



Comparison of chemometric strategies for potential exposure marker discovery and false-positive reduction in untargeted metabolomics: application to the serum analysis by LC-HRMS after intake of *Vaccinium* fruit supplements

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Abstract

Untargeted liquid chromatographic-high-resolution mass spectrometric (LC-HRMS) metabolomics for potential exposure marker (PEM) discovery in nutrikinetic studies generates complex outputs. The correct selection of statistically significant PEMs is a crucial analytical step for understanding nutrition-health interactions. Hence, in this paper, different chemometric selection workflows for PEM discovery, using multivariate or univariate parametric or non-parametric data analyses, were comparatively tested and evaluated. The PEM selection protocols were applied to a small-sample-size untargeted LC-HRMS study of a longitudinal set of serum samples from 20 volunteers after a single intake of (poly)phenolic-rich *Vaccinium myrtillus* and *Vaccinium corymbosum* supplements. The non-parametric Games-Howell test identified a restricted group of significant features, thus minimizing the risk of false-positive retention. Among the forty-seven PEMs exhibiting a statistically significant postprandial kinetics, twelve were successfully annotated as purine pathway metabolites, benzoic and benzodiol metabolites, indole alkaloids, and organic and fatty acids, and five (i.e. octahydro-methyl- β -carboline-dicarboxylic acid, tetrahydro-methyl- β -carboline-dicarboxylic acid, citric acid, caprylic acid, and azelaic acid) were associated to *Vaccinium* berry consumption for the first time. The analysis of the area under the curve of the longitudinal dataset highlighted thirteen statistically significant PEMs discriminating the two interventions, including four intra-intervention relevant metabolites (i.e. abscisic acid glucuronide, catechol sulphate, methyl-catechol sulphate, and α -hydroxy-hippuric acid). Principal component analysis and sample classification through linear discriminant analysis performed on PEM maximum intensity confirmed the discriminating role of these PEMs.

Keywords Untargeted metabolomics · Multivariate analysis · Univariate parametric analysis · Univariate non-parametric analysis · Exposure markers · Nutrikinetic

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Introduction

Untargeted metabolomics has been extensively recognized as the leading approach for the investigation of potential exposure markers (PEMs) of food [1, 2], also in relation to food-health interaction studies [3] (i.e. nutrimentalomics), as it allows in principle the comprehensive overview of the human metabolome. Untargeted metabolomics platforms usually consist in liquid chromatography (LC) hyphenated with high-resolution mass spectrometry (HRMS), which guarantees the determination of a much larger number of biological metabolites and/or a more comprehensive structural investigation, compared to other techniques such as gas chromatography coupled to HRMS or LC coupled to nuclear magnetic resonance [4].

The use of LC-HRMS platforms in nutrimentalomics generally results in very complex outputs of data, which require the implementation of proper workflows for their pretreatment and processing [5]. Currently, no standard protocol has been defined to this aim, even though many workflows of data processing and analysis have been proposed [6]. Data handling is usually performed by chemometric tools, specifically selected according to the study design adopted, which may largely vary depending on a number of factors, such as study design (i.e. cross over or parallel), the number of interventions, sample size, and data acquisition strategies (e.g. standalone full scan or coupled with MSⁿ acquisitions) [5, 7]. In nutrimentalomics studies, food PEM discovery commonly involves the application of multivariate analysis, using partial least square-discriminant analysis (PLS-DA) and the variable importance in projection (VIP) classifier as filtering strategy of statistically significant features from the original dataset [8–10]. However, multivariate methods such as PLS-DA-VIP, due to their probabilistic nature, tend to select false positive (variables not causally related to groups) and/or false negative (missing of relevant features) [11]. On the other hand, the multiple univariate approaches, which have been applied less frequently in nutrimentalomics, although not associated per se with false discovery risks, need the verification of some data distribution pre-requisites, i.e. normality or homoscedasticity [12]. However, it should be emphasized that multiple univariate approaches have been frequently applied as parametric methods (e.g. *t* test and ANOVA), although the sample size was so small to suggest the absence of the aforementioned prerequisite [13, 14], thus involving a high risk of generating false positive. In order to reduce the false-positive risk, an adjustment of the false-positive rate should be performed for the application of multiple inference tests [15]. Conversely, the use of non-parametric tests is much less investigated [16], although the application

of this type of chemometric tools would release the data analysis procedure from the constraints of homoscedasticity and normal distribution of data.

Even though the impact of different chemometric tools in the PEM selection is expected to be remarkable, this issue is poorly investigated in the literature, especially in LC-HRMS nutrimentalomics studies that, as mentioned before, are prone by their nature to generate a large number of features to be tested for their statistical significance [17]. It is therefore important to compare the effects of the use of different chemometric approaches for the selection of statistically significant PEMs. This issue is particularly important in the light of the intrinsic complexity of the biological fluids (e.g. serum and urine) investigated in this type of studies. Among these matrices, serum and plasma are particularly relevant as matrices that contain substances responsible for a direct biological activity, the study of which is of remarkable importance to establish a cause-effect role between the intake of a certain food and the human health benefits.

Based on the aforementioned considerations, this paper aimed at comparing the effectiveness of chemometric protocols for PEM discovery and false-positive reduction, using different data analysis approaches, which included the widely adopted PLS-DA-VIP, the parametric *t* test (before and after *P* value adjustment), and the non-parametric Wilcoxon and Games-Howell tests (*P* value adjusted for both tests), the latter never investigated before in untargeted nutrimentalomics.

The protocols have been tested on the untargeted LC-HRMS analysis of serum samples from an intervention study of *Vaccinium myrtillus* (VM) and *Vaccinium corymbosum* (VC) supplements as a representative application. In this regard, it should be emphasized that this kind of application is of considerable intrinsic importance for nutrimentalomics purposes. In fact, among fruits, small berries represent an abundant source of phenolic compounds in the human diet [18]. In detail, the fruits belonging to the *Vaccinium* genus, above all *V. myrtillus* berries, have been suggested as functional foods and used for supplement and drug preparation [19, 20]. Moreover, in vitro studies have shown anti-proliferative and pro-apoptotic effects of polyphenol-rich berry extracts against different prostate cancer cell lines [21] and the chemopreventive properties of an anthocyanin-rich *V. myrtillus* extract were suggested in a pilot study with patients affected by colon cancer [22]. It should also be noted that few targeted [23, 24] and only one untargeted [1] metabolomics studies have been published so far on human serum and urinary (poly)phenolic metabolites of *Vaccinium* berries. Fig. 1 illustrates a comprehensive and intuitive scheme of the analytical workflow adopted in this study for the PEM discovery in human serum after the administration of bilberry or blueberry supplement.

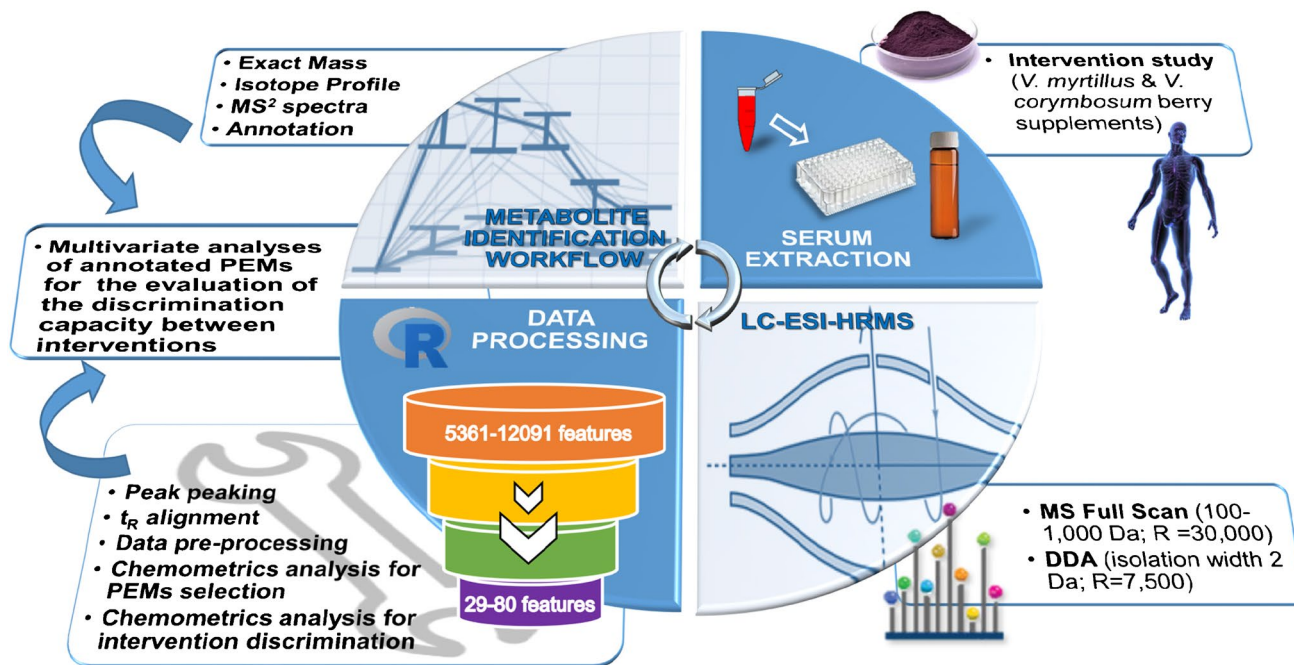


Fig. 1 Graphical workflow of the experimental steps followed in this study

Material and methods

Reagents and standards

Methanol and acetonitrile LC-MS Ultra CHROMA-SOLV™ and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was obtained by purifying demineralized water in a Milli-Q system from Millipore (Bedford, MA, USA). O-Hydroxyhippuric acid, abscisic acid, azelaic acid, 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic, and L-tryptophan-2',4',5',6',7'-d5 (TRI-d5) reference standards were obtained from Sigma-Aldrich, while trans-cinnamic-d5 acid (CIN-d5) and N-benzoyl-d5-glycine (hippuric acid-d5, HIP-d5) were from CDN ISOTOPES Inc.

Vaccinium myrtillus and Vaccinium corymbosum berry supplements

The supplements were obtained by cryo-milling VM and VC freeze-dried berries, as detailed in section 1 of the *Supplementary material*. The supplements were characterized for total soluble polyphenols, total monomeric anthocyanins, and some phenolic compounds, using methods elsewhere described [25]. Full details of the characterization analysis (Tables S1 and S2) and results obtained (Tables S3-S5) are reported in section 2 of the *Supplementary material*.

Study design

The study was a randomized, single-blinded two-arm intervention of a single intake of VM and VC, involving twenty healthy voluntary subjects (11 males and 9 females aged between 25 and 60 years). 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. Ethical approval for freeze-dried VM and VC powdered supplement administration was obtained for a phase I–II study (approval n. SPE 14.178 AOUC, 30th May 2016).

All the subjects convened early in the morning after 10 h of fasting and were randomly divided into two groups according to an electronic randomization key. A single dose of 25 g of VM supplement mixed with 500 mL of water was orally administered to the first group, whereas the same amount of VC berry supplement mixed with 500 mL of water was orally administered to the second group. This dose was chosen according to previous studies, as detailed in section 3 of the *Supplementary material*. Serum was collected at baseline and different sampling times (30, 60, 120, 240, and 360 min) after the supplement consumption, as described in section 3 (Table S6) of the *Supplementary material*. After the collection, samples were divided in aliquots of 500 μL, frozen at – 80 °C, and stored until LC-HRMS analysis was performed.

Serum extraction

Serum extraction was performed on Waters Ostro 96-well plates as reported elsewhere [1]. The quality of the untargeted analysis was guaranteed by the use of quality control (QC) samples and internal and external standards. See details in section 4 of the *Supplementary material*.

LC-MS and LC-MS/MS analyses

LC analysis was performed on a Dionex (Sunnyvale, CA, USA) 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 to a hybrid linear ion trap Fourier Transform (LTQ FT) Orbitrap high-resolution mass spectrometer (Thermo Fisher, Waltham, MA, USA) by an electrospray ionization (ESI) probe for MS and MS/MS analyses both in positive (PI) and negative (NI) ionization. Each sample was analysed under PI and NI, using two different mass acquisition methods for each ionization mode. The first method consisted of a full-scan method (mass range from 100 to 1000 Da) at a mass resolution of 30,000 FWHM (m/z 400) in centroid mode. The second method included data-dependent acquisition (DDA) scans for the three most abundant ions per scan at a resolution of 7500 FWHM. Product ions were generated in the LTQ trap at collision energy of 35 eV using an isolation width of 2 Da. Further details related to MS acquisition are reported in section 5 of the *Supplementary material*.

Data pre-treatment

All raw data were manually inspected using the Qual browser module of Xcalibur version 2.0.7 (Thermo Fisher). The LC-MS raw files were converted to mzML format using the MSConvert utility included in ProteoWizard [26]. Then, the mzML files were processed with the XCMS R package that allows for obtaining detection and retention time alignment of features (i.e. ions characterized by a unique exact mass and retention time) [2, 27]. During this processing, the maximum value for mass accuracy and retention time deviation was set equal to 5 ppm and 2 s, respectively. Data pre-processing of full-scan acquisitions was performed according to Garcia-Aloy et al. 2020 [28]. Briefly, each dataset was filtered to discard noisy and irrelevant features. Firstly, those features exhibiting a mass defect between 0.4 and 0.8 Da were removed from each data set. This procedure is based on the experimental evidence that exact masses mainly associated to metabolites of interest in this study (i.e. approximately 150–500 Da) have mass defects probabilistically distributed outside the aforementioned mass defect interval [29]. Subsequently, the signals that showed a higher

variation coefficient in QC samples than within study samples were also excluded. A random number between 1 and 500 replaced all zero values. These thresholds were fixed empirically according to the intensity level representing the noise of the chromatogram. The principal component analysis (PCA) on log-transformed and Pareto-scaled data was applied to check data quality (i.e. possible batch effect) and to detect the presence of outliers.

Chemometric analyses

PEMs in postprandial responses were selected separately for VM and VC interventions using a selection protocol consisting in a two-step procedure: (i) statistical significance among time points within the same intervention and (ii) nutrikinetic discrimination between interventions.

Statistical significance among time points of the same intervention

For the identification of PEMs suitable for the discrimination among time points, the following approaches were investigated: univariate post hoc non-parametric Games-Howell and Wilcoxon signed-rank tests, univariate parametric t test, and multivariate PLS-DA-VIP. A short overview of the characteristics of these tests is reported in section 6 of the *Supplementary material* (Table S7).

For the univariate approaches, we first selected those features with increasing mean values of chromatographic intensity in at least two consecutive time points, against baseline samples (i.e. time point “0 min”). Normal distribution of data was investigated by the application of Shapiro-Wilk’s test for multiple variables, while homogeneity of variance was evaluated adopting a Bartlett’s test for multiple independent variables, comparing the variances of VM and VC datasets in both polarities. Then, the t test and the post hoc non-parametric Games-Howell and Wilcoxon tests were applied to evaluate the statistical significance between time point “0 min” and all the others, setting the threshold P value at 0.05. For both t test and non-parametric tests, the Benjamini-Hochberg procedure was applied to decrease the false discovery rate (significance level 5%).

The multivariate PLS-DA-VIP approach was performed on (i) the pre-treated VM and VC datasets and (ii) using the *plsda* package implemented with VIP scoring, setting the inclusion cut-off as > 1 , as elsewhere reported [11, 16].

VM and VC discrimination by nutrikinetic data

For each feature selected as significant in VM and/or VC, the baseline sample was subtracted from the intensities of the other time points, and in case of negative values, the replacement by zero was performed. Nutrikinetic significant

features were selected using a two-step procedure based on their nutrkinetic curve behaviour. For that purpose, according to a previous intervention study [28], features that in at least two consecutive points exhibited the 25th percentile of one group ($n = 2$) higher than the 75th percentile of the other group ($n = 8$) were included in the discrimination process based on comparison of the area under the curve (AUC). The AUC of each selected feature was calculated between time 0 and 360 min using the *pracma* R package. Differences of AUCs among diets were tested using the Kruskal-Wallis test, and the obtained P values were adjusted using the Benjamini-Hochberg method. Adjusted P values < 0.05 were considered statistically significant. Descriptive analysis for evaluating the discrimination between the two interventions was carried out by means of PCA using the *FactoMineR* R package. Quality control of PCA was performed using QCs by visually verifying if PCA object scores obtained by replicated injections of the QC sample were close to the origin of PCA coordinates. Linear discriminant analysis (LDA) was performed on QC, VC, and VM sample groups made up by the annotated PEMs discriminating the two interventions, using the *MASS* R package.

Metabolite identification

The identification of features of interest, evidenced by data analysis, was performed according to the criteria previously reported by our research group [19], using the workflow described below. (i) Comparison of the exact mass of the experimental precursor ion with the pseudo-molecular ions proposed by the MzCloud (www.mzcloud.org), Humane Metabolome Database (HMDB, www.hmdb.ca), MassBank of North America (MoNA, <http://mona.fiehnlab.ucdavis.edu>), and Kyoto Encyclopedia of Genes and Genomes (KEGG) libraries, selecting a mass accuracy (Δ) ≤ 5 ppm as tolerance threshold. (ii) Export of the isotopic profile of pseudo-molecular ions selected within libraries at step (i) and subsequent comparison with the isotopic profile of the experimental precursor ions, selecting features providing an isotope ratio percentage difference of 20% as tolerance threshold. (iii) Structural elucidation of features of interest through the evaluation of MS/MS spectra obtained with DDA mass method in comparison with matched library mass spectra. (iv) Feature annotation performed according to golden standards for metabolomics [30].

Results and discussion

Assessment of data quality

Internal standards in study samples and QC samples showed a variation of retention time and mass accuracy below 2 s

and 5 ppm, respectively, thus highlighting the correct data acquisition. 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 samples resulted lower than 20% confirming the repeatability of the extraction and excluding the possibility of signal drift and carryover phenomena during the LC-MS and LC-MS/MS analyses. Moreover, by means of Pareto-scaled PCA performed on raw data (data not shown), it was possible to observe that QC samples are well grouped in component space, showing no batch effect, or trend due to the injection order (i.e. drop in signal intensity). Study samples are distributed homogeneously with no visible trends according to post-prandial time point.

Chemometric workflow for feature selection

After data pre-treatment, complex data sets were obtained for both interventions, the total number of features being 12,091 and 5361 for PI and NI, respectively.

The supervised multivariate PLS-DA-VIP analysis was performed on these groups of features, as commonly done in literature, highlighting a very high number of statistically significant features (2283–8639, depending on the dataset considered). This result is probably due to the tendency of the PLS-DA-VIP approach to select false positives. In fact, this criterion is very reasonable to discard irrelevant variables, but it may have drawbacks if used for assessing the significance of features [11]. Moreover, the model classification error rate of maximized distances was very high, ranging from 0.9 to 0.7, regardless of the number of selected components for PLS-DA modelling.

For the application of univariate data analysis, the total number of features was preliminarily filtered by a selection procedure that preserves only the features showing increasing intensities in at least two consecutive time points. This feature selection resulted in a number of relevant postprandial features ranging from 4245 to 4932, depending on the intervention and acquisition mode (Table 1). The obtained datasets were checked for (i) normal distribution and (ii) homogeneity of variance (i.e. homoscedasticity). Normality was evaluated by the application of the Shapiro-Wilk's test for multiple variables. Shapiro-Wilk's correlation coefficients and P values (in brackets) ranged as follows: (i) VC–NI 0.538–0.897 ($1.0 \cdot 10^{-5}$ – $4.9 \cdot 10^{-2}$); (ii) VC–PI 0.656–0.761 ($3.0 \cdot 10^{-3}$ – $4.9 \cdot 10^{-2}$); (iii) VM–NI 0.366–0.807 ($1.0 \cdot 10^{-7}$ – $3.4 \cdot 10^{-2}$); and (iv) VM–PI 0.609–0.734 ($2.0 \cdot 10^{-4}$ – $5.0 \cdot 10^{-3}$), thus highlighting the absence of normality of the investigated datasets. Homogeneity of variance was evaluated adopting Bartlett's test for multiple independent variables, comparing the variances of VC and VM datasets in both polarities. The results obtained were characterized by P value < 0.05 (confidence interval =

Table 1 Number of relevant postprandial features and potential exposure markers (PEMs) in each dataset (intervention and acquisition polarity) identified using the selection strategies based on univariate non-parametric adjusted post hoc Games-Howell test, pairwise Wilcoxon signed-rank test, and the univariate parametric *t* test, before and after the Benjamini-Hochberg adjustment procedure (BH-adj). VM = *Vaccinium myrtillus* supplement intervention; VC = *Vaccinium corymbosum* supplement intervention

Dataset	Relevant postprandial features	<i>t</i> test	BH-adj	Wilcoxon	Games-Howell
VM ESI (+)	4731	4682	1957	359	29
VC ESI (+)	4932	4395	2154	146	18
VM ESI (−)	4655	3286	1075	224	51
VC ESI (−)	4245	2582	1051	75	11

0.95), thus evidencing lack of homogeneity among variances of the aforementioned datasets.

The aforementioned univariate tests were used for the PEM selection within the aforementioned datasets of relevant postprandial features, obtaining more or less populated groups of statistically significant features in each acquisition polarity (Table 1). The adoption of the *t* test generated the largest group of “statistically significant” PEMs, which surely includes a high number of false positives, even after the *P* value correction using the Benjamini-Hochberg procedure (1051–2154 features, depending on the dataset considered), due to the non-normal and heteroscedastic distribution of data, which should discourage the use of parametric test for PEM selection. The Wilcoxon test was found to be much more restrictive (75–359 features) than the adjusted *t* test, in accordance with its non-parametric character, which does not require compliance with the conditions of normality and heteroscedasticity of data distribution, making it therefore more suitable for the treatment of small sample size, for which the above-mentioned conditions usually do not occur. A significantly reduced number of statistically significant features (i.e. 80 and 29 for VM and VC interventions, respectively) was identified using the Games-Howell test, thus highlighting its strong selectivity, which greatly reduces the probability of including false positives in the PEMs group.

It is worth noting that the group of features selected by the Games-Howell test represents a subset included in all the groups of features selected by the other tests investigated. Moreover, using as representative cases some features that can be easily annotated due to their previous identifications in bilberry and/or blueberry intervention studies, it is interesting to evaluate how these features are treated by the procedures of PEMs selection here tested. For instance, benzoic acid was annotated here ($\Delta = -0.4$ ppm) after the intake of VC supplement and retained in the statistically significant PEM group using the adjusted *t* test. However,

this feature was excluded when PLS-DA-VIP or the non-parametric Wilcoxon and Games-Howell tests for PEM selection were applied. Moreover, hippuric acid was annotated here ($\Delta = -0.6$ ppm) in both interventions and considered statistically significant by PLS-DA-VIP, adjusted *t* test, and Wilcoxon test but excluded by the Games-Howell treatment. It is interesting to note that these features were considered statistically significant in a study investigating plasma after blueberry intake using ANOVA and post hoc Bonferroni correction [24]. As further examples, four metabolites (i.e. hydroxy-methoxy hippuric acid, hydroxy-(hydroxy-methoxyphenyl)-pentenoic acid glucuronide, dihydroxyphenyl propionic acid glucuronide, and hydroxyphenyl propionic acid sulphate) recently annotated as PEMs of bilberry intake [1] resulted here statistically significant by applying the Wilcoxon signed-rank test, while they were discarded by the Games-Howell test, due to the different assumptions made with respect to Wilcoxon test.

All these examples evidenced that the selection of significant features is strongly dependent on the statistical tool adopted. Based on these findings, great caution should be used in reporting metabolites as statistically significant for a given intervention. The application of highly conservative statistical methods, i.e. prone to minimizing the risk of false-positive results, is certainly useful in this sense and should be encouraged, although it involves a certain risk of excluding false negative from the significant dataset. On the contrary, the use of less restrictive tests should be used with great caution in PEM discovery, even though they give a wider overview of features that could become significant when moving from a pilot study, i.e. characterized by a limited number of subjects, to one on a larger validation cohort.

PEMs annotation

Among the features selected by the Games-Howell test, corresponding to 47 PEMs, the annotation protocol allowed for identifying 5 features in PI and 13 features in NI, corresponding to a total of 12 PEMs. Table 2 reports the annotated metabolites, providing their spectral characteristics, formula, time points for statistically significant maximum intensities (T_{\max}) with related *P* values, the annotation level, and for annotation levels I and II, the HMDB/KEGG identification numbers. Among the twelve PEMs identified, eight were annotated only after VM intervention (peaks 2–6, 9, 11, and 12), three only after VC ingestion (peaks 7, 8, and 10), and only one (peak 1) as common response to the consumption of the two supplements.

Peak 1 showed two pseudo-molecular ions in NI full-scan spectra, i.e. at m/z 335.0504 and m/z 167.0214. The second ion was characterized by a fragmentation pattern that matched with uric acid, as reported in mzCloud spectral library and literature findings [31]. *Peak 2* was

Table 2 List of metabolites found in serum samples after acute ingestion of *V. myrtillus* (VM) and *V. corymbosum* (VC) supplements. Retention time (t_R , min), experimental mass in full-scan spectrum (Da), main MS/MS fragments, proposed formula, mass accuracy (Δ , ppm), time point of max intensity (T_{max} , min) and *P* value withininterventions (in brackets), and HMDB/KEGG identification numbers. Roman numbers refer to the level of annotation: (I) spectra and t_R matched with reference standard; (II) spectra matched with reference libraries; (III) spectra matched with literature information. n.a. = not available

Peak	t_R	Full scan	MS/MS	Formula	T_{max} (<i>P</i> value)	Identification	HMDB/KEGG
1	1.2	(⁻) 335.0504 [2M-H] ⁻	167.0214	C ₅ H ₄ N ₄ O ₃	VM: 60 min (0.046)	Uric acid ^(III)	HMDB0000289/ C00366
		(⁻) 167.0214	124.0156 , 96.0207		VC: 30 min (0.037)		
2	1.2	(⁺) 193.0335 [M+H] ⁺	132.1019	C ₆ H ₈ O ₇	VM: 30 min (0.041)	Citric acid ^(II)	HMDB0000094/ C00158
		(⁻) 191.0202 [M-H] ⁻	173.0093, 111.0091		VM: 60 min (0.010)		
3	1.5	(⁻) 267.0744 [M-H] ⁻	135.0313	C ₁₀ H ₁₂ N ₄ O ₅	VM: 60 min (0.013)	Inosine ^(III)	HMDB0000195/ C00294
4	3.1	(⁻) 194.0454 [M-H] ⁻	150.0561	C ₉ H ₉ NO ₄	VM: 240 min (0.0001)	α -Hydroxy-hippuric acid ^(I)	HMDB0013678 HMDB0006116
5	3.9	(⁻) 188.9856 [M-H] ⁻	109.0297	C ₆ H ₆ O ₅ S	VM: 360 min (0.008)	Benzendiol (catechol) sulphate ^(II)	HMDB0059724
6	4.7	(⁻) 203.0014 [M-H] ⁻	188.9860, 123.0454 , 108.0220	C ₇ H ₈ O ₅ S	VM: 360 min (0.028)	Methyl-catechol sulphate ^(II)	HMDB0240663
7	5.23	(⁺) 279.1329 [M+H] ⁺	235.1376, 206.1110, 163.1165	C ₁₄ H ₁₈ N ₂ O ₄	VC: 60 min (0.023 ⁽⁺⁾ , 0.035 ⁽⁻⁾)	Octahydro-methyl- β -carboline- dicarboxylic acid ^(III)	n.a.
		(⁻) 277.1185[M-H] ⁻	233.1232				
		(⁻) 233.1232	189.1336 , 187.1179, 146.0914, 120.0759, 96.0736				
8	5.30	(⁺) 275.1024 [M+H] ⁺	231.1128, 202.0861, 159.0916	C ₁₄ H ₁₄ N ₂ O ₄	VC: 60 min (0.007 ⁽⁺⁾ , 0.008 ⁽⁻⁾)	Tetrahydro-methyl- β -carboline- dicarboxylic acid ^(III)	HMDB0035115
		(⁺) 202.0861	184.0755 , 156.0802				
		(⁻) 273.0875 [M-H] ⁻	229.0982				
		(⁺) 229.0982	185.1084 , 183.0928, 142.0663, 116.0508, 92.0508,				
9	5.9	(⁺) 265.1431	247.1329 , 199.1023	C ₂₁ H ₂₈ O ₁₀	VM: 120 min (0.044)	Abscisic acid glucu- ronide ^(II)	HMDB0036093
		(⁺) 247.1326	229.1431, 211.1327				
		(⁻) 439.1599 [M-H] ⁻	263.1288 , 219.1395, 175.0248, 153.0922				HMDB0035140
10	6.3	(⁺) 145.1220 [M+H] ⁺	127.1130 , 109.1008	C ₈ H ₁₆ O ₂	VC: 30 min (0.021)	Caprylic acid (hydroxy-octanone) ^(II)	HMDB00482/ HMDB0062511 HMDB62511/C06423
11	6.4	(⁻) 187.0980 [M-H] ⁻	125.0973	C ₉ H ₁₆ O ₄	VM: 360 min (0.028)	Azelaic acid ^(I)	HMDB00784/C08261
12	6.5	(⁻) 245.0119 [M-H] ⁻	165.0556	C ₉ H ₁₀ O ₆ S	VM: 120 min (0.038)	Hydroxyphenyl propionic acid sulphate ^(II)	HMDB0094710

Bold MS/MS fragments refer to spectra base peaks

putatively annotated as citric acid, due to the occurrence of precursor ions at m/z 193.0335 and 191.0202, in PI and NI, respectively. The MS/MS fragmentation pattern included as main fragments in NI mode peaks at m/z 173 (loss of a water molecule) and m/z 111.0091 (loss of acetic acid), whilst in PI m/z 132.1019 (loss of acetic acid) was highlighted. This attribution was confirmed by mzCloud spectral library and previous researches [32]. *Peak 3* exhibited [M-H]⁻ ion at m/z 267.0744, characterized by the neutral loss of 132 Da (pentose moiety) that led to the formation of a fragment at

m/z 135.0313 associated to hypoxanthine, in accordance with spectral libraries and previous findings [33]. Thus, this metabolite was putatively identified as inosine, a nucleoside naturally occurring in the pathway of purine metabolism after vegetable and/or fruit intake [34]. *Peak 4* gave rise to a [M-H]⁻ pseudo-molecular ion at m/z 194.0454 Da, which fragmented by losing 44 Da (CO₂) originating the fragment at m/z 150.0561. Since the pseudo-molecular ion matched with high accuracy ($\Delta = -2.6$ ppm) the mass of a hydroxy-hippuric acid and no other fragments were detected in the

MS² spectrum, α -hydroxy-hippuric acid, p-hydroxy-hippuric acid, and o-hydroxy-hippuric acid reference standards were injected, obtaining a t_R of 3.3, 3.8, and 5.4 min, respectively. Accordingly, peak 4 was annotated at level I as α -hydroxy-hippuric acid. Peak 5 was putatively identified as benzodiol (catechol) sulphate owing to the presence of the typical loss of sulphate (m/z 79.9576) and the appearance of the hydroxyphenol ion at m/z 109.0297 in the MS/MS spectrum [1, 35]. An analogous fragmentation pattern was observed for peak 6, putatively identified as methyl-catechol sulphate, due to the presence in the MS/MS spectrum of a peak at m/z 188.9860 (loss of methyl), together with typical losses of sulfonic (m/z 123.0454) and sulphate (m/z 108.0220) groups. Peak 7 (PI: m/z 279.1329, NI: m/z 277.1185) and peak 8 (PI: m/z 275.1024, NI: m/z 273.0875) were recognized as indole alkaloids derivatives, based on their mass accuracy and similar MSⁿ fragmentation patterns (Fig. S1 of the Supplementary material). Spectra of both peaks in PI (Fig. S1, box A and box E) were characterized by the neutral loss of C₂H₃NO₂ (73 Da, iminoacetic acid), typical of carboxylic acid derivatives of carbolines [36]. Moreover, under the NI mode, the pseudo-molecular ions (i.e. m/z 277.1185 and 273.0875) underwent the sequential loss of two CO₂ moieties, indicating the presence of two carboxylic groups (Fig. S1, box B–C and box F–G). In order to confirm the attribution of peaks 7 and 8 to the carbolines class, their fragmentation patterns were compared with the one of 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid reference standard (Fig. S1, boxes D–H). Interestingly, the pseudo-molecular ion of peak 8 in NI mode (m/z 273.0875) lost a carboxylic group, originating the feature at m/z 229.0982 (Fig. S1, box F), the fragmentation pattern of which (Fig. S1, box G) matched the one of the analytical standard 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid (Fig. S1, box D) with good mass accuracy and similar ion intensities. It should also be noted that peak 7 showed in both PI (Fig. S1, boxes A vs. E) and NI (Fig. S1, boxes B vs. F and C vs. G) MSⁿ spectra a constant difference of about 4.03 Da compared to corresponding ions of peak 8. Hence, peak 7 was annotated as octahydro-methyl- β -carboline-dicarboxylic acid. Peak 9 showed the [M–H][–] pseudo-molecular ion in NI at m/z 439.1599, which fragmented through the typical neutral loss of glucuronide-conjugated compounds (176 Da) giving rise to the product ion at m/z 263.1288. This ion further fragmented originating the m/z 219.1391 and 153.0921 ions (Fig. S2-A of the Supplementary material), which can be attributed to abscisic acid, as also highlighted by injecting the abscisic acid reference standard (Fig. S2-B). Peaks 10 and 11 were annotated as caprylic acid and azelaic acid, respectively, by comparing their mass spectra with those present in spectral databases (i.e. mzCloud and HMDB; see Table 2). In addition, azelaic acid was further confirmed at level I by injecting the analytical standard. Finally, peak 12 was putatively annotated as hydroxy-phenyl propionic acid sulphate thanks to the mass

accuracy of its [M–H][–] pseudo-molecular ion (m/z 245.0119, $\Delta = -2.4$ ppm), the comparison with MS² spectral libraries, and based on findings of previous clinical studies on VM intake [1].

Nutrimetabolomics significance

The twelve annotated metabolites reported in Table 2 were categorized as (i) purine pathway metabolites, (ii) benzoic and benzodiol metabolites, (iii) indole alkaloids, and (iv) organic and fatty acids, according to their chemical class and metabolism. Figs. 2, 3, 4, and 5 report the serum postprandial kinetics of the 12 annotated PEMs, expressed as boxplots of the precursor ion intensity as a function of time. Each boxplot represents the interquartile range (75% of the intensity values are less than or equal to the top value of the box, and 25% of the intensity values are less than or equal to the bottom value of the box), the upper and lower whiskers refer to the maximum and minimum data points, respectively; and the line within the box represents the median of the data.

Purine pathway metabolites

Fig. 2 illustrates the kinetic profile of the pseudo-molecular ions of uric acid (peak 1) in VM (Fig. 2A) and VC (Fig. 2B), and inosine (peak 3) in VM (Fig. 2C). According to literature, these metabolites are correlated with the ingestion of fructose, rather than phenolic compounds, since fructose induces acute depletion of ATP and inorganic phosphate and causes increased activity of the enzymes involved in the degradation of purine nucleotides to inosine, hypoxanthine, xanthine, and finally uric acid [37]. Statistically significant variations ($P < 0.05$) of uric acid and inosine were observed at T_{max} 30–60 min, suggesting that the fructose-induced response increases uric acid concentration in plasma in the early stages of metabolism. The increase in uric acid is responsible for the rise of plasma antioxidant activity widely observed in literature after ingestion of fructose-rich fruits [34, 38]. The fast depletion of these metabolites after 2–3 h from food intake is in agreement with the post-prandial trend commonly observed for phase II metabolites of methylxanthines, which are rapidly excreted through the urinary tract within 8 h from the intake [39, 40].

Benzoic and benzodiol metabolites

Fig. 3 shows the kinetic profiles of α -hydroxy-hippuric acid (peak 4, Fig. 3A), the sulphate derivatives of catechol (peak 5, Fig. 3B) and methyl-catechol (peak 6, Fig. 3C), and hydroxyphenyl propionic acid sulphate (peak 12, Fig. 3D), which were detected only in response to the VM intervention. Differently from purine derivatives, the trend observed for these compounds reflected a diverse postprandial

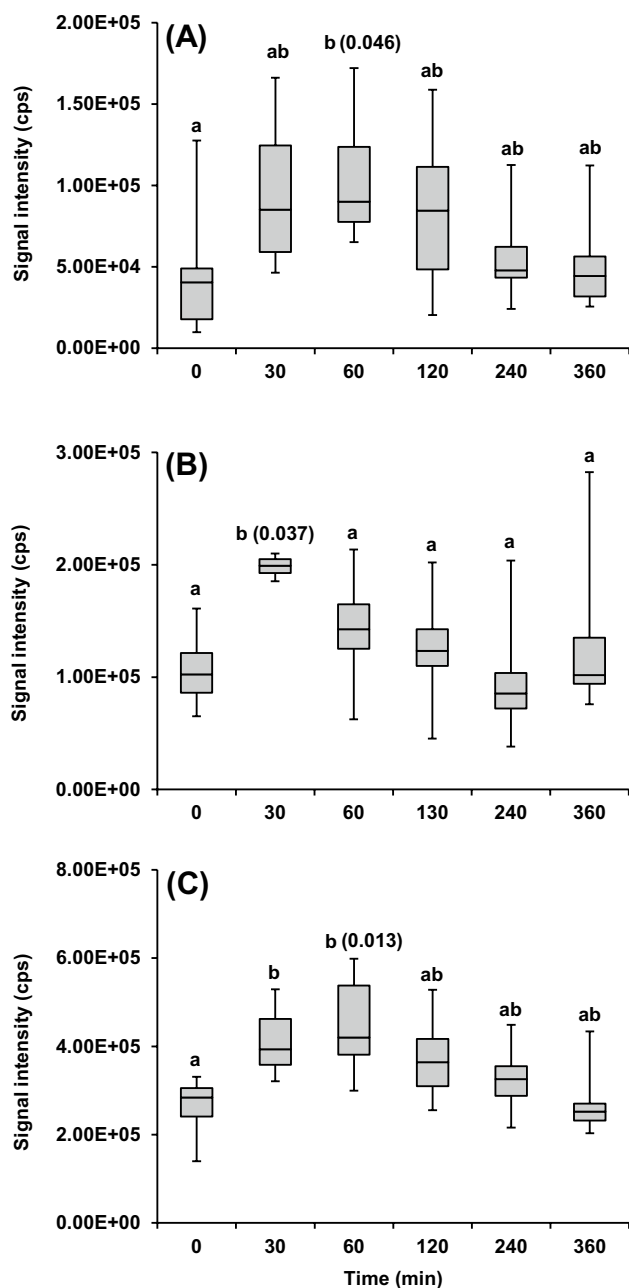


Fig. 2 Kinetic profiles of the pseudo-molecular ions (negative ionization mode) at m/z **A** 335.0504 (uric acid in VM), **B** 167.0214 (uric acid in VC), and **C** 267.0744 (inosine in VM). Boxplots exhibiting different letters are significantly different according to the Games-Howell comparison test ($P < 0.05$). T_{\max} of each pseudo-molecular ion is labelled in brackets with its corresponding P value

scenario. In fact, the increase in signal intensities became statistically significant ($P < 0.05$) starting from 2 h after the supplement ingestion (T_{\max} between 120 and 360 min), suggesting that their occurrence in plasma is associated to microbiota activity, which mediates the degradation of polyphenols in smaller and more soluble molecules [41]. In detail, the occurrence in human plasma of both hippuric acid

and catechol derivatives was already reported in literature [1, 23, 24] as potential markers of acute and/or long-term ingestion of bilberry and blueberry, and their occurrence related to quercetin glycosides, which are dominant flavonols in these fruits [1, 42, 43]. Conversely, phenyl-propionic acid derivatives may derive from caffeic acid degradation and/or from anthocyanins after B-ring cleavage, and C-ring opening and oxidation [44].

Indole alkaloids

Fig. 4 reports the kinetic profiles of the octahydro-methyl- β -carboline-dicarboxylic acid (*peak 7*, Fig. 4A, B) and the tetrahydro-methyl- β -carboline-dicarboxylic acid (*peak 8*, Fig. 4C, D), which have been annotated here for the first time in human plasma in response to the VC intervention. Interestingly, both PEMs showed a significant increase in their intensities ($P < 0.05$) in plasma at T_{\max} 60 min, then decreasing in the following time points, suggesting that they are rapidly absorbed by the human organism and likewise excreted through the urinary tract, as highlighted elsewhere [45]. This group of chemicals is already known to occur in fruits, including berries, as well as in food processed products, suggesting that diet may directly contribute to their presence in human bio-fluids [36, 46, 47]. However, the occurrence of these alkaloids should not be necessarily associated to the intake of fruit, since other foods such as beer, coffee, cereal products, and vegetables contain these alkaloids and may therefore contribute to their presence in human biofluids [36, 45].

Organic and fatty acids

Fig. 5 illustrates the kinetic profiles observed in response to the VM intervention for citric acid (*peak 2*, Fig. 5A, B), abscisic acid glucuronide (*peak 9*, Fig. 5C, D), and azelaic acid (*peak 11*, Fig. 5F). Moreover, the trend of caprylic acid (*peak 10*, Fig. 5E) after VC intervention is also shown.

Citric acid showed a postprandial maximum intensity in between 30 and 60 min. Even though this compound is already known as one of the main organic acids occurring in various fruits, including blueberry and especially bilberry [48–50], these data represent the first evidence of a correlation between fruit intake and the occurrence of a statistically significant increase of citric acid in plasma.

Abscisic acid glucuronide reached a T_{\max} at 120 min after the supplement consumption, suggesting that it is involved in the phase II metabolism, even though the possible endogenous contribution associated with supplement intake cannot be excluded. In fact, abscisic acid has been found at relevant concentrations in bilberry fruits as a well-known plant hormone involved in the regulation of biosynthesis of polyphenols, such as anthocyanins [51].

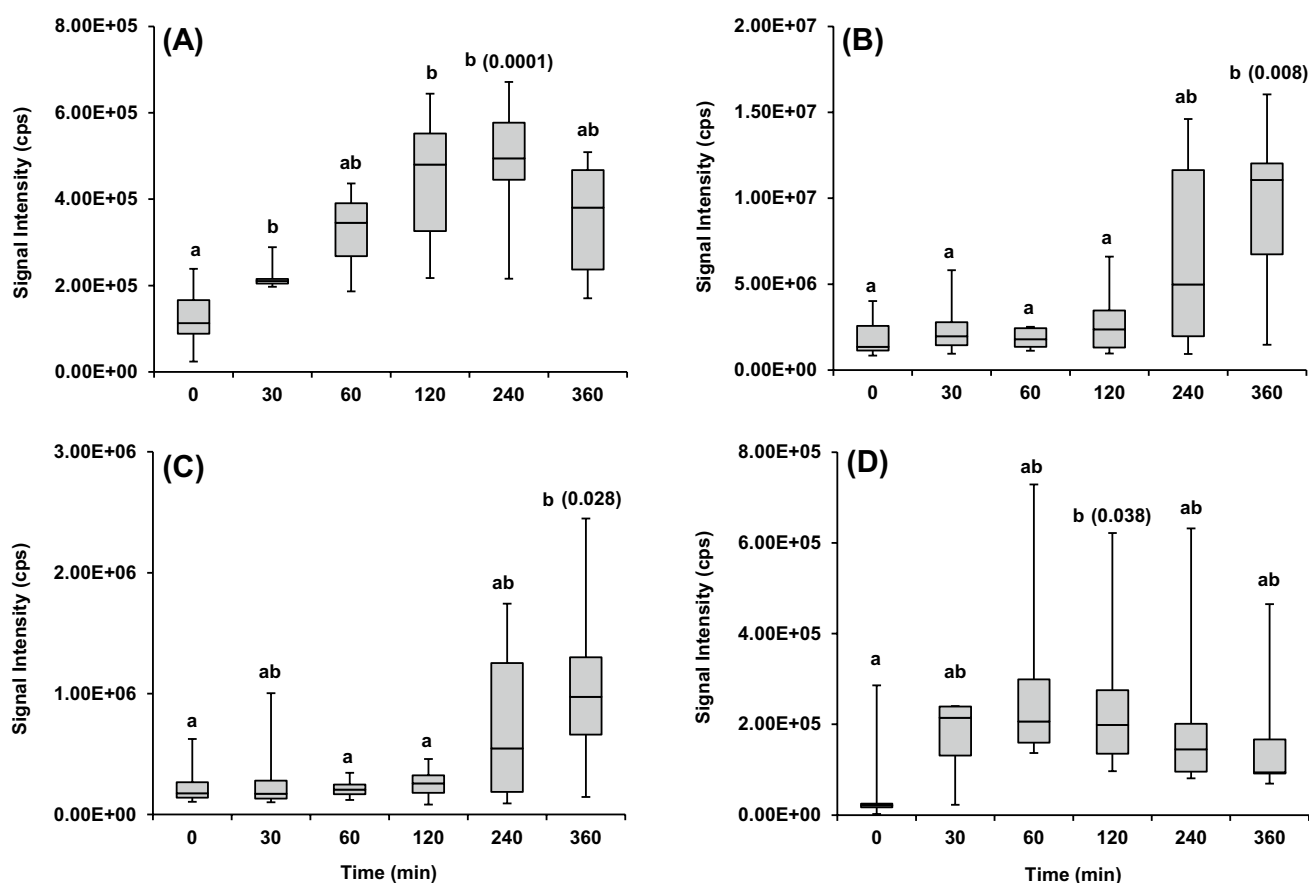


Fig. 3 Kinetic profiles of the pseudo-molecular ions (negative ionization mode) at m/z **A** 194.0454 (α -hydroxy-hippuric acid in VM), **B** 188.9856 (catechol sulphate in VM), **C** 203.0014 (methyl-catechol sulphate in VM), and **D** 245.0119 (hydroxyphenyl propionic acid sul-

phate in VM). Boxplots exhibiting different letters are significantly different according to the Games-Howell comparison test ($P < 0.05$). T_{\max} of each pseudo-molecular ion is labelled in brackets with its corresponding P value

Caprylic acid and azelaic acid showed a postprandial maximum intensity at very different T_{\max} , i.e. 30 min and 360 min after VC and VM interventions, respectively. In detail, caprylic acid, after the very fast absorption with an early post-prandial maximum intensity, exhibited a rapid decrease in the following time points, ascribable to its excretion by urine. On the other hand, the late intensity increase of the azelaic acid kinetic profile suggests a closer dependence on the phase I metabolism than the phase II one. The presence of fatty acids in serum has been poorly investigated in association with phenolic-rich fruit interventions. However, caprylic acid has been detected in human serum after pomegranate ingestion, probably due to its occurrence in seeds [52]. Moreover, fatty acids other than caprylic and azelaic have been detected in human serum after the intake of mulberry and attributed to their occurrence in fruit seeds and peels [53]. Hence, to our knowledge, caprylic acid and azelaic acid have been reported here as PEMs of VM and VC interventions for the first time.

Discrimination between VM and VC interventions

Comparison of serum metabolome profiles between the two berry interventions (performed according to the protocol described in “VM and VC discrimination by nutrikinetic data”) showed ten and fifteen discriminant features in PI and NI, respectively, which correspond to thirteen PEMs. Among these, four metabolites matched the previously annotated PEMs, i.e. abscisic acid glucuronide, α -hydroxy-hippuric acid, catechol sulphate, and methyl-catechol sulphate, which were found to be statistically significant in VM intervention (Table 2).

In order to provide a summarizing description of the discrimination capacity between the two interventions by the thirteen PEMs, it is interesting to perform a multivariate analysis by means of PCA and LDA of the autoscaled intensity data of these PEMs, expressed as the highest intensity value determined for each feature under NI in the kinetic profile of each intervention. Moreover, the intensities of these PEMs in QC samples were included in both PCA and

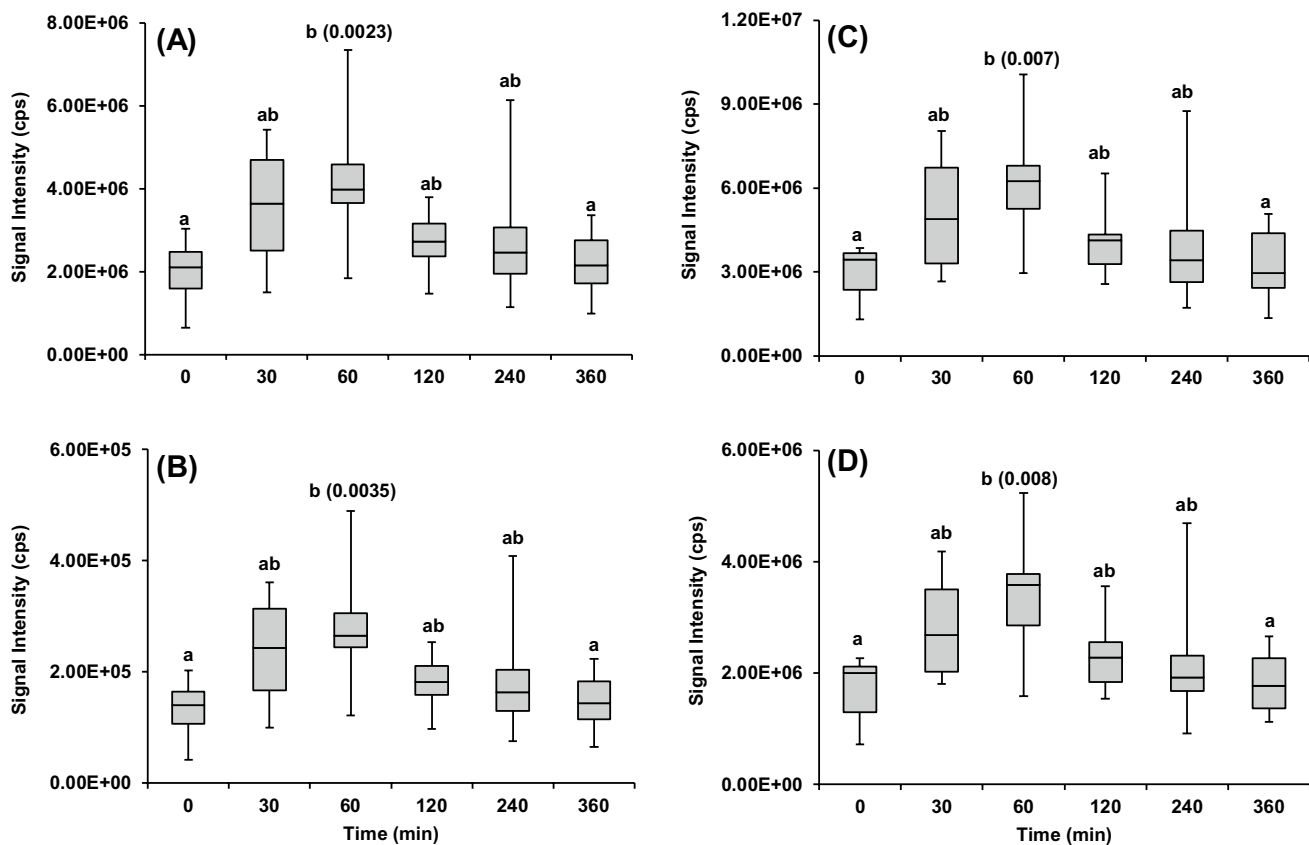


Fig. 4 Kinetic profiles of the pseudo-molecular ions (positive and negative ionization, respectively) at m/z **A** 279.1329 and **B** 277.1185 (octahydro-methyl- β -carboline-dicarboxylic acid in VC), **C** 275.1024 and **D** 273.0875 (tetrahydro-methyl- β -carboline-dicarboxylic acid in

VC). Boxplots exhibiting different letters are significantly different according to the Games-Howell comparison test ($P < 0.05$). T_{\max} of each pseudo-molecular ion is labelled in brackets with its corresponding P value

LDA, in order to make more informative the multivariate analyses, also avoiding misclassifications. Five principal components (PCs) characterized by eigenvalues > 1 and accounting for percentages of explained variance (EV) of 29.9%, 12.9%, 12.2%, 11.2%, and 8.2% were obtained (total EV = 74.6%).

Table S8 in section 6 of the *Supplementary material* illustrates the loading values of the five significant PCs. Most features mainly contributed to only one PC with high absolute values of the loadings, thus highlighting a good separation of the original variables among PCs. For example, abscisic acid glucuronide, α -hydroxy-hippuric acid, catechol sulphate, methyl-catechol sulphate, and the unknown at m/z 287.0224 contributed significantly only to PC1, which alone accounts for about a third of the total variance of the original data. A detailed description of the contribution of the various features to the five significant PCs is reported in section 6 of the *Supplementary material*. **Fig. 6** illustrates the score plots of PC1 vs. PC2, PC3, PC4, and PC5, which highlights a quite good separation of QC, VC, and VM samples in all the four PC spaces.

Moreover, QCs were very close to the origin of the coordinates, suggesting a high accuracy and precision of the entire analytical procedure. LDA has been also carried out on the dataset of the discriminating PEMs in QC, VC, and VM samples, in order to verify the correct assignment of each sample to its own group (i.e. QC, VM, and VC). The summary of the results obtained for the fitted and cross-validated (leave-one-out method) LDA classification is illustrated in **Table 3**. QC samples were correctly classified in all cases, both in fitting and in cross-validation, thus evidencing the robustness of this classification and confirming the results of PCA regarding the reliability of the analytical procedure here adopted. The LDA classification exhibited a very high robustness also for the VM and VC groups, since all samples were correctly classified in fitting and only two (i.e. one for each intervention) were misclassified in cross-validation. It should, however, be remarked that the two misclassified samples were attributed to the QC group, without any misleading attribution to the other volunteer group.

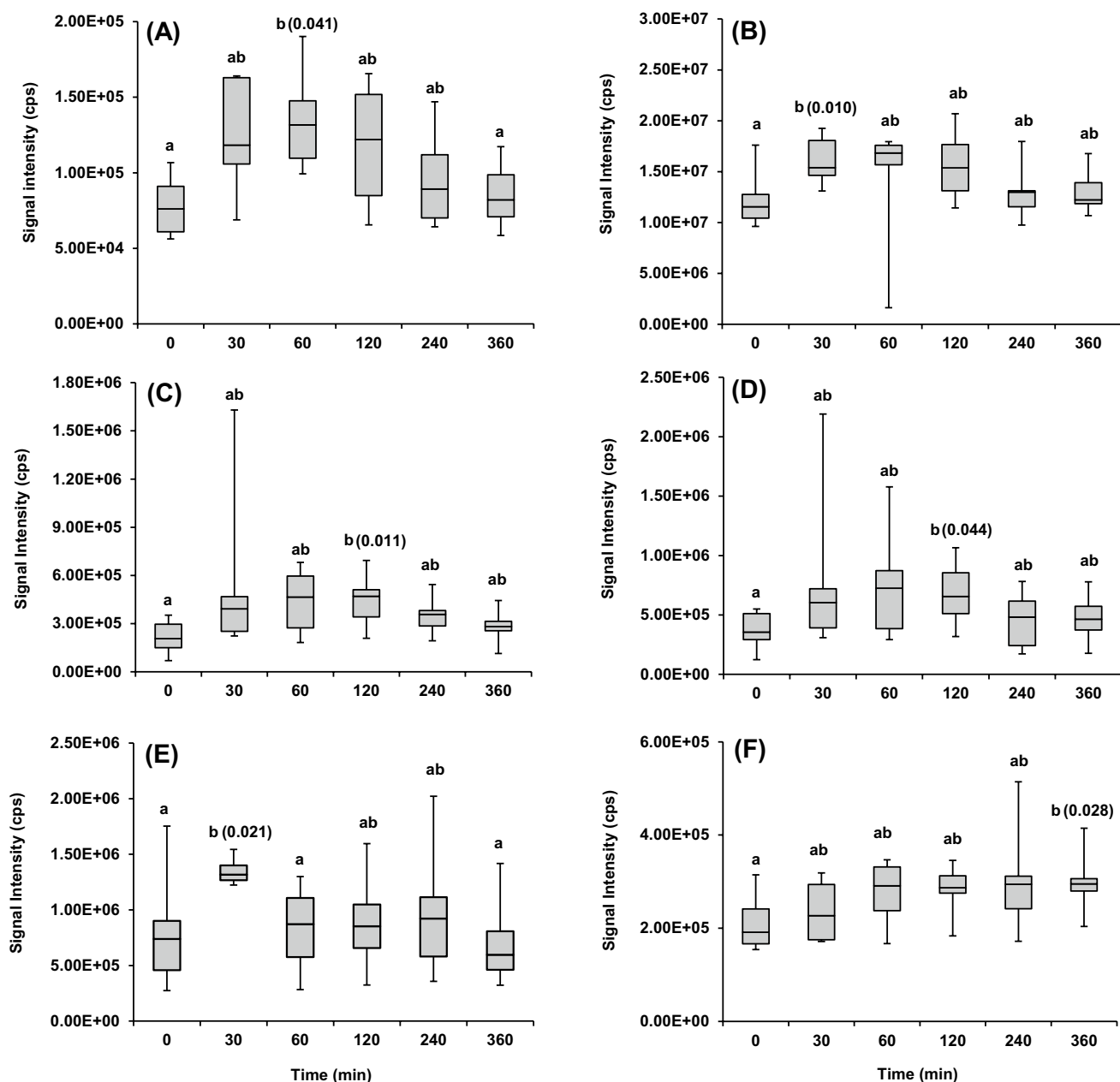


Fig. 5 Kinetic profiles of the pseudo-molecular ions at m/z **A** 191.0202 and **B** 193.0335 (citric acid in VM under negative and positive ionization, respectively), **C** 265.1431 and **D** 439.1599 (abscisic acid glucuronide in VM under positive and negative ionization, respectively), **E** 145.1220 (caprylic acid in VC under positive ioniza-

tion), and **F** 187.0980 (azelaic acid in VM under negative ionization). Boxplots exhibiting different letters are significantly different according to the Games-Howell comparison test ($P < 0.05$). T_{max} of each pseudo-molecular ion is labelled in brackets with its corresponding P value

Conclusions

The comparative evaluation of different multivariate and univariate methods for PEM selection evidenced that the number of significant features is strongly dependent on the statistical tool adopted, thus highlighting the importance of testing multiple statistical approaches for PEM discovery.

The protocol of untargeted metabolomics analysis and chemometric data treatment adopting the non-parametric Games-Howell test for PEM selection within interventions was suitable for selecting a restricted set of statistically significant features, excluding a number of false positive, which were conversely retained in the highly populated group of “significant features”, generated by other statistical methods, commonly adopted in literature. Accordingly, this

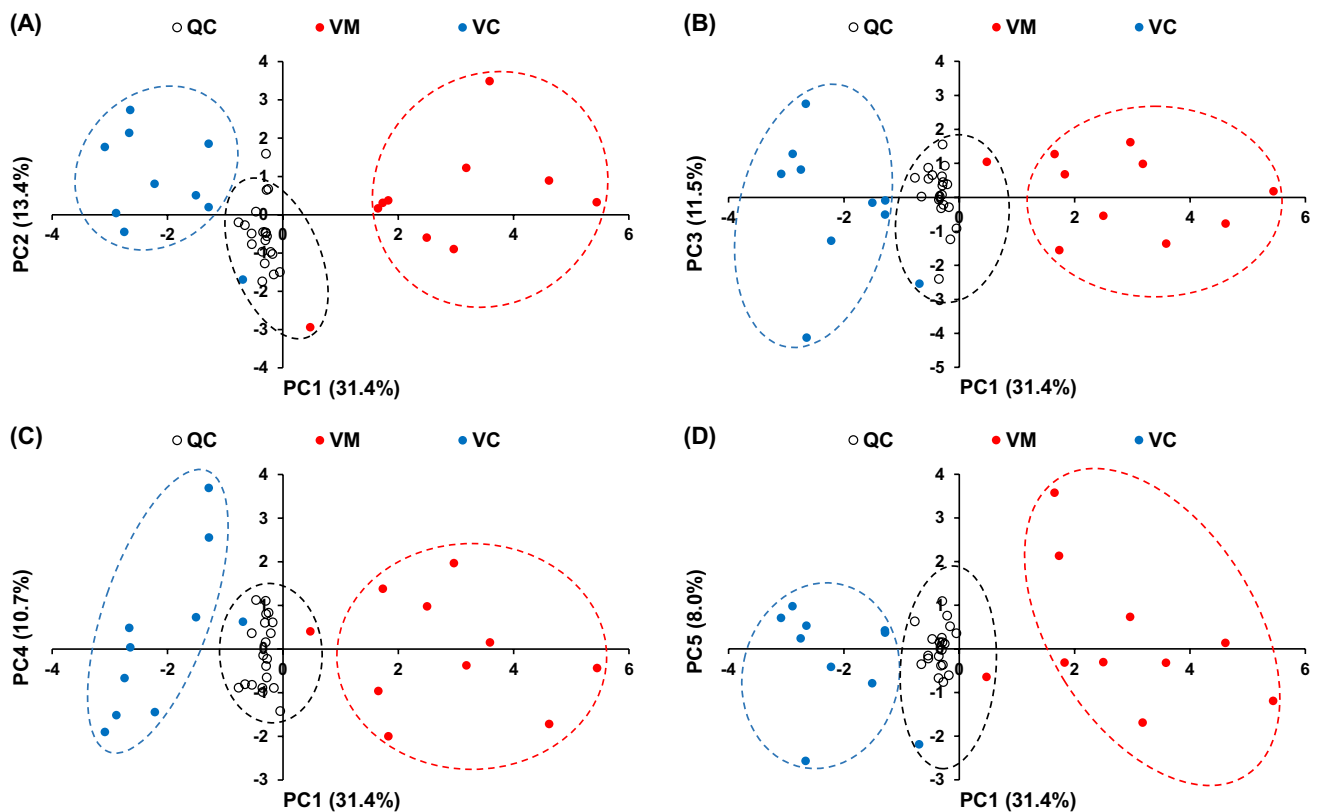


Fig. 6 Score plots of **A** PC1 vs. PC2, **B** PC1 vs. PC3, **C** PC1 vs. PC4, and **D** PC1 vs. PC5. PCA plots were obtained using the autoscaled intensity data of the PEMs discriminating the two interventions in serum samples from each volunteer, expressed as their features measured under NI at the maximum of their kinetic profile in each inter-

vention. Ellipses identify samples assigned to the QC, VM, and VC groups by applying linear discriminant analysis of maximum intensity of each annotated PEMs after cross validation with the leave-one-out method

Table 3 Fitting (FIT) and cross-validation (CV) results of the linear discriminant analysis based on the values of intensity data of the thirteen discriminating PEMs in serum samples from each volunteer, expressed as their features measured at the maximum of their kinetic profile in each intervention. Groups: quality control (QC), intervention with *Vaccinium corymbosum* supplement (VC), and intervention with *Vaccinium myrtillus* supplement (VM)

Put into group		True group		
		QC	VC	VM
QC	FIT	20	0	0
	CV	20	1	1
VC	FIT	0	10	0
	CV	0	9	0
VM	FIT	0	0	10
	CV	0	0	9
Total number of samples		20	10	10
Correct attributions	FIT	20	10	10
	CV	20	9	9
Percentage of correct attribution	FIT	100%	100%	100%
	CV	100%	90%	90%

chemometric strategy could be fruitfully extended to the data treatment of intervention studies characterized by a similar study design (i.e. small sample size, randomized, single-blinded, parallel studies). In fact, it is more useful to identify a smaller set of PEMs, accepting a certain risk of excluding from this group some significant metabolites (false negatives), rather than selecting a larger group of PEMs, which, however, contains a high number of false positive. It should also be noted that with the presented approach, it is possible to concentrate the annotation effort on a smaller but highly discriminant and certainly significant set of PEMs.

Twelve PEMs related to the intake of bilberry and/or blueberry supplements were annotated in serum samples, five of them (i.e. octahydro-methyl- β -carboline-dicarboxylic acid and tetrahydro-methyl- β -carboline-dicarboxylic acid for VC, citric acid for VM, and caprylic acid and azelaic acid for both VC and VM) reported here for the first time as serum metabolites of these interventions.

Based on AUC analysis, thirteen PEMs were found statistically significant for the discrimination of VM and VC interventions. In this regard, it is remarkable that this group of PEMs included four intra-intervention relevant metabolites

(i.e. abscisic acid glucuronide, catechol sulphate, methylcatechol sulphate, and α -hydroxy-hippuric acid), thus confirming the validity of the selection workflow based on the Games-Howell test. PCA data exploration and LDA sample classification were performed on PEM intensity at T_{\max} corroborating the discriminating role of the thirteen PEMs.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00216-021-03815-5>.

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Declarations

Conflict of interest The authors declare no competing interests.

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