Research Article

Improved method for the determination of wax esters in vegetable oils

Amalia A. Carelli, Erica B. Bäumler and Liliana N. Ceci

Planta Piloto de Ingeniería Química (PLAPIQUI, UNS-CONICET), Bahía Blanca, Buenos Aires, Argentina

In this work, a modified International Olive Council (IOC) method for wax determination involving a double-adsorbent layer of silica gel and silver nitrate-impregnated silica gel is presented (SN method). Column chromatography by the SN method did not show retention of wax esters standards with an even number of carbon atoms (C34–C44), observing recovery percentages higher than 90% even for unsaturated wax esters. All wax fractions were lower by the SN method than by the IOC method, resulting in a percentage decrease in the total wax content (olive oils: 20–50%, crude sunflower oil: 38%, crude soybean oil: 58% and crude grape seed oil: 13%). Olive oils analysed by the SN method showed increases of up to 27% in C40 relative percentage with respect to the IOC method. Additionally, decreases were observed by the SN method in the relative percentages for odd-carbon atom waxes for the seed oils in comparison to the IOC method (crude sunflower oil: 27%, crude soybean oil: 28% and crude grape seed oil: 13%). The main advantages of the proposed modification consist in its easy implementation and a better determination of wax esters (C34–C60) by controlling their complete recovery and removing interfering substances. The method is suitable for quality control and for authentication of olive oil and seed oils as well as in processing monitoring.

Practical applications: The proposed method is useful in the quality, authentication and processing control of fruit and seed oils. Moreover, it can be an important tool for vegetable oil industries to control the efficiency of the wax separation process to prevent turbidity in the refined oil.

Keywords: Argentation chromatography / GC / Olive oil / Seed oils / Wax esters

Received: March 7, 2012 / Revised: June 4, 2012 / Accepted: June 26, 2012

DOI: 10.1002/ejlt.201200098

1 Introduction

Vegetable oils contain a number of minor compounds that are removed along with the oil in the extraction process. Among them there is a group of esters of fatty acids and long-chain aliphatic alcohols known as wax esters. Wax esters in the range of 36–60 carbon atoms were reported for several vegetable oils [1–6].

Wax esters with a high melting point and low solubility in the oils contribute to the development of cloudiness when stored at low temperatures. Waxes with chain lengths lower than 40 carbon atoms (C40) are called soluble waxes, those with lengths between C40 and C43 are called partially soluble waxes and those with 44 or more carbon atoms are called crystallisable waxes. The last fraction is reduced by winterization or dewaxing in order to improve the cold stability of the oil. Thus refined oils retain mostly soluble waxes [1, 2].

The content and profile of the waxes depend on the oil origin, seed or fruit variety, pretreatment of the raw material and the temperature and technology used in the extraction process. Oils obtained by solvent extraction have a higher wax content than those obtained by mechanical extraction [6–8]. Therefore, the distribution and amount of waxes in a vegetable oil is a good indicator of its quality and authentication. For example, the sum of C40, C42, C44 and C46 waxes is a parameter used to detect olive-pomace oil in virgin olive oil. This sum has been established as $\leq$250 mg/kg for edible virgin olive oils [9, 10]. Wax esters are very abundant in olive-pomace oil, whereas low concentrations are found in virgin oils [7, 8]. Extra virgin olive oils of premium quality

Correspondence: Dr. Amalia A. Carelli, Planta Piloto de Ingeniería Química (PLAPIQUI, UNS-CONICET), Camino Carrindanga Km. 7, 8000 Bahía Blanca, Buenos Aires, Argentina
E-mail: acarelli@plapiqui.edu.ar

Abbreviations: CC, column chromatography; **CCGC,** capillary column gas chromatography; **CGC,** capillary gas chromatography; **CV,** coefficients of variation; **FID,** flame ionization detector; **GC,** gas chromatography; **GC-MS,** gas chromatography-mass spectroscopy; **IOC,** International Olive Council; **SCS,** second centrifugation sludge

in particular contain predominantly fatty acid esters with diterpenic alcohols such as phytol and geranylgeraniol [3, 8].

The main components of crystallisable waxes in crude sunflower, soybean and grape seed oils are C44 and C46 esters [1]. Monounsaturated waxes containing less than 42 carbon atoms have been identified by gas chromatography-mass spectroscopy (GC–MS) in sunflower, soybean and peanut oils [11]. Crude sunflower oils contain soluble and partially soluble wax esters up to C42, mainly C40–C42, and crystallisable wax esters from C44 to C60, which are fundamentally drawn from the seed hulls during the oil extraction process [2, 4–6]. The study of the wax constituents of crude sunflower oil showed the presence of C14–C30 fatty acids, with 18:2, 18:1, 22:0 and 16:0 being the most prevalent, and C16–C32 fatty alcohols, dominated by C18, C19 and C24 [2].

In recent years, the analysis of wax content in vegetable oils has been carried out using column chromatography (CC) on hydrated silica gel followed by capillary gas chromatography (CGC) with on-column injection, being available as an olive oil-specific method (International Olive Council, IOC) [12]. In CC, the wax fraction is eluted with a mixture of hexane and diethyl ether (99:1 v/v). This fraction contains not only aliphatic waxes, but also other minor components such as steryl and terpenic esters that are not chromatographically well resolved. These compounds interfere in the quantification of the wax esters [7]. Despite this fact, the method is recommended to detect olive-pomace oil in olive oil by the evaluation of C40–C46 waxes. This technique was recently modified to allow simultaneously the determination of the content of waxes, fatty acids methyl esters and fatty acid ethyl esters, but the interference of steryl and terpenic esters was not resolved [13, 14].

In 2010, a new ISO International Standard was established that specifies a gas chromatographic method for determining the amount of waxes in crude, degummed, neutralised, winterised and fully refined vegetable oils [15]. In this method, based on previous studies [1], CC is performed on a double-adsorbent layer of silica gel and silver nitrate-impregnated silica gel, using a mixture of *n*-hexane and dichloromethane as liquid phase, followed by CGC with split injector. By this technique, it is possible to quantify more accurately the content of crystallisable waxes in seed oils, and monitor the dewaxing process. When applied to olive oils, this method also gives better chromatograms and allows more accurate measurements of C40–C46 waxes [1]. However, the ISO method does not use a wax ester as internal standard, thus presenting some difficulties in its application, for example the need for weekly determination of response factors, the use of empirical factors depending on the oil being analysed, and the lack of tools to control the complete elution of wax esters, mainly of those containing unsaturated groups whose elution is delayed when silver nitrate is used.

The objectives of this paper were: (i) the modification of the IOC standard method for a better determination of wax ester content by the introduction, during the column chromatographic step, of a mixed column packaging consisting of silica gel and silica gel impregnated with silver nitrate; (ii) the application of the modified method in vegetable oils, and its comparison with the traditional IOC method.

2 Materials and methods

2.1 Materials

All reagents were of analytical grade, except *n*-hexane, *n*-heptane and diethyl ether, which were of chromatographic grade. Silica gel 60 (Art. 7734) and extra-pure silica gel 60 (Art. 7754), particle size 0.063–0.200 mm, 70–230 mesh for CC (Merck, Darmstadt, Germany) were used as support for the preparation of hydrated silica gel and silver nitrate-impregnated silica gel, respectively. The following wax standards of almost 99% purity (Sigma Chemical Co., St. Louis, MO, USA) were used for chromatographic analysis and recovery assays: arachidyl laurate ($C_{32}H_{64}O_2$), stearyl palmitate ($C_{34}H_{68}O_2$), stearyl stearate ($C_{36}H_{72}O_2$), arachidyl oleate (AO, $C_{38}H_{74}O_2$), oleyl arachidate (OA, $C_{38}H_{74}O_2$), behenyl oleate (BO, $C_{40}H_{78}O_2$), arachidyl behenate ($C_{42}H_{84}O_2$) and behenyl behenate ($C_{44}H_{88}O_2$). The C32 wax standard (arachidyl laurate, $C_{32}H_{64}O_2$) was also used as internal standard for the quantitative analysis of wax esters and recovery studies.

In addition, a filter cake from the dewaxing process of crude sunflower oil consisting of waxes, oil and filter aid was supplied by a local factory and used to obtain crystallised sunflower wax by successive hexane washings, cooling and centrifugation. The purified sunflower wax was used as standard for the identification of waxes with more than 44 carbon atoms. In addition, this wax was both directly injected into the chromatograph (Section 2.6) and processed by the silver nitrate method (SN method, Section 2.5) followed by gas chromatography (GC) (Section 2.6) in order to compare both wax profiles.

2.2 Oil samples

Four monovarietal extra virgin olive oils from NW Argentina (La Rioja and Catamarca provinces) were studied: Arbequina (A1), Barnea (B1 and B2), Coratina (C) and a varietal blend olive oil (VB). Two extra virgin olive oils from the Arbequina variety produced in San Juan province (Cuyo region, Argentina) were also analysed (A2 and A3). Two olive oil samples from the second centrifugation (SC1 and SC2), one oil sample obtained from the second centrifugation sludge (SCS) and one sample of olive-pomace oil (OP) were provided by processor plants. The crude seed oils studied were: sunflower (SF), soybean (SB) and grape seed (GS) oils. Two samples of refined high oleic sunflower oil (RSF1 and RSF2) were also evaluated.

2.3 Recovery of wax standards by the SN method

Two standard solutions were prepared for recovery studies. Standard solution 1 consisted of saturated wax esters

(C34 = 1.02 mg/mL, C36 = 0.98 mg/mL, C42 = 1.08 mg/mL and C44 = 1.10 mg/mL) and wax esters containing an unsaturated acid group (C38, AO = 3.16 mg/mL and C40, BO = 1.52 mg/mL) in *n*-hexane. Standard solution 2 was prepared from a wax ester containing an unsaturated aliphatic alcohol group (C38, OA = 0.4 mg/mL) in *n*-hexane. An aliquot of each standard solution (100 μL of solution 1 and 500 μL of solution 2) was processed separately by the SN method (Section 2.5) using C32 (arachidyl laurate) as internal standard, and analysed by GC (Section 2.6). The percentage ratio of the mass obtained by quantification to that transferred to the chromatographic column represents the recovery percentage of each wax ester.

In addition, a standard solution 3 containing C40–C44 wax esters in a similar proportion to that found in virgin olive oils (C40:C42:C44, 2:1:0.7) was prepared. Standard solution 3 was both directly injected into the chromatograph (Section 2.6) and processed by the SN method (Section 2.5) followed by GC (Section 2.6) in order to compare both profiles.

2.4 Isolation of the wax fraction by IOC method

The wax fraction was isolated according to the IOC method [12]. Briefly, CC was performed in a glass column (i.d. = 15 mm, length = 400 mm) with hydrated silica gel (15 g, 2% water content) as a solid stationary phase. Around 500 mg of oil (weighed exactly), a suitable amount (250–1500 μL) of internal standard solution (0.2% of C32 in *n*-hexane) depending on the oil wax content, and two drops of a 1% solution of the Sudan I dye in *n*-hexane were loaded into the column with the aid of two 2-mL portions of *n*-hexane. Under these conditions, the retention of Sudan I dye lies in between that of the waxes and TAGs. Hence, when the dye reaches the bottom of the chromatographic column, the wax elution is complete. The waxes were eluted with *n*-hexane/ethyl ether (99:1 v/v) at a flow rate of 3 mL/min. The eluted wax fraction was evaporated to dryness and diluted with *n*-heptane for chromatographic analysis.

2.5 Isolation of the wax ester fraction by the SN method

Column chromatography was performed following the IOC method, but changing the stationary phase for a double phase of silver nitrate-impregnated silica gel (3 g) placed in the bottom of the column and silica gel 2% hydrated (12 g) placed on the top. Silver nitrate impregnated silica gel was prepared by pouring a solution of silver nitrate (5 g dissolved in 240 mL of distilled water) onto 100 g of extra-pure silica gel in a ceramic bowl, heating it from room temperature up to 170°C in an electric oven, and activating it overnight. Then, the impregnated silica gel was allowed to cool down slowly to 50°C in the oven (in the dark) and kept in the dark in a sealed bottle. Silica gel 2% hydrated was prepared following the IOC method [12]. The mobile phase, internal standard and dye used to detect the completion of the wax elution were the same as for the IOC method. The technical procedure was similar to that of the IOC method, but in this case the bottom of the column containing the silver nitrate impregnated silica gel was covered with an aluminium foil to protect it from light. A mark was made on the glass 2 cm below the interphase between the 2% hydrated silica gel and the silver nitrate impregnated silica gel. The elution of wax esters was ended when the dye reached that mark. The eluted wax fraction was evaporated to dryness and diluted with *n*-heptane for chromatographic analysis.

2.6 Analysis by capillary column gas chromatography (CCGC)

A Perkin Elmer AutoSystem XL gas chromatograph equipped with an flame ionization detector (FID) detector, a temperature programmable on-column injector and a TotalCrom Workstation Version 6.3.1 data processor (Perkin Elmer, MA, USA) was used for the final analysis of wax fractions. The capillary column was an HP-5 (5% diphenyl and 95% dimethyl-polysiloxane), fused-silica 15 m length × 0.32 mm i.d., 0.25 μm film thickness (Hewlett-Packard, Palo Alto, CA). The operating conditions were: hydrogen at 3 mL/min as carrier gas; oven temperature programming: initial temperature, 80°C hold for 1 min, increase at 20°C/min to 240°C, increase at 5°C/min to 325°C hold for 6 min, increase at 20°C/min to 340°C hold for 27 min; on-column injector programmed from 80 to 320°C at 20°C/min and injection volume of 2 μL; FID at 350°C.

2.7 Statistical analysis

All analyses were carried out in duplicate and each replicate was twice injected in the gas chromatograph. The results are presented as mean value ± 95% confidence interval. Coefficients of variation (CV) were used as a measure of dispersion for the recovery studies of standard waxes by the SN method.

2.8 Results and discussion

Recovery percentages of standard wax esters by the SN method are shown in Table 1. Percentages higher than 90% were obtained with CV below 2%. Neither waxes with oleic acid nor waxes with oleyl alcohol were retained using the SN method.

The chromatographic profiles of standard solution 3 (C40–C44 in a similar proportion to that found in virgin olive oils) obtained by direct injection or previously processed according to the SN method are shown in Table 2. The results confirm that there is not a preferential loss of any standard when the SN method is used. In Table 2, the chromatographic profiles of the crystallised sunflower waxes

Table 1. Recovery of wax standards by SN method

Wax	Recovery[a)] (%)	CV (%)
C34	101	0.87
C36	103	0.13
C38 (OA)	99	1.7
C38 (AO)	91	1.9
C40	96	1.5
C42	105	0.51
C44	99	0.38

[a)] Mean values.
AO, arachidyl oleate; OA, oleyl arachidate.

from the filter cake are also shown. The profiles obtained by direct injection were similar to those obtained by SN method-GC, presenting crystallised wax esters from C42 to C58. One study on the chemical composition of sunflower waxes isolated and purified from tank settlings showed the presence of C14–C30 fatty acids with 18:1, 20:0 and 16:0 being most prevalent, and C18–C34 fatty alcohols, dominated by 24, 26 and 28 carbon numbers [16]. The authors also reported small amounts of odd-carbon fatty acids (C23, C27 and C29) and fatty alcohols (C21, C23, C25, C27 and C29) [16]. These odd compounds could explain the presence of odd waxes in the crystallised sunflower waxes from the filter cake.

Table 2. Profiles of standard waxes (C40–C44) and crystallised sunflower waxes by direct injection and by SN method

	Direct injection		SN method	
Wax	%[a)]	CV (%)	%[a)]	CV (%)
Standard waxes				
C40	54.5	0.6	53.6	0.6
C42	26.9	0.7	27.3	0.9
C44	18.7	0.8	19.1	1.6
Crystallised sunflower waxes				
C42	3.3	5.3	4.6	10
C43	0.7	3.9	0.9	9.1
C44	19.9	2.4	22.4	6.6
C45	2.8	18	2.5	3.3
C46	26.9	2.1	27.4	1.1
C47	2.2	1.2	2.1	2.3
C48	18.1	1.3	17.5	2.9
C49	1.7	14	1.4	7.5
C50	10.1	1.9	9.4	5.9
C51	1.1	16	0.8	7.5
C52	6.6	8.0	5.9	7.9
C53	0.6	10	0.5	11
C54	3.2	2.6	2.8	10
C55	0.3	2.7	0.2	10
C56	2.1	13	1.1	11
C58	0.5	14	0.5	13
Total even waxes	90.6	0.7	91.6	0.2
Total odd waxes	9.4	6.8	8.4	1.7

[a)] Mean values.

Typical chromatograms using the IOC and SN methods are shown in Fig. 1. CC using silver nitrate retains sterol esters (steryl esters), and thus wax esters can be quantified without interferences of these compounds, showing wax peaks accurately defined.

The IOC and the Codex Alimentarius establish C40–C46 wax contents $\leq$250 mg/kg as a purity criterion for edible olive oils and higher values for refined olive oils ($\leq$350 mg/kg) and olive-pomace oils (>350 mg/kg) [9, 10]. In previous studies, extra-virgin olive oils from the variety Arbequina from the hot NW regions of Argentina (La Rioja and Catamarca provinces) have shown wax contents higher than 250 mg/kg using the IOC method [17, 18].

The C40–C46 wax contents for monovarietal extra virgin olive oils using IOC and SN methods are shown in Table 3. Olive oils showed a decrease (between 20 and 50%) in the C40–C46 wax contents when they were analysed by the SN method in comparison to the traditional IOC method. Moreover, a significant decrease in the fractions of individual waxes and an increase in the relative percentage for C40 were observed in all the samples when the SN method was applied. Thus, the traditional IOC method overquantifies the wax ester content. This could be due to the fact that silver nitrate retains fatty acid esters with terpenic alcohols that hinder the right quantification of wax esters by the IOC method. Mariani and Venturini [3] analysed the wax fraction of extra-virgin olive oil by GC–MS and found that the C40 peak consisted of behenyl oleate, palmityl lignocerate and phytyl behenate, while the C42, C44 and C46 peaks were mainly fatty acid esters with diterpenic alcohols such as phytol and geranylgeraniol. As confirmed by the results, a lower reduction of the C40 content with respect to C42–C46 waxes is expected when the SN method is used.

As it can be observed in Table 3, the Arbequina sample (A1) had a wax content above the limit value for edible virgin olive oils even by the SN method (C40–C46 = 287 mg/kg). In contrast, the olive oil obtained from a varietal blend (VB) showed more than 250 mg/kg of C40–C46 waxes (374 mg/kg) by the IOC method and it was adjusted to the normative (241 mg/kg) by the SN method (Table 4). These results suggest that the incorporation of the methodology here proposed could contribute to a better evaluation of wax esters in samples from extra virgin olive oils.

The C40–C46 wax contents obtained by the two methods for olive oils of minor quality are presented in Table 4, including oils from second centrifugation, oil from SCS and olive-pomace oil. The following order was observed for the wax contents: second centrifugation oil < oil from SCS < olive-pomace oil. As observed in virgin oils, a decrease in wax contents and an increase in the relative percentage for C40 were detected by the SN method in comparison with the

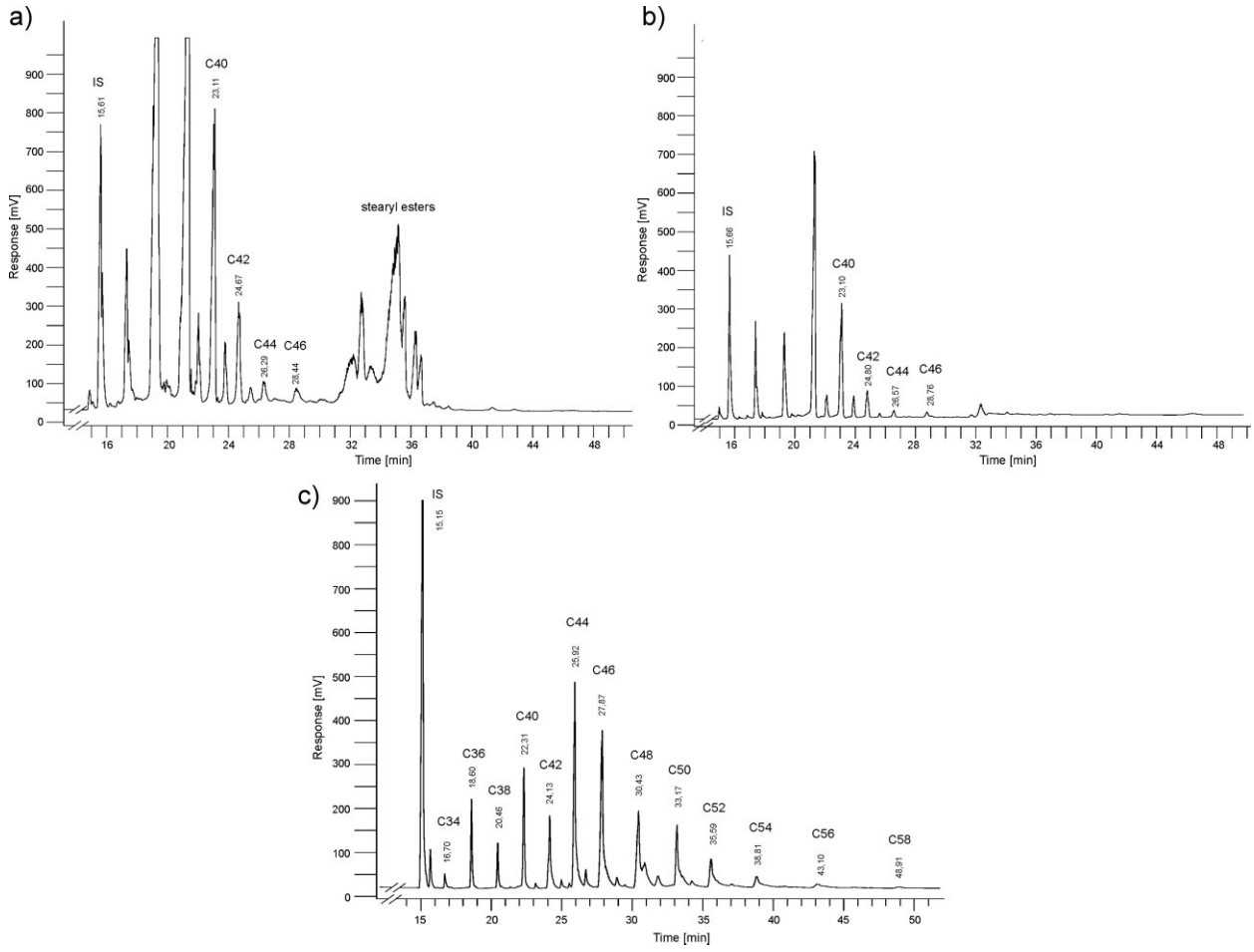

Figure 1. Example of typical chromatograms: (a) virgin olive oil by the IOC method, (b) virgin olive oil by the SN method and (c) crude sunflower oil by the SN method.

results obtained by the IOC method (Table 4). By the SN method, the oils from second centrifugation had a lower C40–C46 wax content than the limit value for lampante virgin olive oil (300 mg/kg), while the oil from SCS and the olive-pomace oil had more than 350 mg/kg. The olive-pomace oil showed a different profile of waxes characterised by higher relative percentages of C42 (IOC: 38%, SN: 34%) and C44 (IOC: 29%, SN: 30%) in comparison to the other olive oil samples, which presented predominantly C40 (IOC: 45–66%, SN: 48–80%) and C42 (IOC: 21–33%, SN: 14–

Table 3. Wax contents (mg/kg) by IOC and SN methods for monovarietal extra virgin olive oils

	A1		A2		A3		B1		B2		C	
Wax	IOC	SN	IOC	SN	IOC	SN	IOC	SN	IOC	SN	IOC	SN
C40	238 ± 2	213 ± 3	143 ± 1	110 ± 4	124 ± 1	120 ± 3	124 ± 1	91 ± 5	114 ± 2	82 ± 2	62 ± 1	54 ± 2
C42	85 ± 7	53 ± 3	46 ± 1	19 ± 3	59 ± 1	27 ± 2	64 ± 1	22 ± 3	57 ± 4	15 ± 0	36 ± 1	20 ± 1
C44	23 ± 6	11 ± 1	14 ± 2	4 ± 1	20 ± 1	7 ± 1	26 ± 2	8 ± 0	24 ± 2	4 ± 0	19 ± 0	13 ± 0
C46	21 ± 3	10 ± 3	13 ± 1	4 ± 1	17 ± 1	6 ± 1	18 ± 1	6 ± 2	20 ± 3	2 ± 0	16 ± 0	13 ± 1
C40–C46	367 ± 13	287 ± 3	216 ± 3	137 ± 5	220 ± 2	160 ± 4	232 ± 3	127 ± 7	215 ± 2	103 ± 2	133 ± 2	100 ± 2

Mean values ± 95% confidence interval.

Table 4. Wax contents (mg/kg) by IOC and SN methods for extra virgin varietal blend olive oil, olive oils from second centrifugation, olive oil from SCS and olive-pomace oil

	VB		SC1		SC2		SCS		OP	
Wax	IOC	SN	IOC	SN	IOC	SN	IOC	SN	IOC	SN
C40	219 ± 3	177 ± 12	251 ± 10	198 ± 1	323 ± 4	213 ± 1	495 ± 2	406 ± 9	704 ± 5	497 ± 23
C42	92 ± 6	47 ± 4	123 ± 4	48 ± 1	178 ± 5	53 ± 1	370 ± 1	264 ± 23	1249 ± 6	773 ± 12
C44	35 ± 3	9 ± 1	39 ± 1	8 ± 1	44 ± 3	10 ± 1	149 ± 1	112 ± 18	948 ± 13	688 ± 25
C46	28 ± 1	8 ± 2	31 ± 1	9 ± 1	34 ± 2	10 ± 2	94 ± 1	58 ± 3	403 ± 2	295 ± 15
C40–C46	374 ± 12	241 ± 15	444 ± 14	263 ± 2	579 ± 11	286 ± 3	1108 ± 3	840 ± 53	3304 ± 24	2253 ± 73

Mean values ± 95% confidence interval.

31%) waxes. Non-extra virgin olive oils are characterized by a very high amount of wax esters consisting of oleic and palmitic acids esterified with fatty alcohols with 22–28 carbon atoms [3]. This increase in wax esters with increasing free fatty acid content may be attributed to a chemical synthesis [3]. The distribution of esters in the olives explains the finding that diterpenic esters are present in all olive oils whereas wax esters vary depending on the extraction technique. Diterpenic esters are primarily in the pulp, while wax esters are mainly in the skin. During the pressing stage, wax esters from the skin are poorly extracted and subsequent solvent extraction causes most of the wax esters to be transferred into the small amount of residual oil [8].

The results for wax composition in crude sunflower oil by the two methods and in two refined sunflower oils by the SN method are presented in Table 5. For crude sunflower oil (SF), it is possible to detect by means of the SN method wax esters up to C58, totalling around 460 mg/kg with about 9% of odd-carbon atom wax esters. The IOC method does not allow quantifying waxes with a carbon number higher than 46 with good resolution due to the interference of other components mentioned above that coelute with waxes during CC. By the IOC method, the crude sunflower oil showed a higher content of total waxes (745 mg/kg) and a higher percentage of odd-carbon atom waxes (36%). The results demonstrate that many compounds assigned as wax esters by the traditional IOC method actually are not such, and moreover, the components identified as odd-carbon atom waxes are less abundant when a support impregnated with silver nitrate that retains interfering compounds is used for CC. The refined sunflower oils (RSF1 and RSF2) had lower contents of all the wax fractions, but the crystallisable waxes with more than 44 carbon atoms only appeared in low amounts or were not detected, demonstrating the effectiveness of the dewaxing processes. Hénon et al. [1] found by GC–MSD that soluble wax esters in sunflower oils are monounsaturated waxes, esters of saturated fatty acids and monounsaturated alcohols, mainly eicosenol (C20:1). As wax esters with monounsaturated alcoholic group are completely recovered by the SN method (Table 1), the reduction of soluble wax esters by the SN method could be due to the presence of other compounds. A GC–MS analysis of the wax ester fraction of sunflower oil obtained by solid-phase extraction with silica gel revealed a significant occurrence of diterpenic esters, mainly geranylgeranyl esters [19]. Therefore, in a similar

Table 5. Wax contents (mg/kg) for sunflower crude oil by IOC and SN methods and for refined high oleic sunflower oils by SN method

	SF		RSF1	RSF2
Waxes	IOC	SN	SN	
C34	9 ± 0	7 ± 0	n.d.	n.d.
C36	135 ± 2	37 ± 3	11 ± 3	18 ± 1
C37	94 ± 3	n.d.	n.d.	n.d.
C38	36 ± 0	22 ± 2	8 ± 2	13 ± 1
C39	27 ± 1	n.d.	n.d.	n.d.
C40	72 ± 3	57 ± 6	40 ± 13	64 ± 5
C41	98 ± 2	3 ± 0	tr	2 ± 0
C42	57 ± 1	37 ± 2	18 ± 4	22 ± 2
C43	34 ± 1	3 ± 0	tr	tr
C44	74 ± 2	75 ± 1	8 ± 1	8 ± 3
C45	18 ± 1	7 ± 0	n.d.	n.d.
C46	91 ± 4	74 ± 2	4 ± 0	6 ± 0
C47	–	5 ± 0	n.d.	n.d.
C48	–	45 ± 2	tr	2 ± 0
C49	–	17 ± 6	n.d.	n.d.
C50	–	27 ± 2	n.d.	n.d.
C51	–	5 ± 2	n.d.	n.d.
C52	–	18 ± 1	n.d.	n.d.
C53	–	2 ± 0	n.d.	n.d.
C54	–	11 ± 2	n.d.	n.d.
C55	–	tr	n.d.	n.d.
C56	–	5 ± 1	n.d.	n.d.
C57	–	tr	n.d.	n.d.
C58	–	3 ± 0	n.d.	n.d.
Total waxes	745	460	89	135
Partially soluble waxes (C40–C43)	261	100	58	88
Crystallisable waxes (C44–C58)	183	294	12	16

Mean values ± 95% confidence interval.
tr, traces (< 2 mg/kg); n.d., not detected.

way as in olive oils, diterpenic esters are quantified simultaneously with wax esters when the IOC method is used.

The crude soybean oil showed a low content of crystallisable waxes, being possible to quantify up to C58 using the SN method (Table 6). As in the case of sunflower oil, the contents of all waxes in soybean oil were lower by the SN method. A lower percentage of odd-carbon atom waxes (11%) was also observed by this method in comparison with the traditional IOC method (39%). Trost [20] analysed a fraction from soybean oil that was less polar than TAGs, and reported the presence of phytyl and geranylgeranyl esters as well as the occurrence of saturated (C16:0, C18:0), unsaturated (C18:1, C18:2, C18:3) and odd (C21:0, C23:0) fatty acids that substantiate the high amount of soluble wax esters, the presence of odd wax esters, and the difference in the results obtained by the ISO and SN methods.

Table 6. Wax contents (mg/kg) for crude soybean and grape seed oils by IOC and SN methods

	SB		GS	
Waxes	IOC	SN	IOC	SN
C34	21 ± 0	20 ± 2	19 ± 0	18 ± 1
C36	94 ± 1	27 ± 6	42 ± 1	14 ± 1
C37	29 ± 1	n.d.	96 ± 1	n.d.
C38	35 ± 0	25 ± 5	19 ± 0	11 ± 2
C39	25 ± 0	8 ± 1	75 ± 1	n.d.
C40	112 ± 1	81 ± 10	62 ± 2	31 ± 2
C41	84 ± 2	16 ± 2	22 ± 0	4 ± 1
C42	74 ± 1	37 ± 5	154 ± 2	84 ± 4
C43	61 ± 1	5 ± 1	39 ± 1	10 ± 1
C44	15 ± 3	6 ± 0	292 ± 2	143 ± 7
C45	37 ± 1	n.d.	40 ± 0	11 ± 1
C46	13 ± 0	7 ± 1	259 ± 2	153 ± 8
C47	–	n.d.	–	11 ± 1
C48	–	5 ± 1	–	127 ± 1
C49	–	n.d.	–	26 ± 3
C50	–	5 ± 1	–	91 ± 4
C51	–	n.d.	–	20 ± 5
C52	–	4 ± 1	–	57 ± 2
C53	–	n.d.	–	11 ± 3
C54	–	3 ± 1	–	43 ± 3
C55	–	n.d.	–	8 ± 3
C56	–	3 ± 1	–	41 ± 0
C57	–	n.d.	–	6 ± 0
C58	–	3 ± 2	–	27 ± 1
C60	–	n.d.	–	22 ± 2
Total waxes	600	255	1119	969
Partially soluble waxes (C40–C43)	331	139	277	129
Crystallisable waxes (C44–C60)	65	36	591	797

Mean values ± 95% confidence interval.
n.d., not detected.

As shown in Table 6, crude grape seed oil had the highest total wax content among the analysed seed oils (1119 and 969 mg/kg by the IOC and SN methods, respectively) and it was highlighted by the high content of crystallisable waxes up to C60; however, the percentage of odd-carbon atom waxes by the SN method was similar to that of other oils (11%). In seed oils, the contents of crystallisable waxes tend to decrease as the homologous series of even-carbon atom waxes progresses. Like soybean and sunflower oil, grape seed oil contains diterpenic esters that interfere with the right quantification of wax esters. Biedermann et al. [8] reported the widespread occurrence of geranylgeraniol esters in various vegetable oils analysed by on-line HLPC–GC–MS and GCxGC-FID.

The proposed modification to the traditional IOC method involving the use of a double-adsorbent layer of silica gel and silver nitrate-impregnated silica gel presents the following comparative advantages:

(i) It allows the analysis of wax esters alone, and the quantification of waxes with more than 46 carbon atoms (up to C60) by removing the interferences of steryl esters and other compounds. This is a remarkable advantage with respect to the currently used IOC method.

(ii) It presents some advantages with respect to the ISO method, such as the use of silica gel with a controlled hydration percentage for the CC step and on-column injection for the GC step. The ISO method applies split/splitless injection and does not establish the humidity control of the hydrated silica gel used as stationary phase for the CC. Therefore, the humidity content can change between lots and through time once the container is opened. Since the activity of the silica gel is related to its water content, this fact affects the separation of the waxes and consequently the repeatability of the assays.

(iii) It uses Sudan I dye to control the completion of the wax elution during CC. The ISO method uses another solvent mixture (hexane/dichlomethane) for elution, and under these conditions it is impossible to see when the elution of wax esters ends since the dye does not have an adequate retention factor. Thus, when the ISO method is applied, the wax esters could be incompletely eluted, or the fractions of tryglycerides and/or steryl esters could be eluted jointly to the wax esters. The ISO method recommends that CC should be repeated if there are overlapping peaks or big peaks at the end of the wax region.

(iv) It uses a wax ($C_{32}H_{64}O_2$) as internal standard, and does not require the calculation of response factors. Previous analysis of wax standards processed by the ISO method (data not shown) showed that the recovery of the waxes with a low number of carbon atoms (soluble and partially soluble waxes) was incomplete. Thus, the ISO method uses the hexatriacontane hydrocarbon ($C_{36}H_{74}$) as internal standard and requires a weekly calculation of response factors relative to C36 wax and empirical factors for C44.

(v) It is easy to implement, being possible to quantify waxes from C34, whereas the ISO method only quantifies waxes from C44. It is possible to know when the wax elution ends, saving time and solvents. In addition, the quantification procedure is the same as that used for the IOC method.

(vi) It presents good repeatability, as evidenced by the confidence intervals included in the Tables, allowing exact wax determinations. An adequate knowledge of wax profiles in vegetable oils can also contribute to the study of the presence of contaminants and adulterations.

3 Conclusions

In this paper, a simple modification to the IOC standard method for wax content determination is proposed, which could be easily applied in edible oil analytical laboratories. By including a double-adsorbent layer of silica gel and silver nitrate-impregnated silica gel in the CC, a more exact determination of wax esters in crude and refined vegetable oils was possible. The proposed modification to the IOC method allows the quantification of wax esters in the range of C34–C60, being useful both for the analysis of waxes in vegetable oils during different stages of processing and for a better classification of their quality. Particularly in some olive oils, this method will help to avoid errors in the assignation of a specific grade and in their marketing.

This work was financially supported by Asociación Argentina de Grasas y Aceites (ASAGA), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Técnica (ANPCyT) and Universidad Nacional del Sur (UNS), Argentina. The authors wish to acknowledge Clarisa Cicchelli for her kind assistance during the analytical work.

The authors have declared no conflict of interest.

References

[1] Hénon, G., Recseg, K., Kovari, K., Wax analysis of vegetable oils using liquid chromatography on a double-adsorbent layer of silica gel and silver nitrate-impregnated silica gel. *J. Am. Oil Chem. Soc.* 2001, *78*, 401–410.

[2] Carelli, A. A., Frizzera, L. M., Forbito, P. R., Crapiste, G. H., Wax composition of sunflower seed oils. *J. Am. Oil Chem. Soc.* 2002, *79*, 763–768.

[3] Mariani, C., Venturini, S., Sulla struttura delle cere degli oli di oliva. *Riv. Ital. Sost. Grasse* 2002, *79*, 49–57.

[4] Baümler, E. R., Crapiste, G. H., Carelli, A. A., Sunflower-oil wax reduction by seed solvent washing. *J. Am. Oil Chem. Soc.* 2007, *84*, 603–608.

[5] Sindhu Kanya, T. C., Jaganmohan Rao, L., Shamanthaka Sastry, M. C., Characterization of wax esters, free fatty alcohols and free fatty acids of crude wax from sunflower seed oil refineries. *Food Chem.* 2007, *101*, 1552–1557.

[6] Carrín, M. E., Carelli, A. A., in: Tomás, M. (Ed.), *Advances in Fats Oil Research*, Editorial Research Signpost, Trivandrum (India) 2010, pp. 25–48.

[7] Cert, A., Moreda, W., Pérez-Camino, M. C., Chromatographic analysis of minor constituents in vegetable oils. *J. Chromatogr. A* 2000, *881*, 131–148.

[8] Biedermann, M., Haase-Aschoff, P., Grob, K., Wax ester fraction of edible oils: Analysis by on-line LC-GC-MS and GCxGC-FID. *Eur. J. Lipid Sci. Technol.* 2008, *110*, 1084–1094.

[9] Codex Alimentarius, Codex Standard for Olive Oils and Olive Pomace Oils. Codex STAN 33-1981, Rev. 2 (2003) Amendment 1 (2009).

[10] International Olive Council (IOC), Trade Standard Applying to Olive Oils and Olive-Pomace Oils, COI/T.15/NC No. 3/Rev. 6 (2011).

[11] Carrín, M. E., Carelli, A. A., Peanut oil: Compositional data. *Eur. J. Lipid Sci. Technol.* 2010, *112*, 697–707.

[12] International Olive Council (IOC), Determination of wax content by capillary column gas chromatography, COI/T.20/Doc. No. 18/Rev. 2 (2003).

[13] Commission Regulation (EU) No 61/2011 of 24 January 2011 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant method of analysis. *Off. J. Eur. Union*, 27 January 2011, L 23, 1-14.

[14] International Olive Council (IOC), Determination of the content of waxes, fatty acid methyl esters and fatty acid ethyl esters content by capillary gas chromatography, COI/T.20/Doc. No. 28/(2010).

[15] International Organization for Standardization (ISO), Vegetable fats and oils – Determination of wax content by gas chromatography, ISO/TS 23647 (2010).

[16] Martini, S., Carelli, A. A., Lee, J., Effect of the addition of waxes on the crystallization behavior of anhydrous milk fat. *J. Am. Oil Chem. Soc.* 2008, *85*, 1097–1104.

[17] Ceci, L. N., Carelli, A. A., Characterization of monovarietal Argentinian olive oils from new productive zones. *J. Am. Oil Chem. Soc.* 2007, *84*, 1125–1136.

[18] Ceci, L. N., Carelli, A. A., in: Tomás, M. (Ed.), *Advances in Fats Oil Research* Editorial Research Signpost, Trivandrum (India) 2010, pp. 71–97.

[19] Reiter, B., Lorbeer, E., Analysis of the wax ester fraction of olive oil and sunflower oil by gas chromatography and gas chromatography-mass spectrometry. *J. Am. Oil Chem. Soc.* 2001, *78*, 881–888.

[20] Trost, V. W., Characterization of corn oil, soybean oil and sunflowerseed oil nonpolar material. *J. Am. Oil Chem. Soc.* 1989, *66*, 325–333.

