

## Superheated liquid extraction of the phenolic fraction from alperujo, a semisolid residue

Oscar F.Mijangos-Ricardez<sup>1,2\*</sup>, Jaime López-Luna<sup>1</sup>

<sup>1</sup>Instituto de Estudios Ambientales, Universidad de la Sierra Juárez, Av. Universidad s/n, Carr. Guelatao-Ixtlán, Ixtlán de Juárez, Oaxaca, C.P. 68725, (MÉXICO)

<sup>2</sup>Instituto Politécnico Nacional CHDIR Oaxaca, Hornos No. 1003. Noche Buena, Santa Cruz Xoxocotlán, Oaxaca, C.P. 71230, (MÉXICO)

E-mail: osramin@hotmail.com

### ABSTRACT

Nature has evolved overtime to produce a bewildering diversity of secondary metabolites. Based on empirical observations and folklore, natural product extracts were the first, and for a long time, the only medicines available to mankind. In this research superheated liquid extraction was applied to isolate phenolic compounds (flavonoids) from alperujo (the semisolid residue generated after extra virgin olive oil processing). Optimization of the variables influencing the extraction step (based on sequential static and dynamic modes) for alperujo and each compounds phenol family was performed by multivariate approaches in all instances. Optimization was based on test for measurement of flavonoids (aluminium chloride colorimetric assay). Under the optimal conditions the extraction kinetics were studied in order to maximize isolation efficiency for each target compound family. Separation and quantification of individual compounds from each phenol was performed by LC—DAD. Flavonoids quercetin (2.82 µg/g), myricetin (2.55 µg/g), catechin (2.23 µg/g) and epicatechin (1.98 µg/g), were identified. © 2013 Trade Science Inc. - INDIA

### KEYWORDS

LC—DAD;  
Flavonoids;  
Natural products;  
Superheated liquid extraction (SLE).

### INTRODUCTION

*Alperujo*—is a semisolid residue from the two—phase decantation process used in the olive—oil industry to obtain extra—virgin olive—oil<sup>[1]</sup>, is characterized for containing important polar and nonpolar compounds<sup>[2]</sup>; thus, a full analysis of this residue necessarily involves leaching its polar and nonpolar fractions. The extraction of the phenolic fraction from alperujo is usually carried out by stirring using methanol—water and ethanol—water mixtures at different ratios depending on the target analytes. Auxiliary energies as ultrasound have been

used to accelerate extraction<sup>[3]</sup>.

On the other hand, superheated liquids can be an attractive industrial alternative for the extraction of these compounds with two fundamental advantages over conventional techniques, namely: (a) raising the temperature above the boiling point of the solvent increases the diffusion rate, solubility and mass transfer of the compounds and decreases the viscosity and surface tension of the solvent. These changes improve the contact of the compounds with the solvent and enhance extraction, which can then be achieved more rapidly and with less solvent consumption as compared with conven-

## Full Paper

tional industrial methods. (b) The absence of light and air significantly reduce both degradation and oxidation of these compounds during extraction<sup>[4-6]</sup>. Additionally, in superheated liquid extraction (SLE), a pressure high enough to maintain the extractant in liquid state above its boiling point is applied: under these conditions the extractant polarity decreases. These facts accelerate the physical–chemical processes of cleaving of bonds, mass transfer, and solubilization of the resulting compounds, increasing the process efficiency<sup>[7]</sup>.

The aim of this research was to develop methods based on the use of superheated–liquid extractants to obtain flavonoids from alperujo for their potential use in medicine, pharmaceuticals, cosmetics and food industries.

## EXPERIMENTAL

### Samples

*Alperujo*—A mixture of alperujo from different cultivars obtained in the 2009/2010 crop season was taken directly from the production line, homogenized, aliquoted and stored at  $-20\text{ }^{\circ}\text{C}$ , until use. Prior to use, the samples were thawed and dried at  $35\text{ }^{\circ}\text{C}$  for 24 h.

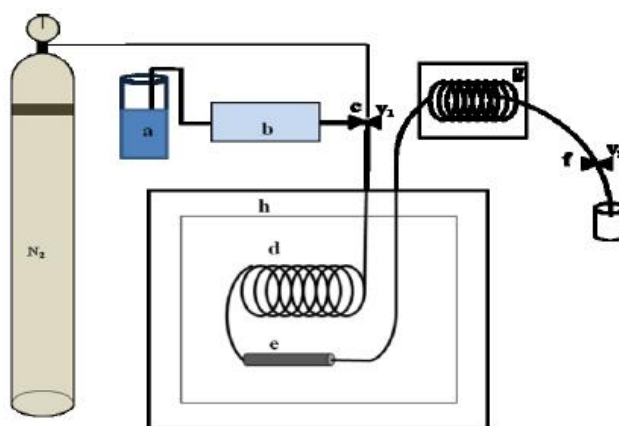
### Experimental setup

Optimization of the extraction step was developed by monitoring the evolution of the study using the test for total determination of the given family of target compounds. Then, characterization of the extracts was carried out by liquid chromatography and DAD detection. The study of alperujo consisted in optimizing the variables that affect the extraction process for flavonoids. The response variable was the total concentration of flavonoids calculated by the conventional test. Then, the separation and quantification of each compound was carried out by LC–DAD.

### Apparatus and instruments

The superheated liquid extractor, designed and constructed in the lab (Figure 1), consist of the following units: (a) an extractant container, (b) a high pressure pump (Shimadzu LD–AC10), which pumps the extractant through the system (c) a selector valve ( $V_1$ ) located after the pump, which selects the entrance of extractant or that of a liquid of gas ( $\text{N}_2$ ) stream to drag the

system, (d) a coil for preheating the extractant at the working temperature before entering the extraction chamber, (e) a cylindrical extraction chamber of stainless steel (200 mm x 10 mm id, 15–mL volume) where the plant material is placed. This chamber is closed at both ends with stainless steel cap nuts inside containing two filters in a stainless steel plate (1 mm thick, 12 mm in diameter) the purpose of which is to prevent the plant material to be dragged by the extractant, (f) a restriction valve,  $V_2$ , which maintains the optimum pressure in the system, (g) a stainless–steel cooling–coil (0.5 m long, 0.4 mm id) immersed in water at room temperature, (h) an oven (Konix, Cromatix KNK–2000) where the extraction chamber is placed and heated.



**Figure 1 :** Experimental set–up for ic–dynamic superheated liquid extraction (SLE). (a) extractant container, (b) high pressure pump, (c) selector valve ( $V_1$ ), (d) coil for preheating, (e) cylindrical extraction chamber of stainless steel, (f) restriction valve ( $V_2$ ), (g) stainless–steel cooling–coil, and (h) oven.

The absorbance of the target compounds in the extracts for the overall tests was monitored by a Thermo Spectronic, Helios gamma UV–visible spectrophotometer (Epsom, Surrey, England).

A HPLC ProStar system (Varian, Palo Alto, California, USA) consisting of a pump (ProStar 240, solvent delivery mixture, a diode array detector (ProStar 330) and an autosampler (ProStar 410) furnished with a column oven and  $20\text{ }\mu\text{L}$  sample loop for separation–quantification of flavonoids was used.

The operational variables were optimized by using the software Statgraphics plus v.5.1. for Windows (Stat Point, Inc., Herndon, VI).

### Reagents

HPLC–grade ethanol, methanol, acetonitrile, so–

dium carbonate and orthophosphoric acid were obtained from Panreac (Barcelona, Spain).

The flavonoids catechin, epicatechin, luteolin-7-glucoside and apigenin-7-glycoside were purchased from Extrasynthese (Genay, France). Myricetin, quercetin, kaempferol and rutin hydrate were from Sigma-Aldrich (St. Louis, USA). The stock-standard solution of each flavonoid was prepared at 1000 µg/mL by dissolving 0.01 g each in 10-mL methanol. The standard solutions, which contained the 8 flavonoids, were prepared by dilution of the appropriate volume of each stock solution in methanol. Solutions were stored at 20 °C in glass vials and kept in the dark until use. *O*-glycol (Sigma-Aldrich, St. Louis, USA) was used as external standard.

### Extraction method

Dried alperujo (1 g) was placed in the extraction cell of the experimental setup. The assembled cell was placed in the oven, pressurized to 10 bar by opening the selecting valve ( $V_1$ ), closing the restrictor valve ( $V_2$ ) and pumping 100% ethanol by the pump and bringing up to the working temperature (150 °C). The restrictor valve was open for short intervals while filling the cell, to avoid pressure increase. Once thermal equilibrium was reached (which usually took no more than 2 min, and was characterized for temperature and pressure stability), the conditions were maintained for 10 min (static extraction time). Dynamic extraction started by opening the inlet valve and controlling the outlet restrictor to keep the pressure at 10 bar; meanwhile the extractant was pumped for 5 min at 1 mL/min. The extract was then diluted to 25 mL in a volumetric flask to determine the total concentration of flavonoids by their respective test.

After collecting the extract, the position of the selecting valve was changed and a  $N_2$  stream purged and dried the system.

### Total flavonoids determination

Total flavonoid content was measured by the aluminum chloride colorimetric assay<sup>[8]</sup>. An aliquot of 1 mL extract or standard solution of catechin (20, 40, 60, 80 and 100 mg/L to run the calibration line) and 0.3 mL 5%  $NaNO_2$  were added to a 10-mL volumetric flask containing 4 mL distilled water. After 5 min 0.3 mL 10%  $AlCl_3$  were added. At min 6<sup>th</sup>, 2 mL 1 M

NaOH were added and the total volume was made up to 10 mL with distilled water. The solution was mixed and the absorbance measured against a blank containing the reagent mixture without extract or standard at 510 nm. The total flavonoid content was expressed as µg catechin equivalents per gram of vege material (µg CE/g).

### Chromatographic separation and quantification of flavonoids

The analytical column used was a reversed-phase Teknokroma mediterranea sea<sub>18</sub> (0.46 x 15 cm, 3 µm), the injection volume was 20 µL, and a binary gradient involved solvents A (methanol) and B (water, 0.066% phosphoric acid). The extracts were injected into the chromatograph and the elution peaks monitored at 230, 280, 325 and 350 nm (total elution time, 45 min). *O*-glycol was used as external standard, added to the extract before being injected into the chromatograph.

## RESULTS AND DISCUSSION

### Optimization of extraction method of flavonoids from alperujo

In the first multivariate screening, the experimental variables optimized were oven temperature, flow rate of the extractant and ethanol percent in the aqueous extractant.

Only one screening study was necessary for the target family of flavonoids in alperujo. The ranges within which the variables were studied are listed in TABLE 1, where also appear the optimum values for the extractant (acidified ethanol, pH 1), flow rate of the extractant (1 mL/min) and oven temperature (150 °C).

**TABLE 1 : Optimization of the superheated liquid extraction of flavonoids of alperujo.**

Variable	Range tested		Optimum value
	First design	Second design	
Oven temperature(°C)	100–150	150	150
Extractant flow rate (mL/min)	0.5–1.5	1	1
Ethanol percent in the extractant(%)	50–100	100	100
Static extraction time(min)	*	5,10,15,20,25	10
Dynamic extraction time(min)	*	5,10,15,20,25	5

\* Univariate optimization with the optimal values of the multivariate study.

## Full Paper

The influence of static and dynamic extraction was studied keeping constant at their optimal values the variables optimized by the multivariate design. First, the static extraction time was optimized fixing the dynamic extraction time at 10 min. The extraction kinetics for total flavonoids in alperujo with optimal extraction times of 10 and 5 min for the static and dynamic modes, respectively. Under these working conditions, the efficiency of the extraction, expressed as total concentration of flavonoids in alperujo, was 30  $\mu\text{g/g}$ .

Conversely, by conventional extraction by maceration with stirring for 24 h, the total concentration of flavonoids in alperujo was 15.23  $\mu\text{g/g}$ . Even though these values were obtained under the very favorable conditions of 2 g of alperujo and 50-mL extractant, the concentrations were very low as compared with those of the proposed method.

### Characterization of flavonoids extracts from alperujo

The extracts obtained under the optimal working conditions were also characterized as proposed by the International Olive Oil Council, adapted for the characterization of flavonoids in olive tissues, based on liq-

uid chromatography (LC) separation and photometric detection using a DAD<sup>[9]</sup>. Identification was based on retention times, absorption spectra and comparison with standards, while quantification was achieved by interpolation of the chromatographic area of each target analyte within the calibration curve obtained with the corresponding standard.

Figure 2 shows the chromatogram of flavonoids from alperujo. The calibration equations of the analytes identified were run using commercial standards and the peak area, according to the standard concentration for each compound. The detection limit (DL) of each analyte was expressed as the concentration that provided a signal that was  $3\sigma$  times the blank signal (where  $\sigma$  is the standard deviation of the blank signal). DLs were between 0.02 and 0.10  $\mu\text{g/mL}$  for all analytes. The quantification limit (QL), expressed as the concentration of analyte that gave a signal  $10\sigma$  times the blank signal, ranged between 0.09 and 0.35  $\mu\text{g/mL}$  for all analytes. The flavonoids quantified in the extracts from alperujo yields the following concentrations in the plant: quercetin 2.82  $\mu\text{g/g}$ , myricetin 2.55  $\mu\text{g/g}$ , catechin 2.23  $\mu\text{g/g}$  and epicatechin 1.98  $\mu\text{g/g}$ .

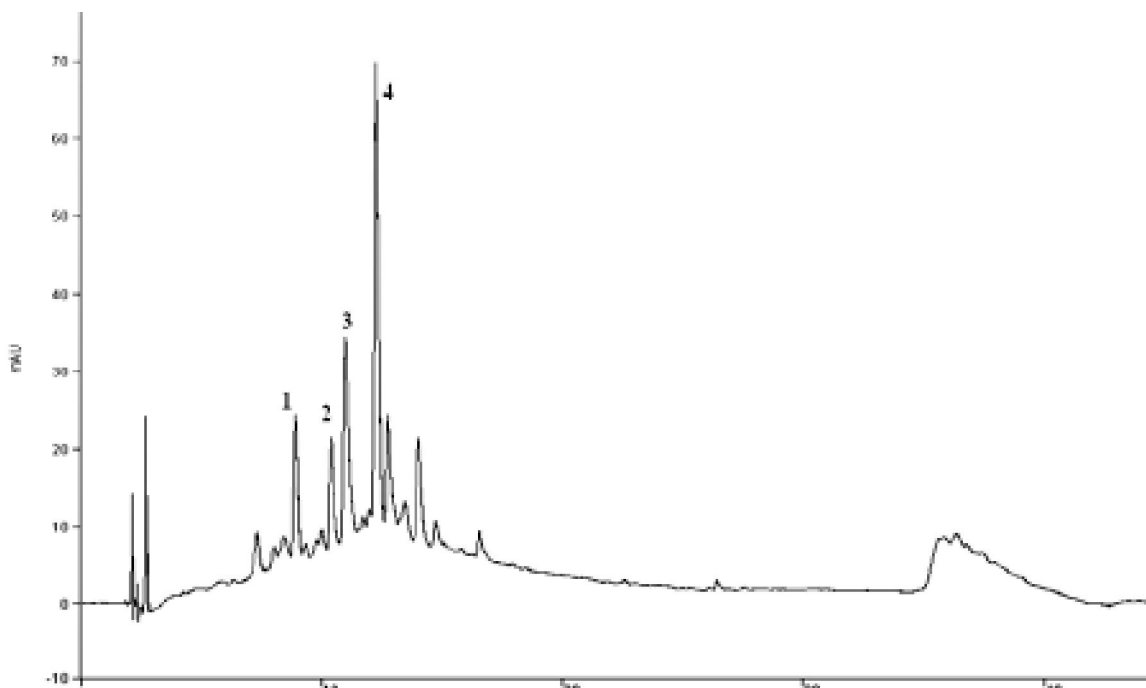


Figure 2 : LC-DAD chromatogram of flavonoids from alperujo. Identified compounds: 1, catechin; 2, epicatechin; 3, myricetin; and 4, quercetin. Monitoring wavelength, 325 nm.

Recent studies have shown that catechin, myricetin and quercetin found in green and black tea work as

antioxidants<sup>[10,11]</sup>. In fact, people who consume products rich in these flavonoids as being the major dietary

antioxidants, statistically have lower risks of cardiovascular diseases<sup>[12,13]</sup>.

### CONCLUSIONS

In alperujo, the use of superheated liquids in static–dynamic modes reduces the extraction time to 15 min (static and dynamic regimes: 10 and 5 min, respectively) compared with 24 h required by the conventional method of maceration by constant agitation for flavonoids. In addition, the efficacy of superheated liquids to extract the target compound from this, very contaminant raw material, the simplicity of the experimental design (with low acquisition and maintenance costs), the use of low–toxic ethanol–water mixtures as extractants (substituting very toxic extractants used to date such as methanol–water mixtures or hexane) and the low costs of the raw material make the industrial implementation of the proposed method advisable.

Finally, the pharmaceutical interest of compounds such as catechin, myricetin and quercetin with antioxidant and antiinflammatory properties and the need for natural dyes, opens a door for extraction from this raw material, which constitutes an interesting way for exploitation of this undesirable waste of the olive—oil production.

### ACKNOWLEDGEMENTS

O.F.M.R. thanks the Consejo Nacional de Ciencia y Tecnología (CONACyT), México, for scholarship, and the Universidad de Córdoba, Spain for technical assistance.

### REFERENTES

- [1] C.Paredes, J.Cegarra, A.Roig, M.A.Sánchez-Monedero, M.P.Bernal; *Biores.Technol.*, **67**, 111-115 (1999).
- [2] J.A.Alburquerque, J.Gonzálvez, D.García, J.Cegarra ; *Biores.Technol.*, **91**, 195-200 (2004).
- [3] J.A.Pérez-Serradilla, R.Japón-Luján, M.D.Luque de Castro; *Anal.Chim.Acta*, **602**, 82-88 (2007).
- [4] J.M.Luque-Rodríguez, M.D.Luque de Castro, P.Pérez; *Biores.Technol.*, **98**, 2705-2713 (2007).
- [5] S.Morales-Muñoz, J.L.Luque-García, M.D.Luque de Castro; *Anal.Chem.*, **74**, 4213-4219 (2002).
- [6] E.Priego-López, M.D.Luque de Castro; *Anal.Chim.Acta*, **511**, 249-254 (2004).
- [7] J.M.Luque-Rodríguez, P.Pérez-Juan, M.D.Luque de Castro; *J.Agr.Food Chem.*, **54**, 8775-8781 (2006).
- [8] D.Marinova, F.Ribarova, M.Atanassova; *J.Univ. Chem.Tech.Metall.*, **40(3)**, 255-260 (2005).
- [9] [IOC] International Olive Council; Identification and determination of phenolic compounds in olive oil, Resolution RES-4/94-/VO6, (2006).
- [10] K.E.Heim, A.R.Tagliaferro, D.J.Bobilya; *Nutritional Biochem.*, **13**, 572-584 (2002).
- [11] D.Roki, N.Menkovic, K.Savikin-Fodulovic, D.Krivokuca-Dokic, M.Ristic, D.Grubic; *J.Herb.Spices Medicinal plants*, **8(4)**, 19-27 (2001).
- [12] I.Kubo, I.Kinst-Hori, S.K.Chaudhuri, Y.Kubo, Y.Sánchez, Y.T.Ogura; *Bioorgan.Med.Chem.*, **8**, 1749-1755 (2000).
- [13] W.Xiao, L.Han, B.Shi; *Sep.Purif.Technol.*, **62**, 616-620 (2008).