Comparison of different extraction procedures for the comprehensive characterization of bioactive phenolic compounds in *Rosmarinus officinalis* by HPLC-DAD-ESI-TOF-MS.

I. Borrás Linares[a,b], D. Arráez-Román[a,b], M. Herrero[c], E. Ibáñez[c], A. Segura-Carretero[a,b], A. Fernández-Gutiérrez[a,b].

[a] Department of Analytical Chemistry, University of Granada, c/Fuentenueva s/n, 18071 Granada, Spain.

[b] Research and Development of Functional Food Centre (CIDAF), Health Science Technological Park, Avda. Del Conocimiento s/n, 18100 Granada, Spain

[c] Department of Bioactivity and Food Analysis, Institute of Food Science Research (CIAL-CSIC), Nicolás Cabrera 9, Campus Cantoblanco, 28049 Madrid, Spain.

*Corresponding author.

Tel.: +34 958 243296; fax: +34 958 249510.

E-mail address: ansegura@ugr.es (A. Segura-Carretero).

ABSTRACT

In the present work, a comparative study between two environmentally friendly and selective extraction techniques, such as supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) have been carried out focusing in the bioactive phenolic compounds present in *Rosmarinus officinalis*. For the analysis of the SFE and PLE extracts, a new methodology for qualitative characterization has been developed, based on the use of reversed-phase high-performance liquid chromatography (RP-HPLC), equipped with two different detection systems coupled in series: photodiode array detector (DAD) and time of flight mass spectrometry (TOF-MS) detector connected via an electrospray ionization interface (ESI). The use of a small particle size C_{18} column (1.8 μm) provided a great resolution and made possible the separation of several isomers. Moreover, UV-visible spectrophotometry is a valuable tool for identifying the class of phenolic compound, whereas MS data enabled to structurally characterize the compounds present in the extracts. The applied methodology was useful for the determination of many well-known phenolic compounds present in *Rosmarinus officinalis*, such as carnosol, carnosic acid, rosmadial, rosmanol, genkwanin, homoplantaginin, scutellarein and cirsimaritin and rosmarinic acid, as well as other phenolic compounds present in other species belonging to Lamiaceae family.

Keywords: *Rosmarinus officinalis*, supercritical fluid extraction, pressurized liquid extraction, phenolic compounds, high-performance liquid chromatography, time-of-flight mass spectrometry.

1. Introduction

Rosemary (*Rosmarinus officinalis* L.) is a shrub that grows wild in the Mediterranean basin. Rosemary has been traditionally used as a culinary spice, mainly to modify or to improve food flavours, as well as in folk medicine, being a greatly valued medicinal herb. Nowadays, it is one of the most appreciated sources for natural bioactive compounds which are of special interest in the functional food industry. In fact, this plant exerts a great number of pharmacological activities, such as hepatoprotective [1, 2], antibacterial [3, 4], antithrombotic [5], antiulcerogenic [6], diuretic [7], antidiabetic [8], antinociceptive [9], anti-inflammatory [10], antitumor [11, 12] and antioxidant [13] activities. Most of these observed effects are linked to the phenolic content of this herb. Specially, its potent antioxidant activity is mainly due to phenolic diterpenes, such as carnosol, carnosic acid, rosmadial or rosmanol, among others. Nevertheless, the presence of other antioxidant phenolic compounds found in rosemary has also been reported, such as flavonoids (genkwanin, cirsimaritin), and phenolic acids (rosmarinic acid)[14, 15].

In the last few years, there has been a growing interest in the use of natural antioxidants in the food industry, not only for their usefulness as a preservation method but also because of their benefits in human health [16]. These natural antioxidants can be considered as products that have a non-synthetic origin and are able to prevent or retard the onset of lipid oxidation without changing the sensory qualities of the food products [17]. Recently, the demand for natural antioxidants for its use as food additives has risen notably because of the growing interest paid to natural food [18] and the restriction in the use of synthetic antioxidants in the food industry due to their toxicological effects of long-term administration, including carcinogenicity [16].

Among natural antioxidants of herbal origin, rosemary is one of the most used and commercialized because of its high content in phenolic.

Polyphenols are secondary metabolites synthesized by plants, both during normal development and in response to stress conditions such as infection, UV radiation, or wounding, among others. The polyphenol content in the plant depends on different factors: such as plant quality, flowering period, geographic origin, harvesting and climatologic conditions [14, 19, 20].

Several traditional methods have been used to extract antioxidants from aromatic plants, such as conventional solvent extraction [15, 21] solid-liquid extraction, aqueous alkaline extraction, extraction with vegetables oils [16], ultrasounds assisted extraction [22, 23], among others. In the last few years more environmentally friendly and selective extraction techniques are preferred, such as supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE). SFE operates at low temperatures, in oxygen absence and typically use CO_2 as extraction agent. These characteristics make SFE an ideal technique for the extraction of natural antioxidants. On the other hand, PLE limits the use of organic solvents while obtaining higher extraction yields and faster extraction processes.

Nowadays, phenolic compound identification in complex plant matrices is a difficult task due to the complexity of the polyphenol structures [24] and the limited standards commercially available. In this sense, the most common separation techniques used to determine these kind of bioactive compounds in plant matrix have been capillary electrophoresis (CE), gas chromatography (GC) and high performance liquid chromatography (HPLC). CE provides high efficiencies in short migration times with small amounts of reagents and sample volumes needed [25], although its main disadvantage is the low concentration sensitivity. GC is the less used technique for this

purpose because a derivatization step is necessary. Lastly, the ability of HPLC to separate polyphenols is well know [15], being the most commonly used separation technique used for determining these compounds [26, 27]. Recently, an improvement in chromatographic performance has been achieved by using columns packed with smaller diamterparticles. This type of columns, packed with particles smaller than 2 μm and operated at a pressures up to 600 bar, allow the attainment of high resolution and efficiency in shorter analysis times [28, 29].These analytical techniques have been coupled to different detection systems. Nowadays, mass spectrometry (MS) is the detection system mainly used due to its high sensitivity and its great capability for identifying compounds.

The aim of this work was to carry out a comprehensive characterization of bioactive compounds present in five rosemary extracts obtained using environmentally friendly extraction procedures (SFE y PLE) by reversed-phase high-performance liquid chromatography (RP-HPLC), coupled to different detection systems, photodiode array (DAD) and time-of-flight (TOF) mass analyzer. DAD has proved to be a valuable tool for identifying the family of these phenolic compounds while TOF-MS allows high resolution acquirements, accurate mass measurements and elemental composition information.

2. Experimental

2.1. Samples and Chemicals

All chemicals were of analytical reagent grade and used as received. Formic acid and acetonitrile for HPLC were purchased from Fluka, Sigma-Aldrich (Steinheim,

Germany) and Lab-Scan (Gliwice, Sowinskiego, Poland) respectively, and ethanol for dissolving the samples was purchased from Panreac (Barcelona, Spain). Solvents were filtered using a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA). Double-deionised water with conductivity lower than 18.2 MΩ was obtained with a Milli-Q system from Millipore (Bedford, MA, USA). Other reagents unmarked were of an analytical grade. The carbon dioxide liquefied at high pressure used in supercritical extraction was supplied by Praxair Inc. (Danbury, CT, USA).

The rosemary leaves were obtained from Herboristeria Murciana (Murcia, Spain) and dried using a traditional method [20]. The dried leaves were grinded under liquid nitrogen and particle size was determined by sieving the ground plant to an appropriate size (500-999 µm). Finally, the sample was stored at -20 ºC until use to avoid any possible degradation.

2.2. Supercritical fluid extraction (SFE)

The SFE system employed was based on a Suprex Prep Master (Suprex Corporation, Pittsburg, PA, USA) with several modifications. It was equipped with a thermostatic oven heated by air convection where the extraction cell (8 mL) containing the sample is placed. A Waters 510 HPLC pump (Waters Corporation, Milford, MA, USA) was used to introduce the modifier in the extraction system. A pre-heater system was employed by placing a heating coil inside a glycerine bath (JP Selecta Agimatic N, JP Selecta S.A., Abrera, Spain) to guarantee that the fluid employed in all the experiences reaches the extraction cell at the target temperature. After the modifier pump, a check valve (Swagelok SS-CHS2-BU-10, Swagelok Corporation, Solon, OH, USA) was used. A

micrometering valve (Hoke SS-SS4-BU-VH, Hoke Incorporated, Spartanburg, SC, USA) was placed after the extraction cell to manually control the flow and a computer-controlled mass flowmeter (EL-FLOW® Mass Flow Meter/Controller F-111C, Bronkhorst High-Tech BV, AK Ruurlo, The Netherlands) was used to adjust the carbon dioxide flow rate at the values selected for each experiment. After depressurization, the extracts were collected in a collection vessel described previously [20]. Inside the collection vessel, 30 mL volume glass vials were placed to recover the extracts.

Two different extraction conditions were tested, including the extraction at 150 bar (SFE 150 extract) and at 400 bar (SFE 400 extract). In both cases, the extraction temperature was maintained at 40 ºC. Once the extracts were collected, the extraction yield was determined and the extracts were dissolved with ethanol to a final concentration of 10 mg/mL and kept at -20 ºC until analysis. INCLUIR QUE LA EXTRACCION A 150 BAR, SE LLEVO A CABO CON 6.6% ETANOL COMO MODIFICADOR

2.3. Pressurized liquid extraction (PLE)

The PLE system consisted in a home-made device described elsewhere [30]. Basically, it consisted of an extraction cell housed in an oven provided with temperature control and regulation, a Hewlett-Packard 1050 series isocratic pump (Agilent, Palo Alto, USA) to deliver and pressurize the solvent in the extraction cell and two six-port Rheodyne valves (model 7000, Rheodyne L.P., Rohnert Park, CA, USA) connected to the inlet and outlet end of the extraction cell. The temperature and the heating rate were set by varying the energy applied to the heating resistances. The temperature programme was manually started at the beginning of each experiment and stopped at the selected extraction time. The extraction cell (8 mL) consisted in a stainless steel holder sealed with 5 µm stainless steel frits (Supelco, Bellefonte, USA). Two different solvents, that

is, water and ethanol were employed to obtain extracts with different chemical composition. Based on previous results [31], the extraction conditions selected consisted of extraction with ethanol at 150 ºC (PLE 150 extract), and with water at 100 ºC (PLE 100 extract) and 200 ºC (PLE 200 extract). The static extraction time was set in all cases at 20 min. For solvent evaporation, a Rotavapor R-210 (Buchi Labortechnik AG, Flawil, Switzerland) was employed for the ethanolic extracts, whereas a freeze-dryer (Labconco Corporation, Missouri, USA) was employed for the water extracts.

2.4. HPLC-DAD-MS analyses.

HPLC analyses were carried out using an Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies, Palo Alto, CA, USA), equipped with a vacuum degasser, an autosampler, a binary pump and an UV-Vis detector (o DAD??). The column used for the chromatographic separation was a Zorbax Eclipse Plus C_{18} (1.8 µm, 150 x 4.6 mm).

In order to obtain the separation of the compounds from the rosemary extracts, the flow rate used was 0.80 mL/min and the analysis was carried out at room temperature. The mobile phases used were water with 0.1 % formic acid (eluent A) and acetonitrile (eluent B). The following linear gradient was applied: 0 min, 5% B; 45 min, 100% B; 55 min, 5% B and finally a conditioning cycle of 5 min with the same conditions for the next analysis. The injection volume in the HPLC system was 10 µL. The compounds

separated were monitored with DAD, peak spectra were recorded between 190 and 450 nm.

Besides, the HPLC system was coupled with a microTOF^{TM} (Bruker Daltonik, Bremen, Germany) instrument, an orthogonal-accelerated TOF mass spectrometer (oaTOFMS), using an ESI interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA) operating in both ionization modes. When this interface is used, in order to obtain a stable spray and consequently reproducible results, the effluent from the HPLC must be splitted because the work flow in HPLC is too high. In this work, a "T" with a split ratio 1:3 was employed, so the flow was reduced from 0.8 to 0.2 ml min^{-1}.

The optimum values of the ESI-TOF source parameters were: capillary voltage, ± 4.5 kV, drying gas temperature, 190 ºC; drying gas flow, 9 l min^{-1}, nebulizing gas pressure, 2 bar and end plate offset, ± 0.5 kV. On the other hand the optimum values of transfer parameters were: capillary exit, ± 150 V; skimmer 1, ± 50 V; hexapole 1, ± 23 V, RF hexapole, 100 Vpp and skimmer 2, ± 20 V. The detection of the compounds of interest was carried out considering a mass range 50-1000 *m/z*.

External mass spectrometer calibration was performed using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, equipped with a Hamilton syringe (Reno, Nevada, USA) containing sodium formiate clusters solution (5 mM sodium hydroxide and water:2-propanol 1:1 (v/v) with 0.2% of formic acid). The calibration solution was injected at the beginning of the run and all the spectra were calibrated prior to the identification for obtaining accurate mass values due to the compensation of temperature drift in the mass analyzer.

The accurate mass data for the molecular ions were processed using the software Data Analysis 3.4 (Bruker Daltonik), which provided a list of possible elemental formulae by using the Generate Molecular Formula^{TM} Editor. This editor uses a CHNO algorithm

providing standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotopic pattern (Sigma-Value™) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions has been established at 5 ppm for most of the compounds.

3. Results and discussion

Rosemary extracts obtained by SFE and PLE were analysed by using HPLC coupled to ESI-TOF-MS in positive and negative ionization modes in order to carry out a complete characterization of bioactive phenolic compounds in rosemary. Peak identification was performed on basis of their relative retention time values, their absorption spectra in UV-visible region, their mass spectra obtained by the TOF-MS, and by using the information previously reported in the literature.

Figure 1 shows the base peak chromatograms (BPC) of the rosemary extracts SFE 150 and PLE 200 of rosemary in negative and positive ionization modes, due to these extracts are representative of each extraction procedure LAS DIFERENCIAS ESTAN TAMBIEN EN EL DISOLVENTE, EN SFE CO2 + 6.6% ETANOL Y EN PLE AGUA A 200C. TOF-MS instrumentation provides excellent mass resolution and mass accuracy in combination with true isotopic pattern, thus TOF-MS is the perfect choice for molecular formula determination using the SmartFormula™ Editor. The HPLC-ESI-DAD-TOF-MS profiles of the analysed extracts showed several peaks corresponding to different polyphenols and other polar compounds, among which 62 compounds were tentatively characterized. These compounds are summarized in **Table 1**, with their

retention time, molecular formula, *m/z* experimental and calculated, sigma value, error, UV-visible bands, ionization mode and extracts where the compounds are found. **Figure 2** (NO SERIA MAS INTERESANTE METER ESPECTROS DE MS?) summarizes the extracted ion chromatograms of the main phenolic compounds present in the extracts, whereas **Figure 3** shows the structures of these compounds. (YO QUITARIA LA FIGURA 3, ESTRUCTURAS REQUETECONOCIDAS)……..

QUIZAS SERIA MEJOR CENTRARSE EN LOS COMPUESTOS MINORITARIOS IDENTIFICADOS, YA QUE LOS MAYORITARIOS SE HAN DESCRITO EN MUCHOS TRABAJOS INCLUSO CON LOS MISMOS EXTRACTOS (REFERENCIA 31).

LA TABLA 1 HABRIA QUE COMPLETARLA CON LOS ESPECTROS UV-VIS DE TODOS LOS COMPUESTOS POSIBLES.....PARA QUE REALMENTE SIRVA COMO METODO DE IDENTIFICACION O CONFIRMACION DE LA FAMILIA DE COMPUESTOS, ES NECESARIO INCLUIR ESTOS DATOS!!!!!

ADEMAS HABRIA QUE INCLUIR UNA COLUMNA CON EL NUMERO DE PICO Y EN LA FIGURA 1, MARCAR EL NUMERO DE PICO CORRESPONDIENTE.....

The main antioxidants compounds previously identified in this herb belong to different families: phenolic diterpenes (carnosol, carnosic acid, rosmadial, rosmanol and isomers), flavonoids (genkwanin, homoplantaginin, scutellarein and cirsimaritin), and other kinds of phenolic compounds such as rosmarinic acid, a phenolic acid. [16, 31].

It is well known that carnosic acid is the strongest antioxidant compound, but the instability of this phenolic diterpene in the presence of oxygen has been demonstrated. This instability gives rise to new compounds resulting from the breakdown of carnosic acid, such as carnosol, rosmanol, epirosmanol, epiisorosmanol, rosmadial and

methylcarnosate [21]. The extraction conditions can influence the stability of carnosic acid, so the presence of this kind of compounds derived from carnosic acid could be an indicator of extreme extraction conditions. NOSOTROS EN EL TRABAJO (CITA 31) Y EN OTROS MUCHOS IDENTIFICAMOS EL CARNOSICO NO SOLO EN LOS EXTRACTOS DE SFE SINO TAMBIEN EN LOS DE PLE CON ETANOL-ADEMAS ES MAYORITARIO (EXTRACTO 4 VUESTRO) Y AGUA A 200 C (EXTRACTO 5 VUESTRO). ADEMAS, APARTE DEL CARNOSOL, AUNQUE ESTOS COMPUESTOS SE HAN DESCRITO COMO PROCEDENTES DEL ÁCIDO CARNÓSICO, SE PRODUCEN EN CIERTAS CONDICIONES AMBIENTALES Y NATURALES, QUE NO TIENEN PORQUE SER LAS DE EXTRACCION.....(POR EJEMPLO: El carnosol llegó a ser detectado como compuesto mayoritario, con un 90% respecto del total de los extractos, pero hoy se cree que su origen puede ser la oxidación del ácido carnósico durante el proceso de extracción (Schwarz K, Ternes W. Antioxidative constituents of Rosmarinus officinalis and Salvia officinalis II. Isolation of carnosic acid and formation of other phenolic diterpenes. Z Lebens Unter Fors1992;195:99). En su degradación, el carnósico puede sufrir también deshidrogenación enzimática a carnosol (Munné-Bosch S, Alegre L, Schwarz K. The formation of phenolic diterpenes in Rosmarinus officinalis L. under Mediterranean climate. Eur Food Res Technol2000;210:263-7.), seguida de ataque por radicales libres dando lugar a diterpenos altamente oxidados como rosmanol o isorosmanol (Luis JG. Chemistry, biogenesis, and chemotaxonomy of the diterpenoids of Salvia. In: Harborne JB, Tomas-Barberan FA, editors. Ecological Chemistry and Biochemistry of Plant Terpenoids. Oxford: Clarendon Press; 1991. p. 63-82.)

NO SE IDENTIFICA EN LA TABLA 1 LA PRESENCIA DE METHYL CARNOSATE....UN COMPUESTO TAMBIEN IMPORTANTE Y QUE SE ENCUENTRA EN BASTANTE CANTIDAD.....

Those breakdown compounds have been found in all the extracts, thus the carnosic acid degradation occurs in all cases. Nevertheless, carnosic acid has only been found in the extracts obtained by SFE. Therefore, we can conclude that this extraction technique is better for extracting this labile compound. NO ESTOY MUY DE ACUERDO EN ESTA AFIRMACION----------VER MI COMENTARIO ANTERIOR

Furthermore rosmaridiphenol, [9]-shogaol, hesperidin, diosmin, the isomers 5-(5,7-dihydroxy-4-oxo-4H-1-benzopyran-2-yl)-2-hydroxyphenylacetate-β-D-glucopyranosiduronic acid, anemosapogenin, augustic acid, benthamic acid, seco-hinokiol, 2,3,4,4a,10,10a-hexahydro-5,6-dihydroxy-1,1-dimethyl-7-(1-methylethyl)-, (4aS,10aR)-9(1H)-Phenanthrenone, nepitrin, 6´´-O-(E)-feruloylnepitrin and its isomer, isorhamnetin 7-glucoside, diosmetin, hispidulin, hinokione, 4´-methoxytectochrysin, salvigenin, ladanein, thymol, among others have also been detected in others studies ¿en romero? [32 (NO ESTAN DESCRITOS EN ROMERO AUNQUE LO ANALIZA....., 33, 34, 35, 36, 37, 38, 39]. HABRIA QUE HACER UNA DESCRIPCION DETALLADA CON SUS REFERENCIAS BIBLIOGRAFICAS PARA LOS COMPUESTOS MINORITARIOS.....NO SE PUEDEN INCLUIR TODOS EN EL MISMO SACO.....

On the other hand the compounds salviol, salvianolic acid A, B and L, yunnaneic acid, notohamosin B, violantin, 5-deoxylamiol, lasiodin, 12-O-methylcarnosol, 12-hydroxyjasmonic acid, and hederagenin, have been reported in another species belonging to the Lamiaceae family [40, 41, 42, 43, 44, 45, 46]. HABRIA QUE HACER UNA MEJOR DESCRIPCION DE LOS DISTINTOS COMPUESTOS QUE SE HAN

ENCONTRADO Y DONDE…....Y SI ESTAN RELACIONADOS CON ALGUNA RUTA ESPECIFICA (QUE PUEDA IMPLICAR QUE SE ENCUENTREN TAMBIEN EN ROMERO.....)

As well as galdosol, safficinolide, carnosol p-quinone, 2-hydroxy-6-(6Z)-6-tridecenylbenzoic acid, 6-O-Caffeoyl-β-D-fructofuranosyl-2(2→ 1)-α-D-glucopyranoside, dehydroabietic acid, dehydro-4-epiabietic acid and 8,11,13-abietatriene-11,12,20-triol, found in sage. [32, 47, 48].

Artepillin C, a phenolic acid contained in *Baccharis dracunculifolia*, and miltipolone, a new diterpenoid tropolone from *Salvia miltiorrhiza*, have been tentatively identified in rosemary extracts [49, 50].

The rest of the compounds have been proposed on the basis of the structures more common in the nature, such as luteolin-7-glucuronide, apigenin-7-O-glucoside, gallocatechin, luteolin-7-O-rutinoside, among others.

PERO ANTES DE CENTRARNOS EN LA DISCUSION Y LA JUSTIFICACION DE LA PRESENCIA DE ESTOS COMPUESTOS MINORITARIOS DEBEMOS CONFIRMAR CUALES SON, ES DECIR, NO PODEMOS PONER VARIAS POSIBILIDADES EN CADA PICO…....DEBEMOS SER CAPACES CON LA INFORMACION DEL MS, DEL DAD Y DE LA BIBLIOGRAFIA, DE IDENTIFICAR TENTATIVAMENTE LA ESTRUCTURA MAS PROBABLE, EN CASO CONTRARIO NO DEBERIAMOS PONER EL NOMBRE.....Y DEBERIAMOS MARCARLOS COMO NO IDENTIFICADOS (AUNQUE DEMOS LAS CARACTERISTICAS EN CUANTO A FORMULA MOLECULAR, UV-VIS, MS, ETC.....)

4. Conclusions

In this study, SFE and PLE, two environmentally friendly extraction techniques have been used in order to obtain different extracts from rosemary leaves. The comprehensive characterization of the extracts obtained by these green extraction processes has been carried out using RP-HPLC coupled to DAD and TOF-MS detection. This powerful analytical technique revealed the existence of a large number of naturally occurring bioactive compounds produced by the secondary metabolism of plants. In this work this kind of compounds has been studied in rosemary leaves extracts. Therefore, the analytical methodology used in the present work has proved to be a useful tool for the separation of a wide range of phenolic diterpenes, flavonoids, phenolic acids, among others classes of phenolic compounds present in rosemary extracts.

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