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Determination of fatty acids in vegetable oil by reversed-phase liquid chromatography with fluorescence detection

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Abstract

The effect of temperature and organic solvent composition (acetonitrile and methanol) on the reversed-phase separation of coumarin-derivatized fatty acids according to their carbon number (C14 to C22), the degree of unsaturation, as well as *cis*/*trans* (C18:1 c/t, C18:2 cc/tt, C18:3 ccc/ttt) configuration was investigated to find out the effective separation condition. Based on the linear plots of the logarithm of the capacity factor of saturated fatty acids versus their carbon number, the equivalent chain length (ECL) of unsaturated fatty acids was calculated. The ECL values were found to be significantly altered and the differentiation between *cis* and *trans* fatty acids was increased when either the temperature or organic solvent composition was decreased. These results generally led to a better resolution at the expense of separation time. A ternary gradient composed of water, acetonitrile, and methanol was then developed to elute the solutes at 55°C within a separation time of 40 min with a minimum resolution of 1.0 for the worst pair. This method was demonstrated to resolve the fatty acids in a vegetable shortening.

Keywords: Vegetable oil; Fatty acids

1. Introduction

The major contents of fatty acids in most vegetable fats and oils include the linear saturated species with the carbon number ranging from 12 to 22 and linear unsaturated species with a carbon number of 18 and the double bond number ranging from 1 to 3 [1,2]. Although the unsaturated fatty acids normally exist as *cis* configuration, the *trans* fatty acids that may cause health problems exist as a relatively small content [3,4]. Determination of different classes of fatty acids under the same condition is desirable for the content quantification of the vegetable oils. However, because of the wide variations in properties between classes of fatty acids, it is not usually possible to resolve members of different classes on the same column. The separation of saturated and unsaturated fatty acids can be easily accomplished by using either gas chromatography with flame ionization detection [1,2,5] or reversed-phase liquid chromatography with fluorescence detection [6–14]. However, the separation involving *cis* and *trans* fatty acids in addition to the saturated and unsaturated fatty acids is more complicated. Among the current separation methods, gas–liquid chromatography with the use of polar stationary phases can resolve the individual fatty acids according to their carbon number and the degree of unsaturation, as well as *cis*/*trans* configuration [1,2,5]. The contents of *cis*

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and *trans* fatty acids can also be determined by silver liquid chromatography in which the silver ion is either added to the mobile phase or impregnated into the stationary phase [15–17]. The silver ions bind reversibly with the double bonds in the unsaturated compounds, resulting in selective retardation. The fatty acids are separated according to the number and *cis*/*trans* configuration of their double bonds. However, such separation mechanism is less dependent on the length of the hydrocarbon chain. Moreover, the silver column often has a limited lifetime.

In order to resolve different classes of fatty acids by using reversed-phase liquid chromatography, their retention behavior should be understood in terms of the number of carbon atoms and double bonds, as well as the *cis*/*trans* configuration. It is often believed that the equivalent chain length (ECL) of fatty acids with N carbon atoms and n double bonds can be expressed as $ECL=N-2n$ and that the fatty acids with higher ECL are retained longer in the liquid chromatography column. Although the ECL rule does not differentiate *cis* and *trans* configurations, the use of ECL to characterize various methods of gas chromatography for the separation of *cis*/*trans* fatty acids has been described [18,19]. In this work, a systematic study of the column temperature and mobile phase composition on the retention of fatty acids in reversed-phase liquid chromatography was carried out. Based on the results obtained from a series of isocratic elutions, a program elution was developed and applied for the analysis of vegetable oil.

2. Experimental

2.1. Chemicals and reagents

The free fatty acid standards with 0 to 3 double bonds, including myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), *cis* oleic acid (18:1c; 9), *trans* olaidic acid (18:1t; 9), *cis* linoleic acid (18:2cc; 9,12), *trans* linoleaidic acid (18:2tt; 9,12), linolenic acid (18:3ccc; 9,12,15), linolenelaidic acid (18:3ttt; 9,12,15), arachidic acid (20:0), behenic acid (22:0) were obtained from Sigma (St. Louis, MO, USA). High-performance liquid chromatography grade acetonitrile and methanol were obtained from Labscan (Ireland). Reagent grade hydrogen chloride, tetrahydrofuran, sodium sulfate anhydrous were from Tedia (OH, USA), sodium hydroxide, potassium hydrogen carbonate were from Showa (Japan), coumarin was from TCI (Japan) and dibenzo-crown-6-ether was from Aldrich (Milwaukee, WI, USA). Water was deionized to 18 MΩ with a Barnstead nanoultrapure water system.

The standards with concentrations ranging from 1 to 1000 ppm were prepared by dissolving the known amount of fatty acids into acetone. The solutions were then derivatized with coumarin according to the procedures described in the literature [12,14]. Briefly, the prepared standards (1 ml) were mixed with an equimolar concentrations of anhydrous mixture (1:1 sodium sulfate and potassium bicarbonate) and dibenzo-18-crown-6. A slight excess amount of coumarin was then added into the mixture and the reaction proceeded at 50°C in the dark. The product was evaporated to dryness by nitrogen and redissolved in acetone for chromatographic injection. The oil sample (0.4 g) was first dissolved in 25 ml of tetrahydrofuran and then saponified with 5 ml of 1 M NaOH at 45°C for 1 h. After the solution was mixed with 10 ml of 1 N HCl, the free fatty acids were extracted three times with 25 ml of chloroform. Anhydrous sodium sulfate (5 g) was added to the combined extracts to remove water from the solution. The solutions were then evaporated to dryness by nitrogen and followed by the coumarin derivatization as described above.

2.2. Chromatographic system

The mobile phase was delivered by a quaternary gradient pump (Model L-7100, Hitachi, Tokyo, Japan) at a flow-rate of 1 ml/min and was used together with a fluorescence detector (Spectroflow 980, Applied Biosystem, USA) at excitation and emission wavelengths of 325 and 375 nm, respectively. The sample was introduced by a 20 μl injection valve (Model 7125, Rheodyne, CA, USA) into the column (ZORBAX Rx-C_{18}, 4.6 mm×25 cm, 5 μm particles) which was placed in a column oven (Model L7300, Hitachi). Data acquisition was performed by a commercial interface (Model 9524, SISC, Taipei, Taiwan) with a Pentium 75 MHz computer.

3. Results and discussion

3.1. Coumarin-derivatized fatty acids

The coumarin-derivatization of fatty acids was chosen for this application because it provided two advantages. First, the fluorescent coumarin moiety allowed for sensitive detection with a broad linear dynamic range. Secondly, the coumarin moiety did not appear to contribute to solute retention because it is not soluble in the octadecylsilica stationary phase. Therefore, the chromatographic retention of the solutes would not be altered by the derivatization. The interaction of the hydrocarbon chain with the octadecylsilica stationary phase arose primarily from dispersion (induced-dipole/induced-dipole) forces; hence, the logarithm of the capacity factor of saturated fatty acids was linearly related to the solute carbon number. As shown in Fig. 1, the plots were found to be linear with R^2 values greater than 0.99 throughout the investigated range of temperatures and organic solvent compositions.

3.2. ECL

For the unsaturated fatty acids, the retention order often depends upon the ECL and the ECL is often calculated based on the retention of two adjacent saturated fatty acids [20]. Alternatively, in this study, the ECL of an unsaturated fatty acid with capacity factor k' was determined based on the linear regression of Fig. 1: ECL=(ln k'−intercept)/slope. In this manner, the calculated ECL values reflected the retention variations of individual unsaturated fatty acid with respect to all solutes in the homologous series as a whole. The ECL implies that unsaturated fatty acids will have an elution time close to their corresponding saturated fatty acids (such as 18:3 versus 14:0 and 16:0 versus 18:1). Moreover, the ECL does not differentiate *cis* and *trans* configurations. Therefore, secondary factors in addition to the

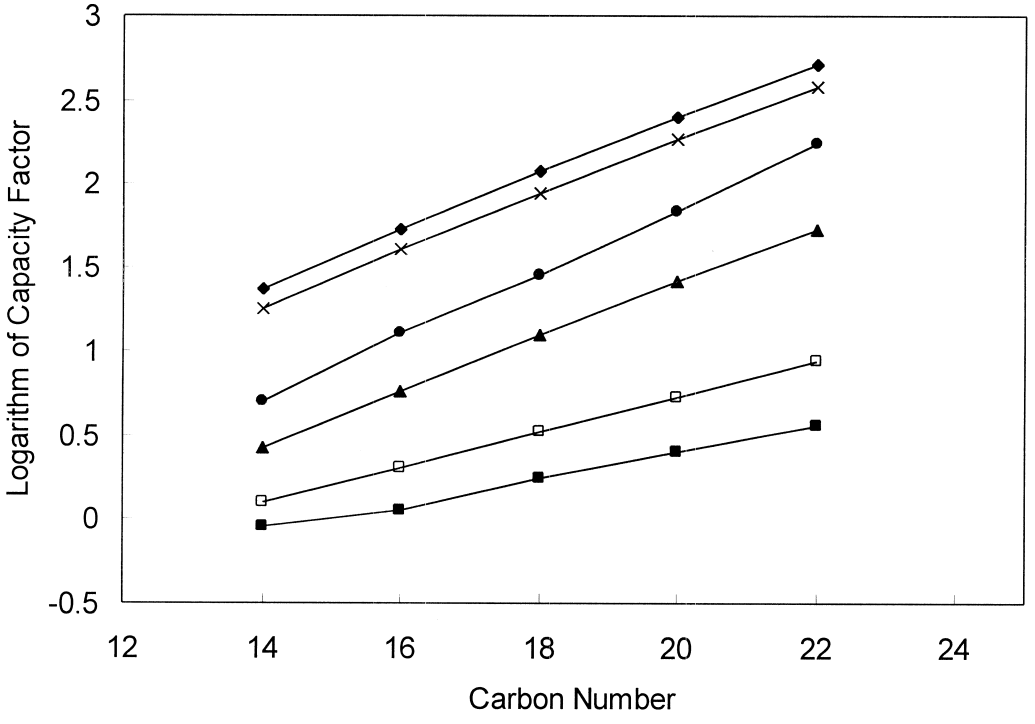

Fig. 1. Plots of logarithm of capacity factor versus carbon number. Mobile phase composition and column temperature: (♦) 85% methanol at 55°C, (×) 85% acetonitrile at 55°C, (●) 100% acetonitrile at 0°C, (▲) 100% methanol at 0°C, (□) 100% acetonitrile at 35°C, (■) 100% methanol at 35°C. Other experimental conditions were given in the text.

dispersion forces were important in causing an effective separation between the unsaturated fatty acid and its corresponding saturated fatty acid as well as between *cis* and *trans* unsaturated fatty acids in reversed-phase high-performance liquid chromatography. For reversed-phase high-performance liquid chromatography, the column temperature and mobile phase composition are the most common factors that effect the separation. Hence, the variation of solute retention and ECL values with respect to temperature and mobile phase composition, were systematically investigated and discussed in the following.

3.3. Temperature effect

Most reversed-phase high-performance liquid chromatography analyses have been done with the column at ambient temperature. However, changes in column temperature can result in changes in chromatographic resolution and separation time. The effect of temperature upon solute retention is described by the well-known Van't Hoff equation

$$\mathrm{Ln}\, k' = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} - \ln \beta \qquad (1)$$

where ΔH, ΔS, and β are the molar enthalpy, the molar entropy, and the volumetric ratio between the mobile and stationary phases, respectively. From Eq. (1), it is apparent that the logarithm of the capacity factor should be linearly related to the inverse temperature if the molar enthalpy and molar entropy are invariant. As illustrated in Fig. 2, the fatty acids generally exhibited the expected linear behavior at temperatures ranging from 0 to 35°C when either acetonitrile or methanol was used as the mobile phase. For saturated fatty acids with a chain length longer than 16, however, the linearity was slightly deviated at low temperatures. The deviation has been noted previously [21–25] and attributed to a phase transition of the octadecylsilica stationary phase from a random liquid-like structure to a more ordered solid-like structure. The brush-like structure of octadecyl chains at low temperatures causes an incomplete insertion for the fatty acids with a chain length longer than 18, resulting in a deviation of retention mechanism. This observation was also consistent with a greater slope for the homologous series at 0°C compared to those at 35 and 55°C as seen in Fig. 1. Apparently, lower temperatures were expected to provide a steric hindrance effect for the separation in addition to the dispersion interaction.

The effect of column temperature on the separation was further explored using the ECL values. The normally used expression for fatty acids with N carbon atoms and n double bonds, $\mathrm{ECL} = N - 2n$, indicates that the reduction of dispersion force of one double bond is equivalent to that of one ethylene group. However, many polyunsaturated fatty acids with three or more double bonds such as 18:3 do not follow the ECL rule [20]. As shown in Fig. 3, except 18:3, the ECL values of 18:1 and 18:2 were close to the expected values 16 and 14, respectively, at high temperatures. Moreover, the ECL was decreased as the temperature was decreased, which resulted in the resolution between the 18:1 and 18:2 fatty acids and their corresponding saturated fatty acids, 16:0 and 14:0, respectively. For 18:3, however, the ECL values at high temperatures were significantly greater than the expected value, 12. Moreover, the decrease of ECL values as the decrease of temperature adversely resulted in the overlapping between 18:3ccc and 12:0. It was also noticeable that the ECL of *trans* fatty acids appeared to be greater than the corresponding *cis* fatty acids and the two values became differentiable at low temperatures. The observed temperature effect on ECL values may be due to the different strength of steric hindrances among the different configurations of fatty acids: saturated < *trans* < *cis*. The saturated fatty acids have a straight configuration and may easily fit into the brush-like C_{18} stationary phase. The *cis* fatty acids which are curved by undeformable double bonds were having a greater hindrance than a saturated fatty acid so that the ECL was decreased with the decrease of the temperature. Compared to *cis* configuration, the *trans* configuration has a less steric hindrance and hence, a smaller ECL value was obtained.

It was possible to achieve a complete resolution at low temperatures. When acetonitrile was used as the mobile phase, a minimum resolution of 0.8 for the worst pair (18:3ccc/ttt) was readily achieved at 0°C. However, an extensive separation time of 200 min was needed to complete the elution of fatty acids up to 22 carbon number. Although a temperature programming may be theoretically applied to speed up

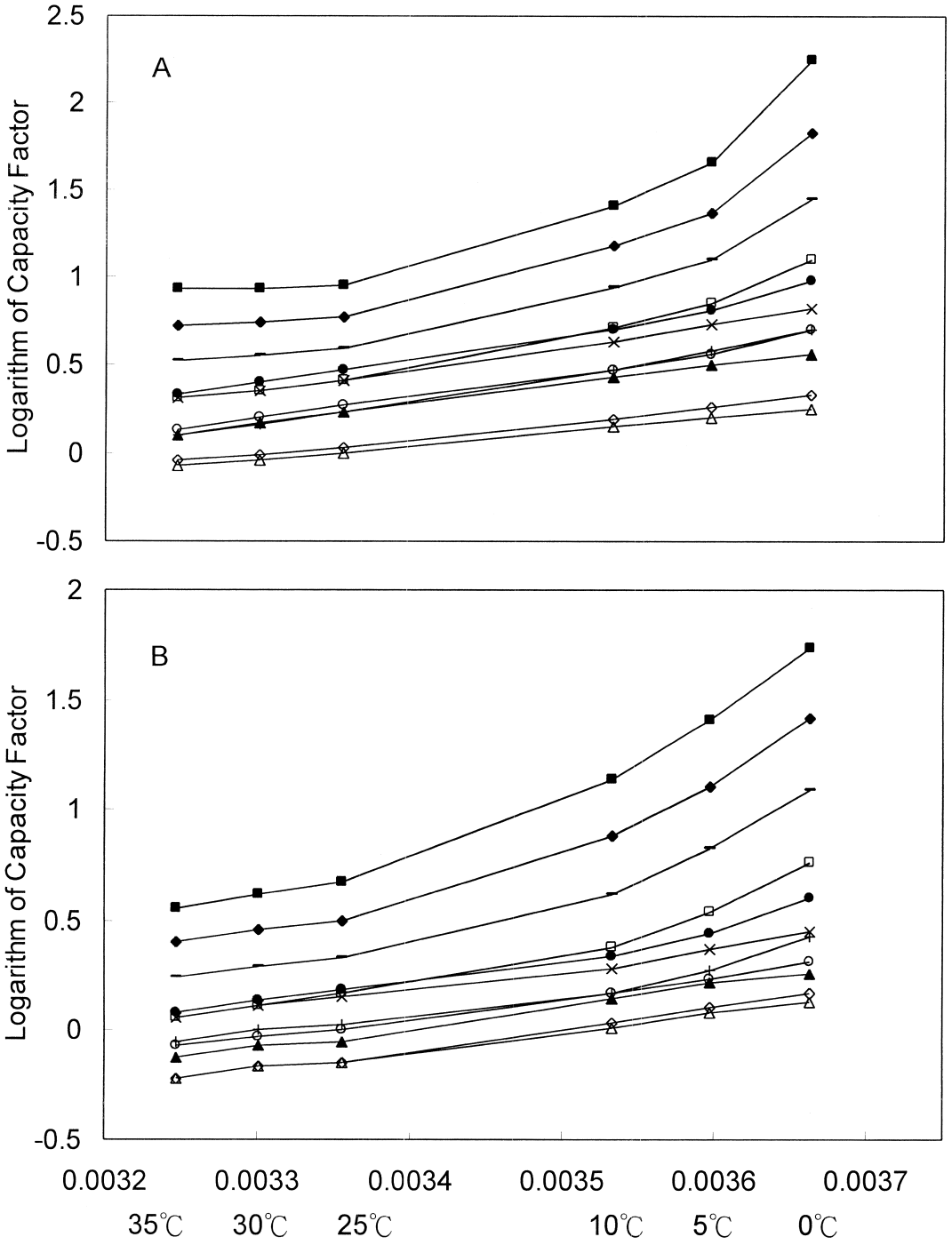

Fig. 2. Van't Hoff plots for the solutes (■) 22:0, (♦) 20:0, (—) 18:0, (□) 16:0, (●) 18:1t, (×) 18:1c, (○) 18:2t, (+) 14:0, (▲) 18:2c, (◊) 18:3t, and (△) 18:3c with the mobile phase of (A) acetonitrile, (B) methanol. Other experimental conditions were given in the text.

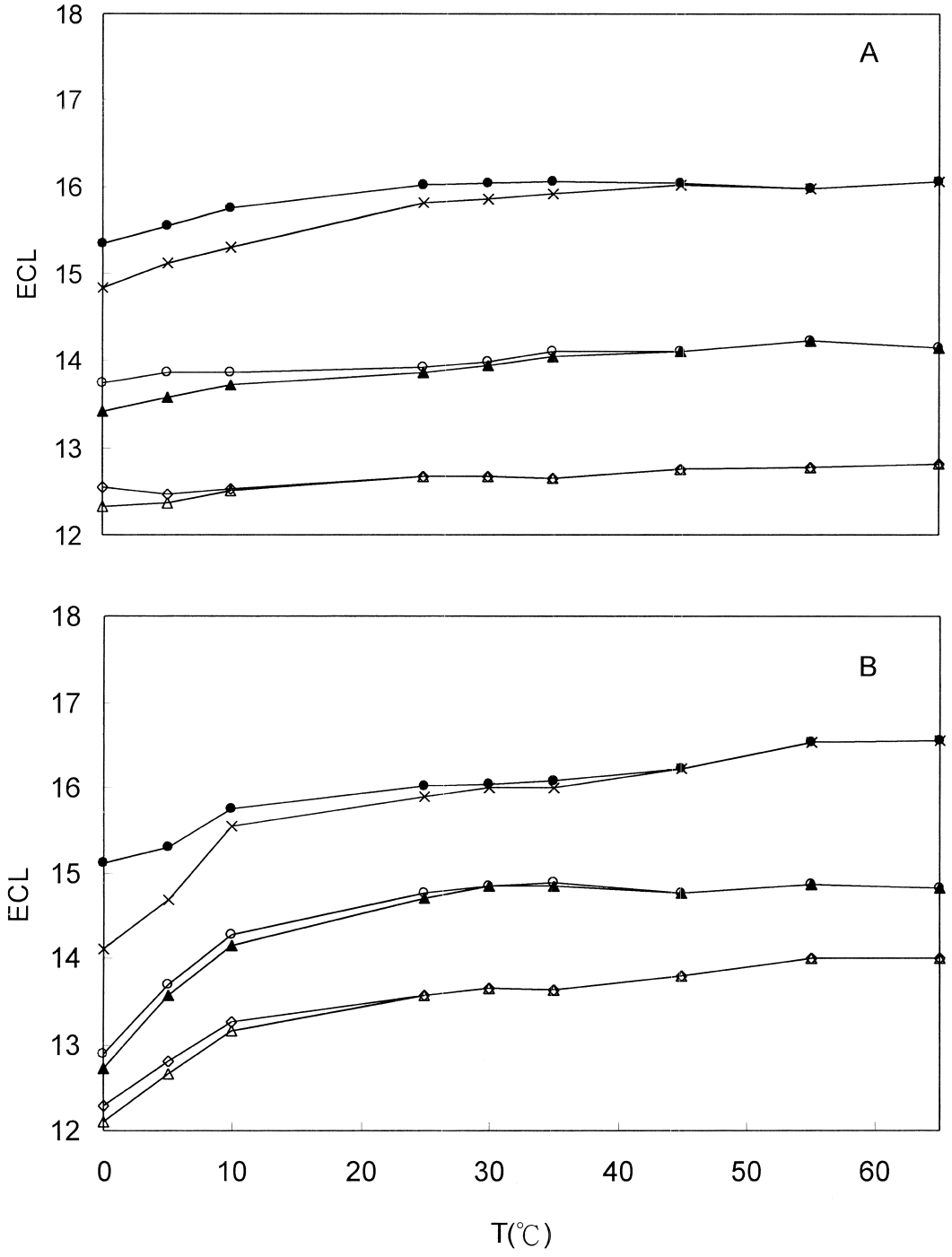

Fig. 3. Effect of temperature on ECL for the solutes (●) 18:1t, (×) 18:1c, (○) 18:2t, (▲) 18:2c, (◊) 18:3t, and (△) 18:3c with the mobile phase of (A) acetonitrile, (B) methanol. Other experimental conditions were given in the text.

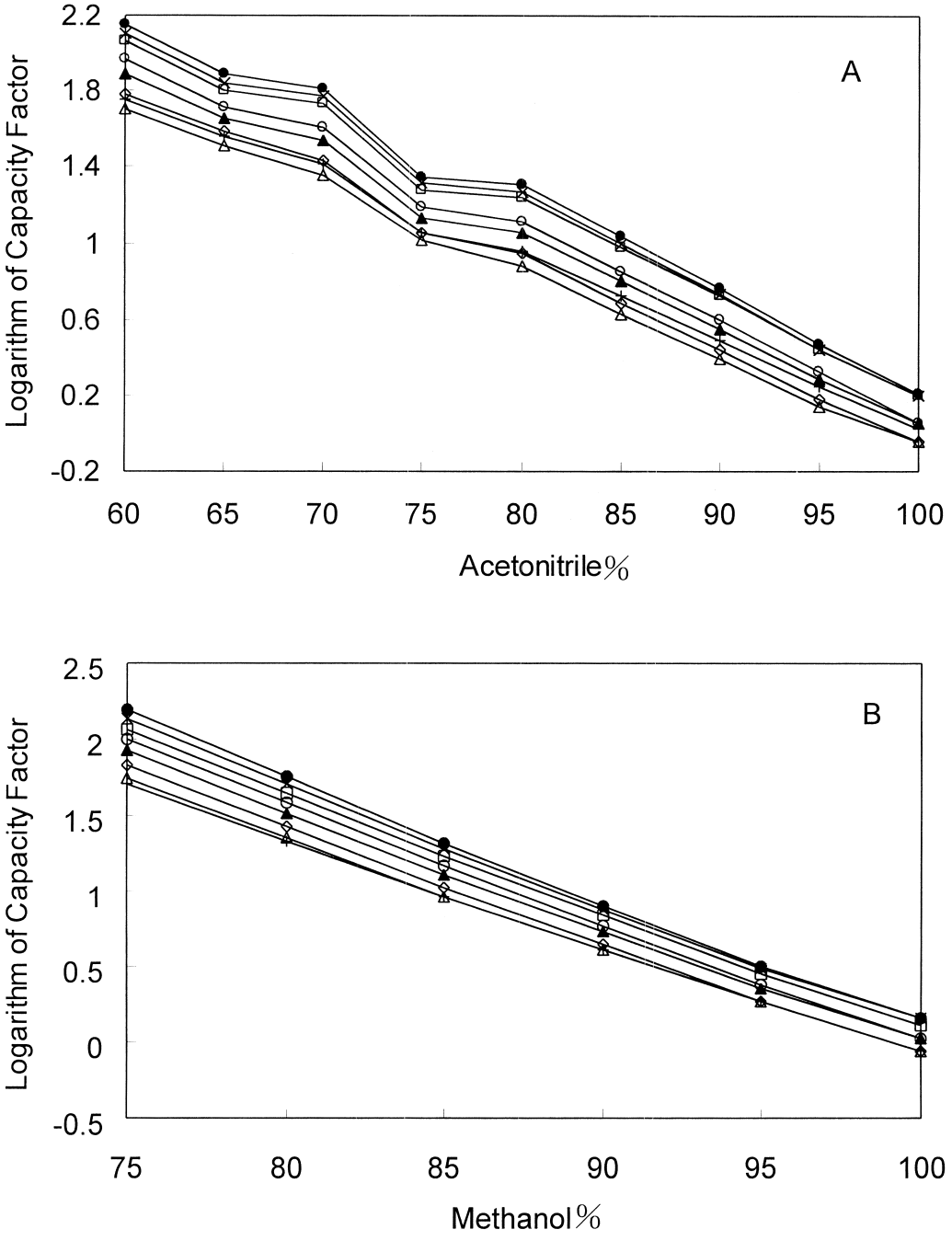

Fig. 4. Plots of logarithm of capacity factor versus (A) acetonitrile (B) methanol composition at 55°C for the solutes (■) 22:0, (♦) 20:0, (—) 18:0, (□) 16:0, (●) 18:1t, (×) 18:1c, (○) 18:2t, (+) 14:0, (▲) 18:2c, (◊) 18:3t, and (△) 18:3c. Other experimental conditions were given in the text.

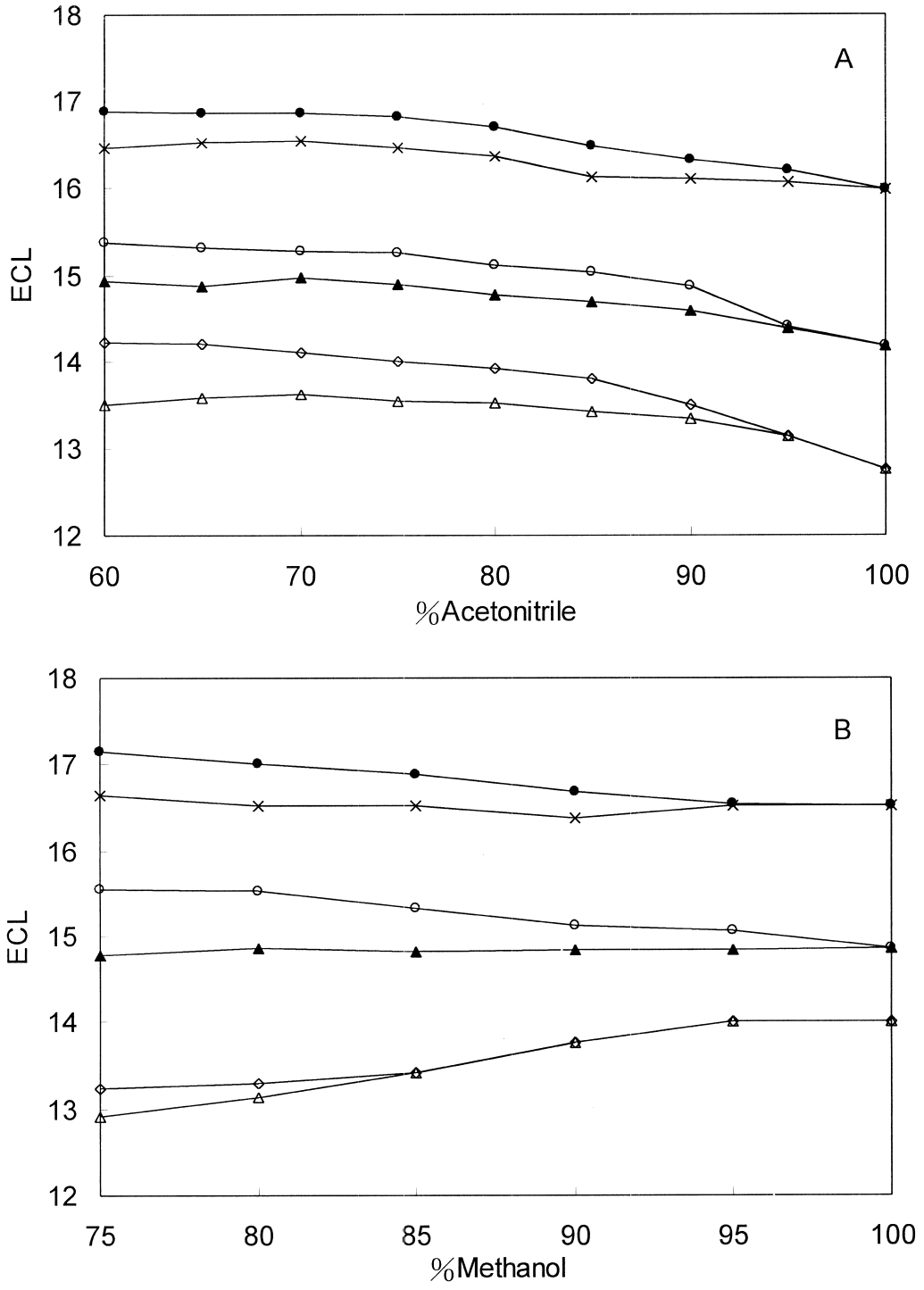

Fig. 5. Effect of (A) acetonitrile (B) methanol composition on ECL at 55°C for the solutes (●) 18:1t, (×) 18:1c, (○) 18:2t, (▲) 18:2c, (◊) 18:3t, and (△) 18:3c. Other experimental conditions were given in the text.

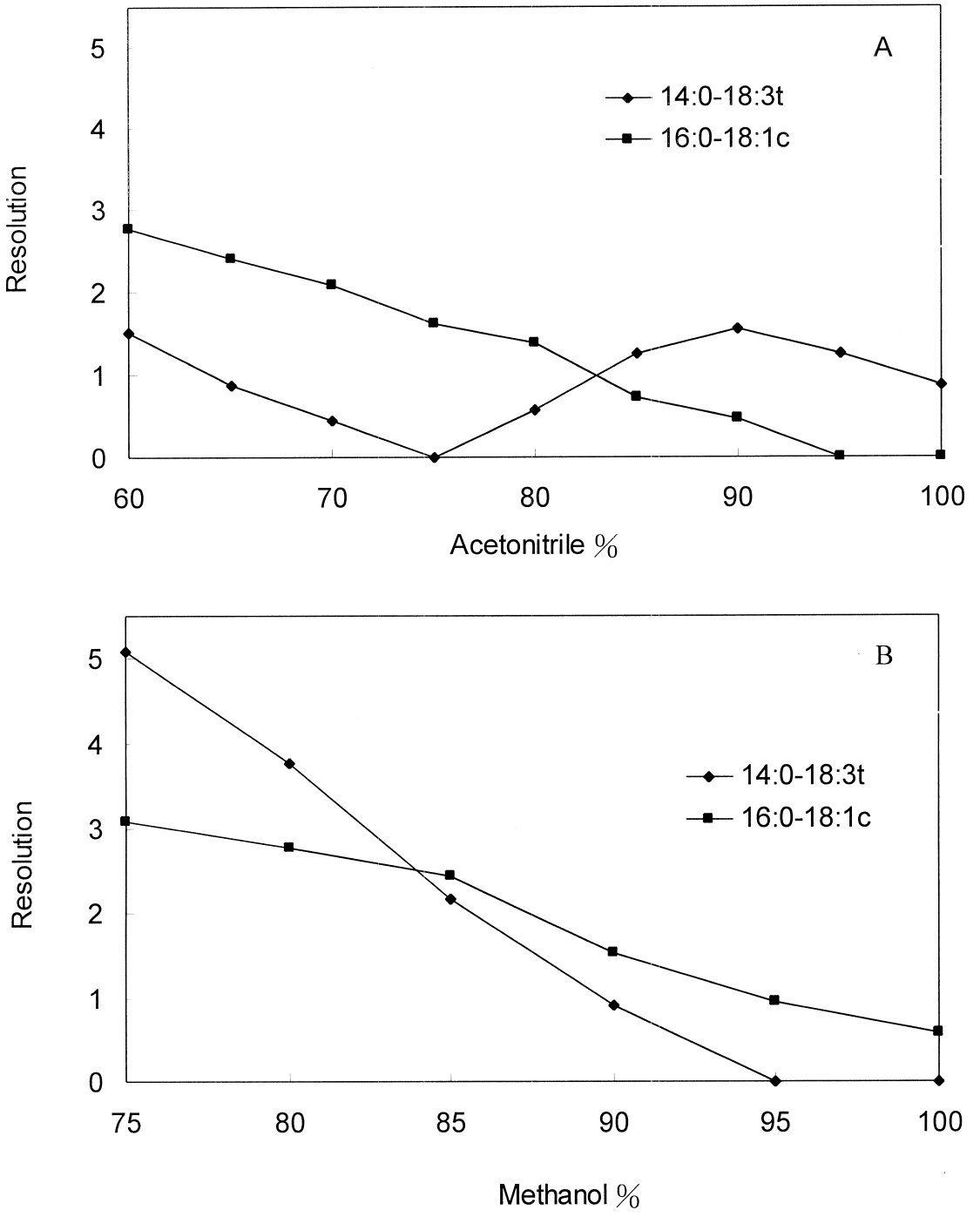

Fig. 6. Plots of the resolution versus (A) acetonitrile (B) methanol composition at 55°C. Other experimental conditions were given in the text.

the separation, the thermal equilibrium in liquid phase is generally not as rapid as in gas phase. Instead, the use of high temperatures can greatly speed up the separation if an effective mobile phase composition can be found. In the following, the mobile phase compositions were systematically studied at a temperature of 55°C to find out the efficient separation.

3.4. Organic solvent effect

Mobile-phase composition is one of the most important chromatographic factors affecting the separation because of the small variability in the types of the stationary phase available. The effect of the percentage of organic modifier, acetonitrile and methanol, on the capacity factor is depicted in Fig. 4. The increase of the methanol composition decreased the polarity of the mobile phase and hence, the capacity factor was linearly decreased. However, for acetonitrile, it is noticeable that a discontinuity was observed for all solutes in the composition range from 70 to 80%. The occurrence of this nonlinear retention behavior is due to nonideal interactions between solute–solvent and solvent–solvent [26,27].

The effect of organic solvent composition on the separation was further explored from the ECL values. As shown in Fig. 5A, the ECL values of unsaturated fatty acids increased with the decrease of acetonitrile composition and the increase was significantly greater for *trans* fatty acid compared to its *cis* counterpart at acetonitrile compositions less than 85%. These observations may be due to a preferential $\pi-\pi$ interaction between acetonitrile and the ethylene group. The $\pi-\pi$ interaction was reduced at low acetonitrile compositions and lead to a relatively greater retention compared to the corresponding saturated fatty acid. Moreover, such interaction is stronger for *cis* configuration than *trans* configuration due to a lesser steric hindrance. These changes at low acetonitrile compositions resulted in the resolution between the unsaturated fatty acids, 18:1 and 18:2, and their corresponding saturated fatty acids,16:0 and 14:0, respectively, as well as between *cis* and *trans* fatty acids. However, the increase of ECL value of 18:3ttt at low organic solvent compositions adversely resulted in its overlapping with 14:0. For methanol as the organic modifier, Fig. 5B shows that the ECL values of 18:1c and 18:2c were relatively constant with the methanol composition. Moreover, in contrast to that observed for acetonitrile, the ECL values for both 18:3ccc and 18:3ttt were decreased as the methanol composition was decreased. These observations were not well understood and may be partially due to the lack of $\pi-\pi$ interaction that was present for acetonitrile. Generally speaking, the decrease of either acetonitrile or methanol composition increased the overall resolution and the change of organic solvent composition was technically more straightforward compared to the change of temperature in liquid chromatography. Although the resolution was greater at low organic solvent compositions, the separation time was increased accordingly.

3.5. Programming method

In seeking the appropriate separation condition, the resolution of the two worst pairs, 16:0/18:1c and 14:0/18:3ttt, was plotted against the composition of acetonitrile (Fig. 6A) and methanol (Fig. 6B), respectively. It was indicated from Fig. 4 that the capacity factor will be less than 10 when the acetonitrile and methanol composition were greater than 80% and 85%, respectively. Within these ranges of organic solvent composition (between 80 and 100%), Fig. 6 indicated that the 14:0/18:3ttt pair was better resolved by acetonitrile while the 16:0/18:1c pair was better resolved by methanol. Based on these understandings, a programming method as shown in Table 1 was attempted to resolve different pairs using different organic modifiers and compositions. In this elution program, the mobile phase composed of 82% acetonitrile and 3% methanol was first used to resolve the 14:0/18:3ttt pair and then the methanol composition was increased to 85% to

Table 1
Progamming method

Acetonitrile	Water	Methanol	Time (min)	Flow rate (ml/min)
82	15	3	0	1
82	15	3	18	1
0	15	85	29	1
0	0	100	32	2
0	0	100	40	2

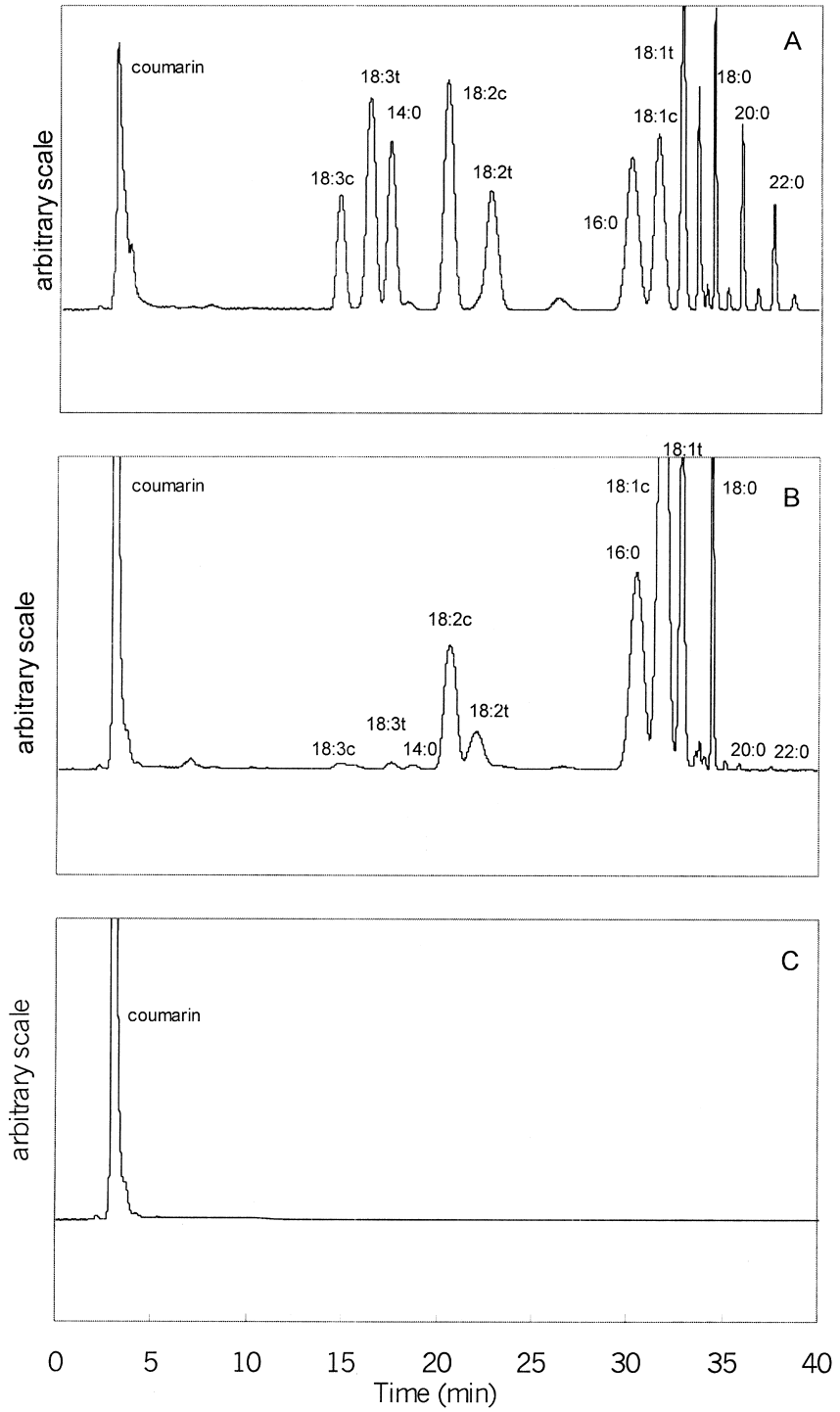

Fig. 7. Chromatograms of (A) standard mixture (B) vegetable shortening (C) solvent blank. Other experimental conditions were described in the text.

resolve the 16:0/18:1c pair. Finally, the methanol composition was increased to 100% and at a flow-rate of 2 ml/min to speed up the elution of the rest of the saturated fatty acids. Under this ternary gradient, a minimum resolution of 1.0 for the worst pair was obtained within a separation time of 40 min (Fig. 7A). The detection limit of the method determined from the three times of the background noise ranges from 0.39 for 14:0 to 3.10 for 22:0. The sensitivity and separation time of this method are comparable to those of the gas chromatography flame ionization detection method [1,2]. Although only all *cis* and all *trans* fatty acids were included in the standard mixture due to the unavailability of other *trans* species, the resolution between all *cis* and all *trans* fatty acids was not limited by this method. Instead, the resolutions between different classes appeared to be the worst pairs. It should also be possible to resolve other *trans* species under proper solvent compositions. The capability of using the developed method for analyzing the real sample was examined. As shown in Fig. 7B and C, the separation of fatty acids in the vegetable shortening was readily achieved. Identifications of individual fatty acids were based on the retention times and some species were further verified by spiking the standards into the real sample.

4. Conclusions

Through a systematic study, a proper solvent system and operating temperature for reversed-phase high-performance liquid chromatography can be found to provide adequate resolution in reasonable time for the analysis of fatty acids according to carbon number and the degree of unsaturation, as well as *cis*/*trans* configuration. It was found that the resolution between *cis* and *trans* configurations was not the limiting factor. Instead, overlapping between different classes was critical and a systematic study would assist the optimization process. Compared to gas chromatography with flame ionization detection the derivatization process of the current method is relatively complicated and the derivatized standards are not commercially available. However, the current method may be a potential alternative when access to gas chromatography is limited. Moreover, under certain circumstances, for example high temperature separation ($>55^{\circ}C$) is not desirable due to the unstability of the sample or fraction collection is necessary for subsequent analysis, the current method using liquid chromatography with fluorescence should be a preference.

Acknowledgements

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References

[1] AOCS Official Method Ce 1c-89.
[2] AOCS Official Method Cd 14c-94.
[3] F.R. Senti, Health Aspects of Dietary *Trans* Fatty Acids, Life Sciences Research Office, FASEB, Bethesda, MD, 1985.
[4] R.P. Mensink, M.B. Katan, New Engl. J. Med. 323 (1990) 439.
[5] K. Eder, J. Chromatogr. B 671 (1995) 113, and references in this review article.
[6] W. Dunges, Anal. Chem. 49 (1977) 442.
[7] S.A. Barker, J.A. Monti, S.T. Christian, F. Benington, R.D. Morin, Anal. Biochem. 107 (1980) 116.
[8] N. Ichinose, K. Nakamura, C. Shimizu, H. Kurokura, K. Okamato, J. Chromatogr. 295 (1984) 463.
[9] M. Ikeda, K. Shimada, T. Sakaguchi, U. Matsumoto, J. Chromatogr. 305 (1984) 261.
[10] W.D. Korte, J. Chromatogr. 243 (1982) 153.
[11] H. Tsuchiya, T. Hayashi, H. Naruse, N. Takagi, J. Chromatogr. 234 (1982) 121.
[12] V.L. McGuffin, R.N. Zare, Appl. Spectrosc. 39 (1985) 847.
[13] V.L. McGuffin, R.N. Zare, Proc. Natl. Acad. Sci. 82 (1985) 8315.
[14] J.S. Yoo, V.L. McGuffin, J. Chromatogr. 627 (1992) 87.
[15] B. Nikolova-Damyanova, W.W. Christie, J. Chromatogr. A 653 (1993) 15.
[16] A. Baillet, L. Corbeau, P. Rafidison, D. Ferrier, J. Chromatogr. 634 (1993) 251.
[17] R.O. Adlof, J. Chromatogr. A 659 (1994) 95.
[18] R.C. Wijesundera, R.G. Ackman, J. Chromatogr. Sci. 27 (1989) 399.
[19] K. Stránsky, T. Jursík, A. Vítek, J. High Resolut. Chromatogr. 20 (1997) 143.
[20] M. Özcimder, W.E. Hammers, J. Chromatogr. 187 (1980) 307.

[21] D. Morel, J. Serpinet, J. Chromatogr. 200 (1980) 95.
[22] D. Morel, J. Serpinet, J. Chromatogr. 214 (1980) 202.
[23] D. Morel, J. Serpinet, J. Chromatogr. 248 (1980) 231.
[24] D. Morel, J. Serpinet, J.M. Letoffe, P. Claudy, Chromatographia 22 (1986) 103.
[25] P. Claudy, J.M. Letoffe, C. Gaget, D. Morel, J. Serpinet, J. Chromatogr. 329 (1985) 331.
[26] H.B. Patel, T.M. Jefferies, J. Chromatogr. 389 (1987) 21.
[27] J.L. Glajch, J.J. Kirkland, K.M. Squire, J.M. Minor, J. Chromatogr. 199 (1980) 57.