Detection of Olive Oils Authenticity by Determination of their Sterol Content using LC/GC

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Abstract

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The content of selected sterols as declared in the EU Commission Regulation was used to prove the authenticity of olive oils. A modified method using the preparative LC with silica gel packed column and gradient elution with three mixtures of hexane and diethyl ether was used to separate undesirable interfering compounds in the unsaponifiable fraction before the determination of sterols using GC. Model experiments based on the determination of Δ-7-stigmastenol and campesterol (addition of sunflower and soybean oils), or brassicasterol (addition of rapeseed oil) were used to verify that this method is capable of identifying adulteration of olive oils by additions of sunflower, soybean or rapeseed oils. An elevated content of these marker sterols, in comparison with their permitted contents, enables the identification of an addition of 5–10% of the above oils to the olive oil. This method was also used to evaluate the authenticity of five samples of olive oils from the SIAL exhibition (Paris) and ten samples of virgin olive oils obtained on the Prague markets. It was revealed that none of the samples showed the signs of adulteration.

Keywords: olive oil; authenticity; adulteration; sterols; sunflower oil; soybean oil; rapeseed oil; liquid chromatography; gas chromatography; sterol determination

This paper follows the publication focused on the detection of adulterated sunflower and soybean oil with the additions of rapeseed oil (BOHAČENKO & KOPICOVÁ 1999). We have considered the risk of that adulteration as the most important for our country since the above oils are the most widely used in the Czech Republic. This time we have focused on the detection of the adulteration of olive oils which are available solely by import in the Czech Republic and whose consumption is not very considerable in our country due to its high price. Their authenticity, however, must also be supervised because even with small import volumes or low sales in our market a relatively high profit can be earned by their adulteration, to the detriment of consumers.

The content of selected sterols is widely accepted as one of the most important markers for the detection of adulterated olive oils. Varying only in a narrow range, the content of sterols is characteristic for these oils. The binding limits for the content of the sterols are set forth in the Commission Regulation (EEC) No. 2568/91 (1991), which can be considered as being the essential standard to assess the quality of all types of olive oils.

Generally, the determination of sterols in olive and other plant oils employing gas chromatography (GC) can be divided into three different parts: isolation from the matrix, separation or pre-purification of the sterol fraction, and the determination of the content of the individual sterols, or their derivatives, using gas chromatography. Several analytical procedures based on this method and differing from each other in practical performance of the above-mentioned steps were published.

Those methods where the sterols are isolated from the unsaponifiable fraction are considered as essential methods. However, this fraction contains a wide spectrum of other compounds in addition to sterols, such as higher hydrocarbons, aliphatic alcohols, tocoferols, triterpene-based alcohols, and waxes (EISNER *et al.* 1963, 1965, 1966.) These compounds may interfere with the following analysis of the sterol fraction using capillary GC and a variety of methods is used for their removal. One of the most frequently used is fractionation using a preparative thin layer chromatography (TLC) (AMATI 1971; ITOH 1973; FREGA 1992; JIMENÉZ & GONZÁLEZ 1996). This method, however, although frequently criticized for its time con-

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sumption and laborious work, continues to be the official method adopted in the above-mentioned Regulation of the Commission Regulation No. 2568/91. Liquid chromatography (LC) is also used to pre-purify the unsaponifiable fraction. This method was probably published for the first time by EISNER *et al*. (1963). They used gradient elution with 700 ml of three mixtures of n-hexane and diethyl ether to separate all the compounds in the Florisil packed column. MOURA FE *et al*. (1975) later used their method in the identification of different compounds of the unsaponifiable fraction of coconut oil.

Included in the second group are methods used to isolate sterols without prior saponification of oils. WORTHINGTON and HITCHCOCK (1984) applied the combination of LC and TLC. They used LC in the silica gel packed column with a gradient elution of 3100 ml of three mixtures of hexane and ethyl acetate with increasing concentration of ethyl acetate to pre-separate sterol esters, triglycerides, and sterols from peanut and sunflower oils without saponification. The main purification was performed with TLC.

The advantage of the up-to-date methods using on-line (HP)LC-GC is a rapid performance since they omit the procedures of oil saponification, extraction of the unsaponifiable fraction, and its pre-purification. In this case, sterols and sterol esters are first separated using the LC directly from the oil in the form of derivatives with pivalic acid (GROB &LAFRANCHI 1989; GROB *et al*. 1991), or in the form of silylated derivatives (ARTHO *et al*. 1993) and then identified using the GC. ALONSO *et al*. (1997) published the method enabling to determine sterols directly by GC after transesterification achieved by alkaline catalysis with mixture of KOH and methanol. They claim that the results are comparable to the procedures employing the isolation of sterols by saponification and determination of their silylated derivatives by the GC. Therefore, the method is suitable for routine use.

Parallel to the development of analytical methods for the determination of sterols in plant oils, it was also suggested to use these methods to prove the authenticity of olive oils. One of the earliest important works on the determination of sterols in vegetable oils was published by ITOH *et al*. (1973). They determined relative retention times (β-sitosterol = 1.00) of nine basic sterols and their percentage share in 19 different types of vegetable oils. JIMENÉZ and GONZÁLEZ (1996) suggested using the sterol contents, in particular Δ-7-stigmastenol and Δ-7-avenasterol, for the determination of individual types of olive oils (virgin oil, refined oil and solvent-extracted oil). GROB *et al*. (1994a) suggested using the determination of Δ-7-stigmastenol, campesterol, stigmasterol and brassicasterol as a proof of the adulteration of the olive oils by additions of sunflower, soybean, or rapeseed oils.

ALONSO *et al*. (1997) similarly used the change in Δ-7-stigmastenol to prove the addition of sunflower oil into the olive oil. LANUZZA and MICALI (1997) monitored the adulteration of olive oils with additions of de-sterolized sunflower oil. They used Δ-8(14)-stigmastenol, which was generated through the isomerization of Δ-7-stigmastenol during the de-sterolization procedure, i.e. in strong bleaching conditions at 150–180°C and with the addition of 5 to 7% of earth.

The main aim of our work was to implement, or modify, the analytical method for the determination of the sterol content in olive oils. Such implementation would enable us to prove their authenticity as a result of their conformity with the defined parameters. In addition, the use of the marker sterols, or their contents, respectively, to prove adulteration of olive oils with additions of the sunflower, soybean, or rapeseed oils was verified.

MATERIAL AND METHODS

Apparatus: Gas chromatograph Hewlett Packard HP 5890 II with auto sampler HP 6890 and FID detector, arrangement for LC: laboratory pump LCP 4020, gradient programmer GP 6 (all ECOM), automatic fraction collector (Development Workshops of the ČSAV).

Chemicals: KOH p.a. (Lachema, CR), n-hexane for trace analysis, methanol p.a. ISO, diethyl ether GR ACS (all from MERCK), ethanol for UV spectroscopy, isopropyl alcohol p.a. (all from Lachema, CR), silica gel for column chromatography SILPEARL sorption capacity 61% H_2O, pH 6–7, particle size 25 μm (Sklárny Kavalier, CR), hexamethyl disilazan (HMDS) purum >98%, trimethyl chlorsilan (TMCS) puriss. >99%, pyridine purum >99 % (all from Fluka).

Standards: Cholesterol purity 99 %, dihydrocholesterol purity 99.2%, β-sitosterol practical, about 60%, from soybeans, also containing dihydrobrassicasterol, campesterol and stigmasterol (all from Sigma).

Vegetable oils: sunflower, soybean and rapeseed oils, checked for authenticity; 5 virgin olive oils obtained from SIAL Exhibition, Paris 1998; 10 virgin olive oils obtained from Prague market.

Method for determination of sterols in olive oil: The method can be divided into the following four steps.

1. Preparation of the unsaponifiable fraction. The preparation procedure was identical to the method described previously (BOHAČENKO & KOPICOVÁ 1999). A modified method was used for preparation (AOAC 1990 – No. 976.26; SLOVER *et al*. 1983).

Four ml of 60% aqueous solution of KOH and 20 ml of the alcohol solution (ethanol:methanol:isopropyl alcohol = 90:5:5) were added to 0.5 g of the oil to be tested. Dihydrocholesterol (0.2 ml) was added as the internal standard (1mg/1 ml of hexane). After one hour of mild boiling under the reflux condenser, the mixture was cooled down, and the condenser was rinsed with 30 ml of alcohol solution. The mixture was extracted using 50 ml of hexane with the

addition of 100 ml of 0.1N aqueous solution of KOH. The aqueous part was separated and mixed with another 30 ml of hexane. The hexane parts were associated together, rinsed with distilled water until the neutral pH was reached, and filtered through the Na_2SO_4 layer on filter paper. Na_2SO_4 was rinsed again with 5 ml of hexane. The solvent fraction was evaporated using a rotary vacuum evaporator with the water bath adjusted to 50–55°C and the rest was removed with a mild nitrogen stream.

2. Purification of the unsaponifiable fraction by preparative LC. The method introduced by EISNER *et al.* (1963) was implemented and modified. Silica gel was used as sorbent instead of Florisil.

The evaporation residue after the saponification was dissolved in approximately 2.5 ml of hexane. A dosing loop was used to apply 2 ml of this sample to the column. The elution was carried out using the gradient method with three mixtures of hexane and diethyl ether.

Working conditions: Glass column packed with regenerated silica, length of column 535 mm, diameter 12.5 mm.

Purification of silica gel: 1 hour extraction on Soxhlet was carried out using the mixture of chloroform and methanol (1:1). Silica gel was activated prior to the first use overnight at 120°C. One silica gel packing could be used 15 to 20 times.

Flow: 1ml per minute, pressure: 0.3–3 MPa, gradient elution: 0–80 ml (hexane:diethyl ether = 4:1); 80–200ml (hexane:diethyl ether = 7:3), 200–400 ml (hexane:diethyl ether = 1:1).

The last fraction of 200–400 ml, containing the pre-purified sterols, was evaporated in the rotary vacuum evaporator with the water bath adjusted to 50–55°C and dried with mild nitrogen stream.

3. Silanization of sterols. The silanization was carried out pursuant to the AOAC No. 976/26 (1990) methodology. A total of 0.2 to 0.3 ml of the silanizing agent (pyridine:HMDS:TMCS = 9 : 3 : 1) was added to the evaporation residue and after 15 min of maintaining at laboratory temperature the sample was ready to be used for the determination of sterols using the GC.

When analyzing the sterol standards, 0.4 ml of the mixed sample (0.5 mg dihydrocholesterol and 0.5 mg of ß-sitosterol practical in 1 ml of hexane) was dried in the nitrogen stream, and 0.5 ml of the silanization agent was added to the evaporation residual. The next procedures were carried out as described above.

4. Identification of sterol contents by GC. The identification was carried out using the method of capillary GC with a non-polar chromatographic column, DB-5 (5% phenyl methyl silicon) 30 m × 0.25 mm × 0,25 µm.

Conditions: injection – 1µl; detection: FID; $T_{detector}$ = 320°C**;** injector: SPLIT 20:1; $T_{injector}$ = 300°C; carrier-gas: nitrogen; flow 1.25 ml/min; temperature program: constant temperature 270°C.

Dihydrocholesterol content (mg/ml) was calculated from the peak areas of its standard.

The percentage share of each of other sterols was calculated from the ratio of the relevant peak area to the total peak area for sterols.

Detection limit of sterols for each sterol monitored = 0.03 mg/ml sample

Yield of the method, found using the tenfold analysis of an olive oil sample with the constant addition of the internal standard: dihydrocholesterol ranged between 98 and 102%.

Repeatability: expressed as RSDr (%) and calculated from seven parallel determinations of the sample of the virgin olive oil was as follows:

Sterol	RSDr (%)	Percentage level
cholesterol	7.8	0.5–1.0
brassicasterol	3.6	4.0–8.0
campesterol	3.3	3.0–5.0
	1.8	16.0–18.0
stigmasterol	7.9	0.5–1.0
	4.5	16.0–18.0
Δ-7-stigmastenol	14.3	0.1–0.3
	8.1	2.0–5.0

RESULTS AND DISCUSSION

Considering the apparatus equipment of this laboratory, the modified methodology suggested by EU Commision Regulation (see above) was used for sterol determination. Sterols were isolated in the unsaponifiable fraction and the pre-purification of the fraction was carried out using the LC on the silica gel column with a gradient elution with three mixtures of hexane and diethyl ether prior to the determination of silylated derivatives using GC. The retention volume containing the sterol was identified using mixed standards.

The method was verified by analyzing the mixture of standard cholesterol, brassicasterol, β-sitosterol, campesterol and stigmasterol. Our relative retention times (RRT) are in very good conformity with those published by JIMENÉZ and GONZÁLEZ (1996), who used the standard method pursuant to the Commission Regulation (EEC) No. 2568/91 and worked with the same type of column. This finding enabled us to use the published relative RT's of other sterols whose standards are not commercially available in our country and which Jimenéz identified using mass spectrometry. Concerning the Δ-7-stigmastenol, a very important marker for the proof of the adulteration of olive oils (see below), the identification of its peak in the sunflower and soybean oil, was confirmed with mass spectrometry. The mass spectrum (Fig. 1) obtained corresponded with the spectrum published by BIEDERMAN *et al.* (1996).

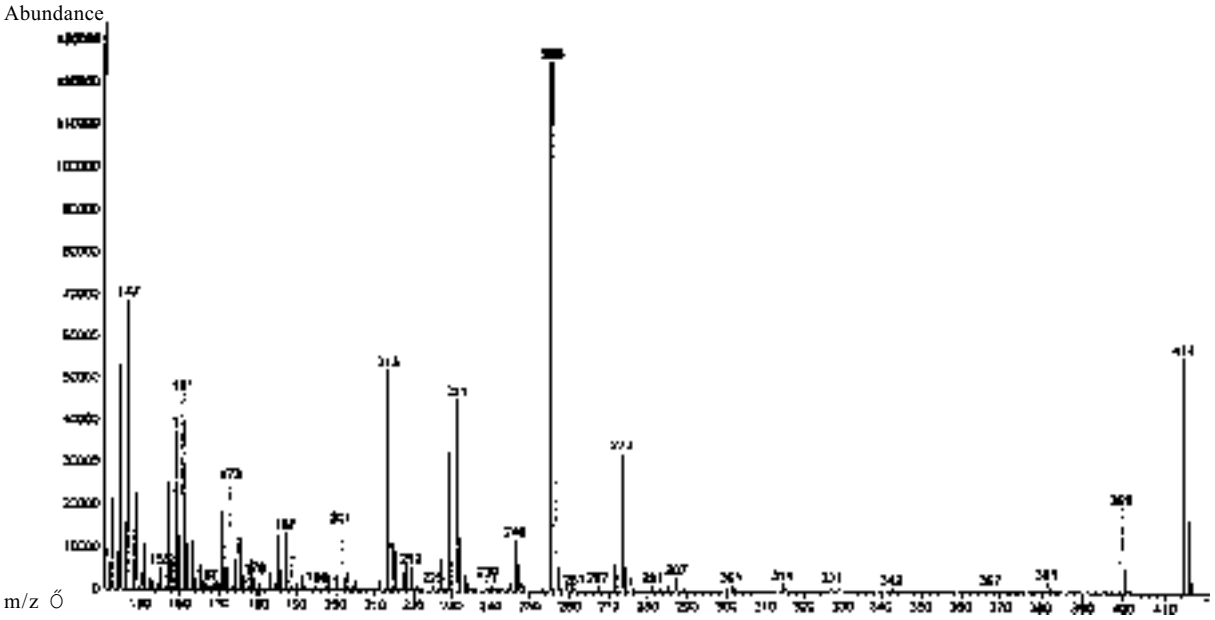


Fig. 1. Mass spectrum of Δ-7-stigmastenol in olive oil

The yield of the method ranged between 98 and 102%. The detection limit for sterols was 0.03 mg/ml of samples. The repeatability expressed as RSDr (%) determination of sterols important for the proof of adulteration has not exceeded 8% with exceptions of Δ-7-stigmastenol where on the percentage level 0.1–0.2 RSDr was 14.3% (see Materials and Methods). The chromatogram of sterol fraction from olive oil (Fig. 2) proves the possibility of good identification of individual sterol peaks.

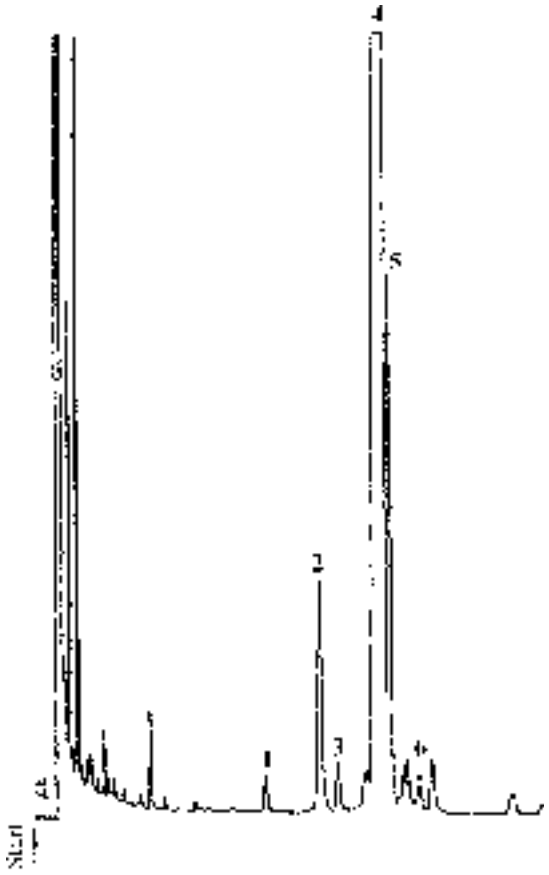
All parameters of the method shown were comparable with other published data, and they met the requirements. Therefore, this method can be used to assess the authenticity of olive oils from the values of the free sterols content included in the Commission Regulation (EEC) No. 2568/91 (Table 1).

The verification of the capability of identifying the adulteration of the olive oil with sunflower, soybean or rape-

Table 1. Limit sterol contents in olive oil (Commision Regulation (EEC) No. 2568/91)

Cholesterol	max. 0.5%
Brassicasterol	max. 0.2%
Campesterol	max 4.0%
Stigmasterol	< campesterol
β-sitosterol*	min. 93%
Δ-7-stigmastenol	max. 0.5%

*sitosterol + Δ-5-avenasterol + Δ-5,23-stigmastadienol + Δ-5,24-stigmastadienol + clerosterol + sitostanol



1 = cholesterol; 2 = campesterol; 3 = stigmasterol; 4 = β-sitosterol; 5 = Δ-5-avenasterol; 6 = Δ-7-stigmastenol

Fig. 2. Gas chromatographic analysis of sterols in olive oil

Table 2. Sterol content (%) in sunflower, soybean and rapeseed oils

	Oil		
	sunflower	soybean	rapeseed
Cholesterol	0.24	0.42	N.D.
Brassicasterol	N.D.	N.D.	9.28
Campesterol	10.29	16.94	34.75
Stigmasterol	7.51	16.64	0.18
β-sitosterol	58.01	54.36	50.28
Δ-5-avenasterol	5.13	3.75	2.53
Δ-5,24-stigmastadienol	1.26	1.19	1.11
Δ-7-stigmastenol	9.72	4.28	0.11
Δ-7-avenasterol	5.54	1.77	0.06
Sum (%)	97.46	98.93	98.3

seed oils was carried out on model samples containing a 5, 10, or 20% addition of these oils to virgin olive oil (sample No. 15 was used, for content of sterols, Table 4). The sterol content in the sunflower, soybean or rapeseed oils added is shown in Table 2. It corresponds well with the data by ITOH *et al*. (1973). The percentage shares of respective sterols in model samples are shown in Table 3.

The most important changes in the content of sterols in increasing additions of sunflower oil were found in Δ-7-stigmastenol and stigmasterol. A 7.6 to 27.6 times, and 2.5 to 6.8 times increase, respectively, was found versus reference olive oil.

The greatest manifestations of changes due to soybean oil additions were found in 4.5 to 13.5 times increase in stigmasterol content; 3.1 to 12.5 times increase of Δ-7-stigmastenol content; and 1.4 to 2.3 times increase of campesterol content. The content of stigmasterol as required under the Commission Regulation, should be lower than the content of campesterol, whose limit is 4% (Table 1). For this reason it was not used as a marker sterol although changes in its content were high, in particular when soybean oil was added. In addition, its content in our olive oils samples was relatively low, i.e. 0.5 to 1.93% (Table 4), and did not exceed the permissible value of campesterol even after the addition of 20% of the sunflower or soybean oil.

In this case, Δ-7-stigmastenol and campesterol can be used to prove an adulteration of olive oils with sunflower or soybean oils, since their increased content in comparison with the limits set forth by the Commission was manifested as of a 5 to 10% addition. Concerning an adulteration with rapeseed oil, the presence of brassicasterol is well detectable as of a 5% addition. Our conclusions correspond well with data recommended to identify this and other methods of adulteration (GROB *et al*. 1994a; ALONSO 1997).

This method, which employs the determination of the content of free sterols, cannot decide in practical terms whether the soybean or sunflower oil was used for an adulteration up to the addition of 20 %. To distinguish among the two oils, GROB *et al*. (1994b) suggested other supportive markers, such as elevated content of γ-tocoferol or linolenic acid for soybean oil additions. Our results also support this conclusion since we have detected a 0.6 to 0.8% content of linolenic acid in olive oils while its content never falls under 6% in the soybean oil.

Finally, the method was used to analyzing 10 samples of virgin olive oils of Italian and Spanish provenance purchased in a Prague market, and 5 samples of olive oils obtained from the food exhibition SIAL (Paris). It is evident from the results shown in Table 4 that levels of campesterol and Δ-7-stigmastenol have not exceeded the limits recommended by the EU commission in any of the

Table 3. Sterol content (%) in model samples of olive oil with addition of other plant oils

	Addition (%) of oil								
	sunflower			soybean			rapeseed		
	5	10	20	5	10	20	5	10	20
Cholesterol	0.35	0.33	0.30	0.35	0.40	0.43	N.D.	N.D.	N.D.
Brassicasterol	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.46	0.93	1.86
Campesterol	4.56	5.24	6.32	5.33	6.58	8.92	5.38	6.92	11.25
Stigmasterol	1.31	2.15	3.63	2.40	4.23	7.14	0.51	0.50	0.45
β-sitosterol	70.72	68.74	63.46	70.28	69.71	64.12	71.41	70.30	67.18
Δ-5-avenasterol	20.54	18.27	16.41	19.69	17.22	15.58	21.30	20.31	17.55
Δ-5,24-stigmastadienol	0.34	0.56	1.07	0.37	0.50	1.01	0.34	0.38	0.49
Δ-7-stigmastenol	1.07	2.21	3.87	0.44	0.93	1.75	0.14	0.14	0.13
Δ-7-avenasterol	0.73	1.41	2.65	0.35	0.50	1.00	0.22	0.21	0.19
Sum (%)	99.62	98.91	97.71	99.21	100.07	99.95	99.76	99.69	99.10

Table 4. Results of sterol analysis of olive oils

	Sterol content (%) in sample No.														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Cholesterol	0.36	0.40	0.57	0.20	0.15	0.56	0.85	0.53	0.27	0.73	0.50	0.37	0.41	0.37	0.35
Campesterol	3.44	3.45	3.42	3.27	3.39	3.45	3.21	3.38	3.64	3.72	4.04	3.57	4.05	3.38	3.83
Stigmasterol	0.74	0.67	1.31	1.89	1.93	0.86	1.02	0.83	0.44	0.79	0.78	0.94	0.50	1.44	0.53
β-sitosterol	81.68	86.81	85.63	87.17	86.53	78.78	79.31	81.62	84.46	80.07	85.72	80.98	70.81	84.88	72.52
Δ-5-avenasterol	11.84	6.67	5.99	3.35	3.88	14.52	13.14	11.68	9.12	12.81	7.11	10.78	22.20	8.22	21.94
Δ-5,24-stigma-stadienol	0.57	0.42	0.84	1.13	1.07	0.60	0.86	0.64	0.58	0.61	0.64	0.70	0.52	0.82	0.30
Δ-7-stigmastenol	0.22	0.18	0.18	0.53	0.52	0.13	0.17	0.14	0.23	0.14	0.36	0.50	0.14	0.30	0.14
Δ-7-avenasterol	0.46	0.40	0.45	0.63	0.61	0.33	0.30	0.36	0.37	0.45	0.23	0.61	0.23	0.27	0.23
Sum (%)	99.31	99.00	98.39	98.17	98.08	99.23	98.86	99.18	99.11	99.32	99.38	98.45	98.86	99.68	99.84

Brassicasterol wasn't identificated; samples No. 1–10 from local market; samples No. 11–15 from SIAL exhibition-Paris

inspected samples. Also, the stigmasterol to campesterol ratio and the content of β-sitosterol comply with the EU directive. Only samples 7 and 10 showed a higher level of cholesterol (0.85% or 0.73%, respectively). The maximum acceptable content of cholesterol is 0.5%. Since the two samples complied with the limits for the most important sterols we have suggested also that both samples are non-adulterated.

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Souhrn

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Pro spolehlivý průkaz autenticity olivových olejů byl použit obsah (procentní zastoupení) vybraných sterolů, který je deklarován nařízením Komise EU. Při implementaci analytické metody na stanovení sterolů pomocí GC byl k oddělení nežádoucích interferujících látek v nezmýdelnitelné frakci modifikován postup využívající preparativní LC s kolonou naplněnou silikagelem a s gradientovou elucí třemi směsmi hexanu a diethyletheru. Dále bylo modelovými pokusy ověřeno, že touto metodou lze identifikovat falšování olivového oleje přídavky oleje slunečnicového, sójového nebo řepkového, a to na základě stanovení obsahu Δ-7-stigmastenolu (přídavek slunečnicového oleje), campesterolu (přídavek sójového oleje), resp. brassicasterolu (přídavek řepkového oleje). Zvýšení obsahu těchto markerových sterolů proti povoleným hodnotám umožňuje rozeznat přídavek již 5–10 % olejů. Touto metodou byla též hodnocena autenticita čtyř olivových olejů z výstavy SIAL a deseti panenských olivových olejů z pražské tržní sítě a bylo zjištěno, že žádný z těchto olejů nevykazoval prvky falšování.

Klíčová slova: olivový olej; autenticita; falšování; steroly; slunečnicový olej; sójový olej; řepkový olej; kapalinová chromatografie; plynová chromatografie; stanovení sterolů

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