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# Development of a targeted method for twenty-three metabolites related to polyphenol gut microbial metabolism in biological samples, using SPE and UHPLC–ESI-MS/MS

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ABSTRACT

An increasing number of studies have concerned the profiling of polyphenol microbial metabolites, especially in urine or plasma, but only a few have regarded their accurate quantification. This study reports on a new ultra-performance liquid chromatography tandem mass spectrometry method with electrospray ionisation (UHPLC–ESI-MS/MS) using a simple clean-up step with solid phase extraction (SPE) and validation on different biological matrices. The method was tested with spiked samples of liver, heart, kidneys, brain, blood and urine.

The purification procedure, after the evaluation of three different cartridges, makes it possible to obtain cleaner samples and better quantification of putative trace metabolites, especially related to dietary studies, with concentrations below ng/g in tissue and for urine and blood, starting from ng/ml. Limits of detection and linear range were also assessed using mixed polyphenol metabolite standards.

Short chromatographic separation was carried out for 23 target compounds related to the polyphenol microbial metabolism, coupled with a triple quadrupole mass spectrometer for their accurate quantification. By analysing different spiked biological samples we were able to test metabolite detection in the matrix and validate the overall recovery of the method, from purification to quantification.

The method developed can be successfully applied and is suitable for high-throughput targeted metabolomics analysis related to nutritional intervention, or the study of the metabolic mechanism in response to a polyphenol-rich diet.

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## 1. Introduction

Nutritional research today deals with health promotion, disease prevention and protection through functional food. By identifying biologically active molecules and their mechanisms, interaction or dietary response in individuals, modern nutrition tries to understand how the biological system can be affected by dietary intervention [1]. The nutritional relevance of polyphenols has been reported in several studies and the role of these natural compounds in a polyphenol-rich diet has been associated with many healthy effects in humans [2], not only related to their antioxidant activity. This wide and heterogeneous class of secondary plant metabolites is distributed in several foods, in particular in fruits and vegetables [3]. Of the most widely consumed fruit, berry fruits represent an unique source of polyphenols, due to their high concentration and variability in terms of the different classes of polyphenols present [2].

Although polyphenol bioactivity has been correlated to bioavailability and catabolism, their biological effects cannot be attributed only to the native forms, as found in food sources, but also and above all to their metabolites [4–7]. The factor most influencing their fate after consumption is the microbial polyphenol metabolism, made up of the gut microbiota, which represents all the microorganisms present in the gastrointestinal tract [8]. The microbial polyphenol metabolism follows a general pattern, in which this extremely diverse group of plant polyphenols is converted to a relatively small number of common metabolites. Hence a relatively small number of metabolites are biotransformed in the colon from a wide group of natural polyphenols [9,10]. For this reason, the compounds that can reach cells, tissues or target organs are chemically and biologically different from the original dietary polyphenols [11]. In recent years, considerable effort has

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been devoted to understanding the fate of polyphenols, their metabolism, the way that polyphenols can be modified by the gut microbiota and what type of lower molecular mass metabolites are produced from each class of polyphenols and released into the cardiovascular system [2,5,12–14]. As a consequence, an increasing number of studies have regarded the determination of polyphenol microbial metabolites, especially in urine or plasma, but only a few have been dedicated to their accurate quantification in different biofluids and tissues [15–19].

Metabolomics makes it possible to characterise the effects of nutrients or diet, analyse many metabolites in a given biological sample at the same time and explore the metabolic effects of nutrients in a global way [20]. Related to this type of study and in nutritional metabolomics in general, target based metabolomics or targeted profiling is aimed at quantitative analysis of a predefined metabolite group associated with a predefined class of compounds or pathway [20]. Targeted metabolomics provides accurate quantitative data and is regarded as a data-driven method [21]. For this purpose different analytical strategies are adopted, but the best choice for this kind of targeted metabolomics analysis is a triple quadrupole mass spectrometer, coupled with a liquid or a gas chromatographic system [22].

New metabolomics or nutrimetabolomics strategies related to intake and health benefits have been proposed for assessing nutritional status, food composition, the consequence of nutritional intervention or the study of the metabolic mechanism in response to diet [21,23].

Several pathways related to the microbial catabolism have been reported in the literature, in vivo with humans and through in vitro digestion with the faecal fermentation system [24–27]. There is therefore a need for rapid and sensitive analytical methods that can quantify such metabolites for a large number of samples, as in the case of clinical studies or long term dietary intervention, in different matrices, with rapid and sensitive targeted metabolomics analysis [20].

The metabolites investigated and used for method development in this work were chosen from the most representative and commercially available, on the basis of a literature survey. The selection was made considering metabolites from different metabolic pathways related to the polyphenol microbial metabolism reported in the literature [5,25,26]. Synthesis was performed for urolithin A and B, as they are specific markers of ellagitannin consumption and are not commercially available as standard references [28].

The main purpose was to develop a unique method, suitable for the analysis of polyphenol microbial metabolites in blood, urine, brain, liver, kidneys and heart, in contrast to the literature, in which specific methods have been developed especially for blood and urine, without considering other organs. As the purpose is to develop a general method for several matrices and chemically diverse metabolites, this could rise to some issues in terms of the optimisation of the SPE procedure, quantification and general recovery in all the samples. General optimisation of the method was performed taking these issues into account and obtaining the best analytical conditions in a holistic manner.

This study reports on a new method using a simple clean-up step for a complex biological matrix, with short chromatographic separation and quantification, using a triple quadrupole mass spectrometer for 23 target metabolites related to the consumption of polyphenols and their microbial metabolism.

## 2. Materials and methods

### 2.1. Chemicals and standards

Phloroglucinol ( > 99%), pyrogallol ( > 98%), gallic acid ( > 99%), protocatechuic acid ( > 97%), 3,4-dihydroxyphenylacetic acid ( > 98%), 4-hydroxyhippuric acid ( > 98%), 4-hydroxybenzoic acid ( > 99%), pyrocatechol ( > 99%), caffeic acid ( > 98%), vanillic acid ( > 97%), 3-hydroxyphenylacetic acid ( > 99%), homovanillic acid ( > 99%), 3-(4-hydroxyphenyl)propionic acid ( > 98%), 3-(3-hydroxyphenyl)propanoic acid ( > 98%), hydroferulic acid ( > 96%), *trans*-ferulic acid ( > 99%), *trans*-isoferulic acid ( > 98%), sinapic acid ( > 98%), *m*-coumaric acid ( > 99%), *o*-coumaric acid ( > 97%) and *p*-coumaric acid ( > 98%) were purchased from Sigma-Aldrich (Saint Luis, Missouri, USA). Isotopically labelled compounds, *trans*-cinnamic acid-*d*5 (IS1) and butyric acid-*d*7 (IS2), were used as internal standards and purchased from C/D/N Isotopes Inc. (Quebec, Canada). Urolithin A and urolithin B were synthesised following a published protocol [28] and characterised using NMR for structure confirmation and purity ( > 99%). LC/MS formic acid, Chromasolv LC/MS methanol and acetonitrile, were purchased from Sigma-Aldrich (Saint Luis, Missouri, USA).

### 2.2. Solutions

Standard stock solutions of 1000 mg/L were prepared in methanol for all compounds. Several further dilutions were prepared in methanol for the different steps involved in the method. All solutions were kept in dark vials at −20 °C.

### 2.3. Biological samples and extraction

This method was developed for different biological samples (liver, kidneys, heart and brain) and biofluids (blood and urine). Biological samples were obtained from previously sacrificed animals, in another experiment already approved by the Ethics Committee and published [29]. Tissue samples were ground with a CryoMill (Retsch, Germany) grinder using liquid nitrogen to ensure the quality of the sample and avoid any melting or degradation reactions. Deep frozen powders were kept at −80 °C before extraction. An aliquot of ground tissue, 1 g, was extracted with 9 mL of methanol 95%. An internal standard (0.1 µg/mL), *trans*-cinnamic acid-$d_5$ (IS1), was added to the extraction solvent to monitor the extraction procedures and further steps in sample preparation. The extract was then shaken at room temperature for 15 min in an orbital shaker and centrifuged for 5 min at 4 °C (5000 g) with a SIGMA 3–30 K centrifuge (Sigma Laborzentrifugen, Osterode am Harz, Germany). The supernatant was transferred into a 10 mL calibrated flask and the volume was adjusted. For blood and urine, the same ratio between volume sample and solvent, 1:9 was maintained and then adjusted after centrifugation to 10 mL. After solvent extraction the samples were ready for the clean-up protocols before injection into the UHPLC–MS/MS system. Further protocol details regarding blood and tissue extraction are reported in Vanzo et al., 2013 [29].

### 2.4. Solid phase extraction (SPE) and purification

For the clean-up phase and purification of the biological matrix, comparison of three different SPE cartridges for sample purification was performed. Waters Sep-Pak C18 cartridge, 1 g (Milford, Massachusetts, USA) is a silica-based bonded phase with strong hydrophobicity. It can be used, as reported by the manufacturer, to adsorb analytes of even weak hydrophobicity from aqueous solutions, including drugs and their metabolites in serum, plasma or urine or organic acids in beverages. Biotage Isolute ENV+, 1 g (Uppsala Sweden) is a hydroxylated polystyrene-divinylbenzene copolymer for non-polar metabolites in aqueous matrix. It can be a good alternative for the extraction of very polar drugs and metabolites that are not retained by C18 cartridges [30]. Phenomenex Strata-X, 1 g (Torrance, California, USA) is based on a polymeric sorbent that contains N-vinylpyrrolidone for a wide range of

metabolites and is suitable for the removal of phospholipids from the biological matrix. Due to the presence of the phenyl ring in the pyrrolidone ligand, the Strata-X cartridge is suitable for compounds with aromatic structures.

In general, the SPE protocol was applied following a procedure developed by Passamonti et al. [31], with some modifications. After solvent extraction with methanol 95%, an aliquot of samples (5 mL) was evaporated and reconstituted with 10 mL of $H_2SO_4$ 0.01 N in water. The conditioning of the cartridges was done with 20 mL of methanol and 20 mL of $H_2SO_4$ 0.01 N in water. After loading the sample, the cartridges were washed with 10 mL of Milli-Q water, dried under a stream of nitrogen and eluted with 20 mL of methanol. Eluates were evaporated to dryness with a rotavapor, and the samples were dissolved in 500 µL of methanol/water (50:50 v/v). The second internal standard, butyric acid-d7 (IS2), was dissolved in methanol/water (50:50 v/v) at a concentration of 1 µg/mL and added to the sample to monitor quantitative recovery during sample reconstitution. The sample was filtered with a 0.22 µm filter and injected into the UPLC–MS/MS system.

SPE recovery efficiency for the three cartridges was calculated for each metabolite by comparing recovery after spiking an aqueous solution with $H_2SO_4$ 0.01 N with the mixed polyphenol metabolite standards at a final concentration of 0.05 and 0.25 µg/mL, after the SPE protocol.

### 2.5. UHPLC–MS/MS conditions

The ultra performance LC system used was a Waters Acquity UPLC (Milford, Massachusetts, USA) equipped with binary pump, autosampler, column compartment and Acquity PDA eλ detector. Separation of the 23 targeted metabolites and 2 deuterated internal standards was performed with a Waters Acquity UPLC column (Milford, Massachusetts, USA), HSS T3 (100 mm × 2.1 mm, 1.8 µm) equipped with the proper guard column. Mobile phases of 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B) were used and chromatographic separation was performed using the linear gradient reported in Vrhovsek et al., 2012 [32]. The injection volume was 10 µL. All the metabolites analysed eluted in 11 min, with a total run time and column equilibration of 17 min. The MS system used was a Waters Xevo TQ (Milford, Massachusetts, USA) triple quadrupole mass spectrometer, coupled with an electrospray interface and polarity switching option during acquisition. To optimise detection, each metabolite was directly infused in the MS system in combined mode with 50/50 v/v of solvents A and B. Characteristic MS conditions were automatically optimised using a Waters Acquity IntelliStart (Milford, Massachusetts, USA), optimising ionisation mode, cone volt energy and collision energy. The two most abundant fragments were selected for each metabolite to establish a MRM (multiple reaction monitoring) quantitative method. The first transition, corresponding to the most abundant fragment, was used as quantifier ion, and the second as qualifier ion. MS parameters for the MRM method and retention time are summarised in Table 1.

### 2.6. Confirmation of the targeted metabolites detected

For each metabolite, the most intense fragment was used for quantification analysis and confirmed on the basis of the second most intense fragment, which was used as the qualifier ion. The presence of the detected metabolites was considered to be confirmed when the conditions agreed with EC document no. SANCO/12495/2011, used for the validation of pesticide analysis [33]. According to this EC document, confirmation is achieved for samples containing one of the targeted metabolites if the

**Table 1**
UHPLC–ESI-MS/MS conditions for quantification and confirmation of polyphenol metabolites and internal standards (IS 1 & 2).

| Compound | RT (min) | ESI mode | Precursor ion (*m/z*) | Cone voltage (V) | Quantifier | | Qualifier | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Product ion (*m/z*) | Collision energy (V) | Product ion (*m/z*) | Collision energy (V) |
| Phloroglucinol | 1.26 | + | 127 | 12 | 53 | 20 | 99 | 16 |
| Gallic acid | 1.39 | + | 171 | 20 | 109 | 14 | 81 | 22 |
| Pyrogallol | 1.62 | + | 127 | 22 | 81 | 22 | | |
| | | − | 125 | 34 | | | 79 | 16 |
| Protocatechuic acid | 2.10 | + | 155 | 20 | 65 | 20 | 93 | 14 |
| 3,4-Dihydroxyphenylacetic acid | 2.33 | + | 168 | 18 | | | 77 | 26 |
| | | − | 167 | 14 | 95 | 18 | | |
| 4-Hydroxyhippuric acid | 2.33 | + | 196 | 6 | 105 | 10 | | |
| | | − | 194 | 18 | | | 73 | 8 |
| 4-Hydroxybenzoic acid | 2.83 | + | 139 | 20 | 77 | 18 | 65 | 24 |
| Butyric acid-d7 (IS2) | 2.85 | + | 96 | 24 | 50 | 12 | 46 | 16 |
| Pyrocatechol | 2.86 | − | 109 | 36 | 81 | 12 | 53 | 14 |
| Caffeic acid | 3.18 | + | 181 | 10 | 145 | 16 | 117 | 22 |
| Vanillic acid | 3.22 | + | 169 | 10 | 93 | 14 | 65 | 22 |
| 3-Hydroxyphenylacetic acid | 3.40 | − | 151 | 18 | 65 | 20 | 79 | 20 |
| Homovanillic acid | 3.40 | + | 183 | 14 | 137 | 14 | 122 | 26 |
| 3-(4-Hydroxyphenyl)propionic acid | 3.81 | + | 167 | 12 | 107 | 10 | | |
| | | − | 165 | 26 | | | 93 | 12 |
| *p*-Coumaric | 4.01 | + | 165 | 8 | 91 | 22 | 65 | 30 |
| Hydroferulic acid | 4.20 | − | 195 | 28 | 136 | 16 | 121 | 24 |
| 3-(3-Hydroxyphenyl)propanoic acid | 4.28 | + | 167 | 8 | 121 | 12 | 107 | 22 |
| *trans*-Ferulic acid | 4.49 | + | 195 | 6 | 145 | 16 | 117 | 22 |
| Sinapic acid | 4.54 | − | 223 | 22 | 208 | 14 | 164 | 16 |
| *m*-Coumaric acid | 4.72 | + | 165 | 14 | 91 | 22 | 65 | 30 |
| *trans*-Isoferulic acid | 4.80 | + | 195 | 8 | 145 | 16 | 117 | 22 |
| *o*-Coumaric acid | 5.67 | + | 165 | 6 | 123 | 12 | 103 | 16 |
| Urolithin A | 6.93 | + | 229 | 10 | 157 | 22 | 128 | 34 |
| *trans*-Cinnamic acid-d5 (IS1) | 7.47 | + | 154 | 6 | 107 | 18 | 135 | 4 |
| Urolithin B | 8.90 | − | 211 | 42 | 139 | 28 | 117 | 28 |

precursor ion and both quantifier and qualifier ions are present with a signal-to noise ratio greater than 3. Confirmation was achieved when the qualifier/quantifier ratios, based on their peak area in samples as compared to the standard, did not differ by more than the fixed percentage reported in the document [33] and related to each individual qualifier/quantifier ratio.

### 2.7. Method validation

The parameters established for validation of the method were performed fully for blood samples, as the most available and commonly used biological samples. The parameters established for blood were selectivity, limit of detection, limit of quantification, linearity, matrix effect, accuracy and precision. In contrast, validation of the purification protocol was performed for all biological matrices, leading to validation of recovery for the SPE protocol.

#### 2.7.1. Selectivity

Selectivity is the ability to differentiate the analytes in the complex mixture of components present in any biological matrix. To ensure selectivity of the method, blank blood samples and spiked blood samples were analysed. With the chromatographic conditions and MRM transition used, all the analytes were resolved without interference from the matrix at the retention time and both mass transitions of the analytes, also as compared to the standards analysed in solvent.

#### 2.7.2. Limit of detection, limit of quantification, matrix effects, linearity and calibration curves

Calibration curves were established using blood for matrix-match calibration and pure solvent (methanol: water, 50:50 v/v) to check the absence of any matrix effects. Calibration curves were performed using a mixture of all the standards spanning from 0.00001 μg/mL to 10 μg/mL. Calibration curves were built using linear regression and not forced to pass through zero. Furthermore, a 1/x statistical weight was applied to obtain the most reliable calibration curves for all the metabolites. The weighting factor typically ensures the best fit of the plot, as determined by visual inspection and the correlation coefficient for both matrix-match and solvent calibration curves. The effective range of calibration curves was obtained on the basis of the linearity of the responses for each individual metabolite. Acceptable linearity was tested using the coefficient of determination ($R^2$) and the p-value of the *lack-of-fit* test. The limits of quantification (LOQ) and limits of detection (LOD) were evaluated at the concentration in which the quantifier transition presented a signal-to-noise (S/N) ratio of $>10$ and $>3$ respectively.

#### 2.7.3. Accuracy and precision

Accuracy and precision were ensured by analysing replicated spiked blood samples at low, middle and high concentration levels. The levels were set at 0.05, 0.25 and 1 μg/mL for each analytes, for the low, middle and high concentration levels respectively. Precision was reported as the relative standard deviation (RSD) between the replicate measurements in spiked blood samples, while accuracy was reported as the relative error (RE), which was calculated as the difference between the measured value and the theoretical value, divided by the theoretical value and expressed as a percentage.

#### 2.7.4. Recovery

In contrast to previous validation parameters, recovery was assessed in all the different biological blank matrices considered (blood, urine, liver, kidneys, heart and brain). Blank samples were spiked with 0.02 ug/mL of each metabolite, close to the LOQ at the low level, and analysed after the SPE clean-up procedure. Recovery was calculated for 5 or 10 replicates, depending on sample availability. The recovery trials were carried out by comparing the peak areas of the spiked samples with the peak areas of the respective pure standards, the ratio of the areas then being expressed as a percentage, and the standard deviation (sd) of the replicate was considered. In some cases, when endogenous amounts of some metabolites were present in the matrix, the known concentration of the endogenous amount was subtracted from the total peak area, thus revealing the concentration of the spiked metabolite. Furthermore, analysis without the purification step was performed and compared with analysis after the clean-up phase.

### 2.8. Statistical analysis

Data processing was done using Waters MassLynx 4.1 and TargetLynx software. Data visualisation and calibration curve processing were done using R software.

## 3. Results and discussion

### 3.1. UHPLC–MS/MS—analytical performance

The UHPLC separation method was developed based on a previously published method optimised for the rapid quantification of 130 polyphenols in fruits [32]. The injection volume was increased from 2 to 10 μL, considering the low amount to be expected in biological samples. The solvent for sample reconstitution before UHPLC injection was changed from methanol to methanol/water (50/50 v/v), with the scope of improving the peak shape and to comply with the retention capacity of the column. All the metabolites were well retained under these conditions, despite the fact that polyphenol metabolites are small, polar molecules compared to polyphenols in their intact form (Fig. 1). Detection of the transitions was performed using IntelliStart software and then manually corrected, although some molecules have a low molecular mass and this can be critical for choosing the transitions (see Table 1). A total of 50 MRM transitions were chosen, covering metabolites and internal standards. Some peak overlap inevitably occurred, due to the shortness of the chromatographic method. Because of this, scheduled MRMs were checked to ensure that there were sufficient data points in each peak. Close retention times between some metabolites were not a problem because of the use of selective MRM transitions for each metabolite. Application of the mass spectrometry rules commonly accepted for the analysis of pesticides in food and feed in the European Union [33] and in particular the strategies used for this method with two MRM transitions for each metabolite made it possible to obtain solid quantitative data. Compared to other methods, in which only one transition is used for identification and quantitative analysis of microbial polyphenol metabolites, the method offered better confirmation of the analytes [15].

A general quantification study covering a variety of different small molecules in different biological matrices and with a long sample preparation would be problematic without the use of internal standards for monitoring the quality of the manual execution and the clean-up reproducibility [20]. For this reason cinnamic acid-$d_5$ was used and added directly to the extraction solvent. Furthermore, a large instrumental variation in analytic response caused by the interference between the ESI source and the matrix can occur in long sequences, leading in most cases to a reduction of the signals. For the monitoring of any signal variation during the analysis, a second isotopically labelled internal standard butyric acid-$d_7$ was added to the sample just before filtration.

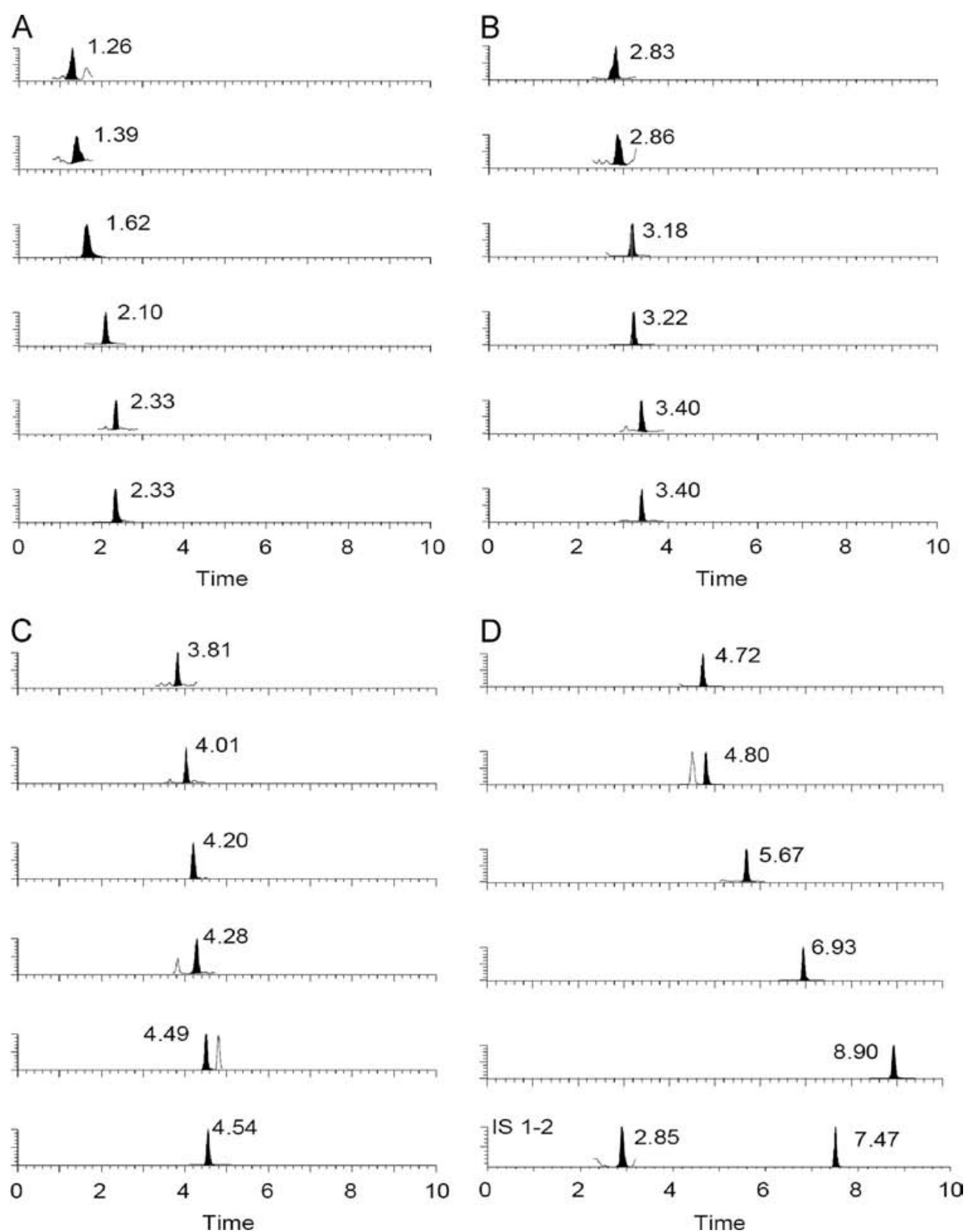


**Fig. 1.** UHPLC–ESI-MS/MS chromatograms of the quantifier MRM transition for the 23 polyphenol metabolites spiked in blood blank matrix at 0.01 ug/mL, listed accordingly to their retention time. *Panel A*: phloroglucinol, gallic acid, pyrogallol, protocatechuic acid, 3,4-dihydroxyphenyl acetic acid, 4-hydroxyhippuric acid; *Panel B*: 4-hydroxybenzoic acid, pyrocatechol, caffeic acid, vanillic acid, 3-hydroxyphenyl acetic acid, homovanillic acid; *Panel C*: 3-(4-hydroxyphenyl)propionic acid, *p*-coumaric acid, hydroferulic acid, 3-(3-hydroxyphenyl)propanoic acid, *trans*-ferulic acid, sinapic acid; *Panel D*: *m*-coumaric acid, *trans*-isoferulic acid, *o*-coumaric acid, urolithin A, urolithin B, butyric acid-$d_7$ and (IS2) *trans*-cinnamic acid-$d_5$ (IS1).

This also made it possible to check the regular injection of each sample. With the use of isotopically labelled internal standards, quantitative data can be monitored when the initial concentration is known.

### 3.2. Enzymatic hydrolysis

An additional step for those interested in the phase II metabolite deriving from the polyphenol microbial metabolite (glucuronides and sulphates forms) could be the enzymatic hydrolysis of the samples, due to the rare availability of this kind of conjugated metabolite. Protocols for hydrolysis are reported in the literature, especially for the analysis of urine and blood [15,34] using β-glucuronidase and sulphatase. However, these approaches provide very indirect information on the metabolites produced and inaccurate quantitative estimates, since there is very little data available on enzymatic hydrolysis efficiency [7].

### 3.3. Sample extraction and solid phase extraction (SPE) to clean up biological samples.

The solvent used for sample extraction was methanol:water (95:5), with a sample-solvent ratio of 1:9, being a rational choice for the extraction of polar and semi-non polar compounds such as the analytes from biological tissue and biofluids investigated. Moreover methanol: water (95:5) is an efficient solvent for protein precipitation and enzyme deactivation for rapid sample quenching [35–37] and the percentage of water can help in the extraction of very polar phenolic acids [38]. Remarkably, when analysing different extraction mixtures for different analytes in different

animal tissue such as liver, kidneys, muscles and brain, it has been reported that methanolic extraction can be the most suitable [39].

After selection of the solvent extraction, the three SPE cartridges selected (Sep-Pak C18, Isolute ENV+, Strata-X), widely used for the clean-up phase and purification of the biological matrix, were tested for the clean up protocols. SPE cartridges are suitable for a wide range of metabolites or drugs, especially for small polar molecules, acids and aromatic compounds, such as the metabolites covered by this method. The selected SPE cartridges have already been used for the purification of polyphenols or polyphenol metabolites from biological samples [31,40–42]. The conditions for the elution of polyphenol and phenolic acid are relatively consistent [38]. The solvents are slightly acidified in order to prevent ionisation of phenolics, which could reduce compound retention [38].

An aqueous solution with $H_2SO_4$ 0.01 N was spiked with the mixed polyphenol metabolite standards and then subjected to the SPE protocol with the different cartridges. However, as shown graphically in Fig. 2, they showed different recovery results for the metabolites investigated, due to their specific chemistry. In Fig. 2, the results of recovery efficiency (%) for the three different cartridges are presented as a box-plot, at two different final spiked concentrations of 0.25 and 0.05 μg/mL, showing the distribution and variability of the individual results for each metabolite. In the case of Strata-X, the overall recovery results for the two spikes were not comparable and were different for the two final concentrations. Indeed with Strata-X, which had a final concentration spike of 0.25 μg/mL most of the metabolites were below 30%, with higher overall recovery for the final concentration spike of 0.05 μg/mL (Fig. 2).

The results on Sep-Pak C18 showed no retention for 4 metabolites at a final concentration of 0.05 μg/mL: phloroglucinol, pyrocathecol, pyrogallol and 3-hydroxyphenylacetic acid. Three of the metabolites not retained were very similar, simple benzenes with different phenol groups which can also be related to their similar negative behaviour with Sep-Pak C18. Phloroglucinol was also not retained with Strata-X.

However, the overall recovery for Sep-Pak C18 was comparable with the different concentrations of the standard mix and the majority of metabolites were within the optimum range of 70–120% for recovery trials [33]. The best results in terms of metabolite retention and recovery for the two concentration levels were achieved with Isolute ENV+, 1 g, as shown in Fig. 2. All the metabolites were retained, with acceptable recoveries. Indeed most of the metabolites, considering both spiked concentrations, were within the optimum range of 70–120% for recovery trials.

In the trials the two last cartridges, Sep-Pak C18 and ENV+, showed a relatively similar affinity to the analytes, as already observed [42]. Moreover, ENV+ has already been observed to have the best recovery at pH 2.0 as compared to the C18 cartridge, obtained in the present protocol with an aqueous solution with $H_2SO_4$ 0.01 N, while the elution of the phenolic fraction with pure methanol is a common procedure for both C18 and ENV+ cartridges [42]. On the basis of these results, Isolute ENV+ cartridges (1 g) were chosen for the clean-up phase and for further validation of the targeted metabolomics method for polyphenol metabolites in the matrix.

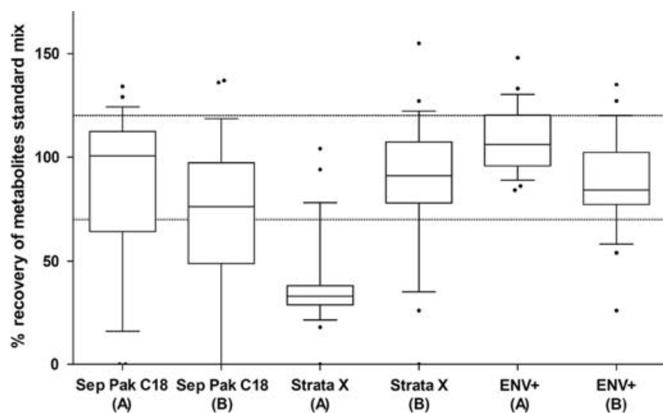


**Fig. 2.** Distribution and variability of the recovery (%) of polyphenol metabolite standard mix dissolved in aqueous solution with $H_2SO_4$ 0.01 N, for three different SPE cartridges at a final spiked concentration of 0.25 (A) and 0.05 μg/mL (B).

### *3.4. Method validation*

#### *3.4.1. Selectivity*

Blank blood samples were extracted using the SPE protocol described above. After injection and MS analysis, the MRM chromatograms obtained were checked for the presence of interference at the metabolite retention time. The blank blood samples were then spiked with mixture of polyphenol microbial metabolites at low concentration to prove the selectivity of the method at the low limit of quantification (Fig. 1). The method was shown to be selective and discriminative in the chromatographic conditions and with the MRM transitions used, with the presence of polyphenol microbial metabolites at low concentration. No interference from the matrix composition al the MRM transition and retention time of the analytes was observed in blood, also in comparison to the standard references injected in solvent (Fig. 1).

#### *3.4.2. Linearity, matrix effects, limit of detection and limit of quantification.*

Calibration curves were performed both in matrix, using blank blood samples, and in solvent with a mixture of all the 23 polyphenol metabolites, at different concentration levels, ranging from 0.00001–10 μg/mL.

Linearity was assessed by studying the level of the calibration curves constructed both in solvent and in blood (matrix-matched). The overall response (see Table 2), in both types of calibration, was characterised by high linearity and a linear dynamic range (LDR) of 3–4 orders of magnitude with a coefficient of determination (R2) of $> 0.99$. Moreover, the linearity of the calibration curves was confirmed by the *p*-values of the *lack-of*-fit test. All the resulting *p*-values were below 0.01, meaning a significant lack of fit. These parameters indicate good linearity within the stated ranges for calibration curves.

The slopes resulting from the matrix-match calibrations and the solvent calibrations in the linear range defined were used to evaluate the percentage of matrix effects for each analyte. The slope ratios were determined as (1-slope in solvent/slope in matrix), expressed as a percentage [43,44]. The percentages of matrix effects (%ME) are listed in Table 2. The %ME values were from $-17\%$ to 1%. %ME in the range of $\pm 20\%$ can be considered not to be relevant, because the variability is close to repeatability values [44]. The overall matrix effects in blood showed a slight suppression. This slight suppression was probably the result and main advantage of using a SPE protocol for sample purification. Indeed, comparison of the %ME and the calibration curve graphs (Supplementary Fig. 1) shows that the two different types of calibration curve, in solvent and matrix-match, are very similar. These %ME results obtained in blood should also be similar for the other matrices considered, as the SPE procedure is most responsible for this low ion suppression. This observation agrees with already published data, in which low limits of detection and quantification were reported in polyphenol microbial metabolites analysis, as well and most importantly a reduction in the matrix effect when SPE was used as the sample purification protocol [45].

**Table 2**
Calibration parameters, linear dynamic range (LDR), coefficient of determination ($R^2$) limit of quantification (LOQ) and limit of detection (LOD) obtained by using polyphenol metabolite standards in solvent and in blood after SPE procedure, with evaluation of the matrix effects (ME).

| | Matrix-match calibration (blood) | | | | | | Solvent calibration (methanol: water, 1:1) | | | | | | ME |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LDR | LOD | LOQ | Curve | | | LDR | LOD | LOQ | Curve | | | |
| | μg/mL | ng/mL | ng/mL | a (slope) | b (offset) | $R^2$ | μg/mL | ng/mL | ng/mL | a (slope) | b (offset) | $R^2$ | % |
| Phloroglucinol | 0.01–1 | 3.0 | 10.0 | 2730 | 112 | 0.991 | 0.01–1 | 3.0 | 10.0 | 2810 | 8 | 0.999 | −3 |
| Gallic acid | 0.01–1 | 3.0 | 10.0 | 44,072 | 475 | 0.997 | 0.01–1 | 3.0 | 10.0 | 43,810 | −197 | 0.998 | 1 |
| Pyrogallol | 0.01–5 | 3.0 | 10.0 | 9708 | −5534 | 0.986 | 0.01–5 | 3.0 | 10.0 | 11,228 | −362 | 0.996 | −16 |
| Protocatechuic acid | 0.005–1 | 1.5 | 5.0 | 127,704 | 33 | 0.998 | 0.005–1 | 1.5 | 5.0 | 142,585 | 312 | 0.999 | −12 |
| 3,4-Dihydroxyphenyl acetic acid | 0.001–1 | 0.3 | 1.0 | 11,742 | −115 | 0.996 | 0.001–1 | 0.3 | 1.0 | 13,408 | 10 | 0.998 | −14 |
| 4-Hydroxyhippuric acid | 0.00025–5 | 0.1 | 0.3 | 91,714 | 24 | 0.999 | 0.00025–5 | 0.1 | 0.3 | 95,567 | −30 | 0.999 | −4 |
| 4-Hydroxybenzoic acid | 0.001–0.5 | 0.3 | 1.0 | 67,773 | 82 | 0.990 | 0.001–0.5 | 0.3 | 1.0 | 78,060 | 65 | 0.997 | −15 |
| Pyrocatechol | 0.01–2.5 | 3.0 | 10.0 | 2243 | 4 | 0.997 | 0.005–2.5 | 1.5 | 5.0 | 2360 | 5 | 0.994 | −5 |
| Caffeic acid | 0.0025–1 | 0.8 | 2.5 | 68,262 | −24 | 0.999 | 0.001–1 | 0.3 | 1.0 | 79,599 | 338 | 0.997 | −17 |
| Vanillic acid | 0.001–1 | 0.3 | 1.0 | 199,368 | 152 | 0.998 | 0.001–1 | 0.3 | 1.0 | 219,717 | 553 | 0.995 | −10 |
| 3-Hydroxyphenyl acetic acid | 0.01–5 | 3.0 | 10.0 | 636 | 1 | 0.991 | 0.01–5 | 3.0 | 10.0 | 734 | −12 | 0.992 | −16 |
| Homovanillic acid | 0.0005–1 | 0.2 | 0.5 | 190,238 | 150 | 0.999 | 0.0005–1 | 0.2 | 0.5 | 210,340 | 145 | 0.998 | −11 |
| 3-(4-Hydroxyphenyl) propionic acid | 0.0025–0.5 | 0.8 | 2.5 | 116,145 | 125 | 0.995 | 0.0025–0.5 | 0.8 | 2.5 | 134,124 | 187 | 0.998 | −15 |
| *p*-Coumaric acid | 0.001–0.5 | 0.3 | 1.0 | 115,839 | 40 | 0.997 | 0.001–0.5 | 0.3 | 1.0 | 132,438 | 35 | 0.999 | −14 |
| Hydroferulic acid | 0.0005–1 | 0.2 | 0.5 | 32,778 | 1 | 0.998 | 0.0005–1 | 0.2 | 0.5 | 35,969 | −11 | 0.998 | −10 |
| 3-(3-Hydroxyphenyl) propanoic acid | 0.001–1 | 0.3 | 1.0 | 86,380 | 194 | 0.999 | 0.001–1 | 0.3 | 1.0 | 92,948 | 97 | 0.998 | −8 |
| *trans*-Ferulic acid | 0.00025–5 | 0.1 | 0.3 | 192,183 | −7 | 0.999 | 0.00025–5 | 0.1 | 0.3 | 205,149 | 78 | 0.997 | −7 |
| Sinapic acid | 0.01–5 | 3.0 | 10.0 | 13,093 | −107 | 0.998 | 0.002–5 | 0.6 | 2.0 | 14,983 | −5 | 0.999 | −14 |
| *m*-Coumaric acid | 0.001–0.5 | 0.3 | 1.0 | 213,837 | −2 | 0.999 | 0.001–0.5 | 0.3 | 1.0 | 241,138 | 27 | 0.999 | −13 |
| *trans*-Isoferulic acid | 0.0005–1 | 0.2 | 0.5 | 145,338 | −24 | 0.998 | 0.0005–1 | 0.2 | 0.5 | 151,884 | 18 | 0.999 | −5 |
| *o*-Coumaric acid | 0.001–0.5 | 0.3 | 1.0 | 162,635 | 92 | 0.999 | 0.001–0.5 | 0.3 | 1.0 | 176,940 | 131 | 0.999 | −9 |
| Urolithin A | 0.001–0.5 | 0.3 | 1.0 | 264,624 | −90 | 0.999 | 0.0001–0.5 | 0.0 | 0.1 | 284,039 | 73 | 0.998 | −7 |
| Urolithin B | 0.0005–0.5 | 0.1 | 0.5 | 27,976 | −4 | 0.998 | 0.00025–0.5 | 0.1 | 0.3 | 30,794 | 18 | 0.996 | −10 |

Therefore, calibration curves in solvent can be used for analyte quantification in these conditions without the need for any matrix match calibration (Table 2). The calibration curves obtained in solvent up to this point were then used for metabolite quantification in the different matrices, for the recovery and comparison of samples with or without SPE purification.

The LODs and LOQs for solvent calibration are listed in Table 2. The metabolite values differed. However as already observed for the matrix effects, LODs and LOQs were comparable between solvent and blood being the SPE procedure an important factor in the reduction of the matrix effect and as consequence also influencing the LODs and LOQs. The lowest LOD and LOQ values in solvent were for *trans*-ferulic acid, 4-hydroxyhippuric acid and urolithin B, with values of 0.1 ng/mL and 0.3 ng/mL respectively. The highest values were for pyrogallol, gallic acid, phloroglucinol and 3-hydroxyphenyl acetic acid with the LOD and LOQ being 3 ng/mL and 10 ng/mL respectively. The LODs and LOQs calculated using this method were below or within the concentration range used with a similar instrumental platform, but with a higher number of microbial polyphenol metabolites as compared to the previously published method [18]. The LODs and LOQs obtained using this method were in agreement or even lower than the results of previous publications [19,46]. Moreover, the concentration of these metabolites in humans is expected to be higher than the LOQs calculated for this method [4], thus showing calibration curves were built using linear regression and not forced to pass through zero. Furthermore, a 1/x statistical weight was applied to obtain the most reliable calibration curves for all the metabolites.

### 3.4.3. Accuracy and precision

Accuracy and precision were evaluated by spiking blank blood samples with 3 different known concentrations: 0.05 μg/mL (low), 0,25 μg/mL (medium) and 1 μg/mL (high) in triplicates. For most of the compounds, accuracy, expressed as RE, was within ± 15% for the three concentration levels. Only pyrogallol and sinapic acid were slightly higher, while precision, expressed as the RSD of the mean concentration, was below 12% for all three concentration levels in all the analytes. The results obtained for accuracy and precision are summarised in Table 3. The results showed that the proposed method is sufficiently accurate and precise to be applied to real samples.

### 3.4.4. Evaluation of analytes recovery

The recovery of the purification protocol was studied by spiking the samples with standard mix solutions containing metabolites at a concentration close to the LOQ, 0.02 μg/mL. In this case, all the matrices proposed for the method development were tested, i.e. liver, kidneys, heart, brain, blood and urine of rats, these being representative matrices used in nutritional studies and bioavailability studies of bioactive compounds for in vivo experiments with mammals. Recovery experiments were performed 10 times, except in the case of heart and urine (5 times). Overall recovery validation was done considering not only the matrix effect but also the SPE clean-up step and instrumental variations. The overall method was checked for recovery, from extraction to quantitative data.

Along with the recovery evaluation of each metabolite, the recovery of *trans*-cinnamic acid-d5 (IS1) was also checked in the different matrices. The results ranged from 80% to 113% in the different matrices and reflected mainly the extraction efficiency and the overall execution of the purification protocol, while the recovery of butyric acid-d7 (IS2) was not evaluated for the SPE procedure, since this was added after purification to monitor analytical performance.

The results of the recovery trial are shown in Table 3 and graphically in the first part of Fig. 3. In the matrix-dependent recovery trials a slightly variability was observed in the results. Tissues are subject to more complex mixture interference, which can significantly affect recovery [47]. However the SPE procedure, reducing the matrix complexity [45] and purifying the analytes, could lead, and as observed in the present method, to obtain very similar results in terms of recovery between the different biological samples considered with few exceptions.

**Table 3**
Recovery (%, ± sd), in different biological matrices, precision (%RSD) and accuracy (%RE) in blood after ENV+ (1 g) for method validation.

| | Recovery | | | | | | | | | | | | Precision | | | Accuracy | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Blood* | | *Brain* | | *Heart* | | *Liver* | | *Kidneys* | | *Urine* | | | | | | | *Blood* |
| | % | ± sd | % | ± sd | % | ± sd | % | ± sd | % | ± sd | % | ± sd | L %RSD | M %RSD | H %RSD | L %RE | M %RE | H %RE |
| Phloroglucinol | 11.0 | *3.9* | 76.5 | *6.4* | 9.0 | *1.9* | 21.8 | *4.6* | 95.4 | *5.8* | 61.7 | *12.6* | 10 | 3 | 4 | 0 | 5 | − 13 |
| Gallic acid | 53.9 | *5.6* | 52.4 | *20.0* | 72.2 | *6.4* | 53.4 | *2.6* | 65.5 | *4.3* | 19.2 | *2.0* | 2 | 1 | 0 | 8 | 2 | 0 |
| Pyrogallol | 42.0 | *2.9* | 50.7 | *5.5* | 48.9 | *6.8* | 54.9 | *2.3* | 82.0 | *4.7* | 31.0 | *3.5* | 4 | 10 | 6 | 13 | − 39 | − 37 |
| Protocatechuic acid | 80.1 | *7.9* | 79.5 | *13.1* | 94.6 | *5.4* | 72.4 | *2.7* | 78.4 | *4.0* | 35.0 | *4.9* | 3 | 1 | 1 | 8 | 2 | 0 |
| 3,4-Dihydroxyphenyl acetic acid | 49.1 | *4.7* | 68.7 | *25.2* | 74.7 | *16.2* | 65.7 | *4.8* | 78.5 | *5.3* | 60.9 | *7.0* | 3 | 5 | 2 | − 16 | − 6 | − 6 |
| 4-Hydroxyhippuric acid | 20.9 | *3.0* | 63.9 | *20.6* | 88.6 | *4.4* | 79.5 | *3.5* | 91.3 | *6.1* | 63.5 | *2.4* | 1 | 1 | 0 | 7 | − 3 | 1 |
| 4-Hydroxybenzoic acid | 93.6 | *6.8* | 86.0 | *6.1* | 119.9 | *11.3* | 90.5 | *1.9* | 94.8 | *5.9* | 66.6 | *17.9* | 7 | 1 | 3 | 0 | − 4 | − 7 |
| Pyrocatechol | 64.7 | *9.5* | 62.0 | *11.3* | 52.2 | *5.1* | 65.9 | *9.7* | 57.6 | *6.6* | 19.8 | *3.4* | 9 | 7 | 7 | 0 | − 17 | − 16 |
| Caffeic acid | 73.4 | *5.1* | 73..9 | *14.9* | 82.6 | *14.1* | 79.4 | *2.6* | 62.5 | *3.5* | 16.3 | *2.0* | 4 | 1 | 1 | − 13 | − 11 | − 11 |
| Vanillic acid | 90.6 | *6.3* | 88.4 | *6.2* | 86.9 | *8.1* | 82.9 | *2.7* | 79.9 | *3.5* | 35.3 | *6.2* | 1 | 1 | 2 | 1 | − 1 | − 2 |
| 3-Hydroxyphenyl acetic acid | 66.8 | *15.0* | 52.7 | *16.8* | 72.8 | *16.8* | 87.7 | *20.3* | 94.3 | *15.3* | – | – | 7 | 7 | 7 | 15 | − 8 | − 4 |
| Homovanillic acid | 85.9 | *8.0* | 110.7 | *8.4* | 83.4 | *7.8* | 84.0 | *1.6* | 95.1 | *5.9* | 103.5 | *3.5* | 3 | 1 | 1 | − 9 | − 4 | − 6 |
| 3-(4-Hydroxyphenyl) propionic acid | 78.7 | *6.1* | 75.6 | *7.7* | 79.1 | *9.9* | 74.3 | *3.4* | 97.6 | *5.2* | – | – | 1 | 2 | 1 | 6 | − 2 | − 5 |
| *p*-Coumaric acid | 88.6 | *5.4* | 85.1 | *4.6* | 48.1 | *9.0* | 88.4 | *2.3* | 94.2 | *8.5* | 67.4 | *31.4* | 6 | 3 | 1 | 4 | − 1 | − 7 |
| Hydroferulic acid | 83.0 | *5.8* | 79.6 | *4.4* | 91.8 | *3.1* | 80.3 | *3.0* | 76.4 | *5.1* | 39.0 | *3.8* | 7 | 1 | 4 | − 10 | − 5 | − 5 |
| 3-(3-Hydroxyphenyl) propanoic acid | 122.5 | *7.8* | 88.4 | *8.1* | 77.6 | *4.2* | 89.0 | *5.7* | 153.5 | *16.5* | – | – | 11 | 2 | 0 | 19 | 4 | 5 |
| *trans*-Ferulic acid | 90.8 | *6.3* | 91.6 | *5.4* | 68.3 | *3.4* | 88.9 | *2.7* | 86.9 | *9.7* | 59.4 | *5.5* | 4 | 2 | 3 | 1 | 1 | − 2 |
| Sinapic acid | 87.9 | *5.9* | 82.4 | *5.4* | 78.4 | *6.6* | 75.3 | *12.5* | 74.6 | *10.2* | 54.4 | *2.5* | 10 | 12 | 7 | − 29 | − 26 | − 11 |
| *m*-Coumaric acid | 86.9 | *5.9* | 84.8 | *6.2* | 106.8 | *5.5* | 89.3 | *3.1* | 89.8 | *6.1* | 84.6 | *2.9* | 2 | 1 | 1 | 3 | 2 | − 4 |
| *trans*-Isoferulic acid | 90.6 | *6.4* | 93.0 | *5.7* | 80.1 | *9.5* | 94.3 | *3.0* | 88.3 | *9.9* | 70.6 | *5.4* | 3 | 4 | 2 | 2 | 3 | 0 |
| *o*-Coumaric acid | 89.1 | *6.0* | 83.2 | *7.2* | 95.7 | *3.0* | 89.7 | *3.1* | 87.9 | *5.7* | 56.6 | *2.3* | 2 | 2 | 2 | 2 | 1 | − 3 |
| Urolithin A | 40.7 | *5.8* | 42.5 | *9.8* | 44.6 | *3.7* | 47.1 | *4.5* | 20.9 | *5.3* | 35.7 | *4.0* | 3 | 2 | 2 | − 1 | − 4 | − 12 |
| Urolithin B | 38.1 | *2.7* | 29.1 | *11.9* | 2.1 | *1.5* | 30.4 | *3.0* | 0.8 | *0.5* | 29.1 | *1.6* | 9 | 2 | 2 | 6 | 2 | − 11 |

The variability related to the type of biological matrix was in few cases very extensive, as in the case of phloroglucinol or 4-hydroxyhippuric acid, ranging from 10 to 95%. These extremely widespread recovery values are nevertheless correlated with acceptable standard deviation of the purification protocol for the repetitions considered in the trials. Only one metabolite, 3-(3-hydroxyphenyl)propanoic acid, had a higher recovery value of 153% in kidneys and 122% in blood, while in the other matrices it was around 77–89%. The weakest recovery data were for urolithin B, with a recovery of 1–2% obtained, again for heart and kidneys. In this case, future data for the analysis of urolithin B clearly cannot be accurate but may at least be semi-quantitative.

With regards to urine samples, the recovery of three metabolites, respectively 3-hydroxyphenylacetic acid, 3-(4-hydroxyphenyl)propionic acid and 3-(3-hydroxyphenyl)propanoic acid did not take place in the experiment because the ratio between the endogenous level of the metabolites in urine and the amount of spiked metabolite was too high. These three metabolites were found in high concentrations endogenously. The endogenous concentrations were estimated at 0.043, 0.034 and 0.131 μg/mL respectively for 3-hydroxyphenylacetic acid, 3-(4-hydroxyphenyl) propionic acid and 3-(3-hydroxyphenyl)propanoic acid.

On average, recovery for the other metabolites in the different matrices was in the range of 40–120% in most cases (Table 3). Considering all the metabolites in the different matrices (23 metabolites in 6 matrices, $n=138$), 58% of them were within the recovery range of 70–120%, 5% of them showed over 120% recovery, 25% of them were within the range 40–70% and 13% of them were below 40% recovery.

Considering the standard deviation of recovery for the SPE protocol for all the metabolites in the different matrices (23 metabolites in 6 matrices, $n=138$), 85,5% of them had a standard deviation below < 10%, while the rest of them were within 10 and 20%. This variability in terms of standard deviation associated with the use of ENV+ has already been observed [42]. In terms of overall recovery, this method would

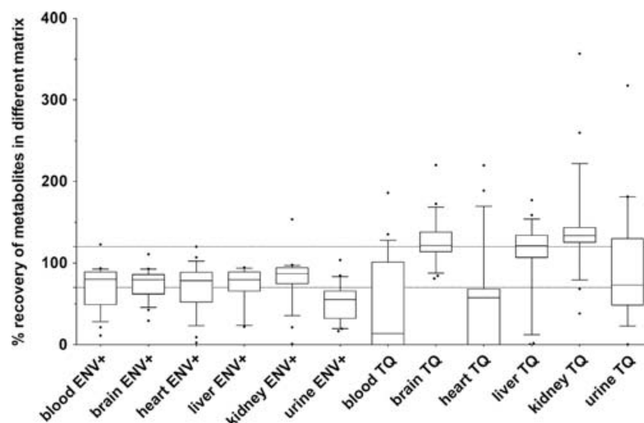


**Fig. 3.** Distribution and variability of the recovery (%) of polyphenol metabolites standard mix in blood, brain, heart, liver, kidney and urine with SPE (ENV+) and without (TQ) at a spiked concentration of 0.02 μg/mL.

again seem to be suitable as a general protocol, with fast sample preparation.

### 3.5. Comparison of recovery with and without matrix purification

In addition to the recovery validation described above, the same sample set was also checked without the SPE clean-up step. The comparison is shown in Fig. 3, in which the first half refers to samples prepared with ENV+ purification, while the right-hand side of Fig. 3 refers to the aliquot of samples with no purification (TQ). The data are expressed as % of recovery after spiking the samples with standard mix solutions containing metabolites at 0.02 μg/mL.

Comparison of the two approaches for the analysis of biological matrices shows clearly that quantitative analysis can be different with and without a sample purification step before injection into

the MS system. This comparison offers further support for validation of the purification protocol in terms of recovery and further supports the need to use an SPE step before MS analysis. In samples without SPE purification, the metabolites were extremely spread out along the recovery axis, meaning that in this case recovery went from 0 to 300%, while most of the metabolites were outside the ideal range of expected recovery (70–120%). Moreover many metabolites were not detected, especially in blood, heart and liver. In blood 8 metabolites were not detected (phloroglucinol, gallic acid, pyrogallol, protocatechuic acid, 3,4-dihydroxyphenylacetic acid, caffeic acid, *trans*-ferulic acid, urolithin A), while in heart 6 metabolites (phloroglucinol, gallic acid, 3,4-dihydroxyphenylacetic acid, pyrocatechol, caffeic acid, 3-(4-hydroxyphenyl)propionic acid) were not detected. An explanation of these results relates to suppression of the signal in the ESI source, due to matrix complexity. The ESI source is highly susceptible to matrix composition and as a result it is possible to observe ion suppression or ion enhancement, due to the by now well-known matrix effect. In addition to ion suppression and signal reduction, in which the worst case scenario is that target compounds are not detected, strong enhancement of the signals of some of the metabolites can be observed (Fig. 3). The strongest ion enhancement was observed in kidneys, especially for 3-hydroxyphenylacetic acid and 3-(3-hydroxyphenyl)propanoic acid, with recovery of 356% and 259%, respectively.

A good strategy for avoiding this kind of problem and reducing the complexity of matrix composition is the use of a SPE step before quantitative analysis. The improvement in the recovery results is evident on looking at Fig. 3. Indeed, recovery seems to be the most important parameter and is clearly most susceptible to matrix compositions and most affected by them observing the extreme variability in the data-point for the samples without SPE purification. With the use of SPE purification the results are more reliable and closer to the real values. Another positive point in the use of "cleaner" samples is the possibility of concentrating samples, 10-fold for this purification protocol. The final concentration detectable is sometimes a major issue, especially for experiments with physiological levels of bioactive compounds. With this preparation protocol, the samples are concentrated 10-fold without affecting the analytical performance for the type of matrix considered, since the negative effect of the matrix was resolved by the clean-up step.

## 4. Conclusion

A high-throughput, sensitive and reproducible method for targeted metabolomics for the quantitative analysis of 23 polyphenol metabolites in six different biological matrices was developed. In contrast to previous quantification methods, which are optimised and developed for a few metabolites and for a specific matrix, the method developed allows simultaneous quantification of many polyphenol metabolites, with a general protocol for different matrices commonly considered in nutritional and bioavailability studies.

The purification procedure made it possible to obtain cleaner and more concentrated samples, with low LOQs and better quantification of possible trace metabolites, especially related to dietary studies with concentrations below ng/g in tissue, and for urine and blood, starting from ng/mL. As compared to the samples without the clean up step, the use of SPE for the samples also serves to concentrate them 10-fold.

Method sensitivity and linear range were assessed using mixed polyphenol metabolite standards. By analysing different biological samples, such as blood, urine, liver, kidneys, heart and brain spiked with target metabolites, we were able to test metabolite detection in the matrix and validate the overall recovery of the method, from purification to quantification. No significant interferences were detected in the different biological matrices. Considering the variety of matrices which can be treated in the same conditions with a single general quantitative analytical protocol for targeted based metabolomics, this method can be considered very flexible and may be widely applied.

Consequently, this method can be used for nutritional studies. in particular with the expected amounts of polyphenol metabolites reported by Manach et al. [4], in which the total plasma concentration of polyphenol metabolites ranges from 0–4 μmol/L, with an intake of 50 mg of polyphenol aglycone equivalent. We can conclude that the method can be applied to targeted based metabolomics analysis of different biological matrices and related to the consumption of polyphenols, also considering the recovery values for adjustment of the quantitative data, if such data are needed.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.04.058.

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