Wax Analysis of Vegetable Oils Using Liquid Chromatography on a Double-Adsorbent Layer of Silica Gel and Silver Nitrate-Impregnated Silica Gel

G. Hénon[a,*], K. Recseg[b], and K. Kovari[b]

Eridania Béghin-Say Group, [a]Lesieur Recherche & Développement, Coudekerque-Branche, France, and [b]Cereol Group Research Centre, Budapest, Hungary

ABSTRACT: A chromatographic method is described to measure the crystallizable wax content of crude and refined sunflower oil. It can also be applied to any other vegetable oil. The preparative liquid chromatography step on a glass column containing a silica gel adsorbent superimposed upon a silver nitrate-impregnated silica gel support is used to isolate a wax fraction which is then analyzed by gas chromatography. The recovered wax fraction contains, in addition to the crystallizable waxes, hydrocarbons and other compounds with gas chromatographic retention times corresponding to waxes with chain lengths C_{34}–C_{42}. These compounds are short-chain saturated waxes in fruit oils, such as grapeseed and pomace. In seed oils such as sunflower, soybean or peanut, the compounds initially referred to as "soluble esters" are identified as monounsaturated waxes, esters of long-chain saturated fatty acids, and a monounsaturated alcohol, mainly eicosenoic alcohol. Such waxes are absent from corn or rice bran oils.

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KEY WORDS: Argentation, chromatography, fruit oil, monounsaturated wax, olive oil, saturated wax, seed oil, soybean oil, sunflower oil.

Sunflower oil contains naturally insoluble high melting point compounds that are removed from the oil during special steps of the refining process (pre-dewaxing by centrifugation, winterization). These components are monoesters of long-chain saturated fatty acids and saturated alcohols (1,2), which we will refer to as crystallizable waxes.

For a long time, wax contents were determined using crystallization procedures followed by filtration and weighing of the solid residue. These methods were more accurate when applied to crude sunflower oils rich in crystallizable waxes than to refined and dewaxed oils (1). In 1986, to avoid the crystallization step and to provide an accurate measurement of the crystallizable waxes, a new chromatographic method was proposed involving liquid chromatography on two superimposed silica supports, one of which was impregnated with silver nitrate (SN) (2). The wax fraction recovered by this technique contains, besides the hydrocarbons and the crystallizable waxes, other components called soluble esters, whose retention times in gas–liquid chromatography were equivalent to those of waxes with chain lengths from C_{36} to C_{42}. The name "soluble esters" reflected their full solubility in oil, even at low temperatures (4°C), and the presence, after methylation, of long-chain saturated fatty acid methyl esters.

Later, Mariani and Fedeli (3) proposed a method based on silica gel hydrated with 2% water and applied it to vegetable oils such as peanut, corn, rapeseed and soybean. The fraction obtained by elution with a mixture of hexane/diethyl ether 99:1 had a large content of sterol esters which coelute in gas chromatography (GC) with waxes having a chain length greater than C_{46}. Despite this fact, the method was able to detect the short-chain waxes C_{40}–C_{46} stemming from pomace oil in olive oil, and it was adopted by the European Economic Community (EEC) as a tool to use to control the purity of olive oil (4,5). Further, Amelio *et al.* (6) proposed the use of a high-performance liquid chromatograph fitted with a fraction collector to improve this standard method. Nota *et al.* (7) suggested that use of a solid-phase extraction (SPE) silica gel column is easier and quicker. In 1997, preparative medium-pressure liquid chromatography was applied using the SN silica gel to isolate the crystallizable waxes from the oil (8). Since it was more difficult to implement than the original one and was less adapted to a series of analyses, it was later abandoned.

The purposes of the present work are to update the SN method, to identify the soluble esters, to apply the method to oils other than sunflower oil, and to compare the SN and EEC methods in the case of olive oil.

MATERIALS AND METHODS

The vegetable oils were supplied by the different refineries of the Eridania Béghin-Say Group, except the rice bran oil, which was provided by RITO (Stuttgart, AR). Silver nitrate, silica gel 60HR extra pure for thin-layer chromatography (Art. 7744), silica gel 60 (Art. 7734), and 60 extra pure (Art. 7754) for column chromatography were Merck products (Darmstadt, Germany). Silica gel 60HR (Art. 7744) impregnated with 5% of SN was prepared by mixing well 100 g of silica gel with a solution of 5 g of SN dissolved in 240 mL of distilled water. After drying overnight at 160°C, the white powder that passed through a 0.125-mm sieve was collected.

*To whom correspondence should be addressed at Lesieur Recherche & Développement, 101 route de Bourbourg, F-59412, Coudekerque-Branche, France. E-mail: ghenon@fr.ebsworld.com

The solvents hexane, heptane, and dichloromethane were obtained from SDS Co. (Peypin, France). The internal standard hexatriacontane from Sigma Aldrich Chimie (St. Quentin Fallavier, France) was used in the experiments at a concentration of 0.1 mg/mL. This solution is prepared by dissolving 20 mg of hexatriacontane in *n*-heptane in a 200-mL volumetric flask. Hexamethyldisilazane (HMDS), trimethylchlorosilane (TMCS), and pyridine were also from Sigma Aldrich Chimie. The short-chain wax standards stearyl myristate (C_{32}), stearyl palmitate (C_{34}), and stearyl behenate (C_{40}) were Nu-Chek-Prep compounds (Copenhagen, Denmark).

Liquid chromatographic column for SN method. The preparative glass column (i.d. = 18 mm, height = 300 mm equipped with a Teflon stopcock) is fitted with a 250-mL solvent chamber topped by a female spherical ground glass joint for a quick connection to a nitrogen regulator. The glass wool plug placed in the bottom of the column partly filled with hexane should be small to avoid creating a dead volume where the separated components can remix. A special sample funnel with a long narrow tube allows the introduction of the sample solution into the hexane layer at the surface of the upper adsorbent and the homogenization of this layer, which is important to get impregnation of the adsorbent by the sample. To concentrate the wax fraction with a rotary evaporator, an adaptor with a soldered internal glass tube is connected to the balloon flask to avoid cross-contamination of the fractions recovered when removing the solvents.

By using a powder funnel, the SN silica is introduced into the column as a hexane slurry. In most cases, for the analysis of crude or pre-dewaxed oils with a wax content higher than 50 mg/kg (ppm), 1 g of this adsorbent is used (option 1). For well-winterized oil with a very low wax content, a quantity of 3 g of the SN silica is suitable to allow into the column the introduction of a higher quantity of sample (option 2). To pack the column, part of the hexane is then eluted with a very slight overpressure of nitrogen. Then, 15 g of silica 7734, also dispersed in hexane, is added over the first adsorbent without disturbing the surface. After filling the column, the solvent is then eluted at a flow rate of about 2–5 mL/min until the level of hexane is *ca.* 0.5–1 cm over the top of the upper adsorbent. The flow rate of the eluent is controlled by setting the nitrogen pressure.

The sample of sunflower oil is weighed into a small flask: 0.5–0.7 g for option 1, 1–1.2 g for option 2. An exact 1-mL vol of the 0.1 mg/mL standard solution is added to the sample before dilution with 2 mL of hexane/dichloromethane (95:5, vol/vol). The sample solution is introduced onto the column using the special sample funnel, and the 250-mL flask is placed under the column. After total elution until the surface of the silica appears mat (no liquid film but no drying), the walls of the column are washed three times with 3 mL of the hexane/dichloromethane 95:5 mixture followed each time by elution as previously. The wax fraction is eluted with a mixture of hexane/dichloromethane (80:20, vol/vol): 150 mL for option 1, 125 mL for option 2. The solvent is evaporated, and the residue is dissolved in dichloromethane (1.5 mL). The crystallizable wax fraction is finally analyzed by GC on a nonpolar capillary column SIMDIST (10 m, i.d. = 0.32 mm, film thickness 0.1 μm; Chrompack, Middelburg, The Netherlands) or equivalent. The chromatograph was a Girdel 30 (Giravions-Dorand, Suresnes, France). The recommended injector is a horizontal Ross with a glass needle. The injector and flame-ionization detector (FID) temperatures are 290°C. The carrier gas is helium at a flow rate of *ca.* 24 mL/min. The temperature program is 150 to 375°C at 10°C/min.

Fatty acid and fatty alcohol composition of the waxes. The wax fraction without hydrocarbon is evaporated to dryness and transferred into a 10-mL glass tube with a screw cap. Then 1 mL of a 0.5 N methanolic solution of anhydrous sodium hydroxide is added. The tube is heated to 80°C for at least 10 min with gentle mixing from time to time. Boron trifluoride solution in methanol (1 mL) is then added, and the tube is heated again to 80°C for at least 10 min. After cooling, brine is added before extracting the methyl esters and fatty alcohols three times with 2 mL of hexane. These fractions are combined, and hexane is removed until dryness. The residue is dissolved in 1 mL of dichloromethane. The alcohols are silylated by adding 1 mL of the silylating reagent (mixture of pyridine 4 mL, HMDS 3.6 mL, and TMCS 2.4 mL), by heating a short time to the boiling point of the dichloromethane, and then allowing the sample to sit at room temperature for 15 min. Excess reagent is removed before dilution with dichloromethane for the GC analysis on the same column as before, using the temperature program 100 to 375°C at 10°C/min.

*GC–mass selective detector (MSD) analysis.*The instrument used was a GC5890 Series II-MSD5972 from Hewlett-Packard (Palo Alto, CA), electron impact mode, with a capillary column CP-SIL5CB from Chrompack (10 m, i.d. = 0.25 mm, film thickness = 0.12 μm). The carrier gas was helium (1 mL/min, constant flow). The injector was an on-column injector. The temperature program was: for methyl esters analysis: 150°C for 1 min, 5°C/min to 300°C, hold for 15 min; for short-chain waxes and soluble esters analysis: 250°C for 1 min, 10°C/min to 300°C, hold for 15 min.

Turbidity measurements. The apparatus is the model 251 turbidimeter from Monitek (Hayward, CA).

RESULTS AND DISCUSSION

Figure 1 is a typical chromatogram of the wax fraction isolated from a crude sunflower oil (crystallizable wax content: 620 ppm) using SN method. Figure 2 is the chromatogram of the corresponding winterized oil (crystallizable wax content: 25 ppm). The hydrocarbons (referred to as H in the figures) are mainly the odd hydrocarbons C_{29} and C_{31}. The chain lengths of the crystallizable waxes in the crude-oil fraction range from C_{40} to C_{60}. The C_{36}–C_{42} soluble esters are eluted between the internal standard C_{36} and the crystallizable waxes with a chain length higher than C_{44}. The interference between the short-chain waxes C_{40} and C_{42} and the soluble esters with the same chain length cannot be avoided and must be considered for the calculation of the crystallizable wax content. Waxes with

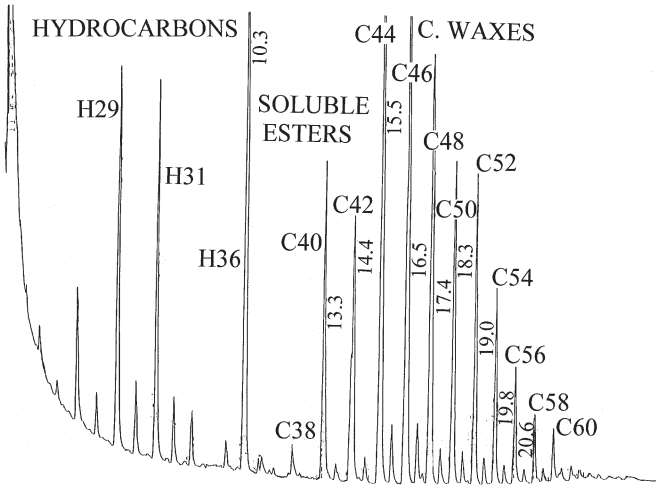

FIG. 1. Chromatogram flame-ionization detection (FID) of the crystallizable wax (c. wax) fraction of a crude sunflower oil.

chain lengths longer than C_{46} do not coelute with the sterol esters retained on the preparative column due to the SN and can be easily quantified. The different stabilizing agents of the dichloromethane (ethanol or 2-methyl-2-butene) have no effect on the elution pattern at their low levels in the solvent. Implemented by many investigators, hydration of the adsorbents to moderate their activities is not recommended for SN method. When both adsorbents are hydrated with 2% of water, the volume of eluent must be increased from 150 to 250 mL to totally recover the crystallizable waxes, without improving the resolution between the crystallizable waxes and the soluble esters. When using silica gel for liquid chromatography without any treatment or after drying at 500°C over hydrated SN silica, 250 mL of eluent is always needed with waxes totally recovered in the 100–250 mL fraction.

In the normal SN method, the activity of each different batch of silica for thin-layer chromatography must be checked and the quantity of SN silica adjusted, especially for the analysis of well-winterized oils. This is done by analyzing a dewaxed sunflower oil containing no more than 50 ppm of waxes using variable quantities of this adsorbent. The mass of SN silica is optimal when the amount of soluble esters eluted in the crystallizable wax fraction represents *ca.* 150 ppm in the oil, guaranteeing total recovery of the crystallizable waxes without elution of the sterol esters.

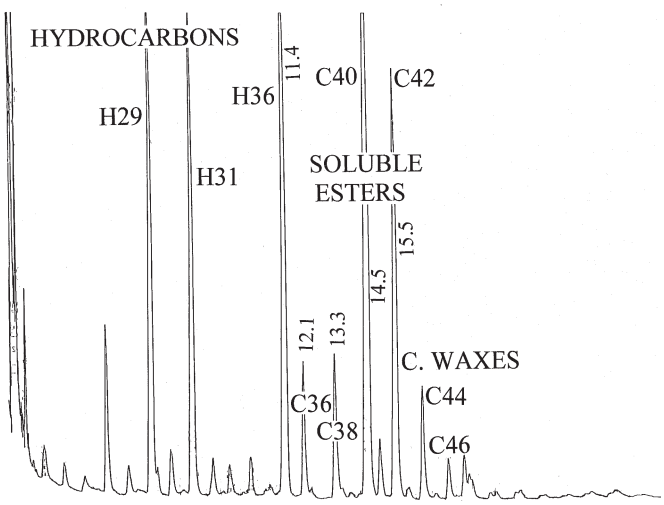

FIG. 2. Chromatogram FID of the crystallizable wax fraction of a winterized sunflower oil. See Figure 1 for abbreviations.

The mass of oil sample introduced into the column can reach 1 g when using 1 g of SN silica but no more than 1.5 g with 3 g of this adsorbent (Table 1). With 2 g of oil, peaks of sterol esters appear and interfere with the C_{48}–C_{50} waxes. In those experiments, if we removed the last measurement with 2 g of oil, the repeatability of the crystallizable wax determination was good: mean value 624 ppm, standard deviation ±26 ppm, and coefficient of variation 4.2%.

The response coefficient K of the waxes relative to the internal standard H_{36} is:

$$K = \frac{C_W}{C_{H_{36}}} \times \frac{I_{H_{36}}}{I_W} \quad [1]$$

where C_W, I_W, $C_{H_{36}}$, $I_{H_{36}}$ are the concentrations C and the integrated peak areas I of the standard wax W and the internal standard H_{36} in the control solution. This coefficient was determined by analyzing with GC a solution containing different standard short waxes mixed in known concentrations with the internal standard hydrocarbon. The measured K values for the waxes—stearyl myristate $K = 1.15$; stearyl palmitate $K = 1.15$; stearyl behenate $K = 1.19$—are in good agreement with the reference value 1.14 defined in the original method (1).

The crystallizable wax content of the oil in mg/kg (ppm) is calculated according to the following equation:

$$C_{\text{c.wax}}\ (\text{ppm}) = 1000 \times K \times \frac{I_{\text{c.wax}}}{I_{H_{36}}} \times \frac{m_{H_{36}}}{M_{\text{oil}}} \quad [2]$$

where $I_{\text{c.wax}}$ is the integrated area of all the crystallizable wax peaks; $I_{H_{36}}$ is the integrated area of the internal standard peak; $m_{H_{36}}$ is the amount of internal standard added to the test portion (mg); M_{oil} is the mass of the test portion (g).

The amount of soluble esters is calculated using the same Equation 2, in which undetermined K is equal to unity and the $I_{\text{c.wax}}$ is replaced by the sum I_{ESol} of the integrated areas of the peaks C_{36}, C_{38}, C_{40}, and C_{42}.

For sunflower oils, the correction factors introduced take into account the unavoidable interferences occurring between the soluble esters and the short waxes whose chain lengths are lower than C_{44}. Especially for dewaxed oils, these correction factors also integrate certain small peaks, in particular odd wax peaks, which are difficult to integrate with accuracy because of their low levels. These factors are based on an average distribution of the waxes prepared by crystallization and purification of the waxes of crude and refined oils.

For sunflower oils, the total integration $I_{\text{c.wax}}$ of the crystallizable waxes is for crude oils

$$I_{\text{c.wax}} = 1.5 \times I_{C_{44}} + I_{>C_{44}} \quad [3]$$

and for dewaxed oils

TABLE 1
Oil Capacity of the Columns According to the Amount of Silica Impregnated with Silver Nitrate (SN) Repeatability[a]

SN silica											
1 g								3 g			
Oil (g)											
0.55	0.52	0.58	0.51	1.15	1.01	1.06	1.05	1.00	1.50	1.51	**2.01**
C. waxes (ppm)											
653	614	588	670	630	622	618	638	582	612	640	**708**

[a]Constant amount of silica for column chromatography Art 7734 Merck (Darmstadt, Germany) = 15 g. C. waxes, crystallizable waxes. Boldface values indicate overloaded column.

$$I_{\text{c.wax}} = 2 \times I_{C_{44}} + I_{\text{even}>C_{44}} \quad [4]$$

For winterized oils with a very low wax content (less than about 20 ppm), only the C_{44} and C_{46} peaks are well defined on the chromatograms; the others are overlapped by some undefined peaks. The crystallizable wax integration in that case can be estimated using Equation 5:

$$I_{\text{c.wax}} = 2 \times I_{C_{44}} + 1.6 \times I_{C_{46}} \quad [5]$$

In these equations, $I_{C_{44}}$, $I_{>C_{44}}$, $I_{\text{even}>C_{44}}$, $I_{C_{46}}$ are the integrations of, respectively, the C_{44} wax, the odd and even waxes with a chain length higher than C_{44}, the even waxes with a chain length higher than C_{44} and the C_{46} wax.

Identification of the soluble esters. Chromatograms obtained according to SN method on the liquid oil and the solid residue prepared by filtration on a 0.6-µm Millipore membrane (Millipore, St. Quentin Yvelines, France) of a crude oil cooled to 4°C are equivalent to the partitioning of the chromatogram of the wax fraction of the crude oil (Fig. 1) into two parts. The solid residue gives the same chromatogram as the crystallizable waxes of the crude oil, without hydrocarbons and soluble esters which are solubilized in the liquid oil. The wax peaks range from C_{42} to C_{60}. The waxes can be characterized by their composition, or preferably by their distribution, which is the amount of each wax expressed in percentage of a reference wax, here C_{46}, and their fatty acid and fatty alcohol compositions (Table 2, minor odd chain length waxes are not reported). The C_{44}–C_{48} even waxes, the saturated fatty acids C_{20}, C_{22}, and the saturated fatty alcohols C_{24}, C_{26} are the most important components of the sunflower solid waxes.

The chromatogram of the wax fraction of the filtered oil is identical to that of the refined oil in Figure 2 and contains the usual saturated hydrocarbons of the sunflower oil, some residual short waxes incompletely removed by the crystallization and the filtration operations in the experiment, and the so-called soluble ester compounds with retention times equivalent to the waxes with chain lengths C_{36}, C_{38}, C_{40}, C_{42}. A partial resolution of the C_{42} wax and the C_{42} soluble ester was observed on the chromatogram. Other small peaks with the same retention times as the sterol esters were present at very low levels. The soluble esters were purified using the SN method, but, in order to remove the hydrocarbons, the first 25 mL was discarded. Sunflower crystallizable waxes and soluble esters were methylated, silylated, and analyzed by GC–MSD. On the sunflower crystallizable wax ester chromatogram (Fig. 3A), silylated alcohols (notated "Ansil," with A = alcohol, *n* = chain length, sil = silylation) are eluted just after the corresponding fatty acids. On the methyl ester chromatogram of the soluble esters (Fig. 3B), saturated fatty acids are predominant with only small peaks of alcohols because of the inability to completely resolve short waxes and soluble esters using the SN method (the only way to obtain pure soluble esters is to use thin-layer chromatography). The fatty acid composition determined using FID is different from that of the crystallizable waxes and is very rich in the C_{22} saturated fatty acid: C_{16}: 7.4%; C_{18}: 10.1%; C_{20}: 15.5%; C_{22}: 48.4%; C_{24}: 18.5%.

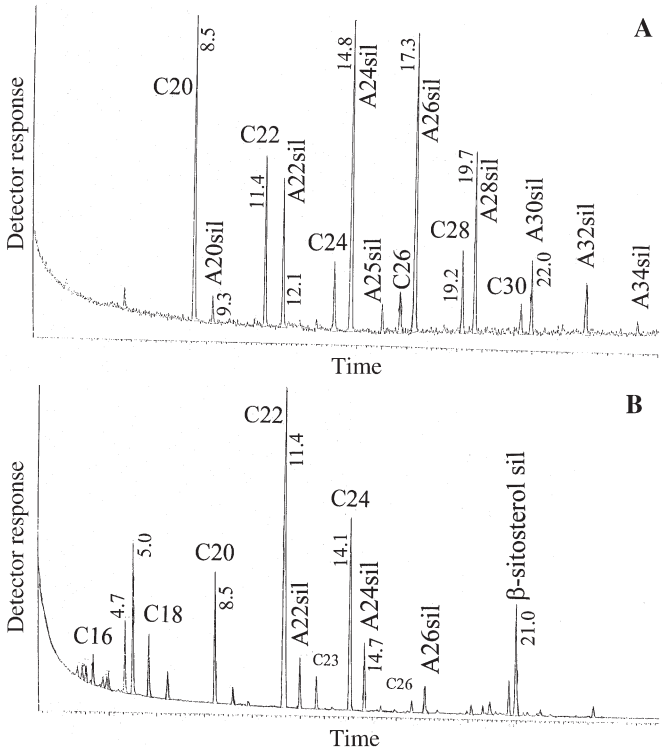

FIG. 3. Chromatogram from GC–MSD5972 of the silylated methyl esters of the c. waxes (A) and the soluble esters (B) of sunflower oil. See Figure 1 for abbreviation. In Ansil notation, A = alcohol, *n* = chain length, and sil = silylation.

TABLE 2
Analysis of the C. Waxes of a Crude Sunflower Oil[a]

C. waxes			Saturated fatty acids		Saturated fatty alcohols	
CL	Composition (%)	Distribution relative to C_{46}	CL	Composition (%)	CL	Composition (%)
C_{42}	3.1	13	C_{16}	2.8	C_{20}	4.2
C_{44}	15.8	67	C_{18}	5.3	C_{22}	15.2
C_{46}	23.6	100	C_{20}	55.3	C_{24}	31.3
C_{48}	17.8	76	C_{22}	20.0	C_{26}	26.0
C_{50}	11.5	49	C_{24}	5.7	C_{28}	12.5
C_{52}	9.3	40	C_{26}	3.3	C_{30}	5.4
C_{54}	5.8	25	C_{28}	5.1	C_{32}	4.0
C_{56}	3.5	15	C_{30}	2.6	C_{34}	1.3
C_{58}	2.3	10				
C_{60}	0.9	4				

[a]CL, chain length of the waxes, fatty acids, or fatty alcohols. See Table 1 for other abbreviation.

The analysis of the standard waxes C_{32} (stearyl myristate), C_{34} (stearyl palmitate), and C_{40} (stearyl behenate) by GC–MSD gives mass spectra in accordance with their chemical composition (Table 3). The same analysis carried out on the soluble ester fraction gives mass spectra that are different for soluble esters and crystallizable waxes. The fragments 313 and 341 of the crystallizable wax peaks at t_R = 9.13 to 14.75 min (Table 3) are due to the major fatty acids of the waxes, respectively C_{20} and C_{22}. The fragments 278 and 296 (minor response) of the soluble ester peaks at t_R = 5.26 to 9.01 min do not correspond to any fatty acids, especially the most important C_{22} fatty acid. Elsewhere, the mass spectra of the soluble esters do not contain the high fragment M − 18 observed for the crystallizable waxes. The fragments 82 and, in particular, 123 reveal a double bond in the structure of the molecule. The fragments 278 and 296 are attributed to the eicosenoic alcohol. We conclude that the soluble esters in sunflower oils are monounsaturated soluble waxes, esters of saturated fatty acids, and monounsaturated alcohols, mainly eicosenol ($C_{20:1}$). This unsaturation explains why their elution volume in the SN method is larger than that for the crystallizable waxes.

The soluble waxes $C_{36:1}$, $C_{38:1}$, $C_{40:1}$, and $C_{42:1}$ in sunflower oils constitute a specific class of compounds, differing from crystallizable waxes by their chemical structure and physical properties (solubility in sunflower oils at low temperature). For these reasons, the soluble waxes must not be integrated as crystallizable waxes and have to be considered separately.

Crude sunflower oils. Nine samples were analyzed, including four samples of high-oleic acid sunflower oil (Table 4). The amount of saturated hydrocarbons varies in a wide area:

TABLE 3
Mass Spectra of Waxes and Soluble Esters[a]

Standard waxes		
CL	Fatty acid fragment $= MW_{FA} + 1$	Wax fragment $= MW_{wax} - 18$
C_{32}	$m/z = 229 \rightarrow MW_{C_{14}} = 228$	$m/z = 480 \rightarrow MW_{C_{32}} = 498$
C_{34}	$m/z = 257 \rightarrow MW_{C_{16}} = 256$	$m/z = 508 \rightarrow MW_{C_{34}} = 526$
C_{40}	$m/z = 341 \rightarrow MW_{C_{22}} = 340$	$m/z = 592 \rightarrow MW_{C_{40}} = 610$

Soluble ester fraction of sunflower oil

		Fragment 1			Fragment 2		
CL	t_R (min)	m/z	MW_{FA}	Identification	m/z	MW_{FA}	Identification
Soluble esters							
C_{36}	5.26	278	277	?	296	295	?
C_{38}	6.20	278	277	?	296	295	?
C_{40}	7.46	278	277	?	296	295	?
C_{42}	9.01	278	277	?	296	295	?
C. waxes							
C_{42}	9.13	313	312	C_{20}	341	340	C_{22}
C_{44}	11.45	313	312	C_{20}	341	340	C_{22}
C_{46}	14.75	313	312	C_{20}	341	340	C_{22}

[a]MW_{wax}, molecular weight of the wax corresponding to m/z (= m/z + 18); MW_{FA}, molecular weight of the fatty acid corresponding to m/z (= m/z − 1). See Tables 1 and 2 for other abbreviations.

TABLE 4
Analysis of Hydrocarbons, Soluble Esters, and C. Waxes of Different Crude Sunflower Oils: Amount and Distribution[a]

	Sunflower high oleic (sample number)				Sunflower high linoleic (sample number)				
	1	2	3	4	5	6	7	8	9
Hydrocarbons (ppm)	128	138	259	160	163	146	127	156	171
H_{29}	100	100	11	100	100	100	100	100	100
H_{31}	82	86	93	94	71	77	72	78	71
Soluble esters (ppm)	149	173	115	214	265	239	235	257	253
C_{36}	21	20	26	19	21	23	19	21	20
C_{38}	17	17	26	16	28	19	17	18	18
C_{40}	100	100	100	100	100	100	100	100	100
C_{42}	52	62	38	40	58	63	68	65	65
C. waxes (ppm)	506	461	597	777	717	578	522	629	658
C_{44}	103	90	67	83	78	88	102	98	96
C_{46}	100	100	100	100	100	100	100	100	100
C_{48}	68	97	93	76	69	66	61	69	71
C_{50}	46	55	63	49	44	45	38	44	47
C_{52}	36	39	47	43	34	36	29	34	37
C_{54}	25	28	31	28	22	23	15	20	21
C_{56}	12	21	20	18	14	18	6	12	12
C_{58}	8	6	6	9	8	8	3	7	7
C_{60}				7	7			3	4

[a]See Table 1 for abbreviation.

125 to 260 ppm, composed essentially of C_{29} and C_{31}. The quantity of soluble esters recovered in the wax fraction is higher for the samples 4 to 9 resulting from a change of the batch of silica gel Art. 7744 during the experiment. Their distribution is the same for high-oleic and high-linoleic sunflower oils and is relatively constant with a maximum for the C_{40} soluble ester. The crystallizable wax content 460–780 ppm is a normal level for crude oils. The chain length of the waxes can be as high as C_{60}, with the highest concentration for the C_{46} wax. The interfering C_{40} and C_{42} waxes are included in the C_{40} and C_{42} soluble esters. The wax distributions are quite similar from one sample to another, which justifies the formula used in the calculations.

Refined sunflower oils. The first series of oils was sampled

TABLE 5
Analysis of Hydrocarbons, Soluble Esters, C. Waxes in Refined Sunflower Oils. Content and Distribution[a]

	Sample number							
	R1	R2	R3	R4	R5	R6	R7	R8
				Content (ppm)				
Hydrocarbons	75	70	83	98	79	108	114	107
Soluble esters	172	115	139	97	124	143	90	110
C. waxes	94	94	91	51	44	41	25	25
				Distribution of soluble esters				
C_{36}	20	15	25	16	21	15	12	12
C_{38}	19	19	23	17	20	16	18	15
C_{40}	100	100	100	100	100	100	100	100
C_{42}	59	66	51	50	52	41	51	43
				Distribution of c. waxes				
C_{44}	219	196	123	143	185	167	182	193
C_{46}	100	100	100	100	100	100	100	100
C_{48}	61	54	75	92	86	82	98	105
C_{50}	29	22	47	46	41	28	38	
C_{52}	21	19	41	41	38	29	34	
C_{54}	12	16	25	30	30			
C_{56}			15		20			
C_{58}			9					

[a]See Table 1 for abbreviation.

after the predewaxing steps by centrifugation during the different refining processes (Table 5). The total hydrocarbon content (70–115 ppm) is lower than that of the previous crude oils (125–260 ppm). Since these dewaxed oils and the previous crude oils do not correspond to each other, we cannot conclude that hydrocarbons are removed during the dewaxing step. The distribution of the soluble esters is about the same as for crude oils with no enrichment in the shorter chain lengths C_{36} and C_{38}, which is a confirmation that they are not involved in the dewaxing operation. The crystallizable wax content of the predewaxed oils is variable: 25–94 ppm. The crystallizable wax distribution is very different from that of the crude oils: the C_{44} wax has become the major wax, and the longest chain length waxes C_{56}, C_{58}, C_{60} have in most cases disappeared.

A second series of refined oils was analyzed for wax content before making turbidity measurements after storage of the oils at 4°C. The crystallizable wax content of the samples ranged from 10 to 72 ppm (Table 6). The linear relationships existing between the wax content and the turbidity at 4°C of the refined oils are similar from 24 to 72 h storage:

$$\text{Monitek turbidity} = 0.5 \times \text{crystallizable wax ppm} - 6.5 \quad [6]$$

Except for one sample for which the low turbidity continued to increase slowly until 7 d, these correlations can be used to forecast the brightness behavior of the oil when submitted to low temperatures.

Ring test. A ring test was organized between some of the laboratories of the EBS Group using the SN method. Values measured by laboratories 4 and 5 on the crude oil A are lower than those of the other laboratories (Table 7) due to the use of too much of the SN silica, resulting in a lack of wax elution. The reproducibility observed for the three other laboratories on this sample is excellent. For the dewaxed oils B to D, including all the laboratories, the reproducibility is worse but is enough to control the efficiency of the dewaxing processes and can be improved with an accurate standardization of the method.

TABLE 6
C. Wax Content and Monitek[a] Turbidity of Refined Sunflower Oils

Wax content (ppm)	Monitek turbidity value		
	24 h	48 h	72 h
10.3	0.4	0.6	0.9
10.8	0.7	0.7	0.8
13.9	0.3	0.6	1.1
13.9	1.1	1.1	1.3
15.0	0.7	1.1	1.4
15.0	1.1	1.3	1.9
15.2	0.3	1.1	1.6
15.5	0.9	1.3	1.6
15.5	0.3	1.2	1.8
16.0	1.0	2.1	0.9
17.5	1.6	2.7	3.3
17.9	2.8	3.2	3.2
22.0	4.3	5.2	5.0
22.4	5.8	7.7	7.8
25.4	0.4	1.0	3.0
26.2	5.5	6.5	5.7
30.6	8.4	8.7	8.7
39.6	12.2	13.0	12.5
44.2	12.0	12.5	12.0
46.6	15.5	16.0	15.0
47.8	15.5	16.5	16.0
53.2	29.0	30.5	29.0
63.5	14.5	14.5	13.5
72.0	34.0	37.0	34.0

[a]Turbidimeter (Monitek, Hayward, CA). See Table 1 for abbreviation.

TABLE 7
C. Wax Content of Sunflower Oils: Results of the Ring Test[a]

Sample	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Mean 2	SD	Variation coefficient
A	468-509-523-513	496-502-502-508-497	531-544-482	417-414-401-414-422	328-371-339-			
Mean 1	503	501	519	414	346	506*	20*	4*
B	34-28-27-23	35-43-46-35-30	31-26-31	36-28-31-30-28-28	27-26-31			
Mean 1	28	38	29	30	28	31	5.5	18
C	60-66-73	82-74-99-82	98-90-91	57-56-63-61-70-80	81-77-73			
Mean 1	66	84	93	65	77	75	13	18
D	78-72-61-59-63-66-64-68	64-70-69-68-71-68	74-77-71-69-73-78	54-55-53-51-50-53-55-53-50	72-64-62-60-63-64			
Mean 1	66	68	74	53	64	64	8	13

[a]Mean 1, average value for each lab; Mean 2, average value for all the labs. *Except labs 4 and 5. See Table 1 for abbreviation. A, crude oil; B, C, predewaxed oils; D, winterized oil.

TABLE 8
Analysis of the C. Wax Fraction of Various Vegetable Oils: Content and Distribution[a]

	Oil samples						
	Crude rice bran	Crude rapeseed	Refined soybean	Peanut	Crude corn	Refined grapeseed	Blend olive pomace
	Peaks equivalent to soluble esters						
ppm:		25	153	50		33	291
C_{36}		51	7	26		13	
C_{38}		124	15	43		46	115
C_{40}		100	100	100		100	100
C_{42}		120	103	47		157	44
	C. waxes						
ppm:	6680	110	31		110	70	12
C_{44}	10.7	80	225		40	100	
C_{46}	44.3	100	100		100	75	
C_{48}	90.3	62			111	64	
C_{50}	85.9	39	54		56	38	
C_{52}	100	33	36		59	66	
C_{54}	99.8	14	24		103	49	
C_{56}	66.6	10	12		76	46	
C_{58}	42.5	4	5		25	24	
C_{60}	14.7						
C_{62}	3.8						

[a]See Table 1 for abbreviation.

Oils different from sunflower oils. The method was applied to rice bran (RBO), rapeseed, soybean, peanut, corn, grapeseed, and olive oils (Table 8). The crystallizable wax contents were calculated on the basis of the total area of the wax peaks using only the response coefficient $K = 1.14$ of waxes. The amount of soluble esters indicated in the table is not the content of the oil but only the quantity recovered in the crystallizable wax fraction.

The crude RBO has a very high content of crystallizable waxes, up to 6700 ppm. Such a wax amount seems to be usual for RBO if we compare it to the wax contents of more than 0.5% determined on refined RBO by the acetone-insoluble method (9). The distribution of the RBO waxes differs from that of the sunflower waxes, with values of more than 85% for the C_{48}–C_{54} waxes. The soluble esters are absent or present at low levels. A high hydrocarbon content was measured (550 ppm).

Crude rapeseed oil is also an oil with a low level of soluble esters. The crystallizable wax content of 110 ppm is high for rapeseed oil, which usually does not contain any waxes. The distribution of these crystallizable waxes is similar to that of crude sunflower oil waxes, suggesting a contamination of the rapeseed oil with sunflower oil or the corresponding waxes. Analyses made on other different samples (Table 9) demonstrate that the method is able to explain why refined rapeseed oils can be unclear (presence of crystallizable waxes) and the possible origin of a problem (contamination of the crude rapeseed oil).

For soybean oil, the chromatogram of the crystallizable wax fraction is very similar to that of the winterized sun-

TABLE 9
Content and Distribution of C. Waxes in Rapeseed Oils[a]

	First series of samples					Second series of samples			
	Crude			Refined		Crude		Refined	
	Waxes (ppm)								
	51	28	27	28	60	65	20	55	67
C_{44}	93	91	91	90	90	88	86	82	88
C_{46}	100	100	100	100	100	100	100	100	100
C_{48}	74	76	78	76	73	72	60	61	70
C_{50}	39	39	36	43	64	35	27	33	33
C_{52}	33	36	29	31	37	43	23	35	36
C_{54}	16	16			31	22	15	15	25
C_{56}					22			8	19

[a]See Table 1 for abbreviation.

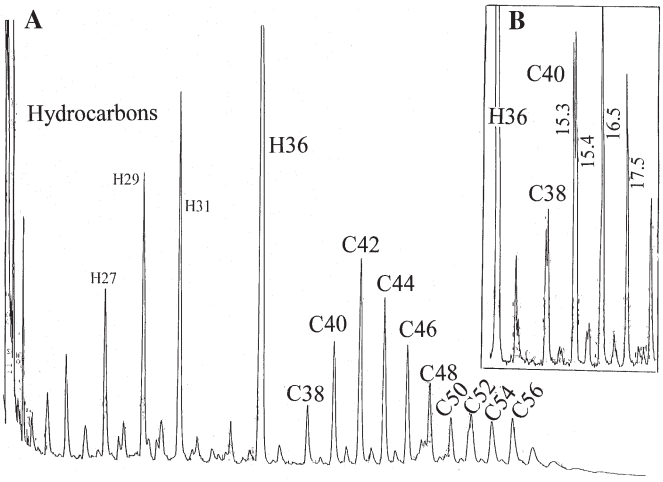

FIG. 4. Chromatogram FID of the c. wax fraction of a pure refined grapeseed oil (A) and a questionable sample (B). See Figure 1 for abbreviations.

flower oil in Figure 2. The hydrocarbons are absent. The analysis of the wax fraction by GC–MSD confirms that the peaks C_{36}–C_{43} with the same fragment 278 as in sunflower oils are soluble monounsaturated waxes. The fragments obtained for the C_{44} wax peak are those of the C_{16}, C_{18}, C_{20} (the most important), and C_{22} saturated fatty acids. The wax content is low (30 ppm), but the distribution is similar to that of sunflower waxes, suggesting also a possible slight contamination with sunflower oil (the distribution of the C_{48} wax is not reported because of some small interfering peaks).

The chromatogram of the crystallizable wax fraction of the peanut oil is similar to that of the soybean oil and contains only 50 ppm of C_{34}–C_{42} soluble esters. These soluble esters are monounsaturated waxes with the same fragment 278 in their mass spectrum as in sunflower and soybean oils.

Hydrocarbons and soluble esters are almost absent in the crude corn oil. The bimodal distribution of the crystallizable waxes (100 ppm) is typical of corn oil.

For refined grapeseed oil (Fig. 4A), GC–MSD analysis of the fraction concludes that the C_{36}–C_{42} peaks are short saturated waxes. The C_{36}–C_{44} waxes contain palmitic acid as the major fatty acid (about the only fatty acid for the C_{40} and C_{42} waxes) while eicosanoic acid C_{20} is the most important fatty acid in the C_{46} and C_{48} waxes. The short chain length of the fatty acids compared to that of the sunflower waxes explains the clarity of the grapeseed oils even containing 100 ppm of total waxes. In some samples (Fig. 4B), there is a splitting of the C_{38} and C_{40} peaks. The same splitting observed with a mixture of sunflower oil (10%) in grapeseed oil can be explained by the fact that peaks C_{36} to C_{42} are monounsaturated waxes in sunflower oils but saturated waxes in grapeseed oils. Further, the method is perhaps a means to identify sunflower oil in grapeseed oil or other seed oils in fruit oils. In the seven other samples of refined grapeseed oils analyzed (Table 10), no splitting of the C_{38} and C_{40} peaks occurred. The total wax content was relatively constant, 90–150 ppm.

In a commercial blend of refined pomace oil and olive oil,

TABLE 10
C. Wax Analysis of Grapeseed Oils

	Sample number							
	1	2	3	4	5	6	7	8[a]
	Waxes (ppm)							
	108	110	90	112	148	115	110	1700
	Distribution							
C_{36}	10	23	20	10	ni	19	20	ni
C_{38}	49	45	56	28	19	53	39	ni
C_{40}	98	99	91	74	54	113	88	88
C_{42}	100	100	100	100	100	100	100	100
C_{44}	68	67	60	56	89	56	58	58
C_{46}	69	46	37	34	71	42	30	30
C_{48}	43	26	18	21	45	29	25	25
C_{50}	28	16	34	49	33	24	19	19
C_{52}	47	23	55	36	33	47	30	30
C_{54}	35	37	40	37	32	40	25	25
C_{56}	39	51	49	59	40	49	25	25
C_{58}	18	23	21	ni	14	13	20	20

[a]Crude oil. ni, small nonintegrated peaks. See Table 1 for abbreviation.

the peaks corresponding to C_{38}–C_{42} soluble esters are present at a 290-ppm level (Table 8) and are also saturated waxes with about only palmitic acid in the C_{38} (the most important) and C_{40} waxes and stearic acid appearing only in appreciable amounts in the C_{42} and higher chain length waxes. For this reason, the SN method can be applied to olive oils to measure the amount of C_{40}–C_{46} waxes noted in the EEC method. Hence, nine samples of virgin olive oil were analyzed using the SN method and the results compared to the EEC method which uses silica hydrated with 2% water as an adsorbent. In the first experiments carried out by the EEC method, only hydrocarbons were recovered in the wax fraction, confirming the observations of other authors who proposed benefits in increasing eluent polarity (7,10). With the EEC method (Fig. 5), squalene and sterol esters are the most important peaks in the chromatogram, and it is difficult to clearly identify the waxes among the numerous small peaks. With the current SN method

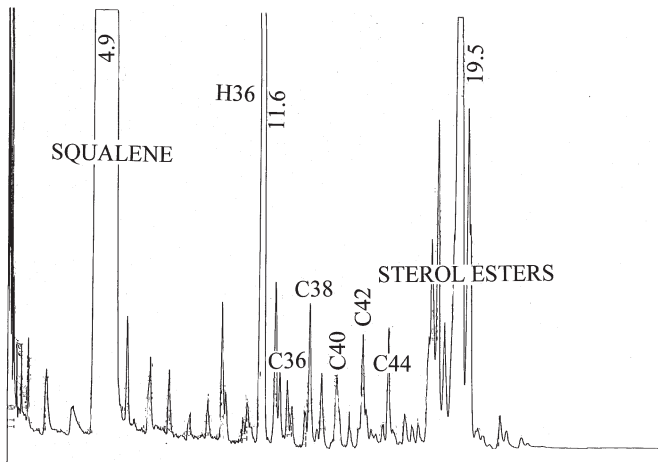

FIG. 5. Chromatogram FID of the wax fraction of an olive oil according to the European Economic Community method. See Figure 1 for abbreviation.

TABLE 11
Comparison of the SN Method and EEC Method (E) on Olive Oils[a]

	Sample																	
	1		2		3		4		5		6		7		8		9	
Method	SN	E	SN	E	SN	E	SN	E	SN	E	SN	E	SN	E	SN	E	SN	E
C_{40}	27	33	0	28	9	10	16	15	11	11	22	17	16	17	9	6	21	13
C_{42}	7	20	4	31	5	10	5	11	3	12	6	12	4	12	3	5	6	7
C_{44}	2	5	3	18	2	3	2	5	1	4	2	4	2	4	1	2	2	4
C_{46}	2	4	2	6	1	2	1	0	1	4	1	ni	1	3	1	ni	1	ni
Total	38	62	8	83	18	26	24	31	17	31	30	33	22	37	14	13	30	24

[a]See Tables 1 and 10 for other abbreviations.

(Fig. 6), squalene and sterol esters disappeared, and the wax peaks are accurately defined. Only waxes C_{40}–C_{46} are quantified as recommended by the EEC method (Table 11). For most samples, an acceptable agreement exists between both methods, but for samples 1 and 2, an important difference exists. This is a consequence of the undefined small peaks interfering with the wax esters when using the EEC method.

It is our opinion that the use of silica impregnated with silver nitrate, as proposed in this SN method, is essential in order to accurately measure the wax content of oils. Such analyses are important for sunflower oils and RBO for which a special treatment of dewaxing is needed to obtain, after refining, a clear oil. The wax fraction recovered after liquid chromatography contains compounds with retention times in GC equivalent to waxes with chain lengths C_{34}–C_{42}, which are soluble monounsaturated waxes in some seed oils, such as sunflower, soybean or peanut, but short saturated waxes in fruit oils such as grapeseed or olive. That difference could possibly be useful to detect certain mixtures of seed oils in fruit oils, such as sunflower oil in grapeseed. These monounsaturated waxes, esters of saturated fatty acids, and a monounsaturated alcohol, mainly eicosenol, should not be considered crystallizable waxes in sunflower oils because they are soluble in the oil at low temperature and are not of concern in the dewaxing operations during refining. The SN method applied to olive oils gives better chromatograms than those obtained with the EEC method and allows more accurate measurements of the C_{40}–C_{46} waxes.

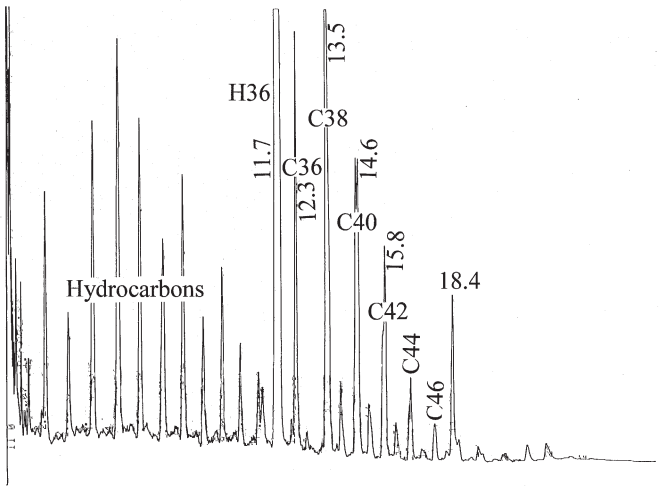

FIG. 6. Chromatogram FID of the c. wax fraction of an olive oil according to the silver nitrate method. See Figure 1 for abbreviations.

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