Untargeted metabolomics strategy based on LC-MS-Orbitrap for discovering new polyphenol metabolites in humans after acute ingestion of *Vaccinium myrtillus* berry supplement

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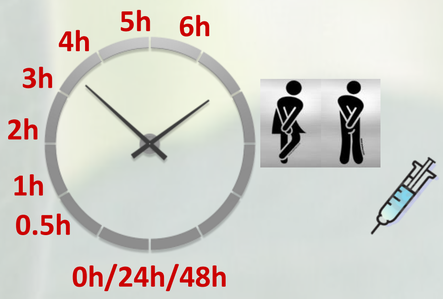
ABSTRACT

In this work, liquid chromatography coupled with 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 *Vaccinium myrtillus* berry supplement. The supplement was obtained by cryo-milling of properly freeze-dried bilberries. 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 thirteen metabolites, among glucuronide (glc) and sulphate (sulf) derivatives of phenylvaleric and phenylpentenoic acids, which have been identified for the first time in human biofluids after ingestion of *V. myrtillus* berries. The identification of these compounds confirmed the key-role of untargeted metabolomics approach in the discovery of new metabolites which could result biologically active.

STUDY DESING

25 g of *V. myrtillus* supplement mixed with 500 mL of water.
Ethical approval n. SPE 14.178 AOUC, 30th May 2016.



EXPERIMENTS

Serum samples were extracted with 96-well plate Ostro (Waters) and diluted 1:2, while urine samples were filtered with 96 well plate with PVDF filter and diluted 1:4. For both urine and serum samples, internal standards (Tryptophan-d5 and Hippuric acid-d5) and external standard (*Trans*-Cinnamic acid-d5) were added. 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 along the injection queue every ten samples.
Samples were analysed by Fourier Transform LTQ FT Orbitrap mass spectrometer (Thermo Fisher) interfaced to a Dionex HPLC system. Mass spectrometer operated under HR-Full Scan mode (30,000 FWHM, centroid mode) and data-dependent-acquisition (DDA) in positive and negative ionization modes. In DDA mode 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.

DATA PROCESSING AND STATISTICAL ANALYSIS

The LC-MS raw files were converted to *mzXML* with the *MSConvert* utility included in *ProteoWizard*. Then, the *mzXML* files were processed with the software XCMS plus (The Scripps Research Institute, La Jolla, CA) that allows for obtaining data processing (feature detection and retention time alignment) and data analysis through statistics tools. 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.
Both for serum and urine samples, the statistic comparison between different sampling times groups were performed using one-way analysis of variance (ANOVA) and the non-parametric Wilcoxon signed-rank test. Accordingly, the *P*-value associated to the comparison among the baseline and the post-ingestion intensities were calculated. Principal Component Analysis was performed by the Compound Discoverer software, version 2.1 (Thermo Fisher Scientific).

RESULTS

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. 6,708 and 13,567, respectively). Statistical analysis allowed to reduce ions of interest to a few hundreds, highlighting in serum and/or urine features with statistically different signals ($P \leq 0.05$) between the baseline level and the maximum intensity observed after supplement ingestion.

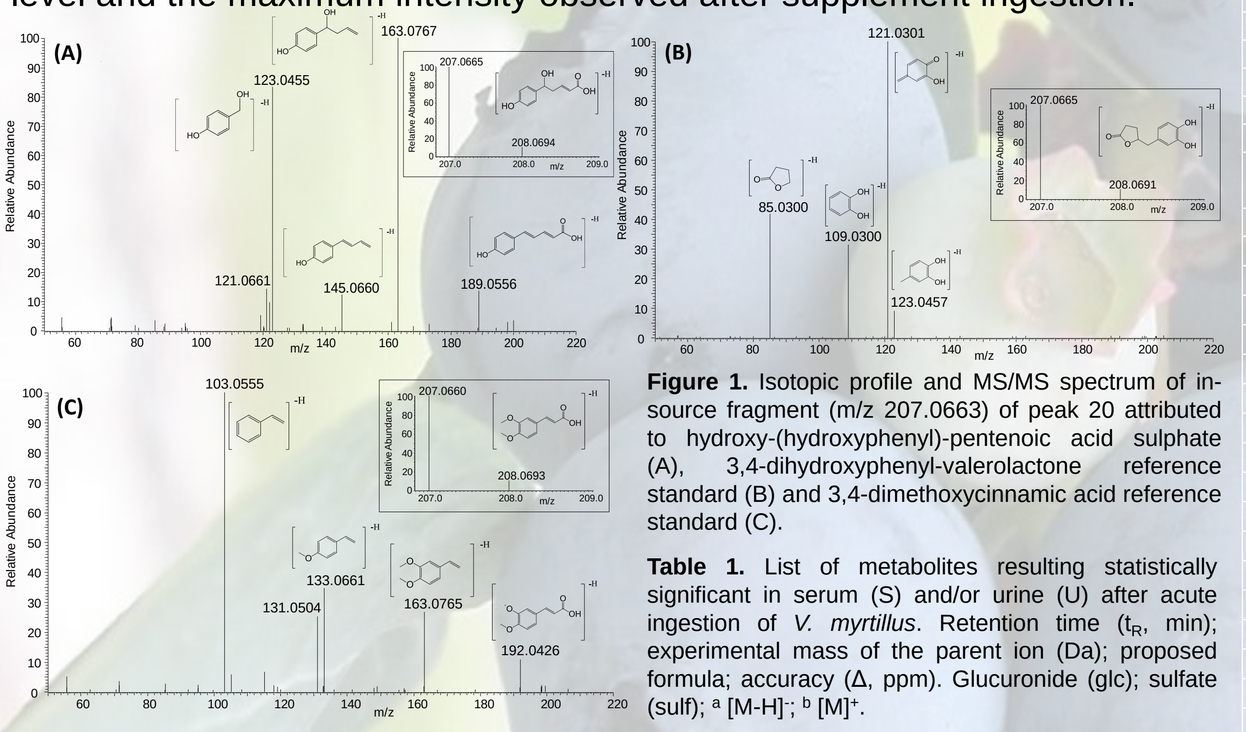


Figure 1. Isotopic profile and MS/MS spectrum of in-source fragment (m/z 207.0663) of peak 20 attributed to hydroxy-(hydroxyphenyl)-pentenoic acid sulphate (A), 3,4-dihydroxyphenyl-valerolactone reference standard (B) and 3,4-dimethoxycinnamic acid reference standard (C).

Table 1. List of metabolites resulting statistically significant in serum (S) and/or urine (U) after acute ingestion of *V. myrtillus*. Retention time (t_R, min); experimental mass of the parent ion (Da); proposed formula; accuracy (Δ, ppm). Glucuronide (glc); sulfate (sulf); [a] $[M-H]^-$; [b] $[M]^+$.

No	t_R	Parent ion	Formula	Δ	Tentative Identification	Biological fluid
1	2.4	343.0663[a]	$C_{14}H_{16}O_{10}$	-2.3	Hydroxy-methoxy benzoic acid glc I (Vanillic acid glc I)	U
2	3.0	274.0019[a]	$C_9H_9NO_7S$	-2.9	o-Hydroxyhippuric acid sulf	S ; U
3	3.3	277.0017[a]	$C_9H_{10}O_8S$	-2.6	Hydroxy-dimethoxy benzoic acid sulf (Syringic acid sulf)	U
4	3.3	373.0768[a]	$C_{15}H_{18}O_{11}$	-2.1	Hydroxy-dimethoxy benzoic acid glc I (Syringic acid glc I)	U
5	3.4	188.9860[a]	$C_6H_6O_5S$	-1.6	Catechol sulf	S ; U
6	3.5	329.0873[a]	$C_{14}H_{18}O_9$	0.9	Hydroxy-methoxy benzoic acid hexoside (Vanillic acid glucoside)	U
7	3.6	343.0663[a]	$C_{14}H_{16}O_{10}$	-2.3	Hydroxy-methoxy benzoic acid glc II (Vanillic acid glc II)	U
8	3.7	399.0924[a]	$C_{17}H_{20}O_{11}$	-2.2	Hydroxy-(dihydroxyphenyl) pentenoic acid glc	U
9	3.8	401.1081[a]	$C_{17}H_{22}O_{11}$	-2.0	Hydroxy-(dihydroxyphenyl) valeric acid glc I	U
10	3.9	373.0767[a]	$C_{15}H_{18}O_{11}$	-2.4	Hydroxy-dimethoxy benzoic acid glc II (Syringic acid glc I)	U
11	3.9	224.0561[a]	$C_{10}H_{11}NO_5$	-1.3	Hydroxy-methoxy hippuric acid	U
12	4.0	303.0173[a]	$C_{11}H_{12}O_8S$	-2.3	Hydroxy-(dihydroxyphenyl) pentenoic acid sulf	U
13	4.0	401.1081[a]	$C_{17}H_{22}O_{11}$	-2.0	Hydroxy-(dihydroxyphenyl) valeric acid glc II	U
14	4.0	465.1029[b]	$C_{21}H_{21}O_{12}$	0.2	Delphinidin-hexoside	U
15	4.2	415.1234[a]	$C_{18}H_{24}O_{11}$	-2.9	Trimethoxy-hydrocinnamic acid glc or Hydroxy-(hydroxy-methoxyphenyl) valeric acid glc	S ; U
16	4.2	305.0328[a]	$C_{11}H_{14}O_8S$	-2.9	Hydroxy-(dihydroxyphenyl) valeric acid sulf	S ; U
17	4.2	285.0609[a]	$C_{12}H_{14}O_8$	-2.4	Catechol glc	S ; U
18	4.2	369.0817[a]	$C_{16}H_{18}O_{10}$	-2.7	Hydroxy-methoxycinnamic acid glc I (Ferulic acid glc I)	S
19	4.3	449.1080[b]	$C_{21}H_{21}O_{11}$	0.5	Cyanidin-hexoside	U
20	4.3	287.0224[a]	$C_{11}H_{12}O_7S$	-2.4	Hydroxy-(hydroxyphenyl) pentenoic acid sulf I	U
21	4.4	353.0870[a]	$C_{16}H_{18}O_9$	-2.3	Chlorogenic acid	U
22	4.5	317.0329[a]	$C_{12}H_{14}O_8S$	-2.5	Hydroxy-(hydroxy-methoxyphenyl)-pentenoic acid sulf	U
23	4.6	399.0925[a]	$C_{17}H_{20}O_{11}$	-2.0	Hydroxy-dimethoxy cinnamic acid glc (Sinapic acid glc)	U
24	4.7	413.1079[a]	$C_{18}H_{22}O_{11}$	-2.4	Hydroxy-(hydroxy-methoxyphenyl)-pentenoic acid glc I	U
25	4.8	383.0977[a]	$C_{17}H_{20}O_{10}$	-1.8	Hydroxy-(hydroxyphenyl) pentenoic acid glc	S ; U
26	4.8	369.0819[a]	$C_{16}H_{18}O_{10}$	-2.2	Hydroxy-methoxycinnamic acid glc II (Ferulic acid glc II)	S
27	4.9	413.1080[a]	$C_{18}H_{22}O_{11}$	-2.2	Hydroxy-(hydroxy-methoxyphenyl)-pentenoic acid glc II	U
28	5.0	287.0224[a]	$C_{11}H_{12}O_7S$	-2.4	Hydroxy-(hydroxyphenyl) pentenoic acid sulf II	S ; U
29	5.1	357.0819[a]	$C_{15}H_{18}O_{10}$	-2.2	Dihydroxyphenyl propionic acid glc	U
30	5.3	455.1549[a]	$C_{21}H_{28}O_{11}$	-2.2	Hydroxy-abscisic acid glc	S ; U
31	5.4	367.1027[a]	$C_{17}H_{20}O_9$	-2.2	Feruloylquinic acid	U
32	5.9	439.1599[a]	$C_{21}H_{28}O_{10}$	-2.5	Abscisic acid glc	S ; U
33	6.1	333.0607[a]	$C_{16}H_{14}O_8$	-2.7	Methyl-dihydromyricetin	U
34	6.4	279.1231[a]	$C_{15}H_{20}O_5$	-2.5	Hydroxy-abscisic acid	U
35	6.5	245.0120[a]	$C_9H_{10}O_6S$	-2.0	Hydroxyphenyl propionic acid sulf	S
36	7.2	263.1283[a]	$C_{15}H_{20}O_4$	-2.3	Abscisic acid	U

CONCLUSIONS

The use of the untargeted metabolomics approach allowed the annotation of a wide range of metabolites belonging to 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. The presence of other metabolites than those deriving from anthocyanins should be emphasized here. 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. 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.

Reference - J. Am. Soc. Mass Spectrom. (2019) 30:381-402

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