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Journal of Chromatography A, 925 (2001) 223–240

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Optimized thiol derivatizing reagent for the mass spectral analysis of disubstituted epoxy fatty acids

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Received 21 February 2001; received in revised form 18 May 2001; accepted 22 May 2001

Abstract

A novel procedure is described for the derivatization of fatty acid epoxides in the presence of their corresponding diols. The acidic character of 2,3,5,6-tetrafluorobenzenethiol promotes favorable mass fragmentation of linoleate and arachidonate derived epoxide derivatives and reduces alkene isomerization to a manageable side reaction, eliminated through the addition of a thiol scavenger. After silylation, regioisomeric mixtures of epoxy- and dihydroxylipids are simultaneously detected and discriminated using gas chromatography with electron impact mass spectral detection. Silylated hydroxysulfanyloctadecanoids yielded instrumental detection limits of 5 pg/μl, sufficient sensitivity for the quantification of endogenous epoxylipids. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Derivatization, GC; Fatty acids; Lipids; Epoxides; Diols; Tetrafluorobenzenethiol

1. Introduction

In vertebrates, invertebrates and plants, fatty acid epoxides and diols are important bioactive compounds. Epoxylipids are produced by either enzymatic [1–6] or autooxidative transformations of unsaturated lipids [7,8]. Hydrolysis of these epoxides under acidic conditions or by enzymatic transformation yields vicinal 1,2-diols. In the plant kingdom, numerous examples of epoxide-containing lipids are known [6] resulting from either alkene epoxidation [9]or internal cyclization of hydroperoxides to yield epoxyhydroxy fatty acids [10]. Some of these compounds play an important role in the host response to pathogen infiltration [9,11]. In the insect realm, the juvenoid hormones are epoxylipids that play essential roles in development and reproduction [12]. In addition, numerous aliphatic epoxides are sex attractant pheromones in lepidopterans [13]. In vertebrate physiology, arachidonic acid epoxides or epoxyeicosatrienoic acids (EpETrEs) affect K^{+} and Ca^{2+} channel activity, vascular tone, hormone secretion, gene transcription, mitogenisis [1] and blood pressure regulation [14]. Complicating matters, the four EpETrE regioisomers differentially stimulate responses in different target tissues, and their corresponding diols (DHETrEs) are also bioactive. While receiving less attention, the linoleate derived epoxides or epoxyoctadecenoic acids (EpOMEs) and dihydroxyoctadecenoic acids (DHOMEs), also per-

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PII: S0021-9673(01)00998-0

turb vascular permeability, renal ion balance [15] and mitochondrial function [16].

Simplification of epoxide and diol analytical methods will accelerate and expand research into these important second messengers. A number of approaches to epoxylipid identification and quantification exist. While high-performance liquid chromatography (HPLC) can resolve many epoxy fatty acids [3,17,18], complete resolution of the EpETrEs from impurities requires the use of both normal- and reversed-phase systems [2]. Advances in atmospheric pressure ionization mass spectrometry (MS) hold the potential for routine quantitative HPLC–MS–MS analyses of epoxide containing lipids [19], however, these procedures will come with a high price in instrumentation.

Classically, free fatty acids are esterified before analysis. The direct analysis of epoxylipid methyl esters using gas chromatography (GC)–MS with electron impact ionization can yield quantitative and structural information [7,20], however the low abundance of characteristic ions results in poor analytical sensitivity [13,20,21]. Alternatively, 3-pyridinylmethyl esters can improve structural information, however sensitivity and chromatographic resolution is limiting [22]. The preparation of pentafluorobenzyl esters with negative chemical ionization GC–MS detection enhances sensitivity, however structural information and chromatographic separation are sacrificed and isomer separation by HPLC is a prerequisite for quantification [2,4]. Similarly, fluorescent esters have dramatically improved the sensitivity of HPLC quantification, but EpETrE regioisomers are only partially resolved and product identification depends on retention time information alone [18].

The epoxide moiety can also be the target for derivatization (Fig. 1). Treating epoxides with trimethylchlorosilane yields silylated chlorohydrins with good analytical properties, however a 60–75% derivatization efficiency was reported [23]. Hydride reduction of epoxides is efficient, but discrimination between endogenous monohydroxylipids and the derivatives requires the use of deuteride, and ketolipids must be accounted for [24]. Similarly, acid catalyzed epoxide hydrolysis can allow quantification of the epoxides as their corresponding 1,2-diols, however the diols are also important analytical targets with distinct biological activity [25].

Fig. 1. Epoxide directed chemical transformations for epoxy fatty acids analysis: (a) trimethylchlorosilane treatment, (b) $LiAlD_4$ reduction, (c) hydrolysis, (d) aminolysis, (e) alcoholysis, (f) thiolysis.

Under anhydrous acidic conditions, amines and alcohols will open epoxides, with epoxide substitution promoting ring opening [26,27]. Although acidic methoxylamine will transform epoxides, these conditions can also convert ketones [28]. Epoxide solvolysis in acidic alcohol allows the sensitive detection of epoxylipids [29,30]. However, competition for charge localization between the alkoxide and silyl ether will reduce the abundance of characteristic ions. Thiolate anions also attack epoxides to produce 1,2-hydroxysulfanyls (i.e., α,β-hydroxythioethers) [21,31–33]. As opposed to the alkoxide, the sulfur should not compete for positive charge localization, thus resulting in higher intensity characteristic fragments.

This study investigates one possible avenue for a unified epoxide and diol analysis, namely thiolate derivatization of epoxides in the presence of diols, followed by hydroxy group derivatization and GC–MS analysis. To our knowledge, a comprehensive evaluation of thiols as epoxide-derivatizing agents does not exist. Here we evaluate a series of thiols with respect to electron impact ionization (EI) MS fragmentation behavior, product resolution by GC, and ultimately derivatization efficiency. In addition, reaction conditions were manipulated to minimize side reactions. We report a novel procedure for the rapid and complete analytical scale transformation of epoxides of linoleic and arachidonic acid in the presence of their corresponding diols.

2. Experimental

2.1. Chemicals

Optima Grade solvents and acids were purchased from Fisher Scientific (Pittsburgh, PA, USA). All other chemical reagents were purchased from either Sigma (St. Louis, MO, USA) or Aldrich (Milwaukee, WI, USA) unless indicated otherwise. Trimethylchlorosilane (TMCS; Supelco, Bellefonte, PA, USA), trimethylsilylimidazole (TMSI; Pierce, Rockford, IL, USA), *N*,*O*-bistrimethylsilyl acetamide (BSA; Supelco), *N*,*O*-bistrimethylsilyltrifluoromethylacetamide (BSTFA; Supelco), trifluoroacetic anhydride (TFAA), trifluoroacetic imidazole (TFAI; Pierce), and *n*-butylboronic acid were evaluated as hydroxy group derivatizing agents.

Unsaturated lipids were purchased from NuChek Prep (Elysian, MN, USA). Epoxyeicosanoid regioisomers were a gift from Dr. Darryl Zeldin at the National Institute of Health. Other lipid epoxides and diols were synthesized as previously described [25,34,35]. To prevent the decomposition of epoxy free fatty acids, residual acetic acid was removed as an azeotrope with toluene (3×1 ml) by rotary evaporation at 60°C. Proton nuclear magnetic resonance (NMR) spectra obtained in C^2HCl_3 on a 300 MHz QE-300 spectrometer (Bruker NMR, Billerica, MA, USA) confirmed complete solvent removal and epoxide, diol and ester presence as previously described [25]. Neat lipids were stored at ≤−25°C under dry nitrogen gas (N_2) until use. All lipid solutions were stored in 300 m*M* (~0.01%) butylated hydroxytoluene (BHT) in *n*-hexane. Solutions were sub-aliquoted into Wheaton prescored gold-band amber ampoules (Fisher), sealed under N_2 and stored at −25°C.

2.2. Chemical nomenclature

Abbreviations of oxidized fatty acids followed published recommendations [36,37]. To aid the reader, the structures, common names, and abbreviations for the analyzed epoxylipids are listed in Table 1. All other chemicals were named using the AutoNom v. 2.1 (Bielstein Informationsysteme) component of CS ChemOffice Ultra 2000 (Cambridge Soft, Cambridge, MA, USA).

2.3. Instrumentation

Hydroxysulfanyllipids were screened on a Fisons Quattro Bio-Q (Altrancham, UK) electrospray ionization mass spectrometry (ESI-MS) system run at unit resolution in the negative mode with an acetonitrile–water (50:50, v/v) mobile phase, 50 V cone voltage and 2.9 eV capillary voltage. The resulting molecular mass data were used for structural verification and evaluation of ester stability. Electrospray results were not evaluated for quantitative applications.

GC with flame ionization detection (FID) was used to track reaction progression and assess product purity during synthesis and purification. The system used was a Hewlett-Packard (HP) 5890A (San Jose, CA, USA) equipped with a 15 m×0.25 m, 0.25 μm DB-5 capillary column (J&W Scientific, Folsom, CA, USA). The initial oven temperature of 250°C was held for 1 min and ramped at 2°C/min to 260°C followed by a 10°C/min ramp to 290°C with a total time of 9 min. The inlet and detector temperatures were 280°C and 300°C, respectively. Helium was used as the carrier gas at a flow of 1 ml/min at 200°C (head pressure=15 p.s.i.; 1 p.s.i.=6894.76 Pa).

Final purity assessment and evaluation of fragmentation behavior was performed with EI-MS on a HP 5973 mass spectral detector tuned with perfluorotributylamine (Scientific Instrument Services, Ringoes, NJ, USA) using the system auto tune parameters. The mass spectrometer was interfaced with a HP 6890 gas chromatograph equipped with a 30 m×0.25 m, 0.25 μm column. Investigated chromatographic stationary phases included DB-23, DB-1701, DB-200, DB-5ms, DB-XLB and DB-17ms (J&W Scientific). Optimized oven programs used inlet, transfer line and quadrapole temperatures of 250°C, 280°C and 160°C, respectively, for all columns. The optimized oven program for the DB-17ms was an initial temperature of 165°C held for 2 min, ramped at 15°C/min to 240°C held 1 min, ramped at 3°C/min to 270°C and held 5 min, ramped at 15°C/min to 320°C and held 30 min. The optimized oven program for the DB-XLB was an initial temperature of 100°C held for 2 min, ramped at 15°C/min to 240°C held 1 min, ramped at 3°C/min to 270°C and held 5 min, ramped at 15°C/min to 340°C and held

Table 1
Structure, common name and IUPAC abbreviation of evaluated epoxylipids

Structure	Common name(s)	IUPAC abbreviation
	10-Epoxyheptadecanoic acid methyl ester	10(11)-EpHep-me
	9-Epoxyoctadecanoic acid methyl ester, epoxystearic acid methyl ester	9(10)-EpO-me
	9-Epoxyoctadec-(12*Z*)-enoic acid methyl ester, 9-epoxylinoleic acid methyl ester, leukotoxin	9(10)-EpOME-me
	12-Epoxyoctadec-(9*Z*)-enoic acid methyl ester, 12-epoxylinoleic acid methyl ester, isoleukotoxin	12(13)-EpOME-me
	5-Epoxyeicosatri-(8*Z*,11*Z*,14*Z*)-enoic acid methyl ester, 5-epoxyarachidonic acid methyl ester 5-EET	5(6)-EpETrE-me
	8-Epoxyeicosatri-(5*Z*,11*Z*,14*Z*)-enoic acid methyl ester, 8-epoxyarachidonic acid methyl ester, 8-EET	8(9)-EpETrE-me
	11-Epoxyeicosatri-(5*Z*,8*Z*,14*Z*)-enoic acid methyl ester, 11-epoxyarachidonic acid methyl ester, 11-EET	11(12)-EpETrE-me
	14-Epoxyeicosatri-(5*Z*,8*Z*,11*Z*)-enoic acid methyl ester, 14-epoxyarachidonic acid methyl ester, 14-EET	14(15)-EpETrE-me

10 min. Helium was used as the carrier gas at a constant flow of 0.8 ml/min for both columns. For selected ion monitoring, ion groups of ≤6 ions were constructed and ion dwell times of 50 ms were used.

2.4. Acid methylation and hydroxy group derivatization

Fatty acid methylation used ethereal diazomethane prepared in 3-ml batches from 1-methyl-3-nitro-1-nitrosoguanidine and sodium hydroxide using an Aldrich MNNG diazomethane generator and accompanying instructions. Hydroxylipid derivatization used 50–100 μl aliquots of ≤10 m*M* lipid dissolved in an appropriate organic solvent, following standard procedures [38].

Various silylation procedures were assessed for hydroxysulfanyl- and dihydroxytrimethylsilyl ether

formation including BSTFA–TMCS (99:1), BSTFA–TMCS (90:10), BSTFA–TMSI (50:50), and BSTFA–pyridine (80:20) at various times, temperatures and relative concentrations. During results and discussion, silylation refers to the dilution of a sample aliquot in ethyl acetate with an equal volume of BSTFA–pyridine (80:20, v/v) and incubation for 2 h at 100°C. Acetylation reactions with TFAA or TFAI were also evaluated: 10 min to 2 h at 60°C, 2× sodium hydrogencarbonate wash (250 μl), back extracted 2× with ethyl acetate (250 μl). Diol *n*-butylboronates were prepared in ethyl acetate using 10 μl of 5 mg/ml *n*-butylboronic acid in acetone–benzene (2:1) incubated 10 min at room temperature, evaporated under N_2, re-dissolved in hexane and silylated as described for prostaglandin F2α [38].

Table 2
Thiols evaluated as epoxylipid derivatizing reagents

Thiol	IUPAC name	Structure	pK_a [a]	σ [b]
I	1-Propanethiol	SH	10.53	–
II	2-Mercaptoethanol	HO SH	9.61	–
III	Cyclohexanethiol	SH	NA [c]	–
IV	(4-Chlorophenyl)methanethiol	Cl SH	NA	–
V	4-Methoxybenzenethiol	CH_3O SH	NA	−0.27
VI	Benzenethiol	SH	6.43	0
VII	4-Chlorobenzenethiol	Cl SH	5.97	0.23
VIII	3-Chlorobenzenethiol	Cl SH	NA	0.37
IX	2,3,5,6-Tetrafluorobenzenethiol	F F SH F F	2.75	0.89

[a] Reported pK_a values from Wilson et al. [39].
[b] Taft–Hammet sigma constants from Hansch and Leo [41].
[c] Thiol pK_a not available (NA) in the literature.

2.5. Thiol selection

The thiols evaluated as derivatizing agents are listed in Table 2. Thiol nucleophilicity correlates to pK_a via independent log–normal relationships for aromatic and aliphatic thiols, respectively [39,40]. Initially, a short thiol series was selected spanning a broad pK_a range (2.75–10.53): 1-propanethiol (**I**), 2-mercaptoethanol (**II**), (4-chlorophenyl)-methanethiol (**IV**), benzenethiol (**VI**), and 2,3,5,6-tetrafluorobenzenethiol (**IX**). To further evaluate the electronic effect of the thiol on sulfanyl fragmentation, the series was later expanded to include cyclohexanethiol (**III**), 4-methoxybenzenethiol (**V**), 4-chlorobenzenethiol (**VII**), and 3-chlorobenzenethiol (**VIII**). Substituent constants for the benzenethiols were gathered from the compilation of Hansch and Leo [41]. For thiol **IX**, parameters from the *para*-fluoro substitution were subtracted from the reported pentafluoro parameters.

For preliminary evaluations, thiolate solutions were prepared in 1:1.1 molar ratios of potassium hydroxide (KOH) to thiol (4 *M*) in methanol as described by Richard et al. [31]. A mixture of 9-epoxyoctadec-(12*Z*)-enoic acid methyl ester and 12-epoxyoctadec-(9*Z*)-enoic acid methyl ester, i.e., 9(10)-EpOME-me–12(13)-EpOME-me (60:40; [Total]=3.4 *M*) was enriched with a 10 molar excess of thiolate and incubated at either 50 or 60°C with a reaction volume of 100 μl under either a nitrogen blanket or normal atmosphere. Exposure to atmosphere did not appear to effect derivatization efficiency but enhanced disulfide levels in solutions of more basic thiols. After solvent removal under a stream of N_2, the residue was re-dissolved in ethyl acetate and silylated. This mixture was diluted to 1 ml in ethyl acetate and 1 μl was analyzed by GC–MS.

*2.6. Thiol **IX** activation*

Thiol **IX** activation was evaluated with various proton transfer agents. Methanolic solutions of thiol **IX** were prepared with or without prior activation with $NaBH_4$, KOH, pyridine, piperidine, or diazabicycloundecene (DBU), as described above with a thiol concentration of 0.65 *M*. A 100-m*M* methanolic solution of 9(10)-EpOME-me–12(13)-EpOME-me (1:1) was exposed to these solutions and incubated at 50°C for ~12 h under normal atmosphere.

2.7. Thiol solution preparation

Thiolate reagents were routinely prepared with sodium borohydride ($NaBH_4$) activated alcohol. The $NaBH_4$ (180 mg, 19 mmol) was placed in a septum-sealed vial with a needle vent and dissolved in 25 ml of the desired alcohol. Following termination of hydrogen gas evolution (~ 2 h, 21°C), 38 mmol of thiol was added dropwise as either a neat liquid or alcoholic solution of crystalline thiols (e.g., 4.55 ml thiol **IX**; 1.52 g/l, 182.14 g/mol). The final solution was brought to 30 ml for a final thiol concentration of ~1.3 *M*. The vial was purged with N_2 and the solution was sub-aliquoted into 1-ml ampoules under N_2. The prepared reagent was stable >1 year at room temperature but was routinely stored at −25°C. Once opened, the ampouled solution was typically transferred to a vial with a PTFE-lined cap and stored under a blanket of N_2. This solution was used during successive experiments (over as much as 3 weeks), purging with N_2 prior to storage. In the case of thiol **IX**, the pale yellow initial solution turns a pale green upon extended exposure to atmosphere. If a green coloration was observed, a new ampoule was opened.

2.8. Ester stability

To evaluate transesterification under the epoxide thiolation conditions, 9-epoxyoctadecanoic acid methyl ester [9(10)-EpO-me; 100 μmol] was incubated in an ethanolic sodium thiolate solution of **IX** (thiol–hydride proton, 1:1) at 60°C for 2 h. Fifty percent of the reaction was spotted onto a 10 cm Kieselgel 60 F_{254} thin-layer chromatography (TLC) plate (EM Science, Gibbstown, NJ, USA) and products were separated using hexane–ethyl acetate (80:20, v/v). The UV active spot (R_F=0.42) was scraped, redissolved in 250 μl of methanol, and

analyzed by ESI-MS and GC–EI-MS. Unreacted thiol and disulfide ran near the solvent front. Spraying developed plates with 4% phosphomolibdic acid in 20% ethanolic water and heating to 100°C for 10 min allowed the destructive visualization of underivatized lipids.

2.9. Thiol buffering, quenching and alkene stability

Alkene isomerization was observed in initial reactions. The derivatization of EpOME was repeated as described above using thiolate solutions prepared from a 2:1 mixture of thiol to hydride exchangeable proton (''buffered'' thiol) at 60°C. To evaluate the effect of thiol scavengers on alkene stability, reactions were cooled at −25°C for 5 min, and spiked with either 2 μl of neat propylene oxide or 150 μl of 0.5 *M* maleic anhydride in acetonitrile. Propylene oxide quenched samples were silylated after evaporative solvent removal. Maleic anhydride exposed reactions were doubled with water to form the diacid and sulfanyl-diacid, and the dihydroxy- and hydroxysulfanyllipid methyl esters were extracted with 3× 500 μl chloroform. The isolated chloroform extract was washed 3× with 1 ml of water, evaporated with N_2, re-dissolved in ethyl acetate and silylated.

2.10. Reaction temperature and alkene stability

To assess the affect of reaction temperature on alkene rearrangement, an EpOME methyl ester mixture [9(10)-EpOME-me–12(13)-EpOME-me, 1:1; 530 m*M* total] was exposed to ''buffered'' thiol (1.5 *M* total thiol) with a reaction volume of 60 μl at 40, 60 and 100°C in sealed vials. The reactions were quenched with propylene oxide as described at 12, 2.5 and 0.5 h, respectively. The solvent was evaporated under N_2 and residues dissolved in 60 μl *n*-hexane. A 10-μl aliquot was diluted to 50 μl with ethyl acetate and silylated with 50 μl BSTFA–pyridine (80:20, v/v). The resulting products were analyzed by GC–MS.

2.11. Reaction temperature and sulfanyl formation

Initially, the temperature dependence of epoxide thiolation was evaluated with methyl epoxy stearate, i.e., 9(10)-EpO-me (100 μmol). This epoxylipid was derivatized with a sodium thiolate of **IX** prepared with a 1:1 ratio of thiol to hydride exchangeable proton in methanol at 20, 50, or 60°C. Reaction rates were then estimated by tracking the transformation of 150 μmol (10000 dpm) of $[^{14}C_1]$9(10)-EpO (Sigma) at 60°C in 100 μl of methanol. At five time points, 2 μl aliquots were removed from the reaction mixture and were separated with hexane–ethyl acetate–acetic acid (80:20:1, v/v/v) on silica TLC and measured using a Bioscan System 200 imaging scanner equipped with a Model 1000 Auto Changer and software version 2.247 (Bioscan, Washington, DC, USA).

2.12. Optimized thiolation reaction

Six matched samples were prepared to test the efficiency of epoxide derivatization under ''optimized'' conditions. These reactions contained 9(10)-EpOME–12(13)-EpOME (1:1) (65 μ*M*), dihydroxyoctadecenoic acid regioisomers [9,10-DHOME–12,13-DHOME (1:1); 61 μ*M*], 10-epoxyheptadecanoic acid [10(11)-EpHep; 91 μ*M*], and 10,11-dihydroxynonadecanoic acid [9,10-DHN; 92 μ*M*]. Samples were methylated with ethereal diazomethane, dried under N_2, redissolved in 60 μl methanol, spiked with the ''buffered'' sodium thiolate solution of **IX** (50 m*M* total thiol) and incubated under normal atmosphere at 100°C for 0, 5, 10, 20, 30 and 45 min, respectively. Each vial was cooled 5 min at −25°C, spiked with 2 μl of propylene oxide and returned to 100°C for 30 min. The reaction solvent was removed under N_2 and the residue was dissolved in 50 μl hexane–ethyl acetate (1:1, v/v) and silylated with 50 μl BSTFA–pyridine (80:20, v/v) 2 h at 100°C.

2.13. Analytical calibration

Analytical standards were prepared from synthesized 17, 18, and 19 carbon fatty acid diols and hydroxysulfanyls. Twelve solutions were prepared with hydroxysulfanyl and diol concentrations ranging between 0.005 and 20 ng/μl (~0.01–40 pmol/μl). The thiol **IX** derivatives of 10(11)-EpHep-me and the 10,11-DHN-me were introduced into the cali-

Table 3
Characteristic ions and retention times for silylated diol fatty acid methyl esters

Compound abbreviation[a]	First ion (m/z)[b]	Second ion (m/z)[c]	RA second ion (%)[d]	t_R (min)[e]
10,11-DHHep-me	187	273	100	12.79
9,10-DHO-12-ME-me	271	213	120	13.71
12,13-DHO-9-ME-me	275	173	150	13.76
9,10-DHO-me	215	259	115	13.82
10,11-DHN-me	273	215	90	14.72
8,9-DHETrEs-me	255	243	55	15.80
11,12-DHETrE-me	295	315	45	15.86
5,6-DHETrE-me	203	215	40	15.99
14,15-DHETrE-me	173	275	60	16.13

[a] Compound abbreviation adapted from Smith and co-workers [36,37]. For example, 14,15-DHETrE-me=**14,15-DiH**ydroxy**E**icosa**T-riE**noic acid **m**ethyl **e**ster.
[b] Ion with highest signal-to-noise ratio proposed for quantification.
[c] Characteristic qualifier ion.
[d] Relative abundance of the second vs. first ions.
[e] DB-XLB retention time using the described conditions for this column. (t_R $C_{18:0}$=12 min; $C_{22:0}$=16.2 min).

bration solutions as potential extraction surrogates, while 10(11)-DHHep-me was included to allow quantification of epoxide hydrolysis during sample preparation. The 9(10)-EpO ethyl ester thiol **IX** derivatives were introduced as an internal standard at 5 ng/μl in each solution. In addition, arachidonic acid methyl ester derived mixtures of dihydroxy- and hydroxy-2,3,5,6-tetrafluorophenylsulfanyls were prepared and analyzed under equivalent conditions. Tables 3 and 4 list two characteristic ions for each of

Table 4
Characteristic ions and retention times for silylated hydroxy(2,3,5,6-tetrafluoro)phenylsulfanyl fatty acid methyl esters

Compound abbreviation[a]	First ion (m/z)[b]	Second ion (m/z)[c]	RA second ion (%)[d]	t_R (min)[e]
10-OTMS-11-TFPS-Hep-me	273	169	10	20.04
10-TFPS-11-OTMS-Hep-me	187	438	10	20.05
9-TFPS-10-OTMS-O-12-ME-me	213	453	90	21.73
9-OTMS-10-TFPS-O-12-ME-me	259	155	20	21.83
12-OTMS-13-TFPS-O-9-ME-me	367	299	80	21.97
9-TFPS-10-OTMS-O-me	215	155	10	21.99
9-OTMS-10-TFPS-O-me	259	155	16	22.01
12-TFPS-13-OTMS-O-9-ME-me	173	283	15	22.06
9-TFPS-10-OTMS-O-ee	215	160	5	22.52
9-OTMS-10-TFPS-O-ee	273	155	12	22.52
8-TFPS-9-OTMS-ETrE-me	253	437	100	25.92
11-OTMS-12-TFPS-ETrE-me	407	283	35	26.30
8-OTMS-9-TFPS-ETrE-me	243	447	20	26.04
11-TFPS-12-OTMS-ETrE-me	213	477	20	26.07
5-TFPS-6-OTMS-ETrE-me	307	397	30	26.10
5-OTMS-6-TFPS-ETrE-me	203	171	25	26.29
14-OTMS-15-TFPS-ETrE-me	367	323	10	26.39
14-TFPS-15-OTMS-ETrE-me	173	103	10	26.52

[a] Compound abbreviation adapted from Smith and co-workers [36,37]. For example, 14-TFPS-15-OTMS-ETrE-me=**14**-2,3,5,6-**T**etra**F**luoro-**P**henyl**S**ulfanyl-**15**-(**O**)**T**ri**M**ethyl**S**ilanyloxy-**E**icosa**T**ri**E**noic acid **m**ethyl **e**ster. Note, ee=ethyl ester.
[b] Ion with highest signal-to-noise ratio proposed for quantification.
[c] Characteristic qualifier ion.
[d] Relative abundance of the second vs. first ions.
[e] DB-XLB retention time using the described conditions for this column (t_R $C_{24:0}$=19.5 min).

analyte and their DB-XLB retention times using conditions described in Section 2.3.

3. Results

Epoxyoctadecenoic acid (EpOME) methyl esters were selected as representative unsaturated epoxylipids for the majority of this method development. These epoxides were transformed to hydroxysulfanyls using a series of thiols (Table 2) to identify chemical properties yielding optimal mass spectral and chromatographic properties after hydroxy group derivatization. Silylation of hydroxysulfanyls improved both chromatographic resolution and fragmentation characteristics. Non-silylated results are not shown. Reaction conditions were adjusted to maximize thiolation efficiency and eliminate alkene isomerization. The thiol producing the EpOME-me derivative with the highest intensity structurally descriptive mass spectrum under electron impact was used to derivatize epoxyeicosatrienoic acid methyl ester (EpETrE-me) regioisomers.

3.1. Hydroxy group derivatization

The complete silylation of diols was significantly slower than the silylation of hydroxysulfanyls. Optimal hydroxylipid silylation used ethyl acetate as a co-solvent and an equal volume of BSTFA–pyridine (80:20). Samples incubated for 2 h at 100±5°C were analyzed without further manipulation. Trifluoroacetic anhydride destroyed the sulfanyl derivatives, while TFAI reactions efficiently derivatized diols and hydroxysulfanyls (<10 min, ~20°C). However, the acetylated products had poor mass fragmentation behavior. Diol boronate formation was also rapid, however the EI-MS fragmentation of boronates produced characteristic ions of lower intensity than the corresponding silyl ethers as described previously [42]. In addition, significant background was observed using silylation after boronate treatment.

3.2. Preliminary thiol selection

Preliminary evaluations of the reaction of thiols with 9(10)-EpO-me at 50°C indicated that the reaction rates increased with increasing thiol pK_a such that **I**>**II**~**IV**>**VI**>**IX**, in general agreement with published thiol nucleophilicity [39,40]. At 60°C, all thiols yielded complete epoxide transformation within 2 h and hydrolysis was not observed. In the EpOME-me thiolation reactions a 40:60±2% ratio of sulfanyls was observed with attack of the epoxide carbon β to the alkene being favored. This product ratio of attack at the β vs. γ carbon was thiol independent.

3.3. Electron impact ionization fragmentation of trimethylsilanyloxysulfanyls

Analysis of EpOME-me and EpETrE-me trimethylsilanyloxysulfanyls (OTMS-SR) revealed that the degree and position of unsaturation(s) influenced

Fig. 2. Systematic description of trimethylsilanyloxysulfanyl (OTMS-SR) products of non-symmetrical polyunsaturated lipid epoxides. R=$CH_3OC(O)CH_2$ for epoxyeicosanoids. R=$CH_3OC(O)(CH_2)_5$ for the epoxy linoleates after removal of the alkenes bound by parentheses. R′=$CH_2CH_2CH_3$ and R″ changes as the derivatizing thiol changes.

the fragmentation behavior. To facilitate a coherent description of the results for both sets of compounds, a stylized representation of the polyunsaturated OTMS-SR products was developed (Fig. 2). Thiolation of each EpOME-me regioisomer will produce a type A and type D product, with either the sulfanyl or the silanyloxy moiety adjacent to a saturated aliphatic chain, respectively. Thiols containing either a second ionizable group (**II**) or producing a labile thioether linkage (**IV**), showed fragmentation influenced by these moieties, decreasing characteristic ion intensity. Excluding sulfanyls derived from these thiols revealed a relationship between the fragmentation behavior and the chemical environment of the hydroxysulfanyl.

Type A compound fragmentation was thiol dependent and showed three dominant decomposition pathways resulting from cleavage around the OTMS-SR moiety (Fig. 3). Cleavage of the carbon–carbon bond between the sulfanyl and silanyl ether (Cα) was >50% of the base peak abundance for all type A OTMS-SR products. The carbon–carbon bond of the silanyloxy bound carbon distal to the sulfur also showed substantial α cleavage (ʻCα) for thiol **IX** sulfanyls, but produced a low intensity fragment for the products of other thiols (Fig. 4). Finally, an ion corresponding to the diunsaturated fatty acid methyl ester was observed from cleavage of both the sulfanyl (Sα) and ether (Oα) linkage with commensurate molecular rearrangement. The notation Sα,Oα will be used to indicate fragmentation leading

Fig. 3. Proposed mechanism of trimethylsilanyloxysulfanyl (OTMS-SR) mass spectral fragmentation. The three decomposition pathways centered on the OTMS-SR function are consistent with radical formation on the sulfur followed by two radical directed pathways. In the first initiation pathway, the ion radical center migrates to the silanyloxy bound carbon. This ion can then decompose along two paths. A single electron transfer to the sulfur-bound carbon will lead to α cleavage (Cα). However, alkene isomerization can promote a single electron transfer away from the sulfur also leading to α cleavage (ʻCα). The second initiation path would involve a proton transfer to the radical sulfur resulting in a distonic ion radical. Cleavage of the sulfanyl bond (Sα) with a two electron transfer would then drive the formation of a new alkene with a proton transfer to the silanyloxy oxygen, maintaining the distonic nature of the ion radical. Cleavage of the silanyloxy bond (Oα) would then release trimethylsilanol and induce alkene migration yielding an aliphatic radical ion stabilized by conjugation.

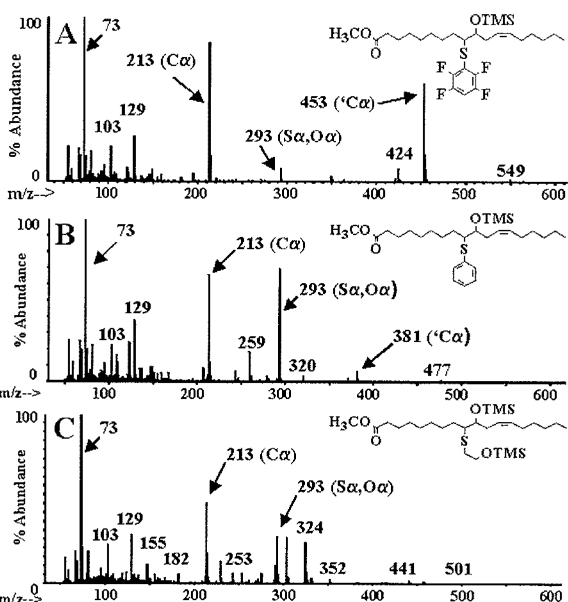

Fig. 4. Electron impact mass spectra of silylated representatives of type A 9(10)-EpOME-me hydroxysulfanyls: 9-sulfanyl-10-trimethylsilanyloxyoctadec-(12*Z*)-enoic acid methyl ester produced by exposure to: (A) 2,3,5,6-tetrafluorobenzenethiol (**IX**), (B) benzenethiol (**VI**), (C) 2-mercaptoethanol (**II**) with subsequent silylation. All spectra show ions indicative of Cα (m/z 213) and Sα,Oα cleavage (m/z 293), as defined in Fig. 3. Fragments from ʻCα-cleavage are apparent in A and B but not C. The presence of a second ionizable group in the thiol **II** derived sulfanyl lead to the formation of other characteristic fragments of relatively low intensity. The corresponding 9-propylsulfanyl derivative produced m/z 213 and 293 fragments at 25 and 54% of the $[Si(CH_3)_3]^+$ m/z 73 base peak, respectively (data not shown).

to this ion. For the products of various thiols, Cα increased while Sα,Oα decreased and ‘Cα increased as thiol acidity increased (Fig. 4), suggesting an electronic effect on fragmentation.

Regression of the Sα,Oα vs. Cα fragment area showed a linear relationship between these two ions ($y=1.49x-1.99+10\cdot^{7}$, $r^2=0.99$, $n=9$). The greater than unit slope of this regression indicates that the relative abundance of the Cα and Sα,Oα cleavage products changed in the spectrum of different sulfanyls. A similar relationship between Cα and ‘Cα was not observed. Regressing the Sα,Oα:Cα ratio vs. the pK_a for a subset of thiols (**I**, **VI**, **VII** and **IX**) revealed a linear relationship ($y=3.85x+2.35$, $r^2=0.99$, $n=4$). Regressing the Sα,Oα:Cα vs. the Taft–Hammet sigma constant for the substituted benzenethiols also revealed a linear relationship ($y=-1.07x+1.13$, $r^2=0.94$, $n=5$). Removal of thiol **IX** altered the magnitude, but not the sign of the observed slope ($y=-1.66x+1.72$, $r^2=0.93$, $n=4$). Regressions with respect to either the hydrophobicity constant π ($r^2=0.53$), or the molar refractivity constant MR ($r^2=0.34$), were considerably weaker and strongly influenced by thiol **IX**.

Analysis of the silylated thiol **IX** sulfanyls (OTMS-TFPS) of EpETrE-me allowed an evaluation of alkene influence on fragmentation. For each of the EpETrE-me regioisomers, at least one of the derivatives produced a characteristic mass fragment with an intensity of 25–100% of the spectral base peak. Like the EpOME methyl esters, both 5(6)-EpETrE-me and 14(15)-EpETrE-me yielded type A and type D sulfanyls. However, 8(9)-EpETrE-me and 11(12)-EpETrE-me produce type B and type C compounds with the hydroxysulfanyl bordered by alkenes on both sides (Fig. 2).

Unlike the Type A EpOME-me products, EpETrE-me Type A sulfanyls showed a low abundance of Cα fragmentation, suggesting an influence from the polyalkene. In addition, while abundant ‘Cα fragmentation was observed, ‘Cα fragments containing both the ester and OTMS functions readily released trimethyl silanol (HOTMS), decreasing the ion mass by 90 u (‘Cα-HOTMS). This is clearly observed in the spectra of the type A product of 5(6)-EpETrE-me (Fig. 5). The sum of the ‘Cα and ‘Cα-HOTMS fragments were nearly equivalent for both EpETrE-

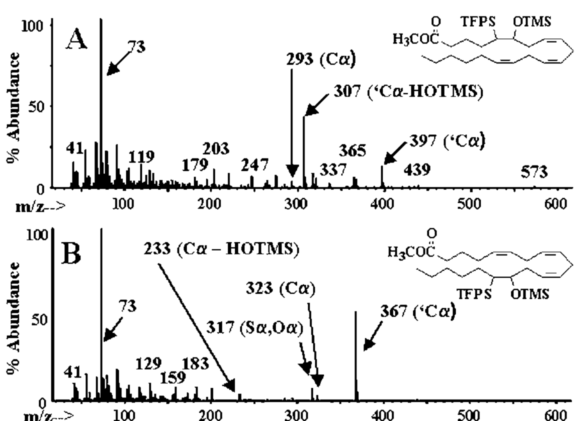

Fig. 5. Electron impact ionization mass spectra of silylated type A hydroxy(2,3,5,6-tetrafluorophenylsulfanyl)s (OTMS-TFPS) of epoxyeicosatrienoic acid methyl ester. (A) 5-TFPS-6-OTMS-eicosatri-(8Z,11Z,14Z)-enoic acid methyl ester. (B) 14-OTMS-15-TFPS-eicosatri-(5Z,8Z,11Z)-enoic acid methyl ester. The molecular ion and the $[M-CH_3]^+$ ions are observed at ~1% abundance for both compounds. Bond notation follows Fig. 3. Fragments containing both the methyl ester and the OTMS decompose further losing trimethyl silanol (HOTMS).

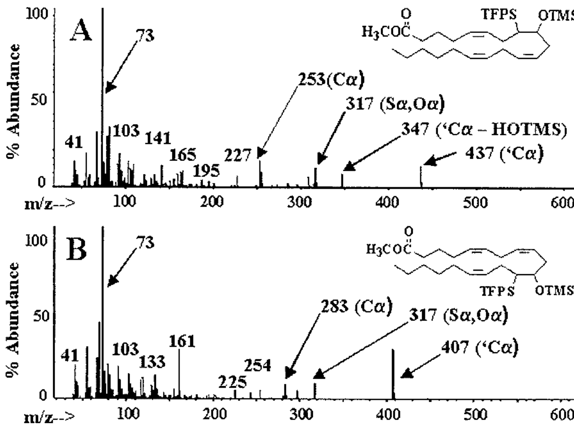

Fig. 6. Electron impact ionization mass spectra of silylated type B hydroxy(2,3,5,6-tetrafluorophenylsulfanyl)s (OTMS-TFPS) of epoxyeicosatrienoic acid methyl ester. (A) 8-TFPS-9-OTMS-eicosatri-(5Z,11Z,14Z)-enoic acid methyl ester. (B) 11-OTMS-12-TFPS-eicosatri-(5Z,8Z,14Z)-enoic acid methyl ester. The molecular ion, and the $[M-CH_3]^+$ ions are observed at ~1% abundance for both compounds. Bond notation follows Fig. 3. Evidence of Cα, Sα,Oα and ‘Cα cleavage is apparent in both spectra. Fragments containing both the methyl ester and the OTMS decompose further losing trimethyl silanol (HOTMS).

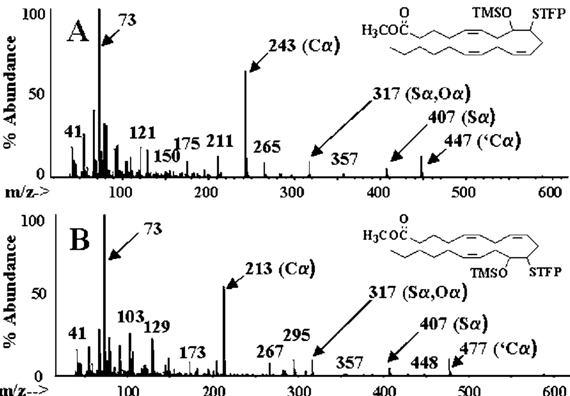

Fig. 7. Electron impact ionization mass spectra of silylated type C hydroxy(2,3,5,6-tetrafluorophenylsulfanyl)s (OTMS-TFPS) of epoxyeicosatrienoic acid methyl ester. (A) 8-OTMS-9-TFPS-eicosatri-(5Z,11Z,14Z)-enoic acid methyl ester. (B) 11-TFPS-12-OTMS-eicosatri-(5Z,8Z,14Z)-enoic acid methyl ester. The molecular ion and the $[M-CH_3]^+$ ions are observed at ~1% abundance for both compounds. Bond notation follows Fig. 3. Evidence of Cα, Sα,Oα and ‘Cα cleavage is apparent in both spectra. Loss of trimethyl silanol from the ‘Cα fragment from A was observed (*m/z* 357). These spectra also show a fragment indicating loss of 2,3,5,6-tetrafluorobenzenethiol (i.e., Sα cleavage).

me type A products at ~50% of the base peak ion intensity. In the spectra of the type B EpETrE-me sulfanyls, both Cα and ‘Cα ions were observed (Fig. 6), however the ion abundance was the lowest of all the sulfanyl types. As seen for the type A product of 5(6)-EpETrE-me, the type B product of 8(9)-EpETrE-me produced a ‘Cα fragment which released trimethyl silanol. Again, the sum of the ‘Cα and ‘Cα-HOTMS fragments are equivalent for both EpETrE-me type B products at ~35% of the base peak while Cα product ion intensity was between 10 and 25%. The relative difference in Cα to ‘Cα fragmentations continued in the type C EpETrE-me sulfanyls. For these compounds, the Cα cleavage product ion was >60% of base peak intensity while the ‘Cα fragment dropped to ~20% (Fig. 7). Finally, in the type D products, fragmentation was dominated by Cα cleavage (Fig. 8), as observed for the type D EpOME-me sulfanyls (data not shown).

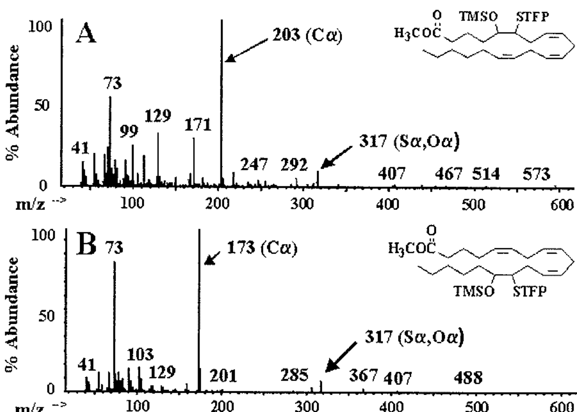

Fig. 8. Electron impact ionization mass spectra of silylated type D hydroxy(2,3,5,6-tetrafluorophenylsulfanyl)s (OTMS-TFPS) of epoxyeicosatrienoic acid methyl ester. (A) 5-OTMS-6-TFPS-eicosatri-(8Z,11Z,14Z)-enoic acid methyl ester. (B) 14-TFPS-15-OTMS-eicosatri-(5Z,8Z,11Z)-enoic acid methyl ester. The molecular ion, $[M]^+$, and the $[M-CH_3]^+$ ions are observed at ~1% abundance for both compounds. Bond notation follows Fig. 3. Both spectra are dominated by fragmentation at Cα with minimal Sα,Oα cleavage and undetectable ‘Cα cleavage. As with type C sulfanyls, a Sα fragment was observed (*m/z* 407).

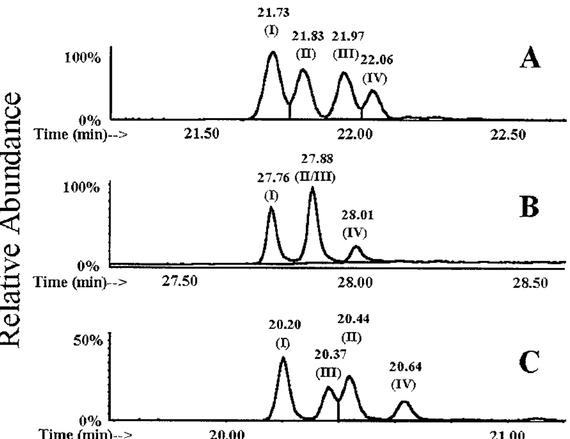

Fig. 9. Total ion chromatograms of epoxylipids derivatized with arylic, benzylic and aliphatic thiols. A 10-mg amount of a mixture of 9(10)-EpOME-me–12(13)-EpOME-me (60:40) was derivatized with base activated solutions of: (A) **IX** – 2,3,5,6-tetrafluorobenzenethiol, (B) **IV** – (4-chlorophenyl)methanethiol, or (C) **II** – 2-mercaptoethanol and silylated, diluted 1000 fold. Approximately 35 nmol of total product was injected in 1 μl on a 30 m×0.25 mm I.D., 0.25 μm DB-17ms column as described. Peak annotation indicates position of thiol attack (I=9-sulfanyl, II=10-sulfanyl, III=13-sulfanyl, IV=14-sulfanyl). The *y*-axes of A, B and C are normalized to peak A-I.

3.4. Chromatographic properties of sulfanyl derivatives

The elution order and chromatographic resolution of the EpOME OTMS-SR products were evaluated on 30 m×0.25 mm I.D. capillary columns with 0.25 μm bonded stationary phases. Noting the OTMS-SR products as their parent thiols, they eluted in the order **I**<**II**<**IX**<**III**<**VI**<**VII**=**VIII**<**V**<**IV** on all columns. In all cases, the type D EpOME-me sulfanyls eluted before the type A products. Optimal resolution was defined as maximal separation of the regioisomers and especially of the 10- and 13-sulfanyls. The DB-17ms column yielded the best absolute performance (Fig. 9), followed by DB-5=DB-XLB>DB-1701, DB-200, and DB-23 (data not shown). In this figure one can see that the nature of the thiol influenced chromatographic separation. While all arylthiol products were reasonably resolved on phenyl-substituted stationary phases, only thiol **IX** produced greater than 80% resolution of products with low Sα,Oα cleavage for the EpOME-me sulfanyls.

Despite the excellent chromatographic performance of the DB-17ms, the high polarity of this column required extended oven programs (>40 min hold at 320°C) due to the appearance of polar high boiling components, like cholesterol, during preliminary evaluations of biological matrices (data not shown). Therefore, after characterizing GC–MS fragmentation on a DB-17ms, the DB-XLB column was used reducing total analysis time to <40 min. Elimination of sterols from samples would allow for additional compression of the chromatographic run. As mentioned above, Tables 3 and 4 list the DB-XLB retention times for the investigated diol and hydroxysulfanyltrimethylsilyl ethers. The C17–C20 diol OTMS ethers elute between the 20 and 22 carbon fatty acid methyl esters, while the OTMS-TFPS derivatives eluted after the 24 carbon methyl esters but before cholesterol. The sulfanyl isomers from unsaturated lipid epoxides were difficult to separate by GC, regardless of the column or thiol used. On the DB-17ms, the 9-EpO-me OTMS-TFPSs eluted before the EpOME-me derivatives but co-eluted among these compounds on other stationary phases. On the DB-5 and DB-XLB the 9-EpO thiol **IX** products co-eluted with the 12-OTMS-13-TFPS product of EpOME-me but have unique fragments (Table 4). While the type B and type C products were only partially resolved, the type A and type D products showed baseline separation.

3.5. Influence of thiol activation reagents

Due to the superior resolution and fragmentation behavior of the OTMS-TFPS products, thiol **IX** was selected as the derivatizing thiol for further procedural optimization. While base activation promotes the nucleophilic properties of thiols through thiolate formation, thiol **IX** is uncharacteristically acidic (pK_a 2.78). Therefore, the thiol activation procedure was re-evaluated. All mixtures produced a single UV dense spot by TLC and a partially resolved mixture by GC–FID. Without activation the reaction reached ~50% completion in 24 h. All proton transfer reagents increased the rate of epoxide transformation. With piperidine and DBU numerous polar side products were observed, which were visualized with phosphomolibdic acid. Reaction efficiency was highest and equivalent with either KOH or $NaBH_4$ activated systems. In situ methoxylate anion generation with $NaBH_4$ was retained for all further reactions.

3.6. Ester stability

Heating esters in alkaline alcohol will result in transesterification [22]. Reacting 9(10)-EpO-me with a thiolate solution of **IX** at 60°C in ethanol produced a UV-active spot with a molecular mass of 508 u, suggesting ethyl ester formation. Subsequent GC–MS analysis confirmed <2% methyl ester and over 98% ethyl ester in the ethanolic reaction. Transesterification of the isolated hydroxysulfanylethyl ester using 1 *M* methanolic NaOH for 2 h produced the methyl ester without side product formation, documenting the base stability of the product.

3.7. Alkene stability under initial conditions

Exposure of unsaturated fatty acids to proton abstracting reagents, including alkoxides and thiolates, can result in alkene(s) isomerization when

heated for extended periods [43,44]. The reactions described above showed evidence of alkene isomerization. Low intensity trailing peaks appeared that showed identical fragmentation to the leading peaks. Methyl linoleate incubated under equivalent conditions, but without the addition of silylating reagents, also showed similar results. The trailing peaks were assumed to be the 10(*E*)ene and 11(*E*)ene compounds derived from the 9(*Z*) and 12(*Z*) alkenes, respectively, based on relative retention times and described alkene rearrangements of linoleic acid [45].

3.8. Thiolate buffering, quenching and alkene stability

To increase the relative pH of the reaction mixture while maintaining high thiolate anion concentrations, a thiol–thiolate ‘‘buffered’’ solution was prepared (thiol–hydride, 2:1, exchangeable proton). Using the ‘‘buffered’’ thiol **IX** solution, reactions run at 60°C were complete within 2 h, polar side products were not observed, and alkene rearrangement was reduced from 20% to less than 5%. Under these conditions, the degree of isomerization increased as thiol pK_a increased: pK_a 2.7, 6.0, 6.4, >9.0, % isomerization 5, 20, 40, 50%, respectively, at 2 h. However, alkene rearrangement continued in reaction mixtures stored at 21°C regardless of the thiol used. Quenching the reactions with a molar excess of either maleic anhydride or propylene oxide halted the progression of alkene rearrangement.

3.9. Reaction temperature and alkene stability

At reaction temperatures of 40, 60 and 100°C, thiol **IX** sulfanyl production was complete when quenched at 12, 2.5 and 0.5 h, respectively. Alkene rearrangement was 20, 5 and <1%, respectively.

3.10. Effect of temperature on sulfanyl formation

Transformations of 9(10)-EpO-me with thiol **IX** at 20, 50 or 60°C were complete in ~5 days, 20 h or 2 h, respectively, as determined by TLC with phosphomolibdic acid visualization. The resulting UV-active spots from plates not treated with phosphomolibdic acid yielded the expected molecular mass of the deprotonated hydroxytetrafluorophenylsulfanyl derivative (i.e., 479 u) when analyzed by negative mode ESI-MS. Transformation of [^{14}C]epoxystearic acid was biphasic, with 20% conversion within 2 min followed by a linear rate of 0.8%/min with a calculated completion time of 1.5 h (% conversion min^{-1}: $y=0.0079x+0.205$, r^2 0.983). Complete conversion was documented at 5 h and no resolvable radioactive side products were observed.

3.11. Optimized epoxide thiolation reaction

Six matched samples containing a mixture of saturated and unsaturated fatty acid epoxides and diols were incubated at 100°C with ‘‘buffered’’ thiol **IX** and quenched with propylene oxide as described. Analysis of these samples by GC–MS revealed complete sulfanyl formation within 30 min for the 10(11)-EpHep and EpOME methyl esters, while alkene rearrangement was non-detectable and diols were unaffected (Fig. 10).

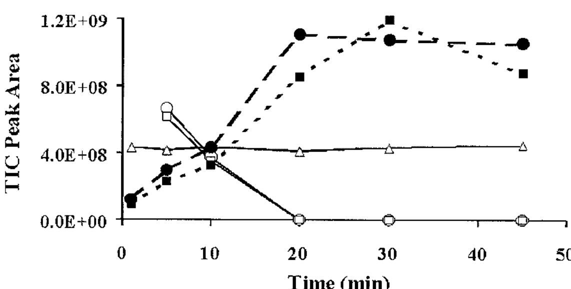

Fig. 10. Time dependence of 2,3,5,6-tetrafluorophenylsulfanyl (TFPS) formation under optimized reaction conditions. A mixture of epoxides and diols exposed to thiol **IX** at 100°C in methanol were quenched with propylene oxide at 0, 5, 10, 20, 30 and 45 min. After evaporative solvent removal, the residue was silylated and analyzed by GC–EI-MS. A plot of the time dependence of total ion chromatogram (TIC) peak areas are shown for 10-epoxyheptadecanoic acid (– ○ –), 10-TFPS-11-OTMS-heptadecanoic acid + 10-OTMS-11-TFPS-heptadecanoic acid (– • –), 9-epoxyoctadec-(12*Z*)-enoic acid (– □ –), 9-TFPS-OTMS-octadec-(12*Z*)-enoic acid + 9-OTMS-10-TFPS-octdec-(9*Z*)-enoic acid (– ■ –), 9,10-dihydroxyoctadec-(12*Z*)-enoic acid (– △ –). The behavior of the 12-epoxyoctadec-(9*Z*)-enoic acid and the 12,13-dihydroxyoctadec-(9*Z*)-enoic acid were identical to the displayed isomers.

3.12. Analytical calibration

Using selected ion monitoring and employing 2 μl injections, instrumental detection limits for silylated diols of saturated and monounsaturated lipid methyl esters were ~20 pg/μl, while the corresponding OTMS-TFPS derivatives could be clearly observed with >3:1 signal-to-noise ratio at 5 pg/μl. These curves appeared linear (r^2>0.997–0.999) through 20 ng/μl, however close inspection of the lines between 0.005 and 2.0 ng/μl revealed a slight positive inflection, which was best fit by a quadratic equation (r^2=0.999–1.000).

4. Discussion

The importance of epoxylipids in biology is evidenced by the diversity of analytical techniques applied to their detection and quantification [2–4,6,7,17–20,22–24,28,30,31]. Unfortunately, epoxy- and dihydroxylipid quantification requires regioisomer separation before analysis [2,4] or the discrimination of some isomers is sacrificed [18]. The goal of this study was to identify a derivatization scheme, allowing the sensitive discrimination of epoxylipid isomers and their corresponding diols in a single GC–EI-MS analytical run. Thiols are soft nucleophiles that readily react with soft electrophiles like epoxides to produce hydroxysulfanyls [21,31,32,38,46]. Here we evaluated the thiols listed in Table 2 as epoxide derivatizing agents, emphasizing their influence on the EI-MS fragmentation, chromatographic resolution and alkene stability in polyunsaturated lipid epoxides. In addition, we examined the effect of these derivatizing agents on the corresponding diols and assessed multiple hydroxy group derivatization procedures to allow the simultaneous detection of hydroxysulfanyls and diols.

Initial thiol evaluations showed that the choice of thiol affected fragmentation behavior, reaction rate and side product formation. For instance, 2-mercaptoethanol (thiol **II**) rapidly transformed epoxylipids but produced weaker characteristic ions than other thiols, and alkene isomerization was difficulty to control for this basic thiol under the initial conditions. On the other hand, all of the thiols listed in Table 2 efficiently derivatized epoxides. Therefore, other thiols and conditions may prove to be optimal for other epoxide containing compounds. For the lipid sulfanyls produced using thiols without secondary ionizable groups, fragmentation around the OTMS-SR moiety yielded three product ions containing either the silanyloxy and/or the methyl ester function, which clearly defined the epoxide position and the molecular mass of the lipid precursor (Fig. 3). In addition, an alkene(s) adjacent to the trimethylsilyl ether promoted ‘Cα cleavage. Specifically, as the silyl ether moved into the zone of unsaturation the Cα:‘Cα ratio was inverted; ~5:60, 20:20, ~65:15, 100:0 for type A, type B, type C and type D, respectively (Figs. 5–8). This behavior is consistent with the proposed isomer stabilization mechanism described in Fig. 3 and provides a partial explanation for the high ‘Cα cleavage in the EpOME-me Type A products. While the low ‘Cα fragmentation in the sulfanyls from other thiols is still unclear, energy dissipation through the Sα,Oα pathway may be higher for less acidic thiols.

While the product of Sα,Oα cleavage identifies the molecular mass of the parent lipid, it is produced by all regioisomers. Therefore, minimization of the Sα,Oα fragmentation pathway will enhance the sensitivity of a selected ion monitoring based detection for these partially resolved derivatives. Identification of the thioether cleavage product (Sα) in the spectra of type C and type D EpETrE-me sulfanyls (Fig. 7, Fig. 8) suggests that sulfanyl cleavage initiates formation of the Sα,Oα ion with subsequent bond rearrangement and expulsion of the silyl ether (Oα). The negative slope of the SαOα:Cα regression with respect to the substituted benzenethiol Taft–Hammet sigma constants indicate that electron withdrawal from the sulfur promotes Cα fragmentation at the expense of Sα,Oα cleavage. This finding is consistent with the proton transfer event proposed in Fig. 3, with acidification of the sulfur by electron-withdrawing R-groups creating an energetic barrier to the entropically favorable Sα,Oα pathway.

The choice of thiol and structure of the resulting product also affected the chromatographic properties of the derivatives (Fig. 9). Sulfanyl elution roughly correlated with product mass but was modified by factors increasing product volatility such as fluorination and hydroxy group silylation. The relative

elution order of the positional isomers was subtler, but is generally described by the EpETrE-me OTMS-TFPS derivatives. First, alkene nested sulfanyls (i.e. types B and C) elute before sulfanyls bordered by unsaturated chains. This was also true for the dihydroxy-OTMS derivatives (Table 3). Secondly, for the products of a single epoxide regioisomer, the sulfanyl with the thiol closest to the zone of unsaturation always eluted first (Table 4).

Based on the fragmentation behavior alone, highly fluorinated benzenethiols are viable reagents for the detection of epoxylipids. However, the observed transesterification indicates that this procedure should not be used if ester integrity is required. In addition, the maintenance of alkene integrity required additional precautions. At room temperature using thiols with a pK_a of ≥ 6.4, sulfanyl generation was rapid and efficient but alkene isomerization was extensive. Using thiols with a $pK_a < 6$ reduced both isomerization and reaction efficiency. A mechanistic evaluation of these two reactions reveals hydroxysulfanyl formation to be a bi-molecular condensation reaction, while proton abstraction drives alkene isomerization. Therefore, isomerization is entropically neutral while sulfanyl formation is entropically unfavorable and temperature sensitive ($\Delta G = \Delta H - T\Delta S$). In addition, the rate of proton abstraction is inversely related to thiol nucleophilicity. Therefore, it was theorized that an acidic thiol in a buffered thiol–thiolate solution at elevated temperatures would maximize sulfanyl formation and minimize alkene isomerization. This was in fact true, however if mixtures were not immediately analyzed isomerization continued. To eliminate alkene isomerization after temperature reduction, maleic anhydride and propylene oxide were evaluated as thiol scavengers. While both reagents halted alkene rearrangement, the use of propylene oxide allowed a simpler sample clean up, consisting of evaporative removal of excess reagent. Finally, using the propylene oxide quenching scheme along with a buffered solution of tetrafluorobenzenethiolate and a reaction temperature of 100°C, complete epoxide derivatization occurred in <30 min and isomerization was kept below 1%.

Using this optimized procedure, 50 mg of each of the tested 17 and 18 carbon epoxides listed in Table 1 was synthesized and calibration solutions were prepared for GC–MS analysis. Using selected ion monitoring an instrumental detection limit of 5 pg/μl was observed for these OTMS-TFPS derivatives. In addition, response factors were essentially linear through 20 ng/μl. Therefore, epoxide derivatization with thiol **IX** yielded instrumental sensitivities for octadecanoids comparable to the 4 pg/μl and 2 pg injected detection limits reported for eicosanoid pentafluorobenzyl esters [2] and fluorescent esters [18], respectively. Considering the similarity in fragmentation between the EpOME-me and EpETrE-me derivatives, it is likely that the EpETrE-me OTMS-TFPS derivatives will yield similar detection limits, with the possible exception of the type B compounds. While the sensitivity for silylated diols was considerably lower, these compounds are generally found at higher concentrations than the epoxides in biological samples [2,4]. In addition, the similarity in derivatization rates for the unsaturated vs. saturated epoxides and diols, and the resolution of the odd chain fatty acid epoxide and diol products from the even chain length products indicate that these compounds will provide suitable analytical surrogates as long as alkene oxidation is prevented.

5. Conclusion

In this study we have identified 2,3,5,6-tetrafluorobenzenethiol as an optimal derivatizing agent for the GC–EI-MS analysis of α,β-disubstituted epoxy fatty acids. The acidic nature of this thiol not only promotes favorable mass spectral fragmentation, but also reduces thiolate induced alkene isomerization to a manageable side reaction, which can be eliminated by the introduction of a thiol scavenger. In addition, at elevated temperatures this reagent efficiently transforms the epoxide to vicinal hydroxysulfanyls and has no effect on the structural integrity of dihydroxy fatty acids present in the reaction mixture. Subsequent silylation of both hydroxysulfanyls and dihydroxy compounds allow the simultaneous identification of both species while clearly discriminating regioisomers. The obtained instrumental detection limits suggest that this approach has the necessary sensitivity for the determination of endogenous

epoxide and diol fatty acids in biological samples, if significant matrix interferences are not encountered. Studies currently underway are applying the described procedure to a diverse array of biological fluids and preliminary results are promising. Therefore this technique has potential as a simplified method for the quantitative analysis of fatty acid epoxides and diols.

Acknowledgements

The authors would like to thank Dr. A.D. Jones of Pennsylvania State University for early inspiration for this study and Drs. C. Morisseau, J. Lango, T. Watanabe and G. Zurek of UC Davis for helpful discussion and critique of this manuscript. NIEHS Training Grant T32 ES07059 supported J.W.N. Research support was supplied by: NIEHS R01 ES02710, NIEHS Superfund Basic Research Program P42 ES04699, NIEHS Center for Environmental Health Sciences P30 ES05707, and USDA Competitive Research Grant 2001-35302-09919.

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