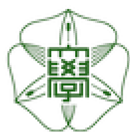

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Enantioseparation of hydroxyeicosatetraenoic acids by hydroxypropyl-γ-cyclodextrin-modified micellar electrokinetic chromatography

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Abbreviations: HETE, hydroxyeicosatetraenoic acid; **HP-γ-CD,** hydroxypropyl-γ-cyclodextrin; **HpETE**, hydroperoxyeicosatetraenoic acid; **LOX**, lipoxygenase

Keywords:

Enantioseparation / *Gracilaria* / Hydroxyeicosatetraenoic acid / Hydroxypropyl-γ-cyclodextrin / Micellar electrokinetic chromatography

(Abstract)

Complete resolution of hydroxyeicosatetraenoic acid (HETE) enantiomers was achieved using hydroxypropyl-γ-cycodextrin (HP-γ-CD)-modified MEKC. The optimum running conditions were determined to be utilizing a 30 mM phosphate-15 mM borate buffer (pH 9.0) containing 30 mM HP-γ-CD and 75 mM SDS as the BGE, application of +30 kV as the effective voltage, and carrying out the experiment at 15°C. The eluents were detected at 235 nm. The method was used successfully for the simultaneous separations of (*S*)- and (*R*)-enantiomers of regioisomeric 8-, 11-, 12-, and 15-HETEs. Subsequently, the optimized method was applied to evaluate the stereochemistry of 8- and 12-HETEs from the marine red algae, *Gracilaria vermiculophylla* and *Gracilaria arcuata,* respectively. The 8-HETE was found to be a mixture of 98% (*R*)-enantiomer and 2% (*S*)-enantiomer, while the 12-HETE was a mixture of 98% (*S*)-enantiomer and 2% (*R*)-enantiomer. The present study demonstrates that the HP-γ-CD-modified MEKC method is simple and sensitive and provides unambiguous information on the configuration of natural and synthetic HETEs.

1 Introduction

Hydroxyeicosatetraenoic acids (HETEs) are a group of endogenous eicosanoids that are generated in mammalian system by the action of multiple cellular oxygenases on arachidonic acid [1-7]. HETEs are widely recognized to have important physiological and pathological functions including angiogenesis [8], cancer growth and metastasis [9,10], neuronal apoptosis [11], and mitogenesis [12]. Lipoxygenases (LOXs) catalyze the conversion of arachidonic acid to hydroperoxyeicosatetraenoic acids (HpETEs) that are then reduced to the corresponding HETEs by glutathione transferases and peroxidases [13-15]. Four different LOXs are responsible for the production of regioisomeric 5-, 8-, 12-, and 15-HETEs [16,17]. Cytochrome P450 isozymes can also metabolize arachidonic acid to HETEs either by *bis*-allylic hydroxylation to generate 7-, 10-, 11-, 12-, 13-, and 15-HETEs [18,19], or by ω hydroxylation to yield 16-, 17-, 18-, 19-, and 20-HETEs [20,21]. Although pathways involving LOXs and cytochrome P450 isozymes are largely responsible for the formation of HETEs, the formation of 11- and 15-HETEs can also be facilitated by cyclooxygenases [22,23]. Reactions involving free radicals can also produce all the HETEs generated by the oxygenases.

A comprehensive analysis of natural and synthetic HETEs often requires separating both regioisomers and enantiomers. The regioisomer separation has frequently been performed using HPLC or GC on achiral stationary phases [5,24,25], whereas the enantiomer separation has been carried out using HPLC on chiral stationary phases [19,26-31]. Using chiral-phase HPLC with ultraviolet detection (HPLC-UV), Brash *et al.* [19] showed that *bis*-allylic HETEs from rat liver microsomes were readily rearranged to conjugated diene-containing HETEs under mildly acidic conditions (1% acetic acid), e.g. when the solvent in a

methanol/water/acetic acid (75:25:1, v/v/v) solution containing10(*S*)-HETE methyl ester was evaporated repeatedly at room temperature with a stream of N_2 gas, the main products were 8(*S*)-HETE methyl ester (*S*:*R* = 79:21) and 12(*R*)-HETE methyl ester (*R*:*S* = 78:22). The analysis of plasma samples obtained from patients before and after extracorporeal photoimmunotherapy was typically characterized by an increase in the total HETE levels, in particular, the level of 5-HETE, which was 80% of the total HETEs, and chiral-phase HPLC-UV revealed that 5-HETE was a racemate, suggesting that the majority of lipid peroxidation products were formed *via* non-enzymatic oxidations [30]. Employing the column-switching technique in the chiral-phase HPLC-MS/MS analysis of 12-HETE in human urine, Suzuki et al. [31] reported that the major enantiomer was 12(*S*)-HETE, indicating its production *via* 12(*S*)-LOX pathway. Furthermore, they also revealed that the levels of 12(*S*)-HETE were gender dependent.

Red algae are a rich source for oxylipins, and various oxidation products of C20 polyunsaturated fatty acids (e.g. arachidonic acid) have previously been found from these organisms [32,33]. Chiral-phase HPLC showed that the chilality of 13-HETE from *Chondrus crispus* was closed to the optical purity with enantiomeric ratio of *R*:*S* = 96:4 [29]. 8-HETE was also found from *Gracilaria vermiculophylla* [34], *Gracilaria chilensis* [35], and *Agardhiella subulata* [36] as the (*R*)-enantiomer, whereas 12-HETE was found from *Murrayella periclados*, *Gracilariopsis lemaneiformis*, *Platysiphonia miniata*, and *Cottoniella filamentosa* [37] as the (*S*)-enantiomer, where the stereochemistry was deduced by comparison of the optical rotations with those of the standards. In addition, 9-HETE was found from *Polyneura latissima* [38] as predominantly the (*S*)-enantiomer by GC-MS analysis of a menthoxycarbonyl derivative of dimethylmalate which was produced from the HETE by ozone degradation and methylation.

CE has proved to be a very powerful and effective analytical tool for chiral separations of real samples [39-44]. When compared to HPLC, CE offers higher enantioseparation efficiency, consumes lesser time, and is flexible, allowing the incorporation of various chiral selectors at different concentrations. Although CE continues to emerge as a useful tool for the analysis of lipids [45,46], the technique has not been used routinely in lipid research. Today enantiomer separation of chiral lipids has been carried out exclusively by chiral-phase HPLC, and so far almost no effective enantiomer separation of chiral lipids, including HETEs [47], has been achieved on CE. Thus, the aim of our study was to show the possibilities of CE for chiral analysis of lipids.

In the present study, we have developed a method for the chiral separation of HETEs using hydroxypropyl-γ-cyclodextrin (HP-γ-CD)-modified MEKC. In addition to the general advantages of CD-MEKC for enantiomer separations, namely, rapid method development, low consumption of analyte, minimal use of expensive chiral reagents and organic solvents, the present method has the merit of providing a simultaneous and complete separation of enantiomeric and regioisomeric 8-, 11-, 12-, and 15-HETEs, which is difficult with chiral-phase HPLC. The optimized method has been successfully applied to the determination of the stereoconfiguration of endogenous HETEs in the marine red algae, *G. vermiculophylla* and *G. arcuate.* The former species is known to produce some oxylipins including prostaglandins and HETEs [33], whereas no data on these compounds have been reported for the later species.

2 Materials and methods

2.1 Chemicals

Racemates of 8-, 11-, 12-, and 15-HETEs (Fig. 1) and the individual enantiomers were purchased from Cayman Chemical (Ann Arbor, MI, USA). SDS (biochemistry grade) and γ-CD (97% purity) were obtained from Wako Pure Chemicals (Osaka, Japan). HP-β-CD (average molar substitution: 0.8) and HP-γ-CD (average molar substitution: 0.6) were obtained from Aldrich (Milwaukee, WI, USA). All other chemicals used were of analytical reagent grade from Wako Pure Chemicals. Purified water obtained from an automatic water distillation apparatus (Aquarius® RFU554CA, Advantec, Tokyo, Japan) was used in the preparation of buffers and samples.

2.2 Purification of γ-CD

When the commercially available γ-CD was used without further purification, many noise peaks appeared in the electropherograms, while no unwanted peaks were detected from the HP-β-CD and HP-γ-CD reagents. Prior to its use, therefore, γ-CD was purified as follows: distilled water (100 mL) containing activated carbons (2.5 g) was boiled and cooled to room temperature. The water was replaced with fresh water and the same operation was repeated, discarding the water at the end. Next, distilled water (80 mL) was added and the mixture was heated to 80°C. Subsequently, γ-CD (25 g) was added to the heated mixture. The solution was vortexed to ensure proper mixing and then boiled for a few minutes. The solution was allowed to cool and then filtered through a 0.2 μm filter under reduced pressure. The residue was washed with water (20 mL). The filtrate was gradually added to a stirred solution of methanol (2 L) and allowed to rest overnight. The resulting turbid solution was centrifuged for 5 min at 3000 rpm. The supernatant was discarded, and the resulting γ-CD precipitate was dried in vacuum to a constant mass.

2.3 Apparatus for CE

Electrophoretic experiments were carried out using a G1600A CE system (Agilent Technologies, Santa Clara, CA, USA). Separation was performed in a fused silica bubble cell capillary (total length 96.5 cm × 50 μm i.d., effective length 88 cm), which increased several times in sensitivity compared to a conventional capillary with the same inner diameter. The capillary was flushed with the BGE for 20 min prior to running the separation experiments. Then, water, a sample solution, and water again were injected at pressure of 50 mbar for 2 sec. Between runs, the capillary was flushed with the BGE for 6 min. The capillary was maintained at 15°C throughout the experiment and the analytes (HETEs) were detected using the conjugated diene chromophore at 235 nm (ε = 23000)[26]. The power supply was operated in the constant-voltage mode (+30 kV) and the substances migrated towards the negative pole. The capillary was flushed with water (20 min) and methanol (20 min) on the completion of separation experiments. The resulting current was approximately 45 μA.

2.4 Buffer and sample preparation

BGE in the electrophoretic experiments, unless stated otherwise, was 30 mM phosphate-15 mM borate buffer (pH 9.0) containing 30 mM HP-γ-CD and 75 mM SDS. Prior to use the buffer was filtered through a 0.2 μm filter. Ethanolic stock solutions (100 μg/mL) of the racemates and enantiomers of 8-, 11-, 12-, and 15-HETEs were prepared separately. These solutions were stored at –30°C and diluted in methanol (final concentration of each enantiomer = 50 μg/mL) before use.

2.5 Red algae samples and preparation of HETEs

Two species of red algae, *Gracilaria vermiculophylla* and *Gracilaria arcuata*, were examined

in this study. Both species were collected from Shinori Beach (Hakodate, Hokkaido, Japan) in July, 2012 and Shiraho Beach (Ishigaki, Okinawa, Japan) in April, 2012, respectively. The algae were separated from epiphytic organisms and directly freeze-dried, following which they were powdered using a grinder. The lyophilized powders were stored at –30°C until use. Eicosanoids including HETEs were obtained from each alga using the procedures described by Nakajima et al. (48) with some modifications. The lyophilized powder (3 g) was suspended in distilled water (50 mL) and the mixture was incubated at 5°C for 1 h. Subsequently, the mixture was filtered under reduced pressure. The eicosanoids present in the aqueous solution were extracted into the organic layer (ethyl acetate, 50 mL). A stream of N_2 gas was used to evaporate the organic solvent and the residue was dissolved in methanol at 5 mg/mL to be used for analysis by HP-γ-CD-modified MEKC described in the previous section.

To determine the absolute configuration, HETEs were purified from the crude extract by conventional TLC and RP-HPLC (28,49,60) with some modifications. TLC was performed on silica gel 60 F_{254} plates (20×20 cm^2, Merck, Darmstadt, Germany) using ethyl acetate/benzene/acetic acid (50:50:2, v/v/v) as the developing solvent. The bands containing HETEs from *G. vermiculophylla* and *G. arcuata* were detected under UV, excised, and then extracted with chloroform/methanol (2:1, v/v). HETEs were purified further by RP-HPLC using D-7000 chromatograph (Hitachi High-Technologies, Tokyo, Japan) equipped with a DAD. A Mightysil RP-18 GP column (150 × 4.6 mm2 i.d., 3 μm particles, Kanto Chemical, Tokyo, Japan) at 10°C was used for the resolution. The mobile phase consisted of water/acetic acid = 100/0.02 (v/v; A) and ACN/acetic acid = 100/0.02 (v/v; B) in the isocratic mode: A/B = 30:70 (v/v) at 0.3 mL/min. The purified HETEs were dissolved in methanol (1.0 mg/mL) for analysis by CD-MEKC.

3 Results and discussion

3.1 Factors influencing separation

VanRollins and VanderNoot [50] investigated the enantioseparation of *cis*-epoxyeicosatrienoic acids (EETs), a class of arachidonate products. Three enantiomer pairs, 8(*S*)-9(*R*) / 8(*R*)-9(*S*), 11(*S*)-12(*R*) / 11(*R*)-12(*S*), and 14(*S*)-15(*R*) / 14(*R*)-15(*S*), were successfully separated by CE using a mixture of β-CD and β-CD-sulfobutyl ether. However, a long analysis time (> 100 min), due to the slow mobility of EOF by the addition of high concentration of organic solvent [45], was a major drawback. To overcome this, we employed another CE-based method, MEKC, for the enantioseparation of 8-, 11-, 12-, and 15-HETEs. As demonstrated by Terabe *et al.* [51,52], introduction of micelles, which serves as a pseudostationary phase, into the separation electrolyte results in the separation of both neutral and ionic species. That is, the distribution coefficient of the solute between the pseudostationary phase and the surrounding aqueous phase determines the relative migration order. Chiral compounds have been successfully separated by MEKC when CDs have been added to the buffer solution [53-55]. CDs and their derivatives have been widely used in CE for the separation of several mixtures of enantiomeric compounds. In this study, a mixture of racemic 8-, 11-, 12-, and 15-HETEs was analyzed by MEKC using 30 mM phosphate-15 mM borate buffer (pH 9.0) containing 75 mM SDS as the BGE in the presence or absence of CDs (Fig. 2). In the absence of CD, three peaks were observed; 15-HETEs at 73 min, 11- and 12-HETEs at 74 min, and 8-HETEs at 74.5 min (Fig. 2A). These longer migration times indicate that these HETEs are practically incorporated into the SDS micelles. The influence of the CD type on the enantioseparation of 8-, 11-, 12-, and 15-HETEs was also investigated by

MEKC using 30 mM phosphate-15 mM borate buffer (pH 9.0) containing 75 mM SDS as BGE in the presence of various CD-based chiral selectors (HP-β-CD, γ-CD, or HP-γ-CD at 30 mM; Fig. 2B-D). With HP-β-CD as the additive, migration times of these HETEs were significantly faster than those in the absence of CD; however, no enantioseparation was observed (Fig. 2B). With the inclusion of γ-CD, 11- and 12-HETEs were enantioseparated, whereas 8- and 15-HETEs were not (Fig. 2C). However, when HP-γ-CD was used as the additive, the four HETEs were effectively resolved (Fig. 2D).

Figure 3 shows the effect of the concentration of HP-γ-CD (20 – 40 mM) in BGE on the resolution of enantiomeric and regioiomeric HETEs. An increase in the concentration of HP-γ-CD reduced the migration times of HETEs. Complete resolution of the 8 isomeric HETEs examined was achieved on both concentrations of 20 mM and 30 mM HP-γ-CD (Fig. 3A and B), whereas lesser resolution was observed for 8(*S*)- and 8(*R*)-HETEs on 40 mM HP-γ-CD (Fig. 3C). Thus, the optimal concentration of HP-γ-CD, i.e., 30 mM, was selected for all subsequent experiments. The (*S*)-enantiomers of these HETEs moved faster than the corresponding (*R*)-enantiomers. Various chiral compounds have been separated by CD-MEKC and the separation mechanism has been discussed [56-59]. Terabe *et al.* [56] have suggested that, in CD-MEKC, an analyte is distributed among three phases, the micellar phase, the CD phase, and the aqueous phase that exclude the micelles and the CD. Here, the CD phase is not a true phase. As HP-γ-CD is electrically neutral, it migrates at the same velocity as the bulk solution. The distribution of the analyte between the micelles and the non-micellar aqueous phase including HP-γ-CD directly affects the resolution. Addition of HP-γ-CD to the micellar solution reduces the quantity of the analyte partitioned into the micelles by increasing the fraction of the analyte in the non-micellar aqueous phase. This indicates that the

(*S*)-enantiomers of HETEs form stronger diastereomer complexes with HP-γ-CD than the corresponding (*R*)-enantiomers.

An increase in the concentration of SDS from 50 to 100 mM significantly increased the migration times of HETEs, while marginally increasing the resolution of HETE enantiomers (Fig. 4). This cannot be attributed to an increase in ionic strength because EOF did not change significantly over the range of SDS concentration. The partition of the solute between the CD and the micelles depends on the SDS concentration. This suggests that the longer migration times are the result of an increase in the ratio of the micellar phase to the aqueous-CD phase.

The effect of pH (8.0−10.0) of the BGE on peak resolution (*Rs*) and the migration times of the HETE isomers was also investigated (Fig. 5). The migration times decreased marginally with increasing pH, while the *Rs* values of HETE enantiomers were not influenced by variations in studied pH range.

A decrease in the capillary temperature from 25°C to 15°C increased the migration times of HETEs as expected and marginally increased the resolution of enantiomers. However, the peaks due to 11(*R*)- and 12(*S*)-HETEs at 20°C were observed to be close, and at 25°C, they overlapped (see Supporting Information). Heuermann and Blaschke [60] rationalized that the observed increase in *Rs* value with a decrease in temperature could be attributed to the decrease in rotational and/or vibrational energies, thereby allowing for one enantiomer to be bound inside or by the rim of CD for a longer duration, thereby, increasing the enantioselectivity. The longer migration time at lower temperature can increase the likelihood of interactions between the analyte enantiomers and HP-γ-CD.

3.2 Analytical characteristics of the method

From the above-mentioned results, the optimum conditions, i.e., the conditions giving both

high resolution and short migration time, were determined to be the use of 30 mM phosphate-15 mM borate buffer (pH 9.0) containing 30 mM HP-γ-CD and 75 mM SDS as the BGE and the application of +30 kV at 15°C. Under these conditions, all enantiomers were separated within 35 min with good resolution (*R*s: 1.45, 5.51, 7.69, and 2.02 for 8-, 11-, 12-, and 15-HETEs, respectively) and high peak efficiency (N > 650000).

Table 1 gives the CD-MEKC data on the HETE racemates obtained under the optimal conditions. The LOD of each enantiomer of HETE, defined as an S/N ratio of 3, was determined to be 0.89−0.99 μg/mL, and the LOQ, defined by an S/N of 10, was 5.95−6.62 μg/mL. Five replicates runs, 50 μg/mL for each enantiomer of HETE, revealed good reproducibility for the peak areas (RSD = 3.2−4.1%) and elution times (RSD < 0.1%). Calibration curves for the four HETEs were plotted using seven different concentrations (10 −400 μg/mL; in triplicate) of racemic mixtures of each regioisomer. Good linearity was observed with all enantiomers, namely the correlation coefficients were > 0.99 ($r^2 > 0.99$).

3.3 HETE profile of red algae

Figure 6 shows the electropherograms of the crude extracts obtained from the marine red algae *G. vermiculophylla* and *G. arcuata*. Unlike the HETE standards, the two extracts gave no clear electropherograms, however, peaks corresponding to 8(*R*)-HETE and 12(*S*)-HETE, confirmed by comparing with the respective standards, were observed in pherograms of the extracts of *G. vermiculophylla* and *G. arcuata*, respectively. The amounts of 8(*R*)- and 12(*S*)-HETEs, determined using calibration curves, were 31.4 μg/g and 3.75 μg/g wet mass (mean, n = 3), respectively, at dry weights of the algae (Table 2). A higher level of 8-HETE (230 μg/g wet mass) in the red alga *Gracilaria asiatica* (= *G. vermiculophyl1a*) has been quantified by reversed-phase HPLC-MS [61]. Such a difference seen between the same

species is probably due to the incubation conditions to produce HETEs or environmental factors such as habitat and season.

Enantiomeric ratios of 8-HETE from *G. vermiculophylla* and 12-HETE from *G. arcuata* were determined by analyzing the pure HETEs isolated from the crude extracts. The 8-HETE was found to be a mixture of 98% (*R*)-enantiomer and 2% (*S*)-enantiomer, while the 12-HETE was a mixture of 98% (*S*)-enantiomer and 2% (*R*)-enantiomer (Fig. 7). A small amount of one enantiomer in the presence of a high amount of the other enantiomer has been reported for 9-HETE (93% *S* and 7% *R*) from the red alga *Polyneura latissimi* [38]. Coexistence of the (*R*)- and (*S*)-enantiomers suggests that these algae possess both of the corresponding (*R*)- and (*S*)-LOXs to produce enantiomeric HpETEs derived from arachidonic acid that is released from glycerolipids (glyco- and phospholipids) by acyl-hydrolase [62].

HPLC-MS has separated and identified phosphatidylcholine esterified hydroperoxides and hydroxides of linoleic and arachidonic acids from oxidized plasma lipoproteins and atheroma tissues [63]. The algal lipids examined may also contain glycerolipid-esterified HpETEs and HETEs, which are hydrolyzed to produce the corresponding free non-esterified HpETEs and HETEs, respectively. In this study, almost no HpETEs were found in the two algal species by reversed-phase HPLC (data not shown), suggesting rapid enzymatic reduction of HpETEs to HETEs under the incubation conditions used. The occurrence of HETE- or HpETE-containing oxo-glycerolipids along with free non-esterified HpETEs in the algal lipids is now under investigation using CE and HPLC.

Concluding remarks

To our knowledge, this is the first report on the separation and quantification of enantiomeric HETEs by CE. Using HP-γ-CD as a chiral selector, simultaneous separation of four racemates

(8-, 11-, 12-, and 15-HETEs) into their enantiomers was achieved under optimized conditions. Parameters optimized include the type and concentration of CD, concentration of SDS, pH of BGE, capillary temperature and applied voltage. The proposed method was used to successfully determine the enantiomeric composition of the HETEs found from red algae. Thus, the present study demonstrates that the HP-γ-CD MEKC method could be utilized widely for the detection, identification and quantification of enantiomeric and regioisomeric HETEs from biological samples.

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The authors have declared no conflict of interest.

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Figure legends

Figure 1. Structures of 8-, 11-, 12-, and 15-hydroxyeicosatetraenoic acids (HETEs). *: asymmetric carbon.

Figure 2. Electropherograms of the HETE enantiomers obtained by MEKC in the presence/absence of CDs. BGE used in these analyses was 30 mM phosphate – 15 mM borate buffer (pH 9.0) containing 75 mM SDS (A), along with 30 mM HP-β-CD (B), 30 mM γ-CD (C) or 30 mM HP-γ-CD (D). **1**: 11(*S*)-HETE, **2**: 11(*R*)-HETE, **3**: 12(*S*)-HETE, **4**: 12(*R*)-HETE, **5**: 15(*S*)-HETE, **6**: 15(*R*)-HETE, **7**: 8(*S*)-HETE, and **8**: 8(*R*)-HETE.

Figure 3. Dependence of resolution and migration times of HETEs on the HP-γ-CD concentration. BGE used in these analyses was 30 mM phosphate – 15 mM borate buffer (pH 9.0) containing 75 mM SDS and varying concentrations of HP-γ-CD: (A) 20 mM, (B) 30 mM, and (C) 40 mM. **1**: 11(*S*)-HETE, **2**: 11(*R*)-HETE, **3**: 12(*S*)-HETE, **4**: 12(*R*)-HETE, **5**: 15(*S*)-HETE, **6**: 15(*R*)-HETE, **7**: 8(*S*)-HETE, and **8**: 8(*R*)-HETE.

Figure 4. Dependence of resolution and migration times of HETEs on SDS concentration. BGE used in these analyses was 30 mM phosphate – 15 mM borate buffer (pH 9.0) containing 30 mM HP-γ-CD and varying concentrations of SDS: (A) 50 mM, (B) 75 mM, and (C) 100 mM. **1**: 11(*S*)-HETE, **2**: 11(*R*)-HETE, **3**: 12(*S*)-HETE, **4**: 12(*R*)-HETE, **5**: 15(*S*)-HETE, **6**: 15(*R*)-HETE, **7**: 8(*S*)-HETE, and **8**: 8(*R*)-HETE.

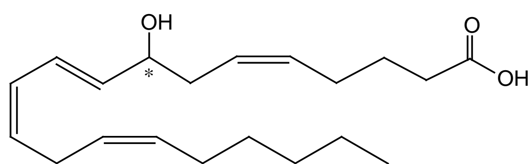
Figure 5. Effect of pH on the resolution and migration times of HETEs. BGE used in these

analyses was a mixture of 30 mM HP-γ-CD, 75 mM SDS and 30 mM phosphate – 15 mM borate buffer at pH 8.0 (A), 9.0 (B), and 10.0 (C). **1**: 11(*S*)-HETE, **2**: 11(*R*)-HETE, **3**: 12(*S*)-HETE, **4**: 12(*R*)-HETE, **5**: 15(*S*)-HETE, **6**: 15(*R*)-HETE, **7**: 8(*S*)-HETE, and **8**: 8(*R*)-HETE.

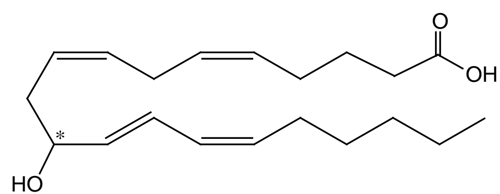
Figure 6. Electropherograms of the crude extracts obtained from the red algae *G. vermiculophylla* (A) and *G. arcuata* (B). **3**: 12(*S*)-HETE, **8**: 8(*R*)-HETE. CD-MEKC conditions used were identical to those used for the identification of electropherogram shown in Figure 2D.

Figure 7. Electropherograms of 8-HETE and 12-HETE isolated from the crude extracts of *G. vermiculophylla* (A) and *G. arcuate* (B), respectively. **7**: 8(*S*)-HETE, **8**: 8(*R*)-HETE; **3**: 12(*S*)-HETE, and **4**: 12(*R*)-HETE. CD-MEKC conditions used were identical to those used for the generation of electropherogram shown in Figure 2D.

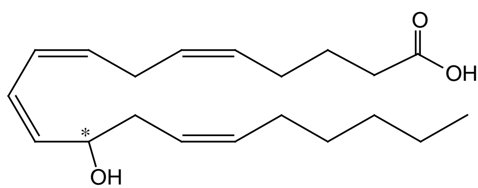
Figure 1S. Effect of column temperature on the separation and migration times of HETE enantiomers. (A) 15°C, (B) 20°C, (C) 25°C. Peak numbers correspond to those on Figure 2.



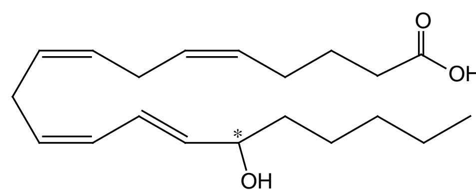
8-HETE



11-HETE



12-HETE



15-HETE

Figure 1

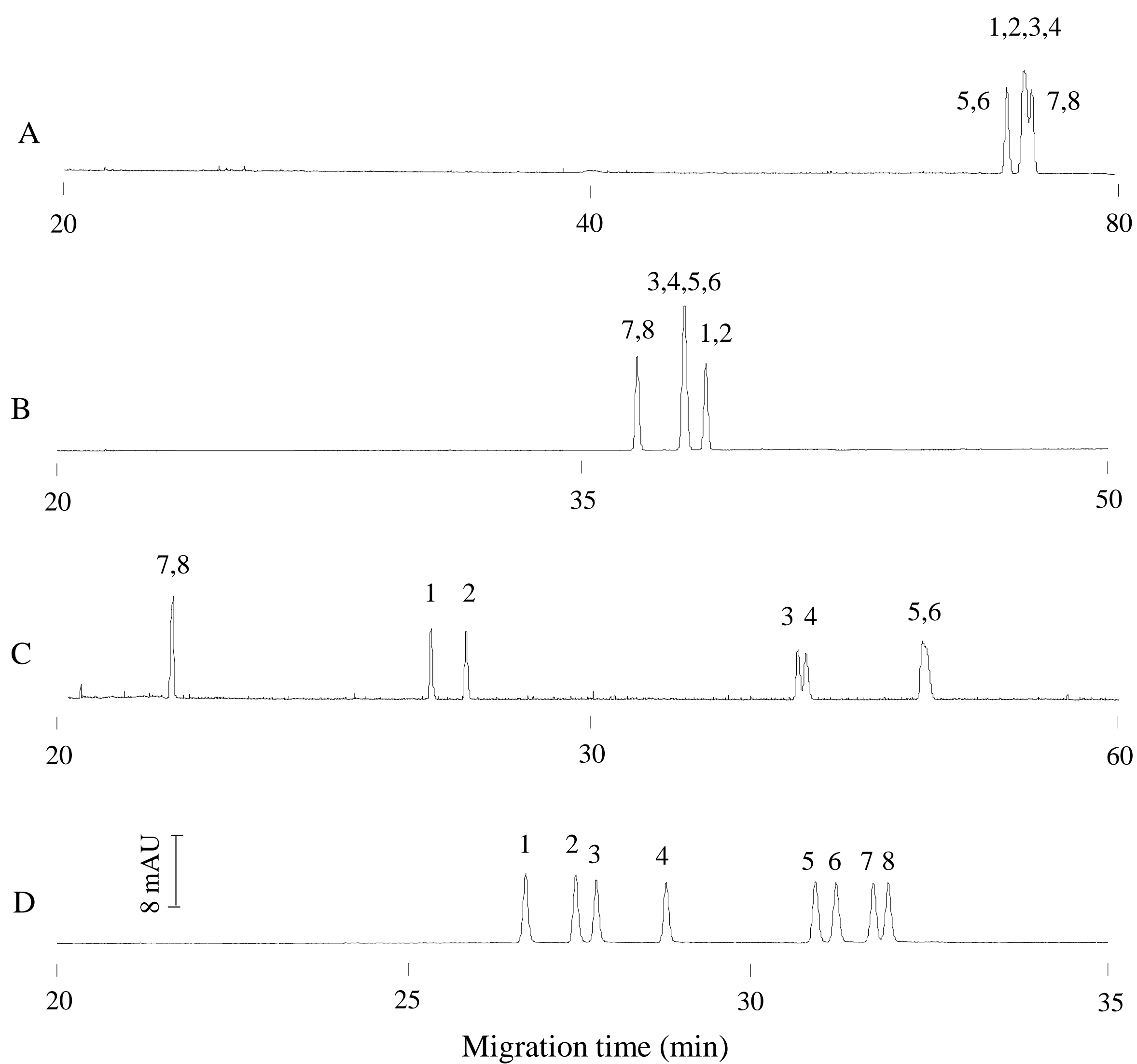


Figure 2

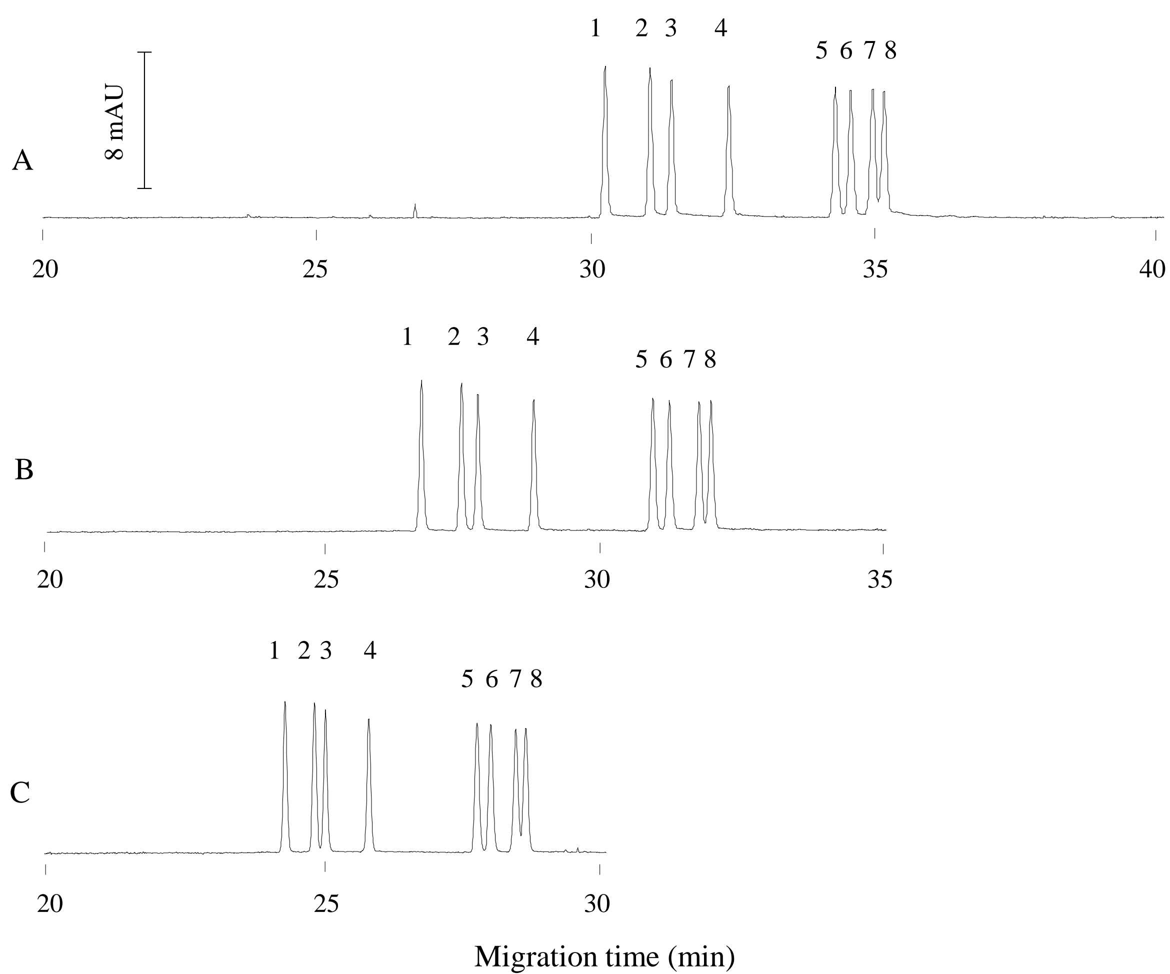


Figure 3

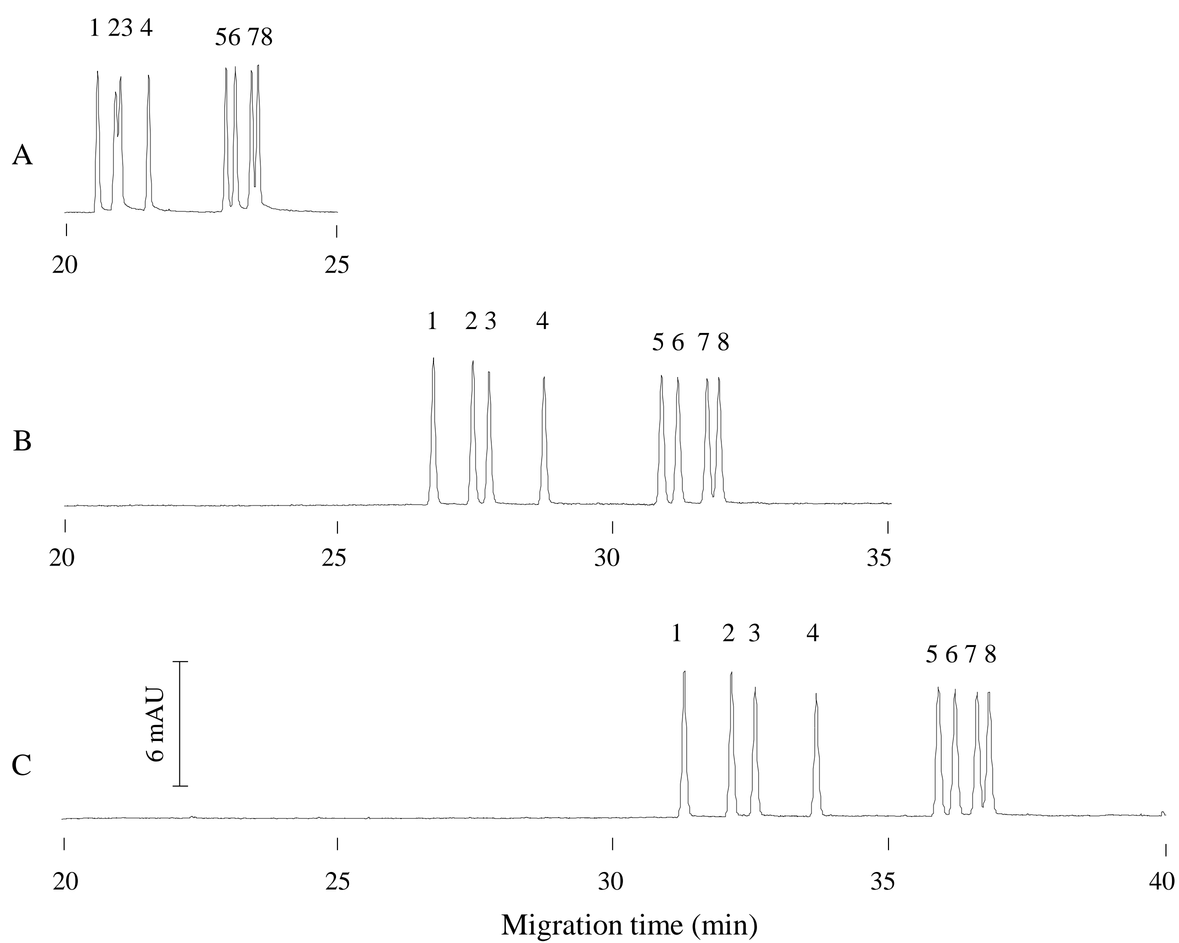


Figure 4

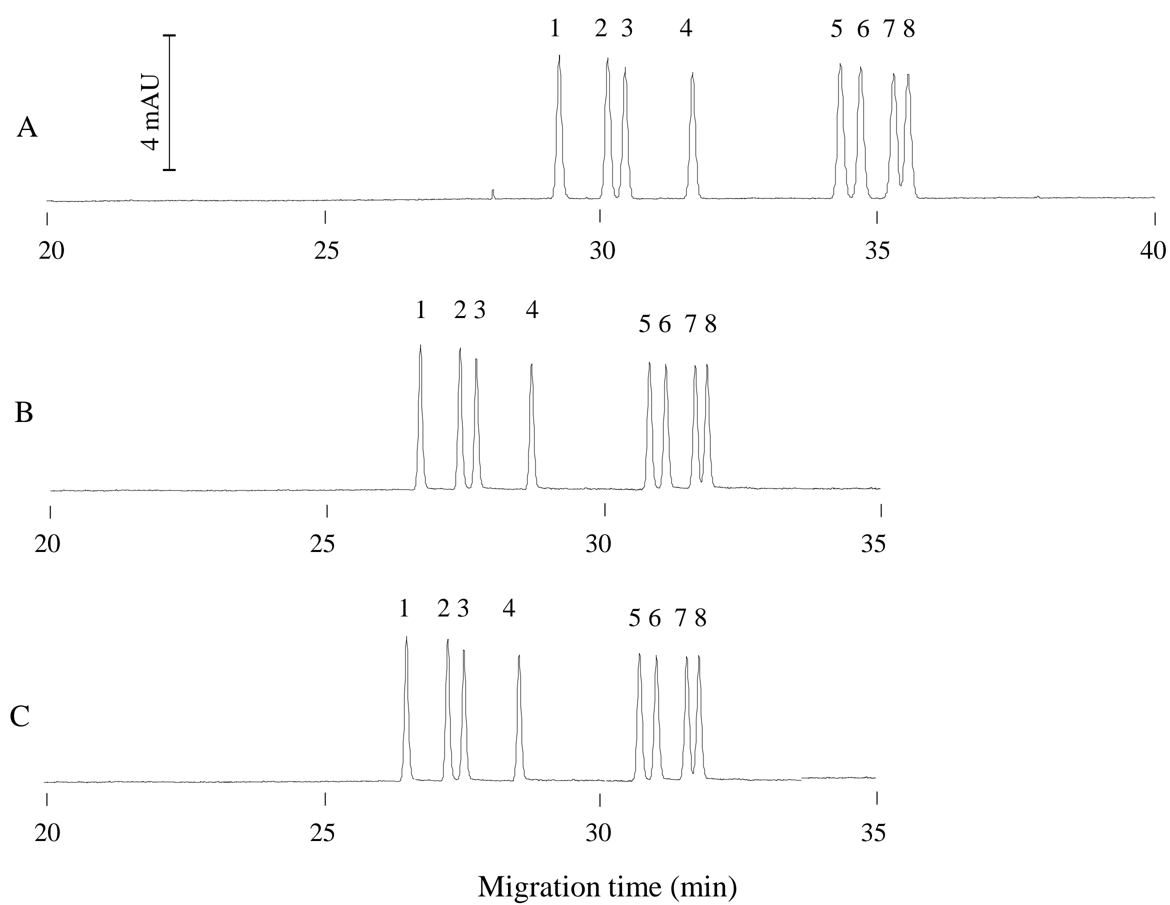


Figure 5

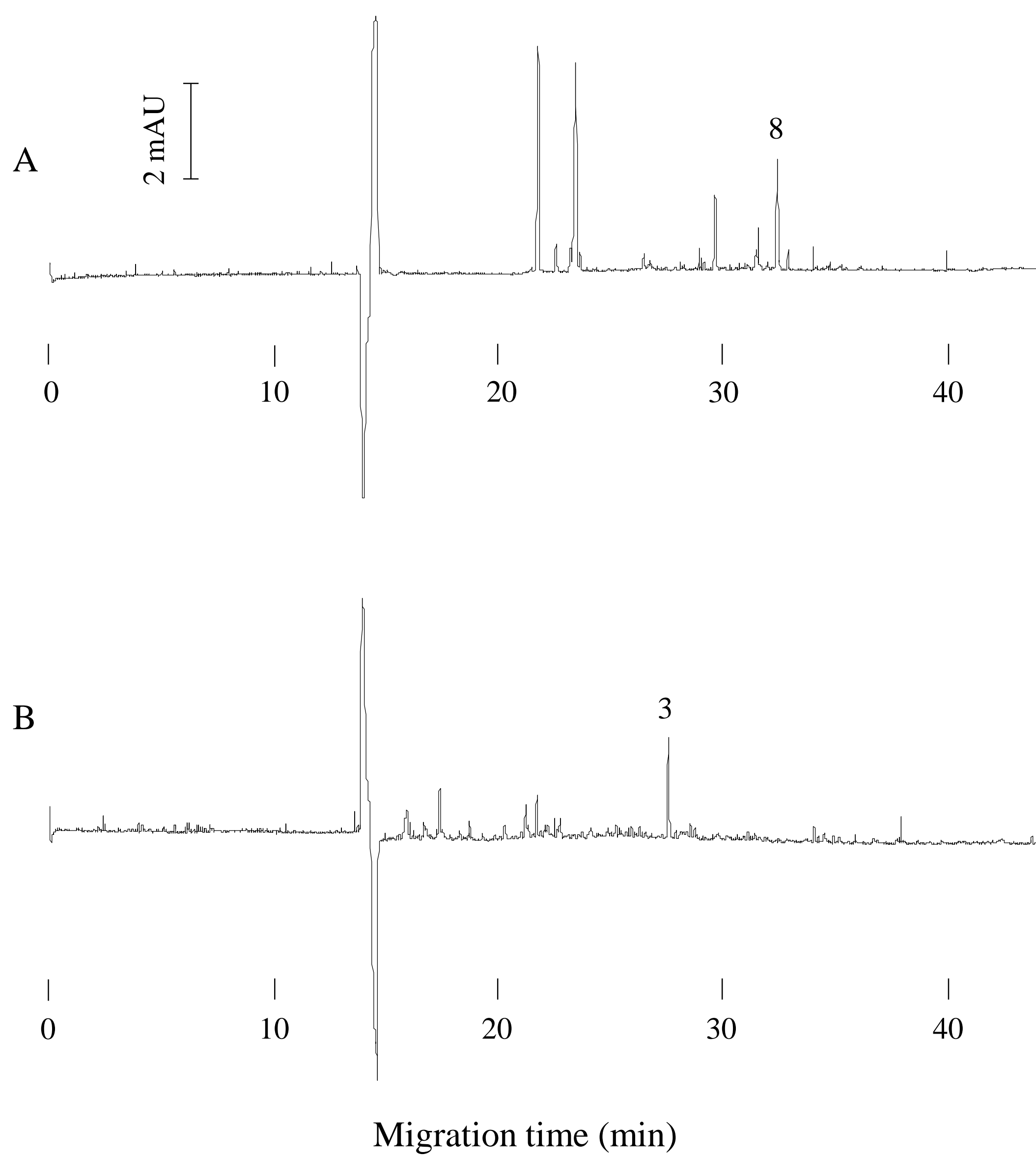


Figure 6

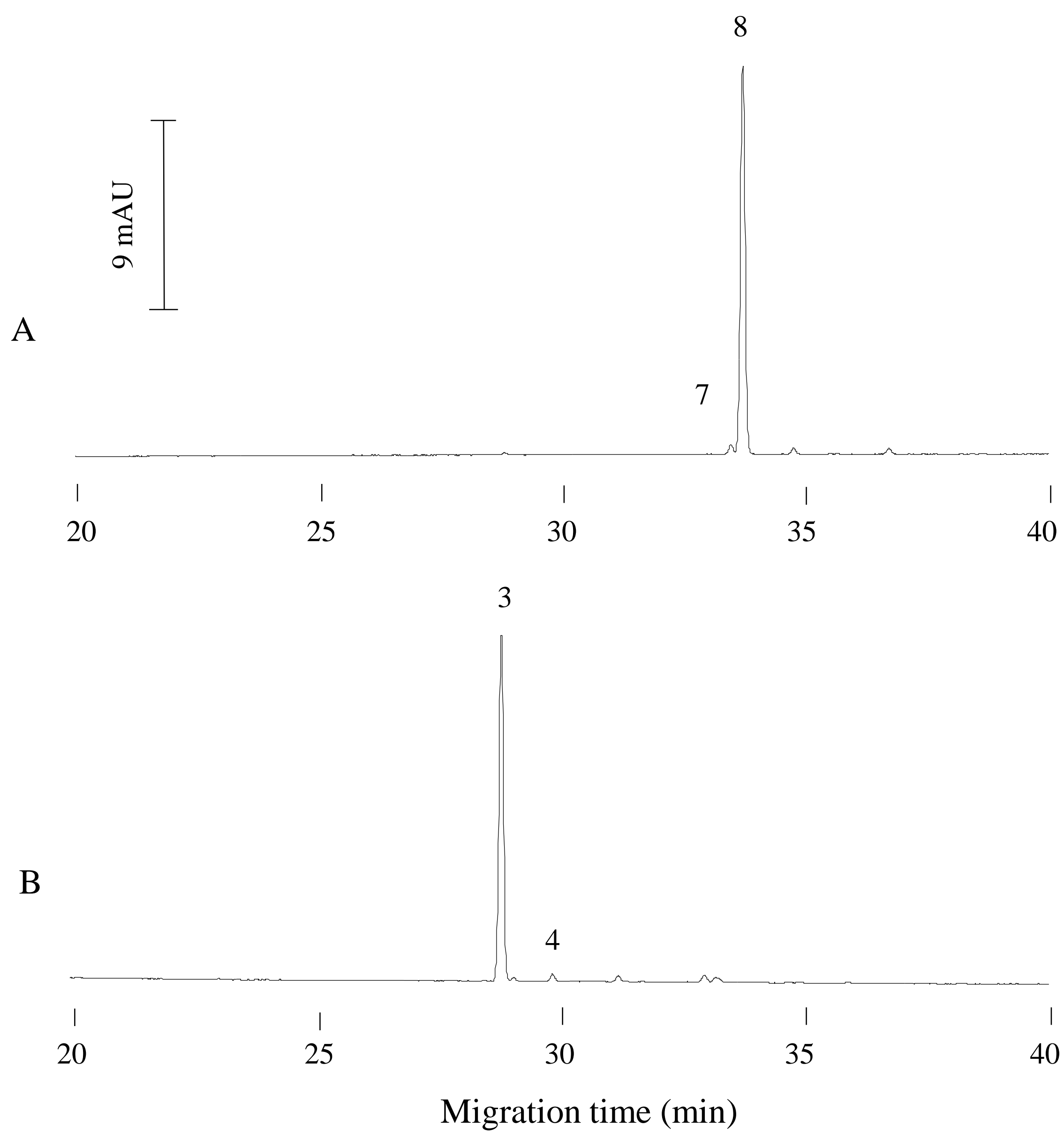


Figure 7

Table 1. Hydroxypropyl-γ-CD-modified MEKC data on HETE racemates.

HETE		Regression equation[a)]	Coefficient of determination (R^2)	LOD (S/N = 3) μg/mL	LOQ (S/N = 10) μg/mL	RSD, % (n = 5)		Peak area %[b]
						Migration time	Peak area	
11-	*S*	y = 0.9809x + 1.4758	0.9991	0.89	2.98	0.065	3.32	49.8 ± 0.3
	R	y = 0.9918x + 1.5568	0.9990	0.92	3.10	0.065	3.25	50.2 ± 0.3
12-	*S*	y = 0.9579x – 0.1304	0.9990	0.95	3.18	0.063	3.79	49.4 ± 0.4
	R	y = 0.9390x – 0.4064	0.9988	0.99	3.31	0.068	3.84	50.6 ± 0.4
15-	*S*	y = 0.9579x – 0.1304	0.9997	0.89	2.98	0.062	3.78	50.0 ± 0.2
	R	y = 0.9884x + 2.2792	0.9997	0.92	3.05	0.065	4.04	50.0 ± 0.2
8-	*S*	y = 0.9895x + 2.2710	0.9992	0.98	3.27	0.067	4.05	49.8 ± 0.2
	R	y = 1.2943x – 1.2514	0.9992	0.99	3.31	0.070	3.88	50.2 ± 0.2

a) y is the peak area, and x is the concentration (5−200 μg/mL).

b) Mean ± SD (n = 21).

Table 2. Quantitative analysis of 8(*R*)- and 12(*S*)-HETEs from *G. vermiculophylla* and *G. arcuata*, respectively, by HP-γ-CD-modified MEKC

Alga	Peak area (mAUs)	μg/mL[a)]	Total extract (mg)	μg/mg extract	μg/g wet alga
G. vermiculophylla	46.23 ± 1.27	36.81 ± 0.96	12.8	7.36 ± 0.19	31.41 ± 0.83
G. arcuata	19.17 ± 0.98	20.84 ± 1.05	2.7	4.17 ± 0.21	3.75 ± 0.19

[a)] Calculated using the calibration curve shown in Table 1. Mean ± SD (n = 3).

Supporting Information