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Journal of Chromatography A, 894 (2000) 145–155

JOURNAL OF CHROMATOGRAPHY A

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Simultaneous micellar electrokinetic chromatographic determination of isomeric fatty acid hydroperoxides and corresponding hydroxy fatty acids

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Abstract

The high selectivity and efficiency of micellar electrokinetic chromatagraphy with a borax–sodium dodecylsulfate (SDS) or meglumin–SDS buffer make possible the rapid separation of hydroperoxy and hydroxy fatty acids and the non-oxidised unsaturated fatty acids from which they are derived. Nearly all the isomers of the hydroperoxides and hydroxy fatty acids derived from oleic, linoleic, α- and γ-linolenic and arachidonic acids can be determined both qualitatively and quantitatively within ca. 10 min. The system has as many as 1×10^{6} theoretical plates, and the detection limits with UV diode array detection at 195 or 234 nm are in the micromolar range. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Fatty acids; Fatty acid hydroperoxides; Hydroxy fatty acids

1. Introduction

Studies of lipid peroxidation (LPO) have in recent years attained a significant position in medicine and biology. Lipid hydroperoxides (LOOHs) and their corresponding hydroxy fatty acids (LOHs), which are readily formed in biologically relevant matrices [1], are particularly important in rheumatism [2–9] and arteriosclerosis [1,10–12], while the hydroperoxides of arachidonic acid are intermediates in the formation of prostaglandins, thromboxanes and leucotrienes.

The analysis of fatty acids, fatty acid hydroperoxides and hydroxy fatty acids is usually carried out by GC [13–19] or HPLC [20–28]. In the case of GC analysis, the polarity and thermal instability of the hydroperoxides necessitate prior derivatisation: the carboxy groups are esterified [14–19] and the hydroperoxy groups silylised [13,18,19]. The double bonds of the hydroxy fatty acids may also be hydrogenated [11,12], although this reduces the information content of the sample. HPLC separation of the hydroperoxide isomers has so far been possible only on normal-phase columns [23–27]. The highly aqueous eluents of reversed-phase HPLC make an enzymatic post-column derivatisation possible, which in combination with fluorescence or chemiluminescence detection improves both the selectivity and the sensitivity [20–22]; none of the HPLC methods are, however, capable of separating the isomers as well as is desired.

Capillary electrophoresis (CE) has recently been added to these methods for LPO analysis [29–34]. Micellar electrokinetic chromatography (MEKC),

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PII: S0021-9673(00)00587-2

with rather complex buffer systems, has been particularly effective in separating structurally very similar isomers. In work reported earlier, we successfully separated the LOOHs with a phosphate buffer containing sodium dodecylsulfate (SDS) and Brij 35 to form micelles, with ethanol and acetone as organic modifiers [34]. That buffer, however, had poor selectivity for LOHs, and there were additional disadvantages in the relatively long analysis times (ca. 30 min) and the complex 6-min conditioning program.

The use of a borax or a meglumin buffer with normal flow has now made it possible to reduce the analysis time to ca. 10 min and the conditioning time to 1 min, while simultaneously improving the efficiency and selectivity for the LOOHs from a variety of fatty acids. For the first time, a complete separation of all the analogous LOH has also been achieved.

2. Experimental

2.1. Apparatus

The results presented here were obtained with a Beckman P/ACE System 5510 equipped with a UV

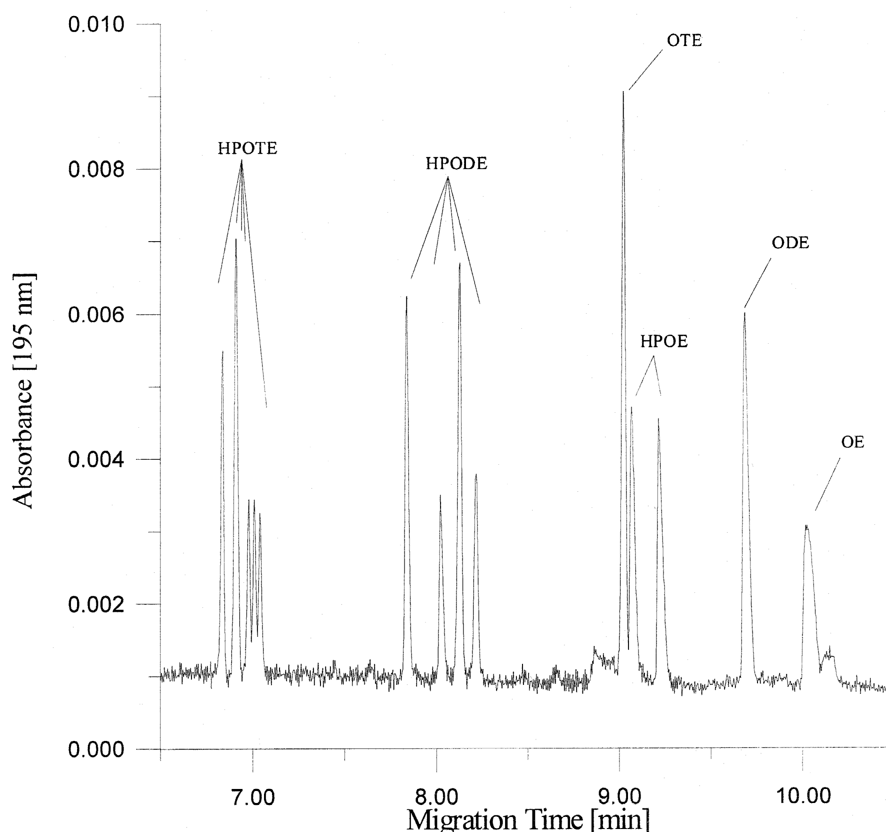

Fig. 1. Separation of oleic (OE; 0.5 mM), linoleic (ODE; 0.3 mM) and linolenic acids (OTE; 0.25 mM) and the hydroperoxides formed from them with singlet oxygen: HPOE (1.1 mM), HPODE (1.9 mM) and HPOTE (1.4 mM); electrolyte: 10 mM borax buffer (pH 9.3), 15 mM SDS, 2.4% (v/v) ACN; fused-silica capillary 67 cm (effective length 60 cm); field strength 448 V/cm; detection UV-DAD at 195 nm.

diode array detection (UV-DAD) system. The fused-silica capillaries, from CS-Chromatographie-Service (Langerwehe, Germany), were 67 cm (effective length 60 cm), 50-μm I.D. and were thermostated at 20°C. The voltage was 30 kV and the field strength accordingly 448 V/cm. Samples were injected hydrodynamically (3 s), and the detector was on the cathode side. Between analyses, the capillary was rinsed for 1 min with buffer. New capillaries were rinsed for 5 min with water and 10 min with buffer.

2.2. Reagents and commercial standards

All chemicals used were of analytical-reagent grade. Fatty acids and lecithin were purchased from Sigma (Deisenhofen, Germany), 5(*S*)-hydroperoxy-eicosa-6E,8Z,11Z,14Z-tetraenoic acid (5-HPETE) and 15(*S*)-hydroperoxy-eicosa-5Z,8Z,11Z,13E-tetraenoic acid (15-HPETE) from Cascade Biochem (Berkshire, UK). The lipase SP 525 was obtained from Novo Nordisk (Denmark). All other chemicals were from Merck (Darmstadt, Germany) and Fluka (Neu-Ulm, Germany). Tridest water (deionised and doubly distilled) was used for the electrolyte solutions, which were passed through a PTFE filter (0.45 μm) and degassed ultrasonically.

The synthesis of the fatty acid hydroperoxides and the corresponding hydroxy fatty acids was described

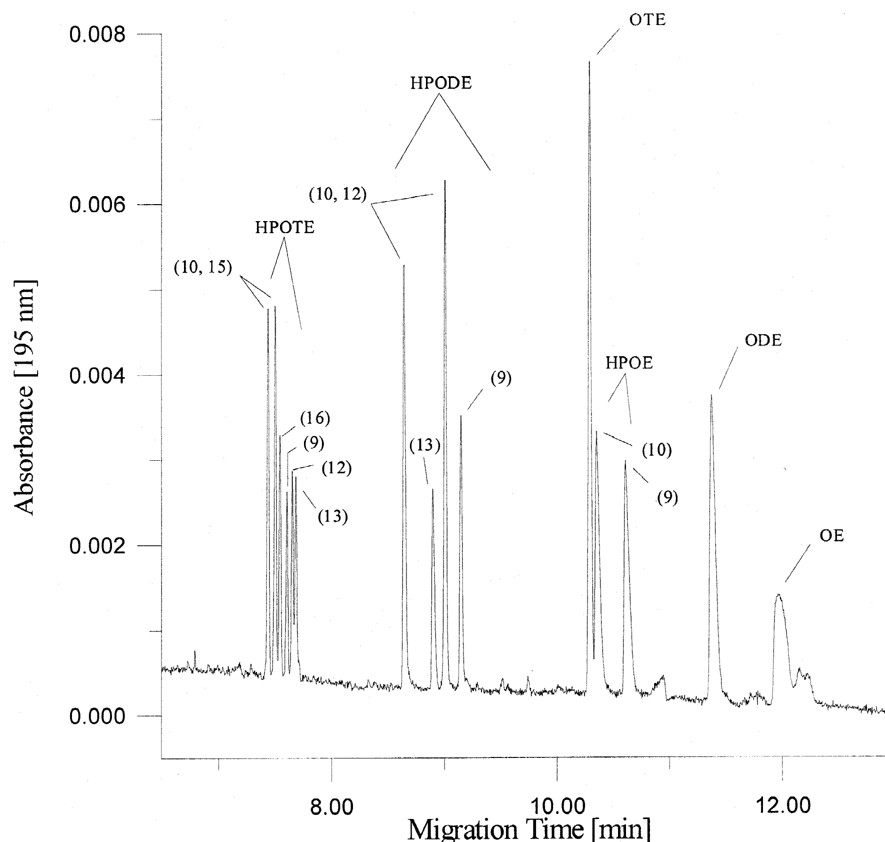

Fig. 2. Separation of oleic (OE; 0.5 m*M*), linoleic (ODE; 0.3 m*M*) and linolenic acids (OTE; 0.25 m*M*) and the hydroperoxides formed from them with singlet oxygen: HPOE (1.1 m*M*), HPODE (1.9 m*M*) and HPOTE (1.4 m*M*); electrolyte: 10 m*M* meglumin buffer (pH 9.0), 15 m*M* SDS, 2.4% (v/v) ACN; fused-silica capillary 67 cm (effective length 60 cm); field strength 448 V/cm; detection UV-DAD at 195 nm; the numbers in parentheses give the position of the hydroperoxide group.

earlier [12,32,33]. In as yet unpublished experiments dihydroperoxides were synthesised for standards by subjecting fatty acids to more prolonged oxidation with singlet oxygen than in the procedure used for the mono-hydroperoxides [32,33]. They were separated from other components of the reaction mixture by column chromatography on silica gel with hexane–diethyl ether–acetic acid 80:20:1, (v/v/v), the isomers eluting together well after the monohydroperoxides. The mixture of dihydroperoxides was examined by ^{1}H- and ^{13}C{^{1}H}-NMR. Most revealing was the number of resonances between 83 and 87 ppm that represent carbon atoms with hydroperoxy groups.

For the photoxidation of egg lecithin, ca. 300 mg were dissolved with ca. 5 mg of methylene blue in 370 ml of methanol and treated with singlet oxygen as in the synthesis of the fatty acid hydroperoxides. Samples (50 μl) were taken at various intervals. The solvent was removed under vacuum from the irradiated lecithin solution, and the fatty acids were liberated enzymatically. A solution consisting of 20 m*M* $CaCl_2$ and 400 m*M* glycyl glycine was buffered at pH 7.5 with 1 *M* NaOH; 200 μl of this solution and 0.4 mg SP 525 were added to the sample, and the reaction mixture was shaken for 90 min at 37°C. The aqueous phase was extracted five times with 1 ml of chloroform. The solvent was removed from the

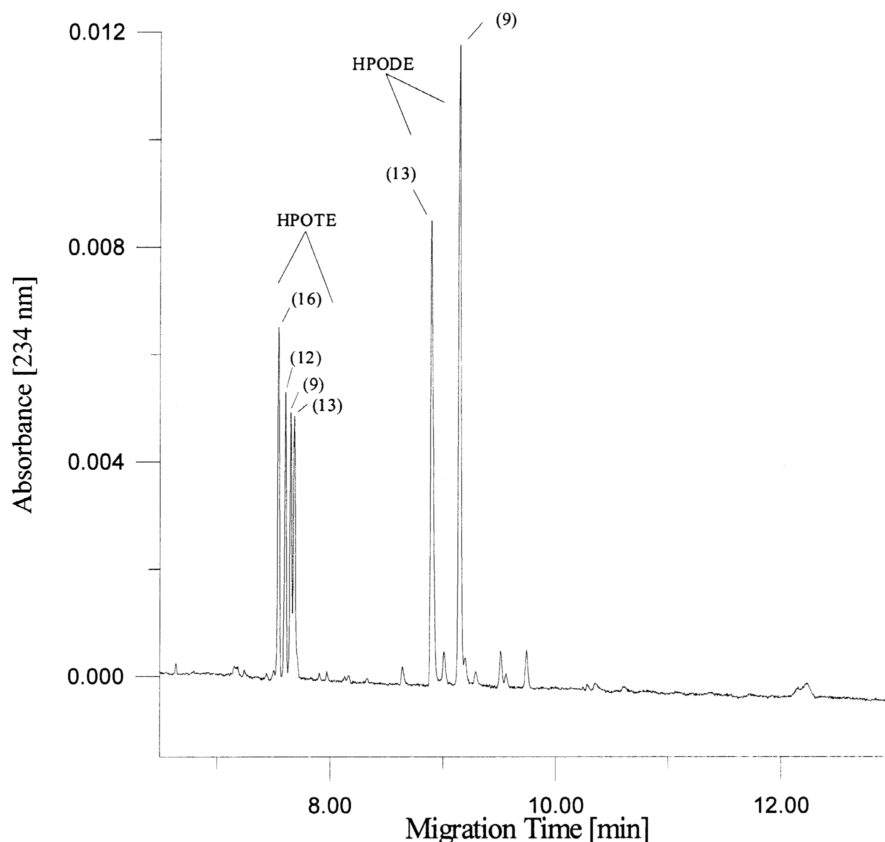

Fig. 3. Conjugated hydroperoxides formed from linoleic and linolenic acids with singlet oxygen: HPODE (1.9 m*M*) and HPOTE (1.4 m*M*); electrolyte: 10 m*M* meglumin buffer (pH 9.0), 15 m*M* SDS, 2.4% (v/v) ACN; fused-silica capillary 67 cm (effective length 60 cm); field strength 448 V/cm; detection UV-DAD at 234 nm; only the conjugated isomers absorb at this wavelength.

combined extracts with a stream of nitrogen, and the residue was taken into 100 μl of methanol for MEKC analysis.

The 10 m*M* meglumin buffer was adjusted to pH 9.0 with 85% (w/w) phosphoric acid. The borax buffer required no pH adjustment.

3. Results and discussion

The reversed-flow system that we used in earlier work had a buffer of 60 m*M* phosphate at pH 6.3, containing 35 m*M* SDS, 750 mg/l Brij 35, 30% (v/v) ethanol and 1% (v/v) acetone [32–34]. The slow migration of the analytes to the anodic detector, against the suppressed electroosmotic flow (EOF), resulted in broad signals and long analysis times (ca. 30 min). In addition, that buffer had poor selectivity for LOHs and required a complex 6-min conditioning program. The system was, however, ideal for the enzymatic post-column derivatisation and fluorimetric detection then used [34].

The composition of the new, normal-flow buffer and the instrumental parameters were optimised empirically to overcome these problems. A voltage of 30 kV, a capillary length of 67 cm (effective

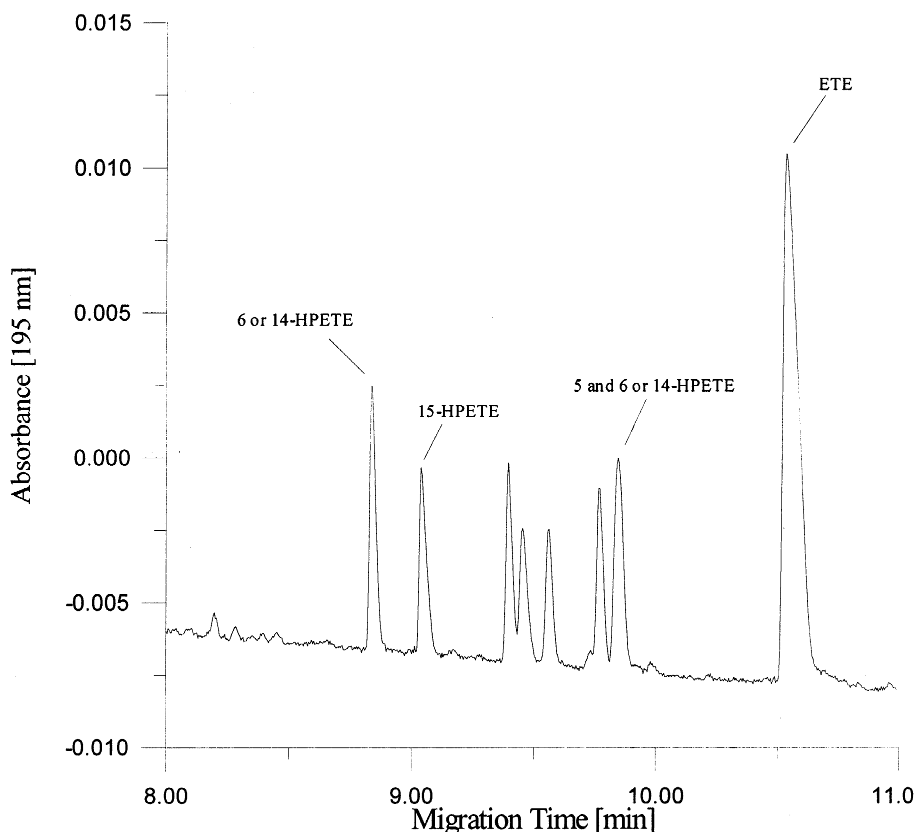

Fig. 4. Separation of arachidonic acid (ETE 0.8 m*M*) and the hydroperoxides formed from it with singlet oxygen (HPETE; 0.9 m*M*); electrolyte: 10 m*M* borax buffer (pH 9.3), 15 m*M* SDS, 2.4% (v/v) ACN; fused-silica capillary 67 cm (effective length 60 cm); field strength 448 V/cm; detection UV-DAD at 195 nm.

length 60 mm) and a temperature of 20°C, with cathodic detection gave the best results. The electrolyte, where not otherwise mentioned, was 10 m*M* borax at pH 9.3 with 15 m*M* SDS and 2.4% (v/v) acetonitrile (ACN).

Fig. 1 shows the separation of the unsaturated fatty acids (LHs) most relevant to biological systems, along with the LOOHs derived from them by photosensitised oxidation: five of the six hydroperoxide isomers expected from linolenic acid (HPOTE), all four isomers from linoleic acid (HPODE) and both of those from oleic acid (HPOE).

Complete separation of all the possible isomers was achieved by replacing the borax buffer with one of meglumin at a slightly reduced pH (Fig. 2), which resulted in a somewhat reduced EOF (ca. 0.5 cm/min) and an increased analysis time of 12 min. The improved separation may be explained by a change in the distribution of the analyte between buffer and micelles. This improved selectivity was unfortunately limited to the fatty acids and hydroperoxides shown in Fig. 2; for the other compounds discussed here, it either had no advantages over the borax system or, as in the case of the hydroxy fatty acids, actually reduced the selectivity.

The components of the mixture were identified

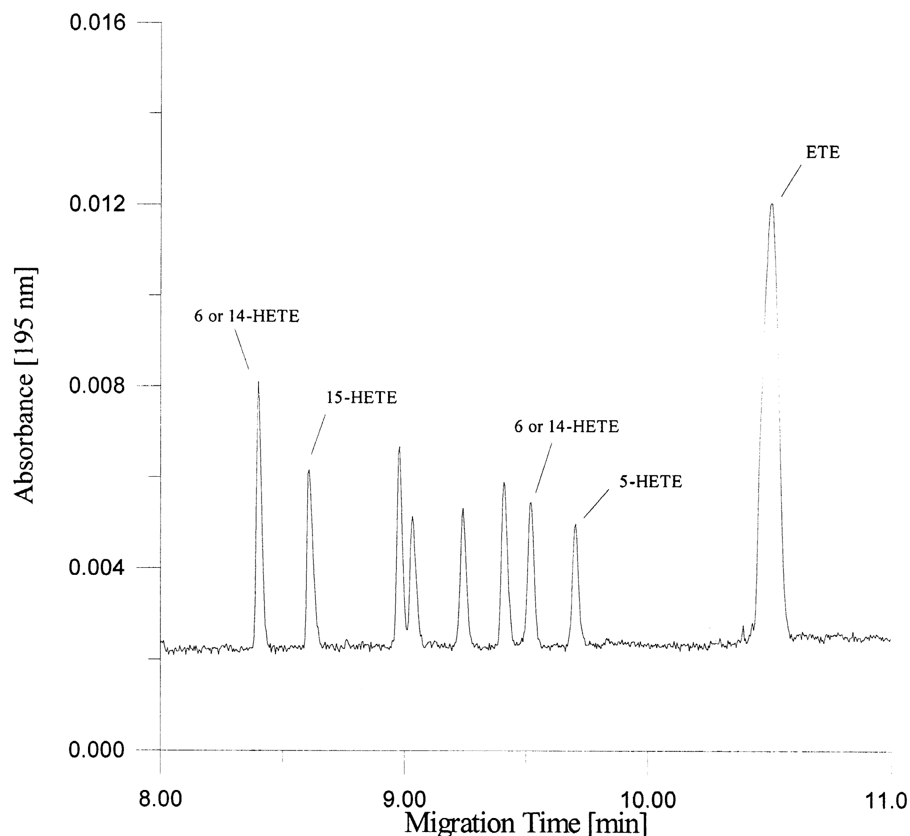

Fig. 5. Separation of arachidonic acid (ETE; 0.8 m*M*) and the hydroxy fatty acids obtained from it by reduction with sodium borohydride (HETE; 0.9 m*M*); electrolyte: 10 m*M* borax buffer (pH 9.3), 15 m*M* SDS, 2.4% (v/v) ACN; fused-silica capillary 67 cm (effective length 60 cm); field strength 448 V/cm; detection UV-DAD at 195 nm.

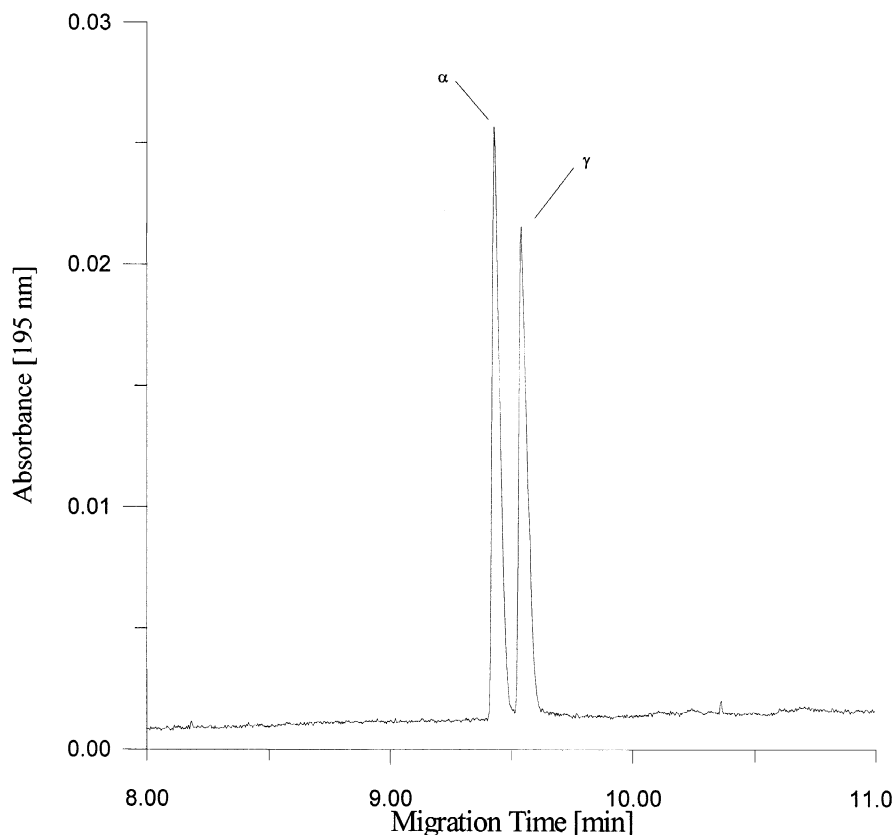

Fig. 6. Separation of α-linolenic acid (α-OTE; 1.0 m*M*) and γ-linolenic acid (γ-OTE; 0.9 m*M*); electrolyte: 10 m*M* borax buffer (pH 9.3), 15 m*M* SDS, 2.4% (v/v) ACN; fused-silica capillary 67 cm (effective length 60 cm); field strength 448 V/cm; detection UV-DAD at 195 nm.

through comparison with synthetic standards prepared in this laboratory as previously described [32,33]. Conjugated and non-conjugated isomers can be distinguished by the fact that conjugated double-bond systems absorb more strongly at 234 nm than isolated systems (cf. Figs. 2 and 3). The conjugated isomers (e.g., 9-, 12-, 13-, 16-HPOTE) were identified by spiking oxidation mixtures with 9- and 13-HPOTE synthesised enzymatically in varying proportions [35,36]. The remaining isomers were assigned by their known yields from singlet oxidation, as reported by Belitz and Grosch [37].

Fig. 4 shows the separation of seven of the eight hydroperoxides (HPETEs) that can be formed on treatment of arachidonic acid with singlet oxygen. The separation of all eight LOHs obtained on reduction of these is displayed in Fig. 5. This MEKC system has not only high efficiency for the LOOHs and LOHs, but high selectivity for the corresponding LHs as well.

Although a number of natural sources for γ-linolenic acid are known, it is relatively rare and always accompanied by the α-isomer [38,39]. A simple analysis of the two isomers is thus of interest.

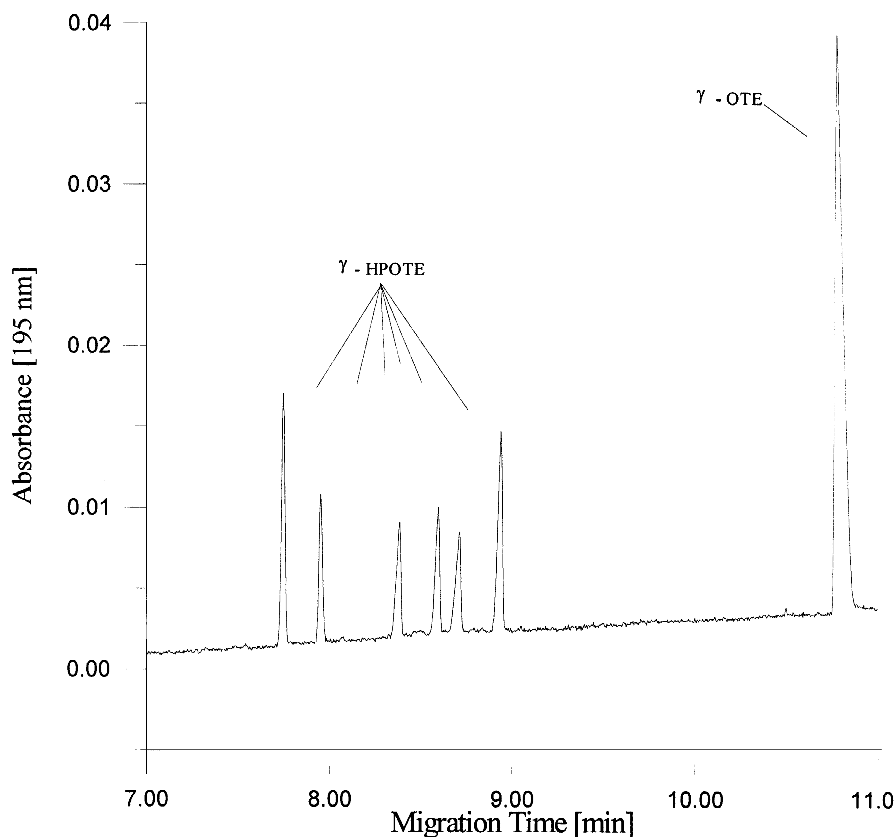

Fig. 7. Separation of γ-linolenic acid (γ-OTE; 1.0 m*M*) and the hydroperoxides formed from it with singlet oxygen (γ-HPOTE; 1.4 m*M*); electrolyte: 10 m*M* borax buffer (pH 9.3), 15 m*M* SDS, 5.7% (v/v) ACN; fused-silica capillary 67 cm (effective length 60 cm); field strength 448 V/cm; detection UV-DAD at 195 nm.

Fig. 6 shows baseline separation of α- and γ-linolenic acids within 10 min with the borax-MEKC system described above. Previously reported analyses [40–42] require derivatisation and/or take several times as long.

The new method was used to follow the synthesis of the hydroperoxides of γ-linolenic acid (γ-HPOTE). Fig. 7 presents a baseline separation of all the six LOOHs expected. The acetonitrile concentration was increased from 2.4% to 5.7% (v/v) in this case to improve the selectivity.

Fig. 8 gives an impression of the efficiency of the borax buffer, with the separation of oleic, linoleic, and linolenic acids and all the LOOHs and LOHs derived from them. The detection limits for OE, ODE, and OTE and the products derived from them were determined according to the German DIN 32645 [43]. Depending upon the number of double bonds, the detection limits of the conjugated compounds ranged from 5 to 12 μmol/l; the non-conjugated isomers had limits of 10–30 μmol/l. The standard deviation of the migration times with the same capillary is less than 1% and with different capillaries under 5%. The somewhat higher standard deviation of the areas when an external standard is used, as much as 8%, is partly due to evaporation of

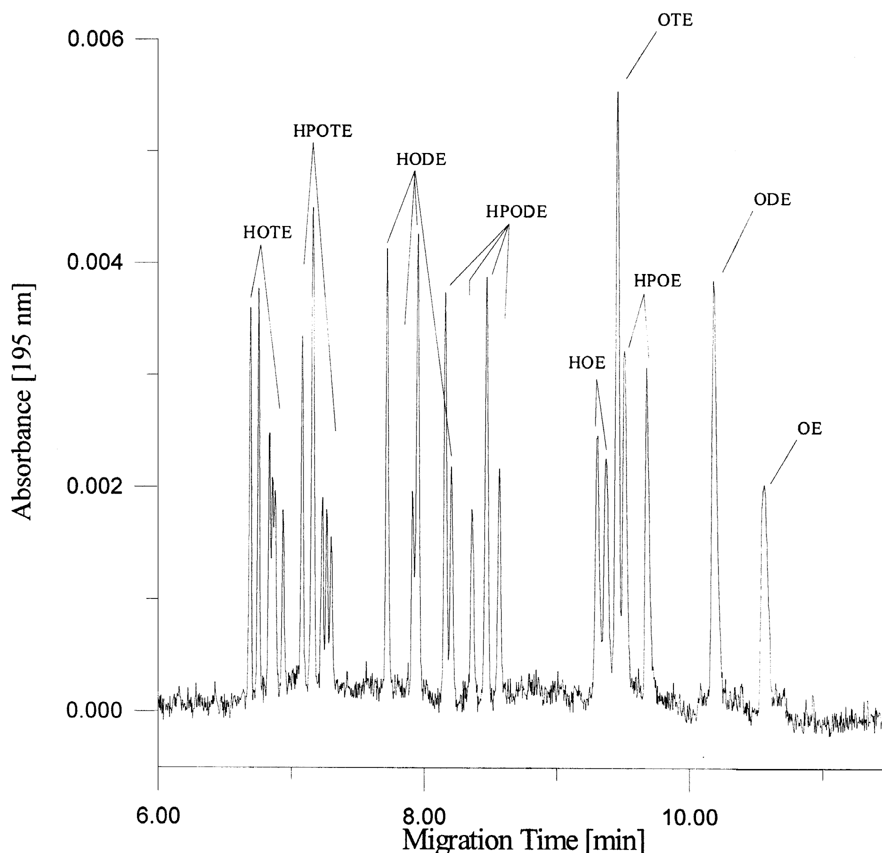

Fig. 8. Separation of oleic (OE; 0.25 m*M*), linoleic (ODE; 0.15 m*M*) and linolenic acids (OTE; 0.13 m*M*) and the hydroperoxides formed from them with singlet oxygen: HPOE (0.5 m*M*), HPODE (0.9 m*M*) and HPOTE (0.7 m*M*) and the corresponding hydroxy fatty acids obtained from these by reduction with sodium borohydride: HOE (0.5 m*M*), HODE (0.9 m*M*) and HOTE (0.7 m*M*); electrolyte: 10 m*M* borax buffer (pH 9.3), 15 m*M* SDS, 2.4% (v/v) ACN; fused-silica capillary 67 cm (effective length 60 cm); field strength 448 V/cm; detection UV-DAD at 195 nm.

the sample in the instrument. The use of C 22:6 as an internal standard can reduce this deviation significantly.

In a practical application, lecithin was oxidised with singlet oxygen, and the phospholipids were hydrolysed with lipase SP 525 to the free fatty acids and their oxidation products. Fig. 9 shows the typical pattern of fatty acids in egg lecithin and the hydroperoxides formed from them. Also to be seen are dihydroperoxy fatty acids [12], whose formation from the monohydroperoxy compounds was discussed as early as 1982 by Carless and Batten [44]; these compounds were also identified by comparison with authentic samples.

4. Conclusions

The MEKC method described here for the separation of unsaturated fatty acids, fatty acid hydroperoxides and hydroxy fatty acids is very selective and highly efficient. The low standard deviations and detection limits make it an excellent alternative to existing analyses by GC or HPLC.

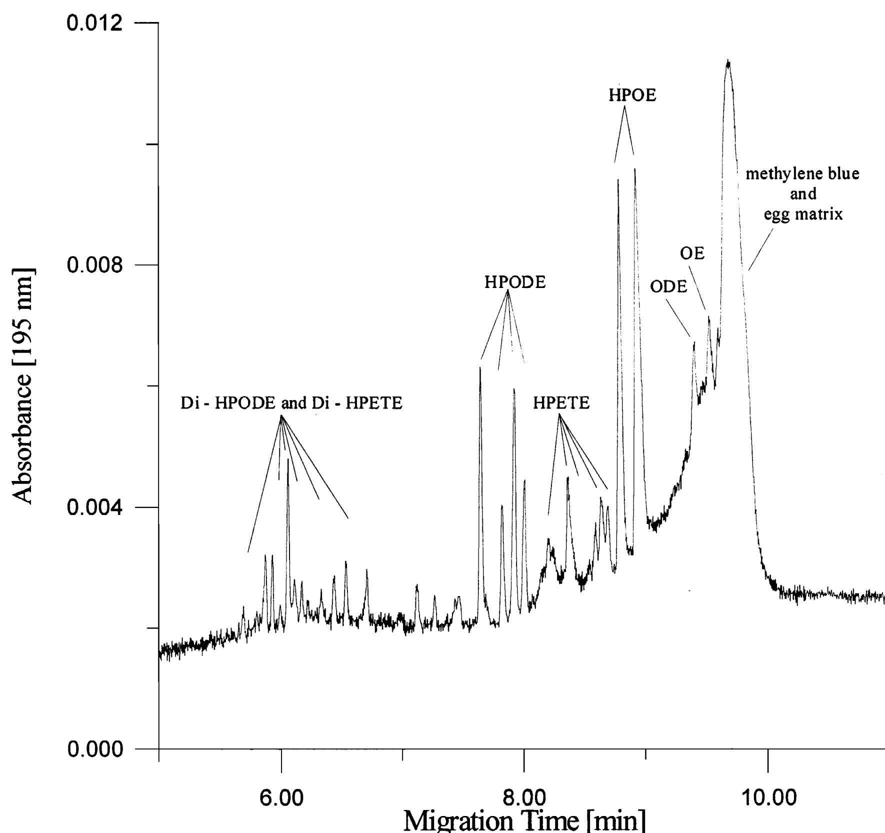

Fig. 9. Typical oxidation pattern of fatty acids contained in egg lecithin. Lecithin was treated with singlet oxygen in methanol for 73 h. The reaction mixture was hydrolysed enzymatically. OE (oleic acid), ODE (linoleic acid), ETE (arachidonic acid), the hydroperoxides formed from them: HPOE, HPODE, HPETE and the dihydroperoxy products di-HPODE and di-HPOTE. Electrolyte: 10 m*M* borax buffer (pH 9.3), 15 m*M* SDS, 2.4% (v/v) ACN; fused-silica capillary 67 cm (effective length 60 cm); field strength 448 V/cm; detection UV-DAD at 195 nm.

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