

Improved method for determination of waxes in olive oils: reduction of silica and use of a less hazardous solvent

Andrea Milani^{1,*}, Paolo Lucci¹, Martina Sedran^a, Erica Moret¹, Sabrina Moret¹ and Lanfranco Conte^b

¹ Dept. of Agro-Food, Environmental and Animal Sciences – University of Udine, Via Sondrio, 2/a, 33100 Udine, Italy

Received 2 February 2020 – Accepted 16 March 2020

Abstract – The evaluation of the content of waxes is requested both by IOC Trade Standard and by Regulation (EEC) 2568/91 and its further amendments. The official method uses 15 g of silicic acid and elutes several fractions by using huge volumes of dangerous solvent (n-hexane). The developed method uses 1 g of silicic acid with a different particle size and less than 20 mL of solvent mixture, substituting n-hexane with less toxic isooctane. Briefly, after spiking with a suitable internal standard, oil sample is fractionated by SPE (Solid Phase Extraction) cartridge with 1 g of silica, waxes are eluted with 14 mL of isooctane/ethyl ether 99/1 (6 mL discarded and 8 mL collected), then, after elution sample is reconstituted in 200 µL of n-heptane and analysed by capillary GC. Data of “In home” validation, (repeatability, accuracy and recovery) and relative chromatograms are reported in this paper.

Keywords: olive oils / oil purity / waxes / Solid Phase Extraction / gas chromatography

Résumé – Amélioration de la méthode de détermination des cires dans les huiles d'olive : réduction de la silice et utilisation d'un solvant moins nocif. L'évaluation de la teneur de cires est demandée à la fois par la norme commerciale de l'IOC (*International Olive Council*) et par le règlement (CEE) n° 2568/91 et ses modifications ultérieures. La méthode officielle utilise 15 g d'acide silicique et des volumes importants de solvant dangereux (n-hexane). La méthode développée utilise 1 g d'acide silicique avec une taille de particules différente et moins de 20 mL de mélange de solvants, en remplaçant le n-hexane par de l'isooctane moins toxique et moins volatil. Ainsi, après l'introduction d'un étalon interne, l'échantillon d'huile est fractionné par cartouche SPE (*Solid Phase Extraction*) avec 1 g de silice, les cires sont extraites avec 14 mL d'isooctane/éthyle 99/1 (6 mL éliminés et 8 mL collectés), puis, après extraction, l'échantillon est reconstitué en 200 µL de n-heptane et analysé par chromatographie en phase gazeuse capillaire. Les données de la validation (répétabilité, précision et récupération) et les chromatogrammes relatifs sont présentés dans cet article.

Mots clés : huiles d'olive / pureté de l'huile / cires / extraction en phase solide / chromatographie en phase gazeuse

1 Introduction

The production chain of oils from olive fruits involves a mechanical extraction, that leads to two edible categories named extra virgin (EVOO) and virgin (VOO) and one not suitable for human consumption, named lampante (LOO), while the by product, named “olive pomace” that still contains some amounts of oil is extracted by means of solvent, as happens for most of the seed oils.

EVOO is the most valuable and, hence the one that had been prone to frauds, even if considered in an historical perspective.

A number of methods had been developed and approved as official worldwide (European Commission, 1991) to

highlight faked oils; this reduced so much the possibility to mix seed oils, thanks to the assessment of fatty acids composition and later of sterols composition; in the past this moved attention of frauders to the use of olive pomace oil to perform frauds by mixing this solvent extracted oil to mechanical extracted ones.

The measurement of the amount of erythrodiol and uvaol, two triterpenic dialcohols mainly present in the fruit skin, was adopted by Italian National law since 1975, after they were studied by Jacini and Fedeli (1972) and the method adopted at national level (NGD method, 1985).

Later, in 1991, the Reg (EEC) 2568/91 (European Commission, 1991) adopted this parameter.

Some technological means were however developed to remove huge amounts of these dialcohols so that Camera (1981–1983) proposed the so called “alcoholic index” as a suitable tool to discover this illegal practice; later, Tiscornia *et al.* (1985)

*Correspondence: andrea.milani.1@spes.uniud.it

^a Part of this work as BSc Diploma thesis.

^b At present retired.

slightly modified the Camera's method by proposing the "Alkanols" content evaluation.

The rationale for this depends on the presence of high concentration of waxes that, after saponification (a step used to prepare the unsaponifiable fraction for the analysis of sterols), give rise to fatty acids and aliphatic alcohols.

The alkanols method was extensively applied and this highlighted the existence of selected oils (mainly from Greece) that presented concentration of alkanols high enough to exceed the legal limit, even if mechanically extracted.

Later, [Mariani *et al.* \(1991\)](#) highlighted the presence of high amounts of alcohols but low amounts of waxes, [Mariani and Fedeli \(1986\)](#) proposed the measurement of the content of waxes as a suitable analytical tool to assess the mixture with solvent extracted oils.

The method involves the pre-separation of the waxes fraction by liquid chromatography by using a silica column, followed by GC analysis.

Some drawbacks of the method are the use of a discrete amount of silica (15 g), the use of relevant volumes of solvent (about 360 mL) and last but not least, the use of n-hexane, that is nowadays considered as healthy risk solvent. Of course, the experimental conditions result as time consuming, in fact, Mariani himself proposed within the International Olive Council (IOC) oil chemists working group, the use of 3 g of silica and the method is present in the IOC as provisional adopted ([International Olive Council, 2012](#)).

In the present work, some modifications of the method are proposed, namely the use of a different silica that admit to reduce its amount at 1 g, as a consequence, a lower volume of solvent (less than 20 mL) is used; furthermore, isooctane was used instead of n-hexane, that is suspected of damaging fertility and that may cause damage to organs (nervous system) (H361f, H373). In a previous paper, [Nota *et al.* \(1999\)](#) proposed the use of 1 g of silica gel, however, the use of high toxic solvent (carbon tetrachloride) was proposed, too.

The establishment of limits underwent to some modifications, depending on poor separation obtained by some laboratories of ester C40 from phityl Behenate that make its measure subject to erroneous data: for this reason IOC decided not to include C40 in the calculation of the total waxes content in the case of extra virgin and virgin oil. For these oil, the limit had been established at ≤ 150 mg/kg, while for lampante oil it is ≤ 300 mg/kg and for pomace (any one) > 350 mg/kg ([International Olive Council, 2018](#)).

A peculiar topic is the possibility of increasing of waxes concentration along oil ageing, as demonstrated by [Mariani and Venturini \(2006\)](#), mainly in oil with high concentration of free fatty acids.

2 Experimental procedures

2.1 Samples

Three different oils were used: an extra virgin olive oil (EVOO), low acidity (0.30%), one year old, Refined Olive Oil (ROO) and Lampante oil (LOO), one year old.

2.2 Chemicals

Silicic acid 60: 0.015–0.04 mm (Macherey-Nagel Duren Germany, Cat. No. 815650)

Isooctane, diethyl ether, n-heptane analytical grade.

Wax C32 (internal standard): Lauryl arachidate (Sigma Aldrich Milan, Italy, Cat No. A8671), n-heptane solution 0.02 mg/mL in the case of virgin and lampante oil or 0.04 mg/mL in the case of olive pomace oil.

2.3 Apparatus

SPE (Solid Phase Extraction) glass tubes 6 mL with PTFA frits, i.d. 12 mm (Sigma Aldrich Milan, Italy, Cat No504394)
SPE manifold (Sigma Aldrich, Milan, Italy).

Gas chromatograph equipped with cold on column injector and FID detector (Fison's MEGA 5300 – Fison's Milan Italy).

Fused silica capillary column 8 m \times 0.32 mm i.d. \times 0.10 μ m stationary phase, coated with MEGA-5 stationary phase (MEGA, Milan, Italy).

Microsyringe for on column injection 10 μ L, with hardened needle.

Rotary evaporator (Büchi, Switzerland).

Ordinary laboratory glassware.

Analytical balance for weighting to an accuracy of within ± 0.1 mg.

2.4 Procedure

2.4.1 Solid phase extraction cartridge preparation

The glass SPE cartridge was prepared transferring 1 g of silica into it between two frits, one above and one below the silica layer, then, the cartridge was attached to the vacuum chamber and conditioned with 5 mL of isooctane and this fraction was discharged.

2.4.2 Sample preparation and analytical conditions

200 μ L of internal standard solution was put in a screw cap tube, then solvent is evaporated by a gentle nitrogen stream and about 50 ± 1 mg of oil are added then dissolved in 0.5 mL of isooctane.

The sample prepared as described above, was quantitatively loaded into the SPE cartridge and the solvent was eluted just above the frit on top of the silica, this volume of solvent was discarded.

Waxes are then eluted with 14 mL isooctane/ethyl ether mixture at 99:1, v/v discarding the first 6 mL and collecting the following 8 mL in a 10 mL ground glass stopper tube. During this step, the column was not allowed to run dry. Vacuum was applied to obtain a flow of about 1 drop every second. The resultant fraction was dried in a rotary evaporator under reduced pressure until all the solvent has been eliminated. Then 200 μ L of n-heptane were added and the solution was analysed by GC-FID applying the following operative conditions: temperature of detector (FID) was set at 350 °C, temperature of the oven programmed from 80 °C (1 minute isotherm), then increased to 240 °C at a rate of 20 °C/min, then to 270 °C at a rate of 7.5 °C/min, lastly to 340 °C at a rate of 10 °C/min. Final isotherm is maintained for 20 minutes.

Helium was used as carrier gas, at a constant pressure of 30 kPa, hydrogen at 50 kPa, air at 80 kPa.

[Figure 1](#) reports the flow chart of the proposed procedure.

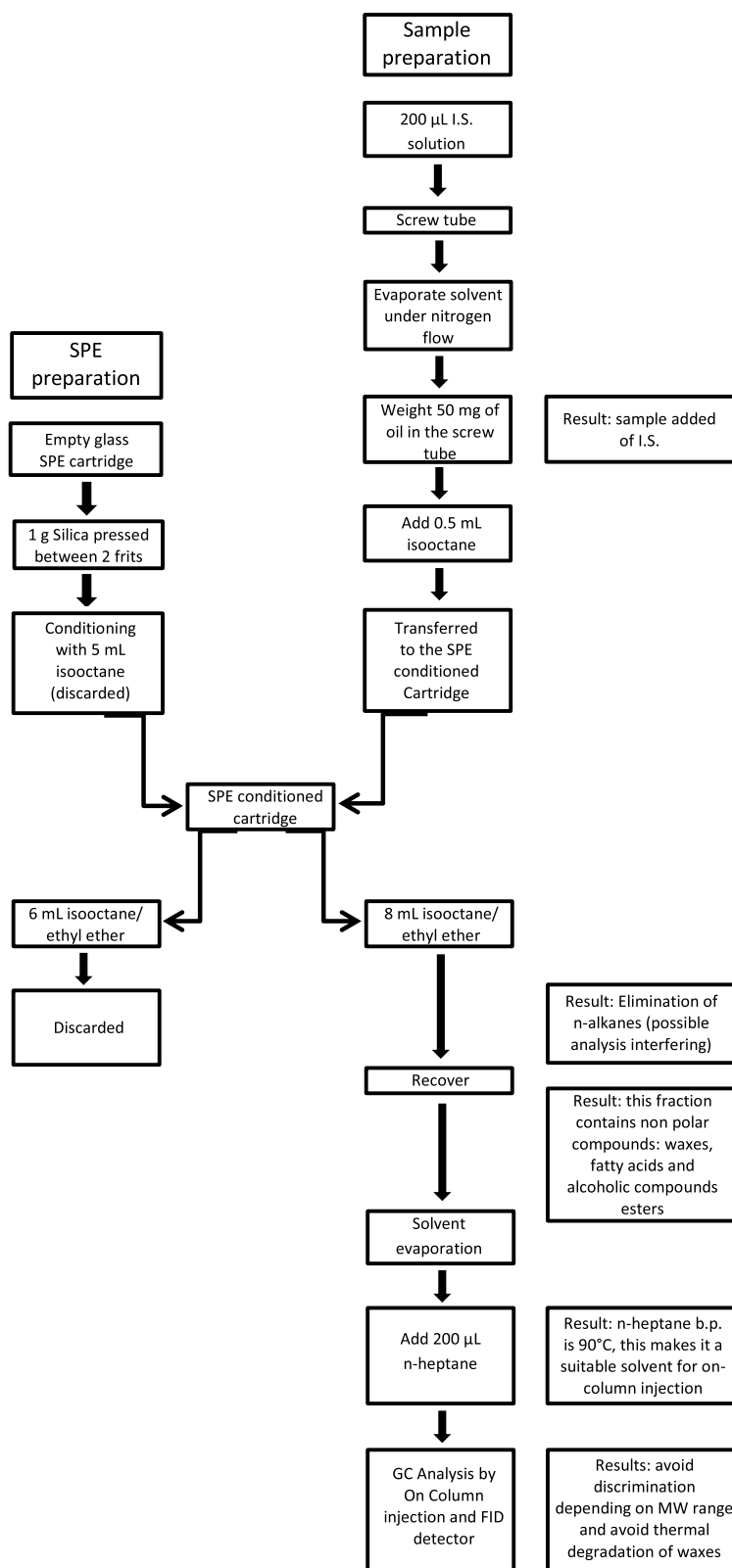


Fig. 1. Flow chart of the proposed method.

Table 1. Validation of the method by using EVOO.

	Waxes (mg/kg)						Mean	SD	RSD
	A	B	C	D	E	F			
C40	10.77	10.22	12.89	8.96	9.72	10.40	10.45	0.62	5.97
C42	11.66	11.32	11.62	10.73	11.39	11.84	11.42	0.39	3.39
C44	4.24	3.97	5.08	5.56	4.13	4.30	4.55	0.63	13.82
C46	7.95	7.25	7.30	6.69	7.55	7.68	7.40	0.43	5.84
∑Waxes C42-C46	23.79	22.54	24.00	22.98	23.07	23.83	23.37	0.59	2.51
∑Waxes C40-C46	34.56	32.76	34.40	32.48	32.78	34.23	33.53	0.95	2.85

2.5 Calculation

The amount of waxes is calculated by the following formula:

$$C_x \text{ Wax, mg/kg} = \frac{A_x * M_s * 1000}{A_s * M}$$

Where:

- A_x = Area of peak of wax x;
- M_s = internal standard mass (mg);
- A_s = Area of peak of internal standard;
- M = mass of the sample (g).

Results are reported with two decimal figures and sum of single wax concentration is reported.

2.6 Method validation

The method had been validated by calculating repeatability according to the following steps:

- Calculation of mean;
- Calculation of standard deviation;
- Calculation of relative standard deviation.

3 Results and discussion

The method had been in house validated by performing six replicated analysis of three different oils.

Mean, standard deviation and relative standard deviation had been calculated and data are reported in the following tables; furthermore, recovery and accuracy were evaluated, by analysing a sample of refined hazelnut oil, spiked with a known amount of synthetic waxes.

Table 1 reports data obtained by analysing an EVOO. The RSD of the proposed method (2.51) is closed to the one reported within the IOC method: bearing in mind that in the case of EVOO only waxes C42, C44 and C46 must be considered to obtain the sum, the value of this method is 2.51%, while the IOC method (International Olive Council, 2017) reports $RSD\% = 2.7\%$ when waxes concentration is 125 mg/kg or 2.29% when waxes concentration is 26 mg/kg: that's to say a comparable concentration.

Even if, as already said, the IOC standard does not consider C40 in the case of EVOO, we did because the sum enclosing C40 will be useful when comparing theoretical and experimental data in the case of ROO/EVOO blends (see later in this paper).

In Figure 2 is reported the GC chromatogram of the EVOO oil analysed with relative enlargement of the waxes area.

Data reported in Table 2 deal with validation carried out using refined olive oil. In this case, the content of waxes is higher than extra virgin; the IOC Trade Standard fixed a limit ≤ 350 mg/kg and the mean of waxes concentration of sample used for the validation in this study is 331.87 mg/kg that makes it comparable to the sample containing 346 mg/kg and 479 mg/kg reported in the validation data of IOC; in this case, too, the RSD obtained in the present study for repeatability (1.90) is comparable to the IOC RSD_r (1.5 and 1.44%).

In Figure 3 is shown the Refined olive oil GC chromatogram.

Data of validation in the case of lampante oil are reported in Table 3. This sample respect the limit established for a Lampante oil (≤ 300 mg/kg); is important to underline the possibility that a lampante oil could cross this limit if aged and characterized by an high free acidity; Mariani and Venturini (2006) highlighted the possibility of a strong increase of waxes concentration in high acidity oils during ageing.

IOC Waxes method that uses 3 g of silica reports data for oils that can be classified as lampante on the basis of ethyl esters so we could compare our results on the basis of concentration only, the closely one to our data is 310 mg/kg with a RSD of repeatability of 2.51%, in this case, our results are better.

In Figure 4 is reported the lampante oil chromatogram where there is also highlighted by a brace the presence of methyl- and ethyl esters.

Recovery and accuracy were evaluated by analysing, with 6 replicates, a sample of refined hazelnut oil spiked with a known amount of synthetic waxes C40–C46 (335 mg/kg of total waxes).

The GC trace is reproduced in Figure 5, while results are reported in Table 4.

Recovery can be considered as quantitative as its mean is $99.51\% \pm 0.85$.

To obtain a further validation of this method two different solutions of ROO spiked with 5% and 10% of EVOO were prepared and the analyses of both of them were performed in triplicate. The means of obtained results are very close to theoretical recovery that are 316.95 mg/Kg for the 5% solution and 302.04 mg/Kg for the 10% solution of EVOO in ROO respectively, as shown in Tables 5 and 6. The theoretical recovery is calculated based on the mean concentration obtained for the six replicates of pure EVOO and ROO with the following equations:

Table 2. Validation of the method by using Refined olive oil.

	Waxes (mg/kg)						Mean	SD	RSD
	A	B	C	D	E	F			
C40	91.65	94.50	88.62	100.78	94.53	100.54	95.10	4.82	5.07
C42	102.23	104.17	105.22	100.25	97.90	100.54	101.72	2.71	2.66
C44	85.51	88.37	87.61	90.66	89.34	93.10	89.10	2.61	2.93
C46	46.84	47.10	44.74	46.05	45.00	45.96	45.95	0.95	2.06
∑Waxes C40-C46	326.23	334.14	326.19	337.73	326.77	340.13	331.87	6.29	1.90

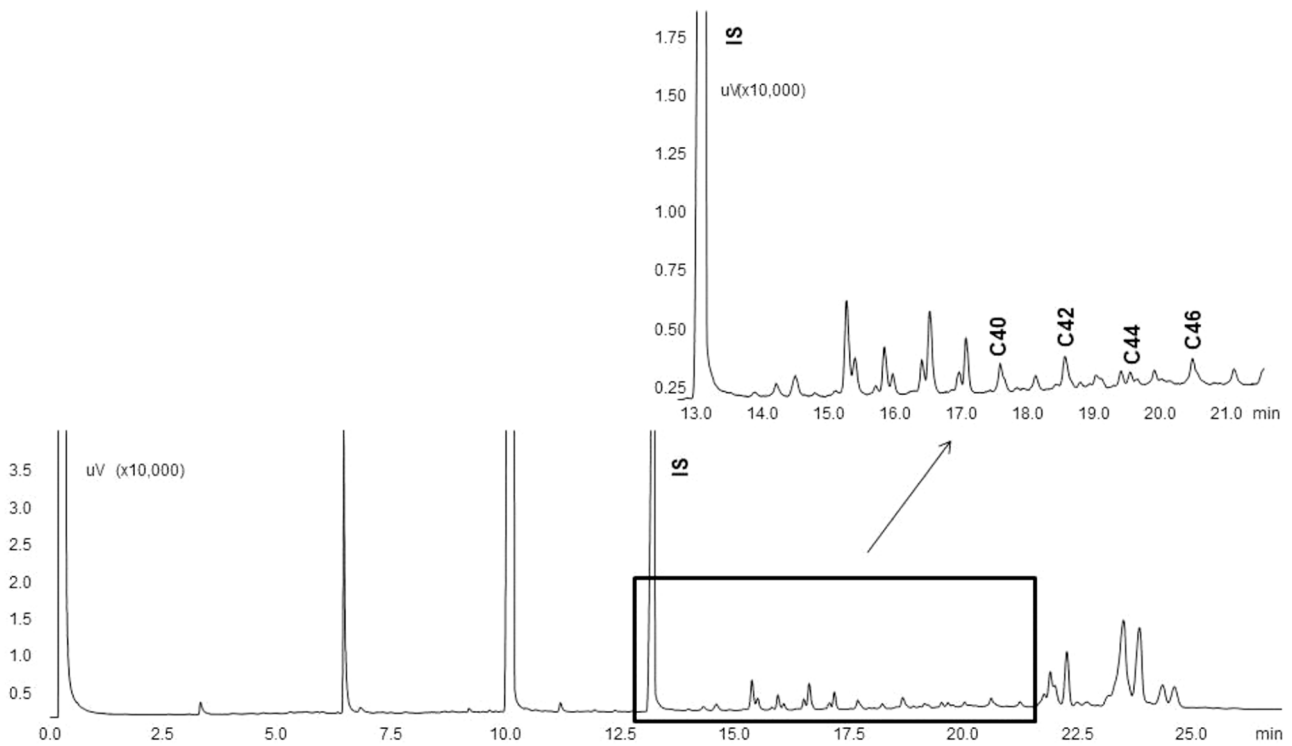


Fig. 2. Gas chromatogram of Extra Virgin Olive Oil. Peaks of waxes are identified on the basis of their carbon number, IS = internal standard, lauryl arachidate.

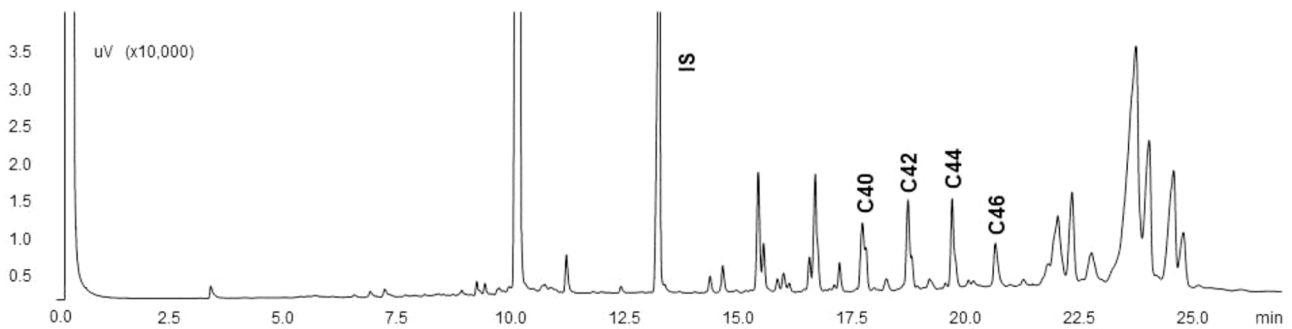


Fig. 3. Gas chromatogram of refined olive oil. Peaks of waxes are identified on the basis of their carbon number, IS = internal standard, lauryl arachidate.

Table 3. Validation of the method by using a Lampante oil.

	Waxes (mg/kg)						Mean	SD	RSD
	A	B	C	D	E	F			
C40	89.96	94.80	89.52	92.46	89.44	95.25	91.91	2.66	2.89
C42	95.44	94.35	92.11	91.70	93.59	93.65	93.47	1.39	1.49
C44	66.64	67.19	64.77	66.37	63.89	66.62	65.91	1.28	1.95
C46	32.27	31.71	29.81	30.57	30.54	32.35	31.21	1.05	3.36
∑Waxes C40-C46	284.31	288.05	276.22	281.11	277.47	287.86	282.50	5.09	1.80

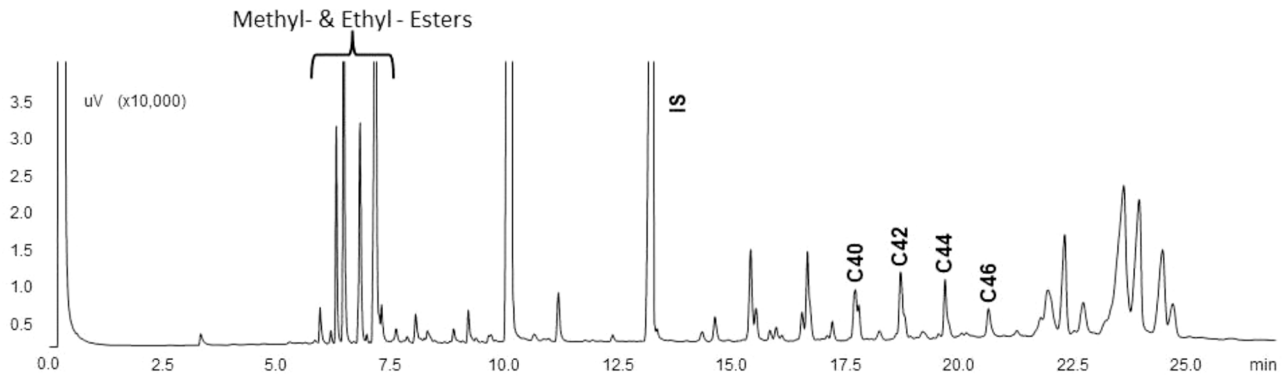


Fig. 4. Gas chromatogram of “Lampante” olive oil. Peaks of waxes are identified on the basis of their carbon number, IS = internal standard, lauryl arachidate, Between 5 and 10 minutes, methyl and ethyl esters of palmitic and oleic acids are eluted.

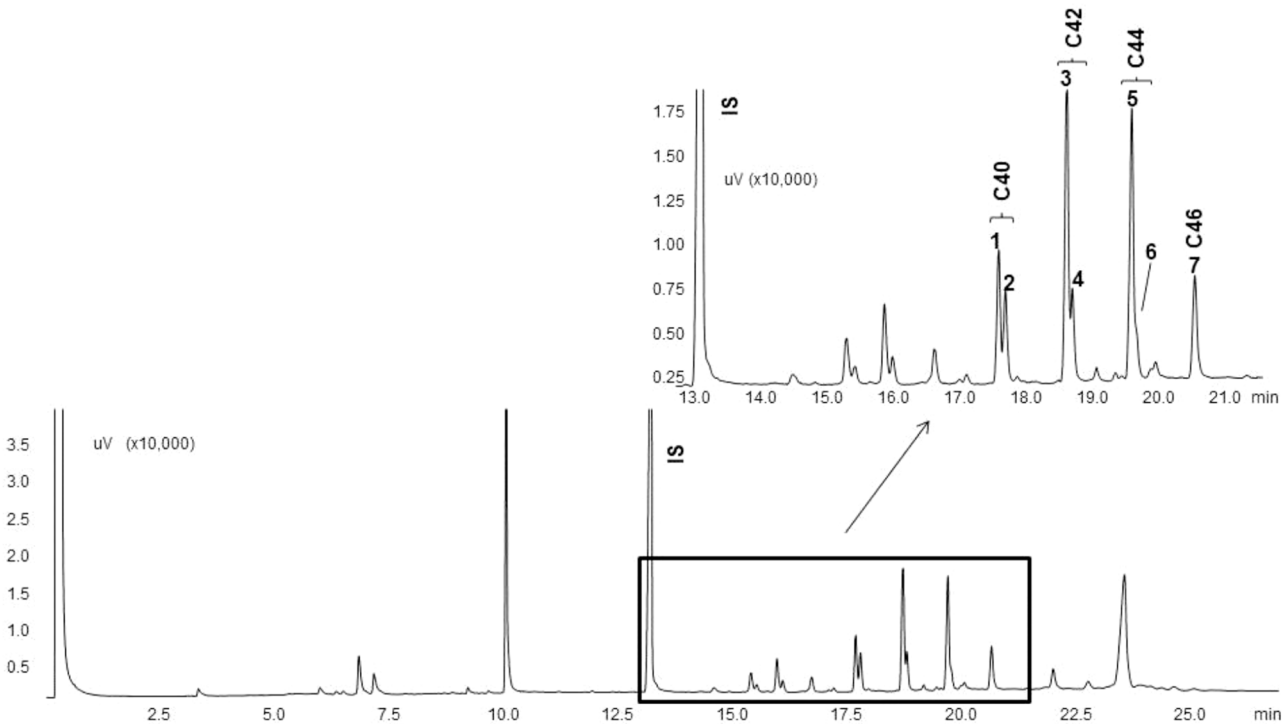


Fig. 5. Gas chromatogram of refined hazelnut oil spiked with a known amount of synthetic waxes C40–C46. Peak identification: 1: C₂₂-OH+C_{18:1}-COOH; 2: C₂₄-OH+C_{16:0}-COOH; 3: C₂₄-OH+C_{18:1}-COOH; 4: C₂₆-OH+C_{16:0}-COOH; 5: C₂₆-OH+C_{18:1}-COOH; 6: C₂₈-OH+C_{16:0}-COOH; 7: C₂₈-OH+C_{18:1}-COOH; IS = internal standard, lauryl arachidate.

Table 4. Results of waxes recovery in spiked sample.

	Waxes (mg/kg)						Mean	SD	RSD
	A	B	C	D	E	F			
C40	70.56	71.70	70.49	70.72	71.52	70.09	70.85	0.63	0.89
C42	123.61	123.33	121.56	123.16	123.61	122.65	122.99	0.79	0.64
C44	100.21	101.55	98.64	101.60	100.88	99.54	100.40	1.17	1.17
C46	38.78	39.28	37.81	39.68	39.58	39.59	39.12	0.72	1.85
∑Waxes C40-C46	333.15	335.86	328.50	335.17	335.59	331.86	333.36	2.84	0.85
Recovery %	99.45	100.26	98.06	100.05	100.18	99.06	99.51	0.85	0.85

Table 5. Waxes concentration for the 5% EVOO/95% ROO solution.

	Waxes (mg/kg)			Mean	SD	RSD
	A	B	C			
C40	96.27	97.14	96.46	96.63	0.46	0.47
C42	94.31	94.26	93.97	94.18	0.19	0.20
C44	81.63	80.87	81.99	81.49	0.57	0.71
C46	43.38	43.98	44.14	43.83	0.40	0.91
∑Waxes C40-C46	315.59	316.24	316.55	316.13	0.49	0.16
Recovery %*	99.57	99.78	99.88	99.74	0.16	0.16

*Recovery is related to the theoretical concentration of the solution obtained by the mixture of ROO and EVOO in the ratio 95:5.

Table 6. Waxes concentration for the 10% EVOO/90% ROO solution.

	Waxes (mg/kg)			Mean	SD	RSD
	A	B	C			
C40	93.12	93.15	92.29	92.85	0.49	0.53
C42	90.87	90.40	90.28	90.52	0.31	0.35
C44	77.90	76.97	77.92	77.60	0.55	0.70
C46	39.88	42.88	40.32	41.03	1.62	3.95
∑Waxes C40-C46	301.78	303.40	300.81	302.00	1.30	0.43
Recovery %*	99.91	100.45	99.59	99.99	0.43	0.43

*Recovery is related to the theoretical concentration of the solution obtained by the mixture of ROO and EVOO in the ratio 90:10.

$WAX_{EVOO} \times 0.05 + WAX_{ROO} \times 0.95$ (for 5% EVOO/95% ROO solution);

$WAX_{EVOO} \times 0.10 + WAX_{ROO} \times 0.90$ (for 10% EVOO/90% ROO solution).

4 Conclusions

The method developed seems reliable to be used for waxes evaluation as a possible alternative to the IOC and EU official ones, in terms of recovery and repeatability.

Advantages are the use of lower amounts of silica and solvent (about 20 mL and 1 g vs 360 mL and 15 g) and the use of a less dangerous solvent (isooctane instead of n-hexane). Consequently, the time required to perform the analysis for what concerns sample preparation is drastically reduced and

the difference can be roughly estimated as 20 min vs more than 300 min: this aspect meets the more recent trends that are moving to a revision of existing methods with the aim to reduce the use of health dangerous solvents in the laboratory routine. Furthermore the use of glass SPE cartridges, compared to polymeric ones, not only avoids the elution of monomers and oligomers but also reduces the plastic waste in the laboratory.

Conflict of interest. Authors declare no conflict of interest.

Funding

This research did not received any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Abbreviation

EVOO	Extra Virgin Olive oil
OPO	Olive Pomace Oil
SPE	Solid Phase Extraction
OC	On Column Injection
GC	Gas Chromatography
SS	Sum of Squares of differences of data minus mean value
SD	Standard deviation
SDM	Standard deviation of mean

Acknowledgements. Authors thank Dr. Carlo Mariani for kindly giving the sample of hazelnut oil spiked with synthetic waxes.

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Cite this article as: Milani A, Lucci P, Sedran M, Moret E, Moret S, Conte L. 2020. Improved method for determination of waxes in olive oils: reduction of silica and use of a less hazardous solvent. *OCL* 27: 20.