Journal of Chromatography B, 905 (2012) 75–84

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Validation of determination of plasma metabolites derived from thyme bioactive compounds by improved liquid chromatography coupled to tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 31 May 2012
Accepted 29 July 2012
Available online 10 August 2012

Keywords:
UPLC–MS/MS
Monoterpenes
Phenolic compounds
Plasma metabolites
Thyme

ABSTRACT

In the present study, a selective and sensitive method, based on microelution solid-phase extraction (μSPE) plate and ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC–MS/MS) was validated and applied to determine the plasma metabolites of the bioactive compounds of thyme. For validation process, standards of the more representative components of the phenolic and monoterpene fractions of thyme were spiked in plasma samples and then the quality parameters of the method were studied. Extraction recoveries (%R) of the studied compounds were higher than 75%, and the matrix effect (%ME) was lower than 18%. The LODs ranged from 1 to 65 μg/L, except for the thymol sulfate metabolite, which was 240 μg/L. This method was then applied for the analysis of rat plasma obtained at different times, from 0 to 6 h, after an acute intake of thyme extract (5 g/kg body weight). Different thyme metabolites were identified and were mainly derived from rosmarinic acid (coumaric acid sulfate, caffeic acid sulfate, ferulic acid sulfate, hydroxyphenylpropionic acid sulfate, dihydroxyphenylpropionic acid sulfate and hydroxybenzoic acid) and thymol (thymol sulfate and thymol glucuronide). The most abundant thyme metabolites generated were hydroxyphenylpropionic acid sulfate and thymol sulfate, their respective concentrations in plasma being 446 and 8464 μM 1 h after the intake of the thyme extract.

1. Introduction

The interest in, and research on, the bioavailability and metabolism of bioactive compounds in leafy spices is well justified. *Lamiaceae* herbs are rich in various phenolic compounds and characterized mainly by the occurrence of phenolic acids, flavonoids and phenolic volatile oils [1]. The contribution of several culinary and medicinal herbs (oregano, sage, thyme, and peppermint) to the total intake of dietary antioxidants has been assessed, and these foods were reported to be a significant source of dietary antioxidants, even superior to many other food groups [2].

Thyme comes originally from the regions around the Mediterranean and is used as cough medicine. It has also been commonly used as a culinary herb for adding flavor. The extensive phytochemical research into thyme has revealed the presence of several classes of bioactive compounds, such as phenolic acids, flavonoids, terpenoids and essential oils [3,4]. Among these compounds, rosmarinic acid (RA) is one of the most important anti-oxidative polyphenols in thyme and is also widely found in *Lamiaceae* herbs [5]. In addition to its potent antioxidant activity [6], many beneficial properties, including anti-inflammation [7], anti-mutagenicity [8] and prevention of Alzheimer's disease [9], have been attributed to rosmarinic acid. There are also large amounts of monoterpenes in thyme and their pharmacological properties have recently been reviewed, they are especially considered promising agents for the prevention or treatment of cardiovascular diseases [10].

Although thyme extracts (TE) and essential thyme oils have been shown to possess various pharmacodynamic activities [11–15], there have been no *in vivo* studies of the oral administration of thyme extract with all its bioactive compounds to clarify its bioavailability and metabolism.

Until now, the only *in vivo* studies reported in the literature that analyzed thyme derivates in plasma samples were based on the oral administration of rosmarinic acid [16–18] or thymol [19]. The analytical separation techniques used were high-performance liquid-chromatography (HPLC) coupled to DAD [18], mass spectrometry (MS) [17] and tandem MS (MS/MS) [16]; and gas chromatography (GC) coupled to a flame ion detector (FID) [19]. The plasma samples were centrifuged [16–18] for the sample pretreatment technique when HPLC was used. Headspace-solid-phase microextraction (SPME) was the chosen method [19] when GC was

Abbreviations: TE, thyme extract.

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http://dx.doi.org/10.1016/j.jchromb.2012.07.040

used. Only the methodology reported by Kohlert et al. [19], based on off-line headspace – SPME-GC-FID, was validated for the analysis of thymol, after its sulfatase and β-glucuronidase enzymatic pretreatment, and its quantification limit (LOQ) was 7.1 μg/L in plasma samples.

In order to improve and enhance the reported methodologies for determining thyme metabolites in plasma samples, this paper presents and validates a rapid, selective and sensitive method to identify and quantify the metabolites generated after an acute intake of thyme extract. The analytical separation technique used is ultra-performance liquid chromatography (UPLC) coupled to tandem MS, and microelution solid-phase extraction (μSPE) plates as the pretreatment technique. The method is validated in terms of linearity, calibration curves, precision, accuracy, recoveries and sensitivity with the most representative analytes from thyme. To observe and understand the future potential benefits of the minor components from thyme, taking their short life in plasma into account, the study was carried out during the postprandial state, which allowed their detection and quantification. This fact may be very useful in future repeated low-dose experiments, facilitating an understanding of long-term interventional studies. To our knowledge, for the first time, a liquid chromatographic (LC) method is validated for the analysis of thyme metabolites, and also for the first time, a thyme extract (TE) is administrated to determine the plasma metabolites generated.

2. Experimental

2.1. Chemicals and reagents

Catechol as internal standard (I.S.), *p*-hydroxybenzoic acid, thymol, hydroxyphenylacetic acid, dihydroxyphenylacetic acid, hydroxyphenylpropionic acid, caffeic acid, dihydroxyphenylpropionic acid, ferulic acid, naringenin, luteolin, eriodictyol, quercetin, rosmarinic acid were purchased from Extrasynthase (Genay, France). *p*-coumaric was purchased from Fluka Co. (Buchs, Switzerland). Acetonitrile (HPLC grade), methanol (HLPC grade), ethanol and acetic acid were all provided by Scharlau Chemie (Barcelona, Spain). The water was of Milli-Q quality (Millipore Corp, Bedford, MA).

2.2. Isolation of thymol sulfate as a standard

Thymol sulfate was isolated by using semi-preparative HPLC (Waters, Milford, MA) in the reversed phase from the μSPE eluted solution of rat plasma after the ingestion of the thyme extract (TE). The HPLC system includes a Waters 1525EF binary HPLC pump, a Waters Flexinject, a Waters 2487 λ absorbance detector (280 nm) and a Waters Fraction Collector II. The HPLC system was operated using Brezze software. 50 μL of the μSPE eluted solution from the rat plasma was injected manually into the injector module (1 mL sample loop). The method used was the same as reported in our previous study [20] except for the column. In this case, an XBridge BEH C18 (2.5 μm, 4.6 mm × 100 mm) analytical column, also from Waters, was used. Prior to chromatographic isolation, the thymol sulfate was identified by MS/MS and its retention time was determined in order to collect the specific fraction. The fraction corresponding to thymol sulfate was collected manually by observing the detector output on the recorder according to its retention time. The organic solvent of the mobile phase was then removed by rotary evaporation (Buchi, Labortechnick AG, Switzerland) under a partial vacuum at 25 °C. Finally, the aqueous extract was placed in a chromatographic vial, which had previously been weighed, and freeze-dried in a Lyobeta 15 lyophilizer (ImaTelstar, Spain). After freeze-drying, the vial was weighed and the difference in the weight was attributed to the isolated thymol sulfate. The thymol sulfate was stored in an N_2 atmosphere at −40 °C until its use as a standard for quantifying thymol metabolites in rat plasma. Fig. 1 shows the precursor ion and the product ions of thymol sulfate obtained in the daughter scan mode and by applying different collision energies, from 5 to 40 eV, after its isolation from plasma samples.

In order to study the quality parameters of this method, the isolated thymol sulfate was dissolved with methanol and spiked in a rat plasma sample obtained under fasting conditions (control plasma).

Table 1
Phenolic and monoterpene composition of the thyme extract (TE) administered to the rats.

Compound	μmol in 1.5 g of TE
Phenolic acids[a]	
p-Hydroxybenzoic acid	4.27
Vainillin	0.59
p-coumaric acid	0.61
Vanillic acid	2.69
Caffeic acid	5.35
Homovanillic acid	4.09
Chlorogenic acid	2.09
Protocatechuic acid	2.75
Rosmarinic acid	236.80
Flavonoids[a]	
Apigenin	1.72
Luteolin	7.60
Luteolin-7-O-glucoside	21.84
Naringenin	10.51
Eriodictyol	11.08
Dihidroxykaempferol	0.36
Quercetin-glucoside	4.54
Eriodictyol-rutinoside	2.96
Thymusin	23.84
Xanthomicrol	17.60
7-Methylsudachitin	13.23
Monoterpernes[b]	
Thymol	44.32
Carvacrol	16.59
α-terpineol	8.42
Borneol	5.58

[a] Compounds analyzed by HPLC–MS/MS.
[b] Compounds analyzed by GC–MS and FID.

2.3. Thyme extract (TE)

TE was prepared from dried thyme (*Thymus Zyguis*) with ethanol:water by accelerated solvent extraction (ASE) following the methodology optimized by Suárez et al. [21]. The bioactive compounds of the TE were analyzed according to the method in Rubió et al. by HPLC coupled to tandem MS [22]. Table 1 shows the results of the bioactive compound contents of TE and these are expressed as μmol in 1.5 g of extract, corresponding to the ingested dose.

Additionally, TE was subjected to gas chromatography (GC) analyses to identify and quantify the monoterpenes. The extract was analyzed using an Agilent 6890N GC interfaced to a 5973N mass spectrometry (MS) selective detector for identification and to a flame ionization detector (FID) for the quantification analysis. The column used was a ZB-50 (50% phenyl and 50% methylpolysiloxane), 30 m × 250 μm internal diameter (ID) and 0.25 μm phase thickness (Pnenomenex, Micron Analítica S.A., Spain). The carrier gas was helium at a constant flow rate of 1 mL/min. One microliter of the extract was injected using splitless mode. The oven temperature was programmed at 100 °C and ramped at 15 °C/min up to a maximum of 280 °C for 15 min. The GC–MS transfer line was held at 280 °C, and the quadrupole analyzer and ion source heaters were maintained at 150 and 230 °C, respectively. MS data were collected in the full-scan mode, with a scan range from 35 to 500 amu and

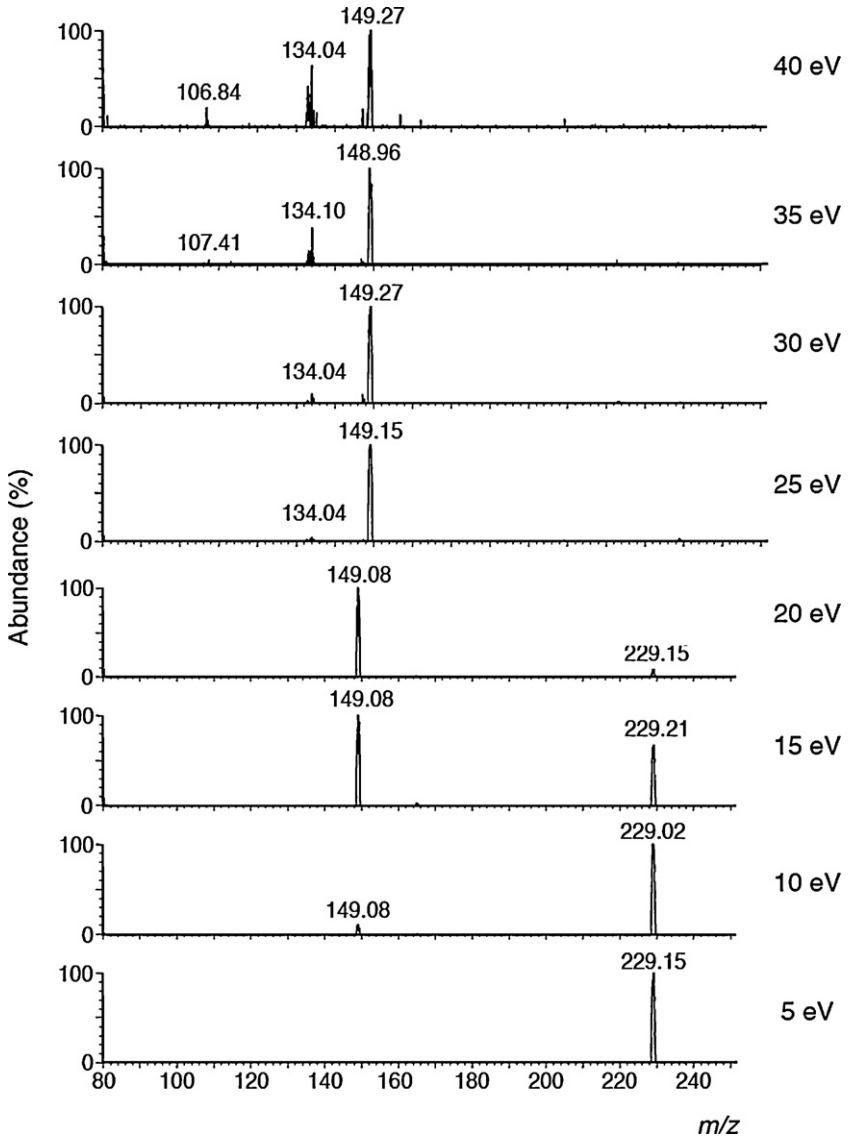

Fig. 1. Precursor ion and product ions of thymol sulfate obtained by daughter scan mode by applying different collision energy, from 5 to 40 eV, and 40 V as the cone voltage. Thymol sulfate was isolated by semi-preparative HPLC from rat plasma sample after TE ingesta. See Section 2.2 for the experimental conditions.

at a rate of 3.5 scans/s. The monoterpene thymol was identified by comparing its mass spectrum with the commercial standard, and this spectrum was obtained under the same analytical conditions. Carvacrol, borneol and α-terpineol were identified by comparing the mass spectra obtained with those found in the Nist/Wiley mass spectra database libraries [23]. Quantification of the monoterpenes was performed by GC-FID, using the same analytical chromatographic parameters. The injector and detector temperatures were 250 and 300 °C, respectively. The calibration curve was prepared by using the external standard of thymol standard. Table 1 also shows the results of the monoterpene contents of TE and these are expressed as μmol in 1.5 g of extract, corresponding to the ingested dose.

2.4. Treatment of rats and plasma collection

Three-month-old male Wistar rats were obtained from Charles River Laboratories (Barcelona, Spain). The rats were housed in cages on a 12 h light–12 h dark schedule at controlled temperature (22 °C). They were subjected to a standard diet of a commercial chow, PanLab A04 (Panlab, Barcelona, Spain), and water *ab libitum*. The animals were then kept in fasting conditions for between 16 and 17 h with only access to tap water. Subsequently, 1.5 g of TE dispersed in 1.5 mL of water was administered to 12 rats. The rats were anesthetized with isoflurane (IsoFlo, VeterinariaEsteve, Bologna, Italy) and killed by exsanguinations 1 h ($n = 3$), 2 h ($n = 3$), 4 h ($n = 3$) and 6 h ($n = 3$) after the ingestion of the TE. Additionally, a control group of rats ($n = 10$) was maintained under fasting conditions without extract ingestion and then similarly euthanized. The study was approved by The Animal Ethics Committee of the University of Lleida (CEEA 04-01/11, 26th January 2011). All experiments with rats were performed in compliance with the relevant laws and University of Lleida guidelines. Blood samples were collected by intracardiac exsanguination. The plasma samples were obtained by centrifuging (2000 × *g*, 30 min at 4 °C) and stored immediately with liquid nitrogen and then at −80 °C until the chromatographic analysis of phenolic and monoterpene metabolites.

2.5. Treatment of plasma samples and UPLC-ESI-MS/MS analysis of thyme metabolites

In order to clean-up the biological matrix and preconcentrate the phenolic and monoterpene compounds and their metabolites, the plasma samples were pretreated by microelution solid-phase

Table 2
Optimized SRM conditions for the analyses of the studied compounds and their plasma metabolites by UPLC–MS/MS.

Compound	SRM 1 (quantification)	Cone voltage (V)	Collision energy (eV)	SRM 2 (confirmation)	Cone voltage (V)	Collision energy (eV)
Standards						
Catechol	108.9 > 90.9	40	15	–	–	–
p-Hydroxybenzoic acid	137 > 93	30	15	–	–	–
Thymol	149 > 134	40	15	–	–	–
Hydroxyphenylacetic acid	151 > 107	20	10	–	–	–
p-Coumaric acid	163 > 119	35	10	163 > 117	35	25
Dihydroxyphenylacetic acid	167 > 123	20	10	167 > 95	20	15
Hydroxyphenylpropionic acid	165 > 121	20	10	165 > 149	20	15
Caffeic acid	179 > 135	35	15	179 > 117	35	20
Dihydroxyphenylpropionic acid	181 > 137	20	10	181 > 93	20	15
Ferulic acid	193 > 134	30	15	193 > 178	30	10
Thymol sulfate[a]	229 > 149	40	20	229 > 134	40	30
Naringenin	271 > 151	40	15	271 > 119	40	20
Luteolin	285 > 133	55	25	285 > 151	55	25
Eriodictyol	287 > 151	40	15	287 > 179	40	25
Quercetin	301 > 151	40	15	301 > 179	40	15
Rosmarinic acid	359 > 161	40	20	359 > 161	40	25
Metabolites						
Coumaric acid sulfate	243 > 163	35	15	163 > 119	35	20
Hydroxyphenylpropionic acid sulfate	245 > 165	35	15	165 > 121	35	20
Cafeic acid sulfate	259 > 179	35	15	179 > 135	35	20
Dihydroxyphenylpropionic acid sulfate	261 > 181	40	15	181 > 137	40	20
Ferulic acid sulfate	273 > 193	35	15	193 > 134	35	20
Thymol glucuronide	325 > 149	20	25	325 > 134	20	30
Luteolin sulfate	365 > 285	35	15	285 > 133	35	20
Luteolin glucuronide	461 > 285	40	15	285 > 133	40	20

[a] Standard isolated by semi-preparative HPLC from rat plasma samples, after ingestion of TE.

extraction (μSPE) plates following the methodology described by Suárez et al. [20]. Briefly the cartridges were firstly conditioned sequentially by using 250 μL of methanol and acidified Milli-Q water at pH 2. Then, 350 μL of plasma mixed with 300 μL of phosphoric acid 4% and 50 μL catechol (IS) at 10 mg/L were loaded onto the plate. After that, the clean-up of the plates was sequentially done with 100 μL Milli-Q water and 100 μL methanol 5% to eliminate any interference that the sample might contain. Finally the elution of the retained phenolic compounds was done with 100 μL of methanol. 2.5 μL of the eluted solution from the μSPE was injected directly into the Acquity UPLC–MS/MS system (Waters, Milford, MA, USA). The UPLC system was equipped with a Waters binary pump system (Milford, MA, USA) using an AcQuity UPLC™ BEH C_{18} column (1.7 μm, 100 mm × 2.1 mm i.d.). The flow rate was 0.4 mL/min using acetic acid 0.2% as solvent A and acetonitrile as solvent B. The elution started at 5% of eluent B for 5 min, then was linearly increased 40% of eluent B in 20 min, further increased to 100% of eluent B in 0.1 min and kept isocratic for 1.9 min. Table 2 shows the quantification and confirmation transitions, and the cone voltage and collision energy for the commercial standards of the compounds studied and the thymol sulfate metabolite, which was isolated from the plasma samples as explained above in Section 2.2.

In order to identify and quantify the bioactive compounds and their metabolites, different MS analyses were performed. These were based on MS (full-scan mode) and MS/MS (based on neutral loss scan and product ion scan). These techniques are excellent tools for verifying structural information about the compounds when standards are not available [24]. Firstly, analyses were carried out in

Table 3
Retention time (RT), extraction recovery (%R), linearity, calibration curves, reproducibility (%RSD), accuracy (%), LODs and LOQs for the determination of the phenolic compounds and the isolated thymol sulfate spiked in plasma samples by UPLC–MS/MS.

Compound	RT (min)	%R	Linearity (μM)	Calibration curve	RSD% (n = 3), inter-day			Accuracy (%) (n = 3) (6 μM)	LOD (μM)	LOQ (μM)
					12 μM	3 μM	0.3 μM			
Dihydroxyphenylacetic	3.00	77	0.30–11.90	$y = 2.1242x - 0.0219$	3.8	5.5	0.1	100	0.09	0.30
p-Hydroxibenzoic acid	3.62	75	0.43–14.50	$y = 39.806x + 1.0159$	6.5	6.5	2.9	103	0.13	0.43
Hydroxyphenylacetic	5.11	79	0.16–13.15	$y = 3.7609x + 0.5972$	0.9	3.0	3.3	103	0.05	0.16
Dihydroxyphenylpropionic	5.29	81	0.50–10.99	$y = 8.7028x + 0.0061$	2.6	1.7	3.2	95	0.14	0.50
Caffeic acid	5.73	80	0.5–11.11	$y = 12.118x + 0.1791$	3.0	1.6	2.5	105	0.36	0.50
Hydroxyphenylpropionic	7.58	85	0.70–12.05	$y = 1.6782x + 0.0298$	1.5	2.8	10.5	101	0.23	0.70
p-Coumaric acid	7.84	90	0.12–12.19	$y = 13.342x + 0.1651$	2.1	3.7	9.8	97	0.03	0.12
Ferulic acid	9.22	92	0.08–10.31	$y = 10.192x - 0.0462$	6.3	3.9	10.3	95	0.02	0.08
Rosmarinic acid	12.77	75	0.008–5.55	$y = 8.0821x + 1.246$	7.9[a]	0.3[b]	2.5[c]	95[d]	0.003	0.008
Quercetin	15.16	77	0.03–6.62	$y = 0.6258x + 0.038$	6.5[a]	0.3[b]	5.5[c]	105[d]	0.009	0.03
Eriodictyol	15.19	98	0.02–6.94	$y = 17.982x + 0.2327$	4.8[a]	5.4[b]	10.2[c]	103[d]	0.007	0.02
Luteolin	15.64	75	0.03–6.99	$y = 8.6544x + 0.0533$	9.9[a]	10.4[b]	0.1[c]	99[d]	0.007	0.03
Thymol sulfate	15.96	75	1.00–8.70	$y = 3.1164x - 2.9851$	5.3[a]	4.9[b]	6.7[c]	101[d]	0.30	1.00
Naringenin	17.65	98	0.02–7.35	$y = 50.64x + 0.9056$	3.7[a]	6.1[b]	9.5[c]	104[d]	0.004	0.02

[a] 6 μM.
[b] 1.5 μM.
[c] 0.15 μM.
[d] 3 μM.

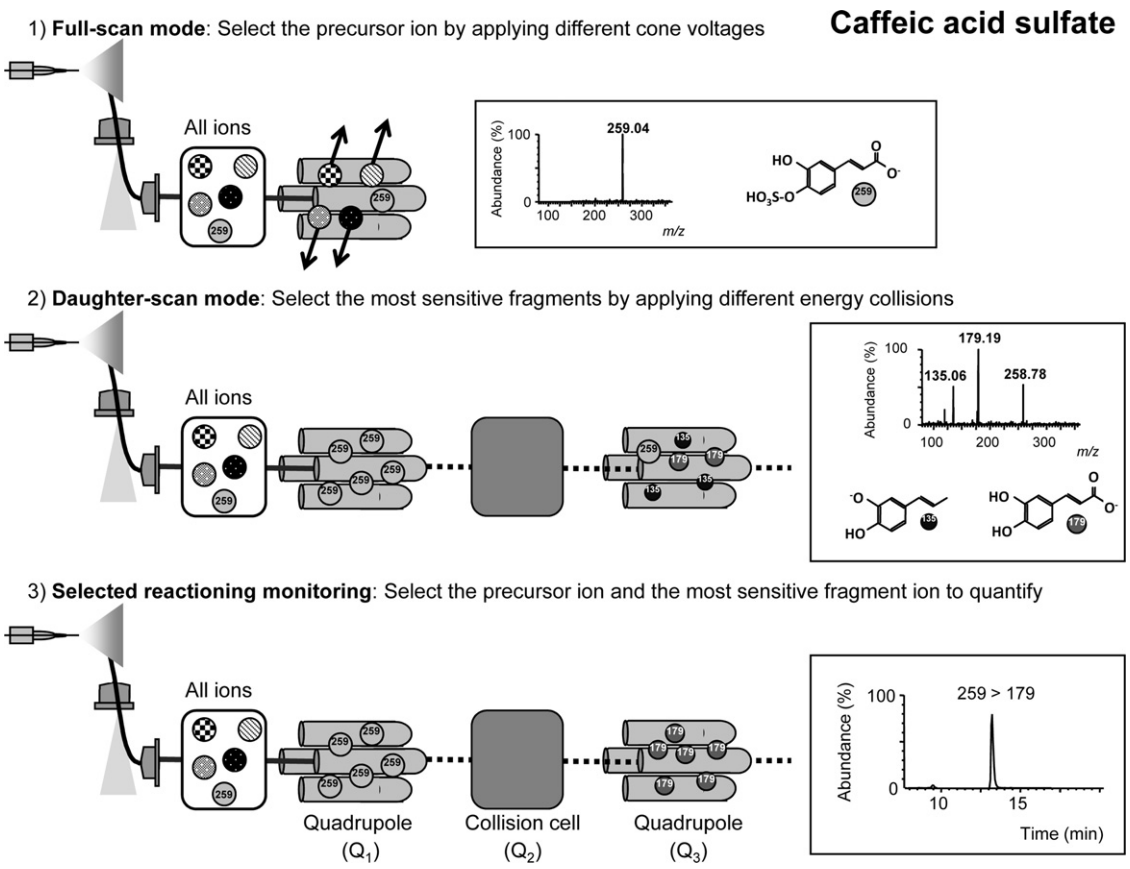

Fig. 2. MS analyses performed to identify and quantify the plasma metabolites, using caffeic acid sulfate as example.

the full-scan mode (from 80 to 800 *m*/*z*) by applying different cone voltages, from 20 to 60 V. When low cone voltages were applied, the MS spectrum gave information about the precursor ion or the $[M-H]^-$. In contrast, when high cone voltages were applied, specific fragment ions were generated and the MS spectrum gave information about their chemical structure. This structural information was also verified in the MS/MS mode by using product ion scan and neutral loss scan. In the product ion scan experiments, the product ions are produced by collision-activated dissociation of the selected precursor ion in the collision cell. Additionally, neutral loss scans of 80 and 176 units were used to characterize the sulfate and glucuronide metabolites, respectively. Then, the detection and quantification of the phenolic and monoterpene compounds and their metabolites were performed based on their ion fragmentation in the MS/MS mode using SRM as the most sensitive. Table 2 also shows the SRM transitions, as well as the cone voltage and collision energy, used to quantify and identify the metabolites generated.

Due to the lack of standards for the great majority of these metabolites, they were tentatively quantified by using the calibration curves corresponding to their phenolic precursors. This way, coumaric acid sulfate, ferulic acid sulfate, and caffeic acid sulfate were quantified using the calibration curves of *p*-coumaric, ferulic and caffeic acids, respectively. Dihydroxyphenylpropionic acid sulfate and hydroxyphenylpropionic acid sulfate were quantified using the calibration curves of dihydroxyphenylpropionic and hydroxyphenylpropionic acids, respectively. Luteolin metabolites were quantified with the luteolin calibration curve. Finally, thymol metabolites were quantified with the calibration curve of thymol sulfate.

2.6. Validation procedure

The instrumental quality parameters of linearity, extraction recovery, calibration curves, precision, accuracy, detection limit (LOD), quantification limit (LOQ), and the study of the matrix effect were evaluated. These parameters were determined by spiking pool basal plasma (obtained in fasting conditions) with the standard phenolic and monoterpene compounds studied at known

Table 4
Metabolites from thyme identified in rat plasma by off-line μSPE and UPLC–MS/MS after ingesta of TE.

Metabolite	RT (min)	$[M-H]^-$ (*m*/*z*)	MS^2 ions (*m*/*z*)	Neutral loss scan
Coumaric acid sulfate	5.45	243	163, 119	80
Ferulic acid sulfate	5.85	273	193, 134	80
Hydroxybenzoic acid	9.12	137	93	–
Luteolin glucuronide	10.41	461	285, 133	176
Dihydroxyphenylpropionic acid sulfate	10.63	261	181, 137	80
Hydroxylphenylpropionic acid sulfate	11.93	245	165, 121	80
Caffeic acid sulfate	13.96	259	179, 135	80
Luteolin sulfate	14.97	365	285, 133	80
Thymol glucuronide	16.94	325	149, 134	176
Thymol sulfate	17.25	229	149, 134	80

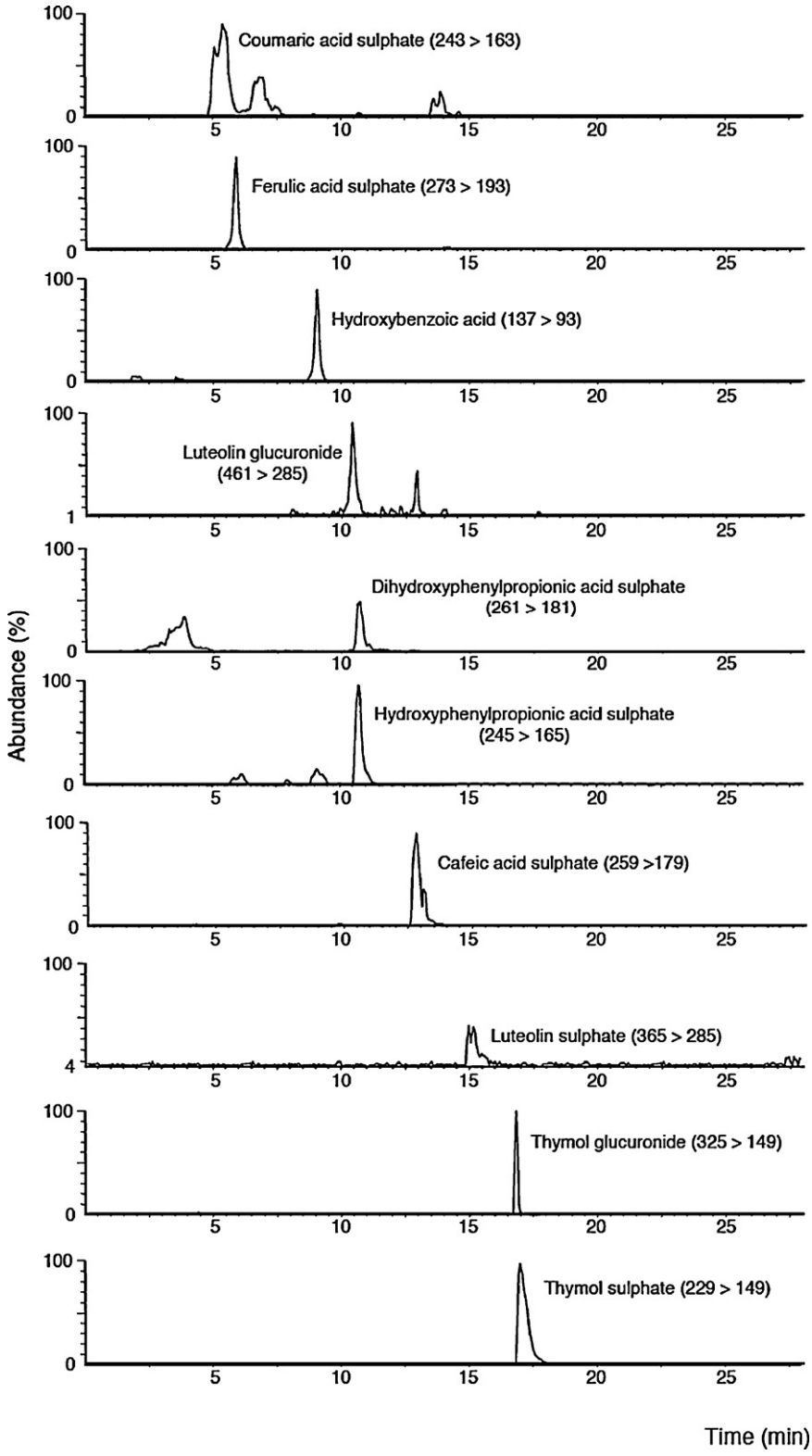

Fig. 3. Extracted ion chromatograms of the 10 generated metabolites detected in rat plasma after the ingestion of TE.

concentrations. This solution was then analyzed by off-line μSPE-UPLC–MS/MS. The linearity of the method was evaluated using these basal plasma samples spiked with the standards (phenols and monoterpenes) at different concentration levels. Calibration curves (based on peak area abundance) were plotted using $y = a + bx$, where y is the analyte/IS peak area abundance ratio and x is the analyte/IS concentration ratio. The concentrations of the metabolites were calculated by interpolating the analyte peak abundance onto their calibration curves. These curves were obtained by analyzing five points at different concentration levels and each standard solution was injected three times.

The precision of the method (inter-day and intra-day precisions) were determined as the relative standard deviation (% RSD) of the concentration ($n = 3$). The accuracy ($n = 3$) was calculated as the ratio

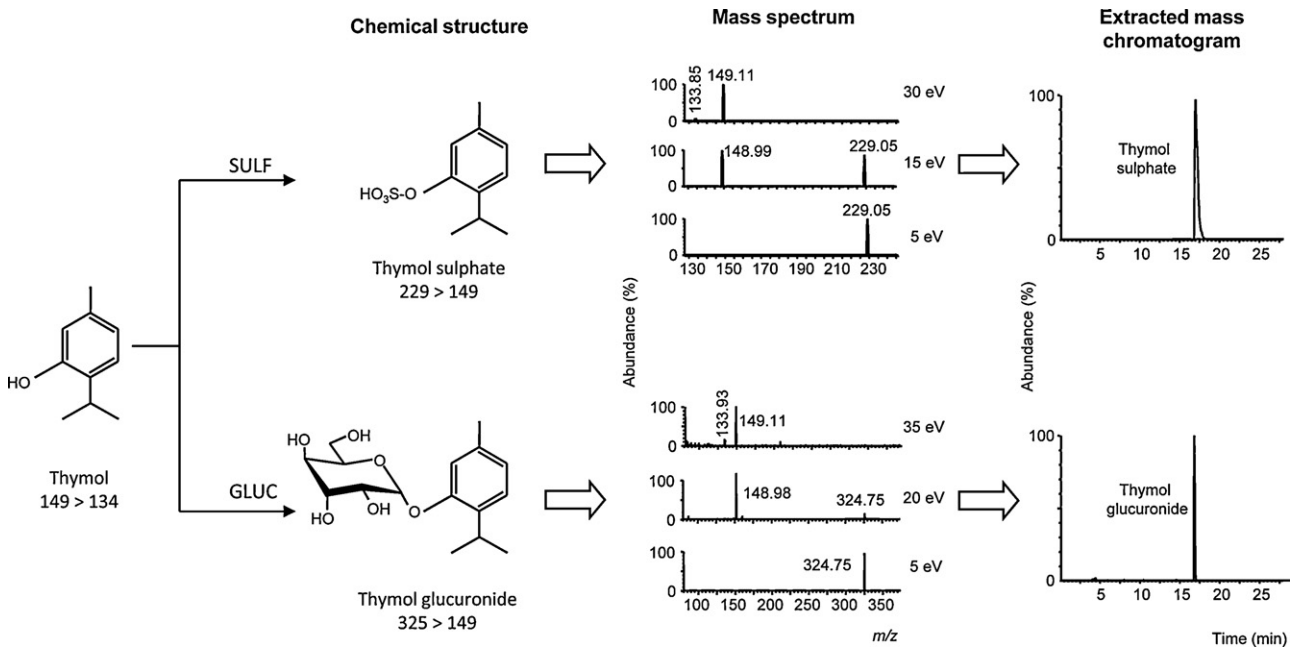

Fig. 4. Chemical structure, mass spectrum (obtained in daughter scan mode) and extracted ion chromatograms of the thymol metabolites, thymol glucuronide and thymol sulfate.

between the mean measured concentration and the nominal concentration multiplied by 100. The extraction recoveries (%R) of the sample pre-treatment method were calculated by comparing the responses of the analytes spiked in the plasma matrices before and after extraction. The matrix effect (%ME) was evaluated by comparing the peak abundances of the analytes spiked in the plasma matrices after the sample pretreatment, with the peak abundances of the analytes spiked in the elution solvent (methanol). The LODs and LOQs were calculated using the signal-to-noise criterion of 3 and 10, respectively.

3. Results and discussion

3.1. Quality parameters

The standards of the phenolic and monoterpene compounds studied were spiked in control plasma samples at known concentrations and analyzed by off-line μSPE-UPLC–MS/MS. Then, the quality parameters of the method, such as linearity, calibration curves, precision, accuracy, LOD, LOQ, extraction recovery and the study of the matrix, were evaluated. These results are shown in Table 3. The functions of the calibration curves were linear, with mean correlation coefficients >0.99. The inter-day and intra-day precisions of the method, expressed as the relative standard deviation (%RSD), were studied at three concentration levels, 0.3, 3 and 12 μM. These were lower than 10.5% and 12%, respectively, for all the compounds analyzed and the accuracy values (intra-day and inter-day) ranged from 95 to 105%. The precision and accuracy results appear to indicate that the methodology for extracting the compounds from the studied plasma matrix is highly reproducible and reliable. The extraction recoveries (%R) of the studied compounds were good, and these were above 75%. The matrix effect (%ME), which is due to the coeluting matrix components that compete for ionization, is observed by a decrease or increase of the analyte signal depending on whether the analyse is prepared in a sample matrix (plasma in our study) or in an organic solvent (methanol) [25]. In our study, the matrix effect (%ME) in absolute values was lower than 18% (values not shown).

Table 5
Concentration of thyme metabolites quantified in rat plasma at different times after ingesta of TE. Phenolic metabolites were quantified in reference to the respective unconjugated form.

	Concentration (μM)				
	Control	1 h	2 h	4 h	6 h
Phenolic metabolites					
Cafeic acid sulfate	n.d.	9.27 ± 2.58	3.32 ± 0.51	6.37 ± 1.53	10.85 ± 2.27
Hydroxybenzoic acid	n.d.	1.16 ± 0.41	1.95 ± 0.46	3.91 ± 0.95	4.11 ± 0.61
Coumaric acid sulfate	n.d.	0.75 ± 0.34	0.30 ± 0.08	0.76 ± 0.26	2.58 ± 0.51
Ferulic acid sulfate	n.d.	4.42 ± 0.95	3.55 ± 0.64	3.67 ± 0.99	3.81 ± 0.66
Ferulic acid glucuronide	n.d.	0.14 ± 0.01	0.13 ± 0.02	0.14 ± 0.02	0.14 ± 0.03
Hydroxyphenilpropionic acid sulfate	n.d.	446.4 ± 109.6	159.3 ± 22.8	172.3 ± 11.3	369.3 ± 91.3
Dihydroxyphenilpropionic acid sulfate	n.d.	24.81 ± 4.13	20.08 ± 2.89	13.51 ± 0.58	20.90 ± 3.44
Luteolin sulfate	n.d.	0.03 ± 0.01	0.05 ± 0.02	0.05 ± 0.05	0.24 ± 0.09
Luteolin glucuronide	n.d.	1.76 ± 0.52	2.16 ± 0.45	2.47 ± 0.86	4.02 ± 1.24
Monoterpene metabolites					
Thymol sulfate	n.d.	8463.9 ± 827.9	3551.5 ± 116.4	5328.3 ± 239.5	6577.1 ± 932.5
Thymol glucuronide	n.d.	2.43 ± 0.23	LOD – LOQ	30.67 ± 4.71	56.92 ± 4.38

n.d.: not detected.
LOD – LOQ: Its concentration is between its LOD value and its LOQ value LOD–LOQ.

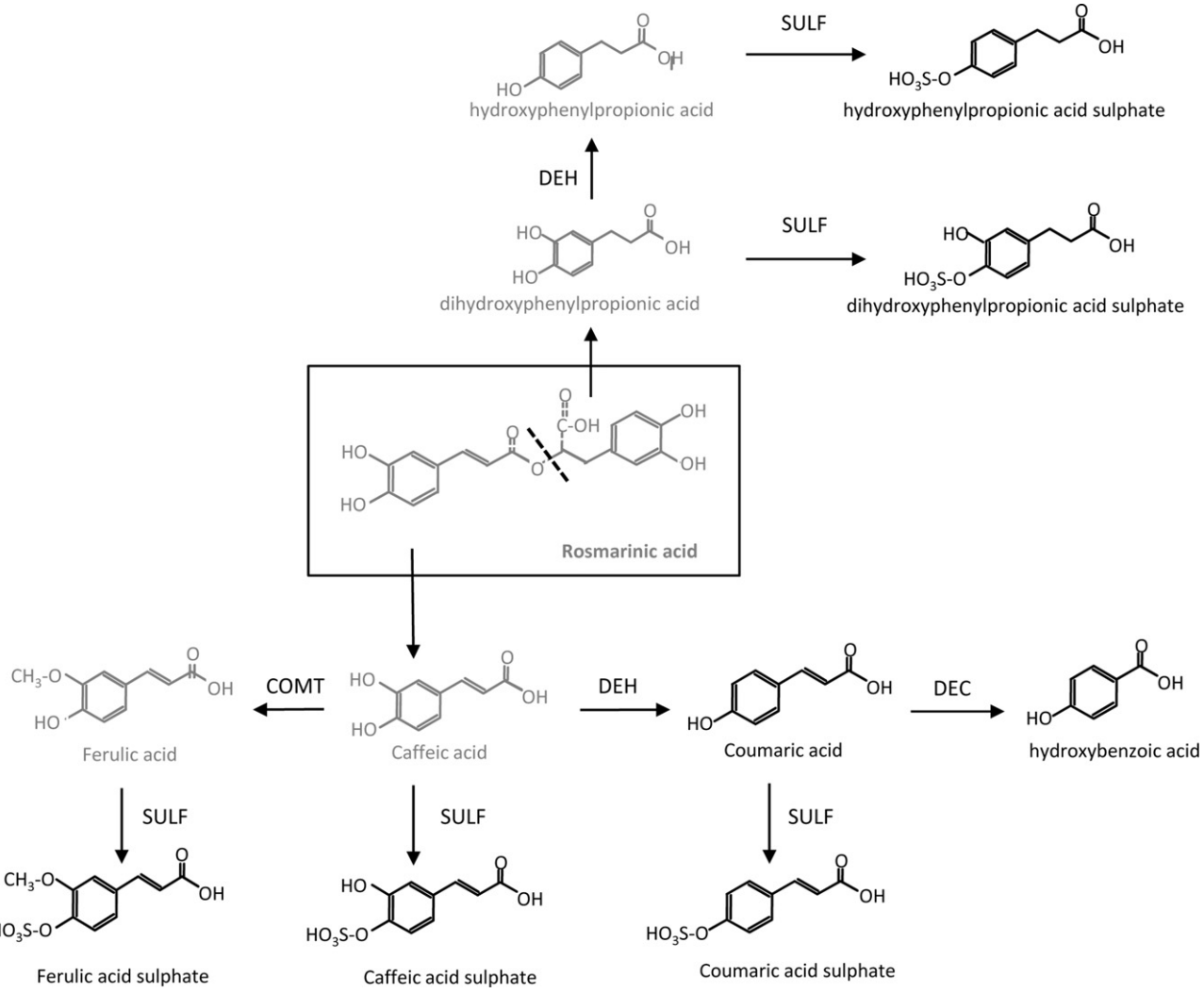

Fig. 5. Proposed metabolic pathway of rosmarinic acid. DEC, decarboxylation; SULF, sulfate conjugation; GLUC, glucuronide conjugation; DEH, *p*-dehydroxylation; COMT, methylation.

The LODs ranged from 0.003 to 0.36 μM. LOQ values ranged from 0.008 to 1.17 μM. These LOD and LOQ values were lower than those reported in the literature when phenolic compounds from olive oil and procyanidins and anthocyanins were studied in spiked plasma [20,26].

3.2. Identification of the plasma metabolites from the thyme extract

The validated method was applied to determine the bioactive compounds from thyme and their metabolites, in plasma samples, after an acute intake of TE. Fig. 2 shows the different MS analyses performed to identify and quantify the plasma metabolites, using caffeic acid sulfate as example. Ten metabolites were generated and these are listed in Table 4. These compounds were not detected in the control plasma samples, showing that all metabolites identified in the plasma samples obtained after the ingesta of TE came from thyme. The native forms of the phenols and monoterpenes present in the TE (Table 1) were not detected in the plasma, except for hydroxybenzoic acid, indicating that the thyme compounds are absorbed and rapidly metabolized into sulfate and glucuronidate conjugates.

The metabolites generated, namely coumaric acid sulfate, ferulic acid sulfate, caffeic acid sulfate, hydroxyphenylpropionic acid sulfate and thymol sulfate, had already been identified in plasma samples after an oral administration of rosmarinic acid [16–18] or thymol [27]. The other metabolites generated (hydroxybenzoic acid, luteolin sulfate, luteolin glucuronide, dihydroxyphenylpropionic acid sulfate and thymol glucuronide) were first detected in our study after an acute intake of thyme extract.

Table 4 also shows the precursor ion, $[M-H]^-$, the MS fragments or product ions (MS^2 ions) and the neutral loss scan, *m/z* 80 or *m/z* 176, according to whether the loss is respectively a sulfate or glucuronide molecule, of the 10 identified and generated metabolites.

The metabolite coumaric acid sulfate was identified because its mass spectra showed a precursor ion of *m/z* 243, and two fragment ions at *m/z* 163 and *m/z* 119. These fragment ions are due to the loss of the sulfate molecule and carboxylic group (CO_2), respectively. The ESI-MS spectra of the compound eluted at 14.96 min obtained in the full-scan mode showed an intense ion at *m/z* 259, which formed two major fragment ions, one at *m/z* 179 and the other at *m/z* 135. These ions could be related to the loss of the sulfate molecule and the caffeic acid rupture, respectively, and this suggests that the metabolite could be caffeic acid sulfate. Another metabolite was observed from the examination of the ESI-MS spectra at a retention time of 5.85 min and this was characterized by an ion at *m/z* 273. This metabolite presents two fragment ions at *m/z* 193 and *m/z*

134, which can be explained by the loss of the sulfate molecule and the ferulic acid rupture, respectively, which suggests that the metabolite could be ferulic acid sulfate. This metabolite can also be generated by methylation of the caffeic acid molecule.

Two luteolin metabolites were identified by the fact that their fragment ions produce ions at *m/z* 285 and *m/z* 133, which demonstrates the existence of the luteolin molecule. The first luteolin metabolite gave a precursor ion of *m/z* 365 and the second one of *m/z* 461. Therefore, these ions could be identified as luteolin sulfate and luteolin glucuronide due to the loss of the sulfate and glucuronide molecules, respectively.

The metabolite dihydroxyphenylpropionic acid sulfate was identified by a precursor ion at *m/z* 261 and two fragment ions, at *m/z* 181 and *m/z* 137 in its MS spectra. These ions can be described by the loss of the sulfate molecule and the carboxylic group (CO_2), respectively. On the other hand, the metabolite with a precursor ion at *m/z* 245 was identified as hydroxyphenylpropionic acid sulfate. This metabolite presents a fragment ion at *m/z* 165 which can be described by the loss of the sulfate molecule, and a fragment ion at *m/z* 121 which can be explained by the loss of the carboxylic group (CO_2).

The ESI-MS spectra of the thymol metabolites eluted at 16.94 and 17.25 min showed intense ions at *m/z* 325, and at *m/z* 229, respectively. These two precursor ions formed a major fragment ion at *m/z* 149, and a second, less-intense, one at *m/z* 134. These ions could be related to the loss of the glucuronide and sulfate molecules, and these could be identified as thymol glucuronide and thymol sulfate, respectively. Although carvacrol sulfate and glucuronide could give the same spectrum as thymol sulfate and glucuronide, the metabolites generated from thyme were thought to be thymol metabolites instead of carvacrol metabolites, because thymol was the most abundant in the TE extract, in comparison with carvacrol (see Table 1).

Fig. 3 shows the extracted ion chromatograms of the 10 metabolites generated, and Fig. 4 shows the chemical structure, mass spectrum (obtained in daughter scan mode by applying different collision energy, from 5 to 35 eV) and the extracted ion chromatograms of the thymol conjugate metabolites.

Once the generated metabolites were identified, these were quantified. Table 5 shows the plasma concentration of the 10 thyme metabolites, expressed as μM, at different times after the ingest of TE, the phenolic acids and monoterpenes being conjugated forms of the main metabolites. Firstly, the two thymol metabolites were tentatively quantified by thymol standard by UPLC–MS/MS. However, due to their volatility we scarcely detected them and were unable to obtain a reliable standard curve. Therefore, in order to quantify these metabolites with a reliable curve, it was proposed to isolate the thymol sulfate metabolite from the plasma samples, and quantify the thymol metabolites compared with thymol sulfate.

Hydroxyphenylpropionic acid sulfate, dihydroxyphenylpropionic acid sulfate, and thymol sulfate metabolites were the most abundant, and their concentrations 1 h after the ingesta of the thyme extract were 446, 25 and 8464, respectively.

The composition analysis of the TE revealed that the rosmarinic acid was the major phenolic compound in the extract (as shown in Table 1), and therefore most of the metabolites detected in the plasma could be linked to its metabolism. Similarly, thymol sulfate and thymol glucuronide were also quantified in the plasma as the main monoterpene metabolites from thyme. According to all this and the generated metabolites that have been identified in the plasma samples, pathways are proposed for both rosmarinic acid and thymol metabolites.

3.2.1. Proposed metabolic pathway for rosmarinic acid

Based on the major phenolic acids (hydroxyphenylpropionic acid, dihydroxyphenylpropionic acid and caffeic acid sulfate conjugates) quantified in the post-prandial plasma samples, we proposed a metabolic pathway for rosmarinic acid, illustrated in Fig. 5. Firstly, the microbial esterases in the digestive tract could hydrolyze the ester linkages into rosmarinic acid. The resulting caffeic and dihydroxyphenylpropionic acids could undergo *p*-dehydroxylation in the lower portion of the digestive tract. This type of transformation has been previously described by Goodwin et al. [28] and Wang et al. [29]. It has been demonstrated that the microflora in the digestive tract has the ability to catalyze the *p*-dehydroxylation of polyphenols with a catechol moiety. The vicinal hydroxy group for this type of biotransformation is emphasized in these studies and that is why rosmarinic acid could be subjected to this reaction. The subsequent hydroxyphenylpropionic acid and coumaric acid derived from the *p*-dehydroxylation of rosmarinic acid could be absorbed, conjugated and methylated in the digestive tract and liver, resulting in a variety of sulfate conjugates of caffeic acid, ferulic acid, coumaric acid, dihydroxyphenylpropionic acid and hydroxyphenylpropionic acid detected in the plasma samples. Some of these, such as coumaric acid, coumaric acid sulfate and hydroxybenzoic acid appeared to have their maximum plasmatic concentration 6 h after the intake of the thyme extract indicating that rosmarinic acid could also be metabolized in the colon, these metabolites appearing as fermentation colonic products as described previously [30].

With regard to the bioavailability of rosmarinic acid, a previous study in rats showed that with an orally administered extract of *Perilla frutescens*, rosmarinic acid was present as intact and degraded and/or conjugated forms, such as hydroxyphenylpropionic acid, coumaric acid, and sulfate conjugates of caffeic acid, coumaric acid and ferulic acid that were subsequently excreted in the urine [18]. Later, Baba et al. [16] determined the absorption and metabolism of rosmarinic acid after a single intake of *P. frutescens* extract but only detected free rosmarinic acid, methylated rosmarinic acid and ferulic acid in plasma, with the maximum levels obtained 0.5, 2 and 0.5 h after the intake of the extract, respectively. In contrast to these previous studies, our results suggest that rosmarinic acid is rapidly degraded into various components with a subsequent conjugation, as we detected no free rosmarinic acid in plasma. Going beyond previous results, we detected more metabolites related to rosmarinic acid metabolism and therefore we could elucidate a possible metabolic pathway, which improves the understanding of the bioavailability of rosmarinic acid.

3.2.2. Proposed metabolic pathway of thymol

The TE administered in the present study contained an important amount of monoterpenes (see Table 1), thymol being the main monoterpene in the extract, followed by carvacrol, α-terpineol and borneol. However, with the exception of thymol, the metabolites of these monoterpenes were not detected in plasma samples after the ingesta of TE. Phase II conjugates, such as thymol sulfate and thymol glucuronide, were identified by means of UPLC–MS/MS analysis. The maximum plasma concentrations were reached 2 and 6 h after the TE intake, respectively (Table 5). By contrast, thymol in its free form was not detected in the post-ingesta plasma samples.

Considerable plasma concentrations of thymol sulfate were detected only 1 h after TE ingesta. This fast absorption indicates that thymol is mainly absorbed in the upper part of the gut. 6 h after the TE ingesta, thymol sulfate was still detectable in plasma. Our results confirm previous observations made by Kohlert et al. [26], who determined the systemic availability and pharmacokinetics of thymol in a clinical trial with 12 healthy male volunteers, where both thymol glucuronide and thymol sulfate were identified in urine with sulfate being the main metabolite again, and no free thymol was detected. Hence, the data available so far (human and animal) indicate that thymol is systemically available mainly as thymol sulfate. Thymol glucuronide was not detected by Kohlert

et al., in plasma, and in our study, it appeared at very low concentrations, which could be the result of lower activity of hepatic UDP-glucuronyltransferases compared with sulfotransferases.

4. Concluding remarks

In the present study, a rapid, selective and sensitive method to identify and quantify thyme metabolites in plasma samples is presented and validated. The use of off-line μ-SPE as the sample preparation with UPLC–MS/MS allowed the rapid determination of the most important thyme bioactive compounds and their metabolites at low concentration levels in complex matrices, such as plasma samples. The analysis of the metabolites revealed that the potential bioactive compounds from thyme are absorbed and rapidly metabolized into simple phenolic acids and thymol as conjugated forms. In the present work, such thyme metabolites as hydroxybenzoic acid, luteolin sulfate, luteolin glucuronide, dihydroxyphenylpropionic acid sulfate and thymol glucuronide were determined for the first time after an acute intake of thyme extract. Additionally, it is the first *in vivo* study with oral administration of TE that proposes two metabolic pathways for rosmarinic acid and thymol, the main potential bioactive compounds from thyme. Given its speed, sensitivity, selectivity and low sample amount needed for the analysis, this method could be proposed as a routine analysis in human intervention studies with a large number of samples.

Acknowledgments

This work was supported by the Spanish Ministry of Education and Science financing the project AGL2009-13517-C03-02/ALI, and the University of Lleida through the L. Rubió grant. The authors thank Carme Piñol (in charge) from the Animal Facility Service of the University of Lleida for their technical support.

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