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PHENANTHRIMIDAZOLES AS FLUORESCENT DERIVATIVES IN THE ANALYSIS OF FATTY ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

In methyl polyphosphate, prepared in chloroform, fatty acids condense with 9,10-diaminophenanthrene to form 2-substituted phenanthrimidazoles. The reaction proceeds to completion within 3–6 min at 85° to give, in general, yields reproducibly in excess of 95%. Exceptions are linolenic acid, from which the yield is reproducibly 91%, and formic acid, from which no derivative is obtained under these conditions.

When the 2-substituent is unconjugated with the nucleus the mean quantum yield of fluorescence from derivatives of seventeen different acids under conditions relevant to the derivatives' chromatography (methanolic solvents) is 0.68 in nitrogen-purged solvents and 0.31 in aerated solvents, both with standard deviations of 0.02. The fluorescence emission exhibits overlapping maxima at 367 and 382 nm, with a width at half maximum peak height of 3120 cm^{-1}. Under nitrogen, but not in the presence of air, quantum yields vary negligibly with the water content of the solvent over the chromatographically useful range. At higher concentrations of water the formation of weakly fluorescent micelles may occur. Above the acetic derivative the molar absorptivities in methanolic solvents are independent of the substituent in the sixteen unconjugated derivatives examined. At 254–255 nm the mean value is 90133. Other weaker absorbance maxima are available at various points up to 354 nm. From these results the intrinsic fluorescence sensitivity value of the derivatives is greater than that of the 4-hydroxymethyl-7-methoxycoumarin ester derivatives by a factor of 12.

The reaction mixture may be injected directly on to reversed-phase (octadecylsilyl) columns. Of the nineteen acids examined, up to erucic in molecular weight, the derivatives of all except the pair *n*-butyric and crotonic are separated from one another in aqueous methanol containing 0.01 *M* ammonium carbonate. On gradient elution, equimolar amounts of different acids give essentially constant peak areas, in contrast to the methoxycoumarins.

INTRODUCTION

Dünges[1], Dünges and Seiler[2], and subsequently other authors[3,4] have applied

4-hydroxymethyl-7-methoxycoumarin esters as derivatives in the analysis of fatty acids by high-performance liquid chromatography (HPLC) with fluorescence detection. Recently, an examination of the ultraviolet (UV) absorbance and the fluorescence emission characteristics of these derivatives in a variety of solvents typically used in HPLC has shown that the fluorescence quantum yields of the derivatives are sometimes low and vary considerably, *e.g.* from less than 0.02 in aprotic solvents to 0.4 in water[5]. Above the caprylic (C_8) derivative, the increase in quantum yield with the water content of methanolic solutions, seen in the lower-molecular-weight derivatives, is offset by the formation of weakly fluorescent micelles[5].

In this paper are reported the characteristics of an alternative series of derivatives based on the well-known[6] condensation of fatty acids with aromatic *ortho*-diamines to give imidazoles. When conducted under acidic aqueous conditions the reaction is often referred to as the Phillips reaction[7]. Examples of these derivatives described below offer a number of advantages over the methoxycoumarins, particularly in their higher molar absorptivities and quantum yields, and in the relative insensitivity of the latter to solvent effects. Unlike many derivatives, where the analyte is incorporated on an already formed chromophore, in the present instance part of the analyte is included within a new chromophore. As a result, interferences due to the absorbance and the fluorescence of the reagent are minimized.

EXPERIMENTAL

Instrumentation

The fluorescence spectra, which are corrected, have been recorded with a Perkin-Elmer MPF-4 spectrometer; and the UV absorbance spectra with a Pye-Unicam SP 8000 spectrometer.

For HPLC, a flow-through cell made from 1 mm I.D. fused synthetic silica is fitted into the fluorescence spectrometer. Columns (length, 250 mm; I.D., 4 mm) are slurry-packed by the balanced density technique with either ODS–Hypersil (Shandon, Runcorn, Great Britain) or Partisil-5 (Whatman, Maidstone, Great Britain) modified with octadecyltrichlorosilane (bonded layer, *ca.* 15%). Similar capacity factors are obtained from the two packings. The columns are replaced when their plate counts fall below 10^4. Solvent is delivered from a Metering Pumps' "M" pump. For gradient elution a second pump feeding a solvent-mixing vessel is added to the system. Provision is made to enable solvents to be deoxygenated with a stream of nitrogen, and to maintain the solvent and pump inlet under *ca.* 5 p.s.i. of nitrogen to inhibit ingress of air.

Methyl polyphosphate and other polyphosphate esters

Chloroform (spectroscopic grade) is freed from ethanol and phosgene by passage through a column of aluminium oxide[8] under nitrogen and with illumination by dim yellow light. To 80 ml of the freshly purified chloroform is added 37 g of phosphoric oxide (Koch-Light, Colnbrook, Great Britain; "puriss.") and then 47 ml of trimethyl phosphate (Aldrich, Gillingham, Great Britain; "97%"). The mixture, which becomes warm at this stage, is allowed to cool to room temperature, sealed against atmospheric moisture and magnetically stirred for 3 days when most of the phosphoric oxide dissolves. The supernatant, which is colourless, is removed as

required; its properties remain negligibly changed over a period of 6 months. It is emphasized that the chloroform must be present when the phosphoric oxide and the trimethyl phosphate are brought together. Otherwise, an uncontrolled, strongly exothermic reaction may develop.

If the reagent is required urgently, 1/10 quantities of trimethyl phosphate and phosphoric oxide may be mixed in the absence of chloroform. The heat evolved may be sufficient to effect solution of the oxide, otherwise the mixture is maintained at 130° until solution is complete. The product is taken up into purified chloroform. This reagent, however, tends to be slightly coloured and to give increased blanks.

The ethyl and *n*-butyl polyphosphates mentioned below were prepared similarly to the methyl ester, in chloroform at room temperature. The ethyl ester was also prepared from diethyl ether according to Schramm *et al.*[9].

Derivatization for HPLC

In 10 ml of the methyl polyphosphate solution are dissolved 20 mg of 9,10-diaminophenanthrene (Aldrich; "97%") to give a straw-coloured solution (a slight cloudiness is sometimes present, which disappears on the subsequent heating). In subdued light the solution is stable over about 4 h and during its manipulation no special precautions to exclude air or moisture are necessary. The sample in, *e.g.*, 10 μl of toluene or chloroform is added to 0.5 ml of the solution in a 1-ml glass ampoule, which is then sealed and heated at 85° for 3–6 min. The actual time varies between different batches of polyphosphate, and is separately determined for each of them.

For reversed-phase separations with solvent mixtures containing 0.01 *M* ammonium carbonate, the solution may be injected directly into the chromatograph. Alternatively, particularly if large volumes are to be chromatographed, *e.g.* 50 μl, 0.5 ml of 5 *M* aqueous ammonium hydroxide is injected into the ampoule, with rapid cooling. After the contents of the ampoule have been mixed and allowed to separate, portions of the lower chloroform layer are removed for use as required.

Isolated phenanthrimidazole derivatives of fatty acids

Typically, 0.2 mmole each of 9,10-diaminophenanthrene and the fatty acid (none of commercially specified purity of less than 98%) were heated under the previously described conditions in 5 ml of the methyl polyphosphate solution. The cooled solution was poured into 5 *M* aqueous ammonium hydroxide (25 ml) and the product collected in chloroform. It was purified by classical column chromatography on aluminium oxide (E. Merck, Darmstadt, G.F.R.; "neutral", deactivated with 3.7% water) in mixtures of *n*-hexane and ethyl acetate, and recrystallized from the same solvents. On occasions, when the product was discoloured, it was rechromatographed on silica gel (Merck; "extra pure", deactivated with 5% water) in similar solvent mixtures. The derivatives of linoleic and linolenic acids are low-melting and could not be satisfactorily recrystallized. They were recovered as their hydrochlorides when the chromatography eluates were treated with hydrogen chloride, rechromatographed, and reprecipitated several times.

Formic acid decomposes under the foregoing conditions. Its derivative was obtained when the diaminophenanthrene was refluxed in 90% formic acid in the presence of phosphoric acid.

All of the derivatives were colourless crystalline solids, each of which gave a single peak on HPLC. They are apparently stable for about a year, in the absence of light. After this time, impurity peaks appeared in the chromatograms of some of them. Derivatives of the following fatty acids were prepared (the indicated melting points are corrected[10]): formic, m.p. 307°, ref. 11: 302°, ref. 12: 292°; acetic, 258°; *n*-butyric, 251°; *iso*-butyric, 264°, ref. 12: 229°; caproic, 218°; caprylic, 170°; *n*-nonanoic, 164°; capric, 167°; lauric, 162°; myristic, 158°; palmitic, 155°; stearic, 154°; arachidic, 151°; oleic, 114°; elaidic, 135°; linoleic (hydrochloride), 122° dec; linolenic (hydrochloride), 101° dec; erucic, 122°; crotonic, 131° dec.

The electron impact mass spectra of the derivatives are consistent with the expected structures. Each derivative gives a strong molecular ion and, above the butyric derivatives, particularly prominent ions at *m/e* values of 245 and 232.

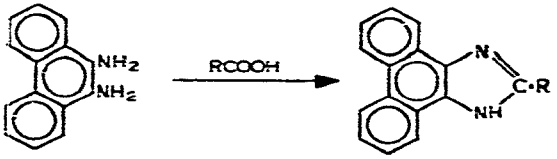
Fluorescence quantum yields

These were determined essentially according to Parker[13], with reference to 9,10-diphenylanthracene in deoxygenated methanol, for which the quantum yield was taken to be 0.95. The reasons for the use of this value and other relevant details have been given before[5].

RESULTS AND DISCUSSION

Attention has been directed mostly to the phenanthrimidazole series because it was apparent initially, from the published data available, that phenanthrimidazole absorbed strongly in the UV[11], and that it was appreciably fluorescent[14]. Preliminary experiments showed that 9,10-diaminophenanthrene, the diamine precursor of the series, was negligibly fluorescent, and that in any case there was no overlap between this fluorescence and that of the derivatives. Hence, any reagent blank directly due to the amine would be small. Experiments with other readily accessible imidazoles (benzimidazoles and naphthimidazoles) indicated that they would be inferior in these respects.

The reaction between 9,10-diaminophenanthrene and a fatty acid produces a 2-substituted phenanthrimidazole:



Reaction conditions

The poor solubility of 9,10-diaminophenanthrene in the aqueous mineral acids often used in the preparation of imidazoles necessitates the presence of an organic solvent. The technique using methyl polyphosphate described under Experimental is based on a preparative technique for benzimidazoles of Kanaoka *et al.*[15], who employed the ethyl polyphosphate ester. However, their preparation of the ester, from diethyl ether and phosphoric oxide in refluxing chloroform[9], yields a slightly coloured product that gives higher reagent blanks than the reagents prepared in the

present work from triethyl phosphate at room temperature, which in turn are poorer than those from trimethyl phosphate. The similarly prepared *n*-butyl ester is likewise unsatisfactory. Above 100° the ethyl ester slowly decomposes with the liberation of ethylene[16], and the butyl ester could decompose similarly. Presumably the methyl ester is more stable, as an analogous decomposition route is not available to it.

If traces of phosgene, derived from the chloroform used, are present in the polyphosphate solution, an intense blue–green colour appears when the diaminophenanthrene is introduced, and the reagent blank increases. Hence, freshly purified chloroform is used in the preparation of the polyphosphate solution. Once the ester has formed, however, the solution is stable with respect to the formation of phosgene, except in bright sunlight.

Formic acid is decomposed in the reaction. But all of the other fatty acids and their derivatives, including the polyunsaturated acids, are stable. Thus, a threefold increase in the heating time of the reaction mixture results in no reduction in the chromatographic response to the derivatives. No intermediate products, *e.g.* acylated diaminophenanthrenes[17], are seen in the chromatograms when the reaction is not taken to completion. A byproduct occurs at levels of up to about 3% of each derivative and at a later point in the chromatograms (these can be seen in Fig. 6). This peak is intensified, indicating that the reagent should be discarded, if the polyphosphate has been prepared from phosphoric oxide that has absorbed a significant amount of moisture, or a reduced ratio of phosphoric oxide to trialkyl phosphate is used in the preparation, or the polyphosphate is old (*e.g.*, more than 6 months). The reagent will, however, tolerate the presence of up to about 1% water introduced with a sample.

A variety of other mixtures including water, methanol, *n*-butanol, dioxan, toluene, methoxyethanol and trimethyl phosphate, permuted with hydrochloric acid, phosphoric acid and polyphosphoric esters, have yielded inferior results in terms both of yields and reagent blanks.

UV absorbance and fluorescence excitation spectral characteristics

In Table I are collected the UV absorbance peak maxima and the molar absorptivities in methanol of the 19 isolated derivatives detailed under Experimental. The corrected fluorescence excitation spectra of the derivatives, of which an example is shown in Fig. 1, exhibit no significant differences from the absorbance spectra.

Beyond the acetic derivative the characteristics of the derivatives of all of the saturated acids are constant and only the means of these are given (Table I). Provided that the substituent derived from an unsaturated acid is not conjugated with the imidazole ring, the characteristics of the derivatives of the unsaturated acids are also expectedly constant, as the Table shows, and not significantly different from their saturated analogues. In the conjugated example, the crotonic derivative, considerable spectral changes occur, although many similarities to the other derivatives remain. The absorbance of the acetic derivative at 253.5 nm might be expected to lie between that of the formic derivative and the remainder in this region. Although the presence of impurities could be responsible, separate preparations of the acetic derivative, and repeated recrystallisations of it have not produced a significantly higher result.

Results of other workers are available only for the formic derivative (phenanthrimidazole). These[11,14] are in general agreement with the present results.

In highly aqueous solvents (Table II), the UV absorbance spectra of the lower-

TABLE I

UV ABSORBANCE DATA OF PHENANTHRIMIDAZOLE DERIVATIVES OF SOME ALIPHATIC CARBOXYLIC ACIDS IN METHANOL

Molar absorptivity data are given in parentheses.

Derivatized acid	*Wavelength of absorbance maxima (nm) and molar absorptivity*						
Formic*	246 (63347)	252.5(86483)	280 (14040)	298 (8210)	319 (871)	333 (1543)	349 (1913)
Acetic**	248 (60078)	253.5(80749)	279 (15375)	303 (9005)	324 (1110)	338 (1711)	353 (2054)
$C_nH_{2n+1}COOH$***	248.2(66341)	254.8(90295)	279.8(17339)	303.5(9831)	324.5(1113)	338.8(1879)	354.5(2284)
Oleic	248 (66494)	255 (89394)	280 (18486)	303 (10429)	325 (1380)	338 (2125)	354 (2536)
Elaidic	248 (66676)	254 (90863)	280 (17737)	303 (9979)	325 (1223)	338 (1995)	354 (2505)
Linoleic	247 (64543)	254 (87835)	279 (17613)	303 (9571)	325 (1298)	338 (1970)	354 (2433)
Linolenic	248 (63625)	254 (89307)	280 (17396)	303 (9693)	325 (1220)	338 (1953)	354 (2353)
Erucic	248 (67609)	254 (91647)	279 (17682)	303 (9985)	325 (1294)	338 (1964)	354 (2496)
Crotonic	252 (46715)	260 (61321)	280 (15934)	306 (19603)		342 (5915)	358 (5673)

* Means of 3 determinations.

** Means of 5 determinations on 2 separately prepared samples of the derivative.

*** Means of individual determinations on derivatives from: *n*-butyric, isobutyric, caproic, caprylic, *n*-nonanoic, capric, lauric, myristic, palmitic, stearic and arachidic acids. The wavelength values are distributed with a standard deviation of 0.4 nm; the standard deviations of the absorptivities vary from 117, for the highest absorptivity, to 73 for the lowest.

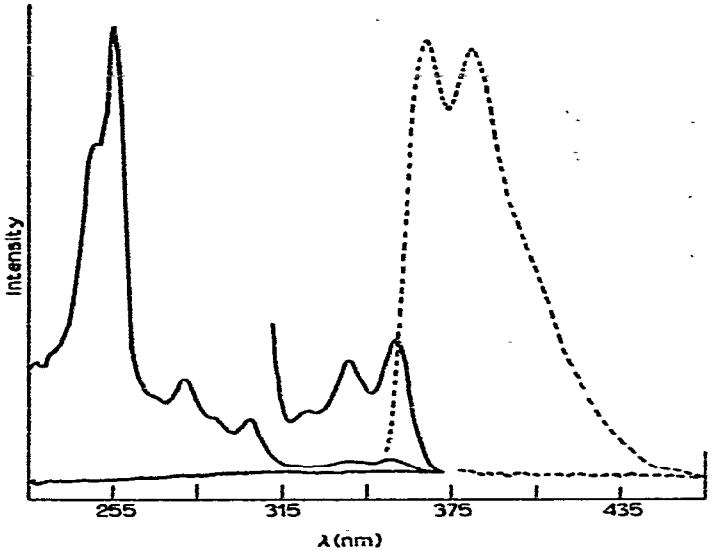


Fig. 1. Corrected fluorescence excitation (full line) and emission (dotted line) spectra of 2-*n*-pentadecylphenanthrimidazole, the palmitic derivative, at a concentration of 1 μM in methanol. In each is shown the solvent blank. In the near-UV region, the excitation spectrum is shown additionally at a tenfold increased sensitivity.

molecular-weight derivatives are similar to the spectra in methanolic solvents except for some peak-broadening and loss of amplitude. With the caprylic (*n*-C_8) derivative and above, however, the spectra become extensively broadened and weakened; and although the peak maxima are still identifiable to those in methanol, they tend to be red-shifted. Similar effects occur in the fluorescence excitation spectra except their onset is delayed to the capric–lauric (C_{10}–C_{12}) region.

Fluorescence emission spectra and quantum yields

All of the derivatives, in methanol, produce an intense blue fluorescence. The fluorescence emission spectrum is independent of the 2-substituent on the derivatives, with the exception of the crotonic and formic derivatives. These exhibit slight but predictable differences (Table III). A typical corrected spectrum is shown in Fig. 1. The fluorescence quantum yield (Table III) is also constant in either nitrogen-purged or aerated methanol, although sensitive to oxygen quenching. Aeration of a deoxygenated solution reduces the quantum yield, on average, from 0.68 to 0.31 (Table III). Even so, the fluorescence is still relatively intense. A value of 0.35 for the quantum yield of phenanthrimidazole in ethanol has been reported[14], which is consistent with the value in aerated methanol given in Table III. Whether the former value refers to an aerated solution is not clear however.

The fluorescence emited from strongly aqueous solutions (Table III) is slightly red-shifted and usually exhibits a single maximum, except for the higher molecular weight compounds. As the series is ascended the quantum yield falls abruptly in the region of the caprylic and capric derivatives, depending on concentration, as the table shows. Beyond this point the quantum yields are negligibly affected by oxygen quenching.

TABLE II

UV ABSORBANCE DATA OF PHENANTHRIMIDAZOLE DERIVATIVES OF SOME ALIPHATIC CARBOXYLIC ACIDS IN 90% (v/v) WATER IN METHANOL

Molar absorptivity data are given in parentheses. Some of the spectra are concentration-dependent, these data refer to solutions at 4 μM. Shoulders are indicated by *s*. The 325 nm absorption seen in methanol is largely obscured.

Derivatized acid	*Wavelength of absorbance maxima (nm) and molar absorptivity*					
Formic	247 (61740)	253 (79678)	280 (14642)	299 (7676)	336 (1418)	349 (1585)
Below C_8 (Table I), except formic, crotonic*	248 (61223)*s*	254 (75399)	279 (16350)	303 (7179)	342 (1314)	352 (1288)
Caprylic (n-C_8)	253 (39280)	263 (33905)*s*	285 (24110)	310 (18334)	342 (7283)	358 (6731)
Nonanoic	253 (28162)	266 (30576)	287 (24427)	311 (20691)	342 (8161)	358 (7357)
Above C_9 (Table I)**	252.4(46334)*s*	260.4(56911)	283.0(26463)	308.0(18331)	341.4(6130)	356.9(5594)
Crotonic	252 (34041)*s*	259 (43456)	288 (12168)	304 (14727)	341 (3718)	354 (3235)

* The results given are the means; the standard deviations of the wavelength maxima vary from 3.4 to 0.5 nm; for the molar absorptivity values the variation is from 3422 to 474, according to the magnitude of the values.

** The results are the means; the standard deviations of the wavelength maxima vary from 1.9 to 0.5 nm; for the molar absorptivity values the variation is from 9312 to 2148, according to magnitude.

TABLE III

FLUORESCENCE EMISSION AND QUANTUM YIELD (Q) DATA OF THE PHENANTHRIMIDAZOLE DERIVATIVES UNDER VARIOUS CONDITIONS

Derivatized acid	*Solvent*	*Conc. (μM)*	*Emission maxima ± S.D. (nm)*		$Q_{N_2} \pm$ S.D.	$Q_{air} \pm$ S.D.
Formic	Methanol	0.4	358	374	0.698	0.418
All except formic, crotonic*	Methanol	0.4–4.0	366.9 ± 0.6	382.3 ± 0.7	0.683 ± 0.020	0.314 ± 0.017
Crotonic	Methanol	0.4		387	0.645	0.370
Formic	90% aq.	4.0		377	0.598	0.569
Below C_8 except formic, crotonic*	90% aq.	0.4–4.0		384.5 ± 0.5	0.549 ± 0.012	0.520 ± 0.015
Caprylic (n-C_8)	90% aq.	0.4		384	0.614	0.594
		4.0		387	0.240	0.237
n-Nonanoic	90% aq.	4.0		386.5	0.117	0.114
Capric (n-C_{10})	90% aq.	0.4		387	0.223	0.207
		4.0	372**	387	0.084	0.081
Lauric (n-C_{12}) and above*	90% aq.	4.0	370.8 ± 0.9	383.6 ± 0.8	0.097 ± 0.027	0.092 ± 0.026
Crotonic	90% aq.	0.4		395	0.448	0.440

* As given in Tabel I.

** The peak is apparent in the earlier members as a shoulder, which tends to increase in intensity through the series, although the trend is rather irregular.

The influence of varying concentrations of water on the intensity of fluorescence from the formic and stearic derivatives, chosen to represent extremes of behaviour, is shown in Fig. 2. In deoxygenated solutions the addition of water up to 30% (v/v) produces a slight fall in intensity of about 5% in both cases. Thereafter a sudden decrease in the stearic derivative's fluorescence occurs, whereas only a slightly accelerated decrease occurs in the formic case. The behaviour of the other derivatives depends on the length of the side chain, with the sigmoid curve developing in the capric–caprylic region. As in the methoxycoumarin series[5] it is apparent that molecular aggregation to produce weakly fluorescent micelles occurs in the higher derivatives.

In contrast to deoxygenated solutions, in aerated solutions the initial effect of the addition of water is to produce a steadily rising fluorescence intensity, which eventually becomes parallel to and practically coincident with that from deoxygenated solutions. Only the case of the stearic derivative is illustrated (Fig. 2), but the formic and all of the other derivatives examined do likewise: the two curves become closely parallel in the region of 60% water. As the sensitivity of a fluorescence to quenching is dependent on the lifetime of the fluorescence, it is probable that here the addition of water reduces the fluorescence lifetime and, therefore, the sensitivity to quenching by oxygen.

A slight quenching effect is exerted on the formic derivative's fluorescence by ammonium carbonate, but the effect is negligible on the others. Thus, the quantum yields of the formic, acetic and stearic derivatives in deoxygenated 10% (v/v) water in methanol containing 0.01 *M* ammonium carbonate are found to be 0.60, 0.67 and 0.65 respectively. As this concentration of water is without significant effect (Fig. 2), these values are directly comparable with the methanol values in Table III.

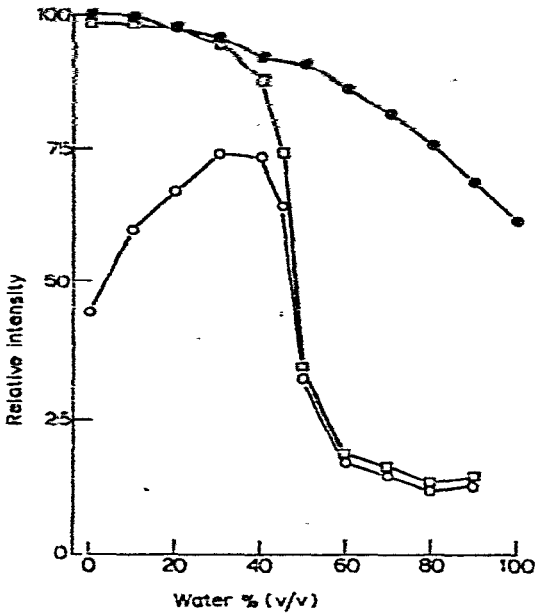


Fig. 2. Variation in fluorescence intensity, excited at 255 nm and monitored at 385 nm, with water content of aqueous methanol solutions, of the formic derivative (deoxygenated, filled circles) and the stearic derivative (deoxygenated, squares; aerated, open circles). The concentration of the derivatives was 1 μM.

In non-polar solvents, *e.g.* cyclohexane, the shoulder present on the long-wave side of the emission (Fig. 1) becomes resolved. Otherwise the general features of the spectra are unchanged, but the quantum yields are reduced. For the formic and stearic derivatives the values found in deoxygenated cyclohexane are 0.31 and 0.26 respectively. Because aqueous methanolic solvents are clearly to be preferred for the chromatography of this group of compounds, other non-polar solvents have not been examined.

The photolability of the derivatives is relatively small, although on irradiation in the sample compartment of a spectrometer for 1–2 min some changes in the fluorescence occur. The effect is more pronounced in aerated solvents, but unlikely to be of significance under the usual HPLC conditions.

Absorbance and fluorescence features with reference to HPLC detection

Under conditions applicable to their chromatography, the maximum molar absorptivity of the phenanthrimidazoles (90143 at 254.6 nm, means of 16 derivatives in Table I) is exceptionally high, and virtually coincident with the strong emission, at 254 nm, from low-pressure mercury lamps. For the methoxycoumarins[5] the maximum absorptivity is 14602 at 322.5 nm. The mean fluorescence quantum yield of the phenanthrimidazoles is high (0.68, Table III) and, in deoxygenated solvents, relatively insensitive to solvent effects; whereas the methoxycoumarins vary considerably, from 0.09 to 0.4 (but they are insensitive to oxygen quenching). The mean bandwidth of the phenanthrimidazole emission is 3120 cm^{-1}, hence the intrinsic fluorescence sensitivity (IFS) value[5] is 19.7. For the methoxycoumarins the value is 1.6.

Chromatographic characteristics

Fig. 3 summarizes the variation in capacity factor of the derivatives, on ODS–Hypersil, with the water content of methanolic solvents containing 0.01 *M* ammonium carbonate. (In the absence of ammonium carbonate some peak-broadening is evident.) The same type of variation occurs in other fatty acid derivatives[2,3,18]: capacity factors increase as the series is ascended; the *n*-isomer is more strongly retained than its branched chain isomers; retention decreases with an increase in unsaturation of a given carbon chain; and *trans*-isomers are more strongly retained than *cis*-isomers.

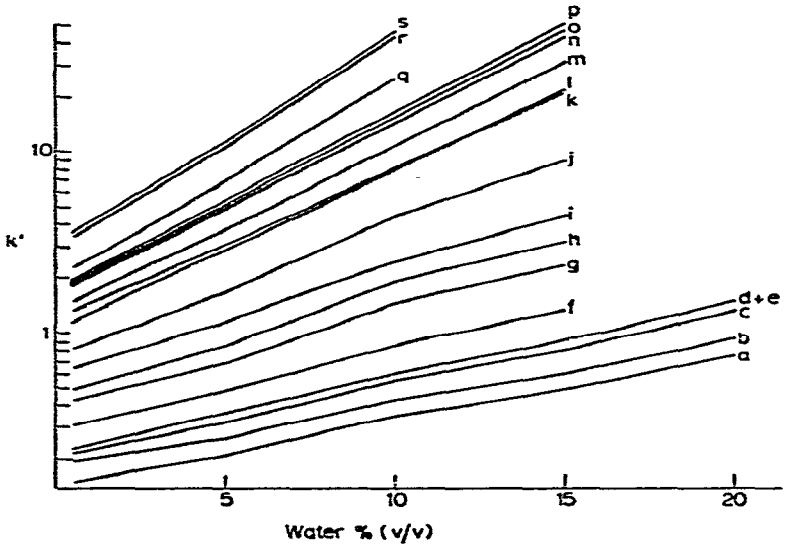


Fig. 3. Variation of capacity factor (k') on ODS–Hypersil with varying concentration of water in methanol, containing 0.01 *M* ammonium carbonate, of the phenanthrimidazole from formic (a), acetic (b), isobutyric (c), *n*-butyric (d), crotonic (e), caproic (f), caprylic (g), nonanoic (h), capric (i), lauric (j), myristic (k), linolenic (l), linoleic (m), palmitic (n), oleic (o), elaidic (p), stearic (q), arachidic (r), and erucic (s) acids.

For the closely eluting linolenic and myristic derivatives, a marked change in relative selectivity occurs as the solvent is changed, as Figs. 3 and 4 show. In the presence of 15% water the former derivative is slightly the more strongly retained. At 10% water the order is reversed, although the two are not resolved (Fig. 4); but as the water content is further reduced the resolution continues to increase, despite the reduced retention of both derivatives. Their resolution becomes complete in the range 1–5% water. Only the crotonic and *n*-butyric derivatives, out of all of those examined, remain unresolved under the conditions used.

Sensitivity, range and linearity

The results of some experiments in which varying concentrations of fatty acids in the methyl polyphosphate and the diaminophenanthrene reagent were used are summarized in Table IV. In general, the usable range is covered by a factor of 10^4. Log–log plots of peak areas against concentration are in most cases significantly

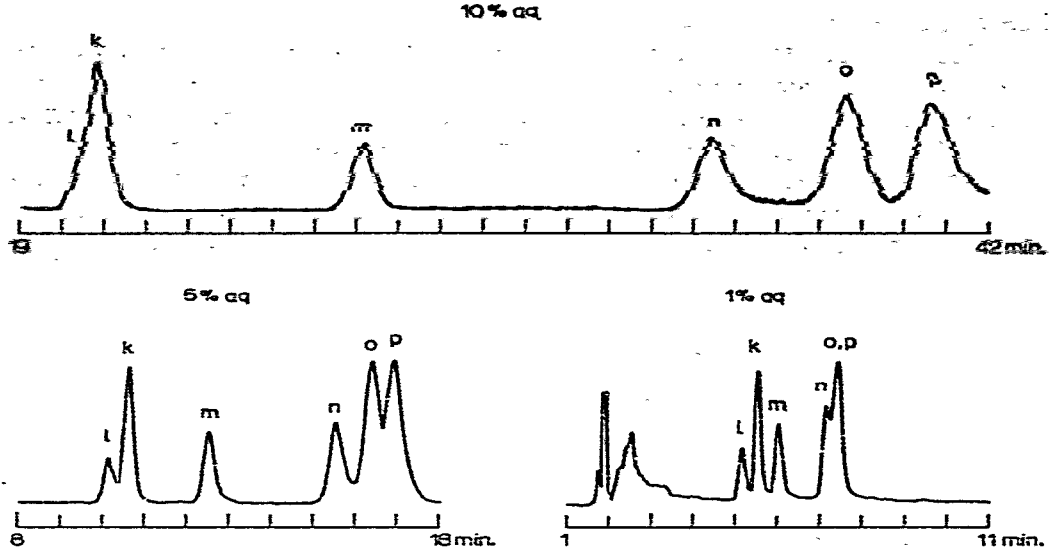


Fig. 4. Chromatograms on ODS–Hypersil, 25 cm, in the indicated concentrations of water in methanol (0.01 *M* ammonium carbonate), of the phenanthrimidazoles from linolenic (l), myristic (k), linoleic (m), palmitic (n), oleic (o), and elaidic (p) acids.

greater than 1, possibly because some losses occur at the lower concentration levels. Here, the quantities chromatographed correspond to picogram amounts of the derivatized acid. Some chromatograms from this region, for palmitic acid, are shown in Fig. 5. In these the offscale peaks due to the reagent correspond to the acetic region, as the gradient elution chromatograms in Fig. 6 show, and generally constitute no significant interference except for the acetic derivative. The lower concentration range of the latter (Table IV) is evidently limited by the reagent blank.

In Table IV are also shown the results of experiments in which the recovery of the derivative from various acids, at an intermediate concentration level of 50 μM

TABLE IV

LINEARITY (LOG–LOG PLOTS) AND YIELD IN THE FORMATION OF PHENANTHRIMIDAZOLES FROM SOME ALIPHATIC CARBOXYLIC ACIDS

c.l. = confidence limits. S.D. = standard deviations calculated from the results of six independent determinations.

Derivatized acid	*Conc. range of linearity of log–log plot (μM)*	*No. of points*	*Linear regression coefficient of slope ± 95% c.l.*	*Yield ± S.D. at 50 μM (%)*
Acetic	3.4 –1038	9	1.033 ± 0.017	95.5 ± 4.7
Caproic (n-C_6)	0.74–2874	12	1.040 ± 0.024	95.7 ± 4.4
Capric (n-C_{10})	0.78–3011	13	1.027 ± 0.017	98.0 ± 3.7
Palmitic	1.6 –2000	10	1.041 ± 0.020	95.5 ± 2.8
Erucic	0.40–3823	14	1.033 ± 0.013	101.3 ± 5.5
Oleic	2.1 –10177	13	1.042 ± 0.022	97.7 ± 3.2
Linoleic	0.20–3119	15	1.001 ± 0.020	99.2 ± 4.8
Linolenic	0.38–3111	15	1.002 ± 0.013	91.5 ± 1.5

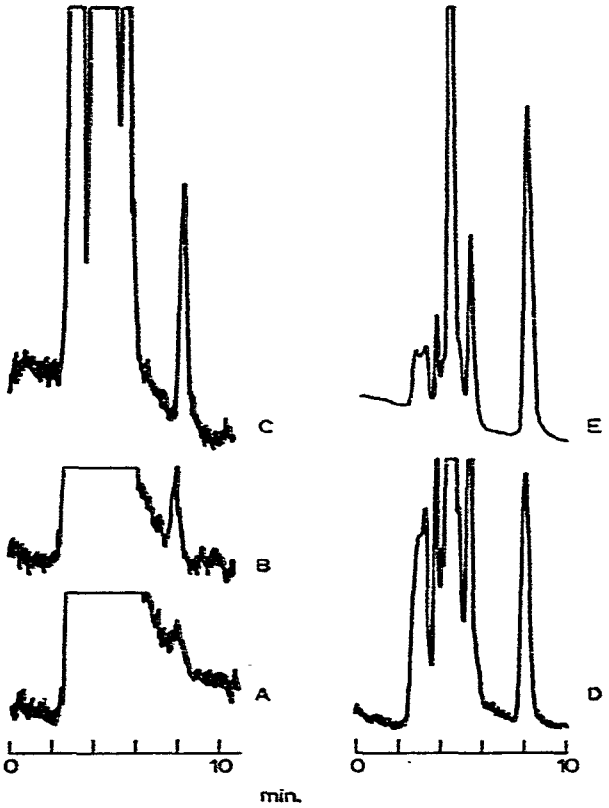


Fig. 5. Chromatograms, on 25 cm ODS–Hypersil in deoxygenated 1% water in methanol (0.01 *M* ammonium carbonate) at 0.8 ml min^{-1}, showing peaks derived from 0 (A), 8 (B), 64 (C), 256 (D), and 1024 (E) pg of palmitic acid in the injected (0.4 μl) reagent. The relative fluorescence detector sensitivities are: 10 (A, B, C), 3 (D), and 1 (E); with 255 nm excitation, 382 nm emission, and with both bandwidths at 3 nm.

in the reagent, was determined by HPLC with reference to known amounts of the isolated derivative. The recovery values are reproducibly in excess of 95% except for the linolenic acid, where the slightly lower recovery is at least as reproducible as the others.

Solvent effects and gradient elution

Because of their relative insensitivity to solvent effects, gradient elution chromatography of the derivatized fatty acids yields practically constant peak areas. This is shown in Fig. 6, where each peak is due to 0.1 μmole of each even-numbered fatty acid from C_2 to C_{20} with a variation in solvent composition of 30% to 1% water. The peak areas, indicated over each peak, are distributed with a coefficient of variation of 6.1%. This, particularly in view of the increased running time of the chromatogram and consequent variation in instrumental sensitivity, is negligibly greater than most of the values obtained from replicate measurements on the individual fatty acids (Table IV). In the methoxycoumarin derivatives of fatty acids in the range C_8–C_{18} apparently a fourfold variation in peak area occurs[2].

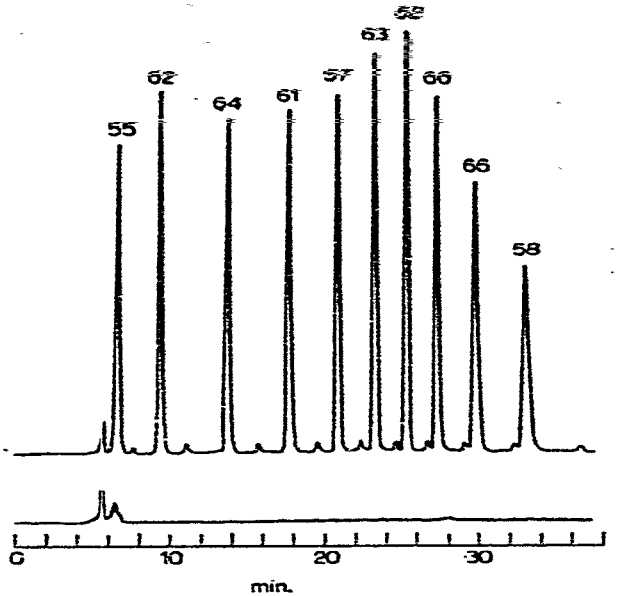


Fig. 6. Gradient elution chromatograms, with water concentration in methanol varying from 30% to 1% (v/v) throughout the chromatograms. The conditions are otherwise as in Fig. 5. The lower chromatogram is a reagent blank. The upper is from a derivatization of equimolar amounts of all the even-numbered, linear, saturated fatty acids from C_2 to C_{28}. Each peak corresponds to 0.1 μmole of fatty acid. The numbers indicate peak areas.

CONCLUSIONS

The phenanthrimidazoles offer a high level of sensitivity for fatty acids, *e.g.*, down to the 30 femtomole level as in Fig. 5. Their use should enable very small amounts of fatty acid-containing materials and carboxyl-substituted drugs, for instance, to be identified and determined. Against this must be set the requirement that the reaction, like the methoxycoumarin derivatization, requires essentially non-aqueous samples; and molecules carrying other functional groups, *e.g.* hydroxyl groups, may be phosphorylated and possibly degraded by the reagent. In this instance a methoxycoumarin derivatization might be preferable. However, a variety of other imidazole-forming reactions occur under aqueous conditions, and on a variety of substrates[6]; thus, other derivatives of this type could probably be developed fairly readily to deal with such circumstances.

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