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Review

Recent advances in the high-performance liquid chromatography of fatty acids

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Introduction

Ever since the pioneering work of James and his co-workers, chromatography and in particular gas–liquid chromatography (GLC) has been a mainstay for the analysis of mixtures of fatty acids. Recent advances in stationary phases and glass capillary columns for GLC have maintained this interest and have enabled many previously unresolvable positional and geometric isomers to be separated.

The development of high-performance liquid chromatography (HPLC) has enabled triglycerides and involatile fatty acids to be separated. Despite these possible advantages, the application of this method has been slow, primarily because of the lack of a sensitive universal HPLC detector. Nevertheless, there has been a steadily expanding literature on the use of HPLC, with many developments in derivatization methods to enhance detection and in techniques for selective separation.

Methods using GLC and HPLC for the separation of fatty acids and their derivatives published up to 1977 have been compared [1]. This review will concentrate on advances in HPLC methods since then. Only a limited coverage of applications of these techniques to the study of lipids is included as specific reviews of this area have recently appeared [2, 3].

Detection of Fatty Acids

Because the free fatty acids and lipids lack a significant ultraviolet chromophore at 254 nm, their detection following separation has always posed a problem. Two solutions have been adopted: either an alternative method of detection has been employed, or the acids have been converted into more readily detected derivatives usually possessing absorbing or fluorescent groups.

(a) Precolumn derivatization

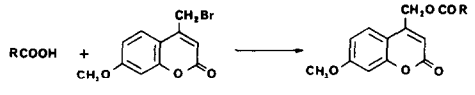
This has been the most popular method for fatty acid analysis. A large number of derivatives have been used, including benzyl, nitrobenzyl, dansyl, phenacyl and naphthacyl and their modifications. The methods of preparation and use are generally well established [1, 4–7]. As well as improvements in the reaction conditions of existing methods, recent advances have included a number of new derivatives with fluorescence and electrochemical responses.

(i) Phenacyl and related derivatives. Recently *p*-chlorophenacyl esters have been added [8] to the well established unsubstituted, *p*-nitro, *p*-phenyl, *p*-bromophenacyl esters and related naphthacyl esters. A number of methods have been used for the synthesis of this group of derivatives, principally using crown ethers as catalysts. It has been reported that these expensive reagents can be replaced by potassium fluoride with no loss in yield [9].

Although lipids have usually been converted by alkaline hydrolysis to give the free fatty acids, which are then isolated, dried and derivatized, a one-pot reaction can be used. After hydrolysis the solution is neutralized to phenolphthalein and derivatized directly. The glycerol released during hydrolysis does not interfere with the subsequent HPLC analysis and need not be separated. The combined method has been successfully applied to lipids from soybean [10], grain [11] and rapeseed [12].

(ii) Methoxymethylcoumarins. In a series of studies Dünges and his co-workers pioneered the reaction of carboxylic acids with 4-bromomethyl-7-methoxycoumarin to give fluorescent methoxycoumarin derivatives (Fig. 1), which can be separated by chromatography. The first studies on fatty acids used potassium carbonate in acetone as the catalyst and separated the products by TLC [13]. The reaction conditions were subsequently improved by using crown ethers as the catalyst [14, 15]. The separation of the fatty acids was extended to HPLC by Dünges [16] and other groups [15, 17] using fluorescence detection. UV-spectrometric detection can also be used, but with lower sensitivity [16].

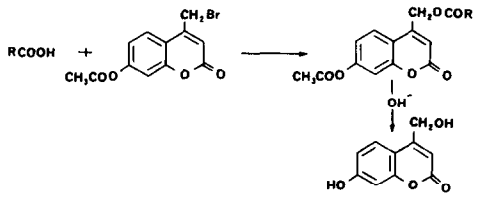
Figure 1



An important critical analysis of the use of this derivative has been carried out by Lloyd [18]. He pointed out that the fluorescence yield of the derivatives varied with chain length, particularly if the number of carbon atoms was greater than eight, as micelle formation could occur. The yield was also sensitive to the solvent employed, with implications for programmed elution separations. The previously reported fluorescence excitation and emission spectra [15] were found to have been distorted by an inner filter effect and an inappropriate excitation wavelength had been used. Optimum conditions were found to be $\lambda_{Excitation}$ 320–325 nm and $\lambda_{Emission}$ 390–400 nm, small changes in wavelength occurring with solvent [18]. More recently the reaction has been applied to the detection of trace levels of fatty acids and of the thermally-sensitive oxirane fatty acids [19].

A related derivative has been prepared using 4-bromomethyl-7-acetoxycoumarin (Fig. 2) [20]. Following separation the derivative is hydrolysed by the post-column introduction of alkali to give the free hydroxycoumarin which is detected fluori-

Figure 2



metrically. As the product in each case is the same, different acids all give the same response. The fluorescence of the free coumarin is insensitive to solvent and gradient elution can be used.

(iii) Further derivatives. A number of other methods have been proposed to enhance the detectability of fatty acids. Unsaturated acids can be halogenated and the products detected at 265 nm [21]. Fatty acids will react with 1-chloromethylisatin to give methylisatin derivatives (Fig. 3) [22], while *N*-chloromethylphthalimides and their 4-nitro analogues will react with carboxylic acid groups to give esters (Fig. 4) [23]. Both systems give enhanced ultraviolet spectrometric detection following separation.

Figure 3

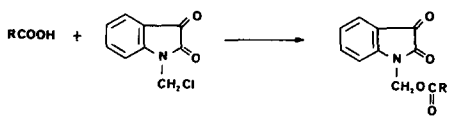
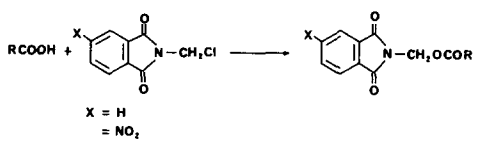


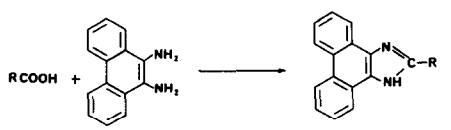
Figure 4



Diazomethane has been used for a long time as a reagent to prepare methyl esters for GLC, although in HPLC these derivatives have only a weak chromophore. However, 1-diazomethylnaphthalene, 2-(1-diazoethyl)-naphthalene and 1-(4-biphenylyl)-diazoalkanes can also be used to form the corresponding aromatic esters and give enhanced ultraviolet detection [24]. The same reaction using 9-diazomethylanthracene can be used to yield a fluorescent derivative [25]. An alternative route to the same product using the more readily available 9-halomethylanthracenes has also been suggested [26].

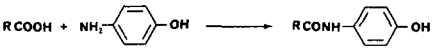
As an alternative to the methoxycoumarin derivatives, Lloyd has explored the reaction with phenanthrenediamine to give the fluorescent phenanthrimidazoles (Fig. 5). These were found to give quantum yields virtually independent of the chain length of the acid and to be insensitive to changes in the solvent [27].

Figure 5



A further method of detection was proposed by Ikenoya and co-workers, who reacted fatty acids with *p*-aminophenol in the presence of 2-bromo-1-methylpyridinium iodide and trimethylamine to give *p*-hydroxyanilides (Fig. 6). After separation these compounds can be detected using a glassy carbon electrochemical detector [28].

Figure 6



(b) Detectors

Little work has been carried out on the HPLC of free fatty acids, but their readily prepared methyl esters have been studied by a number of groups, frequently during comparative studies with GLC. They have been detected using conventional refractive index detectors [1], moving wire systems, and ultraviolet detectors at 210–215 nm, but few advances have been made in these techniques. However, a number of more specialized detectors suitable for simple esters have recently been proposed.

(i) Nephelometry. Novotny and co-workers, by adding aqueous buffer to the eluent from a column, were able to detect triglycerides and vegetable oils by nephelometry because of micelle formation [29]. However, although the method also worked with argentation chromatography, it was not applied to individual fatty acids or esters.

(ii) Mass detector. By separating the eluent solvent as a vapour the mass or evaporative detector has been used to detect the remaining particles of a sample by light scattering [30]. Although its main application has been for the detection of carbohydrates, a few chromatograms were presented for lipids and methyl palmitate, but no more detailed studies have been reported.

(iii) HPLC–Mass spectrometry. Although the direct coupling of mass spectrometry to HPLC has enormous potential, its implementation has been limited by practical problems. One promising development is the use of microbore columns. Such a system coupled with a jet separator has been used for the separation and detection of the C_{12} to C_{18} methyl esters [31].

Separation of Fatty Acids and Derivatives

Almost all the recent separations of fatty acids and related compounds have been carried out using alkylbonded reversed-phase columns (see (a) below). An important development has been the use of normal and reversed-phase systems containing silver ions (argentation chromatography) to modify the separation of unsaturated compounds (see (b) below).

(a) Bonded phase chromatography

A wide range of bonded-phase columns and conditions have been reported during the period of this review for the separation of fatty acids and their derivatives (Table 1). The eluent has usually been methanol–water or acetonitrile–water, the latter being favoured by some authors because it can resolve palmitate and oleate derivatives [37]. Because of the wide range of the fatty acid hydrophobicities, gradient elution has frequently been used, sometimes continuing with non-aqueous systems (e.g. [38]).

In common with other HPLC studies, commercial octadecylsilyl bonded silicas have been the most popular stationary phases, with octylsilyl bonded phases as an alternative. Some authors have prepared their own bonded phases and a tricontyl (C_{30}) bonded silica was considered to be particularly suitable for long chain acids [35]. A commercially available column based on a phenyl bonded phase has been used for free fatty acids

Table 1
Separation of fatty acids and derivatives on bonded-phase chromatography*

Fatty acids†	Derivative	Column	Mobile phase	Detector	Reference
C 18:3 isomers	Methyl	ODS-Zorbax	Acetonitrile–water (80:20 v/v)	UV	32
Mono-unsaturated isomers	Methyl	Nucleosil C-18	Methanol–water (89:11 v/v)	RI	33
Cyclopropenoic and cyclopropanoic	Methyl	Lichrosorb RP-18	Acetonitrile–water (90:10 or 95:5 v/v)	UV 206 nm	34
C_{12}–C_{22}	*m*-Methoxyphenacyl	μ-Bondapak C-18	Acetonitrile–water (40:60 to 100:0 v/v)	UV 254 nm/280 nm	35
C_6–C_{22}	Phenacyl	C_8 + Lichrosorb	Acetonitrile–water (70:30 to 100:0 v/v)		36
C_{12}–C_{24}	Phenacyl	μ-Bondapak C-18	Acetonitrile–water (67:33 to 97:3 v/v) or acetonitrile–water (80:20 to 100:0 v/v)	UV 254 nm	37
C_2–C_{24}	*p*-Br-phenacyl	μ-Bondapak C-18	Acetonitrile–water (40:60 to 100:0 v/v)	UV 254 nm or λ_{max}	8
	p-NO_2-phenacyl	or μ-Bondapak Fatty Acid	Acetonitrile–water (40:60 to 100:0 v/v)		
	p-Cl-phenacyl				
	2-Naphthacyl				
C_1–C_{40}	*p*-Br-phenacyl	C_{30} + μ-Porasil‡	Acetonitrile–water (40:60 to 100:0 v/v)	UV 254 nm	38
			to acetonitrile–dioxan (0:100 v/v)		39
C_{12}–C_{22}	*p*-Br-phenacyl				
C_{14}–C_{20}	*p*-Br-phenacyl	Supelcosil LC-18	Acetonitrile–water (91:9 v/v)	UV 254 nm	40
		Nucleosil ODS	Methanol–water (91:9 v/v) or methanol–acetonitrile–water (82:9:9 v/v)		

Table 1 (contd)

Fatty acids†	Derivative	Column	Mobile phase	Detector	Reference
C_8–C_{24}	Methyl	Lichrosorb RP-8	Methanol–water (93:7 to 97:3 v/v)	RI	41
C_8–C_{18}	Esters of C_3–C_8 alcohols				
C_8–C_{18}	Methoxycoumarin	Nucleosil ODS	Methanol–water (40:60 to 100:0 v/v)	Fluorescence	15
C_1–C_{16}	Methoxycoumarin	C_{18} + Partisil 10	Methanol–water (85:15 v/v)	Fluorescence	18
Oxirane fatty acids	Methoxycoumarin	Lichrosorb RP-8	Acetonitrile–buffer (pH 2.7) (30:70 to 100:0 v/v)	Fluorescence	19
C_{10}–C_{18}	Methylnaphthyl	μ-Bondapak C-18	Acetonitrile–water (85:15 v/v) Methanol–water (75:25 v/v)	UV	24
C_8–C_{20}	Methylanthracene	MCH 10 C-18	Acetonitrile–water (95:5 v/v)	UV & fluorescence	25
C_1–C_{18}	Methylisatin	Hibar RP-8	Methanol–water (50:50 to 100:0 v/v)	UV 254 nm	22
C_2–C_{20}	Phenanthrimidazoles	ODS-Hypersil	Methanol–water (70:30 to 90:10 v/v)	Fluorescence	27

* Studies which include single fatty acids have not been included.
† Unsaturated fatty acids can include monoenes, polyenes and positional isomers.
‡ Bonded phase not commercially available.

Table 2
Argentation chromatography of fatty acids

	Fatty acids	Derivative	Column	Mobile phase	Detector	Reference
a. Normal phase	C 18:1 isomers	*p*-Br-phenacyl	Silica modified with alumina $+Ag^+$	0.01% acetonitrile in hexane–chloroform (13:1 v/v)	UV 254 nm	42
	C 18:1–C 18:2	Methyl	Silica $+Ag^+$	Benzene	RI	43
	C 18:1 and C 18:2 isomers	Methyl	Partisil 10 + 2% $AgNO_3$ or Spherisorb S5W + 5% $AgNO_3$	Hexane + 1% tetrahydrofuran	UV 205 nm	44
	C 14:0–C 22:6	Methyl	Partisil 10 + Ag^+	Hexane	Moving-wire	46
b. Bonded phase	C 18:1–C 22:1	*p*-Br-phenacyl	Partisil 10-ODS or Spherisorb S5-ODS	Methanol–water (90:10 v/v) + 0.8% m/v $AgNO_3$	UV 257 nm	47
	C:18 unsaturated	Methyl	Nucleosil 5C-18	Methanol–water (80:20 v/v) + 10^{-2}M $AgClO_4$	R.I.	48
c. Ion exchange	C 18:2	Methyl	Amberlite XE-284 Ion exchange + Ag^+	Methanol	R.I.	49

[8]. Many of the studies are very similar and considerable duplication of earlier work is evident, although often minor improvements in selectivity are noted. A recent study has examined the *p*-bromophenacyl esters in detail and proposed a set of rules to determine elution order [39].

(b) Argentation chromatography

Silver ions have been widely used to enhance the separation of unsaturated fatty acids on TLC. This technique has also been applied to normal phase HPLC (Table 2a). A useful improvement in the technique was made by Lam and Grushka who modified the silica surface before coating with the silver ion to give a silver aluminosilicate which was more stable than the directly coated column [42]. Argentation chromatography has also been employed in separations using bonded-phase systems by incorporating silver nitrate in the eluent (Table 2b). Both *cis/trans* [48] and positional isomers can be resolved. An interesting development studied by Schofield was to use a macroreticular ion-exchange column coated with silver ions to separate C 18:1 *cis*- and *trans*-isomers [49].

Applications

The methods discussed in this review have been applied to the analysis of fatty acids from a wide range of sources (Table 3). Of particular interest is the work of Wheals and co-workers on the fatty acid anilides present as the suspected toxic agents in contaminated Spanish cooking oil [53]. Also noteworthy is the measurement of free fatty acids in serum, using an Extrelut column instead of conventional liquid–liquid extraction to isolate the fatty acids before derivatization and separation [56].

Table 3
Applications of HPLC analysis of fatty acids

Sample	Fatty acid derivative	Reference
Oral streptococci	*m*-Methoxyphenacyl	50
Oral bacteria	*m*-Methoxyphenacyl	35
α-Mycolic acids	*p*-Br-phenacyl	38
River water	Phenacyl	51
Alkanolamides	Ethanolamides	52
Grains and feeds	*p*-Br-phenacyl	11
Fish oils	Methyl	46
Soybean oil	*p*-Br-phenacyl	10
Cooking oil	Anilide	53
Margarine	Methyl	45
Oil and alkyl resins	Free acids	54
n-Alkanes	Phenacyl	55
Serum	*p*-Br-phenacyl	56
Monkeys	Methyl	57

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