

Gas and liquid chromatography-mass spectrometry of aldehydic products from lipid peroxidation

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The present report describes the identification of aldehydic products of lipid peroxidation by two chromatographic methods coupled to mass spectrometry, GC/EI-MS and LC/ESI-MS. The easy detection of molecular ions in LC/ESI-MS and fragmentation pattern in GC/EI-MS illustrate the complementarity of these two techniques. Several carbonyl compounds, other than the usual reported breakdown products of lipid peroxidation, e.g. malondialdehyde and 4-hydroxynonenal, were identified as 2,4-dinitrophenylhydrazine derivatives in the Fe²⁺/ascorbate-catalyzed peroxidation of linoleic and linolenic acids.

Introduction

Reactive species, especially those deriving from oxygen, produced during either physiological enzymatic reactions or particular conditions involving xenobiotics metabolism and pathological disorders, generate oxidative stress when physiological antioxidant defense mechanisms are overloaded. Oxidative stress leads to damages of biomolecules, such as lipids, proteins and nucleic acids. Lipid peroxidation is a degradation chain reaction of polyunsaturated fatty acids, which are easy targets of free radical attack. It further leads to conjugated dienes, hydroperoxides and aldehydes [1]. Aldehydes are secondary products of peroxidation, more stable and diffusible than hydroperoxides. Their composition is very complex and depends on the fatty acid content of the lipids and on the free radical generating system [2]. Aldehydes act as "second toxic messengers", responsible for damage of targets which are distant from the initial free radical event [3,4]. Among them, 4-hydroxyalkenals, in particular 4-hydroxynonenal, were found to have high toxic effects on cell membranes and on numerous cell functions, being involved in the aetiology of many human diseases, e.g. atherosclerosis and cancer [3,5].

The present study is focused on the development of methods devoted to the identification of aldehydes resulting from polyunsaturated fatty acids oxidation. Lipid peroxidation is usually monitored by colorimetric reaction of thiobarbituric acid with malondialdehyde, a dicarbonyl compound produced in relatively high amount during autoxidation of fatty acids. Nevertheless, the thiobarbituric acid-reactive substances assay is criticized because of many interfering side reactions [6]. Moreover, major products of lipid peroxidation, such as alkanals and 4-hydroxyalkenals, are not detected by the thiobarbituric acid-reactive substances assay; thus, it is not a reliable index of peroxidation and toxicity [7].

Further, analysis of 4-hydroxynonenal has been performed by HPLC [8] or by GC/MS after derivatization [8,9,10]. As a result, 4-hydroxynonenal being a better indicator than malondialdehyde, it was assumed as a model molecule for oxidative stress [3,5].

Characterization of individual breakdown products of lipid peroxidation requires high sensitive derivatization methods of carbonyl groups and the use of mass spectrometry (MS) techniques for their identification. Among various derivatization techniques, reaction with 1,3-cyclohexanedione produces fluorescent dehydroacridine derivatives which are separated by HPLC. Such a method is sensitive, but has a low specificity [11]. Oxime derivatives, obtained with either hydroxylamine or pentafluorobenzylhydroxylamine, were successfully used for carbonyl products identification by GC/MS, directly or after trimethylsilylation [12, 13]. However, these derivatives are colourless and some difficulties arise for their specific detection during the preliminary thin layer chromatography (TLC) step. 2,4-dinitrophenylhydrazine (DNPH) derivatization presents several advantages: high reaction specificity, sensitive UV detection (λ_{\max} : 360-380 nm, ϵ : 25.000-28.000 M⁻¹ cm⁻¹), good stability and easy visualisation of dinitrophenylhydrazones by TLC [14]. Moreover, these derivatives can be analysed either by HPLC or GC coupled to MS. Despite the interferences of traces of contaminating aldehydes from solvents, from reagents and from laboratory air, they have been extensively used for the characterization of lipid peroxidation products.

Concerning structural studies of DNPH derivatives by MS, chemical [15] and electrospray [16] ionization (CI and ESI) easily supply molecular ions, but fragments are not sufficiently abundant for their precise identification. In order to assess structures by fragmentation, electron impact (EI) [17] or MS/MS [18] techniques have to be tested.

Therefore, we selected in the present work the DNPH derivatization method followed by TLC separation and LC/ESI-MS and GC/EI-MS analysis of the corresponding derivatives. Two fatty acids were selected as peroxidation substrates, linoleic and linolenic acids, because of their wide distribution in biological samples and their high susceptibility to autoxidation.

Materials and methods

Chemicals and standards

Linoleic (9,12-octadecadienoic) and linolenic (9,12,15-octadecatrienoic) acid methylesters and aldehyde standards were purchased from Sigma-Aldrich (St. Quentin-Fallavier, France) and used without further purification. 2,4-dinitrophenylhydrazine (Merck, Darmstadt, Germany) was purified by recrystallisation (*n*-butanol).

Oxidation of polyunsaturated fatty acids

20 mg linoleic or linolenic acid methylester was suspended in 30 mL 0.1 M Tris buffer pH 7.4 and 60 mL 0.15 M KCl. Autoxidation was started by adding 5 mL 20 mM ascorbic acid and 5 mL 0.8 mM FeSO₄ [19]; the resulting mixture was incubated at room temperature for either 24 h (linolenic acid) or 48 h (linoleic acid). Aldehydes were extracted with CH₂Cl₂ (3 × 20 mL), then organic layers were concentrated to dryness under a gentle nitrogen stream (≤ 35 °C).

Derivatization of aldehydes with DNPH

10 mL 2.5 mM DNPH in 1 M HCl was added to the above residue then incubated for 24 h in the dark at room temperature under nitrogen atmosphere stirring gently during the first 2 h. Derivatives were extracted with CH₂Cl₂ (3 × 20 mL), then organic layers were concentrated to dryness under nitrogen (≤ 35 °C).

Thin layer chromatography

DNPH derivatives were dissolved in 1 mL CH₂Cl₂, applied on a home made TLC plate (silica gel G 60 Merck, layer thickness 0.25 mm), then successively developed with CH₂Cl₂ (5 cm) and toluene (15 cm). Three zones with different R_f: 0-0.2 (zone I), 0.3-0.5 (zone II) and 0.6-0.8 (zone III) or well defined bands in each zone were scraped off and eluted with CH₃OH (2 × 1 mL). The methanolic extracts were dried under nitrogen and the resulting residue was dissolved in 0.5 mL CH₃CN.

LC/ESI-MS

Mass spectrometry was carried out using a Micromass Platform II (Micromass Ltd., Manchester, UK). Cone voltage was set at 30 V and the temperature source was 80 °C. Data were collected in negative mode and reprocessed by means of Mass Lynx software (Micromass). The HPLC system coupled to the electrospray mass spectrometry consisted of a solvent delivery pump (model PU 980; Jasco, Prolabo, Fontenay-sous-Bois, France), an injection valve (model 7725i, Rheodyne) fitted with a 20-μL loop, an analytical column (125 × 2 mm i.d.) prepacked with LiChrospher RP 18 end-capped (5 μm; Merck) and a UV spectrophotometric detector (model UV 975; Jasco) set at 360 nm. Elution was operated using CH₃CN-H₂O with a linear gradient (50 to 95 % CH₃CN in 30 min), at a flow rate 0.2 mL.min⁻¹. A postcolumn 1:20 splitter reduced the eluent flow rate entering the mass spectrometry source.

GC/MS

A GC (Carlo Erba) apparatus was equipped with a methylsilicone SE-30 capillary column (25 m × 0.32 mm i.d., 0.20 μm). Injector and interface temperatures were 250 °C; column oven temperature: 200 °C to 250 °C (5 °C/min); helium gas carrier (0.2 mL.min⁻¹). The GC system was coupled to EI-MS (Nermag R 10-10c) set at an electron energy of 70 eV.

Results and discussion

Oxidation of polyunsaturated fatty acids produces a great number of carbonyl products with different chain lengths, unsaturation degrees and substitutions, even when a single and pure fatty acid is used as an oxidation substrate. Therefore, the complex mixture of 2,4-dinitrophenylhydrazones requires preliminary TLC separation. We focused on the identification of compounds from zones I and III, which are hereinafter designed as "polar" and "non-polar" fractions, respectively. Zone II, according to literature data [2,7,14], contains osazones and was not presently studied. GC and HPLC analysis of zones I and III, even when considering individual bands, showed an important number of components. Moreover, as shown by MS, some compounds coelute in HPLC.

Non-polar aldehyde derivatives

The GC/MS analysis of the non-polar fraction (TLC zone III) of oxidized linolenic acid allowed to identify dinitrophenylhydrazones of alkanals, alkenals and alkadienals, using the following molecular ions: acetaldehyde (224), propanal (238), butanal (252), pentanal (266), hexanal (280), hexenal (278), hexadienal (276) heptadienal (290), octadienal (304) and nonadienal (318) (Fig. 1). Typical fragments corresponding to the DNPH residue were observed: M-30 (NO), M-46 (NO₂) and the ion m/z 182 ascribed to the cleavage of the nitrogen-nitrogen bond in DNPH moiety.

GC/MS of a non-polar TLC fraction

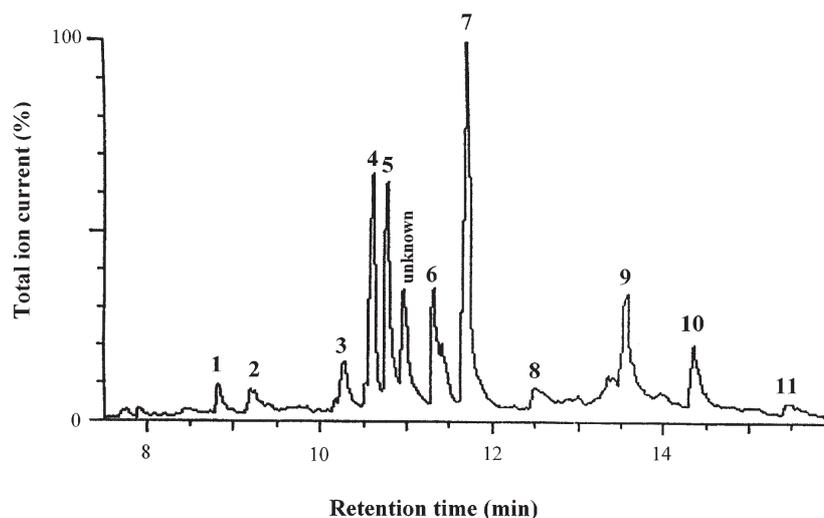


Figure 1. GC/EI-MS of a non-polar TLC fraction (zone III; R_f 0.7) of DNPH carbonyls resulting from oxidized linolenic acid: 1: pentanone; 2: butanal; 3: hexanal (*syn*); 4: hexanal (*anti*); 5: 2-hexenal; 6: 3-hexenal (*syn*); 7: 3-hexenal (*anti*); 8: hexadienal; 9: heptadienal; 10: octadienal; 11: nonadienal.

LC/ESI-MS of a non-polar TLC fraction

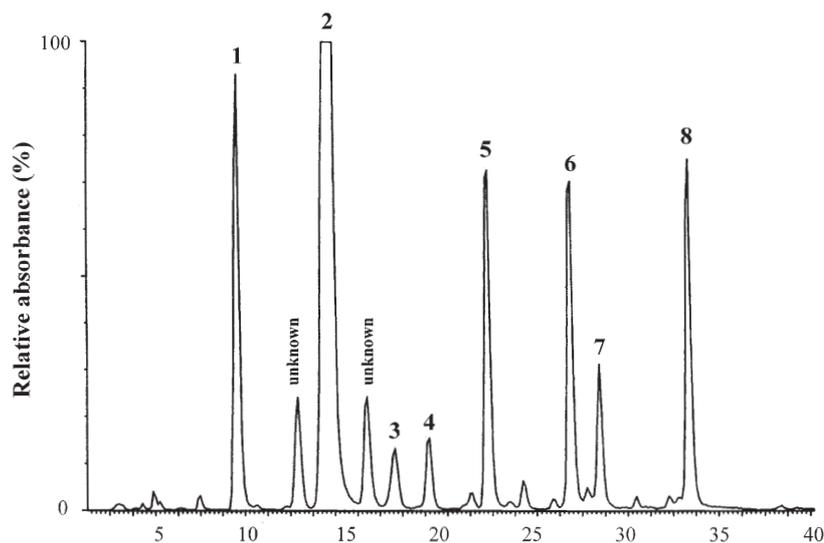


Figure 2. DNPH derivatives of aldehydes identified using LC/ESI-MS and monitored by UV detection ($\lambda = 360$ nm) in a non-polar TLC fraction (zone III; R_f 0.6) of oxidized linolenic acid: 1: acetaldehyde; 2: propanal; 3: butanal; 4: butanal; 5: pentanal; 6: hexenal; 7: heptadienal; 8: nonadienal.

This indicates the presence of DNPH carbonyls as described previously [15,18]. Ions corresponding to fragmentation of the side chain are also useful.

Since DNPH derivatives exist as *syn* and *anti* isomers, they appear as double GC peaks. Their identification relied upon literature data indicating successive elution of *syn* and *anti* isomers [20]. The isomerization is promoted by increasing the column temperature (elevation of *syn/anti* ratio), whereas LC/ESI-MS supplies simpler chromatograms by avoiding formation of *syn* isomers. The identification of products cited above was also performed using LC/ESI-MS technique (Fig. 2).

Similarly, using GC/MS and LC/ESI-MS, we identified in the case of linoleic acid a wide number of saturated and unsaturated aldehydes with C_3 to C_{10} side-chain in the non-polar zone; m/z values of molecular ions are for acetaldehyde: 224, propanal: 238, butanal: 252, butenal: 250, pentanal: 266, hexanal: 280, heptanal: 294, heptenal: 292, octanal: 308, octenal: 306, nonanal: 322, nonenal: 320, nonadienal: 318, decenal: 334 and decadienal: 332. Ketones, such as butanone and pentanone, which are also formed as secondary products of lipid peroxidation, give the same m/z values of molecular ions as the corresponding aldehydes. Their identification can be ascertained by comparison with HPLC profiles of standards.

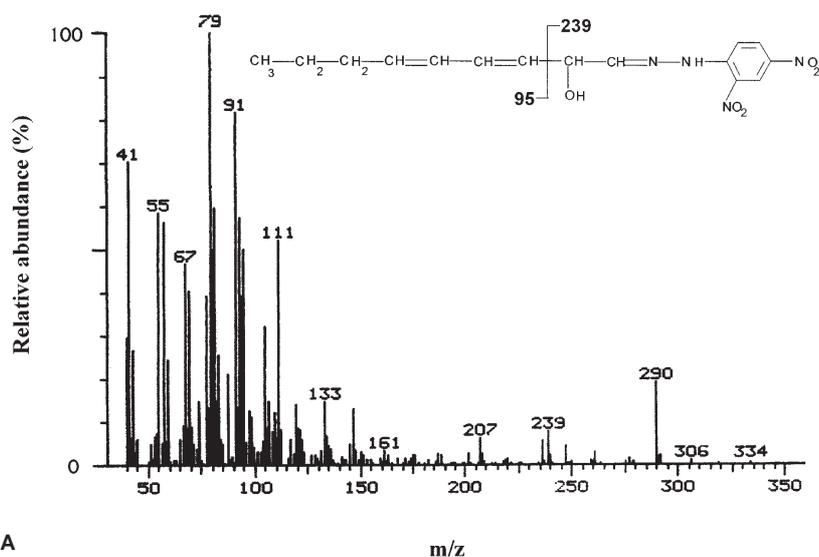
The most abundant non-polar aldehydes were propanal (60 % of the fraction Rf 0.6) and hexanal (68 % of the fraction Rf 0.7), for linolenic acid and linoleic acid oxidation, respectively, as already reported [1,7].

Polar aldehyde derivatives

Identification of 2,4-dinitrophenylhydrazones in the polar fraction (TLC zone I) is very important because they might

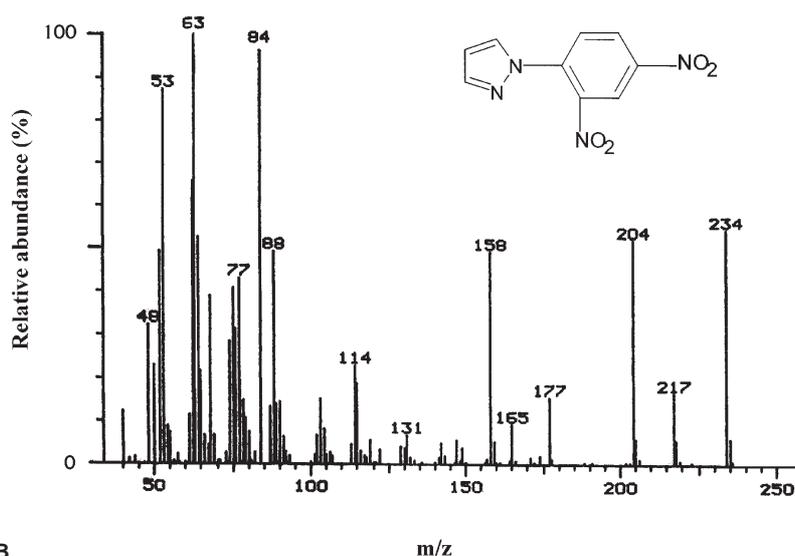
be hydroxyaldehydes, which show a high toxicity. Their identification is however more difficult using GC/MS because molecular ions are frequently unseen. Nevertheless, when the molecular ion can be detected, EI-MS supplies useful data to assess structures. For example, Fig. 3A represents the EI-MS of the dinitrophenylhydrazone derived from 2-hydroxy-nona-3,5-dienal (m/z 334); the localization of the OH group relied upon the ion m/z 239. Only few α -hydroxyalkanals have been described as lipid peroxidation secondary products. It was suggested that most α -hydroxyaldehydes react with biomolecules due to their high

EI-MS of 2-hydroxy-nona-3,5-dienal-DNPH



A

EI-MS of malondialdehyde-DNPH



B

Figure 3. EI-MS of 2,4-dinitrophenylhydrazones of: A: 2-hydroxy-nona-3,5-dienal (m/z 334). The fragment at m/z 239 is essential for the localization of OH group. The fragment at m/z 290 corresponds to M-17-27 (OH, HCN). B: malondialdehyde (m/z 234). Key fragments correspond to M-17 (OH), M-30 (NO), M-30-27 (NO, HCN), M-30-46 (NO, NO₂).

reactivity and thus escape detection [12,19]. Owing to its structure, such as OH in α position which considerably enhances the reactivity of carbonyl and conjugated double bonds, the α -hydroxyalkadienal has probably a very high reactivity and toxic effects similar to 4-hydroxyalkenals.

In order to improve thermal stability of hydroxyaldehydes, we performed trimethylsilylation of the OH group. Indeed, it was reported that DNPH derivatives of hydroxyaldehydes can be trimethylsilylated without altering their recovery [15]. As N-methyltrimethylsilyl-trifluoroacetamide (MSTFA) was successfully used for the trimethylsilylation of oxime derivatives [12,19], we used this reagent in the case of DNPH-carbonyls. We obtained a derivative resulting from the replacement of the DNPH residue with formation of a typical aldehyde-MSTFA adduct, as in the case of direct MSTFA derivatization of aldehydes [21]. Such an adduct gave an abundant fragment (m/z 228) corresponding to the MSTFA derivatized carbonyl group after α cleavage of the side chain (data not shown). However, molecular ions are frequently missing in MSTFA derivatives.

Hydroxyaldehydes were mainly detected using LC/ESI-MS and in part confirmed using GC/EI-MS, because of difficulties exposed above. For example, oxidation of linoleic acid resulted in a wide range of mono- and dihydroxy-substituted alkanals (1), alkenals (2) and alkadienals (3). The [M-H]⁻ values of their pseudomolecular ions are (1) hydroxyhexanal: 295; hydroxyheptanal: 309; hydroxydecanal: 351; hydroxyundecanal: 365; dihydroxyheptanal: 325; dihydroxyoctanal: 339; dihydroxydecanal: 367; dihydroxyundecanal: 381; dihydroxydodecanal: 395; (2) hydroxyheptenal: 307; hydroxynonenal: 335; hydroxydecanal: 349; dihydroxyundecenal: 379; dihydroxydodecanal: 393; dihydroxytetradecenal: 421 and (3) hydroxynonadienal: 333; dihydroxyundecadienal: 377, dihydroxydodecadienal: 391; dihydroxytetradecadienal: 419. 4-hydroxynonenal was a main hydroxyaldehyde originated from the linoleic acid oxidation, as already reported [1,3]. In the zone I, we also found DNPH derivatives corresponding to the fatty acid long chain substituted with polar groups (hydroxy, keto), *i.e.* 9-oxo-13-hydroxy-10-octadecenoic acid methylester (m/z 506).

Malondialdehyde was found to be a major product resulting from linolenic, but not linoleic acid oxidation. It does not form a dinitrophenylhydrazone, but a cyclic and colourless pyrazole derivative (λ_{\max} : 307 nm [22]; m/z : 234 [23]) (Fig. 3B).

Conclusion

Analysis of aldehydic breakdown products reflects the balance between the rate of lipid peroxidation and the metabolism of peroxidized products. It is therefore the most reliable indicator for the measurement of the *in vivo* occurrence of lipid peroxidation [1]. Contrary to the currently used thiobarbituric acid-reactive substances assay, which affords a

global evaluation of peroxidation, the reported methods including DNPH derivatization allow the identification of individual aldehydes and a better estimation of its toxicological consequences.

MS techniques presently used are complementary for the identification of DNPH derivatives of aldehydes. The GC/MS technique allows to assign structures in numerous cases. Nevertheless, difficulties in identification of high molecular mass and polar compounds and frequent absence of molecular ions limit this technique. ESI-MS easily supplies molecular ions and enables an accurate study of aldehydes resulting from lipid peroxidation. Both MS techniques permit the single ion monitoring. Correlation between EI-MS and ESI-MS data led us to the identification of most of aldehydic products of linoleic and linolenic acids oxidation. The peroxidation breakdown products are very complex. We found numerous compounds corresponding to previously described categories: *n*-alkanals, 2-alkenals, 2,4-alkadienals, α -hydroxyaldehydes, 4-hydroxyalkenals, MDA, ketones [1,2,7]. In addition, the two MS techniques permitted us to identify an important number of carbonyl products substituted with OH group, which possess high reactivity and may contribute to damages during lipid peroxidation, as described for 4-hydroxynonenal.

These preliminary experiments permitted to define a useful tool to study lipid peroxidation resulting from a specific enzymatic reaction (GGT), *i.e.* cleavage of glutathione by gamma-glutamyltransferase in the presence of iron. As a matter of fact, we have recently demonstrated that this enzyme located in cell membrane has a prooxidant effect by producing reactive oxygen species during glutathione metabolism [24]. The analysis of lipid peroxidation products generated by GGT/glutathione system is under current study, using mainly LC/ESI-MS. Furthermore, comparison of HPLC retention times of these products with chromatograms of DNPH aldehydes identified during oxidation by Fe²⁺/ascorbate is quite useful, especially in the case of compounds produced in very small amounts. We detected thus an important number of non-polar, as well as polar dinitrophenylhydrazones of aldehydes which are formed in GGT-dependent oxidation of linoleic and linolenic acids [25], most of them being common to the Fe²⁺/ascorbate-catalyzed lipid peroxidation. The analysis of products formed with the enzymatic system will now be performed using a simpler methodology, *i.e.* HPLC with UV detection. Their respective proportion will be determined using an internal standardization method, because DNPH derivatives have very similar molar absorbance.

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