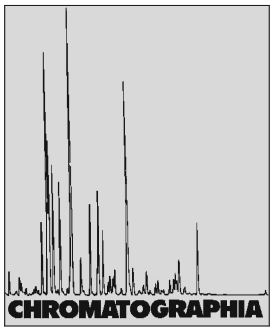
A Versatile GC Method for the Analysis of Alkylglycerols and Other Neutral Lipid Classes



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Abstract

The present study focuses on the GC analysis of alkylglycerols and other neutral lipid classes. For that matter, a simple and rapid method based on the direct on-column injection has been developed for the simultaneous analysis of numerous fatty samples from different sources. Chemical transformation or treatment of the sample before the injection of samples is not required. In addition, quantification of different lipid classes was also evaluated with satisfactory results. Excellent results regarding reproducibility and resolution were observed. This method can be utilized for multiple purposes such as analyses of edible oils, shark liver oils, deodorizer distillates, time course analyses of lipase-catalyzed reactions, etc.

Keywords

Gas chromatography
On-column injection
Non-polar lipids
Sterols
Alkylglycerols

Introduction

Lipid classes occur in nature as a complex mixture of molecular species in which the fatty acids and other aliphatic moieties are present in different combinations. One excellent method of resolving this complexity is to use high-temperature gas chromatography [1]. In some lipids, such as cholesterol esters, only the single fatty acid moiety will vary; in others, for example triacylglycerols, every position of each molecule may be esterified by a different fatty acid. Ideally, it would be preferable if lipids could be separated into individual molecular species without being modified in any way. The chromatographic methods used for the analysis of molecular species of lipids differ little in principle from those used for simpler aliphatic molecules such as the fatty acids. When they are applied to the isolation of molecular species of more complicated lipids, the separations achieved depend on the combined physical properties of all the aliphatic residues. If triacylglycerols are considered to illustrate the magnitude of the analytical problem, a triacylglycerol with only five different fatty acid constituents may consist of 75 different molecular species.

In addition to the separation of lipid classes, it is also possible to separate each particular lipid class according to their molecular weight but there is no useful resolution by degree of unsaturation, although some partial separations may be attained. High-temperature GC has until recently been used largely to separate molecular species simply according to the combined chain-lengths of the fatty acid moieties. However, improvements in technology have also led to separations according to the degree of unsaturation. In essence, high temperature GC is simply an analytical technique, but one which is capable of a high degree of precision. It can be married well with mass spectrometry.

The length of column used plays a crucial role and a compromise between

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Table 1. Retention times (t_R), resolution, and response factors (R_F) of the different lipids under study

	t_R (min)	Resolution	R_F
Dodecane	1.8	21.3	1.000
Linoleic acid methyl ester	4.3	89.0	1.370
Oleic acid	4.4	3.9	1.073
Batyl alcohol	5.5	24.0	1.074
2-Monolein	5.7	5.1	1.388
Squalene	6.1	7.8	0.869
α-Tocopherol	6.5	8.9	1.153
β,γ-Tocopherol	6.9	8.7	1.153
δ-Tocopherol	7.4	7.4	1.153
Stigmasterol	8.2	8.6	0.901
Palmityl palmitate	9.4	11.2	0.857
1,2-Di-*O*-hexadecyl glycerol	16.5	44.9	1.182
1,2 Diolein	21.5	35.5	1.919
1,3 Diolein	22.1	5.9	1.919
Cholesteryl ester	23.6	17.4	1.271
Cholesteryl ester	24.9	18.0	1.271
Cholesteryl oleate	26.4	13.0	1.271
1-*O*-Palmityl-2,3-dipalmitoyl-glycerol	27.8	9.4	0.772
Triolein	37.9	30.0	1.111

the optimum in terms of resolution with a need to limit the exposure time of the solute to high temperatures to the minimum has to be found.

Another important aspect is the precision that can be attained in quantification. It is virtually essential that electronic integration be applied for peak area measurements, ideally with some form of automatic base-line correction. Many different gas chromatographs have been used for the purpose, and most modern instruments appear suitable, but it is evident that the nature of the injection system can be of crucial importance. The minimum requirement is for some form of on-column injection. Grob [2], for example, demonstrated that techniques based on sample vaporization in the injector are not suitable for intact lipids as discrimination in favor of the less volatile constituents occurs. With splitless injection, most losses were found to be a consequence of insufficient elution from the syringe needle; split injection gave even worse results, although the reasons for this were not clear, and only cold on-column injection gave acceptable recoveries. Any involatile material (''dirt'') on the column from previous analyses affected the injection because of adsorption effects [3, 4]. In addition, the flow-rate of the carrier gas can have a marked effect on sample loss and discrimination, and thermal decomposition takes place to change the sample composition [5]. Cold on-column injection eliminates many of these problems, although other factors then come into play [6].

A method combining a baseline separation of the different components present in deodorizer distillate has been published [7]. Analysis of the tocopherol and sterol content of deodorizer distillate usually requires a laborious sample preparation procedure including saponification and derivatization. Determination of vitamin E compounds along with other important chemicals in vegetable oils and fats has been accomplished by capillary GC–FID with continuous on-line removal of triglycerides via transesterification [8]. Numerous GC methods for the analysis of sterols and steryl esters have been reviewed recently [9]. In addition, direct injection without derivatization for the analysis of free and esterified sterols has also been reported [10].

Analysis of alkylglycerols requires numerous steps including saponification, fractionation, silylation, and subsequent analysis [11]. For that matter identification and quantification of samples containing free and esterified alkylglycerols frequently combines GC and LC analyses and results laborious and very time consuming. Therefore, a method for simultaneous analysis of neutral lipid classes occurring in shark liver oil and its derivatives enormously facilitates the monitoring of processes involving these substances such as fractionation, purification, chemical and/or enzymatic modifications, and biological determinations.

Previously in our lab, an LC method with evaporative light scattering detection (ELSD) for the simultaneous analysis of alkoxyglycerols and other neutral lipid classes [12] has been developed. However, the separation achieved was not influenced by the nature of the aliphatic residues. In other words, normal phase chromatography is based on the polar groups of the molecule regardless of the non-polar side chain of the lipid class under study. In an attempt to improve the separation of the different lipid classes attained by the mentioned LC-ELSD, we have developed an easy and rapid GC methodology for the simultaneous analysis of neutral lipid classes including alkylglycerols.

The present study is based on the direct on-column injection without chemical transformation of the sample, for the analysis of numerous fatty samples from different sources. With this methodology, separation of numerous lipid classes according to both structure and chain length is attained. Hence, a higher number of chemical species from each lipid class can be simultaneously identified and quantified. This method is intended to be used for multiple purposes such as simultaneous analyses of alkylglycerols and other neutral lipids, edible oils, deodorizer distillates, lipase-catalyzed reactions, etc.

Experimental

Materials

Hexadecane and dodecane were purchased from Merck (Hohenbrunn, Germany) and stigmasterol, palmityl palmitate, cholesteryl oleate, squalene, linoleic acid methyl ester, 1,2-di-*O*-hexadecyl-*rac*-glycerol, 1-oleyl-*rac*-glycerol, oleic acid, 1-*O*-palmityl-2,3-dipalmitoyl-*rac*-glycerol, α and δ-tocopherol, trioleine, batyl alcohol and diolein were purchased from Sigma-Aldrich (Bornem,

Belgium) and were at least 90% pure except cholesteryl oleate that was technical grade. All solvents were LC-grade from Lab-Scan (Dublin, Ireland).

Gas Chromatography

Separations were performed on a Hewlett-Packard 5890 series II gas chromatograph with on-column injection using a 7 m 5% phenyl methyl silicone capillary column (Quadrex Corporation, New Haven, CT, USA) (0.25 μm I.D.). 12 cm of a 530 μm I.D. deactivated column was used as pre-column. An injector and detector temperature of 43 and 360 °C respectively were utilized. The temperature program was as follows: starting at 40 °C and then heating to 250 °C at 42 °C min^{-1} with 10 min hold, followed by heating from 250 to 325 °C at 7,5 °C min^{-1} with 30 min hold. Helium was used as a carrier gas at a pressure of 5,2 Psi. The peaks were computed using GC chemstation software.

Results and Discussion

Response Factors

Two different internal standards namely dodecane and hexadecane were utilized for the quantification of the different neutral lipids under study. Response factors were calculated accurately weighing the pure component and by means of five successive injections. Good repeatability of the response factors was observed over the period of time of the present study. Linoleic acid methyl ester, oleic acid, batyl alcohol (1-*O*-octadecylglycerol), 2-monolein, α-tocopherol, stigmasterol, palmityl palmitate, 1,2-di-*O*-hexadecyl glycerol, diolein, cholesteryl oleate, 1-*O*-palmityl-2,3-dipalmitoyl-glycerol, and triolein were used as the estimated response factors for fatty acid methyl/ethyl esters, fatty acids, non-esterified alkylglycerols, monoacylglycerols, tocopherols, sterols, waxes, dialkylglycerols, diacylglycerols, sterol esters, and triacylglycerols respectively. The response factors obtained are shown in Table 1. This table also shows the resolution of the different peaks and it can be observed that in all cases it was higher than 3.5, which indicates complete separation of all compounds under study.

Table 1 also shows the retention times and resolution of β,γ-tocopherol and δ-tocopherol that were obtained injecting a standard δ-tocopherol with a purity of ca. 90%. It should also be indicated that the cholesteryl oleate utilized in the lipid mixture was technical grade and contained also two other cholesteryl esters (t_R 23.6 and 24.9).

Table 2. Relative standard deviation (RSD) of the intra-day and inter-day response times for the different lipid classes analyzed

	Intra-day		Inter-day	
	SD	RSD	SD	RSD
Dodecane	0.003	2.06	0.002	1.81
Linoleic acid methyl ester	0.002	2.05	0.002	2.36
Oleic acid	0.003	2.26	0.004	3.00
Batyl alcohol	0.002	1.96	0.003	2.78
2-Monolein	0.004	5.64	0.004	5.30
Squalene	0.004	1.94	0.004	2.28
α-Tocopherol	0.001	1.86	0.020	23.33
δ-Tocopherol	0.001	2.30	0.022	22.94
Stigmasterol	0.003	1.90	0.003	2.31
Palmityl palmitate	0.003	2.03	0.003	2.25
1,2-Di-*O*-hexadecyl glycerol	0.002	2.10	0.002	2.32
1,3 + 1,2 Diolein	0.007	9.25	0.004	5.30
Cholesteryl ester	0.003	3.04	0.004	3.54
1-*O*-Palmityl-2,3-dipalmitoyl-glycerol	0.002	2.39	0.002	2.11
Triolein	0.004	3.00	0.004	2.72

Table 3. Relative standard deviation (RSD) of the intra-day and inter-day retention times for the different lipid classes analyzed

	Intra-day		Inter-day	
	SD	RSD	SD	RSD
Dodecane	0.004	0.24	0.003	0.15
Linoleic acid methyl ester	0.022	0.53	0.052	1.23
Oleic acid	0.026	0.59	0.060	1.36
Batyl alcohol	0.050	0.91	0.108	1.96
2-Monolein	0.054	0.96	0.117	2.06
Squalene	0.056	0.93	0.120	1.98
α-Tocopherol	0.057	0.89	0.123	1.90
γ-Tocopherol	0.058	0.85	0.123	1.79
δ-Tocopherol	0.059	0.80	0.128	1.74
Stigmasterol	0.060	0.74	0.125	1.53
Palmityl palmitate	0.066	0.71	0.130	1.39
1,2-Di-*O*-hexadecyl glycerol	0.048	0.29	0.079	0.48
1,2-Diolein	0.012	0.05	0.016	0.07
1,3-Diolein	0.016	0.07	0.016	0.07
Cholesteryl ester	0.008	0.03	0.009	0.04
Cholesteryl ester	0.008	0.03	0.008	0.03
Cholesteryl oleate	0.012	0.04	0.015	0.06
1-*O*-Palmityl-2,3-dipalmitoyl-glycerol	0.013	0.05	0.018	0.06
Triolein	0.053	0.14	0.048	0.13

Reproducibility

Variability intra-day and inter-day of both retention times and response was evaluated for the different lipid classes under study. The relative standard deviation of intra-day variation for retention times and areas never exceeded of 1 and 10, respectively (Tables 2 and 3).

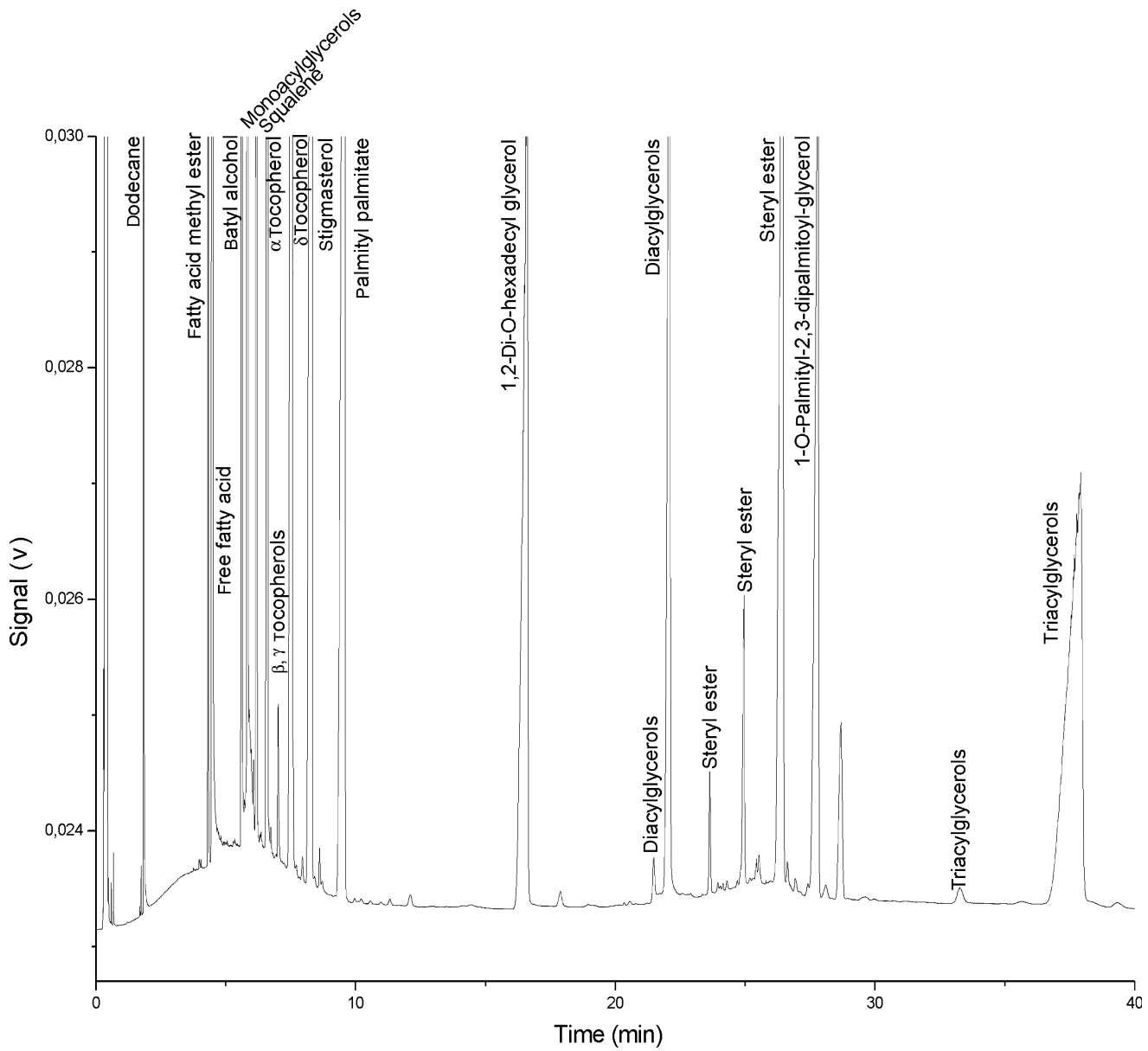

Fig. 1. Separation of the different lipid classes

To determine inter-day variation, six injections of the mixture ($n = 6$) of lipids under study were performed in five separate days during a total period of time of 1 month. Inspection of Table 3 indicates a very good reproducibility regarding the retention times and an acceptable precision in the response. It should be noted that variation inter-day of the response was smaller than 6 except for tocopherols (ca. 20). This result besides the apparition of a slight color in the solution containing the lipid mixture indicates than these compounds are partially oxidized over time. The rest of the compounds were stable along the study and provide very small variation in both response and retention time.

Quantification of Samples

In order to validate the proposed GC method, the lipid mixture utilized in the present study (Fig. 1) was also quantified. For that matter the response factors for each lipid class were utilized. Quantification of ca. 96% (*w*/*w*) of the total lipids analyzed over a month period was attained. Taken into consideration the lack of derivatization procedures in the present study and the partial degradation of tocopherols, the results obtained were excellent.

In addition, analyses of a deodorizer distillate from soybean oil, a mixture of steryl esters produced via lipase-catalyzed esterification of butteroil fatty acids with sterols, and an intermediate product of ethanolysis of shark liver oil were effected.

The results are shown in Figs. 2, 3, and 4. Quantification of these samples was also carried out (Table 4). The weight balance obtained for the three product mixtures 1, 2, and 3 was ca. 81, 110, and 75%, respectively.

Figure 3 shows fatty acid ethyl esters that can be separated by their corresponding chain length, non esterified alkylglycerols (separated in two peaks including batyl alcohol), monoesterified alkylglycerols (that possess a similar retention time to those of diacylglycerols), and diesterified alkylglycerols with retention times among those of 1-*O*-palmityl-2,3-dipalmitoyl-*rac*-glycerol and triacylglycerols.

In order to identify the different peaks, in some cases, such as the mixture of steryl esters, the product was first analyzed via LC according to our previously published method [11]. Hence, it is possible to know the composition of our product in lipid classes. Then, peak assignation can be easily effected considering two main assumptions: (i) the number of sterols and (ii) the number of fatty acids, according to chain length, contained in the product mixture. Figure 3 shows that ca. 24 different steryl esters can be separated. If one takes into consideration than three main sterols and eight different fatty acids were separated up to 24 different sterols could be obtained. This result indicates the versatility of the present methodology and how well it can work together with other analytical techniques such as LC.

In order to provide more accuracy in the identification of peaks this methodology is intended to be coupled to a mass spectrometrometer in the near future.

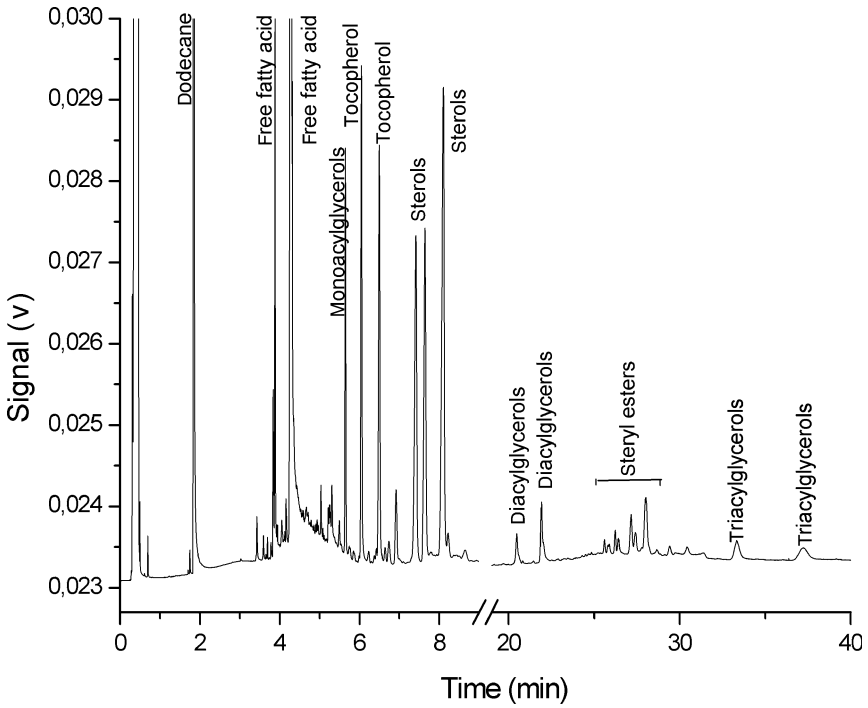

Fig. 2. GC analysis of a deodorizer distillate from soybean oil

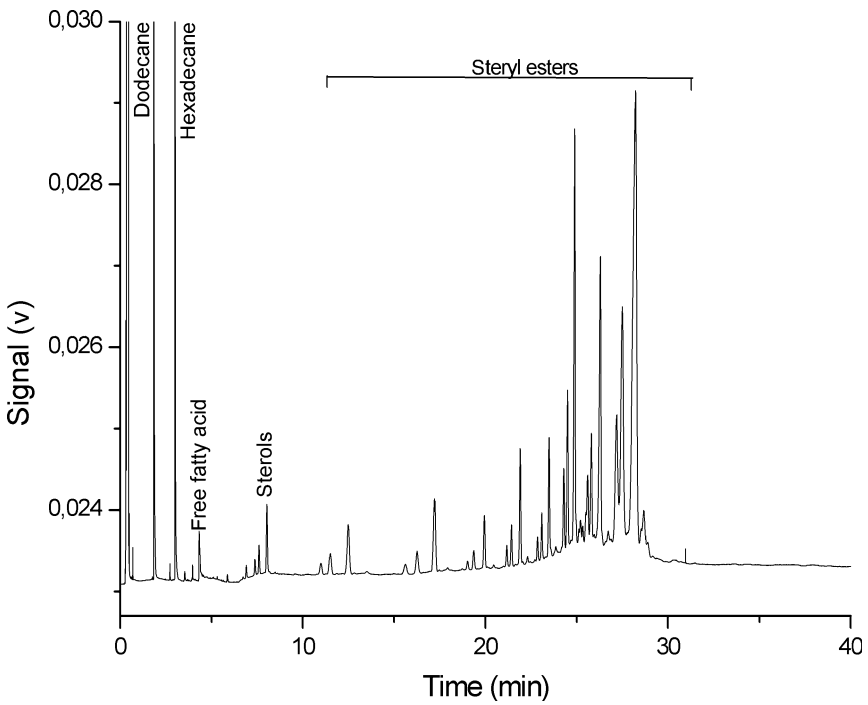

Fig. 3. GC analysis of steryl esters produced via lipase-catalyzed esterification of butteroil with sterols

Conclusions

The methodology herein described can be utilized for the analysis of numerous lipid classes without any treatment by simple on-column injection. Good reproducibility regarding the retention times and responses can be obtained for the different lipid classes studied. The response factors calculated can be utilized for quantification of major and minor constituents of numerous fats and oils. In addition, monitoring the kinetics of lipase-catalyzed reactions involving sterols, alkoxyglycerols and acylglycerols is also possible.

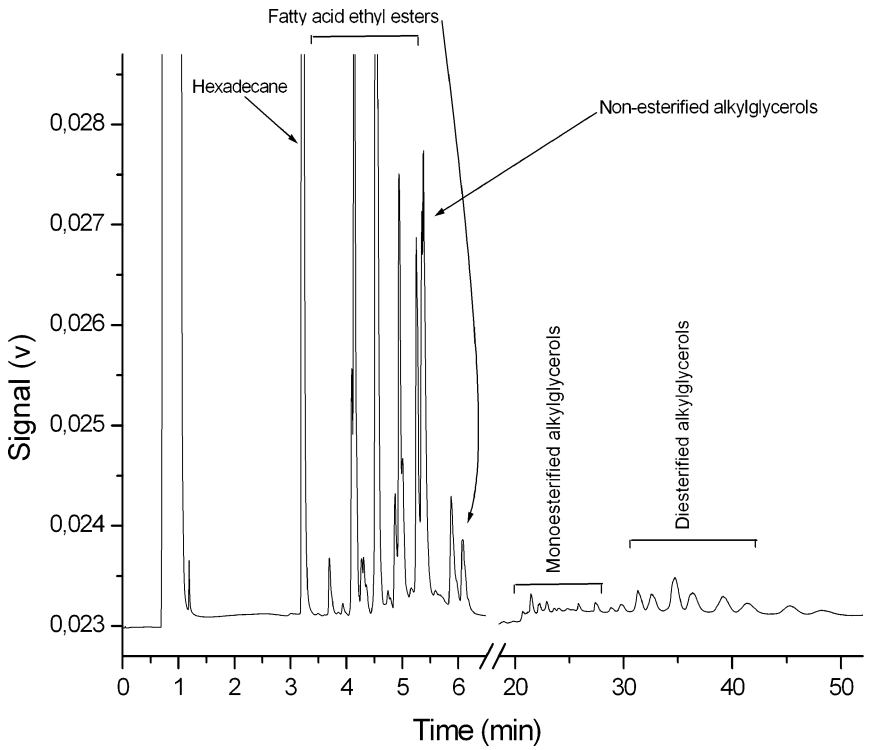

Fig. 4. GC analysis of an intermediate product obtained via ethanolysis of shark liver oil

Table 4. Quantification of a deodorizer distillate from soybean oil (1) a mixture of steryl esters produced via lipase-catalyzed esterification of butteroil fatty acids with sterols (2), and an intermediate product of ethanolysis of shark liver oil (3)

w/*w* (%)	1	2	3
Dodecane	23.4	4.6	0.0
Hexadecane	0.0	8.4	12.6
Fatty acid ethyl esters	0.0	0.0	35.4
Non-esterified alkylglycerols	0.0	0.0	5.5
Free fatty acids	26.0	1.0	0.0
Monoacylglycerols	5.8	0.0	0.0
Tocopherols	4.7	0.0	0.0
Sterols	10.3	1.3	0.0
Monoesterified alkylglycerols	0.0	0.0	4.4
Diacylglycerols	3.2	0.0	0.0
Steryl ester	6.5	95.4	0.0
Diesterified alkylglycerols	0.0	0.0	18.6
Triacylglycerols	1.5	0.0	0.0

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