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BUTTEROIL RM 164: ASSESSMENT OF PURITY OF TRIGLYCERIDE CALIBRANTS

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Section Carbohydrates and lipid chemistry

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SUMMARY

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Ten saturated and three unsaturated triglycerides have been controlled on their purity. Fatty acid and triglyceride analyses of the individual samples and of mixtures of the short chain respectively the long chain fatty acids have been done. The gaschromatographic purity was judged to be acceptable for further use in the certification trial of BCR butterfat reference material RM 164. From the results of the mixtures it was concluded that none of the samples contains impurities of non-eluting materials. Some problems were found in preparing and handling the long chain fatty acid mixtures since some crystallization was observed, leading to increasing calibration factors (fig 2).

1. INTRODUCTION

For the certification of RM 164, the BCR (Bureau Communautaire de Reference) was interested in the purity of triglyceride (TG) standards with fatty acids C4 till C20. The RIKILT was asked to control the purity by gaschromatographic (GC) analysis of the triglycerides and fatty acids. Problem of this study is that impurities had to be discovered which are not detected with the GC system for fatty acid or TG analyses.

All samples were analysed individually for control on their GC purity of the triglycerides and the fatty acids. For control on any other impurity also mixtures with respectively the high and low carbonnumbered triglycerides were made from these samples and analysed. The TG and fatty acid methyl ester (FAME) analyses were done and the area percentages have been compared with the expected values leading to calibration factors which should have been used when the standards were fully pure. The exactness of these calibration factors will be defined by: the purity of the samples; the theoretical respons of the fatty acids in the Flame Ionization Detector (FID) and the inevitable non ideal GC technic, column and apparatus. The theoretical respons factors of FAMEs in the FID have been discussed by Ackman (1964), Badings (1983) and Bannon (1985). The reasons leading to non ideal respons factors are being investigated from the beginning of the gaschromatography till now. The principal ones are: discrimination of the TG or FAME by selective evaporation from the injection needle (Badings (1981, 1983), Grob (1978); discrimination caused by the splitted injection technique; nonlinear detection in the FID caused by apparatus lack; integration problems due to peak broadening etc.. All these effects are reflected in the calibration factors which are normally used in GC fatty acid analyses. However since these imperfects of the GC analyses are continuously dependent of the chain length and saturation degree, any discontinuous abbreviation in the calibration factors must be due to involatile noneluting materials in the sample.

Summarised: the triglyceride standard samples of BCR have been controlled on their gaschromatographic purity of the triglycerides and fatty acids and have been controlled on noneluting materials by investigation abbreviations in the expected calibration factors from the FAME analyses.

2. MATERIALS AND METHODS

2.1 Samples.

Thirteen triacylglyceride samples of 200-300 mg each were obtained from the Laboratory of the Government Chemist (UK). The TG consist resp. of the saturated fatty acids C4, 5, 6, 8, 10, 12, 14, 16, 18, 20 and of the unsaturated fatty acids C16:1, 18:1 and 18:2. From these samples one mixture was prepared with the short chain fatty acids C4-14 and two mixtures were prepared with the long chain fatty acids of C12-20. The mixtures were prepared by using a special 5 decimal balance (0.01 mg precision) and by solvation in n-hexane.

2.2 Fatty acid analyses.

The individual samples and the mixtures therefrom were analysed on their fatty acid composition by means of a CPWAX 57CB capillary column and split injection. The fatty acid methylesters (FAME) have been prepared by KOH/MeOH transmethylation first described by Christopherson and Glass (1970).

2.3 Triglyceride analyses.

The triglycerides were analysed on a special 25m triglyceride capillary column with TAP (Triglyceride Analysis Phase) and on a 10m CPSIL 5CB column. As injection technique a split injector was used.

2.4 Elaboration of the results.

All calibrations and integrations were made with computated systems. The results of obtained calibration factors from the mixtures have been plotted against the fatty acid chain length to discover any discontinuity.

3. RESULTS

3.1 Fatty acid composition of the samples.

The data of the fatty acid analyses are given in table 1 and the chromatogrammes of the samples are given in annex I for the lower triglycerides and in annex II for the higher triglycerides. The values are confirmed by duplicate analyses. The results were obtained as area percentages but, since the influence of the calibration factors may be neglected in these cases, the results can be read as expressed in percentages by mass of FAME on total FAME.

From table 1 it may be concluded that all triglyceride samples can be used for further studies when the small impurities are taken into account. Only stearic acid C18:0 contains more than 1% other fatty acids. In earlier studies in our lab comparable results were found (RIKILT report 82.42).

Tabel 1.

Fatty acid composition of the triglyceride samples, expressed as percentages by mass of FAME/total FAME.

Fatty acid:	C4:0	C5:0	C6:0	C8:0	C10:0	C12:0	C14:0	rest
Sample								
TG C 4:0	99.9							0.1
TG C 5:0	0.1	99.7						0.2
TG C 6:0			99.9					0.1
TG C 8:0				99.7				0.3
TG C10:0					100.0			-
TG C12:0					0.1	99.2	0.6	0.1
TG C14:0							100.0	

Fatty acid:	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C20:0	rest
Sample								
TG C16:0	0.1	99.5		0.3				0.1
TG C16:1	0.4	0.2	99.3*					0.1
TG C18:0		0.3		98.8			0.5	0.4
TG C18:1				0.3	99.5**			0.2
TG C18:2					0.2	99.8		
TG C20:0							100.0	

* including 0.9% of a sub component (see annex II)

** including a non-determined isomer of C18:1 (see annex II)

3.2 Triglyceride composition of the samples.

In table 2 the data are given of the content of the main triglyceride in the samples. It seems that the samples are less pure than was concluded from the fatty acid study. One should keep in mind however that different fatty acids may lead to a one up to a threefold higher content of different triglycerides depending on the position of the different fatty acids in the triglyceride.

Tabel 2. Content of the main triglycerides in the samples, expressed as percentages by mass of triglyceride on total triglycerides.

Sample	%m/m
TG C 4:0	98.7
TG C 5:0	97.5
TG C 6:0	99.6
TG C 8:0	98.8
TG C10:0	99.7
TG C12:0	98.0
TG C14:0	99.8
TG C16:0	98.6
TG C16:1	97.6
TG C18:0	97.3
TG C18:1	98.9
TG C18:2	99.4
TG C20:0	100.0

Analyses on a special triglyceride capillary column with TAP (Triglyceride Analysis Phase) were only possible for the higher triglycerides above C36 since the lower triglycerides are too volatile at the lowest working temperature (260'C) of this phase. The higher triglyceride samples of fatty acids C12-20 showed secundary peaks in all samples as is illustrated in the chromatogrammes in annex III.

The lower triglyceride samples with fatty acids C4-C16 were analysed on the CP sil 5CB capillary column with split injection. In all these samples secundary peaks were also found, as is shown in annex IV.

Some of the peaks in annex III and IV are supposed to be mono and diglycerides with the same fatty acids as the main peak, the others are probably caused by the secundary fatty acids in the samples.

3.3 Mixtures of the samples.

With the results of the fatty acid composition of the samples three mixtures were prepared, one composed with the triglyceride samples C4-C14 and two with the samples C12-C20. The triglycerides were divided in these two groups on account of the unnatural broad range of carbonnumbers and consequently great differences in behaviour of these triglycerides with carbon numbers from 12-60! Especially the saturated triglycerides with high carbon numbers gave crystallization problems during the solution .These problems were solved by carrying out the methylester preparation at about 40'C.

3.3.1 Mixture with short chain fatty acids C4-14.

With the results of table 1 the composition of the mixture with fatty acids C4-C14 has been calculated and tabulated in table 3. Three separate determinations of the fatty acid profile have been performed with temperature programmed GCC and lead to a composition expressed as area percentages of the total area. Calibration factors relative to C12 have been calculated from these results and are also shown in table 3.

Table 3.
Calibration factors calculated from the area percentages (FA/FA) of the different groups of analyses (A,B and C) and the originally known content of the fatty acids C4-C14 in a mixture.

	Mass % in FA/FA	Area percentages FA/FA A	B	C	Calibration factors A n=2	B n=3	C n=2
C 4:0	14.13	12.27	12.18	11.56	1.230	1.252	1.358
C 5:0	14.38	13.32	13.17	12.69	1.153	1.178	1.260
C 6:0	14.19	13.82	13.65	13.38	1.097	1.121	1.179
C 8:0	14.03	14.40	14.37	14.40	1.041	1.053	1.082
C10:0	14.86	15.77	15.85	16.06	1.007	1.011	1.028
C12:0	15.46	16.52	16.68	17.18	1.000	1.000	1.000
C14:0	12.96	13.89	14.08	14.71	0.997	0.993	0.979

These calibration factors have been plotted in figure 1. From these figures eventually occurring noneluting materials in the samples might have been observed as discontinuities in the lines. Since this was not the case, it was conluded that the triglyceride samples of C4-C14 did not contain noneluting impurities. Chromatogrammes of the fatty acid and the triglyceride profile of the mixture are given in annex V. All data of the calibration factors with mean and standard deviation are given in annex VI.

3.3.2 Mixtures with long chain fatty acids C14-20.
With the results of table 1 the composition of two mixtures with fatty acids C12-C20 have been calculated and tabulated in table 4. The fatty acid profile of the two mixtures has been determined in simplo with isothermal GCC and leads to area percentages. From these results the calibration factors have been calculated relative to C16:0.

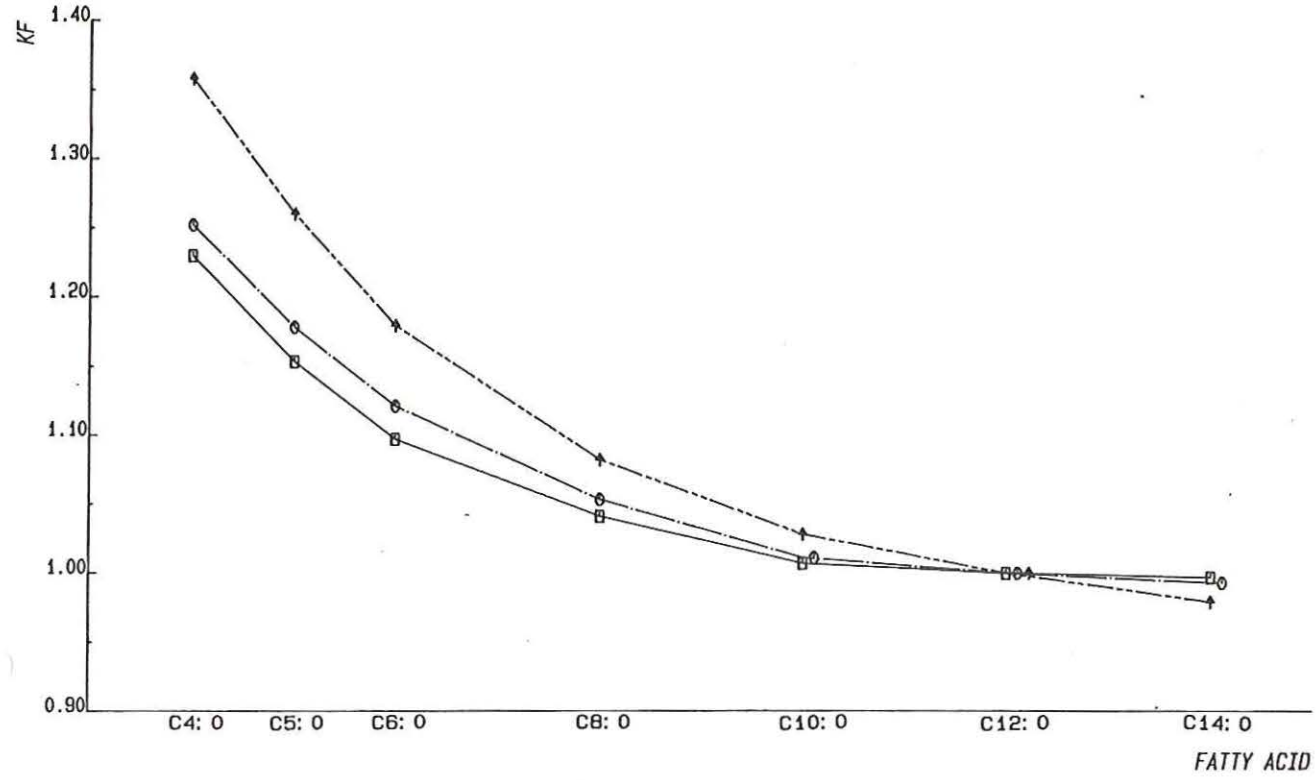

Figure 1: Calibration factors in relation to the fatty acids
Plot of data from table 3.
The analyses have been performed in three different periods.

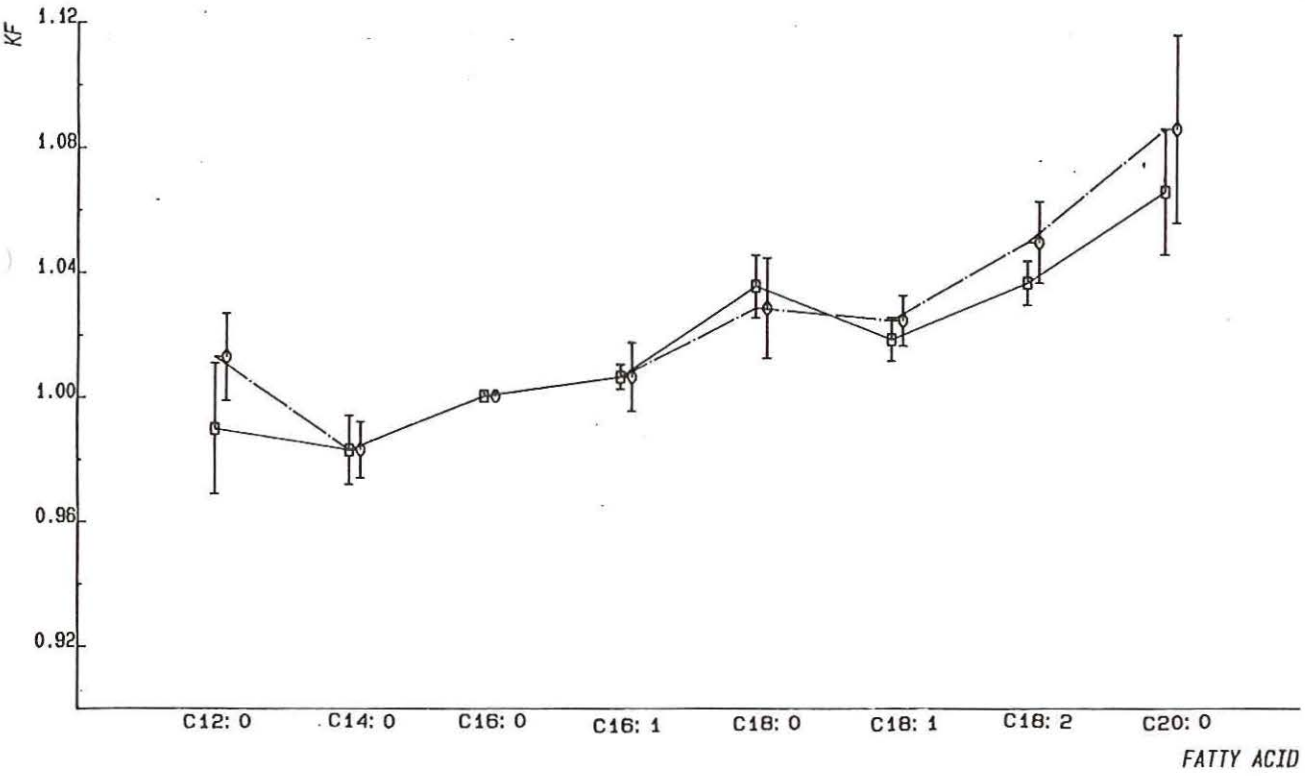

Figure 2: Calibration factors (with SD) in relation to the fatty acids with succeeding retention time. Plot of data from table 4.
The analyses have been performed in 9-fold on two mixtures.

Table 4.
Mean calibration factors of mixture I and II of the long chain fatty acids C12-C20, calculated from the area percentages FAME/FAME and the originally known content in mass percentages FAME/FAME.

	Mass % in FAME/FAME	Area % in FAME/FAME	Calibration Factor
MIXTURE I			
C12:0	12.94	13.21	0.990
C14:0	15.86	16.30	0.983
C16:0	13.32	13.46	1.000
C16:1	11.65	11.71	1.006
C18:0	9.34	9.12	1.035
C18:1	19.27	19.12	1.018
C18:2	11.58	11.30	1.036
C20:0	6.03	5.72	1.065
MIXTURE II			
C12:0	12.14	12.27	1.013
C14:0	11.26	11.74	0.983
C16:0	12.72	13.03	1.000
C16:1	10.81	11.01	1.006
C18:0	9.73	9.69	1.027
C18:1	12.08	12.09	1.024
C18:2	18.66	18.20	1.049
C20:0	12.61	11.92	1.085

The results of table 4 have been plotted in figure 2. Possibly occurring noneluting impurities in the samples might have given discontinuities in the lines. The unsaturated fatty acids seem to give slightly lower calibration factors than the corresponding saturated fatty acids which might be caused by the mentioned crystallization problems and or different behaviour of these fatty acids during the gaschromatographic separation. Chromatogrammes of the fatty acid and triglyceride profile of the mixture is given in annex V. All data of the calibration factors with mean and standard deviation are given in annex VII.

4. DISCUSSION AND CONCLUSIONS.

All samples were of high purity degree. No indications have been found of non eluting materials. Carefull precautions have been made for the preparation of especially the higher triglyceride mixtures since some crystallization of the triglycerides may occur in hexane at room temperature.

All or nearly all samples contain small amounts of secundary peaks probably caused by mono and diglycerides of the corresponding fatty acids as present in the main component and by triglycerides of the main fatty acid combined with adhering fatty acids.

Compared to earlier studies in our laboratory no indications of impurities have been found.

The increasing calibration factors of the long chain fatty acids in fig 2 may indicate lack of the chromatographic system. The slightly higher calibration factors of the saturated long chain fatty acids relative to the corresponding unsaturated ones indicate still some problems like crystallization, but the found differences were too small for a firm conclusion (the unsaturated triglycerides do not crystallize while the saturated do).

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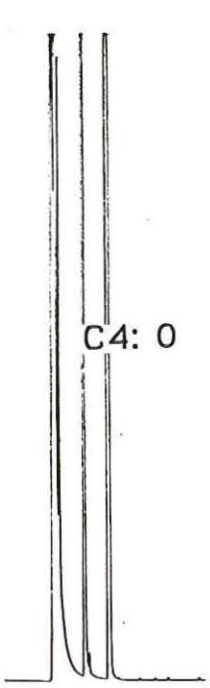
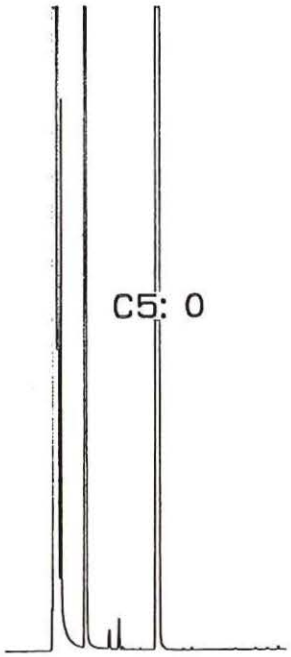
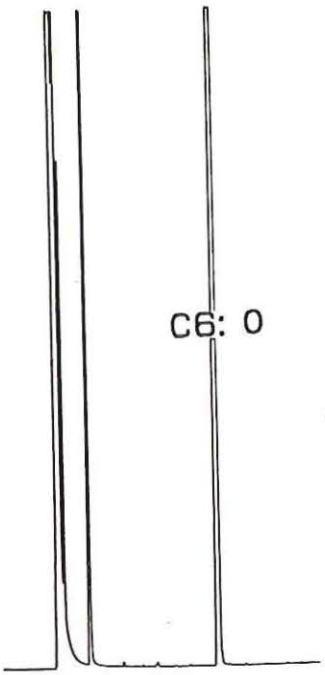
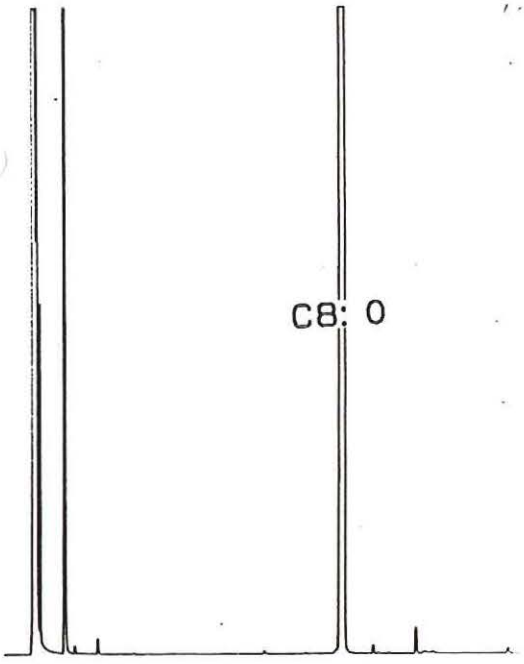
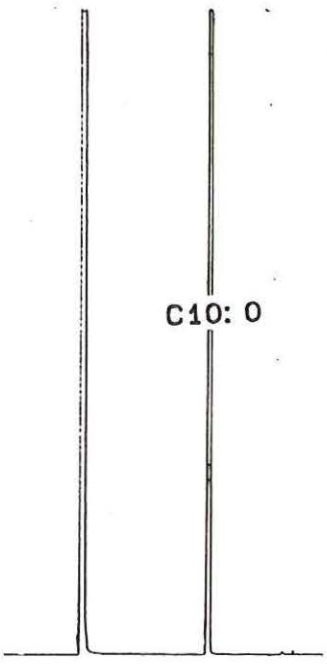
Fatty acid chromatogrammes of samples C4-C12

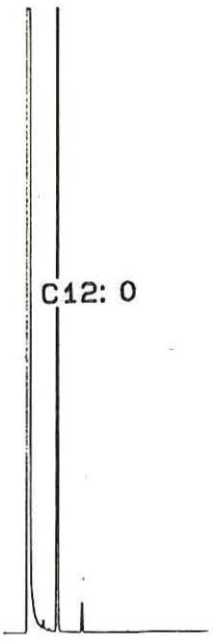




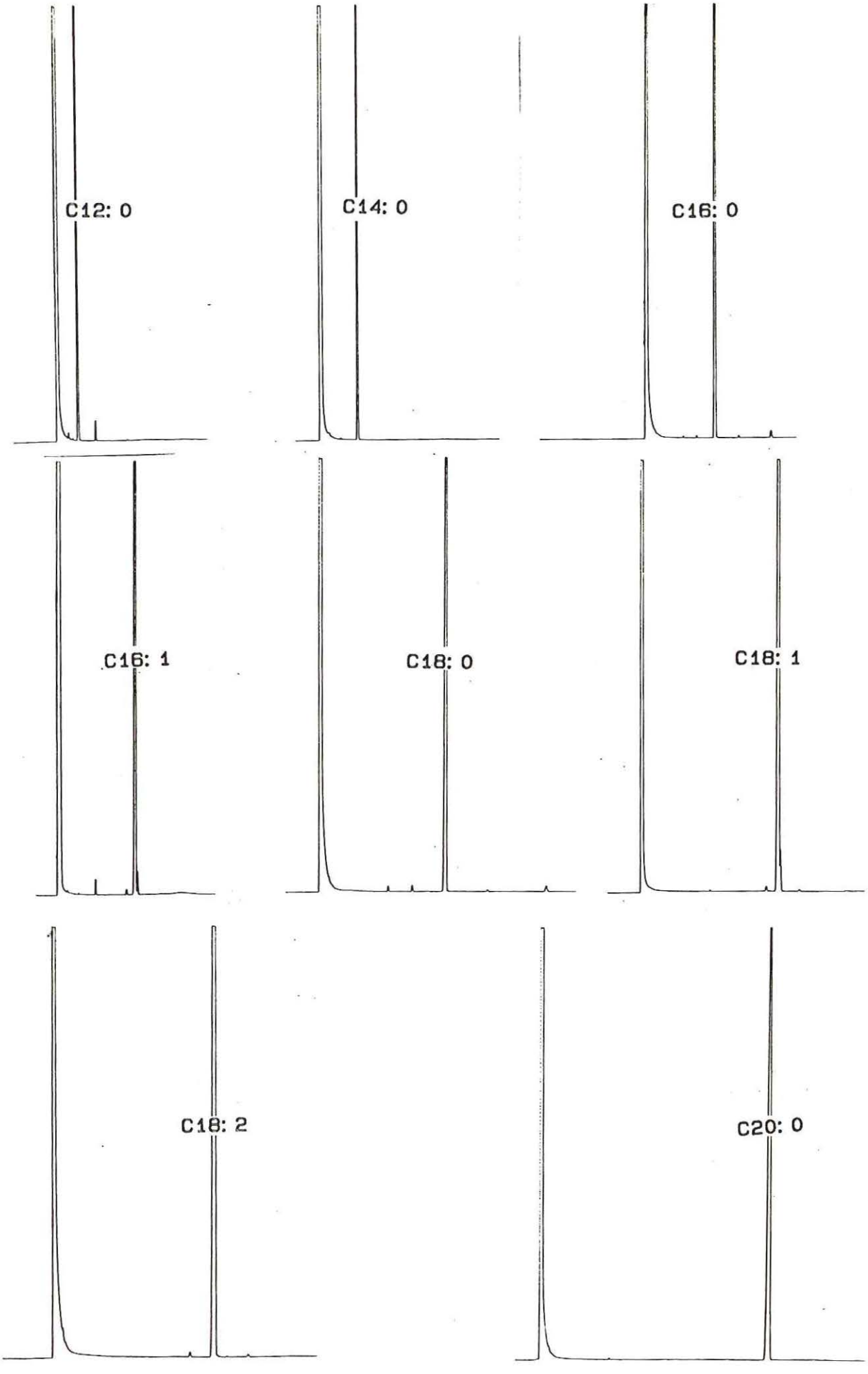




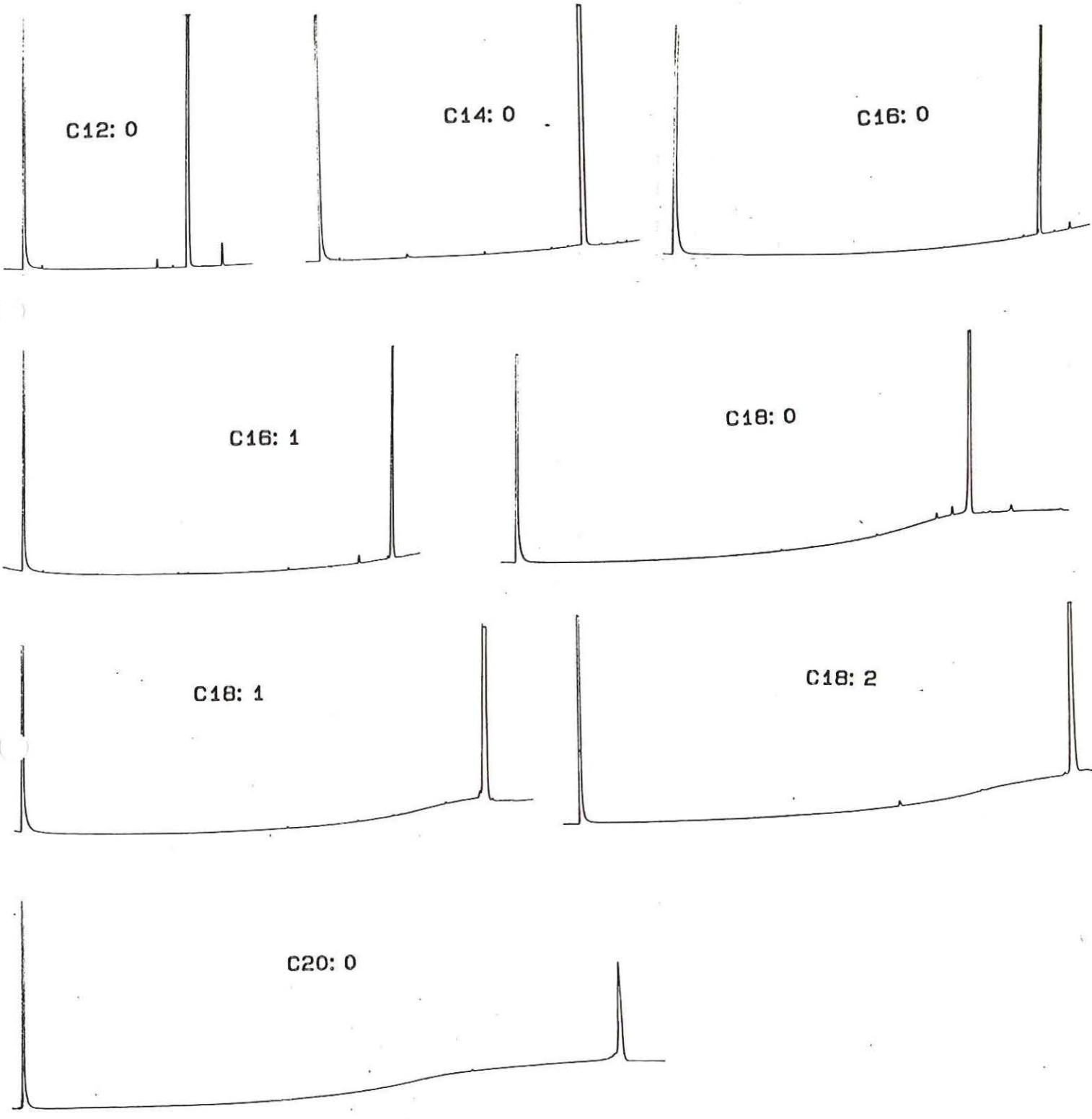




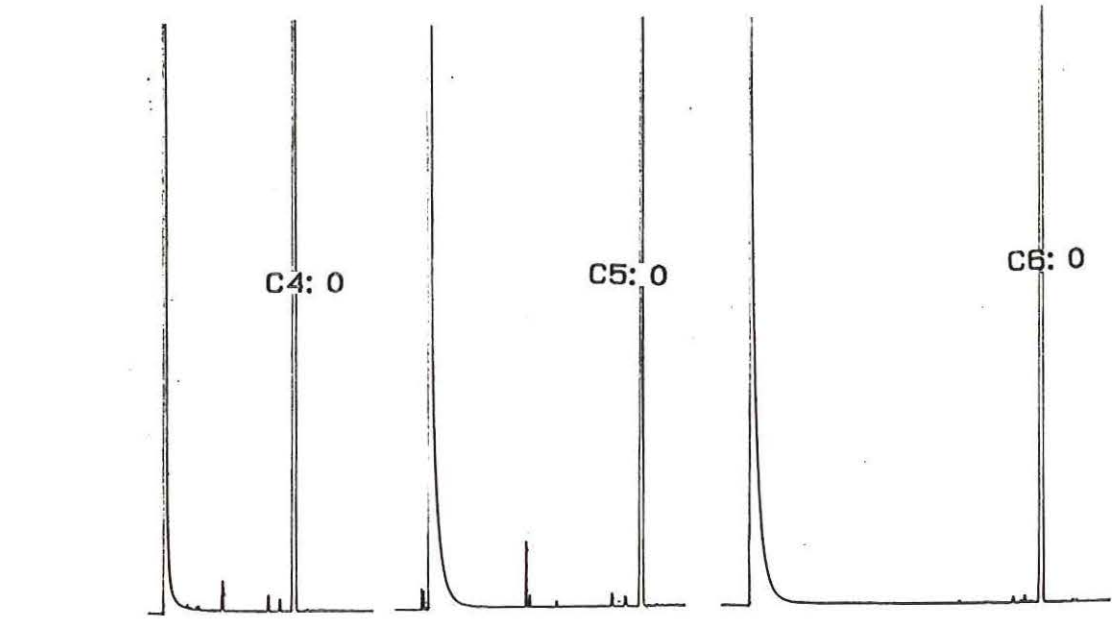
Fatty acid chromatogrammes of samples C12-C20

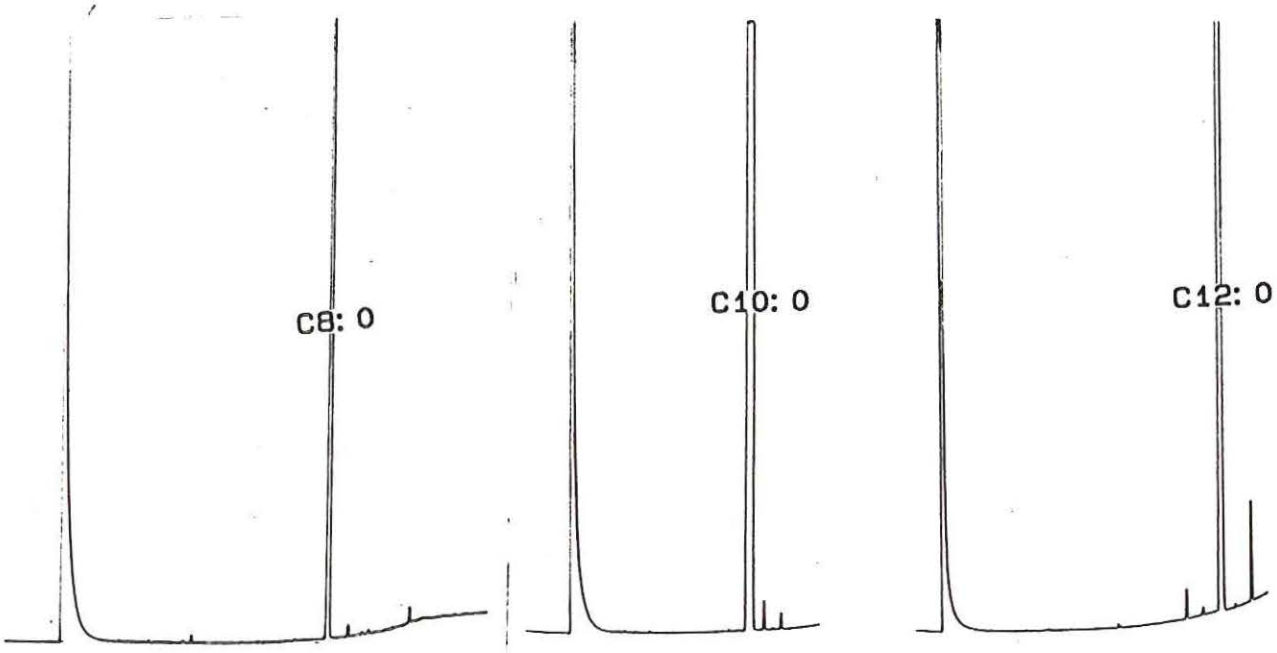


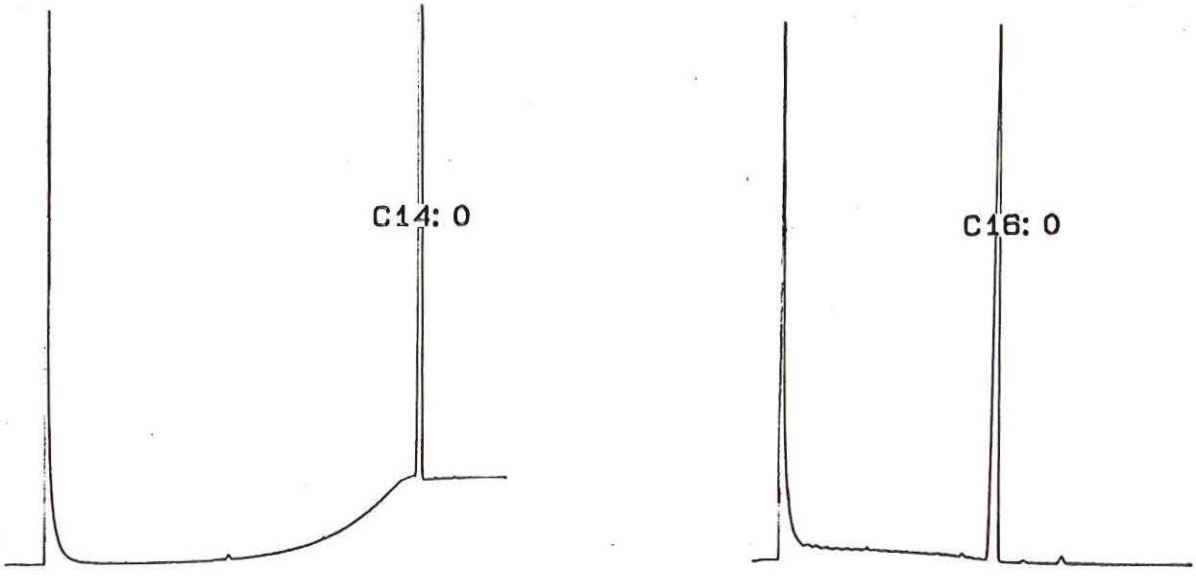
Triglyceride chromatogrammes of samples C12-C20 (TAP column)



Triglyceride chromatogrammes of samples C4-C16
(CP Sil 5 CB column)

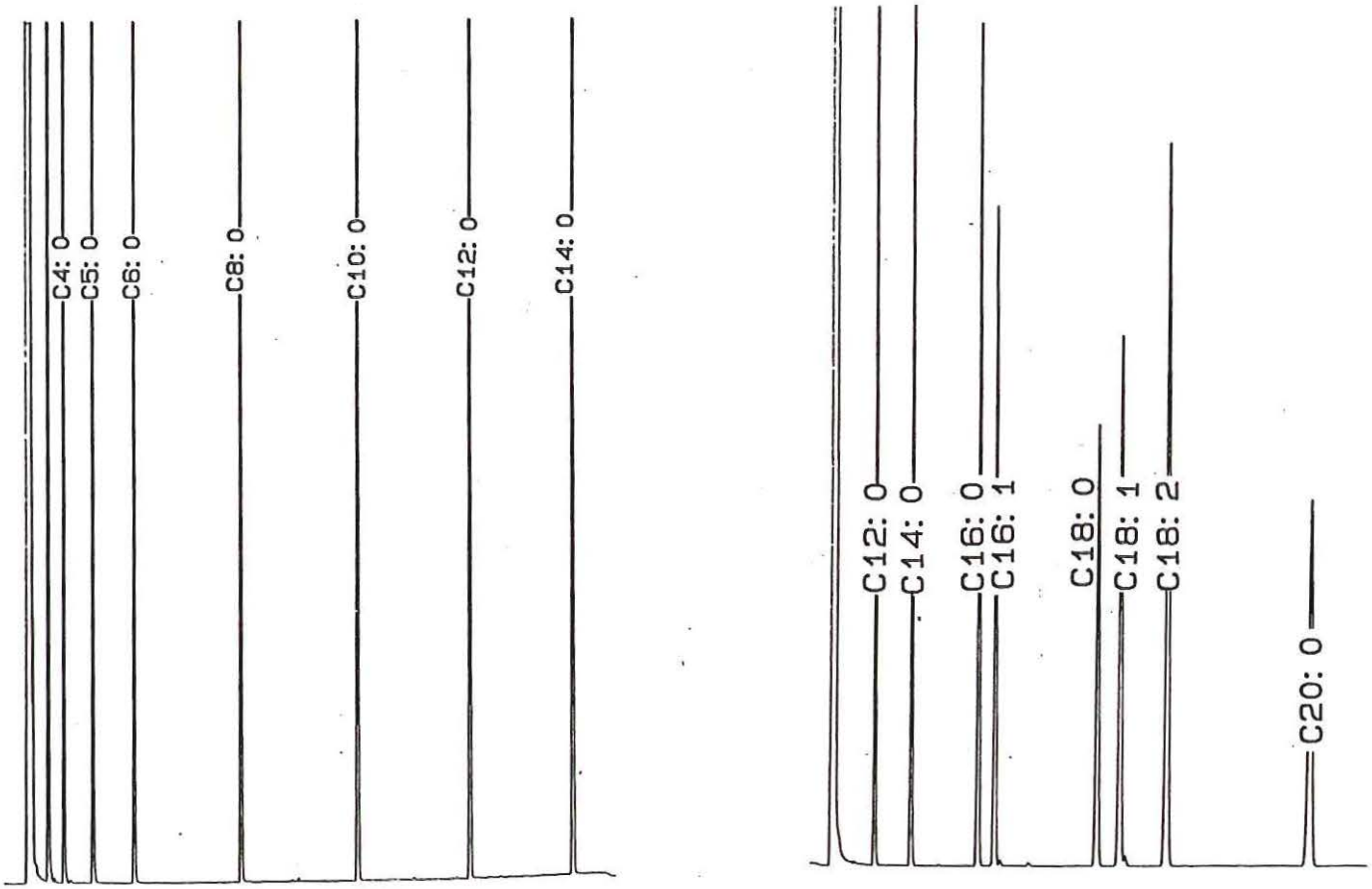




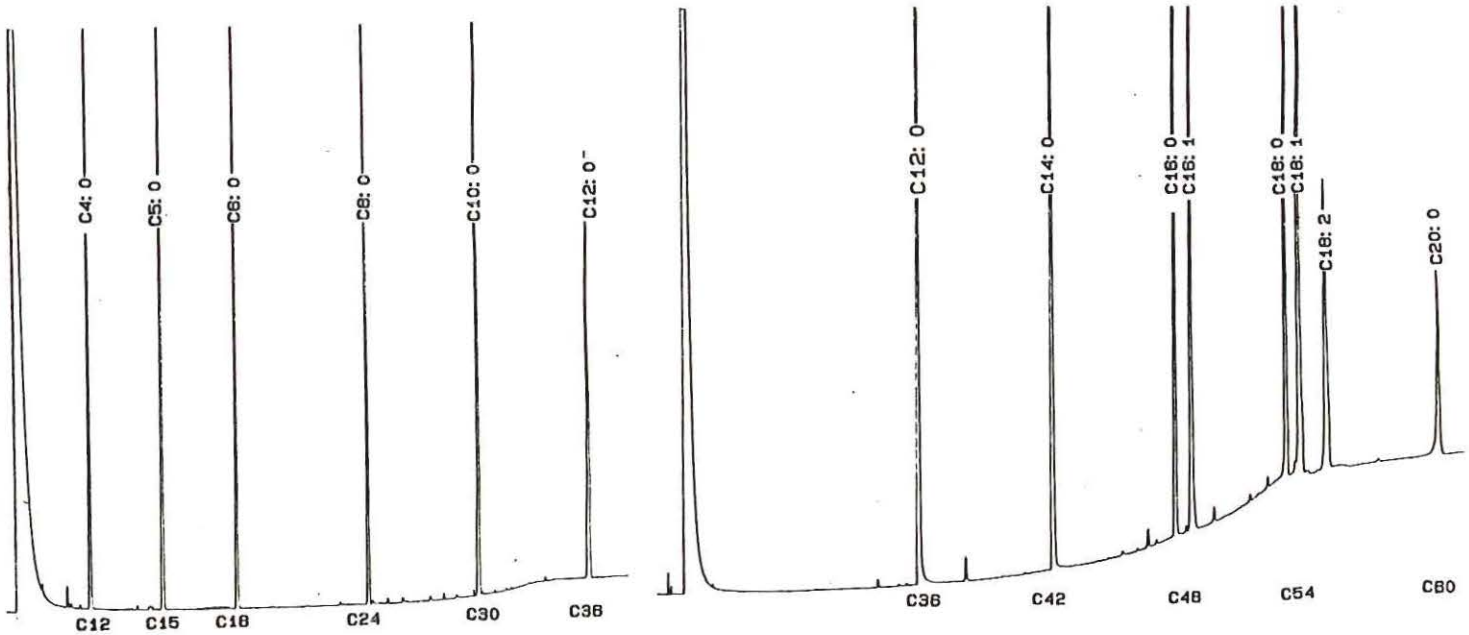


Fatty acid and triglyceride chromatogrammes of the mixtures of respectively C4-C14 and C12-C20.

Fatty acid profile



Triglyceride profile



Data of calibration factors (KF) of C4-C12 with mean and standard deviation (SD) per group of analyses (A,B,C).

Fatty acid	C4:0	C5:0	C6:0	C8:0	C10:0	C12:0	C14:0
A	1.228	1.156	1.100	1.043	1.011	1.000	0.993
A	1.232	1.150	1.094	1.038	1.003	1.000	1.000
Mean	1.2300	1.1530	1.0970	1.0405	1.0070	1.0000	0.9965
St.Dev.	0.0028	0.0042	0.0042	0.0035	0.0057	0.0000	0.0055
B	1.263	1.180	1.118	1.049	1.008	1.000	0.993
B	1.249	1.184	1.123	1.052	1.012	1.000	0.994
B	1.243	1.169	1.122	1.057	1.014	1.000	0.991
Mean	1.2517	1.1777	1.1210	1.0527	1.0113	1.0000	0.992
St.Dev	0.0103	0.0078	0.0027	0.0040	0.0031	0.0000	0.0015
C	1.352	1.252	1.171	1.075	1.025	1.000	0.978
C	1.363	1.268	1.186	1.089	1.031	1.000	0.979
Mean	1.3575	1.2600	1.1785	1.0820	1.0280	1.0000	0.9785
St.Dev.	0.0078	0.0113	0.0106	0.0099	0.0042	0.0000	0.0007

ANNEX VII

Data of calibration factors (KF) of C14-C20 with mean and standard deviation obtained from the analyses of mixtures 1 and 2.

Fatty acid	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C20:0
M	0.973	0.971	1.000	1.002	1.047	1.024	1.037	1.093
I	0.970	0.974	1.000	1.007	1.047	1.028	1.046	1.079
X	1.012	0.994	1.000	1.001	1.030	1.014	1.032	1.080
T	1.002	0.987	1.000	1.012	1.039	1.027	1.041	1.079
U	0.993	0.984	1.000	1.011	1.026	1.015	1.036	1.052
R	0.965	0.973	1.000	1.002	1.043	1.021	1.036	1.062
E	1.013	0.999	1.000	1.005	1.021	1.006	1.022	1.043
	1.015	0.996	1.000	1.009	1.029	1.016	1.039	1.069
1.	0.967	0.972	1.000	1.001	1.027	1.013	1.031	1.030
Mean	0.9900	0.9832	1.0000	1.0056	1.0345	1.0184	1.0356	1.0653
St.Dev.	0.0215	0.0113	0.0000	0.0044	0.0097	0.0074	0.0066	0.0203
M	0.994	0.975	1.000	0.999	1.022	1.020	1.048	1.073
I	0.999	0.972	1.000	0.998	1.041	1.028	1.056	1.106
X	1.031	0.986	1.000	1.004	1.026	1.017	1.049	1.079
T	1.004	0.976	1.000	1.000	1.042	1.031	1.060	1.112
U	1.002	0.974	1.000	1.008	1.050	1.039	1.066	1.114
R	1.028	0.985	1.000	1.019	1.013	1.015	1.054	1.075
E	1.009	0.984	1.000	0.992	1.037	1.024	1.051	1.105
	1.028	0.997	1.000	1.012	1.023	1.024	1.038	1.079
2.	1.021	0.996	1.000	1.024	0.997	1.016	1.021	1.019
Mean	1.0128	0.9827	1.0000	1.0064	1.0278	1.0238	1.0493	1.0846
St.Dev.	0.0141	0.0092	0.0000	0.0107	0.0164	0.0078	0.0132	0.0296

Total results of MIX 1+2

(n=18)

Mean	1.0014	0.9829	1.0000	1.0060	1.0312	1.0211	1.0425	1.0749
St.Dev.	0.0212	0.0100	0.0000	0.0080	0.0135	0.0079	0.0123	0.0265