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Short communication

Improved procedure for the determination of malonaldehyde by gas-chromatography with electron-capture detection as 2,4,6-trichlorophenylhydrazine derivative

Lorenzo Sangalli [a], Luca Maria Chiesa [a], Elena Passerò [a], Ada Manzocchi [b], Giovanni Maffeo [a], Pier Antonio Biondi [a,*]

[a] *Department of Veterinary Science and Technology for Food Safety, Faculty of Veterinary Medicine, University of Milan, Via Celoria 10, Milan 20133, Italy*
[b] *Department of Chemistry, Biochemistry and Medical Biotechnologies, Faculty of Medicine and Surgery, University of Milan, Via Saldini 50, Milan 20133, Italy*

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Abstract

A previously described derivatization method using trichlorophenylhydrazine was developed for the estimation of malonaldehyde measured by gas-chromatography (GC) with electron-capture detection. The precision and reliability of the procedure are improved here by the use of methylmalonaldehyde as internal standard and by the introduction of a diverter valve at the end of the capillary column to protect the electron-capture detector, respectively.

The method was applied to determine malonaldehyde content in bovine plasma samples.

Keywords: Derivatization; GC; Malonaldehyde

1. Introduction

Malonaldehyde (propandial, MA) is a terminal product of polyunsaturated fatty acid peroxidation, often used as a marker of oxidative damage associated with degenerative phenomena and various diseases [1]. The most widely used method for MA estimation, based on the reaction with thiobarbituric acid (TBA), has been shown to lack selectivity in comparison to other derivatization procedures [2]. More specific methods involving HPLC and gas-chromatography (GC) techniques have been introduced; as regards the reagents used for GC determinations hydrazine based ones have been often preferred because of its capability to form stable pyrazole derivatives [3]. Among the chromatographic methods, the gas chromatography–mass spectrometry (GC–MS) one can be considered the technique of choice [4], as verified in our experience too [5]. A recently reported "reference method" involves the determination of the derivative with phenylhydrazine by isotope dilution GC–MS using di-deuterated MA as internal

* Corresponding author. Tel.: +39-02-5031-7930; fax: +39-02-5031-7941.
E-mail address: pierantonio.biondi@unimi.it (P.A. Biondi).

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standard [6]. As the GC–MS apparatus is not always available in analytical laboratories, an alternative cheaper detection system would be desirable without lacking the sensitivity of the MS one. In this connection, gas chromatoraphy–electron capture detector (GC–ECD) has been often exploited for MA estimation, using different halogenated reagents, such as pentafluorophenylhydrazine (PFPH) [7], pentafluorobenzylhydroxylamine (PFBHA) [8], and recently trichlorophenylhydrazine (TCPH) [9]. PFBHA reacts with MA in a 2:1 ratio to give three isomeric oximes, while N-arylhydrazines (PFPH and TCPH) react with MA in a 1:1 ratio to give a single product, N-arylpyrazole. Although, PFBHA derivatives were found to be more sensitive than the PFPH ones [7], no data are available regarding the comparison between PFBHA and TCPH derivatives of MA. A comparison of these halogenated reagents with respect to their ECD response is the preliminary aim of this work.

Methylmalonaldehyde (2-methylpropandial, MMA) has been recently introduced as internal standard for the analysis of MA by different techniques such as HPLC [10], capillary electrophoresis [11] and GC–MS, where it can be used instead of the more expensive isotope labelled MA [12]. Another aim of this communication is to test the suitability of MMA as internal standard also in a GC–ECD method.

Regarding the use of ECD apparatus, the main drawback is the poor reproducibility in detector response, generally due to the bleeding of stationary phase and to the interfering substances, which reach the ECD. The last aim of this work was to improve the GC–ECD method by the use of a diverter valve to maintain the stability of the ECD response. The improved procedure has been applied to the determination of MA in bovine plasma.

2. Experimental

2.1. Chemicals

2,4,6-Trichlorophenylhydrazine (TCPH), *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA), 2,6-di-*tert*-butyl-*p*-cresol (BHT), 2,4-dinitrochlorobenzene (DCNB) and MA tetrabutylammonium salt were purchased from Fluka (Buchs, Switzerland). TCPH was recrystallized twice from acetonitrile before use. 2-methyl-1,1,2,2-tetraethoxypropane was purchased from Seratec (Epinoy sur Seine, France). All the other chemicals of analytical grade were obtained from Merck (Darmstadt, Germany).

2.2. Standard solutions

A 10 mM stock solution of MA was obtained by dissolving MA tetrabutylammonium salt in 150 mM pH 7.0 phosphate buffer.

A 10 mM stock solution of MMA was prepared by hydrolyzing 25.97 μl of 2-methyl-1,1,3,3-tetraethoxypropane with 10 ml of 10 mM HCl at 50 °C for 60 min.

Working solutions of both standards were prepared by appropriate dilutions with 150 mM, pH 7.0 phosphate buffer and were stored at 4 °C in the dark. Their concentrations were verified before use by UV absorbance (for MA at 267 nm ($\varepsilon = 31800\,M^{-1}\,cm^{-1}$); for MMA at 275 nm ($\varepsilon = 23000\,M^{-1}\,cm^{-1}$)).

2.3. TCPH derivatization

In a 10 ml PTFE lined screw-capped glass tube, 100 μl of standard solutions or samples, 50 μl of 20 μM MMDA solution, 290 μl of 150 mM pH 7.0 phosphate buffer and 200 μl of TCPH solution in acetonitrile (3 mg ml^{-1}) were mixed and the pH was adjusted to 2.0–3.0 range with 1 N HCl. After 60 min at 30 °C, 3 ml of *n*-hexane were added. To extract the derivatives, the mixture was shaked, kept in an ice-water bath and added with six drops of concentrated sulphuric acid. After vigorous shaking, the hexane layer was removed and filtered on anhydrous sodium sulphate. The organic phase was brought to dryness under a stream of nitrogen and the residue dissolved in 100 μl of *n*-hexane. A 0.5 μl aliquot was injected into the GC system.

In the experiment carried out to compare the ECD response of TCPH and PFBHA derivatives, 1 μM and 0.5 μM MA solutions were treated according to the described procedure with the exception of the addition of 500 pmol of DNCB as internal standard in *n*-hexane used for the derivative extraction.

2.4. PFBHA derivatization

A procedure based on a previously described method [8] was followed. Briefly, 400 μl of 5 mg ml^{-1}

solution of PFBHA in 1.5 M pH 5.0 acetate buffer were added either to 100 μl of 1 μM or to 100 μl of 0.5 μM MA solutions. After 60 min at room temperature, 3 ml of *n*-hexane (containing 500 pmol of DNCB) were added. To extract the derivatives, the mixture was shaked, kept in an ice-water bath and added with 12 drops of concentrated sulphuric acid. The mixture was then treated as above described for TCPH derivative.

2.5. Quantification, reproducibility and accuracy

100 μl aliquots of standard solutions with MA content in the range of 0.1–10 μM were treated as described in TCPH derivatization. Measurements of MA concentration were calibrated by comparing the ratios of the areas of MA to MMA versus the MA concentration.

To evaluate the precision of the method, five separate preparations of a same bovine plasma sample were analysed. The same sample was spiked with two different concentration of MA standard and each sample was analysed three times in order to evaluate method accuracy.

2.6. Apparatus and chromatographic conditions

The gas-chromatograph was a HP 6890 (Hewlett-Packard, Palo Alto, CA, USA) equipped with a programmable temperature vaporization (PTV) inlet system and a 15 mCi ^{63}Ni micro-cell ECD. A fused silica capillary column (30 m × 0.25 mm i.d.) containing the 5% phenyl methylpolysiloxane stationary phase Rtx-5 (0.25 μm film thickness; Superchrom, Milan, Italy), coupled to a deactivated fused-silica retention gap (1.5 m × 0.32 mm i.d.; MEGA, Legnano, Italy), was used. The end of the analytical column was linked by a three way fused silica connector either to the ECD by a narrow bore deactivated capillary (1 m × 0.1 mm i.d.) or to a Swagelok on-off bull valve (mod. B-41S2) (GSG Nuclear, Bresso, Italy) by a short wide bore deactivated capillary (0.15 m × 0.53 mm i.d.). The valve was positioned so as to allow only the fraction containing MA and MMA (eluting between 5.0 and 6.3 min retention times) to be transferred to the ECD.

The GC operating conditions were as follows: injector temperature 250 °C, detector temperature 300 °C, oven temperature programmed from 120 to 290 °C at 20 °C min^{-1}. The flow rate of helium as carrier gas was maintained at 0.9 ml min^{-1} for 4.5 min, then increased at 3.0 ml min^{-2} to 3.0 ml min^{-1}, maintained at this value for 2 min and finally decreased again to 0.9 ml min^{-1}. Nitrogen was used as make-up gas at a flow-rate of 30 ml min^{-1}. The split injection technique was used with a 1:20 ratio and the injection volume was 0.5 μl. The valve was closed 5.0 min after the injection and opened again after another 1.3 min.

MS spectra were obtained by a 5988A MS detector (Hewlett-Packard, Palo Alto, CA) after electron impact ionization at 70 eV.

2.7. Plasma sample preparation

Blood samples were withdrawn from male limousine 10 months old bovines by Vacutainer vials containing heparin (Becton Dickinson, Rutherford, UK). Five specimens were from animals at rest, while other five specimens were from animals suffering for a 12 h of transportation by lorry.

Blood samples were centrifuged at 3000 × *g* for 10 min at 4 °C and the plasma was added with 50 μl ml^{-1} of a 2% BHT ethanolic solution. 2 ml aliquots of samples were vortexed with 2 ml of ethyl ether and, after centrifugation at 3000 g for 5 min, the organic solvent was discarded. 100 μl aliquots of defatted samples were analysed as described above.

3. Results

In order to choose the reagent suitable for MA analysis, we compared the derivatives with PFBHA and TCPH and evaluated which gives the better ECD response. Using DNCB as internal standard, the peak area ratios for 50 and 100 pmol of MA treated with TCPH resulted 38 and 43% higher than those obtained for the same amounts treated with PFBHA, respectively.

The chromatographic profile of a standard mixture of trichlorophenylpyrazole and trichlorophenyl-4-methylpyrazole, obtained from MA and MMA, respectively, is shown in Fig. 1. The 1.3 min window corresponds to the closure of the on-off valve, during which the eluate can reach the EC detector. In preliminary experiments, in order to achieve the best resolution in the final chromatographic profile of the

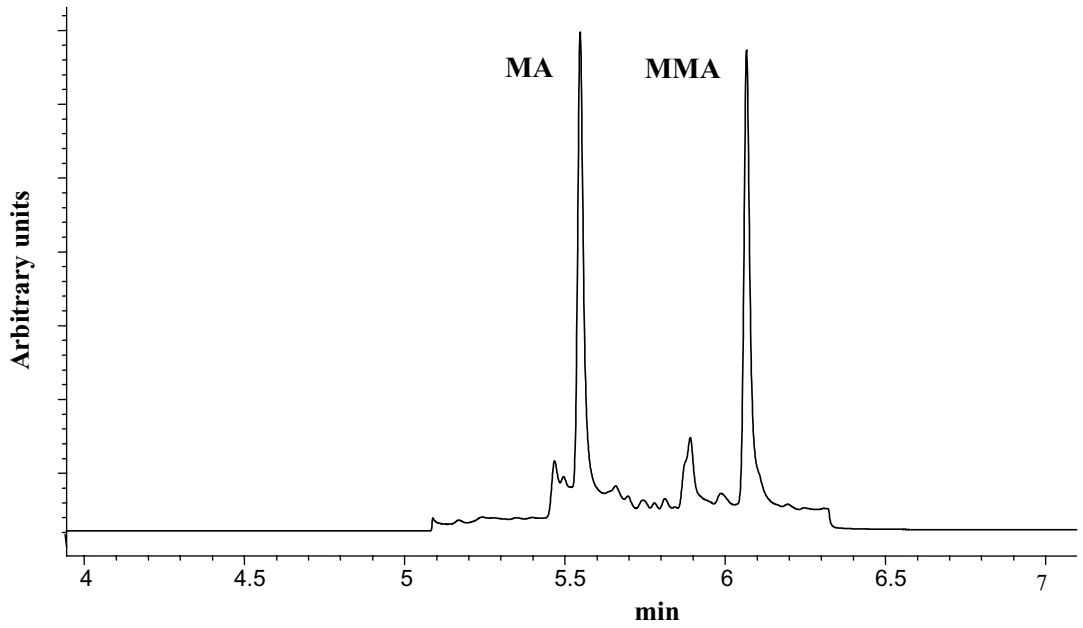

Fig. 1. GC profile obtained from a standard solution containing MA and MMA.

analysed window, different operating conditions were tested, such as final flow, flow increase rate and delay time between the valve closing and the start of the flow increase.

The identity of the derivatives was confirmed by their MS analysis, shown in Fig. 2. The spectrum of MA derivative ($M_r = 246$) is very similar to the previously reported one [9]; in the spectrum of MMA derivative ($M_r = 260$) the higher fragments are similar to those of MA derivative with the only expected difference of 14 in *m*/*z* value.

Using MMA 10 μM as internal standard the calibration curve of area ratio (Ra) against MA concentration in the range 0.1 (limit of quantitation, LOQ) −10 μM resulted Ra = 0.088 μM MA + 0.023 ($R^2 = 0.9987$). A peak giving a signal-to-noise ratio of five resulted from a standard MA solution 0.05 μM, which can be considered the limit of detection (LOD) in the described conditions. As injected amounts 50 and 25 fmol of MA derivative are the LOQ and LOD, respectively.

Table 1
Precision and accuracy data of MA estimates in bovine plasma

MA added (nmol ml^{-1})	MA found (nmol ml^{-1})	R.S.D. (%)	Recovery (%)
0	1.364 ± 0.075[a]	5.4	–
0.395	1.742 ± 0.123[b]	7.1	96
0.791	2.148 ± 0.083[b]	3.9	99

[a] based on five independent determinations.
[b] based on three independent determinations.

Table 2
MA (nmol ml^{-1}) content in bovine plasma from animals at rest and from animals after transportation by lorry

Animals at rest	Animals after transportation
1.245	1.892
1.544	2.165
1.267	2.009
1.784	2.285
1.644	2.627

A chromatogram corresponding to a bovine plasma sample is shown in Fig. 3. The results regarding the precision and the accuracy of the method are reported in Table 1. The MA content in the tested plasma samples are shown in Table 2.

4. Discussion

Our aim was to test an alternative cheaper detection technique compared to the MS one allowing to

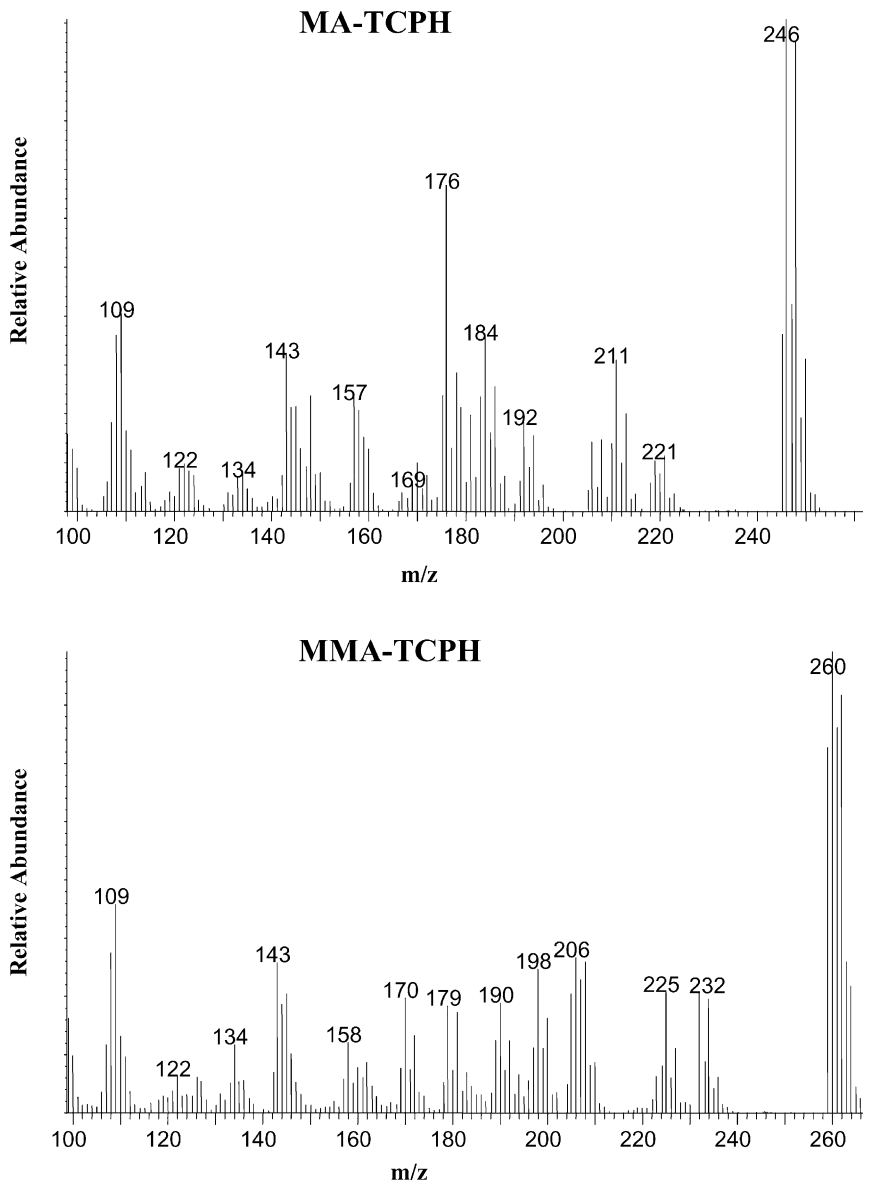

Fig. 2. MS spectra of MA and MMA derivatives with TCPH.

evaluate MA without losing in measurement reliability. In this view the ECD appears a suitable tool due to its selectivity and sensitivity; for the MA determination both these properties can be assured only by the addition of a halogenated group to the analyte structure. In previous works PFPH, PFBHA, and TCPH were used without a systematic comparison of their derivatives ECD response. Tomita et al. [7] introduced PFPH as reagent for human urine MA determinations, using *p*-dibromobenzene as internal standard and a packed column for the chromatographic separation. In our experience, preliminary experiments suggested that the high volatility of PFPH derivative does not allow the evaporation of final sample without the risk of a derivative loss. On the other hand, De Zwart et al. [8] estimated

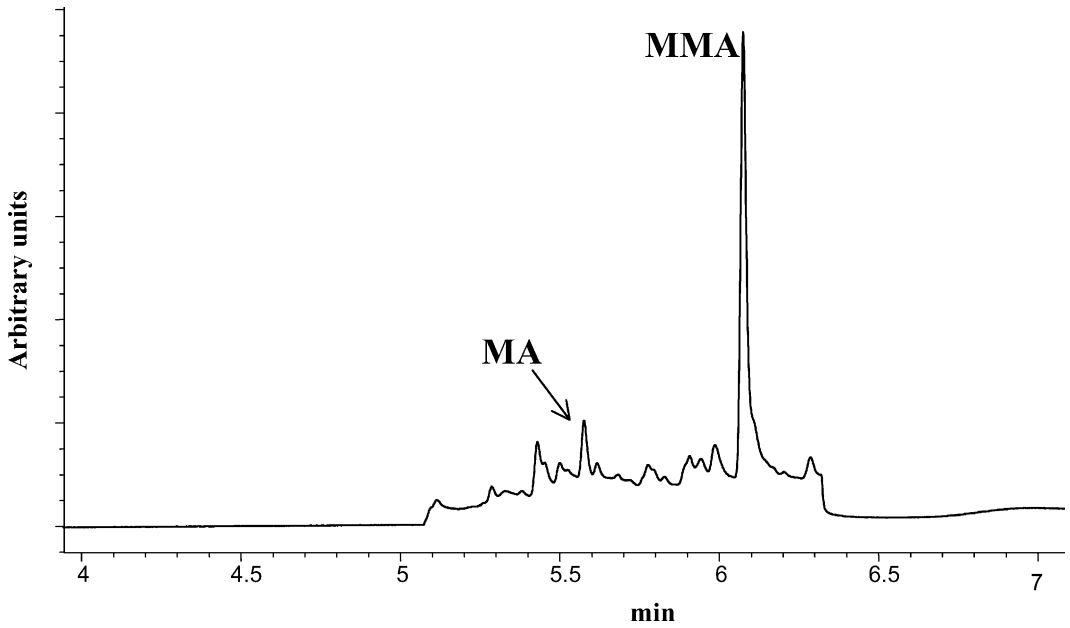

Fig. 3. GC profile obtained from a bovine plasma sample added with MMA.

MA in rat urine preferring PFBHA for ECD detection, because of its higher sensitivity with respect to PFPH; nevertheless, PFBHA generates three isomers, thus making the quantification step more difficult. Really, in the reported chromatogram corresponding to an untreated sample, MA peaks appear not well resolved from the background profile. Finally Stalikas and Konidari analysed human plasma MA using TCPH which gives a single peak as PFPH but a higher boiling derivative with respect to PFPH [9]. Consequently, we compared the response of TCPH and PFBHA derivatives in order to choose the better reagent as concerns the ECD sensitivity. Using DNCB as internal standard we found that the response of the trichlorophenylpyrazole single peak resulted larger than that of the MA bis-pentafluorobenzyloximes three isomer peaks.

Regarding the quantitative analysis, the use of MMA as internal standard has already been demonstrated to assure an acceptable measurement precision in methods adopting different techniques. Claeson et al. suggested MMA also for GC–ECD technique using PFPH as halogenated reagent, but without any application to a real sample [13]. Here we confirm that MMA can be considered the internal standard of choice also using TCPH in the GC–ECD analysis of MA in a biological matrix.

The reluctance to use ECD in GC methods is generally due to its lack of stability over a long period of time. In order to achieve a better reproducibility of the ECD response, a simple valve-fitting tool has been added to a common gas-chromatographic system to preserve the detector from either the excess of halogenated derivatizing reagent or highly boiling components, which can decrease its specific response. The analytical column was linked by a three way connection both to a on-off bull valve by a wide bore capillary segment and to the detector by a narrow bore capillary segment. The lengths of two segments were chosen in order to force the eluate to go to waste without reaching ECD, when the valve was open. On the other hand, with the valve closed, the eluate was directed toward the detector, thus allowing only the fraction containing the analytes to be detected and quantified. In this way, the ECD response did not change even after several injections of biological samples. Therefore the ECD apparatus can be used without any heavy trouble in routine MA determinations by adding a simple fitting.

We applied the described procedure to evaluate for the first time the MA content in bovine plasma. Among

the procedures suggested by Stalikas and Konidari for free MA [9], we used the procedure not involving a preliminary separation of proteins, because of its simplest handling . According to the comments of Pilz et al. [14] about MA content in human plasma, the obtained data probably correspond not only to the effectively MA present in the free form, but also to a small aliquot of MA weakly bound to proteins and peptides and liberated in the mild acidic derivatization conditions. The right definition of these MA contents could be “not strongly bound MA”, instead of really “free” one. As our aim was to compare different homogenous populations, also the estimation of this composite MA fraction allowed to induce interesting preliminary findings. In fact, we found a difference in the analysed specimens from different animals either at rest or under stress due to a long distance transportation by lorry. As expected, the animals under stress showed increased levels of MA in plasma. Works are in progress to verify the significance of these preliminary results on a higher number of test animals.

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