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DETERMINATION OF TRIGLYCERIDES ACCORDING TO THEIR DEGREE OF UNSATURATION USING MERCURY(II) ACETATE ADDUCTS AND THIN-LAYER CHROMATOGRAPHY WITH FLAME-IONIZATION DETECTION

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SUMMARY

A rapid technique has been developed for the analysis of triglycerides with respect to their degree of unsaturation. This was achieved by preparing mercury(II) acetate adducts, which were separated on silica gel sintered rods and quantified with the flame-ionization detector of an Iatroscan TH-10 analyser.

The developing solvent was chloroform–light petroleum–acetic acid–methanol (25:25:1.5:0.15–0.40). The range of linearity found for different model substances was approximately 0.25–3.0 μg, giving a ratio of upper to lower limits of linearity of 12. Therefore, in some oils and fats it was necessary to carry out analyses with two different total amounts (two-level analysis). Different responses were found for different degrees of unsaturation, but not between carbon numbers. These responses were determined and found to bear a linear relationship with degree of unsaturation.

Analyses were carried out on palm oil, shea butter and mango kernel oil. Good agreement was achieved with results obtained by independent techniques. The standard deviation for the method varied between 0.19 and 0.71 wt.-% depending on the level determined.

INTRODUCTION

The triglyceride (TG) composition of oils and fats is normally very complex. To establish the complete composition is very difficult and for some isomers impossible in practice. However, in many practical instances limited information concerning the composition would be sufficient.

In, for example, cocoa butter equivalents, the degree of unsaturation of the TGs is very important. The traditional means of establishing this is by Ag^+ thin-layer chromatographic (TLC) separation and gas chromatographic (GC) quantification (using an internal standard) of the separated bands. Separations on silver-impregnated plates have several difficulties. The silver ions are sensitive to light and prepared plates have very limited durability. For degrees of unsaturation higher than 2 the π-bond localization also influences the separation, causing overlapping between dif-

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ferent degrees of unsaturation. The greatest disadvantage, however is, that the combination of Ag^+ TLC and GC is very time consuming and a faster method is very desirable.

The combination of TLC with a flame-ionization detector (FID) for quantification purposes was developed by Okumura and Kadano[1], the instrument being the Iatroscan TH-10 analyser. The TLC separation is achieved on sintered silica gel rods. Okumura *et al.*[2] have also shown that the technique could have several applications. Several papers[3–10] concerning quantitative separations of lipids have been published. Silver-impregnated rods have been used for the separation of olive oil TGs according to their degree of unsaturation[11]. The rods can be re-used after boiling in concentrated nitric acid for about 2 h and re-impregnated.

This paper reports the separation of TGs according to degree of unsaturation on silica rods utilizing the quantitative advantages of an Iatroscan instrument. However, instead of using silver ion-impregnated rods, the TGs are derivatized prior to the separation. This is achieved by well known reaction, *viz.*, the formation of methoxyacetoxymercuri adducts, which change the polarity considerably and enhance polarity differences between the different degrees of unsaturation.

The formation of mercuri adducts was first reported by Hofmann and Sand[12,13] in 1900, and later used in chromatographic separations of olefinic compounds by Inouye *et al.*[14]. Many workers[15–22] have studied the preparation of methoxyacetoxymercuri adducts and related compounds. The reaction is not simple and several by-products, depending on the reaction conditions, have been reported.

EXPERIMENTAL

Standards and natural samples

The model substances (purity ⩾99%) used are listed in Table I. Fatty acid residues are abbreviated as follows: Oc = octanoic, D = decanoic, La = lauric, M = myristic, P = palmitic, 17:0 = heptadecenoic, St = stearic, Ad = arachidic, Be = behenic, O = oleic, El = elaidic and L = linoleic acid.

Commercially available oils (palm, shea butter and mango kernel oil) were obtained from AB Karlshamns Oljefabriker (Karlshamn, Sweden). The TGs from these oils were isolated by silicic acid column chromatography[23] and their purity was checked by TLC.

Reagents

The solvents used were methanol (Merck, Darmstadt, G.F.R.), chloroform containing 0.6–1.0% of ethanol as a stabilizer (Merck), acetic acid (Merck), light petroleum (b.p. 35–60°C) (Mallinckrodt) and benzene (Fisher Scientific, Pittsburgh, PA, U.S.A.), all of analytical-reagent grade and used without further purification. Mercury(II) acetate (Merck) was of p.a. quality.

Adduct formation

Adduct formation was carried out by refluxing, with magnetic stirring, in a 5-ml round-bottomed flask. Different reaction conditions (time, temperature, chemicals, etc.) were tested. On completion of the reaction, the solvents were removed in a Rotavapor and the residue was dissolved in three 1-ml portions of chloroform; these

TABLE I

MODEL SUBSTANCES

Lipid class	*Structure*	*Abbreviation*	*Supplier*
Triglycerides	C24:0	OcO:Oc	Larodan Lipids, Malmö, Sweden
	C30:0	DDD	Larodan Lipids
	C36:0	LaLaLa	Nu-Chek-Prep, Elysian, MN, U.S.A.
	C42:0	MMM	Larodan Lipids
	C48:0	PPP	Nu-Chek-Prep
	C51:0	3 × 17:0	Hormel Institute
	C66:0	BeBeBe	Nu-Chek-Prep
	C52:1	StOP	Larodan Lipids
	C52:2	OOP	Larodan Lipids
	C54:2	StLSt	Larodan Lipids
	C54:3	OOO	Larodan Lipids
	C54:3	ElElEl	Hormel Institute, Austin, MN, U.S.A.
	C56:6	LLL	Larodan Lipids
Diglycerides	C24:0	1,3-LaLa	Nu-Chek-Prep
	C44:0	1,3-BeBe	Nu-Chek-Prep
Monoglycerides	C12:0	MG-La	Nu-Chek-Prep
	C20:0	MG-Ad	Nu-Chek-Prep
	C18:1	MG-O	Analabs, North Naven, CT, U.S.A.
Free fatty acids	C16:0	FFA-P	Hormel Institute
	C18:2	FFA-Ln	Hormel Institute

aliquots were combined in a test-tube and the mercury salts were separated from the solution by centrifugation. The clear solution was then ready for chromatographic separation.

Separation on TLC rods

The separation was performed on Chromarods (type S; sintered silica gel). Chloroform solutions of adducts (3–6 μg substance/μl) were spotted on the rods in volumes of 0.5–10.0 μl with a 10-μl Hamilton syringe. A glass frame containing the spotted rods was placed in a paper-lined glass tank. Development with appropriate solvents was performed until the solvent front reached approximately 1 cm from the top (this took 20–30 min, depending on the rods). The frame was placed in an oven (at 100°C for 2 min) to dry the rods, which were then placed in the Iatroscan TH-10 TLC analyser and scanned.

Instrument and operation conditions

An Iatroscan TH-10 TLC analyser (Iatron Labs., Tokyo, Japan) equipped with a flame-ionization detector was used for quantification, together with a Varian CDS 111 electronic integrator and a Varian Model 9176 recorder. The hydrogen and air flow-rate were 160 and 2000 ml/min, respectively. The scanning speed was 32 sec/scan. The recorder had a chart speed of 10 cm/min and a 1-mV full-scale deflection. The recorder attenuation on the integrator was normally 16.

Comparison with results from independent analytical methods

Results obtained with the reported method were correlated with two other

independent methods, namely (a) Ag^+ TLC–GC and (b) multi-step separation with HPLC and Ag^+ TLC in combination with GC quantification.

Ag^+ TLC–GC. The sample was dissolved in chloroform (10%, w/v) and placed on an Ag^+-impregnated thin-layer plate (Merck, silica gel 60, 0.25 mm thick). Chloroform was used as the eluent. The separated TG bands could be seen under UV light after spraying with 2,7-dichlorofluorescein in ethanol. An internal standard (trimargarin) was added to each band in appropriate amounts. Each band was scraped off, extracted with two 2.0-ml volumes of chloroform, transesterified with dimethyl carbonate–sodium methylate and analysed by GC for its fatty acid composition.

HPLC and Ag^+ TLC–GC. The multi-step separation and quantification of intact triglycerides by GC has been described elsewhere[24].

RESULTS AND DISCUSSION

Adduct formation

The adduct formation reaction to be performed was

$$-CH = CH- \xrightarrow[CH_3OH]{Hg(OCOCH_3)_2} -CH(OCH_3)-CH(HgOCOCH_3)-$$

Some difficulties arose in obtaining a pure reaction, *i.e.*, free from by-products, which showed up as extra peaks when chromatographed on the rods. Some workers[21,22] recommend benzene as a reaction medium, which was found to be successful. The conditions finally adopted were as follows. A 10-mg amount of sample was weighed into a round-bottomed flask and dissolved in 300 µl of benzene, then 300 µl of methanol and 40.0 mg of mercury(II) acetate were added. The mixture was refluxed for 20 min, all solvents were removed by evaporation and the mercury(II) acetate adducts were dissolved in chloroform.

Model substances treated in this way showed no extra peaks (Fig. 1). Even highly unsaturated TGs, e.g., trilinolein, reacted completely (Fig. 1).

Developing solvents

The rods were developed in a solvent mixture containing 25 ml of chloroform, 25 ml of light petroleum, 1.5 ml of acetic acid and 0.15–0.40 ml of methanol. The volume of methanol added varied slightly depending on the different sets of rods and

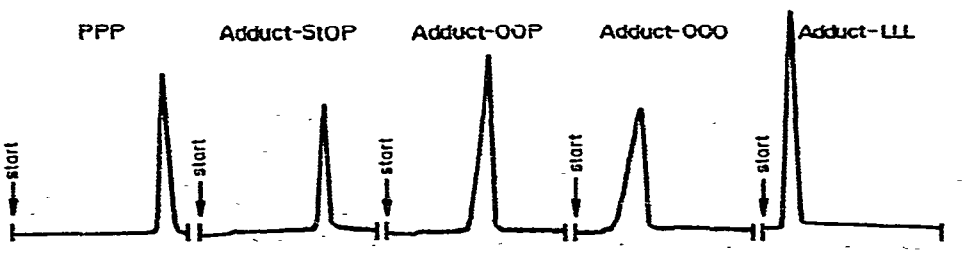

Fig. 1. Adduct formation with benzene, showing no extra peaks. Reaction conditions: 10 mg of substance, 300 µl of benzene, 300 µl of methanol and 40 mg of mercury(II) acetate were refluxed for 20 min. Even a highly unsaturated TG (LLL) reacted completely under these conditions.

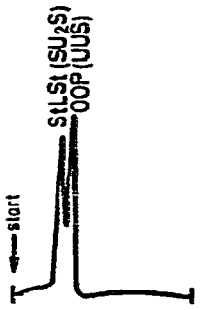

Fig. 2. Separation of diunsaturated TGs into TGs with two monoene acids (SUU) and one diene acid (SU_2S), using adducts from OOP and StLSt.

how many times the rods had been scanned earlier by the FID. Probably even variations in the ethanol content of the chloroform will have an effect. The methanol content of the eluent was found to be very critical, with the separation being governed by slight variations. An increase in the methanol content gave poorer separations between saturated and monounsaturated TGs but better resolution between compounds with higher degrees of unsaturation. For lower levels of methanol the situation was reversed. Separations were unsatisfactory in the absence of acetic acid.

Interferences were found between mercury(II) acetate adducts of partial glycerides and TGs. Depending on the amount and the accuracy required, these have to be removed before TG adduct formation.

Adducts of triolein and trielaidin were separated in this system. To ensure no interference caused by *trans*-double bonds present in partly hydrogenated TGs such samples should not be analysed.

With adducts of diunsaturated TGs, the influence of the positions of the π-bonds is considerable. This causes a separation giving a diunsaturated TG with two monoene acids (normally oleic acid) and a diunsaturated TG with one diene acid (normally linoleic acid) (Fig. 2). It is probable that the positions of the π-bonds in higher unsaturated TGs will have the same effect and probably result in interference with triunsaturated TGs. Because of this risk of overlap and because of the bad separation, all TGs with more than two π-bonds were taken as one group, referred to as "others".

Linearity

The range of linearity was determined using a mixture of PPP, StOP, OOP and OOO in equal amounts. Five rods were used for each total amount (varied by spotting different volumes on the rods). A view of the complete range tested can be seen in Fig. 3 for the adduct of StOP. The other model substances showed similar curves. The most suitable range was found to be in the lower part, and by controlling this range especially (Fig. 3; four insets), the upper and lower limits of linearity were obtained: PPP, 0.25–2.6 μg; StOP, 0.24–3.0 μg; OOP, 0.25–2.6 μg; and OOO, 0.22–2.9 μg. These give a ratio of upper to lower limits of linearity of approximately 12. In some oils and fats, *e.g.*, palm oil, this will be sufficient, but not in others, *e.g.*, shea butter. The problem with higher ratios could easily be solved by using a two-level analysis (see shea butter, Fig. 4 and Table II).

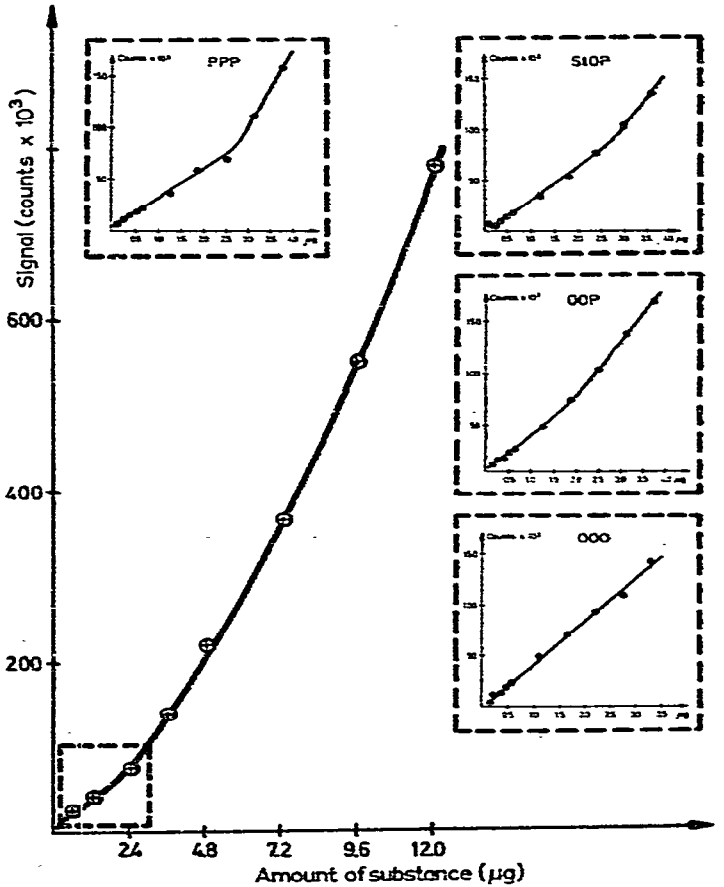

Fig. 3. Linearity of response for adduct of StOP (main curve) showing linearity over a wide range. Adducts of PPP, OOP and OOO gave similar curves. The lower part of the curve (broken line area) is that most suitable for good chromatographic separation and also the best linearity; this part of the scale is expanded for StOP and also for PPP, OOP and OOO in the four inset squares.

Response

A number of saturated TGs with different carbon numbers (C_{24}–C_{48}) showed no significant deviation in response with this method, but this was not so with the adducts with different degrees of unsaturation. Four mixtures with different ratios of PPP, StOP, OOP and OOO were used to examine these responses and the values found were: PPP = 1.00, StOP = 0.82, OOP = 1.05 and OOO = 1.34.

A plot of response *versus* degree of unsaturation showed approximate linearity (Fig. 5). The responses for tetra- and pentaunsaturation were calculated to be 1.60 and 1.90, respectively, by extrapolation. All responses found were inverted to give correction factors. This could be taken for all TGs with the same degree of unsaturation because of the independence from carbon number shown earlier, giving saturated TG = 1.00, monounsaturated TG = 1.22, diunsaturated TG = 0.95, triunsaturated TG = 0.75, tetraunsaturated TG = 0.63 (extrapolated value) and pentaunsaturated TG = 0.54 (extrapolated value).

TABLE II

CALCULATION OF THE COMPOSITION, ACCORDING TO THE DEGREE OF UNSATURATION, OF SHEA BUTTER BASED ON A TWO-LEVEL ANALYSIS

Ratios from 1.5 μl of spotted solution of shea butter adduct ($n = 5$) (all peaks used within the linear range): monounsat./diunsat. (SU_2S) = 4.77, 4.68, 4.66, 4.63, 4.56 (mean = 4.66); diunsat. (SUU)/diunsat. (SU_2S) = 3.99, 4.22, 4.21, 4.08, 4.15 (mean = 4.13); "others"/diunsat. (SU_2S) = 3.18, 3.28, 3.39, 3.34, 3.49 (mean = 3.34). Ratios from 3.0 μl of spotted solution of shea butter adduct ($n = 5$) (all used peaks within the linear range): saturated/diunsat. (SU_2S) = 0.21, 0.24, 0.22, 0.21, 0.19 (mean = 0.21). Take diunsat. (SU_2S) = 1.00, which gives all degrees of unsaturation equal to their mean values. Multiply by the correction factors and normalize to 100%.

TG	*Normalized mean value*	*Correction factor*	*Corrected value*	*Normalized to 100%*
Saturated	0.21	1.00	0.21	1.6
Monounsaturated	4.66	1.22	5.69	43.5
Diunsaturated (SUU)	4.13	0.95	3.92	30.0
Diunsaturated (SU_2S)	1.00	0.95	0.95	7.3
"Others"	3.34	0.70	2.30	17.6

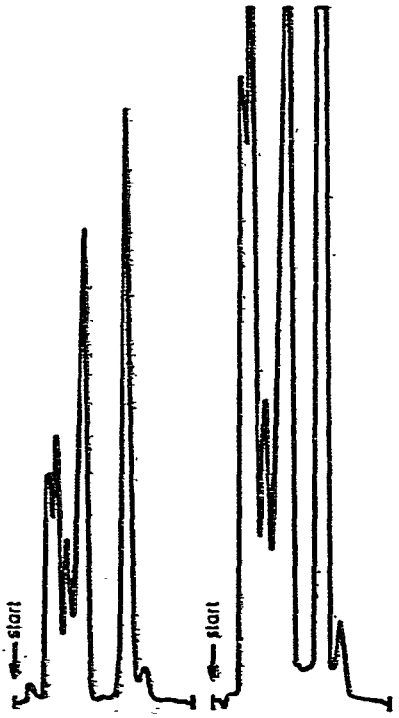

Fig. 4. Two-level analysis of shea butter adduct. By spotting 1.5 and 3.0 μl of the prepared adduct solution all peaks came into the linear area. Eluent: chloroform–light petroleum–acetic acid–methanol (25:25:1.5:0.18).

Oils and fats with a high content of highly unsaturated TGs will not be suitable for analysis by this technique because of poor or no separation. Samples with a low content of "others" have to be roughly estimated using the extrapolated correction factors and an approximated distribution. In these instances errors in "others" will have little effect on the results on saturated, mono- and diunsaturated TGs. The correction factor used for "others" under *Applications* (see below) was 0.7.

Applications

Palm oil was converted into mercury(II) acetate adducts and subjected to one-level analysis (Fig. 6). Calculations, with correction factors, showed good agreement with the results of an independent technique[24] (Table III).

Shea butter had to be subjected to two-level analysis (Fig. 4). The results were in good agreement with those from the independent Ag^+ TLC–GC technique (Table III). Even mango kernel oil was analysed in this way and gave similar results (Table III).

Reproducibility and accuracy

Shea butter was analysed six times (including adduct formation) and the standard deviation (SD) and coefficient of variation (CV) were calculated (Table IV). Good reproducibility was found. A significantly higher CV (11.3%) was found for saturated TG, which is related to its low absolute concentration.

As mentioned under *Applications*, no significant deviations were found on comparing the results for palm oil, shea butter and mango kernel oil with those

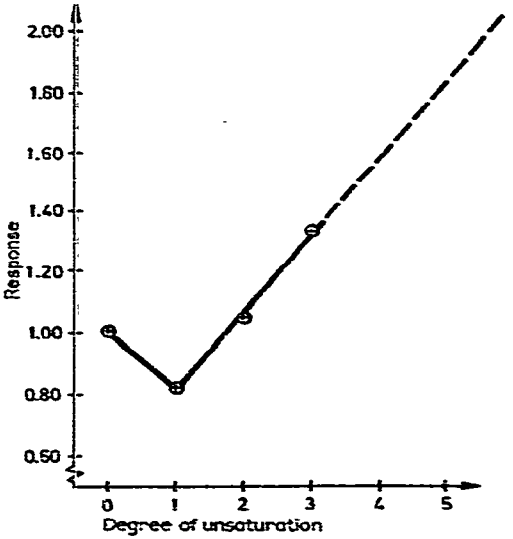

Fig. 5. Plot of response *versus* degree of unsaturation. Approximate linearity was found for the TG adducts.

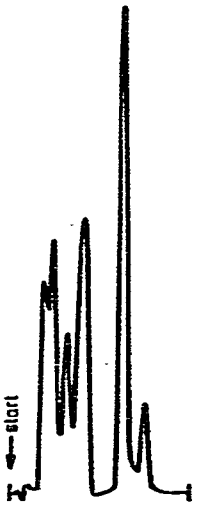

Fig. 6. One-level analysis of palm oil adducts. Eluent: chloroform–light petroleum–acetic acid–methanol (25:25:1.5:0.15).

TABLE III

PALM OIL, SHEA BUTTER AND MANGO KERNEL OIL ANALYSED BY THE PROPOSED METHOD AND INDEPENDENT TECHNIQUES

Palm oil (one-level analysis) as mean values from two determinations. Shea butter (two-level analyses) as mean values from six determinations. Mango kernel oil (two-level analyses) as mean values from two determinations.

Degree of unsaturation (TG)	*TG content (wt.-%)*					
	Palm oil		*Shea butter*		*Mango kernel oil*	
	Proposed method	*Independent technique*	*Proposed method*	*Independent technique*	*Proposed method*	*Independent technique*
Saturated	4.7	5.0	1.7	1.4	1.5	1.2
Monounsaturated	39.4	36.3	43.3	40.2	55.8	51.7
Diunsaturated (SUU)*	24.5	26.0	29.8	41.5	20.3	29.8
Diunsaturated (SU_2S)*	11.5	9.2	7.6		6.8	
"Others"	20.1	23.7	17.6	16.9	15.7	17.2

* U = monoene acid; U_2 = diene acid.

TABLE IV

REPRODUCIBILITY

Six analyses (including derivatisation) on shea butter.

Degree of unsaturation (TG)	*Individual results for TGs in shea butter (wt.-%)*	*Mean value (wt.-%)*	*SD (wt.-%)*	*CV (%)*
Saturated	2.0, 1.4, 1.7, 1.7, 1.8, 1.7	1.7	0.19	11.3
Monounsaturated	44.3,43.2,42.3,42.7,43.4,43.7	43.3	0.71	1.7
Diunsaturated (SUU)*	29.2,29.5,30.4,30.3,29.3,30.0	29.8	0.52	1.7
Diunsaturated (SU_2S)*	7.7, 7.8, 7.9, 7.4, 7.5, 7.5	7.6	0.20	2.6
"Others"	16.8,18.2,17.7,18.0,18.0,17.1	17.6	0.51	2.9

* U = monoene acid; U_2 = diene acid.

obtained by independent techniques (Table III). A true test of the accuracy of the method, by the standard additions technique, has not been carried out, however.

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