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Liquid chromatography–tandem mass spectrometry analysis of oleuropein and its metabolite hydroxytyrosol in rat plasma and urine after oral administration

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Abstract

We describe a liquid chromatography–electrospray ionisation tandem mass spectrometry method for the qualitative and quantitative determination of the secoiridoid oleuropein and its bioactive metabolite hydroxytyrosol in rat plasma and urine. Samples were prepared by liquid–liquid extraction using ethyl acetate with a recovery for both compounds of about 100% in plasma and about 60% in urine. The chromatographic separation was performed with a RP-ODS column using a water–acetonitrile linear gradient. The calibration curve was linear for both biophenols over the range 2.5–1000 ng/ml (LOD 1.25 ng/ml) for plasma and 5–1000 ng/ml (LOD 2.5 ng/ml) for urine. Plasma concentrations of oleuropein and hydroxytyrosol were measured after oral administration of a single dose (100 mg/kg) of oleuropein. Analysis of treated rat plasma showed the presence of unmodified oleuropein, reaching a peak value of 200 ng/ml within 2 h, with a small amount of hydroxytyrosol, whereas in urine, both compounds were mainly found as glucuronides.
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Keywords: Oleuropein; Hydroxytyrosol

1. Introduction

Epidemiological studies indicate that the consumption of natural phenolic compounds produces beneficial health effects and these substances are now considered as being potentially therapeutic [1,2]. There is an increasing body of evidence, from in vitro studies, showing that polyphenolic compounds possess strong radical-scavenging activity [3–7]. Dietary antioxidant intake plays an important role in protection from the oxidative damage and aging of cells [8]. In the Mediterranean diet, rich in fresh fruit and vegetables, extra virgin olive oil represents the principal source of fat [9,10]. This alimentary behaviour has been associated with a reduced incidence of cardiovascular disease and cancer, in part due to the presence of bioactive constituents [11–14]. Oleuropein and hydroxytyrosol, 2-(3,4-dihydroxy-

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phenyl)ethanol, are inherent components of the diet in populations consuming olive fruit and olive oil [15,16]. Oleuropein is a heterosidic ester of elenolic acid and 3,4-dihydroxyphenylethanol, containing a molecule of glucose, the hydrolysis of which yields elenolic acid glucoside and hydroxytyrosol [17]. In vitro studies have demonstrated that the polar fraction of olive oil has the ability to inhibit prooxidative processes on human low density lipoproteins (LDL) [18,19]. Moreover, in vivo studies on rabbits have revealed that extra virgin olive oil biophenols, in particular oleuropein and hydroxytyrosol, are effective in preventing oxidation. In fact, supplementation of the diet with oleuropein decreased susceptibility to LDL peroxidation and reduced the plasmatic levels of total, free, and ester cholesterol [20]. Many studies, carried out both in vitro and in vivo, have indicated that hydroxytyrosol is able to reduce the amount of isoprostane excreted in urine [21] and possesses strong antioxidant-scavenging ability [22], contributing to the prevention of cardiovascular diseases.

Although in vivo studies have been performed to verify the antioxidant efficacy of oleuropein, reports of its bioavailability and biological fate are very scarce [20] and data indicating the circulating concentration of oleuropein and its bioactive metabolites after oral administration are absent. In a recent study [23], the absorption of oleuropein was investigated in isolated perfused rat intestine, determining its plasma concentration by HPLC with fluorescence detection.

Analytical methods suitable for the measurement of oleuropein and hydroxytyrosol from biological tissues are mainly based on HPLC and GC–MS, the latter being used particularly for hydroxytyrosol [24–27]. Recently, several studies have measured the level of hydroxytyrosol in human plasma using an HPLC–UV system [28] and in rat plasma either by LC–MS or GC–MS analysis [29,30]. More recently, Vissers et al. [31] described the absorption of olive oil phenols in humans by measuring oleuropein and hydroxytyrosol by HPLC and GC–MS, respectively.

To better characterise the precise pharmacokinetic properties of oleuropein, it is important to develop a highly sensitive and simple analytical method for its quantitation in biological samples. Therefore, we have developed an accurate LC–MS–MS methodology for the simultaneous detection of oleuropein and hydroxytyrosol in rat plasma and urine following oral ingestion of oleuropein. The method was validated and applied in vivo. Oleuropein was determined in plasma, then metabolised to hydroxytyrosol as the major product, and as glucuronide derivatives in urine. These results demonstrate that oleuropein is absorbed and converted enzymatically into hydroxytyrosol, the latter having relevant antioxidant properties.

2. Experimental

2.1. Chemicals and materials

Oleuropein and taxifolin were obtained from Extrasynthese (Genay, France) and from Sigma (St. Louis, MO, USA), respectively. 2-(3,4-Dihydroxyphenyl)ethanol (hydroxytyrosol) was synthesised in our laboratories by the $LiAlH_4$ reduction of 3,4-dihydroxyphenylacetic acid [32]. Acetonitrile and methanol (both HPLC grade) were purchased from Carlo Erba Reagenti (Milan, Italy), formic acid (analytical grade) was purchased from Merck (Darmstadt, Germany), ethyl acetate was obtained from Aldrich (Steinheim, Germany) and sterile deionised water was purchased from Laboratori Diaco Biomedicali (Trieste, Italy). β-Glucuronidase (EC 3.2.1.31) type X-A, G-7896 and β-glucosidase (EC 3.2.1.21) were purchased from Sigma. Heparin was purchased from Roche (Milan, Italy). Heparinised plasma and urine, for blank matrices, were prepared in our laboratories from CD-COBS rats, before oleuropein administration.

2.2. Stock solutions

Standard stock solutions of oleuropein, hydroxytyrosol and taxifolin, the latter used as the internal standard (I.S.), were prepared in methanol with a final concentration of 1 mg/ml. These solutions were stored at −20 °C until use.

The I.S. stock solution was diluted in blank rat plasma and urine to achieve a final concentration of 1 μg/ml; this solution was prepared immediately before sample preparation.

2.3. Instrumental conditions

HPLC separations were achieved using a Perkin-Elmer 200 micro LC pump system (Norwalk, CT, USA) and were performed at room temperature using a reversed-phase Hypersil-5 ODS column (100×3.0 mm; C_{18}, 5 μm, 120 Å) provided with a Chromsep guard column (10×2.0 mm, C_{18}, 5 μm, 120 Å), both purchased from Chrompack (Middelburg, Netherlands).

Samples were injected (20 μl) using a Perkin-Elmer 200 autosampler (thermostated at 4 °C) with a 20 μl injection loop. Separations were carried out using a linear gradient of acetonitrile (solvent A) in water (solvent B), both with 0.5% formic acid (10% A for 1 min; to 35% A in 6 min; to 100% A in 1 min; isocratic at 100% A for 3 min) at a flow-rate of 500 μl/min.

The HPLC system was coupled directly to a Sciex API 365 triple-quadrupole mass spectrometer (Toronto, Canada) equipped with a TurboIonSpray source (Sciex). Instrument control and data acquisition were performed using Masschrom 1.1.1 software (PE Sciex, Foster City, CA, USA) run on a Power Macintosh G4 (Apple, Cupertino, CA, USA). Analytical conditions were optimised with direct infusion of standard solutions of oleuropein, hydroxytyrosol and taxifolin [at a concentration of 10 μg/ml in water–acetonitrile (50:50, v/v) with 0.5% formic acid] using a Model 11 syringe pump (Harvard Apparatus, South Natick, MA, USA) at a flow-rate of 5 μl/min.

The negative ion mode and multiple reaction monitoring (MRM) for tandem MS was used. The mass spectrometer monitored the deprotonated molecules $[M-H]^-$ at *m/z* 539 (oleuropein), 153 (hydroxytyrosol) and 303 amu (taxifolin) via the first quadrupole filter, and collisionally activated dissociation (CAD) was performed (collision gas N_2 at 0.36 Pa and collision energy at 25, 17 and 29 eV for oleuropein, hydroxytyrosol and taxifolin, respectively) at the second quadrupole. The product ions *m/z* 275, 125 and 123 amu were monitored via the third quadrupole for oleuropein, hydroxytyrosol and taxifolin, respectively. The TurboIonSpray voltage was set at −4400 V for all the analytes and the orifice and ring voltages at −20 and −200 V for oleuropein, −26 and −210 V for hydroxytyrosol, and −31 and −220 V for taxifolin, respectively. The TurboIonSpray source was heated at 450 °C and the flow-rates of nebulizer gas (air), curtain gas (nitrogen) and auxiliary gas (air) were set at 2.5, 2.7 and 8 l/min, respectively. Dwell time was set at 400 ms for each compound.

2.4. Extraction procedures

2.4.1. Plasma samples

The I.S. (20 μl) and 1 ml of ethyl acetate were added to 100 μl of rat plasma. After 10 min vortexing and 10 min centrifugation at 20 800 *g*, the supernatant was evaporated to dryness under a stream of nitrogen. The dried residue was stored at −20 °C and reconstituted immediately before analysis with 200 μl of water–acetonitrile–formic acid (89.5:9.5:1, v/v).

2.4.2. Urine samples

Extractions were performed on 500 μl of urine. The I.S. (20 μl) and 3 ml of ethyl acetate were added and after 10 min vortexing and 10 min centrifugation at 2500 *g*, the supernatant was evaporated to dryness under a stream of nitrogen. The dried sample was stored at −20 °C and reconstituted immediately before analysis with 200 μl of water–acetonitrile–formic acid (89.5:9.5:1, v/v).

2.5. Validation procedures

2.5.1. Linearity, precision and accuracy

Calibration standards of oleuropein and hydroxytyrosol were prepared by adding appropriate amounts of stock solutions to a blank matrix and then serially diluting the standards with additional blank matrix in order to obtain concentrations ranging from 1000 to 1.25 ng/ml for both plasma and urine. Lower concentrations were also prepared for the determination of the limit of detection (LOD).

In order to determine the assay linearity, accuracy and precision, five samples at each concentration were extracted and analysed to construct five independent calibration curves for both plasma and urine. Peak integration of the extracted ion chromatograms (539→275 amu for oleuropein, 153→123 amu for hydroxytyrosol and 303→125 amu for taxifolin) and all the calculations of con-

centrations and regression parameters were performed using PE sciex TurboQuan 1.0 software. Other statistical calculations were performed using Excel 98 software (Microsoft, Redmond, WA, USA).

The internal ratios (oleuropein peak area/I.S. peak area and hydroxytyrosol peak area/I.S. peak area) were calculated for each point, and calibration curves were constructed by weighted ($1/y$) least squares linear regression analysis of internal ratios vs. concentrations. After back-calculations of the concentrations from the regression curve, precision was expressed as the relative standard deviation (RSD %) of the recalculated concentrations, whereas accuracy was calculated as [(mean calculated concentration − nominal concentration)/nominal concentration]·100.

The limit of quantitation (LOQ) was considered the lowest level of each analyte that could be determined with an accuracy and precision of ≤20%, while the limit of detection (LOD) was considered the lowest concentration with a signal intensity at least three times greater than the background level.

2.5.2. Intra- and inter-batch assays

Variability studies were performed using quality controls (QC) obtained by spiking plasma and urine with oleuropein and hydroxytyrosol at low, mid and high concentrations. To determine the intra-batch precision and accuracy of the assay, three replicates of each QC sample in plasma and urine were analysed. Inter-batch precision and accuracy were evaluated on three different days ($n=9$). Precision and accuracy were calculated as described for standard curves.

2.5.3. Extraction recovery

Extraction recoveries of the analytes were determined by comparing the mean peak area of extracted plasma and urine samples ($n=3$) at low (25 ng/ml), mid (250 ng/ml) and high (500 ng/ml) levels of the calibration range with the mean peak area of the standard at the same concentrations. Analyte extracts and standards were dissolved in the initial HPLC mobile phase. The extraction recovery of taxifolin was determined at the adopted concentration. The reproducibility of the extraction procedure was determined as RSD %.

2.5.4. Stability

The stability of oleuropein and hydroxytyrosol in the matrices was evaluated after storage of spiked rat plasma and urine at 100 ng/ml in the dark for 24 h, both at room temperature (RT) and 4 °C, and after three freeze–thaw cycles. The stability of both analytes was expressed as recovery of the found concentrations compared with the analyte concentration of samples extracted and analysed immediately after spiking.

2.6. Animal treatment

Oleuropein was administered orally to 14 week old CD-COBS rats weighing about 350 g as a single dose of 100 mg/kg using soya oil and distilled water (1:1) as vehicle. Heparinised blood (1 ml) was collected from the femoral vein 0, 10, 30, 60, 120 and 300 min after administration and centrifuged at 20 800 *g* for 6 min. Plasma samples were then frozen and stored at −20 °C. Urine samples were collected before treatment and 24 h after treatment and frozen and stored at −20 °C.

Aliquots of plasma (100 μl) and urine (500 μl), with 125 μl of 1 *M* phosphate buffer added (pH 6.8), were treated with 100 U/ml β-glucuronidase at 37 °C for 1 h, in order to investigate the possible glucuronidated metabolites of oleuropein and hydroxytyrosol. Plasma and urine samples after enzymatic treatment were extracted as described in Sections 2.4.1 and 2.4.2, respectively.

3. Results and discussion

3.1. Liquid chromatography–tandem mass spectrometry

Optimisation of the analytical conditions was carried out in a three-step process. First, infusion of the standards was performed in negative scan mode to investigate the $[M-H]^-$ ion of oleuropein (m/z 539 amu) and hydroxytyrosol (m/z 153 amu), as shown in Fig. 1a and b, respectively. Therefore, product ion scan mode was used to determine the most abundant product ion for each analyte. Among the product ions the most abundant were the ion at

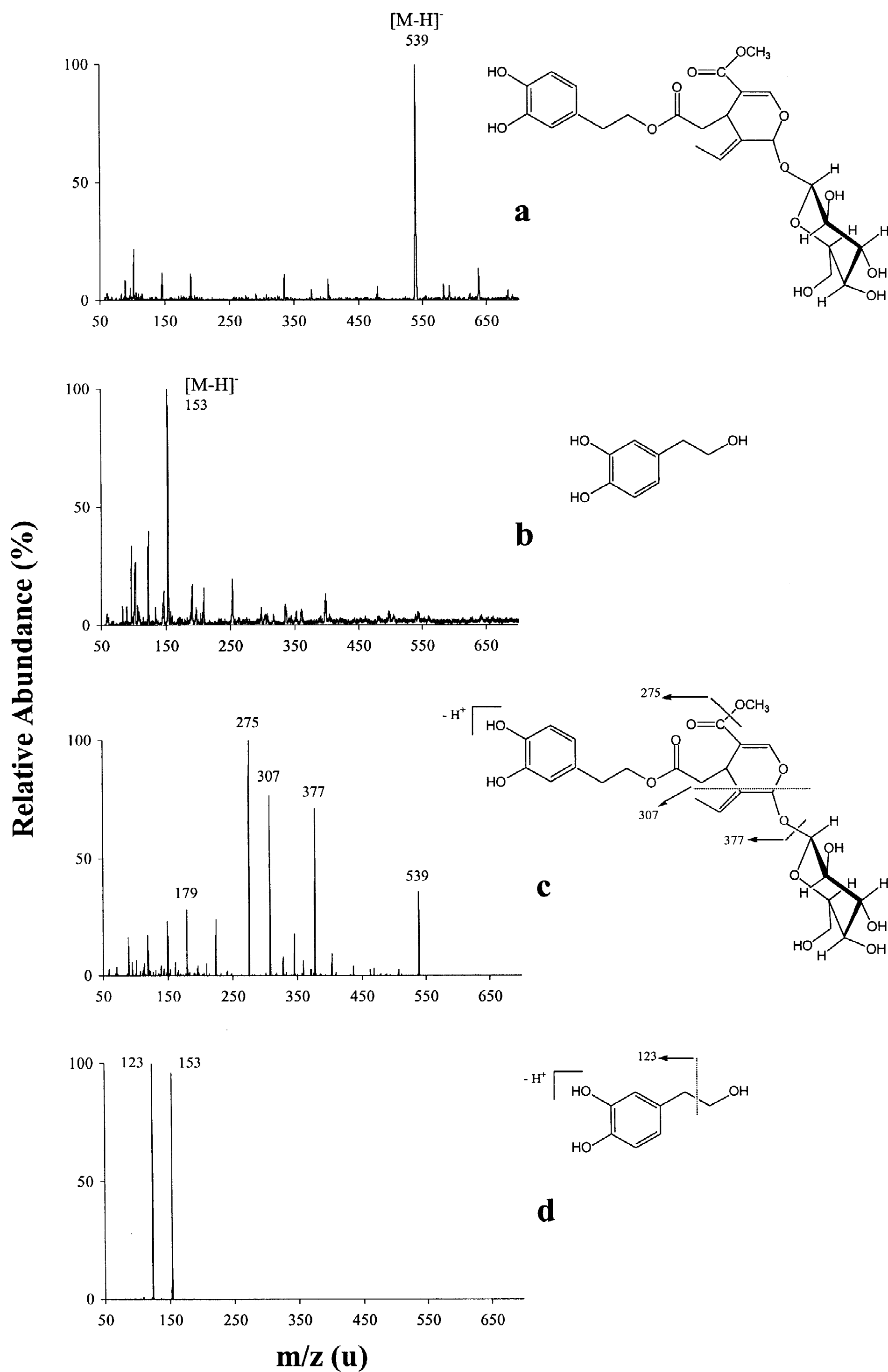

Fig. 1. Mass spectra of oleuropein and hydroxytyrosol obtained by infusion of the standards. Negative ion scan spectra of oleuropein (a) and hydroxytyrosol (b); full scan product ion spectra of oleuropein (c) and hydroxytyrosol (d).

m/z 275 amu for oleuropein (Fig. 1c), the ion at *m/z* 123 amu for hydroxytyrosol (Fig. 1d) and the ion at *m/z* 125 amu for taxifolin used as I.S. Consequently, the transitions *m/z* 539→275, 153→123 and 303→125 amu were chosen for the MRM analytical mode. Finally, LC–MS–MS analysis by the MRM mode combined with liquid chromatography separation was performed with a RP-ODS column using a water–acetonitrile linear gradient. Fig. 2 shows a representative chromatogram of spiked rat plasma and urine, both containing analytes at a concentration of 250 ng/ml. High assay specificity was achieved using liquid chromatographic separation in addition to MRM detection. Control rat plasma and urine did not contain any endogenous compound that could interfere with the assay.

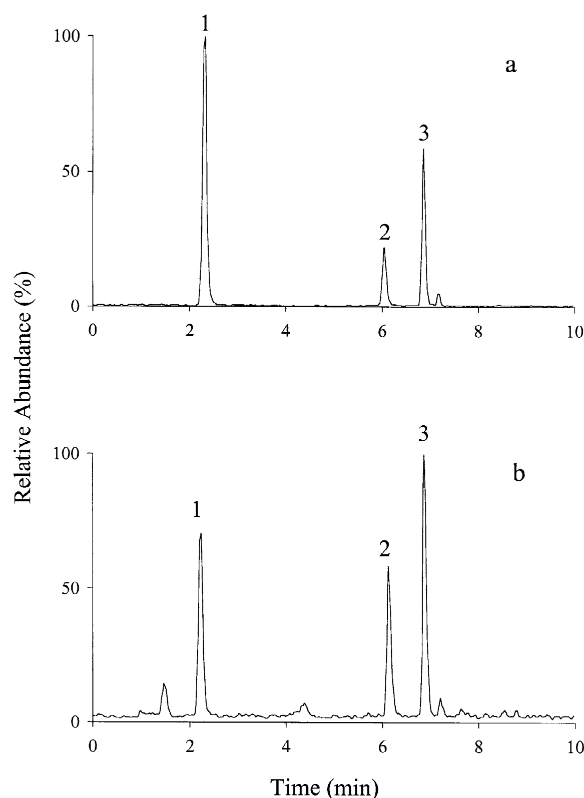

Fig. 2. MRM chromatogram of spiked rat plasma (a) and urine (b). Peaks 1 and 3, oleuropein and hydroxytyrosol, respectively (250 ng/ml); peak 2, taxifolin.

3.2. Validation

3.2.1. Linearity, precision and accuracy

The LC–MS–MS method for the quantitation of oleuropein and hydroxytyrosol in rat plasma and urine extracts was linear over the range 2.5–1000 ng/ml for plasma and 5–1000 ng/ml for urine.

The r^2 values of the calibration curves for oleuropein and hydroxytyrosol in plasma were 0.997 and 0.996, respectively, whereas the r^2 value in urine was 0.993 for both analytes. Precision and accuracy data for spiked rat plasma and urine are reported in Tables 1 and 2, respectively. The precision was ≤7.2% for plasma and ≤12.9% for urine, and the accuracy (excluding the LOQ) was below 7.5 and 14.5% for plasma and urine, respectively.

The limit of detection (LOD) was 1.25 ng/ml for plasma and 2.5 ng/ml for urine for both analytes.

The intra- and inter-batch variabilities are shown in Table 3. For all samples, precision and accuracy were below 15%.

3.2.2. Recovery

The extraction recovery was determined at low (25 ng/ml), mid (250 ng/ml) and high (500 ng/ml) concentrations of oleuropein and hydroxytyrosol in spiked rat plasma and urine. As shown in Table 4, the mean recovery for plasma samples was about 100% for both compounds, whereas the mean recovery for urine samples was about 60% for oleuropein and about 65% for hydroxytyrosol. The mean recovery ($n=3$) of the I.S. was 94% (RSD 2.7%) and 64% (RSD 7.3%) for plasma and urine, respectively.

3.2.3. Stability

The stability of oleuropein and hydroxytyrosol in biological matrices was determined using four replicate samples for each assay. In this study, samples of spiked rat plasma and urine at a concentration of 100 ng/ml were used. Samples were separated into three groups; one group was extracted and analysed immediately, the second group was stored in the dark at RT and 4 °C for 24 h and then analysed, and the third group underwent three freeze–thaw cycles before extraction. As shown in Table 5, oleuropein was stable in urine samples both at 4 °C and after the

Table 1
Precision and accuracy data for oleuropein and hydroxytyrosol after extraction from spiked rat plasma ($n=5$)

Nom. conc. (ng/ml)	Mean calc. conc. (ng/ml)		Precision (RSD %)		Accuracy (%)	
	Oleuropein	Hydroxy-tyrosol	Oleuropein	Hydroxy-tyrosol	Oleuropein	Hydroxy-tyrosol
2.5	2.89	2.98	7.2	3.7	15.6	19.2
12.5	11.92	12.50	4.2	4.3	−4.6	0.0
25	24.84	23.40	4.2	3.1	−0.6	−6.4
50	47.02	46.26	7.1	5.7	−5.9	−7.5
125	127.00	121.80	1.5	4.8	1.6	−2.6
250	234.10	238.10	5.2	3.4	−6.4	−4.7
500	521.50	502.00	6.5	6.5	4.3	0.4
1000	1003.00	1024.00	3.4	4.4	0.3	2.4

Table 2
Precision and accuracy data for oleuropein and hydroxytyrosol after extraction from spiked rat urine ($n=5$)

Nom. conc. (ng/ml)	Mean calc. conc. (ng/ml)		Precision (RSD %)		Accuracy (%)	
	Oleuropein	Hydroxy-tyrosol	Oleuropein	Hydroxy-tyrosol	Oleuropein	Hydroxy-tyrosol
5	5.15	5.85	6.5	11.0	3.0	17.0
10	9.75	11.45	11.4	9.6	−2.5	14.5
25	23.93	23.52	12.9	8.5	−4.3	−5.9
50	46.16	45.34	6.7	10.0	−7.7	−9.3
250	245.00	241.50	4.9	2.4	−2.0	−3.4
500	481.70	494.90	9.6	4.5	−3.7	−1.0
1000	1040.00	1030.00	5.3	9.0	4.0	3.0

Table 3
Intra-batch[a] and inter-batch[b] precision and accuracy for plasma and urine samples

Nom. conc. (ng/ml)	Intra-batch				Inter-batch			
	Precision (RSD %)		Accuracy (%)		Precision (RSD %)		Accuracy (%)	
	Oleuropein	Hydroxy-tyrosol	Oleuropein	Hydroxy-tyrosol	Oleuropein	Hydroxy-tyrosol	Oleuropein	Hydroxy-tyrosol
Plasma								
2.5	9.7	14.1	−11.5	−11.9	10.1	8.8	−10.3	−15.4
125.0	2.2	2.1	8.1	0.9	7.4	5.5	6.5	2.9
500.0	2.2	10.4	−11.4	−14.9	14.2	14.0	−0.8	−3.2
Urine								
25.0	7.2	6.9	−1.9	12.1	14.2	10.9	−5.1	6.7
250.0	8.4	4.1	−0.5	10.0	8.0	10.3	4.0	10.1
500.0	10.4	2.2	0.5	−6.0	13.8	12.6	−7.2	−1.2

[a] Samples ($n=3$) analysed on a single day.
[b] Samples ($n=9$) analysed on three different days.

Table 4
Extraction recovery for plasma and urine

	Low conc., recovery (%)		Mid conc., recovery (%)		High conc., recovery (%)	
	Oleuropein	Hydroxy-tyrosol	Oleuropein	Hydroxy-tyrosol	Oleuropein	Hydroxy-tyrosol
Plasma	92.9±5.3	104.9±5.8	101.0±6.0	106.4±0.8	96.9±2.1	100.0±8.4
Urine	58.5±3.1	78.0±7.3	65.2±12.7	60.5±9.4	60.6±5.3	56.1±13.0

Data reported as mean recovery±RSD % ($n=3$).

freeze–thaw cycles, whereas it underwent degradation at RT. On the other hand, hydroxytyrosol was stable only after the freeze–thaw cycles, whereas loss after degradation of about 50% at 4 °C and about 70% at RT was observed. Furthermore, both compounds were stable in plasma at 4 °C and after the freeze–thaw cycles; a decrease of 54 and 48% in recovery was observed at RT for oleuropein and hydroxytyrosol, respectively.

3.3. Method application

The method was used to analyse plasma and urine of rats treated with a single oral dose of oleuropein (100 mg/kg). Fig. 3a shows the total ion chromatogram (TIC) of taxifolin (peak 1), oleuropein (peak 2) and hydroxytyrosol, obtained from a rat plasma sample 1 h after oral administration. The extracted ion chromatogram of hydroxytyrosol (Fig. 3b) shows that it is not detectable, whereas oleuropein (Fig. 3c) reaches a concentration of 137 ng/ml. A second peak with a longer retention time (Fig. 3c) is also present for the oleuropein standard solution. When the two peaks were collected and reinjected separately, they eluted as single peaks with their own retention times showing the same mass spectrum. These results exclude a configurational isomeric equilibrium and a constitutional form of oleuropein can be hypothesised. Urine samples treated with β-glucuronidase before extraction, as described in Section 2.6, show (Fig. 4) that both oleuropein and hydroxytyrosol were present as glucuronide conjugates at about 91 and 97%, respectively. Enzymatic treatment of plasma samples did not reveal the presence of conjugated derivatives (data not shown). The sulfate conjugates of oleuropein were not investigated, because oleuropein underwent complete

Table 5
Stability of the analytes in biological matrices

	Plasma				Urine			
	Oleuropein		Hydroxytyrosol		Oleuropein		Hydroxytyrosol	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Control	100.0	1.6	100.0	4.9	100.0	10.1	100.0	9.4
After three freeze–thaw cycles	96.3	5.3	105.3	12.7	89.7	3.2	77.9	12.2
After storage for 24 h at 4 °C	83.3	8.6	84.5	12.0	75.3	7.6	54.5	6.2
After storage for 24 h at RT	46.6	10.9	52.2	8.4	35.0	13.2	25.0	16.0

Data reported as mean recovery %±RSD % ($n=4$).

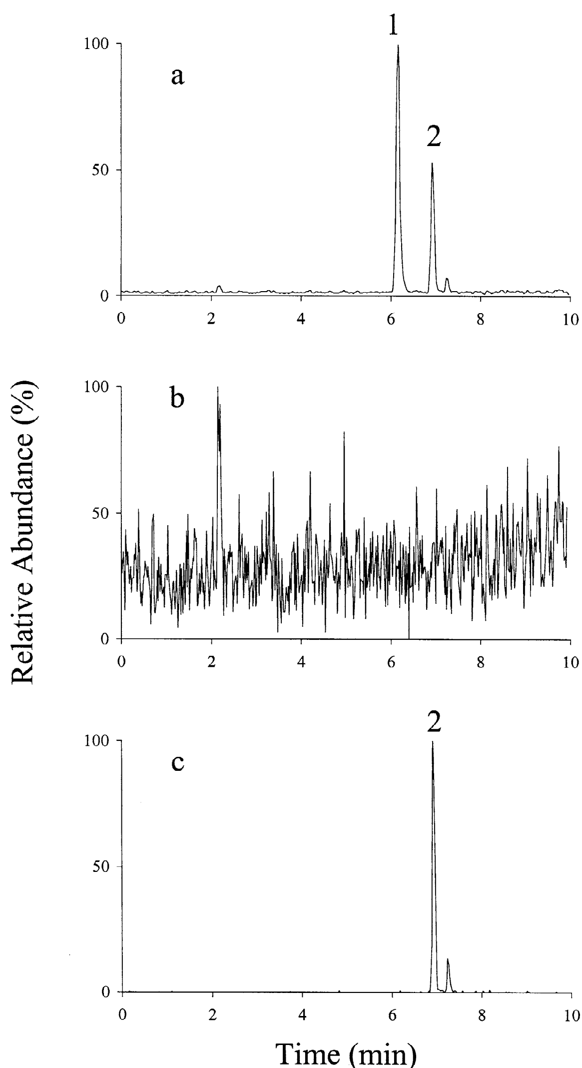

Fig. 3. (a) Total ion chromatogram obtained from rat plasma 1 h after oral administration of oleuropein (100 mg/kg). Peak 1, taxifolin; peak 2, oleuropein. (b, c) Extracted ion chromatograms of hydroxytyrosol (m/z 153→123 amu) and oleuropein (m/z 539→275 amu), respectively.

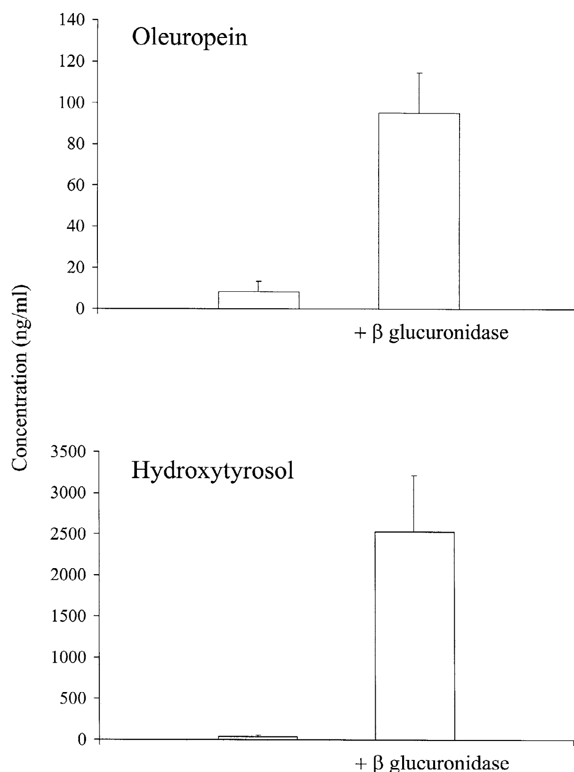

Fig. 4. Oleuropein and hydroxytyrosol concentrations before and after β-glucuronidase hydrolysis in rat urine samples 24 h after administration of oleuropein (100 mg/kg; $n=3$).

degradation when treated with all available commercial deconjugating enzymes.

As shown in Fig. 5, oleuropein could already be observed in plasma 10 min (59 ng/ml) after oral administration and the maximum peak value (t_{max} = 200 ng/ml) was reached at 2 h.

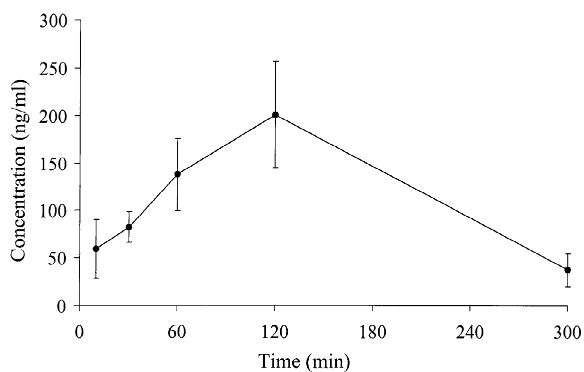

Fig. 5. Rat plasma oleuropein concentrations 10, 30, 60, 120, and 300 min after administration of oleuropein at 100 mg/kg ($n=3$).

4. Conclusions

We have described a new LC–MS–MS method for the simultaneous measurement of oleuropein and hydroxytyrosol in rat plasma and urine by monitoring the ion transitions m/z 539→275 and 153→123 amu. It appears that oleuropein is absorbed after oral administration, since it was found in plasma and in urine, and hydroxytyrosol was its most relevant metabolite. Oleuropein and hydroxytyrosol could be quantified as low as 2.5 and 5 ng/ml in plasma and urine, respectively. Moreover, it appears that oleuropein is absorbed rapidly considering the t_{max} of 2 h. Oleuropein and hydroxytyrosol were recovered in urine mainly as glucuronides, and in very low concentrations as free forms. This is due to the instability of these free forms in urine, as demonstrated. In plasma samples, oleuropein was present only as the glucoside, whereas hydroxytyrosol was found only in traces as the free form. The simultaneous quantification method using LC–MS–MS is specific, sensitive and accurate for the determination of these important dietary components and for investigating their bioavailability and metabolism, in view of their consideration as potential therapeutic agents.

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