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Static–Dynamic Superheated Liquid Extraction of Hydroxytyrosol and Other Biophenols from Alperujo (a Semisolid Residue of the Olive Oil Industry)

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Hydroxytyrosol and other olive biophenols (OBPs) such as tyrosol, verbascoside, apigenin-7-glucoside, and α-taxifolin have been extracted from alperujo by using static−dynamic superheated liquids. Multivariate methodology has been used to carry out a detailed optimization of the extraction. Under the optimal working conditions no further extraction of the target analytes was achieved after 27 min (up to 2800 and 1500 mg/kg of hydroxytyrosol and tyrosol, respectively), so complete removal of them within this interval was assumed. The extract was injected into a chromatograph−photodiode array detector assembly for individual separation−quantification. The efficacy of ethanol/water mixtures to extract OBPs from alperujo has been demonstrated and compared with that of a conventional stirring-based method. These less toxic extractant mixtures are of interest with a view to future human uses of OBPs.

KEYWORDS: Alperujo; olive waste; olive wastewater; hydroxytyrosol; tyrosol; verbascoside; phenols; biophenols; OBPs; superheated liquid extraction

INTRODUCTION

Alperujo, a semisolid residue from the two-phase decanter process used in the olive oil industry, has in the past years been a contamination source owing to its high content of organic substances, including sugars, tannins, phenols, polyalcohols, pectins, and lipids. The time when alperujo was dumped in evaporation pools is over as this residue is used at present as a heating source in industries and, more interesting, it is the source for providing nutraceutical industries with high-added-value olive biophenols (OBPs) to be used for human applications (*1*).

Olive biophenols are common ingredients of the Mediterranean diet, in which virgin olive oil is the principal source of fats. OBPs also comprise a major group of metabolites that display a wealth of both structural variety and diversity of important activities. Hydroxytyrosol, a well-known derivative of oleuropein, shows better results in scavenging and/or antioxidant capacity tests than antioxidants such as vitamins C and E or 2,6-di-*tert*-butyl-4-methylphenol (BHT) (*2*, *3*), which endow this biophenol with prevention effects on numerous cardiac and tumoral diseases, among others (*4*). Olive biophenols such as tyrosol (*5*), verbascoside (*6*, *7*), and α-taxifolin (*8*) provide potential health benefits demonstrated by potent in vitro activities, in addition to a synergistic effect of their antioxidant capacity when in mixtures (*9*, *10*). Recent investigations on the use of apigenin-7-glucoside (*11*, *12*) to fight against Alzheimer's (*13*) or liver diseases (*14*) support and call for continuing research on the healthy effects of OBPs.

Table 1. Optimization of the Static–Dynamic Extraction of Biophenols from Alperujo

variable	tested range: factorial design	tested range: response surface design	optimum value
temperature (°C)	150–200	200	200
extractant flow rate (mL/min)	0.5–1.5	1	1
ethanol (%)	80–100	80	80
static extraction time (min)	5–10	5–15	12
dynamic extraction time (min)	5–15	5–15	15

Laboratory-scale methods for the extraction of OBPs from olive leaves or alperujo have used extractants such as methanol/water mixtures (*15*−*17*), ethyl acetate, propanol, acetone, or acetonitrile (*15*, *17*), but the increased interest in these compounds for human uses makes mandatory the development of methods based on the use of less toxic extractants such ethanol or ethanol/water mixtures (*15*, *17*).

Superheated liquid extraction (SHLE) uses aqueous or organic solvents at a high pressure and temperature without reaching the critical point and can be implemented in three modes: static (with a fixed volume of extractant), dynamic (where the extractant flows continuously through the sample), and static−dynamic (a combination of the static and dynamic modes) (*18*). The most significant negative feature of the static mode is partition of the analytes between the solid and the fixed

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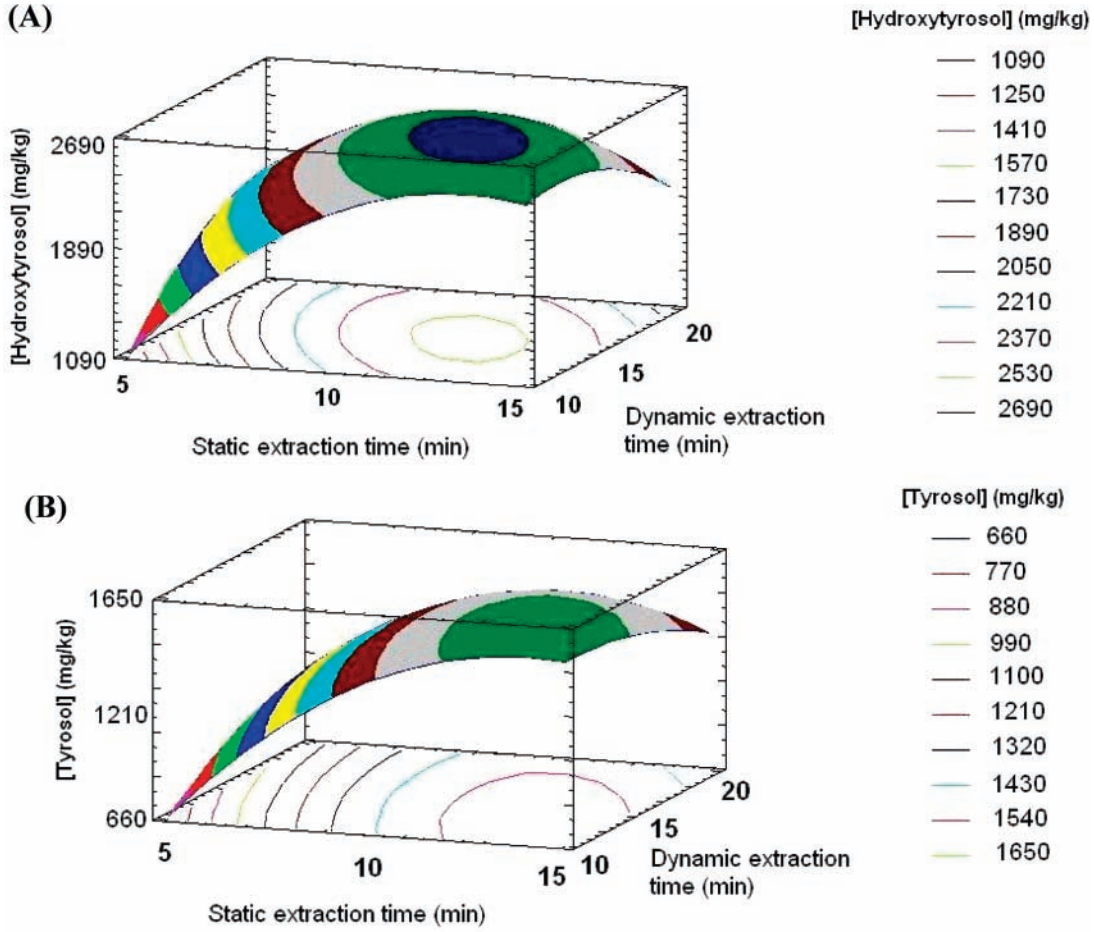

Figure 1. Static and dynamic extraction times and concentration relationship for hydroxytyrosol (**A**) and tyrosol (**B**). The maximum of the response surfaces is located at 12 and 15 min (static and dynamic extraction, respectively) for hydroxytyrosol and at 12 min for tyrosol by both extraction modes.

extractant volume, and that of the dynamic mode is dilution of the analytes in the extract, so a combination of static and dynamic modes was found to reduce the extraction time and provide better extraction efficiency (*19*, *20*). In addition, SHLE has been demonstrated to save solvents, reduce manipulation, improve selectivity, and increase automatability. All of these advantageous characteristics have allowed different analytes to be extracted from a number of samples by applying SHLE (*21*−*33*). Although SHLE has also been used to extract phenols from different matrices [e.g., apple (*34*), carob pod (*35*), and rosemary (*36*)], this is the first time this technique is applied to the extraction of phenols from alperujo.

The overall aim of this research was the development of an approach for the extraction of OBPs from alperujo assisted by high pressure and high temperature for favoring and accelerating the process using nontoxic extractants such as ethanol/water mixtures. A multivariate optimization design has been used to discover the best working conditions for extraction.

MATERIALS AND METHODS

Samples. A mixture of alperujo from different cultivars obtained in the 2005/2006 crop season was taken directly from the production line, homogenized, and stored at −18 °C until use. The samples were dried at 35 °C for 24 h prior to use.

Reagents. Ethanol, methanol, ethyl acetate, acetonitrile, and acetic acid were from Panreac (Barcelona, Spain). Eighteen microohm deionized water from a Millipore Milli-Q water purification system was used to prepare both the water/ethanol extractant mixtures and chromatographic mobile phases.

The most representative phenolic compounds in alperujo, which are commercially available, were purchased from Extrasynthese (Genay, France) in the case of tyrosol, hydroxytyrosol, apigenin-7-glucoside, α-taxifolin, and verbascoside, whereas gallic and vanillic acids were from Sigma (Madrid, Spain).

Apparatus and Instruments. The static and dynamic superheated extractions were performed using a laboratory-made extractor described elsewhere (*37*).

A mechanical electrical stirrer (Selecta, Barcelona, Spain) was used for conventional extraction, and a rotary evaporator (R-200 Büchi, Flawil, Switzerland) was used to evaporate the ethanol prior to HPLC analysis.

An Agilent 1100 liquid chromatograph consisting of a G1322A vacuum degasser, a G1315A diode array detector (DAD), and a Rheodyne 7725 high-pressure manual injection valve (20 μL injection loop) was used for the chromatographic analysis. The analytical column was a Lichrospher 100 RP-18 (250 × 4 mm i.d., 5 μm) from Análisis Vínicos (Ciudad Real, Spain). A Kromasil 5 C-18 column (15 × 4.6 mm i.d., 5 μm) protected with a steel holder, both from Scharlab (Barcelona, Spain), was also used.

Proposed Extraction Procedure. Two grams of dried alperujo was placed in the extraction cell of the experimental setup. After the cell had been assembled and located in the oven, this was pressurized with 10 bar by opening the inlet valve, closing the restrictor valve, and pumping 80:20 ethanol/water by the pump and brought up to the working temperature (200 °C); these conditions were maintained for 12 min. Dynamic extraction starts by opening the inlet valve and controlling the outlet restrictor to maintain the pressure; meanwhile, the extractant is pumped for 15 min at 1 mL/min. Therefore, the overall static−dynamic extraction takes 27 min, and around 20 mL of extract is obtained.

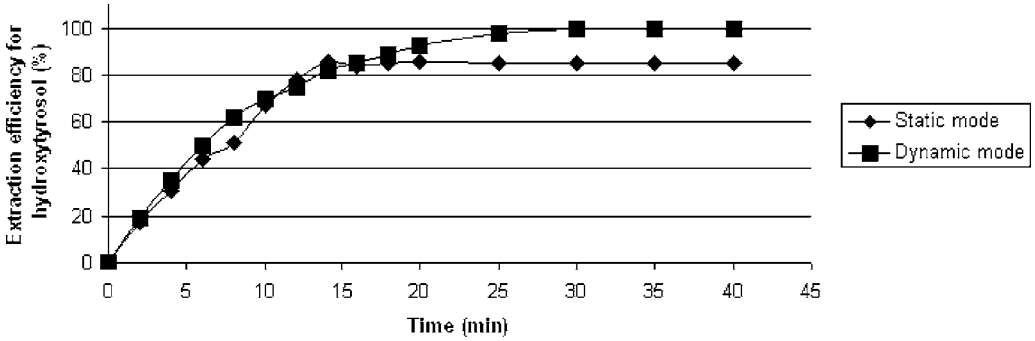

Figure 2. Extraction of hydroxytyrosol by the static and dynamic modes, separately. Other OBPs showed a similar behavior.

Conventional Extraction Procedure. Ten grams of dried alperujo and 40 mL of 80:20 ethanol/water were placed in a beaker and subjected to stirring at 25 °C for 24 h.

HPLC-DAD Separation−Detection. The elution solvents used were as follows: A (6% acetic acid, 2 mM sodium acetate, in water) and B (acetonitrile). The samples were eluted according to the following gradient: 0−19 min, 100−97% A and 0−3% B, flow rate = 0.8 mL/min; 19−27 min, 97−89.6% A and 3−10.4% B, flow rate = 0.8−1 mL/min; 27−28 min, 89.4−87% A and 10.4−13% B, flow rate = 1.0 mL/min; 28−58 min, 87−47% A and 13−53% B, flow rate = 1.0 mL/min; 58−59 min and 47−0% A and 53−100% B, flow rate = 1.0−0.8 mL/min. The chromatograms were acquired at 280, 330, 340, and 350 nm because no single wavelength is appropriate to monitor all target phenolics as they display absorbance maxima at different wavelengths in the UV region, where they show high absorption.

Table 2. Precision of the Proposed Method, Expressed as Repeatability Relative Standard Deviation (s_r) and Within-Laboratory Intermediary Precision Relative Standard Deviation (s_{WR}), for Each Analyte and Limits of Detection and Quantification, Expressed as Concentration and Mass Units

compound	s_r (%)	s_{WR} (%)	LOD (mg/kg; ng)	LOQ (mg/kg; ng)
hydroxytyrosol	8.33	12.10	1.42; 29	3.90; 77
tyrosol	5.60	8.35	1.94; 38	5.01; 100
α-taxifolin	5.13	7.38	1.61; 31	4.32; 85
verbascoside	8.27	13.11	2.30; 46	6.41; 128
apigenin-7-glucoside	2.28	18.90	3.28; 65	9.21; 185
gallic acid	3.72	4.21	1.88; 36	5.33; 106
vanillic acid	8.53	9.17	1.99; 39	5.24; 104

RESULTS AND DISCUSSION

Optimization of the Extraction Step. The five potentially more influential variables on the extraction step (i.e., temperature, static and dynamic extraction time, extractant flow rate, and extractant composition) were optimized using as response variable the extraction efficiency expressed as the peak area of each compound under the chromatographic conditions previously optimized.

A half-fraction two-level factorial design allowing three degrees of freedom and involving 16 randomized runs plus three center points was built for a screening study of the behavior of the five factors affecting the extraction process. The upper and lower values given to each variable were selected from the available data and experience gathered in the preliminary experiments; the variables tested and the optimum values obtained for each are shown in **Table 1**.

The conclusions of this screening study were that the extractant flow rate, dynamic extraction time, and extractant composition were not statistically influential factors within the ranges under study; however, better extraction efficiencies were obtained with the maximum values of the dynamic extraction time, the minimum value of ethanol in the extractant, and an intermediate value of the extractant flow rate. Thus, 15 min of dynamic extraction time, 80% ethanol, and a 1 mL/min flow rate were selected for further experiments. It is worth emphasizing that the extraction efficiencies are very similar for 80:20 ethanol/water and 100% ethanol.

Higher values of the temperature were not tested, despite its positive effect on the extraction, to avoid degradation on the target analytes; therefore, 200 °C was selected for further experiments. The static extraction time was the most statistically influential factor, and it was studied in depth (see Kinetics Studies).

Extract Treatment. The extracts obtained by the proposed method were very dense, so direct injection caused overpressure in the chromatographic column. Pretreatments consisting of drying the extract and reconstituting it with different volumes (between 10 and 20 mL) of solvents such as water, methanol, methanol/water, or ethyl acetate/water mixtures were assayed, and the best results were obtained by reconstituting the extracts with 10 mL of a 1:1 methanol/water mixture prior to HPLC analysis.

Other cleanup−concentration steps such as solid-phase extraction or liquid−liquid extraction are not recommended in the literature dealing with these analytes (*11*, *12*, *17*).

Kinetics Studies. With the aim of establishing the optimal values of both static and dynamic extraction times, two response surface central composite 2^2+star designs allowing 10 degrees of freedom and involving eight center points for hydroxytyrosol and tyrosol were constructed. The optimum values of hydroxytyrosol extraction were obtained after 12 and 15 min of static and dynamic extraction times, respectively; tyrosol required 12 min by each extraction mode. Small variations of both extraction times do not decrease extraction significantly, as can be seen in **Figure 1**.

The static and dynamic extractions were also studied independently. The kinetics study of the static mode showed that after 13 min the amounts of hydroxytyrosol and tyrosol extracted were 85% (taking the yield of the static−dynamic extraction as 100%); longer extraction times did not improve the extraction efficiency, so this can be taken as corresponding to the solid−liquid partition equilibrium under the working conditions used. In the kinetics study of the dynamic mode, 100% efficiency (also taking the yield of the static−dynamic extraction as 100%) was achieved after 27 min for hydroxytyrosol and tyrosol. The other OBPs extracted showed a behavior similar to that of hydroxytyrosol and tyrosol (the differences were never higher than 3%). The plots of the kinetics of static and dynamic extractions are in **Figure 2**. Note that the volume of extract obtained by the proposed method is lower than that provided by 27 min of dynamic extraction (16 vs 31 mL, approximately).

Table 3. Efficiency of the Proposed Method As Compared with the Conventional Method and the Proposed Approach Using Pure Water

compound	proposed method (mg/kg of dry weight)	proposed approach (water as extractant)[a] (%)	conventional method (80:20 ethanol/water as extractant)[a] (%)	conventional method (water as extractant)[a] (%)	conventional method (ethanol as extractant)[a] (%)
hydroxytyrosol	2872	100	11	26	11
tyrosol	1565	12	<LOD	<LOD	<LOD
apigenin-7-glucoside	30	93	100	<LOD	100
verbascoside	21	33	100	<LOD	100
α-taxifolin	147	100	100	<LOD	100
gallic acid	56	100	5	100	<LOD
vanillic acid	33	<LOD	22	<LOD	<LOD

[a] The amount extracted with the proposed method, expressed as mg/kg (second column), is taken as 100% efficiency of the extraction, to which columns 3–6 are referred.

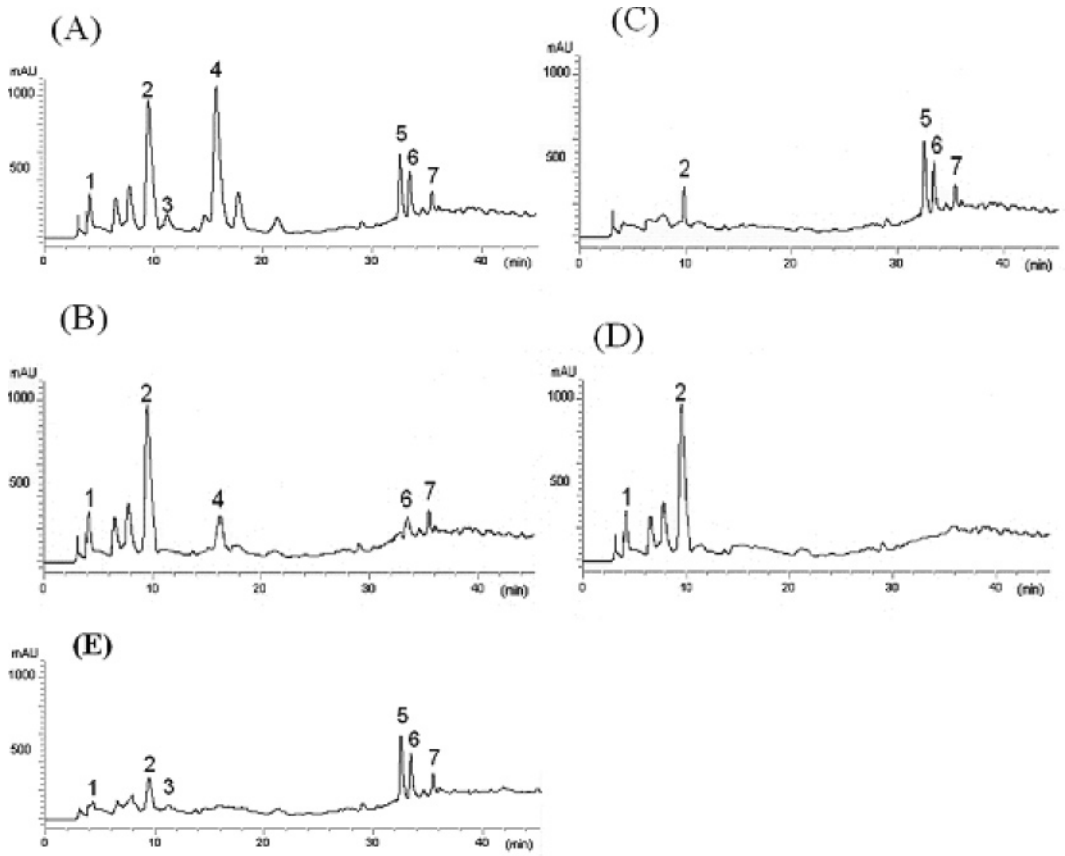

Figure 3. Chromatogram at 280 nm of an extract from alperujo (HPLC-DAD conditions given under HPLC-DAD Separation–Detection): (**A**) proposed method (see Proposed Extraction Procedure); (**B**) identical conditions as in the proposed method but changing 80:20 ethanol/water to 100% water as extractant; (**C**) conventional stirring-based extraction using 100% ethanol as extractant (see Conventional Extraction Procedure); (**D**) conventional stirring-based extraction using 100% water as extractant (see Conventional Extraction Procedure); (**E**) conventional stirring-based extraction using 80:20 ethanol/water as extractant (see Conventional Extraction Procedure). Peaks: 1, gallic acid; 2, hydroxytyrosol; 3, vanillic acid; 4, tyrosol; 5, α-taxifolin; 6, verbascoside; 7, apigenin-7-glucoside.

Determination of OBPs from Alperujo and Characterization of the Method. Calibration curves were obtained by plotting the peak area of each OBP as a function of standard concentration. All of the measurements were done within a working day. The regression coefficients ranged between 0.9970 and 0.9999 for all analytes. The limit of detection (LOD) was expressed as the mass of analyte which gives a signal that is 3σ above the mean blank signal (where σ is the standard deviation of the blank signal). The LODs obtained ranged between 1.4 and 3.4 mg/kg. The limits of quantification (LOQs), expressed as the mass of analyte which gives a signal that is 10σ above the mean blank signal, ranged from 3.9 to 9.2 mg/kg. LODs and LOQs were estimated from alperujo extracts and standard solutions of these compounds. Also, LODs and LOQs have been calculated in mass units by taking into account the volume injected into the chromatograph (see **Table 2**).

Precision of the Method. The precision of the proposed method was calculated by a within-laboratory intermediary precision and repeatability study (*38*). The extractions were carried out using 2 g of dried alperujo under the optimal working conditions. Two measurements of each compound per day were performed on 7 days. The results obtained, listed in **Table 2**, show that the repeatability, expressed as relative standard deviation, was from 2.28 to 10.18%; meanwhile, within-laboratory intermediary precision ranged from 7.07 to 18.90%.

Efficiency of the Proposed Method. Additional Studies of the Extractant. The efficacy of pure water for the extraction of phenolics from alperujo was compared with that of the 80:

20 ethanol/water mixture. With this aim, an experiment was carried out under the optimum working conditions fiven in **Table 1**, but using water as extractant.

Also, different extraction times between 1 and 48 h were tested by the conventional method (see Conventional Extraction Procedure) using ethanol or water as extractant. Extraction times longer than 24 h did not improve the extraction in any case, which was always lower than that of the proposed method.

The efficiency of the proposed extraction method (an 80:20 ethanol/water mixture as extractant) was compared with that obtained using the same approach and pure water as extractant and that provided by the conventional method using 80:20 ethanol/water and pure water or ethanol, and the results for the quantifiable OBPs, taking as 100% the amount obtained by the proposed method, are listed in **Table 3**. **Figure 3** shows the chromatogram of the extract obtained in each case.

It is worth emphasizing that no degradation of the target analytes occurs under the optimal working conditions as no new peaks appear in the chromatogram from the extracts obtained by the proposed method as compared with that from an extract obtained by the conventional method based on stirring at 30 °C.

Other OBPs such as oleuropein, apigenin, luteolin and luteolin-7-glucoside, rutin, syringaldehyde, vanilline, catechin, *trans*-cinnamic acid, *p*-hydroxyphenylacetic acid, gentisic acid, ferulic acid, tannic acid, 3,4-dimethoxybenzoic acid, *o*-coumaric acid, *p*-coumaric acid, quercetin, caffeic acid, and protocatechuic acid, if present in the extracts, were below their detection limits.

The higher extraction efficiency obtained by the proposed method as compared with the conventional one can be attributed to the higher capacity to disrupt matrix−analyte interactions of the former due to the high temperature and pressure. Moreover, the surface tension and the viscosity of the extractant are reduced by increased temperature (keeping elevated pressures to maintain the solvent in the liquid state), leading to improved sample wetting and matrix penetration, thus increasing mass transfer and enhancing extraction (*18*).

The efficacy of ethanol/water mixtures to extract OBPs from alperujo has been demonstrated, so these mixtures can replace the very toxic extractants used to date. In this way, this research opens a door to a further extension of the extraction process to industrial exploitation of alperujo BPs for human uses, a growing present trend.

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