Oxidized and nitrated oleic acid in biological systems: Analysis by GC–MS/MS and LC–MS/MS, and biological significance

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Identification of cis-EpOA in biological samples

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Oleic acid, both non-esterified and esterified to lipids, undergoes non-enzymic oxidative metabolism, which leads to formation of the free and esterified forms of *cis*-EpOA **(Fig. 1**). The detection of racemic *cis*-EpOA in lipids of human leukocytes suggests a free radical-catalyzed epoxidation of esterified oleic acid in lipids. The cytochrome P450 (CYP) system also catalyses the epoxidation of oleic acid to *cis*-EpOA. First, the presence of *cis*-EpOA in human blood and urine has been shown indirectly, i.e., after reduction of the epoxy and carboxy groups with $LiAlH_4$ to generate 1,9-dihydroxyoctadecane and 1,10-dihydroxyoctadecane. However, the oxirane ring of *cis*-EpOA is thermally sufficiently stable for GC–MS analysis of intact *cis*-EpOA as its methyl or pentafluorobenzyl (PFB) ester. Indeed, the first accurately measured basal concentration of intact *cis*-EpOA in plasma of humans was achieved by a validated ECNICI GC–MS/MS method after derivatization of *cis*-EpOA to its PFB ester by 2,3,4,5,6-pentafluorobenzyl bromide (PFB-Br) in anhydrous acetonitrile and *N,N*-diisopropylethylamine (DIPEA) as the catalyst and HPLC separation.

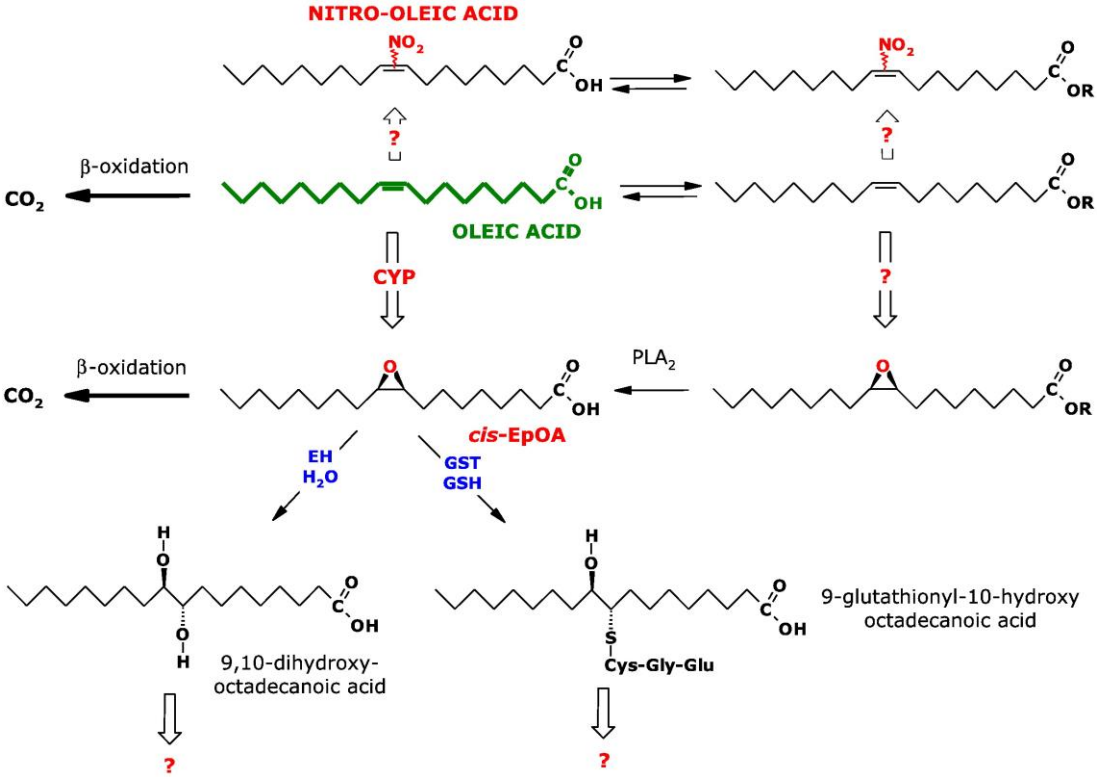

Fig. 1. Chemical structures of oleic acid and some of its oxidized and nitrated metabolites and their major known and putative enzymatic pathways. The arrows on the middle of the double bond indicate a nitro group at carbon-9 or carbon-10. The question marks indicate that the underlying mechanisms, reactions and enzymes are unknown. Abbreviations: CYP, cytochrome P 450; EH, epoxide hydratase; GST, GSH S-transferase; PLA2, phospholipase A2. R, alkyl.

It is worth mentioning that plants are capable of synthesizing enzymically *cis*-EpOA from oleic acid. Also, *cis*-EpOA may be present in various plastic laboratory materials. GC–MS/MS revealed that contaminating *cis*-EpOA may reach levels that approach and even exceed physiological concentrations as they occur in human urine (see below). Therefore, nutrition and contaminating *cis*-EpOA are likely to be additional sources for circulating and excretory *cis*-EpOA in humans. These pre-analytical factors may render difficult the search for the origin and the physiological role(s) of *cis*-EpOA in humans and need to be considered in quantitative analyses.

Identification of nitro-oleic acid species in biological samples

For the unequivocal identification and artifact- and interference-free quantification of nitrated oleic acid species, specifically 9-NO_2-OA and 10-NO_2-OA, 9-$^{15}NO_2$-OA and 10-$^{15}NO_2$-OA were synthesized, purified and thoroughly standardized prior to use as internal standards. Nitrated oleic acid species in plasma of healthy humans and their externally added synthetic analogues 9-$^{15}NO_2$-OA and 10-$^{15}NO_2$-OA were extracted from slightly acidified plasma samples (1 mL) by solid-phase extraction (SPE). Then, analytes present in the eluate were chromatographed by reversed phase HPLC without preceding derivatization. The HPLC fractions with the retention times of synthetic 9-NO_2-OA and 10-NO_2-OA were collected and the analytes were extracted from the slightly acidic mobile phase by solvent extraction with ethyl acetate. Subsequently, fatty acids were derivatized under mild conditions to the PFB ester derivatives which were then analysed by ECNICI GC–MS/MS by selected-reaction monitoring (SRM) of a highly specific mass transition. This analysis reproducibly yielded two GC peaks with the retention time of the PFB derivatives of synthetic unlabelled and ^{15}N-labelled standards of 9-NO_2-OA and 10-NO_2-OA. A reversed order of elution of 9-NO_2-OA and 10-NO_2-OA on reversed phase HPLC columns and on chemically bonded fused silica GC columns was observed. After thorough method validation 9-NO_2-OA and 10-NO_2-OA were quantified in plasma from freshly drawn blood of fifteen healthy subjects. Their concentrations were measured to be 0.88 ± 0.29 nM for 9-NO_2-OA and 0.96 nM for 10-NO_2-OA.

Derivatization of oxidized and nitrated oleic acid from biological samples

For GC–MS analysis, long-chain fatty acids and their oxidized metabolites can easily be converted to their methyl esters, e.g., by diazomethane, and to their PFB esters by PFB-Br under gentle derivatization. Thus, esterification of *cis*-EpOA by PFB-Br proceeds quantitatively without opening of the oxirane ring, if derivatization is performed in anhydrous acetonitrile (e.g., dried over molsieve) and in the presence of a base such as DIPEA (purified over Al_2O_3) as the catalyst for 30 to 60 min at room temperature or at 30 °C (**Fig. 2**). Under similar conditions, nitro-oleic acids can also be converted to their PFB esters without losing their nitro group or undergoing other changes such as conversion of 9-NO_2-OA to 10-NO_2-OA or inversely (**Fig. 2**). The PFB esters of *cis*-EpOA, 9-NO_2-OA to 10-NO_2-OA are stable against hydrolysis, so that they can be chromatographed by reversed phase HPLC in aqueous mobile phases, for instance in mixtures of acetonitrile and water (e.g., 85:15, v/v).

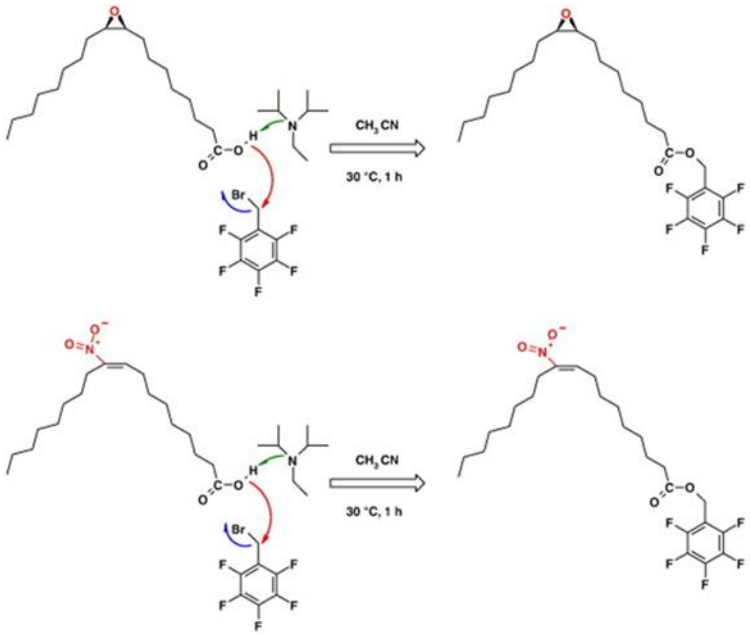

Fig. 2. Derivatization of *cis*-EpOA (upper panel) and 10-NO_2-OA to their pentafluorobenzyl esters by 2,3,4,5,6-pentafluorobenzyl bromide in anhydrous acetonitrile using *N*,*N*-diisopropylethylamine (DIPEA) as the catalyst.

The methyl or PFB esters of hydroxy-groups containing compounds including fatty acids are further derivatized. The most frequently used derivatization reaction of hydroxyl groups is etherification by silylating agents such as *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The trimethylsilyl (TMS) ether derivatives are thermally stable, considerably more volatile and have improved GC properties compared to their precursors. For GC–MS analysis of the PFB ester of *cis*-EpOA, i.e., *cis*-EpOA-PFB, silylation of the sample is not required because of the lack of OH groups. However, sample silylation greatly improves GC, because OH-groups containing compounds are allowed to emerge from the GC column. Sample silylation with BSTFA, which usually requires elevated temperatures, e.g., 60 °C, does not affect the oxirane ring of *cis*-EpOA-PFB.

The issues outlined above for *cis*-EpOA-PFB also apply to the PFB esters of 9-NO_2-OA (i.e., 9-NO_2-OA-PFB) and 10-NO_2-OA (i.e., 10-NO_2-OA-PFB). In addition to the vinylic nitro-oleic acids 9-NO_2-OA and 10-NO_2-OA, their allylic homologues, i.e., 9-NO_2-10-octadecenoid acid and 10-NO_2-8-octadecenoic acid, as well as the corresponding hydroxyl-nitro octadecanoic acids, i.e., 9-NO_2-10-hydroxy-octadecanoic acid and 10-NO_2-9-hydroxy-octadecanoic acid, have also been analysed by GC–MS and LC–MS methods. Derivatization of the allylic 9-NO_2-10-octadecenoic acid and 10-NO_2-8-octadecenoic acid but not of the vinylic 9-NO_2-OA and 10-NO_2-OA with PFB-Br/DIPEA (30 min, room temperature) and subsequent GC–MS analysis have been reported to yield an additional derivative. Formation of this isoxazole derivative suggests dehydration of these compounds during derivatization. Interestingly, consecutive derivatization of the allylic 9-NO_2-10-octadecenoic acid with PFB-Br/DIPEA and BSTFA and GC–MS analysis produced an *N*-trimethylsilyl oxazole derivative. 9-NO_2-OA and 10-NO_2-OA and the other mentioned above nitro derivatives were formed by NO_2-mediated nitration of oleic acid in hexane.

In recent years, electrospray ionization (ESI) has emerged as the most readily applied ionization technique for eicosanoids analysis by LC–MS/MS. ESI in the negative mode, i.e., negative electrospray ionization (NESI), is the most frequently used ionization technique for the analysis of non-derivatized eicosanoids and other classes of compounds that can form anions. NESI of carboxylic acids yields carboxylate anions $[M - H]^-$ analogous to their PFB esters (see below). Analysis of substances without any derivatization enables measurement of highly labile compounds such as prostaglandin H_2 (PGH_2), prostaglandin I_2 (PGI_2, prostacyclin) and leukotrienes A_4 (LTA_4) , which would not survive the hard derivatization conditions required in GC–MS.

Mass spectrometry of *cis*-EpOA

GC–MS and GC–MS/MS of *cis*-EpOA

Mass spectra and tandem mass spectra of the PFB ester derivatives from various unlabelled stable-isotope labelled *cis*-EpOA are summarized in Table 1, Table 2 and in Fig. 3, Fig. 4, Fig. 5, respectively. The electron ionization (EI) and ECNICI mass spectra of the PFB ester of unlabelled *cis*-EpOA (Fig. 3) contain very few and characteristic mass fragments. The ions at *m/z* 155 and *m/z* 365 are complementary and provide evidence for the intact 9,10-oxirane group of *cis*-EpOA (Fig. 3A). The most intense mass fragment in the ECNICI mass spectrum of the *cis*-EpOA-PFB ester is *m/z* 297 due to the carboxylate anion $[M - PFB]^-$ (Fig. 3B). Obviously, this anion loses one water molecule (H_2O) to form *m/z* 279 $[M - PFB - H_2O]^-$. Interestingly, no ion with *m/z* 279 is formed due to loss of D_2O (20 Da) from *m/z* 299 of d_2-*cis*-EpOA-PFB ester. This observation suggests that the deuterium atoms at C-9 and C-10 are not involved in the dehydration process. Subjection of the most characteristic ions of *cis*-EpOA-PFB ester observed in the EI and ECNICI mode to collision-induced dissociation (CID) at *m/z* 365 and *m/z* 297, respectively, yields quite different product ion mass spectra (Fig. 4). In the EI mode, CID of *m/z* 365 ($M^{+\bullet}$) fragments completely and yields a single intense cation at *m/z* 181 which is due to the PFB cation and therefore not characteristic for *cis*-EpOA. In the ECNICI mode, on the other hand, CID of *m/z* 297 ($[M - PFB]^-$) yields several intense product ions, of which *m/z* 171 and *m/z* 155 are complementary and characteristic for *cis*-EpOA (Fig. 5, upper panel).

Table 1. Summary of the $[M - PFB]^-$ ions in the ECNICI GC–MS mass spectra of the pentafluorobenzyl (PFB) ester derivatives of unlabelled and stable-isotope labelled oxidized and nitrated oleic acid species and of the $[M - H]^-$ ions in the NESI LC–MS mass spectra of the non-derivatized species.

***cis*-Oleic acid metabolite**	**Abbreviation**	**$[M - PFB]^-$**	**Reference**	**$[M - H]^-$**	**Reference**
cis-9,10-Epoxyoctadecanoic acid	*cis*-EpOA	297	[7]	297	This study
cis-9,10-Epoxy-[9,10-$^2H_2$]octadecanoic acid	d_2-*cis*-EpOA	299	[7]	299	This study

cis-Oleic acid metabolite	Abbreviation	$[M - PFB]^-$	Reference	$[M - H]^-$	Reference
9-Nitro-oleic acid	9-NO_2-OA	326	[22], [34]	326	[18], [21], [22]
10-Nitro-oleic acid	10-NO_2-OA	326	[22], [34]	326	[18], [21], [22]
9-[^{15}N]Nitro-oleic acid	9-$^{15}NO_2$-OA	327	[34]	Not reported	
10-[^{15}N]Nitro-oleic acid	10-$^{15}NO_2$-OA	327	[34]	Not reported	
9-[^{15}N]Nitro-[10-$^2H_1$]oleic acid	9-$^{15}NO_2$-[10-^{2}H]OA	328	[34]	Not reported	
10-[^{15}N]Nitro-[9-$^2H_1$]oleic acid	10-$^{15}NO_2$-[9-^{2}H]OA	328	[34]	Not reported	

Table 2. Summary of the major[a] product ions generated by collision-induced dissociation of the $[M - PFB]^-$ of the pentafluorobenzyl (PFB) ester derivatives of unlabelled and labelled 9- and 10-NO_2-OA species.[b]

NO_2-OA species	$[M - PFB - O^*NOH]^-$[a] *m/z* (intensity, %)			$[^*NO_2]^-$[a]
9-NO_2-OA	279 (55)	197 (65)	195 (64)	46 (100)
10-NO_2-OA	279 (100)	N.D.	N.D.	46 (55)
9-$^{15}NO_2$-OA	279 (100)	197 (30)	195 (20)	47 (58)
10-$^{15}NO_2$-OA	279 (100)	N.D.	N.D.	47 (59)
9-$^{15}NO_2$-[10-^{2}H]OA	280 (100)	198 (31)	196 (22)	47 (67)
10-$^{15}NO_2$-[9-^{2}H]OA	280 (100)	N.D.	N.D.	47 (59)

N.D., not detected.

a *N indicates ^{14}N or ^{15}N.
b Table was constructed with data from Ref.

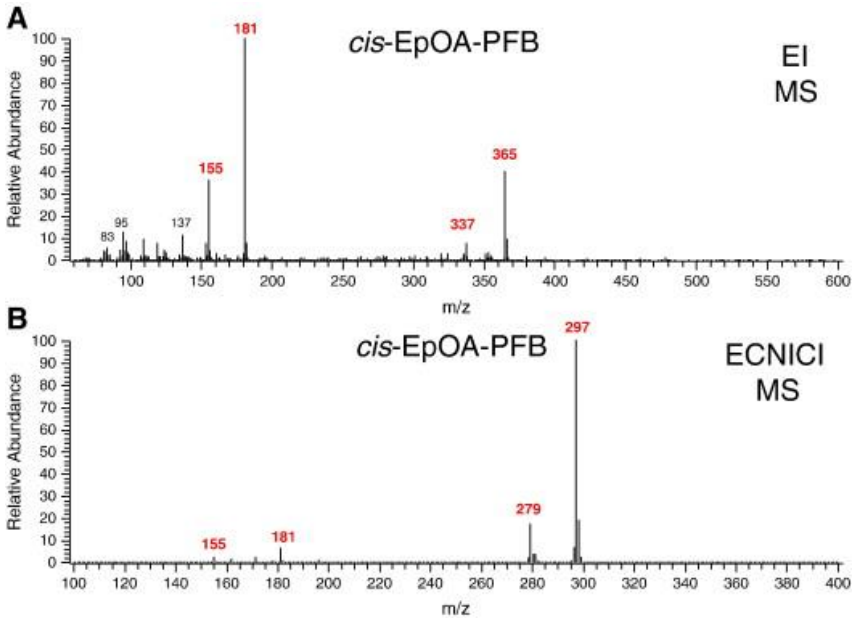

Fig. 3. (A) EI and (B) ECNICI GC–MS spectra of unlabelled *cis*-EpOA-PFB ester.

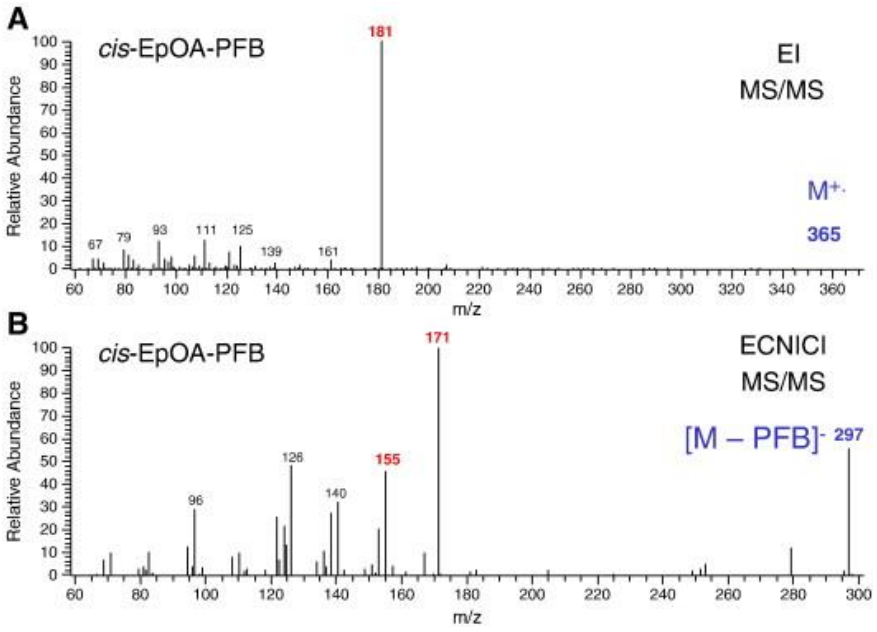

Fig. 4. (A) EI and (B) ECNICI GC–MS/MS spectra of unlabelled *cis*-EpOA-PFB ester.

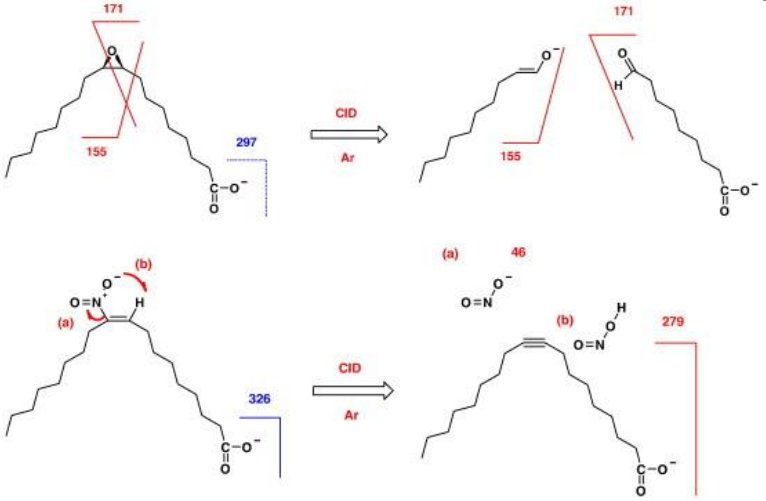

Fig. 5. Proposed mechanisms for the CID of the PFB esters of *cis*-EpOA and 10-NO_2-OA. Upper panel: CID of *m/z* 297 ($[M - PFB]^-$) of *cis*-EpOA-PFB ester to produce the anions *m/z* 155 and *m/z* 171. Lower panel: CID of *m/z* 326 ($[M - PFB]^-$) of 10-NO_2-OA-PFB ester to produce the anions *m/z* 46 and *m/z* 279 via two different pathways, i.e., (a) and (b).

At present, no stable-isotope labelled analogues of *cis*-EpOA are commercially available. A way to obtain this material is to start from a commercially available precursor such as [9,10-$^2H_2$] octadecenoic acid which is chemically epoxidized to *cis*-9,10-[9,10-$^2H_2$] octadecenoic acid (*cis*-d_2-EpOA), for instance by using the peracetic acid method. The most intense ions in the mass spectrum of *cis*-d_2-EpOA-PFB ester in the ECNICI mode are *m/z* 299 ($[M - PFB]^-$) and *m/z* 281 ($[M - PFB - H_2O]^-$). CID of *m/z* 299 generates the product ions at *m/z* 281 that contains two deuterium atoms, and the complementary ions *m/z* 172 and *m/z*156 which contain each one deuterium atom. For the quantitative determination of *cis*-EpOA in biological samples, *cis*-d_2-EpOA is an appropriate internal standard. Quantification is performed for instance by SRM of the mass transition *m/z* 297 → *m/z* 171 for *cis*-EpOA and *m/z* 299 → *m/z* 172 for *cis*-d_2-EpOA.

LC–MS and LC–MS/MS of *cis*-EpOA

Unlike the nitro-oleic acids, *cis*-EpOA has not been quantitated in biological samples by LC–MS or LC–MS/MS thus far. NESI is a useful ionization method for non-derivatized *cis*-EpOA. Interestingly, the LC–MS and LC–MS/MS NESI spectra of *cis*-EpOA very much resemble those of the *cis*-EpOA-PFB ester in the ECNICI mode in

GC–MS and GC–MS/MS (**Fig. 6 Table 1**). Deprotonation of *cis*-EpOA yields the carboxylate anion at *m*/*z* 297 $[M - H]^-$. CID of *m*/*z* 297 $[M - H]^-$ generates the product ions at *m*/*z* 279 $[M - H - H_2O]^-$, *m*/*z* 171 and *m*/*z* 155. The corresponding product ions from CID of *m*/*z* 299 $[M - H]^-$ for *cis*-d_2-EpOA are 281 $[M - H - H_2O]^-$, *m*/*z* 172 and *m*/*z* 156. Similar CID spectra were obtained from epoxyeicosatetraenoic acids in the NESI mode.

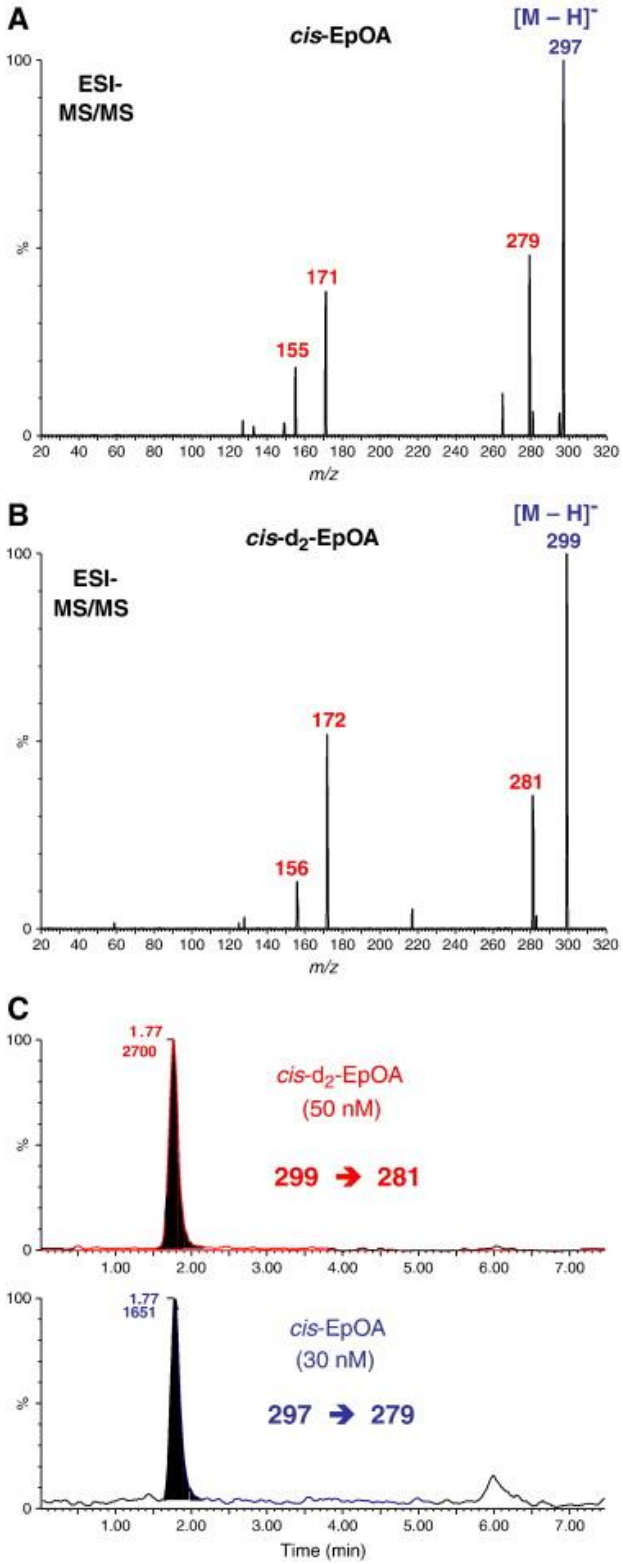

Fig. 6. LC–MS/MS spectra of non-derivatized *cis*-EpOA (A) and *cis*-d_2-EpOA (B) in the NESI mode. Chromatogram from the LC–MS/MS analysis of a mixture of synthetic *cis*-EpOA (30 nM) and *cis*-d_2-EpOA (50 nM) generated by SRM of *m*/*z* 279 from *m*/*z* 297 for *cis*-EpOA and of *m*/*z* 281 from *m*/*z* 299 for *cis*-d_2-EpOA.

In LC–MS/MS, quantitative determination of *cis*-EpOA using *cis*-d_2-EpOA as internal standard can be performed by SRM of the mass transition *m*/*z*297 → *m*/*z* 279 for *cis*-EpOA and of *m*/*z* 299 → *m*/*z* 281 for *cis*-d_2-EpOA (**Fig. 6 Table 1**). LC–MS/MS analysis of mixtures of synthetic *cis*-d_2-EpOA at the fixed concentration of 50 nM and varying concentrations of *cis*-EpOA (0–1000 nM) in the mobile phase was performed. Linear regression analysis of measured (*y*) versus nominal *cis*-EpOA concentration (*x*) resulted in a straight line with the regression equation: $y = 1.4 + 1.2x$, $r = 0.9993$. The lowest quantifiable *cis*-EpOA concentration was 1 nM. Thus, LC–MS/MS should allow quantitative determination of *cis*-EpOA in human plasma.

CID of anions $[M - PFB]^-$ and $[M - H]^-$ is likely to proceed via the same mechanism that needs to be elucidated. In particular, the loss of H_2O remains to be investigated. It is unclear which O and H atoms leave the *cis*-EpOA anion upon CID. One possibility could be the loss of the oxirane group O atom together with two H atoms other than those on C-9/C-10, for instance from the methylene groups at C-7/C-8 or C-11/C-12.

Mass spectrometry of nitrated oleic acid

GC–MS and GC–MS/MS of nitro-oleic acid

The mass spectra and tandem mass spectra of the PFB ester derivatives from various unlabelled and stable-isotope labelled 9-NO_2-OA and 10-NO_2-OA are summarized in **Table1, 2 and 3** and in **Fig. 7**. The ECNICI mass spectra of the PFB esters of the allylic nitrated oleic acid species contain each a single intense ion due to $[M - PFB]^-$. The finding suggests that the nitrated species are thermally stable like the epoxidized oleic acid and can be analysed by GC. In the ECNICI mass spectra of the PFB esters of 9-NO_2-OA and 10-NO_2-OA there were only minor differences. Unlike *cis*-EpOA, 9-NO_2-OA and 10-NO_2-OA do not lose a water molecule or their nitro groups. CID of $[M - PFB]^-$ of the 9-NO_2-OA- and 10-NO_2-OA-PFB esters yields distinct product ion mass spectra (**Fig. 7**). Both 9-NO_2-OA and 10-NO_2-OA fragment to produce the characteristic nitrite anion $[NO_2]^-$ at *m*/*z* 46. Also, both 9-NO_2-OA and 10-NO_2-OA lose nitrous acid (HONO) to produce the anions $[M - PFB - HONO]^-$ at *m*/*z* 279. These product ions are suited for specific GC–MS/MS quantification of 9-NO_2-OA and 10-NO_2-OA. Besides this common fragmentation, there is a major difference in the CID behaviour of 9-NO_2-OA and 10-NO_2-OA. For instance, CID of *m*/*z* 326 ($[M - PFB]^-$) yields an intense paired product ion at *m*/*z* 195 and *m*/*z*197 only from 9-NO_2-OA (**Fig. 7**) and can be utilized to differentiate between 9-NO_2-OA and 10-NO_2-OA. As mentioned above, allylic nitro-oleic acids can be discriminated from their vinylic homologues by GC–MS analysis of the PFB derivatives as only the vinylic compounds form isoxazol derivatives. Hydroxylated-nitrated oleic acid species have also been analysed qualitatively as their PFB-TMS derivatives by GC–MS (**Table 1**).

Table 3. Measured and calculated GC–MS- and LC–MS-related data for derivatized and native vinyl- and allyl-nitro-fatty acids from major unsaturated fatty acids.[a]

Parent fatty acid	**Molecular mass**	**Nitro-fatty acid**	**Molecular mass**	**$[M - PFB]^-$ *m*/*z***	**Transition *m*/*z* → *m*/*z***	**$[M - H]^-$ *m*/*z***	**Transition *m*/*z* → *m*/*z***
Palmitoleic acid	254.4	*v*-Nitro-palmitoleic acid[b]	299.4	298.4	298.4 → 46.0	298.4	298.5 → 251
Linoleic acid	280.5	*v*-Nitro-linoleic acid	325.5	324.5	324.5 → 46.0	324.5	324.5 → 277
Linolenic acid	278.4	*v*-Nitro-linolenic acid	323.4	322.4	322.4 → 46.0	322.5	322.5 → 275
Oleic acid	282.5	*v*-Nitro-oleic acid	327.5	326.5	326.5 → 46.0	326.5	326.5 → 279
Oleic acid	282.5	*a*-Nitro-oleic acid[c]	327.5	326.5	Not reported	326.5	326.5 → 46

Parent fatty acid	Molecular mass	Nitro-fatty acid	Molecular mass	$[M - PFB]^-$ *m/z*	Transition *m/z* → *m/z*	$[M - H]^-$ *m/z*	Transition *m/z* → *m/z*
Oleic acid	282.5	*v*-[^{15}N]Nitro-oleic acid	328.5	327.5	327.5 → 47.0	327.5	Not reported
Arachidonic acid	304.5	*v*-Nitro-arachidonic acid	349.5	348.5	348.5 → 46.0	348.5	348.5 → 301

a This table was constructed with data reported in Refs.
b v, vinyl.
C a, allyl.

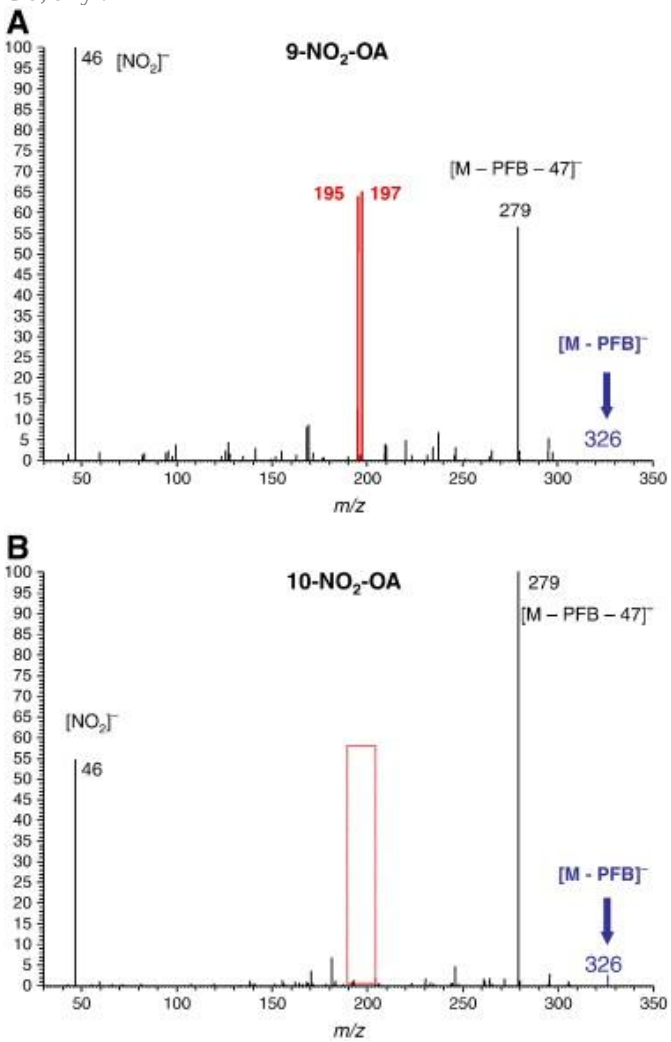

Fig. 7. GC–MS/MS spectra of (A) 9-NO_2-OA and (B) 10-NO_2-OA. The anions at *m/z* 326 ($[M - PFB]^-$) generated by ECNICI were subjected to CID. The product ions at *m/z* 195 and 197 were obtained only from 9-NO_2-OA. The dotted rectangle in (B) indicated the region where *m/z* 195 and 197 would appear.

Chemical synthesis of stable-isotope labelled nitro-oleic acid

As outlined above for *cis*-EpOA, there are no commercially available stable-isotope labelled analogues of nitrated oleic acid species. However, they can be easily synthesized in the laboratory by nitration of a commercially available precursor such as oleic acid or [9,10-$^2H_2$] octadecenoic acid (e.g., 8.5 mg, 30 μmol). A useful synthetic approach is to use unlabelled or ^{15}N-labelled nitrate (e.g., 5.1 mg) together with concentrated sulphuric acid (300 μL) in ice-cold dimethyl formamide (600 μL). Reaction products need to be extracted, isolated by HPLC and structurally characterized and standardized by HPLC, GC–MS and/or LC–MS. The molar absorptivity (ε) at the maximum wavelength (each at 263 nm in acetonitrile) was determined to be 4600 mM^{-1} cm^{-1} for 9-NO_2-OA and

4780 mM^{-1} cm^{-1} for 10-NO_2-OA using the commercially available compounds and is a useful mean for rough content estimation. By this synthetic route, oleic acid is nitrated to the 9- and 10-nitro-oleic acid isomers with a molar ratio of about 1:1 (**Fig. 8**). It is worth mentioning that nitration yield is of the order of only 7% for each isomer, most likely due to the low reactivity of the olefinic group of oleic acid as compared to the highly reactive phenolic ring of tyrosine.

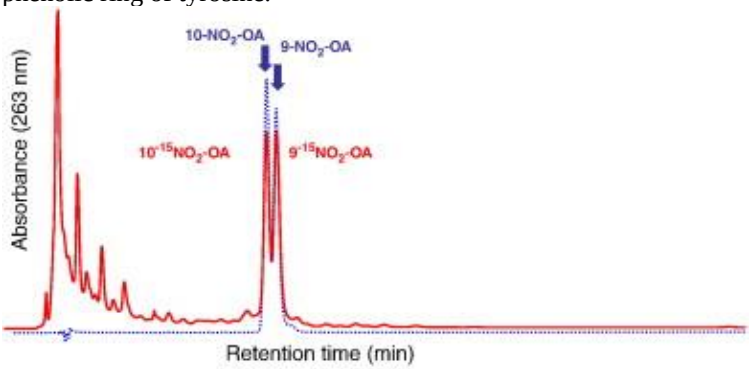

Fig. 8. Superposed HPLC chromatograms from analyses of commercially available (dotted line) non-derivatized 9-NO_2-OA (retention time, 10.9 min) and 10-NO_2-OA (retention time, 10.1 min) and of 9-$^{15}NO_2$-OA and 10-$^{15}NO_2$-OA prepared (solid line) by reacting for 30 min at 0 °C (ice bath) unlabelled oleic acid (8.5 mg) with a two-molar excess of sodium [^{15}N]nitrate (5.1 mg) in dimethyl formamide (600 μL) and concentrated sulphuric acid (300 μL) serving as the catalyst. This chromatogram was reconstructed by using the individual chromatograms published elsewhere.

For the quantitative determination of 9-NO_2-OA and 10-NO_2-OA in biological samples such as human plasma the ^{15}N-labelled analogues, i.e., 9-$^{15}NO_2$-OA and 10-$^{15}NO_2$-OA are appropriate internal standards. Thus, quantification of 9-NO_2-OA and 10-NO_2-OA can be performed for instance by SRM of the mass transition of *m*/*z* 326 → *m*/*z* 46 for 9-NO_2-OA and 10-NO_2-OA and of *m*/*z*327 → *m*/*z* 47 for 9-$^{15}NO_2$-OA and 10-$^{15}NO_2$-OA.

LC–MS and LC–MS/MS of nitro-oleic acid

The LC–MS and LC–MS/MS spectra for 9-NO_2-OA and 10-NO_2-OA are summarized in Table 1 and 2. When non-derivatized nitrated oleic acids are analysed by reversed phase HPLC, the order of elution is 10-NO_2-OA and 9-NO_2-OA (**Fig. 8 and 9**), i.e., opposite to the elution order of their PFB esters in GC–MS. CID mass spectra of the carboxylate anion *m*/*z*326 of 9-NO_2-OA and 10-NO_2-OA and of the allylic 9-nitro-10-octadecenoic acid are virtually identical (**Fig. 9**). Therefore, LC–MS/MS discrimination between 9-NO_2-OA and 10-NO_2-OA requires preceding LC separation. On the other hand, CID of the carboxylate anions *m*/*z* 326 produced by ECNICI from the PFB esters and by NESI from the non-derivatized 9-NO_2-OA and 10-NO_2-OA generates a common highly characteristic product at *m*/*z* 46 due to the nitrite anion (**Fig. 9**). Thus, both in NESI LC–MS/MS and in ECNICI GC–MS/MS the same transition, i.e., *m*/*z* 326 → *m*/*z* 46, can be monitored for the quantification of nitrated oleic acid species.

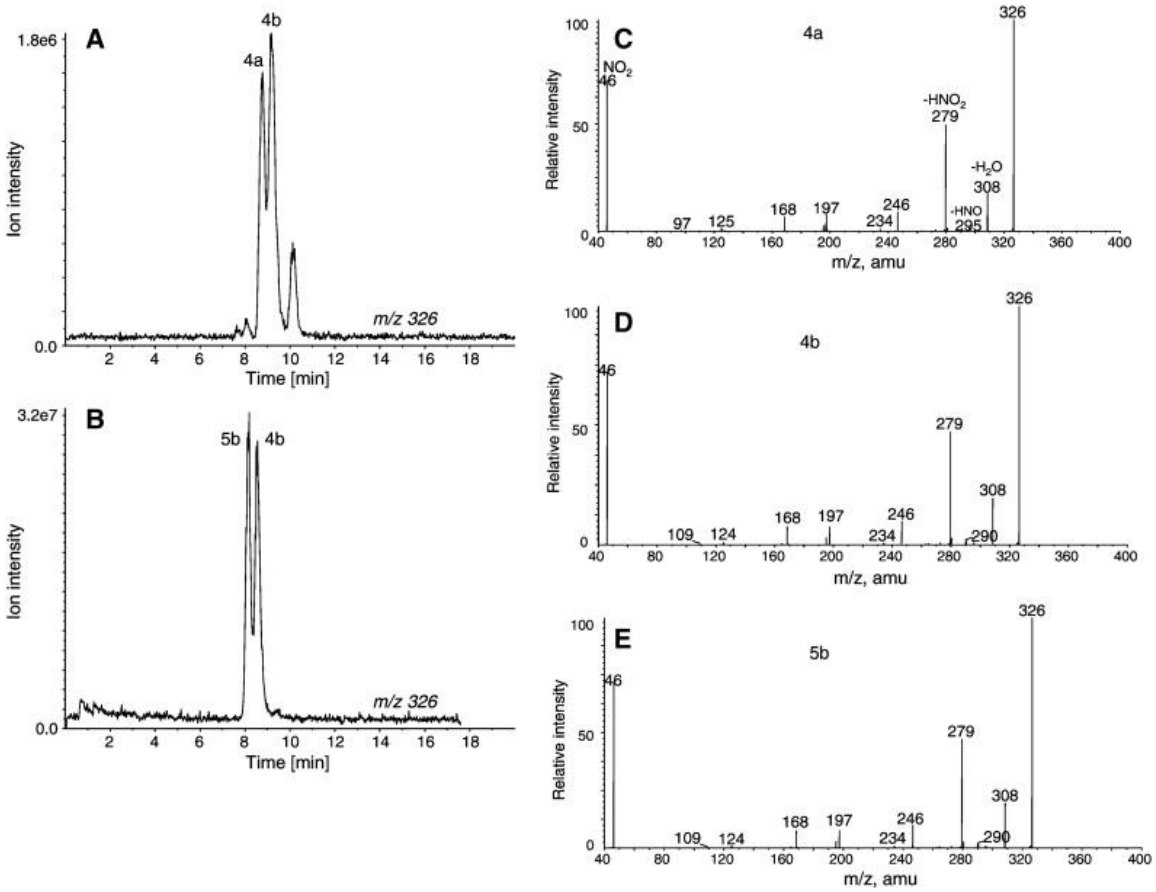

Fig. 9. LC–MS chromatograms and LC–MS/MS spectra of non-derivatized 9-NO_2-OA, 10-NO_2-OA and 9-nitro-10,11-octadecenoic acid. Explanation: 4a, allyl 9-NO_2-OA; 4b, vinyl 9-NO_2-OA; 5b, vinyl 10-NO_2-OA.

Concentration of oxidized and nitrated oleic acid in biological samples and method applications

Until the present day, *cis*-EpOA, 9-NO_2-OA, 10-NO_2-OA and other nitrated fatty acids have not been quantified in biological samples by validated stable-isotope dilution LC–MS/MS methods. Nevertheless, concentrations of nitrated fatty acids including nitro-oleic acid in biological samples such as human plasma and blood have been reported. On the other hand, *cis*-EpOA, 9-NO_2-OA and 10-NO_2-OA have been quantified in human plasma by fully validated stable-isotope dilution GC–MS/MS methods. Below, the application of LC–MS/MS and GC–MS/MS methods for the quantification of *cis*-EpOA, 9-NO_2-OA, 10-NO_2-OA and other nitrated fatty acids are briefly discussed. Representative chromatograms from the quantitative determination of *cis*-EpOA, 9-NO_2-OA and 10-NO_2-OA in human plasma by ECNICI GC–MS/MS as PFB esters are shown in **Fig. 10, Fig. 11**, respectively.

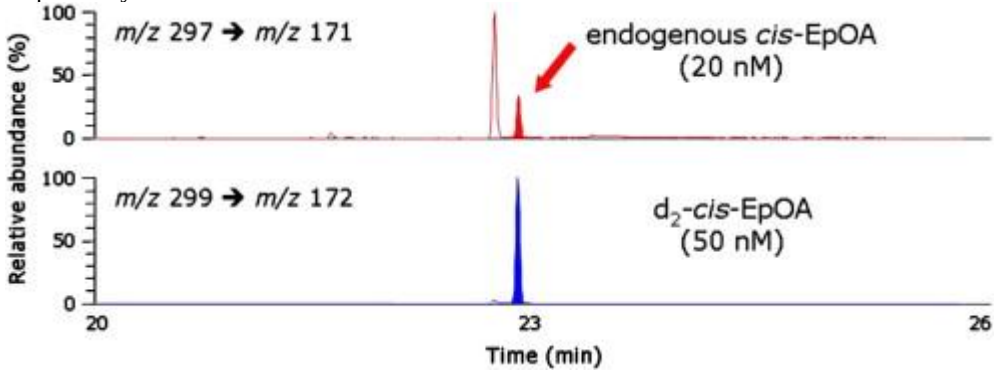

Fig. 10. Partial chromatograms from the quantitative GC–MS/MS analysis of *cis*-EpOA in a plasma sample from a healthy volunteer. *cis*-d_2-EpOA was used as the internal standard at 50 nM. SRM of *m*/*z* 171 and *m*/*z* 172 produced by CID of the parent ions ($[M - PFB]^-$) at *m*/*z* 297 for endogenous *cis*-EpOA and *m*/*z* 299 for *cis*-d_2-EpOA. The peak in front of the *cis*-EpOA-PFB peak coelutes with its *trans*-isomer, i.e., *trans*-EpOA-PFB.

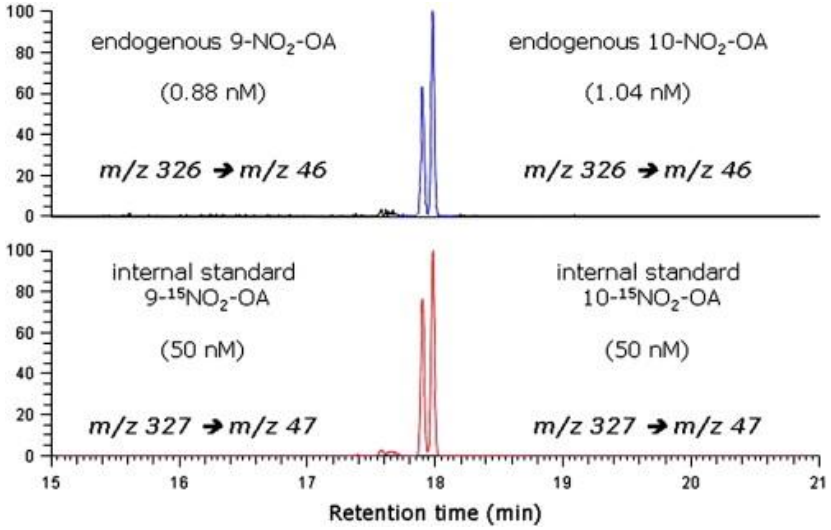

Fig. 11. Partial chromatograms from simultaneous quantitative GC–MS/MS analysis of 9-NO_2-OA and 10-NO_2-OA in a plasma sample from a healthy volunteer. 9-$^{15}NO_2$-OA and 10-$^{15}NO_2$-OA were used as the internal standards at 50 nM each. SRM of *m*/*z* 46 and *m*/*z*47 produced by CID of the parent ions at *m*/*z* 326 for the endogenous 9-NO_2-OA and 10-NO_2-OA and *m*/*z* 327 for 9-$^{15}NO_2$-OA and 10-$^{15}NO_2$-OA. The GC–MS/MS method used to obtain the chromatograms is reported in Ref.

Epoxidized oleic acid

In EDTA plasma of healthy humans, *cis*-EpOA occurs in its free, non-esterified form at concentrations within the range of 30 to 45 nM, whereas end-stage liver disease patients have lower *cis*-EpOA plasma concentrations (i.e., 10 to 40 nM) as measured by ECNICI GC–MS/MS. The lowest *cis*-EpOA plasma concentrations were measured in patients suffering from cirrhosis. This finding suggests that the liver is the main *cis*-EpOA synthesizing organ. Various CYP450 isoforms including CYP2C9 and CYP3A4 have been shown to epoxidize oleic acid to *cis*-EpOA. Remarkably, *cis*-EpOA concentrations in serum may be considerably higher than in plasma and may increase in dependence upon phospholipase A_2 (PLA_2) activity. Addition of PLA_2 to human serum increased both *cis*-EpOA and the F_2-isoprostane 15(*S*)-8-*iso*-prostaglandin $F_{2\alpha}$ {15(*S*)-8-*iso*-$PGF_{2\alpha}$}, which is mainly esterified to lipids. There was a close correlation ($r = 0.974$) between serum *cis*-EpOA (y) and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ (x): $y = 84 + 1.5x$. These observations suggest that an appreciable fraction of *cis*-EpOA is esterified to lipids. EDTA plasma is recommended for quantification of *cis*-EpOA in blood.

cis-EpOA has been identified in human urine. However, whether *cis*-EpOA in urine samples is endogenously produced or merely results from contamination is unclear. Similar to *cis*-EpOA contamination in laboratory plastic ware, *cis*-EpOA concentration in urine of healthy volunteers is of the order of 1 to 2 nM. The contribution of contaminating *cis*-EpOA can be reduced by using glass ware instead of plastic ware, notably glass SPE cartridges, as well as by using water of HPLC grade where applicable such as in SPE procedures and in HPLC mobile phases (**Fig. 12**).

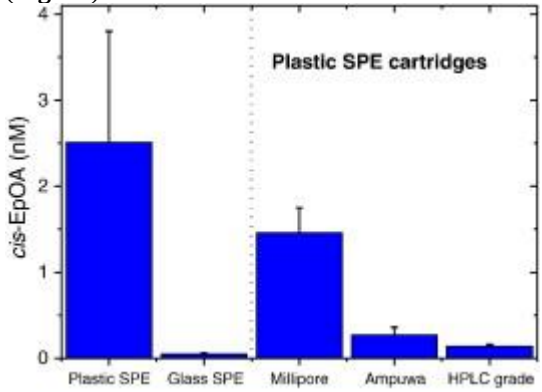

Fig. 12. Contaminating *cis*-EpOA in plastic and glass laboratory ware and in potassium phosphate buffer prepared by water of different sources. Plastic SPE, Chromabond-LV C18ec columns made of polypropylene; Glass SPE, Chromabond C18ec columns made of glass. Millipore, water prepared in the institute by using a Millipore apparatus. Ampuwa, commercially available water (Fresenius, Bad Homburg, Germany). HPLC grade,

commercially available water (Baker, Deventer, The Netherlands) of HPLC quality. This figure was constructed with previously reported data.

Nitrated oleic acid and other nitrated fatty acids

Nitrated linoleic acid was quantified in red blood cells and plasma from healthy humans by NESI LC–MS/MS using a ^{13}C-labelled nitro-linoleic acid as internal standard. In that study, the mean concentration of all non-esterified nitro-linoleic acid isomers was reported to be 79 nM in plasma and 50 nM in packed red cells. The mean concentration of all esterified nitro-linoleic acid isomers was reported to be 550 nM in plasma and 199 nM in packed red cells. In whole human blood, the total concentration of all nitro-linoleic acid isomers was reported to be 477 nM.

By using the same NESI LC–MS/MS method and a ^{13}C-labelled nitro-oleic acid as internal standard the same group reported later on the occurrence of nitro-oleic acid in plasma, red cells and urine of healthy humans. In that study, the mean concentration of all non-esterified nitro-oleic acid isomers was reported to be 619 nM in plasma and 59 nM in packed red cells. The mean concentration of all esterified nitro-oleic acid isomers was reported to be 302 nM in plasma and 155 nM in packed red cells. Thus, nitrated linoleic acid and nitrated oleic acid were estimated to be 1000 nM in the blood of healthy humans. In human urine, nitrated oleic and linoleic acid were found at about 0.6 and 0.3 nmol/mol creatinine, respectively.