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Short communication

9- and 10-Nitro-oleic acid do not interfere with the GC–MS quantitative determination of nitrite and nitrate in biological fluids when measured as their pentafluorobenzyl derivatives☆

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Abstract

The nitrated lipids 9-nitro-oleic acid (9-NO_2-OA) and 10-nitro-oleic acid (10-NO_2-OA) have been reported to be present in blood of healthy humans. Free and esterified forms of 9-NO_2-OA and 10-NO_2-OA have been detected in human plasma at about 600 and 300 nM, respectively. These concentrations are of the same order of magnitude of circulating nitrite. In theory, 9-NO_2-OA and 10-NO_2-OA may interfere with the analysis of circulating nitrite and nitrate. In the present study, we investigated a possible interference of 9-NO_2-OA and 10-NO_2-OA with the GC–MS method of analysis of nitrite and nitrate involving derivatization by pentafluorobenzyl (PFB) bromide in aqueous acetone at 50 °C for 5 min (nitrite) or for 60 min (nitrite and nitrate). Our results show that 9-NO_2-OA and 10-NO_2-OA do not interfere with the GC–MS analysis of nitrite and nitrate as PFB derivatives in plasma and phosphate buffered saline when added to these matrices at supraphysiological concentrations of 1–10 μM. Thus, nitrated lipids such as 9-NO_2-OA and 10-NO_2-OA can be excluded as potential interfering substances in the GC–MS quantitative determination of nitrite and nitrate as their PFB derivatives.

Keywords: Circulation; Derivatization; GC–MS; Interferences; Nitrated lipids; Pentafluorobenzyl bromide

1. Introduction

Nitric oxide (NO) from endogenous and exogenous sources partly autoxidizes to nitrite (NO_2^-), whereas by far the major fraction of NO is oxidized to nitrate (NO_3^-) within the erythrocytes by oxyhemoglobin ($HbFe^{(II)}O_2$). Nitrite and nitrate circulate in blood and are excreted in the urine. Under certain conditions, circulating and excretory nitrite and nitrate may serve as indicators of endogenous NO synthesis, with circulating nitrite presumably reflecting NO synthesis in the endothelium (reviewed in [1]). Accurate and interference-free analysis of NO-derived nitrite in biological fluids represents an analytical challenge. Important factors potentially influencing quantitative analysis of nitrite include various pre-analytical and analytical parameters. (1) Occurrence of NO-derived nitrite at relatively low concentrations (e.g. 200–2000 nM, in the circulation, with the great variation of concentrations measured being mainly dependent upon the analytical method used [1]. (2) The ubiquitous presence of contaminating nitrite in virtually all laboratory materials, including blood monovettes and distilled water, at concentrations similar to or even higher than those of NO-derived nitrite in the circulation. (3) Endogenous and exogenous NO-containing substances with a potential to contribute to nitrite upon analysis. Endogenous NO-containing compounds include *N*-nitroso amines, *S*-nitrosothiols and 3-nitrotyrosine. Exogenous substances potentially contributing to nitrite and nitrate include the drug class of organic nitrates as well as N^G-nitro-L-arginine, the most frequently used exogenous inhibitor of NO synthases, both as free acid (L-NNA) and as methyl ester (L-NAME) (discussed in [2]).

In recent years, new classes of NO-containing, endogenous, simply and multiply unsaturated fatty acids have been identified in human plasma and urine. 9-Nitro-oleic acid (9-NO_2-OA) and 10-nitro-oleic acid (10-NO_2-OA) (for structure see Fig. 1) belong to the most abundant known nitrated lipids [3,4].

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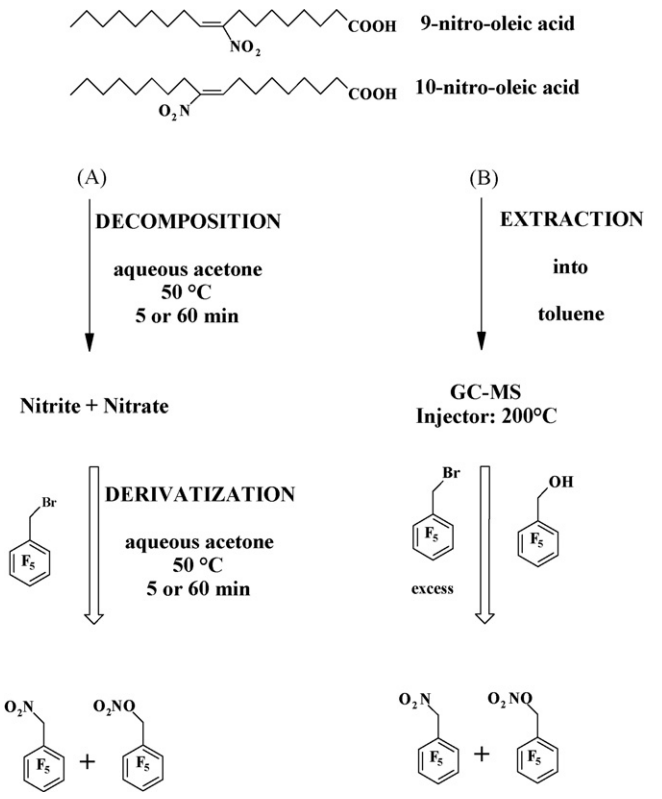

Fig. 1. Chemical structures of 9-nitro-oleic acid (9-NO_2-OA) and 10-nitro-oleic acid (10-NO_2-OA) and two mechanisms by which these nitrated lipids could contribute to nitrite and nitrate detection in a GC–MS method that involves simultaneous derivatization of nitrite and nitrate by pentafluorobenzyl bromide (PFB-Br) in aqueous acetone to PFB-NO_2 and PFB-ONO_2, respectively, their extraction into toluene and injection of the toluene phase into the GC–MS instrument (injector port kept at 200 °C). For more details see the text and [5].

9-NO_2-OA and 10-NO_2-OA have been reported to occur in human plasma at basal concentrations of about 600 nM (free acids) and 300 nM (esterified to lipids), i.e. of the same order of magnitude of circulating nitrite [1]. 9-NO_2-OA and 10-NO_2-OA have also been detected in human urine at 0.57 pmol/mmol creatinine [4]. This excretion rate is about three orders of magnitude smaller than that of nitrite [1]. Nitrated linoleic acid has also been detected in human plasma (i.e. at about 90 nM for free acid and 550 nM for esterified to lipids forms) [4].

Pentafluorobenzyl (PFB) bromide (PFB-Br) is a versatile derivatization agent in GC. We have shown that PFB-Br is useful for the simultaneous derivatization and quantitative determination of nitrite and nitrate in various biological matrices by GC–MS [5]. In a S_N2 reaction, nitrite reacts with PFB-Br to form PFB-NO_2, whereas the reaction of nitrate with PFB-Br forms PFB-ONO_2 (Fig. 1). These reactions are best performed in aqueous acetone at 50 °C. Maximum derivatization of nitrite occurs within 5 min, whereas nitrate derivatization requires 60 min [5]. For simultaneous nitrite and nitrate analysis, incubation time is 60 min at 50 °C. PFB-NO_2, PFB-ONO_2 and excess of PFB-Br are extracted with toluene (usually 1 ml) from which 0.5- or 1-μl aliquots are injected into the GC–MS instrument at an injector temperature of 200 °C. Under such derivatization and GC conditions this method has been shown to be highly specific for nitrite and nitrate. NO-containing substances such as *N*-nitroso-morpholine, 3-nitrotyrosine, the organic nitrate pentaerythrityl tetranitrate, L-NNA and L-NAME, have found not to interfere with the GC–MS analysis of nitrite and nitrate as their PFB derivatives [5,6]. Endogenous *S*-nitrosothiols are potential contributors to nitrite. However, because of their low concentration in biological fluids [7], their interference with nitrite is considered rather very minor, at least when analyzed in plasma or serum.

9-NO_2-OA and 10-NO_2-OA could interfere with the GC–MS analysis of nitrite and nitrate as PFB derivatives by two mechanisms (Fig. 1). 1) One mechanism could involve decomposition of 9-NO_2-OA and 10-NO_2-OA to nitrite and/or nitrate during derivatization at 50 °C in aqueous acetone (Fig. 1, route A). 2) Upon injection of the toluene phase, co-extracted 9-NO_2-OA and 10-NO_2-OA could react with co-extracted PFB-Br or with PFB-OH, the hydrolysis product of PFB-Br [5], within the injector port and/or on column as long they co-elute to form PFB-NO_2 and PFB-ONO_2. The stability of 9-NO_2-OA and 10-NO_2-OA in plasma, urine, buffers and solvents is poorly investigated [4]. The potential of these substances to contribute to nitrite and nitrate detection in analytical methods is unknown. The aim of the present study was to investigate a possible interference of 9-NO_2-OA and 10-NO_2-OA with the GC–MS quantitative determination of nitrite and nitrate as PFB derivatives in human plasma and aqueous buffer. For this purpose, 9-NO_2-OA and 10-NO_2-OA were added at concentrations covering published and supraphysiological concentrations, i.e. up to 10 μM of 9-NO_2-OA or 10-NO_2-OA, samples were derivatized with PFB-Br for 5 or 60 min at 50 °C, reactants were extracted by toluene, and analyzed by GC–MS.

2. Experimental

2.1. Materials and chemicals

2,3,4,5,6-Pentafluorobenzyl bromide, sodium [^{15}N]nitrate (declared as 98 at% at ^{15}N) and sodium [^{15}N]nitrite (declared as 98 at% at ^{15}N) serving as internal standards were obtained from Sigma–Aldrich (Steinheim, Germany). 9-Nitro-9-octadecenoic acid (9-NO_2-OA) and 10-nitro-9-octadecenoic acid (10-NO_2-OA) were obtained as ethanolic solutions (both declared as 500 μg/ml, 1527 μM, and purity ≥98%) from Cayman Chemical (Ann Arbor, MI, USA) and were stored at −20 °C. Toluene was purchased from Baker (Deventer, The Netherlands) and acetone was from Merck (Darmstadt, Germany).

2.2. Sample preparation and derivatization procedures

Phosphate buffered saline (PBS, 100 mM potassium phosphate, 500 mM NaCl, pH 7.4) and human pooled plasma were used. PBS (1 ml) and plasma (300 μl) were spiked separately with 9-NO_2-OA and 10-NO_2-OA to achieve final added concentrations of 0, 1, 2, 5 and 10 μM each. For this purpose, the stock solutions of 9-NO_2-OA and 10-NO_2-OA were freshly diluted with acetonitrile. Each two 300-μl aliquots of 9-NO_2-OA- or 10-NO_2-OA-containing plasma or PBS were spiked with a mix-

ture of both internal standards to achieve final concentrations of 4 μM for [^{15}N]nitrite and 40 μM for [^{15}N]nitrate. Derivatization of nitrite and nitrate in all matrices investigated was performed as described elsewhere [5]. Briefly, to the samples (100 μl) was added acetone (400 μl) and PFB-Br (10 μl), and the reaction mixtures were incubated at 50 °C either for 5 min (nitrite analysis) or for 60 min (nitrite and nitrate analysis). After derivatization acetone was removed under nitrogen and reaction products were extracted by vortex mixing with toluene (1 ml) for 1 min. Samples were then centrifuged (5 min, 800 × *g*, 4 °C) and aliquots (700 μl) of the toluene phase were transferred into 1.5-ml autosampler glass vials for GC–MS analysis.

2.3. Gas chromatography–mass spectrometry

GC–MS was performed on a ThermoElectron DSQ quadrupole mass spectrometer connected directly to a ThermoElectron Focus gas chromatograph and to an autosampler AS 3000 (ThermoElectron, Dreieich, Germany). A fused-silica capillary column Optima-17 (15 m × 0.25 mm i.d., 0.25-μm film thickness) from Macherey-Nagel (Düren, Germany) was used. Aliquots (1 μl) were injected in the splitless mode and quantification was performed by selected-ion monitoring (SIM) of *m/z* 46 for nitrite, *m/z* 47 for [^{15}N]nitrite, *m/z* 62 for nitrate, and *m/z* 63 for [^{15}N]nitrate with a dwell time of 50 ms for each ion. The following oven temperature program was used with helium (constant flow of 1 ml/min) as the carrier gas: 1 min at 70 °C, then increased to 135 °C at a rate of 30 °C/min, and to 280 °C at a rate of 70 °C/min, where it was held for 1 min. Interface, injector and ion source were kept at 260, 200 and 250 °C, respectively. Electron energy and electron current were set to 70 eV and 100 μA, respectively, for negative-ion chemical ionization (NICI) with methane (2.4 ml/min) as the reagent gas.

3. Results

3.1. Analysis of nitrite and nitrate in aqueous buffer in the presence of 9-NO_2-OA

Using the respective optimal derivatization time, i.e. 5 min for nitrite and 60 min for nitrate [5], mean blank nitrite and nitrate concentrations in the PBS buffer used were measured to be about 340 nM and 3.1 μM, respectively (Table 1). Addition of 9-NO_2-OA to the buffer at final concentrations within the range 0–10 μM, derivatization with PFB-Br in aqueous acetone and GC–MS analysis did not change the concentration of nitrite or nitrate in PBS (Table 1). These findings suggest that 9-NO_2-OA does not contribute to nitrite or nitrate detection in this GC–MS method, even when 9-NO_2-OA is heated for 60 min at 50 °C. Different nitrate concentrations were measured after 5 and 60 min of derivatization. This collaborates with previous findings of our group [5], and can be explained by the low derivatization yield of PFB-ONO_2 for short incubation times [5]. Indeed, the mean peak area of *m/z* 63 for the internal standard [^{15}N]nitrate (at 40 μM) was 4.6 times greater in samples incubated for 60 min as compared with those derivatized for 5 min. Also, the very comparable nitrite concentrations measured after 5 and 60 min of derivatization collaborate with previous findings [5], and can be explained by the complete derivatization of nitrite after an incubation time of 5 min. After that time PFB-NO_2 (from nitrite) and PFB-$^{15}NO_2$ (from the internal standard [^{15}N]nitrite) hydrolyze slowly and to the same extent. Indeed, the mean peak area of *m/z* 47 for the internal standard [^{15}N]nitrite (at 4 μM) was only 1.07 times greater in the samples incubated for 5 min as compared with those derivatized for 60 min.

Table 1
Nitrite and nitrate concentrations simultaneously measured by GC–MS in phosphate buffered saline in the presence of varying concentrations of 9-nitro-oleic acid (9-NO_2-OA)

9-NO_2-OA (μM)	Nitrite (nM, mean ± SD, *n* = 2)		Nitrate (μM, mean ± SD, *n* = 2)	
	5 min	60 min	5 min	60 min
0	341 ± 13	320 ± 8	6.79 ± 0.39	3.10 ± 0.13
1	322 ± 4	327 ± 4	6.48 ± 0.03	3.03 ± 0.02
2	326 ± 7	328 ± 5	5.87 ± 0.19	3.00 ± 0.08
5	337 ± 10	336 ± 9	5.84 ± 0.21	3.51 ± 0.17
10	343 ± 7	352 ± 1	6.25 ± 0.11	3.20 ± 0.01
Mean ± SD	334 ± 12	333 ± 13	6.25 ± 0.42	3.17 ± 0.21

3.2. Analysis of nitrite and nitrate in human plasma in the presence of 9-NO_2-OA or 10-NO_2-OA

The results from the quantitative determination of nitrite and nitrate in human plasma before and after addition of varying amounts of 9-NO_2-OA or 10-NO_2-OA to achieve final added concentrations of 1, 2, 5 and 10 μM each are summarized in Table 2 and Table 3. Representative GC–MS chromatograms from the simultaneous analysis of nitrite and nitrate (derivatization for 60 min) in an unspiked plasma sample and in a plasma sample spiked with 10 μM of 9-NO_2-OA are shown in Fig. 2. These data show that neither 9-NO_2-OA nor 10-NO_2-OA contribute to nitrite and nitrate detection in human plasma, even when added at concentrations more than 10-fold those reported to physiologically occur in human plasma [4]. Thus, 9-NO_2-OA and 10-NO_2-OA do not interfere with the simultaneous analysis of nitrite and nitrate as PFB derivatives by this GC–MS method [5].

Table 2
Nitrite concentrations measured by GC–MS in human plasma samples in the presence of varying concentrations of 9-nitro-oleic acid (9-NO_2-OA) or 10-nitro-oleic acid (10-NO_2-OA)

NO_2-OA added (μM)	Nitrite (nM, mean ± SD, *n* = 2)			
	9-NO_2-OA		10-NO_2-OA	
	5 min	60 min	5 min	60 min
0	788 ± 40	892 ± 6	748 ± 34	820 ± 23
1	802 ± 8	902 ± 48	650 ± 8	780 ± 74
2	804 ± 45	828 ± 6	668 ± 11	764 ± 0
5	794 ± 37	794 ± 25	714 ± 14	754 ± 42
10	776 ± 17	818 ± 59	658 ± 42	776 ± 40
Mean ± SD	793 ± 1	847 ± 48	688 ± 42	779 ± 25

Table 3
Nitrate concentrations measured by GC–MS in human plasma samples in the presence of varying concentrations of 9-nitro-oleic acid (9-NO_2-OA) or 10-nitro-oleic acid (10-NO_2-OA)

NO_2-OA added (μM)	Nitrate (μM, mean ± SD, $n = 2$)			
	9-NO_2-OA		10-NO_2-OA	
	5 min	60 min	5 min	60 min
0	54 ± 0	40.4 ± 0.2	50.5 ± 0.8	41.9 ± 0.0
1	51.9 ± 1.3	41.4 ± 0.1	50.9 ± 0.7	41.3 ± 0.2
2	51.3 ± 1.3	41.9 ± 0.4	49.7 ± 0.7	41.6 ± 0.6
5	51.9 ± 1.8	41.5 ± 0.1	53 ± 1.4	43.1 ± 1.3
10	50.4 ± 0.8	41.9 ± 0.1	50.2 ± 2.1	42.4 ± 0.1
Mean ± SD	51.9 ± 1.3	41.4 ± 0.6	50.9 ± 1.3	42 ± 0.7

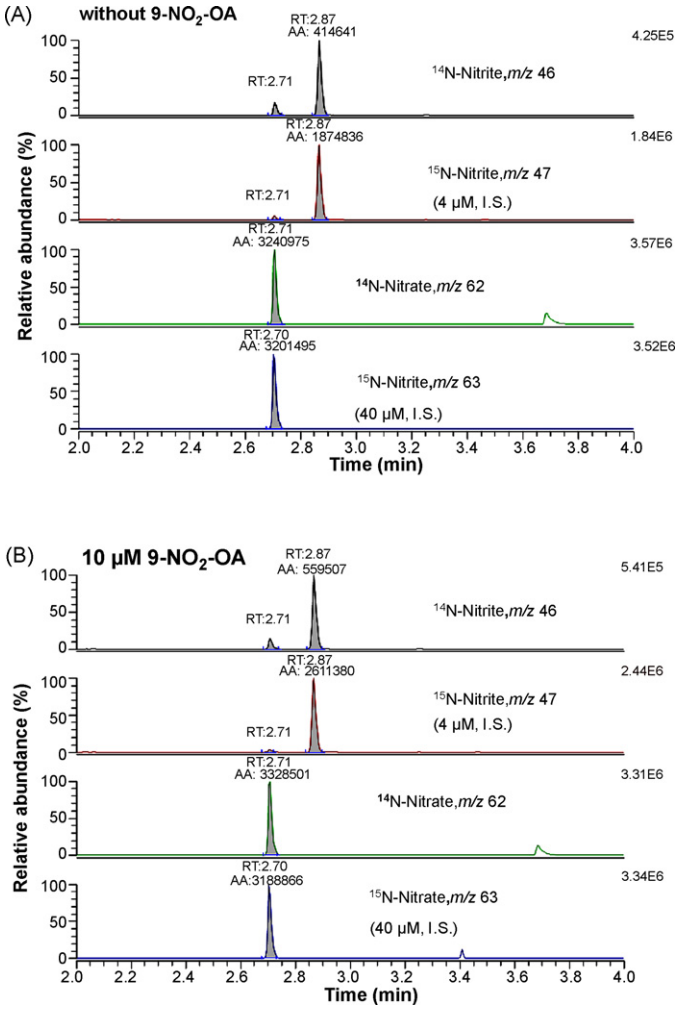

Fig. 2. Partial GC–MS chromatograms from the simultaneous analysis of nitrite and nitrate in human plasma samples before (A) and after (B) addition of 9-NO_2-OA at a final concentration of 10 μM. Both plasma samples were spiked with [^{15}N]nitrite (at 4 μM) and [^{15}N]nitrate (at 40 μM) and derivatization time was 60 min each in unspiked and spiked samples. Selected-ion monitoring of *m/z* 46 ($^{14}NO_2^-$), *m/z* 47 ($^{15}NO_2^-$), *m/z* 62 ($^{14}NO_3^-$), and *m/z* 63 ($^{15}NO_3^-$) was performed. The PFB derivatives of unlabelled and labelled nitrate and nitrite emerged from the GC column at 2.71 min and 2.87 min, respectively. The small peaks with the retention time of the nitrate PFB derivatives (PFB-O$^{14}NO_2$ and PFB-O$^{15}NO_2$, 2.71 min) at *m/z* 46 and *m/z* 47 result from the weak ionization of PFB-O$^{14}NO_2$ and PFB-O$^{15}NO_2$ to form $^{14}NO_2^-$ (*m/z* 46) and $^{15}NO_2^-$ (*m/z* 47) in addition to O$^{14}NO_2^-$ (*m/z* 62) and O$^{15}NO_2^-$ (*m/z* 63), respectively [5].

Different concentrations of nitrate were measured in plasma after 5 and 60 min of derivatization. The mean peak area of *m/z* 63 for the internal standard [^{15}N]nitrate (at 40 μM) was 10.2 times greater in plasma samples incubated for 60 min as compared with those derivatized for 5 min. Very comparable nitrite concentrations were measured after 5 and 60 min of derivatization, with the mean peak area of *m/z* 47 for the internal standard [^{15}N]nitrite (at 4 μM) being 0.7 times lower in plasma samples incubated for 60 min as compared with those derivatized for 5 min. In collaboration with previous findings [5], optimum derivatization time with PFB-Br in aqueous acetone at 50 °C is 5 min for nitrite and 60 min for nitrate. For simultaneous analysis of nitrite and nitrate, the derivatization time should be chosen to 60 min.

4. Discussion

Previous work suggests that endogenous and exogenous compounds containing *N*-nitroso and *N*-nitro functionalities such as *N*-nitroso-morpholine and N^G-nitro-L-arginine, respectively, *O*-nitro and *C*-nitro functionalities such as pentaerythrityl tetranitrate and 3-nitrotyrosine, respectively, do not contribute to nitrite and nitrate detection under the derivatization conditions of the GC–MS method that uses PFB-Br [5]. By contrast, other derivatization procedures that require stronger conditions such as the use of concentrated sulfuric acid or reduction with cadmium were found to artifactually contribute to nitrite and nitrate concentrations ([8]; for a discussion see [9]). The aim of the present study was to investigate the potential interference of the newly discovered nitrated lipids 9-NO_2-OA and 10-NO_2-OA [3,4] with the GC–MS analysis of nitrite and nitrate as PFB derivatives [5].

Nitrated lipids such as 9-NO_2-OA and 10-NO_2-OA represent a special class of nitro-compounds. In contrast to nitrated amino acids such as N^G-nitro-L-arginine (L-NNA or L-NAME) and 3-nitro-tyrosine, 9-NO_2-OA and 10-NO_2-OA are highly lipophilic. Preliminary investigations indicated that 9-NO_2-OA and 10-NO_2-OA can be analyzed by GC–MS as their PFB esters (data not shown), suggesting that oleic acid metabolites such as 9-NO_2-OA and 10-NO_2-OA as well as the oleic acid epoxide (9,10-epoxy-octadecanoic acid, EODA) [10] exert sufficient thermal stability enabling GC–MS analysis of these apparently thermally labile compounds. We also found (data not shown) that the free fatty acids of 9-NO_2-OA and 10-NO_2-OA are readily extractable from aqueous buffered solution of neutral pH into toluene which is mainly used in the GC–MS method that utilizes PFB-Br as the derivatization agent [5].

Theoretically, 9-NO_2-OA and 10-NO_2-OA could contribute to nitrite and/or nitrate detection by two different mechanisms (Fig. 1). In accordance with the first mechanism the free acids as well as the esters of 9-NO_2-OA and 10-NO_2-OA could hydrolyze to nitrite and/or nitrate during the derivatization step, i.e. heating at 50 °C for 5 or 60 min. Nitrite and/or nitrate produced from decomposed 9-NO_2-OA and 10-NO_2-OA would then readily react with PFB-Br to form the PFB derivatives, i.e. PFB-NO_2 and PFB-ONO_2, respectively (Fig. 1, route A). The second mechanism could involve reaction of 9-NO_2-OA

and 10-NO_2-OA, co-extracted into toluene, with excess PFB-Br and/or its hydrolysis product PFB alcohol (PFB-OH) [5] in the injection port of the GC (kept at 200 °C) and/or on column (initially kept at 70 °C) during co-elution to produce PFB-NO_2 and PFB-ONO_2 (Fig. 1, route B). Our results show that 9-NO_2-OA and 10-NO_2-OA do not contribute to nitrite and nitrate detection in the GC–MS method involving derivatization of nitrite and nitrate with PFB-Br in aqueous acetone [5]. Our results suggest that 9-NO_2-OA and 10-NO_2-OA are quite stable in aqueous acetone under the derivatization conditions at least they do not decompose to form nitrite and nitrate. Also, 9-NO_2-OA and 10-NO_2-OA seem to possess sufficient thermal stability under the GC conditions both as free acids and as PFB derivatives. It is likely that other nitrated lipids such as nitro-linoleic acid would also not interfere with the GC–MS analysis of nitrite and nitrate as PFB derivatives in plasma and other matrices containing such species. However, the potential of nitro-linoleic acid and other nitrated polyunsaturated fatty acids to interfere with the nitrite and nitrate analysis by this method remains to be investigated.

Nitrite and nitrate have been shown to nitrate tyrosine, thus yielding falsely too high concentrations of 3-nitrotyrosine in human plasma (reviewed in [11]). The potential interference of nitrated lipids such as 9-NO_2-OA and 10-NO_2-OA with the analysis of 3-nitrotyrosine by methods involving sample derivatization, such as GC–MS and GC–MS/MS or even some LC–MS/MS [11], should be investigated. From the mechanistic point of view, the reaction of nitrated lipids such as 9-NO_2-OA and 10-NO_2-OA and nitrated linoleic acid forms with reactive aromatic rings such as that in tyrosine and tyrosine derivatives in the heat, e.g. in the injector port at 280 °C [11], seems to be favored.

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