Published in final edited form as:

J Chromatogr A. 2007 May 18; 1149(2): 305–311.

Direct and indirect high-performance liquid chromatography enantioseparation of *trans*-4-hydroxy-2-nonenic acid

Jiri Brichac^{a,1}, Ales Honzatko^a, and Matthew J. Picklo Sr.^{a,b,*}

^a Department of Pharmacology, Physiology, and Therapeutics, University of North Dakota, Grand Forks, ND 58203-9024, USA

^b Department of Chemistry, University of North Dakota, Grand Forks, ND 58203-9024, USA

Abstract

trans-4-Hydroxy-2-nonenic acid (HNEA) is a marker of lipid peroxidation resulting from the metabolism of *trans*-4-hydroxy-2-nonenal (HNE). Direct and indirect RP-HPLC methods for the separation of HNEA enantiomers were developed and compared. The indirect method involved pre-column derivatization with a chiral amino agent, (1*S*,2*S*)-(+)-2-amino-1-(4-nitrophenyl)-1,3-propanediol, and subsequent separation of diastereomers on a Spherisorb ODS2 column. The direct separation of HNEA enantiomers was performed using the chiral stationary phase, Chiralpak AD-RH. Validation parameters including limit of quantification, linear range, accuracy and precision were determined. The indirect separation method was successfully applied for the determination of enantiomeric ratio of HNEA in rat brain mitochondrial lysate, and showed that HNEA was formed (*R*)-enantioselectively from HNE.

Keywords

Chiral separation; Chiral derivatization; LC; Enantioselectivity; *trans*-4-hydroxy-2-nonenic acid; *trans*-4-hydroxy-2-nonenal; Chiralpak AD-RH; (1*S*, 2*S*)-(+)-2-Amino-1-(4-nitrophenyl)-1,3-propanediol

1. INTRODUCTION

trans-4-Hydroxy-2-nonenic acid (HNEA) is a marker of lipid peroxidation formed from *trans*-4-hydroxy-2-nonenal (HNE) [1–3] (Fig. 1A). HNE and HNE-protein adducts are implicated in diseases involving oxidative stress, including Alzheimer's disease and Parkinson's disease [4,5]. Detoxification of HNE to HNEA, mediated by aldehyde dehydrogenases (ALDHs) [6], is a major route of metabolism in many systems [6,7]. HNE and HNEA possess chiral centers at the C4 carbon and can exist as two enantiomers, (*R*)- and (*S*)-, with potentially different biochemical reactivities. We showed previously that HNE is detoxified (*R*)-enantioselectively to HNEA by respiring rat brain mitochondria [8]. However, further study of the role of enantioselectivity in HNE metabolism is restricted by the fact that no method is available for the enantioseparation of HNEA.

* Corresponding author. Tel.: +1-701-777-2293; fax: +1-701-777-0387. E-mail address: mpicklo@medicine.nodak.edu (M. J. Picklo)

¹On leave from Department of Analytical Chemistry, Charles University, Prague 128 43, Czech Republic.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Chiral carboxylic acids can be determined indirectly by reversed-phase (RP) chromatography as diastereomeric amides using derivatization with chiral amines [9–12], including (*1S,2S*)-(+)-2-amino-1-(4-nitrophenyl)-1,3-propanediol (ANPAD, “dextrobase”) [13,14]. For direct enantioseparation of carboxylic acids, a polysaccharide based chiral stationary phase (CSP) Chiralpak AD-RH can be used [15–17]. Chiralpak AD-RH is a tris(3,5-dimethylphenylcarbamate) derivative of amylose and is used for enantioseparation of HNE [18] and profens [19–21].

In this work, we describe two RP-HPLC methods for enantioseparation of HNEA. The indirect separation method is based on the derivatization of HNEA enantiomers with the chiral derivatization agent ANPAD (Fig. 1B) in a phosphate buffer - methanol (MeOH) mixture in the presence of the coupling agent *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and the additive 1-hydroxybenzotriazole (HBT) [22]. The direct separation method is based on the enantioseparation of HNEA using CSP Chiralpak AD-RH.

2. EXPERIMENTAL

2.1 Chemicals and reagents

HNEA, HNE dimethylacetal and HNE enantiomers were synthesized as described previously [8,23,24]. HNEA enantiomers were synthesized by oxidation of the individual HNE enantiomers in the presence of sodium chlorite and sulfamic acid and were purified by solid-phase extraction (SPE). Optical purity was assessed by HPLC using CSP Chiralpak AD-RH and was >97% for (*R*)-HNEA and >95% for (*S*)-HNEA. HNE and HNEA stock solutions were stored in –20°C. ANPAD (99% purity), HBT hydrate, EDC, NAD⁺, sodium chlorite and sulfamic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile (ACN) and MeOH were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Water was purified by Purelab Ultra water purification system from ELGA LabWater (Lowell, MA, USA). Mobile phases were filtered through a Millicup-HV filter, 0.45 µm (Millipore, Billerica, MA, USA) and degassed under reduced pressure.

2.2 Derivatization procedure

Solutions of derivatization agents were prepared in MeOH daily. Samples and derivatization agents, except ANPAD, were kept on ice. 180 µL of HNEA in 40 mM sodium phosphate buffer, pH 5.0, was placed into 1.5 mL eppendorf tube. Derivatization agents were added strictly in the order described below, and the mixture was vortexed after each particular addition. 10 µL of 0.080 M HBT (final concentration 2 mM), 50 µL of 1.60 M EDC (final concentration 200 mM) and 160 µL of 0.050 M ANPAD (final concentration 20 mM) were added. Derivatization reactions proceeded for 1 hour. Samples were centrifuged using a Biofuge fresco centrifuge (Hanau, Germany) for 10 min at 16,000 g and stored in the HPLC autosampler at +4°C until analysis. The mass spectra of HNEA-ANPAD derivatives were measured by electrospray API 3000 MS/MS (MDS Sciex, Concord, Canada) in positive mode using direct infusion.

2.3 Chromatographic system and screening of RPs for separation of HNEA-ANPAD

The HPLC system consisted of a SCL-10A system controller, DGU-14A degasser, FCV-10AL mixing unit, LC-10AD HPLC pump, SIL-10AD auto injector, SPD-M10A diode array detector, and Class VP 7.2 software (Shimadzu, Kyoto, Japan).

The size of all HPLC columns used for screening was 150 mm × 2.00 mm I.D. The Synergi polar-RP 4 µm (ether-linked phenyl with polar end capping), Synergi fusion-RP 4 µm (C18 with polar embedded group), Luna C8(2) 3 µm, Luna C18(2) 3 µm columns were obtained from Phenomenex (Torrance, CA, USA) and Spherisorb ODS2 column 3 µm was purchased from Waters (Milford, MA, USA). Mobile phase consisted of MeOH - 5 mM ammonium

acetate, pH 7.0 (40:60, v/v) and was run at a flow rate of 0.1 mL/min. The hold-up time t_M was estimated using the equation $t_M \approx \frac{0.5L d_c^2}{F}$, where L is length of column, d_c is internal diameter and F is mobile phase flow rate [25]. Chromatographic parameters were calculated according to the equations: $R_s = 1/2(t_{r2} - t_{r1})/(w_1 + w_2)$, peak resolution; $\alpha = k_2/k_1$, separation factor; $k = (t_r - t_M)/t_M$, retention factor; $A_s = w_A/w_B$, peak asymmetry determined at 10% peak height.

2.4 Chromatographic conditions of indirect and direct separation methods

Indirect separation of HNEA-ANPAD diastereomers was performed using an RP Spherisorb ODS2 250 mm $\times$ 4.6 mm I.D., 5 μ m (Waters). HNEA-ANPAD diastereomers were separated isocratically in a mobile phase consisting of MeOH - 5 mM ammonium acetate, pH 7.0 (39:61, v/v), followed by a washing gradient program. The mobile phase flow rate was 1.5 mL/min and absorbance was monitored at 211 nm. The injection volume was 80 μ L.

Direct separation of HNEA enantiomers was performed using a CSP Chiralpak AD-RH 150 mm $\times$ 2.1 mm I.D., 5 μ m (Chiral Technologies, West Chester, PA, USA). The mobile phase consisted of MeOH - ACN - 5 mM ammonium acetate, pH 3.5 (52:5:43, v/v/v) and the flow rate was 0.1 mL/min. Absorbance was monitored at 210 nm. The injection volume was 20 μ L.

Separations were performed at ambient temperature ($23 \pm 1^\circ\text{C}$). The order of elution was determined by injecting standards of individual enantiomers or diastereomers. The enantiomeric ratio (ER) was calculated according to the equation

$$ER(R - HNEA, \%) = \frac{A_R}{A_R + A_S} \times 100, \text{ where } A_R \text{ is the peak area of } (R)\text{-HNEA or } (R)\text{-HNEA-ANPAD, respectively, and } A_S \text{ is the peak area of } (S)\text{-HNEA or } (S)\text{-HNEA-ANPAD, respectively.}$$

2.5 Validation parameters

The limit of quantification (LOQ) was determined using the equation $LOQ = 10\sigma/S$, where σ is the standard deviation of responses and S is the slope of the calibration curve. S and σ were determined from the calibration curve of the analyte in the low concentration range close to LOQ. The standard deviation σ was determined as the residual standard deviation of a regression line [26].

The repeatability of derivatization of HNEA by ANPAD and the repeatability of the injection was determined by triplicate experiments ($n = 3$). The stability of samples at $+4^\circ\text{C}$ was tested by periodical injection of HNEA-ANPAD prepared by derivatization of 200 μ M rac-HNEA in the case of indirect separation method and by analyzing 200 μ M rac-HNEA on Chiralpak AD-RH in the case of direct separation method. Enantiomerically enriched samples containing various ER of HNEA were prepared by mixing solutions of (*S*)-HNEA and (*R*)-HNEA enantiomers. Concentration of the major enantiomer was 250 μ M.

2.6 Preconcentration by SPE

Rac-HNEA was spiked into 40 mM sodium phosphate buffer, pH 2.0 containing lysed liver mitochondria (protein concentration 0.2 mg/mL) and 1 mM NAD^+ . Oasis HLB extraction cartridges (6 mL, 200 mg) were purchased from Waters. Solvents were passed through SPE cartridges using a vacuum manifold (Alltech, Deerfield, IL, USA). The cartridges were conditioned with 3 mL of MeOH and equilibrated with 3 mL of deionized water. The sample was loaded on the cartridge and washed with 4 mL of water. MeOH was used to elute HNEA.

The first 0.3 mL of eluent was discarded and the next 2.7 mL fraction containing HNEA was collected. The MeOH extract was evaporated under a gentle stream of nitrogen and the residue was dissolved in 300 μ L of MeOH - 40 mM sodium phosphate buffer, pH 5.0 (50:50, v/v) and stored in -20°C . Before analyses using Chiralpak AD-RH, samples were acidified to pH 3.5 by addition of 3.8 μ L of 0.05 M H_3PO_4 .

2.7 Enantioselective formation of HNEA in rat brain mitochondria

Experimental protocols were in accordance with the NIH guidelines for the use of live animals and were approved by the University of North Dakota Institutional Animal Care and Use Committee. Sprague–Dawley rats (male, 250–300 g) were purchased from Charles River (Wilmington, MA, USA). Brain and liver mitochondria were isolated as described previously [6,27]. Protein concentration was determined using Protein Assay reagent (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. Rat brain mitochondria at a final concentration 0.2 mg/mL were incubated in 40 mM sodium phosphate buffer (pH 7.4) containing 160 μ M rac-HNE and 1 mM NAD^+ at 37°C . The 3 mL aliquots were taken at defined time intervals. To stop the reaction, the mixture was immediately mixed with 1/10 sample volume of cold 10%_w phosphoric acid and was chilled on ice [6,8,27]. 2.8 mL of acidified reaction mixture (pH 2) was passed over SPE cartridge in order to concentrate HNEA. HNEA was derivatized and resulting HNEA-ANPAD diastereomers were analyzed as described above. A 165 μ L aliquot of the reaction mixture was used for quantification of the total amount of HNEA formed by the oxidation reaction. The total amount of HNEA was quantified using the RP-HPLC as described previously [6,8,27].

3. RESULTS AND DISCUSSION

3.1 Optimization of HNEA derivatization by ANPAD

Derivatization of HNEA using ANPAD was performed in a MeOH – phosphate buffer mixture, which provides several advantages. For example extraction into an organic solvent is not necessary, the derivatization can be performed directly in a biological sample and the resulting mixture is fully compatible with RP chromatography.

The derivatization conditions for generation of HNEA-ANPAD were optimized. The formation of HNEA-ANPAD diastereomers was pH dependent (Fig. 2A). The HNEA-ANPAD signal was decreased with the increased ionic strength of derivatization mixture (Fig. 2B). In further experiments, 40 mM phosphate buffer, pH 5.0 was used in respect to the composition of biological samples. Derivatization of HNEA ANPAD depended on the concentration of EDC (Fig. 2C) and HBT (data not shown).

To confirm the formation of the HNEA-ANPAD diastereomers, the derivatization mixture purified by SPE was analyzed by electrospray MS/MS in positive mode using direct infusion (Fig. 3). The peak of 367.3 amu corresponding to $[\text{HNEA-ANPAD-H}^+]$ molecular ion was found in mass spectra. The fragmentation pattern was the same for rac-, (*R*)- and (*S*)-HNEA-ANPAD (data not shown).

3.2 Screening of RPs for separation of HNEA-ANPAD

Various RPs were screened for efficiency to separate HNEA-ANPAD diastereomers (Table 1). Separation factor α was in linear correlation ($y = 0.1111x + 0.9181$, $r^2 = 0.96$) with hydrophobicity of RP reported by Eurby and Petersson [28], suggesting that hydrophobic interactions play role in separation of HNEA-ANPAD diastereomers.

By comparing C18 RPs only, Spherisorb ODS2 and Luna C18(2) showed similar hydrophobicity, but the total silanol activity is higher for Spherisorb ODS2 (Waters column

selectivity chart 2005 and [28]). In this work, the separation factor of HNEA-ANPAD was the highest using RP Spherisorb ODS2. On the other hand, the peak shape was better on the RP Luna C18(2) than on the Spherisorb ODS2.

3.3 Chromatographic conditions of indirect and direct separation methods

Mobile phase and temperature suitable for the separation of HNEA-ANPAD were optimized using the RP Spherisorb ODS2 250 mm $\times$ 4.6 mm I.D. (data not shown). Optimum separation conditions were achieved using a mobile phase MeOH - 5 mM ammonium acetate, pH 7.0 (39:61, v/v). (*R*)- and (*S*)-HNEA-ANPAD eluted at 40 min and 43 min, respectively, with resolution $R_s = 2.26$.

Direct separation of HNEA was performed on a CSP Chiralpak AD-RH. As mentioned above, HNE is the metabolic precursor of HNEA and therefore interference of HNE was also monitored. Baseline separation of (*R*)- and (*S*)-HNEA was achieved and HNE enantiomers eluted as a non-separated peak in mobile phase ACN - 5 mM ammonium acetate, pH 3.5 (26:74, v/v) (Fig. 4A). If MeOH, instead of ACN was used, baseline separation was achieved as well. However, coelution of (*S*)-HNEA and (*S*)-HNE occurred (Fig. 4B). Finally, an application of ternary mobile phase MeOH - ACN - 5 mM ammonium acetate, pH 3.5 (58:4:38, v/v/v) led to the shortest analysis time and no interference of HNE (Fig. 4C). Retention times of (*R*)-HNEA and (*S*)-HNEA were approximately 13 min and 16 min, respectively and retention times of (*S*)-HNE and (*R*)-HNE were 21 min and 23 min, respectively. The elution order of HNEA enantiomers was the opposite of the elution order of HNE enantiomers. Resolution R_s was as follows: (*R*)- and (*S*)-HNEA 1.78, (*S*)-HNEA and (*S*)-HNE 2.81 and (*S*)-HNE and (*R*)-HNE 1.07. The separation factor α of HNEA enantiomers was 1.23.

3.4 Linear range and LOQ of indirect and direct separation methods

Calibration curves of both direct and indirect separation methods were measured in buffer-based matrixes (Table 2). Excellent linearity was observed for both methods. However, LOQs represented as absolute amount injected were lower for the direct separation method. The derivatization reaction was also performed successfully in a complex biological matrix. In this case, rac-HNEA was spiked and derivatized in cold (+4 °C) 40 mM phosphate buffer, pH 5.0 containing lysed liver mitochondria lysate (protein concentration 0.2 mg/mL) and 1 mM NAD^+ .

3.5 Precision and stability of indirect and direct separation methods

Repeatability of derivatization of 50 μM rac-HNEA by ANPAD was measured showing an RSD of peak area < 5%. The repeatability of injection at LOQ was <9 % for indirect separation method and <5% for direct separation method. The samples were stable during analysis. An increase of signal of no more than 15% was observed during 48 hours of measurement, and the ER of the racemic mixture was constant, namely 50.2 ± 0.7 for indirect separation method and 50.2 ± 0.2 for direct separation method.

3.6 Accuracy of ER

The accuracy of ER was estimated by analyzing enantiomerically enriched samples containing various ER of HNEA (Table 3). Difference in ER obtained by direct and indirect separation method was less than 0.7%, showing excellent accuracy. These data clearly demonstrate, that kinetic resolution (for example ANPAD preferentially reacted with one enantiomer) or racemization did not occur during derivatization procedure, and that the influence of chiral impurities in the derivatization agent was negligible.

3.7 Preconcentration by SPE

The concentration of HNEA necessary for precise determination of ER is higher than the concentration of HNEA formed in brain mitochondrial lysate by enzymatic oxidation of HNE. Moreover, a cleaning step is necessary to protect relatively expensive CSP Chiralpak AD-RH from biological sample matrix influence. Therefore, we developed an SPE method for the purification and preconcentration of HNEA using Oasis HLB extraction cartridges. Recovery and preconcentration by SPE after dissolving the evaporated extract in 10-fold lower solvent volume was determined by direct separation method (Table 4). The LOQ was 8-fold lower after preconcentration by SPE (Table 2).

3.8 Enantioselective formation of HNEA in rat brain mitochondria

The indirect separation method was applied for determination of ER of HNEA formed in rat brain mitochondria by enzymatic oxidation of HNE. HNEA-ANPAD was successfully separated (Fig. 5A) and no interfering compounds were observed in the blank (Fig. 5B). Oxidation of 160 μ M rac-HNE by aldehyde dehydrogenases present in rat brain mitochondria led to (*R*)-enantioselective formation of HNEA (Fig. 5C). HNEA did not undergo further reaction when incubated with mitochondrial lysate supplemented with NAD⁺ demonstrating that selective formation of (*R*)-HNEA was not result of further enantioselective consumption of HNEA (data not shown). These results are the first to demonstrate enantioselective formation of HNEA when rac-HNE, rather than individual HNE enantiomers, was used. Because of the long analysis time and no internal standard available, the chiral separation was used for determination of ER only. The total amount of HNEA formed by oxidation was quantified by a non-chiral RP-HPLC method routinely used in our laboratory [27].

4. CONCLUSION

Direct and indirect RP-HPLC methods were developed for the separation of HNEA enantiomers. To best of our knowledge, no method allowing derivatization of carboxylic acid by chiral amine directly in buffer-based biological matrix has been reported previously. Enantiomerically enriched samples containing various ERs of HNEA enantiomers were analyzed showing excellent agreement between the direct and indirect methods. The direct method provides better precision and lower LOQ. On the other hand, HNEA-ANPAD diastereomers can be separated using widely available C18 columns. The indirect separation method was successfully applied for determination of ER of HNEA formed by oxidation of HNE in rat brain mitochondria lysate. Rac-HNE was detoxified (*R*)-enantioselectively to corresponding (*R*)-HNEA showing that chirality plays important role in HNE metabolism.

Acknowledgements

The authors gratefully acknowledge NIH grants P20 RR17699-05 COBRE from the NCRR and AA15145-01 from NIAAA. We thank Ms. Laura Leiphon for the preparation of mitochondria.

References

1. Guichardant M, Chantegrel B, Deshayes C, Doutheau A, Moliere P, Lagarde M. Biochem Soc Trans 2004;32:139. [PubMed: 14748733]
2. Alary J, Debrauwer L, Fernandez Y, Paris A, Cravedi JP, Dolo L, Rao D, Bories G. Chem Res Toxicol 1998;11:1368. [PubMed: 9815199]
3. Esterbauer H, Schaur RJ, Zollner H. Free Radical Biol Med 1991;11:81. [PubMed: 1937131]
4. Picklo MJ, Montine TJ, Amarnath V, Neely MD. Toxicol Appl Pharmacol 2002;184:187. [PubMed: 12460747]
5. Yoritaka A, Hattori N, Uchida K, Tanaka M, Stadtman ER, Mizuno Y. Proc Natl Acad Sci U S A 1996;93:2696. [PubMed: 8610103]

6. Murphy TC, Amarnath V, Gibson KM, Picklo MJ Sr. *J Neurochem* 2003;86:298. [PubMed: 12871571]
7. Kubatova A, Murphy TC, Combs C, Picklo MJ Sr. *Chem Res Toxicol* 2006;19:844. [PubMed: 16780364]
8. Honzatko A, Brichac J, Murphy TC, Reberg A, Kubatova A, Smoliakova IP, Picklo MJ Sr. *Free Radical Biol Med* 2005;39:913. [PubMed: 16140211]
9. Lunn, G.; Hellwig, LC. *Handbook of Derivatization Reactions for HPLC*. Wiley; New York: 1998.
10. Toyo'oka T. *J Biochem Biophys Methods* 2002;54:25. [PubMed: 12543490]
11. Gorog S, Gazdag M. *J Chromatogr B* 1994;659:51.
12. Goto J, Goto N, Nambara T. *J Chromatogr* 1982;239:559.
13. Ladanyi L, Sztruhar I, Slegel P, Vereczekey-Donath G. *Chromatographia* 1987;24:477.
14. Slegel P, Vereczekey-Donath G, Ladanyi L, Toth-Lauritz M. *J Pharm Biomed Anal* 1987;5:665. [PubMed: 16867463]
15. Okamoto Y, Yashima E. *Angew Chem, Int Ed* 1998;37:1021.
16. Yashima E. *J Chromatogr A* 2001;906:105. [PubMed: 11215883]
17. Tachibana K, Ohnishi A. *J Chromatogr A* 2001;906:127. [PubMed: 11215884]
18. Schneider C, Porter NA, Brash AR. *Chem Res Toxicol* 2004;17:937. [PubMed: 15257619]
19. Perrin C, Matthijs N, Mangelings D, Granier-Loyaux C, Maftouh M, Massart DL, Heyden YV. *J Chromatogr A* 2002;966:119. [PubMed: 12214686]
20. de Oliveira AR, Cesarino EJ, Bonato PS. *J Chromatogr B* 2005;818:285.
21. Teng XW, Wang SW, Davies NM. *J Pharm Biomed Anal* 2003;33:95. [PubMed: 12946535]
22. Klausner YS, Bodansky M. *Synthesis* 1972:453.
23. Chandra A, Srivastava SK. *Lipids* 1997;32:779. [PubMed: 9252968]
24. Alary J, Bravais F, Cravedi JP, Debrauwer L, Rao D, Bories G. *Chem Res Toxicol* 1995;8:34. [PubMed: 7703364]
25. Snyder, LR.; Kirkland, JJ.; Glajch, JL. *Practical HPLC Method Development*. Wiley; New York: 1997.
26. Anonymous. *Guidance for Industry - Q2B Validation of Analytical Procedure: Methodology*. US Food and Drug Administration (FDA); Rockville, MD: 1996.
27. Murphy TC, Amarnath V, Picklo MJ Sr. *J Neurochem* 2003;84:1313. [PubMed: 12614331]
28. Euerby MR, Petersson P. *J Chromatogr A* 2003;994:13. [PubMed: 12779216]

Fig 1. Formation of HNEA by ALDH-mediated oxidation of HNE (A). Derivatization of (*R*)- and (*S*)-HNEA by ANPAD to corresponding diastereomers (*R*)- and (*S*)-HNEA-ANPAD (B).

Fig 2.

Optimization of derivatization procedure. 150 μ M rac-HNEA in 40 mM phosphate buffer, pH 5.0 was derivatized at ambient temperature (23 ± 1 °C) using 20 mM ANPAD, 20 mM HBT and 20 mM EDC. The yield of derivatization depended on the pH of 200 mM phosphate buffer (A) and on the concentration of phosphate buffer (B). The yield of derivatization of 75 μ M rac-HNEA depended on the concentration of EDC (C). HNEA-ANPAD diastereomers were analyzed as a non-resolved peak using RP Spherisorb ODS2. Results are expressed as mean $\pm$ SD ($n = 2$)

Fig 3. Mass spectra of rac-HNEA-ANPAD obtained by electrospray MS in positive mode using direct infusion.

Fig 4.

Separation of (*R*)- and (*S*)-HNEA using CSP Chiralpak AD-RH. 20 μ L of mixture containing rac-HNEA and rac-HNE (both 400 μ M) was injected and analysis at flow rate 0.1 mL/min was monitored at 210 nm. Mobile phases used: ACN - 5 mM ammonium acetate, pH 3.5 (26:74, v/v) (A), MeOH - 5 mM ammonium acetate, pH 3.5 (65:35, v/v) (B) and MeOH - ACN - 5 mM ammonium acetate, pH 3.5 (58:4:38, v/v/v) (C).

Fig 5.

Enantioselective oxidation of HNE by rat brain mitochondria. HNEA formed by oxidation of 160 μ M rac-HNE by rat brain mitochondrial lysate (48 min, 37°C) was analyzed as HNEA-ANPAD (A). Blank of brain mitochondrial lysate incubated for 48 min at 37°C did not contain any interfering peaks co-eluted with the compounds of interest (HNE was not added) (B). Time dependence of enantioselective formation of HNEA by rat brain mitochondria from lysate treated with 160 μ M HNE (C). Results are expressed as mean $\pm$ SD ($n = 3$).

Table 1

Screening of RPs for efficiency to separate of (*R*)- and (*S*)-HNEA-ANPAD diastereomers.

RP	<i>R</i>	α	α_{CH2}	t_{R, I^*} min	t_{R, II^*} min	$A_{s, II}$
Synergi Polar	1.37	1.055	1.22	124.4	131.1	0.79
Synergi Fusion	1.59	1.068	1.37	137.3	146.4	0.95
Luna C8(2)	2.17	1.081	n.r.	138.8	149.7	1.00
Luna C18(2)	2.44	1.079	1.47	140.4	151.3	1.11
Spherisorb ODS2	1.79	1.089	1.51	98.0	106.4	1.42

600 μ M rac-HNEA was derivatized and purified using SPE and 5 μ L of purified solution was injected.

α_{CH2} is the hydrophobicity of RP as reported [28]; n.r. - not reported.

Table 2

Parameters of developed direct and indirect separation methods.

Matrix	Method	Enantiomer	r ²	Linear range	LOQ
PB	Indirect	(R)-	0.9994	pmol 340 – 51200	μM 4.2
PB	Indirect	(S)-	0.9993	620 – 51200	7.7
MA	Direct	(R)-	0.9997	50 – 6400	2.6
MA	Direct	(S)-	0.9996	60 – 6400	3.0
LM	Indirect	(R)-	0.9986	1110 – 25600	13.9
LM	Indirect	(S)-	0.9998	1370 – 25600	17.1
LM	Direct after SPE	(R)-	0.9990	5.6 – 400	0.3
LM	Direct after SPE	(S)-	0.9989	7.2 – 400	0.4

Matrix used for preparation of standards: PB (40 mM phosphate buffer; pH 5.0), MA (MeOH - 5.0 mM ammonium acetate, pH 3.5 (60:40, v/v)); LM (liver mitochondria lysate). The injection volume for direct and indirect method was 20 and 80 μL, respectively. Standards were prepared at least at 5 concentrations in duplicate (*n* = 2), except for direct separation method after SPE, where was *n* = 1.

Table 3

Precision and accuracy of ER

ER _R by separation method		
Direct	Indirect	Δ ER, %
4.7 ± 0.2	5.4 ± 0.9	−0.7
10.1 ± 0.1	10.7 ± 0.8	−0.6
21.7 ± 0.1	21.0 ± 1.2	+0.7
43.6 ± 0.1	43.3 ± 0.2	+0.3
49.8 ± 0.1	49.6 ± 0.4	+0.3
75.2 ± 0.1	74.6 ± 1.5	+0.6
98.2 ± 0.2	98.2 ± 0.1	0.0

Δ ER %, difference in ER determined by direct and indirect separation method

Table 4

Preconcentration of HNEA by SPE using Oasis HLB extraction cartridges (6 mL, 200 mg). The concentration of HNEA is expressed as single enantiomer. The recovery is expressed as mean $\pm$ SD ($n = 3$).

Matrix	c (HNEA), μ M	Expected	Recovery		ER _{R-} , %
			(<i>R</i>)-HNEA	(<i>S</i>)-HNEA	
	160.00	1600.0 *	76 $\pm$ 8	75 $\pm$ 9	49.8 $\pm$ 0.2
	10.00	100.0	72 $\pm$ 2	72 $\pm$ 2	49.6 $\pm$ 0.1
	1.25	12.5	74 $\pm$ 3	81 $\pm$ 3	46.9 $\pm$ 0.9

* analyzed after dilution