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Determination of neutral lipids from subcutaneous fat of cured ham by capillary gas chromatography and liquid chromatography

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Abstract

The determination of neutral lipids in fat of cured ham is reported. Fat samples were extracted with chloroform–methanol (2:1) and neutral lipids and free fatty acids were separated on an aminopropyl minicolumn, the first fraction with chloroform–2-propanol (neutral lipids) and the second fraction with 2% acetic acid in diethyl ether (free fatty acids). Neutral lipids were fractionated with minicolumns, with aminopropyl and silica stationary phases. Two fractions were obtained with the first column: (A) triglyceride and cholesteryl esters and (B) cholesterol and mono- and diglycerides. Fraction A was applied to the silica column to obtain two new fractions: (C) cholesteryl esters and (D) triglycerides. Fractions B and C were analysed by capillary gas chromatography (cGC) and fraction D by cGC and HPLC. The R.S.D.s obtained were below 5% except for the monoglycerides (8%). Cholesteryl esters were determined by cGC in 5 min with R.S.D. 5%. The main triglycerides identified were PPO, POS, POO, POL and OOO (P = palmitic acid, O = oleic acid, L = linoleic acid; S = stearic). Monoglyceride and diglycerides having 18, 34 and 36 carbon atoms were the most abundant. The determination of triglycerides by HPLC was more difficult than by cGC because the linearity with HPLC was concentration dependent. The procedure allowed the determination of neutral lipid classes without derivatization of mono- and diglycerides.

1. Introduction

Neutral lipids are the main components of fat tissues. Triglycerides are the most abundant class of neutral lipids (90% or more), but minor constituents such as cholesterol, cholesteryl esters and mono- and diglycerides must be considered when characterizing a fat and studying lipolysis during the production of fatty foods. Fatty acids have been used to establish the fat composition and to evaluate lipid degradation in some kinds of food products, and in particular in cured ham and sausages fatty acids have been determined in order to study changes produced by lipid oxidation and hydrolysis [1–3]. However, fatty acid profiles do not reveal specific changes in a neutral lipid class as they are an indirect measure of the true molecular species. Biochemical changes during the production of meat products (cured ham, sausages) can be better characterized on the basis of the depletion

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of some triglycerides and the subsequent increase in mono- and diglyceride production [4]. The determination of neutral lipid classes is generally carried out only to measure the total percentage of each lipid class by TLC or HPLC without separating them into individual components [5–8], although there are methods for determining the molecular species of diglycerides or monoglycerides by HPLC or GC [9–11]. However, with meat products a suitable method for determining neutral lipid classes has not been reported. An important aspect of lipid analysis is the prior fractionation to separate neutral lipids from polar and complex lipids, and different materials (silica, silicic acid, Florisil) have been used for this purpose [12–14].

The development of thermally stable stationary phases has allowed the analysis of neutral lipids by capillary GC (cGC) on the basis of carbon number (CN) and the number of double bonds (NDB) in the same CN [15–17]. HPLC analysis with light-scattering detection is an alternative technique for determining neutral lipids as gradient elution can be used to increase the peak resolution [18–20]. The separation of triglycerides by HPLC is carried out on the basis of the equivalent carbon number (ECN) defined as $ECN = CN - 2NDB$ or $ECN = CN\text{-}a NDB$, where a is calculated by linear regression [21]. The peak assignment is easier in cGC as the elution pattern with medium-polarity stationary phases is related to the NDB; the higher the NDB, the greater is the retention time in the same CN. Quantification is more complicated in HPLC because the linearity changes with the concentration range [18,22].

This paper describes a method for determining neutral lipids in ham fat by cGC and HPLC using prior separation of neutral lipids into three fractions: triglyceride, cholesteryl esters and cholesterol and mono- and diglycerides.

2. Experimental

The following abbreviations are used: MG = monoglycerides; DG = diglycerides; CP = cholesteryl palmitate; CS = cholesteryl stearate; fatty acids: P = palmitic (C16:0); S = stearic (C18:0); O = oleic (C18:1); L = linoleic (C18:2) and araquidonic (C20:4); triglycerides: POS = palmitoyloleoylstearoyl. This nomeclature does not indicate the position of the fatty acid in triglyceride molecules.

2.1. Samples and extraction

Subcutaneous fat from cured ham, at 5 months of ageing, was taken for analysis. Lipids were extracted from subcutaneous fat (1 g) with 50 ml of chloroform–methanol 2:1 [23]. The extract was evaporated to dryness and the residue was dissolved in a 10 ml of chloroform.

2.2. Lipid fractionation

The method used is a combination of previous methods [13,14,24] to achieve a complete separation of cholesteryl esters from triglycerides. An alliquot (10 mg of extractable fat) of the dissolved extract was applied to an aminopropylsilica minicolumn (100 mg; Analytichem). A prior fractionation was applied to obtain free fatty acids, first with 2 ml of chloroform–2-propanol (neutral lipids) and second with 3 ml of 2% acetic acid in diethyl ether (free fatty acids). The first fraction was applied to a second aminopropylsilica minicolumn (500 mg; Analytichem) and neutral lipid classes were collected under the following conditions: (A) 5 ml of 1% diethyl ether and 10% dichloromethane in hexane (triglycerides and cholesteryl esters) and (B) 5 ml of chloroform–methanol (2:1) (mono- and diglycerides and cholesterol). Fraction A was evaporated to dryness and the residue was dissolved in 1 ml of isooctane, then the solution was applied to a silica minicolumn (500 mg; Millipore) to separate triglycerides from cholesteryl esters by the following procedure: (C) 4 ml of hexane–diethyl ether (98:2) (cholesteryl esters) and (D) 5 ml of hexane–diethyl ether (90:10) (triglycerides).

2.3. Chromatographic analysis

Fraction D was analysed by HPLC and cGC. The HPLC conditions were as follows: LiChrospher RP-18 column (250 × 4 mm I.D.) (Merck)

at ambient temperature (<20°C); mobile phase: gradient from 30 to 60% dichloromethane in acetonitrile in 50 min at a flow-rate of 1.00 ml/min; detection, light-scattering detector (ACS) at 70°C; and injection volume 10 μl via a Rheodyne valve. The pump system was an LKB 2152 HPLC pump controller and an LKB 2150 HPLC controller (Pharmacia). For cGC analysis, two fused-silica open tubular (FSOT) columns, 10 m × 0.2 mm I.D. (0.1 μm film thickness) coated with methylsilicone (MS) and 25 m × 0.2 mm I.D. (0.15 μm film thickness) coated with 50% phenyl–methylsilicone (PMS) (Rescom, Belgium), were used, connected to a deactivated retention gap (1 m × 320 μm I.D.) (Supelco, Bellefonte, PA, USA). The MS column was used for cholesterol and mono- and diglyceride analysis and the PMS column for triglyceride analysis. The carrier gas was helium at 50 cm/s (MS) and 60 cm/s (PMS), with temperature programming from 150 to 310°C at 5°C/min for the MS and from 330 to 360°C at 1.5°C/min for the PMS column. A flame ionization detector at 380°C and on-column injection with high-temperature secondary cooling were used. The gas chromatograph used was a Carlo Erba HRGC 5300HT Mega series.

The analysis of free fatty acids as methyl esters (FAMEs) was carried out by cGC. A 1-ml volume of 14% BF_3 in methanol was used to obtain methyl esters (20 min at 50°C), which were recovered in 2 ml of hexane. A FSOT column (25 m × 0.25 mm I.D.) coated with 60% cyaonopropylsilica (SH-90) (Rescom) (0.20 μm) was used to separate FAMEs under the following conditions: temperature, programmed from 140 to 180°C at 3°C/min, held at 180°C for 5 min; detector and injector temperatures, 250°C; carrier gas helium at 1.00 ml/min at constant flow; split injection mode (splitting ratio 1:40); and gas chromatograph, Hewlett-Packard 5890 Series II with electronic pressure control.

2.4. Standard solutions

Three standard solutions of monolein (MG18), distearin (SS), cholesterol (40 ng/μg), cholesteryl palmitate and stearate (CP, CS) (20 ng/μl) and triglycerides (PPO, POS, POO, OSO and SOS), PPO (1,2-dipalmitoyl-3-oleoyl-*rac*-glycerol), POS (1-palmitoyl-2-oleoyl-3-stearoyl-*rac*-glycerol), POO (1,2-dioleoyl-3-palmitoyl-*rac*-glycerol) OSO (1,3-dioleoyl-2-stearoyl-*rac*-glycerol) and SOS (1,3-distearoyl-2-palmitoyl-*rac*-glycerol) (20 and 500 ng/μl) were prepared in isooctane. Standard solutions of pentadecanoic and heptadecanoic acids (50 ng/μl) were used for the determination of free fatty acids. All the compounds were obtained from Sigma (St. Louis, MO, USA).

2.5. Repeatibility and linearity

Standard and sample fraction (A, B, C and D) solutions were injected seven times and subjected to the different chromatographic techniques described. Linearity, using four points, was established with standard solutions.

2.6. Fraction repeatibility

A fat sample was fractionated five times and each fraction was analysed by the procedure described. Peak areas obtained for the selected compounds (Table 1) were used to determine the fraction repeatibility.

3. Results and discussion

3.1. Sample preparation

The complete determination of neutral lipids in the same run is possible, but with fat samples the high concentration of triglycerides (>90%) limited this possibility because it was necessary to concentrate the extract to allow the analysis of minor neutral lipid classes which caused the simultaneous concentration of triglycerides, resulting in large amounts being introduced into the gas chromatograph (*e.g.*, 500 ng/μl). To avoid this problem, it was necessary to preseparate triglycerides from the other neutral lipid classes. In the first instance the use of the aminopropyl column to obtain three fractions, (1) cholesteryl esters, (2) triglycerides and (3) cholesterol and mono- and diglycerides, was evaluated, but it was not possible to separate triglycerides from cholesteryl esters owing to the

Table 1
Selected compounds evaluated for determination of fraction repeatibility

Fraction	Compound
B	Monolein, cholesterol, diolein
C	Cholesteryl palmitate and stearate[a]
D	Triglycerides (PPO, POS, POO, POL, OOO)
Free fatty acids	C16:0, C18:0, C18:1, C18:2, C20:4

[a] Spiked sample.

high content of triglycerides. The silica column allowed the separation of cholesteryl esters from triglycerides. The fraction repeatibilities showed R.S.D.s below 10% for fractions B, C and D obtained from fat samples and considering the selected compounds used to evaluate them (Table 1), but in fraction B the R.S.D. obtained for monoglyceride was 15%. The more difficult analysis of underivatizated monoglyceride could contribute to this poorer repeatibility. On the other hand, cross-contamination of fractions was not observed. When standards of each neutral lipid class were added to samples the recovery was >90%. Free fatty acids showed a high repeatibility (R.S.D. < 10%).

3.2. Triglycerides

Triglycerides were analysed by HPLC and cGC. The repeatibility of both techniques was good, showing R.S.D.s below 5% (Tables 2 and 3). cGC allowed easier peak assignment and the calibration graph was not concentration dependent as in HPLC with light scattering detection. However, the peaks of POO and PSL were not completely separated. It was possible to identify eight peaks by this technique (Fig. 1) on the basis of NDB in a same CN. Linearity for plots of certain selected triglyceride peak areas *versus* concentration showed the following correlation coefficients (r): 0.999 (POP), 0.998 (POS), 0.999 (POO), 0.999 (SOS) and 0.999 (OSO). Retention gaps of 530 μm showed greater peak broadening than 320 μm. HPLC analysis was easier from the point of view of temperature analysis but the calibration graphs were concentration dependent, the slope obtained in the range 50–20 μg being 2649 (SOS) and that in the range 20–5 μg being 749 (SOS); the r values obtained in the range 50–20 μg were 0.989 (POP), 0.991 (POS), 0.999 (POO), 0.989 (SOS) and 0.976 (OSO). The analysis time was longer using HPLC than cGC and the peak assignment was more complicated. A tentative identification

Table 2
Repeatibilities of retention times[a] and concentrations[b] of triglycerides[c] in capillary GC

Compound	x^a	s^a	(%) R.S.D.[a]	x^b	s^b	(%) R.S.D.[b]
PPO	7.49	0.03	0.40	32.20	0.98	3.04
POS	9.03	0.04	0.44	108.10	5.75	5.32
POO	9.19	0.03	0.32	230.00	12.80	5.57
POL	9.44	0.04	0.42	62.10	1.99	3.20
SOO	11.06	0.03	0.27	39.10	1.97	5.04
OOO	11.23	0.03	0.27	46.00	1.94	4.21

[a] Minutes.
[b] Concentrations in mg/g extractable fat.
[c] Ham fat sample.

Table 3
Repeatibilities of retention times[a] and concentrations[b] of triglyceride[c] analysis of HPLC

Triglyceride	x^a	s^a	(%) R.S.D.[a]	x^b	s^b	(%) R.S.D.[b]
POL	19.43	0.07	0.36	66.10	2.87	4.34
OOO	21.56	0.12	0.56	61.70	2.00	3.25
POO	22.64	0.13	0.57	220.40	5.00	2.26
PPO	24.02	0.16	0.67	33.10	1.53	4.60
SOO	25.73	0.16	0.62	30.80	1.27	4.13
POS	27.06	0.17	0.63	105.80	3.42	3.24

[a] Minutes.
[b] Concentrations in mg/g extractable fat.
[c] Ham fat sample.

was made with known standards and by the ECN; thus, ten peaks were identified based on the ECN and by comparison with the results reported by other workers [25] (Fig. 2). HPLC with UV detection could be also used to identify low percentages of polyunsaturated triglycerides (peaks, 1, 2, 3 and 4 in Fig. 2) because of their high relative responses in this detector. Good agreement was observed for concentrations determined by HPLC and cGC expected for OOO, which showed a higher value in HPLC, reflecting the more critical separation of OOO by cGC (Fig. 1).

3.3. Cholesteryl esters

The analysis time for separating cholesteryl esters of 16 and 18 CN by cGC was 5 min (Fig. 3). The R.S.D.s of the retention times and absolute peak areas were <5% (Table 4) for

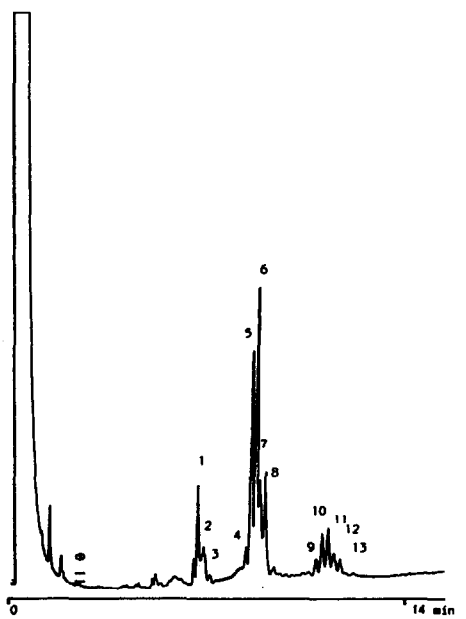

Fig. 1. cGC analysis of triglycerides from ham fat. Peaks identified: 1 = PPO; 5 = POS; 6 = POO; 7 = PLS; 8 = POL; 9 = SOS; 10 = SOO; 11 = OOO; 12 = SOL. For conditions, see text.

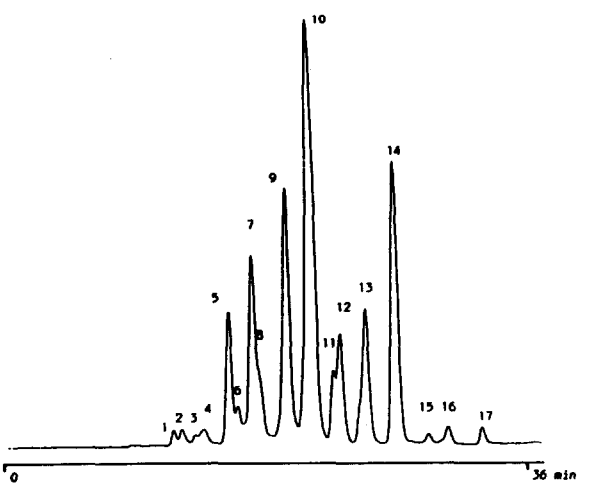

Fig. 2. HPLC analysis of triglycerides from ham fat. Peaks identified: 5 = OOL; 7 = POL; 9 = OOO; 10 = POO; 11 = PSL; 12 = PPO; 13 = SOO; 14 = POS; 16 = SSO; 17 = PSS. For conditions see text.

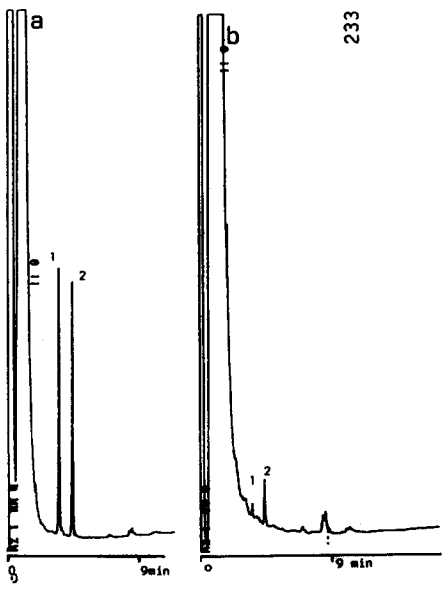

Fig. 3. cGC analysis of fraction B. (a) Ham fat spiked with cholesteryl esters. Peaks: 1 = cholesteryl palmitate (CP); 2 = cholesteryl stearate (CS). (b) Unspiked sample. Peaks: cholesteryl esters of (1) CN 16 and (2) CN 18. For conditions, see text.

standard solutions, but higher values were obtained for fat samples. Cholesteryl ester concentrations are low in fats, and to facilitate the evaluation of the repeatibility the samples were spiked with CP and CS to increase the peak heights. In this instances the R.S.D.s obtained were lower (Table 4) and similar to those obtained with standard solutions. Cholesteryl esters detected in fat samples were associated with 16 and 18 CN, only single peaks were detected and the esterified fatty acid was not assigned. However, the retention time observed for the main compound (CN 18) was shorter than that of CS standard, suggesting the possibility of cholesteryl oleate (OC). Linear plots of concentration in ng/μl (y) *versus* peak area (x) *were* obtained for CP and CS standards: $y = -0.84 + 6.49 \cdot 10^{-5}x$ ($r = 0.999$) and $y = -0.67 + 6.53 \cdot 10^{-5}x$ ($r = 0.998$), respectively.

3.4. Cholesterol and mono- and diglycerides

This fraction was determined without derivatization (Fig. 4). Underivatizated monoglycerides were difficult to analyse and the peak symmetry was poorer than for cholesterol and diglycerides. These difficulties were reflected by the higher R.S.D. (8%) obtained for the repeatibility of the absolute peak area of monoolein. The other compounds showed R.S.D.s below 5% for absolute peak area and retention time repeatibilities (Table 5). Monoglyceride analysis could be improved by derivatization to obtain trimethylsilyl derivatives. The peak identification was based on the retention times of standards and the NDB. Monoolein was the main monoglyceride and diglycerides with 34 and

Table 4
Repeatibilities of retention times[a] and concentrations[b] of cholesteryl esters

Compound	x^a	s^a	(%) R.S.D.[a]	x^b	s^b	(%) R.S.D.[b]
CP[c]	3.51	0.02	0.62	356 140[c]	11800	3.31
CS[c]	4.42	0.03	0.61	342 667[c]	10896	3.18
CE16[d]	3.57	0.01	0.35	94.80	4.93	5.20
CE18[d]	4.37	0.02	0.44	437.30	25.37	5.80
CP[e]	3.51	0.03	0.89	2350.90	81.57	3.47
CS[e]	4.41	0.04	0.89	246.17	55.14	2.24

[a] Minutes.
[b] Concentrations in μg/g extractable fat.
[c] Absolute peak areas corresponding to a concentration of 20 ng.
[d] Sample CE 16 and CE 18 are cholesteryl esters of CN 16 and CN 18, respectively, in fat samples.
[e] Spiked sample.

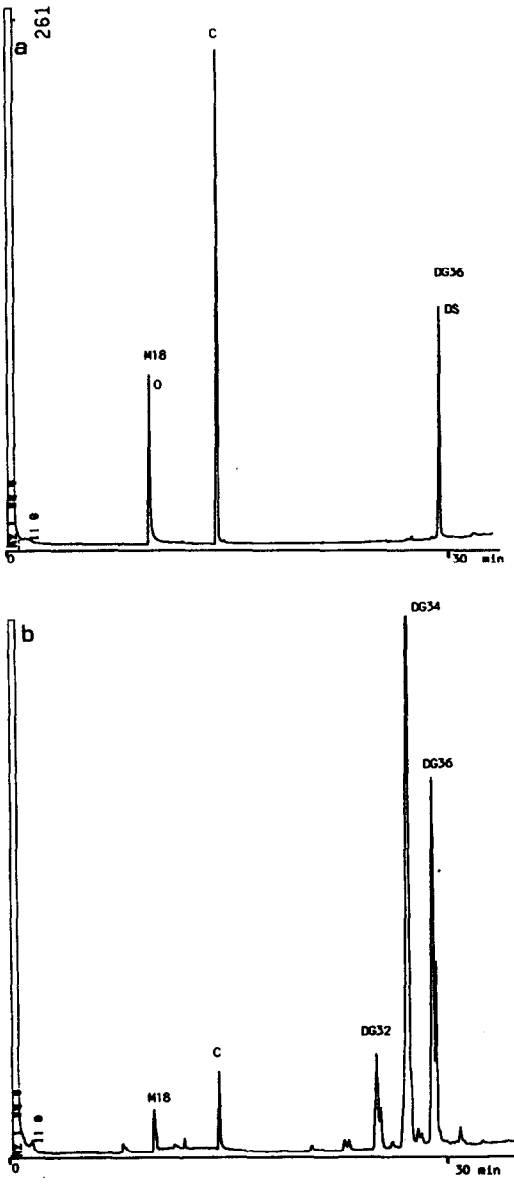

Fig. 4. cGC analysis of fraction B. (a) Standard solution. Peaks: M18 = monoleine; C = cholesterol; DS = distearin. (b) Ham fat. Peaks: M18 = monoglyceride; DG32, DG34 and DG36 = diglycerides of CN 32, 34 and 36, respectively. For conditions, see text.

36 CN were the most abundant. Tentative identifications considering the NDB were made (Fig. 4) and PO, OO and SO were considered the most probable. Linear regression for concentration in ng/μl (y) *versus* peak area were $y = 9.97 + 1.16 \cdot 10^{-4}x$ ($r = 0.990$) for monoolein, $y = 1.99 + 6.36 \cdot 10^{-5}x$ ($r = 0.997$) for cholesterol and $y = 7.29 + 1.14 \cdot 10^{-4}x$ ($r = 0.990$) for distearin.

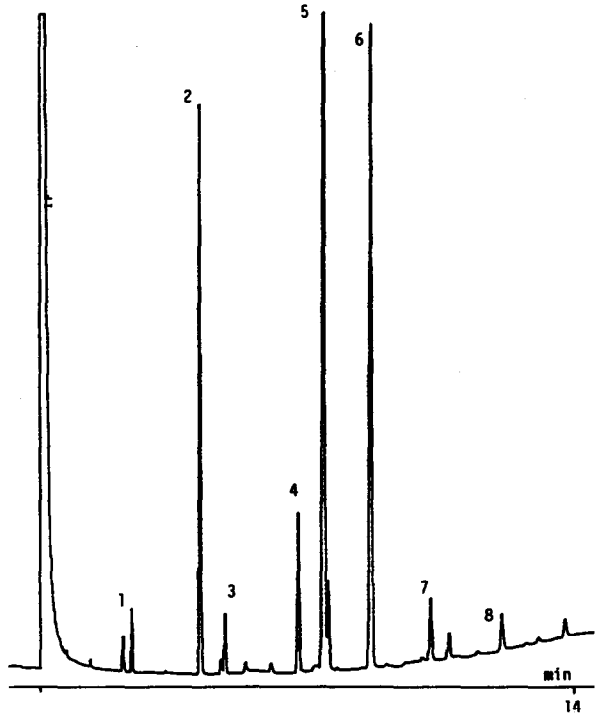

Fig. 5. cGC of free fatty acid fraction. Peaks: 1 = myristic; 2 = palmitic; 3 = palmitoleic; 4 = stearic; 5 = oleic; 6 = linoleic; 7 = linolenic; 8 = arachidonic acid. For conditions, see text.

3.5. Free fatty acid fraction

The separation of fatty acids as FAMEs by GC has been intensively investigated and they were included in this work because they are an important fraction related to lipid changes in food products. The origin of free fatty acids in cured ham is not only associated with neutral lipids; phospholipid breakdown produces free fatty acids but in fat samples this contribution is smaller because of the low percentage of phospholipids. The use of bonded and stabilized polar stationary phases allows the effective separation of FAMEs on the basis of the NDB in the same CN (Fig. 5). The R.S.D.s obtained for

Table 5
Repeatibilities of retention times[a] and concentrations[b] of cholesterol and mono- and diglycerides

Compound	x^a	s^a	(%) R.S.D.[a]	x^b	s^b	(%) R.S.D.[b]
MG18	9.49	0.04	0.44	2286.50	176.97	7.74
Cholesterol	13.73	0.01	0.09	768.35	30.58	3.98
DG32	24.05	0.02	0.07	1975.07	66.17	3.35
DG32	24.26	0.01	0.04	1844.47	76.91	4.16
DG34	26.06	0.01	0.06	26655.05	927.59	3.48
DG36	27.68	0.01	0.03	20243.85	734.85	3.63

MG18 = monolein; for diglycerides DG32, DG32, DG34 and DG36, see Fig. 4.
[a] Minutes.
[b] Concentrations in μg/g extractable fat.

Table 6
Repeatibilities of retention times[a] and concentrations[b] of free fatty acids[c] in capillary GC

Fatty acid	x^a	s^a	(%) R.S.D.[a]	x^b	s^b	(%) R.S.D.[b]
C14:0	2.81	0.01	0.35	1.15	0.02	2.13
C16:0	4.38	0.01	0.23	18.11	0.07	0.40
C16:1	4.88	0.01	0.20	2.13	0.07	3.28
C18:0	6.50	0.01	0.15	6.25	0.04	0.62
C18:1	7.05	0.01	0.14	34.81	0.16	0.47
C18:2	8.08	0.01	0.12	27.21	0.19	0.73
C18:3	9.31	0.01	0.10	2.80	0.03	0.94
C20:4	12.37	0.01	0.08	1.89	0.12	6.17

[a] Minutes.
[b] Concentrations in mg/g extractable fat.
[c] Ham fat sample.

both absolute peak areas and for concentrations were below 5% (Table 6). The concentration of free fatty acids can vary as a function of lipolysis intensity and of ageing time.

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5. References

[1] C. Cantoni, M.A. Bianchi, S. D'Aubert, P. Renon and F. Cerutti, *Arch. Vet. Ital.*, 21 (1970) 213.
[2] J. Flores, P. Nieto, S. Bermell and J. Arberola, *Rev. Agroquim. Tecnol. Aliment.*, 27 (1987) 599.
[3] J. Flores, P. Nieto, S. Bermell and J. Arberola, *Rev. Agroquim. Tecnol. Aliment.*, 28 (1988) 90.
[4] J.A. García Regueiro, I. Díaz, F. David and P. Sandra, in *Proceedings of the 35th Congress of Meat Science and Technology*, Vol. III, Danish Meat Research Institute, Copenhagen, 1989, p. 719.
[5] J.E. Storry and B. Tuckley, *Lipids*, 2 (1967) 501.
[6] V.P. Pchelkin and G. Vereshchagin, *J. Chromatogr.*, 209 (1981) 49.
[7] W.W. Christie, *J. Lipid Res.*, 26 (1985) 507.
[8] O.S. Privett, K.A. Douherty, W.L. Erdahl and A. Stolyhwo, *J. Am. Oil Chem. Soc.*, 50 (1973) 516.
[9] J.J. Myher and A. Kuksis, *Can. J. Biochem.*, 60 (1982) 638.
[10] L. Motta, M. Brianza, F. Stanga and G. Amelotti, *Riv. Ital. Sostanze Grasse*, 60 (1983) 625.
[11] H. Takamura, H. Narita, R. Urade and M. Kito, *Lipids*, 21 (1986) 356.

[12] K.K. Carroll, J.H. Cutts and G.D. Murray, *Can. J. Biochem.*, 46 (1968) 899.
[13] K.K. Carroll, in G.V. Marinetti (Editor), *Lipid Chromatographic Analysis*, Vol. 1, Marcel Dekker, New York, 2nd ed., 1976, pp. 173–214.
[14] G. Rouser, G. Kritchevsky and A. Yamamoto, in G.V. Marinetti (Editor), *Lipid Chromatographic Analysis*, Vol. 3, Marcel Dekker, New York, 2nd ed., 1976, pp. 713–776.
[15] E. Geeraert and D. De Schepper, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 5 (1982) 80.
[16] E. Geeraert and D. De Schepper, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 6 (1983) 123.
[17] E. Geeraert and P. Sandra, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 415.
[18] A. Stolywho, H. Colin and G. Guiochon, *Anal. Chem.*, 57 (1985) 1342.
[19] B. Herslof and G. Kindmark, *Lipids*, 20 (1985) 783.
[20] O. Podlaha and B. Töregrad, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 5 (1982) 553.
[21] B. Herslöf, O. Podlaha and B. Töregard, *J. Am. Oil Chem. Soc.*, 56 (1979) 864.
[22] A. Stolywho, H. Colin, M. Martin and G. Guiochon, *J. Chromatogr.*, 288 (1984) 253.
[23] J. Folch, M. Lees and O.H.S. Stanley, *J. Biol. Chem.*, 266 (1957) 497.
[24] M.A. Kaluzny, L.A. Duncan, M.V. Merritt and D.E. Epps, *J. Lipid Res.*, 26 (1985) 135.
[25] J.-L. Perrin and A. Prévot, *Etud. Rech.*, 33 (1986) 437.