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Validation of a New LC-MS/MS Method for the Detection and Quantification of Phenolic Metabolites from Tomato Sauce in Biological Samples

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ABSTRACT: Tomato is a good source of bioactive molecules such as vitamin C, carotenoids, and phenolic compounds. Up to now, only a few studies have evaluated the bioavailability of phenolic compounds from tomato. This paper presents the optimization of a method for the determination of phenolics in tomato and their metabolites in human urine and plasma after ingestion of tomato sauce. The sample preparation includes a SPE step to obtain cleaner extracts for injection in the LC-MS/MS system. The mean recovery of analytes ranged from 73 to 104% in plasma and from 65 to 106% in urine, the accuracy was between 90.3 and 115.0% in urine and between 85.7 and 115.0% in plasma, and the precision coefficient of variation was <15%. The method allowed detection and quantification limits of 0.5−29 and 2.0−90 ng mL^{-1} in urine, respectively, and 0.5−30 and 2.0−105 ng mL^{-1} in plasma, respectively, for the same phenolic compounds.

KEYWORDS: *tomato, phenolic metabolites, LC-MS/MS, plasma, urine*

INTRODUCTION

Tomato (*Solanum lycopersicum*, formerly *Lycopersicon esculentum*) is one of the most popular and extensively consumed vegetable crops worldwide[1−3] and the most exported vegetable from Spain (880630 tons annually, according to FAO data). Tomato fruit is widely consumed either fresh or after processing into various products.[4,5]

Phenolic compounds found in tomato sauce belong to the class of phenolic acids, including hydroxycinnamic acids and their ester conjugates (mono- and dicaffeoylquinic acids, glucosides of caffeic, ferulic, dihydroferulic, and coumaric acids), with chlorogenic acid being the most abundant phenolic acid.[6,7] Tomato products also have a high content of the flavanone naringenin, which has been reported as the most abundant compound in tomato sauce.[6] In addition, recent results from our group show that rutin is a major flavonoid present in tomato products.[8,9]

There is today a growing awareness that the health effects derived from phenolic compound consumption depend on their bioavailability (absorption, distribution, metabolism, and elimination), a factor that is also influenced by their chemical structure.[10−14] Once ingested through food, phenolics are absorbed and then subjected to phase I (hydrolysis) and phase II (conjugation) metabolism in the small intestine and liver, which produces a series of water-soluble conjugate metabolites (methyl, glucuronide, and sulfate derivatives) that may pass into the bloodstream, accumulate in tissues, and then be excreted in urine.[15]

For the determination of phenolics and their metabolites in human urine and plasma, it is necessary to ensure an efficient and reproducible extraction in the sample treatment process. Optimization of this step is key to obtaining a method of analysis sensitive enough to determine these substances at low concentration. One of the most widely used techniques for the preconcentration and cleanup of analytical samples is solid-phase extraction (SPE). Tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS) has been demonstrated to be highly suitable for the analysis of phenolic metabolites[11,16−18] due to its high sensitivity, allowing lower detection limits and adequate selectivity versus interfering substances of the matrix, compared to LC with UV or electrochemical detection.

Previous studies by our group have established that the tomato sauce-making process (for instance, the addition or not of an oil matrix during sauce preparation) may affect the phase II metabolism of tomato phenolics and consequently the urinary excretion and plasma bioavailability. We here present the optimization of a new simple and rapid method for the determination of phenolic compounds and their metabolites in urine and human plasma and additionally report a screening of the occurring conjugated metabolites, which were identified by MS/MS experiments.

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EXPERIMENTAL PROCEDURES

Reagents and Materials. Phenolic standards caffeic acid, chlorogenic acid, dihydrocaffeic acid, ferulic acid, hippuric acid, isoferulic acid, and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA); 3-hydroxyphenylpropionic acid, 4-hydroxyphenylpropionic acid, naringenin, and ethyl gallate (internal standard (IS)) were from Extrasynthèse (Genay, France); and 4-hydroxyhippuric acid was from Phytolab (Vestenbergsgreuth, Germany).

All reagents were of HPLC grade: acetonitrile (MeCN), methanol (MeOH), and formic acid (HCOOH) were purchased from Scharlau Chemie S.A. (Barcelona, Spain); hydrochloric acid 37% (HCl) and orthophosphoric acid 85% (H_3PO_4) were supplied by Panreac Quimica SA (Barcelona, Spain). Human plasma was purchased from Sigma-Aldrich. Ultrapure water was generated by a Millipore System (Bedford, MA, USA).

The SPE cartridges used in this study were Oasis HLB 96-Well Plate 30 μm (30 mg) supplied by Waters (Milford, MA, USA).

Subjects and Study Design. Eight volunteers aged between 27 and 46 years (33 ± 5.9 years) and with a mean body mass index of 23 ± 0.93 kg/m^2 were selected. On the day of intervention, baseline blood and urine samples were collected from the subjects, who then consumed 250 mL of tomato sauce. Blood and urine samples were collected after the ingestion of the sauce. Blood was immediately centrifuged at 1500*g* for 20 min at 4 °C and stored at −80 °C until analysis. The urine bottles were stored at 4 °C and, immediately after the participants had started to collect the next fraction, the amount of urine in each fraction was measured, acidified with 0.2 M HCl, and stored at −80 °C until analysis. The study protocol was approved by the Ethics Committee of Clinical Investigation of the University of Barcelona (Spain), and the clinical trial was registered at the International Standard Randomized Controlled Trial Number (ISRCTN20409295). Informed consent was obtained from all participants.

Preparation of Standard and Stock Solutions. Individual stock solutions of caffeic acid, chlorogenic acid, dihydrocaffeic acid, dihydrophenylpropionic acid, ethyl gallate, ferulic acid, hippuric acid, isoferulic acid, naringenin, quercetin, and 4-hydroxyhippuric acid were prepared at a concentration of 1 mg mL^{-1} in 80% methanol.

The working solution, used to spike plasma and urine samples, was prepared by mixing the individual standard solutions and diluting it with H_2O. A solution of ethyl gallate at a concentration of 400 ng mL^{-1} was used as the internal standard. All standard solutions were stored at −80 °C.

Synthetic human urine was prepared by dissolving 0.65 g/L of $CaCl_2{\cdot}2H_2O$, 0.65 g/L $MgCl_2{\cdot}H_2O$, 4.6 g/L NaCl, 2.3 g/L Na_2SO_4, 0.65 g/L $Na_3C_6H_5O_7{\cdot}2H_2O$, 2.8 g/L KH_2PO_4, 1.6 g/L KCl, 1.0 g/L NH_4Cl, 25 g/L urea, and 1.1 g/L creatinine in Milli-Q water.

Instrumentation. *LC-ESI-MS/MS.* An HP Agilent Technologies 1100 LC system equipped with an autosampler and a column oven set to 30 °C and coupled to an API 3000 triple-quadrupole mass spectrometer (PE Sciex) with a turbo ion spray source was used to identify and quantify the corresponding phenolic metabolites. Chromatographic separation was achieved on a Luna C18 (50 × 2.0 mm (5 μm)) from Phenomenex (Torrance, CA, USA) using a precolumn Phenomenex security guard C18 (4 × 3 mm i.d.).[6] The system was controlled by Analyst v. 1.4.2 software supplied by Applied Biosystems (Foster City, CA, USA).

Procedures. *Extraction of Phenolic Compounds from the Tomato Sauce.* For the extraction of phenolic compounds from the tomato sauce, 5 mL of 80% ethanol acidified with 0.1% HCOOH was added to 0.5 g of sample. The mixture was vortexed for 1 min and then sonicated for 5 min on an ice bath to prevent degradation of the compounds. After centrifugation at 900*g* for 20 min at 4 °C, the supernatant was collected, another 5 mL of the acidified 80% ethanol solution was added to the pellet, and the extraction procedure was repeated. Both supernatants were combined and concentrated to dryness on a sample concentrator (Techne, Duxford, Cambridge, U.K.) at room temperature under a stream of nitrogen gas. The samples were redissolved in 2 mL of H_2O (0.1%HCOOH). After filtration of the aqueous extracts with 4 mm 0.45 μm PTFE syringe filters (Waters Corp.), 20 μL of the resulting filtrate was injected into the LC-MS/MS system, in triplicate.[6,8]

Sample Preparation Method for Plasma and Urine. On the day of the analysis, synthetic urine and plasma samples were defrosted on ice in the dark. For the preparation of the calibration curves, the samples were spiked with increasing concentrations for each phenolic standard. The IS, ethyl gallate, was added at a level of 400 ng mL^{-1}. Twenty-five microliters of H_3PO_4 was added to 2 mL of plasma and 17 μL of HCl to 2 mL of urine, which gave the samples a pH of 2. Before SPE, urine samples were centrifuged at 15000*g* for 4 min at 4 °C.

Solid-Phase Extraction. Solutions of 1.5 M HCOOH, 5% MeOH, MeOH (0.1% HCOOH), and H_2O (0.1% HCOOH) were prepared to be used in the SPE.

HLB plate activation was achieved by adding 2 mL of MeOH and 2 mL of 1.5 M HCOOH, consecutively. After the sample loading into the 96-well plate (2 mL), sample cleanup was performed with 2 mL of 1.5 M HCOOH followed by 2 mL of 5% MeOH solution. Phenolic compounds were then eluted with 2 mL of MeOH acidified with 0.1% HCOOH (v/v). The elution fraction obtained was evaporated to dryness by a sample concentrator (Techne) at room temperature under a stream of nitrogen. Two hundred microliters of water acidified with 0.1% HCOOH (v/v) was added to dissolve the residue to be injected into the LC system.

Chromatographic Conditions. The mobile phase used in LC-MS/MS for the tomato sauce and biological samples was water (A) and MeCN (B) with 0.1% HCOOH in both solvents. For the tomato sauce an increasing linear gradient (v/v) of B was used (*t* (min), %B), as follows: (0, 5), (10, 18), (13, 100), (14, 100), and (15, 5), followed by a 5 min re-equilibration step, at a constant flow rate of 400 μL min^{-1}. The flow rate for the biological samples was 0.6 mL min^{-1}, and the gradient used (*t* (min), % B) was (0, 5), (2, 25), (10, 90), (11, 100), and (12, 100), followed by a 5 min re-equilibration step. Twenty microliter aliquots of the extracts were injected in the LC-MS/MS system.

LC-ESI-MS/MS Parameters. The LC-ESI-MS/MS conditions were optimized by direct injection of 50:50 (v/v) water (0.1% HCOOH)/MeCN (0.1% HCOOH) of each phenolic standard individually at a concentration of 1 μg mL^{-1} into the mass spectrometer using a model syringe pump (Harvard Apparatus, Holliston, MA, USA) at a constant flow rate of 5 μL min^{-1}. The turbo ion spray source was in negative mode with the following settings: capillary voltage, −4500 V; nebulizer gas (N_2), 10 (arbitrary units); curtain gas (N_2), 12 (arbitrary units); drying gas (N_2) was heated to 400 °C and introduced at a flow rate of 4500 mL min^{-1}. Table 1 shows the declustering potential (DP), focusing potential (FP), and entrance potential (EP) optimized to detect phenolics with the highest signals.

Full-scan data were acquired by scanning from *m*/*z* 100 to 800 in profile mode using a cycle time of 1 s. Multiple reaction monitoring (MRM) experiments in the negative ionization mode were performed using a dwell time of 154 ms. The ions in MRM mode were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple quadrupole and analyzed with the second analyzer of the instrument.[14,19,20] The optimum collision-activated dissociation (N_2) was 4 (arbitrary units). Two transitions were followed for each phenolic compound; one was used for quantification and the other for identification. Table 1 shows these transitions with their optimum collision energy.

To identify the phenolic metabolites, we carried out different experiments such as product ion scan (PIS), neutral loss (NL), and precursor ion scan (PrIS). Only PIS experiments were successfully used for identification purposes due to the lower concentration of the phenolic metabolites investigated and, consequently, the need for greater sensitivity. The PIS experiments were carried out using a cycle time of 2 s.

Quality Parameters. The quality parameters established for the correct validation of the method were recovery, selectivity, limit of detection, limit of quantification, linearity, accuracy, and precision.[11,19,21]

Table 1. Declustering Potential (DP), Focusing Potential (FP), and Entrance Potential (EP) Optimized[a]

	DP (V)	FP (V)	EP (V)	quantification transition	collision energy (V)	identification transition	collision energy (V)
caffeic acid	−40	−170	−11	179 → 135	−20	179 → 107	−30
chlorogenic acid	−40	−180	−11	353 → 191	−20	353 → 179	−30
dihydrocaffeic acid	−40	−170	−11	181 → 137	−20	181 → 121	−30
3- and 4-hydroxyphenylpropionic acid	−30	−200	−10	165 → 121	−20		
ethyl gallate (IS)	−60	−200	−10	197 → 169	−25	197 → 124	−30
ferulic acid	−50	−220	−11	193 → 134	−20	193 → 178	−20
hippuric acid	−40	−170	−10	178 → 134	−20		
isoferulic acid	−40	−170	−11	193 → 178	−20	193 → 134	−20
naringenin	−50	−190	−11	271 → 151	−30	271 → 119	−40
quercetin	−50	−210	−11	301 → 151	−30	301 → 179	−30
4-hydroxyhippuric acid	−40	−170	−10	194 → 100	−20	194 → 150	−30

[a]Quantification and identification transitions with the optimum collision energy.

RESULTS AND DISCUSSION

Chromatographic Separation and Ion Mass Detection. The best compromise between keeping chromatographic run times as short as possible and allowing a convenient resolution of plasma and urine sample extracts was found to be a C18 column applying a gradient elution from 5 to 100% in organic component using LC-MS/MS. Peak resolution was higher than 1.5 except for the separation of hippuric acid, dihydrocaffeic acid, and chlorogenic acid. Peak width was acceptable for all of the compounds, ranging between 5.2 and 13.2 s. The multistep gradient elution yielded an optimum separation of the 11 phenolic compounds in <12 min using LC-MS/MS. Figure 1 shows spiked blank plasma at a level 16 times the LOQ.

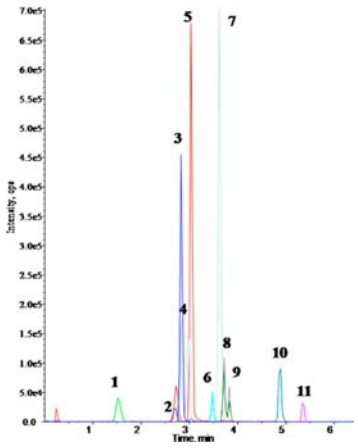

Figure 1. Separation of the 11 phenolics by LC-MS/MS. Peaks: (1) 4-hydroxyhippuric acid; (2) hippuric acid; (3) dihydrocaffeic acid; (4) chlorogenic acid; (5) caffeic acid; (6) ethyl gallate (IS); (7) 3- and 4-hydroxyphenylpropionic acid; (8) ferulic acid; (9) isoferulic acid; (10) quercetin; (11) naringenin.

The coupling of LC with MS is a powerful tool for the identification and quantification of analytes in biological samples.[11,16,17] Optimization of the methods was achieved by selecting the best ionization mode and mass spectrometer parameters. MRM mode exhibited the highest selectivity and sensitivity using LC-ESI-MS/MS.

Method Validation. The described method was fully validated by the criteria of the AOAC International: recovery, selectivity, limit of detection, limit of quantification, linearity, accuracy, and precision.[21]

Recovery. The recovery is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the same concentration of the pure authentic standard. The recovery obtained is illustrated in Tables 3 and 4. The results showed comparable values of recovery in fortified urine and plasma samples, with values being >95% in urine, except for 4-hydroxyhippuric acid and quercetin, which had recoveries of 75 and 65%, respectively, and values >96% in plasma, except for 4-hydroxyhippuric acid, which had 73% recovery. The recoveries of quercetin and 4-hydroxyhippuric acid obtained in the extraction were in agreement with the results of previous studies.[11,22]

Selectivity. Selectivity is the ability of an analytical method to differentiate and quantify the analytes in the presence of other components in the sample. To ascertain selectivity, six

Table 2. Contents of the Quantified Phenolic Acids, Flavanones, and Flavonols in the Administered Sauce

compound	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	concn range (ng mL^{-1})	linear fit	sauce (μg/g FW[a])
protocatechuic acid	8.01	30.4	30−400	0.996	129.2 ± 6.7
3-caffeoylquinic acid	23.9	75.8	75−1600	0.997	60.3 ± 1.1
5-caffeoylquinic acid	23.9	75.8	75−1600	0.997	814.3 ± 15.0
4-caffeoylquinic acid	23.9	75.8	75−1600	0.997	213.7 ± 6.9
dicaffeoylquinic acid	23.9	75.8	75−1600	0.997	54.3 ± 5.1
caffeic acid glucoside II	9.15	40.2	40−800	0.991	437.1 ± 9.4
caffeic acid glucoside IV	9.15	40.2	40−800	0.991	150.7 ± 1.3
caffeic acid glucoside II	9.15	40.2	40−800	0.991	6.1 ± 0.1
ferulic acid glucoside	18.0	50.2	50−2000	0.997	238.8 ± 12.2
naringenin	6.05	28.4	30−2000	0.992	1347 ± 63.6

[a]FW, fresh weight.

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Table 3. Urine Validation: Recovery, LOD, LOQ, Concentration Range, Concentration Added, Accuracy, Intraday, and Interday

					concn added			accuracy (%)			intraday 1 (%RSD)			intraday 2 (%RSD)			intraday 3 (%RSD)			interday (%RSD)		
	recovery (%)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	concn range (ng mL^{-1}) (r^2)	L[a] (ng mL^{-1})	M[b] (ng mL^{-1})	H[c] (ng mL^{-1})	L[a]	M[b]	H[c]	L[a]	M[b]	H[c]	L[a]	M[b]	H[c]	L[a]	M[b]	H[c]	L[a]	M[b]	H[c]
caffeic acid	97 ± 4	1.5	6	6−3450 (0.990)	12	290	1730	1.7	2.8	0.7	2	3	6	2	2	4	2	5	5	3	15	13
chlorogenic acid	103 ± 2	0.5	2	2−1150 (0.999)	4	96	576	1.9	1.0	0.6	7	2	3	7	3	3	9	3	3	15	4	10
dihydrocaffeic acid	101 ± 2	6.3	25	25−14400 (0.993)	50	1200	7200	0.9	15.0	7.1	3	5	6	3	3	2	9	2	4	12	7	7
3- and 4-hydroxyphenylpropionic acid	97 ± 2	6	20	20−11520 (0.995)	40	960	5720	6.4	1.9	9.1	2	3	4	1	3	2	7	5	5	9	7	7
ferulic acid	95 ± 2	15	50	50−28800 (0.990)	100	2400	14400	9.7	15.0	0.3	6	5	9	6	8	8	9	3	4	11	9	10
hippuric acid	106 ± 2	25	80	80−51840 (0.990)	160	4320	25920	1.4	5.9	1.6	2	6	6	3	4	5	2	5	7	4	9	11
isoferulic acid	99 ± 2	29	90	90−28800 (0.997)	180	2400	14400	0.5	1.1	1.4	4	4	7	2	6	3	8	6	3	9	8	9
naringenin	104 ± 4	0.5	2	2−1150 (0.999)	4	96	576	1.6	0.5	0.6	7	5	8	9	8	7	7	7	6	12	9	11
quercetin	65 ± 3	1.7	6	6−3450 (0.991)	12	290	1730	2.7	7.1	0.5	3	9	3	6	7	8	9	6	9	10	15	14
4-hydroxyhippuric acid	75 ± 3	15	50	50−28800 (0.990)	100	2400	14400	2.2	8.3	3.2	9	8	10	4	3	5	7	8	5	10	14	12

[a]L, low concentration. [b]M, middle concentration. [c]H, high concentration.

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Table 4. Plasma Validation: Recovery, LOD, LOQ, Concentration Range, Concentration Added, Accuracy, Intraday, and Interday

					concn added			accuracy (%)			intraday 1 (%RSD)			intraday 2 (%RSD)			intraday 3 (%RSD)			interday (%RSD)		
	recovery (%)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	concn range (ng mL^{-1}) (r^2)	L[a] (ng mL^{-1})	M[b] (ng mL^{-1})	H[c] (ng mL^{-1})	L[a]	M[b]	H[c]	L[a]	M[b]	H[c]	L[a]	M[b]	H[c]	L[a]	M[b]	H[c]	L[a]	M[b]	H[c]
caffeic acid	98 ± 3	1.8	6	6−3450 (0.993)	12	290	1730	6.4	0.6	0.7	7	8	7	10	6	4	8	4	4	12	11	8
chlorogenic acid	99 ± 4	0.6	2	2−1150 (0.999)	4	96	576	8.4	1.5	0.7	2	6	5	2	4	3	7	10	5	9	11	7
dihydrocaffeic acid	104 ± 5	4.4	15	15−14400 (0.991)	50	1200	7200	13.5	2.3	0.9	1	7	6	3	7	5	1	7	4	5	15	8
3- and 4-hydroxyphenylpropionic acid	99 ± 5	6	20	20−11520 (0.994)	40	960	5720	0.2	14.5	0.4	9	6	5	8	6	5	8	6	5	11	7	6
ferulic acid	98 ± 4	18	60	60−28800 (0.996)	100	2400	14400	14.3	14.6	0.8	5	5	7	4	2	3	2	3	9	6	9	11
hippuric acid	99 ± 4	25	90	90−51840 (0.992)	160	4320	25920	4.6	15.0	3.0	5	5	7	6	7	4	6	8	4	13	11	9
isoferulic acid	99 ± 4	30	105	105−28800 (0.994)	180	2400	14400	3.7	14.4	0.6	2	2	6	3	7	5	3	9	3	4	12	7
naringenin	96 ± 3	0.5	2	2−1150 (0.995)	4	96	576	12.6	0.5	1.0	8	8	5	6	6	6	5	7	7	9	13	9
quercetin	100 ± 3	1.4	5	5−3450 (0.990)	12	290	1730	5.9	9.7	6.0	5	4	7	3	3	8	5	4	13	6	11	15
4-hydroxyhippuric acid	73 ± 3	15	50	50−28800 (0.992)	100	2400	14400	14.3	2.9	1.6	4	12	3	7	5	5	5	14	7	10	15	11

[a]L, low concentration. [b]M, middle concentration. [c]H, high concentration.

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plasma and urine samples of six volunteers were analyzed at 3 h after ingestion of tomato sauce.

Under the chromatographic and MS/MS conditions used for the assay, analytes were well resolved with resolutions >1.5 for almost all of the compounds, and no interferences from matrix components at the mass transition and retention time of the analytes were observed in plasma and urine, compared with standards dissolved in water.

Limit of Detection (LOD) and Limit of Quantification (LOQ). The LOD was estimated for a signal-to-noise ratio of 3 from the chromatograms of spiked blank plasma and urine samples at the lowest analyte concentration tested. Similarly, the LOQ was determined for a signal-to-noise ratio of 10. Spiked plasma and urine samples at five different concentration levels were prepared in triplicate to establish the LOD and LOQ in the different mass spectrometric systems. Tables 3 and 4 present the LOD and LOQ obtained. These data indicated considerable differences in sensitivity between polyphenols but not variation in the biosamples. Hippuric acid and isoferulic acid presented higher LOD and LOQ than the other compounds in both plasma and urine. The concentrations obtained were in agreement with previous methodology described in the literature,[11,23] and for some compounds such as caffeic acid, chlorogenic acid, and quercetin, the limits were lower than previously reported values.[24]

Linearity. Linearity refers to the response of an instrument to the concentration of an analyte. In our case the response held a linear relationship with the analyte concentration. Linearity was tested by assessing signal responses of target analytes from plasma and urine samples spiked at a concentration ranging from the LOQ for each analyte to 52 μg mL^{-1}, depending on the LOQ of the phenolic compound. Spiked plasma and urine samples at eight different concentrations were prepared in duplicate. Calibration curves were constructed using the internal standard method (analyte/internal standard peak area ratio versus concentration of analyte/internal standard ratio), using 400 ng mL^{-1} of the internal standard. All calibration data are presented in Tables 3 and 4. The analytical procedure was linear over the concentration range tested with the correlation coefficient exceeding 0.990 for all compounds in plasma and urine samples, demonstrating the excellent linearity of the curves.

Accuracy and Precision. Accuracy is the closeness of agreement between the measured value and the accepted "true" or reference value. Experiments were evaluated by repetitively spiking the matrix with known levels of analyte standards. The accuracy of the method was expressed as (mean observed concentration/added concentration) × 100. The accuracy of the method was acceptable at each concentration level: in plasma, 85.7−114.3% for the low, 97.1−115.0% for the medium, and 98.4−103.0% for the high concentration levels; and in urine 90.3−106.4% for the low, 97.2−115.0% for the medium, and 98.4−109.1% for the high concentration levels.

Precision expresses the closeness of agreement among a series of measurements obtained from multiple testing of a homogeneous test sample under the method's established conditions. Precision was determined by independently processing 18 spiked plasma and urine samples at three different levels on three different days (six samples per day and level). The corresponding results of intra- and interday precision variabilities, summarized in Tables 3 and 4, show the good precision of the proposed method, with RSD values lower than 15%.

Analysis of Tomato Sauce. The most abundant type of phenolic compounds found in the studied tomato sauce were phenolic acids, particularly hydroxycinnamic acids and their ester conjugates (mono- and dicaffeoylquinic acids, caffeic acid glucoside, and ferulic acid glucoside), with chlorogenic acid being the most abundant, as previously described by Vallverdú-Queralt et al. The most abundant phenolic compound in the sample was the flavanone naringenin, confirming the high content previously reported in tomato and tomato products.[6] Table 2 shows the contents of the quantified phenolic acids, flavanones, and flavonols in the administered sauce.

Analysis of Plasma and Urine Samples. The developed method was applied to determine the concentration of the studied phenolics and identify their metabolites in plasma and urine samples after human consumption of nutritionally regular doses of tomato sauce. Phenolic compounds in plasma and urine were identified and quantified by comparing their MRM transition, retention time, and product ion scan with those of authentic standards.[11] The phenolic metabolites were identified by a product ion scan, neutral loss, or precursor ion scan in the absence of standards.

The MRM allowed 22 transitions to be monitored (Table 5), corresponding to 11 standard phenolic compounds and their expected metabolites. A chromatogram of urine sample at 4 h after the administration of tomato sauce is shown in Figure 2. Naringenin was detected in urine and plasma, although at very low concentrations. Naringenin was confirmed by the presence of a peak in the transition 271 → 151, at the same retention time as the standard. The compound appeared to a lower extent than its glucuronide metabolite, confirming its rapid metabolism, and was rapidly excreted after ingestion.

Naringenin glucuronide, caffeic acid glucuronide, and ferulic acid glucuronide were confirmed by the presence of peaks in transitions 447 → 271, 355 → 179, and 369 → 193, respectively. To ensure the identity of the conjugated metabolites, PIS was applied as a second experiment. The PIS of naringenin glucuronide shows two characteristic ions, m/z 271 belonging to the aglycone naringenin and m/z 176 belonging to the glucuronyl unit. Another fragmentation ion at m/z 113 is typical of the fragmentation of the glucuronide unit.[6,14] Caffeic

Table 5. Transitions of Phenolic Compounds and Their Metabolites in Plasma and Urine Samples

compound	MRM transitions (m/z, amu)	compound	MRM transitions (m/z, amu)
caffeic acid	179 →135	hippuric acid	178 → 134
caffeic acid glucuronide	355 → 179	hydroferulic acid	195 → 136
caffeic acid sulfate	259 → 179	hydroferulic acid glucuronide	371 → 195
chlorogenic acid	353 → 191	hydroferulic acid sulfate	275 → 195
dihydrocaffeic acid	181 → 137	isoferulic acid	193 → 178
dihydrocaffeic acid glucuronide	357 → 181	naringenin	271 → 151
dihydrocaffeic acid sulfate	261 → 181	naringenin glucuronide	447 → 271
3- and 4-hydroxyphenylpropionic acid	165 → 121	quercetin	301 → 151
ferulic acid	193 → 134	quercetin glucuronide	477 → 301
ferulic acid glucuronide	369 → 193	quercetin sulfate	381 → 301
ferulic acid sulfate	273 → 193	4-hydroxyhippuric acid	194 → 100

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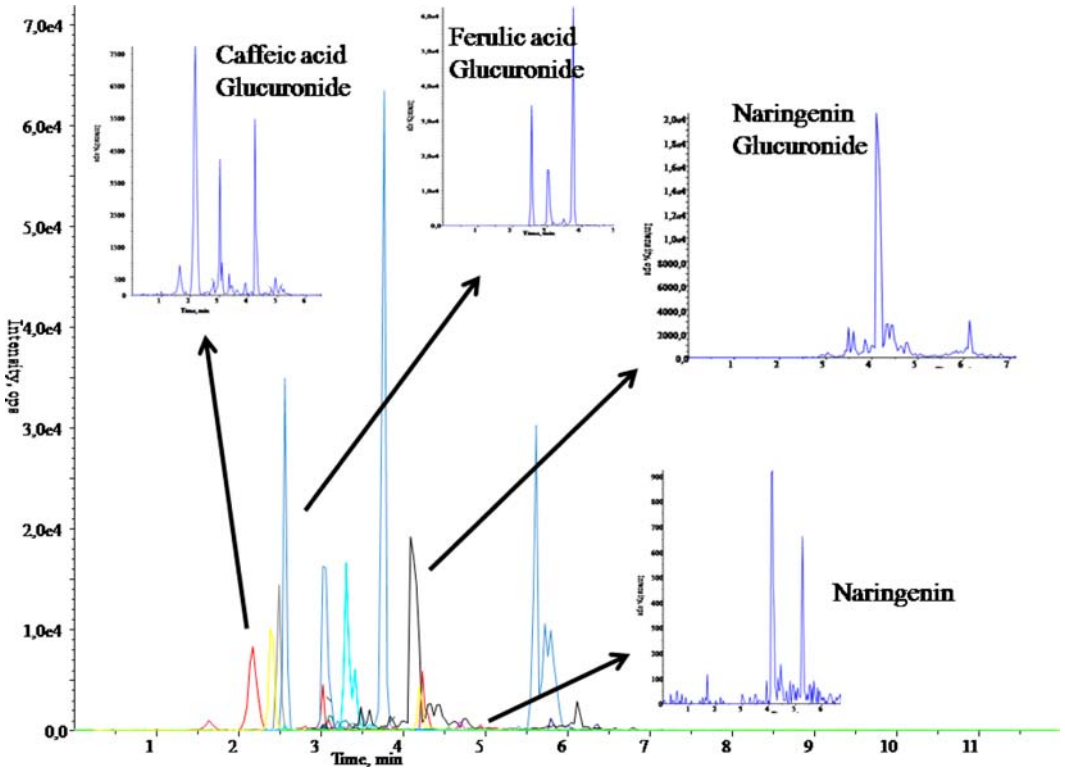

Figure 2. Chromatogram of urine sample at 4 h after the administration of tomato sauce.

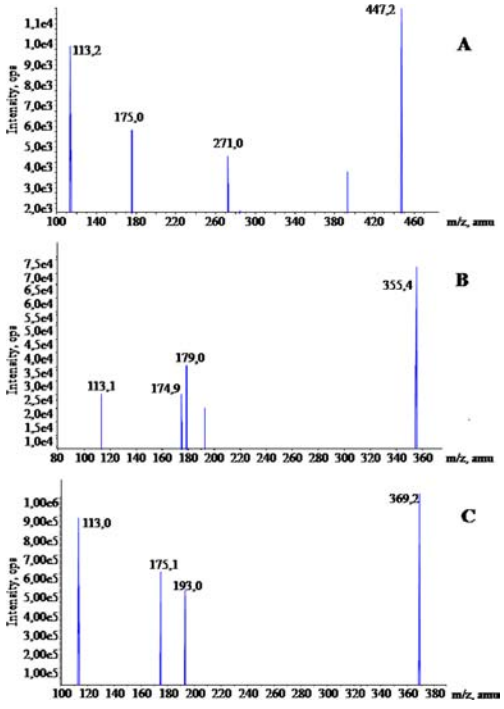

Figure 3. PIS of (A) naringenin glucuronide, (B) caffeic acid glucuronide, and (C) ferulic acid glucuronide.

acid glucuronide and ferulic acid glucuronide were also confirmed by PIS by the presence of m/z 176 amu, corresponding to the glucuronide unit, and by the ions corresponding to the aglycone at m/z 179 for caffeic acid and at m/z 193 for ferulic acid.[6] Figure 3 shows the PIS of naringenin glucuronide, caffeic acid glucuronide, and ferulic acid glucuronide. Ferulic acid glucuronide presented a slower rate of appearance in plasma, which corresponds with the longer and more complex metabolism leading to this compound.[6] On the other hand, the metabolite remained detectable in plasma for a longer period than the other compounds.

We have developed and validated a new LC-MS/MS procedure that determines tomato phenolics and their metabolites in biological samples with an extraction method capable of simultaneously analyzing a high number of samples. To our knowledge, this is the first reported method for the rapid detection and quantification of tomato sauce phenolics and their metabolites in plasma and urine. The procedure offers excellent selectivity and sensitivity. The use of LC-MS/MS to obtain the fragmentation patterns of the phenolics and phenolic metabolites led to a confident assignment of their structural classes. This method can be applied in future clinical and epidemiological studies with a high number of subjects to identify the real active compounds of tomato sauce and, consequently, to study their physiological effects in animal and human populations.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

MeCN, acetonitrile; CVD, cardiovascular disease; CAD, collision-activated dissociation; CE, collision energy; DP, declustering potential; FP, focusing potential; HCOOH, formic acid; FS, full scan; HCl, hydrochloric acid 37%; IS, internal standard; LOD, limit of detection; LOQ, limit of quantification; MeOH, methanol; MRM, multiple reaction monitoring; NL, neutral loss; H_3PO_4, orthophosphoric acid 85%; PrIS, precursor ion scan; PIS, product ion scan; RSD, relative standard deviation; SPE, solid-phase extraction; SEM, standard error; LC-MS/MS, tandem mass spectrometry coupled to liquid chromatography; H_2O, water.

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