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Simple quantification of phenolic compounds present in the minor fraction of virgin olive oil by LC–DAD–FLD

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ABSTRACT

This paper presents the results of the study on the extraction, identification and quantification of a group of important phenolic compounds in virgin olive oil (VOO) samples, obtained from olives of various varieties, by liquid chromatography coupled to UV–vis and fluorescence detection. Sixteen phenolic compounds belonging to different families have been identified and quantified spending a total time of 25 min. The linearity was examined by establishing the external standard calibration curves. Four order linear ranges and limits of detection ranging from 0.02 to 0.6 μg mL^{-1} and 0.006 to 0.3 μg mL^{-1} were achieved using UV–vis and fluorescence detection, respectively. Regarding the real samples, for the determination of the phenolic compounds in higher concentrations (hydroxytyrosol and tyrosol) a simple liquid–liquid extraction with ethanol was used to make the sample compatible with the mobile phase. Recovery values close to 100% were obtained. However, a previous solid phase extraction with Diol cartridges was necessary to concentrate and separate the minor phenolic compounds of the main interferences. The parameters affecting this step were carefully optimized and, after that, recoveries near 80–100% were obtained for the rest of the studied phenolic compounds. Also, the limits of detection were improved 15 times. Finally, the standard addition method was carried out for each of the analytes and no matrix effect was found, so the quantification of the 16 phenolic compounds from different monovarietal VOO was carried out by using the corresponding external standard calibration plot.

1. Introduction

Under the denomination "phenolic compounds" there are more than 4000 compounds divided in 12 subclasses [1]. Currently, these compounds are receiving considerable attention, fundamentally due to their antioxidant activity, and strong relation to cancer prevention, inflammatory disorders and cardiovascular diseases [2,3]. They are part of the minor components of virgin olive oil (VOO), one of the most important foods in the Mediterranean diet which has many associated benefits on the human health fundamentally due to its content in phenolic compounds [4]. In addition, phenolic compounds and their strong natural antioxidant activity contribute to the stability of virgin olive oil (VOO) against oxidation and influence its organoleptic characteristics and nutritional qualities [5]. The composition of phenolic compounds in VOO is related to agronomic and technological aspects [6]. Considering the importance of this kind of analytes and the complexity of the oil samples, it is very interesting to develop simple and rapid analytical methods for the characterization and quantification of these compounds. In addition, the price of VOO depends on olive variety and analytical tools are necessary to authenticate monovarietal oils.

A large number of procedures to isolate the polar phenolic fraction of the olive oil sample have been employed, although two different extraction techniques have been mainly used: liquid–liquid extraction (LLE) using methanol:water mixtures [7] and solid phase extraction (SPE) using different types of sorbents, although the best results have been obtained when Diol cartridges are employed [8]. Although good results have been obtained, this methodology requires the use of high amounts of olive oil and solvents. In addition, studies of cartridge saturation and recovery assays using the VOO matrix have not been carried out. Regarding the determination, many methods have been developed for the detection and quantification of phenolic compounds in different types of samples liquid chromatography (LC) being the most used technique [1]. Reversed-phase columns (RP) are the most commonly used, mainly C_{18}, ranging from 150 to

Abbreviations: FL, Fluorescence; VOO, Virgin olive oil; GAL, Gallic acid; 3,4-DHPEA or HYTY, hydroxytyrosol; DOPAC, 3,4-dihydroxyphenylacetic acid; GEN, Gentisic acid; p-HPEA or TY, Tyrosol; 4HB, 4-hydroxybenzoic acid; 4HP, 4-hydroxyphenylaceitc acid; VAN, Vanillic acid; CAF, Caffeic acid; SY, Syringic acid; *p*-CUM, *p*-coumaric acid; *m*-CUM, *m*-coumaric acid; FER, Ferulic acid; *o*-CUM, *o*-coumaric acid; LUT, Luteolin; APIG, Apigenin; LOD, Limit of detection; LOQ, Limit of quantification

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250 mm in length with ID from 4.6 mm, and particle sizes of 5 μm. In specifying the determination of compounds in VOO, traditional methods have been replaced by separation techniques, such as gas chromatography (GC), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) coupled to different detectors [9–12]. However most of the proposed methodologies have been focused on the optimization of LC methods. A summary of the LC methodologies is shown in Table 1 and to complete this information the review published in 2005 by Carrasco-Pancorbo et al. is recommended to be consulted [12]. C_{18} columns followed by UV–vis detection have been fundamentally employed. Nevertheless, the use of C_{18} stationary phases results in high analysis time, as a consequence of its inherent nonpolarity. To reduce the total analysis time different alternatives have been used, such as ultra high performance liquid chromatography (UHPLC) [13,20], rapid resolution liquid chromatography (RRLC) [14,15] or chemometric tools [16]. Another interesting alternative to reduce the total analysis time could be the use of more polar stationary phases and, to the best of our knowledge, no paper in this sense has been published. Regarding the detection, the most used detectors have been the diode array (DAD) and the mass spectrometer (MS) detectors and only a few recent papers show how to perform the determination of phenolic compounds using a fluorescence detector (FLD) [25,27]. MS detectors are being increasingly used after many forms of separation, due to their near-universality, their ability to elucidate or confirm structures and their high sensitivity. However, it is not always possible to dispose of an MS detector, fundamentally due to its cost, which is not affordable for many laboratories. In addition, the matrix effects may become very important when an MS detector with electrospray ionization (ESI) is used [20]. Matrix effects result from co-eluting matrix components that compete for ionization capacity. This competition produces significant errors in the accuracy and precision of the analytical method [30]. Regarding the determination of phenolic compounds using FLD, Selvaggini et al. [25] evaluated the hydrophilic phenols (phenolic acids, secoiridoid derivatives and lignans) of VOO based on the direct injection (DI) in HPLC with the use of FLD and also carried out a comparison with the traditional LLE and between DAD and FLD. Good results were obtained for the compounds in higher concentrations in olive oil, since 2 g of VOO was diluted to 10.00 mL with acetone for the DI. On the other hand, García et al. [27] have researched the phenolic composition of the most commonly sold VOO varieties, *picual*, *hojiblanca*, *cornicabra* and *arbequina*, with the aim of obtaining a database on phenolic compounds by HPLC–DAD–FLD. A chemical classification of VOOs by chemometric procedures was also undertaken.

The aim of the present work is to develop simple, rapid and effective methods for the identification and quantification of phenolic compounds at different concentration levels in VOO samples by liquid chromatography. We highlight the advantages of the use of a more polar column, as well as the possibility of detection and quantification of trace phenolic compounds using an SPE with Diol cartridges, and a simple LLE (for phenolics in higher concentrations) by UV–vis and FL detection coupled to LC, without making necessary the use of more expensive detectors. Variables related both to the SPE and the LLE have been carefully optimized and good extraction recoveries have been obtained. No matrix effect was found and the quantification of the phenolic compounds was successfully performed by the external standard calibration plot of each of them.

2. Experimental

2.1. Chemicals, solutions and samples

For all experiments, analytical reagent grade chemicals and solvents were used. Ultrapure water was obtained from a Millipore Milli-QA10 System (Waters, Germany). Hydroxytyrosol (HYTY), luteolin (LUT) and apigenin (APIG) were obtained from Extrasynthèse (Genay, France); gallic acid (GAL), 3,4-dihydroxybenzoic acid (DOPAC), tyrosol (TY), 4-hydroxybenzoic acid (4HB), 4-hydroxyphenylacetic acid (4HP), vanillic acid (VAN), caffeic acid (CAF), syringic acid (SY), *p*-coumaric acid (*p*-CUM), *m*-coumaric (*m*-CUM), ferulic acid (FER) and *o*-coumaric acid (*o*-CUM) from Sigma-Aldrich Chemie GmbH (Steinheim, Germany); and gentisic acid (GEN) from Aldrich (Gillingham-Dorset, England). All solvents employed were HPLC grade; ethanol and

Table 1
Comparison between the analytical techniques employed for the analysis of phenolic compounds in VOO by LC.

Analyte	Analytical technique	Stationary phase	Total analysis time of phenolic compounds (min)	Reference
Phenolic compounds	UHPLC-MS/MS	UPLC BET C_{18}	7.5	[13]
Phenolic compounds	RRLC-ESI-TOF-MS	Zorbax C_{18}	35	[14,15]
Four phenolic acids	HPLC-DAD and chemometrics	Varian Inertsil-5 C_{18}	20	[16]
Minor components (phenolic compounds, α-tocopherols and pigments) and fatty acids	HPLC-DAD	Spherisorb S3 ODS-2	65	[17]
Simple phenols, phenolic acids and flavonoids	HPLC-DAD	C_{18}	85	[18]
Major (triacylglycerols and fatty acids) and minor compounds (squalene, tocopherols, chlorophylls, carotenoids and phenols)	HPLC-DAD/MSD	Luna C_{18}	75	[19]
Phenolic compounds	HPLC-FLD	Inertsil ODS-3 AcQuity	55	[20]
Phenolic compounds	UPLC-DAD-MS/MS	UPLC™ BEH (C_{18})	29	[20]
Phenolic compounds	HPLC-DAD-MS	Luna RP18	70	[21]
Phenolic compounds	HPLC-TOF-MS	C_{18}	45	[11]
Phenolic compounds and phospholipids	MLC-DAD	Nucleosil 120 C_{18}	57	[22]
Phenolic compounds metabolites	HPLC/ESI-MS/MS	Luna C_{18}	50	[23,24]
Major phenolic compounds	HPLC-DAD-FLD	ChromSep Inertsil ODS-3 and Spherisorb ODS-1	73	[25]
Phenolic compounds	HPLC-DAD HPLC-MS/MS	Luna C_{18}	45	[26]
Phenolic compounds	HPLC-DAD-FLD	Spherisorb ODS-2	80	[27]
Phenolic compounds	HPLC-DAD	Spherisorb S3 ODS-2	65	[28]
Phenolic compounds	HPLC-DAD	Spherisorb ODS-2	85	[29]
Phenolic compounds	HPLC-ECD	Spherisorb ODS-2	85	[29]

acetonitrile were provided by Panreac (Spain), acetic acid by Romil Chemicals LTD (England) and 1-propanol by Sigma-Aldrich (USA).

1.00 mg mL^{-1} stock solutions of each compound were prepared in 1-propanol. These solutions were stored at 4 °C, avoiding exposure to direct light. Fresh solutions of lower concentrations were prepared daily by appropriate dilution of the stock solution with the selected solvent. VOO samples were acquired from the market and kept at 4 °C avoiding exposure to direct light. Monovarietal olive oils analyzed were obtained from the olive varieties: *Manzanilla cacereña*, *cornicabra*, *arbequina*, *picual*, *hojiblanca* and *morisca*. It is important to clarify that these samples are characterized as a particular group of VOO, called extra virgin olive oil (EVOO), since they present an acidity value $\leq 2.0\%$.

2.2. Instrumentation and software

The chromatographic studies were performed using an Agilent Model 1100 LC instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with a degasser, quaternary pump, column oven, autosampler Agilent 1290 infinity thermostated at 5 °C, UV–vis diode-array detector, rapid scan fluorescence spectrophotometer detector, and the Chemstation software package to control the instrument, and for data acquisition and data analysis. The analytical column employed was a Hypersil MOS 100 mm × 2.1 mm id and 5 μm particle size (Agilent Technologies). The column temperature was set at 25 °C. The mobile phase components were high-purity water with 0.5% acetic acid and 1% acetonitrile (A) and acetonitrile with 0.5% acetic acid (B) and were degassed by ultrasonication before use. The gradient program was as follows: 0–10 min, 0% B; 10–20 min, 25.6% B; 20–22 min, 27.8% B; 22–23 min, 40% B; 23–24 min, 98% B; 24–27 min, 100% B, 27–30 min, 100% B. Finally, the B content was decreased to the initial conditions (0%) and the column re-equilibrated for 15 min. The flow rate was set constant at 0.5 mL min^{-1} and the injection volume was 10 μL. Calibration curves and analytical figures of merit were performed by means of the ACOC program, in Mat Lab code.

2.3. Procedures

2.3.1. Calibration curves

To build the calibration curves, standards solutions containing all phenolic compounds in variable concentrations (Tables 3 and 4) were prepared in triplicate, in water with 0.5% acetic acid and 1% acetonitrile, taking the corresponding volumes of more concentrated stock solutions in 1-propanol and evaporating this solvent to dryness. 10 μL of the resulting solutions was injected in the chromatographic system and separation and detection were carried out under the optimized conditions. Once the chromatograms were obtained, the retention time and the analytical signals (peak area or height) were measured using the ChemStation package.

2.3.2. Analysis of HYTY and TY in virgin olive oil samples: Liquid–liquid extraction with ethanol

HYTY and TY are extracted from the VOO matrix by LLE with ethanol [31]. For this, 1 g of VOO is weighed in a centrifuge tube and 2 mL of ethanol is added. The mixture is stirred for 2 min in an ultrasonic bath and the tube is centrifuged. After that, the ethanolic phase is separated and 0.10 mL of it is diluted with water, with acetic acid (0.5%) and acetonitrile (1%) until 1.00 mL and filtered through 0.20 μm PTFE filter. Finally, this solution is injected in the chromatographic system for its analysis in the optimized conditions.

2.3.3. Analysis of minor phenolic compounds in virgin olive oil samples: Solid phase extraction with Diol cartridges

A Diol-SPE procedure previously described elsewhere [15] was slightly modified and applied with the aim of obtaining recoveries values from the VOO matrix close to 100% for all the studied phenolic compounds. Isohexane was used instead of hexane due to its lower toxicity and good behavior in the recovery of all the phenolic compounds. Firstly, the cartridges are conditioned with 10 mL of methanol and 10 mL of isohexane, and then 15 g of VOO dissolved in 15 mL of isohexane is slowly passed though the column. After removing the non-polar fraction with 15 mL of isohexane, the phenolic compounds are eluted with 40 mL of methanol. The final volume is dried in a rotary evaporator under reduced pressure at 40 °C and the residue is reconstituted in 1.00 mL of methanol:water 50:50 v:v, with the aim of solving only the polar fraction, and filtered through 0.20 μm PTFE filters. Subsequently, 0.20 mL of this solution is diluted with 0.20 mL of water/0.5% acetic acid/1% acetonitrile. This is to make the composition of the sample similar with the composition of the mobile phase at the beginning of the gradient and guarantee a good shape of the peaks. Finally, the solution thus obtained is injected in the system for its chromatographic analysis.

3. Results and discussion

3.1. Previous chromatographic studies

A selection of phenolic compounds of different families has been made that, according to literature, can be present in virgin olive oil and for which standards are available (Table 2). First, chromatographic and detection (DAD and FLD) conditions were optimized in order to get suitable sensitivity and reduced analysis time for these 16 phenolic compounds.

Typically, the conventional LC methods employ C_{18} columns. In the case of phenolic analysis, it sometimes involves long run times, even more than 60 min, when DAD and FLD detection is used (Table 1). In this work, a Hypersil MOS column was used to achieve a shorter run time, given the polarity of this column regarding the traditional C_{18} columns. Hypersil MOS has monolayer coverage of dimethyloctyl silane chemically bonded to the silica surface and it is suitable for the analysis of non-polar to moderately polar solutes, like the studied phenolic compounds, so the analytes are more weakly retained and more quickly eluted resulting in shorter run times. On the other hand, most of the previously proposed chromatographic methods for the analysis of phenolic compounds are reverse phase methods, and carry out

Table 2
Studied phenolic compounds and their spectral parameters.

Analyte	λ_{max} (nm)	λ_{exc}	λ_{em}
Gallic acid (GAL)	275	205, 270	370
Hydroxytyrosol (HYTY)	280	220, 290	330
3,4-Dihydroxyphenylcacetic acid (DOPAC)	280	227, 245	320, 380
Gentisic acid (GEN)	325	230	450
Tyrosol (TY)	275	225, 290	315
4-Hydroxybenzoic acid (4HB)	255	220, 270	330
4-Hydroxyphenylaceitc acid (4HP)	275	230, 290	320
Vanillic acid (VAN)	260	225, 275	360
Caffeic acid (CAF)	320	–	–
Syringic acid (SY)	275	230, 290	370
p-Coumaric acid (*p*-CUM)	310	215, 240	420
m-Coumaric acid (*m*-CUM)	280	–	–
Ferulic acid (FER)	320	300	450
o-Coumaric acid (o-CUM)	275, 320	220	440
Luteolin (LUT)	350	–	–
Apigenin (APIG)	340	–	–

the elution by using gradient elution, being mobile phase mixtures of diluted acid solutions and organic solvents. Acetic, formic and phosphoric acids, and methanol and acetonitrile, are the most common acidic and organic components of mobile phases, respectively. Based on these previous literature data and taking into account that to separate 16 different polarities phenolic compounds a gradient elution is required, the mobile phase was selected; it was constituted by water/0.5% acetic acid/1% acetonitrile (phase A) and acetonitrile/0.5%acetic acid (phase B). Different gradients were tested to achieve a good separation of the 16 phenolic compounds in a time as small as possible using a flow rate of 0.5 mL min^{-1} and finally the following was selected: 0–10 min, 0% B; 10–20 min, 25.6% B; 20–22 min, 27.8% B; 22–23 min, 40% B; 23–24 min, 98% B; 24–27 min, 100% B, 27–30 min, 100% B. Finally, the B content was decreased to the initial conditions (0%) and the column re-equilibrated for 15 min. Once the column was selected and the mobile phase gradient was optimized, the column temperature was set at 25 °C to avoid variations in the ambient temperature that may have influence on the retention times and shape of the peaks.

To select the best conditions for the DAD and FLD for these compounds, studies in relation to their spectral behavior in the chromatographic conditions were carried out. The absorption, fluorescence excitation and emission spectra of these compounds were obtained and their maximum absorption, excitation and emission wavelengths are shown in Table 2. According to these results, 254 nm, 280 nm, 310 nm and 350 nm were initially selected in the DAD and the FLD was performed by measuring of the sample emission using an excitation wavelength of 300 nm. Emission wavelengths of 330 nm, 350 nm, 380 nm and 450 nm were selected.

Once the chromatographic and detection parameters were set, a standard solution containing all the phenolic compounds was injected in the chromatographic system. In the chromatogram, the peaks were assigned by comparison of the retention times and the UV–vis and FL spectra in the apex with those obtained for solutions of each analyte. On the other hand, it has been observed that when the standards and samples are prepared in different media to mobile phase, such as 1-propanol and methanol, an important deformation in the first peaks of the chromatogram is produced. Therefore, the samples were prepared in the aqueous initial mobile phase of the gradient. Also, it is important to take into account the behavior of the phenolic compounds regarding the material of the used filters. Different filters were tested to inject the phenolic compounds standards in the chromatographic system with the aim of using one in which all of the studied phenolic compounds were not retained. It was observed that nylon retained some of the phenolic compounds of interest whereas all the phenolic compounds cross the hydrophobic polytetrafluoroethylene (PTFE) filters without being retained. Thus, hydrophobic PTFE filters, 0.20 μm pore size, were finally selected. Fig. 1 shows the chromatogram obtained in the optimum conditions for a standard sample of the 16 phenolic compounds. A good separation is obtained in a time not higher than 25 min. Regarding the detection, not all of the studied

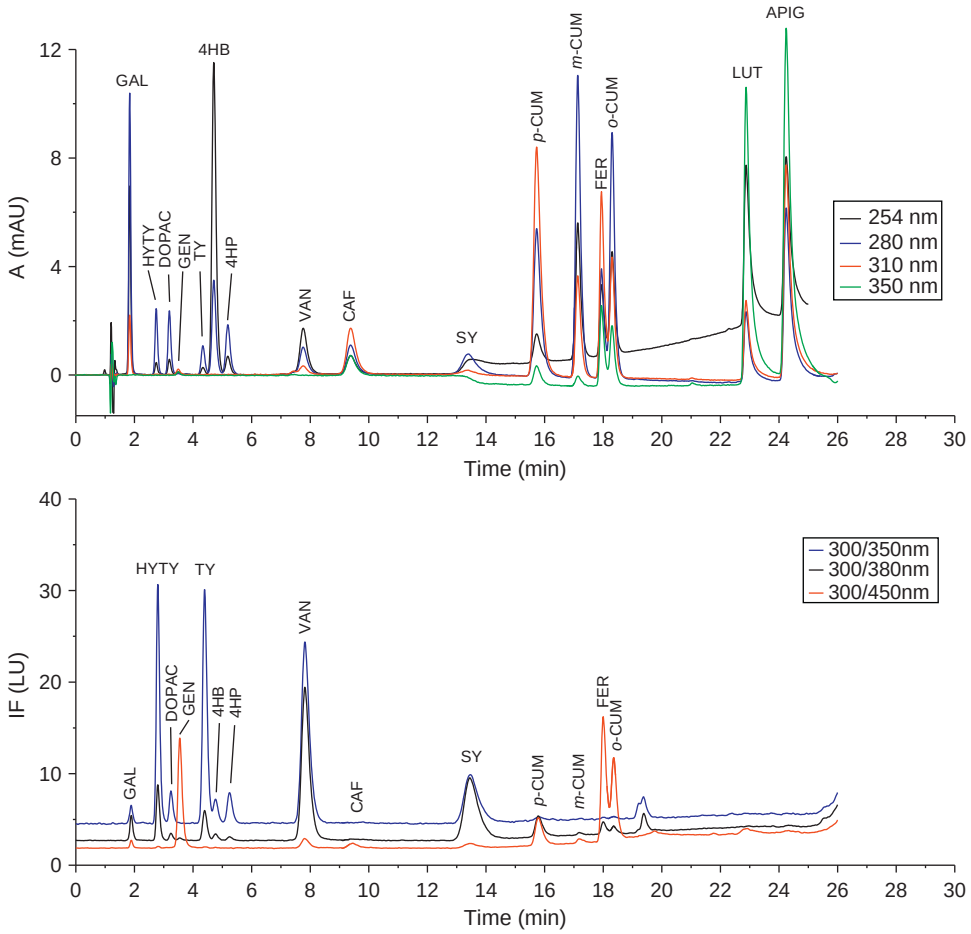

Fig. 1. Chromatogram of the standard of 16 phenolic compounds in the optima separation conditions. DAD signals (upper); 254 nm (—) 280 nm (—), 310 nm (—) and 350 nm (—), and FLD signals (lower): 300/350 nm (—), 300/380 nm (—) and 300/450 nm (—). Phenolic compounds concentrations in water with 0.5% acetic acid and 1% acetonitrile: 1 μg mL^{-1}, GAL, HYTY, DOPAC, TY, 4HB, *p*-CUM, *m*-CUM, FER, *o*-CUM; 0.1 μg mL^{-1}, GEN; 2 μg mL^{-1} 4HP, 0.5 μg mL^{-1}, VAN, CAF, SY; 5 μg mL^{-1}, LUT, APIG. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

phenolic compounds are fluorescent in these conditions. In fact CAF, *m*-CUM, LUT and APIG are practically not fluorescent (Table 2 and Fig. 1). In contrast, GEN, VAN, SY, HYTY and TY exhibit a good fluorescent signal. The fluorescent behavior of the rest of the compounds is between these two situations. In conclusion, the use of one or another signal will depend on the inherent characteristics of the problem sample, such as selectivity and sensitivity.

3.2. Method validation: Analytical parameters

For the validation of the method, the calibration curves of each compound were established, according to the procedure described in Section 2.3.1, and the analytical figures of merit were calculated employing the peak areas and heights, using both the UV–vis and the FL signals. The obtained results using the peak areas are shown in Tables 3 and 4 for the UV–vis and FL signals, respectively. Very good analytical parameters were also obtained when the peak heights were employed (data not shown). The limits of detection (LOD) and quantification (LOQ) were calculated as the concentrations corresponding to three and 10 times the standard deviation of the lower concentration standard signal, respectively. The evaluation of the precision of the optimized method was done by analyzing standard solutions of the phenolic compounds, in the same day (intra-day precision, $n=6$; two different concentration levels) and in successive days before the beginning of the experimental work (inter-day precision, $n=10$). The RSD values of the analytical signals and retention times were determined considering the 16 studied compounds. Data obtained in this study are summarized in Table 5. Both intra-day and inter-day repeatability values are lower than 6.3%, so they may be considered as a guarantee of the goodness of the proposed method.

3.3. Selection of the conditions for extraction of the phenolic compounds from olive oil samples

Lastly, studies related to the applicability of the proposed method for the determination of these compounds in real samples of VOO were carried out. The concentration range of phenolic compounds in VOO is very broad [6], HYTY and TY being two of the most important and major compounds in these samples [7,15,23], relative to the studied phenolic compounds. The high content in these compounds allows carrying out their detection without preconcentration steps being necessary [10]. The rest of the studied phenolic compounds, in general are present in VOO

Table 3
Analytical figures of merit. UV–vis detection and peak area as analytical signal.

Analyte	Wavelength (nm)	Lineal range ($\mu g\ mL^{-1}$)	Intercept ± SD	Slope ± SD ($mL\ \mu g^{-1}$)	Determination coefficient (r^2)	LOD[a] ($\mu g\ mL^{-1}$)	LOQ[b] ($\mu g\ mL^{-1}$)
GAL	280	0.263–263	17 ± 64	49.9 ± 0.6	0.997	0.04	0.06
HYTY	280	0.212–212	−43 ± 22	22.4 ± 0.3	0.997	0.03	0.04
DOPAC	280	0.290–290	26 ± 14	11.9 ± 0.1	0.998	0.05	0.2
GEN	310	0.0560–11.1	−1.2 ± 0.5	20.1 ± 0.1	0.999	0.03	0.06
TY	280	0.210–210	−5 ± 5	10.25 ± 0.07	0.999	0.09	0.2
4HB	254	0.268–268	40 ± 120	104 ± 2	0.998	0.03	0.05
4HP	280	0.515–515	0.4 ± 0.8	0.740 ± 0.005	0.999	0.02	0.2
VAN	254	0.0550–55.0	−11 ± 12	61.4 ± 0.5	0.999	0.03	0.04
CAF	310	0.159–159	−25 ± 47	89.7 ± 0.8	0.999	0.07	0.1
SY	280	0.158–158	−13 ± 27	55.1 ± 0.5	0.999	0.04	0.07
p-CUM	310	0.550–550	202 ± 202	129 ± 1	0.999	0.06	0.2
m-CUM	310	0.260–260	−7 ± 55	46.7 ± 0.6	0.997	0.1	0.2
FER	310	0.253–253	6 ± 108	85 ± 1	0.997	0.1	0.2
o-CUM	310	0.255–255	−13 ± 36	53.3 ± 0.4	0.999	0.1	0.3
LUT	350	0.500–100	−48 ± 51	36 ± 1	0.990	0.1	0.2
APIG	350	0.580–116	16 ± 32	30.9 ± 0.7	0.995	0.6	0.7

SD: Standard deviation.
[a] Limit of detection.
[b] Limit of quantification.

Table 4
Analytical figures of merit. Fluorescence detection and peak area as analytical signal.

Analyte	$\lambda_{exc}/\lambda_{em}$	Lineal range ($\mu g\ mL^{-1}$)	Intercept ± SD	Slope ± SD ($mL\ \mu g^{-1}$)	Determination coefficient (r^2)	LOD[a] ($\mu g\ mL^{-1}$)	LOQ[b] ($\mu g\ mL^{-1}$)
GAL	300/380 nm	0.263–26.3	0 ± 2	19.1 ± 0.2	0.998	0.06	0.3
HYTY	300/350 nm	0.212–5.30	−2 ± 3	212 ± 1	0.999	0.02	0.04
DOPAC	300/350 nm	0.290–29.0	−2 ± 4	29.9 ± 0.3	0.998	0.04	0.2
GEN	300/450 nm	0.0110–1.11	15 ± 8	1274 ± 15	0.999	0.006	0.009
TY	300/350 nm	0.210–5.25	−9 ± 3	262 ± 2	0.999	0.05	0.08
4HB	300/350 nm	0.268–26.8	0 ± 4	29.1 ± 0.3	0.998	0.2	0.5
4HP	300/330 nm	0.515–22.0	11 ± 8	59.8 ± 0.8	0.998	0.3	0.4
VAN	300/350 nm	0.0550–5.50	14 ± 22	652 ± 9	0.998	0.03	0.06
SY	300/380 nm	0.158–5.25	−5 ± 22	466 ± 9	0.995	0.02	0.04
p-CUM	300/450 nm	0.550–55.0	−10 ± 4	57.1 ± 0.2	0.999	0.09	0.2
FER	300/450 nm	0.550–10.1	−10 ± 18	182 ± 4	0.995	0.1	0.3
o-CUM	300/450 nm	0.255–10.2	12 ± 13	114 ± 3	0.993	0.05	0.1

SD: Standard deviation.
[a] Limit of detection.
[b] Limit of quantification.

Table 5
RSD (%) values of the migration time and the peak area for each compound.

Analyte	Intra-day ($n=6$) (low concentration standard[a])		Intra-day ($n=6$) (high concentration standard[b])		Inter-day[c] ($n=10$)	
	Migration time	Peak area	Migration time	Peak area	Migration time	Peak area
GAL	1.2	0.46	0.14	0.30	0.70	0.38
HYTY	0.38	2.4	0.13	1.7	0.93	3.4
DOPAC	0.60	1.6	0.24	0.21	1.4	1.7
GEN	0.73	2.4	0.35	0.58	1.7	3.3
TY	0.53	1.4	1.5	0.19	1.3	0.93
4HB	0.74	0.38	0.31	0.20	1.8	0.49
4HP	0.76	0.91	0.31	0.98	1.8	2.6
VAN	0.80	2.0	0.35	0.58	2.0	2.1
CAF	1.1	0.86	0.46	0.21	2.4	1.4
SY	0.92	1.6	0.39	0.85	2.1	2.0
p-CUM	0.53	0.13	0.20	0.22	3.0	1.1
m-CUM	0.35	0.78	0.13	0.25	1.5	1.0
FER	0.26	0.68	0.077	0.30	0.74	0.59
o-CUM	0.28	0.97	0.095	0.16	1.7	0.93
LUT	0.12	2.7	0.038	2.2	0.26	6.3
APIG	0.13	1.2	0.052	3.8	0.27	5.8

[a] Low concentration standard containing 0.5 μg mL^{-1} GAL, HYTY, DOPAC, TY, 4HB, *m*-CUM, FER, *o*-CUM; 0.05 μg mL^{-1} GEN; 1 μg mL^{-1} 4HP, LUT, APIG; 0.1 μg mL^{-1} VAN; 0.3 μg mL^{-1} CAF, SY; 0.75 μg mL^{-1} *p*-CUM.

[b] High concentration standard containing 5 μg mL^{-1} GAL, HYTY, DOPAC, TY, 4HB, 4HP, *p*-CUM, *m*-CUM, FER, *o*-CUM; 0.25 μg mL^{-1} GEN; 1 μg mL^{-1} VAN, CAF, SY; 10 μg mL^{-1} LUT, APIG.

[c] Ten different days. Standard containing 1 μg mL^{-1} GAL, HYTY, DOPAC, TY, 4HB, *p*-CUM, *m*-CUM, FER, *o*-CUM; 0.1 μg mL^{-1} GEN; 2 μg mL^{-1} 4HP; 0.5 μg mL^{-1} VAN, CAF, SY; 5 μg mL^{-1} LUT, APIG.

samples in lower concentrations [12]. Therefore, to carry out their determination, preconcentration stages are required. Two of the most used extraction and preconcentration procedures are LLE of olive oil solved in n-hexane with methanol:water 60:40 v:v, in successive steps [7], and SPE using Diol cartridges [8]. In the present work, two different extraction methods were tested and applied to extract and quantify the studied phenolic compounds from different monovarietal VOO samples. Below, the optimization and the obtained results with each of them are shown.

3.3.1. Liquid–liquid extraction with ethanol for TY and HYTY analysis

The hydroxytyrosol or (3,4-dihydroxyphenyl)ethanol (3,4-DHPEA or HYTY) and the tyrosol or (*p*-hydroxyphenyl)ethanol (*p*-HPEA or TY) are two of the main phenolic alcohols in VOO [6] and in this work they have been found to be the most abundant of the studied phenolic compounds. For this reason, for their determination a preconcentration stage is not necessary, so the direct injection of the olive oil sample was tested. The injection of the sample was initially assayed, dissolved in tetrahydrofuran (THF) and 1-propanol, in the chromatographic system. The obtained chromatograms after dissolving 0.05 g of VOO in 1 mL of each one of these solvents and filtration through 0.20 μm PTFE hydrophobic filters are shown in Fig. 2**A**. The TY and HYTY peaks were detected; however, an important peaks deformation was observed, maybe as a consequence of the different compositions between the sample (olive oil in an organic solvent) and the mobile phase (water/0.5% acetic acid/1% acetonitrile). Subsequently according to previous results [31] a simple LLE of these compounds with ethanol was employed. Different olive oil amounts were tested and finally the extraction of 1 g of VOO with 2 mL of ethanol was selected, since an adequate sensitivity and selectivity for the determination of these two compounds

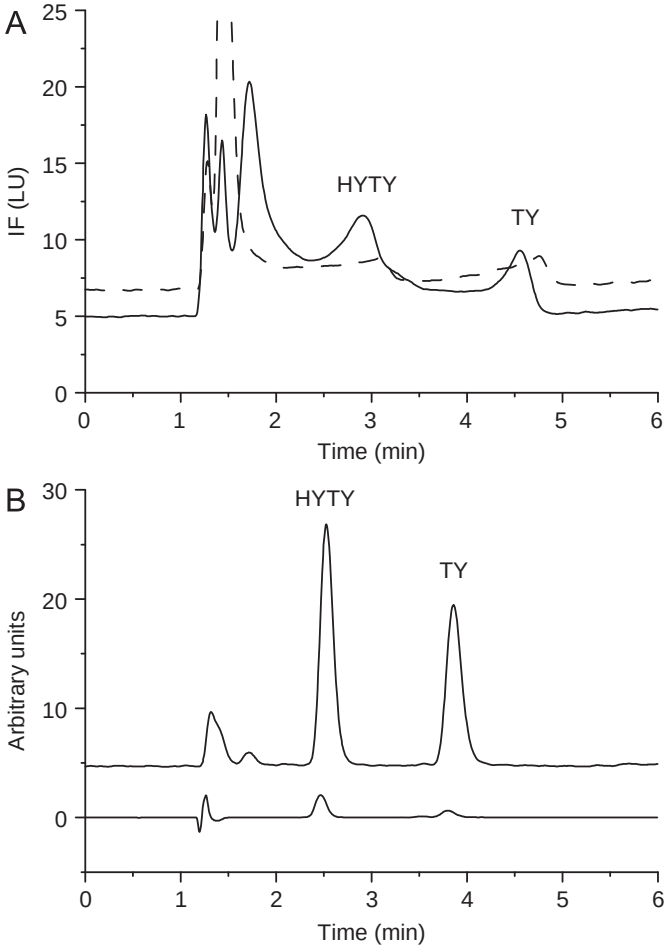

Fig. 2. (**A**) Chromatogram of a VOO sample (0.05 g) dissolved in 1 mL of THF (solid line) and in 1-propanol (dashed line). (**B**) Chromatogram of a VOO sample (1 g) subjected to the LLE with ethanol; UV–vis signal at 280 nm (lower) and FL signal at $\lambda_{exc}/\lambda_{em}$ 300/350 nm (upper).

was obtained. The extraction was performed by stirring the olive oil ethanolic mixture in an ultrasound bath for 2 min and then centrifuging it (Section 2.3.2). After that, 0.10 mL of the ethanolic phase was diluted until 1.00 mL with water/0.5% acetic acid/1% acetonitrile to obtain similar sample and mobile phase composition, filtered through 0.20 μm PTFE filters and injected in the chromatographic system (Fig. 2**B**). The peaks corresponding to HYTY and TY present a very good shape and both the sensitivity and the selectivity are adequate for their determination. In addition, the separation was very simple, since it has been performed in a time less than 5 min with water/0.5% acetic acid/1% acetonitrile as mobile phase in isocratic mode. Finally, recovery assays of these two compounds were carried out. For this the VOO samples were spiked with HYTY and TY in different concentration levels before and after the LLE and the obtained results were compared. The recovery values obtained were between 80% and 100% in both cases (Table 6).

3.3.2. Solid phase extraction with Diol cartridges for the phenolic compounds analysis

One of the most used extraction procedures found in the literature to carry out the isolation of phenolic compounds from VOO is the solid phase extraction (SPE) using Diol cartridges [8,11,14,15]. Studies related to this extraction procedure were carried out with the aim of obtaining a complete extraction of the

Table 6
Recovery values obtained with the extraction procedures utilized, LLE with ethanol for HYTY and TY, and Diol-SPE for the rest of the studied phenolic compounds.

Analyte	Extraction recovery (%)
GAL	91
DOPAC	80
HYTY	81
GEN	80
TY	92
4HB	96
4HP	89
VAN	96
CAF	84
SY	87
p-CUM	95
m-CUM	93
FER	86
o-CUM	76
LUT	91
APIG	58

16 phenolic compounds. Initially, different capacity (500 mg and 1 g) Diol cartridges were tested to achieve higher recuperation. For this, 30 g of VOO was spiked before and after extraction with the analytes and the SPE was carried out according to the procedure employed by García-Villalba et al. [15] using different capacity cartridges. The obtained results showed that when 500 mg cartridges were employed low extraction recovery values were obtained. However the extraction recoveries undergo a substantial increase with the 1 g cartridges, so it was finally decided to use them. Later, saturation studies of these cartridges were carried out. For this, different VOO amounts (10, 15, 20 and 30 g) spiked with a given concentration of all phenolic compounds, except the most abundant (HYTY, TY, VAN, LUT and APIG) were extracted. Most of the compounds showed a linear increase of peak area with the increase of the olive oil amount; however, for TY, VAN and SY a curvature of the signal, probably related to the cartridge saturation, was observed from 20 g of olive oil. Then, according to these results, recuperation assays of the SPE with Diol cartridges were again carried out, but now employing 15 g of olive oil, spiking before and after the extraction with the phenolic compounds and comparing the obtained signals in both cases. The obtained values are shown in Table 6. Recovery values near 80–100% have been obtained for all the studied phenolic compounds, except for the APIG with a recovery of 58%. Regarding HYTY and TY, as mentioned before, it was tested that they are present in olive oil at enough levels to make their direct determination possible, without needing a preconcentration step. Therefore, these compounds were quantified by the simpler extraction method already described. Finally, it is important to take into account that the recovery assays have been carried out using the extra virgin olive oil matrix, in contrast to the employed procedures by many authors, where refined olive or other vegetable oils spiked with the phenolic compounds are employed as matrix.

In Fig. 3, a chromatogram corresponding to a VOO sample (15 g), subjected to the Diol-SPE procedure previously optimized and described in Section 2.3.3, is shown. The peaks were identified by the retention time and comparing the UV–vis or FL apex spectra of a determined peak in the sample with the corresponding spectra in a standard of all analytes. In addition, enrichment of the olive oil with the compounds, in the concentration level in which they may be found in the sample, was carried out to confirm the assignation of the peaks. Once the peaks were identified, different UV–vis and FL wavelengths were used for their determination, according to the selectivity and sensitivity they provide. Regarding the LUT peak, there is another olive oil peak overlapping with it when detection was at 350 nm, its optimum detection wavelength (Table 2). This unknown compound presented an absorption spectrum very similar to that of LUT, but the small difference between both allowed us to determine LUT in presence of the other, making use of the DAD possibilities. In this way, the contribution of the unknown compound was eliminated by detecting the LUT at 348 nm with a reference wavelength of 293 nm. On the other hand, as regards the FLD, a wavelength program was necessary to avoid the detector saturation in some moments. Thus, the excitation wavelength was maintained at 300 nm, while the emission wavelength changed as follows: 0–4.5 min, λ_{em} 450 nm; 4.5–6 min, λ_{em} 330 nm; 6–11.5 min, λ_{em} 350 nm; 11.5–14.5 min, λ_{em} 380 nm; 14.5–30 min, λ_{em} 450 nm. In addition to this program, λ_{em} 450 was also selected. In these conditions, many of the phenolic compounds were found as can be observed in Fig. 3**A** and **B**, where the signals at the selected wavelengths in each case have been shown. Finally, it is pointed out that the use of the Diol-SPE as previous cleaning and preconcentration stage implies improving the LOD of the method 15 times, resulting in intervals between 2 and 40 ng $g^{-1}_{\text{olive oil}}$ and between 0.4 and 20 ng $g^{-1}_{\text{olive oil}}$ for UV–vis and FL detection, respectively.

3.4. Quantification of the phenolic compounds in virgin olive oil

After the optimization of the conditions for the extraction and separation of the 16 phenolic compounds, the method was checked by analyzing different monovarietal VOO samples. To carry out the quantification of the 16 phenolic compounds, firstly the influence of the matrix over the chromatographic separation was evaluated. For this, the standard addition calibration curves were obtained for each one of the phenolic compounds after the extraction procedure and the calibration slopes were compared with the corresponding slopes of the external standard calibration plots of each analyte. In all cases, no matrix effect was found since similar slopes were obtained and, finally, the external calibration method was applied. Six monovarietal VOO samples were analyzed (Table 7). To determine the phenolic compounds in lower concentrations, the Diol-SPE previously optimized and described in Section 2.3.3 was applied for each one of the varieties, and for the determination of HYTY and TY the LLE with ethanol, described in Section 2.3.2, was also employed for each one of the varieties. In both cases, the analyses were performed in triplicate for each olive oil variety. Many of these phenolic compounds were found and the concentrations are shown in Table 7. The concentration values are in the order of those found by other authors [13,15,18,27,32] taking into account that the phenolic content in olive oil is influenced by different factors, such as the olive variety, location, environmental conditions or degree of ripeness [33], as well as agronomic and technological aspects of production [6]. Regarding the total concentration of these phenolic compounds, these values are 386 mg kg^{-1}, 298 mg kg^{-1}, 254 mg kg^{-1}, 453 mg kg^{-1}, 261 mg kg^{-1} and 301 mg kg^{-1} for *picual*, *arbequina*, *hojiblanca*, *cornicabra*, *morisca* and *manzanilla cacereña* olive oil varieties, respectively. According to these results, *picual* and *cornicabra* olive oils have a higher amount of them, which is in accordance with the results previously published by other authors [27].

4. Conclusions

The present study demonstrates the ability of the liquid chromatography coupled to UV–vis and fluorescence detection to separate, identify and quantify a group of 16 phenolic compounds, from different monovarietal VOO samples. The use of a Hypersil MOS column allows employing a shorter elution

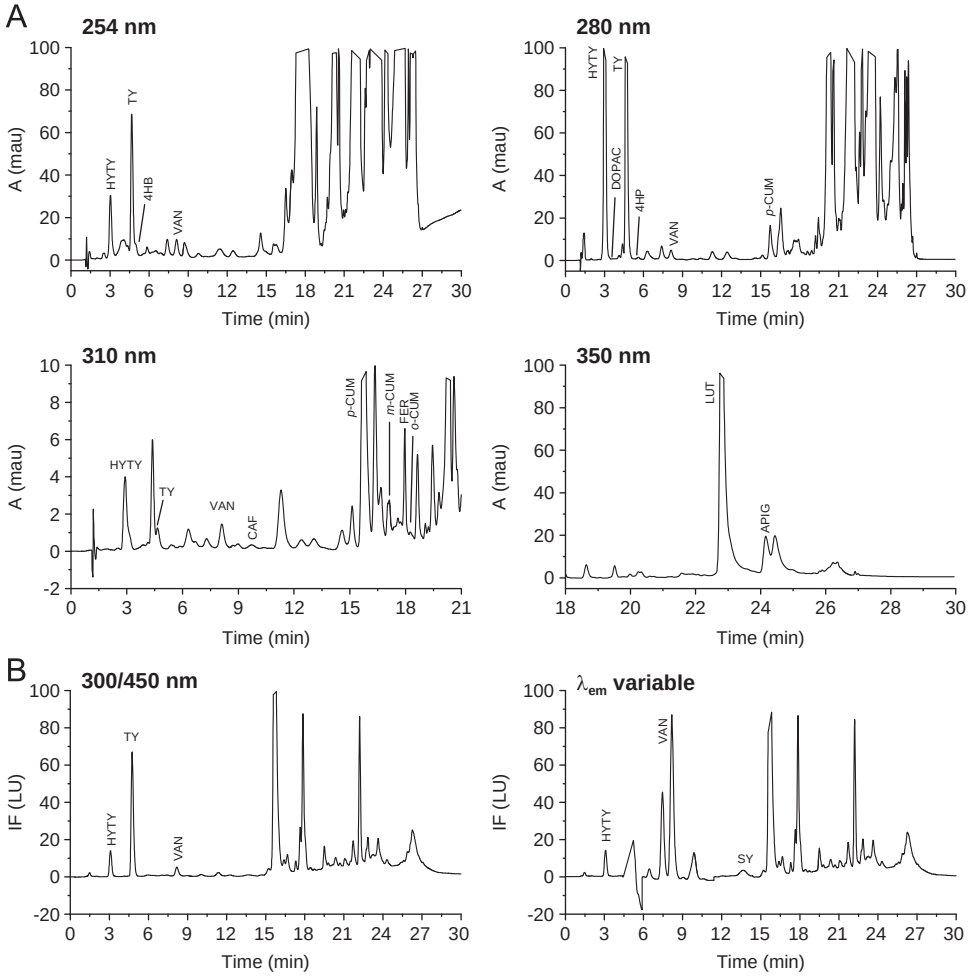

Fig. 3. Chromatogram of a VOO sample (15 g) subjected to the Diol-SPE procedure. (**A**) DAD signals and (**B**) FLD signals. Wavelengths emission program: 0–4.5 min, λ_{em} 450 nm; 4.5–6 min, λ_{em} 330 nm; 6–11.5 min, λ_{em} 350 nm; 11.5–14.5 min, λ_{em} 380; 14.5–30 min, λ_{em} 450 nm.

Table 7
Results of the analysis of real VOO samples. Phenolic compound concentrations (standard deviation).

Analyte ($\mu g\ g^{-1}$)	**Cornicabra**	**Morisca**	**Arbequina**	**Hojiblanca**	**Picual**	**Manzanilla Cacereña**
HYTY	21.2 (0.3)	9.1 (0.3)	10.0 (0.3)	10.5 (0.3)	20.8 (0.3)	9.0 (0.3)
TY	20.8 (0.2)	10.4 (0.2)	11.5 (0.2)	8.7 (0.2)	13.2 (0.2)	16.3 (0.2)
Analyte ($\mu g\ kg^{-1}$)	**Cornicabra**	**Morisca**	**Arbequina**	**Hojiblanca**	**Picual**	**Manzanilla Cacereña**
GAL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
DOPAC	n.d.	n.d.	68 (8)	68 (8)	68 (8)	68 (8)
GEN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4HB	n.d.	69 (7)	n.d.	78 (7)	18 (8)	37 (8)
4HP	n.d.	1610 (20)	1550 (20)	1910 (10)	1310 (20)	n.d.
VAN	155 (3)	391 (6)	589 (9)	628 (10)	156 (4)	147 (3)
CAF	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
SY	10 (3)	42 (3)	53 (4)	22 (4)	36 (4)	17 (4)
p-CUM	62 (7)	564 (8)	186 (6)	62 (7)	139 (6)	163 (6)
m-CUM	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
FER	n.q.	89 (7)	67 (7)	n.q.	n.q.	n.q.
o-CUM	n.d.	n.d.	n.d.	n.d.	n.d.	n.q.
LUT	2800 (80)	3520 (110)	5110 (160)	2870 (90)	2640 (80)	3990 (130)
APIG	278 (42)	295 (41)	677 (41)	530 (40)	223 (42)	386 (41)

n.d., not detectable.
n.q., not quantifiable.

time and consequently to spend less time in the analysis, even when a relatively high number of phenolic compounds were studied. In addition, a good separation has been obtained in these conditions.

The detection has been carried out using the UV–vis and FL detectors, this research showing the capabilities of these detection modes. The use of DAD and FLD in series offers high selectivity and sensitivity, without assuming a high acquisition

cost, and they are easy to use and do not need any maintenance. These results are interesting when other detectors as MS, usually considered better, are not available. In addition, the coupling with a more polar stationary phase than C_{18} allows performing the determination in shorter periods of time. Two simple extraction procedures to isolate, and also to concentrate the minor phenolic compounds, have been carefully optimized and applied. Recovery values from near 80% up to 100% have been obtained in most cases with these two procedures.

Finally, the applicability of the proposed method to the analysis of phenolic compounds in real VOO samples has been examined and encouraging results have been obtained. No matrix effect was found in the chromatographic analysis of the phenolic compounds and their quantification was carried out in a simple way by using the external standard calibration plots. Reliable results have been obtained in all cases.

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