180	−2.3	Hydroxy-(dihydroxyphenyl) pentenoic acid sulphate	U ($P = 0.006$)
13	4.0	401.1081 [a]	***225.0610***, 175.0245	207.0660, 163.0764, **101.0245**	$C_{17}H_{22}O_{11}$	401.1089	−2.0	Hydroxy-(dihydroxyphenyl) valeric acid glucuronide II	U ($P = 0.008$)
14	4.0	465.1029 [b]	**303.0501**	NA	$C_{21}H_{21}O_{12}$	465.1028	0.2	Delphinidin-hexoside	U ($P = 0.006$)
15	4.2	415.1234 [a]	**239.0923**, 175.0249, 113.0245	NA	$C_{18}H_{24}O_{11}$	415.1246	−2.9	Trimethoxy-hydrocinnamic acid glucuronide *or* Hydroxy-(hydroxy-methoxyphenyl) valeric acid glucuronide	S ($P = 0.008$); U ($P = 0.008$)
16	4.2	305.0328 [a]	***225.0764***, 207.0659	207.0659, 163.0764, 123.0453, **101.0246**	$C_{11}H_{14}O_8S$	305.0337	−2.9	Hydroxy-(dihydroxyphenyl) valeric acid sulphate	S ($P = 0.019$); U ($P = 0.008$)
17	4.2	285.0609 [a]	175.0245, **113.0244**, 109.0245	NA	$C_{12}H_{14}O_8$	285.0616	−2.4	Catechol glucuronide	S ($P = 0.006$); U ($P = 0.014$)
18	4.2	369.0817 [a]	*193.0501*, 175.0243, **113.0243**	178.0275, **149.0611**, 134.0377	$C_{16}H_{18}O_{10}$	369.0827	−2.7	Hydroxy-methoxycinnamic acid glucuronide I (Ferulic acid glucuronide I)	S ($P = 0.006$)
19	4.3	449.1080 [b]	**287.0544**	NA	$C_{21}H_{21}O_{11}$	449.1078	0.5	Cyanidin-hexoside	U ($P = 0.006$)
20	4.3	287.0224 [a]	***207.0663***	189.0556, **163.0767**, 145.0660, 123.0455	$C_{11}H_{12}O_7S$	287.0231	−2.4	Hydroxy-(hydroxyphenyl) pentenoic acid sulphate I	U ($P = 0.006$)
21	4.4	353.0870 [a]	**191.0558**, 179.0350	NA	$C_{16}H_{18}O_9$	353.0878	−2.3	Chlorogenic acid	U ($P = 0.019$)
22	4.5	317.0329 [a]	302.0095, ***237.0764***, 222.0528	**222.0533**	$C_{12}H_{14}O_8S$	317.0337	−2.5	Hydroxy-(hydroxy-methoxyphenyl)-pentenoic acid sulphate	U ($P = 0.006$)
23	4.6	399.0925 [a]	***223.0610***, 175.0247	**208.0378**, 179.0716, 164.0479	$C_{17}H_{20}O_{11}$	399.0933	−2.0	Hydroxy-dimethoxy cinnamic acid glucuronide (Sinapic acid glucuronide)	U ($P = 0.006$)
24	4.7	413.1079 [a]	***237.0763***, 175.0243	222.0533	$C_{18}H_{22}O_{11}$	413.1089	−2.4	Hydroxy-(hydroxy-methoxyphenyl)-pentenoic acid glucuronide I	U ($P = 0.006$)
25	4.8	383.0977 [a]	**207.0658**, 175.0244, 113.0243	**163.0763**, 122.0374	$C_{17}H_{20}O_{10}$	383.0984	−1.8	Hydroxy-(hydroxyphenyl) pentenoic acid glucuronide	S ($P = 0.006$); U ($P = 0.008$)
26	4.8	369.0819 [a]	*193.0502*, 175.0246, **113.0244**	178.0272, **149.0610**, 134.0375	$C_{16}H_{18}O_{10}$	369.0827	−2.2	Hydroxy-methoxycinnamic acid glucuronide II (Ferulic acid glucuronide II)	S ($P = 0.006$)
27	4.9	413.1080 [a]	**237.0765**, 222.0528, 175.0246, 113.0245	NA	$C_{18}H_{22}O_{11}$	413.1089	−2.2	Hydroxy-(hydroxy-methoxyphenyl)-pentenoic acid glucuronide II	U ($P = 0.006$)

Table 2 (continued)

Peak	t_R	Parent ion	Mass Fragments		Formula	Exact Mass	Δ	Identification	Biological fluid
			MS^2	MS^3					
28	5.0	287.0224 [a]	**207.0659**	**163.0764**, 123.0451, 122.0376	$C_{11}H_{12}O_7S$	287.0231	−2.4	Hydroxy-(hydroxyphenyl) pentenoic acid sulphate II	S ($P = 0.011$); U ($P = 0.011$)
29	5.1	357.0819 [a]	**181.0502**	**137.0610**	$C_{15}H_{18}O_{10}$	357.0827	−2.2	Dihydroxyphenyl propionic acid glucuronide	U ($P = 0.006$)
30	5.3	455.1549 [a]	**279.1231**, 217.1229, 175.0244, 151.0762	NA	$C_{21}H_{28}O_{11}$	455.1559	−2.2	Hydroxy-abscisic acid glucuronide	S ($P = 0.006$); U ($P = 0.006$)
31	5.4	367.1027 [a]	193.0500, **191.0557**, 173.0454	NA	$C_{17}H_{20}O_9$	367.1035	−2.2	Feruloylquinic acid	U ($P = 0.025$)
32	5.9	439.1599 [a]	**263.1289**, 219.1387, 175.0245, 153.0919	NA	$C_{21}H_{28}O_{10}$	439.1610	−2.5	Abscisic acid glucuronide	S ($P = 0.006$); U ($P = 0.006$)
33	6.1	333.0607 [a]	183.0296, **165.0191**, 137.0243	NA	$C_{16}H_{14}O_8$	333.0616	−2.7	Methyl-dihydromyricetin	U ($P = 0.006$)
34	6.4	279.1231 [a]	**217.1230**, 151.0763	NA	$C_{15}H_{20}O_5$	279.1238	−2.5	Hydroxy-abscisic acid	U ($P = 0.006$)
35	6.5	245.0120 [a]	**165.0556**	**121.0663**	$C_9H_{10}O_6S$	245.0125	−2.0	Hydroxyphenyl propionic acid sulphate	S ($P = 0.006$)
36	7.2	263.1283 [a]	219.1391, **153.0921**	NA	$C_{15}H_{20}O_4$	263.1289	−2.3	Abscisic acid	U ($P = 0.006$)

NA not available
[a]Quasi-molecular ion $[M\text{-}H]^-$
[b]Molecular ion $[M]^+$

metabolites. Following, the identification of each one of the 36 features selected is discussed with full details.

Feature Identification

All features showing statistically significant differences in intensity between the baseline sample and at least one of the samples collected after supplement administration were putatively identified according to the identification criteria reported in the paragraph "Data processing and statistical analysis". For each feature, Table 2 illustrated retention times (t_R, min), mass to charge ratio (m/z) of parent ion found by the HR full scan experiment, main mass fragments, the proposed formula and the corresponding exact mass, the mass accuracy (Δ, ppm), the putative identification, the biological fluid (i.e. serum and/or urine) in which the metabolite was identified and the lowest P value resulting from the Wilcoxon signed-rank test. In detail, mass fragments can be produced during both MS^2 and MS^3 experiments, being the latter the result of an in-source fragmentation or a dedicated MS^3 method.

In addition, a more robust identification was provided for glucuronide and sulphate conjugates for which the MS^3 fragments can be compared with the MS^2 spectrum of the reference standard of the non-conjugate metabolite. For these peaks, a more informative attribution was hypothesized and reported in bracket in Table 2.

Almost all the features reported in Table 2 were detected in negative ionization mode as pseudo-molecular ion $[M\text{-}H]^-$ and for most of these ions, this finding was in accordance with their putative annotation as phenolic acids. The only two exceptions were represented by the two compounds putatively identified as delphinidin-hexoside (peak *14*) and cyanidin-hexoside (peak *19*). Indeed, these two ions were detected in positive ionization mode as molecular ions $[M]^+$ in agreement to their chemical structure, which is characterized by a positive charge at pH ≒ 3 [40]. It should also be noted that the mass of detected molecular ions was in very good agreement with the exact mass of the proposed formula being the mass accuracies the highest among all the identified features (Table 2). The mass fragments found for peaks *14* and *19* corresponded to the cleavage of the sugar and formation of aglycones at m/z 303.0501 and 287.0544 for delphinidin and cyanidin, respectively. The loss of only the glycoside unit during the mass fragmentation, as well as the earlier elution of delphinidin-3-glucoside in respect to cyanidin-3-glucoside, were typically reported in literature for these compounds [29–31]. In addition, the fragmentation spectra of peaks *14* and *19* matched perfectly with those of the reference standards of delphinidin-3-glucoside and cyanidin-3-glucoside, respectively (data not shown). Nevertheless, the same fragmentation spectra were obtained for the corresponding galactoside standards and therefore the only chance to discriminate the two glycosides is based on their chromatographic separation. Unfortunately, the elution gradient of the chromatographic run commonly suggested for untargeted metabolomics analysis and herein adopted is too fast for obtaining the resolution of anthocyanidin galactoside and

glucoside. Thus, due to the impossibility of discriminating the type of bound sugar, peaks *14* and *19* were identified as delphinidin-hexoside and cyanidin-hexoside, respectively.

The presence of these anthocyanins was in agreement with the high content of galactoside and glucoside derivatives of delphinidin and cyanidin in *V. myrtillus* fruits [29, 30]. It is important to note that these two compounds were the only native anthocyanins found in the studied biofluids, although nearly 60 anthocyanidin glycosides were identified in bilberry [31]. Moreover, a list of predicted anthocyanin metabolites (i.e. glucuronides, sulphate and methylated conjugates) were investigated both in serum and urine, but none of these compounds were found in our samples. Conversely, many literature studies reported anthocyanin-predicted metabolites, even if at very low concentrations (i.e. 1–2% of the ingested dose) [17, 21–24, 41]. Nevertheless, the difference between our results and those reported elsewhere can be due to some variables of the study design, such as the form of the anthocyanin dose ingested (fruits, juice, supplement or enriched-extract), the ingestion of the dose with or without a meal, the duration of administration (long-term intake compared to an acute ingestion) and in particular the sampling time. Furthermore, the quantitative data about cyanidin 3-glucoside distribution in rat biofluids and organs, including brain, have clearly shown that once reaching the bloodstream, an extremely high rate of cellular uptake and metabolism occurs, thus strongly lowering the biofluids concentration of native compounds and related products of the human metabolism [42, 43].

In addition, the use of targeted approach, even though unable to discover unknown metabolites, is characterized by higher sensitivity compared to the untargeted one, thus allowing for the detection of low concentrations of predicted anthocyanin conjugates.

Among the features listed in Table 2, a group of compounds were putatively identified as benzoic acids conjugates (peaks *1*, *3*, *4*, *6*, *7* and *10*). More in detail, peaks *1* and *7* were characterized by the quasi-molecular ion $[M\text{-}H]^-$ at *m/z* 343.0663 and showed the neutral loss of 176.0321 giving rise to the fragment at *m/z* 167.0349 (Figure 3A). In addition, the glucuronate "fingerprint" ions found at *m/z* 175.0246 and 113.0245 (formed by the elimination of H_2O and CO_2 from the ion at *m/z* 175.0246) suggested that the pseudo-molecular ion was a glucuronide-conjugated compound [44]. It should also be noted that for peaks *1* and *7* the loss of glucuronate in the ion source provided the MS^2 spectrum of the non-conjugated ion at *m/z* 167.0349 (Figure 3B) that showed fragments at *m/z* 152.0114 (loss of methyl radical), 123.0452 (loss of CO_2) and 108.0216 (further loss of methyl radical), in agreement with the fragmentation pattern of hydroxy-methoxy benzoic acids, such as vanillic or isovanillic acids (Scheme 1) [45, 46]. Furthermore, these ions matched perfectly the mass fragments obtained for the vanillic acid reference standard. The presence of the glucuronide derivatives of vanillic acid and of one of its isomers (other than isovanillic acid) was reported by Pimpão and coworkers in human urine of healthy volunteers after the ingestion of a mixture of five small berries [14]. These compounds were tentatively attributed to two vanillic acid glucuronides in which the glucuronidation occurs either at the hydroxyl group or at the carboxylic group, as pointed out by Piazzon et al. [47]. Accordingly, peaks *1* and *7* might be attributed to vanillic acid glucuronides. However, due to the lack of conjugated reference standards, peaks *1* and *7* were reported in Table 2 as hydroxy-methoxy benzoic acid glucuronides I and II.

Peak *6* was ascribed to hydroxy-methoxy benzoic acid hexoside, being characterized by the neutral loss of 162.0526 and the same fragmentation pattern (*m/z* 167.0347 and 123.0451) found for the previously described hydroxy-methoxy benzoic acid glucuronides (Table 2). It should also be noted that peak *6* eluted immediately earlier than peak *7*, thus suggesting for this compound the putative identification as vanillic acid glucoside. In fact, under reversed-phase chromatographic conditions, glucoside derivatives typically elute right before the corresponding glucuronides [31]. In accordance with these considerations, peak *6* could be tentatively ascribed to vanillic acid glucoside, whose presence in human urine after bilberry consumption is suggested herein for the first time.

Peak *3* exhibited the loss of 79.9565 Da, which is characteristic for a sulphate-conjugated compound [12], leading to the formation of the ion at *m/z* 197.0452 as the most intense fragment in MS^2 spectrum (Table 2). In addition, the ion at *m/z* 233.0121 deriving from the loss of CO_2 from the pseudo-molecular ion confirmed the presence of the carboxylic unit, thus suggesting for peak *3* the attribution to a sulphate conjugate of a carboxylic acid. The MS^3 fragmentation spectrum of the ion at *m/z* 197.0452 highlighted the presence of ions at *m/z* 182.0221 and 153.0554, due to the loss of methyl radical and CO_2 units, which are consistent with the occurrence of a hydroxy-dimethoxy benzoic acid (Table 2). More specifically, since this fragmentation pattern matched perfectly the mass fragments of syringic acid standard, the tentative identification of syringic acid sulphate is proposed. Based on similar considerations, peaks *4* and *10* that showed the same MS^3 daughter ions of syringic acid, together with the typical MS^2 fragments of a glucuronide conjugate, have been reported as glucuronide derivatives of hydroxy-dimethoxy benzoic acids (Table 2), even though their putative identification as syringic acid glucuronides is suggested. The presence of syringic acid was elsewhere detected in enzymatically hydrolysed serum and urine from volunteers assuming a *Vaccinium* berry-based diet, thus supporting our identification of peaks *3*, *4* and *10* [25, 48].

Peak *2* was putatively identified as hydroxyhippuric acid sulphate on the basis of the exact mass of the pseudo-molecular ion (*m/z* at 274.0019), as well as the loss of sulphate unit, which gave rise to the ion at *m/z* 194.0455 (Table 2). Moreover, the further fragmentation of the latter ion resulted in the loss of CO_2 with the consequent formation of the ion at *m/z* 150.0561, already suggested in literature for the identification of 2-hydroxyhippuric and 3-hydroxyhippuric acids as urinary metabolites of quercetin-rutinoside [49]. However, in order to attempt a more specific level of identification of peak *2*, the LC-MS/MS analysis of α-HA, p-HA and o-HA reference standards was performed. Interestingly, the three isomers were

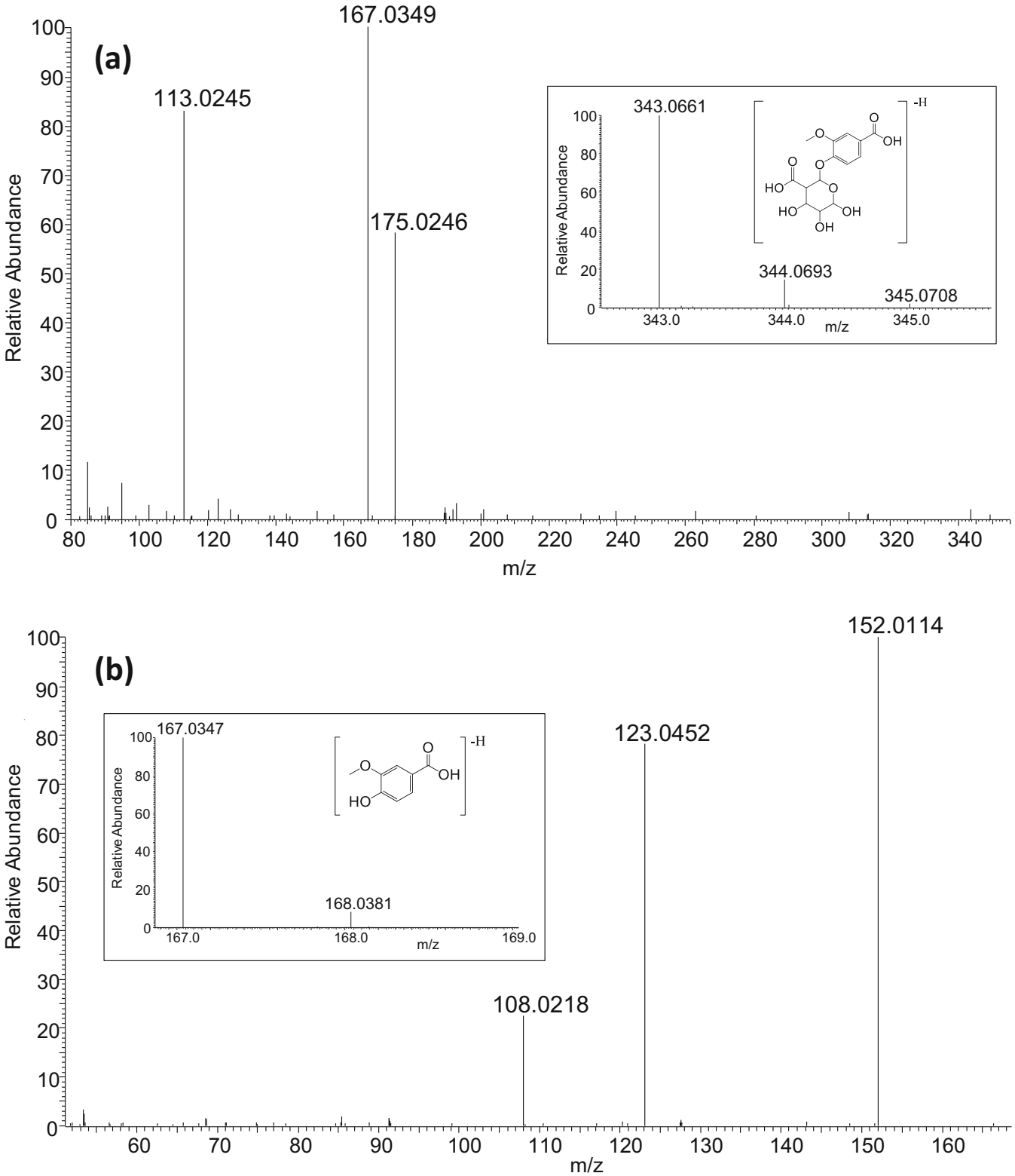
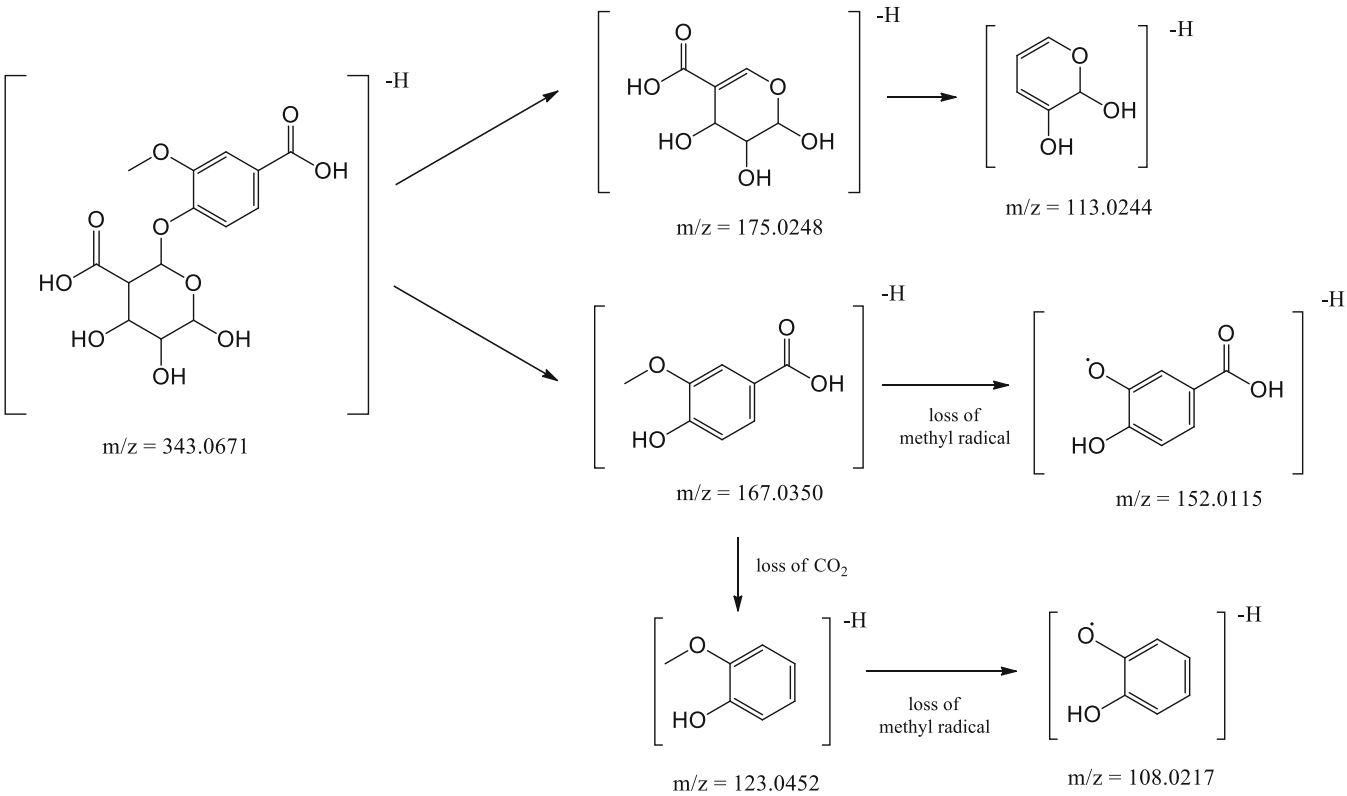


Figure 3. Isotopic profile and MS/MS spectrum of peak 7 (**a**) and its in-source fragment at *m/z* 167.0347 (**b**) identified as hydroxy-methoxy benzoic acid glucuronide and hydroxy-methoxy benzoic acid, respectively

characterized by different retention times and main mass fragments (t_R=3.7, 3.9, 5.6 min and *m/z* 72.9933, 165.0507, 150.0558, for α-HA, p-HA and o-HA, respectively). From the comparison with the mass fragments of reference standards, the presence of the ion at *m/z* 150.0558 in the MS^3 experiment of peak *2* suggested the putative attribution to o-HA for the ion 194.0455. Moreover, the conjugation of hydroxyhippuric acid with a sulphate unit increases the molecule polarity, thus



Scheme 1. Hypothesized structure and fragmentation scheme for peaks 1 and 7 ([M-H]$^-$ = 343.0663)

explaining the earlier retention time observed for peak *2* (Rt=3.0 min) compared to that of the *o*-hydroxyhippuric acid reference standard (Rt=5.6 min). For this reason, peak *2* was putatively ascribed to *o*-hydroxyhippuric acid sulphate (Table 2).

Interestingly, both hydroxyhippuric acids and their sulphate conjugates were found in urine after the ingestion of different foods, such as tomato juice, coffee [49, 50], as well as wild blueberry [51]. The presence of a derivative of the quercetin catabolism is not unexpected considering that quercetin glycosides are the dominant flavonols in both blueberry and bilberry, ranking among the top edible vegetables and fruits in terms of average quercetin content [52, 53].

Peak *11* was ascribed to hydroxy-methoxy-hippuric acid because of the loss of CO_2 unit (ion at *m/z* 180.0661), as well as the presence of the fragments at *m/z* 123.0452 (corresponding to the methoxy-phenol unit) and at *m/z* 100.0040, being the latter commonly reported for hydroxyhippuric acid identification [50].

Peaks *5* was putatively identified as catechol sulphate owing to the presence in the MS^2 spectrum of the typical accurate masses of a sulphate (*m/z* at 79.9576) and hydroxyphenol (*m/z* at 109.0297) ions. Similarly, peak *17* was ascribed to catechol glucuronide because of the aforementioned characteristic mass fragments of a glucuronide-conjugated compound (i.e. *m/z* 175.0245 and 113.0244) and the presence of the exact mass of catechol in its MS^2 spectrum. In this regard, it is worth noting that the presence of catechol derivatives in human urine was already reported in literature after berry and tea consumption [14, 50, 51].

Another group of putatively identified metabolites was represented by cinnamic acid derivatives (peaks *18*, *21*, *23*, *26* and *31*, see Table 2). Among them, peaks *18* and *26* were putatively attributed to hydroxy-methoxy cinnamic acid glucuronides, due to the presence in the MS^2 spectra of the typical fragments of a glucuronide conjugate (*m/z* 175.00243 and 113.0243), together with the exact mass of the ion at *m/z* 193.0501, corresponding to a hydroxy-methoxy cinnamic acid. Moreover, the further fragmentation of this last ion produced the individual losses of methyl radical (*m/z* 178.0272) and CO_2 (*m/z* 149.0610), as well as the loss of both units with formation of the ion at *m/z* 134.0375. Based on these findings, peaks *18* and *26* were ascribed to hydroxy-methoxy cinnamic acid glucuronides, such as ferulic acid. Interestingly, the fragmentation pattern described above matched exactly the MS^2 spectrum of the ferulic acid reference standard. It should also be noted that ferulic acids were already found in human serum and urine after small berry ingestion [14, 48, 51].

Among the other cinnamic acid derivatives, peak *21* was identified as caffeoylquinic acid being its fragmentation spectrum characterized by the signal at *m/z* at 191.0558 and 179.0350, corresponding to the quasi-molecular ions of quinic and caffeic acid, respectively. Moreover, the reference standard of chlorogenic acid showed the same retention time and fragmentation spectrum, confirming the level-I identification of peak *21*. Based on similar considerations, peak *31* was ascribed to feruloylquinic acid because of the characteristic fragments of quinic and ferulic acids [50]. It is interesting to note that among the non-anthocyanin native polyphenols of *V. myrtillus* fruits [31], only caffeoylquinic and feruloylquinic acids were found in the urines of volunteers involved in the present study.

Peak *23* was attributed to the glucuronide of the ion at *m/z* 223.0606 (Figure 4A) that corresponds to the exact mass of the pseudo-molecular ion of three different compounds: (i) hydroxy-dimethoxy cinnamic acid, (ii) hydroxy-(dihydroxyphenyl) pentenoic acid and (iii) trihydroxy-phenyl valerolactone. Each one of these compounds could be a metabolite of bilberry

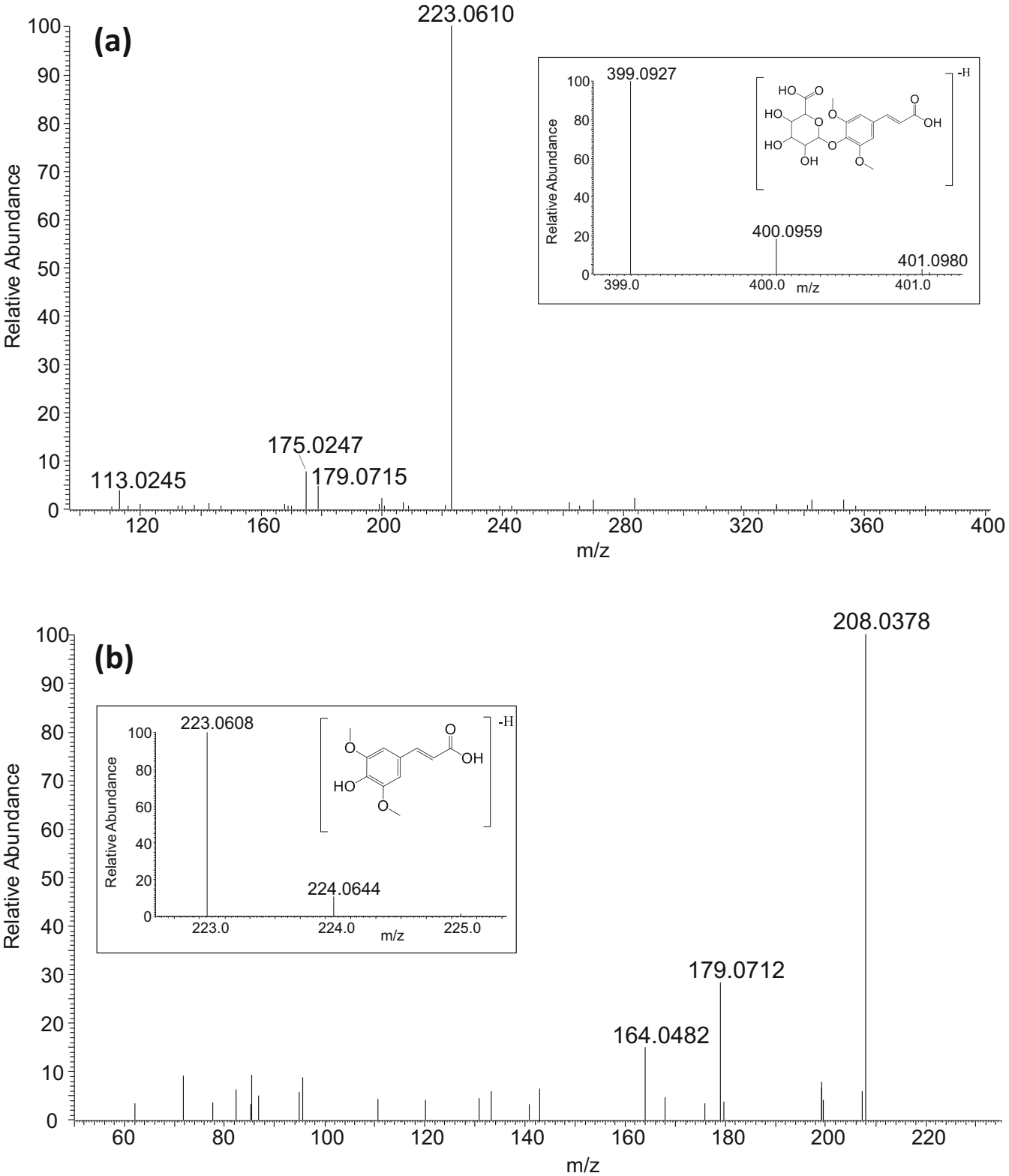
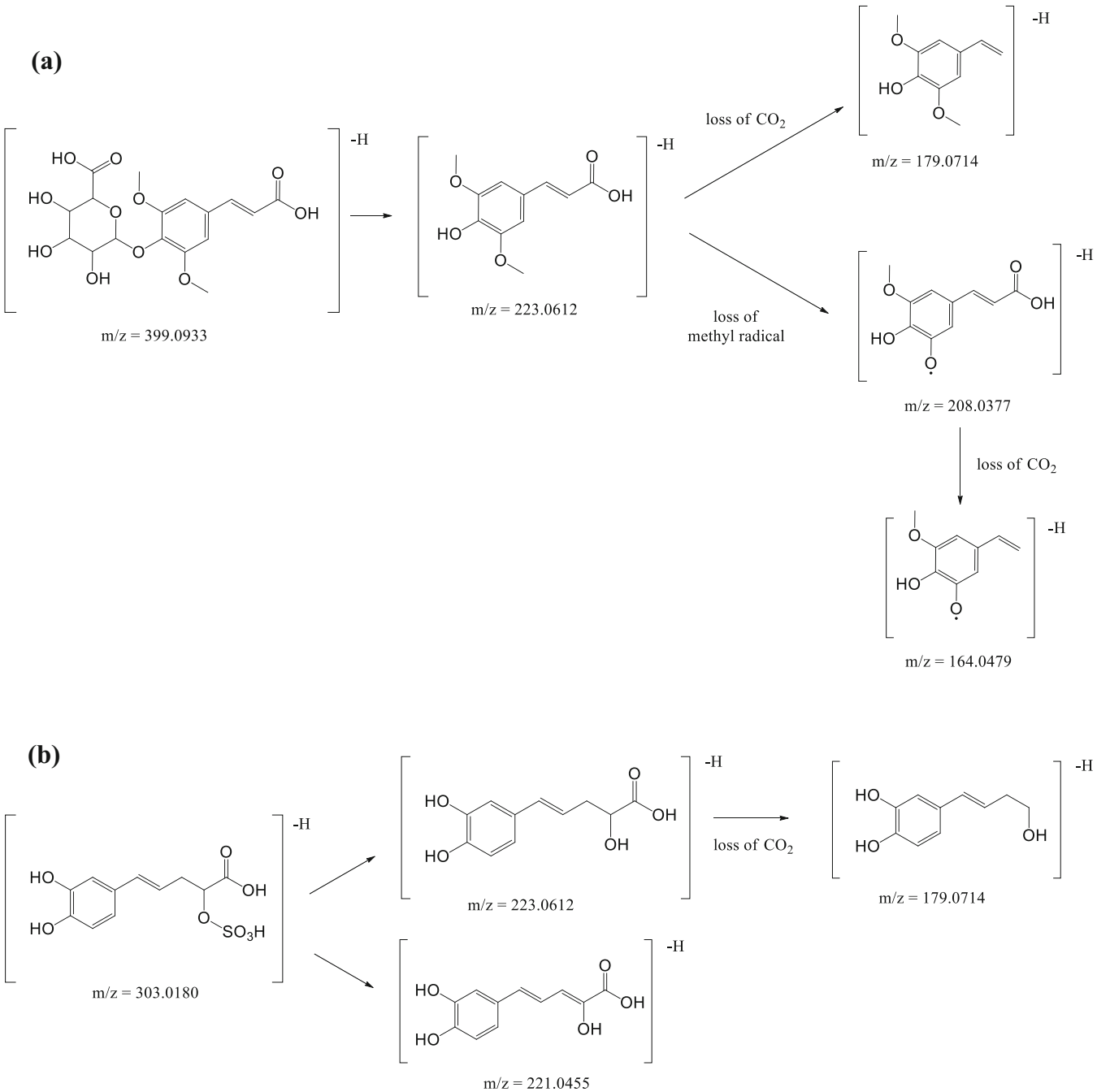


Figure 4. Isotopic profile and MS/MS spectrum of peak 23 (**a**) and its in-source fragment of *m/z* 223.0310 (**b**) identified as hydroxy-dimethoxy cinnamic acid glucuronide and hydroxy-dimethoxy cinnamic acid, respectively



Scheme 2. Hypothesized structure and fragmentation scheme for peak *23* (**a**) attributed to hydroxy-dimethoxy cinnamic acid glucuronide and peak *12* (**b**) putatively identified as hydroxy-(dihydroxyphenyl) pentenoic acid sulphate

consumption because both cinnamic and valeric acid derivatives have been reported as products of polyphenol metabolism [27, 54]. Nevertheless, the in-source loss of glucuronate unit of peak *23* allowed for obtaining the fragmentation spectrum of non-conjugated compound at *m/z* 223.0610 that showed the ion deriving from the loss of methyl radical (*m/z* 208.0378) as the most abundant one (Figure 4B). In addition, the other ions at *m/z* 179.0716 and 164.0479 corresponded to the fragments obtained for the reference standard of sinapic acid, in agreement with data reported in literature for the determination of this compound in vegetal matrices [55, 56]. In fact, the fragments at *m/z* 208.0378 and 164.0479 can be explained only through the attribution of the ion at *m/z* 223.0610 to a hydroxy-dimethoxy cinnamic acid, since the latter is the only compound able to provide the loss of the methyl radical unit (Scheme 2A). For this reason, peak *23* was putatively ascribed to hydroxy-dimethoxy cinnamic acid glucuronide.

Peak *12* showed a quasi-molecular ion at *m/z* 303.0173, which fragmented in the ions at *m/z* 223.0608 (loss of the sulphate unit) and *m/z* 221.0452 (Figure 5A), the latter being not consistent with the attribution to a hydroxy-dimethoxy cinnamic acid (Scheme 2B). However, a MS^3 experiment dedicated to the fragmentation of ion at *m/z* 223.0608 clearly

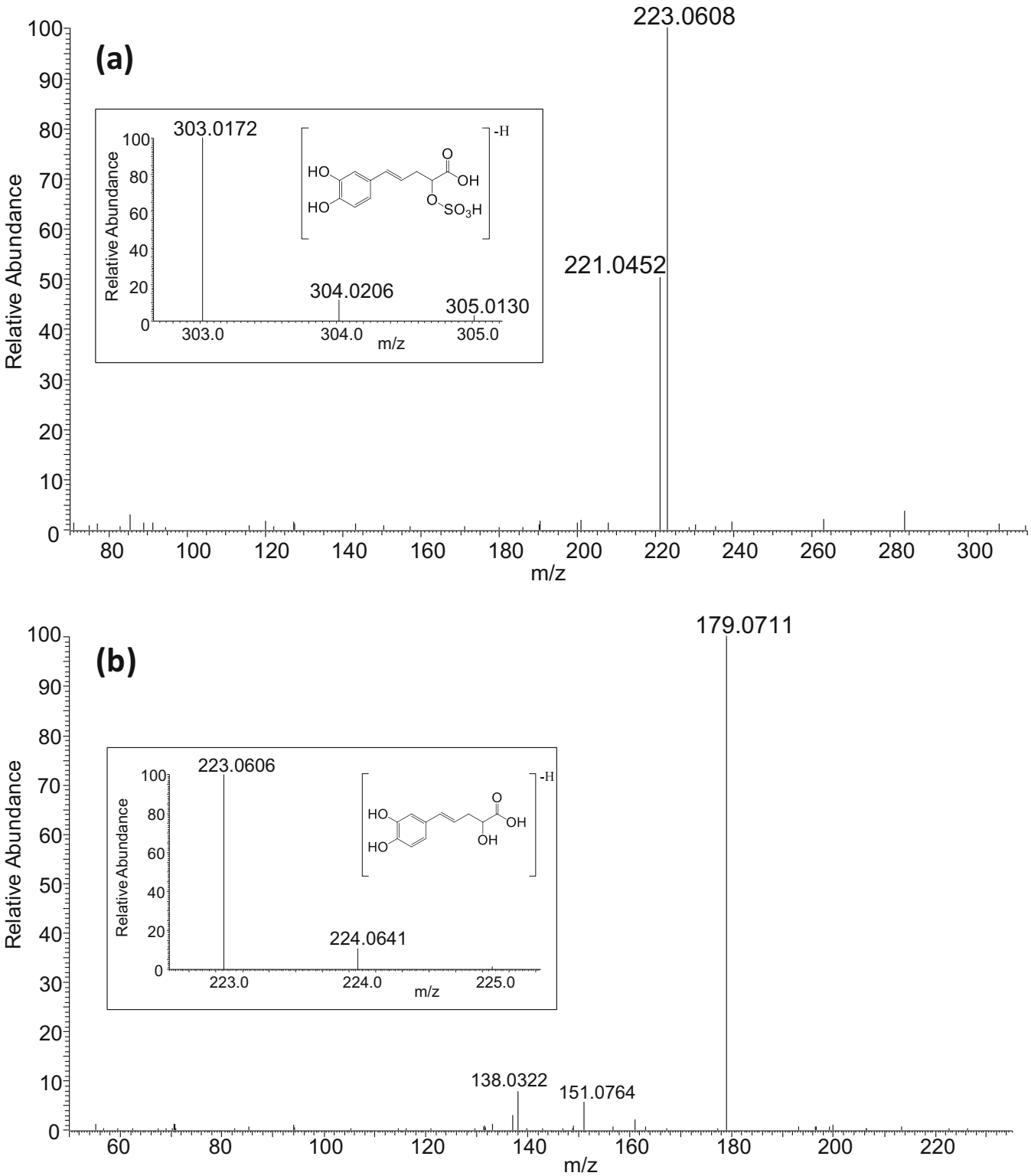


Figure 5. Isotopic profile and MS/MS spectrum of peak 12 (**a**) and its in-source fragment of *m/z* 223.0310 (**b**) identified as hydroxy-(dihydroxyphenyl) pentenoic acid sulphate and hydroxy-(dihydroxyphenyl) pentenoic acid, respectively

highlighted a very different behaviour (Figure 5B) compared to the one previously discussed for peak 23 (Figure 4B). In fact, the absence of the fragments related to the loss of methyl radical (*m/z* 208.0377 and 164.0479) suggested for peak *12* the attribution to a hydroxy-(dihydroxyphenyl) pentenoic acid sulphate, rather than a cinnamic acid derivative. Interestingly, peak *8*, which showed a pseudo-molecular ion at *m/z* 399.0924, fragmented with the loss of a glucuronide unit, originating the MS^2 fragment at *m/z* 223.0606. The further fragmentation of this latter ion gave rise to the same product (*m/z* 179.0711) mentioned above for

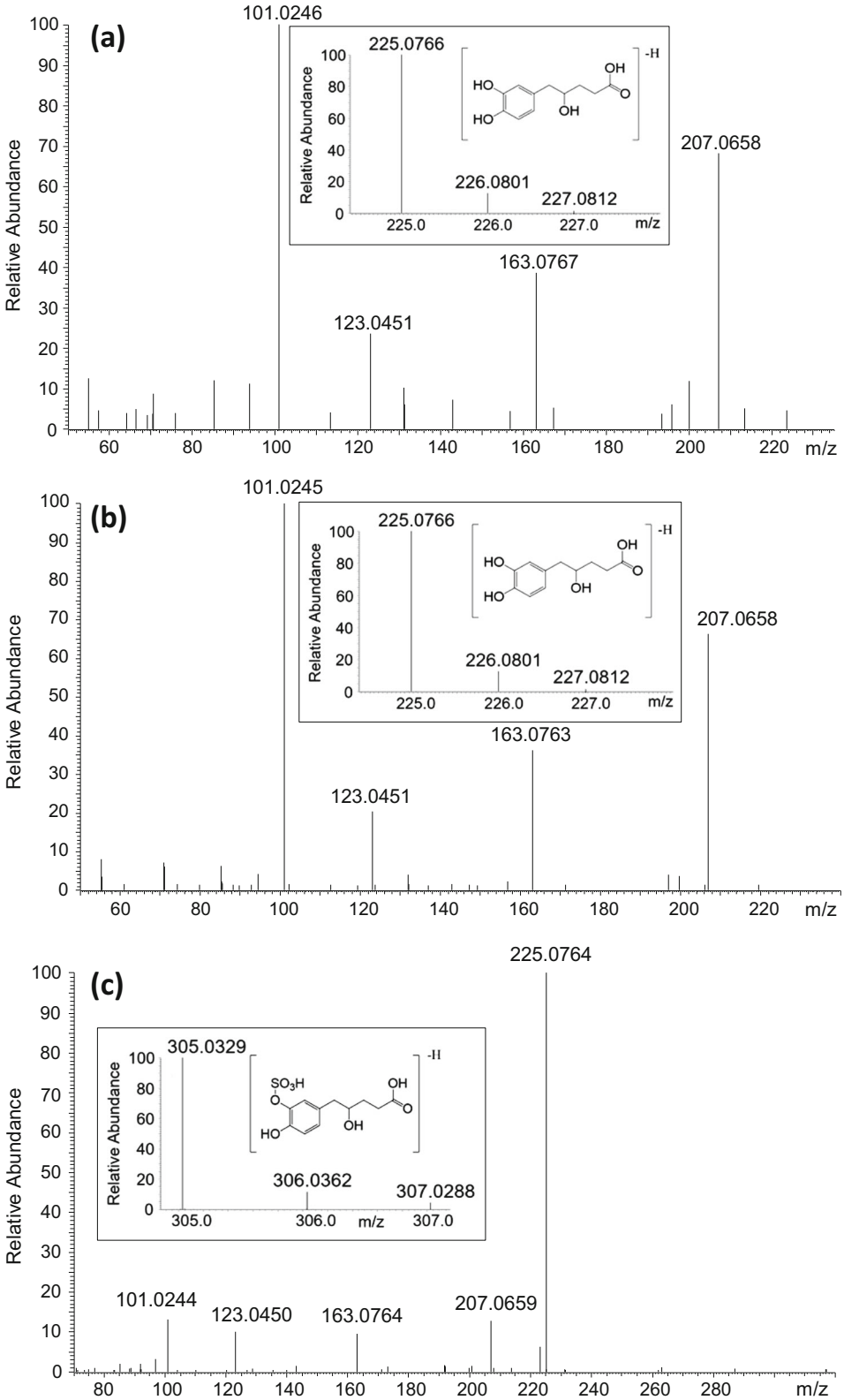
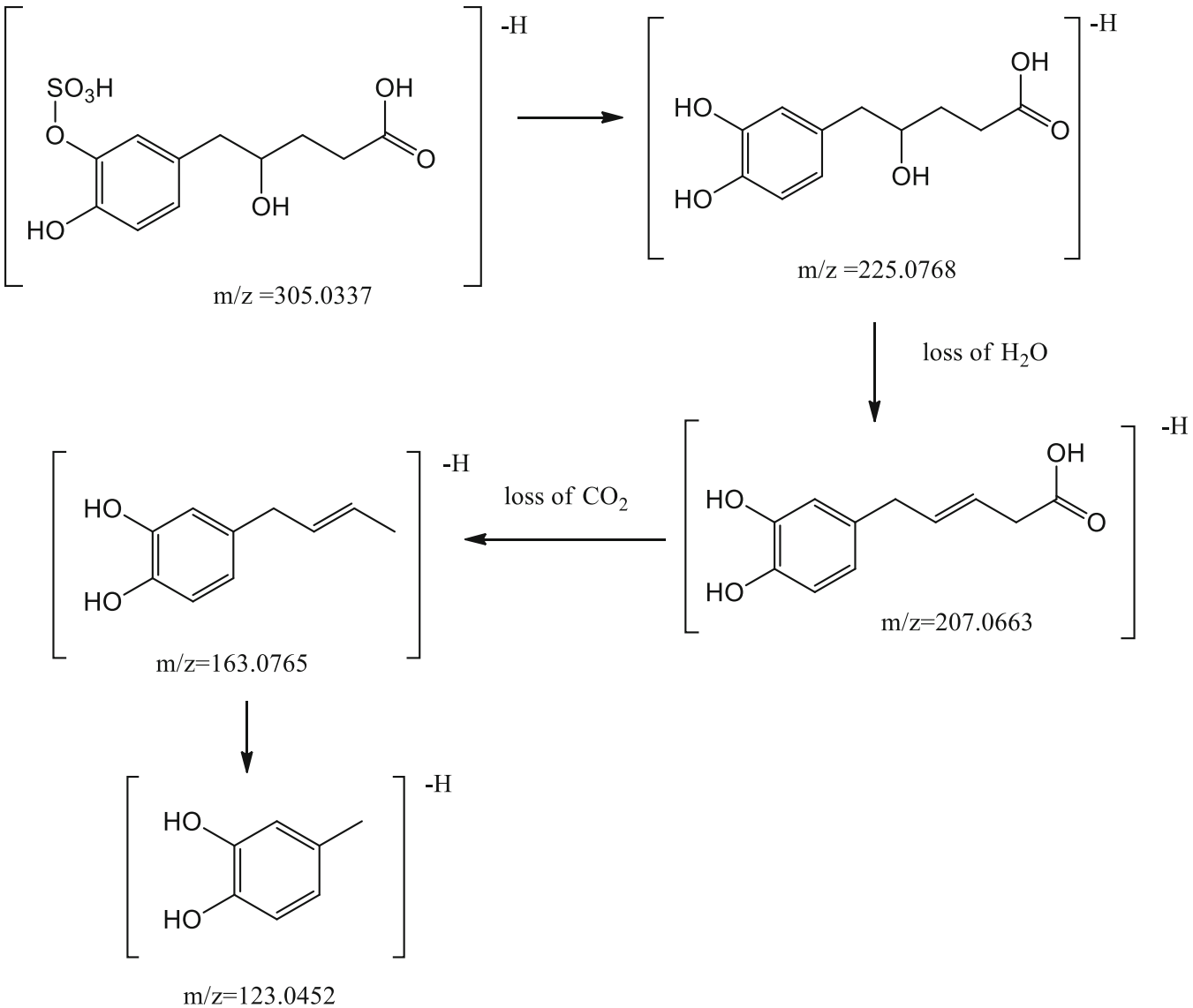


Figure 6. MS^3 spectrum of peak 9 attributed to hydroxy-(dihydroxyphenyl)-valeric acid glucuronide (**a**), MS^2 spectrum of the reference standard of hydroxy-(dihydroxyphenyl)-valeric acid (**b**), MS^2 spectrum of peak 16 attributed to hydroxy-(dihydroxyphenyl)-valeric acid sulphate (**c**)



Scheme 3. Hypothesized structure and fragmentation scheme for peak *16* ($[M-H]^-$ = 305.0328) attributed to hydroxy-(dihydroxyphenyl)-valeric acid sulphate

peak 12, thus suggesting for peak *8* the attribution to a hydroxy-(dihydroxyphenyl) pentenoic acid glucuronide.

Peaks *9* and *13* showed a pseudo-molecular ion $[M-H]^-$ at *m/z* 401.1081 corresponding to a mass 2 Da higher than peak *23*. This finding could suggest the presence of glucuronides of the hydroxy-dimethoxy hydrocinnamic acid according to the formation of the dihydro form of the phenylpropionic acid during the polyphenol catabolism [27]. Nevertheless, the dedicated MS^3 experiment evidenced a different scenario for peaks *9* and *13* (Figure 6A) because of the absence of the methyl radical losses (i.e. ions at *m/z* 210.0534 and 166.0635) observed for the corresponding cinnamic acid derivative (Figure 4B). Moreover, the non-conjugated unit (*m/z* 225.0768) produced the same fragments found for the reference standard of (4R)-5-(3,4-dihydroxyphenyl)-γ-hydroxyvaleric acid, analyzed in the same condition of serum and urine samples (Figure 6B). Analogously, the MS^2 spectrum of peak *16* (Figure 6C) highlighted the same ions related to the consequent losses of sulphate (*m/z* at 225.0764), water (*m/z* 207.0659) and CO_2 (163.0764) (Scheme 3), suggesting also the presence of the sulphate derivative of hydroxy-(dihydroxyphenyl) valeric acid in the investigated samples. For this reason, the presence of two glucuronides (peaks *9* and *13*) and one sulphate (peak *16*) of hydroxy-(dihydroxyphenyl) valeric acid in the investigated biological fluids was claimed. It is interesting to note that phenylvaleric acid derivatives were reported in literature as by-products of flavanol and proanthocyanidin metabolism [54, 57], thus evidencing that also metabolites deriving from polyphenols different from anthocyanins can occur at significant concentrations as a consequence of bilberry consumption.

Peaks *20* and *28* showed the characteristic loss of a sulphate unit with the consequent formation of the ion at *m/z* 207.0663 (Table 2). This feature could be attributed to a dihydroxyphenyl valerolactone, which was previously reported in literature as a

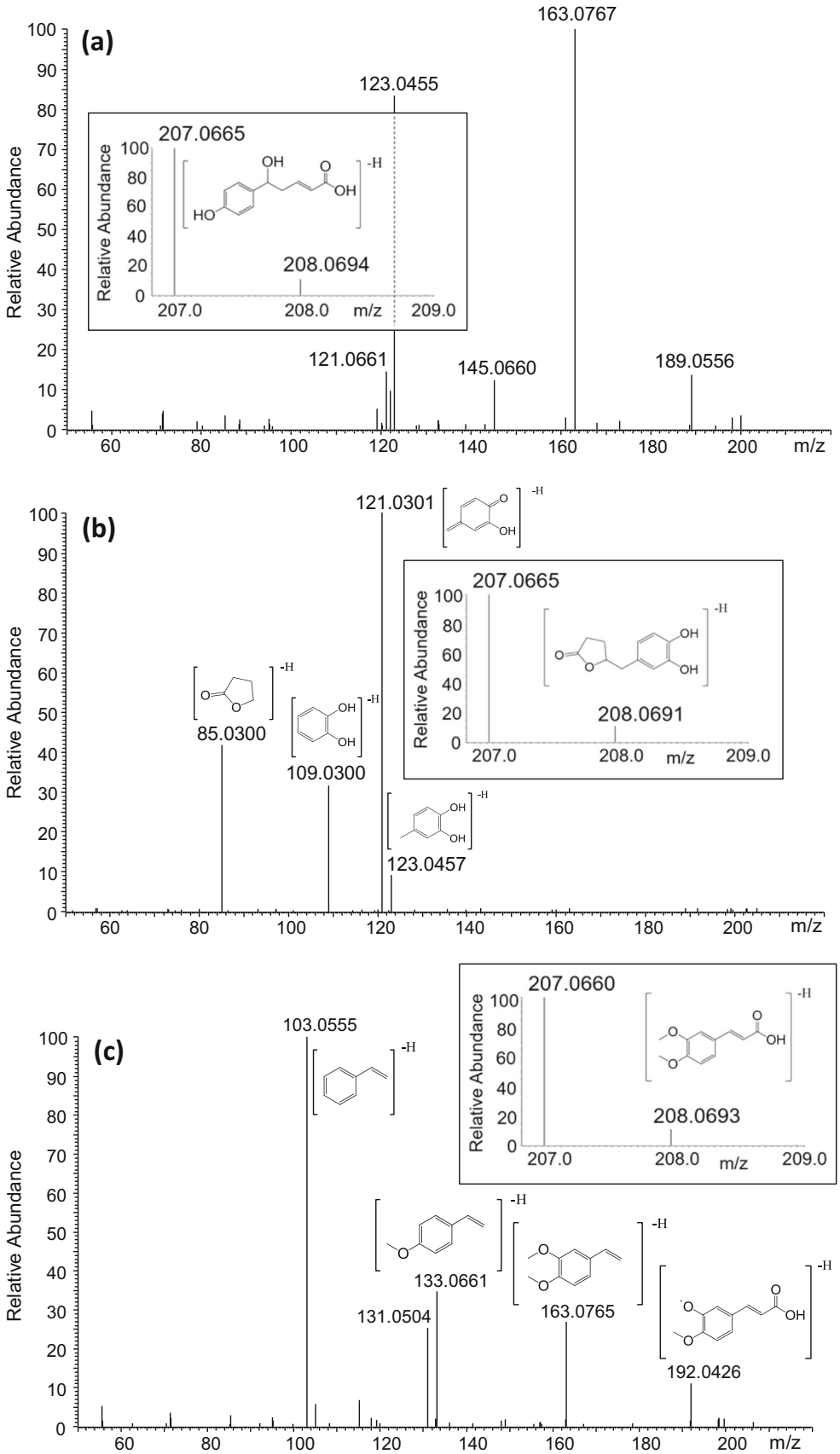
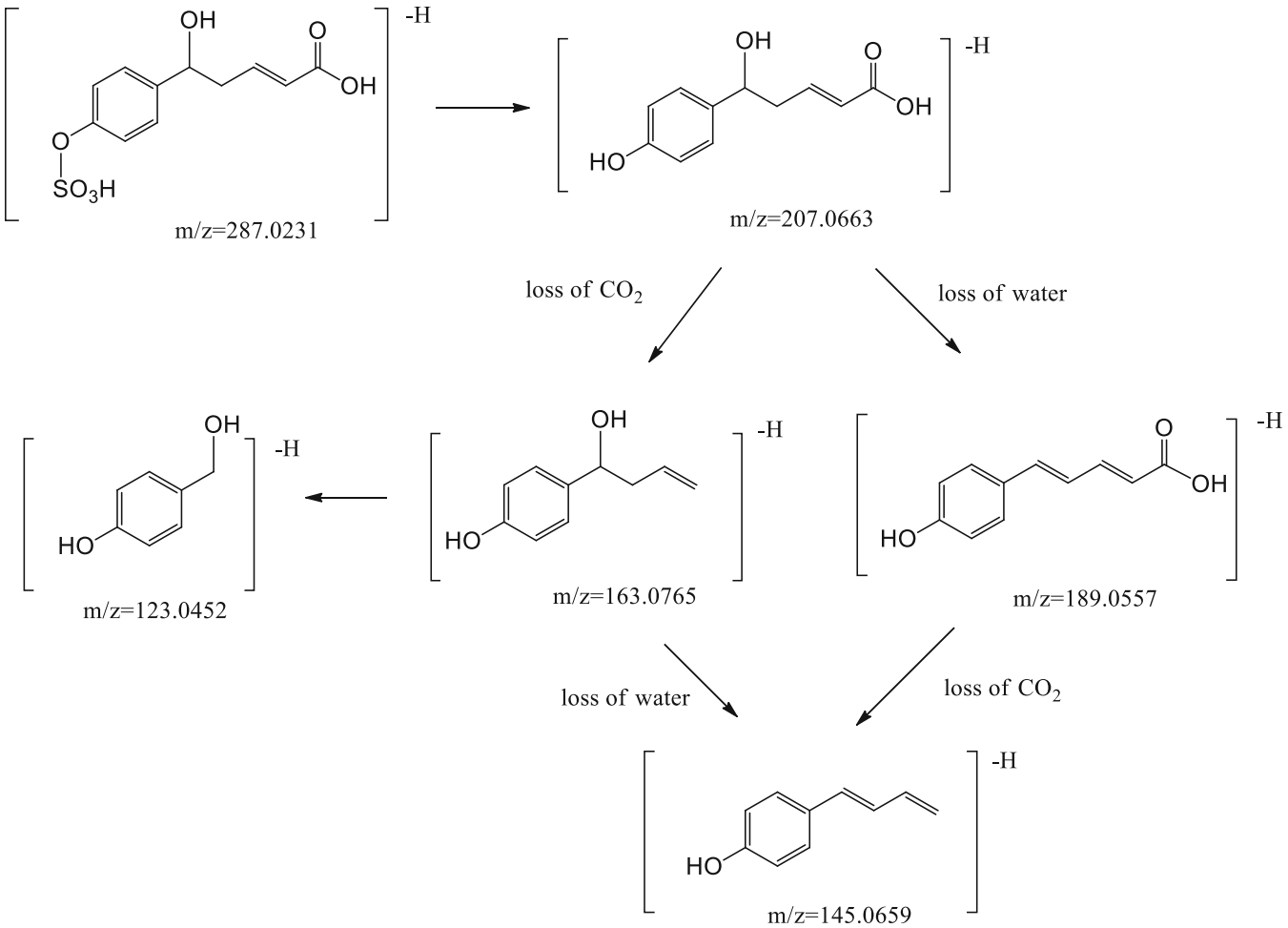


Figure 7. Isotopic profile and MS/MS spectrum of in-source fragment (*m/z* 207.0663) of peak 20 attributed to hydroxy-(hydroxyphenyl)-pentenoic acid sulphate (**a**), 3,4-dihydroxyphenyl valerolactone reference standard (**b**) and 3,4-dimethoxycinnamic acid reference standard (**c**)



Scheme 4. Hypothesized structure and fragmentation scheme for peaks *20* and *28* ([M-H]$^-$ = 287.0224) attributed to two isomers of hydroxy-(hydroxyphenyl)-pentenoic acid sulphate

metabolite of the ingestion of flavanol-rich food [50, 57]. However, the exact mass of the pseudo-molecular ion at *m/z* 207.0663 could be also ascribed to dimethoxycinnamic acid deriving from the methylation of dihydroxycinnamic acid. In fact, dimethoxy cinnamic acid has been elsewhere reported as a polyphenol metabolite in humans [51]. Nevertheless, in-source fragmentation of peaks *20* and *28* gave rise to the MS^2 spectrum of the non-conjugated compound at *m/z* 207.0663 (Figure 7A) that showed different ions in respect to those obtained after the fragmentation of 3,4-dihydroxyphenyl valerolactone (Figure 7B) and 3,4-dimethoxy cinnamic acid (Figure 7C) reference standards. The fragmentation pattern of the ion at *m/z* 207.0663 highlighted the loss of CO_2 (*m/z* 163.0767) and water (*m/z* 145.0660), as well as other characteristic ions, thus matching well a hydroxy-(hydroxyphenyl) pentenoic acid (Scheme 4). Based on these considerations, peaks *20* and *28* were attributed to hydroxy-(hydroxyphenyl) pentenoic acid sulphates and peak *25* was ascribed to hydroxy-(hydroxyphenyl) pentenoic acid glucuronide. To the best of our knowledge, these polyphenol metabolites have been reported herein for the first time as metabolites of bilberry intake.

Peak *15* exhibited a quasi-molecular ion at *m/z* 415.1234 and a main fragment at *m/z* 239.0923 (loss of glucuronide unit), which did not fragment further. Accordingly, peak *15* could be identified either as glucuronide derivative of trimethoxy-hydroxycinnamic acid or hydroxy-(hydroxy-methoxyphenyl) valeric acid (Table 2).

Peaks *22*, *24* and *27* could also be ascribed to both cinnamic and phenylvaleric acid derivatives, on the basis of their exact mass of quasi-molecular ions and their fragmentation, which highlighted the attribution to one sulphate (peak *22*) and two glucuronide (peaks *24* and *27*) derivatives. For all three peaks, the most intense MS^2 fragment was the ion at *m/z* 237.076. More in detail, peaks *24* and *27*, which could be attributed to trimethoxycinnamic acid or hydroxy-(hydroxy-methoxyphenyl)-pentenoic acid glucuronides, showed the presence of the ion at *m/z* 222.0528 in the MS^2 spectrum, clearly evidencing the loss of a methyl radical (Figure 8A). In addition, the further fragmentation of the deconjugated ion at *m/z* 237.076 produced only the fragment at *m/z* 222.0533 (Figure 8B), thus evidencing the loss of a methyl radical as the sole significant product ion. However, the comparison of this last spectrum with that of 3,4,5-trimethoxycinnamic acid reference standard (Figure 8C) excluded the possibility of identifying peaks *24* and *27* as trimethoxycinnamic acid glucuronide because of the absence of the cinnamic acid fragments. Moreover, MS^2 spectrum of peak *22* was also characterized by the fragment at *m/z* 302.0095, which is consistent with the loss of a

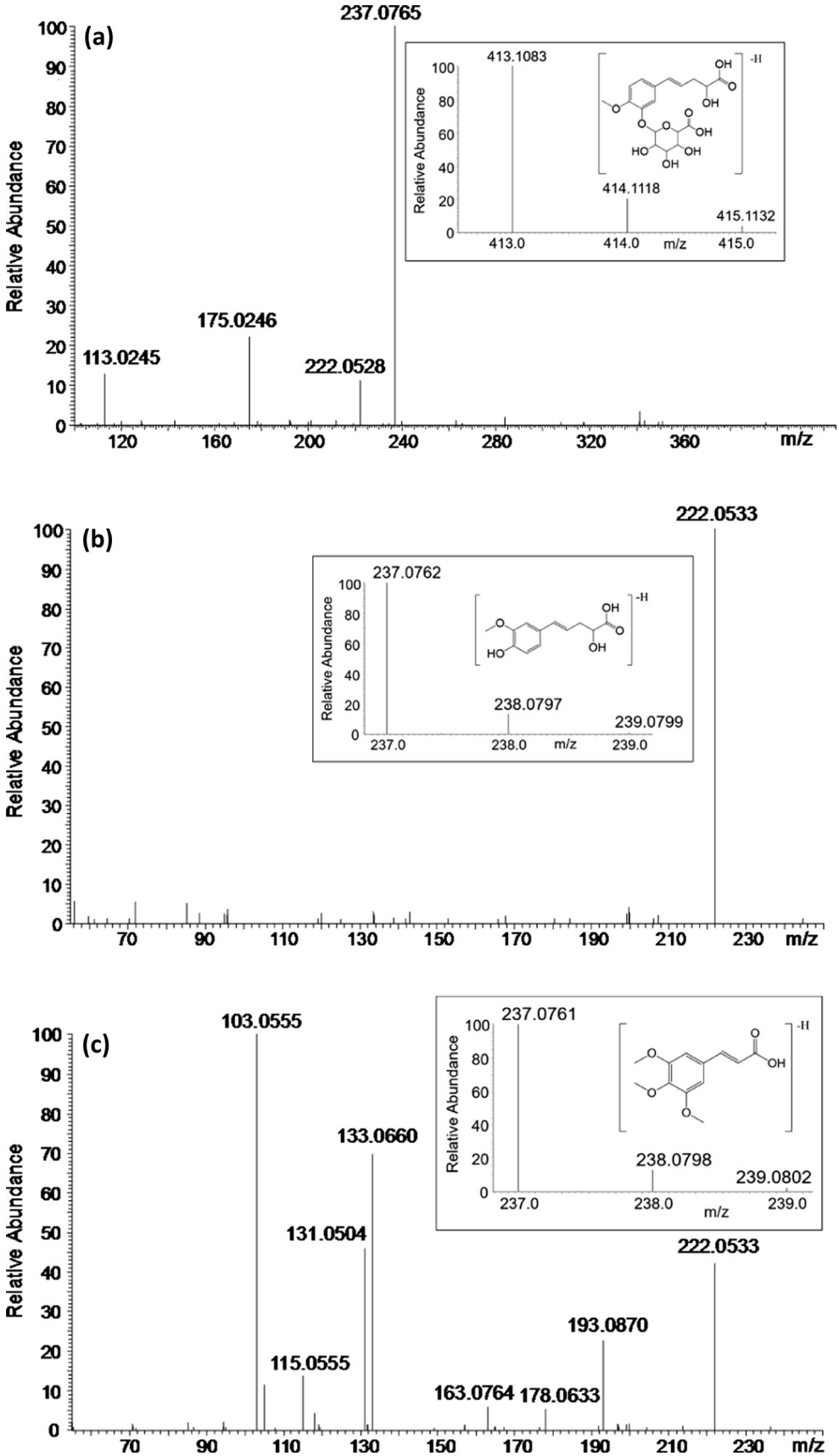


Figure 8. Fragmentation spectrum of peak 27 (**a**), its in-source fragment (*m/z* 237.0762) (**b**) and the reference standard of 3,4,5-trimethoxycinnamic acid (**c**)

methyl radical from the pseudo-molecular ion $[M-H]^-$ at *m/z* 317.0329 (Table 2). For these reasons, peaks *24* and *27* were attributed to hydroxy-(hydroxy-methoxyphenyl)-pentenoic acid glucuronides and peak *22* was putatively identified as hydroxy-(hydroxy-methoxyphenyl)-pentenoic acid sulphate. To the best of our knowledge, none of these compounds were previously reported in literature as metabolites of *V. myrtillus* berry species consumption.

Peaks *29* and *35* were tentatively attributed to phenylpropionic acid derivatives. More in detail, based on their exact mass, isotopic profile and mass fragments, peak *29* was ascribed to dihydroxyphenyl propionic acid glucuronide, whereas peak *35* was identified as hydroxy-phenyl propionic acid sulphate (Table 2). Moreover, the identification of peaks *29* and *35* was confirmed by the comparison with the fragmentation spectra of 3-(3,4-dihydroxyphenyl) propionic acid and 3-(4-hydroxyphenyl) propionic acid, respectively. The presence of phenylpropionic acids in serum and urine deriving from anthocyanin metabolism after berry ingestion was in agreement with recent literature studies [25, 51].

Among features listed in Table 2, a group of compounds (peak *30*, *32*, *34* and *36*) were attributed to abscisic acid derivatives. More in detail, the exact mass of the pseudo-molecular ion $[M-H]^-$ (*m/z* 263.1283), the isotopic profile and fragments (*m/z* 219.1391 and 153.00921) obtained for peak *36* exactly matched those of abscisic acid standard. In addition, peak *32* showed the typical neutral loss of glucuronide-conjugated compounds (*m/z* 176.031) together with the same fragmentation pattern of peak *36*. It is also remarkable that peak 32 eluted earlier than peak 36, the latter being characterized by a retention time difference of only 2 s compared to the reference standard of abscisic acid. Based on these considerations, peaks *32* and *36* were identified as abscisic acid glucuronide and abscisic acid, respectively. Similarly, peaks *30* and *34* were ascribed to hydroxyabscisic acid and hydroxyabscisic acid glucuronide, based on the ions found in their MS^2 spectra at *m/z* 217.1230 and 151.0763 already reported in literature for hydroxyabscisic acid fragmentation [58]. It is worth noting that abscisic acid is a plant hormone involved in the regulation of polyphenol biosynthesis [59] and abscisic acid glucuronide was recently reported for the first time as a marker of low-flavonoid diet [46].

Finally, peak *33* was putatively identified as methyl-dihydromyricetin on the basis of the exact mass and the isotopic profile of the quasi-molecular ion $[M-H]^-$ at *m/z* 333.0607, as well as the fragments deriving from the C ring fission (i.e. *m/z* 183.0299 and 165.0193). This is the only compound clearly deriving from flavonol metabolism even if literature researches often reported also conjugated glucuronide and sulphate derivatives as markers of a diet rich in flavonols [60].

Conclusions

LC-HRMS and LC-HRMS/MS analyses, performed both in positive and negative modes, allowed for identifying the most relevant serum and urinary polyphenol metabolites after the ingestion of *V. myrtillus* berry supplement. In particular, compounds putatively attributed to conjugates of benzoic acids, hydroxyhippuric acids, cinnamic acids, phenylpropionic acids, phenylvaleric acids, phenylpentenoic acids and abscisic acid, together with two fruit native anthocyanins, one flavonol metabolite and two catechol derivatives, were identified based on their chromatographic behaviour and in-depth evaluation of mass spectrometric information.

Interestingly, the application of the untargeted metabolomics approach allowed the annotation of compounds never reported elsewhere as phenolic biomarkers of the intake of *V. myrtillus* fruits. More in detail, some glucuronide and sulphate of hydroxy-(dihydroxyphenyl) valeric acid and hydroxy-(hydroxyphenyl) pentenoic acid were evidenced after bilberry ingestion. The presence of these compounds is consistent with their origin from fruit native flavanol monomers and oligomers. In this regard, the presence of metabolites other than those deriving from anthocyanins should be emphasized. In fact, non-anthocyanin metabolites are often ignored, even though flavanols represent one of the most occurring polyphenol class in bilberry, with both A-type and B-type trimers, tetramers and pentamers, as recently highlighted by our research group [31]. It is important to underline that these new identified metabolites could be biologically active and might partially explain the healthy properties of *V. myrtillus*, elsewhere evidenced in clinical trials.

For this reason, further studies focusing on the undoubted identification and accurate quantification of these compounds should be carried out together with in vitro studies in order to evaluate their potential efficiency against specific pathologies.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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