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Separation of isomeric hydroperoxides of unsaturated fatty acids by capillary electrophoresis

Oliver Schmitz, Siegmar Gäb*

Department of Analytical Chemistry, Bergische Universität-Gesamthochschule Wuppertal, Gauss-Strasse 20, 42119 Wuppertal, Germany

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Abstract

Nearly all the isomeric hydroperoxides from the reaction of oleic, linoleic and linolenic acids with singlet oxygen are separated in a single analysis by micellar electrokinetic chromatography with polyoxyethylene lauryl ether (Brij 35) as surfactant. Fatty acids tend to adsorb strongly onto the silica, so that coating of the capillary is essential. Brij 35 is used for dynamic coating of either octadecylsilane-derivatised or fused-silica capillaries. A diode array is used for the detection.

Keywords: Fatty acids; Hydroperoxides

1. Introduction

It is well established that autoxidation of the unsaturated fatty acids of lipids proceeds by a free radical chain reaction and leads to products including hydroperoxides [1,2]. Similar hydroperoxides result from the treatment of these fatty acids with singlet oxygen, although the isomeric composition of the product mixture is somewhat different [3,4]. The rancid aroma of old fats and oils is largely due to thermal degradation products of these lipid hydroperoxides [5]. The role of oxidised lipids in atherosclerosis has been discussed in recent years [6–8].

Gas chromatography and HPLC are often used to carry out the analysis of fatty acid hydroperoxides. GC requires derivatisation because the hydroperoxides themselves are destroyed in the injector [9,10]. Although normal-phase HPLC can lead to relatively good separation [11,12], conditioning the column is problematic. Reversed-phase HPLC allows the use of enzymatic reactions for post-column derivatisation, which increases sensitivity with fluorescence or chemiluminescence detection [13,14]. This method is very mild and can be carried out in a relatively short time, but it gives only a poor separation of the isomeric hydroperoxides of a particular fatty acid [11,13]. In the present work the isomeric hydroperoxide derivatives of oleic, linoleic and linolenic acids were separated by micellar electrokinetic chromatography (MEKC) and a UV-photodiode array was used to detect them.

2. Experimental

2.1. Apparatus

A Beckman P/ACE System 5510 (Munich, Germany) equipped with a photodiode array detector and Beckman System Gold V810 software was used for analysis. Samples were injected under pressure over 5 s. The fused-silica capillary was conditioned

*Corresponding author.

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before each run by rinsing for 1 min with 0.1 m*M* HCl and 5 min with the buffer to be used for the separation. The C_{18}-coated capillary was rinsed for 1 min with 0.1 m*M* NaOH and 5 min with buffer. The critical micellar concentration (CMC) of Brij 35 was determined with a Hitachi UV spectrophotometer Model 100 (Tokyo, Japan).

An uncoated fused-silica capillary [57 cm(50 cm to detector)×50 μm I.D.] from Laser 2000 (Wessling, Germany) and a C_{18}-coated capillary [CElect H250, 57 cm(50 cm to detector)×50 μm I.D.] from Supelco (Deisenhofen, Germany) were used for the separations, which were carried out at 25°C. The capillary outlet was the anode in all analytical runs.

2.2. Reagents and standards

All chemicals used were purchased from Merck (Darmstadt, Germany) and were of p.a. quality. Tridest water was used for the buffer solution, which was sterilised by filtration through a 0.45-μm cellulose-nitrate filter and degassed in an ultrasonic bath. Uncharged polyoxyethylene lauryl ether (Brij 35) was used as a surfactant and methanol and ethanol as organic modifiers.

The hydroperoxides were prepared by treatment of the fatty acids with singlet oxygen [3,4]. Methylene blue was used as photosensitiser and a halogen lamp ($\lambda_{max} \approx 500$ nm) for excitation. The hydroperoxide mixtures were purified of starting materials and by-products by chromatography on a cooled silica-gel column with hexane–diethyl ether–acetic acid (80:20:1, v/v/v) as eluent and were dissolved in methanol for standard solutions.

The hydroperoxides were characterised by ^{1}H- and ^{13}C{^{1}H}-NMR spectroscopy, the well-resolved signals of the olefinic carbon-atoms being indicative of the number of isomers present [15]. Fig. 1 shows the isomeric hydroperoxides from oleic, linoleic and linolenic acids expected in the oxidised fatty acid mixtures. In addition to these mixtures, 13*S*-hydroperoxyoctadeca-9(*Z*),11(*E*)-dienoic (**6**) and 13*S*-hydroperoxyoctadeca-9(*Z*),11(*E*),15(*Z*)-trienoic acids (**10**) were prepared by enzymatic oxidation of linoleic and linolenic acids [16,17] in order to define more closely two of the peaks observed.

2.3. CMC determination

To be certain of forming micelles, it was necessary to determine the CMC of Brij 35 in the buffer system used. Methods for determining the CMC include spectrophotometric measurements [18–21] as well as measurement of surface tension [22] or

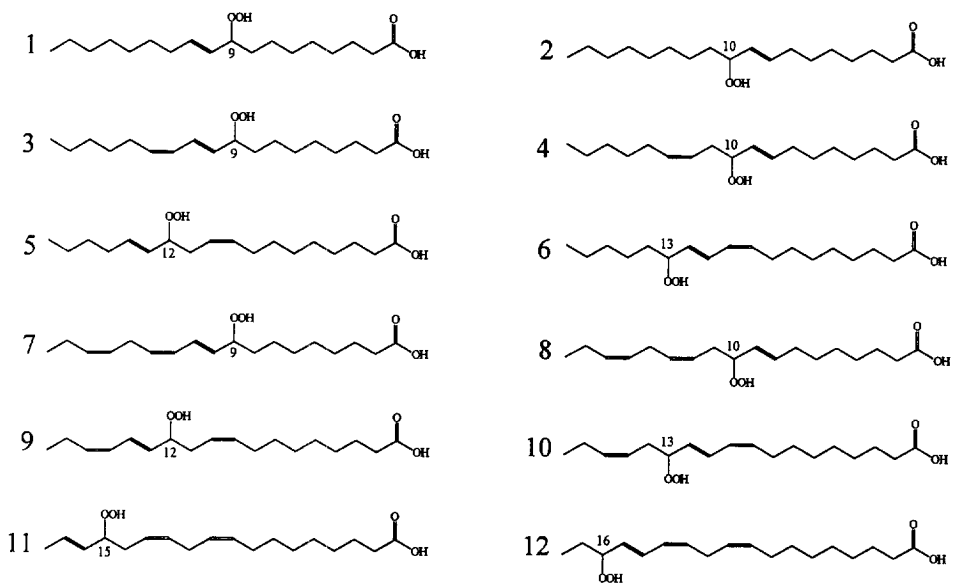

Fig. 1. Structures of the hydroperoxides from oleic (**1**, **2**), linoleic (**3**–**6**) and linolenic acids (**7**–**12**).

conductivity [20,23]. In this work the CMC of Brij 35 was determined by the measurement of the transmission of solutions of bromophenol blue (20 μ*M*) [21], whose interaction with the micelle is a function of the concentration of the surfactant. Spectra were made at a scan rate of 80 nm/min over the wavelength range 560 to 620 nm, and the transmission at λ=586 nm was plotted against the Brij 35 concentration. The concentration at which the transmission of bromophenol blue increased sharply was taken as the CMC of Brij 35. This spectrophotometric method yielded a CMC of approximately 0.25 g/l for both the methanolic and the ethanolic buffer systems used here.

3. Results and discussion

3.1. Uncoated fused-silica capillary

The best separation in the uncoated fused-silica capillary was achieved with a background electrolyte consisting of 1.1 g/l Brij 35 in a solution of 70% phosphate buffer (50 m*M*, pH 4.85) and 30% methanol. Brij 35 at concentrations over 100 mg/l in the buffer strongly suppressed the EOF, so that it was necessary to work with an anodic outlet to transport the acids in the desired direction. At a lower Brij 35 concentration the acids were transported by EOF to the cathode. A plausible mechanism for the suppression of the EOF is strong hydrogen bonding of the polyoxyethylene chains of Brij 35 to silanol groups, as has been proposed for polyoxyethylene itself [24]. The field strength was 526 V/cm. The mono-unsaturated hydroperoxides from oleic acid and the non-conjugated hydroperoxides from linoleic and linolenic acids were detected with the greatest sensitivity at 195 nm, the conjugated hydroperoxides from linoleic and linolenic acids at 234 nm. The detection limit for each isomer was about 90 μ*M*.

There was unfortunately a shift to shorter migration times from run to run under these analytical conditions. This shift decreased with time, however, and after approximately ten runs the migration times were relatively constant. Fig. 2 shows the migration times and the separation of the isomeric hydroperoxides from linolenic acid as a function of the run

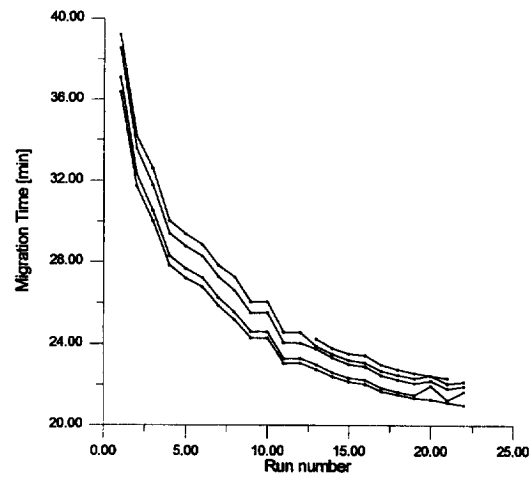

Fig. 2. Variation of the migration time of the isomeric hydroperoxides from linolenic acid in an uncoated fused-silica capillary.

number. All runs showed good separation of the conjugated isomers (**7**, **9**, **10**, **12**) from the non-conjugated (**8**, **11**). There were at least two peaks for the conjugated isomers from the first run on. Only after repeated analysis could three peaks be observed; hydroperoxide **10** and one other isomer remained unresolved. The electropherogram in Fig. 3 is run number 16, which was recorded at a wave-

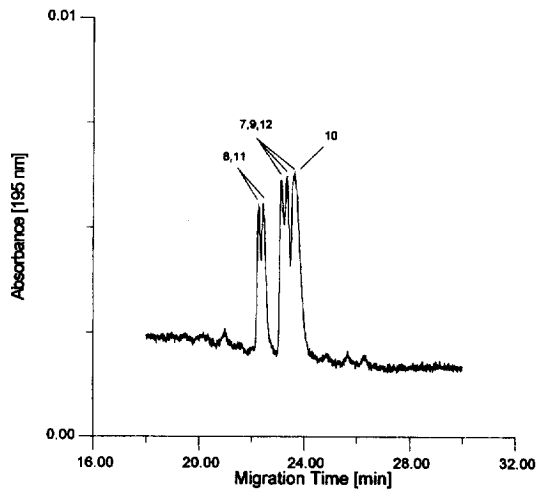

Fig. 3. Electropherogram showing the separation of the isomeric hydroperoxides from linolenic acid with a 70% potassium phosphate buffer (50 m*M*, pH 4.85), 30% methanol and 1.1 g/l Brij 35 in a 57 cm(50 cm to detector)×50 μm uncoated fused-silica capillary.

length of 195 nm. The pH of the buffer changed by about 0.2 units over twenty runs, but each time the buffer solution was replaced, either with fresh buffer or with fresh buffer corrected by 0.2 pH units, this cycle began again with the long migration times. These observations suggest that the dynamic coating of the capillary is influenced by changes in the buffer composition that can not be explained by the simple decrease in pH. The shift of the migration times with run number was observed at all pH values. The best resolution was obtained at pH 4.85. Varying the concentrations either of Brij 35 or methanol led to deterioration of the separation.

3.2. C_{18}-coated fused-silica capillary

It has previously been determined that non-ionic surfactants can be hydrophobically adsorbed onto an alkylsilane-derivatised surface to create a hydrophillic layer that will prevent adsorption of analyte [25]. Comparison of a number of Brij and Tween surfactants has shown that dynamic coating of the C_{18}-column with Brij 35 gives the best results [26].

In the C_{18}-coated fused-silica capillary the fatty acid hydroperoxides were separated most effectively with a background electrolyte consisting of 1.1 g/l Brij 35 in a solution of 70% phosphate buffer (60 m*M*, pH 6.30) and 30% ethanol rather than methanol. The outlet was anodic for these conditions as well; the field strength was 491 V/cm. With this background electrolyte reproducible migration times were achieved (R.S.D.=0.4%). Either varying the pH or changing the buffer concentration made the migration times less reproducible. With other Brij 35 concentrations the separation was poor. The longer migration times in the C_{18}-coated capillary are probably due to the use of ethanol rather than methanol, which leads to a significant increase in the viscosity of the buffer [27].

The detector wavelengths were the same as with the uncoated capillary. The detection limit for each isomer was about 190 μ*M*. This is in agreement with the observation of improved sensitivity with uncoated fused-silica capillaries [28,29]. Figs. 4 and 5 show the electropherograms of a mixture containing the hydroperoxides from oleic (1.8 m*M*), linoleic (3.9 m*M*) and linolenic acids (3.8 m*M*) as detected at 234 and 195 nm, respectively.

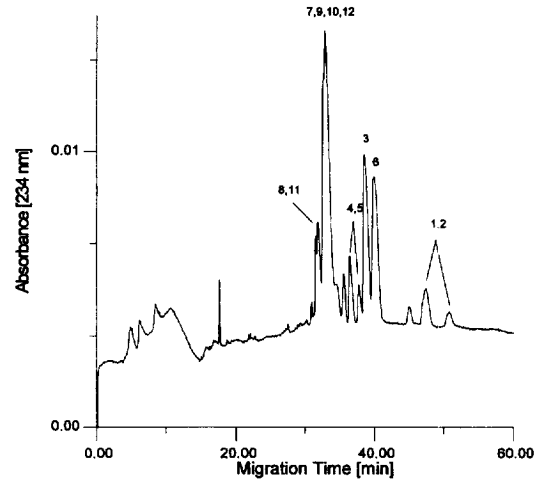

Fig. 4. Electropherogram showing the separation of the isomeric hydroperoxides from oleic, linoleic and linolenic acids with a 70% potassium phosphate buffer (60 m*M*, pH 6.30), 30% ethanol and 1.1 g/l Brij 35 in a 57 cm(50 cm to detector)×50 μm C_{18}-coated capillary, as detected at 234 nm.

4. Conclusion

The separation of fatty acid hydroperoxides by capillary electrophoresis has considerable advantages. Because this method is mild, it is not neces-

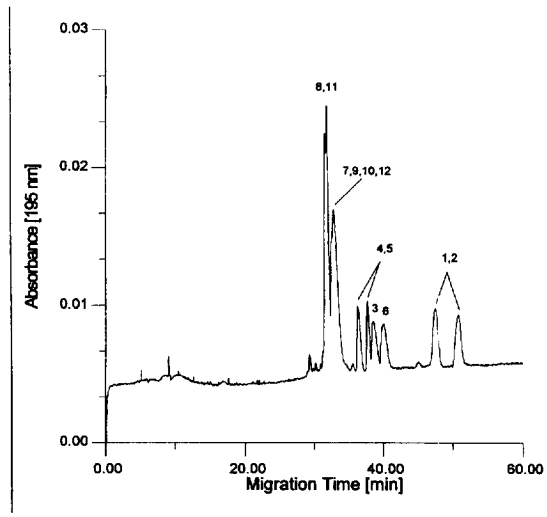

Fig. 5. Electropherogram showing the separation of the isomeric hydroperoxides from oleic, linoleic and linolenic acids with a 70% potassium phosphate buffer (60 m*M*, pH 6.30), 30% ethanol and 1.1 g/l Brij 35 in a 57 cm(50 cm to detector)×50 μm C_{18}-coated capillary, as detected at 195 nm.

sary to derivatise the hydroperoxides before analysis, and the separation is far better than with RP-HPLC. The use of polyoxyethylene lauryl ether makes it possible to separate nearly all the isomeric hydroperoxides from oleic, linoleic and linolenic acids. Reproducible migration times were achieved with a C_{18}-coated capillary, but the separation and the detection limit were better with uncoated fused-silica.

Acknowledgments

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