Journal of Chromatography, 471 (1989) 397–405
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 294

DETERMINATION OF FREE AND ESTERIFIED STEROLS AND OF WAX ESTERS IN OILS AND FATS BY COUPLED LIQUID CHROMATOGRAPHY–GAS CHROMATOGRAPHY

KONRAD GROB* and MAURO LANFRANCHI
Kantonales Labor, P.O. Box, CH-8030 Zürich (Switzerland)
and
CARLO MARIANI
Via Scheiwiller 1, I-20139 Milan (Italy)

SUMMARY

A liquid chromatographic–gas chromatographic (LC–GC) method is described for the rapid determination of free sterols, esterified sterols and wax esters in oils and fats in a single GC run, circumventing saponification and any off-line preseparation. The free sterols are esterified with pivalic acid in the oil. The diluted oil is injected into the LC system and the fraction containing the above classes of compounds is transferred to the gas chromatograph (carried out by a fully automated LC–GC instrument). The information obtained with this analysis allows a better identification of oils and fats and a better characterization of the treatment of an oil (*e.g.*, distinguishing between cold-pressed and extraction oils and between different refining methods).

INTRODUCTION

Small-bore liquid chromatographic columns with silica

There is a new field, namely coupled liquid chromatography–gas chromatography (LC–GC), making wide use of the small-bore LC columns initiated by Scott and Kucera in 1976[1] and subsequently reviewed[2,3]. In addition to other arguments brought forward by users of small-bore LC columns, in coupled LC–GC there is a strong interest in keeping the LC fraction volumes small in order to facilitate transfer, to shorten solvent evaporation times and to keep the peaks due to solvent impurities as small as possible. Column diameters down to 0.25 mm have been used[4]. Many applications, however, call for larger column diameters to provide the capacity required by the sample (*e.g.*, in the application considered below), and 2 mm I.D. LC columns have proved to be well suited. In addition to the small-bore LC columns, coupled LC–GC has profitted from the work on silica gel carried out by Scott and Traiman[5]. As in sample preparation for conventional LC, normal-phase chromatography is far more important when using LC for sample preparation than for the final analytical determinations.

0021-9673/89/$03.50 © 1989 Elsevier Science Publishers B.V.

Sterol determination

As the fatty acid composition of fats and oils often does not allow one to distinguish between different oils or different qualities of a given oil, trace components are determined in order to obtain the information required. The most important groups of these compounds are the sterols and the triterpene alcohols. Their presence and concentrations vary over a far wider range than those of the fatty acids.

The sterol fraction is analysed for the identification of a fat or an oil (*e.g.*, to distinguish between sunflower oil and other oils of similar fatty acid composition[6,7]), for the detection of the addition of non-declared cheap oils to more expensive oils (*e.g.*, rapeseed oil in olive oil) or to distinguish between different qualities of the same oil (*e.g.*, between virgin, *i.e.*, cold-pressed, and extraction olive oils[8], or oils subjected to different treatments[9]). Wax ester contents are measured during the raffination of certain oils[10,11], but also serve to distinguish between virgin and extraction olive oil[12]. For the latter analysis an LC–GC method has been described[13].

Considerable efforts have been made to develop rapid methods for the analysis of the sterol fraction. Nearly all methods start with the saponification of the triglycerides. The unsaponifiable matter is extracted and analysed directly (with considerable problems concerning interfering peaks) or after preseparation, classically carried out by thin-layer chromatography[14–17]. Thin-layer chromatography can be replaced with chromatography on silica gel columns[18,19] or by off-line high-performance liquid chromatography (LC)[20]. Finally, adsorption on aluminium oxide has been used to circumvent the often tedious extraction of the saponified fat[21].

We propose here a fundamentally new method, greatly reducing manual operations and providing more information at the same time. Saponification of the triglycerides is avoided; instead, the triglycerides are removed by LC. Simultaneously, interfering components are removed. Previous esterification of the free sterols (and other alcohols) with pivalic acid allows the determination of the free and the esterified sterols and of the wax esters within the same GC run. On-line coupling of LC to GC allows full automation of the analysis except for the derivatization carried out in the fat or oil.

CONCEPT OF THE METHOD

When simultaneously determining several components by coupled LC–GC, the major problem in LC concerns not separating these components at the maximum separation efficiency in order to obtain a narrow fraction containing the components of interest. This is often a problem. For instance, when determining sterols in an unsaponified fat, both free and esterified sterols should be isolated from the triglycerides and transferred to the GC system. However, free sterols elute from LC columns far removed from the sterol esters. Esterified sterols elute from silica gel columns nearly together with the wax esters. Hence, all three classes of compounds of interest, the free and esterified sterols and the wax esters, can be transferred to the GC system within the same fraction if we succeed in derivatizing the free sterols in such a way that their LC retention time corresponds to the esterified sterols and the wax esters.

Esterified sterols are eluted from apolar GC capillary columns at *ca.* 350°C, clearly separated from the wax esters. As they are split into several peaks (each sterol can be esterified with all the fatty acids available) and their quantitation is based on

the summed peak areas, this separation from the wax esters is important. The derivatized free sterols form a single GC peak for each sterol and should again be positioned such that no other peaks (wax ester peaks) interfere. To keep the maximum analysis temperature at an acceptable level, they should elute before the esterified sterols. As there is not sufficient "space" in the chromatogram between the wax esters and the sterol esters, this necessitates elution even before the wax esters.

Esterification of the free sterols with a short-chain acid (*e.g.*, acetylation) causes these components to be eluted from the GC capillary column well before the wax esters. However, their polarity is so high that in LC they are eluted excessively far behind the esterified sterols and wax esters. On elongating the chain length of the acid used for esterification, the situation improves in LC, but shifts the corresponding GC peaks into the zone where the wax ester peaks are located. To solve this problem, the free sterols were esterified with pivalic acid. The bulky apolar part of this 2,2-dimethylpropanoic acid protects the polar ester group, reducing the LC retention time. The molecular weight is still low enough to keep the GC retention time clearly below that of the wax esters.

EXPERIMENTAL

Derivatization of the free sterols

Sterols were derivatized with pivalic anhydride, primarily because pivalic chloride produces a strong odour. The conditions required for the derivatization were investigated using the following experiment. Sitosterol was added to an olive oil to increase its concentration to 1%. To 100 μl of this oil, 10–50 μl of pivalic anhydride were added. This mixture was heated at various temperatures for 15 min. Then the oil was diluted 1:100 with *n*-heptane and 0.3 μl of the mixture was injected on-column on to a 17 m × 0.27 mm I.D. glass capillary column coated with SE-52 of 0.15-μm film thickness. Free sitosterol eluted clearly before the corresponding pivalate ester, allowing a rapid determination of the esterification yield.

In a first step, it was found that the addition of pyridine (10 μl) to the reaction mixture did not accelerate esterification. Nevertheless, pyridine was used as a solvent for adding the internal standard. The amount of anhydride and the reaction temperature required can be derived from Table I.

For the determination of the sterols, the following procedure was applied. To

TABLE I

YIELDS OF ESTERIFIED SITOSTEROL UNDER DIFFERENT CONDITIONS

Temperature (°C)	*Pivalic anhydride (μl)*	*Yield (%)*
120	10	35
120	50	92
140	20	82
140	50	100
160	10	62
160	20	95
160	50	100

100 mg of the oil to be analysed, weighed into a 10-ml screw-capped flask, 10 μl of a 1% cholesterol solution in pyridine were added as an internal standard, representing a 0.1% concentration. Pivalic anhydride (50 μl) was added and the mixture was heated at 150°C on a hot-plate. After 15 min, the flask was filled with *n*-heptane, resulting in a 1:100 dilution.

Instrumentation

LC–GC was performed on a prototype of the fully automated Carlo Erba LC–GC instrument[22]. This instrument included a 20-ml syringe pump (Phoenix 20), a fully automated valve system for the interface and the solvent vapour exit, and a GC oven equipped with a loop-type and an on-column interface.

LC conditions

LC preseparation was carried out on a 10 cm × 2 mm I.D. silica gel column (Spherisorb S-5-W) from Knauer, using *n*-hexane–dichloromethane (80:20) containing 0.05% of acetonitrile as the eluent. Volumes of 20 μl were injected through a Valco six-port valve; detection was performed at 220 nm. Derivatized oils produced a small, broad peak (representing many compounds) at the position of the esters analysed Fig. 1). The fraction transferred to the GC system (750 μl) was slightly extended towards higher retention times (as indicated in the chromatogram) to include all material of the originally free sterols (pivalate esters). The LC column was backflushed (first removing the triglycerides) with 1 ml of dichloromethane–acetonitrile (95:5), followed by normal eluent, 2 min after transfer of the ester fraction to the GC system. The backflush valve returned to stand-by 10 min later. There were 45 min between two injections. The regularity of this cycle ensured stable LC retention times.

LC–GC transfer

Concurrent eluent evaporation[23] was applied with the loop-type LC–GC interface[24]. A solvent vapour exit was used at the column exit, diverting most of the

Fig. 1. Liquid chromatogram of an olive oil after esterification with pivalic anhydride. The fraction indicated was transferred to the GC system (Fig. 2). The triglycerides would be eluted later, but were removed by backflushing (not shown).

solvent before it entered the detector. The carrier gas inlet pressure behind the flow regulator was 3 bar; the regulated flow-rate 3 ml/min; GC column temperature during transfer 2.7 min after injection, 130°C. The sample and the carrier gas valves switched to transfer, and the solvent vapour exit opened at the same time. Then, 5 min later, the sample and the gas valve returned to stand-by and the GC oven was heated at 30°C/min to 200°C, then at 10°C/min to 350°C. With a delay of 40 s on the reduction of the inlet pressure at the end of the transfer, the solvent vapour exit automatically switched to a high resistance (2 m × 75 μm I.D. fused-silica capillary), leaving a small purge flow.

GC separation

GC separation was carried out on a 15 m × 0.32 mm I.D. glass capillary column coated with PS-225 (a methylsilicone) of 0.15-μm film thickness. This column was equipped with a 3 m × 0.32 mm I.D. fused-silica precolumn deactivated by phenyldimethylsilylation (connection: press-fit, after opening of the glass capillary butt[25]). The column exit, equipped with a short piece of 0.32 mm I.D. fused-silica tubing, was mounted in the stainless-steel T-piece of the solvent vapour exit, which in turn was connected to the flame ionization detector through a 15 cm × 100 μm I.D. fused-silica capillary.

RESULTS

Fig. 2 shows the gas chromatogram obtained from the fraction of the olive oil shown in Fig. 1. The small peaks eluted before the cholesterol (internal standard) have not been identified, but probably represent originally free fatty alcohols (now esterified with pivalic acid). The group of the originally free sterols clearly shows the

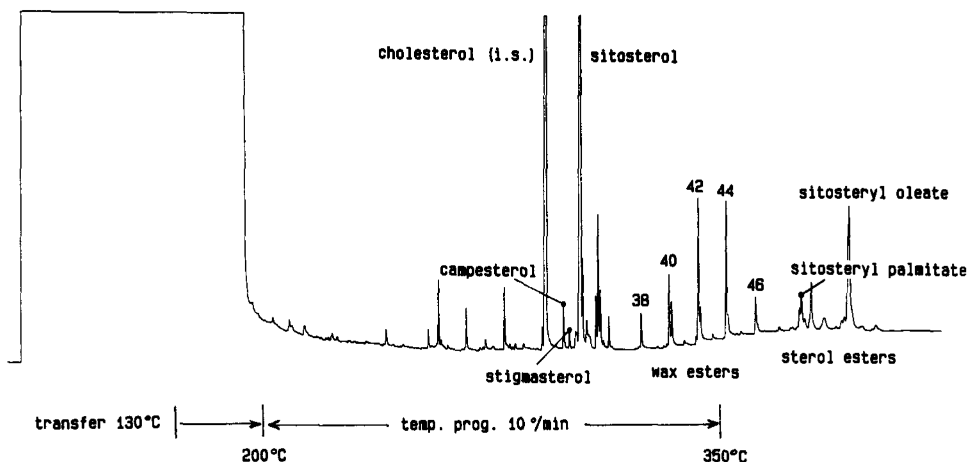

Fig. 2. Gas chromatogram of the olive oil fraction shown in Fig. 1. Cholesterol was added as an internal standard (0.1% referred to the oil). Wax esters are indicated by the total number of carbon atoms. The solvent peak has a width of 7.5 min. The analysis lasted 31 min. The olive oil shown, a commercial oil not declared as "extra virgin" (cold-pressed), contains some extraction oil, as shown by the moderately high concentration of the wax esters and by the fact that the wax esters 42 and 44 are most abundant. On the other hand, the concentration of free sitosterol (740 ppm) is low.

abundance of β-sitosterol (740 ppm) and the two much smaller campesterol and stigmasterol peaks typical of olive oil. The peaks immediately following sitosterol have not been identified. Next are the peaks of the wax esters, characterized by total carbon numbers. Finally, the sterol esters form a multiplet of peaks. The major peak represents sitosteryl oleate, combined with slightly later eluted sitosteryl stearate and the esters of avenastenol. Some tentative identifications suggested that there are some compounds other than sterol esters, forming peaks primarily between the C_{16} and C_{18} sitosteryl esters.

Available information

The following information is available from the described analysis:

(1) The total sterol content is calculated by addition of the concentrations of the originally free sterols and of the esterified sterols. We found good agreement with results obtained by the classical method involving saponification.

(2) The sterol composition (individual concentrations relative to the total sterol concentration, as commonly used for characterizing a fat or oil) is calculated from the free sterols, assuming that the compositions of the free and the esterified esters are identical (small deviations are negligible as the esterified sterols represent only small proportions of the total sterol content).

(3)The ratio of the (originally) free to the esterified sterols is probably a sensitive indicator of oil treatments. For instance, extraction oils contain considerably higher concentrations of free sterols than cold-pressed oils, whereas the esterified sterols are nearly completely recovered by the cold-pressed oil. Treatments such as filtration or crystallization affect the free and esterified sterols to different extents.

(4) The amounts and the composition of the wax esters are different for cold-

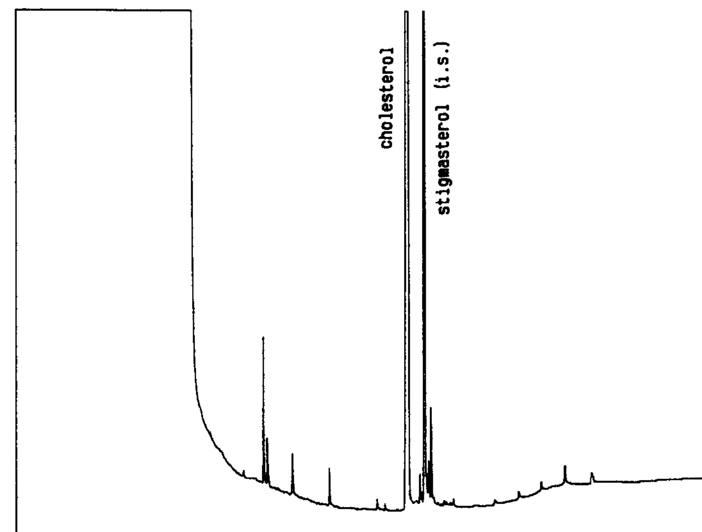

Fig. 3. Rapid determination of the cholesterol concentration in a butter fat, using stigmasterol as internal standard.

pressed (virgin) and extracted olive oil, and are again influenced by the methods applied for refining the oil.

Application of the new technique to routine analysis has only just started, and a more thorough study on the recognition of differently treated olive oils is in progress. Some preliminary results are given below.

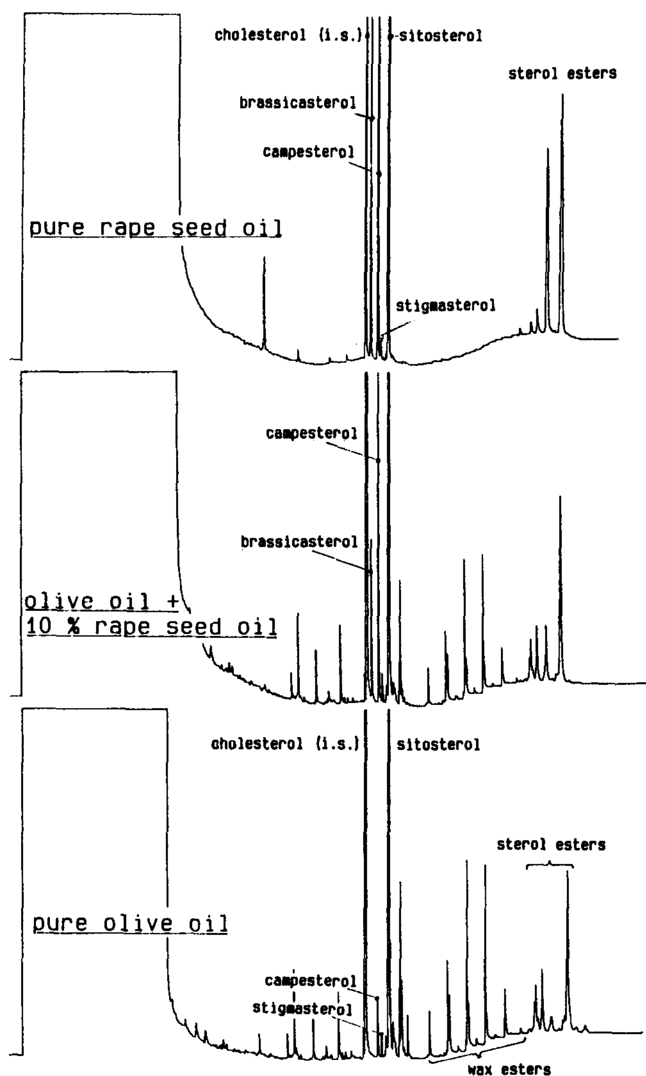

Fig. 4. Determination of rapeseed oil in olive oil. Rapeseed oil contains high concentrations of brassicasterol and campesterol (top chromatogram), whereas these sterols are absent or present at only low concentration in olive oil (bottom chromatogram). The detection limit for rapeseed oil in olive oil is below 1% (middle chromatogram).

Butter fat

Butter fat produced the simplest chromatogram of the fats analysed (Fig. 3). Stigmasterol (0.1%) was added as an internal standard (instead of the cholesterol added to the plant fats and oils). Most of the peaks near this stigmasterol peak represent impurities in the stigmasterol. Hence, cholesterol (present at a concentration of 0.28%) is almost the only peak in the chromatogram. There is no significant concentration of esterified cholesterol. The small peaks at the rear of the chromatogram were not identified, but eluted at higher retention times than the wax esters found in plant fats and oils.

Rape seed oil in olive oil

Fig. 4 shows the sterols and wax esters of a rapeseed oil, an olive oil and an olive oil containing 10% rapeseed oil. Rapeseed oil is characterized by a high total sterol

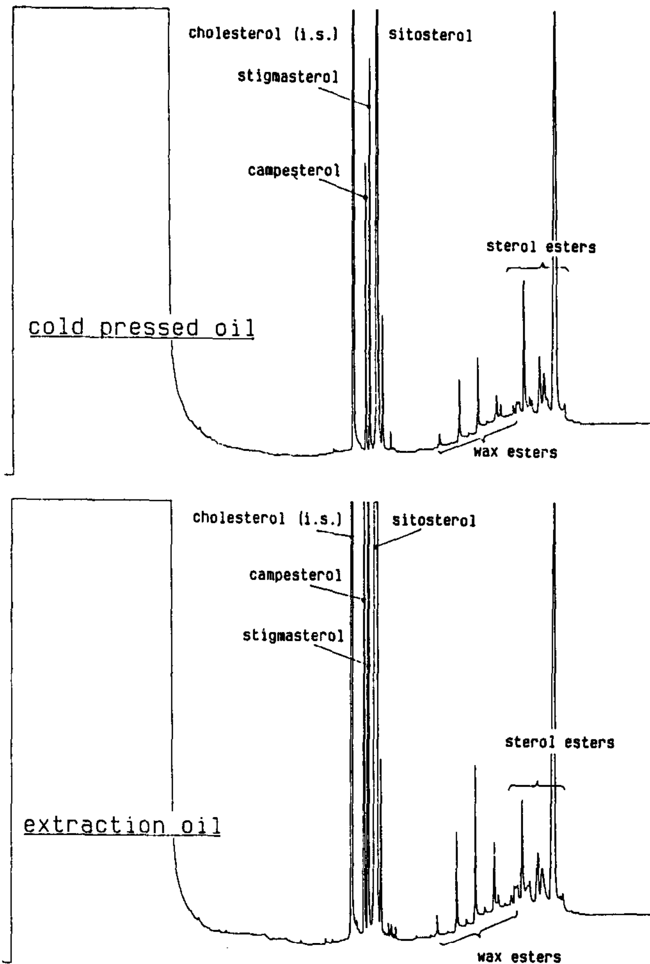

Fig. 5. Differentiation between cold-pressed and extracted sunflower oil.

content (0.43% for our oil), a high concentration of campesterol and the presence of brassicasterol. This is the basis for the classical method used for detecting rapeseed oil in olive oil. With the method described above, the analysis becomes rapid and easy. Rapeseed oil additions below 1% can be detected.

Cold-pressed versus extracted sunflower oil

Fig. 5 compares the chromatograms obtained from a cold-pressed and an extracted sunflower oil. The sterol ester concentrations are almost identical, which indicates that the sterol esters pressed out of the seeds at about the same yield as the oil. The concentrations of free sterols, however, are more than doubled in the extraction oil (as for olive oil [26]). Wax ester concentrations also increased, although much less than for extracted olive oil. The distribution of the individual wax esters is shifted slightly towards higher molecular weight.

REFERENCES

1 R. P. W. Scott and P. Kucera, *J. Chromatogr.*, 125 (1976) 251.
2 R. P. W. Scott, *Adv. Chromatogr.*, 22 (1983) 247.
3 R. P. W. Scott, *J. Chromatogr. Sci.*, 23 (1985) 233.
4 H. J. Cortes, C. D. Pfeiffer and B. E. Richter, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 469.
5 R. P. W. Scott and S. Traiman, *J. Chromatogr.*, 196 (1980) 193.
6 E. Homberg, *Dtsch. Lebensm.-Rdsch.*, 81 (1985) 12.
7 F. Mordret, A. Prévot and J.-P. Wolff, *Ann. Falsif. Expert. Chim.*, 70 (1977) 87.
8 D. Firestone, K. L. Carson and R. J. Reina, *J. Am. Oil. Chem. Soc.*, 65 (1988) 788.
9 C. Gertz, *Fat Sci. Technol.*, 90 (1988) 45.
10 C. Mariani, A. Lanzani and E. Fedeli, *Riv. Ital. Sostanze Grasse*, 64 (1987) 13.
11 V. Paganuzzi, *Riv. Ital. Sostanze Grasse*, 60 (1983) 489.
12 C. Mariani and E. Fedeli, *Riv. Ital. Sostanze Grasse*, 63 (1986) 3.
13 K. Grob and T. Läubli, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 593.
14 G. Morchio and R. De Andreis, *Riv. Ital. Sostanze Grasse*, 60 (1983) 427.
15 G. Lercker and M. F. Caboni, *Riv. Ital. Sostanze Grasse*, 62 (1985) 193.
16 G. Amelotti, A. Griffini, M. Bergna and P. Montofano, *Riv. Ital. Sostanze Grasse*, 62 (1985) 459.
17 G. Bonaga, A. Antonelli, L. S. Conte and G. Lercker, *Riv. Ital. Sostanze Grasse*, 63 (1986) 583.
18 R. E. Worthington and H. L. Hitchcock, *J. Am. Oil Chem. Soc.*, 61 (1984) 1085.
19 P. Horstmann and A. Montag, *Fette-Seifen-Anstrichm.*, 88 (1986) 262.
20 M. C. Iatrides, J. Artaud and M. Derbesy, *Analusis*, 12 (1984) 205.
21 E. Homberg, *Fat Sci. Technol.*, 89 (1987) 215.
22 F. Munari and K. Grob, *J. High Resolut. Chromatogr. Chromatogr. Commun.*,11 (1988) 172.
23 K. Grob, Ch. Walder and B. Schilling, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 95.
24 K. Grob and J.-M. Stoll, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 518.
25 B. Schilling, K. Grob, P. Pichler, R. Dubs and B. Brechbühler, *J. Chromatogr.*, 435 (1988) 204.
26 C. Mariani, E. Fedeli and V. Bovio, *Riv. Ital. Sostanze Grasse*, in press.