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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF FATTY ACIDS: QUANTITATIVE ANALYSIS OF SATURATED, MONOENOIC, POLYENOIC AND GEOMETRICAL ISOMERS

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SUMMARY

A procedure is described for the rapid preparation of phenacyl and naphthacyl derivatives of fatty acids. These derivatives of saturated, monounsaturated, and poly-unsaturated fatty acids were analyzed by high-performance liquid chromatography (HPLC) on a C_{18} reversed-phase column at nanogram sensitivity. Standard mixtures of fatty acids gave quantitative mole percentages. HPLC analyses of phenacyl and naphthacyl derivatives of fatty acids from several seed oils compared well with values obtained by gas–liquid chromatography (GLC). Oleic and elaidic, geometrical oc-tadecenoate isomers, were well resolved. Three of the four $\Delta^{9,12}$-octadecadienoic acids, geometrical isomers of linoleic acid, were also resolved. The rapid quantitative analysis of fatty acid phenacyl derivatives by HPLC hold some advantages over GLC, especially with samples that contain heat labile and short-chain fatty acids.

Phenacyl derivatives of monoenoic fatty acids were also shown to undergo *cis–trans* isomerization when exposed to ultraviolet light.

INTRODUCTION

The use of high-performance liquid chromatography (HPLC) for the analysis of lipids has lagged primarily because most lipids do not contain chromophores to facilitate detection. Generally, the refractive index detector has been used for most applications, but its stability and sensitivity leave something to be desired. The variable-wavelength ultraviolet–visible detector is frequently used in the region between 195 and 206 nm, but the response is affected by degree of unsaturation and the nature of non-acyl moieties[1–3] and one is limited to a very few solvents that are transparent in this region of the spectrum. Another reason for the limited use of HPLC for the analysis of lipids is that fatty acids, the major constituents of most complex lipids, have traditionally been analyzed quantitatively by gas–liquid chromatography (GLC) while the analysis of fatty acids by HPLC, up to now, was not quantitative. The efforts of those pioneers who have analyzed fatty acids by HPLC have been directed at improving resolution of components and improving the sensi-

tivity of detection. Attempts to improve sensitivity have included a variety of deriva-tives and these are covered in a review by Cooper and Anders[4]. Since then, a variety of long-chain fatty acids have been analyzed as their phenacyl esters[5], p-bromophen-acyl esters[6,7] and m-methoxyphenacyl esters[8] by reversed-phase HPLC. These studies emphasized the resolving power of the system and the sensitivity of derivative detec-tion. Quantitation was either not examined or found non-existent.

The present paper describes the rapid preparation of phenacyl and 2-naph-thacyl derivatives of standard and natural fatty acid mixtures and the quantitative analysis by reversed-phase HPLC. A preliminary report of this work has appeared[9].

MATERIALS AND METHODS

Materials

Individual fatty acids >99 % pure and mixtures of methyl ester standards were purchased from Nu-Chek-Prep (Elysian, MN, U.S.A.). Raw unprocessed seed oils were from our supplies stored at 4°C or lower. α-Bromoacetophenone (98 %) was purchased from Aldrich (Milwaukee, WI, U.S.A.). This material had a greenish brown color and was distilled at reduced pressure to give a white solid (m.p. 50°C) when cooled. This compound is also available from Fluka (Huappauge, NY, U.S.A.) as white crystals with a purity of. >99 % according to the manufacturer. All the phenacyl derivatives used in this study were prepared from the redistilled Aldrich product. 2-Bromo-2′-acetonaphthone was purchased from Pfaltz and Bauer (Stam-ford, CT, U.S.A.) and used without further purification. Triethylamine and acetone (spectro grade) were obtained from Eastman-Kodak (Rochester, NY, U.S.A.) and redistilled before use. All other organic solvents were glass distilled and were pur-chased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Water was first distilled from a Loughborough all glass water still and then redistilled from an all-glass still containing alkaline permanganate. The final distillate was collected in liter glass bottles, heated in a microwave oven until boiling, degassed under vacuum while hot, head space flushed with helium, and sealed with a PTFE-lined cap. Water ob-tained from a multibed ultrapurification apparatus was unusable.

Derivative preparation

Standard fatty acids or fatty acids resulting from the saponification of natural fats (100 μg or less) were brought to dryness in a 100 × 16 mm PTFE-lined screw cap culture tube. 25 μl of an α-bromoacetophenone solution (10 mg/ml in acetone) and 25 μl of a triethylamine solution (10 mg/ml in acetone) were added to the tube, capped tightly, and placed 25 mm deep in a boiling water-bath. After 15 min, 35 μl of an acetic acid solution (2 mg/ml in acetone) were added to the tube and heated for an additional 5 min. The tube contents were then evaporated to dryness with dry pre-purified nitrogen while being heated at 40°C. A 100-μl volume of acetonitrile was added to the tube and a 5–10-μl aliquot of the derivatives was used for analysis. Except for a proportional increase in the 2-bromo-2′-acetonaphthone concentration, the naphthacyl derivatives were prepared by the same procedure.

Methyl esters of the fatty acids from seed oil samples were prepared by sulfuric acid catalyzed esterification or transesterification as described previously[10].

HPLC analyses

Analyses were carried out using an IBM Model 9533 liquid chromatograph (IBM Instruments, Danbury, CT, U.S.A.) equipped with a variable-wavelength ultraviolet detector. This is a microprocessor-controlled triple-piston pump instrument capable of developing linear ternary gradients. Analyses were made at 242 nm and 254 nm. The detector signals were sent to a Spectra-Physics Minigrator (Spectra-Physics, Santa Clara, CA, U.S.A.) for quantitation and attenuation to a 5-mV recorder. Analyses were carried out on a IBM 250 × 4.5 mm column packed with 5-μm octadecyl-bonded spherical silica. An octadecyl column guard was also used. All solvents were degassed under vacuum and maintained under 6 p.s.i. of helium. A solvent flow-rate of 2 ml/min was used for all analyses. A solvent system of acetonitrile–water (80:20) was used for isocratic analyses of the phenacyl derivatives that usually produced a pressure of 140 bar. To obtain the same approximate elution time of the naphthacyl derivatives as the corresponding phenacyl derivatives, a 87:13 mixture of acetonitrile–water was required. Most analyses were performed by gradient elution programmed linearly from 80:20 to 90:10 acetonitrile–water over 45 min. Some analyses were performed by starting the change in the solvent proportions linearly from 80:20 to 85:15 or 90:10 over the last 20–25 min of a 40–45-min run.

GLC analyses

Methyl esters of fatty acids were analyzed with a Varian Aerograph Model 3700 chromatograph equipped with flame ionization detectors and a digital integrator. A glass column (1.54 m × 2 mm) packed with 10% SP 2330 was used for the analyses under the operating conditions described previously[11].

Absorbance spectra

The spectra between 200 and 350 nm were obtained for α-bromoacetophenone, 2-bromo-2'-acetonaphthone and phenacyl derivatives of olive oil fatty acids in acetonitrile using a Cary Model 219 spectrophotometer.

RESULTS AND DISCUSSION

Derivative preparation

Initially, the phenacyl and naphthacyl derivatives were prepared by the procedure described by Borch[5] for the preparation of phenacyl esters. After several phenacyl samples were run, large peaks with broad base widths began emerging during sample runs. Our first approach was to separate the phenacyl derivatives from the excess reagent and any free fatty acids using thin-layer chromatography (TLC) [silica gel G, hexane–diethyl ether (90:10), double development]. The phenacyl derivatives purified in this manner are stable and the extraneous broad peaks were eliminated, but another problem appeared. Small peaks following only unsaturated fatty acids appeared in the chromatogram where there had previously been no peaks. The appearance of these small peaks is illustrated in Fig. 2 where they are seen after major peaks 2, 5, 7, 11 and 17. These unexpected peaks were traced to the production of *trans* isomers during the visualization of the phenacyl derivatives with ultraviolet (UV) light on the TLC plate. Silica gel was shown not to be a participant in the isomerization by showing that phenacyl oleate could be partially isomerized to a

compound that co-eluted with phenacyl elaidate, the *trans* isomer, with only UV light. Myristoleate, palmitoleate and oleate derivatives all produced 1–2% *trans* isomers on TLC when exposed to low-intensity (0.12 A) short-wavelength UV light for 1–2 min. No detectable isomerization of unsaturated fatty acid phenacyl derivatives was observed when TLC was used to purify the derivatives without exposure to UV light.

Although the phenacyl derivatives could be purified by TLC that produced very clean HPLC chromatograms, this was a time-consuming process that resulted in the loss of samples from the outer edges of the TLC plate exposed to UV light while the major portion of the located phenacyl derivative band was covered. An alternative to this process was sought. It was felt the large excess of 2-bromoacetophenone might be reacting with the column material, or any other compounds trapped in the column that contained a carboxyl group, as it passed through the column producing broad interfering peaks. Another reason for removing the excess reagent was to improve the stability of the phenacyl derivatives. Samples that contained a large excess of reagent deteriorated noticeably within 1 week and were more than 75% destroyed within 1 month, even when stored at −20°C. The problem of the broad baseline peaks and derivative deterioration was alleviated when acetic acid was used to neutralize the excess α-bromoacetophenone and form phenacyl acetate. The latter elutes quickly at the beginning of the run. Phenacyl derivatives prepared in this way appear to be as stable as those purified by TLC.

Heating the reaction mixture in a boiling water-bath produced no detectable changes in a large variety of fatty acids, including sterculic acid that contains a
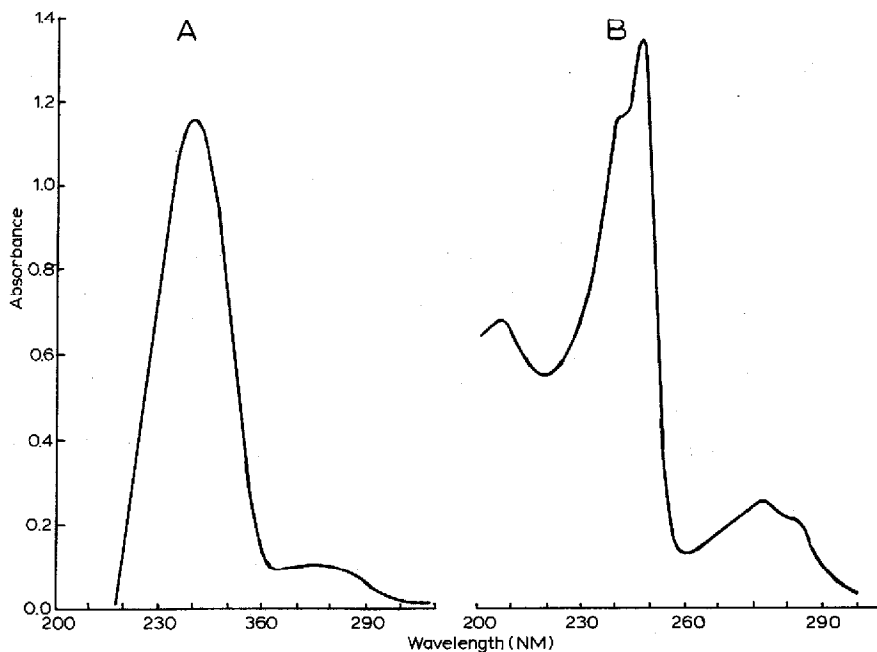

Fig. 1. Ultraviolet absorbance spectrum of 2-bromoacetophenone (A) and 2-bromo-2′-acetonaphthone (B) in acetonitrile.
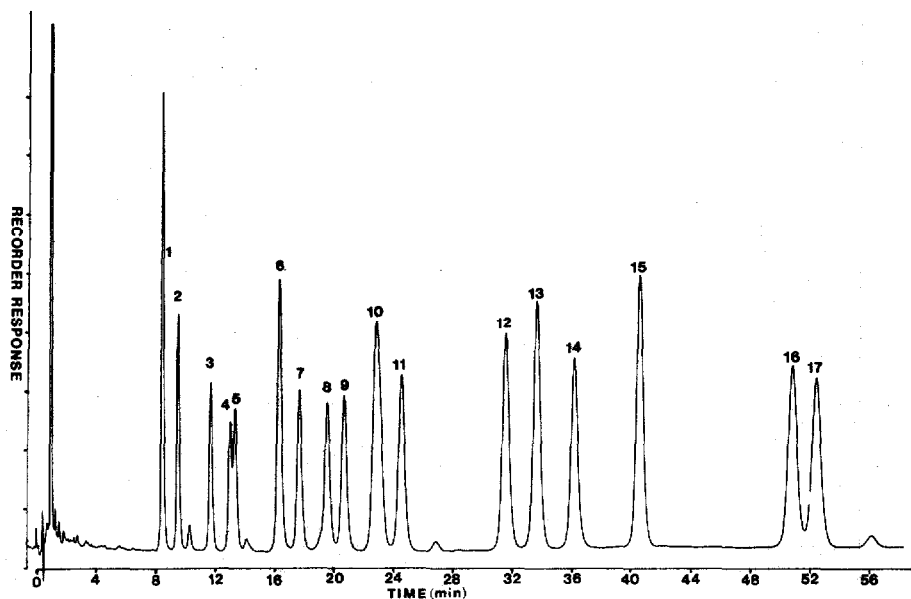
Fig. 2. A chromatogram showing the typical separations of phenacyl esters of fatty acids by HPLC. Analysis was made on a 250 × 4.5 mm octadecyl column at a solvent flow-rate of 2.0 ml/min. The column was run isocratically with acetonitrile–water (80:20) for 25 min then programmed to 85:15 in 15 min. Peaks: 1 = lauric; 2 = myristoleic; 3 = tridecanoic; 4 = cis-Δ^{10}-pentadecenoic; 5 = linolenic; 6 = myristic; 7 = arachidonic; 8 = $trans$-Δ^9-hexadecenoic; 9 = linoleic; 10 = pentadecanoic; 11 = all-cis-$\Delta^{8,11,14}$-eicosatrienoic; 12 = palmitic; 13 = oleic; 14 = elaidic; 15 = heptadecanoic; 16 = stearic; and 17 = cis-Δ^{11}-eicosenoic.

cyclopropene ring, when compared to phenacyl derivatives prepared overnight at room temperature. Heating reduces the time required to produce the derivatives to minutes. Although we have not determined how quickly the reaction proceeds, the 15 min reaction time chosen for convenience is probably much longer than required as judged from the absence of reagent and free fatty acids on charred TLC plates 5 min after acetic acid was added to neutralize excess reagent.

Naphthacyl derivatives of long-chain fatty acids do not appear to have been prepared and analyzed by HPLC previously. Stotz and Hassing[12] have indicated in a paper describing the impurities of 2-bromo-2'-acetonaphthone they have prepared naphthacyl derivatives of prostaglandins.

Absorbance spectra and sensitivity

Most substituted and unsubstituted phenacyl esters analyzed previously by HPLC[5–8,12] were detected at 254 nm. In most cases this probably resulted from the use of fixed wavelength detectors. The variable-wavelength detector allows detection at 242 nm, which is the maximum for 2-bromoacetophenone and 2-bromo-2'-acetonaphthone as indicated in Fig. 1. Phenacyl derivatives of olive oil fatty acids gave a similar spectrum indicating the attached fatty acyl chain has little or no effect, as might be expected. The only difference between chromatograms of the same sample obtained at 254 nm and 242 nm was the several-fold increase in the size of the peaks at the latter wavelength. One of the main advantages of the phenacyl and
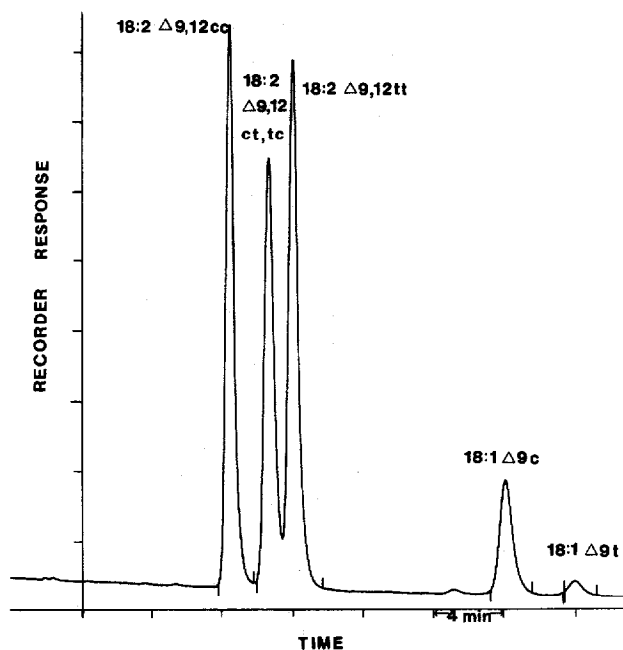
Fig. 3. A HPLC chromatogram showing the resolution of the $\Delta^{9,12}$-octadecadienoic and Δ^9-octadecenoic geometrical isomers as their phenacyl derivatives. The analysis was performed isocratically with acetonitrile–water (80:20) at 2.0 ml/min.

naphthacyl derivatives is the high sensitivity of detection. We injected 10 μg of sample that contained five of more major components. This quantity of mass allows fatty acid derivatives that represent 0.1% or 10 ng to be measured accurately without difficulty. It is estimated that by increasing the detector sensitivity, 1 ng or less could be measured.

Resolution of phenacyl derivatives of fatty acids

The order of elution and the resolution of a mixture of phenacyl derivatives of saturated, monoenoic and polyenoic fatty acids are given in Fig. 2. Saturated fatty acids are separated according to chain length with the longer chains eluting last. The introduction of a double bond in the hydrocarbon chain changes the partition coefficient to make it roughly equivalent to that of a hydrocarbon chain containing two fewer carbon atoms. These critical pairs, 1 and 2, 3 and 4, 12 and 13, and 16 and 17, are well resolved. As the number of double bonds in the hydrocarbon chain increases, the elution time continues to decrease. This is well illustrated with stearic (peak 16), oleic (peak 13), linoleic (peak 9) and linolenic (peak 5) acids all of which contain eighteen carbon atoms with zero, one, two and three double bonds, respectively. This changes the elution order dramatically from that normally observed on polar GLC columns. Arachidonic acid (peak 7) is the only common fatty acid that can occur at high levels in biological samples that overlaps another common fatty acid, namely palmitoleic acid. Even a partial separation of these two fatty acids sufficient for quantitation can be achieved by starting with a more polar system [acetonitrile–water

(70:30)]: palmitoleic is eluted ahead of arachidonic. The separation of oleic (peak 13) and elaidic (peak 14) acids illustrates geometrical isomers of unsaturated fatty acids can also be analyzed. This also applies to three of the four geometrical $\Delta^{9,12}$-octadecadienoic isomers as illustrated in Fig. 3. Analysis of this 18:2 sample by capillary GLC (unpublished data) revealed it contained both the *cis trans* and *trans cis* isomers which eluted here as a single peak. Fig. 3 illustrates that the solubility of the geometrical isomers in the mobile phase decreases as the number of *trans* bonds in the fatty acids increase. This figure also shows that oleic and elaidic acids can be separated with retention times as much as 4 min apart when analyzed isocratically.

The resolution of C-18 geometrical isomers by reversed-phase HPLC is not unique to this paper. Some excellent separations of substituted and unsubstituted phenacyl derivatives of *cis* and *trans* monoene isomers have been reported by Borch[5] and by Bussell *et al.*[8] on very long columns (60–90 cm) that required up to 4 h for analysis. We have demonstrated that equally good resolution can be achieved with one 25-cm column in a time period that is practical, in addition to being quantitative. Halgunset *et al.*[7] recently described the resolution of nine common fatty acids as *p*-bromophenacyl esters by HPLC. The mixture did not contain geometrical isomers and quantitation was not examined. Critical pairs were not resolved by the acetonitrile–water solvent system used in this study, but were well resolved by a three-component solvent system. Svensson *et al.*[13] have recently reported retention times for a series of *cis* and *trans* octadecenoate positional isomers separated on a single column as methyl esters with nearly the same degree of resolution as reported in this paper. Under identical conditions, methyl esters elute several minutes ahead (*ca.* 4 min for linoleic acid) and exhibit slightly improved resolution between components relative to the corresponding phenacyl derivatives. This positive aspect of the methyl esters is offset by the difficulty in trying to obtain quantitative data on mixtures of esters using either the UV detector in the region of 195–206 nm[1–3] or the refractive index detec-

TABLE I

COMPARISON OF VALUES OBTAINED BY HPLC AND GLC ANALYSES OF STANDARD MIXTURES OF FATTY ACYL DERIVATIVES

GLC analyses were made on a glass column (1.54 m × 2 mm) packed with 10 % SP 2330. The temperature was programmed from 140°C to 210°C at 3°C/min. See Fig. 2 for HPLC conditions.

Standard	Data base and type of analysis	Percentage					
		14:0	*16:0*	*16:1*	*18:0*	*18:1*	*18:2*
NIH	Weight %	11.8	23.6	6.9	13.1	44.6	
Mix D	Mole %	13.8	24.6	7.2	12.3	42.1	
	HPLC (phenacyl)	14.2	24.4	7.6	11.3	42.1	
	HPLC (naphthacyl)	13.4	24.2	7.4	12.7	42.3	
	GLC (methyl esters)	12.0	25.2	6.6	12.2	44.0	
Nu-	Weight %		20.0	20.0	20.0	20.0	20.0
Chek	Mole %		21.1	21.3	19.1	19.2	19.3
XA	HPLC (phenacyl)		21.3	21.7	18.1	19.5	19.4
	HPLC (naphthacyl)		21.0	21.2	18.9	19.3	19.6
	GLC (methyl esters)		20.3	20.6	19.7	20.2	19.2

tor[13]. As will be seen, the problem of quantitation is overcome with the use of the phenacyl and naphthacyl derivatives.

Quantitation

The comparisons of values obtained from two standard mixtures of fatty acids analyzed as phenacyl and naphthacyl esters by HPLC and as methyl esters by GLC are given in Table I. Phenacyl percentages were nearly identical to the values obtained with the naphthacyl esters. Values obtained by HPLC gave quantitative mole percentages, while percentages obtained by GLC agreed more closely with weight percentages. The two standard mixtures were purchased as methyl esters which required saponification to yield the free fatty acids before phenacyl and naphthacyl derivative preparation. Both the saponification and derivatization procedures gave quantitative results when the sample contained a relatively low level of palmitoleic and a high oleic acid content (NIH Mix D) and when the sample contained a polyunsaturated fatty acid (Nu-Chek XA).

TABLE II

COMPARISON OF ANALYSES OF FATTY ACYL DERIVATIVES FROM NATURAL SOURCES BY HPLC AND GLC

Abbreviations used: Me = methyl; BAP = phenacyl; BAN = naphthacyl. Methyl esters were analyzed on 1.54 m × 2 mm column packed with 10% SP 2330. Temperature was programmed from 140 to 210°C at 3°/min. Phenacyl and naphthacyl derivatives were analyzed on a 250 × 4.5 mm octadecyl column. Phenacyl and naphthacyl derivatives were analyzed isocratically using acetonitrile–water 80:20 and 87:13 for 25 min, then programmed to 85:15 and 92:8, respectively in 15 min at a flow-rate of 2 ml/min.

Source of fatty acids	Analysis and derivative	Percentage								
		12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	Other
Coconut oil	GLC Me	54.1	20.6	10.2		2.4	7.0	1.7		4.2*
	HPLC BAP	48.3	16.3	7.7		1.8	4.5	1.2		20.2**
	HPLC BAN	49.4	16.4	8.6		1.8	4.9	1.3		17.6***
Corn oil	GLC Me			9.7		1.5	28.0	60.8		
	HPLC BAP			14.0		2.2	28.0	54.8	0.7	
	HPLC BAN		0.1	12.9		1.7	26.8	57.3	0.7	0.5
Cotton seed oil	GLC Me		0.1	17.1	0.7	3.1	19.0	57.8		2.2
	HPLC BAP		1.1	26.9	0.5	2.5	17.6	51.1		0.3
	HPLC BAN		1.1	25.2		2.3	18.0	52.0		1.4
Olive oil	GLC Me			8.0	Trace	2.7	81.6	7.7		
	HPLC BAP			12.2		3.7	76.0	7.0		1.2
	HPLC BAN			11.4		3.4	75.8	8.2		1.2
Safflower oil	GLC Me			5.1		1.9	11.7	81.3		
	HPLC BAP			7.9		2.3	12.6	75.1		3.0
	HPLC BAN			7.4		2.1	12.6	76.3		1.6

* C-10 = 4.2%.
** C-8 + C-10 = 19.6%.
*** C-8 + C-10 = 17.5%.

Analysis of natural oil samples

Lipids from natural sources containing high percentages of medium-chain fatty acids, high percentages of monoenes and high percentages of polyunsaturated fatty acids were examined by HPLC and GLC and are compared in Table II. Except for some minor differences, phenacyl and naphthacyl esters gave similar results. Generally, the fatty acid compositions of corn, cotton, olive and safflower oils determined by HPLC showed only minor differences between the percentages determined by GLC. These differences are probably attributable to differences between weight and mole percentages mentioned earlier. Corn oil analyzed by HPLC contained 0.7% 18:3, whereas measurable quantities were not detected by GLC. The short elution times of the polyunsaturated fatty acids and the absence of heat (which can cause decomposition of labile compounds) probably accounts for the difference in the 18:3 content obtained by the two analytical systems. Clearly, the composition of coconut oil analyzed by HPLC and GLC showed the largest differences. Although care was taken not to over-evaporate the solvent during the processing of the methyl esters used for the TLC analysis, only 4.2% of decanoic acid was found, whereas HPLC analyses showed approximately 10.5% and 7.5% octanoic and decanoic acids, respectively. The use of the non-volatile derivatives with HPLC to analyze lipid samples containing short to medium-chain fatty acids has an unquestionable advantage over the use of GLC to analyze such samples. The comparisons of the data from the two systems show that phenacyl and naphthacyl derivatives of fatty acids from natural sources can be analyzed quantitatively by HPLC. This appears to represent the first report to describe the quantitative analysis of fatty acid mixtures from natural source byHPLC.

The phenacyl derivatives are more soluble in the mobile phase than the corresponding naphthacyl derivatives and therefore have shorter retention times. This results in being able to use a higher percentage of the less expensive solvent (water) and the lower-molecular-weight derivative gives slightly greater resolution than the naphthacyl derivatives, somewhat like the methyl esters giving slightly better resolution than the phenacyl derivatives. Because of these advantages of the phenacyl derivatives they are used routinely in this laboratory.

Historical

Phenacyl esters have been known for more than a century. Graebe[14] was apparently the first to prepare the phenacyl esters of acetate in 1871. Between 1917 and 1920 Reid and co-workers[15–17] prepared phenacyl and halo- and nitro-substituted phenacyl esters of a large variety of mono-, di- and tricarboxylic acids, including long-chain fatty acids. These derivatives were used to characterize the carboxylic acids by their melting points. Determining melting points of substituting and unsubstituted phenacyl derivatives has given way to more modern methods of identification and characterization such as mass spectrometry, nuclear magnetic resonance spectroscopy, infrared spectroscopy, etc., and these derivatives have been used in recent times by Umeh[18] for the analysis of short-chain fatty acids by GLC. Now, the reintroduction of an old derivative has been shown useful for the quantitative analysis of fatty acids by HPLC, a modernized version of an old technique. A new application for old compounds strike a sympathetic note for the old chemists and lends credence to the old adage, "the more things change the more they stay the same".

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