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Short communication

Pyrrolidides as derivatives for the determination of the fatty acids of triacylglycerols by gas chromatography

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Abstract

Triacylglycerols can be converted quantitatively into the pyrrolidides of their constituent fatty acids. On the basis of this reaction, an easy and highly accurate gas chromatographic method for the determination of the fatty acids from triacylglycerols has been developed.

1. Introduction

Pyrrolidides have been recognized as useful derivatives of fatty acids for characterization by gas chromatography–mass spectrometry (GC–MS) [1–4]. We have recently worked out a convenient one-step procedure for the conversion of free fatty acids into their pyrrolidides [5]. This procedure consists in the treatment of the fatty acids with a mixture of trimethylsilylimidazole (TMSI) and pyrrolidine for several hours at room temperature, whereby quantitative derivatization results. No workup, except dilution with ethyl acetate, is necessary before GC analysis because all reagents and side-products (imidazole, TMSI, TMS-pyrrolidine and TMS-OH) are sufficiently volatile to be easily separated from the pyrrolidides of the common fatty acids by GC.

The ease of preparation and the thermodynamic stability of the pyrrolidides {$\Delta\Delta H$ of the reaction $CH_3COOCH_3 + HN(CH_2)_4 \rightarrow CH_3CON(CH_2)_4 + CH_3OH$ is calculated to be -1 ± 5 kJ mol^{-1} [6]} suggest that they might be useful not only for structure analysis by GC–MS, but also for the quantitative analysis of mixtures of fatty acids by GC.

Fats and oils are the most common source of mixtures of fatty acids. We introduce here a facile one-step method for the conversion of triacylglycerols into the pyrrolidides of their constituent fatty acids. In close analogy to the method described above for the free acids [5], triacylglycerols are reacted with a mixture of pyrrolidine and imidazole for a few hours at 60°C. After dilution with an inert solvent, the reaction mixture can be used directly for GC analysis.

2. Experimental

2.1. Sample preparation

The triacylglycerol (1 mg) is dissolved in a solution of 20 μl of pyrrolidine and 20 mg of

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imidazole and left at 60°C overnight to ensure quantitative reaction (shortening the reaction time by increasing the temperature is not recommended, because of a progressively intense yellow coloration of the reaction mixture above 60°C). The reaction mixture is diluted with 1 ml of ethyl acetate. Using the on-column injection technique, 1 μl of this solution is injected into the gas chromatograph.

2.2. Instrumentation

A Carlo Erba Vega 6000 gas chromatograph equipped with a flame-ionisation detector and an on-column injector was employed.

2.3. Gas chromatography

Two fused-silica columns, coated with different stationary phases were used: column A, 23 m × 0.32 mm I.D., coated with SE-30 [poly(dimethylsiloxane) gum, cross-linked] with film thickness 0.25 μm (laboratory made) and column B, 15 m × 0.25 mm I.D., coated with Stabilwax (Crossbond Carbowax-PEG) with film thickness 0.25 μm (Restek, Bellefonte, PA, USA). The carrier gas was hydrogen at a linear velocity of 50 cm s^{-1}. The injection port temperature was ca. 30°C and the detector temperature was 250°C for column A and 350°C for column B.

3. Results and discussion

Fig. 1 shows the results of a kinetic study on the conversion of glycerol trioleate into oleic acid pyrrolidide using the proposed reagent. For comparison, some other reagent mixtures are also shown, including a 1:1 (v/v) mixture of pyrrolidine and trimethylsilylimidazole used previously for the conversion of fatty acids into pyrrolidides [5]. Comparison of the reaction rates of the different imidazole-containing mixtures in Fig. 1 indicates that imidazole functions as a catalyst, presumably by the formation of a hydrogen bond to the carbonyl group of the ester, facilitating the nucleophilic attack of the

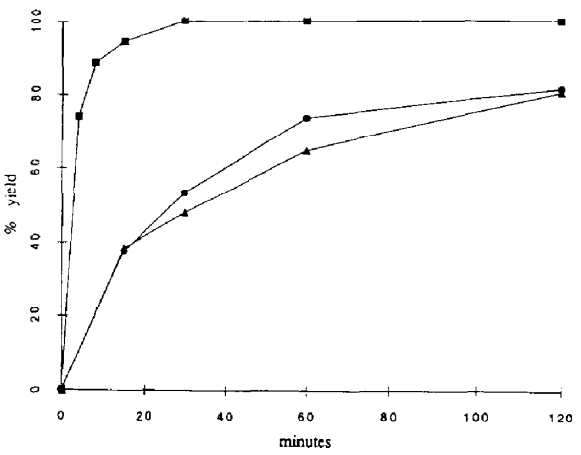

Fig. 1. Kinetics of the reaction of triolein with excess neat pyrrolidine at 60°C, catalysed in various ways. ■ = Pyrrolidine–imidazole (1:1, w/w); ● = pyrrolidine–imidazole (1:0.1, w/w); ▲ = pyrrolidine–trimethylsilylimidazole (1:1, w/w).

pyrrolidine. The intermediacy of an imidazolide is excluded because methyl stearate remains essentially unchanged in the presence of a large excess of imidazole in dimethylformamide at 60°C.

The series of chromatograms in Fig. 2 show the progressing conversion of glycerol trioleate into the pyrrolidide of oleic acid, passing through the intermediate diacylglycerols and the monoacylglycerols. (To facilitate the GC analysis, the samples were trimethylsilylated before injection into the gas chromatograph.) The series of chromatograms shows that the conversion of the fat becomes complete after about 40 min under the conditions given in the caption of Fig. 2.

The GC retention times of the pyrrolidides are expected to be longer than those of the methyl esters owing to three additional carbon atoms and the higher polarity of the amide group. This difference in retention times was quantified by determining the Kovats retention indices of both derivatives of selected fatty acids on a column with a poly(dimethylsiloxane) coating (column A). Table 1 shows the result of this analysis. As can be seen, the difference between the Kovats retention index of the pyrrolidides and the methyl esters is ca. 600 units in all three cases:

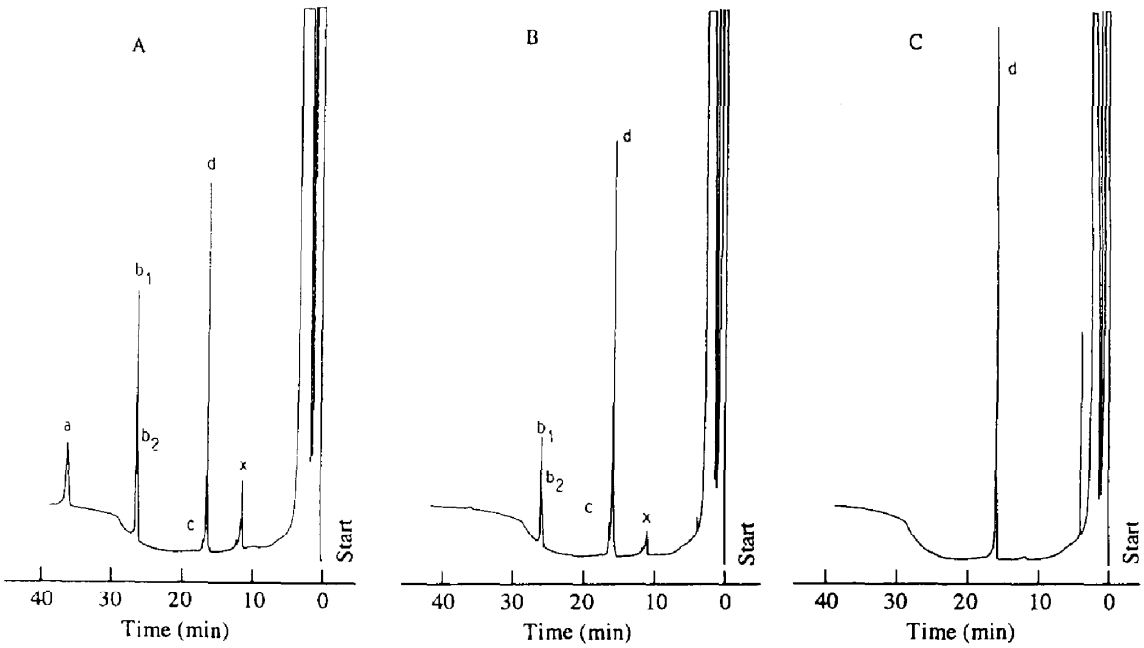

Fig. 2. Progress of the reaction of triolein with pyrrolidine–imidazole (1:1, w/w) at 60°C, (A) after 5 min, (B) after 10 min and (C) after 30 min. The mixture was diluted with bis(trimethylsilyl)trifluoroacetamide before injection into the gas chromatograph for better separation of the intermediates. Peaks: a = trioleine; b_1 = 1,3-dioleyl-2-TMS-glycerol; b_2 = 1,2-dioleyl-3-TMS-glycerol; c = 1,2-di-TMS-3-oleylglycerol; d = oleyl pyrrolidide; x = contaminant. GC temperature programme: column A, from 50 to 200°C at 25°C min^{-1}, 200 to 330°C at 5°C min^{-1}, held at 330°C for 10 min.

ca. 300 for the additional CH_2 groups and ca. 300 for the increased polarity.

We used silicon-based columns for the determination of the pyrrolidides. Carbowax columns, which are sometimes used for the separation of closely related methyl esters, were employed in an experiment designed to demonstrate a similar separating efficiency for methyl esters and pyrrolidides (Fig. 3). Owing to the lower volatility of the pyrrolidides, the bleeding of the column is more significant than it is for the corresponding methyl esters. Columns with increased thermal stability would be desirable for the pyrrolidides.

Table 1
Kovats retention indices of the pyrrolidides and the methyl esters of three selected fatty acids on SE-30 stationary phase

Acid	Pyrrolidide	Methyl ester
Palmitic acid	2520	1920
γ-Linolenic acid	2650	2050
Tetracosanoic acid	3350	2730

In order to assess this procedure with regard to its use for the determination of the fatty acids in fats and oils, we prepared first an equimolar mixture of methyl esters of fatty acids from C_{12} to C_{24} (because they are available from many fatty acids in highly pure form) and then a mixture of triglycerides from C_{12} to C_{20}. To achieve the highest possible accuracy, all esters were checked for homologues and other impurities by GC and capillary supercritical fluid chromatography (SFC), and the masses in the mixture were corrected where necessary. The mixtures were analysed by GC after conversion of the esters into the pyrrolidides as described under Experimental section. The results are reported as response factors (*RF* values) in relation to that of stearoylpyrrolidide whose *RF* was set to 1.000 (Table 2, column 3).

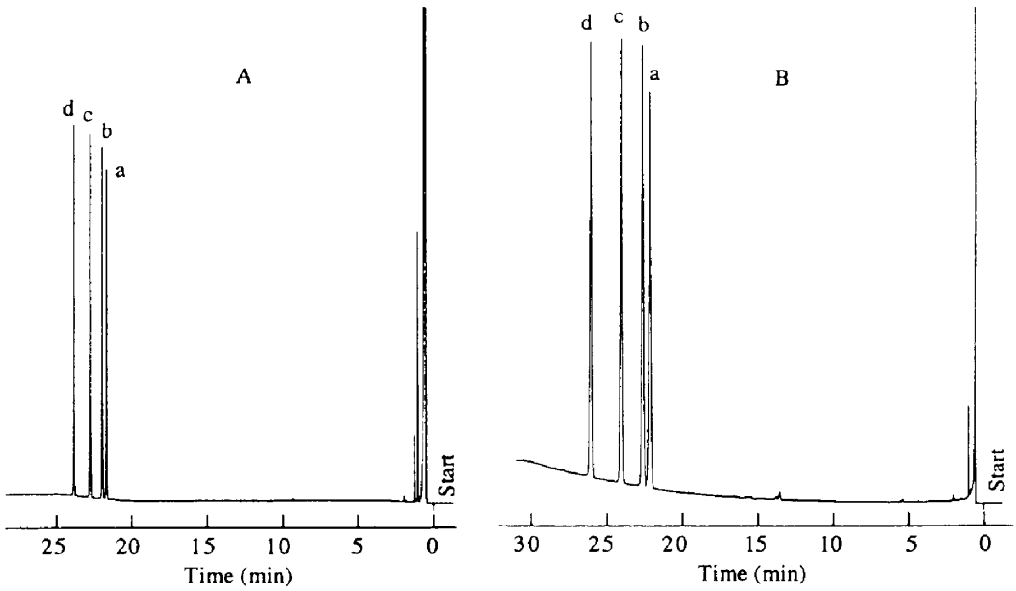

Fig. 3. Comparison of the separation of (A) the methyl esters and (B) the pyrrolidides of four C_{18} fatty acids on a Carbowax stationary phase. Peaks: a = stearic; b = oleic; c = linoleic; d = linolenic acid. GC temperature programme: column B, 50 to 190°C at 25°C min^{-1}, 190 to 250°C at 2°C min^{-1}, held at 250°C for 10 min.

For the methyl esters of the common saturated and unsaturated fatty acids it has been established in careful studies [7,8] that experimental relative response factors can be calculated by the following equation:

$$RF_{(n)} = \frac{M_n}{EC_n} \cdot \frac{EC_s}{M_s} \quad (1)$$

where M = molecular mass of methyl esters (n) and methyl stearate (s) and EC = mass of all "effective carbons", i.e. those in $-CH_2-$, $=CH-$ or $-CH_3$ groups (the degree of saturation is not relevant).

Taking the same approach, it should also be possible to calculate the relative RF values of pyrrolidides, provided the values of the effective carbons in the pyrrolidine ring are properly taken into account. From studies of secondary

Table 2
Determination of pyrrolidides produced from a mixture of fatty acid methyl esters

Compound	RF (calc.)[a]	RF (found)[b]	S.D. (%)[c]	S.D. (%)[d]
C_{12}	1.072	1.070	±0.1	±0.1
C_{14}	1.042	1.037	<±0.1	<±0.1
C_{16}	1.019	1.019	<±0.1	<±0.1
C_{18}	1.000	1.000		
C_{20}	0.985	0.982	<±0.1	±0.3
C_{22}	0.972	0.973	<±0.1	±0.3
C_{24}	0.961	0.962	±0.5	±0.3

[a] RF values calculated with Eq. 1.
[b] RF values found, mean of six samples, each analysed six times.
[c] Standard deviation of the mean of column 3.
[d] Average standard deviation of six GC injections from each sample.

amines, it is known that carbon atoms in the α-position must only be taken at half of their mass, i.e., 6 instead of 12 each [9]. Assuming that pyrrolidides behave in the same fashion as secondary amines and using Eq. 1, the values in column 2 in Table 2 are obtained.

Columns 3–5 in Table 2 show the results for the pyrrolidides obtained from the mixture of methyl esters. The average peak areas of the various pyrrolidides taken from six samples, each analysed six times, normalized with respect to that of stearic acid are given in column 3. They represent the *RF* values based on 1.000 for stearic acid. The standard deviations of the means are given in column 4. The standard deviations obtained for six parallel GC injections of one reaction mixture, representing the GC error, are given in the last column. It is seen that both errors are very satisfactory and, except for one at 0.5%, all are ≤0.3%. Comparing columns 2 and 3 it is seen that the experimental and the calculated values all agree very well also. The difference in the worst case is 0.5%.

The results for the pyrrolidides produced from the mixture of triglycerides are presented in Table 3. The experimental values in column 3 represent eight samples, each analysed twice by GC. The standard deviations (last column) are those of the overall error, consisting of derivatization and GC analysis. Comparing the results with those of Table 2 shows that they are of comparable quality. The higher deviation of the average values from the theoretical values for certain acids, e.g., palmitic acid, may be due to an impurity present in the sample in question, which raises the *RF* value found.

On the basis of these results, we are confident that the pyrrolidide method can be developed into a method for the analysis of fats and oils which is equally as accurate but much easier than all present methods using methyl esters.

The reagent mixture used in this analysis, pyrrolidine and imidazole, is of course not suitable to convert free fatty acids into pyrrolidides. If free fatty acids have to be dealt with, trimethylsilylimidazole has to be added to the reagents in order to convert the acids into trimethylsilyl esters for consecutive attack by pyrrolidine [5]. Longer reaction times are then required, as shown in Fig. 1. The same measure has to be taken if water is present in the oil to ensure that saponification of the triglyceride does not impair the results.

4. Conclusions

Fats and oils are quantitatively converted into the pyrrolidides of the constituent fatty acids under mild conditions using a mixture of pyrrolidine and imidazole. The diluted reaction mixture can be applied without further workup to the gas chromatograph for quantitative analysis. The accuracy and reproducibility of the results are comparable to those of the methods using methyl esters [8]. The whole procedure is considerably simpler and thus more easily amenable to automatic handling than any of the

Table 3
Determination of pyrrolidides produced from a mixture of fatty acid triacylglycerols

Compound	*RF* (calc.)[a]	*RF* (found)[b]	S.D. (%)[c]
C_{12}	1.072	1.066	±0.3
C_{14}	1.042	1.050	±0.3
C_{16}	1.019	1.028	±0.2
C_{18}	1.000	1.000	
C_{20}	0.985	0.988	±0.1

[a] *RF* values, calculated with Eq. 1.
[b] Average of eight samples, each analysed twice.
[c] Overall standard deviation of all sixteen analyses.

conventional schemes using methyl esters. Problems may arise when Carbowax columns are necessary for the separation of unsaturated fatty acids above C_{18}, because of the insufficient thermal stability of these columns at the required temperatures.

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References

[1] W. Vetter, M. Vecchi and W. Walther, *Helv. Chim. Acta*, 54 (1971) 1599.
[2] B.A. Andersson and R.T. Holman, *Lipids*, 9 (1974) 185.
[3] B.A. Andersson, *Prog. Chem. Fats Lipids*, 16 (1978) 279.
[4] W.W. Christie, *Gas Chromatography and Lipids*, Oily Press, Ayr, 1988, p. 166.
[5] W. Vetter and W. Walther, *J. Chromatogr.*, 513 (1990) 405.
[6] J.B. Pedley, R.D. Naylor and S.P. Kirby, *Thermochemical Data of Organic Compounds*, Chapman and Hall, London, 1986.
[7] R.G. Ackman and J.C. Sipos, *J. Am. Oil Chem. Soc.*, 41 (1964) 377.
[8] J.D. Craske and C.D. Bannon, *J. Am. Oil Chem. Soc.*, 64 (1987) 1413.
[9] R.G. Ackman, *J. Gas Chromatogr.* 6 (1968) 497.