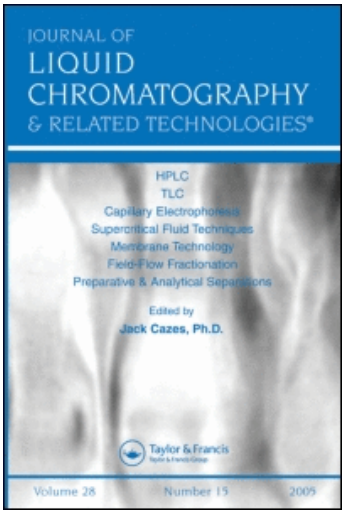

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ISOCRATIC SEPARATION OF FATTY ACID DERIVATIVES BY REVERSED PHASE LIQUID CHROMATOGRAPHY. INFLUENCE OF THE SOLVENT ON SELECTIVITY AND RULES FOR ELUTION ORDER.

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ABSTRACT

The p-bromophenacyl esters of 16 fatty acids (C_{12}-C_{22}) have been separated by isocratic chromatography on a Radial Pak A cartridge (Reverse phase C_{18} material). The separation factors α were measured using two solvent mixtures of comparable strength and the superiority of methanol-water to acetonitrile-water becomes evident.

Five precise rules are established, which indicates the retention of every fatty acid. They explain the chromatographic process i.e. elution order, resolution and selectivity.

INTRODUCTION

HPLC is now widely employed instead of GLC to separate long chain fatty acid mixtures. After some experiments with Corasil II (1,2) or ion-exchange (3) columns the reverse mode, more versatile and of higher efficiency, became commonly used with C_8,C_{18} and in some cases C_{30} stationary phases (4-12).

The use of an UV detector, sensitive and ideal in gradient elution, is required for trace amounts of fatty acids (in the nanogram range). Then, several simple, rapid and exhaustive methods of derivatization are available giving benzyl, naphtacyl,

phenacyl and substituted phenacyl esters, all UV-absorbing. Another advantage of these derivatives is to permit direct quantitation of molar ratios based on peak areas. (1,4,6,13,14).

In the first analyses only simple mixtures were separated but in 1975 BORCH, employing a 90 cm μ Bondapak C-18 column, was able to resolve 24 fatty acids with a step-change program of solvent using a mixture of acetonitrile and water.

This mixture was also used by many authors and seems superior to tetrahydrofuran-water or dioxane-water, but PEI et al (7) or CHAN et al (8) employed methanol-water and D'AMBOISE et al (11) a ternary solvent mixture of acetonitrile, methanol and water. In most cases the gradient elution technique permitting the separation of acids from C_2 to C_{24} (9) has been used, but the isocratic mode is also possible (11).

The most important features of these separations are now well known :

The retention volume is :

- widely increased by chain enlargement,
- decreased by unsaturation,
- larger for the trans-unsaturated acid

Some authors also noted that small changes in position of the double bond affect the interaction between the fatty acid ester and the reverse phase.

Some separations are particularly difficult : those of isomers (α and γ linolenic...) but also palmitoleic (16:1) arachidonic (20:4) and myristic (14:0) acids. We note the bad resolution of oleic and vaccenic acids (18:1), arachidic (20:0) and erucic (22:1) acids, behenic (22:0) and nervonic (24:1) acids. In some cases elaidic (18:1 9t) and vaccenic (18:1 11c) acids co-elute and the separation of oleic and palmitic (16:0) acids is not always satisfactory.

No search has been done to obtain a precise evaluation (in given conditions) of the chromatographic factors k' or α which

are of considerable importance in the understanding of the retention rules. Such a determination needs the isocratic mode with a sufficiently wide variety of compounds ; this is now possible with a column of low back pressure and high efficiency (5-7,000 plates) such as the Radial Pak cartridge (Waters Associates).

MATERIALS AND METHODS

Reagents :

Fatty acids were purchased from Sigma Chemical Company and used without further purification ; p-bromophenacyl bromide and diisopropyléthylamine obtained from Fluka were also of satisfactory grade.

Acetonitrile and methanol of good quality were carefully rectified and a fraction of very low absorbance employed. Water was distilled from a glass still and all solvents filtered through a HA 0.45 (or FH 0.5) filter (Millipore Corp.).

Preparation of the chromatographic samples :

The derivatization was made according to the method of COOPER and ANDERS (4,14) using p-bromophenacyl bromide as reagent. Complete conversion requires heating at 60°C for 2 hr. For analysis of a standard mixture the only treatment before injection was a filtration on Millipore FH 0.5 µ. For application to a very small amount of saponified lipid a sample preparation by treatment on a Sep Pak C_{18} cartridge (Waters Associates) eliminates the high polarity compounds arising from the derivatization reaction and provides a large improvement of the baseline.

Instrumentation :

We used a Waters Associates Model 204 U/45 chromatograph equipped with a M.45 solvent delivery system, a U6K injector and a M 440 absorbance detector operating at 254 nm. The column was a

Radial Pak A cartridge (10μ reverse phase C_{18}) pressurized in a compression module RCM 100.

The efficiency of our columns was 5,000-7,000 plates and v_o from 1.7 to 2.0 ml.

Eluents :

Two solvent mixtures of comparable "strength" (15) have been used : 87-13 acetonitrile-water (azeotropic mixture) and 90-10 methanol-water.

Although k' values for linoleic acid were close ($V_R \simeq 45$-50 ml) these solvents are different in polarity (P' = 6.4 for acetonitrile-water and 5.6 for methanol-water) and present large differences in interaction with the solute.

Flow rate, 2 ml/min, was increased to 4 ml/min. after elution of oleic acid. Experiments with other flow rates gave no modification of results.

Analyses :

Mixtures of 4-6 sufficiently different fatty acid derivatives were injected into the chromatograph and elution times carefully noted. α values were firstly related to oleic acid for convenience, that acid eluting after 35-45 minutes. Recalculations gave α related to stearic acid. When two acids co-eluted they were reinjected separately, but always with the reference.

RESULTS

Experimental results :

Although the capacity factor k' of a particular fatty acid derivative differs from a column to another and decreases when after some analyses degradation has occurred, the separation factor α (relative retention) of two species never varies on a given

column and does not differ significantly when measured with another one. Reproducibility is better than 1%.

Table I shows the separation factors related to stearic acid in each case (α_A and α_M). Evidence of the greater selectivity of acetonitrile-water appears and is better noted with the results shown in tables 2 and 3 (dividing two values of table 1 gives the separation factor between the chosen acids). In all cases chain lengthening and increase in unsaturation cause larger modifications of retention when using acetonitrile-water as solvent.

But another feature of the separation of fatty acid derivatives using acetonitrile-water in isocratic mode is the very bad resolution between two major components of all lipids : palmitic (16:0) and oleic (18:1 9c) acids ; in our conditions 15,000 plates

TABLE 1

Separation Factors Related to Stearic Acid.

Num. Ref.	Acid derivatives	α_A	α_M	α_A/α_M
12 : 0	Lauric	0.138	0.148	0.932
14 : 0	Myristic	0.264	0.278	0.950
16 : 0	Palmitic	0.510	0.527	0.968
18 : 0	Stearic	1	1	1
14 : 1	Myristoleic	0.145	0.173	0.838
16 : 1	Palmitoleic	0.270	0.309	0.874
18 : 1 9c	Oleic	0.519	0.560	0.927
18 : 1 9t	Elaidic	0.571	0.610	0.936
18 : 1 6c	Petroselenic	0.551	0.593	0.929
18 : 2	Linoleic	0.303	0.372	0.814
18 : 3 ω_3	α Linolenic	0.192	0.265	0.725
18 : 3 ω_6	γ Linolenic	0.196	0.258	0.760
20 : 3 ω_3	Eicosatrienoic 11.14.17	0.357	0.479	0.745
20 : 3 ω_6	Eicosatrienoic 8.11.14	0.356	0.451	0.789
20 : 4 ω_6	Arachidonic	0.243	0.338	0.719
22 : 6 ω_3	Docosahexaenoic	0.194	0.314	0.618

would be required for R_s = 0.6. The separation of myristic (14:0) and palmitoleic (16:1) acids is also difficult.

These resolutions being excellent with methanol-water the better solvent is thus the less "selective".

That anomaly is in fact due to the similar changes in retention caused by a first unsaturation (table 3) or by decrease in chain length. Methanol-water being more sensitive to the second factor provides a better result.

A more precise survey of tables 1, 2 and 3 shows other interesting features which suggest many questions :

- Methanol-water resolves perfectly the two isomers of eicosatrienoic acid (20:3), not the linolenic acids (α values are 1.06 and 1.03 respectively, the second one being too low for a convenient resolution). With acetonitrile-water the isomers are always unseparable.

- The ω_3 family seems more sensitive to chain lengthening than the ω_6 one (table 2).

- A change in position of the double bond affects the retention (as in case of oleic and petroselenic acids). This result is apparent in table 3 when we remind us that myristoleic (14:1)

TABLE 2

Relative Retentions Between Homologs. Effect of Chain Lengthening.

Numeral representations	MeCN-H_2O	MeOH-H_2O
14 : 0 / 12 : 0	1.91	1.88
16 : 0 / 14 : 0	1.93	1.89
18 : 0 / 16 : 0	1.96	1.89
16 : 1 / 14 : 1	1.86	1.79
18 : 1 / 16 : 1	1.92	1.81
20 : 3 ω_3 / 18 : 3 ω_3	1.86	1.81
20 : 3 ω_6 / 18 : 3 ω_6	1.82	1.75

TABLE 3

Relative Retentions Between Acids of Same Chain-Length. Effect of *Unsaturations*.

Numeral representations	MeCN-H_2O	MeOH-H_2O
14 : 0 / 14 : 1	1.82	1.61
16 : 0 / 16 : 1	1.89	1.71
18 : 0 / 18 : 1 9c	1.93	1.79
18 : 0 / 18 : 1 6c	1.81	1.69
18 : 1 / 18 : 2	1.71	1.51
18 : 2 / 18 : 3 ω_3	1.58	1.40
18 : 2 / 18 : 3 ω_6	1.55	1.44
20 : 3 ω_6 / 20 : 4 ω_6	1.47	1.33

and palmitoleic (16:1) acids have not the double bond in the middle of their chain.

- The largest effect produced by the introduction of a third unsaturation into a C_{18} chain (i.e. the transformation of 18:2 into 18:3) is obtained with the ω_3 compound (α linolenic) when using acetonitrile-water. Elution with methanol-water reverses that result (table 3).

Rules for elution order :

We suggest to lay down five simple rules providing a satisfactory interpretation to all these experimental results. The first three are readily perceived.

First Rule

Other things being equal the trans unsaturated derivative elutes after the cis isomer.

For instance elaidic acid is more retained than oleic acid. PEI et al (7) also noted the case of palmitelaidic acid (16:2 9t).

Second Rule

For saturated acids, all chain lengthening by two carbon atoms causes the retention volume to be multiplied by a nearly constant factor. (Nevertheless that factor slightly increases with chain-length when using acetonitrile-water : table 2).

Third Rule

Unsaturation lowers retention. That effect is decreasing when unsaturation increases (table 3).

The other rules relate to position isomerism and necessitate further developments. Let us consider that the double bonds as a whole divide the fatty chain in two parts : the internal chain I and the terminal chain II :

$(CH_3 \ldots .)\ (CH = CH - CH \ldots CH = CH)\ (\ldots .)COOH$

Chain II Chain I

TABLE 4

Lengths of the Internal (I) and Terminal (II) Chains of Unsaturated Fatty Acids.

Num. Ref.	Fatty acid derivative	I	II
14 : 1 9c	Myristoleic	7	4
16 : 1 9c	Palmitoleic	7	6
18 : 1 9c	Oleic	7	8
18 : 1 6c	Petroselenic	4	11
18 : 2 9c 12c	Linoleic	7	5
18 : 3 ω_3	α Linolenic	7	2
18 : 3 ω_6	γ Linolenic	4	5
20 : 3 ω_3	Eicosatrienoic 11.14.17	9	2
20 : 3 ω_6	Eicosatrienoic 8.11.14	6	5
20 : 4 ω_6	Arachidonic	3	5
22 : 6 ω_3	Docosahexaenoic	2	2

These chains are shown in table 4. We find :

Fourth Rule

Other things being equal the retention is generally minimal if chains I and II are equal. As a consequence the retention is more increased by an enlargement of the longest chain.

That rule applies perfectly to interpret a previously noted difference between ω_3 and ω_6 families : a more important α factor for 20:3 versus 18:3 is observed in the first case in which the longest chain is involved (table 4)

One fact, however, needs interpretation : as previously noted in table 3, the α values between linoleic acid and the linolenic acid isomers are inverted by a change of solvent. That result suggests the last rule :

Fifth Rule

When eluting with acetonitrile-water a very short terminal chain II may cause a large decrease in retention. In some cases that effect prevails (ω_3 effect).

DISCUSSION

The foregoing rules may receive some theoretical support in considering the interactions involved in the chromatographic process.

In reversed phase chromatography the eluent is frequently a binary mixture of water and of a miscible less-polar solvent ; thus a change in that second solvent generally provides limited modifications in selectivity.

However some significant changes related to solute-solvent interactions may be observed. In our work, methanol-water, less-polar, elutes more quickly the saturated species but the retentions of polyunsaturated acids are larger. Table 5 shows an example to illustrate this fact.

TABLE 5

Retention Volumes of some Fatty Acid Derivatives (Consecutive Analyses using the same Column).

Numeral reference	$(v-v_o)$ ml CH_3CN-H_2O	$(v-v_o)$ ml CH_3OH-H_2O
18 : 0	162	127
16 : 0	83	67
14 : 0	43	35
18 : 1	84	71
18 : 2	49	47
18 : 3 ω_3	31	34
20 : 4 ω_6	39	43
22 : 6 ω_3	31	40

Methanol is essentially a strong hydrogen acceptor and has no important specific interaction with the fatty acid moiety of the solute. Thus the main effect of unsaturation is to decrease the fatty chain responsible for retention.

Rule 4 shows the large importance of a long continuous chain and, for example, the change from 14:0 to 14:1 generates less modification in retention than from 18:0 to 18:1, 9c (see tables 3 and 4).

Although that effect remains while using acetonitrile-water, we must consider the dramatic change in α values for unsaturated species.

In the last column of table 1 is shown the α_A/α_M ratio which is an interesting factor in such a survey. Taking into account the foregoing considerations the α_M factor may be accepted as "normal," thus a decrease in α_A/α_M ratio reveals some specific dipole-dipole interaction between the solute and acetonitrile. We observe that α_A/α_M ratio is increased by chain lengthening, the solute becoming less polar, and decreased largely by increasing unsaturation. The ω_3 effect (rule 5) significantly lowers α_A/α_M as compared to ω_6

isomers, showing an enhancement of the solvent-solute interaction at the level of the terminal double bond permitted by a lack of steric hindrance.

This effect is strong enough to make α linolenic acid slightly inferior to γ linolenic in retention. In case of eicosatrienoic acid isomers the chain lengthening prevails and rule 4 applies but the compounds are extremely close and no separation occurs whereas methanol-water provides an efficient resolution.

CONCLUSIONS

Although giving larger separations between homologs or between differently unsaturated fatty acids, acetonitrile-water provides less satisfactory resolution than methanol-water in analysis of a complex mixture using the isocratic mode. Giving a better repartition of peaks in a shorter analysis methanol-water is the best solvent in these conditions (fig. 1)

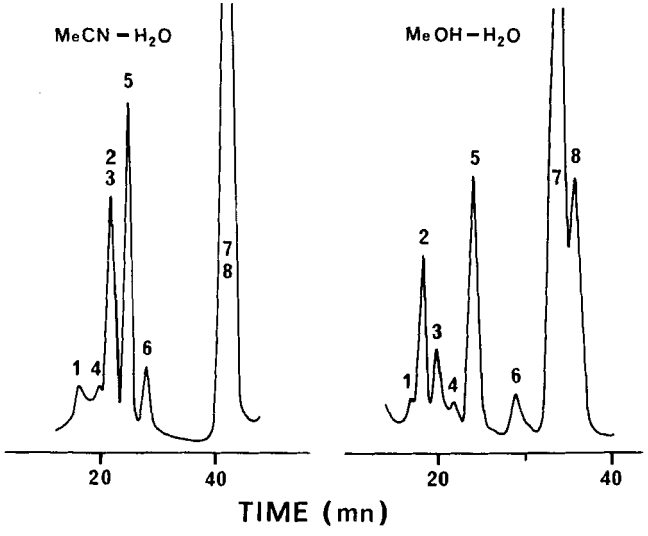

FIGURE 1 : Parts of the actual chromatograms of fatty acids extracted from the phospholipids of a fungus (unpublished results).

1: α linolenic - 2:myristic - 3:palmitoleic - 4:arachidonic
5: linoleic - 6:eicosatrienoic (ω_6) - 7:palmitic - 8:oleic.

Some simple empiric rules, confirmed by theoretical condiderations upon solute-solvent interactions, may permit to predict elution order and separation, facilitating the choice of solvent.

Use of our technique for the analysis of fatty acids from phospholipids of fungi will be reported elsewhere.

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