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Journal of Chromatography A, 807 (1998) 111–119

JOURNAL OF CHROMATOGRAPHY A

Microemulsion electrokinetic chromatography of fatty acids as phenacyl esters

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Abstract

A mixture of saturated fatty acids containing even number of carbon atoms was successfully separated as phenacyl esters by microemulsion electrokinetic chromatography. Separations were done by using an untreated fused-silica capillary [57 cm (effective length 50 cm)×75 μm] at 15 kV; detection was done by UV at 243 nm. The microemulsion system was composed of 10 m*M* borate buffer (87.93%, w/w), cholate (4.87%, w/w), heptane (0.66%, w/w) and *n*-butanol (6.55%, w/w), pH 10.2. Separation by microemulsion electrokinetic chromatography was characterized by high efficiency (theoretical plates ranged from $142 \cdot 10^3$ to $428 \cdot 10^3$) with peak resolution 1.25 and better. Micellar electrokinetic chromatography under the standard conditions with sodium dodecyl sulfate as micellar phase was unsuccessful in separating all long-chain fatty acids. The described method can be used for monitoring of saturated fatty acids containing even number of carbon atoms in the molecule in a broad spectrum of chain length (C_2–C_{20}). © 1998 Elsevier Science B.V.

Keywords: Microemulsion electrokinetic chromatography; Fatty acids

1. Introduction

Fatty acids, the principal components of lipids, can be separated by a number of chromatographic methods of which gas chromatography (GC) is most frequently the method of choice. In GC fatty acids are determined mainly as methyl esters employing capillary columns and flame-ionization detection [1,2]. High-performance liquid chromatography (HPLC) methods represent the other alternative, however, successful separations require pre-column derivatization. On the contrary to GC, where practically only methyl esters are used, in HPLC a plethora of the carboxyl group derivatives was described and successfully used for separation. Of these many derivatives phenacyl esters are used most frequently; phenacylbromide (2-bromoacetophenone) as the derivatizing agent with triethylamine as catalyst [3,4].

Capillary electrophoresis methods represent a category of the most rapidly developing analytical techniques at the present time, however their potential for the separation of long-chain fatty acids has not yet been fully exploited. The main problem in the separation of long-chain fatty acids stems from their poor solubility in water and aqueous buffers. Until now there have been three ways how to solve this problem: separation in (i) aqueous–organic systems, (ii) non-aqueous systems, or (iii) micellar systems. As an example of the first approach it is possible to mention the separation of saturated and unsaturated fatty acids using a diethylbarbiturate carrier electrolyte (5 m*M*) with trimethylammonium propanesulfonate (0.5 *M*) at a pH between 10 and 11 in 70% ethyleneglycol monomethyl ether [5]. The second system was developed by Drange and Lun-

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0021-9673/98/$19.00
PII: S0021-9673(98)00071-5

danes [6] who separated long-chain fatty acids (*n*-C_{14}–*n*-C_{26}) in a system consisting of anthraquinone-2-carboxylic acid (2.5 m*M*) and Tris (40 m*M*) in *N*-methylformamide–dioxane (3:1, v/v) (anthraquinone-2-carboxylic acid served here as a probe for negative detection). The last mentioned system was developed by Erim et al. [7] who separated saturated C_8–C_{20} fatty acids in sodium dodecyl benzenesulfonate (10 m*M*), acetonitrile (50%) and Brij 35 (30 m*M*). All these separations were monitored at indirect UV detection mode. Separation of derivatized long-chain fatty acids has not been described so far. Also there is to our best knowledge no information available about microemulsion electrokinetic chromatography (MEEKC) of this category of compounds.

MEEKC has emerged only recently. It was used by Watarai [8] in 1991 for the first time for the separation of fluorescent aromatic compounds; Terabe et al. [9] used this system for separation of test mixtures of some aromatics and drugs and compared this separation mode with micellar electrokinetic chromatography (MEKC) [with sodium dodecyl sulfate (SDS)]. Boso et al. [10] used this system for separation of fat and water soluble vitamins, Vomastová et al. [11] separated steroids, Fu et al. [12] separated antipyretic analgesic ingredients, Mikšík et al. [13] separated diphenylhydrazones of dicarbonyl sugars, and Debusschère et al. [14] successfully separated cardiac glycosides. All above mentioned papers described excellent resolution of analytes in comparison with classical MEKC (with SDS as the micellar pseudophase), in which the separation is frequently poor if not totally unsuccessful.

In general the microemulsions (oil-in-water, o/w) are formed from oil (named the core phase, usually a hydrocarbon or other hydrophobic substance), water (buffer), surfactant and co-surfactant (such as a medium alkyl-chain alcohol). It appears that the structure of the microemulsion is similar to the structure of the micelle – the oil droplet is stabilized by the surfactant and co-surfactant located on the droplet surface [9].

In this work, we attempted to develop a fast and reliable electrokinetic method for the separation of saturated even carbon fatty acids (containing even number of carbon atoms in their molecule) as phenacyl esters.

2. Experimental

2.1. Capillary electrophoresis

Separations of fatty acid mixtures were done with Beckman P/ACE 5500 (Fullerton, CA, USA) using untreated fused-silica capillary (CElect; Supelco, Bellefonte, PA, USA) 57 cm (50 to the detector)×75 μm capillary mounted in cartridge with 200×100 μm aperture and run at 15 kV and 30°C, monitored by diode-array detection at 243 nm (auto zero at 5 min). Between runs the capillary was washed with Milli-Q water (1 min), 1 mol/l NaOH (7 min), Milli-Q water (1 min), methanol (1 min) and finally with Milli-Q water again (4 min). Before each run the capillary was equilibrated with the run electrolyte (2 min). Samples were injected hydrodynamically (3.45 kPa, 1 s). Instrument was managed by P/ACE Station software (Beckman).

2.1.1. Microemulsion electrokinetic chromatography

Microemulsions were prepared by mixing the microemulsion forming organic solutes (heptane) (0.66%, w/w), cholate (4.87%, w/w), *n*-butanol (6.55%, w/w) with 10 m*M* borate buffer (87.93%, w/w), the pH of which was adjusted to 9.2–11.7 with 0.1 *M* NaOH where necessary.

2.1.2. Micellar electrokinetic chromatography

Separations were carried out in the same manner as in case of MEEKC but without organic additives, i.e., with 10 m*M* borate buffer (94.75%, w/w) and cholate (5.25%, w/w) at pH 10.2.

2.2. Chemicals

Cholic acid (sodium salt) was purchased from Sigma (St. Louis, MO, USA) and SDS was from Merck (Darmstadt, Germany). Saturated fatty acids (even carbon), benzoic acid, phenacyl bromide (2-bromoacetophenone) and triethylamine were purchased from Sigma. All other chemicals were obtained from Lachema (Brno, Czech Republic) and were of the highest available purity. All buffers were prepared in Milli-Q water.

2.3. Preparation of derivatives and standard solutions

Phenacyl esters of fatty acids were prepared by the method described by Wood and Lee [3] and Hanis et al. [4]. Fatty acids (100 μg) were placed in Nalgene PTFE (FEP) tubes with PTFE screw closure (Oak Ridge tubes; Nalge, Rochester, NY, USA), then 25 μl of a phenacyl bromide solution (10 mg/ml in acetone) and 25 μl of a triethylamine solution (10 mg/ml in acetone) were added, capped under N_2 and heated in a boiling water-bath for 5 min. The excess of phenacyl bromide was reacted with acetic acid (40 μl of a 2 mg/ml solution in acetone) and, after evaporation of solvents under a stream of N_2 at laboratory temperature, the derivatization products were reconstituted in methanol.

The standard solution for calibration was prepared from a stock solution (in methanol): 2.84 m*M* butyric acid, 0.33 m*M* benzoic acid, 2.15 m*M* caproic acid, 1.73 m*M* caprylic acid and 1.16 m*M* for higher fatty acids (capric, lauric, myristic, palmitic, stearic and arachidic acid).

2.4. Calculations

The effective mobility (μ_{eff}) was calculated according equation $\mu_{eff}=\mu_{app}-\mu_{eof}$ where μ_{app} is apparent mobility of the analyte and μ_{eof} is electroosmotic flow mobility. Mobilities were calculated as: $\mu=(L_dL_t)/Vt$, where L_d is the capillary length to the detector; L_t, total capillary length, t is the migration time and V is the applied voltage. The number of theoretical plates N was calculated according to the equation $N=5.54(t_m/W_h)^2$, where t_m is the migration time and W_h is the peak width at half height of the peak (in min); resolution R was calculated from the equation $R=2\Delta t(W_1+W_2)^{-1}$, where Δt is the difference in migration times of the two solutes involved and W_1 and W_2 are the peak widths (in time units) at the baseline.

3. Results and discussion

Optimized separation of fatty acids as phenacyl esters by MEEKC is shown at Fig. 1. The electrophoretic mobilities of the set of investigated fatty acids decreased with the increasing alkyl chains, which is a just in reverse in comparison to the situation in MEKC separation of free fatty acids (underivatized) described by Drange and Lundanes [6]. The system presented here offers also possibility of separating aromatic (benzoic) acid and straight-chain fatty acids in a single run.

Optimization procedure included selection of core phase, pH and solvent strength. From the numerous core phases which were examined, in particular octanol, hexane, heptane and iso-octane we have finally selected heptane. Octanol, which yielded good results in MEEKC (with SDS) of vitamins [10], steroids [11] and diphenylhydrazones of dicarbonyl sugars [13] did not show in this case (however with cholate) optimal separation characteristics (results not shown), and was therefore replaced by heptane. Cholate was selected as surfactant on the basis of previous unsuccessful results with SDS. Optimization of pH is demonstrated in the Fig. 2. It is evident that at higher pH (above 10.5) the peaks are broader with a decreasing peaks area. On the contrary, separation at lower pH values (lower than 10) leads to sharp peaks, however, with limited resolution.

Comparison of the MEEKC and MEKC is shown in Fig. 3. It is obvious that MEKC is capable to separate higher fatty acids only to C_{12}, higher acids were not eluted within a reasonable period of time. The separation characteristics are poor too, e.g., number of theoretical plates varies from $0.7\cdot10^5$ to $1.6\cdot10^5$.

With MEEKC the high efficiencies (from $1.0\cdot10^5$ to $3.3\cdot10^5$ plates) with relatively high sample concentration (from 1.9 m*M* to 0.6 m*M* for butyric and higher fatty acids respectively) were observed. This magnitude increased further up to $4.3\cdot10^5$ if lower concentration was applied (from 0.355 m*M* for butyric acid to 0.145 m*M* for higher fatty acids) (Table 1). These values are at least two- or three-times higher than the plate numbers reported by Drange and Lundanes [6] for a 75 μm I.D. capillary (with which the best results were reportedly obtained).

The concentration dependence of the separation on the analyte concentration is shown in Fig. 4. It is obvious that the retention time remains unaffected by changed concentration, while resolution, as expected, is influenced very strongly, in particular at high concentrations (more than 1 m*M*). Calibration curves were in principle linear in the region 0.6–0.010 m*M*,

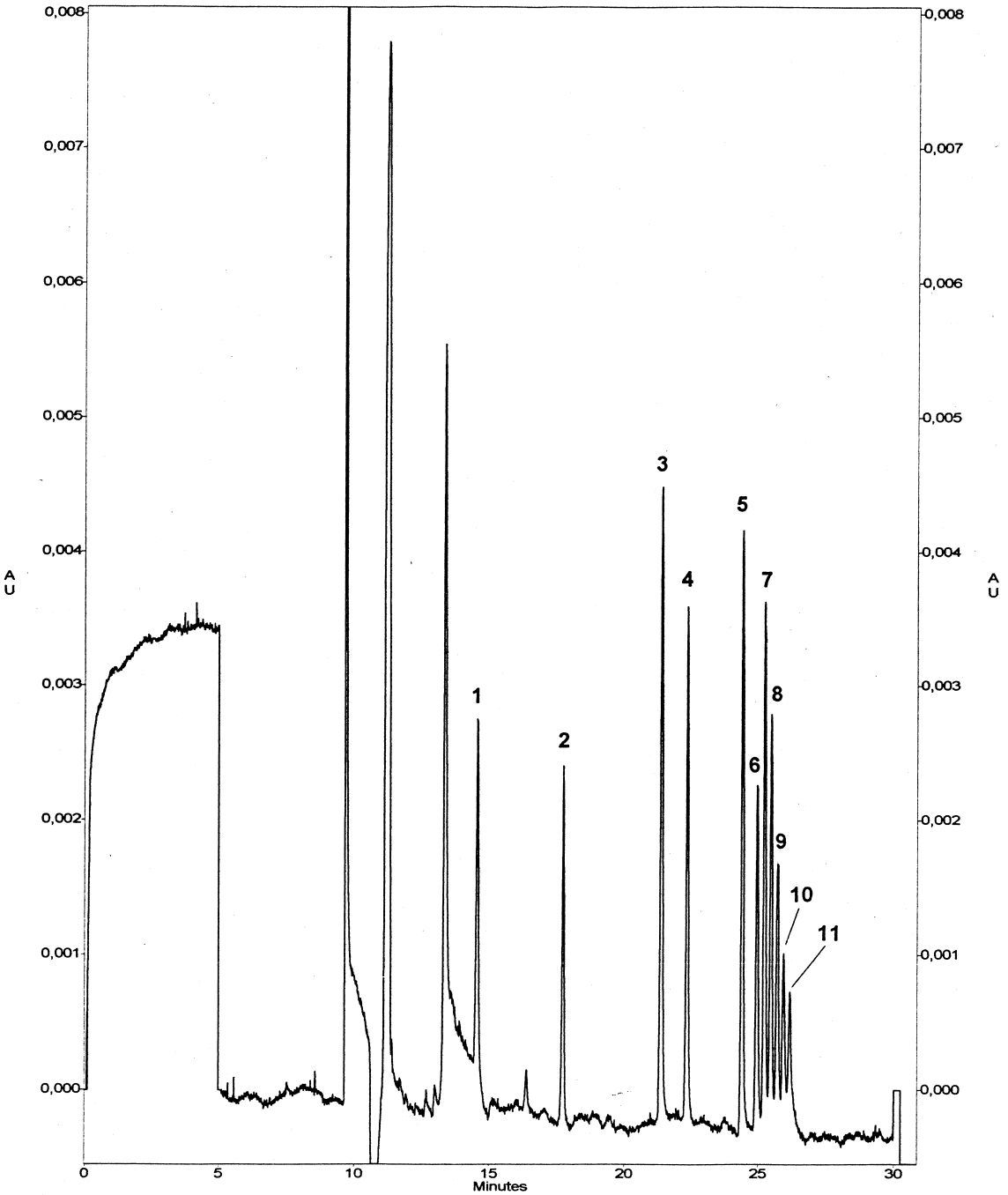

Fig. 1. Separation of phenacyl esters of fatty acids by microemulsion electrokinetic chromatography at pH 10.2. For separation conditions see Section 2.1.1. Peak identification: 1=acetic acid, 2=butyric acid (1.14 m*M*), 3=benzoic acid (0.13 m*M*), 4=caproic acid (0.86 m*M*), 5=caprylic acid (0.69 m*M*), 6=capric acid (0.46 m*M*), 7=lauric acid (0.46 m*M*), 8=myristic acid (0.46 m*M*), 9=palmitic acid (0.46 m*M*), 10=steric acid (0.46 m*M*) and 11=arachidic acid (0.46 m*M*).

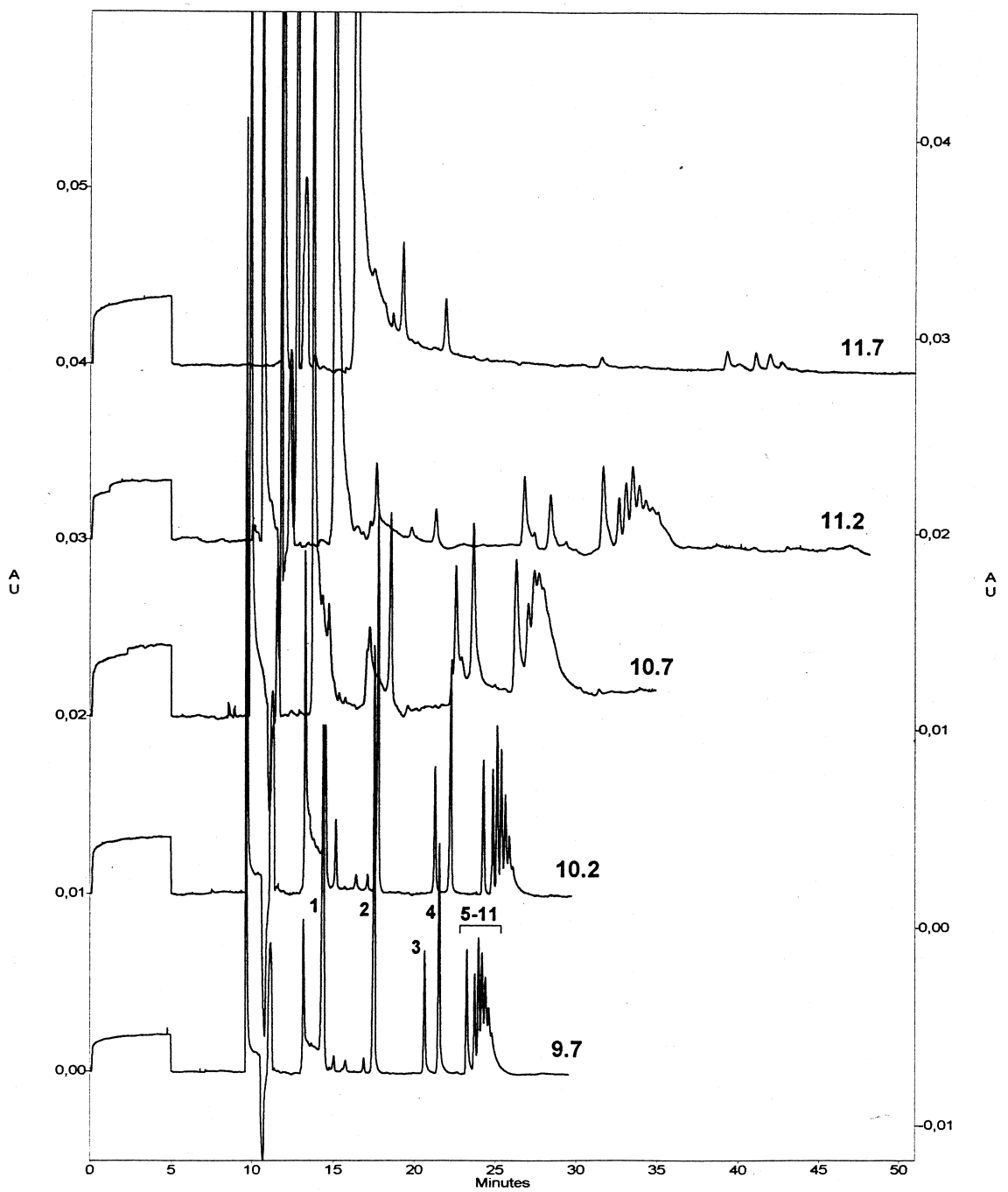

Fig. 2. Influence of pH on separation of derivatized fatty acids separated by microemulsion electrokinetic chromatography. For detailed peak identification see Fig. 1.

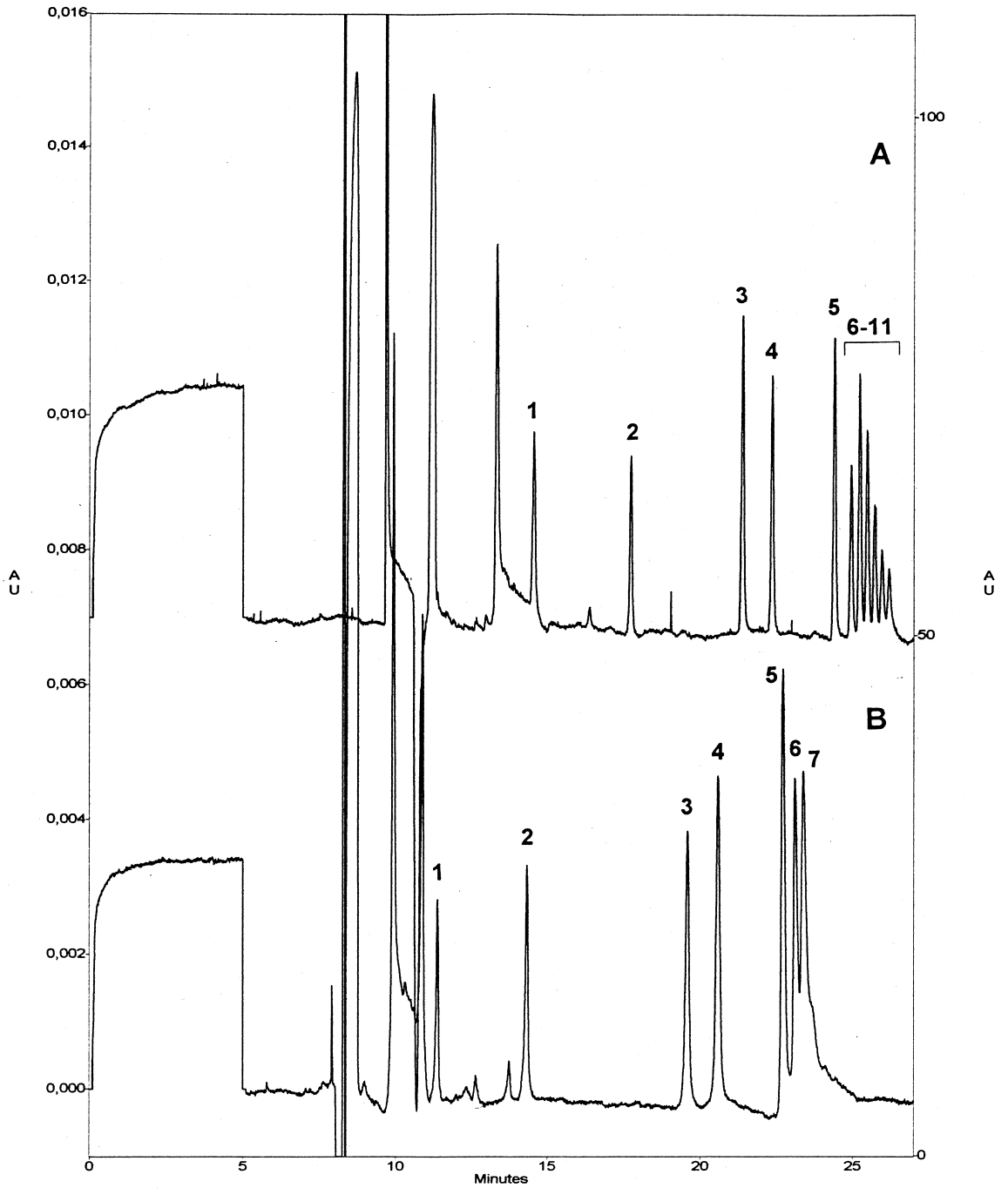

Fig. 3. Separation of phenacyl esters of fatty acids by microemulsion electrokinetic chromatography (A) and micellar electrokinetic chromatography (B).

Table 1
Electrophoretic characteristics of separated compounds by microemulsion electrokinetic chromatography – effective mobility μ_{eff}, number of theoretical plates N ($\cdot 10^3$) and resolution R (to the preceeding peak)

Fatty acid	No. of carbons	Mobility μ_{eff} (10^{-5} cm^2 V^{-1} s^{-1})	Theoretical plates ($\cdot 10^3$)	Resolution
Acetic acid	2	−6.44	142	7.07
Butyric acid	4	−10.24	195	10.62
Benzoic acid	7 (aromatic)	−13.31	269	22.50
Caproic acid	6	−13.91	269	5.45
Caprylic acid	8	−15.15	428	13.19
Capric acid	10	−15.45	301	3.57
Lauric acid	12	−15.60	342	1.69
Myristic acid	14	−15.72	313	1.36
Palmitic acid	16	−15.84	280	1.28
Stearic acid	18	−15.96	295	1.25
Arachidic acid	20	−16.07	298	1.26

except for benzoic acid (0.2–0.001 m*M*), caprylic acid (0.6–0.005 m*M*), lauric acid (0.6–0.005 m*M*), stearic acid (0.6–0.030 m*M*) and arachidic acid (0.6–0.050 m*M*). Correlation coefficients were in all cases larger than 0.985.

The concentration limit of detection (cLOD) and the mass limit of detection (mLOD) were found to be generally in the range around 0.008 m*M* and 30 fmol, respectively. The best sensitivity was found for benzoic acid (0.001 m*M* or 4 fmol, respectively), caprylic acid (0.003 m*M* or 12 fmol) and lauric acid (0.003 m*M* or 12 fmol), the poor sensitivity was observed in the case of high fatty acids, i.e., for stearic acid (0.024 m*M* or 90 fmol, respectively) and arachidic acid (0.040 m*M* or 150 fmol). It is possible to conclude, that for higher fatty acids sensitivity decreased with the length of the carbon chain. Limits of detection reported here are higher than previously reported cLODs for fatty acids by Drange and Lundanes [6] for C_{16}–C_{26} (0.025 m*M*).

4. Conclusions

The advantage of using a microemulsion system for the separation of fatty acids as phenacyl esters was clearly demonstrated: the separation obtained was considerably better than results obtained with MEKC. Use of the microemulsion pseudophase together with a more hydrophobic derivatization reagent resulted in better selectivity and rather high plate numbers. While cholate–micellar separations were capable of resolving only fatty acids to C_{12} (with good separation to C_8 only), using of the microemulsion pseudophase resulted in a practically complete separation of all members of the test mixture. It is feasible to assume that the better results obtained are due to the more hydrophobic nature of the derivatizing reagent along with the more hydrophobic nature of the pseudophase used. It appears that the use of more hydrophobic derivatives with microemulsion pseudophases can generally result in good separations of mixtures which are difficult to separate by MEKC as was conclude in our previous work [13]. It is possible to use the described method for monitoring saturated fatty acids possessing even number of carbon atoms in the molecule in a broad spectrum of chain length (C_2–C_{20}) and for the simultaneous separation of aromatic and straight-chain fatty acids.

Acknowledgements

This work was supported by the Grant Agency of the Academy of Sciences of the Czech Republic (grant No. A7011713/1997) and Grant Agency of the Czech Republic (grant No. 203/96/K128.

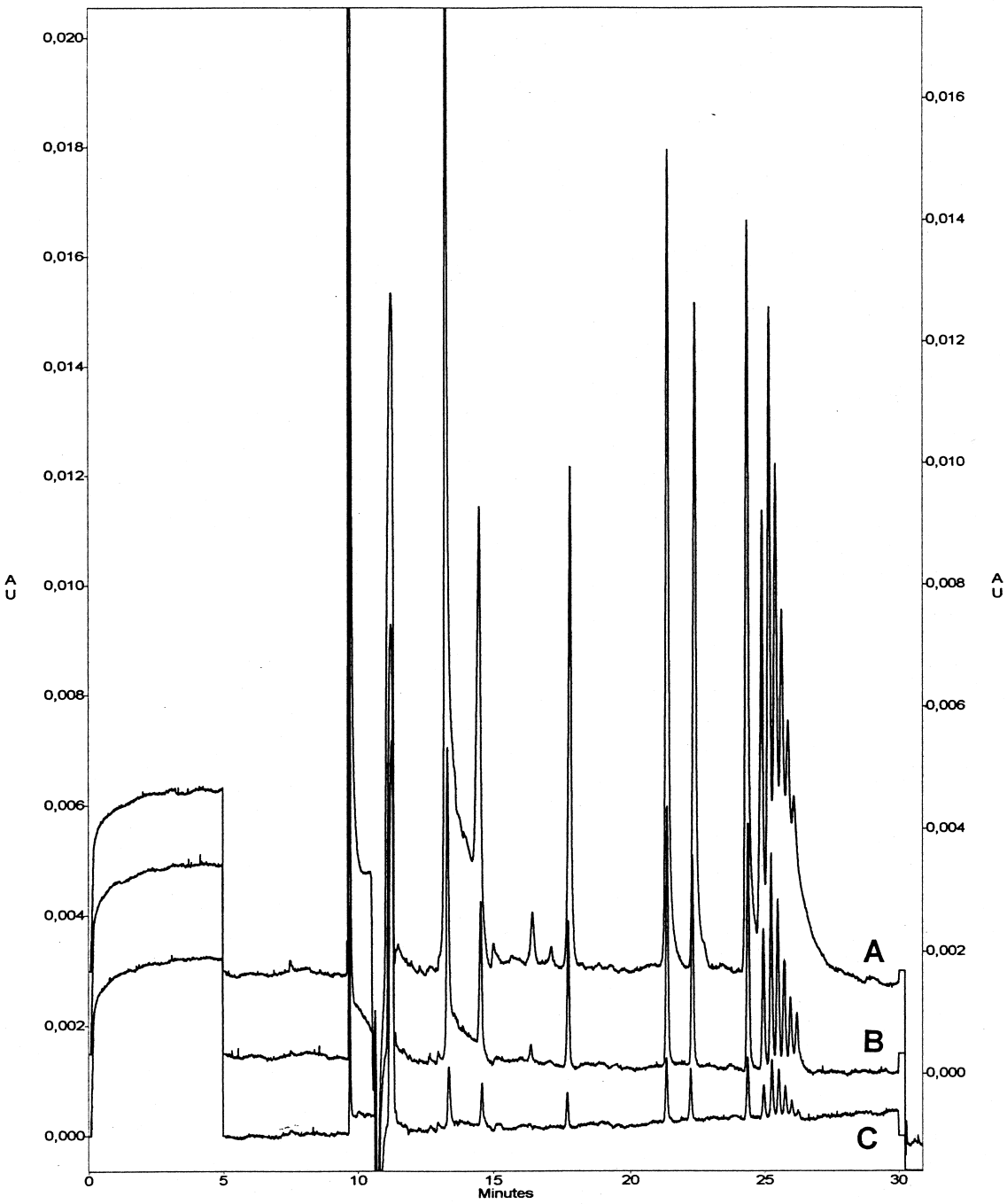

Fig. 4. Influence of sample concentration on separation of derivatized fatty acids by microemulsion electrokinetic chromatography. (A) Stock solution, (B) 2.5-times diluted stock solution, (C) 10-times diluted (i.e., from 0.284 m*M* for butyric acid to 0.116 m*M* for higher fatty acids).

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