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Analysis of fatty acid epoxidation by high performance liquid chromatography coupled with evaporative light scattering detection and mass spectrometry

Cecilia Orellana-Coca, Dietlind Adlercreutz*, Maria M. Andersson, Bo Mattiasson, Rajni Hatti-Kaul

Department of Biotechnology, Center for Chemistry & Chemical Engineering, Lund University, Box 124, S-221 00 Lund, Sweden

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Abstract

Conventionally, epoxidation of unsaturated fatty acids has been studied either with titrimetric methods or in a lengthy procedure involving derivatization followed by gas chromatography (GC). We have developed a more rapid and descriptive analysis procedure for the substances using high performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD). Chemo-enzymatic epoxidation of unsaturated fatty acids (oleic, linoleic and linolenic acid, respectively) has been performed using hydrogen peroxide and immobilized lipase from *Candida antarctica* (Novozym 435). The fatty acids and their epoxidation products were separated by HPLC on a C-18 reversed-phase column using methanol–water containing 0.05% acetic acid as mobile phase. The method facilitated the simultaneous determination of fatty acids and epoxides differing from each other in the number of epoxide rings, the degree of unsaturation and the position of the epoxide rings and double bonds. An important aspect of the method development was the use of electrospray ionization and tandem mass spectrometry to confirm the structure of the epoxide products. It is suggested that the HPLC method, providing more information about the kind and concentration of fatty acids and their epoxides, represents a powerful complement to the existing methods for monitoring epoxidation processes on fatty acids.

Keywords: Epoxidation; Unsaturated fatty acids; HPLC analysis; Mass spectrometry; Lipase

1. Introduction

The utilization of oils and fats for the production of chemicals and materials that can act as a replacement for those derived from petroleum is becoming increasingly important (Biermann et al., 2000). Vegetable oils

* Corresponding author. Tel.: +46 46 222 7363; fax: +46 46 222 4713.
E-mail address: dietlind.adlercreutz@biotek.lu.se (D. Adlercreutz).

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and their unsaturated fatty acids have, for instance, been converted to epoxides (Klaas and Warwel, 1999; Ruesch gen. Klaas and Warwel, 1996) that are useful building blocks in organic synthesis as they participate in numerous reactions owing to their highly reactive oxirane ring. Oleochemical internal epoxides are industrially applied as PVC-stabilizers, plasticizers, as reactive diluents for paints and as intermediates for polyurethane–polyol production (Ramirez-de-Arellano-Aburto et al., 2002; Wilkes et al., 2004). Epoxidized vegetable oils and their derivatives are also suitable as cross-linkers in environmentally friendly coatings, where they serve as a substitute for triglycidyl isocyanurate, a putative mutagenic substance (Overeem et al., 1999).

Fatty epoxides are currently produced on an industrial scale by the Prileshajev epoxidation reaction in which a peracid is used for oxygen transfer to the double bonds (Swern, 1947). (Warwel and Klaas, 1995) have earlier described a selective epoxidation process, which involves the use of a lipase to catalyze the formation of peracid from H_2O_2 and the corresponding fatty acid. The reaction scheme for the lipase-mediated epoxidation of oleic acids is presented in Fig. 1.

A combination of two titrimetric methods is commonly employed to monitor the epoxidation of fatty acids and oils (Klaas and Warwel, 1999). These include the determination of iodine value, which is a measure of the number of double bonds (Wijs, 1929), and oxirane number, which is a measure of the amount of epoxide oxygen (Jay, 1964). The epoxidation process should go hand-in-hand with a decrease in the iodine value and an increase in the oxirane number. The degree of epoxidation can in principle be calculated from these two values. Generally, the quality of the epoxidized fatty acid or oil is better, the higher the oxirane number and the lower the iodine value (Carlson and Chang, 1985; Hang and Yang, 1999).

This analysis, however, is afflicted with an error, arising from the fact that the iodine value is typically strongly dependent on the method by which it is determined (Kyriakidis and Katsiloulis, 2000). Moreover, these titration methods are inadequate for providing information on the fatty acid composition and the degree of epoxidation of individual fatty acids, characteristics that can be expected to have a large impact on the applicability of the epoxide product (Klaas and Warwel, 1999; Overeem et al., 1999). In order to obtain more detailed information on the composition of epoxidized fatty acids and oils, these have been converted to methyl esters and then been analyzed by GC in combination with FID and/or mass spectrometric detection (Overeem et al., 1999; Ruesch gen. Klaas and Warwel, 1996; Warwel and Klaas, 1995). However, as samples are exposed to high temperatures, derivatization of the fatty epoxides cannot be excluded completely. Methyl esters of epoxidized fatty acids have also been analyzed by HPLC with evaporative light scattering detection (ELSD) using two symmetry C-18 reversed-phase columns connected in series and acetonitrile and water as the mobile phase (Piazza et al., 2003). HPLC-electron impact mass spectrometric (EI-MS) detection and atmospheric pressure chemical ionization mass spectrometric (APIC-MS) detection were used for characterization of the fatty epoxides.

In this report we present a simple HPLC method with ELSD and electrospray ionization (ESI) identification, which provides simultaneous analysis and structural identification of both the fatty acids and the corresponding epoxides, including the regio-isomers. In contrast to the other methods, derivatization of the free fatty acids prior to analysis was not required. External standards were used for calibration of the ELSD, and the synthesis and purification of these standards is also described. Finally, the method has been applied for monitoring the chemo-enzymatic process of epoxidation of fatty acids with different degrees of unsaturation.

2. Materials and methods

2.1. Materials

Candida antarctica lipase (Novozym®435) was a gift from Novozymes (Bagsvaerd, Denmark). Oleic acid, linoleic acid, linolenic acid, stearic acid, palmitic acid, heptadecanoic acid and epoxy stearic acid were purchased from Sigma–Aldrich (St. Louis, USA). Hydrogen peroxide (30%, w/w) in water, glacial acetic acid, toluene, sulphuric acid, ethanol, *para*-anisaldehyde, silica gel 60 (0.04–0.063 μm), TLC aluminium sheets, silica gel 60 F_{254}, heptane, methanol of HPLC grade and ethyl ether (SeccoSolv®) were procured from Merck (Darmstadt, Germany). The water used was purified with a Milli-Q system from Milli-

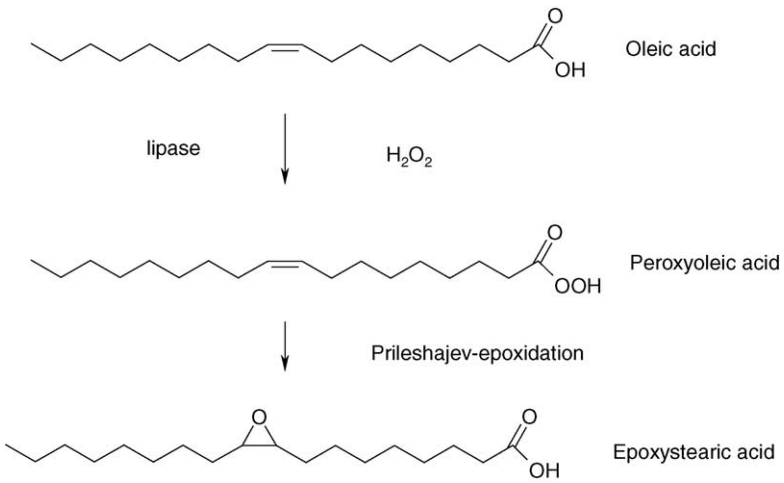

Fig. 1. Reaction scheme for the chemo-enzymatic epoxidation of oleic acid. Oleic acid is first converted by the lipase to yield the corresponding peracid, which then epoxidizes the double bond of oleic acid in a predominantly intermolecular reaction (Warwel and Klaas, 1995).

pore (Billerica, USA). All the reagents were of analytical grade.

2.2. HPLC analysis of fatty acids and epoxides

HPLC analyses were carried out on a LaChrom HPLC system, equipped with an L-2500 pump used in low gradient mode, an L-2400 automatic sample injector, a D-7000 Interface (all from Merck Hitachi) and an evaporative light scattering detector , model 500 with a laser diode light source from Alltech (Deerfield, IL). The ELSD was operated at 87 °C with a gas flow of 2.37 standard litres/min. Air was used as nebulizer gas. The fatty acids and their epoxidation products were separated on a Lichrospher RP-C18 column (5 μm, 250 mm × 4 mm) with an RP-C18 guard cartridge (5 μm, 4 mm × 4 mm) from Merck (Darmstadt, Germany). The injection volume was 20 μl. The mobile phase solvents were: solvent A, methanol–acetic acid (99.95:0.05, v/v) and solvent B, water–acetic acid (99.95:0.05, v/v). The gradient program employed was as follows: segment 1—isocratic 75% A, 25% B for 12 min; segment 2—linear gradient from initial conditions to 90% A; 10% B for 8 min; segment 3—linear gradient to 98% A; 2% B for 15 min; segment 4—linear gradient from 98% A; 2% B to initial condition 75% A; 25% B for 2 min; and segment 5—isocratic 75% A, 25% B for 3 min. The flow rate used was 1 ml/min at room temperature. The commercially available pure fatty acids were used as standards; the epoxide standards, except for epoxy stearic acid, had to be prepared since they are not commercially available in pure form.

2.3. Lipase-mediated epoxidation of fatty acids: monitoring by HPLC

The reaction medium consisted of a 0.5 M solution of fatty acid (oleic acid, linoleic acid and linolenic acid, respectively) in 5 ml of toluene (10 ml for linoleic acid) and 100 mg immobilized *C. antarctica* lipase. Hydrogen peroxide (30%, w/w) was added stepwise in order to prevent the enzyme from being inactivated. The rate of addition was adjusted for every fatty acid substrate (oleic acid: 30 μl every 15 min during 2.5 h; linoleic acid: 50 μl every 15 min during 6 h; and linolenic acid: 40 μl every 15 min during 5 h). The reaction mixtures were stirred with a magnetic stirrer bar. At appropriate time intervals (about every hour), the stirring was stopped and the two phases were allowed to separate for about 1 min. A 50 μl (or 100 μl) sample was then withdrawn from the organic phase with a syringe and diluted 1:100 (or 1:200) with methanol before being analyzed by HPLC. Once the sample was withdrawn, no further conversion occurred, since the enzyme, being immobilized on a solid support, remained in the reaction vessel.

2.4. Preparation of epoxide standards

The fatty acid epoxides, except epoxystearic acid, were prepared by chemo-enzymatic epoxidation of the fatty acids using the reaction conditions described above. The reaction was terminated after 21.8 and 10.2 h for linoleic and linolenic acid, respectively. Diethyl ether (30 ml) was added to the reaction mixture, and the enzyme preparation was filtered off to terminate the reaction. If necessary, the reaction mixture was stored at −20 °C before further processing the next working day. The organic layer, containing the epoxidized fatty acids, was washed with water to remove traces of H_2O_2, and concentrated in a rotary evaporator. The oily residue obtained after evaporation, consisting of the epoxides and possibly traces of solvent and water, was then purified by flash chromatography. The purity and identity of the epoxides were confirmed by thin layer chromatography (TLC) and electrospray MS.

2.5. Flash chromatography

The flash chromatography equipment, fabricated in-house at the glass workshop of the Engineering Faculty of Lund, consisted of a glass column (290 mm × 20 mm), a 500 ml solvent reservoir and a flow controller valve. Air, supplied at a pressure of 0.2 bar, was used to force the eluent through the column. Thirty grams of silica gel 60 (0.04–0.063 μm) was suspended in the eluent and poured into the column to a height of 200 mm. The concentrated epoxide sample (300 mg) obtained above was applied on top of the silica column, and the sample components were then eluted from the sorbent with the mobile phase at a flow rate of approximately 17 ml/min. The eluent consisted of heptane–diethyl ether–acetic acid 75:25:1 (v/v/v) to elute the mono-epoxidation products from linoleic and linolenic acid, respectively. The di-epoxidation products (di-epoxy octadecanoic acid and di-epoxy octadecenoic acid, respectively) were eluted with heptane–diethyl ether–acetic acid 50:50:1 (v/v/v), and tri-epoxy octadecanoic acid was eluted with a mixture of heptane–diethyl ether–acetic acid 40:60:1 (v/v/v). The effluent from the column was collected in 10 ml fractions. From these fractions, samples of about 5 μl were withdrawn for analysis by TLC. The fractions showing a single band on the TLC plate were pooled, dried by evaporation and analyzed by MS. Di-epoxy octadecanoic acid was further purified by crystallization to yield two diastereomers.

2.6. Thin layer chromatography analysis

TLC of the fractions obtained from flash chromatography was done on aluminium sheets coated with silica gel 60 F_{254}. The samples were spotted on the sheets using a 5 μl micro glass pipette and eluted with a mobile phase system comprising heptane, ethyl ether and acetic acid (50:50:1). After elution, the sheets were sprayed with *para*-anisaldehyde reagent (ethanol/sulphuric acid/*para*-anisaldehyde/acetic acid (338:12.5:9.2:3.75, v/v/v/v), dried and heated at 105 °C. TLC provided separation of fatty acids and their mono-, di- and tri-epoxides, with the R_f values being 0.5, 0.4, 0.2 and 0.1, respectively. Saturated and unsaturated fatty acids containing the same number of epoxy groups were not distinguished.

2.7. Acquisition of MS and MS/MS data

All the experiments were conducted on a hybrid QSTAR Pulsar quadrupole TOF mass spectrometer (Applied Biosystems Sciex Instruments, Toronto, Canada) equipped with an electrospray ionization source. Initial separation was achieved on a Perkin Elmer Series 200 capillary LC system. Samples were prepared by dissolving the products from each reaction in methanol and filtering through 0.2 μm GHP membrane filters from Gelman Laboratory, supplied by Merck (Darmstadt, Germany). Samples of 10 μl were injected and separated on a Lichrospher RP-C18 column at a flow rate of 1 ml/min, using the same separation parameters as described in Section 2.2 except that formic acid (0.05%) was used in the mobile phase instead of acetic acid. Prior to entering the mass spectrometer, the mobile phase flow was split (1:10) with a Valco valve placed on the instrument.

MS and MS/MS spectra were recorded in the mass range of *m/z* 50–400 amu with the electroionspray operating at 350 °C and using negative mode (despite the acidic pH of the eluent phase) with an electrospray voltage of −4400 V and the Q1 resolution set to LOW. Nitrogen was used as curtain gas at 20.0 psi and as nebulizing and drying gas at 20 and 35 psi, respectively. Optimized compound parameters proved to

be: declustering potential, −65 V; focusing potential, −245 V and collision energy, −40 eV. Argon with a purity of 99.996% was used as the collision gas. Precursor ions were chosen with $m/z > 250$ and exceeding 10 counts. Data were acquired and processed with Analyst™ QS software.

2.8. Melting point determination

The melting points of the two diastereomers 9-10, 12-13 diepoxy octadecanoic acid were measured in a melting point device from KIFA (Reichert, Austria).

3. Results and discussion

3.1. HPLC analysis

Vegetable oils contain a number of saturated and unsaturated fatty acids, the concentrations of which vary depending on the plant source. It is only the unsaturated fatty acids that undergo epoxidation. The epoxide products formed may differ in the number of epoxy groups, the degree of unsaturation and in the position of epoxy groups and double bonds. Hence, a method that can be employed for simultaneous analysis for the variety of components present in a reaction mixture would provide valuable information on the epoxidation process.

A prerequisite for evaluating the scope and sensitivity of the analytical method was the preparation of standard compounds due to their lack of availability from commercial sources. The epoxide standards were produced by chemo-enzymatic epoxidation, since this method is known to give fewer unwanted by-products than any other method (Klaas and Warwel, 1999).

All the fatty acids and their various epoxidation products, including regio-isomers, were separated using HPLC on a reverse phase column and detected by an ELSD (Fig. 2). The saturated fatty acids, palmitic (C16:0) and stearic (C18:0) acid were eluted after 30.7 and 34.2 min, respectively. Heptadecanoic acid (C17:0), eluting between palmitic and stearic acid, is not found in nature and might thus serve as an internal standard. The unsaturated fatty acids, oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acid eluted after 30.7, 28.1 and 26.0 min, respectively. Palmitic and oleic acid thus co-eluted and their separation based on retention time was not possible. But the compounds can be distinguished by MS and MS/MS. Table 1 gives the retention times for the epoxide products along with the MS and tandem MS data used for the identification of the epoxides.

As expected, the polarity of the compounds had a major influence on their retention time. The unsaturated fatty acids eluted before saturated ones with the same number of epoxy groups, while highly epoxidized fatty acids eluted before those containing fewer numbers of epoxy groups. The retention time was also influenced by the position of the double bond and epoxy group, though to a minor extent; regio-isomers thus eluted close to each other.

Peracids, the reactive intermediates in the lipase-mediated epoxidation of fatty acids, comprising <2% of the total fatty acids (Warwel and Klaas, 1995), were not detected in the concentration range investigated.

3.2. Identification of the products

Products formed upon partial epoxidation of linoleic and linolenic acid (e.g., mono-epoxy octadecanoic acid, mono-epoxy octadidecenoic acid and di-epoxy octadecenoic acid) exhibit different regio-isomeric forms, hard to distinguish by ELSD alone. The identities of these products were further confirmed by mass spectrometric and tandem mass spectrometric detection. The ion source was operated in the negative mode to produce $[M-H]^-$ precursor ions, which are prone to charge-remote fragmentation (CRF) (Cheng and Gross, 2000). CRF of fatty acids occurs as H_2 elimination along the acyl chain to give both an alkene (invisible in the mass spectrum) and a charged species possessing the carboxylic group (Cheng et al., 1998). Accordingly, saturated fatty acids fragment into an array of product ions being separated by 14 mass units (57, 71, 85, etc.). Interruption of the alkyl chain by double bonds or epoxy groups causes a deviation from that peak pattern. Moreover, the specific fragment obtained after the interruption provides information on the kind of substitution. ESI–MS/MS spectra obtained upon CRF are usually straightforward to interpret and have been widely used for structural determination of fatty acids and related compounds (Hsu and Turk, 2004; Perret et al., 2004). In contrast, positively charged ions fragment in a more complex manner, which makes structural information more difficult to extract (Cheng and Gross, 2000).

Table 1
Retention time of fatty acid epoxidation products and the MS and MS/MS data used for their identification

Fatty acid substrate	Retention time (ELSD) of epoxidation product(s)	Precursor ion $[M-H]^-$	No. of epoxy groups	Major fragments $m/z > 155$	Epoxidation product
C18:1	23.6	297	1	171	C18:1/1(9)
C18:2	10.4	311	2	171, 199	C18:2/2(9,12)
	21.2	295	1	195	C18:2/1(12)
	21.5	295	1	171	C18:2/1(9)
C18:3	4.2	325	3	171, 199	C18:3/3(9,12,15)
	6.3	309	2	171, 185, 251	C18:3/2 (9,15)
	8.1	309	2	171, 199, 211	C18:3/2 (9,12)
	8.5	309	2	183, 195, 211, 223	C18:3/2(12,15)
	18.1	293	1	171	C18:3/1(9)
	18.5	293	1	235	C18:3/1(15)
	19.0	293	1	183,195, 211, 223	C18:3/1(12)

Abbreviations for the epoxidation products are the same as in Fig. 2, with the position of the epoxy groups being given in parentheses.

The total ions chromatogram (TIC) from an HPLC–ESI–MS/MS experiment involving the epoxidation products of linolenic acid is shown in Fig. 3. Peaks around 18–19 min corresponded to precursor ions with a mass to charge ratio (*m*/*z*) of 293 amu, which is 16 units more than the *m*/*z* of the linolenic acid precursor ion. It can thus be concluded that these peaks originate from mono-epoxy octadidecenoic regio-isomers. The peaks around 9 min originate from the precursor ion with an *m*/*z* of 309 amu, thus repre-

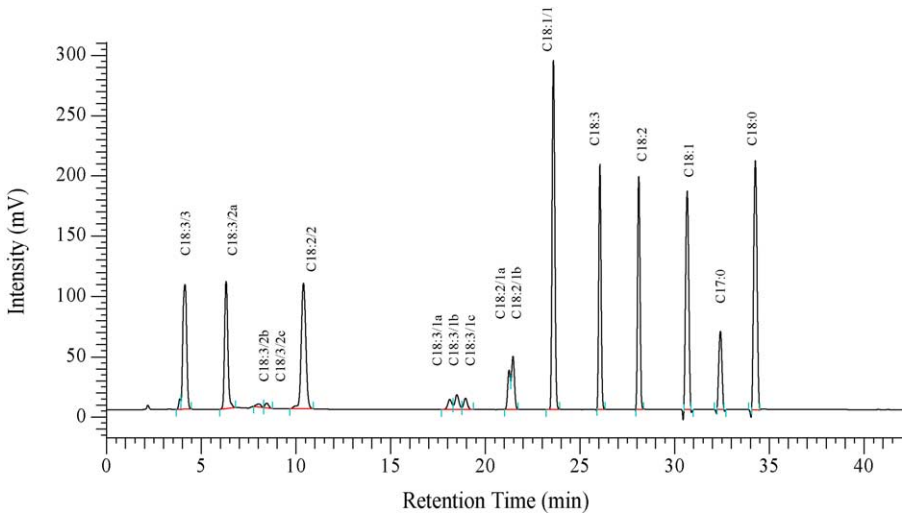

Fig. 2. HPLC chromatogram of fatty acids and fatty acids epoxides, obtained by chemo-enzymatic epoxidation of oleic, linoleic and linolenic acid, respectively. Mobile phase: methanol/water containing 0.05% acetic acid, as described in Section 2.2, flow rate 1 ml/min, temperature ambient, injection volume 20 μl. Peaks corresponding to commercial external standards: epoxy stearic acid (C18:1/1), linolenic acid (C18:3), linoleic acid (C18:2), oleic acid (C18:1), heptadecanoic acid (C17:0) and stearic acid (C18:0). Peaks corresponding to epoxy fatty acids, prepared by chemo-enzymatic epoxidation and identified by HPLC tandem mass spectrometry: 9-10, 12-13, 15-16 tri-epoxy octadecanoic acid (C18:3/3); 9-10, 15-16 di-epoxy 12 octadecenoic acid (C18:3/2a); 9-10, 12-13 di-epoxy 15 octadecenoic acid (C18:3/2b); 12-13, 15-16 di-epoxy 9 octadecenoic acid (C18:3/2c); 9-10, 12-13 di-epoxy octadecanoic acid (C18:2/2); 9-10 mono-epoxy, 12, 15 octadidecenoic acid (C18:3/1a), 15-16 mono-epoxy 9, 12 octadidecenoic acid (C18:3/1b), C18:3/1 c corresponds to 12-13 mono-epoxy 9,15 octadidecenoic acid; C18:2/1a: 12-13 mono-epoxy 9 octadecenoic acid and C18:2/1b: 9-10 mono-epoxy 12 octadecenoic acid. The concentration of the analytes was between 0.25 mM (di-epoxy octadecanoic acid) and 2.4 mM (stearic acid).

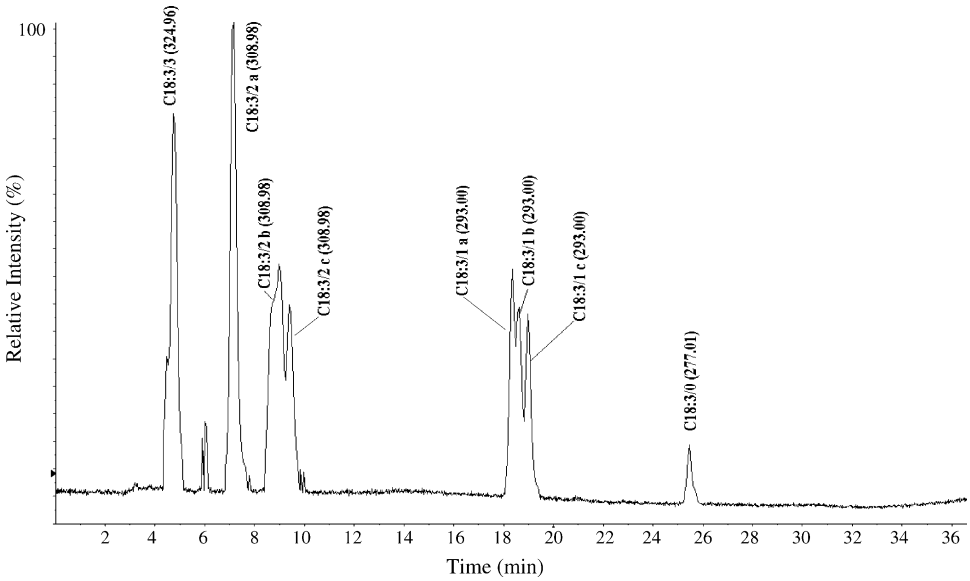

Fig. 3. Total ion chromatogram of the epoxidation products of linolenic acid where each peak is represented with negatively charged molecular ion $[M-H]^{-1}$. The three peaks with *m/z* 293 amu represent the mono-epoxy octadidecenoic acid regio-isomers, the peaks 309 represent the di-epoxy octadecenoic acid regio-isomers, and peak 325 represents 9-10, 12-13, 15-16 tri-epoxy octadecanoic acid. The experimental details are provided in the text.

senting the di-epoxy octadecenoic acid regio-isomers while the peak around 4 min, with an *m/z* of 315 amu represents 9-10, 12-13, 15-16 tri epoxy octadecanoic acid.

Fig. 4a–c shows the ESI–MS/MS spectra of the three regio-isomers of mono-epoxy octadidecenoic acid, having retention times on HPLC of 18.3, 18.6 and 19.0 min, respectively. As expected, it was found that fragments comprising the first nine carbon atoms ($m/z \leq 155$ amu; e.g., 71, 97 and 127 amu) were not useful for structural elucidation. Larger fragments, on the other hand, contained either oxygen or double bonds and could thus be used to differentiate between different regio-isomers. The peak with *m/z* of 171 amu for instance was characteristic for the epoxy group being between C9 and C10. It was thus concluded that ESI–MS/MS spectra recorded at 18.3 min originate from 9-10 mono-epoxy 12, 15 octadidecenoic acid (18:3/1a; Fig. 4a). The peak with *m/z* of 235 amu, on the other hand, was characteristic for a fragment containing two double bonds as shown in the MS/MS spectra of 15-16 mono-epoxy 9, 12 octadidecenoic acid (18:3/1 b; Fig. 4b). Peaks with *m/z* of 183 and 195 amu were found in epoxidation products with a double bond between C9 and C10 and peaks with *m/z* of 211 and 223 amu were characteristic for fragments containing oxygen and are shown in the MS/MS pattern of 12-13 mono-epoxy 9,15 octadidecenoic acid (C18:3/1 c; Fig. 4c). Spectra obtained with other epoxy fatty acids were interpreted in a similar way. The ESI–MS and ESI–MS/MS data used for the determination of their molecular structure are given in Table 1.

3.3. Calibration of the ELSD

Standards for the epoxides of linoleic and linolenic acid were prepared by chemo-enzymatic epoxidation and purified by flash chromatography. Purification of the reaction mixture allowed a good resolution of mono-, di- and tri-epoxides, but unfortunately not good resolution of the regio-isomers. To solve this problem, it was assumed that regio-isomers give the same response. The validity of this assumption is supported by several studies, showing that the response of the ELSD is the same for structurally similar analytes; for a review see (Young and Dolan, 2004). The calibration curves for each fatty acid and epoxide were made with 10 concentrations in a range of 0.5–5 mM. For

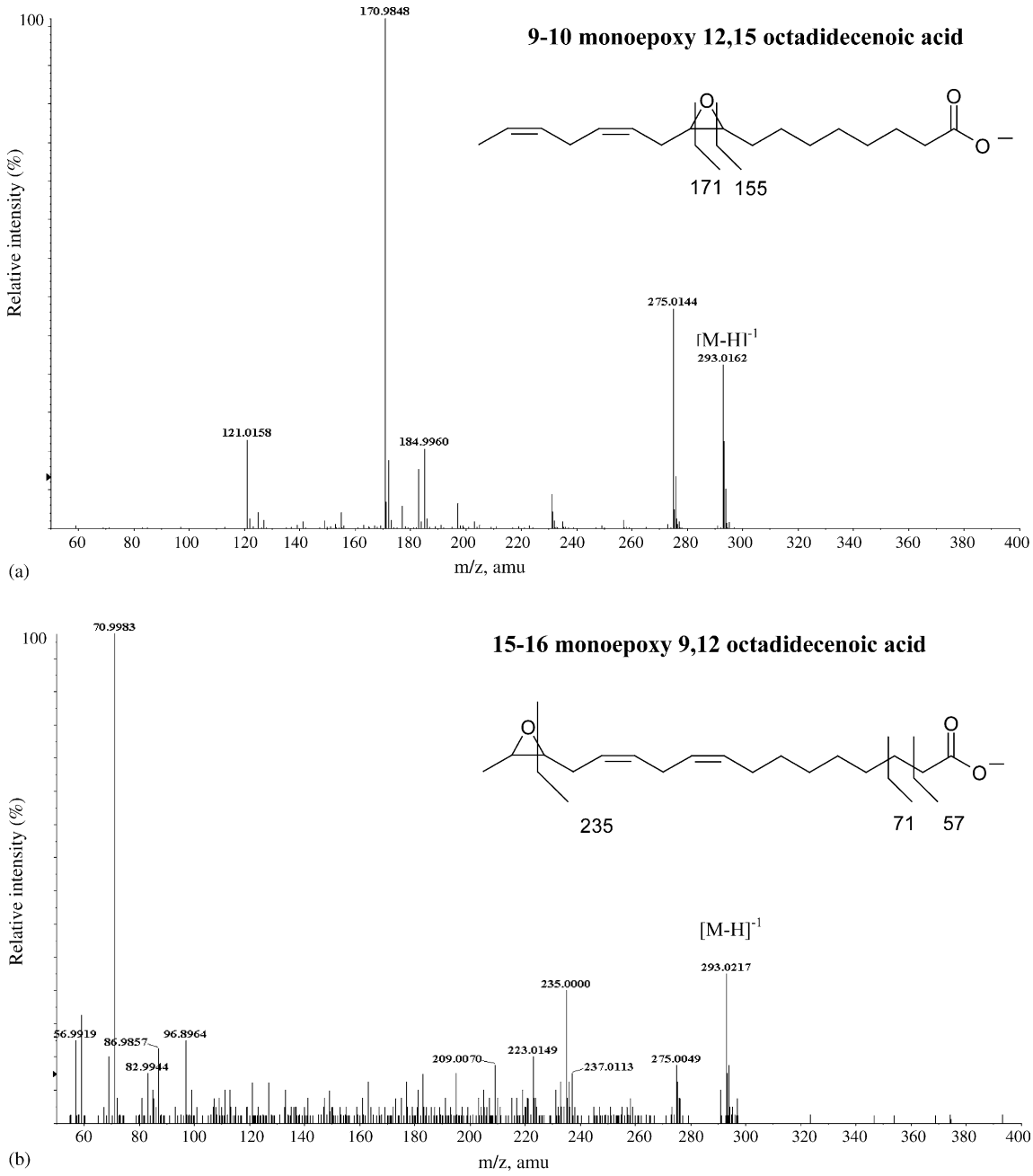

Fig. 4. MS/MS spectrum of epoxidized products of linolenic acid: (a) 9-10 monoepoxy 12, 15 octadidecenoic acid, (b) 15 monoepoxy 9,12 octadidecenoic acid and (c) 12-13 monoepoxy 9,15 octadidecenoic acid. The experimental details are provided in the text.

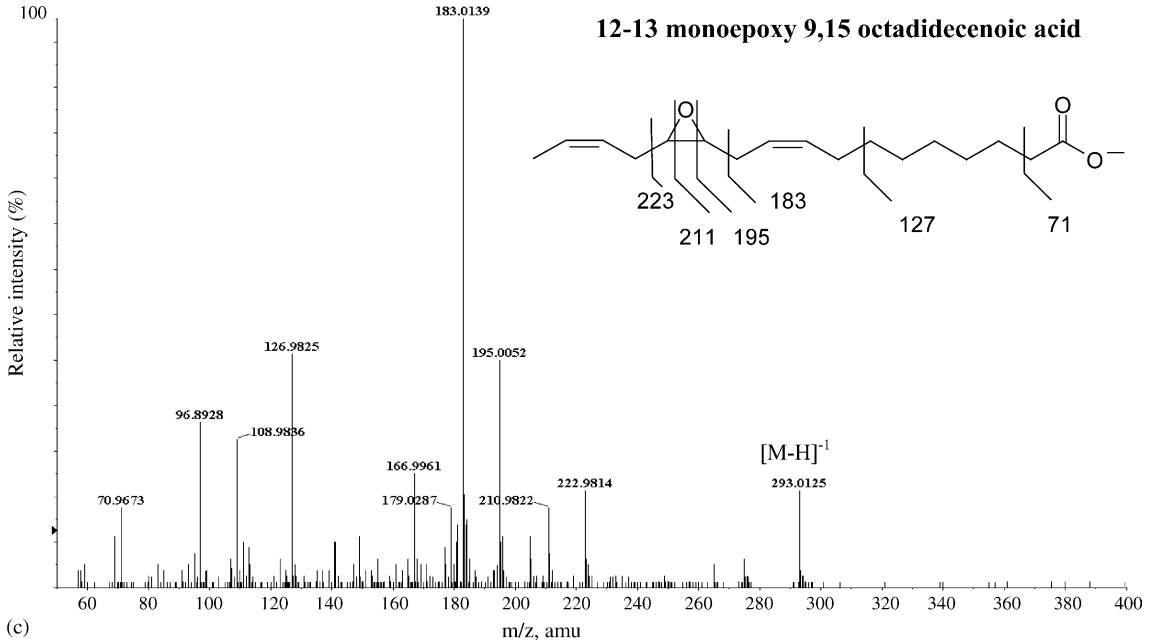

Fig. 4. (*Continued*).

regio-isomers, the total area covered by the peaks of the isomer products was taken into consideration. The detector response could be described by a quadratic equation. The non-linear response is quite typical for the ELSD (Heron et al., 2004). The equation for each of the substances and the corresponding correlation coefficients are shown in Table 2. The detection limits, defined as three times the noise levels, are also given in Table 2.

3.4. Monitoring of epoxidation reactions by HPLC

The applicability of the HPLC method was then evaluated for monitoring the lipase-mediated epoxidation of unsaturated fatty acids, by using the ELSD. The progress of the epoxidation of oleic acid is shown in Fig. 5. The reaction yielded epoxy stearic acid as the only product and was complete within 10 h. The epoxidation of linoleic acid yielded first mono-

Table 2
Standard curve constants from the calibration curve

Compound	a_0	a_1	a_2	R	Detection limit (μg)
C18:3/3	2.076E−1	1.286E−6	−6.445E−14	0.997	2.1
C18:3/2	3.743E	2.318E	−1.162E−13	0.987	2.9
C18:2/2	2.555E	8.807E	−3.254E−14	0.995	2.8
C18:3/1	5.423E	9.908E	−2.005E−14	0.996	2.3
C18:2/1	4.172E	5.627E	−1.958E−5	0.997	4.1
C18:1/1	1.914E	5.044E	−2.059E−15	0.997	1.9
C18:3	3.750E	8.044E	−2.542E−14	0.993	3.9
C18:2	3.109E	6.734E	−1.637E−14	0.993	3.4
C18:1	3.782E	6.164E	−1.587E−14	0.989	3.1
C18:0	5.054E	5.304E	−1.303E−14	0.997	1.2

The data were fitted to a quadratic equation $C = a_0 + a_1X + a_2X^2$, where C represents the concentration measured in millimolar, a_0, a_1 and a_2 are constants for the equations and X is the peak area.

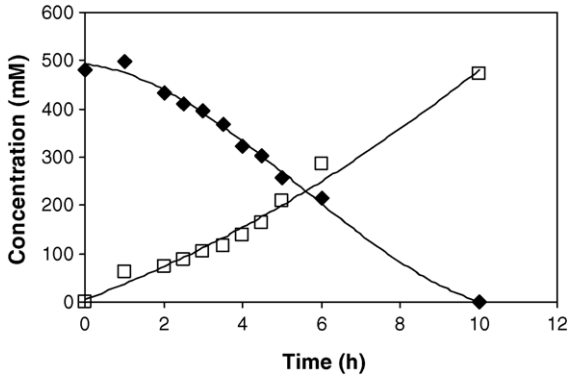

Fig. 5. Progress of the chemo-enzymatic epoxidation of oleic acid monitored by HPLC. The reaction mixture consisted of 5 ml of a 0.5 M solution of oleic acid in toluene and 100 mg of Novozym 435, to which hydrogen peroxide (30%, w/w) was added in 30 μl aliquots every 15 min during 2.5 h. Samples of 50 μl were withdrawn and diluted 200 times in methanol. (♦) Oeic acid and (□) epoxy stearic acid.

epoxy octadecenoic acid, which was then further converted to di-epoxy octadecanoic acid (Fig. 6). Di-epoxy octadecanoic acid was the major product (almost 60%) after 22 h reaction time. The HPLC chromatogram revealed that the mono-epoxide of linoleic acid consisted of two regio-isomers, one with the epoxy group positioned between C9 and C10 and another with the epoxy group positioned between C12 and

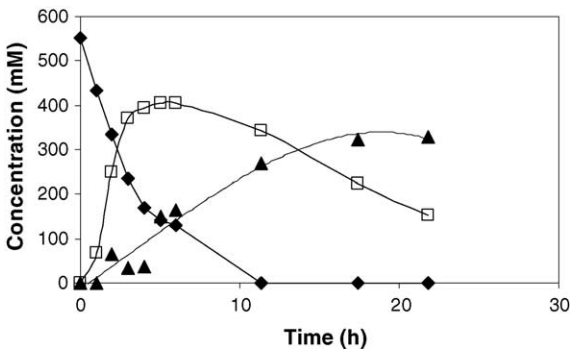

Fig. 6. Progress of the chemo-enzymatic epoxidation of linoleic acid monitored by HPLC. The reaction mixture consisted of 10 ml of a 0.5 M solution of linoleic acid in toluene and 100 mg of Novozym 435, to which hydrogen peroxide (30%, w/w) was added in 50 μl aliquots every 15 min during 6 h. (♦) Linoleic acid, (□) monoepoxy octadecenoic acid and (▲) diepoxy octadecanoic acid. Isomers are not distinguished. Information concerning the isomers is provided in the text.

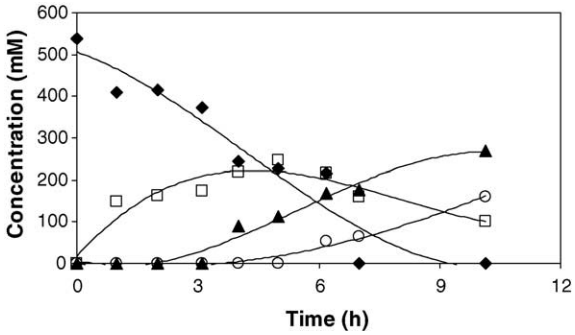

Fig. 7. Progress of the chemo-enzymatic epoxidation of linolenic acid monitored by HPLC. The reaction mixture consisted of 5 ml of a 0.5 M solution of linolenic acid in toluene and 102 mg of Novozym 435, to which hydrogen peroxide (30%, w/w) was added in 40 μl aliquots every 15 min during 5.25 h. Samples of 100 μl were withdrawn every hour and diluted 100 times in methanol. (♦) Linolenic acid, (□) monoepoxy octadidecenoic acid, (▲) diepoxy octadecenoic acid and (◯) triepoxy ocatadecanoic acid. Isomers are not distinguished. Information concerning the isomers is provided in the text.

C13. There was no preference for the formation of either isomer. Also di-epoxy octadecanoic acid eluted in a double peak. The two peaks were found to be two diastereomers, one with a melting point of 78 °C and another with a melting point of 36 °C. The isomer with a melting point of 78 °C was preferentially formed.

The progress of the epoxidation of linolenic acid is shown in Fig. 7. The reaction yielded first mono-epoxy octadidecenoic acid (three regio-isomers), then di-epoxy octadecenoic acid (three regio-isomers) and finally tri-epoxy octadecanoic acid. The final product contained about 30% of tri-epoxy octadecanoic acid, ∼50% di-epoxy octadecenoic acid and ∼20% of mono-epoxy octadidecenoic acid (Fig. 7). The HPLC chromatograms revealed that in linolenic acid the positions 9-10 and 15-16 were more easily epoxidized than position 12-13, as seen in both the mono- and di-epoxy product intensities. The total concentration of the di-epoxy octadecenoic acid formed was constituted by 75% 9-10, 15-16 di-epoxy 12-octadecenoic acid, 18% of 9-10, 12-13 di-epoxy 15-octadecenoic acid and 7% of 12-13, 15-16 di-epoxy 9-octadecenoic acid. Interestingly, a similar observation has been made in a study dealing with peroxygenase-catalyzed epoxidation of unsaturated fatty acids (Piazza et al., 2003).

4. Conclusion

The study shows clearly that HPLC in combination with ELSD and ESI–MS/MS detection provides a great deal of information on the components formed during the epoxidation of fatty acids found in vegetable oils. The method is applicable for monitoring of epoxidation processes on fatty acids in general and can also be used for analyzing epoxidized vegetable oils of different origin. Studies on the effect of the molecular composition of the epoxidized products on their applicability in surface coatings are currently under progress.

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