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Factorial design of electrolyte systems for the separation of fatty acids by capillary electrophoresis

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Abstract

In this work, a capillary zone electrophoretic methodology using UV indirect detection (224 nm) for the analysis of fatty acids (FAs) in saponified oils is proposed. The electrolyte consisted of a 5 mmol l^{-1} phosphate buffer, pH 7, containing 4 mmol l^{-1} sodium dodecylbenzenesulfonate (SDBS) as chromophore, 4 mmol l^{-1} dimethyl-β-cyclodextrin and 45% acetonitrile (ACN). The composition of the electrolyte was optimized by a 2^3 factorial design with triplicate at the central point. The design established practical concentration boundaries for SDBS and ACN. In a defined concentration range of 2–4 mmol l^{-1}, SDBS can certainly be used as a chromophore for indirect detection without imparting excessive baseline noise. For ACN, a suitable interval of 45–55% was found to enhance FAs solubilization without overflowing the system with bubble formation and current interruption. Additionally, the design revealed the importance of dimethyl-β-cyclodextrin in the resolution of difficult pairs and its function as a solubilizing agent for long chain FAs. At the optimized conditions, nine FAs from C_{10} to C_{20}, including mono- di- and tri-unsaturated C_{18} fatty acids were baseline separated in less than 10 min. The proposed method was applied to the separation of FAs in edible oils and polyunsaturated fatty acid enriched margarine. Additionally, spectral monitoring at 206 nm was used to confirm peak identity in the samples.

Keywords: Factorial design; Oils; Fats; Buffer composition; Fatty acids

1. Introduction

Oils and fats are water insoluble substances, derived from animal, vegetable or microbial sources, formed by the condensation of glycerol and fatty acids, resulting in the so-called triglycerides or triacylglycerols, when all three of the glycerol –OH groups have been esterified by fatty acids. In the chemical composition of oils and fats, minor components are also present such as hydrocarbons, terpenes, waxes, steroids and vitamins, among others. The difference between oils and fats reside in their physical appearance: fats are solid (m.p.>20°C) and oils are liquid at room temperature.

Fatty acids (FAs) are aliphatic carboxylic acids with carbon numbers between C_6 and C_{24}. Short chain FAs have <8 carbons, middle chain FAs have 8–11 carbons, intermediate chain FAs have 12–15 carbons and long chain FAs have >16 carbons. FAs are divided into two groups according to saturation degree: the saturated FAs (SAFAs) and those with different degrees of unsaturation, from monounsaturated fatty acids (MUFAs) to polyunsaturated fatty acids (PUFAs) [1]. The physical and chemical characteristics of FAs, for instance, freezing point and solubility, as well as their nutritional properties (digestibility, metabolic effects, etc.) depend upon

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carbon number, number of double bonds, positions of the double bonds and *cis–trans* isomer conformations. FAs that occur naturally in oils and fats are long chain carboxylic acids with even numbers of carbons and no ramification, varying from 12 to 22 carbon atoms.

The determination of FAs is important for the chemical characterization of edible oils and fats and in the quality control of dairy products. Classical methodologies for FAs analysis include gas chromatography (GC) [2,3] and more recently, high-performance liquid chromatography (HPLC) [4]. In the former technique, more volatile methyl or trimethylsilyl ester derivatives [3,5] are commonly required, especially for the longer chain FA solutes, although specifically designed columns that tolerate high temperatures are also available and can be used for the analysis of FAs in a flame ionization detector [6]. In HPLC analysis, UV-absorbing derivatives are usually employed, such as phenacyl [7] or naphthacyl [8] esters and 2-nitrophenylhydrazides [9]. Alternatively, less convenient direct detection schemes might be used, such as refractive index [10], conductivity [11] and chemiluminescence detectors [12]. Derivatization reactions often produce incomplete conversion of the analyte and undesirable interfering side products. In addition, for solutes having surfactant properties as is the case for the long chain FAs, adsorption phenomena onto silica-based stationary phases are likely to occur, resulting in poor resolution and peak tailing.

In recent years, capillary electrophoresis has been strongly considered for the analysis of FAs. Separation is generally conducted in the zone electrophoresis mode, under indirect UV detection. Several chromophores have been considered for this purpose including *p*-anisate [13], diethylbarbiturate [14] and benzenesulfonate [15]. It is a common practice to use large amounts of organic solvents to enhance solute solubility. Particularly in the case of benzenesulfonate, which is also a surfactant, organic solvents have an additional impact: they decrease micelle size and under certain circumstances they even suppress micelle formation [15]. Therefore, the solute elution order, despite the presence of a surfactant above its critical micelle concentration (CMC), follows the order found in a zone electrophoresis mechanism, i.e. longer chain FAs elute first [15]. Another feature of the electrolyte systems for FA analysis is the use of cyclodextrin and its derivatives as selector additives to improve resolution of difficult solute pairs, especially when PUFAs are present in the sample [13].

Since the FA solubilities decrease considerably as the carbon chain length increases, the use of surfactants and, therefore, micellar electrokinetic chromatography (MEKC), appeared to be an interesting alternative for their separation. Pure MEKC mechanism for the analysis of FAs has been demonstrated with SDBS and also SDBS–Brij35 mixed micelle systems [15], but the separation scope of such electrolytes was limited to the middle chain length FAs, C_{11}–C_{15}. Additionally, high concentration of Brij 35 decreases the charge density of the mixed SDBS–Brij micelles in such a way that micelle migration approaches t_0, the electroosmotic flow time. As a result, the elution window decreases and the solute elution order suffers an inversion, the larger FAs elute first and the selectivity is attributed to solute mobility differences rather than micelle interaction. Separation of C_{16}–C_{24} is only possible using a high content of organic solvent. Again, in this situation, partition into the micelle phase, if it exists, is no longer the basis of selectivity since intrinsic solute electrophoretic mobility is more important. The use of indirect detection schemes using neutral surfactants has also been considered, as is the case for *p*-anisate–Brij–methanol based electrolytes [16]. This latter electrolyte system was found to be very advantageous for the separation of C_{18}–C_{20} solutes, however, whether Brij neutral micelles persist in 40–60% methanolic solutions and the separation mechanism is dictated by micellar interactions is at least disputable. It is well established that short chain alcohols and some additives, such as urea, increase the CMC of surfactants through their influence on the structure of the solvent [17,18]. They are not incorporated into the micelle structure, unlike long chain alcohols, which tend to decrease CMC through their solubility in the micelles and by reduction of the electrostatic repulsion between ionic head groups, when ionic surfactants are considered. At relatively low organic solvent concentration ($<$25%, v/v), aggregation of surfactant monomers in aqueous solutions may be totally inhibited [19,20].

Other analytical possibilities to approach FA separation and analysis by capillary electrophoresis

include non-aqueous electrolytes [21], microemulsion electrokinetic chromatography [22] and the use of laser-induced fluorescence [23] or mass spectrometric detection [24].

In this work, a zone electrophoretic method for the separation of FAs is proposed using phosphate buffered benzenesulfonate electrolytes, containing dimethyl-β-cyclodextrin and acetonitrile (ACN). A 2^3 factorial design [25,26] was used to optimize the electrolyte composition and to identify factors that affect characteristics of the electropherograms.

2. Experimental

2.1. Reagents and solutions

All reagents were of analytical grade; solvents were of chromatographic purity. Dimethyl-β-cyclodextrin (DM-β-CD) was obtained from Beckman-Coulter (Fullerton, CA, USA), and SDBS was obtained from Aldrich (St. Louis, MO, USA). Buffer electrolyte solutions (composition specified in the figure captions) were prepared fresh daily. The fatty acid standards capric acid ($C_{10:0}$), lauric acid ($C_{12:0}$), myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$) and arachidic acid ($C_{20:0}$) and the omega-9 unsaturated FAs *cis* oleic acid ($C_{18:1}$), *cis* linoleic acid ($C_{18:2}$) and *cis* linolenic acid ($C_{18:3}$) were obtained from Sigma (St. Louis, MO, USA). Stock solutions of the standards were prepared in MeOH at a 170 mmol l^{-1} concentration and stored in a freezer.

2.2. Samples

Locally available commercial samples of soya oil, olive oil and PUFA enriched margarine (Becel) were saponified by treating 200–500 mg of the sample with 5 ml of a methanolic NaOH solution (0.5 mol l^{-1}) at 75–80°C for 5 min.

2.3. Instrumentation

All experiments were conducted in a capillary electrophoresis system (model $HP^{3D}CE$, Agilent Technologies, Palo Alto, CA, USA), equipped with a diode array detector set at 224 nm (206 nm to monitor PUFAs, working within 20–50 mV full scale), a temperature control device, maintained at 25°C and an acquisition and treatment data software (HP CHEMSTATION, rev A.06.01). Samples were injected hydrodynamically (50 mbar, 1 s) and the electrophoretic system was operated under normal polarity and constant voltage conditions of +25 kV. A fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) 58.5 cm (50 cm effective length)×75 μm I.D.×375 μm O.D. was used.

2.4. Analytical procedures

At the beginning of the day, the capillary was conditioned by a pressure flush of 1 mol l^{-1} NaOH solution (5 min), deionized water (5 min) and electrolyte solution (10 min), followed by an electrokinetic flush of electrolyte (25 kV for 10 min). In between runs, the capillary was just replenished with fresh electrolyte solution (2 min, pressure flush).

3. Results and discussion

The variables and their selected levels as well as the values of two response functions [inverse chromatographic resolution statistic (CRS) and signal-to-noise ratio, S/N] used to assess the quality of separation during execution of the 2^3 factorial design of electrolyte systems are compiled in Table 1. The corresponding electropherograms are presented in

Table 1
2^3 Factorial design levels and results

Run	SDBS	ACN	DM-β-CD	CRS^{-1}	S/N
A	−	−	−	0.0022	10.4
B	+	−	−	0.0047	5.1
C	−	+	−	0.0409	13.4
D	+	+	−	0.0174	8.7
E	−	−	+	0.3545	1.0
F	+	−	+	0.3770	0.69
G	−	+	+	0.3608	0.53
H	+	+	+	0.3698	1.5
CP[a]	0	0	0	0.5270	6.8

Levels: SDBS: 2 mmol l^{-1} (−); 6 mmol l^{-1} (+) ACN: 35% (−); 55% (+) DM-β-CD: 2 mmol l^{-1} (−); 6 mmol l^{-1} (+).
[a] Central point (using average values for each factor).

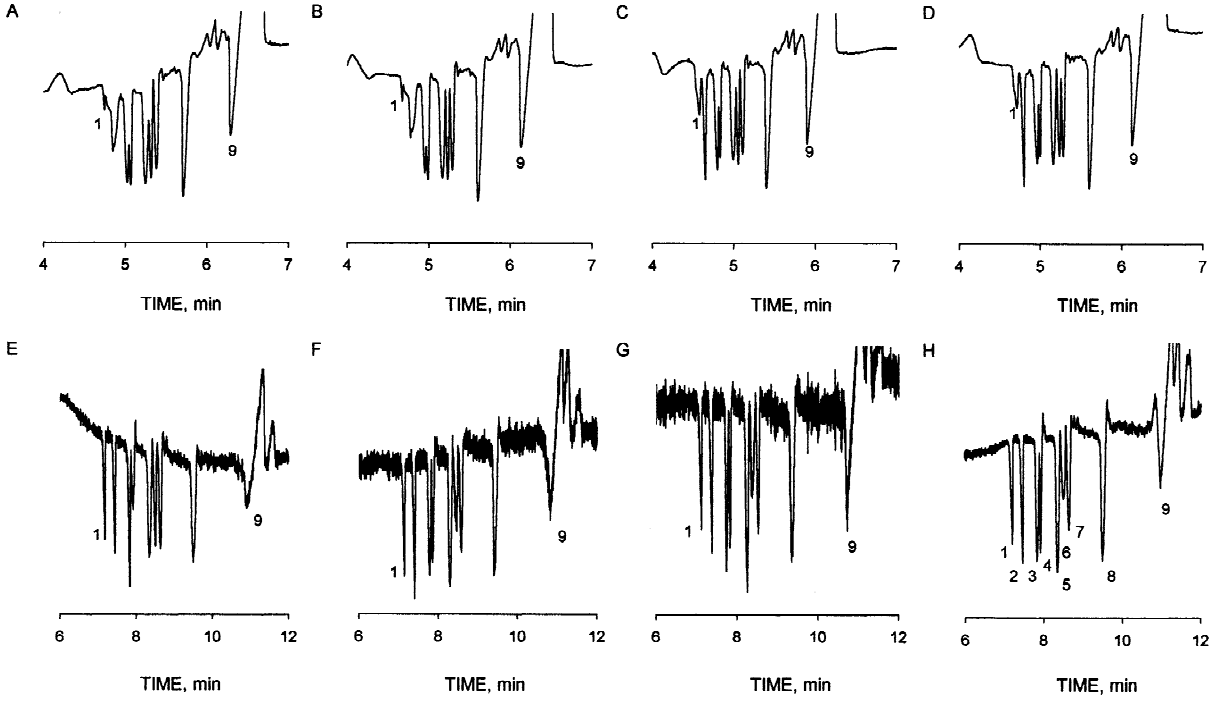

Fig. 1. Factorial design of electrolyte systems for the capillary zone electrophoresis separation of arachidic acid (1), stearic acid (2), palmitic acid (3), *cis* oleic acid (4), *cis* linoleic acid (5), myristic acid (6), *cis* linolenic acid (7), lauric acid (8) and capric acid (9). Fatty acids standard solution concentration: 0.5 mmol l^{-1}. Electrolytes A–H: 5 mmol l^{-1} phosphate buffer, pH 7, with additives as described in Table 1. Other conditions: 1-s injection at 50 mbar pressure, +25 kV applied voltage, 25°C cartridge temperature and indirect detection at 224 nm.

Fig. 1. The estimates of the effects of single variables and the effects resulting from variable interactions are given in Table 2.

The CRS [27,28] is a mathematical function developed originally for chromatographic separations:

$$\mathrm{CRS} = \left\{ \sum_{i=1}^{n-1} \left[\frac{(R_{i,i+1} - R_{\mathrm{opt}})^2}{(R_{i,i+1} - R_{\mathrm{min}})^2 R_{i,i+1}} \right] + \sum_{i=1}^{n-1} \frac{R_{i,i+1}^2}{(n-1)R_{\mathrm{av}}^2} \right\} \cdot \frac{t_n}{n} \quad (1)$$

Table 2
Estimates of effects for the 2^3 factorial design

Estimates	CRS^{-1} as response function	S/N as response function
Main effects		
1 (SDBS)	0.0260	−2.35
2 (ACN)	0.0126	1.70
3 (DM-βCD)	0.349	−8.48
Variable interactions		
12	−0.099	0.45
13	0.131	2.63
23	−0.131	−1.57
123	0.0031	0.18

where $R_{i,i+1}$ is the resolution between adjacent solute pairs, R_{av} is the average resolution of all solute pairs, R_{opt} is the optimum or desired resolution, R_{min} is the minimum acceptable resolution, t_n is the migration time of the last eluting solute and n is the number of solutes in the sample. The CRS considers the resolution of all solutes in the sample, rather than solely the least-resolved pair, and incorporates three important aspects of the separation. The first term of Eq. (1), named the resolution term, evaluates the resolution between all adjacent solute pairs in comparison to defined values for optimum and minimum resolution, 1.5 and 1, respectively. The resolution term decreases as $R_{i,i+1}$ approaches R_{opt} and reaches the minimum value of zero when $R_{i,i+1}$ is exactly equal to R_{opt}. Any further increase in resolution offers no additional improvement in the quality of the separation: hence the resolution term is maintained at a constant value close to zero. The resolution term increases rapidly as $R_{i,i+1}$ approaches R_{min} and becomes undefined when $R_{i,i+1}$ is exactly equal to R_{min}. The second term of Eq. (1), named the distribution term, considers the relative spacing of the solute zones. The distribution term approaches a minimum value of 1 when the resolution of each solute pair is equal to the average resolution, which is the case when all zones are uniformly spaced. The final multiplier term in Eq. (1) takes into consideration the analysis time and the number of analyte peaks to be separated.

Table 1 shows that the inverse CRS values are higher for experiments using 6 mmol l^{-1} of DM-β-CD than those using 2 mmol l^{-1}. This can be easily seen in Fig. 1 where the electropherograms E–H show much better resolution than A–D. Resolution does not depend on the DM-β-CD concentration in a linear manner. Use of an intermediate DM-β-CD concentration of 4 mmol l^{-1} (central point) results in the largest inverse CRS value, 0.53. This is confirmed by the data in Table 2, where it is shown that the only significant effect on inverse CRS and consequently resolution, comes from the cyclodextrin additive. The levels of SDBS and ACN have much smaller effects on the inverse CRS values than the DM-β-CD concentration. The DM-β-CD concentration also affects the S/N ratio. The 2 mmol l^{-1} concentration level results in the largest S/N value, independent of the levels of SDBS and ACN. This ratio becomes much smaller for electropherograms E–H. Although the low concentration for DM-β-CD used here results in desirable S/N, corresponding inverse CRS values and resolutions are not acceptable. Increasing the DM-β-CD concentration improves resolution at the expense of introducing more noise in the electropherograms. DM-β-CD also had an impact on the solubility of the larger FAs. Electropherograms A–D in Fig. 1 show that the the peak corresponding to $C_{20:0}$ is hardly visible, in contrast with electropherograms E–F, where the same peak is well defined and separated from the others.

The best resolution obtained occurred for the central point of the factorial using intermediate levels of SDBS, ACN and DM-β-CD. The central point shows the largest inverse CRS value and accepted S/N ratio. Since SDBS and ACN levels have small effects on resolution and S/N values, their concentrations can be adjusted to satisfy other electrophoresis criteria, such as to improve solute solubilization and also to modulate electroosmotic flow.

Fig. 2 illustrates the optimized separation of a standard mixture of the FAs under investigation at two different wavelengths. The choice of the best wavelength for analysis is supported by the spectral data in Fig. 3, where the absorption profiles of the PUFAs in the standard mixture in contrast to the absorption of SDBS, the electrolyte chromophore, are presented. At 206 nm the absorption spectrum of SDBS presents a minimum. At this wavelength, the absorption of linoleic and linolenic acids surpasses that of SDBS. Therefore, if an electropherogram is recorded at 206 nm, as depicted in Fig. 2B, a peak inversion of these solutes with respect to the background signal is expected. Thus, 206 nm can be a useful wavelength to confirm the presence of linoleic and linolenic acids in real samples. At 224 nm, SDBS presents a large absorption with respect to the PUFAs solutes and most certainly to other non absorbing species considered in the standard mixture. Therefore, 224 nm is the wavelength of choice for indirect detection analysis (Fig. 2A). Another possibility for indirect detection is to set the optical device around 190 nm, however, this is not a very convenient wavelength to use because most commercial equipment lack appropriate optics.

The performance of the proposed methodology to

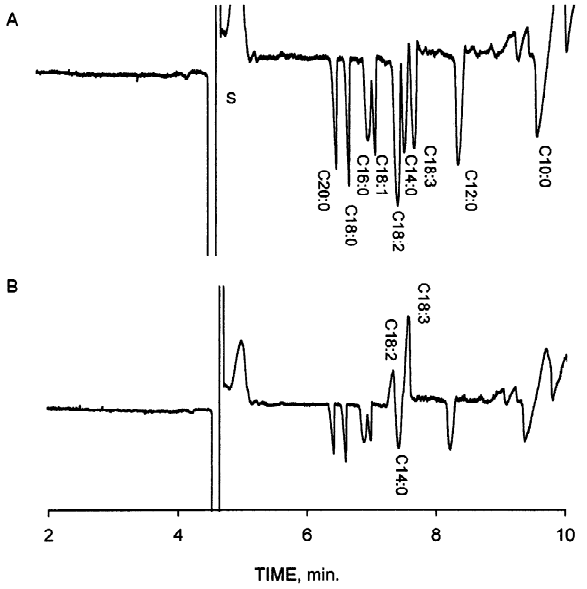

Fig. 2. Optimal conditions for the separation of FAs under indirect detection at 224 nm (A) and 206 nm (B). Fatty acids standard solution concentration: 0.5 mmol l^{-1}. Electrolyte: 5 mmol l^{-1} phosphate buffer, pH 7, containing 4 mmol l^{-1} SDBS, 35% ACN and 4 mmol l^{-1} DM-β-CD. Other operational conditions as in Fig. 1.

assess the FAs contents of real samples is illustrated in Fig. 4 with the analysis of olive oil, soya oil and a PUFA enriched margarine (Becel), under the optimized separation conditions. Possible applications of

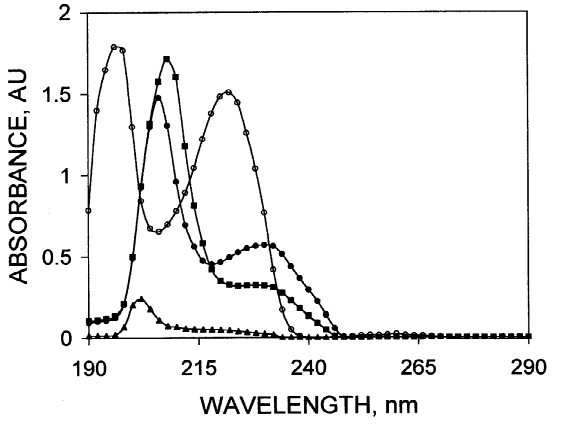

Fig. 3. Spectral features of SDBS (○), oleic acid (▲), linoleic acid (●) and linolenic acid (■). FAs were dissolved in pure methanol at 10^{-4} mmol l^{-1} concentration. The SDSB solution was prepared at 10^{-4} mmol l^{-1} in 50% acetonitrile.

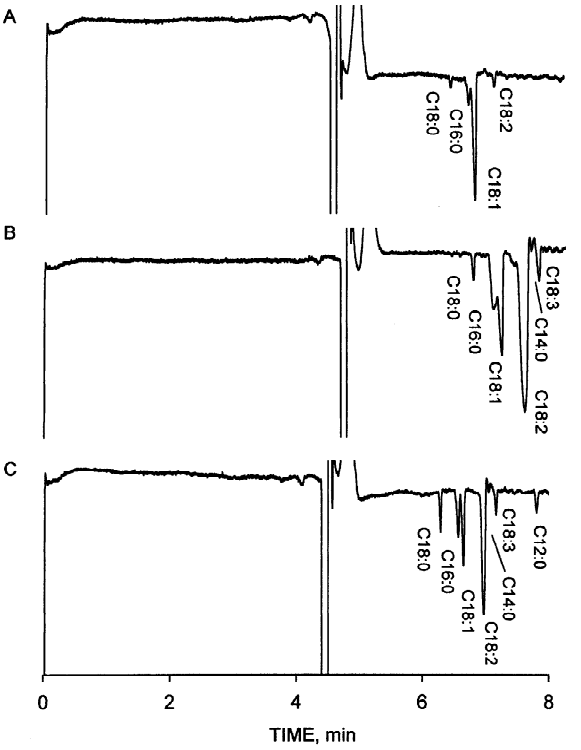

Fig. 4. Capillary zone electrophoresis separation of crude olive oil (A), soya oil (B) and PUFAs enriched margarine (C) after preliminary saponification in methanolic sodium hydroxide solution. Electrophoretic conditions as in Fig. 2. Indirect detection at 224 nm.

the proposed methodology include quality control of edible oils and fats and evaluation of food adulteration. As is well established and demonstrated in Fig. 4, olive oil contains a large amount of $C_{18:1}$ and minor amounts of $C_{18:0}$, $C_{16:0}$ and $C_{18:2}$. An adulteration of olive oil by soya oil, for instance, or other less expensive edible oils, would increase the amount of $C_{18:2}$ in olive oil, which could be clearly monitored by the proposed method.

4. Conclusions

The optimization of complex electrolyte systems in capillary zone electrophoresis can be readily achieved by factorial design of experiments. Besides providing more reliable results than conventional univariate techniques, factorial designs are very

economical, permitting the determination of optimum conditions with the minimum number of experiments.

In this work, factorial design was shown to be an elegant mathematical tool to optimize the separation of FAs. With eight experiments it was possible to establish the role of each variable in the attainment of optimum conditions and to determine whether variables interact to promote optimal results.

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