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Assay of Urinary Excretion of Polyphenols after Ingestion of a Cup of Mountain Tea (*Sideritis scardica*) Measured by HPLC-DAD-ESI-MS/MS

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Supporting Information

ABSTRACT: Flavonoids and phenolic acid metabolites excreted in human urine after ingestion of *Sideritis scardica* decoction with characterized polyphenolic composition were studied. A feeding study was carried out with 10 human volunteers, and urine samples were collected for 24 h after ingestion of the *Sidertis* decoction. Polyphenol metabolites were identified and quantified in urine samples by HPLC with tandem mass spectrometric detection. Thirty-one different metabolites of hypolaetin, methylhypolaetin, isoscutellarein, methylisoscutellarein, and apigenin and 32 phenolic acid metabolites were detected and quantified using a method validated for this purpose. The urinary excretion of polyphenol metabolites corresponded to 5% (n/n) of the intake of polyphenols from the *Sideritis* decoction. Flavonoid metabolites were dominant in urine samples with 87−94% of total polyphenolic metabolites content. The most abundant metabolites were methylhypolaetin and methylisoscutellarein glucuronides. Urinary excretion of isoscutellarein (35.61%) was 10 times higher than that of hypolaetin (3.67%). Apigenin also showed high urinary excretion (32.46%).

KEYWORDS: *Sideritis scardica, polyphenols, flavonoids, phenolic acids, metabolites, urinary excretion, hypolaetin, isoscutellarein*

INTRODUCTION

The protective effects of a diet rich in fruits, vegetables, beverages, and teas are due not only to fiber, vitamins, and minerals but also to a diversity of plant secondary metabolites, in particular, phenolic compounds and, especially, flavonoids. The bioavailability of these compounds after dietary intake has been a topic of increasing research in recent years. A number of clinical trials based on dietary interventions have been performed to establish the bioefficacy of distinct subclasses of polyphenols.[1] There are many publications with results from studies on the bioavailability of flavan-3-ols, isoflavones,[2] proanthocyanidins, flavonols (especially quercetin derivatives),[3] and hydroxycinnamic acids.[4]

In the past decade, the interest in and research focused on the bioavailability and metabolism of phenolic substances from leafy spices is well justified. Lamiaceae herbs are rich in various phenolic compounds.[5−7] Because of the antioxidant activity of these herbs in laboratory test models,[6,8,9] they have been suggested to have beneficial effects on human health.

In the Mediterranean and Balkan countries, the dried inflorescences of a number of species of the genus *Sideritis* L., which belongs to the Lamiaceae family, are used to prepare a traditional beverage, the so-called "mountain tea", and they are widely used as a refreshing herbal tea as well in the folk medicine for the treatment of cough, bronchitis, and asthma and for curing gastrointestinal disorders. This type of drink is usually obtained for domestic use and consumption. Pharmacological studies indicate that *Sideritis* samples have various bioactivities, such as as antiulcer, anti-inflammatory[10] and antioxidant activities.[11−13] *Sideritis* decoctions are known for their high content of polyphenols, especially flavonoids[14−19] and phenylethanoid glycosides.[18−20] The dominant flavonoids in *Sideritis* are glucosides of hypolaetin and isoscutellarein and their methylated analogues,[15,21−24] which is a challenge for studying their bioavailability because such data for the this group of 5,7,8-OH flavones are scarce. The high phenylethanoid glycoside content[18,25] also makes this plant a good sample for testing the bioavailability of this class of compounds.

In recent years, many research groups are focused on the studies of flavonoids' bioavailability by different mechanisms to elucidate the health-promoting properties of different teas, foods, and beverages. However, as far as we are aware, the effect of the ingestion and nature of the phenolic compounds on the bioavailability of the target compounds (flavonoids and phenylethanoid glycosides) from *Sideritis* decoctions remains unknown.

The aim of this study was to investigate the metabolism of polyphenols in humans from *Sideritis scardica* decoction using liquid chromatography coupled to mass spectrometry (HPLC-ESI-MS/MS) to identify and quantify all of the urinary metabolites of flavonoids, phenylethanoid glycosides, and hydroxycinnamic acids and give an insight into their bioavailability. For that purpose, a study with 10 human volunteers was carried out in two phases. In the first phase, the volunteers followed a polyphenol-restricted diet and consumed the *Sideritis* decoction, after which daily urine samples were collected. In the second phase, the volunteers did not follow the polyphenol-restricted diet, but they drank the *Sideritis* decoction, and urine was collected for analysis 24 h after ingestion of the drink. Analysis of metabolites of flavonoids, phenylethanoid glycosides, and hydroxycinnamic acids in the

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collected urine samples revealed the urinary excretion and implied the metabolic transformations of the various classes of polyphenolic compounds from *Sideritis* in the human organism.

■ MATERIALS AND METHODS

Chemicals and Reagents. Formic acid, methanol, and water, all of analytical grade, were purchased from Merck KGaA (Darmstadt, Germany). 5-Caffeoylquinic acid, luteolin 7-*O*-glucoside, and rutin (quercetin 3-*O*-rutinoside) were purchased from Sigma (St. Louis, MO, USA). Authentic samples of verbascoside, forsythoside B, leucoseptoside A, hypolaetin 7-*O*-[6‴-*O*-acetyl]allosyl(1→2)glucoside, isoscutellarein 7-*O*-[6‴-*O*-acetyl]allosyl(1→2)glucoside, hypolaetin 7-*O*-[6‴-*O*-acetyl]allosyl(1→2)glucoside, 3′-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl]allosyl(1→2)glucoside, 4′-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl]allosyl(1→2)glucoside, apigenin 7-(6″-*p*-coumaroylglucoside), isoscutellarein 7-*O*-[6‴-*O*-acetyl]allosyl(1→2)[6″-*O*-acetyl]-glucoside, 4′-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl]allosyl-(1→2)[6″-*O*-acetyl]glucoside, and 4′-*O*-methylisoscutellarein 7-*O*-[6‴-*O*-acetyl]-allosyl(1→2)[6″-*O*-acetyl]glucoside were previously isolated and identified in the laboratory of the Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria, using NMR (^{1}H and ^{13}C). Reference standards were isolated in 2009 from cultivated sample of *S. scardica* collected in 2008 on Pirin Mountain, Bulgaria. The isolation and purification of all reference standards were in accordance with previous work[7,16,17] and literature data.[10,12,13] All compounds were identified by ^{1}H and ^{13}C Bruker AV 600 in DMSO-d_4. The purity of every reference standard was checked by NMR, and values between 96 and 98% were obtained. The stability of standards was followed by HPLC, and it was found that they are stable as solid substances within two years, and their methanol solutions were stable within 2 months stored at 4 °C.

Instruments and Analytical Conditions. The native phenolic compounds present in *S. scardica* samples and the phenolic metabolites in urine samples were identified by HPLC-ESI-MS/MS analysis.

Liquid chromatography separations were performed on an Agilent Technologies 1100 LC system (Waldbronn, Germany) equipped with a diode array and mass detector in series. It consisted of a G1312A binary pump, a G1329A autosampler, a G1379B degasser, and a G1315D photodiode array detector, controlled by ChemStation software (Agilent, v. 08.03). Spectral data from all peaks were accumulated in range 190−600 nm, and chromatograms were recorded at 280, 300, and 330 nm.

For analysis of both plant and urine sample extracts an XDB-Eclipse (Agilent, USA) C18 (150 mm × 4.6 mm, 5 μm) column was used at 38 °C, with the sample injection volume of 20 μL. Gradient elution was performed with a binary system consisting of (A) 1% aqueous formic acid (v/v) and (B) methanol. A linear gradient starting with 20% B (v/v) was installed to reach 80% B at 50 min and 100% B at 60 min. The flow rate was 0.4 mL min^{-1}.

The mass detector was a G2449A ion-trap mass spectrometer equipped with an electrospray ionization (ESI) system and controlled by LCMSD software (Agilent, v. 6.1.). Nitrogen was used as nebulizing gas at pressure of 50 psi, and the flow was adjusted to 12 L min^{-1}. The heated capillary and the voltage were maintained at 350 °C and 4 kV, respectively. MS data were acquired in the negative ionization mode. The full scan covered the mass range at m/z 100−1200. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycle from 0.3 to 2 V. Maximum accumulation time of the ion trap and the number of MS repetitions to obtain the MS average spectra were set at 300 ms and 5, respectively.

Identification and peak assignment of the native phenolic compounds present in *S. scardica* decoction and the phenolic metabolites in urine samples were based on comparison of their retention times and UV and mass spectral data with those of standards and published data.

Quantification of all phenolic compounds present in the *Sideritis* decoction was made using UV-DAD. Hydroxycinnamic acids were quantified using caffeic acid as external standard at 330 nm, phenylethanoid glycosides were quantified and expressed as verbascoside equivalent at 330 nm, and hypolaetin glucosides were quantified with 4′-*O*-methylhypolaetin 7-*O*-[6‴-*O*-acetyl]allosyl(1→2)glucoside at 290 nm, whereas isoscutellarein glucosides were quantified and expressed as isoscutellarein 7-*O*-[6‴-*O*-acetyl]allosyl(1→2)glucoside equivalent at 300 nm. Apigenin and luteolin derivatives were quantified using luteolin 7-*O*-glucoside at 350 nm.

Quantification of the phenolic metabolites in urine samples was made with MS detection using a full scan MS method, covering the mass range m/z 100−1200, and peak areas from extracted ion current (EIC) chromatograms were used for the quantification.[26] All metabolites were quantified using luteolin 7-*O*-glucoside as external standard. The ratio of peak area (from EIC chromatogram) of analyzed compound versus peak area of IS-rutin (from EIC chromatogram) was used for quantification. Because standards of the metabolites are not commercially available, and isolation is not possible due to their low abundance, quantification, which is in fact a semiquantitative estimation, is performed with these two standards chosen as representative compounds for the group of flavonoid metabolites (luteolin 7-*O*-glucoside) and hydroxycinnamic acid metabolites (caffeic acid).

Preparation of *S. scardica* Decoction. The flowering plant of *S. scardica* (wild growing) was collected from Kozjak Mountain, Republic of Macedonia, in July 2011. One kilogram of dried plant material (herba) was cut and mixed, and portions of 8 g were extracted with 400 mL of water during 10 min of boiling to the final volume of 300 mL. The phenolic composition of the sample was determined by HPLC-DAD-ESI-MS/MS.

Study Design. The study protocol was in accordance with WMA Declaration of Helsinki for ethical principles for medical research involving human subjects.[27]

Three male and seven female volunteers (19−26 years of age; mean body index = 24.2, range = 18.4−29.7), who were healthy, nonsmokers, and not on any medication, gave their written consent for participation in the study. They participated in the study divided in two phases. In phase I, the volunteers followed a polyphenol-restricted diet (without fruits and vegetables; high-fiber products; tea, coffee, fruit juice beverages; wine and alcoholic drinks; cigarettes; but without following same meals and water intake) for 2 days before and 5 days during the study. After the wash period of 2 days, they consumed 300 mL of the *Sideritis* decoction every morning in the next five days. Urine was collected over 24 h after ingestion of the drink. In phase II, volunteers did not follow the flavonoid-restricted diet, but continued drinking the *Sideritis* decoction and collecting urine 24 h after ingestion of the drink. Urine samples were immediately frozen after collection and stored at −80 °C until analysis. Tests performed on a sample right after collection and after 1 week, 3 weeks, and 3 months kept at −80 °C indicated no significant differences in the polyphenolic metabolite content.

Extraction Procedure for *S. scardica* Phenolic Metabolites in Urine. *Sideritis* source phenolic metabolites in urine were extracted by solid-phase extraction (SPE). Frozen urine samples were thawed at ambient temperature. Prior to SPE, 20 mL urine samples were centrifuged at 15000*g* for 10 min at 4 °C and filtered. A 10 mL urine sample (containing 100 μL of IS with a concentration of 100 μmol/L) was vortex-mixed for 5 min and then applied to the preconditioned C18 Sep-Pack cartridge with a 500 mg stationary phase (Waters, USA) with 5 mL of methanol and 5 mL of water and dried with 5 mL of air. Sample cleanup was performed with 3 mL of water, followed by drying with 3 mL of air. Elution was performed with 3 mL of methanol. The eluted fraction was evaporated to dryness, and residues were dissolved in 500 μL of methanol, filtered through a 0.45 μm filter (UPTIDISC RC 13 mm), and analyzed.

Preparation of Standard and Quality Control Samples. Accurately measured solid portions of caffeic acid and luteolin 7-*O*-glucoside were dissolved in 70% methanol to prepare stock solutions with a concentration of 1000 μmol/L. The stock solutions were stored at 4 °C and were stable for at least 2 months.

The stock solution of rutin (IS) was prepared by dissolving approximately 5 mg of accurately weighed sample (to 0.01 mg) in 10

mL of 70% methanol. A working solution of the internal standard (100 μmol/L) was prepared by diluting the stock solution with 70% methanol.

Frozen blank urine samples (collected after the 2 day wash period) were thawed at ambient temperature. Prior to preparation of the calibration standards and quality control samples, blank urine was checked for the presence of caffeic acid and luteolin 7-*O*-glucoside, and none of the two compounds was detected. The calibration standards of caffeic acid and luteolin 7-*O*-glucoside were prepared in blank urine (collected after the 2 day wash period), and they were processed in the same way as urine samples using SPE; the final concentrations in the extracts were in a series of 0.1, 1, 10, 20, 30, 40, 50, 100, and 250 μmol/L. The final concentration of the IS after SPE was 20 μmol/L in all samples. Quality control (QC) samples (5, 20, and 100 μmol/L) were prepared in the same way.

Method Validation. Linearity was tested using nine concentration levels of caffeic acid and luteolin 7-*O*-glucoside (0.1−250 μmol/L) in blank urine by plotting the ratio of peak areas of the standard compounds and peak area of the internal standard of rutin (20 μmol/L) versus the standard's concentration. The precision (coefficient of variation (CV%)) and accuracy (% recovery) of the intra- and interday assays were tested for the method developed. For evaluation of intra- and interday precision, three concentrations of caffeic acid and luteolin 7-*O*-glucoside (5, 20, and 100 μmol/L) were tested. The analytes' concentrations were determined with daily calibration curves. To assess the precision and accuracy of the method, three replicates per specimen and three runs per day were carried out on three independent days. The CV% was calculated as (standard deviation/mean) × 100. The recovery after standard additions was used to assess the accuracy of the method.[28] The sensitivity of the analytical method was evaluated by determining the low limit of detection (LLOD) and low limit of quantification (LLOQ). LLOD was established at a S/N ratio of 3:1 and LLOQ was set at S/N ratio of 10:1.[28] It was performed by taking the average area (counts) of the lowest detectable peaks in EIC chromatograms, which was multiplied by 3 (10) and the corresponding concentration calculated from the calibration curve.

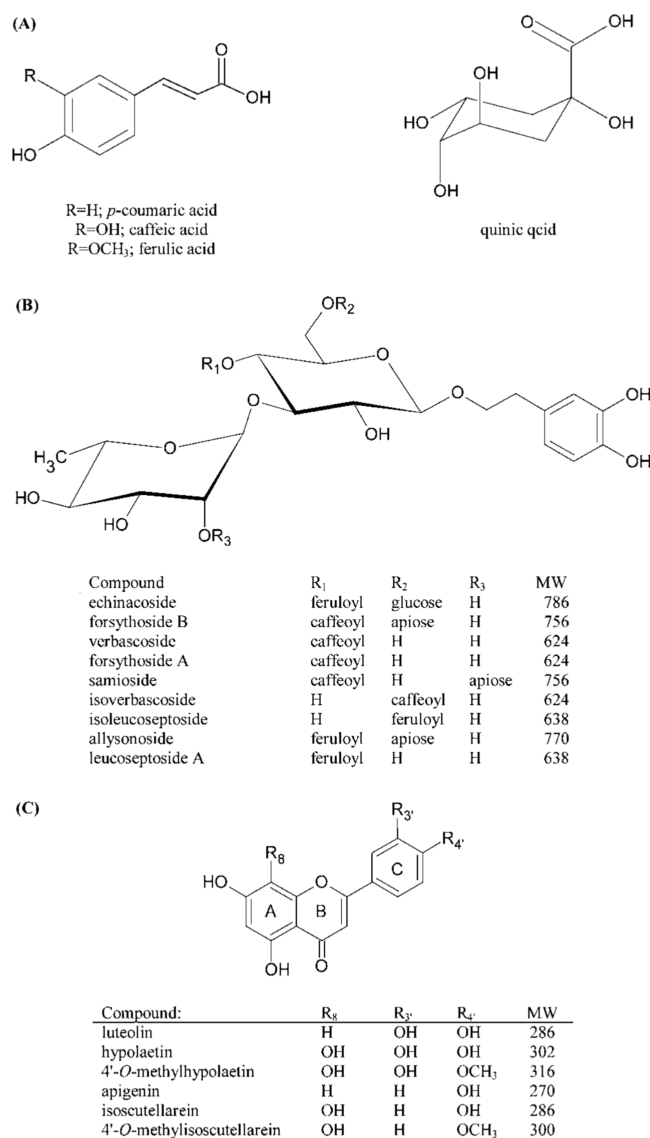
Statistical Analysis. Statistical treatment including calculations of means and standard deviations were performed using Excel (Microsoft Office, 2003). Samples were analyzed in triplicate, and one-way analysis of variance was performed using STATISTICA, version 7. The Newman−Keuls post hoc test (at $p < 0.05$) was used to evaluate the significance of differences in the results between two experimental phases (see study design).

RESULTS AND DISCUSSION

Analysis of *S. scardica* Decoction. The phenolic compounds present in *Sideritis* decoction were analyzed by HPLC-DAD-ESI-MS/MS. In total, 31 compounds were identified on the basis of their mass spectra and cochromatography with reference compounds, when available, and by reference to the literature; details for the identification are available in earlier publications.[18,19] The data for identification and quantification are summarized in Supporting Information Table S1. The content of phenolic compounds was expressed as the sum of the content of hydroxycinnamic acid derivatives, phenylethanoid glycosides, luteolin, hypolaetin, apigenin, and isoscutellarein derivatives and the total content of phenolic compounds was found to be 1450 ± 8 mg in 8 g of dried aerial part of *S. scardica* used for preparation of the decoction.

The total content of hydroxycinnamic acid derivatives (Figure 1A) was 72.24 ± 0.70 mg, which represents around 5% (m/m) of total phenolic compounds.

In the *Sideritis* decoction, the most abundant compound was isoverbascoside (296.3 ± 7.2 mg) followed by verbascoside (197.3 ± 5.2 mg) and 3′-*O*-methylhypolaetin 7-*O*-[6″-*O*-acetyl]allosyl(1→2)glucoside (139.9 ± 3.5 mg).



Compound	R1	R2	R3	MW
echinacoside	feruloyl	glucose	H	786
forsythoside B	caffeoyl	apiose	H	756
verbascoside	caffeoyl	H	H	624
forsythoside A	caffeoyl	H	H	624
samioside	caffeoyl	H	apiose	756
isoverbascoside	H	caffeoyl	H	624
isoleucoseptoside	H	feruloyl	H	638
allysonoside	feruloyl	apiose	H	770
leucoseptoside A	feruloyl	H	H	638

Compound:	R8	R3'	R4'	MW
luteolin	H	OH	OH	286
hypolaetin	OH	OH	OH	302
4'-*O*-methylhypolaetin	OH	OH	OCH3	316
apigenin	H	H	OH	270
isoscutellarein	OH	H	OH	286
4'-*O*-methylisoscutellarein	OH	H	OCH3	300

Figure 1. Structures of (A) hydroxycinnamic acids, (B) phenylethanoid glycosides, and (C) flavone aglycones present in *Sideritis scardica* samples.

The total content of phenylethanoid glycosides in *Sideritis* decoction was in the range of 831.0 ± 1.9 mg. The contribution of phenylethanoid glycosides to the total phenolic content was around 57% (m/m). Only the content of verbascoside and isoverbascoside was around 59% of total phenylethanoid content and around 34% of total phenolic content present in the *Sideritis* decoction. The structures of phenylethanoid glycosides present in *Sideritis* decoction are given in Figure 1B.

The total content of flavonoid 7-*O*-allosyl(1→2)glucosides was in the range of 17.47 ± 0.54 mg for luteolin derivatives, 399.7 ± 1.4 mg for hypolaetin derivatives, 13.68 ± 1.77 mg for apigenin derivatives, and 116.3 ± 1.1 mg for isoscutellarein derivatives. The structures of these flavones are given in Figure 1C. The most abundant flavonoid 7-*O*-allosyl(1→2)glucoside derivative was 3′-*O*-methylhypolaetin 7-*O*-[6″-*O*-acetyl]allosyl-(1→2)glucoside, and its content was around 34% (m/m) of total hypolaetin derivatives content and around 10% of total phenolic content present in the *Sideritis* decoction. The total flavonoid content represented around 38% of total phenolic compounds. This result makes this plant a rich source of 8-

hydroxy flavones (hypolaetin and isoscutellarein). A similar flavonoid composition has been reported for pulps and seeds of *Theobroma grandiflorum* (cupuassu) growing naturally in the Brazilian rainforests.[29]

Characterization of Urinary Metabolites. Flavonoid metabolites and profiles were identified in urine collected for 24 h after the consumption of 300 mL *Sideritis* decoction on five consecutive days. HPLC eluents were monitored by HPLC-ESI-MS/MS, and the combined chromatographic trace and mass spectra suggested the presence of more than 30 flavonoid metabolites and more than 30 phenolic acid metabolites (Table 1). Figure 2 illustrates typical HPLC-DAD and total ion current (TIC) chromatograms of urine collected in the third day, 24 h after the consumption of 300 mL of *Sideritis* decoction. The characterization of these metabolites was achieved by first full scan over a mass range of m/z 100−1200 and detecting peaks that corresponded to mass values of all possible metabolites. These peaks were then selected for MS/MS fragmentation and fragment ion monitoring.

A total of 63 different metabolites were detected in urine samples, implying that these compounds are metabolized mainly by glucuronidation and/or sulfation. As presented in Table 1, the metabolites can be summarized in four groups as follows: hypolaetin, isoscutellaerin, and apigenin derivatives and phenolic acid derivatives (quinic acid, caffeic acid, chlorogenic acid, ferulic acid, and coumaric acid). Details about the characterization and classification into these groups are presented elsewhere.[30]

The TIC chromatogram of urine collected 24 h after the consumption of 300 mL of *Sideritis* decoction and extracted ion current (EIC) chromatograms for most abundant compounds are presented in Figure 3.

Validation of the Method for Quantification of Urinary Metabolites. *Linearity.* Calibration curves using nine concentration levels of caffeic acid and luteolin 7-*O*-glucoside (0.1−250 μmol/L) (dissolved in blank urine and subjected to SPE) were constructed by plotting y , the ratio peak area (from EIC chromatogram) of the standard compound/peak area of the internal standard of rutin (20 μmol/L, EIC chromatogram), versus x, the corresponding concentrations (μmol/L). Linear response profiles were obtained for both standards with the following equations: $y = 0.0498x - 0.0344$ and $y = 0.0289x + 0.1483$ for caffeic acid and luteolin 7-*O*-glucoside, respectively. The correlation coefficients, R^2, obtained were 0.9986 and 0.9997 for caffeic acid and luteolin 7-*O*-glucoside, respectively.

Precision. Both intraday (instrumental) precision and interday (overall) precisions were tested by analyzing control samples (caffeic acid and luteolin 7-*O*-glucoside, dissolved in urine samples and extracted with SPE) at all concentration levels (from 0.1 to 100 μmol/L) with rutin as IS (20 μmol/L). The values for the average relative standard deviation obtained for the respective concentration ranges for each standard compound were very low and varied from 0.5% for standard mixture with concentrations 100 μmol/L to 7.5% for concentrations of 0.1 μmol/L.

Accuracy. The recovery test was used to evaluate the accuracy of the method. Accuracy was determined by standard addition at three different concentration levels of caffeic acid and luteolin 7-*O*-glucoside (low, medium, and high: 5, 20, and 100 μmol/L) to the urine sample. Satisfactory values for recoveries were obtained for both compounds, and in all cases they were in the range from 90.1 ± 2.1 to 92.2 ± 1.2% for low level, from 93.2 ± 1.5 to 94.5 ± 0.5% for medium level, and from 95.3 ± 1.1 to 96.1 ± 0.5% for high level for caffeic acid and luteolin 7-*O*-glucoside, respectively ($n = 3$).

Table 1. MS/MS Identification of Flavonoids and Phenolic Acid Metabolites in Human Urine Collected 0−24 h after Ingestion of 300 mL of Mountain Tea

	$[M - H]^-$ (m/z)	MS^2	no. of isomers detected in urine[a]
hypolaetin			
sulfate	381	301	1
glucuronide	477	459, 301	3
diglucuronide	653	477, 459, 301	1
glucuronide-sulfate	557	447, 301	1
methylhypolaetin			
sulfate	395	315	2
glucuronide	491	473, 447, 315	2
glucuronide + pentose	623	447, 315	1
isoscutellarein			
sulfate	365	285	1
glucuronide	461	443, 285	2
glucuronide-sulfate	541	461, 285	4
methylisoscutellarein			
disulfate	459	379, 299	2
glucuronide	475	457, 299	2
diglucuronide	651	475, 299	2
apigenin			
disulfate	429	349, 269	1
glucuronide	445	269	2
diglucuronide	621	445, 427, 269	1
glucuronide-sulfate	525	445, 269	3
quinic acid			
trisulfate	431	351, 271, 191	1
glucuronide	367	191	2
glucuronide-sulfate	447	367, 191	2
caffeic acid			
caffeic acid	179	161	1
glucuronide-sulfate	435	259, 179	1
dimethylcaffeic acid			
glucuronide	383	207, 179	2
caffeoylquinic acid			
sulfate	433	353, 179	2
disulfate	513	433, 353	2
glucuronide	529	353, 179	3
ferulic acid			
sulfate	273	193	1
glucuronide	369	193	4
glucuronide-sulfate	449	369, 193	3
dimethylferulic acid			
sulfate	301	273, 193	1
glucuronide	397	221, 193	2
feruloylquinic acid			
disulfate	527	447, 367	1
glucuronide	543	367	1
dimethylferuloylquinic acid glucuronide	571	395, 367	1
coumaric acid			
glucuronide	513	337	2

[a]More details about the identification of each metabolite are available in ref 30.

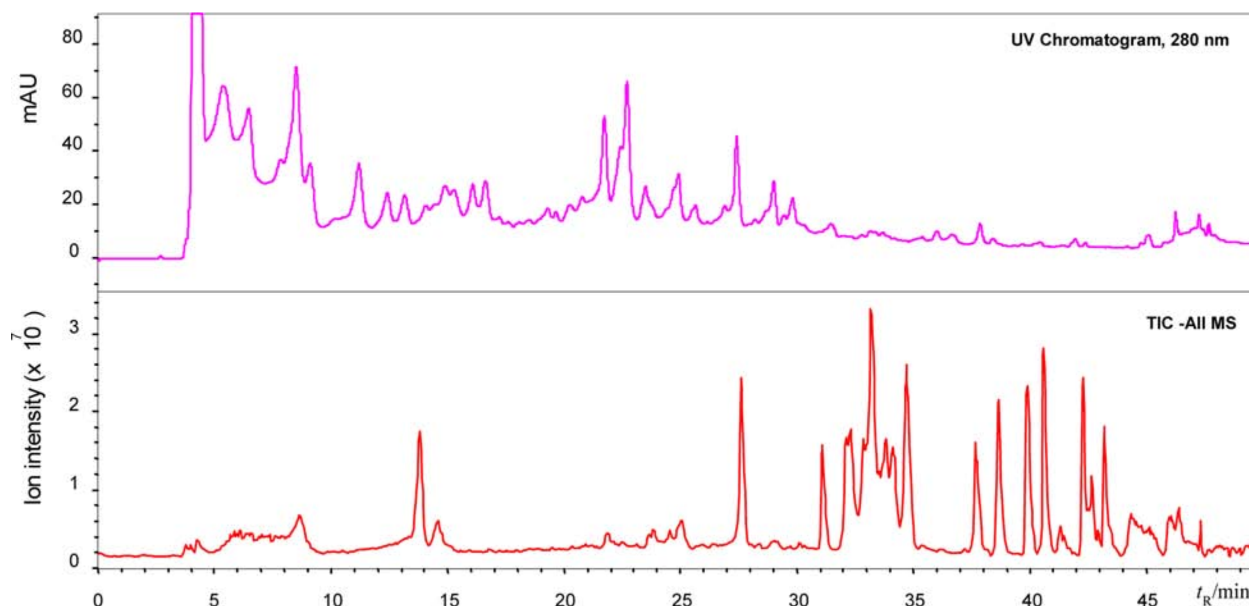


Figure 2. HPLC-DAD and total ion current (TIC) chromatograms obtained for urine sample collected on the third day, 24 h after the consumption of 300 mL of *Sideritis* decoction.

Sensitivity. The sensitivity of the method was evaluated by determining the LLOD and LLOQ at *S/N* ratios of 3:1 and 10:1, respectively, as described above.[28] LLOD and LLOQ were evaluated by analyzing six urine samples spiked with standard solutions of caffeic acid and luteolin 7-*O*-glucoside at final concentrations of 0.1, 1, and 10 μmol/L and rutin as IS. The lowest LLOD and LLOQ values were 60 and 200 nmol/L for caffeic acid and 15 and 50 nmol/L for luteolin 7-*O*-glucoside, respectively.

Quantification of Urinary Metabolites. After identification and method validation, urine samples were analyzed by MS, and EIC were used to quantify metabolites of hydroxycinnamic acids, phenylethanoid glycosides, and flavonoids from *Sideritis* excreted in urine collected 24 h after ingestion.

The total content of phenolic metabolites in urine samples was determined as a sum of total content of metabolites from hydroxycinnamic acid derivatives, apigenin, hypolaetin, and isoscutellarein. Total metabolite content in urine after 24 h was in the range from 32.73 to 184.9 μmol (Table 2), which represents 1.46−8.24% (n/n) of the total phenolic content present in the 300 mL of *Sideritis* decoction ingested (results for the content of polyphenols in the tea, Table S1, and for all metabolites in urine samples, Tables S2 and S3, are given in the Supporting Information).

Hydroxycinnamic Acid Metabolites. In comparison with *Sideritis* decoction, in which only 4 different hydroxycinnamic acid derivatives were found (3-caffeoylquinic acid, 5-caffeoylquinic acid, *p*-coumaric acid 4-*O*-glucoside, and feruloylquinic acid), in the urine a total of 32 hydroxycinnamic acid metabolites were detected (5 quinic acid derivatives, 5 caffeic acid derivatives, 7 caffeoylquinic acid derivatives, 8 ferulic acid derivatives, 3 dimethylferulic acid derivatives, 3 feruloylquinic, acid derivatives, and 2 coumaric acid derivatives (Table 1)).

As found in previous research, in urine and plasma, phenolic acids are found to be intact or conjugated as glucuronide, sulfate, or sulfoglucuronide.[31] Most of the studies focused on the bioavailability of ferulic, caffeic and chlorogenic acid indicate that if hydroxycinnamic acids were ingested in their free form, they were rapidly absorbed in the stomach or the small intestine and conjugated by the intestinal and/or hepatic detoxification enzymes.[4]

The total amount of hydroxycinnamic acid derivatives in urine samples ranged from 1.714 to 33.62 μmol (Table 2), which corresponds to 5.24−18.18% (n/n) of total metabolites excreted in urine and around 1.5% of the ingested dose of total phenolics present in 300 mL of the *Sideritis* decoction.

In urine samples, caffeoylquinic and feruloylquinic acids were detected as sulfate, disulfate, and glucuronide (Table 1) but also as free caffeic acid or caffeic acid glucuronide and glucuronide-sulfate, quinic acid trisulfate, glucuronide, glucuronide-sulfate, ferulic acid sulfate, and glucuronide-sulfate. This situation can be due to different mechanisms of absorption of chlorogenic acids.

Olthof et al.[32] found that the absorption of caffeic acid esterified with quinic acid is 3 times lower than that of caffeic acid itself. They recovered 0.3% of chlorogenic acid and 11% of caffeic acid in urine after ingestion. The reason for the lower absorption of caffeic acid esterified with quinic acid was attributed to the different absorption mechanisms.

Moreover, hydroxycinnamic acid metabolites may be derived not just from hydroxycinnamic acid derivatives present in *Sideritis* decoction but also from phenylethanoid derivatives.

Whereas the occurrence of phenyl glucuronide and sulfate derivatives of caffeic and ferulic acids in human plasma and urine has been widely reported, little or no attention has been paid to the metabolic pathway of phenylethanoid glycosides. Jia et al.[33] isolated and identified eight metabolites of echinacoside from rat bile collected after oral or intravenous administration of echinacoside, and they found that four of them were *O*-dimethyl conjugates, two were *O*-dimethyl glucuronides, and two were *O*-dimethyl sulfates.

Funes et al.[34] found verbascoside as the only metabolite identified by HPLC-ESI-MS/MS in the plasma samples from rats fed a lemon verbena extract.

Metabolites of phenylethanoid glycosides in urine samples after oral ingestion of *Sideritis* decoction were not found in our experiment.

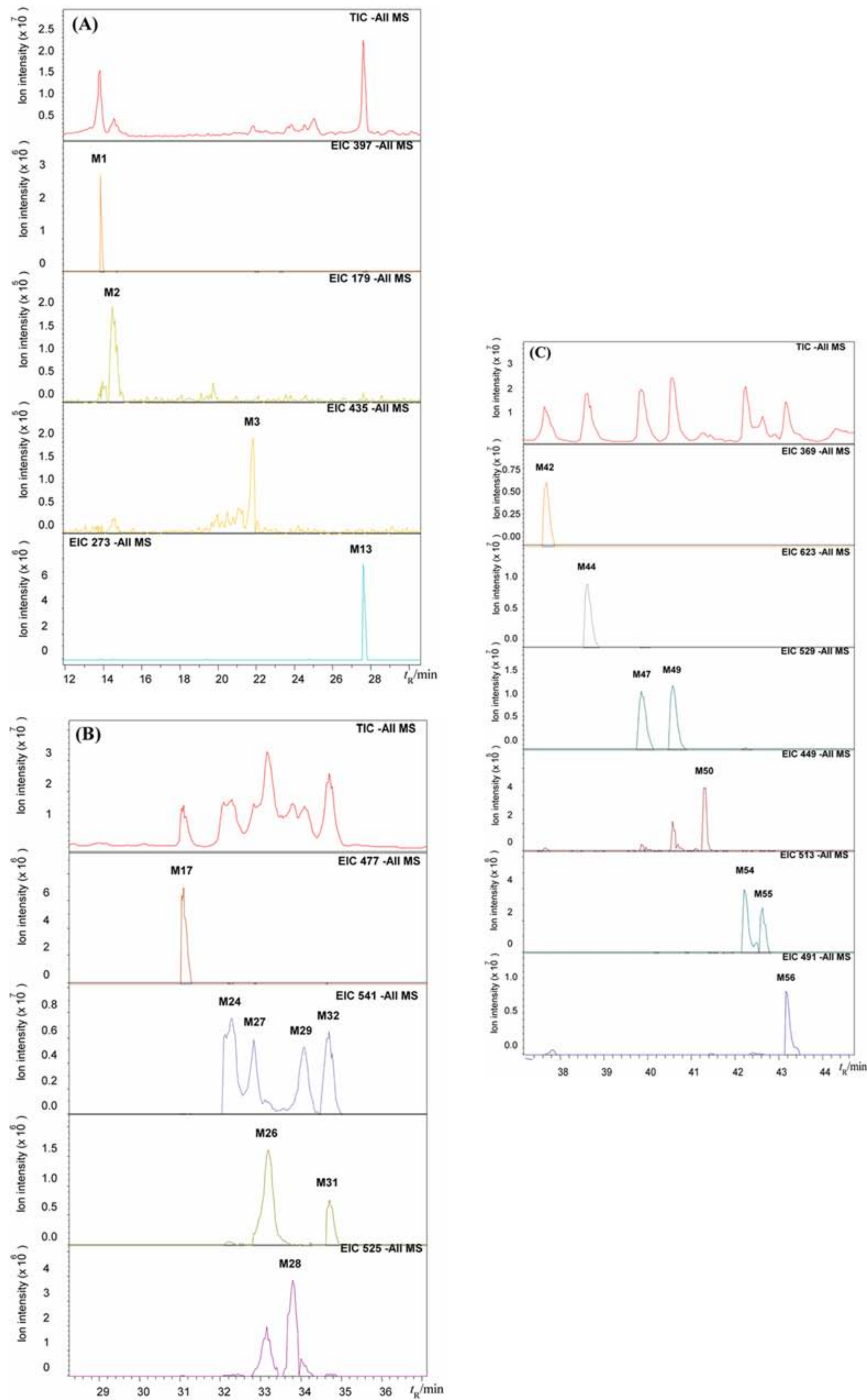


Figure 3. TIC chromatograms obtained for urine sample collected 24 h after the consumption of 300 mL of *Sideritis* decoction and EIC chromatograms of (A) M1, dimethylcaffeic acid glucuronide; M2, caffeic acid; M3, methylhypolaetin sulfate; M13, ferulic acid sulfate; (B) M17, hypolaetin glucuronide; M24, M27, M29, M32, isoscutellarein glucuronide-sulfate; M26, M31, chlorogenic acid sulfate; M28, apigenin glucuronide-sulfate; (C) M42, ferulic acid glucuronide; M44, methylhypolaetin glucuronide + pentose; M47, M49, chlorogenic acid glucuronide; M50, ferulic acid glucuronide-sulfate; M54, M55, chlorogenic acid disulfate; and M56, methylhypolaetin glucuronide.

Table 2. Urinary Excretion of Flavonoid and Phenolic Acid Metabolites Collected over 24 h after Ingestion of *Sideritis* Decoction (Expressed in Micromoles)

derivatives of	day 1	day 2	day 3	day 4	day 5	all days
total flavonoids	35.539−90.04	66.97−173.2	39.35−121.5	40.88−87.92	28.49−72.48	28.49−173.2
H + MH	3.829−33.51	10.36−41.50	12.69−41.84	6.549−31.40	5.802−21.98	3.829−41.84
hypolaetin (H)	1.440−6.222	1.892−11.25	0.241−7.788	1.575−5.586	1.399−5.068	0.241−11.25
methylhypolaetin (MH)	0.503−27.29	8.394−33.50	9.149−36.29	4.797−28.51	5.102−17.51	0.503−36.29
I + MI	31.71−74.73	56.61−137.8	26.66−103.6	29.96−77.59	16.85−61.80	16.85−137.8
isoscutellarein (I)	13.29−47.58	19.61−67.34	16.34−54.20	15.69−44.31	12.26−27.17	12.26−67.34
methylisoscutellarein (MI)	0.583−44.84	8.279−70.50	8.831−69.65	4.417−57.23	4.592−39.62	0.583−70.50
apigenin	0.058−11.94	0.244−20.84	0.373−17.31	1.999−13.48	0.459−10.13	0.058−20.84
total acids	1.714−15.17	3.332−17.67	3.930−33.62	2.847−18.49	1.905−9.889	1.714−33.62
quinic acid	0.177−5.184	0.456−1.708	0.305−1.644	0.126−1.440	0.213−3.141	0.126−5.184
caffeic acid	0.104−5.175	0.229−4.818	0.313−5.531	0.078−3.408	0.130−3.833	0.078−5.531
caffeoylquinic acid	1.029−6.683	1.081−15.77	2.797−32.51	0.893−16.64	0.228−6.056	0.228−32.51
ferulic acid	1.756−14.82	2.685−11.12	1.819−12.67	1.003−12.97	1.905−17.05	1.003−17.05
coumaric acid	0.642−1.342	0.214−2.179	0.117−0.409	0.097−1.284	0.117−0.584	0.097−2.179
total phenolics	50.71−98.29	70.30−184.9	44.29−132.6	44.35−95.76	32.73−79.24	32.73−184.9

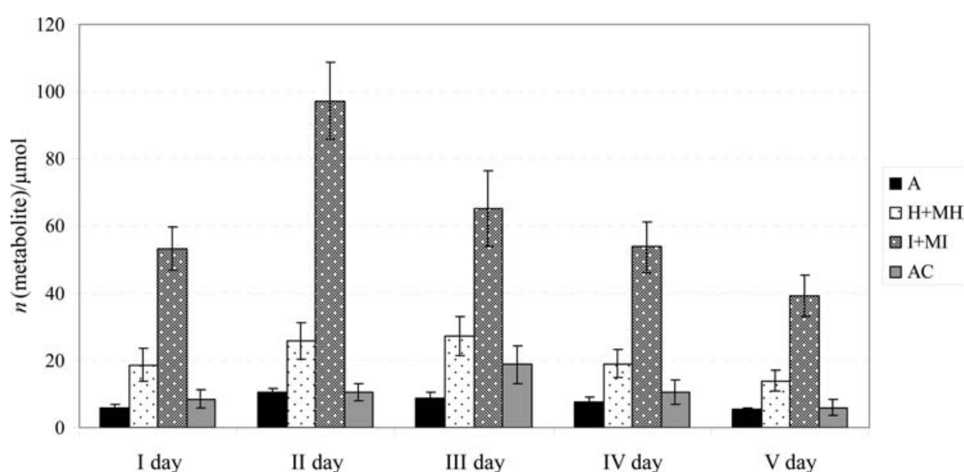


Figure 4. Excretion profiles of the four dominant groups of phenolic metabolites expressed in micromoles per day. A, apigenin derivatives; H+MH, hypolaetin + methylhypolaetin derivatives; I+MI, isoscutellarein + methylisoscutellarein derivatives; AC, total acid derivatives.

It is interesting that the content of hydroxycinnamic acid derivatives in the *Sideritis* decoction is around 5% of total phenolic content, whereas phenylethanoid content is around 57% of total phenolic content. This fact could indicate the low bioavailability of phenylethanoids after oral administration[35] as a result of poor absorption of phenylethanoids through the gastrointestinal tract. Jia et al.[33] suggested that the low concentration of phenylethanoids in urine can be due to the difficulties in identification of these metabolites only by LC−multiple tandem mass spectrometry. Therefore, preparation of metabolites and further identification based on NMR data should be done.

Flavonoid Metabolites. Although the flavonoids present in the *Sideritis* decoction have very complex structure (aglycone-diglucoside-(mono- or di-) acetyl), they were conjugated to glucuronides and sulfates and also transformed to methylated forms and excreted in urine.

Flavonoid metabolites were the most abundant group of polyphenol metabolites in the studied urine samples with the highest content, and their contribution to total metabolite content was from 87 to 94% even though they constitute 20% of total phenolic content in the *Sideritis* decoction.

The total hypolaetin metabolite content in urine samples (expressed as a sum of total content of metabolites of hypolaetin and methylhypolaetin) ranged from 3.829 to 41.84 μmol (Table 2), representing 22.63% (n/n) of total metabolite content and 3.67% of hypolaetin derivatives, and it corresponds to 1.86% of total phenolic content present in the *Sideritis* decoction.

The isomers of methylhypolaetin glucuronide were the most abundant metabolites detected in the urine samples of volunteers on the different days of the study and composed up to 95% (n/n) of total hypolaetin metabolite content.

The total isoscutellarein metabolite content in urine samples (expressed as a sum of total content of metabolites of isoscutellarein and methylisoscutellarein) ranged from 16.85 to 137.8 μmol (Table 2). The contribution of these compounds in the total polyphenol metabolites content was around 74.53% and around 6.14% of total phenolic content present in the *Sideritis* decoction.

Methylisoscutellarein glucuronide and isoscutellarein glucuronide-sulfate were found in all studied urine samples and represent around 85% of total isoscutellarein metabolites.

Apigenin metabolites content in the urine samples ranged from 0.058 to 20.84 μmol, representing from 0.17 to 11.27% of total metabolite content and corresponding to 1% of total phenolic content present in the *Sideritis* decoction.

Table 3. Quantification of Phenolic Compounds in Tea (Micromoles per 300 mL), Total Excretion of Metabolites in Urine, and Urinary Excretion of These Compounds after Ingestion of 300 mL of *Sideritis* Decoction by 10 Human Volunteers

		I phase experiment		II phase experiment	
derivatives of	*Sideritis* decoction (300 mL)	U, 24 h (μmol)	UE (% mol fraction)	U, 24 h (μmol)	UE (% mol fraction)
total flavonoids	806.97	91.67	11.36	93.21	11.55
H + MH	**581.3**	21.36	3.67	22.48	3.87
hypolaetin (H)	224.96	4.246	1.89	4.391	1.95
methylhypolaetin (MH)	356.37	17.11	4.80	18.09	5.08
I + MI	**175.9**	62.63	35.61	62.98	35.80
isoscutellarein (I)	96.12	31.78	33.06	32.01	33.30
methylisoscutellarein (MI)	79.82	30.85	38.65	30.97	38.80
apigenin	**23.67**	7.683	32.46	7.753	32.75
total phenolics	**2245**	102.5	4.57	103.2	4.60

By observing the excretion profiles of the four dominant groups of phenolic metabolites (Figure 4), it was clear that most of the metabolites showed their peak excretion during the second day after oral ingestion of 300 mL of *Sideritis* decoction. Urinary excretion was calculated as a ratio between total excretion and total intake (in μmol) of polyphenolic compounds (Table 3).

In total, 184.9 μmol of metabolites was excreted, corresponding to 8.24% (n/n) of the ingested dose of polyphenols present in the 300 mL of *Sideritis* decoction. The value for urinary excretion for phenolic metabolites from *Sideritis* decoction (8.24%) is comparable to that of urinary excretion of metabolites after ingestion of tea (ready-to-drink) flavan-3-ols (7.2%).[36]

Overall, urinary excretion of isoscutellarein (35.61%) is 10 times higher than that of hypolaetin (3.67%). In both cases, the dominant metabolites were glucuronides of methylhypolaetin and methylisoscutellarein, and they contribute to total urinary excretion of hypolaetin and isoscutellarein with 95 and 85%, respectively. Apigenin also showed high urinary excretion of 32.46%. A challenge of the study is the absence of literature data on the bioavailability and urinary excretion of hypolaetin and isoscutellarein and their methoxy derivatives.

Most of the published studies focused on glucuronidation in flavonoid metabolism (using glucuronosyltransferases) have demonstrated that glucuronidation is regiospecific and isoform-dependent.[37,38] Simons et al.[39] observed that hydroxyl groups at the 5-, 7-, and 4′-positions of flavonoids are important structural characteristics for optimal flavonoid degradation by human gut microflora. The flavonoid degradation rate ranking they found is as follows: genistein = apigenin = kaempferol = naringenin > chrysin > daidzein = 5,3′-dihydroxyflavone = 5,4′-dihydroxyflavone = 6,4′-dihydroxyflavone = 7,4′-dihydroxyflavone = 4′-hydroxyflavone = flavone. Genistein, apigenin, naringenin, and kaempferol were degraded more rapidly than the other flavonoids without hydroxyl groups at the 5-, 7-, and 4′-positions. Therefore, genistein, apigenin, naringenin, and kaempferol may not be as bioavailable in the colon as compared to more slowly degraded flavonoids, because they have less time to be absorbed before they are degraded by the gut microflora. The other studied flavonoids may be more bioavailable because the gut microflora degrades them at a slower rate, which gives these flavonoids a greater opportunity to be absorbed.

This might suggest that when the hydroxyl group in the 4′-position in isocutellarein and hypolaetin is changed with a methoxy group in methylisoscutellarein and methylhypolaetin, their bioavailability will increase as observed in our case (evident domination of methylisoscutellarein and methylhypolaetin glucuronides).

Robotham and Brodbelt[40] have shown, in the series 7-hydroxyflavone, chrysin, apigenin, and luteolin, how additional hydroxyl groups affect glucuronidation site selectivity. Comparing the results obtained for chrysin and apigenin, they conclude that a hydroxyl group at the 4′-position of a flavone has little to no effect on the selectivity of the uridine 5′-diphosphoglucuronosyltransferase (UGT) isozymes as there is virtually no difference in the product distributions for chrysin and apigenin for all of the UGT isozymes. Luteolin, on the other hand, provides a unique opportunity to observe how UGT selectivity changes upon addition of another hydroxyl group at the 3′-position. This resulted in modification of both B-ring sites for all active UGT isoforms and produced patterns with variable distribution of all three 3′-, 4′-, and 7-glucuronides. Isoscutellarein and hypolaetin, which are 8-OH apigenin and 8-OH luteolin, follow this pattern by producing two isoscutellarein glucuronides (7 and 4′) and three hypolaetin glucuronides (7, 3′, and 4′), as indicated in Table 1 and in previous data.[30]

Tang et al.[41] suggested that the sulfation pathway will not compensate for the glucuronidation pathway or vice versa in limiting the bioavailability of flavonoids. In other words, there is no relationship between a flavonoid's susceptibility to sulfation versus glucuronidation. The sulfation reaction results for all monohydroxy flavonoids had shown that 7-OH was more active than 4′-OH and, therefore, the formation of the 7-*O*-sulfate of 4′,7-dihydroxy flavonoids was greater than that of the 4′-*O*-sulfate, whereas the sulfation at the 5-OH position of dihydroxy flavonoids was below the detection limit. On the other hand, the hydroxyl group susceptibility of dihydroxy flavonoids for glucuronidation had the same order as for monohydroxy flavonoids (7-OH > 3-OH > 4′-OH ≥ 6-OH). Both sulfation and glucuronidation pathways showed stronger preference for the 7-OH group. Besides the 7-OH group, glucuronidation also showed a similar preference for 3-OH, whereas sulfation also showed a preference for the 4′-OH position.

Two stages of the experiment were carried out to evaluate the effect of the diet on the urinary excretion of polyphenolic metabolites. The Newman−Keuls test (at $p < 0.05$) revealed no significant differences in the type and content of phenolic metabolites between the two phases of the experiment (with and without a flavonoid restricted diet) (Table 3). The reason for that can be found in the specificity of hypolaetin and isoscutellarein as dominant flavonoids in *Sideritis* that are not abundant in the usual diet.

In conclusion, these are the first results elucidating the type of metabolites of polyphenols excreted in urine after ingestion of a *Sideritis* decoction. In vivo experiments performed in this work showed that phenolic compounds present in *Sideritis* decoction are extensively conjugated to glucuronides and sulfates and also transformed to methylated forms after their oral administration. In the analyzed urine samples, after consumption of a standardized extract of mountain tea, 31 different metabolites of hypolaetin, methylhypolaetin, isoscutellarein, 4′-*O*-methylisoscutellarein, and apigenin and 32 phenolic acid metabolites were detected.

Hypolaetin and isoscutellarein were the dominant groups present in the urine with the highest number of different metabolites. These two types of flavones are quite specific for the genus *Sideritis* that are not so abundant in the plant-derived food in contrast to apigenin and luteolin, so there were not significant differences in the samples when the nonflavonoid diet was or was not followed.

From these results it was clear that the flavonoid metabolites are dominant in urine samples, and they present from 87 to 94% (n/n) of total polyphenolic metabolites content. This can be attributed to the fact that only flavonoid glycosides with hydroxyl and methoxy groups available at specific positions, such as the 3′-, 4′-, and 7-positions, showed significant urinary excretion. An interesting finding of this study was the higher urinary excretion of the 4′-methoxy derivatives of the corresponding flavones (hypolaetin and isoscutellarein), implying their higher bioavailability. In total, the urinary excretion of the phenolic compounds from *Sideritis* decoction was found to be 4.57% from the ingested total polyphenols.

■ ASSOCIATED CONTENT

Supporting Information

Data for identification and quantification of polyphenolic compounds present in *Sideritis* decoction (Table S1); results from HPLC-MS/MS quantification of flavonoids (Table S2) and phenolic acid metabolites (Table S3) in urine collected 0–24 h after ingestion of mountain tea. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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