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1. Introduction

Malondialdehyde (MDA) is considered a marker of lipid peroxidation and it was determined for this purpose in different biological samples (1–6). In addition to different diseases also stress has been linked to lipid peroxidation and plasma MDA has been estimated as biochemical stress marker (7). Among the analytical methods so far used the formation of a volatile derivative and the separation by gaschromatography (GC) have been recently preferred to the well known spectrophotometric assay of complex with thiobarbituric acid (TBA) (8–13). As detection technique mass spectrometry (MS) allows the best selectivity to be reached (9–12). Hydrazones and oximes have been the derivatives mostly used and the pentafluorobenzyl moiety has been exploited in order to achieve the highest sensitivity by negative-ion chemical ionization (NICI) (10–12). In particular a NICI-GC-MS method has been validated for the analysis of MDA and 4-hydroxy-2-nonenal in plasma by single ion monitoring (SIM) technique (14). Mass spectral analysis can be improved by tandem mass spectrometry (MS-MS) which allows the isolation of a single ion produced by an analyte and its fragmentation giving daughter spectrum characteristics of the intact molecule; the highest specificity is reached by multiple reaction monitoring (MRM). Since no examples of application of tandem mass spectrometry have been presented until now for the MDA detection, in this work the pentafluorobenzyl oxime of MDA (MDA-PFBO) has been analysed in pig plasma as stress marker by GC-NICI-MS-MS.

2. Experimental

2.1 Materials

10 mM standard solution of MDA was prepared by hydrolysis in 0.01 M HCl of tetraethoxypropane (TEP) (Sigma, St.Louis, MO, USA) at 50 °C for 60'. The MDA final concentration was determined by absorbance at 245 nm. 10 µM solutions were obtained by dilution with 0.01 M HCl. Standard solutions of glutaraldehyde (GA) were prepared by dilution of commercial 25 % aqueous solutions (Sigma) with 0.01 M HCl. Pentafluorobenzylhydroxylamine (PFBHA) (Fluka, Buchs, Switzerland) was recrystallized from ethanol. Blood samples were drawn directly into preheparinized Vacutainer blood collection tubes (Becton Dickinson, Rutherford, NJ, USA) and centrifuged. The plasma was divided in 0.2 ml portions, added with 40 µl of 3 mM diterbuthylhydroxytoluene (BHT) in ethanol and frozen at –20 °C until analysis.

2.2 Apparatus and chromatographic conditions

A Finnigan GCQ instrument consisting of a Finnigan MAT A200S GC Autosampler, a Finnigan MAT/Tremetrics high-performance capillary GC, a capillary split/splitless injector with electronic pressure control and a Finnigan MAT Quadrupole Ion Trap Analyzer was used. The separations were carried out on a fused silica capillary column (30 m x 0.22 mm) containing the BPX-35 stationary phase (0.25 µm film thickness) (SGE, Austin,TX, USA). The column temperature increased from 160 °C to 300 °C at a rate of 15 °C/min.

The GC-MS-MS analysis was performed on a Finnigan GCQ instrument in the multiple reaction monitoring (MRM) mode. The analytes were ionised with negative ion chemical ionisation (NICI). Methane was used as a reagent gas at ion source pressure of $1{,}1 \times 10^{-4}$ torr. The temperature of injector and of transfer line were 240 °C and 300 °C respectively. The emission current was 250 µA and the collision energy of 50 eV. For data acquisition and processing the Finnigan GCQ 2.2 with MS-MS software implemented on a GCQ-PLUS with turbo molecular pump was used.

2.3 Derivatisation procedure

To plasma (0.2 ml) 10 µM aqueous solution of GA (20 µl) and 15 mg/ml solution of PFBHA in acetate buffer 1.5 M pH 5.0 (0.5 ml)

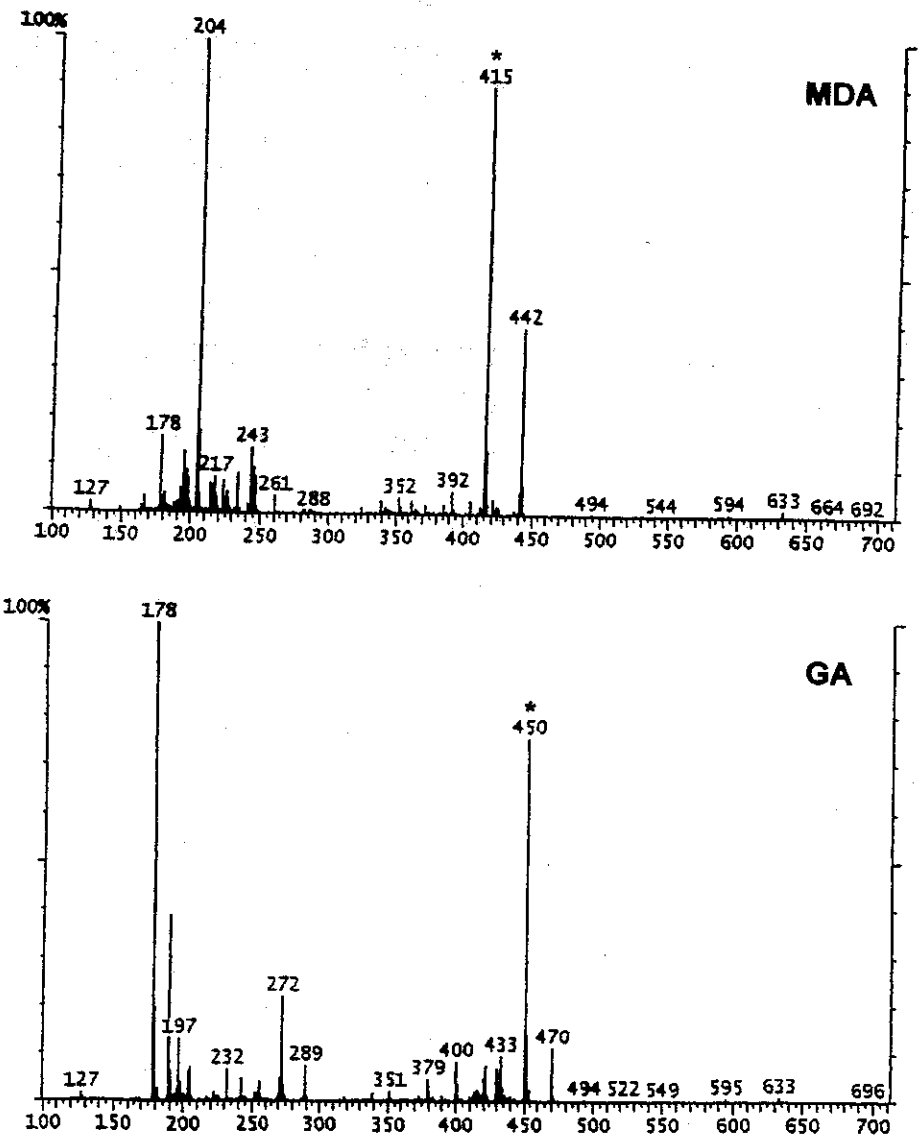

Fig. 1: NICI-MS spectra of pentafluorobenzyloximes of MDA and GA. Parent ions chosen for the subsequent fragmentation are indicated.

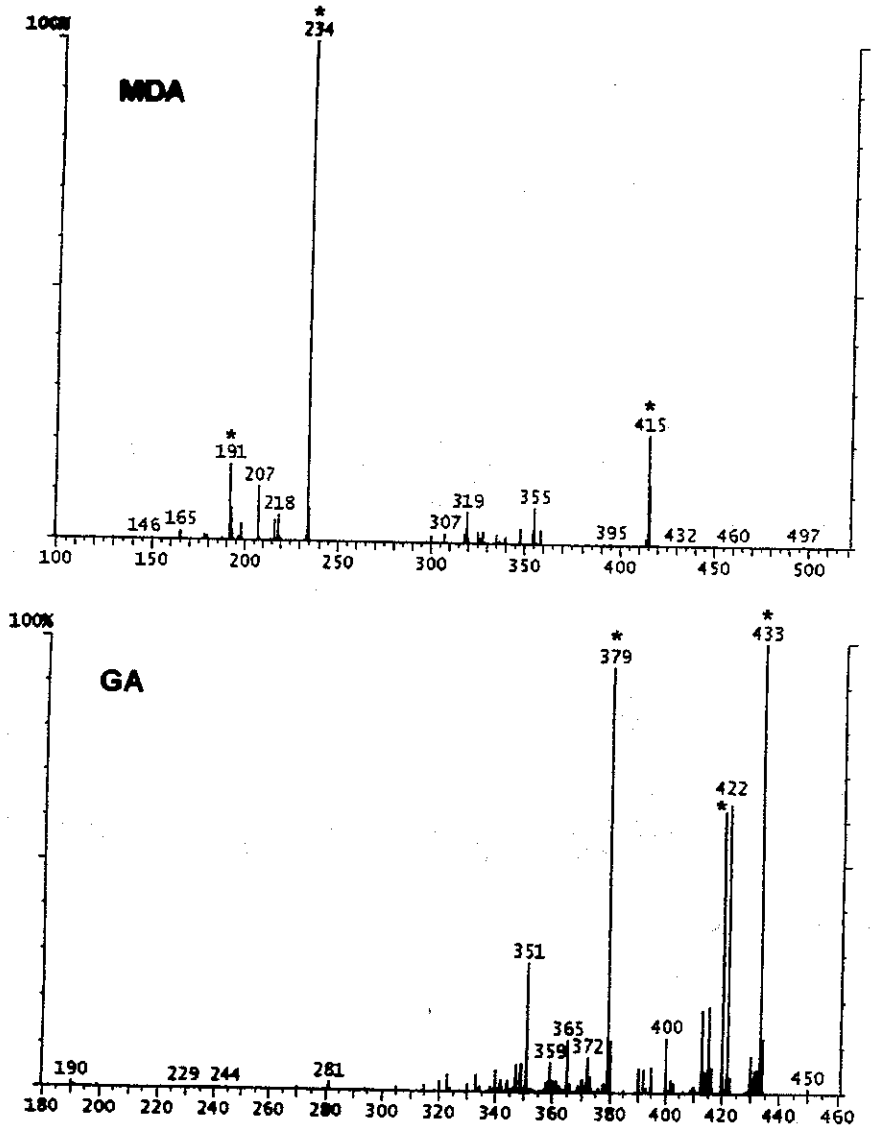

Fig. 2: NICI-MS-MS spectra of MDA and GA derivatives, The ions chosen for MRM detection are indicated.

were added. The mixture was kept at room temperature for 30 min. Methanol (1 ml) was then slowly added under mixing. Hexane (3 ml) and, after vortexing, 12 drops of concentrated sulphuric acid were added while keeping the mixture in ice. The sample was finally centrifuged, filtered on anhydrous sodium sulphate and dried under a stream of nitrogen. The residue was redissolved in hexane (100 µl) and aliquots were injected into the GC apparatus.

2.4 Quantitative analysis

Different amounts of MDA (40-1000 pmoles) and a same amount of GA (200 pmoles) were treated according the described procedure. Ratios between areas of MDA and GA obtained in MRM mode, were reported against MDA amounts resulting in the calibration curve.

3. Results and discussion

In order to exploit the NICI technique PFBHA was chosen as specific reagent for aldehyde groups of MDA. In fact one pentafluorobenzyl (PFB) moiety only is involved in the final MDA derivative when pentafluorobenzylhydrazine is used, while two PFB moiety are present in the derivative obtained with PFBHA, so increasing its electron capture power.

In Fig. 1 the NICI-MS spectra of MDA and GA derivatives are shown. Peaks at m/z 415 for MDA (M^+-HF-C_2H_3) and at m/z 450 for GA (M^+-HF-HF) appeared suitable as parent ions for MS-MS detection, because of their high molecular weight, which assures the detection specificity. In Fig. 2 the MS-MS spectra of MDA and GA are reported: the daughter ions at m/z 415, 234 and 191 for MDA and at m/z 433, 420 and 379 for GA were chosen for MRM analysis.

Four theoretical different derivatives are possible for dialdehyde compounds as MDA and GA, if the syn/anti isomerism is considered. While in the cited NICI-GC-MS determination of MDA as PFBO derivative three peaks were detected (14), in our chromatographic conditions only a minor peak and a major one for MDA and a single sharp peak for GA appear in the final MRM chromatogram reported in Fig. 3 (top) and corresponding to a standard mixture. The quantitative analysis has been carried out by considering the MDA major peak only. Really when the ratios (R) between areas of MDA major peak and GA one (corresponding to 200 pmoles) were plotted versus amounts of MDA (40-1000 pmoles) a linear relationship resulted according to the following equation:

$R = 0.00475 \times \text{pmoles MDA} - 0.116$ ($r = 0.999$)

In Fig. 3 a typical NICI-GC-MS-MS profile of a pig plasma sample, containing 44 pmole/0.2 ml (middle) and the MS-MS spectrum corresponding to the major peak of MDA (bottom) are reported. On one hand the comparison between the secondary fragmentation obtained with plasma sample (Fig. 3) and that obtained with standard MDA (Fig. 2) assures the identity of the analyte; on the other hand the higher relative area of the MDA minor peak compared to the major one confirms the findings elsewhere reported (14) regarding the occurrence of interferents at retention time of MDA minor peak, which was not considered for quantita-tive analysis.

The selectivity of the separation cannot be compared with that of the cited NICI-GC-MS method (14) because in that paper no chromatograms corresponding to real plasma samples were reported. In the present work the background due to the biological sample appears almost completely eliminated.

In Table 1 the contents of MDA in analysed pig samples are reported. The range (220–1015 pmoles/ml) is higher than that found in man (10,14), also probably due to the increased stress in these subjects whose blood collection was carried out at slaughter-house.

Table 1: **MDA content in pig plasma samples.**

Sample	MDA content (pmoles/ml)
Female large white	805
Female large white	1015
Female durok	560
Female durok	220
Male large white castrate	730
Male large white castrate	740
Male large white castrate	695
Male durok castrate	965
Male durok castrate	400

4. Conclusions

For the first time a NICI-GC-MS-MS determination of MDA in a biological matrix was performed. The presence in the final volatile derivative of ten fluorine atoms allows the NICI mode to be exploited; furthermore, in order to reach the maximum specificity in the MRM detection mode, representative fragments with high molecular weight can be used as parent ions for the secondary ionisation. Work is in progress in the view of linking the MDA pig plasma content to the features of the obtained meat. However the described method, due to its high selectivity, appears suitable for the determination of MDA in other biological specimens.

Abstract

Malondialdehyde (MDA) has been estimated in pig plasma by GC with NICI-MS-MS detection after derivatisation with pentafluorobenzylhydroxylamine. Glutaraldehyde was used as internal standard. MDA gave two peaks, the major one only was considered for the quantitative analysis. In multiple reaction monitoring (MRM) mode the minimum detectable amount was 40 pmoles/sample. In nine pig plasma samples, collected at slaughter-house, the MDA contents found were between 220–1015 pmoles/ml.

Zusammenfassung

Malondialdehyd (MDA) wurde gaschromatographisch (GC) mit NICI-MS-MS-Bestimmung nach Derivatisation mit Pentafluorobenzylhydroxylamin im Schweineplasma gemessen. Als innerer Standard wurde Glutaraldehyd verwendet. MDA erreichte zwei Höchstwerte, davon wurde nur der höchste quantitativ analysiert. Bei der Technik der Vielfachreaktionsüberwachung (MRM) konnte man eine Minimalmenge von 40 pmoles/Muster nachweisen. In neun Schweineplasmaproben aus dem Schlachthaus lagen die MDA-Werte zwischen 220–1015 pmoles/ml.

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Luca Maria Chiesa is the recipient of Italian Ministry of University and Scientific and Technological Research (M.U.R.S.T.) fellowship as Ph. D. student in „Control of total quality in zootechnic production of foods from animal source". Katia De Wasch is researcher in the department of Veterinary Food Inspection, Laboratory of Chemical Analysis, Veterinary Faculty of the University of Ghent, Belgium. Hubert De Brabander is professor of Analytical Chemistry of Food in the same department. Alessandro Maria Pecile is the recipient of a Italian M.U.R.S.T. fellowship as Ph.D. student in „Bovine clinics". Pier Antonio Biondi is professor of Propaedeutics Biochemistry in the Faculty of Veterinary Medicine of University of Milan, Italy.

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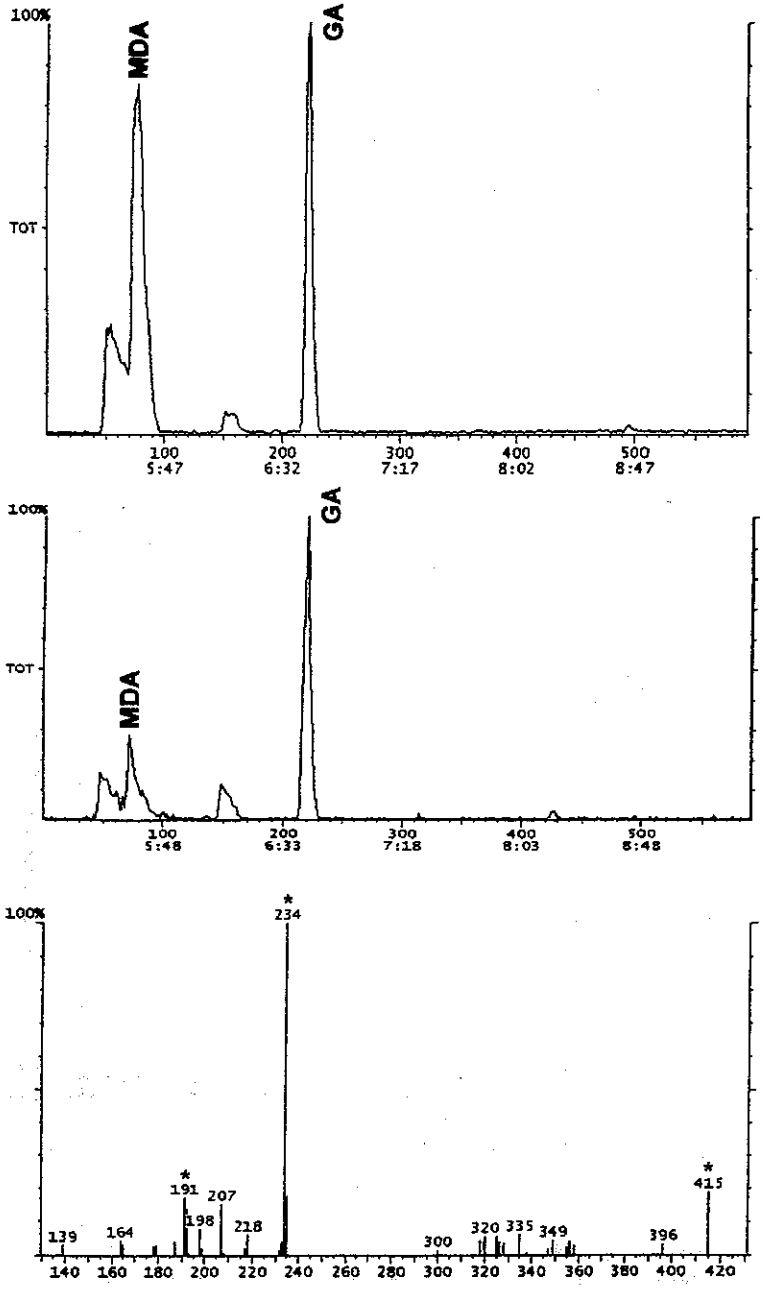

Fig. 3: NICI-GC-MS-MS chromatographic profiles of a standard mixture (top) and of a pig plasma sample (containing 44 pmoles MDA/0.2 ml) (middle), and MS-MS spectrum (bottom) collected at MDA major peak retention time (the ions chosen for MRM detection are indicated).

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