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A dispersive liquid – liquid aerosol phase extraction method has been applied optimized and validated for the determination of polyphenols and metals in extra virgin olive oils.

Total polyphenol content and metals determination in Spanish virgin olive oils by means of a dispersive liquid-liquid aerosol phase extraction method and ICP-MS

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Abstract

This report presents a study on the determination of total polyphenols together with metals in several samples of olive oil produced in Spain. The results provided by applying a conventional extraction method were compared against those encountered by means of the so-called Dispersive Liquid-Liquid Aerosol Phase Extraction method. The novel method is based on the dispersion of the extracting solution in the sample. To accomplish this, an aerosol is pneumatically generated and directed against the surface of the oil sample. The aerosol was generated in order to increase as much as possible the surface area of the interface between the two involved non-miscible phases. As a result, the partition equilibrium was quickly achieved. The critical variables dictating the characteristics of the obtained aerosols as well as those influencing the partition equilibrium state were studied. With the aerosol phase extraction method, the values corresponding to the total polyphenols and metals in real samples were not statistically different as compared to those obtained by the conventional liquid – liquid extraction method. The new method provided shorter extraction times and lower mass of consumed reagents than the conventional one, thus giving rise to a more environmentally friendly method. For polyphenols, calculated limits of detection and quantification were 0.48 and 1.5 mg of gallic acid kg⁻¹, respectively. The absorbance linearity, in turn, was kept from 0 to 50 mg kg⁻¹ ($R^2 = 0.998$). In the case of metal and metalloid quantification, the limits of detection found with a sample digestion method ranged from 1.3 (Cu) to 291 (Na) ng mL⁻¹. Meanwhile, because of the lower dilution factor, this parameter was one order of magnitude lower when these elements were extracted according to the new method. The new extraction method was applied to the analysis of 42 extra virgin olive oils both bottled and directly collected from the oil press, containing single cultivars or blends and produced from different areas. Fresh oils

were analyzed and a preliminary study on the oil thermal degradation was also done. Dispersing the extracting solution as an aerosol into the sample can be considered a versatile method able to provide extensive oil chemical information in a rapid way what is especially important in the case of polyphenols.

Keywords

Dispersive Liquid-liquid Aerosol Phase Extraction; Polyphenols; Metals; Metalloids; Olive Oil; Inductively Coupled Plasma Mass Spectrometry

1. Introduction

One of the most prominent ingredients included in the Mediterranean diet is extra virgin olive oil (EVOO) which is characterized by its health benefits [1] as well as organoleptic properties [2]. Polyphenols, precluding both antioxidant as well as characteristics such as bitterness, astringency and pungency [3], can be useful to differentiate among high-quality EVOOs [4]. It is interesting to notice that only around 10% of commercially available EVOOs have a suitable phenolic content for the application of the health claims [5]. It is widely accepted that high quality oil is obtained by early harvesting the olives and carefully controlling the variables involved in the oil extraction process (exposure to oxygen [6], centrifugation process [7] and temperature of the olive paste [8,9]).

A common and critical step in most of the proposed methods for polyphenols measurement in olive oil is the preliminary sample preparation. Described EVOOs pre-treatment approaches include: (i) hydrolysis of the oil polar fraction [10]; (ii) supercritical fluid extraction [11]; (iii) liquid-liquid extraction, LLE; and, (iv) solid-phase extraction, SPE, using C₈, C₁₈ [12] or amino [13] sorbents.

Extraction techniques have advantages related to the simplicity of the methodology. Particularly, LLE provides better recoveries than SPE [14]. In order to isolate the oil polar fraction, the EVOO sample, previously diluted with a non-polar solvent (*e.g.*, hexane, petroleum ether or chloroform), is mixed with several portions of water : methanol [15], N,N-dimethylformamide [16] or tetrahydrofuran : water [17]. Once obtained, the extract is analyzed, the most common method being absorbance measurement after reaction of the polyphenol hydroxyl functional groups with the Folin-Citocalteu reagent [18]. This method gives an indication of the total phenolic groups and the antioxidant capacity of the olive oil extracts [19,20]. The main

drawbacks of this straightforward protocol are its low specificity and the dependence of the measured absorbance on the structure of the phenolic molecule.

Another important group of species that affect the EVOO oxidation degree are trace metals [21]. They may originate from the soil and atmospheric contamination, incorporation of fertilizers, pesticides and from the instruments used during the oil production and storage. Inductively coupled plasma mass spectrometry (ICP-MS) has been largely recommended in order to determine the elemental concentration in oil samples. [22]. However, the direct introduction of organic samples in the ICP-MS instrument leads to spectral and non-spectral interferences as well as the formation of soot deposits in the torch and the interface [23,24]. In order to overcome these problems, oil pre-treatment methods have been proposed such as microwave – assisted acid digestion in closed high – pressure vessels [25].

Classical LLE methods require a large volume of sample and reagents for the elemental analysis and they are, in some instances, inefficient. A good approach leading to the separation of the elements of interest from the matrix is acid LLE assisted by ultrasounds [26]. Schiff bases have been proposed as possible metal chelating agents [27]. In the last times, a new Aerosol Phase Extraction (DLLAPE), initially called Aerosol Phase Extraction (APE) has been applied to the analysis of water [28] and biodiesel [29]. Basically, the DLLAPE belongs to the dispersive liquid – liquid extraction category [30] but, in this case, one of the involved phases is nebulized over the other phase and, hence, it is not necessary to use a dispersing agent. Comparatively speaking, the DLLAPE method shortens the extraction time by more than one order of magnitude with respect to a conventional LLE method. Moreover, it lowers the total amount of reagents consumed and provides good accuracy. These advantages are based on the high surface area of the liquid – liquid interface (on the order of $1 \text{ m}^2 \text{ per cm}^3$).

Furthermore, the new aerosol phase extraction assay can be easily automatized and implemented in a routine laboratory.

So far, the DLLAPE has been applied to the determination of molybdenum [28] and Na, K, Ca and Mg [29] through ICP-OES. Up to date, neither the determination of organic components nor the application of this method to olive oil analysis has been performed. Therefore, the goal of the present study was to develop, for the first time, an extraction method based on the DLLAPE principle to extract separately total phenolic compounds and metals from virgin olive oil samples. The extraction method was first optimized for the determination of each one of these species and, then, it was compared with conventional extraction protocols. Finally, under optimized conditions, the DLLAPE was applied to the analysis of several EVOOs and to a preliminary study of oil thermal degradation.

2. Materials and methods

2.1. Reagents and samples

Ultrapure water supplied by a three-step ion-exchange system, Milli-Q, fed by reverse osmosis, Elix 3, both from Millipore (El Paso, TX) was used throughout. Methanol analytical grade (Panreac, Barcelona, Spain) was also selected to prepare the extracting solutions. HNO_3 65%, Suprapur[®] (Merck, Darmstadt, Germany) was added to the metals extracting solutions. Standards of gallic acid (Merck) were prepared by proper dilution. Sodium carbonate (analytical grade, Panreac) was used to basify the extracted solutions prior to the addition of the Folin-Citocalteu reagent (Merck) in the assay to determine the total polyphenol content.

An ICP multielement standard solution (Merck IV) containing 1000 mg of element·L⁻¹ was used to prepare the standards by serial dilution. Stock and standard solutions were preserved in 2% (v/v) HNO₃.

A total of 42 samples were studied containing Manzanilla, Alfafara, Blanqueta, Arbequina, Genovesa, Villalonga, Picual and Changlot Real cultivars. Twenty-three of these EVOOs were mono varietal, whereas the remaining ones were obtained through blending some of the aforementioned varieties. Some of them were bottled whereas others were directly taken from the oil presses. In the second case, the EVOO was obtained from green olives not subjected to heating during the oil production process and stored under controlled oxygen conditions. Once in the laboratory, the samples were preserved in a closed dark medium in order to prevent the oxidation of polyphenols. Table 1 contains information about the codification of the olive oils studied together with their varietal composition.

Table 1. Extra virgin olive oil samples analyzed in the present work.

Oil code	Varieties contained	Origin
1	Blanqueta	Oil press
2	Blanqueta	Oil press
3	Blanqueta	Bottled
4	Blanqueta	Oil press
5	Blanqueta	Oil press
6	Blanqueta	Oil press
7	Blanqueta	Oil press
8	Blanqueta	Bottled
9	Blanqueta	Oil press
10	Alfafara	Oil press
11	Alfafara	Bottled
12	Alfafara	Bottled
13	Alfafara	Bottled
14	Manzanilla	Bottled
15	Manzanilla	Oil press
16	Manzanilla	Bottled
17	Arbequina	Bottled
18	Arbequina	Bottled
19	Picual	Bottled

20	Picual	Bottled
21	Picual	Oil press
22	Changlot Real	Bottled
23	Villalonga	Bottled
24	20% Blanqueta 35% Manzanilla 45% Alfafara	Oil press
25	20% Blanqueta 40% Manzanilla 40% Alfafara	Oil press
26	50% Cornicabra 50% Blanqueta	Bottled
27	70% Manzanilla	Oil press
28	95% Blanqueta 5% Manzanilla	Oil press
29	59% Blanqueta 33% Alfafara 8% Manzanilla	Oil press
30	65% Blanqueta 16% Alfafara 19% Manzanilla	Oil press
31	43% Blanqueta 23% Alfafara 34% Manzanilla	Oil press
32	52% Blanqueta 30% Alfafara 18% Manzanilla	Oil press
33	27% Blanqueta 45% Alfafara 28% Manzanilla	Oil press
34	15% Blanqueta 10% Alfafara, Genovesa y Arbequina 75% Manzanilla	Oil press
35	20% Blanqueta 10% Alfafara, G y Arbequina 70% Manzanilla	Oil press
36	Alfafara + Blanqueta	Oil press
37	Manzanilla, Villalonga, Arbequina, Blanqueta, Changlot Real, Alfafara and Genovesa	Bottled
38	Manzanilla, Villalonga, Arbequina, Blanqueta, Changlot Real, Alfafara and Genovesa	Bottled
39	Manzanilla, Villalonga, Arbequina, Blanqueta, Changlot Real, Alfafara and	Bottled

	Genovesa	
40	Blend	Bottled
41	Blend	Oil press
42	Blend	Oil press

2.2. Conventional procedure for determining polyphenols

The reference method for the determination of total polyphenols was inspired in procedures described elsewhere [31,32]. A first LLE of the oil polar fraction was performed. 1 g of oil was weighed with a 0.1% precision balance. Then, 1 mL of hexane was added to lower the viscosity of the sample. The diluted sample was mixed with 1 mL of a 50% (v/v) methanol solution using pure water as a solvent and the resulting mixture was manually agitated for 2 min. Once this step was accomplished, the mixture was placed on a platform shaker (Heidolph Promax 1000, Schwabach, Germany) for 30 min at 150 rpm and finally centrifuged at 3000 rpm for 10 min. The water : methanol extract was separated from the rest of the matrix and preserved in a closed polyethylene tube. The remaining oily fraction was re-extracted two more times and the fractions were recovered in the previously closed tube. The total volume of the extract was led to 10 mL with the methanolic solution. 0.4 mL of sodium carbonate 2.5% were added to basify the solution followed by the addition of 0.2 mL of the Folin-Citocalteu reagent, FCR. The mixture was kept in the dark for 30 min at room temperature. The absorbance of the mixture was measured at 760 nm with a Thermo Fisher Scientific spectrophotometer (OrionTM AquaMate 7000 Vis, Valencia, Spain).

2.3. Conventional procedure for metals determination

In order to evaluate the efficiency of the DLLAPE method, a conventional protocol was applied that consisted of the olive oil microwave sample digestion in PTFE reactors. A Milestone oven (Sart D, Sorisole, Italy) was used. 0.5 g of oil were poured

into PTFE reactors together with 7 g of nitric acid and 0.5 g of ultrapure water. The reactors were sealed and adapted to the oven carousel. Sample digestion was performed at a 200°C temperature and 75 bar maximum pressure for 30 min. To achieve these conditions, the microwave power was set at 1500 W. Once the samples were digested, the solutions were allowed to cool down for 30 additional minutes and they were subsequently diluted with water up to 10 mL. All these experiments were done by triplicate.

The digested solutions were analyzed by means of an ICP-MS instrument (Agilent, 7700x, Santa Clara, California, USA). Internal standardization was applied to compensate for possible ICP-MS signal drift. Table 2 summarizes the instrumental operating conditions.

Table 2. ICP-MS Agilent 7700x operating conditions.

Variable	Value
Plasma gas /L min ⁻¹	15.0
Auxiliary gas/ L min ⁻¹	0.9
RF power/W	1600
Sampling depth/mm	10
He Collision cell flow rate/ mL min ⁻¹	4.3
OctP Bias/V	-18
Oct RF/V	200
Energy discrimination/V	3.0
HMI System Conditions	
Ar HMI flow rate/ L min ⁻¹	0.56
Measured nuclides	
²³ Na; ²⁴ Mg; ²⁷ Al; ³⁹ K; ⁴³ Ca; ⁵² Cr; ⁵⁵ Mn; ⁵⁶ Fe; ⁶⁰ Ni; ⁶³ Cu; ⁶⁶ Zn; ⁷⁵ As; ⁸⁷ Se; ⁸⁸ Sr; ⁹⁵ Mo; ¹⁰⁷ Ag; ¹¹¹ Cd; ¹¹⁸ Sn; ¹²¹ Sb; ¹³⁷ Ba; ²⁰⁸ Pb	

2.4. Aerosol Phase Extraction (DLLAPE) procedure

The experimental setup required to perform the DLLAPE has been described elsewhere [28,29] and is depicted in Figure 1. In this figure, the processes favoring the extraction of the analytes are also schematized. First, droplets having a given initial velocity penetrate into the sample and, second, the gas stream leaving the nebulizer causes agitation of the mixture that generates turbulences promoting the agitation of both phases.

Figure 1. Experimental setup of the DLLAPE together with the involved processes (a) and detailed picture showing the extraction with a water : methanol mixture (b).

In the present study, 1 g of oil was weighed in a 5 mL polypropylene tube, 1 mL of hexane was added and the tube weighed again. The nebulizer gas and liquid flow rates were adjusted at desired values by using a mass flow controller (58505, Brooks Instruments, Hatfield, PA, EEUU) and a peristaltic pump (Perimax, Spetec, Ending, Alemania), respectively. The extracting solution was aspirated for a measured period of time. Once this solution reached the nebulizer (Model TR-30-A2, Meinhard, Golden, CO, EEUU) it was dispersed into fine droplets that were directed against the surface of the solution contained in the plastic tube. When the nebulization ceased, the tube containing the sample, hexane and the emulsified extracting solution was weighed in order to measure the mass of extracting solution actually trapped by the sample. The tubes were left stand for 30 s. Afterward, the methanolic solution, deposited at the bottom of the extraction tube, was taken with a pipette and inserted into a new open polypropylene tube.

Slight differences between the procedures followed for the determination of polyphenols and metals should be pointed out. First, the extracting solution used for

polyphenols determination only contained water and methanol. Meanwhile, nitric acid was added to promote the migration of the metallic species from the oil sample to the extracting solution containing methanol. Second, the extracts were diluted with water : methanol prior the polyphenols measurement, whereas for metals, a different scheme was selected. In order to avoid plasma degradation caused by the presence of methanol, the samples were allowed to evaporate at 40°C in a laminar extraction hood in order to avoid external contamination from the air. Then, the residues were re-dissolved with 2 mL of a 2% nitric acid aqueous solution and, finally, analyzed through ICP-MS.

3. Results and Discussion

A general comment should be made regarding the mass of extracting solution to sample mass ratio, r . As it will be demonstrated in this section, in the present work, the optimum ratio was close to 1. In the case of polyphenols, the high sensitivity of the measurement method, the narrow dynamic range and the high concentration of polyphenols in the oil samples, made it necessary to dilute the sample and, thus, low values of r could cause a saturation of the absorbance obtained. For the determination of metals, an evaporation step was applied and, thus, the r – value was not relevant from the point of view of detection power.

Furthermore, it is worth to notice that the main goal of the present work was to test the DLLAPE method by comparing the results with those provided by conventional procedures. Note that the detection methods used for the determination of polyphenols and metals were the same in both cases.

3.1. Determination of polyphenols

According to some literature reports, the FCR overestimates the actual concentration of polyphenols, since other species such as ascorbic acid or additional organic acids also yield the typical blue complex. For this reason, it has been claimed that this method is an indication of the total antioxidant (or reducing) capability of an EVOO [33].

In order to assess the extent of these effects that could be attributed to the matrix, an experiment was carried out in which the concentration found according to the external calibration procedure was compared against that measured when applied a standard additions procedure. Oil with ref. #14 was selected, because its total phenolic content was a good average of all the samples analyzed. The obtained concentrations were 192 ± 15 and $176 \pm 12 \text{ mg kg}^{-1}$, respectively. The slopes ($\pm 3 \sigma$) of the calibration line obtained for a set of four standards going from 5 to 40 mg kg^{-1} and the corresponding line obtained when applied the standard additions method were not significantly different (*i.e.*, 0.0110 ± 0.0004 and 0.0104 ± 0.0004 , respectively). It was thus concluded that, for olive oil samples, the matrix had a slight influence.

Another problem associated with the Folin-Citocalteu assay lies in the poor specificity. In fact, several purification methods have been proposed to improve this attribute [34]. Therefore, the absorbance was measured after the addition of the Folin-Citocalteu reagent in presence and in absence of sodium carbonate. According to the literature, under acidic conditions, some matrix constituents could react with the polyphosphotungstate reagent thus giving rise to a blue colored solution. The experimental data revealed that, for the oil with reference #14, absorbance was 0.042 ± 0.002 , whereas it increased up to 0.232 ± 0.003 upon the addition of sodium carbonate. In other words, the absorbance caused by the matrix compounds extracted from oils was 18% of the total measured absorbance. This result could reveal a significant lack of

specificity. However, the same experiment was performed for a standard and it was verified that the absorbance measured in absence of sodium carbonate was 8% of the absorbance measured in its presence. Therefore, it could be concluded that the matrix components only varied the absorbance by a 10% what, taking into account the confidence intervals supplied by the used method, suggested an acceptable specificity. In any case, the method based on the use of the Folin-Citocalteu reagent could be considered as to be useful for providing good indication of the antioxidant capability of a particular olive oil and not merely of the polyphenol content.

Finally, a comparison between the visible spectra for the standards and the oil samples revealed no differences in terms of absorption maxima thus providing additional proofs on the specificity of the assay.

3.1.1. Preliminary considerations with the DLLAPE method

A point that had to be initially considered was that, once the aerosol was generated at the nebulizer nozzle, solvent evaporation from the droplets surface begun and, hence, a fraction of the extracting solution did not enter in contact with the sample. Furthermore, the argon stream also promoted the evaporation of a fraction of the hexane added to the oil sample. An experiment was performed in order to characterize the total mass evaporated. Thus, for a 1 min extraction period of time, it was found that 0.2 out of 0.8 g of hexane left the diluted oil. Meanwhile, around 30% of the methanol : water aerosol evaporated before it reached the sample surface and was not available for extracting polyphenols. All these losses were compensated for by weighing the tubes with their contents before and after the extraction step.

Another important point was related to the role of methanol in terms of its effect on the characteristics of the aerosols generated by the nebulizer. The addition of methanol

causes a decrease in the solution surface tension and viscosity thus leading to the generation of finer aerosols as compared to water [35]. Thus, for instance, working at 0.6 L min^{-1} and 1.2 mL min^{-1} nebulizer gas and liquid flow rate, respectively, the aerosol liquid volume contained in droplets with diameters lower than $2 \text{ }\mu\text{m}$ was two times higher for methanol than for water. Therefore, the addition of this alcohol promoted the generation of fine droplets and the extraction of the analytes of interest.

3.1.2. Optimization of the DLLAPE

The variables studied were those affecting the characteristics of the aerosols generated by the pneumatic nebulizer (*i.e.*, liquid and gas flow rates) as well as those having an impact on the partition equilibrium (*i.e.*, the mass of extracting solution and methanol concentration, nebulizer tip – sample surface gap, d in Figure 1, and extraction time).

The liquid flow rate was first varied for a given extraction time (*i.e.*, 1 min) and it was found that the determined polyphenol concentration steeply increased from 0.35 to 0.87 mL min^{-1} . This was clearly due to the increase in the mass of the extracting solution. According to previous studies, it was found that, for a given mass of extractant, the liquid flow rate had a detrimental effect on the extraction yield because of the generation of coarse aerosols with the concomitant decrease in the total aerosol liquid surface [29]. In the present work, this problem was circumvented by setting the extraction time at 1 minute, because it was sufficient to perform the efficient extraction of polyphenols from EVOOs. In fact, it was found that above 0.7 g of 50% methanol, the extraction efficiency remained constant.

As regards the nebulizer gas flow rate, an increase in this variable had two beneficial consequences: first, the aerosols became finer and, second, the eddy

formation in the sample during the extraction was favored (see Figure 1). However, for excessively high values of this variable, it was found that a fraction of the liquid contained in the polyethylene tubes was expelled and the extraction was inefficient. An additional negative effect of increasing the nebulizer gas flow rate was related to the intensification of solvent evaporation that also contributed to the degradation of the extraction yield. Indeed, by weighing the tube before and after the extraction, it was found that the mass of extracting solution decreased from 0.60 g to 0.38 g as the gas flow rate increased from 0.1 to 0.4 L min⁻¹. This phenomenon had to be corrected for by using, in the calculations, the mass of methanol : water solution actually available to perform the extraction. By applying a correction factor, it was observed that the extraction efficiency increased as the gas flow rate went up from 0.1 to 0.4 L min⁻¹. Therefore, a 0.3 L min⁻¹ gas flow rate was taken as the optimum value of this parameter in order to avoid sample ejection from the extraction tube. At this flow rate, the normalized gallic acid concentration was around 0.95.

Regarding the methanol concentration, it was realized that the extraction yield increased from 10 to 30% methanol content and then reached a plateau. According to previous reports, it was indicated that 80% of methanol concentration provided the highest polyphenols recoveries [36]. Meanwhile, others mentioned the formation of emulsions in the presence of water in the extracting solution and, thus, neat methanol was suggested as the best suitable extracting agent [37]. These results were in clear contrast with those encountered when the DLLAPE was applied. It was concluded that, since an aerosol was formed, the liquid – liquid interface area was higher than when conventional procedures were applied. Therefore, less methanol was necessary to achieve an efficient extraction. The methanol content finally selected was 50%.

Another studied variable was the distance between the nebulizer tip and the surface of the sample (see d in Figure 1). It was observed that, because of the action of the gas stream, a decrease in this parameter below 15 mm led to the projection of a sample fraction towards the walls of the tube thus degrading the precision of the measurement. Furthermore, it should be mentioned that, as the extraction proceeded, the level of the sample raised virtually reaching the tip. This fact disturbed the nebulization process itself. In contrast, above 15 mm, the extraction efficiency went down. Because of the conical geometry of the aerosol, if the nebulizer was excessively recessed with respect to the sample, a fraction of the generated droplets impacted against the tube walls before entering in direct contact with the sample. For these reasons, 15 mm corresponded to the optimum d value.

Finally, experiments were carried out with and without the addition of hexane to the oil samples. It was found that the determined total polyphenols were similar irrespectively of the use of this solvent. Previous studies have described the same results for a conventional method [36]. However, the addition of hexane promoted the separation of the phases once the extraction step was completed. For this reason, 1 mL of hexane was added to all the analyzed samples.

The optimum operating conditions for the determination of polyphenols are summarized in Table 3. A comment should be made concerning the mass of extracting solution. As it may be observed, the optimum amount of methanolic solution was slightly lower than the mass of olive oil. This gave rise to a moderate analyte enrichment factor. This was appropriate to determine polyphenols, because their concentrations were high and hence the sensitivity of the spectrophotometer was strong enough to perform these determinations.

Table 3. Optimum operating conditions for the determination of polyphenols and metals through the DLLAPE method.

Variable	Polyphenols determination	Metals determination
Extraction time/ s	60	90
Methanol concentration in the extracting solution/ % methanol:water (w/w)	50:50	50:48
Nitric acid concentration in the extract/ %	---	2
Mass of extracting solution/ g	0.7	0.9 – 1.2
Hexane added/ mL	1.0	
Nebulizer tip – sample level gap, d / mm	15	
Nebulizer liquid flow rate/ mL/min	0.9	
Nebulizer gas flow rate/ L/min	0.3	

3.1.3. Method validation

The obtained calibration line when applying the Folin-Citocalteu assay ($R^2 = 0.998$) corresponded to the following equation:

$$A = (0.0110 \pm 0.0004) \text{ Concentration} + (2.122 \pm 0.002) \times 10^{-3} \quad (1)$$

The limit of detection was calculated according to the $3 \sigma_b$ criterion, where σ_b corresponded to the standard deviation of the intercept of the calibration line ($n=4$). A value of 0.48 mg of gallic acid kg^{-1} was finally obtained. The limit of quantification, in turn, took a value of 1.5 mg of gallic acid kg^{-1} .

The precision of the DLLAPE for the determination of polyphenols was based on twenty consecutive replicates of the same sample. It was observed that the RSD of the measurements took a value of 8% that corresponded to an acceptably repeatable method.

In order to test for accuracy, the DLLAPE was compared against the conventional method (Section 2.2). Table 4 gathers the polyphenol concentrations found for 10

different oil samples together with the confidence intervals. In general terms, it may be indicated that there were not significant differences between the concentrations found by applying the two methods.

Further tests for trueness were undertaken and the IUPAC³⁸ and Eurachem^{39,40} criteria were applied. Nonetheless, as there was not any CRM available for polyphenols in EVOO, the data provided by the reference method were taken as target values.

For a given sample, the difference between the concentration found by applying each one of the methods (Δ_m) was calculated according to:

$$\Delta_m = |C_{polyphenols\ APE} - C_{polyphenols\ conventional}| \quad (2)$$

Besides, the uncertainty, u_Δ , was calculated from the standard deviations measured both for the DLLAPE and the conventional method by means of:

$$u_\Delta = \sqrt{s_{APE}^2 + s_{Conventional}^2} \quad (3)$$

Finally, for a 95% confidence level, the combined uncertainty of the DLLAPE and conventional methods, U_Δ , corresponded to the u_Δ value multiplied by a coverage factor that, in our case, was 2 [41]. Then, U_Δ was compared with Δ_m and it was verified that, for virtually all the evaluated samples, $U_\Delta < \Delta_m$ (Table 4), thus revealing that there were no statistically significant differences between the results provided by both methods.

Table 4. Comparison of the polyphenols content (expressed as mg of gallic acid equivalents kg⁻¹ of sample) found with the conventional method and the DLLAPE.*

Olive oil sample	Conventional method	DLLAPE	Δ_m	U_Δ	U_{DLLAPE} (%)
1	193±13	155±36	38	60	3.26
4	187±9	206±22	19	38	3.32
7	227±27	195±15	32	49	2.75
10	132±11	148±11	16	24	4.74
17	173±19	209±10	36	37	3.69
21	198±5	242±25	44	47	3.98
22	115±13	117±10	32	25	5.47
24	164±7	171±14	7	25	3.85
27	243±16	218±8	25	28	3.23
29	214±11	240±19	26	34	2.94

*Confidence intervals have been calculated according to: $\pm \frac{t s}{\sqrt{n}}$ where n=5 and t for a 95% confidence level was 2.015.

The expanded uncertainty, U, for a coverage factor (k) of 2, was determined by considering the contribution of calibration (u_{cal}), repeatability (u_{rep}) and trueness (u_t) according to:

$$U = k \sqrt{u_{\text{cal}}^2 + u_{\text{rep}}^2 + u_t^2} \quad (4)$$

Table 4 summarizes the relative expanded uncertainty, U(%), obtained for the DLLAPE. It was verified that, for all the evaluated samples, the major source of uncertainty was calibration. Trueness and repeatability only had a marginal influence corresponding to a percentage of the total uncertainty from 0.3 to 5%.

3.1.4. Analysis of real samples

This section is aimed at demonstrating the applicability of the DLLAPE method to the analysis of real EVOO samples and performing a preliminary test on oils thermal degradation.

The acidity of the samples was determined (Figure 2) and the obtained values were lower or very near to 0.2 g oleic acid/100 g oil indicating that all of the tested samples complied with this condition for high quality EVOOs. Only for one of the samples, this parameter surpassed the critical value (0.8 g oleic acid/100 g oil), although it was declared in the label.

The analyzed samples provided gallic acid concentrations included within the 93 to 375 mg gallic acid equivalents kg^{-1} range. These values were in full agreement with those previously reported in the literature for similar oils [42]. Figure 2 shows the data obtained for all the analyzed samples. Among the monovarietal oils, samples labelled as #18 and #22 had the lowest values of gallic acid concentration. These two EVOOs corresponded to bottled oils and contained the Arbequina and Changlot Real olive cultivars. Previous reports have already suggested that the total reducing capacity for oils containing the Arbequina variety was moderate [42].

Taking into account the cultivar (colored bars), it was verified that, in average, Arbequina was the variety providing the lowest values of gallic acid concentration. As indicated in previous reports, Picual (samples from #19 to #21) provided higher polyphenolic content than Arbequina [43]. The remaining monovarietal oils provided quite similar values of the reducing capability, although the varieties specific of the geographical area located to the North of the Province of Alicante (Blanqueta, Manzanilla and Alfafara) contained quite high average gallic acid concentrations (Figure 2).

Figure 2. Acidity (black line, right scale) and gallic acid concentration (bars, left scale) found by applying the DLLAPE method to EVOOs containing a single olive variety. Green: Blanqueta; red: Alfafara; blue: Manzanilla; brown: Arbequina; purple: Picual; yellow: Changlot Real; pink: Villalonga; grey: blends of different varieties. Operating conditions defined in Table 3 were applied. Error bars correspond to the standard deviation ($n=5$).

A common practice in the EVOO industry is based on the blending of autochthonous olive fruits to achieve improved organoleptic characteristics [43]. The data shown in Figure 2 clearly revealed the wide range of concentration in gallic acid found as a function of the blend.

In the present work, mixtures of the local cultivars (Blanqueta, Alfafara and Manzanilla) were analyzed. The obtained data are summarized in Table 5. This table also contains the corresponding values for the monovarietal oils as well as the values in the blends predicted according to the content of polyphenols in EVOOs containing a single variety. It may be observed that, as expected [43], by considering a tolerance of

$\pm 3s$, the predicted values of gallic acid concentration were in good agreement with those experimentally measured. Only in one out of the ten cases studied, the predicted values according to the monovarietal oils differed from the measured ones.

Table 5. Total polyphenol content (n=5), expressed as mg equivalent to gallic acid per kg of oil obtained for different proportions of autochthonous olive cultivars.*

Oil code	% Blanqueta	% Alfafara	% Manzanilla	$C_{\text{gallic acid}}/\text{mg kg}^{-1}$	$C_{\text{gallic acid}}/\text{mg kg}^{-1}$ (predicted) $\pm 3s$
#24	20	45	35	168 $\pm$ 10	204 $\pm$ 34
#25	20	40	40	171 $\pm$ 15	215 $\pm$ 48
#26	50	50	0	160 $\pm$ 16	201 $\pm$ 55
#29	59	33	8	214 $\pm$ 18	240 $\pm$ 61
#30	65	16	19	277 $\pm$ 11	279 $\pm$ 35
#31	43	23	34	302 $\pm$ 14	258 $\pm$ 45
#32	52	30	18	374 $\pm$ 25	245 $\pm$ 83
#33	27	45	28	240 $\pm$ 19	206 $\pm$ 62
#34	15	10	75	218 $\pm$ 11	279 $\pm$ 37
#35	20	10	70	230 $\pm$ 22	280 $\pm$ 74

* Average values (in mg kg^{-1}) for monovarietal EVOOs produced in the same oil press as the blends: Blanqueta: 244 $\pm$ 17; Alfafara: 197 $\pm$ 60; Manzanilla: 218 $\pm$ 9.

3.1.5. Olive oil thermal degradation

Finally, a preliminary study concerning the thermal degradation of EVOOs was undertaken with five selected oils. The experiments were done in two different ways: first the chosen oils were inserted into a heater at 100 °C and the samples were analyzed at four different times in presence of oxygen (Figure 3.a) and, second, 50 mL of EVOO

were poured into a beaker and put into a sand bath at 180 °C. Then, 10 – 15 g of 1 cm wide 10 cm length potato strips were added and cooked until a brownish color appeared. The oils were subjected to four frying cycles with fresh potatoes strips (Figure 3.b).

(a)

(b)

Figure 3. Gallic acid concentration versus heating time at 100°C (a) and subject from one to four potatoes frying cycles at 180°C (b). Operating conditions defined in Table 3 were applied for the polyphenols extraction. Error bars correspond to the standard deviation (n=5).

It was first observed that the thermal degradation was produced in a different way depending on the olive oil. Thus, as Figure 3.a reveals, oil #24 did not appreciably degrade 1 h after treatment at 100°C whereas a period of 2.5 h was long enough to cause a 46% decrease in total polyphenols. This oil was a blend of three different varieties: Blanqueta, 20%, Alfafara, 45%, and Manzanilla, 35%. Another sample containing a combination of these three cultivars with a higher proportion of Blanqueta (#29, Blanqueta, 52%, Alfafara, 39%, Manzanilla, 18%) was also quite resistant to the thermal degradation and 1 h of heating only caused an 18% decrease in the polyphenols content. Oil #15, containing only the Manzanilla cultivar, and oil #5, a Blanqueta variety oil, lost appreciably a significant part of its reducing capability heating them for just 1 h. In the case of the other monovarietal oil from Picual cultivar (# 21), the initial degradation was also produced but, apparently, thermal decomposition of polyphenols was less significant. It has been claimed that this cultivar resists better thermal degradation than others such as Arbequina [44]. For all the tested EVOOs, the thermal degradation proceeded and after a 12 h heating period, the measured gallic acid concentration was from 60% to 80% lower than that measured in the case of fresh oils.

When a cooking process was explored (Figure 3.b), it was evidenced that oils #15, #5 and, in a less extent, oil #21 degraded appreciably after the first frying cycle. Oils #24 and #29 were more refractory to thermal degradation. The presence of water from foods during the cooking process causes hydrolysis of secoiridoid aglycons. Furthermore, a fraction of polyphenols is extracted in the aqueous phase and then retained in the food surface. Globally, all these results appear to suggest that blending oil cultivars also resulted beneficial from the point of view of their thermal stability in terms of total polyphenols content. The observed trends are undoubtedly linked to the evolution of the

polyphenols profiles. Thus, for instance, it is indicated that thermal treatment at 180°C cause a decrease in the contents of hydroxytyrosol and tyrosol whereas minor changes in the lignans concentration are found. Thermal oxidation also induces rapid degradation of α -tocopherol [44]. Therefore, more studies are required to evaluate the type and concentration of the found polyphenols. Furthermore, more control on the oil extraction and storing conditions is necessary in order to draw useful conclusions on the benefits that EVOOs of different varieties and blends can have in terms of antioxidants.

3.2. Determination of metals

The liquid – liquid extraction of elements such as Cu, Fe, Ni and Zn from edible oils has been already made by using diluted nitric [45] or hydrochloric [46] acid solutions. In a first instance, experiments demonstrated that, in the absence of methanol, the concentrations of metals extracted were much lower than in the presence of this alcohol. Therefore, at least 50% of methanol was present together with nitric acid.

3.2.1. Optimization of the DLLAPE

An optimization similar to that undertaken for the determination of polyphenols was done in terms of quantification of metals and metalloids in EVOOs. The experiments revealed that optimum values of variables such as nebulizer liquid and gas flow rates and nebulizer tip – sample surface gap had the same values as those previously indicated (Table 3). Also for metals, the addition of hexane to the oil sample did not modify appreciably the extracted concentration.

However, in the case of metals, interesting observations were made. First, it was found that the optimum conditions depended on the element considered. This fact is illustrated in Figure 4. It may be observed that an increase in the extraction time (or, in

other words, the mass of the extracting solution) led to a rise in the measured elemental concentration especially pronounced for Zn and Mn (Figure 4.a). Meanwhile, for As, Ca and Sr the impact of the extraction time was less marked and 30 s were enough to achieve normalized concentrations higher than 0.8. According to these findings, and taking into account the flow rate selected in Figure 4, it was concluded that the mass of methanolic solution required to complete the metal extraction went from 0.9 to 1.2 g (Zn).

Different trends were also found as a function of the methanol content in the polar solution. Again, Zn and Mn were the most influenced analytes, since it was found that the higher the methanol content, the lower the normalized concentration (*i.e.*, the lower the extraction efficiency). Normalized concentrations for the remaining analytes did not modify appreciably with this variable (Figure 4.b).

The nitric acid concentration was evaluated from 1 to 10% and it did not have a significant impact on the elemental concentration in the extracts. This trend was in marked contrast with other reports that recommend a 10% nitric acid concentration [45]. The dispersion of the aqueous solution as an aerosol and the generation of an emulsion apparently promoted the efficient extraction of the elements tested.

(a)

(b)

Figure 4. Effect of the extraction time for a 50% methanol concentration in the extracting solution (a) and methanol concentration for a 90 s extraction step (b) on the concentration of metals in olive oil ref # 34. Liquid flow rate: 0.6 mL min^{-1} ; nebulizer tip – sample surface gap: 15 mm; nitric acid concentration in the extraction solution: 2% (w/w). Error bars correspond to $\pm\sigma$ (n=5).

Taking into account the data summarized in Table 3, it may be observed that the mass of the extracting solution was higher than the sample mass. Some of the determined elements were present at actually low concentrations. Fortunately, the detection technique (ICP-MS) provided high sensitivities that allowed quantification of more than twenty elements. Pre-concentration of analytes would be interesting for ultratrace analysis. A further modification of the DLLAPE, consisting of lowering the extracting solution flow rate and using a micronebulizer, could lead to analyte pre-concentration.

3.2.2. Comparison of the DLLAPE with a microwave digestion method

The concentrations reported by the DLLAPE method were compared against those provided by a conventional procedure in which the samples were first digested and then analyzed through ICP-MS. The obtained results are summarized in Table 6 for six different oil samples. It could be claimed that both methods provided statistically similar concentrations for ten elements, thus giving evidence that the DLLAPE was efficient in obtaining information on the total metal content in olive oil. Finally, it was observed that, as the sample dilution factor was one order of magnitude lower for the DLLAPE than when the digestion procedure was applied (2 and 20, respectively), there were some elements whose concentration fell below the procedural LOQ for the latter approach (see last column in Table 6) and, hence, no results were provided for those analytes when the conventional method was applied. Note that the blank in the case of the MW digestion corresponded to a highly concentrated nitric acid solution and the standard deviation of its signal was from one to two orders of magnitude higher than when the DLLAPE method was used.

As it has been explained in Section 2, once the samples were extracted, the obtained methanolic solutions were completely evaporated. This fact increased the analysis time. Obviously, this step was applied in order to avoid the problems associated with the introduction of a high amount of carbon into the ICP-MS instrument [23,24]. Further modifications of the method could include the removal of the evaporation step in order to increase the actual sample throughput.

Table 6. Elemental concentrations found with the DLLAPE and a conventional microwave digestion method.*

Sample code	2		14		17		20		37		39		Procedural LOQ [@] / ng mL ⁻¹
Element	Aerosol	MW	Aerosol	MW	Aerosol	MW	Aerosol	MW	Aerosol	MW	Aerosol	MW	MW
Na	1047±1	1070±86	1070±24	846±20	1045±22	951±70	1020±22	838±52	1050±36	1570±80	1021±5	1200±300	291
Mg	194±6	182±16	221±3	190±16	194±6	182±16	100±2	91±6	201±14	373±20	192±13	313±60	52
Al	37±2	-	38±4	46±5	36±6	-	90±1	-	35±3	-	17±4	-	29
Ca	184±22	184±40	248±20	209±22	143±10	173±30	148±16	137±60	152±12	171±80	140±14	130±25	67
Cr	2.1±0.06	1.8±0.4	0.89±0.14	-	0.9±0.2	-	1.2±0.4	-	1.02±0.16	-	1.6±0.5	-	4.9
Mn	3.5±0.4	5.4±0.4	2.28±0.08	-	2.4±0.2	2.8±0.5	2.33±0.06	2.0±0.2	2.1±0.8	1.04±0.02	2.19±0.06	-	2.3
Fe	30±0.8	84±14	23±2	20±7	28.2±1.6	35±8	29±6	30±12	24±2	18±7	28±3	17±9	9.6
Ni	4.2±0.1	4.2±0.1	3.7±0.2	2.6±0.2	3.8±0.2	4.2±1.2	3.9±0.2	3.6±0.1	5.4±1.2	6.8±0.8	7.4±1.4	8±3	6.8
Cu	3.0±0.2	5.5±0.5	3.6±0.4	2.9±0.2	3.4±0.1	3.3±1.3	3.3±0.6	-	2.9±0.8	-	3.6±0.1	-	1.3
Zn	68±3	73±10	48±2	44±4	39±1	51±8	51±2	45±4	62±8	65±7	68±8	76±20	16
Pb	6.5±0.2	5.5±2	5.9±0.6	4.2±2	4.9±0.8	5.5±3	6.2±0.3	7.4±1	4.5±0.4	4.0±0.7	1.28±0.05	-	3

* Confidence intervals expressed as: $\pm \frac{t s}{\sqrt{n}}$ for a 95% level. Number of replicates: 5 for the DLLAPE method and 3 for the microwave digestion.

@ Limit of quantification in the sample, determined according to:

$$LOQ = \frac{10 s_b}{Sensitivity} \times Dilution\ factor$$

where s_b corresponded to the standard deviation of twenty consecutive blank measurements, Sensitivity was the slope of the calibration line and the dilution factor was 20.

3.2.3. Analysis of real samples

Besides their toxicological and nutritional importance, metals play a very important role in the stability of olive oils. Elements such as Fe, Cr, Pb and Cd contribute to the lipid peroxidation [47]. All the samples selected were analyzed according to DLLAPE – ICP-MS and a total of 21 elements were encountered and quantified above the procedural quantification limit (see Table 7). The obtained concentrations were in agreement with previously published work. Table 7 also contains the range of the concentrations found in the present work and compares the values with those encountered in the literature for olive oil samples as well as with international legislations. In general terms, it may be concluded that there was high variability in elemental concentration as a function of the sample considered. In the case of elements such as Pb and Zn, Angioni et al. [48] attributed these differences to the different cultivars and ripening of olives, whereas elements such as Cd and Cu have been determined at levels that are independent of these two factors.

Table 7. Elemental concentration range found for the EVOOs analyzed in the present work and comparison with the values reported in the literature for similar samples.

Element	Procedural limit of quantification (ng g ⁻¹) [@]	Concentration range in the present work (ng g ⁻¹) ^{\$}	Concentration range found in the literature (ng g ⁻¹)	Reference
Na	4.8	1000 – 4500	< 11000 [#]	[51]
Mg	0.1	180 – 380	< 5800 [#]	[51]
			36 – 125	[49]
			56 – 1032	[50]

Al	0.1	28 – 280	500 [#]	[52]
K	8	14 – 853	< 8100 [#]	[51]
Ca	1.8	120 – 270	<25 [#] 1853 – 12220	[51] [50]
Cr	0.04	0.8 – 2.4	83 [#] 0.69 – 2 0.35 – 1.3 18 – 114	[52] [51] [49] [22]
Mn	0.03	2 – 22	LOQ – 3.6 [#] 4.4 – 25.2 8 – 60	[51] [50] [22]
Fe	0.2	23 – 1200 (3000)*	LOQ - 7900 [#] 4.4 – 48 89.3 – 550 40 – 120	[51] [49] [50] [53]
Ni	0.02	3 – 15	0.18 – 0.53 10.6 – 46.9	[49] [50]
Cu	0.02	2 – 17 (100)*	LOQ – 4 [#] 0.73 – 63.1 15.94 – 58.51	[51] [49] [54]
Zn	0.04	50 – 113	< 180 [#] 100 [#] 4.25 – 14.3 25.5 – 68.3 157 – 385	[52] [51] [49] [55] [54]

As	0.06	0.2 – 0.5 (100)*	1.25 – 26.7	[50]
Se	0.36	0.03 – 0.31	< 0.1 [#] 1.47 – 6.78	[51] [50]
Sr	0.06	4.8 – 7.3	1.52 – 48.9	[50]
Mo	0.04	0.4 – 11.3		
Ag	0.006	56 – 111		
Cd	0.008	0.1 – 1.3	0.08 – 0.16 0.088 – 0.366	[49] [50]
Sn	0.06	0.4 – 1.2	14.4 – 53.6	[49]
Sb	0.04	0.1 – 0.4	0.194 – 0.411	[50]
Ba	0.02	1.2 – 2.8		
Pb	0.02	1.1 – 27.2 (100)*	0.6 – 0.9 32.6 – 156	[49] [54]

[@] Limit of quantification in the sample, determined according to:

$$LOQ = \frac{10 s_b}{Sensitivity} \times \text{Dilution factor}$$

where s_b corresponded to the standard deviation of twenty consecutive blank measurements, Sensitivity was the slope of the calibration line and the dilution factor was 2.

^{\$} Number of replicates: 5

* Maximum residue levels (MRL) of the legislated elements in olive oil.⁵³

[#] Values included in international legislation (see references).

The data reported in Table 7 also indicated that the EVOOs analyzed contained metals and metalloids concentrations below the maximum levels allowed by the legislation [53].

4. Conclusions

The present work shows, for the first time, the potential of an efficient liquid – liquid aerosol phase extraction (DLLAPE) method for the determination of organic as well as inorganic species in extra virgin olive oil (EVOO). This is a straightforward procedure that involves the generation of an aerosol by means of a pneumatic nebulizer and the further phases spontaneous separation.

Although the method for detecting polyphenols was subject to some limitations, the main goal of the present study was to evaluate the applicability of the aerosol phase extraction methodology in comparison with a conventional extraction procedure. The DLLAPE consumes extremely short sample preparation times. Thus, the extraction time recommended in the conventional method is close to 45 minutes. However, the DLLAPE only requires on the order of 1 – 2 min to complete the extraction step. This is extremely important in order to avoid polyphenol degradation during the analysis procedure, with the subsequent underestimation of the oil reducing capability. Moreover, unlike the conventional method, the DLLAPE can be easily automatized. An additional advantage of the aerosol phase extraction includes the reduction in reagents consumption.

The application of the DLLAPE to the determination of a long list of elements in EVOO has also been demonstrated in the present study. In this case, it was necessary to add to the extracting solution a low proportion of nitric acid in order to fully solubilize the metals contained in the olive oil samples. Again, a dramatic shortening in the

sample preparation step compared with a microwave digestion procedure was noticed. Indeed, the preparation time to perform the analysis of ten different samples went from around 180 minutes to 20 minutes (2 minutes per sample) for the DLLAPE. Additionally, procedural quantification limits were much lower when the analytes were extracted in aerosol phase, because the blank provided a lower standard deviation and the sample was diluted by a lower factor than when applying a microwave digestion procedure.

In the present work, there were no problems related to the lack of sensitivity. Phenolic compounds were present at actually high contents and, for the elemental determination, a sensitive technique was used (*i.e.*, ICP-MS). Nevertheless, many applications would require a previous analyte pre-concentration in order to have access to ultra-trace information. It would be necessary to determine the maximum pre-concentration factor attainable with the DLLAPE. Likewise, dealing with high sample volumes (*i.e.*, on the order of hundreds of milliliters) to achieve high pre-concentration factors seems to be actually difficult with the aerosol phase extraction procedure and more studies are required in this field.

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A dispersive liquid – liquid extraction method was used for analyzing olive oils.

Only 2 minutes were required for a complete extraction of polyphenols and elements..

The method allows performing quickly studies on oil variety and its thermal degradation.

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The authors declare that they have not conflicts of interest.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: