HPLC–Tandem Mass Spectrometric Method to Characterize Resveratrol Metabolism in Humans

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Background: Nutritional biomarkers are alternatives to traditional dietary assessment tools. We sought to develop a method for nutritional analysis of resveratrol, a phenolic compound with purported health-promoting properties, and to determine all resveratrol metabolites. Method: We obtained LDL and urine samples from 11 healthy male volunteers who had consumed 250 mL of Merlot red wine. We measured resveratrol and its metabolites with 96-well solid-phase extraction plates coupled with HPLC-tandem mass spectrometry. Hexestrol was used as the internal standard. Gradient chromatography in multiple reaction monitoring mode was performed on a Luna C18 column, maintained at 40 °C; m/z transitions were as follows: resveratrol, 227/185; resveratrol glucosides, 389/227; resveratrol glucuronides, 403/227; resveratrol sulfates, 307/227; taxifolin, 303/285; and hexestrol, 269/134.

Results: Standard calibration curves were linear at 4.4–3289.5 nmol/L. Residual analyses were 100% (3.2) for trans-resveratrol and 100% (11.1) for trans-piceid. In both matrices, imprecision (CV) was <10.8% at all concentrations. Detection limits for resveratrol were 0.2 nmol/L (LDL), 0.3 nmol/L (synthetic urine), and 4.0 nmol/L (blank urine). Resveratrol and metabolites were checked for stability, and no degradation was observed.

Conclusions: The HPLC–tandem mass spectrometry method enabled us to identify resveratrol sulfates in human LDL and to characterize the complete profile of resveratrol metabolism in human LDL and urine. This method provides an accurate index of exposure to resveratrol and its metabolites, which can be used as nutritional biomarkers for evaluating the biological effects of moderate wine intake on human health.

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Resveratrol is a phenolic compound that has been linked to the beneficial effects of red wine (1) (Fig. 1), which have been proposed to be mimetic of caloric restriction in mammals (2). In red wine, resveratrol occurs predominantly as its glucoside derivative, piceid. Several in vitro studies have demonstrated that resveratrol acts as an antioxidant (3), reduces the synthesis of proatherosclerotic substances (4), is a potential cancer preventative (5), and acts as a neuroprotector (6). Few authors, however, have studied resveratrol metabolism in humans. As with many polyphenols, resveratrol is reasonably well absorbed but has low bioavailability (7). Therefore, the health benefits attributed to the ingestion of resveratrol are most likely related to biologically active metabolites. In vivo characterization of resveratrol’s metabolic profile may reveal which metabolites act as signaling molecules within tissues (6) or reach target organs and account for the health benefits of resveratrol (8).

Nutritional biomarkers of nutrient exposure may be useful alternatives to traditional dietary assessment tools but require a clear understanding of the metabolism of the specific phytochemical. The metabolism of resveratrol has been partially characterized (9–13). After resveratrol ingestion, the main metabolites found in biological fluids are glucuronide and sulfate conjugates (9–12). Resveratrol glucuronide was reported to be a nutritional biomarker of wine consumption (13), but underestimation of
sulfate conjugates due to poor chromatographic behavior has limited the analytical methods used for the analysis of resveratrol metabolites (9–13). Other drawbacks included rather laborious sample preparation (14–16), long total analysis time (9–20), and the use of enzymatic hydrolysis that precluded direct detection of conjugates (14, 19, 20).

We describe an HPLC–tandem mass spectrometry (HPLC–MS/MS) method to characterize the metabolic profile of resveratrol in human urine and LDL after sample clean-up with solid-phase extraction (SPE).

**Materials and Methods**

**STANDARDS AND REAGENTS**

All samples and standards were handled with no exposure to light. Standards of **trans**-resveratrol (99% purity), **trans**-3,4',5-trihydroxystilbene-3-β-D-glucopyranoside (**trans**-piceid) (97% purity), diethylstilbestrol (3% purity), diethylstilbestrol dipropionate, 4-nitrobenzoic acid, 4-nitrophenol (98% purity), and human blank LDL were purchased from Sigma-Aldrich. Triethoxy resveratrol (98% purity) was purchased from Cayman Chemical, diethylstilbestrol-d6 from RIVM, taxifolin (>90% purity) from Extrasynthese, and creatinine from Fluka.

Methanol, acetone, and acetonitrile of HPLC grade were purchased from SDS. Glacial acetic acid, ethyl acetate, and o-phosphoric acid were purchased from Panreac. Ultrapure water (MilliQ) was obtained from Millipore. Synthetic urine was prepared as previously described (21).

We purified standard resveratrol metabolites from the livers of male Wistar rats raised at the Institut für Versuchstierzücht und-haltung (University of Vienna). Ethics Review Board approval was obtained for the animal studies. The animals were humanely treated. The livers were perfused with 20 μmol/L of **trans**-resveratrol in a recirculating system as previously described (22). We purified resveratrol metabolites from multiple bile samples collected over a time period of 60 min. After collection the samples were pooled and lyophilized. Chemical structures were confirmed by nuclear magnetic resonance (10).

**STUDY DESIGN AND SAMPLES**

We obtained human LDL samples from 11 healthy male volunteers (ages 18–50) before and 24 h after the consumption of 250 mL of Merlot red wine (10). All volunteers were considered healthy based on the results of physical examination and standard biochemical and hematological tests. The study was performed in accordance with the Helsinki Declaration of 1975, as revised in 1996. The Ethics Committee of our institution (Comité Ético de Investigación Clínica–Institut Municipal d’Investigació Médica) approved the protocol, and all the participants provided signed informed consent. Exercise was monitored with the Minnesota Leisure Time Physical Activity Questionnaire (23).

Before administration, the volunteers followed a 10-day washout period in which they consumed a controlled diet from days 1 to 7, avoiding excess intake of antioxidants. During the immediate 3 days before and on the intervention day, the volunteers consumed a standardized low phenolic compound diet. On the intervention day they drank a single dose of 250 mL of red wine. We collected EDTA blood at baseline and at 24 h after wine consumption. LDL was isolated by sequential flotation ultracentrifugation (24). We immediately froze all LDL samples at −80°C, with thawing immediately before analysis. Protein content was determined with the red pirogalol method (Sigma-Aldrich).

We obtained urine samples from 5 healthy male volunteers (ages 25–28 years). The study design and conditions were similar to those of Meng et al. (9), with the exception that urine was collected at baseline and during the 4 h after wine consumption. Urine creatinine was measured by a colorimetric assay using picric acid (25).

We used the same red wine in both studies and analyzed resveratrol by HPLC (26). The mean (SD) amount of total resveratrol consumed was 5.4 (0.4) mg, corresponding to 2.6 (0.0) mg of **trans**-piceid, 2.0 (0.2) mg of **cis**-piceid, 0.4 (0.1) mg of **trans**-resveratrol, and 0.4 (0.1) mg of **cis**-resveratrol.

**SAMPLE EXTRACTION**

LDL (1 mL) was treated with 20 μL of o-phosphoric acid (850 mL/L) and vortex-mixed. Urine was centrifuged at 10 000g at 4°C for 3 min and then vortex-mix mixed after addition of 20 μL of the hexestrol as internal standard (92.6 μmol/L) to 1 mL of sample. Samples were then loaded onto a Waters Oasis® HLB 96-well SPE plate (30 mg) that had been preconditioned with 1 mL of methanol and equilibrated with 1 mL of 2 mol/L acetic acid in
water. The plate was washed with 1 mL of 2 mol/L acetic acid in water and 1 mL of 2 mol/L acetic acid in water/methanol (85/15 v/v). Elution was achieved with 0.5 mL of 1 mol/L acetic acid in methanol and 2 × 0.75 mL of 1 mol/L acetic acid in ethyl acetate. The eluate was evaporated to dryness. We reconstituted the residue with 100 μL of taxifolin (1.64 μmol/L) dissolved in mobile phase as an additional the external standard.

HPLC-MS/MS ANALYSES
We performed liquid chromatography (LC) analyses using a Perkin-Elmer series 200 system equipped with a quaternary pump and a refrigerated plate autosampler. An Applied Biosystems API 3000 triple quadrupole mass spectrometer, equipped with a Turbo IonSpray source ionizing in the negative mode, was used to obtain the mass spectrometry (MS) and MS/MS data. A Phenomenex Luna C18 column, 50 × 2.0 mm i.d., 3 μm, maintained at 40 °C, was used for chromatographic separation. The injection volume was 15 μL, and the flow rate was 550 μL/min. Gradient elution was carried out with 0.5 mL/L acetic acid as mobile phase A and 700 mL/L acetone, 300 mL/L acetonitrile with 0.4 mL/L acetic acid as mobile phase B. We applied a linear gradient profile with the following proportions (v:v) of phase B ([min], %B): (0, 15), (1, 15), (1.5, 40), (2.5, 100), (4.5, 100), (4.8, 15), (10, 15). The column was reequilibrated for 6 min. The MS and MS/MS parameters were as previously described (10).

The identification of metabolites in biological samples was based on 3 indicators (10, 27): (a) comparison of retention time of available standard, (b) multiple reaction monitoring (MRM) of metabolite and resveratrol transitions [with higher declustering potential (DP) in collision-induced dissociation MS/MS conditions], or (c) product ion spectra. For MS/MS, a product ion scan was used at a cycle time of 2 s. The product ion spectra of metabolites showed the deprotonated molecule (m/z 403 or m/z 307, respectively) and the ion corresponding to resveratrol (m/z 227) through the neutral loss of the glucuronide or sulfate unit (−176 u or −80 u, respectively) from the glucuronide or sulfate. MRM mode was used with a dwell time of 200 ms, monitoring 6 transitions for each analysis: resveratrol (227/185), resveratrol glucosides (389/227), resveratrol glucuronides (403/227), resveratrol sulfates (307/227), taxifolin (303/285), and hexestrol (269/134). The concentrations of resveratrol metabolites were expressed as trans-resveratrol equivalents (10, 20).

EVALUATION OF INTERNAL STANDARDS
Several compounds, structurally similar to resveratrol, were evaluated as possible internal standards. MRM transitions were 267/237 for dihydrostilbestrol, 273/254 for dihydrostilbestrol-d6, 269/134 for hexestrol, and 265/93 for dienestrol. Trismethoxy resveratrol and dihydrostilbestrol dipropionate were not ionizable in negative mode.

ASSAY VALIDATION
We assessed endogenous interference by analyzing blank human LDL, synthetic urine, and blank urine samples (n = 5) collected from volunteers after the washout period. Recovery and linearity were investigated by adding trans-resveratrol and trans-piceid, at 10 concentrations, to blank urine (Table 1). The limit of detection was defined as the concentration of analyte that produced a signal-to-noise ratio of 3. The lowest standard on the calibration curve was accepted as the limit of quantification (28). Within- and between-day imprecision and recovery were evaluated with use of 10 different concentrations of resveratrol and piceid (n = 3) over a 10-day period. We evaluated stability during the analytical process, after freeze and thaw cycles, and after short-term and long-term storage. Control materials with resveratrol concentrations of 219.3 nmol/L and 2193.0 nmol/L, and piceid concentrations of 140.8 nmol/L and 1145.6 nmol/L, in the proper matrices, were stored under the same conditions (−80 °C) as biological samples. We assessed the stability of metabolites with urine from volunteers who had consumed red wine.

After we had validated the analytical method for routine use, we used resveratrol at concentrations of 21.9, 219.3, and 2193.0 nmol/L and piceid at concentrations of 12.8, 128.2, and 1282.0 nmol/L in duplicate as QC samples (28).

STATISTICAL ANALYSIS
SPSS statistical software, Windows version 11.5.1, was used. Kolmogorov–Levene and a paired Student t-test were employed. A weighted least-squares regression analysis was used to obtain correlation coefficients and slopes. Statistical significance was defined as P <0.05. Data are shown as the mean (SD).

Results
SELECTIVITY
Under the chromatographic and MS/MS conditions used for the assay, metabolites and standards were well resolved (Fig. 2, Table 2). Endogenous peaks at the retention time of the analytes of interest were not observed in blank human LDL or in synthetic urine. Blank urine from volunteers showed some endogenous peaks, but none at the same retention time of the analytes.

EXTRACTION RECOVERY AND LINEARITY
The mean (SD) recoveries of known amounts of trans-resveratrol and trans-piceid added to blank matrices were 92 (11.5)% and 89 (6.3)% respectively. The 9-point calibrator concentrations showed a linear and reproducible curve for standards. Weighted (1/x²) least-square regression analysis yielded equation regression lines and residual analysis [mean range (SD)] as follows: y = 35.2x − 0.07 (r² = 0.996) and 100% (3.2) for trans-resveratrol and y = 19.3x + 1.3 (r² = 0.967) and 100% (11.1) for trans-piceid.
Table 1. Within- and between-day precision and recovery data obtained from the LC-MS/MS of \textit{trans}-resveratrol and \textit{trans}-picneid in blank human urine.

<table>
<thead>
<tr>
<th>Imprecision</th>
<th>Added, mmol/L</th>
<th>Mean, mmol/L</th>
<th>Precision (RSD), %</th>
<th>Recovery (error), %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within-day (n = 3)</td>
<td>4.4</td>
<td>4.4</td>
<td>4.5</td>
<td>99.8</td>
</tr>
<tr>
<td></td>
<td>21.9</td>
<td>22.8</td>
<td>2.1</td>
<td>104.0</td>
</tr>
<tr>
<td></td>
<td>43.9</td>
<td>40.9</td>
<td>3.2</td>
<td>93.2</td>
</tr>
<tr>
<td></td>
<td>87.7</td>
<td>90.6</td>
<td>2.8</td>
<td>103.3</td>
</tr>
<tr>
<td></td>
<td>219.3</td>
<td>226.1</td>
<td>3.7</td>
<td>103.1</td>
</tr>
<tr>
<td></td>
<td>329.0</td>
<td>318.7</td>
<td>10.5</td>
<td>96.9</td>
</tr>
<tr>
<td></td>
<td>438.6</td>
<td>475.6</td>
<td>6.0</td>
<td>108.4</td>
</tr>
<tr>
<td></td>
<td>1096.5</td>
<td>1106.4</td>
<td>8.1</td>
<td>100.9</td>
</tr>
<tr>
<td></td>
<td>2193.0</td>
<td>2022.6</td>
<td>6.8</td>
<td>92.2</td>
</tr>
<tr>
<td></td>
<td>3289.5</td>
<td>3441.1</td>
<td>3.1</td>
<td>104.6</td>
</tr>
<tr>
<td>Between-day (n = 10)</td>
<td>4.4</td>
<td>4.7</td>
<td>10.8</td>
<td>106.6</td>
</tr>
<tr>
<td></td>
<td>21.9</td>
<td>24.2</td>
<td>10.5</td>
<td>110.3</td>
</tr>
<tr>
<td></td>
<td>43.9</td>
<td>39.4</td>
<td>8.1</td>
<td>89.9</td>
</tr>
<tr>
<td></td>
<td>87.7</td>
<td>95.5</td>
<td>10.1</td>
<td>108.9</td>
</tr>
<tr>
<td></td>
<td>219.3</td>
<td>227.2</td>
<td>4.4</td>
<td>103.6</td>
</tr>
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<td></td>
<td>328.9</td>
<td>295.7</td>
<td>10.2</td>
<td>89.9</td>
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<td></td>
<td>438.6</td>
<td>475.7</td>
<td>9.2</td>
<td>108.5</td>
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<tr>
<td></td>
<td>1096.5</td>
<td>1076.5</td>
<td>8.4</td>
<td>98.2</td>
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<tr>
<td></td>
<td>2193.0</td>
<td>1994.1</td>
<td>8.5</td>
<td>90.9</td>
</tr>
<tr>
<td></td>
<td>3289.5</td>
<td>3605.9</td>
<td>10.4</td>
<td>109.6</td>
</tr>
</tbody>
</table>

**PRECISION, RECOVERY, AND DETECTION LIMIT**

Precision and recovery (Table 1) met acceptance criteria (28) at all concentrations. According to these criteria, the lowest standards of \textit{trans}-resveratrol and \textit{trans}-picneid, 4.4 and 12.8 mmol/L, respectively, were accepted as the limit of quantification in human blank urine, and 0.4 and 1.9 mmol/L, respectively, in the LDL matrix (10). Limits of detection for \textit{trans}-resveratrol and \textit{trans}-picneid were 0.2 and 1.2 mmol/L, respectively, in LDL matrix, 4.0 and 8.4 mmol/L, respectively, in human blank urine, and 0.3 and 1.9 mmol/L, respectively, in synthetic urine.

**STABILITY**

To evaluate short-term temperature stability, 3 aliquots of each concentration were thawed at room temperature, maintained at this temperature for 3 h, and then analyzed. This represents the average sample preparation time for 96-well plates. The aliquots were then put in a refrigerated autosampler and analyzed at 10 and 25 h, the average time required to analyze 96 samples. Under these conditions, and after freeze and thaw cycles, we observed differences <5% for \textit{trans}-resveratrol and \textit{trans}-picneid. Evaluation of the long-term stability of resveratrol glucuronide stored at −80 °C for 5 years yielded an observed CV of 10.8% (n = 5). After testing the stability of human urine after moderate consumption of red wine, we observed no statistically significant differences in glucuronidated and sulfated metabolites at freeze and thaw.

**Fig. 2.** MRM trace chromatogram of sulfated and glucuronidated standards of resveratrol and MRM of 227/185 (DP = −80) in LC-MS/MS conditions as described in the text. Numbered peaks refer to Table 2.
Table 2. Description of relative molecular mass, retention times, negative mode multiple reaction monitoring transitions, mean concentrations (SD) of 24-h LDL and 4-h urine after moderate consumption of red wine, and percentage of volunteers who demonstrated each metabolite.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Peak no.</th>
<th>Mz</th>
<th>Rn min</th>
<th>MS/MS ions</th>
<th>m/z</th>
<th>LDL protein, mean (SD)</th>
<th>Volunteers, %</th>
<th>Urine samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>trans-Resveratrol-4'-O-glucuronide</td>
<td>1</td>
<td>404</td>
<td>1.6</td>
<td>403/227</td>
<td>403/227</td>
<td>37.8 (43.6)</td>
<td>27</td>
<td>59.6 (88.7)</td>
</tr>
<tr>
<td>Taxifolin</td>
<td>ES*</td>
<td>304</td>
<td>2.4</td>
<td>303/285</td>
<td>ES</td>
<td>ES</td>
<td></td>
<td>ES</td>
</tr>
<tr>
<td>trans-Resveratrol-3-O-glucuronide</td>
<td>2</td>
<td>404</td>
<td>3.3</td>
<td>403/227</td>
<td>403/227</td>
<td>111.7 (126.0)</td>
<td>36</td>
<td>179.2 (276.0)</td>
</tr>
<tr>
<td>trans-Resveratrol</td>
<td>3</td>
<td>228</td>
<td>5.5</td>
<td>227/185</td>
<td>227/185</td>
<td>3.5 (4.6)</td>
<td>73</td>
<td>ND</td>
</tr>
<tr>
<td>cis-Resveratrol-4'-O-glucuronide</td>
<td>4</td>
<td>404</td>
<td>5.6</td>
<td>403/227</td>
<td>403/227</td>
<td>ND</td>
<td>ND</td>
<td>355.8 (567.4)</td>
</tr>
<tr>
<td>cis-Resveratrol-3-O-glucuronide</td>
<td>5</td>
<td>404</td>
<td>4.8</td>
<td>403/227</td>
<td>403/227</td>
<td>7.1 (5.8)</td>
<td>27</td>
<td>893.5 (894.6)</td>
</tr>
<tr>
<td>trans-Resveratrol-4'-sulfate</td>
<td>6</td>
<td>308</td>
<td>6.2</td>
<td>307/227</td>
<td>307/227</td>
<td>2.0 (1.9)</td>
<td>36</td>
<td>2.4 (14.8)</td>
</tr>
<tr>
<td>Hexestrol</td>
<td>IS</td>
<td>270</td>
<td>6.7</td>
<td>269/134</td>
<td>IS</td>
<td>IS</td>
<td></td>
<td>IS</td>
</tr>
<tr>
<td>trans-Resveratrol-3-sulfate</td>
<td>7</td>
<td>308</td>
<td>6.8</td>
<td>307/227</td>
<td>307/227</td>
<td>4.0 (5.4)</td>
<td>36</td>
<td>74.7 (339.0)</td>
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<tr>
<td>cis-Resveratrol-4'-sulfate</td>
<td>8</td>
<td>308</td>
<td>7.3</td>
<td>307/227</td>
<td>307/227</td>
<td>7.1 (5.2)</td>
<td>64</td>
<td>9294.2 (8219.2)</td>
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<tr>
<td>cis-Resveratrol-3-sulfate</td>
<td>9</td>
<td>308</td>
<td>7.5</td>
<td>307/227</td>
<td>307/227</td>
<td>5.4 (2.9)</td>
<td>36</td>
<td>221.2 (1010.1)</td>
</tr>
</tbody>
</table>

* ES, additional external standard; IS, internal standard; ND, not detected.

cycles and after short- and long-term stability. We concluded that the metabolites were stable under the storage and sample handling conditions used for this assay.

INTERNAL STANDARD EVALUATION
Diethylstilbestrol and diethylstilbestrol-d6 showed 2 unstable peaks over time. Hexestrol and dienestrol, both veterinary synthetic products, were absent in human nutritional and body fluids. Their mean recoveries (n = 11) at the concentrations used in the assay procedure (1851.8 and 1879.7 nmol/L, respectively) were 96% and 89%, respectively. Although the mean recoveries were acceptable for both, dienestrol showed a higher variability (CV >15%) than hexestrol (CV = 11.2%). Hexestrol was selected as the internal standard.

QUALITY CONTROL RESULTS
trans-Resveratrol showed that 83% of QC were within 15% of their nominal value. trans-Piceid showed that 67% of QC were within 15% of their nominal value.

APPLICATION TO LDL SAMPLES
To identify sulfated metabolites of resveratrol and to complete its metabolic profile (10), we analyzed LDL samples with this LC-MS/MS method. Three different profiles of 24-h LDL glucuronide and sulfate conjugates of resveratrol after a single dose of red wine are shown in Fig. 3. Six metabolites were identified in volunteer A, 5 in volunteer B, and 4 in volunteer C. Volunteer B showed several peaks with 403/227 transition, but only 2 of them were positively identified as resveratrol glucuronides. Mean (SD) concentrations are shown in Table 2.

In addition to the well-described phase II metabolites of resveratrol, we also screened phase I metabolites, such as methylated (241/227) and hydroxylated (243/159) resveratrol, and their respective phase II metabolites, such as hydroxyresveratrol-glucuronide (419/243) and hydroxyresveratrol-sulfate (323/243). We also screened microflora metabolites, such as dihydroresveratrol-glucuronide (405/229) and dihydroresveratrol-sulfate (309/229) (12). After checking for these

![Fig. 3. MRM trace chromatogram of LDL after the intake of 250 mL of red wine (volunteers A, B, and, C). Numbered peaks refer to Table 2.](image-url)
transitions, some peaks were observed but were below the limit of detection.

APPLICATION TO URINE SAMPLES
Glucuronidated and sulfated metabolites were characterized in human urine by LC-MS/MS. MRM chromatograms of sulfates (307/227) and glucuronides (403/227) in urine from 4 volunteers are shown in Fig. 4. As can be seen in Fig. 4, the application of a higher DP (−80) in the collision-induced dissociation MS/MS experiment allowed the confirmation of all the metabolites through the characteristic 227/185 transition for resveratrol. The means (SD) of the metabolites for these volunteers are presented in Table 2 as nmol resveratrol/g creatinine.

Discussion
We have developed a new method to evaluate resveratrol metabolism in human samples. With this HPLC-MS/MS method, we determined the resveratrol metabolic profile in 10 min in different types of matrices. We emphasize that because of the observed differences in limits of detection, human blank urine is a better tool than synthetic urine because it shows the real matrix effect (29).

Investigations on human resveratrol metabolism have only recently been performed. In 2003, Goldberg et al. (30) were the first to administer resveratrol to humans. Subsequent published studies have shown glucuronides and sulfates to be the main metabolites of resveratrol. Only the glucuronide metabolites have been well characterized because of the poor chromatographic behavior of resveratrol sulfates (12).

We have circumvented the drawbacks of previous methods. To improve the resolution of the sulfates (10, 12), acetone was incorporated into mobile phase B. Acetone allows better resolution of sulfates by improving the peak shape and reducing the relative retention time. The incorporation of a shorter chromatographic column also reduced the chromatographic time to 10 min (9–20). The use of a 96-well SPE plate helped avoid laborious sample preparation (14–16), requiring −3 h of preparation per plate. The use of LC-MS/MS avoids the need to perform enzymatic hydrolysis (14, 19, 20), thus simplifying the procedure.

**Resveratrol Sulfates in Urine Samples**

![Graph showing resveratrol sulfates in urine samples for Volunteer W, Volunteer X, Volunteer Y, and Volunteer Z.](image)

**Resveratrol Glucuronides in Urine Samples**

![Graph showing resveratrol glucuronides in urine samples for Volunteer Y and Volunteer Z.](image)

Fig. 4. MRM trace chromatogram of resveratrol sulfates (307/227), resveratrol glucuronides (403/227), and resveratrol (227/185; DP −80) in urine samples of representative volunteers after the consumption of 250 mL of red wine.

Numbered peaks refer to Table 2.

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ing the quantitative and qualitative profiling of the resveratrol metabolites.

Another highlight of the present method is the ability to differentiate between the trans and cis isomers of resveratrol-4′-O-glucuronide, resveratrol-3′-O-glucuronide, resveratrol-4′-sulfate, and resveratrol-3′-sulfate. This method is the first to identify the entire profile of resveratrol sulfates in human LDL and urine (Figs. 3 and 4).

There was variability between volunteers (Table 2), but all sulfates were found in similar concentrations in LDL. The main sulfate in LDL was the cis-resveratrol-4′-sulfate, and the main glucuronide was trans-resveratrol-3′-O-glucuronide. The trans-resveratrol-0-glucuronides were in greater concentrations than sulfates. Resveratrol can be glucuronidated at 2 positions on the molecule. Although the 3 position seemed to be the preferential glucuronida-
tion site in vitro in human liver microsomes, the 4′ position is also a possible site of metabolism in humans in vivo (11). Considering activity, the presence of the 4′-OH is a requisite for inhibition of cell proliferation (31). Our results show major glucuronidation of resveratrol in 3-position at 24 h maintaining the 4′-OH free. Although the glucuronide metabolites of resveratrol have previously been described in LDL (10), this new method is able to determine resveratrol sulfates without reducing the resolution of glucuronides.

After successful characterization of the resveratrol metabolite profile in LDL, we applied the method to urine samples. Urine is a more adequate sample to be used in large-scale population studies to establish nutritional biomarkers (32). Meng et al. (9) described the rapid excretion of resveratrol in urine (after 2-3 h) when low amounts are consumed. In this study, the urine was collected during the 4 h after moderate red wine intake. When absorbed, resveratrol is rapidly cleared through the glucuronidation and sulfation pathways, and metabolites are principally excreted in urine (9, 12). All the resveratrol metabolites previously described were found in these urine samples. Concerning the stereoselectivity of glucuronidation, cis-isomers were glucuronidated faster than trans-isomers (15). This observation is in accordance with our results of our study, in which greater amounts of cis-O-glucuronide are obtained. Because this is the first time that sulfates of resveratrol have been well characterized, there are no published data about sulfate stereoselectivity. Taking into account the concentration results (Table 2), however, the behavior of sulfates seems similar to that of glucuronides, showing higher amounts for cis isomers. The variability shown in these results has been seen previously in LDL (10) and is attributable to polymorphisms of intestinal enzymes (33) or to interactions with other compounds (34). Further investigations on resveratrol variability with more volunteers are needed.

This method can be used in future epidemiological and clinical intervention trials. In studies aimed at evaluating the biological effects of resveratrol intake via moderate wine consumption, knowledge of the resveratrol profile may facilitate a better estimation of resveratrol consumption than dietary data obtained by food frequency questionnaires.

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References


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