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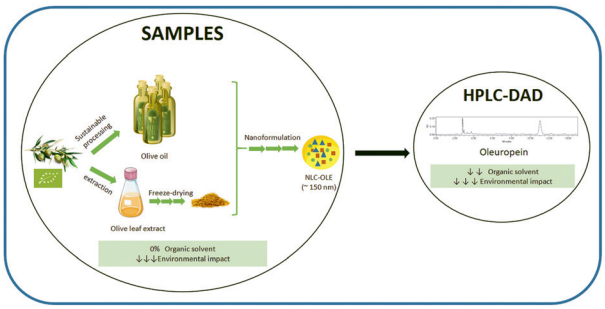
1

Development and validation of an eco-friendly HPLC-DAD method for the determination of oleuropein and its applicability to several matrices: olive oil, olive leaf extracts and nanostructured lipid carriers

Amaia Huguet-Casquero, Tania Belén López-Méndez, Eusebio Gainza and Jose Luis Pedraz

An alternative HPLC approach for oleuropein determination in olive oil, *Olea europaea* leaf extracts and nanoparticles with minimized environmental impact.



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First (given) and middle name(s)	Last (family) name(s)	ResearcherID	ORCID iD
Amaia	Huguet-Casquero		0000-0001-9724-0921
Tania Belén	López-Méndez		0000-0001-5029-6549
Eusebio	Gainza		0000-0002-7449-5232
Jose Luis	Pedraz		0000-0002-3938-2267

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Development and validation of an eco-friendly HPLC-DAD method for the determination of oleuropein and its applicability to several matrices: olive oil, olive leaf extracts and nanostructured lipid carriers

Amaia Huguet-Casquero, [a,b] Tania Belén López-Méndez, [a] Eusebio Gainza [b] and Jose Luis Pedraz [a,c]

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Oleuropein is a natural ingredient largely used in nutritional supplements. This study reports on the development, validation and application of a HPLC method based on UV-vis detection for determining oleuropein in olive oil, olive leaf extracts and nanoparticles. The principles of green chemistry were taken into account for both sample manufacturing and HPLC method development. A Zorbax C18 column was used on which a mobile phase containing acetonitrile–water was applied in isocratic elution mode injecting 10 µl of sample at 1.2 ml min^{-1} constant flow-rate, 30 °C temperature and 15 minutes run time. Method linearity (r^2 > 0.999) was assessed in the range of 50 to 420 µg ml^{-1}. Precision expressed by RSD% was always better than 2%. Accuracy was in all cases within 98-102% of the expected concentration value. The sensitivity of the method was at a level of 0.08 µg ml^{-1} as the limit of detection and at 0.25 µg ml^{-1} as the limit of quantification. The results show that the method is suitable for the quantification of oleuropein in a variety of samples with reduced environmental impact.

1. Introduction

Olives and their juice are considered the cornerstone of the Mediterranean diet, which has been historically linked with higher longevity and the reduced incidence of some chronic diseases in the Mediterranean population. These health benefits have been attributed by the scientific evidence to some of the 20 types of olive polyphenols found in olive trees.[1] Among these, oleuropein (OLE) (Fig. 1), the major constituent of the sercoiridoid family, has gained special attention. Mainly due to its hydrophilic nature as well as other factors (*i.e.* olive harvesting time, processing method) it is normally found in high quantities in olive leaves (*Olea europaea* L.) but lower (sub-ppm) in olive oil.[2–4] Apart from being considered one of the main ones responsible for the organoleptic properties of food it is also used for chemical standardization of marketed olive-derived products (*i.e.* olive leaf extracts, supplements and olive oil). Beyond this, several pharmacological benefits have been attributed to this natural polyphenol during the last few decades. Even the European Medicines Agency (EMA) and the European Food Safety Authority (EFSA) have submitted their own assessment reports about its health-promoting properties in human health.[5,6] In particular, oleuropein has been reported to exert antiinflamatory,[7] platelet anti-aggregant,[8] antimicrobial,[9] hypoglycemic,[10] anticancer,[11,12] antiviral,[13] hypolipidimic,[14] antioxidant and neuroprotective[15] effects among others. The literature offers a long list of other natural compounds that

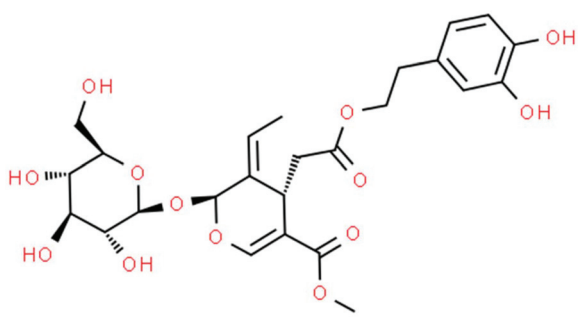

Fig. 1 Oleuropein.

[a]*NanoBioCel Group, Laboratory of Pharmaceutics, University of the Basque Country (UPV/EHU), School of Pharmacy, Paseo de la Universidad 7, Vitoria- Gasteiz 01006, Spain*
[b]*Biosasun S.A., Iturralde 10, Etxabarri-Ibiña, Zigoitia, 01006, Spain*
[c]*Biomedical Research Networking Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Vitoria-Gasteiz, Spain*

have also been proved to possess important pharmacological activities.[16] All these pieces of evidence, together with the recently increased environmental awareness, have promoted a trend towards the consumption of natural-origin products and thus, the use of bioactive compounds like olive polyphenols has gained the attention of the pharmaceutical, food and chemical industries. Most of these natural molecules are obtained from agro-industrial waste through extraction processes in which not only high energy consumption but also the use of large amounts of organic solvents contributes to its overall environmental impact. Therefore, both from a toxico-pharmacological and ecofriendly point of view, those harmful aspects should be minimized through the application of green chemistry.

Another issue that must be addressed to achieve the required properties of olive polyphenol based by-products is their lability against both external and systemic factors. Specifically, OLE is rapidly degraded after light and oxygen exposure and once in the human organism its bioavailability is compromised by enzymatic degradation and limited absorption in the target site.[17–19]

Taking into account the aforementioned facts, polymeric and lipid-based nanoparticles have been proposed as innovative drug delivery systems (DDS) that could protect and improve the efficacy of the encapsulated compounds until their release in the target site. Among these DDS, nanostructured lipid carriers (NLC) offer several advantages such as the avoidance of organic solvents during the manufacturing process, higher drug loading, long-term stability and scaling-up feasibility.[20,21] In order to determine the yield of the nanoformulation process, the amount of encapsulated drug should be quantified and thus, precise analytical methods are needed.

High performance liquid chromatography (HPLC) coupled with a diode array detector (DAD) is one of the most common analytical methods in the pharmaceutical and food industries for routine quantification analysis. More specifically, reverse phase (RP)-HPLC has emerged as a sensitive, rapid and accurate technique that allows the separation and determination of analytes in several nutraceutical and pharmaceutical forms including nanoparticles.

As a result, several HPLC methods have been reported for oleuropein quantification in olive leaves and olive leaf extracts,[22,23] olive oil,[24,25] olive stems and roots,[4] and olive pomace and fruit.[26] However, they have some limitations such as the use of large amounts of organic solvents due to long analysis time, lack of validation results, more complex methods using gradients and mass detectors, tedious sample preparation techniques and lack of method applicability for the quantification of oleuropein in nanoparticle formulations.

Taking the aforementioned, the goal of the present work was to validate a rapid and versatile RP-HPLC-DAD method for the determination of oleuropein in olive oil, olive leaf extracts and nanoparticles obtained through eco-friendly processes.

2. Materials and methods

2.1. Materials

An oleuropein reference standard (>98% purity) was purchased from Sigma-Aldrich (St Louis, MO). Oleuropein (>80% purity) for nanoencapsulation was kindly donated by Nonaherbs Bio (Tech) (China). Olive leaf, organic extra virgin olive oil (EVOO) and organic extra virgin olive oil rich in olive polyphenols (EVOO-ph) were donated by Biosasun S.A. (Álava, Spain). HPLC-grade methanol (MeOH), acetonitrile (ACN) and orthophosphoric acid (85%) were purchased from Scharlab®. Water (H_2O) was purified in a Milli-Q Plus system (Millipore®) with a conductivity of 18.2 MÙ.cm at 25 °C. Precirol® ATO 5 (Glycerol distearate) was a kind gift from Gattefosé (France). Polysorbate, Tween ®80 was purchased from Panreac Química (Castellar del Vallès, Barcelona, Spain). Poloxamer 188 was kindly provided by Merck (Darmstadt, Germany). All other reagents and solvents used in this study were of analytical grade.

2.2. Instrumentation

A Waters 2795 Alliance HPLC system (Waters Corporation, Milford, MA, USA) equipped with a binary HPLC pump (Waters 2487), a diode array detector (Waters 2489), a column oven (Waters Column Heater Module), and an auto sampler (Waters 717 plus Autosampler) was used. Data acquisition, analysis and reporting were performed using Empower 2 chromatography software (Milford, MA, USA). Analysis was performed using a Zorbax® C18 column (Agilent, Wilmington, DE, USA) with 5 μm in particle size, 4.6 mm in internal diameter and 250 mm in length.

2.3. Chromatographic conditions

Separation of OLE was performed in isocratic mode with a mobile phase consisting of a mixture of ACN–H_2O (20 : 80, v/v). The pH of the mobile phase was adjusted to 3.0 using orthophosphoric acid. The mobile phase was degassed in an ultrasonic bath for 15 minutes before analysis. The analysis was carried out at 1.2 mL min^{-1} flow rate with a detection wavelength of 230 nm and the injection volume was arranged as 10 μL. Column temperature was set to 30 °C. Method run time was 15 min. All tested samples were filtered through a 0.45 μm pore-size polyvinylidene fluoride (PVDF, Scharlau®) filter prior to analysis. Each working day injection was carried out after pre-conditioning of the column at the optimized temperature (30 °C) and flow rate for 15 min.

2.4. Preparation of standard solutions

Standards were prepared in deionized water and filtered through a 0.45 μm pore size PVDF-filter before injection. All standards were protected from light. A stock standard solution of 1 mg ml^{-1} was prepared in Milli-Q water. Seven working standard solutions (50, 75, 100, 280, 350, 385 and 420 μg ml^{-1}) were prepared by diluting in Milli-Q water the corresponding aliquots of stock standard solution in 5 ml amber volumetric flasks.

2.5. System suitability

System suitability testing is essential for the assurance of the quality performance of the chromatographic system. For this purpose, six replicates of the OLE reference standard at a concentration of 280 μg ml^{-1} were analyzed. System performance and chromatographic parameters such as the number of theoretical plates (N), tailing factor (T), resolution (R_s) and capacity factor (k') were taken into consideration.

2.6. Method validation

The analytical method for the quantification of OLE was validated according to the International Conference on Harmonization (ICH) guidelines Q2 (R1)[27] in terms of specificity, selectivity, linearity, range, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ).

2.6.1. Linearity. Linearity was determined at seven different concentration levels (50, 75, 100, 280, 350, 385 and 420 μg mL^{-1}). Experiments were carried out in triplicate. Peak areas were plotted against the analyte concentration to obtain a regression line. The linear calibration function was fitted by the least-squares methodology.

The range of the method was validated by checking the interval between the upper and lower concentrations (amounts) of the analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

2.6.2. Specificity. In order to demonstrate that no interference occurred due to the other sample constituents, a specificity test was carried out by comparing the chromatograms obtained from the EVOO hydroalcoholic extraction solution (MeOH–H_2O, 50 : 50, v/v), deionized water, mobile phase and supernatant of blank nanoparticles (without OLE) with the OLE reference standard solution and samples containing OLE.

2.6.3. LOD and LOQ. In this study a calibration curve was prepared at low concentrations (0.2 μg mL^{-1}, 0.4 μg mL^{-1}, 0.6 μg mL^{-1}, 1 μg mL^{-1} and 6 μg mL^{-1}). This method can be applied in all cases, and it is most applicable when the analysis method does not involve background noise. It uses a range of low values close to zero for the calibration curve, and with a more homogeneous distribution will result in a more relevant assessment. The LOD and LOQ were assessed by applying the following equations:

$$\mathrm{LOD} = \frac{3.3\sigma}{S}$$

$$\mathrm{LOD} = \frac{10\sigma}{S}$$

Where σ is the standard deviation of the response of ten blank samples and S is the slope of the calibration curve.

2.6.4. Precision. Precision is a measure of how close results are to one another. Precision of the method was determined by repeatability (intra-day precision) and intermediate precision (inter-day precision) studies. Intra-day precision was assessed by analyzing three replicates of the standard solutions: at three levels of low (50 μg mL^{-1}), medium (280 μg mL^{-1}) and high (420 μg mL^{-1}) concentrations on the same day. Inter-day precision was evaluated through the analysis of three levels of the standard solutions at low (50 μg mL^{-1}), medium (280 μg mL^{-1}) and high (420 μg mL^{-1}) concentration levels in three different days. Results were expressed as relative standard deviation (RSD).

2.6.5. Accuracy. Accuracy is the closeness of the test results obtained by the analytical method to the true value. With this aim and following ICH Q2(R1)[27] recommendations, accuracy of the method was studied by the addition of known amounts of OLE to blank samples, working at the three concentration levels in the linear range previously established for the analyte (50, 280 and 420 μg ml^{-1}). From the obtained data the average recovery values of the lowest, intermediate and upper concentration levels were calculated.

2.7. Method applicability and sample preparation

All samples were prepared by dilution in deionized water with the exception of EVOO and EVOO-ph for which an extraction process was carried out.

2.7.1. Organic extra virgin olive oil rich in polyphenols (EVOO-ph). Organic *Olea europaea* fruit juice rich in olive polyphenols (EVOO-ph) was obtained from organic olives of Arroniz and Arbequina varieties following the protocols of Biosasun. Briefly, olives were harvested in early November, when the fruit is yet in a low ripening index, and leaves were discarded. Immediately after, olives were ground and centrifuged in a horizontal centrifuge (Baby 2-l, Pieralisi MAIP SPA, Zaragoza, Spain) at low temperature (<27 °C). The obtained product was kept in High Density Polyethylene (HDPE) containers for decantation for 2 months at low temperature (<27 °C) and finally, packaged in amber bottles. EVOO was also obtained through the same methodology but only Arroniz variety olives were employed.

With the aim to determine the OLE content in EVOO-ph through the validated HPLC method, it was processed through a simple and rapid method. Briefly, EVOO-ph was subjected to sonication in a water-bath for 15 minutes in order to obtain a homogeneous sample. Then, 4 grams of the sample were diluted in 20 ml of a MeOH–H_2O (50 : 50, v/v) solution and left to mix under magnetic stirring for 4 hours. After that, the obtained solution was filtered through a 0.45 μm pore-size PVDF-filter prior to its analysis by the validated HPLC method. As a blank-control, pure EVOO was also processed as aforementioned and analysed.

2.7.2. Organic *Olea europaea* L. extracts. Organic olive leaves discarded during the olive oil manufacturing process were used for OLE extraction. Briefly, *Olea europaea* dried leaves (10 kg) were ground and introduced into a brew bag. Solid–liquid extraction was conducted by immersing the brew bag into a pre-heated water bath (50 L, 80 °C) for 4 hours. After extraction, the aqueous solution of olive polyphenols was stored at 4 °C and protected from light, until use. The *Olea europaea* L. extract was also subjected to a freeze-drying process for 42 hours (Telstar Lyobeta freeze-dryer, Terrasa,

Table 1 Freeze-drying process steps for *Olea europaea* L. extracts

Freeze-drying step	Temperature (°C)	Time (hh mm)	Pressure (mBar)
Freezing	−50	3 h 00 min	—
Chamber vacuum			0.2
Primary Drying	−50	5 h 00 min	0.2
	20	7 h 00 min	0.2
Secondary Drying	20	24 h 00 min	—

Spain). For this purpose, the following drying cycle was programmed: freezing for 3.0 h, primary drying for 12 h, and secondary drying for 24 h. The pressure was set to 0.2 mBar during the primary drying. Table 1 summarizes the exact parameters of the process.

Enumeration of moulds and yeast in the obtained *Olea europaea* L. aqueous and freeze-dried extracts was conducted following the ISO 21527-1:2008 and ISO 21527-2:2008 guidelines.

In order to quantify the OLE content in the obtained aqueous and freeze-dried extracts, they were diluted 1 : 20 and 1 : 1 in deionized water, respectively, and filtered through a 0.45 μm pore-size PVDF-filter prior to its analysis by the validated HPLC method.

2.7.3. Oleuropein-loaded nanostructured lipid carriers (NLC-OLE). For OLE nanoencapsulation, a commercial OLE powder (purity > 80%, Nonaherbs®) from olive leaves was employed. OLE-loaded nanoparticles were obtained by the hot melt homogenization method as previously described by our group.[28] Briefly, EVOO (liquid at room temperature) and the solid lipid Precirol ATO® 5 (melting point: 56 °C) were mixed and melted 5 °C above the solid lipid melting point until a clear and homogeneous phase was obtained. The aqueous phase was prepared by dispersing Tween® 80 and Poloxamer 188 in purified water. Oleuropein was also dissolved in purified water (0.66 mg ml^{-1}). When the lipid phase was melted and the aqueous phase was heated to the same temperature, oleuropein solution was added to the lipid phase and immediately after, the hot aqueous phase was added to the melted oily phase, and then sonicated at 50 W (Branson Sonifier 250, Danbury, CT, USA). The obtained nanoemulsion was mixed by magnetic stirring at room temperature and then stored for 2 h at 4 °C to allow the re-crystallization of the lipids and NLC formation. Afterwards, particles were collected using a 100 kDa molecular weight cut-off centrifugal filter unit (Amicon, "Ultracel-100 k", Millipore, Spain) by centrifugation at 2500 rpm and washed three times with MillliQ water. The obtained supernatants were collected to determine by HPLC the encapsulation efficiency. Blank-NLCs without OLE were also prepared and used as the control.

OLE content in nanoparticles was assessed by an indirect method. The supernatant obtained from the ultracentrifugation process was diluted 1 : 10 in water, and filtered through a 0.45 μm pore-size PVDF-filter and the analyte concentration was determined by the validated HPLC method. Blank-NLCs without OLE were analysed as the control. Encapsulation efficiency was calculated following the equation:

$$\mathrm{EE}\,(\%) = \frac{\text{Total amount of OLE} - \text{Amount of free OLE}}{\text{Total amount of OLE}} \times 100$$

2.8. Greenness evaluation of the HPLC method for olive-derived samples

With the aim to assess the impact of the proposed method on health and environment the Environment Assessment Tool (EAT) free software developed by Gaber *et al*. was employed.[29] This tool is based on the type and amount of organic solvents used in sample/standard pre-treatment and mobile phase preparation, as well as the elution program employed (isocratic or gradient). Other factors involved in the energy-consumption are not taken into account (*i.e.* temperature, type of detector). Therefore, to conduct this assessment the type and amount of solvents employed for each sample preparation were introduced in the tool, as well as, the chromatographic conditions (flow rate, mobile phase composition, analysis time) and the safety (*S*), health (*H*) and environment (*E*) impacts were obtained. The sum of *S*, *H* and *E* gave the total EAT value. For this metric, the higher the score, the greener the method. Calculation is performed according to the following equation:

$$\begin{aligned}\mathrm{HPLC-EAT} = {} & S1m1 + H1m1 + E1m1 + S2m2 + H2m2 \\ & + E2m2 + \ldots + Snmn + Hnmn + Enmn\end{aligned}$$

where *S*, *E* and *H* are safety, environmental and health factors, respectively, for *n* number of solvents and *m* is the mass of the solvent. Mobile phases composed of either pure H_2O or H_2O containing buffer salts and/or analytical modifiers are considered to have zero *E*, *H* and *S*, values due to the outweighing effect of organic solvents. Detailed information is provided elsewhere.[29] Furthermore, we employed the EAT tool to rank the greenness of the different HPLC methods for OLE quantification found in the literature and we compared them with our method.

2.9. Statistical analysis

The statistical analysis of the validation data was performed using EMPOWER PRO 2 software (Milford, MA, USA). The Environment Assessment Tool (EAT) was employed for the greenness evaluation of the HPLC method.

3. Results and discussion

3.1. Method development

The proposed RP-HPLC-DAD method was developed with the aim to provide a simple and versatile technique for OLE quantification in different samples derived from organic certified olive trees and obtained by green methods, with shortened time and cost analysis.

HPLC is a powerful analytical technique and one of the most widely employed ones in industry. Among these, the most popular is RP-HPLC which usually uses mixtures of water

and ACN. Despite the toxicity of ACN, replacing it with another non-toxic solvent such as alcohols is not an easy work due to the advantages that H_2O–ACN mixtures can offer in efficient RP-HPLC separation methods (*i.e.* better separation efficiencies, lower viscosity, and higher transparency in the UV region). Therefore, with the aim to reduce the volume of organic waste by shortening the retention time of OLE and reducing the amount of ACN employed in the mobile phase as well as effectively separating the OLE peak in compliance with ICH Q2(R1)[27] requirements, a new RP-HPLC-DAD method for OLE determination was developed, based on previous work of our group and from an eco-friendly perspective.

Previously, our group defined a gradient method for OLE determination (ultrapure water with orthophosphoric acid *pH = 3 as eluent A and ACN as eluent B) (data not published). The gradient started at 5% of eluent B and increasing to 20% in 6 min, and remained constant until minute 10. At this time eluent B was increased to 100% and maintained for 3 minutes. At minute 13, eluent B was decreased to 5% and kept constant until minute 18. The injection volume was 20 μl, the flow rate was 1 ml min^{-1} and the detector wavelength was set to 280 nm. Acetic acid was also assayed for pH adjustment in eluent A but since it gave a small signal in the baseline it was discarded. With this method, OLE eluted at minute 9.3 but no resolution was achieved in real samples and high noise was detected in the baseline. Since OLE eluted when the mobile phase composition was ACN–H_2O (20 : 80 v/v) we decided to try it in isocratic mode, keeping the other chromatographic parameters constant. Baseline noise was eliminated and oleuropein displayed the same retention time as before. The Zorbax extended C-18 column was employed for these tests and lack of reproducibility was shown, probably due to the fact that this column is more suitable for high pH values. Therefore, due to its long-term stability and suitability at low pH values, as well as its properties we decided to try a Zorbax SB-CN C18 column and better peak shape and reproducibility were achieved. Taking the aforementioned, ACN and H_2O were selected as mobile phase solvents and pH was always adjusted to 3.0 with orthophosphoric acid. Initially, several ACN–H_2O proportions were assayed with the OLE reference standard, commercial OLE extract and olive leaf aqueous extract and results were studied to obtain acceptable retention times, good symmetry, shape and separation between peaks. Results are displayed in Table 2. Despite the fact that shortened retention time was obtained with ACN-H_2O (25 : 75, v/v, *pH 3.0), retention factor (k′) and resolution values were lower or too close to the acceptance criteria (R_s > 1.5) in the studied samples. Therefore, the mobile phase composed of ACN–H_2O (20 : 80, v/v, *pH 3.0) was selected for the following optimisation steps. The detector wavelength was set to 255 and 230 nm, and as 230 nm gave a significantly higher area value for the same oleuropein concentration it was selected as the best one. All the development processes were carried out with the analyte dissolved in water. However, theory states that better results are obtained when a sample is dissolved in the mobile phase and based on bibliography, oleuropein seemed to have better results when dissolved in methanol and thus, both solvents were assayed as well as water. No significant differences were obtained in the peak quality parameters (data not shown) and since one of the objectives of this method was to keep the organic solvent use to a minimum, water was selected as the sample dissolution medium. Finally, column temperature was adjusted to 30 °C in order to slightly improve the resolution and reduce the retention time from 12 to 11 minutes.

3.2. Method validation

Method validation was carried out following ICH Q2 (R1) guidelines.[27]

3.2.1. System suitability. Once the method was optimized with the best conditions, the system suitability parameters were investigated. Since ICH Q2 (R1) guidelines[27] do not indicate any acceptance criteria to evaluate the system suitability, US Food and Drug Administration (FDA) guidelines and European Pharmacopoeia (EP) established criteria for HPLC methods of pharmaceutical analysis were followed.[30] Therefore, the analyte capacity factor (k'), peak symmetry or tailing factor, number of theoretical plates (N) and resolution (R_s) were investigated. As shown in Table 3, the developed method accomplished the acceptance criteria for all the studied parameters in the OLE reference standard.

3.2.2. Specificity. The specificity test allows to verify if the method is selective enough to quantify the analyte of interest in the presence of other substances that can interfere in the analysis of a complex sample. To assess this, the retention times of OLE standard and samples solutions' chromatograms were compared (Fig. 2). Moreover, blank samples' chromato-

Table 2 HPLC method development results

Mobile phase composition (ACN : H_2O)	Sample	Results			
		k' ($2 > k < 10^b$	Resolution (>1.5) [b]	Symmetry (>1) [b]	R_T (min)
25 : 75	Oleuropein reference standard	1.53[a]	—	1.14	7.4
	Olea europaea L. extract	1.59[a]	1.54	1.14	7.5
	Oleuropein for nanoencapsulation	1.59[a]	1.56	1.19	7.4
20 : 80	Oleuropein reference standard	2.74	—	1.21	11.0
	Olea europaea L. extract	2.83	2.62	1.16	11.1
	Oleuropein for nanoencapsulation	2.79	2.84	1.17	11.0

[a] Results that do not accomplish acceptance criteria. [b] Acceptance criteria. r_T (retention time).

Table 3 Results from the system suitability study

Parameter	Acceptance criteria	Results
Theoretical plates (N)	EP: ≥1000	11 474.47
	FDA: >2000	11 270.17
USP tailing (T)	FDA: ≤2	1.21 ± 0.01
Symmetry factor EP	EP: 0.8 to 1.5	
Capacity factor (k)	EP: ≥2	2.743
	FDA: >2	
R_T %RSD	EP: ≤2	0.04
	FDA: ≤2	

grams were studied. Similar retention times (~11 min) were observed in the chromatograms of the standard and sample solutions. Furthermore, no interfering peaks were observed in the blank chromatograms (Fig. 2B, D, and F). The chromatograms of the samples also showed other peaks of unknown compounds that absorb in the same wavelength as OLE. Nevertheless, good resolution was shown for these peaks that did not co-elute with the OLE peak, so they did not interfere in the analysis (Fig. 2A, C, E and G). Therefore, the proposed chromatographic method offered an adequate selectivity for OLE analysis in EVOO, olive leaf extracts and NLCs.

3.2.3. Linearity. Based on the analysis of seven concentrations from 50 to 420 μg ml^{-1}, in triplicate, the linearity of the developed analytical method was evaluated (Fig. 3). The square of correlation coefficient (r^2) value was found to be over the acceptance criteria (>0.999) indicating that the developed method is linear in the considered range. Therefore, a good linearity was established over the studied range (50–420 μg ml^{-1}) demonstrating the suitability of the proposed method.

3.2.4. LOD and LOQ. The lowest concentration at which an analyte can be detected (LOD) or quantified (LOQ) with acceptable precision and accuracy was calculated from the standard deviation of the response and the slope obtained from the linear regression of a calibration curve with concentrations close to zero (Table 4). To assess these parameters, the following eight concentrations were prepared and analysed in triplicate: 0.2 μg mL^{-1}, 0.4 μg mL^{-1}, 0.6 μg mL^{-1}, 1 μg mL^{-1}, and 6 μg mL^{-1}. According to the obtained results, the LOD was found to be 0.08 μg mL^{-1}, whereas the LOQ was found to be 0.25 μg mL^{-1}.

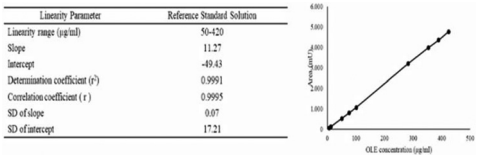

Fig. 3 Regression data and curve of the calibration curve for quantitative determination of OLE by HPLC.

3.2.5. Precision. For precision analysis, OLE standard solutions (50, 280 and 420 μg mL^{-1}) were prepared in triplicate, and analyzed on the same day (repeatability) or in three different days (intermediate precision). As shown in Table 5 both repeatability and intermediate precision did not exceed the maximum established RSD value: ≤2. The highest RSD value was found to be 1.92%. Therefore, the good precision of the method was confirmed.

3.2.6. Accuracy. In the present work, the accuracy of the method was studied using the average recovery values of the lowest, intermediate and upper concentration levels of the cali-

Table 4 Curve calibration parameters for LOD and LOQ determination

Linearity parameter	Reference standard solution
Linearity range (μg ml^{-1})	0.2–6.0
Slope	7.98
Intercept	0.44
Determination coefficient (r^2)	0.9994
Correlation coefficient (r)	0.9997
SD of slope	0.06
SD of intercept	0.20

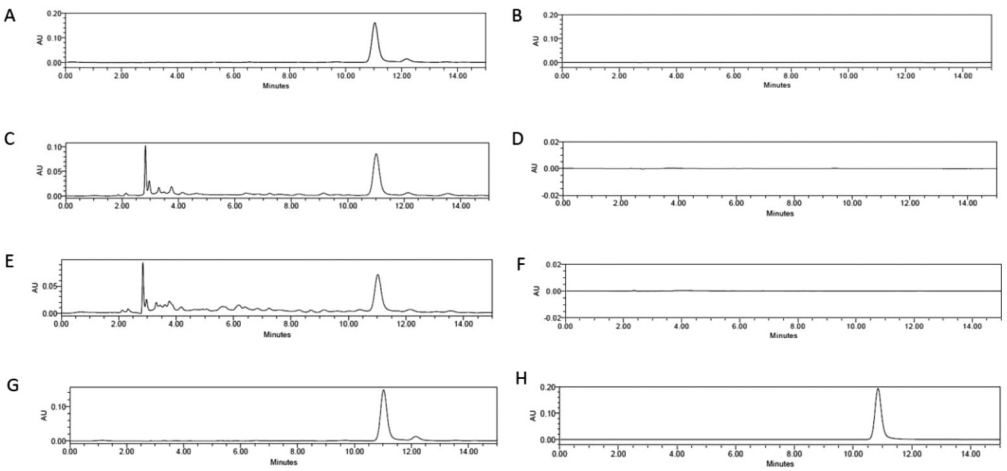

Fig. 2 Chromatograms of the NLC-OLE supernatant (A), Blank-NLC supernatant (B), *Olea europaea* L. aqueous extract (C), Water (D), EVOO-ph (E), EVOO (F), OLE for nanoencapsulation (purity > 80%, Nonaherbs®) (G), and OLE reference standard (H).

Table 5 Results from intra-day (repeatability) and inter-day (intermediate) precision studies

	Intra-day		Inter-day	
Level	% of recovery	RSD (%)	% of recovery	RSD (%)
Low	100.3	0.53	101.0; 99.2; 101.8	1.34
Medium	98.8	1.92	101.1; 99.1; 102.5	1.72
High	102.9	0.71	100.5; 99.5; 102.9	1.72

bration curve (50, 280 and 420 μg ml^{-1}), covering the linear range of the analyte. The supernatant of empty nanoparticles, distilled water and EVOO extraction solution were spiked with OLE standard solutions and analysed after three repeated injections for each sample, in triplicate. Results were expressed as the percentage of recoveries with their respective RSD (Table 6). For all samples at the three concentration levels tested, recovery was within 100 ± 1.89%. Since RSD values were ≤2, accuracy of the method was confirmed.

3.3. Method applicability to real samples

The developed and validated method in this work was proposed to study the presence of OLE in different sample matrices obtained through environmentally friendly processes. More specifically, we looked for an analytical method that with reduced organic solvent use could be able to quantify the OLE content in EVOO, *Olea europaea* L. extracts and EVOO based nanoparticles. Results are summarized in Table 7. The European Food Safety Authority has released a claim concerning the effectiveness of the ingestion of olive polyphenols on protecting low-density lipoprotein cholesterol (LDL) from oxidative stress (Commission Regulation (EU) 432/2012). On this basis, marketed olive oils should have a minimum content of 5 mg of oleuropein (or related polyphenols) per 20 grams of product in order to be acknowledged with the proposed health claim (*"Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress"*). However, these polyphenols are rarely found in regular olive oils and thus, it is difficult to find these products in the market labelling the claim. In fact, we have only detected three of them in the market: Oleohealth®, Oliveheart® and Secret to live®. Nevertheless, the last one corresponds to a food supplement composed of olive oil with the addition of olive leaf extract. Evidence suggests that specific factors such as the ripening state of the olive on harvest time, olive variety and olive oil processing method (temperature and pressure) have an important influence on that.[31–33] Other authors state that it is a consequence of the lack of adequate analytical methods to quantify OLE and related hydroxytyrosol and tyrosol in olive oils.[34] Routine analysis techniques for olive oils employ two-step extraction methods. Firstly, an extraction process with n-hexane is normally conducted to discard fat components followed by one or two hydroalcoholic extraction steps. In this work, the first step was avoided and extraction with MeOH–H_2O (50 : 50, v/v) seemed to be enough for OLE isolation. Other authors have proved that the defatting step led to a difference of 3% in the obtained results and thus, from an environmental point of view, in our case, it was not an essential step to carry out. Following our validated method, EVOO-ph showed a concentration of 0.52 mg per gram of oil (Table 6) whereas EVOO did not exert any signal. Taking into account these results, the intake of at least 10 gr of EVOO-ph might be enough for protecting LDL from oxidation and thus, the product accomplishes the requirements for labelling the aforementioned claim. Beyond this health claim, the truth is that currently there is no recommended dose for oleuropein or toxicity established for this compound and related phenols. Evidence in clinical trials suggests that oleuropein is safe in high doses up to 240 mg per person per day. Susalit *et al.* reported in a clinical study with hypertensive patients that the intake of 200 mg per person per day of oleuropein could effectively lower blood pressure in subjects with stage-1 hypertension and that the only side effects that might be related to this polyphenol may include headache and muscle discomfort.[35] In other clinical trials it was demonstrated that a daily intake of 51.1 mg of oleuropein combined with 9.7 mg of hydroxytyrosol for 12 weeks significantly improved insulin sensitivity and pancreatic β-cell secretory capacity in men at risk of developing metabolic syndrome.[36] In contrast, Castañer *et al.* also demonstrated the effectiveness of the intake of only 25 ml per day of high polyphenol content olive oil (around 8 mg of total polyphenols/person per day) to reduce systemic cardiovascular risk factors and the possibility to modulate genes involved in some chronic degenerative diseases.[37] Therefore, clinical data suggest that most of the pharmacological effects of these poly-

Table 7 OLE quantification from different samples. Results are expressed as mean ± standard deviation

Sample	Oleuropein content
EVOO-ph (mg g^{-1})	0.52 ± 0.03
Olea europaea L. aqueous extract (mg g^{-1})	5.18 ± 0.1
Olea europaea L. freeze-dried extract (mg g^{-1})	115.01 ± 1.94
NLC-OLE supernatant (mg ml^{-1})	30.62 ± 0.06

Table 6 Results from the accuracy study

			EVOO extraction solution			
OLE (μg ml^{-1})	Distilled water Recovery (%)	NLC-Supernatant RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
50	100.3	0.53	99.0	1.43	102.5	1.08
280	98.8	1.89	97.3	1.62	99.0	0.79
420	102.9	0.73	98.0	0.95	97.8	1.80

phenols need the intake of such an amount that could not be reached with the maximum recommended intake of olive oil per person per day. As a result, *Olea europaea* L. extracts emerge as an alternative source of olive polyphenols. In this work, *Olea europaea* L. aqueous extract was obtained through a green extraction method, using high temperatures and distilled water. The use of any organic solvent was avoided during the extraction process. Unknown peaks (probably other minor polyphenols) were found in the chromatograms of the studied extracts but the OLE peak showed good resolution (>1.5) and thus, no peak interfered in OLE quantification, indicating that the method was valid for OLE analysis in these samples. Aqueous extracts offer several advantages such as better bioactivity and less toxicity than their organic equivalents. However, they are most likely to be contaminated with yeast and molds because of their high water content and, at the same time, low concentration of the bioactive compounds is normally found in these kinds of extracts, especially when obtained through solvent-free extraction processes.[38] In industrial processes extraction is normally followed by a purification step, in which the main disadvantage is not only the high energy consumption but also the excessive use of organic solvents. Whilst several efforts have been made to minimize the use of hazardous solvents during extraction, this is not possible for the purification steps which are usually based on industrial-scale chromatography methods. Aimed to address this issue, we proposed the application of freeze-drying as a greener alternative for the purification of OLE which offers less harmful residues and lower waste amounts than conventional large-scale chromatography. Freeze-drying is a common process in the pharmaceutical, biotechnological and nutraceutical industries normally employed for improving the shelf life and storage capabilities of drugs and natural compounds. From a biological point of view, freeze-drying is also used for yeast preservation with the addition of cryoprotectants. However, some authors have also postulated the possibility to inactivate or even reduce those cells' viability by this drying technology. More specifically, the freezing step followed by sublimation is thought to influence yeast and mould inactivation whereas if freezing slowly, large ice crystals might be formed damaging cell membranes.[39] Given this scenario, with the aim of both decreasing risks of microbial contamination as well as increasing OLE purity in the final product, *Olea europaea* L. extract was freeze-dried for 36 hours. As a result, OLE content was increased from 5.18 to 115.01 mg g^{-1} and the amount of yeast and molds was reduced from 18000 to <100 UFC per g after the lypophilization process (Table 7). Therefore, the proposed procedure in this work offers a greener alternative for OLE extraction and purification through the avoidance of organic solvent use in both steps which could be applied to other natural compounds and might be easily implemented in industrial-scale processes meeting current Good Manufacturing Process (cGMP) regulations. Finally, with the aim to protect and improve the efficacy of OLE after oral administration, EVOO based NLCs were proposed in this work. The use of organic solvents was avoided during the nanoformulation process and a biodegradable matrix composed of glyceryl distearate (Precirol® ATO 5) and organic EVOO was employed. Given this, the HPLC method of the present work was also aimed to indirectly quantify the encapsulation efficiency of the nanoencapsulation process. Formulation components from the NLC supernatants (residual lipids and surfactants) did not interfere in OLE determination, as shown in the specificity assay. Therefore, the developed RP-HPLC-DAD method could be applied for OLE quantification in nanoparticles. OLE content in the NLC-OLE supernatant was found to be 30.62 ± 0.06 mg ml^{-1} and thus, the encapsulation efficiency was 94.09 ± 0.01%. In light of these results, it could be assumed that the analyte was successfully encapsulated in the EVOO-based NLCs, through the hot-melt emulsification method and therefore, it was validated as an adequate system for OLE encapsulation. We have only found one research article where a HPLC-DAD method was used for OLE determination in nanoemulsions, but the retention time was high (30 min) and no data about linearity nor system suitability were reported.[40] Therefore, to the best of our knowledge this is the first RP-HPLC-DAD method that has been validated for the quantification of OLE in NLCs, which is also applicable to *Olea europaea* L. extracts and olive oil.

3.4. Greenness assessment of the validated RP-HPLC-DAD method

Apart from validating a HPLC method for OLE quantification in the reported samples, offering the conventional standard parameters of good accuracy, precision and selectivity, greenness of the method was also taken into account. With this aim and from an eco-friendly perspective the well-known and established principles of the green chemistry were taken into account to optimize the developed HPLC method.[41] Green chemistry can be summarized by three words: Reduce, Replace and Recycle but when applying it to HPLC techniques only reduce and replace are viable options and thus, in this work, the amount of organic solvent employed for OLE quantification was reduced by shortening the analysis time and increasing the water proportion in the mobile phase composition and sample preparation process. The EAT[29] was used for greenness assessment of the validated HPLC method with regard to its potential health, safety and environmental impact. For this purpose, the EAT was applied to our method and those found in the literature for OLE quantification. The obtained results were used for comparison of our method with those from the literature (Table 8). As shown in Table 8, the total EAT value was strongly influenced by the use of organic solvents for sample pre-treatment and for instance, method D exhibited an EAT value of 69 for samples prepared in water and a value of 97 for those prepared with MeOH. The same occurred for our method (G) in which samples prepared in water (olive leaf extracts and nanoparticles) showed an EAT value of 12.87 whereas for samples of EVOO in which an hydroalcoholic extraction step was conducted, the EAT value was increased to 34.02. Nevertheless, it is worth mentioning that our method displayed the lowest EAT values for samples

Table 8 Summary of HPLC methods from the literature used for HPLC-EAT ranking

Method	No. of analytes	Type of sample	OLE R_T (min)	Analysis time (min)	Mobile phase	Elution method		Flow rate (ml min^{-1})	Validation	Sample preparation for HPLC	Total EAT
						Isocratic (v/v)	Gradient (steps)				
A[26]	2	EVOO and Olive fruit	10.6	~25	H_2O–ACN	70 : 30	—	0.5	No	*n*-Hexane, EtOH	67.20
B[23]	12	Olive leaf	18.0	62	H_2O–ACN/MeOH (50 : 50)	—	5	1.0	No	MetOH 80%	77.35
C[24]	7	Olive leaf	24.9	70	H_2O–ACN	—	5	1.0	No	MetOH 50%	135.56
D[22]	1	Olive leaf	27.0	60	H_2O–ACN	—	8	1.0	No	H_2O	69.62
										Or MetOH	97.79
E[4]	3	Stems and roots	50.0	100	H_2O–MeOH	—	12	1.0	No	MetOH	157.90
F[42]	2	Dietary supplement	11.0	15	H_2O–ACN	—	6	0.8	Yes	MetOH 50%	114.2
G[a]	1	EVOO, Olive leaf, and NLC	11.0	15	H_2O–ACN	80 : 20	—	1.2	Yes	H_2O	12.87
										Or MeOH 50%	34.02

[a] G is our validated method.

pre-treated with or without organic solvents. From the studied analysis, F was found to be the most similar to our method. The total EAT value for *F* was found to be 114 because they employed 100 ml of MeOH–H_2O (50 : 50, v/v) for chromatographic sample preparation. However, EAT could be reduced to ~19 by only decreasing the amount of solvent to 10 ml. Focusing on our method the overall EAT values were decreased to 12.87 for *Olea europaea* L. extracts and nanoparticle analysis whereas the total EAT value for EVOO samples was found to be 34.02. As aforementioned EVOO samples' EAT impact was higher due to the use of MeOH during the sample pre-treatment process.

Nevertheless, it has to be taken into account that other published methods usually employ another extraction step with n-hexane and even double-extraction step with ethanol which led to a higher EAT value. Therefore, our method was demonstrated to have the lowest impact on health, safety and environment for OLE quantification.

Beyond olive polyphenols, several other natural compounds have recently gained special attention due to their pleiotropic pharmacological activities and low toxicity. There is a clear trend towards the study of isolated natural polyphenols such as curcumin, resveratrol or quercetin among others. A number of preclinical data state their potential therapeutic effects as well as their unfortunately, typical limiting factors (*i.e.* low bioavailability, poor stability). As a result, many researchers are developing novel formulations to improve the stability and bioavailability of these promising compounds in which analytical techniques are mandatory. RP-HPLC is one of the main preferred techniques for that purpose and researchers take advantage of the amount of published methods for the determination of this kind of molecule. However, most of these methods were developed for the simultaneous determination of a number of molecules in a complex extract with long analysis time and thus, special attention should be paid to precisely adapt the analytical method to the determination of the isolated molecule of interest, with the aim to optimize time and avoid unnecessary waste of organic solvents. We have validated a RP-HPLC-DAD method for the determination of OLE in the NLC supernatant, aqueous extracts and EVOO samples with a low environmental impact which could be also applied for the determination of other phenolic compounds in a variety of formulations.

4. Conclusions

The RP-HPLC-DAD method developed and validated in this work represents an alternative approach for the quantification of OLE in several matrices such as olive oil, *Olea europaea* leaf extracts and lipid nanoparticles with a minimized environmental impact. The validation procedure was carried out according to ICH Q2(R1) guidelines and from an environmental perspective evaluating specificity, linearity, accuracy, precision and greenness. The method proved to be reliable and simple, complying with the requirements of these parameters in the range of 50–420 μg mL^{-1}. The LOD was found to be 0.08 μg mL^{-1} and LOQ 0.25 μg mL^{-1}. RSD obtained in the assays indicates that this method is adequate to quantify OLE in polyphenol rich-olive oil and aqueous and lyophilized extracts of olive leaves as well as nanoparticulated systems obtained through solvent-free methods. Besides, the method was proved to reduce the toxic impact against in regard to the environment, health and safety compared to other HPLC methods. The proposed method could be applied to the determination of other phenolic compounds in a variety of samples.

Abbreviations

ACN	Acetonitrile
DAD	Diode array detector
DDS	Drug delivery systems
EAT	Environmental Assessment Tool
EVOO	Extra virgin olive oil
EVOO-ph	Extra virgin olive oil rich in polyphenols
HDPE	High Density Polyethylene

H_2O	Water
HPLC	High performance liquid chromatography
k'	Capacity factor
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol
N	Number of theoretical plates
NLC	Nanostructured lipid carrier
NLC-OLE	Oleuropein loaded nanostructured lipid carrier
OLE	Oleuropein
PVDF	Polyvinylidene fluoride
R_S	Resolution
R_T	Retention time
RP-HPLC-DAD	Reverse phase high performance liquid chromatography coupled with a diode array detector
RSD	Relative standard deviation
T	Tailing factor

Conflicts of interest

The authors declare no competing conflicts of interest.

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