CHROM. 24 299

Determination of sterols, erythrodiol, uvaol and alkanols in olive oils using combined solid-phase extraction, high-performance liquid chromatographic and high-resolution gas chromatographic techniques

Mauro Amelio, Renzo Rizzo and Flavio Varazini

Fratelli Carli SpA, Via Garessio 11/13, 18100 Imperia (Italy)

ABSTRACT

A method is described for the determination of the sterol, erythrodiol, uvaol and alkanol content in olive oils by means of solid-phase extraction and high-performance liquid chromatography, instead of liquid–liquid and thin-layer chromatographic separations, the following step being high-resolution gas chromatographic separation. This type of procedure allows the simultaneous analysis of a larger number of samples and a substantial reduction in manual operations. Comparisons were made between the two methods on 100 different olive oils and with a statistical analysis of the results (Student's t-test).

INTRODUCTION

The sterol, erythrodiol, uvaol and alkanol contents are very important for the investigation of the quality of olive oil. The recent EEC Regulation No. 2568/91 requires analyses for this type of investigation and fixes the physico-chemical characteristics of the product. Sterols, erythrodiol, uvaol and alkanols are present in the unsaponifiable fraction and are separated from it by suitable techniques. Unfortunately, they are very time consuming and require substantial manual operations, which limits the number of samples that can be analysed daily.

Several papers have described alternative methods to replace the official methods. In particular, solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) have been proposed for the determination of both free and combined sterols and for the total sterols in unsaponifiable matter.

The use of SPE to separate sterols from biological matter has been applied for a long time [1]. In contrast, it is not very common in fat analysis and most official methods do not take into account this type of separation.

Worthington and Hitchcock [2] used semi-preparative HPLC to separate free and combined sterols from seed oils. The collected fractions were then separated and analysed by means of thin-layer (TLC) and gas chromatography (GC). Horstmann and Montag [3] suggested the use of silica gel cartridges and different eluents of increasing polarity to obtain sterols from fats or unsaponifiable matter. They achieved a better separation by HPLC (direct phase) and discussed some instrumental problems. Schuster [4] suggested a multi-component analysis (triglycerides, hydroperoxides, sterols and vitamins) by means of HPLC with diode-array detection.

More recently, Grob *et al.* [5] used an "on-line" LC–GC system to determine the sterol (free and

---

Correspondence to: Dr. Mauro Amelio, Fratelli Carli SpA, Via Garessio 11/13, 18100 Imperia, Italy.

combined), alkanol and wax contents in olive oil. However, the quality control routine requires sterol and alkanol contents to be determined in the unsaponifiable matter. In this case, they must be separated from the soap solution. This separation is normally performed by means of a liquid–liquid extraction.

Cortesi and co-workers [6,7] used HPLC to separate sterols, alkanols, erythrodiol and uvaol in the unsaponifiable matter from olive oil. Further, they investigated the possibility of determining these analytes directly by HPLC.

In order to collect a suitable amount of samples from HPLC for further analyses and achieve good separations, Iatride et al. [8] chose repeated cycles of separation of unsaponifiable from rapeseed oil, using an analytical column, automated injection and a fraction collector. Further, sterols were well separated from triterpenic alcohols. Holen [9] optimized the isolation and identification by reversed-phase HPLC of eight sterols from rapeseed oil and mayonnaise. Mordret et al. [10] compared separations of unsaponifiable matter carried out by means of TLC and HPLC. They obtained comparable results and investigated the advantages of the HPLC method. Homberg [11] carried out rapid separations of sterols and other non-polar compounds in the unsaponifiable matter from the soap solution using aluminia columns. Perrin and Raoux [12] investigated the effects of the mobile phase and temperature on the reversed-phase HPLC separation of unsaponifiable matter. They achieved good separations of sterols from different oils.

Nowadays disposable SPE cartridges are available that absorb large amounts of solutions that allow liquid–liquid extractions to be replaced. For this reason, we used SPE cartridges (3 ml) only for the clean-up of the unsaponifiable solutions. We tried to develop a single procedure that would combine the advantages of SPE, HPLC and high-resolution GC (HRGC) to allow us to perform the largest possible number of analyses daily. Further, if the laboratory is equipped with an automatic HPLC injector and collector, it is possible to perform the separation and collection automatically. Each component is then determined using HRGC, as it is when using the conventional method described in the NGD collection [13]. The aim of this work was to verify that the suggested method gives

results that are not significantly different from those given by the NGD method. Comparisons were made on numerous different olive oils: crude olive oil (45), extravirgin oil (30), refined oil (20) and crude pomace olive oil (5).

EXPERIMENTAL

Apparatus and materials

The extraction columns used were a Bakerbond SPE, quaternary amine (N^+) No. 7091-03 (3 ml) (40 μm average particle diameter, 60 Å) and a Varian Chem Elut 2050 (50 ml). Suitable flasks for the saponification of about 2 g of olive oil were used.

For HPLC separations the following were used: gradient pump, LDC Analytical CM 4000; UV detector, Milton-Roy SpectroMonitor 3100; column, Supelcosil LC-Si, 15 cm × 4.6 mm I.D., 5 μm (Supelco); flow-rate, 1.00 ml/min; detection wavelength, 210 nm; range, 0.10 a.u.f.s.; response time, 0.10 s; loop, 10 μl; chart speed, 0.5 cm/min; and elution gradient (Fig. 1) with *n*-hexane–diethyl ether, 0 min 80:20, 15–20 min 60:40, 20.1–30 min 80:20 and 30 min ready for next run.

For HRGC separations, the following were used: gas chromatograph, Carlo Erba Mega Series HRGC 5300; capillary column, SPB-5 (5% diphenyl–94% dimethyl–1% vinylpolysiloxane), fused silica, 30 m × 0.25 mm I.D., 0.25 μm film thickness (Supelco) oven temperature, for sterols isothermal at 263°C, for alkanols programmed from 180°C (5 min) to 260°C (25 min) at 5°C/min; injector temperature, 290°C; detector (flame ionization) temper-
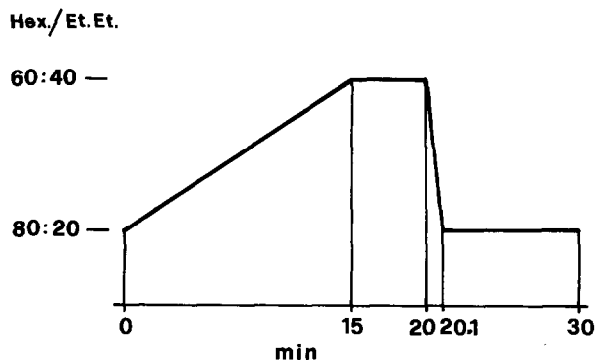
Hex./Et.Et.

Fig. 1. Gradient profile for the HPLC separation. Hex./Et.Et. = *n*-Hexane–diethyl ether.

ature, 300°C; carrier gas, hydrogen at 4.5 ml/min; split ratio, 2.8; and injection volume, 1–2 μl.

Computing was performed using Maxima 820 software (Water Dynamic Solutions, Millipore) installed on an IBM PS2/H21 personal computer.

Reagents

Analytical reagent-grade chemicals were used unless indicated otherwise.

Diethyl ether, methanol, chloroform, pyridine, anhydrous sodium sulphate and potassium hydroxide were obtained from Fluka. *n*-Hexane (HPLC grade) and diethyl ether (HPLC grade) from Fluka were used for HPLC separations. Trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) were supplied by Supelco.

The saponification solution [20% (w/v) potassium hydroxide in methanol] was prepared by dissolving potassium hydroxide (40 g) in distilled water (24 ml) and diluting 200 ml with methanol in a volumetric flask.

Internal standard solutions were 2 mg/ml 5α-cholestan-3β-ol (cholestanol) (>99%, Fluka) in chloroform and 1 mg/ml 1-eicosanol (C_{20}) (>98%, Fluka) in chloroform.

The derivatization reagent was pyridine–hexamethyldisilazane (HMDS)–trimethylchlorosilane (TMCS) (9:3:1).

Procedure

As regards the NGD methods, we refer to the NGD method collection for sterols, method No. C71-1989 and C72-1989; alkanols, C75-1989 and C76-1989; and erythrodiol and uvaol, C52-1985.

The method tested was the following. Add 200 μl of each internal standard solution to the saponification flask (if pomace oil has to be tested, add 600 μl) and evaporate the solvent. Weight exactly a sample of about 2.000 g, add 5 ml of saponification solution, fit the condenser and boil over a water-bath or another suitable device for 25 min. Add 15 ml of distilled water, pour the hot solution into the Chem Elut 2050 column, wait for about 15 min, add 50 ml of diethyl ether, wait for about 15 min, add 20–25 ml of diethyl ether and collect the solution in a 50-ml flask containing about 1 g of anhydrous sodium sulphate. Purify the solution by passing it through a Bakerbond quaternary amine (N^+) column, already "conditioned" with 3 ml of diethyl ether. Col-

lect the solution in a 50-ml flask containing about 1 g of anhydrous sodium sulphate. Filter the solution through paper into a small flask, evaporate the solvent, weight the unsaponifiable matter and dissolve it in chloroform to give a 10% solution. Inject the solution into the HPLC system and collect the fractions which are of interest, evaporate the solvent, derivatize and inject into the HRGC system.

RESULTS AND DISCUSSION

Several methods for performing the saponification and separation of unsaponifiable material have been suggested, as surveyed by Homberg [11]. However, saponification is usually carried out using a suitable alkali solution.

We preferred not to change the method, but to choose the saponification conditions in order to minimize the solution volumes and to decrease the reaction time. For this reason, the concentration of the KOH solution was increased and, consequently, the reaction time was investigated.

First, we waited only 1 min after the disappearance of the phases (10 min in all). This period is too short and erythrodiol and uvaol were wrongly determined. To solve this problem, four tests using 10, 20, 40, 60 min were carried out and the results were compared with the results obtained with the conventional method (NGD). Table I shows that at least 20 min are needed to determine the erythrodiol and uvaol contents exactly.

Homberg [11] replaced the liquid–liquid extraction with a separation on a laboratory-filled alumina column. For the reasons mentioned earlier, we used the "ready-for-use" Chem Elut 2050 column.

TABLE I

RECOVERY OF ERYTHRODIOL + UVAOL WITH DIFFERENT SAPONIFICATION TIMES

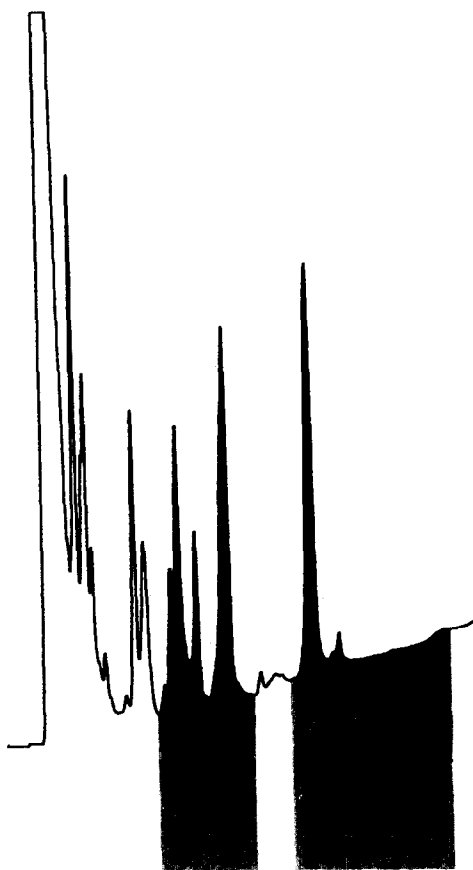| Saponification time (min)	Erythrodiol + uvaol (%)[a]
10	1.9
20	2.8
40	2.7
60	2.8

[a] Value obtained using NGD = 2.8%.

Fig. 2. HPLC of extra-virgin olive oil unsaponifiable and collected fractions. A = Fraction containing alkanols (6 and 9 min); S = fraction containing sterols (10 and 18 min).
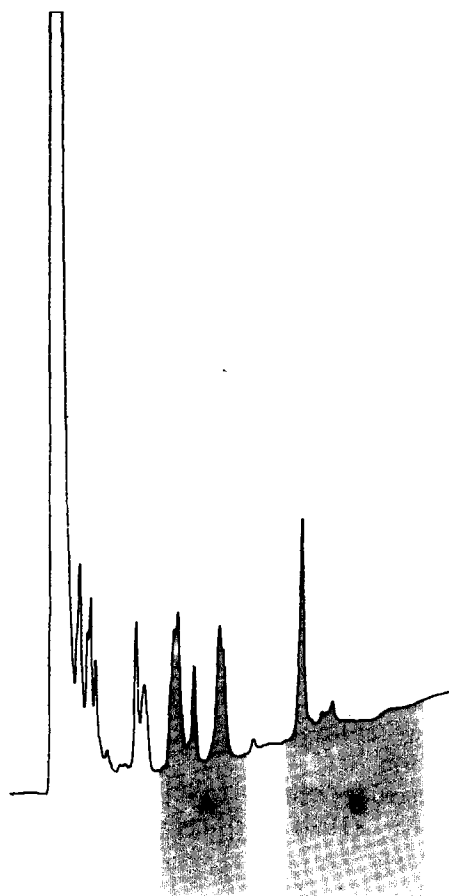

Fig. 3. HPLC of crude olive oil unsaponifiable and collected fractions. A and S as in Fig. 2.

This type of highly efficient, disposable column is made for rapid and easy sample preparation for HPLC or HRGC analyses. It allowed emulsions and several manual operations to be avoided. This is why it is possible to process numerous samples at the same time.

After saponification, it is necessary to add water and to pour the hot solution into the Chem Elut 2050 column at once, in order to facilitate the solid-phase adsorption. It is advisable to wait for about 15 min, instead of 3–5 min as suggested in the instructions, because of the high soap concentration.

The collected solution contains water, which is partially absorbed by sodium sulphate, and a small amount of soaps that has to be removed as it could interfere in the following steps. In order to do this,

the solution is passed through the Bakerbond quaternary amine (N^+) column, which removes all the remaining soaps (clean-up). A small amount of water is again separated and absorbed by sodium sulphate.

The subsequent HPLC separation is carried out in a way similar to that described by Cortesi and co-workers [6,7]. We chose gradient elution to achieve complete separation of the less polar compounds from alkanols and sterols and, at the same time, to keep the analysis time short.

The collection of sterols and alkanols is very easy; the fractions which have to be collected are clearly shown in Figs. 2–5. These fractions give sufficient analytes for the subsequent HRGC determinations. Hence it is not necessary to perform repeat-
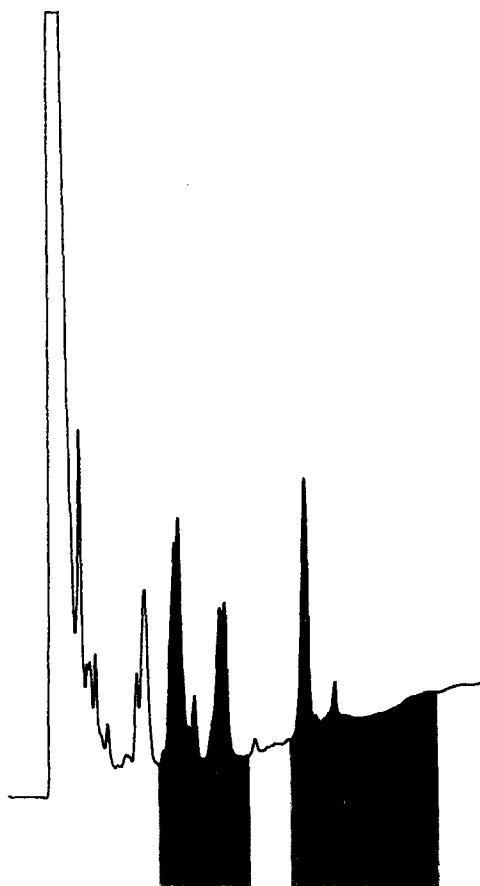
Fig. 4. HPLC of refined olive oil unsaponifiable and collected fractions. A and S as in Fig. 2.
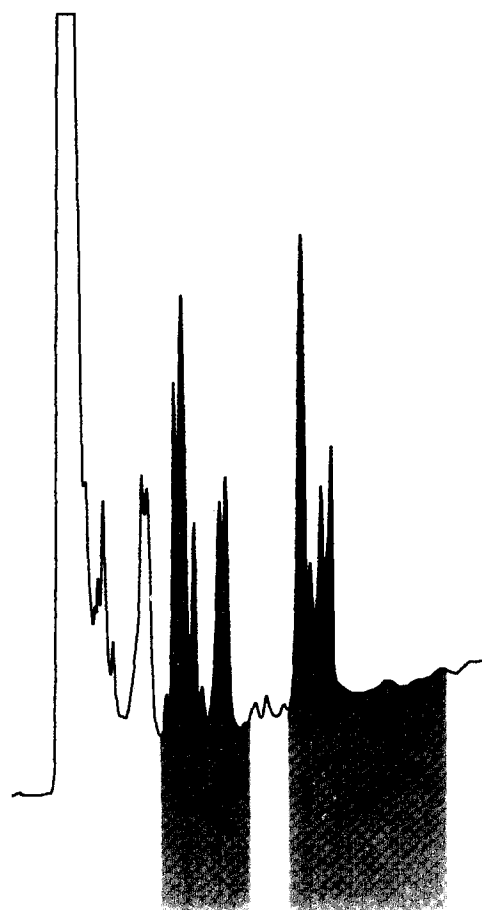

Fig. 5. HPLC of crude pomace oil unsaponifiable and collected fractions. A and S as in Fig. 2.

ed cycles of injections as was done by Iatrides et al. [8]. This allows us to choose a gradient profile that gives a good separation of unsaponifiable material (useful, for instance, for future applications) instead of very fast, but worse, separations. The resolution achieved by several workers [9,10,12] is not necessary here because the quantification is to be performed by HRGC. In our case, alkanols are eluted between about 6 and 9 min and sterols and erythrodiol + uvaol between 10 and 18 min.

Each fraction is then evaporated and the solutes are derivatized with a suitable reagent and injected for HRGC separation.

CONCLUSION

As mentioned above, official methods are very time consuming, but a faster method can be chosen only if it gives results that are not significatively different from those obtained using the official methods.

Tables II and III compare the mean values and the standard deviations of the results obtained by the two methods. In order to verify whether the differences between the results are random or not, Student's t-test was applied to check the H_O hypothesis, i.e., random differences [17,18]. For a 95% confidence level and $N - 1 = 99$ degrees of freedom, the t-value is 1.984. The t values calculated from the

TABLE II

STATISTIC ANALYSIS OF STEROL DATA (STUDENT'S t-TEST)

Method	Parameter	Compound[a]													
		a	b	c	d	e	f	g	h	i	l	m	n	o	p
Conventional (NGD method)	Mean (%)	0.328	0.117	3.630	1.769	0.090	0.855	80.391	0.817	10.057	0.658	0.299	0.465	3.020	1576.530
	S.D. (%)	0.131	0.619	2.724	1.047	0.226	0.185	5.820	0.486	5.991	0.331	0.154	0.137	2.221	928.450
This work	Mean (%)	0.375	0.123	3.491	1.761	0.074	0.818	80.455	0.835	9.992	0.704	0.299	0.462	3.015	1580.950
	S.D. (%)	0.210	0.620	2.571	0.989	0.218	0.151	5.837	0.455	5.876	0.335	0.180	0.145	2.153	919.588
	t	-1.888	-0.068	0.369	0.055	0.506	1.540	-0.077	-0.268	0.077	-0.972	0.000	0.149	0.016	-0.033

[a] a = Cholesterol; b = brassicasterol; c = campesterol; d = stigmasterol; e = $\Delta^{5,23}$-stigmastadienol; f = clerosterol; g = β-sitosterol; h = sitostanol; i = Δ^5-avenasterol; l = $\Delta^{5,24}$-stigmastadienol; m = Δ^7-stigmastenol; n = Δ^7-avenasterol; o = erythrodiol + uvaol; p = total sterols (mg/kg).

TABLE III

STATISTICAL ANALYSIS OF ALKANOL DATA (STUDENT'S t-TEST)

Method	Parameter	Alkanol[a]							
		a	b	c	d	e	f	g	h
Conventional (NGD method)	Mean (mg/kg)	83.695	7.190	142.187	9.362	130.476	6.992	50.584	429.456
	S.D. (mg/kg)	187.982	12.896	269.367	15.099	214.025	10.581	75.458	780.711
This work	Mean (mg/kg)	82.691	7.183	137.675	9.183	128.109	6.251	51.296	422.313
	S.D. (mg/kg)	175.717	11.945	249.974	13.236	198.039	8.943	65.204	718.363
	t	0.038	0.003	0.121	0.088	0.080	-0.184	-0.070	0.066

[a] a = 1-Docosanol (C_{22}); b = 1-tricosanol (C_{23}); c = 1-tetracosanol (C_{24}); d = 1-pentacosanol (C_{25}); e = 1-hexacosanol (C_{26}); f = 1-heptacosanol (C_{27}); g = 1-octacosanol (C_{28}); h = total alkanols.
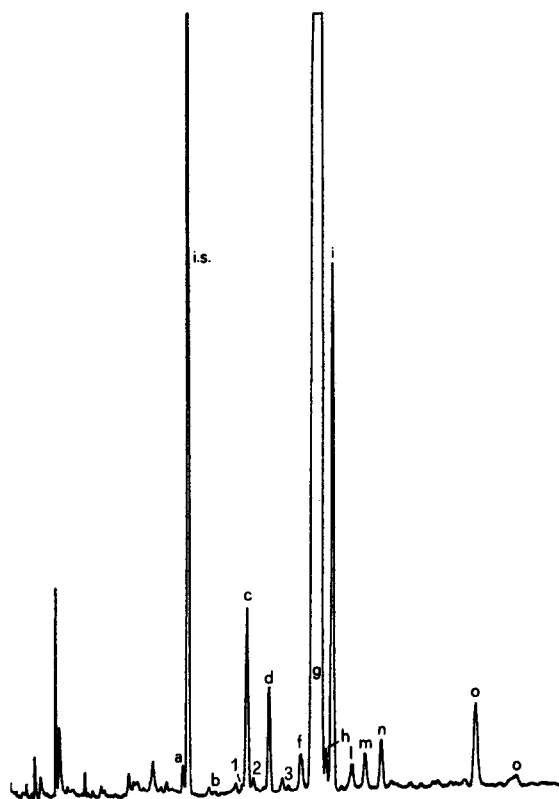
Fig. 6. Gas chromatogram showing olive oil sterol, erythrodiol and uvaol composition. Peaks: 1 = 24-methylenecholesterol; [retention time (t_R) = 10.6 min]; 2 = campestanol (t_R = 11.0 min); 3 = Δ^7-campesterol (t_R = 12.2 min); i.s. = internal standard (cholestanol) (t_R = 8.8 min). See Table II for the identification of the other sterols.

experimental data are less than 1.984 for each component; this means that H_0 is always true (random differences).

The statistical analysis does not take into account 24-methylenecholesterol, campestanol and Δ^7-campesterol (Fig. 6) because, as is known [14,15], severe interferences can occur which make it difficult to determine these sterols. Further, we have never observed interference at the retention time of Δ^7-stigmastenol, due to 24-methylenecycloartanol not being well separated by TLC [16]. The examination of the t values shows $\mid t \mid$ = 1.888 for cholesterol, which is quite close to 1.984.

We suspect that sometimes interferences occurred during the cartridge separation of the unsaponifiable matter from the soap solution. In these cases we "washed" the cartridge using diethyl ether and dried it at room temperature before use.

With regard to the erytrodiol, uvaol and alkanols, the examination of the t values shows good agreement between the two methods.

Overall we believe that the tested method gives the same results as the NGD method and can replace it, especially when many samples have to be analysed daily. On the other hand, we think the method could be improved; for instance, a more suitable elution solvent for the Chem Elut 2050 column is desirable, and the HPLC separations could be performed more efficiently with a significant decrease in the sampling time.

ACKNOWLEDGEMENTS

REFERENCES

1 Application Notes SS-AN020, 1982, PH-009 and PH-010, 1989, J. T. Baker Chemicals, Phillipsburgh, NJ.
2 R. E. Worthington and H. L. Hitchcock, J. Am. Oil Chem. Soc., 61 (1984) 1085.
3 P. Horstmann and A. Montag, Fette Seifen Anstrichm., 88 (1986) 262.
4 R. Schuster, HPLC Application Note No. 12-5954-6269, Hewlett-Packard, Avondale, PA, 1987.
5 K. Grob, M. Lanfranchi and C. Mariani, J. Am. Oil Chem. Soc., 67 (1990) 626.
6 N. Cortesi, E. Fedeli, A. Gasparoli and E. Tiscornia, Riv. Ital. Sostanze Grasse, 54 (1977) 16.
7 N. Cortesi, E. Fedeli and E. Tiscornia, Riv. Ital. Sostanze Grasse, 55 (1978) 168.
8 M. C. Iatrides, J. Artaud and M. Derbesy, Analusis, 12 (1984) 205.
9 B. Holen, J. Am. Oil Chem. Soc., 62 (1985) 1344.
10 F. Mordret, H. Ajana and C. Gauchet, Rev. Fr. Corps Gras, 32 (1985) 305.
11 E. Homberg, Fat Sci. Technol., 89 (1987) 215.
12 J. L. Perrin and R. Raoux, Rev. Fr. Corps Gras, 35 (1988) 329.
13 NGD, Norme Grassi e Derivati, Stazione Sperimentale per le Industrie degli Oli e dei Grassi, Milan, 1976, and subsequent revisions.
14 G. Morchio, G. Amelotti, A. Bocca, V. Bocio, L. Conte, O. Cozzoli, L. Cremonesi, R. Fascioli, L. Giro, D. Grieco, G. Lercker, C. Mariani, G. Pierattine, E. Sarti and P. Zunin, Riv. Ital. Sostanze Grasse, 66 (1989) 531.
15 G. Amelotti, Riv. Ital. Sostanze Grasse, 62 (1985) 337.
16 G. Amelotti, A. Griffini, M. Bergna and P. Montorfano, Riv. Ital. Sostanze Grasse, 62 (1985) 459.
17 G. Bussetti, Esercitazioni Pratiche di Fisica, Univ. Levrotto e Bella, Turin, 1980, p. 669.
18 M. R. Spiegel, Statistica, ETAS Libri, Milan, 1982, p. 357.