Journal of Chromatography, 269 (1983) 81–90
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,059

4-BROMOMETHYL-6,7-DIMETHOXYCOUMARIN AS A FLUORESCENT LABEL FOR CARBOXYLIC ACIDS IN CHROMATOGRAPHIC DETECTION

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(First received September 2nd, 1982; revised manuscript received June 9th, 1983)

SUMMARY

The UV absorbance, corrected fluorescence spectra, quantum yields and lifetimes of 4-bromomethyl-6,7-dimethoxycoumarin derivatives of carboxylic acids as well as of a few fatty esters are presented. The fluorescence quantum yields in methanol are not affected by the number of carbon atoms (C_2–C_5) of the carboxylic acid. Among various solvents, water gives the highest quantum yield (0.64), whereas in non-hydrogen-bonding solvents the yields are less than 0.23. The yield from the C_2 derivative rises rapidly from 0.43 to 0.60 as 20% water is added to methanol, and from 0.23 to 0.61 if 50% water is added to acetonitrile. The fluorescence spectra and quantum yields are only slightly affected by the pH, ionic strength and the nature of electrolytes used in the mobile phase. The results show that fatty acid esters of 4-hydroxymethyl-6,7-dimethoxycoumarin have higher quantum yields and intrinsic fluorescence sensitivities than homologous 7-methoxycoumarin derivatives. Furthermore they offer a better possibility for gradient elution chromatography. Derivatization of fatty acids with 4-bromomethyl-6,7-dimethoxycoumarin is a very sensitive method for the evaluation of picomole amounts by liquid chromatography using fluorescence detection.

INTRODUCTION

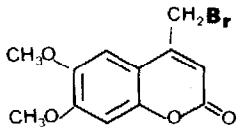
The analysis of physiological samples is important in providing new insights

0021-9673/83/$03.00

into metabolic reactions. Short chain fatty acids are intermediates in the metabolism of amino acids such as leucine, isoleucine and valine in mammals, and are secreted by some bacteria. Thus, great interest has been shown in their chromatographic analysis for the study of genetic disease and of the physiology and taxonomy of micro-organisms. Short chain fatty acids also affect the flavour and aroma in cheese, wine and fruits. Low detection limits are satisfactory in the chromatography of these compounds.

The first method used for their analysis was gas–liquid chromatography (GLC), either by direct injection[1,2] or after derivatization[3]. In liquid chromatography (LC), compounds can be detected by using UV, fluorescence or electrochemical detection. For compounds that are transparent to these detectors, labelling with UV adsorbents[4–10], fluorescents[11] or electro-active nuclei[12] before or after[13] chromatography may provide a solution. The most commonly used derivatizing reaction for organic acids is the formation of phenacyl esters, which have been used to analyse short chain fatty acids[7], prostaglandins[14] and penicillins[15].

Specificity and sensitivity are greatly improved by use of coupled fluorimetric liquid chromatography. Dünges[11] reported a very sensitive fluorescence labelling technique for the thin-layer chromatographic analysis of picomole amounts of fatty acids using 4-bromomethyl-7-methoxycoumarin. This method was extended to LC analysis[16,17]. Lloyd[18] observed that the use of fatty acid esters of 4-hydroxymethyl-7-methoxycoumarin in determinations by gradient liquid chromatography was limited by the fluorescence yields in non-polar solvents and the possible formation of micelles at high water contents in the eluent. Two types of solutions were proposed: a post-column reaction to hydrolyse 7-acetoxycoumarin derivatives[19], or other labels such as 9,10-diaminophenanthrene[20] or anthryldiazomethane[21,22]. Post-column reaction after previous derivatization is rather tedious. The reagents proposed by Lloyd[20] and Barker *et al.*[22] are characterized by a low polarity and a high molecular weight implying the use of mobile phases of low polarity. In order to avoid these difficulties, we have looked for a polar fluorescent label, which can be used with an elution gradient. Our previous studies[23] on differently substituted 7-methoxycoumarins led to the choice of 4-bromomethyl-6,7-dimethoxycoumarin (I). In the present paper the UV and fluorescence spectral properties, quantum yields and fluorescence lifetimes of some carboxylic acid esters in different solvents are reported. The influence of various liquid chromatographic parameters (nature, pH and ionic strength of mobile phase) on these properties is presented, and a separation on a reversed-phase column is proposed.



I

MATERIALS AND METHODS

Apparatus

The fluorescence spectra were recorded with an Aminco SPF 500 spectrofluo-

rometer (band width 2 nm) and UV spectra with a Jobin Yvon JY 201 spectrophotometer. A SLM Instruments phase fluorometer was used to measure fluorescence lifetimes. A microcomputer (Kontron) coupled to the spectrofluorometer calculated corrected spectra and quantum yields.

The liquid chromatograph comprised a Chromatem 380 pump (Touzart et Matignon), a 20-μl sample loop, a Schoeffel 970 spectrofluorometer and a (250 × 4.6 mm) 5-μm ODS ultrasphere column (Altex).

Reagents

18-Crown-6 and 4-bromomethyl-7-methoxycoumarin were obtained from Aldrich (Milwaukee, WI, U.S.A.), methanol, hexane, chloroform, dimethylformamide, acetone and potassium carbonate Prolabo (France), acetonitrile UV grade from Fisons and dimethyl sulphoxide and tetrahydrofuran for spectroscopy from Merck (F.R.G.) Fatty acids were used as received (Merck).

Synthesis of reagent

4-Bromomethyl-6,7-dimethoxycoumarin (Brmdmc) was synthesized in three steps: 1,2,4-triacetoxybenzene[24] was condensed with 3-oxo-glutaric acid, the 6,7-dihydroxycoumarin-4-acetic acid formed was methylated to 6,7-dimethoxycoumarin-4-acetic acid and finally this compound was brominated.

6,7-Dihydroxycoumarin-4-acetic acid. This synthesis is performed by the Pechmann reaction. Triacetoxybenzene (0.1 mole) was condensed with 3-oxo-glutaric acid (0.1 mole) at 0°C in concentrated sulphuric acid. After 24 h at 0°C, 6,7-dihydroxycoumarin-4-acetic acid was precipitated with ice. After washing until neutral pH, the product was crystallized in water–methanol (25:75, v/v); m.p. = 230°C, yield = 85%.

6,7-Dimethoxycoumarin-4-acetic acid. 6,7-Dihydroxycoumarin-4-acetic acid (0.1 mole) was dissolved in 300 ml of 1.5 *M* sodium hydroxide and 0.25 ml of dimethyl sulphate were added dropwise. The solution was heated at 60°C for 1.5 h. Concentrated sulphuric acid (60 ml) was added and the precipitate obtained after 24 h at +4°C was crystallized in tetrahydrofuran–ethanol (5:95, v/v); m.p. = 218°C, yield = 60%.

4-Bromomethyl-6,7-dimethoxycoumarin. 6,7-Dimethoxycoumarin-4-acetic acid (0.1 mole) was dissolved in 60 ml of acetic acid and heated at reflux. 200 ml of a 0.075 *M* solution of bromine in acetic acid were added dropwise. The solution was refluxed for 1 h and cooled. The precipitate formed was washed with acetic acid and with acetone; m.p. = 208°C, yield = 70%.

Synthesis of samples

For fluorescence study. 7-Methoxycoumarin-4-acetic acid was synthesized according to Secrist *et al.*[25]. 4-Acetoxymethyl-7-methoxycoumarin (Ia) and 4-acetoxymethyl-6,7-dimethoxycoumarin (Ib) were prepared according to Dünges[11]. A semi-preparative thin-layer chromatography was carried out for final purification: Ia, m.p. = 165°C; Ib, m.p. = 182°C.

4-Acetyl-6,7-dimethoxycoumarin esters were prepared as follows. 6,7-Dimethoxycoumarin-4-acetic acid (10^{-2} mole) was dissolved in 20 ml of an alcohol together with a trace amount of sulphuric acid. The alcohol is chosen according to the ester to be prepared. The mixture was refluxed for 8 h, then cooled to allow precipitation of the ester. The latter was crystallized from ethanol.

For chromatographic study. In a 3-ml flask protected from light with aluminium foil, the fatty acids were dissolved in 100 μl of anhydrous acetone and neutralized to a phenolphthaleine end-point with a 10% methanolic potassium hydroxide solution. A three-fold excess of Brmdmc (in acetone), 0.2 mole of 18-crown-6 (in acetonitrile) and 2 mg of anhydrous potassium carbonate were added. The flask was stoppered and heated at 70°C for 30 min. The solution was then cooled and an aliquot injected in the chromatograph.

The structures of all synthesized molecules were assigned by IR spectroscopy, nuclear magnetic resonance (NMR) spectrometry and elemental analysis.

Quantum yields and fluorescence sensitivity

Quantum yields were determined with reference to quinine bisulphate in 0.1 *N* sulphuric acid according to Parker and Rees[26]. Measurements of quantum yields[18] and lifetimes were done at room temperature without deoxygenation.

Fluorescence lifetimes

Fluorescence lifetimes were measured on the SLM 4800 phase fluorometer using the principle of Spencer and Weber[27]. The modulation frequency was chosen to be 30 MHz. This instrument allows the determination of lifetimes either by measurement of the phase lag between the incident and emitted light, τ_ϕ, or of the relative modulation of the emitted light, τ_M. These parameters should be identical when the emitting population is single, homogeneous and with a unique lifetime of the excited state.

RESULTS AND DISCUSSION

The UV and fluorescence spectral properties in methanol of 4-substituted 7-methoxycoumarin and 6,7-dimethoxycoumarin are reported in Table I. The bromomethyl derivatives show a lower fluorescence quantum yield due to quenching by bromine. Introduction of an electron-donating group (methoxy) at the 6-position in coumarin results in a red shift and induces an almost 1.5-fold increase in the quantum yield. Based on these results, 4-bromomethyl-6,7-dimethoxycoumarin was employed as the fluorescence reagent.

Spectral properties of coumarin esters: influence of chain length of fatty acids

Preliminary investigations carried out in our laboratory have shown (Table I) that 4-acetoxymethyl (Ia, Ib) and 4-methoxycarbonylmethyl (IIa, IIb) coumarin derivatives exhibit similar quantum yields in methanol; the chain length has a very slight effect on the wavelength of the absorption and emission maxima. This fact is in close agreement with Lloyd's results[18]. Fluorescence lifetimes measured by phase delay and by relative modulation indicate the purity of the samples and the homogeneity of the fluorescence emission spectra (Table II).

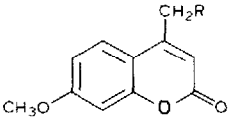
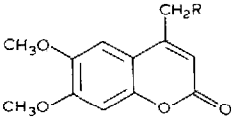
Spectral characteristics and solvent effects

Fluorescence spectra and quantum yield were measured in solvents usable in chromatography. The solubility of 4-acetoxymethyl-6,7-dimethoxycoumarin allowed determinations in different media, such cyclohexane and water. A red shift of the emission wavelength and an increase in Stokes shift with solvent polarity is shown

TABLE I

UV ABSORPTION AND FLUORESCENCE DATA OF SOME 7-METHOXY- AND 6,7-DIMETHOXYCOUMARIN DERIVATIVES IN METHANOL

Q = Quantum yield.

Compound	*R*	*UV absorption*		*Fluorescence*	
		λ_{max} *(nm)*	ε *(cm^{-1} M^{-1})*	λ_{em} *(nm)*	*Q*
	–COOH	320	11,820	380	0.18
	–Br	325	12,080	396	0.02
	–$OCOCH_3$ (Ia)	322	12,821	395	0.11
	–$COOCH_3$ (IIa)	325	12.587	384	0.14
	–COOH	340	12,800	415	0.36
	–Br	348	10,631	425	0.24
	–$OCOCH_3$ (Ib)	340	12,942	425	0.42
	–$COOCH_3$ (IIb)	343	12,880	425	0.43

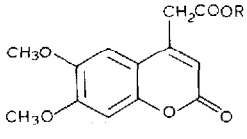
in Table III. The spectral maxima are shifted from 404 nm in cyclohexane to 429 nm in water. This phenomenon may be explained in terms of solvent relaxation. The coumarin molecules have a higher dipolar moment in the excited state than in the ground state when they are dissolved in a polar solvent.

The orientational polarizability factor used to characterize solvent polarity was calculated as follows[28]:

$$f = \frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}$$

TABLE II

UV ABSORPTION AND FLUORESCENCE DATA OF SOME 4-SUBSTITUTED 6,7-DIMETHOXYCOUMARINS IN METHANOL

Compound	*R*	*UV absorption*		*Fluorescence*		
		λ_{max} *(nm)*	ε *(cm^{-1} M^{-1})*	λ_{em} *(nm)*	*Q*	τ *(ns)**
	–CH_3	343	12,880	425	0.43	4.2 (4.1)
	–C_2H_5	341	10,990	423	0.45	4.1 (4.0)
	–$(CH_2)_2CH_3$	346	10,320	423	0.46	4.1 (4.1)
	–$CH(CH_3)_2$	345	11,780	427	0.44	4.1 (4.0)
	–$(CH_2)_3CH_3$	344	13,537	426	0.48	4.3 (4.0)
	–$CH_2CH(CH_3)_2$	343	11,720	423	0.47	4.2 (4.0)

* The first value corresponds to the lifetime measured by phase delay, that in parentheses to the lifetime measured by relative modulation (30 MHz).

TABLE III

FLUORESCENCE EMISSION AND QUANTUM YIELDS OF 4-ACETOXYMETHYL-6,7-DIMETHOXYCOUMARIN IN DIFFERENT SOLVENTS

f = orientational polarizability.

Solvent	f	λ_{em} *(nm)*	Q	τ *(ns)*	*Stokes shift* (cm^{-1})	*Intrinsic fluorescence sensitivity (IFS)*
Cyclohexane	0.001	404	0.14	0.5 (−)	3839	0.07
Cloroform	0.185	415	0.47	2.9 (2.8)	4313	0.95
Tetrahydrofuran	0.209	415	0.10	1.0 (1.3)	4313	0.34
Dimethyl sulphoxide	0.260	424	0.26	2.8 (2.8)	4969	0.97
Dimethylformamide	0.276	425	0.61	2.5 (2.3)	5042	1.50
Acetonitrile	0.304	419	0.23	2.0 (1.9)	4935	0.57
Methanol	0.308	425	0.43	4.2 (4.1)	5206	1.14
Water	0.320	429	0.64	5.6 (5.8)	5002	2.36

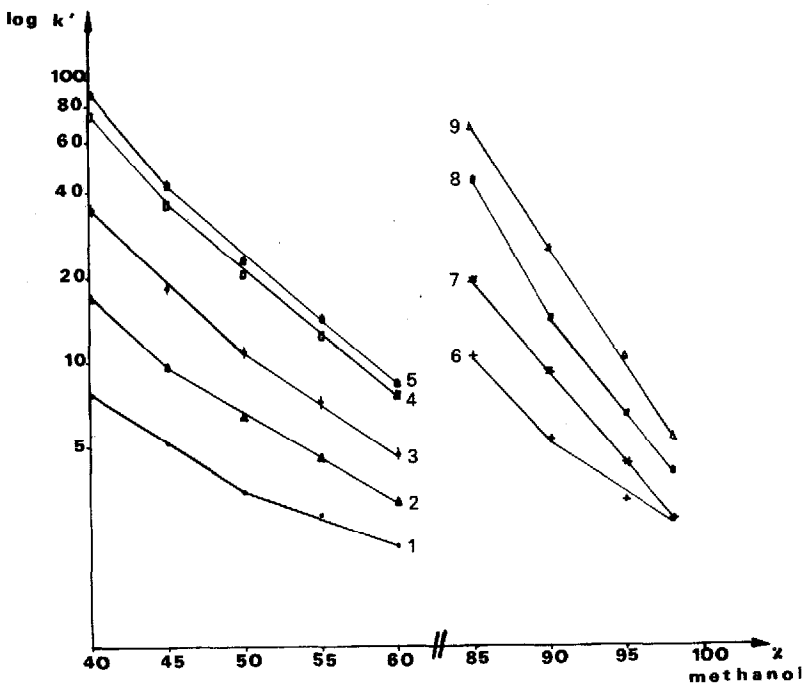


Fig. 1. Variation of log k' of 6,7-dimethoxycoumarin derivatives *versus* % of methanol in the mobile phase. Acids: 1, acetic; 2, propionic; 3, butyric and isobutyric; 4, isovaleric; 5, valeric; 6, myristic; 7, palmitic; 8, stearic; 9, arachidic.

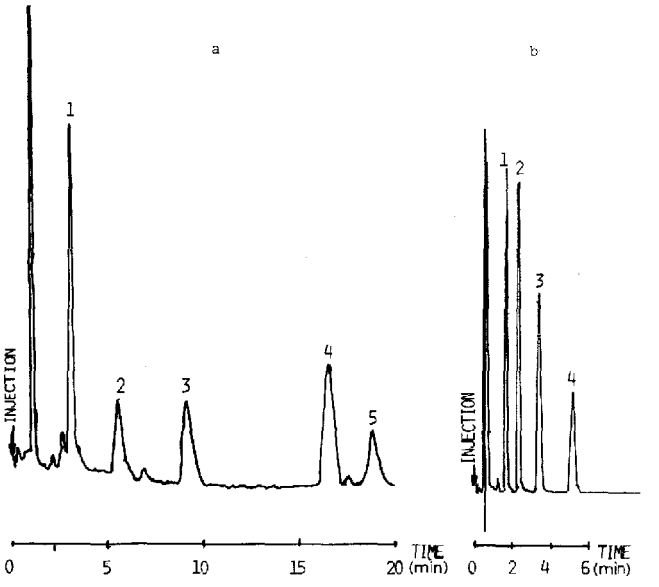


Fig. 2. a, Separation of some 6,7-dimethoxycoumarin derivatives of carboxylic acids. Mobile phase: water–methanol (50:50), flow-rate 2 ml min^{-1}. Peaks: 1 = acetic acid; 2 = propionic acid; 3 = butyric acid; 4 = isovaleric acid; 5 = valeric acid. b, Separation of some 6,7-dimethoxycoumarin derivatives of fatty acids. Mobile phase: water–methanol (5:95), flow-rate 3 ml min^{-1}. Peaks: 1 = myristic acid; 2 = palmitic acid; 3 = stearic acid; 4 = arachidic acid.

n and ε are the refractive index and the dielectric constant of the solvent respectively. The quantum yield varies with solvent polarity, the maximum being found in water. The yields are several fold lower in non-hydrogen-bonding solvents such as cyclohexane and acetonitrile. The fluorescence lifetimes reported for the different solvents do not accurately parallel the quantum yields. According to these results, a reversed-phase system will be more suitable for the analysis of derivatives of this type.

Chromatographic applications

In order to optimize separations, chromatographic parameters such as the nature, pH and ionic strength of the mobile phase must be investigated. We have studied the influence of these factors on the retention and fluorescence properties of 6,7-dimethyoxycoumarin derivatives.

Retention properties. Fig. 1 summarizes the variations in capacity factor of C_2–C_5 and $C_{14:0}$–$C_{20:0}$ derivatives on C_{18} reversed-phase chromatography, with the polarity of the mobile phase. The capacity factors are typical of those obtained in reversed-phase system, and increased with the number of carbon atoms. The linear C_5 derivative is more strongly retained than its branched chain isomer. The isobutyric and butyric derivatives remain unresolved under the conditions used, it is clear that a gradient elution would allow the separation. As an example, Fig. 2 demonstrates the isocratic separations of short chain acid derivatives (C_2–C_5) and long chain fatty acid esters. The principal by-products of the alkylation procedure are eluted rapidly at the start of both separations and do not interfere. The efficiency of the separation is not readily improved either by variation of pH or of the ionic strength of the

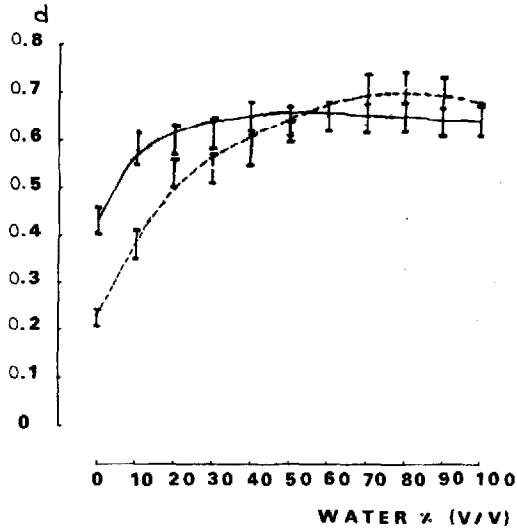


Fig. 3. Variation of quantum yield of 4-acetoxymethyl-6,7-dimethoxycoumarin in different methanol–water (———) and acetonitrile–water (---------) mixtures.

mobile phase: lowering the pH to 4 decreased the k' value of each derivative by about 25%; changing the ionic strength from $5 \cdot 10^{-3}$ to 10^{-2} and $5 \cdot 10^{-2}$ have no effect on the capacity factors.

Fluorescence properties. The influence of the water content in the mobile phase

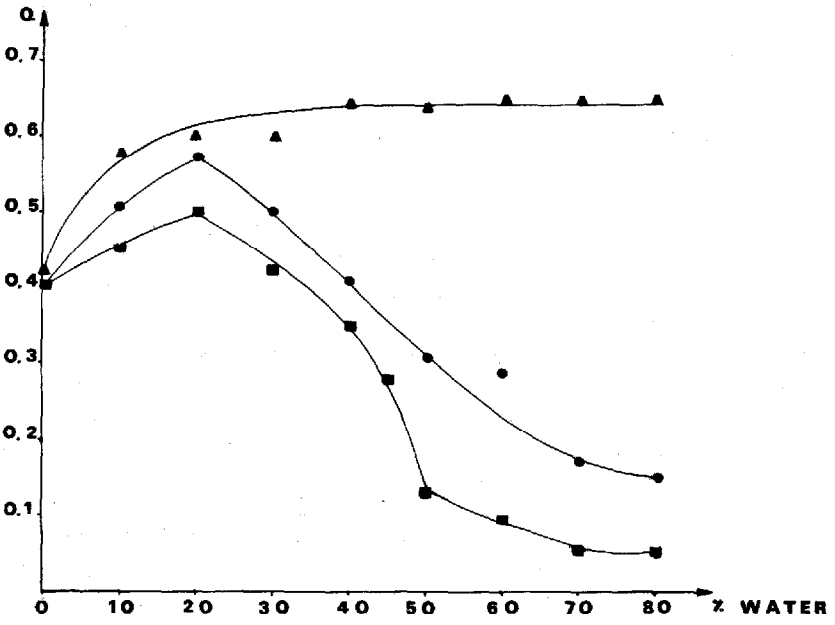


Fig. 4. Variation of fluorescence quantum yield of 6,7-dimethoxycoumarin derivatives with the percentage of water in methanol. ▲, Acetic acid; ●, myristic acid; ■, stearic acid.

TABLE IV

pH INFLUENCE ON FLUORESCENCE PROPERTIES OF 4-ACETOXYMETHYL-6,7-DIMETHOXYCOUMARIN

Solvent: water. Ionic strength: 10^{-2}. Anion: chloride.

pH	λ_{em} *(nm)*	*Width at half-peak height (nm)*	*Q*
2.2	429	71	0.61
3	429	70	0.69
3.7	431	70	0.62
4.5	431	71	0.68
6.5	429	70	0.64

systems (water–methanol and water–acetonitrile) employed on the fluorescence properties of 4-acetoxymethyl-6,7-dimethoxycoumarin was studied. The emission is red-shifted (420 to 429 nm) and the width at half-peak height broadened (66 to 71 nm) when the water ratio is increased in the mobile phase and the quantum yield reached a mean value of 0.64 in pure water (Fig. 3). Therefore, depending on the nature of the solvent, acetonitrile or methanol, the maximum value of the quantum yield is obtained for a water:organic solvent ratio of 0.5 and 0.2 respectively. This low water:methanol ratio, confirmed with 4-butyryl-6,7-dimethoxycoumarin, represents an improvement compared to 7-methoxycoumarin derivatives. With the latter compounds, a maximum is reached only with a water:methanol ratio of 0.80[18]. Therefore, methanol is suitable for gradient elution in reversed-phase chromatography of 6,7-dimethoxycoumarin derivatives. However, for long chain fatty acids, the fluorescence is strongly suppressed in more aqueous solvents (Fig. 4), presumably owing to the formation of molecular aggregates such as micelles[18].

Tables IV and V show that the fluorescence spectra and quantum yields of 4-acetoxymethyl-6,7-dimethoxycoumarin are not affected by changes in pH, ionic strength or the nature of the electrolytes used to optimize chromatographic conditions.

Sensitivity. To ascertain the detection limit a sample containing each acid (C_2–C_5) at a concentration of 1 mg/ml was diluted successively in methanol and

TABLE V

INFLUENCE OF IONIC STRENGTH AND NATURE OF ELECTROLYTE ON FLUORESCENCE PROPERTIES OF 4-ACETOXYMETHYL-6,7-DIMETHOXYCOUMARIN

Solvent: methanol–water (80:20).

Ionic strength	*Electrolyte*	λ_{em} *(nm)*	*Width at half-peak height (nm)*	*Q*
10^{-1}		427	70	0.66
10^{-2}	Cl^-	430	69	0.66
10^{-3}		425	70	0.64
	$H_2PO_4^-/HPO_4^{2-}$	422	68	0.59
	CH_3COO^-	430	70	0.65

injected on to the column. Considering a signal-to-noise ratio of 3, the detection limit is about 0.5 picomole.

CONCLUSIONS

Derivatization of carboxylic acids is necessary to obtain a greater sensitivity in their measurement. Also in liquid chromatography, the choice of label is governed by its UV or fluorescence spectral properties. However, other factors must be considered such as chemical properties (kinetics of derivatization), polarity, stability, ionizability and solubility in the usual mobile phases.

4-Bromomethyl-6,7-dimethoxycoumarin appears to be an interesting label. The derivatization reaction is rapid and gives good yields, and the physico-chemical and fluorescence properties of the derivatives in apolar solvents are very similar to those of 7-methoxycoumarin derivatives. This reagent has two main advantages: a higher quantum yield for derivates in water (0.64 *versus* 0.44), and an intrinsic fluorescence sensitivity of 2.36 *versus* 1.6 as reported by Lloyd[18] for 7-methoxy derivatives. A higher polarity allowing a shorter analysis time than with phenanthrene, anthracene and 7-methoxycoumarin derivatives. 4-Bromomethyl-6,7-dimethoxycoumarin might be an interesting tool as a label for proteins, nucleic acids, bile acids, prostaglandins and in drug investigations.

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