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# Semi-Targeted Ultra-High-Performance Chromatography Coupled to Mass Spectrometry Analysis of Phenolic Metabolites in Plasma of Elderly Adults

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**Citation**

Muñoz-Bernal, Ó.A., Vazquez-Flores, A.A., Alvarez-Parrilla, E., Martínez-Ruiz, N.R., de la Rosa, L.A. Semi-Targeted Ultra-High-Performance Chromatography Coupled to Mass Spectrometry Analysis of Phenolic Metabolites in Plasma of Elderly Adults. *J. Vis. Exp.* (182), e63164, doi:10.3791/63164 (2022).

**Date Published**

April 22, 2022

**DOI**

10.3791/63164

**URL**

jove.com/video/63164

## Abstract

A group of 23 elderly persons was given functional meals (a beverage and a muffin) specially formulated for the prevention of sarcopenia (age-related loss of muscle mass). Plasma samples were taken at the beginning of the intervention and after 30 days of consuming the functional meals. A semi-targeted ultra-high-performance chromatography coupled with tandem mass (UPLC-MS/MS) analysis was carried out to identify phenolic compounds and their metabolites. Plasma proteins were precipitated with ethanol and the samples were concentrated and resuspended in the mobile phase (1:1 acetonitrile: water) before injection into the UPLC-MS/MS instrument. Separation was carried out with a $C_{18}$ reverse-phase column, and compounds were identified using their experimental mass, isotopic distribution, and fragment pattern. Compounds of interest were compared to those of data banks and the internal semi-targeted library. Preliminary results showed that the major metabolites identified after the intervention were phenylacetic acid, glycitin, 3-hydroxyphenylvaleric acid, and gomisin M2.

## Introduction

Sarcopenia is a progressive skeletal disorder related to an accelerated loss of muscle in the elderly population. This condition increases the risk of falls and leads to limited activities of daily living. Sarcopenia is present in about 5%-10% of persons over 65 years old and about 50% of persons aged 80 years or older[1]. No specific drugs have been approved for the treatment of sarcopenia, so prevention with physical activity and a well-balanced diet is important[1,2]. Nutritional interventions with specially formulated foods enriched with dairy protein and essential amino acids have shown positive results in preventing sarcopenia[2]. In other studies, authors have included vitamins and antioxidants, like

vitamin E and isoflavones, in the diet, increasing the benefits for muscle gain on the waist and hips[3].

*Brosimum alicastrum* Sw. (Ramón) is a tree that grows in the Mexican tropical regions; it has been consumed by Mayan cultures due to its high nutritional value[4]. It is a good source of protein, fiber, minerals, and phenolic antioxidants, such as chlorogenic acid[5]. Since it can be ground into powder and used in baking products or consumed in beverages, recent studies have evaluated the incorporation of Ramón seed flour (RSF) into different foods to improve their nutritional value. An RSF-supplemented cappuccino-flavored beverage was formulated, which was high in dietary fiber and had more than 6 g of protein per serving, and was highly accepted by consumers; thus, it was considered a potential alternative for meeting special dietary requirements[6]. In a follow-up study, RSF was also used to formulate a muffin and a new beverage rich in protein, dietary fiber, micronutrients, and phenolic antioxidants. The muffin and beverage were used in a dietary intervention for elderly individuals, who consumed both products twice per day for 30 days. After this period, the nutritional and sarcopenic status of the participants improved, and the total phenolic content of plasma increased[7]. However, the determination of total phenolic compounds in plasma was carried out by a spectrophotometric method, so identification of the actual phenolic compounds that were absorbed was not possible; moreover, this method is not completely specific for phenolic compounds, so some overestimation may occur[8].

Identification and quantification of the phenolic compounds that are absorbed after consumption of foods rich in these antioxidants is a difficult task but is necessary to demonstrate the biological activity of these phytochemicals. The bioavailability of most phenolic compounds is low; less than 5% of them can be found without structural transformation in plasma. Phenolic compounds undergo several biotransformations, such as methylation, sulfonation, or glucuronidation, which are carried out by enterocytes and hepatocytes[9]. Phenolic compounds are also biotransformed by the microbiota into bacterial catabolites that may exert their beneficial effects in the body after being absorbed into the plasma[10]. For example, phenylacetic acid is a product of the bacterial transformation of flavonoids and oligomeric proanthocyanidins, which can inhibit up to 40% of bacteria (*Escherichia coli*) adhesion in the urinary tract after cranberry consumption[11].

The structural diversity of naturally occurring phenolic compounds, added to the diversity of their metabolites and their low bioavailability, makes their identification in plasma even more challenging. Metabolomic profiling, using spectroscopic analysis platforms like nuclear magnetic resonance (NMR) and tandem mass spectroscopy (MS/MS), is probably the best approach to achieve this goal; unfortunately, the equipment is not easily accessible, and the development of analysis protocols is still limited[12]. Several studies have reported MS/MS coupled with a separation system (such as liquid chromatography) as a strategy for reducing the complexity of mass spectra in metabolomic studies. The recent introduction of ultra-high-performance liquid chromatography (UPLC) separation methods has reduced the time of analysis and increased the resolution and sensitivity compared with conventional high-performance liquid protocols, so UPLC-MS/MS systems have rapidly been widely accepted by the analytical metabolomics community[13]. In this way, some studies have investigated phenolic metabolites and detected glucuronidated derivatives from caffeic acid, quercetin, and ferulic acid, as well as sulfonated derivatives from syringic and vanillic acid in the plasma of individuals after cranberry intake[14]. Previous protocols have intended to find phenolic compounds and phenolic metabolites in biofluids such as plasma. These

protocols were based on identification and quantification by high-performance liquid chromatography (HPLC) coupled to a UV-vis detector[15]. Nevertheless, such protocols require the use of authentic standards to assess absolute identification and accurate quantification. A wide range of studies have identified the most common metabolites in biofluids (sulphonated, glucuronidated, and methylated forms) by UPLC-MS and UPLC-MS/MS; however, a large part of the bacterial metabolites has not been reported due to the lack of databases that contain their complete information[16]. Metabolite identification is complicated by the cost and commercial availability of metabolite standards. Therefore, the best strategy may be untargeted or semi-targeted MS/MS metabolite analysis, which relies on the use of molecular feature information (m/z, monoisotopic exact mass, isotopic distribution, and fragmentation pattern) to determine the chemical identity and compares it with freely available online databases that contain polyphenol metabolites identified in biofluids after the consumption of polypolyphenol-richts[12]. The most important databases used in UPLC-MS/MS studies for the identification of phenolic compounds and their metabolites are the Human Metabolome Database (HMDB), LipidBlast Library, METLIN Library, and other complementary databases, such as PubChem, ChemSpider, and Phenol Explorer[17].

In the present study, a semi-targeted UPLC-MS/MS method was developed to analyze the plasma samples of the group of elderly persons involved in the RSF-containing muffin and beverage consumption study[7]. Data from different free online databases of plasma metabolites were collected and integrated into a specialized database. This database can be accessed automatically by the equipment software to identify the polyphenolic metabolites in the five plasma samples before and after the 30-day nutritional intervention. This is done to identify the main phenolic compounds, or their metabolites, that are absorbed from the specially formulated functional foods designed for the prevention of sarcopenia.

## Protocol

The plasma samples used in this protocol were collected in a previous study following all the ethical guidelines and approved by the Institutional Ethics and Bioethics Committee (CIEB-2018-1-37) from the Universidad Autónoma de Ciudad Juárez. The complete protocol for the extraction and identification of the phenolic compounds and metabolites in plasma by UPLC-MS/MS is represented in **Figure 1**.

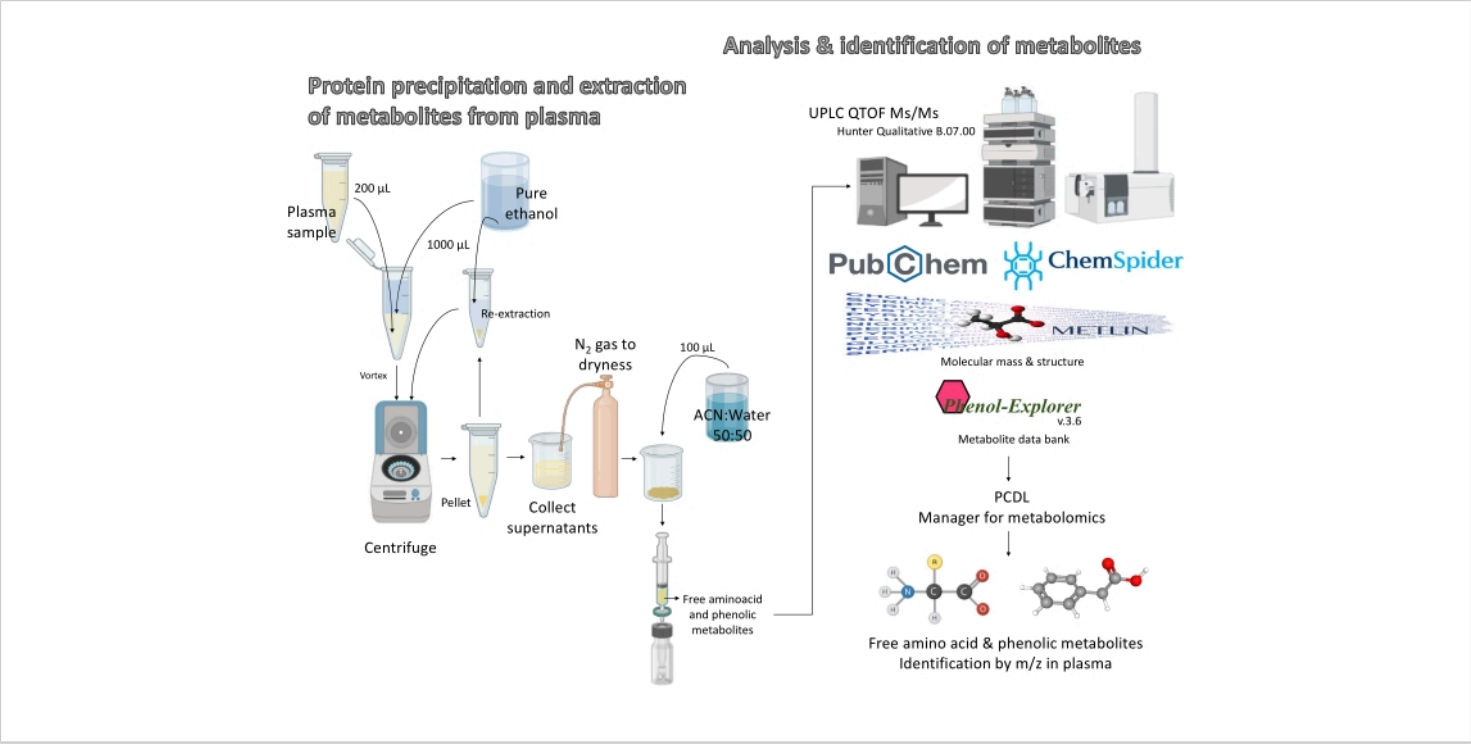


**Figure 1: Schematic representation of the extraction and identification of phenolic compounds and metabolites in plasma by the semi-targeted UPLC-MS/MS method.** Please click here to view a larger version of this figure.

## 1. Sample preparation

1. Store the plasma samples at -80 °C until analysis.
2. Defrost the plasma samples at room temperature for 15 min.
   NOTE: The samples can be placed in a water bath at 37 °C to accelerate the process (5 min).
3. Place 200 µL of plasma sample in a 2 mL microtube and mix with 1,000 µL of pure ethanol. Vortex the plasma sample for 30 s.
   NOTE: Always use gloves when working with plasma samples.
4. Centrifuge the sample at 6,580 x *g* for 5 min. After centrifugation, collect the supernatant with a micropipette or Pasteur pipette and place it in a new microtube. Store the supernatant at 4 °C.
5. Mix the pellet from the previous step with 1,000 µL of 100% ethanol, vortex for 30 s, and then centrifuge at 6,580 x *g* for 5 min.
   NOTE: The pellet is strongly packed and needs to be resuspended well to ensure contact between the sample and pure ethanol. The use of a micropipette to flush the pellet with ethanol is recommended.
6. After centrifugation, collect the supernatant and mix with the supernatant previously obtained from Step 1.4. in a 2 mL microtube.
7. Remove ethanol from the sample by using pure nitrogen (99.997%) at 135 psi. Keep the needle 1 cm away from the top of the microtube to prevent sample loss and flush

until the sample is dry. No heat is needed to evaporate the ethanol.

NOTE: The nitrogen flow must be low to prevent sample loss. Once the ethanol is dried, keep the nitrogen flow for at least 5 min to ensure sample dryness. The protocol can be paused at this point; samples must be stored at -20 °C. Avoid storing the samples for more than 12 h.

8. Resuspend the dry samples in 100 µL of a mixture of acetonitrile: water at a proportion of 50:50 (v:v).

9. Filter the sample through a 0.45 µm nylon syringe membrane directly into an HPLC vial micro insert.

NOTE: The samples in the vial can be stored at -20 °C before analysis. Store the samples for no more than 8 h. It is recommended to inject the samples into the UPLC system just after filtration.

## 2. UPLC-MS/MS analysis

1. Inject 3 µL of sample onto a UPLC equipped with a $C_{18}$ reverse-phase column (50 mm x 2.1 mm; 1.8 µm). Set the autosampler temperature at 20 °C and the column thermostat at 25 °C. Inject each sample in triplicate.

2. Use 0.1% (v:v) formic acid in water as solvent A, and 100% acetonitrile as solvent B. Set the flow rate at 0.4 mL/min and a gradient program as follows: 0-1 min 10% B, 1-4 min 30% B, 4-6 min 38% B, 6-8 min 60% B, 8-8.5 min 60% B, 8.5-9 min 10% B (**Table 1**).

3. Set the mass spectrometer to negative mode ionization. Use nitrogen as a drying gas at 340 °C and a flow rate of 13 L/min. Set the nebulizer pressure at 60 psi. Set the capillary voltage at 4,000 V, the fragmentor voltage at 175 V, and the Skimmer voltage at 65 V. Use collision energy at 20 V (**Table 2**).

4. Scan the masses between 100-1100 mass to charge ratio (*m*/*z*) and, for MS/MS, scan masses between 50-1000 *m*/*z* (**Table 2**). Set data acquisition to Auto MS/MS mode. Use the following reference mass: 119.036 and 966.0007.

| Time (min) | Solvent A (0.1 % formic acid in HPLC water) | Solvent B (100 % acetonitrile) |
|---|---|---|
| 0 to 1 | 90 | 10 |
| 1 to 4 | 70 | 30 |
| 4 to 6 | 62 | 38 |
| 6 to 8 | 40 | 60 |
| 8 to 8.5 | 40 | 60 |
| 8.5 to 9 | 90 | 10 |

**Table 1: Mobile phase gradient used for the separation of phenolic compounds by UPLC.**

| Ionization mode | Negative |
|---|---|
| Drying gas | Nitrogen at 340 °C, flow rate 13 L/min |
| Nebulizer pressure | 60 psi |
| Capillary voltage | 175 V |
| MS scan masses | 100-1100 m/z |
| MS/MS scan masses | 50-1000 m/z |

**Table 2: Ionization parameters for the MS/MS analysis.**

## 3. Database construction

1. Search for phenolic compounds, phenolic metabolites, or other compounds of interest in the scientific literature.
2. Open the database management software included in the UPLC system. Select **File | New Personal Database Compound Library (PCDL) | Create New PCDL**. Select the type of PCDL: **LC/MS Metabolomics**. Set a name for the PCDL. Then select **Create**.
3. In the toolbar, select **PCDL** and then the **Allow editing** option. Then click the **Find compounds** button.
   NOTE: Since it is a new PCDL, the table results will be empty. This will change once new compounds are added into the PCDL.
   1. Add compounds to the specialized personal database compound library by copying them from the instrument's general library. Open the instrument's existing database included in the database management software. Click the button **Find compounds**. In the **Single search** option, enter the compound search criteria to find the compound of interest.
      NOTE: Compounds can be found by name, molecular formula, exact mass, and retention time.
   2. In the compound results table, select the compound of interest. To select more than one compound, click the first compound, hold down the **CTRL** key, and then click each compound of interest. Then, right-click on all the highlighted compounds and select **Append to PCDL**.
   3. In the new window, search and select the specialized personal database file. Mark the boxes **Include spectra for compounds if present** and **Include ion mobility info for compounds if present**. Click the **Append** button. In the new dialog box, select **Yes** to check the new compounds added. Select **No** to keep searching for more compounds of interest.
4. If the compounds of interest are not available in the instrument's general library, add new compounds manually.
   1. Open the specialized personal database. Once opened, follow Step 3.3. Select the **Edit compounds** option. Click the **Add new** button.
   2. In the upper section of the window, complete the information for the new compound. Fill in

the formula, name, IUPAC name, CAS number, Chemspider ID, and other identifiers.

3. Use the information available in the free online libraries (Chemspider, PubChem, and Phenol Explorer) to fill in the information for the new compound of interest. Once finished, click the button **Save as new** to save the new compound information in the specialized personal database.
   NOTE: When adding information from free libraries, be sure to include the compound information without the presence of chloride or iodide ions. This may modify the exact mass and molecular formula of the compound of interest.

5. Repeat the process with all the compounds of interest to complete the specialized personal database.

### 4. Data analysis

1. Use the instrument´s qualitative manager software to identify the phenolic compounds and phenolic metabolites present in the samples.
2. Open the sample file. In the Chromatogram panel, select **Define Chromatograms** and extract the total ion chromatogram (TIC), the extracted ion-chromatogram of MS (EIC), and the EIC of MS/MS. Select the integrate chromatogram option.
3. In the **Find Compounds** panel, select **Find by Formula-Options**. In the new window, select **Formula Source** and then the **Database/Library** option. Find the personal database previously created and click on **Open**.
4. Select the **Formula Matching** option and set a mass match tolerance of 5 parts per million (ppm).
   NOTE: A different match tolerance of masses can be set at 10 ppm; this difference depends on the mass spectrometer used.
5. Select the **Negative Ions** option and select only the **-H** dialog box. In the **Results** option, mark the **Extract EIC, Extract Cleaned Spectrum, Extract Raw Spectrum**, and **include Structure** dialog boxes.
6. Select the **Result Filters** option. Mark **Warn if score is** and set the score match at 80.00%. Mark **Do not match if the score is** and set the score at 75.00%.
   NOTE: The match/not match scores can be changed to lower values if needed. This will reduce the accuracy of identification.
7. Click on the **Find Compounds by Formula** to identify compounds of interest in the sample.

## Representative Results

The step-by-step process for the identification of phenolic metabolites through the semi-targeted UPLC-MS/MS analysis, in negative mode, of plasma samples is depicted in **Figure 2**. First, the total ion chromatogram (TIC) from the plasma phenolics extract (obtained after protein precipitation of the total plasma sample) was obtained through the instrument's qualitative software. Then, the extracted ion chromatogram was used, and the exact mass and fragmentation pattern (MS/MS analysis) of each signal (or molecular feature) were compared with those of a specific personal database created also in the instrument's software. Signals with a mass match of less than 5 ppm were assigned a molecular formula from the database. Finally, the isotopic distribution of each signal was compared to that of the assigned molecular formula to achieve the final tentative identification. Compounds that a) were identified only in one replicate or b) presented an area

lower than 10,000 were treated as false identifications. From this analysis, a total of 25 phenolic compounds and metabolites were identified in the plasma samples (**Table 3**). In this list, both phenolic compounds and their metabolites, such as 3-hidroxyphenylvaleric acid and isopropyl 3-(3,4-dihydroxyphenyl)-2-hydroxypropanoate, were found. Since the negative ionization mode is best suited for all classes of phenolic compounds, except anthocyanins, these compounds could not be detected with the present method. If anthocyanins are important components of the food matrix, the positive mode should be also used.

| Phenolic metabolites | R.T. (min) | Formula | Precursor | Experimental mass | Theorical mass | Difference (ppm) |
|---|---|---|---|---|---|---|
| 2,3-Dihydroxybenzoic acid | 0.622 | $C_7H_6O_4$ | 153.0203 | 154.0273 | 154.0266 | 4.2 |
| 2-Hydroxyhippuric acid | 8.631 | $C_{18}H_{33}NO_4$ | 410.1648 | 411.1725 | 411.1717 | 1.8 |
| 3,4-Dihydroxytoluene | 2.239 | $C_7H_8O_2$ | 123.0451 | 124.0524 | 124.0524 | -0.25 |
| 3-Hydroxyphenylvaleric acid | 6.717 | $C_{11}H_{14}O_3$ | 193.0874 | 194.0947 | 194.0943 | 2.12 |
| 5-(3',4'-dihydroxyphenyl)-valeric acid | 4.293 | $C_{11}H_{14}O_4$ | 209.0823 | 210.0894 | 210.0892 | 0.68 |
| 6-Hydroxyenterodiol | 9.201 | $C_{18}H_{22}O_5$ | 317.1387 | 318.1465 | 318.1467 | -0.65 |
| Ajugol | 3.889 | $C_{15}H_{24}O_9$ | 347.134 | 348.1418 | 348.142 | -0.59 |
| Benzoic acid | 3.915 | $C_7H_6O_2$ | 121.0296 | 122.0367 | 122.0368 | -0.28 |
| Carnosic acid | 6.785 | $C_{20}H_{28}O_4$ | 331.1905 | 332.1979 | 332.1988 | -2.58 |
| Carnosol | 6.347 | $C_{20}H_{26}O_4$ | 329.1764 | 330.1842 | 330.1831 | 3.43 |
| Catechol | 0.892 | $C_6H_6O_2$ | 109.0297 | 110.037 | 110.0368 | 1.91 |
| Glycitin | 6.01 | $C_{22}H_{22}O_{10}$ | 445.1155 | 446.1228 | 446.1213 | 3.4 |
| Hesperetin | 6.01 | $C_{16}H_{14}O_6$ | 301.0718 | 302.0796 | 302.079 | -1.81 |
| Hippuric acid | 1.396 | $C_9H_9NO_3$ | 178.051 | 179.058 | 179.0582 | -1.16 |
| Homovanillic acid | 0.823 | $C_9H_{10}O_4$ | 181.0503 | 182.0576 | 182.0579 | -1.88 |
| Isopropyl 3-(3,4-Dihydroxyphenyl)-2-hydroxypropanoate | 6.177 | $C_{12}H_{16}O_5$ | 239.0926 | 240.0999 | 240.0998 | 0.48 |
| Phenylacetic acid | 5.666 | $C_8H_8O_2$ | 135.0444 | 136.0518 | 136.0524 | -4.92 |
| Phloretic acid | 2.811 | $C_9H_{10}O_3$ | 165.0556 | 166.0626 | 166.063 | -2.41 |
| Protocatechuic aldehyde | 1.094 | $C_7H_6O_3$ | 137.0247 | 138.0311 | 138.0317 | -4.5 |
| Secoisolariciresinol | 8.837 | $C_{20}H_{26}O_6$ | 361.1656 | 362.1729 | 362.1729 | -0.23 |
| Vanillin | 2.508 | $C_8H_8O_3$ | 151.04 | 152.0471 | 152.0473 | -1.82 |
| Epicatechin 3'-O-glucuronide | 9.342 | $C_{21}H_{22}O_{12}$ | 465.1024 | 466.109 | 466.1111 | -4.64 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Gomisin M2 | 5.234 | $C_{22}H_{26}O_6$ | 385.1676 | 386.1746 | 386.1729 | 4.38 |
| Irisolidone | 6.145 | $C_{17}H_{14}O_6$ | 313.0727 | 314.0798 | 314.079 | 2.33 |
| Urolithin C | 6.753 | $C_{13}H_8O_5$ | 243.0294 | 244.0368 | 244.0372 | -1.69 |

**Table 3: Tentative identification of phenolic compounds and metabolites in plasma samples by the semi-targeted UPLC-MS/MS method.**

To evaluate the effectiveness of the designed method for the identification of the main phenolic compounds, or their metabolites, that were absorbed from the RSF-containing muffin and beverage, five aleatory samples of the study participants, obtained before and after the 30-day intervention, were analyzed. The relative abundance of each compound was calculated by dividing the area under the curve (AUC) after treatment by the AUC before treatment. From this analysis, it was possible to observe that some compounds only appeared in the samples obtained before the treatment, others remained unchanged, while some of them increased after consumption of the functional foods. **Table 4** shows the list of 12 phenolic compounds that showed an increase in plasma after the 30-day consumption of the RSF-containing foods. Phenylacetic acid was the only metabolite found consistently in higher concentrations after the treatment. Glycitin, a glycosylated isoflavone, and 3-hydroxyphenylvaleric acid (a phenolic metabolite) increased in three of the five samples but decreased in the other two. Gomisin M2, a lignan, was detected in three of the five samples only after the nutritional intervention. The other phenolic compounds (such as hesperetin, secoisolariciresinol, and vanillin) and metabolites (such as 2-hydroxyhippuric acid) were found only in one sample and only after the treatment.

| | | Sample (AUC after treatmet/AUC before treatment) | | | | |
|---|---|---|---|---|---|---|
| **Compound** | **Formula** | **1** | **2** | **3** | **4** | **5** |
| 2-Hydroxyhippuric acid | $C_{18}H_{33}NO_4$ | T | nd | nd | nd | nd |
| 3-Hydroxyphenylvaleric acid | $C_{11}H_{14}O_3$ | 1.30 | 2.69 | 2.69 | 0.62 | 0.62 |
| 6-Hydroxyenterodiol | $C_{18}H_{22}O_5$ | nd | nd | T | nd | nd |
| Glycitin | $C_{22}H_{22}O_{10}$ | 1.88 | 1.07 | 1.07 | 0.43 | 0.45 |
| Hesperetin | $C_{16}H_{14}O_6$ | T | nd | nd | nd | nd |
| Phenylacetic acid | $C_8H_8O_2$ | 4.06 | T | T | T | 1.28 |
| Phloretic acid | $C_9H_{10}O_3$ | T | nd | nd | nd | nd |
| Protocatechuic aldehyde | $C_7H_6O_3$ | T | nd | nd | nd | nd |
| Secoisolariciresinol | $C_{20}H_{26}O_6$ | T | nd | nd | nd | nd |
| Vanillin | $C_8H_8O_3$ | T | nd | nd | nd | nd |
| Gomisin M2 | $C_{22}H_{26}O_6$ | nd | T | T | T | nd |

**Table 4: List of phenolic compounds that increased in the plasma of elderly individuals after 30-day consumption of RSF-containing foods.** Data are the ratios of the abundance (AUC) of each compound after treatment compared to their abundance before the treatment. T indicates that the compound was only identified in the sample after the treatment. Nd: not detected.

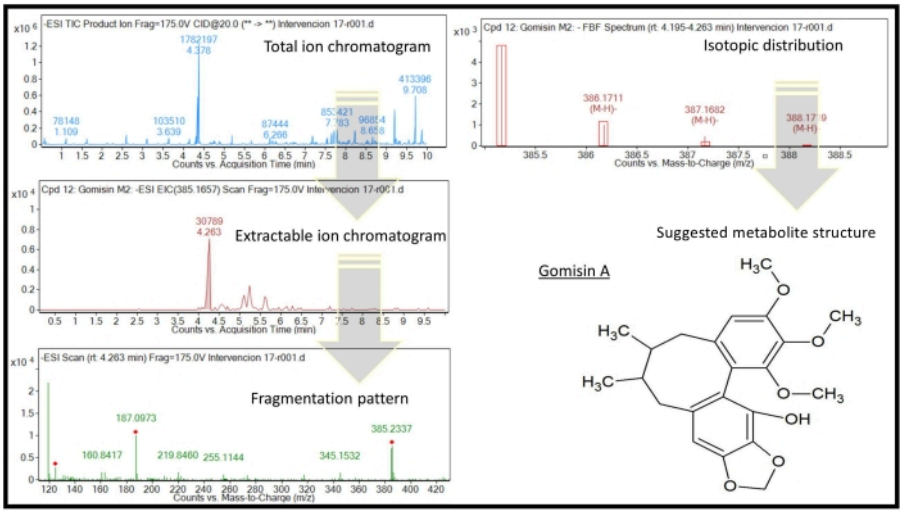


**Figure 2: Protocol for the identification of phenolic compound metabolites by semi-targeted UPLC-MS/MS.** Please click here to view a larger version of this figure.

## Discussion

The identification and quantification of the bioactive phytochemicals that are absorbed after consumption of a food or food supplement are crucial for demonstrating and understanding the health benefits of these compounds and the foods containing them. In the present work, the UPLC-MS/MS method was developed, aimed only at the identification of the main phenolic compounds and their metabolites that increased in concentration in plasma after a 30-day nutritional intervention with two food products specially formulated for the elderly. It was assumed that, if one compound increased or appeared in plasma only after the intervention period, that compound had been absorbed and/or biotransformed from the foods used in the intervention.

UPLC-MS/MS is one of the preferred technologies for metabolomic studies in which many low molecular weight compounds are analyzed simultaneously in complex samples to assess the effect of some treatment or environmental alteration[13]. This can be done through targeted and non-targeted (or global) methods. Targeted analyses focus on a known, small number of metabolites of interest. They are the best option for quantifying compounds with available standards, providing the best specificity and sensitivity; however, these methods must be thoroughly optimized for the selected metabolites and are incapable of detecting unexpected compounds in the samples[12]. In other studies, HPLC coupled with a UV-vis detector has been used to identify phenolic acids and metabolites in plasma, but this type of study has used analytical standards to identify and quantify the compound of interest[15]. In another study, the addition of an internal standard was proposed[18]; however, only ferulic and caffeic acids and their metabolites were analyzed in this work. On the other hand, the synthesis of internal standards of phenolic metabolites has been proposed as an alternative to the lack of commercial

analytical standards[19]. Nevertheless, the synthesis of a large variety of phenolic compounds is difficult, time-consuming, and expensive. Non-targeted methods attempt to detect and identify as many molecular compounds as possible and are primarily hypothesis-generating[12]. They can identify several hundreds of compounds in one sample by assigning formulas to detectable chromatographic signals with characteristic features, such as m/z or measured accurate mass, chromatographic retention time, isotopic fingerprint, etc., so the data generated by these analyses are very abundant and can be difficult to interpret[20]. Since the objective of the present method was not to obtain a full metabolic profile of the plasma samples (which would be an untargeted approach) but to identify the major phenolic compounds and phenolic metabolites (previously unknown) in them, a semi-targeted approach was designed. For this, all the identified signals were extracted automatically with the instrument's software and then compared with a self-created database that contained 645 phenolic compounds and their metabolites, which were obtained from free online databases. The database included reference MS/MS fragmentation patterns of the compounds that were used for the assignment of the compound name and formula, which allows for more accurate identification of compounds in the sample[12].

The most critical steps in the protocol were 1) sample pretreatment (extraction and concentration of phenolic compounds and metabolites from plasma samples); 2) the construction of a complete and specific database for the semi-targeted analysis of the samples and identification of all the possible compounds belonging to a specific compound class; and 3) the selection of the parameters used by the instrument's qualitative software (mass match tolerance ppm, and score%) to accurately identify the compounds in the sample. In sample pretreatment, caution must be taken to avoid loss of the compounds of interest, recover them at a high final concentration, and be able to eliminate interfering compounds. In the creation of the specific database, it is critical to conduct a thorough literature search and then use free online databases to obtain specific chemical data that will be used for compound identification in the sample. The selection of the best values for mass match and score required previous validation of the analytical method using standards and known samples[21,22].

Once the protocol was implemented, the common problems and their recommended solutions were the following: 1) No compounds were identified in the samples. This was due to the low concentration of the compounds and could be solved by drying the sample and re-dissolving it in a smaller volume. 2) Signals appeared in the TIC, but compounds were not identified. This could be due to a failure in mass calibration, so the difference between experimental and theoretical masses was high. In this case, the instrument's mass calibration should be performed according to the manufacturer and model of the equipment. A second reason could be an incomplete personal database, so it is critical to constantly check and maintain the database. Masses of compounds suspected to be of interest can be checked and compared with freely available online databases or new scientific literature. 3) Signals appeared in blank runs. This indicates contamination and can be solved by cleaning the autosampler needle and column. The standard cleaning protocol is to perform some runs using methanol, then isopropanol, and finally acetonitrile as the mobile phase. Then the column is re-equilibrated, running the mobile phase for at least 30 min.

The major difficulties in the development of this kind of method are 1) the low bioavailability, in general terms, of all phytochemicals, including phenolic compounds, which

translates to very low plasma concentrations[16]; 2) the high diversity of phenolic compounds present in foods, which is increased by their metabolism and biotransformation that occur in human enterocytes and hepatocytes and the colonic microbiota[23]; 3) the lack of standards (some standards exist but are very difficult to obtain) for many compounds, especially for the metabolites, and the existence of some unknown or uncharacterized compounds[23]; and 4) variable responses among individuals, both in absorption and metabolism of phytochemicals[14]. To overcome the problem of low plasma concentrations of phenolic metabolites, they should be extracted and concentrated. This can be achieved by micro solid-phase extraction[14] or, as in the present work, by the addition of solvents that precipitate proteins and dissolve phenolic compounds and metabolites, followed by solvent evaporation and resuspension in a smaller volume[19]. This technique is simple, economic, and adequate for a small number of samples. However, the recovery of compounds of interest could be easily improved by using larger plasma volumes, so the final concentration of all the compounds would be higher. The high diversity of phenolic metabolites and lack of standards is the reason why a semi targeted approach was used to identify the compounds increased by the intervention before attempting to quantify them. By using a specially created database instead of a completely untargeted analysis, it was possible to ignore the numerous primary metabolites in plasma, focusing only on the phenolic compounds and their metabolites. An important limitation of this database, specific for phenolic compounds and their human metabolites, was the lack of mass spectral information for many compounds and their metabolites, which reduces the accuracy of the compound identification.

Large variability among individuals is regularly observed in studies evaluating absorption and metabolism of phenolic compounds, both quantitively and qualitatively. In the present results, one individual presented 18 compounds in the plasma sample taken after the intervention, while the others showed only 9 or 10. Moreover, many phenolic compounds and metabolites were found only in the baseline samples (before the intervention), and they also varied among individuals. Overall, more than half of the identified compounds showed higher concentrations (AUC) before than after the intervention or were found only in the pre-intervention samples. Therefore, it was necessary to compare individual samples taken before and after the intervention to understand which compounds were actually increased by it. The only metabolite that consistently increased after the treatment was phenylacetic acid, a microbial catabolite of flavan-3-ols[24] that has been found in the plasma of individuals in acute studies of the consumption of phenolic-rich foods[14,25]. Most of the studies that have identified and quantified phenolic metabolites in plasma were acute studies, in which the compound concentrations were monitored during the 24 h after consumption of the phenolic-rich meal, observing that, after 24 h, their concentrations were near the basal value[19,25]. Therefore, it is understandable that the samples analyzed in the present work showed low numbers and concentrations of phenolic metabolites. Recently, Zhang et al.[25] evaluated the consumption of red raspberry, both in acute studies and after 4 weeks of supplementation. They observed that both acute and chronic interventions increased phenolic compounds and their metabolites, while after the 4-week supplementation, the basal plasma concentration of some metabolites increased (urolithins, cinnamic, phenylpropionic, and hippuric acids) and of others decreased (conjugated anthocyanin derivatives).

A final examination of the method developed in the present paper shows that, in most parts, it was well suited for the objective for which it was created. The sample pretreatment

was simple and effective, and the UPLC separation and semi-targeted MS/MS identification of phenolic metabolites were achieved. This semi-targeted method can be useful as a first screening approach to identify phenolic compounds that can be absorbed from different food matrixes, as well as the phenolic metabolites present in human plasma. Moreover, the protocol is useful because it allows identification when no standards from phenolic compounds or metabolites are available. Finally, the method uses a small plasma quantity, which is critical in most *in vivo* studies where plasma samples are scarce. However, it is recommended that, for future studies, an acute study should be carried out before the chronic intervention to better identify the main phenolic compounds and metabolites absorbed from the specially formulated foods.

## Disclosures

All authors declare no conflict of interest.

## Acknowledgments

The authors are grateful for the financial support from CONACYT, Mexico (CB- 2016-01-286449), and UACJ-PIVA (Projects 313-17-16 and 335-18-13). OAMB wishes to thank CONACYT for his Ph.D. scholarship. Technical support from the Multimedia Production office from UACJ is gratefully acknowledged.

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