Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 103–108

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Short communication

Quantification of phenolic antioxidants in rat cerebrospinal fluid by GC–MS after oral administration of compounds

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ARTICLE INFO

Article history:
Received 28 October 2009
Received in revised form 22 February 2010
Accepted 1 March 2010
Available online 7 March 2010

Keywords:
Cerebrospinal fluid
Phenolic antioxidants
Gas chromatography–mass spectrometry

ABSTRACT

A gas chromatographic-mass spectrometric (GC–MS) method for qualitative and subsequent quantitative analysis of phenolic antioxidants compounds, presents in olive oil, in rat cerebrospinal fluid (CSF) after oral administration of compounds is proposed. The procedure involves the extraction of compounds from the samples by a traditional microliquid–liquid extraction method, followed by a silylation step before the GC–MS analysis. The chromatographic separation was performed by using a low bleed DB5-MS fused-silica capillary column. The presence of 21 phenolic compounds was tested in CSF extracts and only free tyrosol, hydroxytyrosol and ferulic acid were detected. Those compounds were then quantitatively determined using the proposed methology. The molecular ion for silylated compounds appears at 370 m/z for hydroxytyrosol, 282 m/z for tyrosol and 338 m/z for ferulic acid respectively, while the base peak appears at 267 m/z, 179 m/z and 338 m/z. α-Naphthol was used as a surrogate (216 and 201 m/z). The detection capabilities obtained were 74, 92 and 79 ng/mL respectively. The method was applied to the determination of trace amounts of compounds in rat cerebrospinal fluid after oral administration. The animals were fed with a standard chow diet (free of phenolic antioxidants) in order to avoid the influence of any other component of the diet on the CSF of the animals.

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1. Introduction

Hippocrates, the father of medicine, first mentioned the health and therapeutic benefits of olive oil. For centuries, the people of the Mediterranean region [1] have recognized the nutritional, cosmetic and medicinal benefits of olive oil. It has been used both internally and externally for health and beauty [2].

One of the most important factors in olive oil benefits is the presence of phenolic compounds. These compounds are a group of chemicals that act as strong antioxidants and radical scavengers. Olive oil is a source of a large amount of phenolic compounds. The most abundant compounds in olive oil are oleuropein, hydroxytyrosol and tyrosol [3–5].

In the last decade, the number of publications devoted to studies of natural and synthetic biologically active compounds has sharply increased. The studies of the antioxidant activity or biological activity of, in particular, phenolic derivatives attract special attention of researchers. Those compounds protect cells and body chemicals against damage caused by free radicals, reactive atoms that contribute to tissue damage in the body [6,7]. It has been postulated that the components in olive oil in the Mediterranean diet can contribute to the lower incidence of coronary heart disease and prostate and colon cancers [8].

In the last years, some authors have also described that brain aging and the most diffused neurodegenerative diseases of the elderly proceed with oxidative damage, redox metals homeostasis impairment and inflammation [9]. The compounds have been described as potential inhibitors of amyloid aggregation and significance to Alzheimer's disease has been suggested [10]. Much evidence suggests that oxidative stress induced by reactive oxygen species (ROS) is involved in neuronal loss in neurodegenerative diseases [11,12].

Despite the huge and increasing amount of the in vitro studies trying to unravel the mechanisms of action of dietary antioxidants, the research in this field is still incomplete, and questions about bioavailability, biotransformation and mechanisms of the antioxidant activity are still unanswered. However, is a proven fact that a healthy diet, including the Mediterranean one, is highly recommended throughout the entire life, and the use of food additives is becoming increasingly common, to prevent the onset and to slow down development of aging-associated neuropathies [13–15].

In this context, it is of crucial importance to have analytical methodology to check the presence of this group of antioxidants into the different biological matrices (body fluids or tissues), and to study the bioavailability of these compounds.

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doi:10.1016/j.jpba.2010.03.003

Not many bibliographic references have been found in the literature for the analysis of phenolic antioxidants in biological fluids. Spectroscopy, chromatography and electrophoresis have been used for the study of the presence of phenolic antioxidants saliva, serum, blood or urine [16]. Liquid chromatography with ultraviolet detection [17–19], fluorescence detection [20] or electrochemical detection has been used for the detection of phenolic antioxidants from plants in biological samples [21–23]. Some of them have been determined in human serum and blood by liquid chromatography [24] or gas chromatography [25] coupled mass spectrometry. Some references have also been found related to the use of capillary electromigration techniques for polyphenol analysis [26–28]. Some acids, as ferulic, homovanilic and some other biologically important acids has been previously studied in brain and cerebrospinal fluid [29–34]. De Jong proposes the determination of catecholamine metabolites in plasma and cerebrospinal fluids [35]. However, these methodologies do not include the analysis of the most important antioxidants present in olive oil (hydroxytyrosol or tyrosol) that are important for our work. As well, the sample treatment and the derivatization step uses to be tedious and time consuming, and a large amount of different reagents and steps are necessaries with the subsequent increase in costs. For example, the derivatization procedure used by De Jong's et al. is very complicated. In this work, the phenolic hydroxy groups are previously acetylated in aqueous solution, and then extracted with an organic solvent. In a second step, the acetyl derivatives are converted into their pentafluorobenzyl esters (PFB) for CG analysis. However, in our work, we have obtained a good extraction yield without the previous acetylation step, and a simple silylation step after extraction of analytes at room temperature is enough to get our objective. As consequence, a very simple, quick and low cost sample treatment and derivatization procedure of compounds is proposed. This allows applying the proposed methodology in routinely work.

The present work has been focused, in the qualitative detection of the presence of the most important metabolites of oleuropein and a wide group of natural phenolic antioxidants in cerebrospinal fluid (CSF), including acids and alcohols, after oral administration to rats and then in the quantification of the detected ones. Cerebrospinal fluid is a clear bodily fluid that occupies the subarachnoid space and the ventricular system around and inside the brain. It constitutes the content of all intra-cerebral ventricles, cisterns and sulci, as well as the central canal of the spinal cord.

The application of gas chromatography coupled mass spectrometry (GC–MS) previous derivatization combined with a simple microliquid–liquid extraction step for qualitative and quantitative analysis of some natural phenolic compounds in cerebrospinal fluid is proposed.

2. Experimental

2.1. Chemicals and reagents

All reagents were analytical grade unless specified otherwise. Water was purified with a Milli-Q plus system (Millipore, Bedford, USA). p-Vanillin, 3-hydroxyphenyl acetic acid, protocatetic aldehyde, p-hydroxybenzoic acid, vanillol, p-hydroxyphenyl acetic acid, caffeic acid, vanillic acid, protocatetic acid, gallic acid, syringic aldehyde, hydroxytyrosol, tyrosol, syringic acid, p-hydroxyphenylpropionic acid, 3,4,5-trimetoxibenzoic acid, p-coumaric acid, ferulic acid, esculetin, sinapic acid and epicatequine were supplied by Sigma–Aldrich (St. Louis, MO, USA). Stock solutions of compounds containing 500 μg/mL, were prepared in absolute ethanol (99%, v/v) from Panreac (Barcelona, Spain). These solutions were stored in dark glass bottles at 4 °C, remaining stable for at least 3 months. Working solutions containing 100 μg/mL of each compound were prepared by diluting stock solutions in distilled water. These solutions remain stable for at least 1 week.

A solution of 1000 μg/mL of α-naphthol, Sigma–Aldrich (St. Louis, MO, USA), prepared by dissolution of 10 mg of the pure standard in 10 mL of in ethanol was used as surrogate (internal standard) after adequate dilution to a final concentration of 71 μg/mL in samples (5 μL/70 μL of sample). Pyridine and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) by Sigma–Aldrich (St. Louis, MO, USA) were used as the silylation reagent.

2.2. Apparatus and software

Gas chromatographic analysis was performed using an Agilent 6890 Series GS System gas chromatograph fitted with a splitless injector for a low background with an injector liner split/splitless. A capillary column DB5-MS fused-silica J&W Scientific Inc. (30 m × 0.25 mm i.d.; 0.25 μm film) was used. Detection was carried out with a 5973 mass-selective single quadrupole detector (Agilent technologies). Operation control and the data process were carried out by Agilent Technologies ChemStation software (Santa Clara, CA, USA). The mass spectrometer was calibrated everyday before use with perfluorotributylamine (PFTBA) as a calibration standard.

Statgraphics Centurion XV, vs 15.1.02 software package (1982–2006 Statpoint Inc.) was used for the statistical analysis of data [36].

2.3. Animal manipulation

Male rats of the Wistar strain used were purchased from University of Granada (Animal facilities of University of Granada, Spain), weighing around 250 g/rat. The animals were fed with a standard chow diet (PANLAB, Barcelona, Spain). The meaning of using this diet was to avoid the influence of any other component of the diet on the CSF of the animals.

The different amounts of the phenolic antioxidant (depending of the experiment) were directly administered in a water suspension by using an oral gavage tube (1 mL/rat) in order to get a high homogeneity in replicates.

The Ethical Committee of the University of Granada (Spain) regulated the manipulation and sacrifice of the animals. All the animals were fed with food and water ad libitum, maintained on a 12:12 h light dark cycle beginning at 8.00 a.m. Wistar rats were euthanased by stunning followed by decapitation. The animals were anaesthetised using isofluorane (Sigma–Aldrich, Barcelona, Spain) and CSF removed by puncturing the cisterna magna with a 21G butterfly cannula and then euthanased by decapitation [37]. All samples were collected into sterile Eppendorf tubes and routinely centrifuged twice at 14,000 rpm to remove any cells or debris from the fluid, which was decanted into another sterile tube. Samples were frozen on dry ice and stored at −80 °C until use. CSF samples with visible blood contaminants were discarded.

2.4. Preparation of fortified samples

Fortified cerebrospinal samples were prepared by spiking 50 μL (accurately measured) of different blanks (tested prior to recovery assay) adding a maximum of 15 μL of a concentrated standard solutions of the phenolic compounds and 5 μL of α-naphthol solution, used as surrogate. The final concentrations for recovery assays were 0.2, 1.0 and 10.0 μg/mL.

Before sample treatment and analysis, all samples were allowed to stand in the dark for 60 min at room temperature to permit the total interaction between the phenolic compounds and cerebrospinal fluid.

2.5. Basic procedure

An aliquot of 50 μL of CSF blank sample or natural sample obtained from in vivo studies was placed in 1.5 mL Eppendorf tube. 5 μL of the ethanolic surrogate solution (1000 μg/mL) and 15 μL of distilled water for in vivo samples and of standard solutions of compounds for the spiked ones were added and then shaked for 10 s in vortex mixer. 70 μL of a mixture of acetonitrile/hydrochloric acid (1.0 M) 1:1 (v/v) were added. After shaking for 10 s in vortex mixer, samples were centrifuged at 3500 rpm (2630 × *g*) for 5 min in order to remove precipitated proteins. After protein precipitation, the supernatant was transferred to a 2 mL Eppendorf tube and then mechanically extracted twice using 140 μL of ethyl acetate every time. The mixture was shaken for 1 min and the upper organic phase was transferred into a new Eppendorf tube. Next, the sample was evaporated to near dryness under nitrogen and transferred to a 150 μL microvial, where the evaporation was completed. The vial was stoppered, because silyl derivatives tend to be sensitive to moisture, and 25 μL of a mixture containing 10 μL of N,O-bis(trimethylsilyl)acetamide, 5 μL of pyridine and 10 μL of ethyl acetate was added and the mixture was mechanically shaken for 1 min at room temperature. At this point, the sample is ready to be injected into the GC–MS.

2.6. Chromatographic conditions

The injector temperature was set at 100 °C (1 min) and then increased to 300 °C at a rate of 200 °C/min. The oven temperature was held at 45 °C for 2 min, then increased to 150 °C at a heating rate of 20 °C/min and held for 1 min and then to 270 °C at 10 °C/min and held for 0.75 min. The total run time was 21.0 min. The MS detector temperature was maintained at 280 °C. The carrier gas used was helium (purity 99.999%) at a flow rate of 1.0 mL/min. The samples were injected in the splitless mode and the splitter was opened after 5 min (delay time). The sample volume in the direct injection mode was 1 μL. The conditions for electron impact ionization (EI) were: ion energy of 70 eV and the mass range scanned was 140–465 *m*/*z*. The MS was tuned everyday to *m*/*z* 69, 219 and 502 with calibration standard. Single ion monitoring (SIM) adquisition mode (dwell time 100 ms/ion) was used.

3. Results and discussion

The number of studies published on the bioavailability of phenolic antioxidants in cerebrospinal fluid is limited, mainly due to the lack of validated methods. Consequently, the aim of the study was to develop an extraction process that allowed the determination and quantification of compounds in this matrix using gas Chromatography coupled mass spectrometry detection. The extraction of compounds from CSF was attempted through liquid–liquid extraction, which is a basic sample preparation method. The effects of experimental and chromatographic variables were evaluated to develop a rapid, specific and accurate method for measuring phenolic antioxidants in cerebrospinal fluid. Consequently, method development was focused on the optimization of sample preparation as well as chromatographic separation.

3.1. Liquid–liquid microextraction procedure

Quantitative extraction of phenolic compounds in biological matrices is difficult. Therefore, tests of recovery were carried out to ensure effectiveness of the extraction procedure of phenolic compounds from matrix. A simple microliquid–liquid extraction procedure was selected as appropriate for analyte isolation.

The influence of the pH of the medium in the extraction efficiency of phenolic antioxidants was studied. It was observed that the extraction efficiency is maximum for acid pH values. This behaviour could be attributed to a drop in the extraction efficiency of compound because the dissociated form remains in aqueous phase. Therefore, compounds extractions were carried out at pH 2–3 by adding hydrochloric acid solution.

For protein precipitation, the addition of an organic solvent at low pH was studied. A decrease in the dielectric constant with the addition of an organic solvent causes that the solubility also decreases. Different ratios sample/dissolution of chlorhydric acid (1.0 M) in acetonitrile were assayed. Due to the good recovery obtained and the low analyte lost, 1:1 (v/v) ratio was selected as the most adequate.

Organic extracting solvent ethyl ether, n-hexane, dichloromethane, ethyl acetate and trichloromethane were also tested. Due to the good recovery obtained for all compounds and the lower boiling point for solvent removing, ethyl acetate was selected as the most adequate extracting agent.

3.2. Qualitative analysis of phenolic compounds

The presence of phenolic compounds including p-vanillin; 3-hydroxyphenyl acetic acid; protocatetic aldehyde; p-hydroxybenzoic acid; vanillol; p-hydroxyphenyl acetic acid; caffeic acid; vanillic acid; protocatetic acid; gallic acid; syringic aldehyde; hydroxytyrosol; tyrosol; p-hydroxyphenylpropionic acid; syringic acid; 3,4,5-trimetoxibenzoic acid; p-coumaric acid; ferulic acid; esculetin, sinapic acid and epicatequine, were qualitatively determined in CSF extrats using SIM mode GC–MS. The gradient was adapted to obtain a sharp peak separation for all the compounds in the matrix. By means of the chromatographic settings, all compounds were adequately separated and eluted. Table 1 shows the most important fragments for each one used for qualitative analysis in SIM mode for silylated compounds.

The mixture of compounds for qualitative analysis was administered directly as aqueous suspension using an oral gavage tube (50 mg of compound/100 g of animal weight). Following a similar methodology to the one proposed by Juan et al. [38], samples were taken at 5, 15, 30, 60, 120, 240, 360, 480 and 720 min after oral administration. Only ferulic acid, 4-dihydroxyphenylethanol and tyrosol were clearly detected in the great majority of the samples after 30 min and before 120 min being maximum at 60 min in all cases. These compounds were the selected analytes for quantitative analysis and for the analytical method establishment.

Table 1
SIM mode for phenolic compounds identification.

Compound	*m*/*z*
p-Vanillin	224 + 209 + 194
Tyrosol	282 + 267 + 193 + 179
3-Hydroxyphenyl acetic acid	296 + 281 + 252 + 164 + 147
Protocatetic aldehyde	282 + 267 + 193 + 165
p-Hydroxybenzoic acid	282 + 267 + 223 + 193
Vanillol	298 + 209 + 179
p-Hydroxyphenyl acetic acid	296 + 281 + 252 + 179
Syringic aldehyde	254 + 239 + 224 + 195
Hydroxytyrosol	370 + 267 + 193 + 179
p-Hydroxyphenyl propionic acid	310 + 295 + 192 + 179
Vanillic acid	312 + 297 + 282 + 267
Protocatetic acid	370 + 355 + 311 + 193
3,4,5-Trimetoxibenzoic acid	284 + 269 + 225 + 195
Syringic acid	342 + 327 + 312 + 297
p-Coumaric acid	308 + 293 + 279 + 219
Gallic acid	458 + 459 + 443 + 444
Ferulic acid	338 + 323 + 308 + 293
Esculetin	395 + 322 + 307
Caffeic acid	396 + 381 + 219 + 191
Sinapic acid	368 + 353 + 338 + 323
Epicatequine	368 + 369 + 370 + 356 + 357
α-Naphthol (ISTD)	216 + 201

Table 2
Analytical and statistical parameters.

	n	a	S_a	b (mL/μg)	S_b (mL/μg)	R^2 (%)	$S_{y/x}$	LDR (μg/mL)	CC_α (ng/mL)	CC_β (ng/mL)	RSD (%)	P_{lof} (%)
Tyrosol	30	5.2×10^{-3}	1.1×10^{-3}	5.7×10^{-2}	6.0×10^{-4}	99.9	7.6×10^{-3}	0.05–10.0	45	74	5.0	30.1
Hydroxytyrosol	30	1.2×10^{-2}	3.6×10^{-3}	1.5×10^{-1}	1.7×10^{-3}	99.8	2.2×10^{-2}	0.09–10.0	56	92	4.8	56.2
Ferulic acid	30	6.8×10^{-3}	1.3×10^{-3}	6.3×10^{-2}	8.3×10^{-4}	99.8	1.1×10^{-2}	0.08–10.0	48	79	4.2	39.8

n: number of measurements; a: intercept; S_a: intercept standard deviation; b: slope; S_b: slope standard deviation; R: determination coefficient; LDR: linear dynamic range; $S_{y/x}$: regression standard deviation; $CC_{\alpha,0.05}$: decision limit; $CC_{\beta,0.05}$: detection capability; RSD: relative standard deviation; P_{lof}: P value for *lack-of-fit* test.

Table 3
Recovery assay for phenolic compounds in spiked CSF.

Compound	Added (μg/mL)	R^a (%)	Added (μg/mL)	R^a (%)	Added (μg/mL)	R^a (%)
Tyrosol	0.20	108.5	1.00	94.1	10.0	96.1
Hydroxytyrosol	0.20	94.3	1.00	106.9	10.0	102.2
Ferulic acid	0.20	92.1	1.00	96.2	10.0	97.1

[a] Mean of five determinations.

3.3. Quantitative analysis: analytical characteristics of the method

A calibration curve for the three compounds found in CSF samples was built by injecting 1 μL of different standard solutions at concentrations ranging from CC_β to 10.0 μg/mL by using standard addition calibration methodology. Table 1 shows the most important fragments used for SIM mode analysis. Analytical performance was established according to the Analytical Methods Committee [39], the *lack-of-fit* test was applied to two replicates and three injections of each standard (five concentration levels). The results for the intercept (a), slope (b) and correlation coefficient (R^2) are summarised in Table 2.

3.4. Method validation

3.4.1. Selectivity

The specificity of the method was determined by comparing the chromatograms of blank with the corresponding spiked cerebrospinal fluid. Typical chromatograms in SCAN mode of blank and spiked CSF are shown in Fig. 1. No interferences from endogenous substances were observed at the retention time of the analytes. A good separation was obtained under the described conditions and tyrosol, DOPET and ferulic acid eluted at 11.47 min, 13.55 min and 16.90 min respectively. α-Naphthol appears at 11.30 min. These suggest that the extraction procedure was capable of obtaining highly purified samples, which in turn ensured a high selectivity of the GC–MS method.

3.4.2. Precision and accuracy

The precision and accuracy data for the analytical procedures are shown in Table 2. Intra-day and inter-day precision (%RSD) of the method were lower than 5% and were within the acceptable limits to meet the guidelines for bioanalytical method validation which is considered to be ≤20%. The accuracy of the method was also good with the deviation between the nominal concentration and calculated concentration. Precision and accuracy data indicated that the methodology to extract the compounds from CSF is highly reproducible and robust.

3.4.3. Recovery

A recovery assay with spiked samples free of analytes was carried out. The recovery values for five replicate samples at three concentration levels of each compound are shown in Table 3.

The concentration of each phenolic compound was determined by direct interpolation in the standard addition calibration curve within their linear dynamic range. Recoveries were from 90.0% to 110%. The results pointed out the high extraction efficiency of the procedure.

3.4.4. Sensitivity

Limits of detection were calculated in order to determine analytes present in real samples. In this paper, criteria for method performance have been proposed that include the decision limit, CC_α, and the detection capability, CC_β [40]. The decision limit, CC_α, is the limit from which it can be decided that a compound is present in the sample with an error probability of α. The detection capability, CC_β, is the smallest content of the analyte that may be detected, identified and/or quantified in a sample with an error probabil-

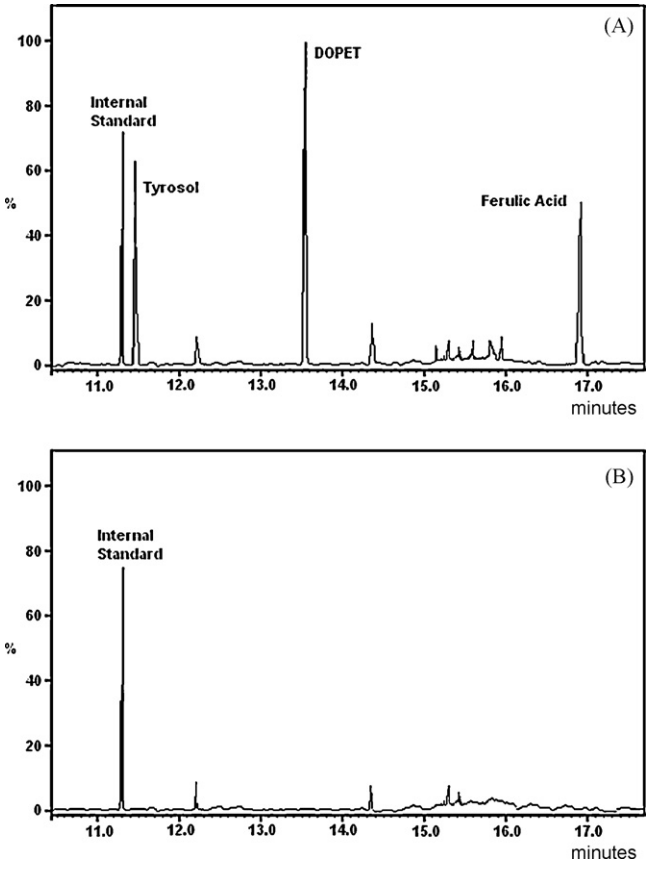

Fig. 1. Representative chromatogram of (A) cerebrospinal fluid sample containing naphtol (71 μg/mL), tyrosol, hydroxytyrosol and ferulic acid and (B) blank containing α-naphthol (71 μg/mL).

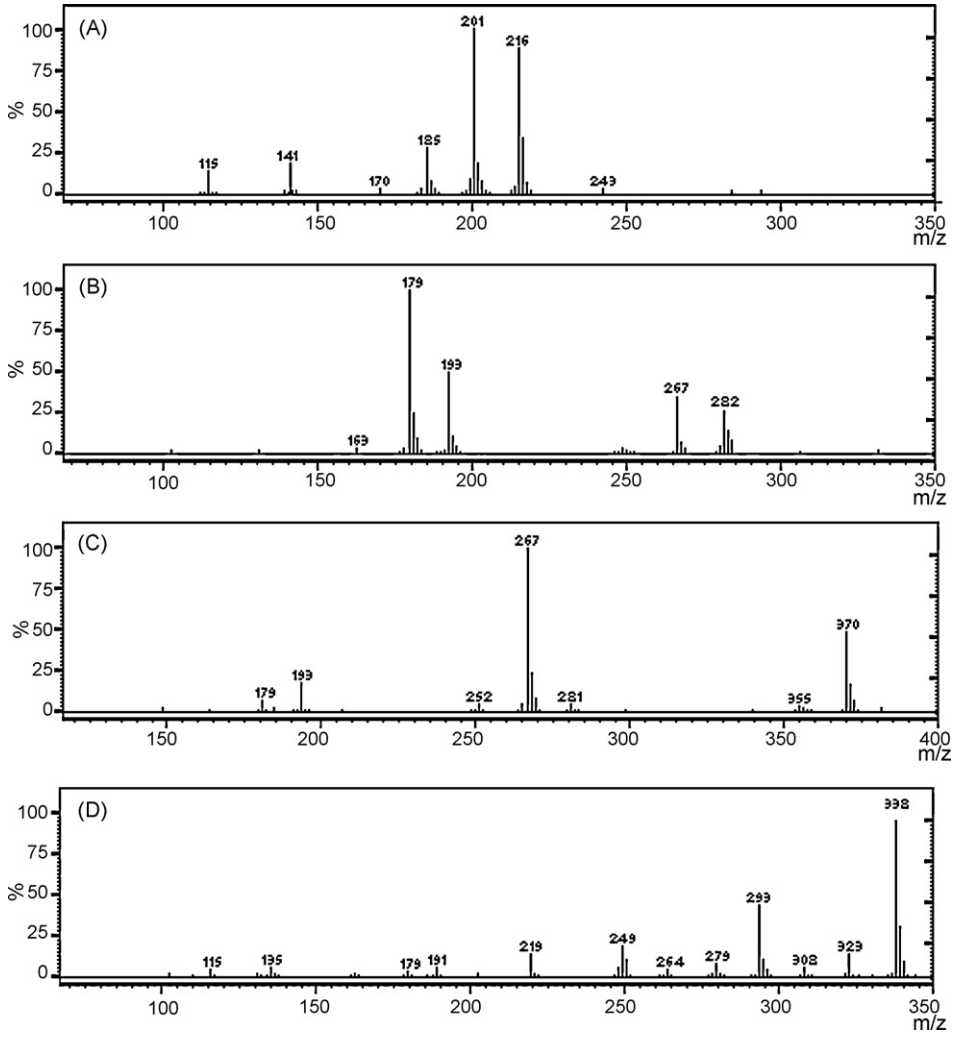

Fig. 2. Mass spectra of phenolic compounds: (A) α-naphthol, (B) tyrosol, (C) hydroxytyrosol and (D) ferulic acid.

Table 4
Polyphenolic concentrations in cerebrospinal fluid.

	Administered amount	Found amount in CSF (μg/mL)[a]		
		Tyrosol	Hydroxytyrosol	Ferulic acid
Sample 01 (Control)	0.0 mg	ND	ND	ND
Sample 02 (Control)	0.0 mg	ND	ND	ND
Sample 03 (Control)	0.0 mg	ND	ND	ND
Sample 04 (Control)	0.0 mg	ND	ND	ND
Sample 05 (Group 01)	1.0 mg	0.085 ± 0.003	ND	ND
Sample 06 (Group 01)	1.0 mg	0.096 ± 0.002	0.112 ± 0.005	ND
Sample 07 (Group 01)	1.0 mg	0.095 ± 0.004	0.108 ± 0.005	ND
Sample 08 (Group 01)	1.0 mg	0.087 ± 0.002	0.106 ± 0.003	ND
Sample 09 (Group 02)	10.0 mg	0.565 ± 0.021	0.596 ± 0.012	0.335 ± 0.013
Sample 10 (Group 02)	10.0 mg	0.542 ± 0.018	0.505 ± 0.015	0.352 ± 0.010
Sample 11 (Group 02)	10.0 mg	0.536 ± 0.022	0.559 ± 0.013	0.361 ± 0.016
Sample 12 (Group 02)	10.0 mg	0.506 ± 0.025	0.520 ± 0.011	0.346 ± 0.015
Sample 13 (Group 03)	50.0 mg	2.556 ± 0.105	4.827 ± 0.128	1.901 ± 0.073
Sample 14 (Group 03)	50.0 mg	2.670 ± 0.098	4.703 ± 0.143	1.980 ± 0.083
Sample 15 (Group 03)	50.0 mg	2.752 ± 0.102	4.825 ± 0.161	1.803 ± 0.085
Sample 16 (Group 03)	50.0 mg	2.692 ± 0.093	4.795 ± 0.149	1.885 ± 0.079
Sample 17 (Group 04)	100.0 mg	5.356 ± 0.155	9.199 ± 0.203	3.626 ± 0.103
Sample 18 (Group 04)	100.0 mg	5.479 ± 0.162	9.072 ± 0.192	3.912 ± 0.131
Sample 19 (Group 04)	100.0 mg	5.535 ± 0.183	9.210 ± 0.199	3.856 ± 0.123
Sample 20 (Group 04)	100.0 mg	5.625 ± 0.142	8.810 ± 0.163	3.635 ± 0.141

[a] Mean of five determinations.

ity of β. Decision limit and detection capacity which are better adjusted to a statistical evaluation are implemented. Thus, CC_α (α = 5%) and CC_β (β = 5%) were calculated and the results obtained are summarised in Table 2.

3.4.5. Linearity

Linear calibration curves were obtained over the concentration range from CC_β to 10 μg/mL in rat CSF. The correlation coefficients obtained of 99.8–99.9% and probability level of the *lack-of-fit* test, P_{lof} (%) indicate a good linearity within the stated ranges. The results obtained are also summarised in Table 2.

3.5. Application of the analytical method

Once the method was established and validated, it was applied to the detection of tyrosol, hydroxytytosol and ferulic acid in rat CSF. A representative chromatogram of a CSF sample of an animal fed with a 50 mg of pure compounds and the corresponding mass spectra are depicted in Figs. 1 and 2 respectively. Table 4 shows the evolution of found polyphenol concentrations in analysed samples.

Samples were collected 60 min after oral administration of compounds. It can be observed a lineal increment in detected amounts when dose is increased.

4. Conclusions

The presence of 21 phenolic antioxidants in rat cerebrospinal fluid, including acids, and alcohols usually presents in natural olive oil were analysed by GC–MS after protein precipitation, microliquid–liquid extraction and derivatization of the extracts. The extraction solvent employed was ethyl acetate, a mixture of acetonitrile/hydrochlorohydric acid was used for protein removing and BSTFA was used as derivatization reagent. The presence of phenolic compounds was determined by working in SIM mode (three fragments at least for identification). The great majority of the 21 studied compounds were not detected in assayed samples. However, the presence of hydroxytyrosol, tyrosol and ferulic acid was confirmed and the compounds were also quantitatively determined. These phenolic compounds displayed linear calibration curves over the concentration range of CC_β–10.0 μg/mL, and could be determined accurately and precisely at the concentrations studied.

The appropriate sample collection and conservation in conjunction with an addecuate treatment allows good recovery values in all cases as demonstrated by the validation procedure employed.

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