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Methyl malondialdehyde as an internal standard for the determination of malondialdehyde

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Abstract

Methyl malondialdehyde (Me-MDA) is suggested as an internal standard for the determination of the lipid peroxidation product, malondialdehyde (MDA). A procedure for synthesising the Me-MDA sodium salt is described in detail. The purity and identity of the synthesised Me-MDA have been confirmed using nuclear magnetic resonance and UV spectroscopy, and by micellar electrokinetic chromatography. The applicability of Me-MDA as an internal standard has been demonstrated for rat brain homogenate samples. These samples were purified solely through ultrafiltration. The preferred analytical technique was capillary zone electrophoresis (CZE) with UV detection at 267 nm. The limits of detection (3 S/N) for the CZE separations of Me-MDA and MDA were 0.5 and 0.2 μM, respectively, and the total analysis time was approximately 10 min. Details of separations are also presented using high-performance liquid chromatography (HPLC) with UV detection at 245 nm, and gas chromatography, together with either electron capture or mass spectrometric detection. The GC separations require derivatisation of MDA and Me-MDA with pentafluorophenylhydrazine while the CZE and HPLC separations can be performed on the native molecules.

Keywords: Internal standard; Methyl malondialdehyde; Malondialdehyde; Capillary electrophoresis

1. Introduction

There is increasing evidence supporting the suggestion that lipid peroxidation has a causative role in several clinically significant disease states, including neurodegenerative and cardiovascular diseases [1–5]. During lipid peroxidation in vivo a multitude of degradation products is formed [6]. Measuring lipid peroxidation is therefore a complex task and there is a need to develop improved analytical tools for reliably determining markers associated with it. One of the molecules most commonly quantified is malondialdehyde (propanedial, MDA). MDA is formed during the decomposition of various primary lipid peroxidation products. The literature dealing with theories about MDA formation and methods for its quantification is extensive [7–9]. The correlation between MDA and various disease states is discussed in [6].

The most widely used analytical techniques involve the reaction between MDA and thiobarbituric acid (TBA), which generates a red, fluorescent complex [7]. This approach has several drawbacks, including lack of selectivity and harsh derivatisation conditions. Apart from the TBA-based methods, gas chromatography (GC) with electron capture (EC)

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[10,11] or mass spectrometric (MS) detection [11–15] has been used to determine MDA in various sample types. Both HPLC [16–19] and capillary zone electrophoresis (CZE) [20,21] have also been used, in conjunction with UV-absorbance detection. CZE with UV detection has proved to be an excellent technique for direct determination MDA owing to its good separation efficiency and low sample volume requirements [20,21].

Isotope-labelled molecules can be used as internal standards in MS, although they are generally expensive, but there is a lack of suitable internal standards for all the other methods. Bull and Marnett suggested in 1985 the use of methyl malondialdehyde (2-methyl-propanedial, Me-MDA) as an internal standard for MDA determination in an HPLC method [18]. However, they failed to separate the MDA and the Me-MDA peaks, and they presented no simple synthesis procedure for Me-MDA. In 1993 Traoré et al. also used Me-MDA for the same purpose but the suggested procedure, derivatisation with lumarin 3 and separation by HPLC with fluorescence detection, did not seem to be applicable to real samples [22].

In this paper, a fast and simple synthesis procedure for Me-MDA is presented, and its potential for use as an internal standard in MDA determinations is demonstrated. The main focus is on separations using CZE, but its applicability for chromatographic techniques, such as GC (with EC or MS detection) and HPLC (UV detection), is also shown.

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade and solutions were prepared with water from an Elgastat UHQII (Elga, High Wycombe, UK). 3-dimethylamino-2-methyl-2-propenal (DMP), 1,1,3,3,-tetramethoxypropane (TMP), ferrous ammonium sulphate, butylated hydroxytoluene (BHT), pentafluorophenylhydrazine (PFPH) and Sudan III were obtained from Sigma (St. Louis, MO, USA). Borax and cetyl trimethyl ammonium bromide (CTAB) were purchased from Merck (Darmstadt, Germany).

2.2. Animals

Three adult, female Sprague–Dawley rats (B&K International, Sweden) were used in the study.

2.3. Synthesis of standards

The procedure for synthesising the sodium salt of Me-MDA was adapted from Arnold and Sorm [23]. DMP (0.5 g), 0.2 g sodium hydroxide and 0.7 ml water were mixed in a 10 ml flask. The mixture was then heated in a water bath at 70°C until the two initial phases merged into one (approx. 25 min). The liquid obtained was evaporated under reduced pressure until white crystals formed, which were carefully washed with a mixture of acetone and ethanol. The crystals were then dried in an excicator over P_2O_5. Solutions of Me-MDA were prepared with 5 m*M* phosphate buffer, pH 7.0.

The MDA standard solution (10 m*M*) was prepared as described by Esterbauer and Cheeseman [9], i.e., by acid hydrolysis of TMP in 1% H_2SO_4 at room temperature for 2 h. This solution was then further diluted with 5 m*M* phosphate buffer, pH 7.0, to prepare the working standard solution.

2.4. Sample preparation

The heads of the rats were stored at −70°C immediately after decapitation. After thawing at room temperature the brains were quickly excised and put on ice. An ultraturrax set at 800 rpm was used to prepare the tissue homogenates (approx. 20%) in 5 m*M* phosphate buffer, pH 7.0. In some of the samples oxidative stress was induced by the addition of 100 μ*M* ferrous ammonium sulphate (mixing for 30 min at room temperature). Me-MDA was added to some samples to a final concentration of 5.5 μg/g brain. BHT (0.075%), an antioxidant, was added to prevent unwanted oxidation. The homogenates were stored at −80°C until further use.

2.5. CZE

Prior to injection into the CZE system the homogenates were centrifuged at 14 000 *g* for 3 min in order to remove particles, and proteins were removed by ultrafiltration using Microcon centrifugal

filtration devices (Millipore, Bedford, MA, USA) with a 30-kDa molecular mass cut-off. Using this sample pretreatment method only the free fraction of MDA remains in the sample since the protein bound fraction is removed. An evaluation of the sample pretreatment procedure is presented in Ref. [21]. The capillary electrophoresis system (HP^{3D}CE, Wilmington, DE, USA) was equipped with a diode array detector, monitoring the eluate at 267 nm, which could also be operated in UV scanning mode. Untreated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA), 65 cm long (55 cm to the detection window), 50 μm I.D., 375 μm O.D., were used. New capillaries were preconditioned with 0.1 *M* NaOH for 10 min, distilled water for 10 min and running buffer for 15 min. The electrophoresis buffer, 10 m*M* borax and 0.5 m*M* CTAB, pH 9.3, was degassed and filtered through a 0.25-μm filter before use. Samples corresponding to an injection volume of 20 nl (equivalent to1.6% of the capillary length) were injected by pressure. The running voltage was −25 kV. To maintain capillary performance, conditioning cycles were applied comprising rinsing for 2 min with 0.1 *M* NaOH, 2 min with methanol and 2 min with running buffer.

2.6. HPLC

The sample pretreatment procedure was identical to that used for CE. Before injection, the filtrate was diluted to 50% with methanol. HPLC analysis was performed with a Varian 9012 gradient pump (Palo Alto, CA, USA) equipped with a 20-μl sample loop and a HP 1050 UV detector set at 245 nm (Wilmington, DE, USA). A GL Sciences 5 μm, 250×4.6 mm Inertsil ODS 3 column (Tokyo, Japan) was used for separation and the mobile phase comprised 2% methanol in 20 m*M* phosphate buffer at pH 3.0 for 10 min, followed by a 5-min linear gradient to 50% methanol. The flow-rate was 1 ml/min.

2.7. GC

The PFPH derivatisation was performed at room temperature. An acetic acid buffer (20 m*M* at pH 3.2) was used to maintain constant pH during the derivatisation reaction. To 100 μl of sample solution in a 5 ml borosilicate glass tube, 100 μl of buffer and a surplus of PFPH were added (50 μl of a 10 m*M* PFPH solution). The tube was then sealed and the derivatisation reaction was allowed to proceed for 30 min. In order to remove unreacted reagent the pH was then lowered by adding 10 μl of 9 *M* H_2SO_4. This charged the excess reagent, so it remained in the aqueous phase when the reaction products were extracted in 200 μl of hexane. The hexane phase was then analysed by GC. Two Thermoquest Trace GC 2000 (CE Instruments, Austin, TX, USA) instruments were used, one equipped with an electron capture detector set at 310°C, the other with a Termoquest Automass III. The capillary columns used were DB 5 MS columns supplied by J&W Scientific (Folsom, CA, USA), 30 m long, with 0.25 mm I.D. and 0.1-μm film thickness. MS analysis was performed using electron impact ionization (EI) at 70 eV after injecting (1 μl) samples in the splitless mode (2 min split time). The reaction products were separated by a temperature program, in which the column passed from 60°C (after a 2-min hold) to 200°C at 10°C/min and then at 25°C/min to 300°C.

2.8. NMR

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra of the synthesis product (Me-MDA) were recorded on a Varian Mercury spectrometer (Palo Alto, CA, USA) using D_2O as solvent and dioxane as a reference.

2.9. Micellar electrokinetic chromatography

The micellar electrokinetic chromatography (MEKC) was run on the same apparatus as the CZE. Methyl-MDA and MDA were separated in a system using a 20 m*M* phosphate buffer adjusted to pH 7.0 and 50 m*M* sodium dodecyl sulphate as surfactant. The running voltage was +20 kV and the sample volume injected was 20 nl. Methanol was used as a neutral marker for the electroosmotic flow. The migration time for the micelles was estimated by injecting Sudan III, which was assumed to be fully partitioned into the micelles.

2.10. UV-spectroscopy

Absorption spectra were obtained for Me-MDA, MDA and DMP using a Cary 3Bio UV–Vis spectrophotometer (Varian, Palo Alto, CA, USA).

3. Results and discussion

3.1. Characterisation of Me-MDA

The carbon and proton NMR spectra were obtained for the synthesis product. These spectra confirmed the identity of 2-methyl-propanedial, i.e., Me-MDA. The NMR results were: ^{1}H NMR (400 MHz, D_2O) δ: 1.4 (s, 3H, CH_3), 4.6 (s, 1H, CH), 8.2 (s, 2H, COH). ^{13}C NMR (100 MHz, D_2O) δ: 5.53 (CH_3), 117.0 (CH), 191.2 (C=O).

Absorption spectra were obtained for MDA and Me-MDA solutions at pH 7. Absorption maxima were found at 267 and 275 nm, respectively. The molar absorptivities (mol $l^{-1}cm^{-1}$) were estimated to be 23 000 at 275 nm and 19 000 at 267 nm for Me-MDA, and 31 000 at 267 nm for MDA. At acidic pH, the absorption maxima shift to shorter wavelengths. The sodium salt of Me-MDA appears to be stable since no degradation was detected during several months of storage at room temperature in an exciccator. During this period the molar absorptivity was determined regularly and found to remain constant.

3.2. Purity of the synthesised Me-MDA

After the synthesis procedure it was verified that all the DMP was consumed using UV-spectroscopy. Further, the remaining traces of NaOH were carefully washed away by rinsing the crystals with an acetone–ethanol mixture. The yield was, after this rinsing step, approximately 80%.

Only one peak, corresponding to Me-MDA, was found in a CZE analysis of a standard solution. MEKC, a technique relying on a different separation mechanism, was then used to verify the results from the CZE experiment. MEKC is primarily considered to be a chromatographic method, in which separation is based on differential partitioning between charged micelles, acting as a pseudo-stationary phase, and the surrounding separation electrolyte [24].

According to the pertinent literature [25] the contribution of the retention factor, k', to the resolution equation in MEKC has an optimum in the range $k'=1$ to $k'=5$.

The calculated retention factors for a standard mixture of MDA and Me-MDA were 2.5 and 5.4, respectively. The selectivity, calculated as for chromatographic systems, was 2.16 and the resolution obtained between MDA and Me-MDA was 14. Thus, the MEKC separation was performed under near optimal conditions. No impurities were detected in the chromatograms produced. Since no other UV-absorbing compounds were detected in solutions of Me-MDA using either the CZE or MEKC separation systems described above (or, later, HPLC), it was concluded that the synthesised Me-MDA was essentially free of contaminating compounds. This conclusion is further supported by the NMR results, which showed no other signals beside those originating from Me-MDA.

3.3. The merits of Me-MDA as an internal standard for MDA determination

3.3.1. In capillary electrophoresis

An electropherogram of a standard mixture of 10 μ*M* MDA and 12 μ*M* Me-MDA is shown in Fig. 1. The Me-MDA peak migrates shortly after the MDA peak, yet it is well separated and the analysis time is below 5 min. The identity of the molecules was confirmed by comparing the diode array spectra with the spectra obtained for the pure standards. Fig. 2 shows electropherograms obtained from a rat brain. In (a) BHT was added in order to create a control sample. In this analysis, only a very small peak from MDA can be discerned. In (b) the electropherogram is of a sample where oxidative stress was induced by the addition of iron. It can be seen that the amount of MDA in the sample increased dramatically. The sample in electropherogram (c) was identical to that in (b), except for the addition of Me-MDA (5.5 μg/g brain). The limit of detection (LOD, 3 S/N) was found to be 0.2 μ*M* for MDA and 0.5 μ*M* for Me-MDA. The linearity of the response was studied between LOD and 100 μ*M* for both species. The r^2

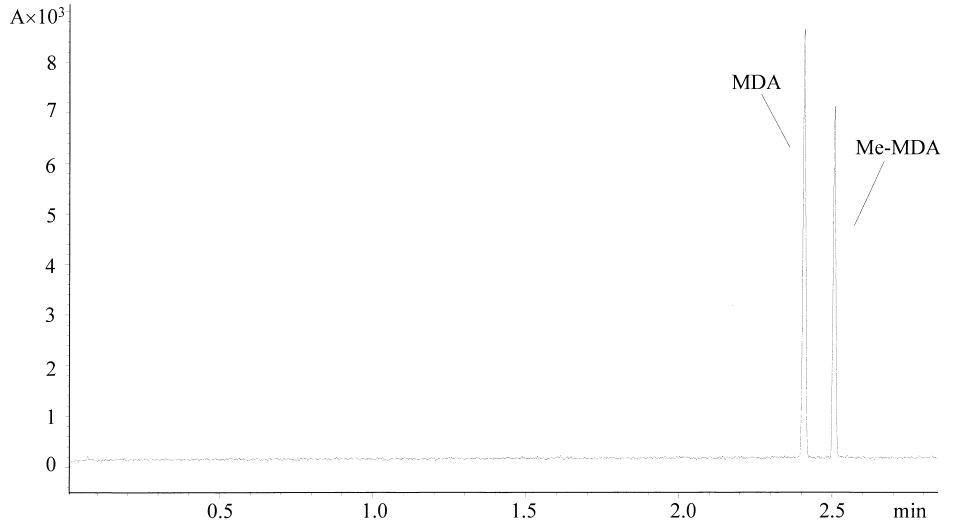

Fig. 1. Electropherogram obtained for a standard solution containing 10 μ*M* MDA and 12 μ*M* Me-MDA.

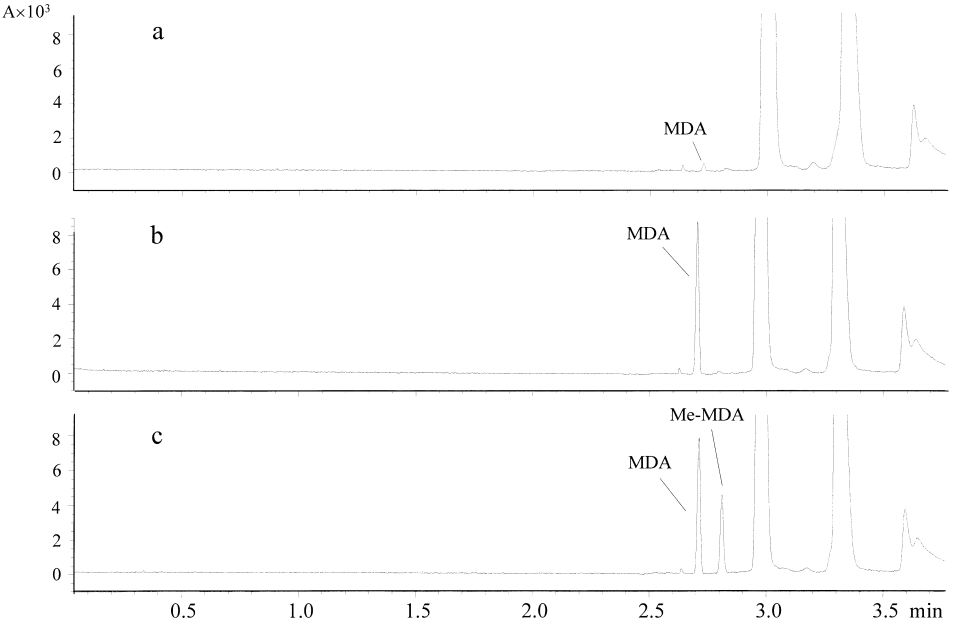

Fig. 2. Electropherograms obtained for ultrafiltered rat brain homogenates: (a) treated with BHT, (b) oxidised by the addition of iron, (c) same sample as in (b), but with the addition of Me-MDA.

values obtained were above 0.999. The response factor, F was calculated using the equation:

$$\frac{A_X}{[X]} = F\left(\frac{A_S}{[S]}\right)$$

where A is the area, X the analyte and S the internal standard. The average F value calculated from eight runs was 2.1 ($s = 0.07$).

The amount of MDA in the BHT-treated rat brain homogenate was determined to be 0.2 μg/g brain. This value is in accordance with our earlier results, which were obtained with a standard addition methodology [21]. In the sample subjected to lipid peroxidation the amount of MDA was 4.3 μg/g brain, thus a more than 20-fold increase.

In order to verify that no discrimination occurred between MDA and Me-MDA during the ultrafiltration procedure, the ratios between the two peak areas in the electropherograms were calculated both before and after filtration. No significant difference (*t*-test, $n = 5$, 95% confidence level), in terms of peak ratios was found. This test was performed with standard solutions of Me-MDA and MDA in a 5 m*M* phosphate buffer at pH 7.0.

3.3.2. In HPLC

In the HPLC-separation method developed by Bull and Marnett [18] an ion-pairing reagent was used at neutral pH to increase k' for MDA and Me-MDA. Our alternative approach is to use a low pH to increase the degree of protonation of MDA and Me-MDA, and then to separate the compounds on an HPLC column with a high carbon load. Separation of MDA and Me-MDA was achieved using a 20 m*M* phosphate buffer at pH 3.0 with 2% methanol for 10 min and then a 5-min linear gradient up to 50% methanol (Fig. 3a). Fig. 3b shows a chromatogram of a homogenate treated with BHT, similar to sample (a) in the CZE analyses described above, in which no MDA peak can be seen. In Fig. 3c the sample used in (b) was spiked with MDA and Me-MDA to concentrations of 20 and 25 μ*M*, respectively. The peaks are fully separated and the elution time is below 20 min. Separation could also be achieved under isocratic conditions, but this took longer. When acetonitrile was used instead of methanol, MDA could not be separated from unknown peaks, implying that there was a significant difference in selectivity. The

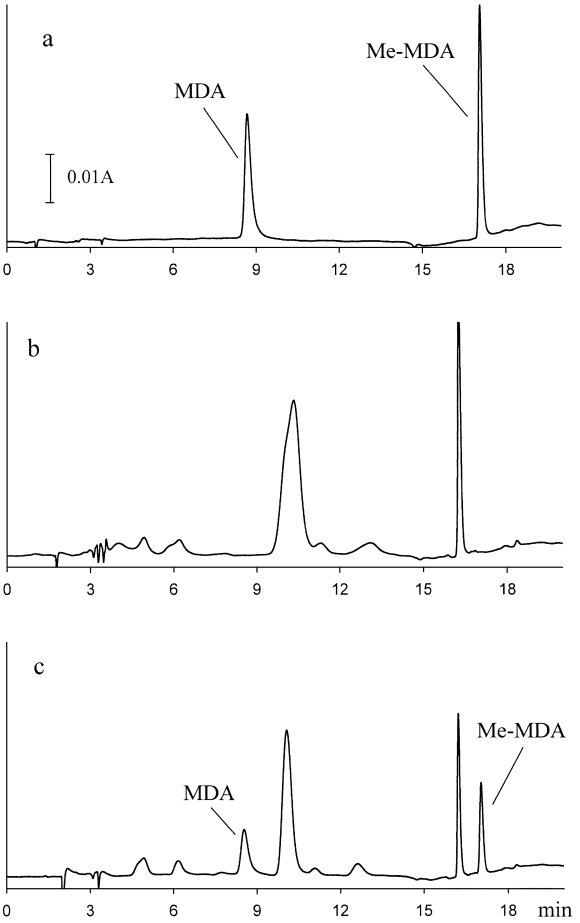

Fig. 3. HPLC chromatograms (a) for a standard solution containing MDA and Me-MDA, 50 μ*M* of each (b) for a rat brain homogenate sample treated with BHT, (c) same sample as in (b), but spiked with 20 μ*M* MDA and 25 μ*M* Me-MDA.

UV detector was set at 245 nm since the absorption maxima for the species appear in that region in the acidic conditions of the HPLC separation.

3.3.3. In GC–ECD

Compounds containing carboxyl functional groups, such as steroids, aldehydes, and ketones, can be derivatised with PFPH for GC analysis. The resulting derivatives have favourable detection properties using gas chromatography with either electron capture detection or negative ion chemical ionisation mass spectrometry (GC–NICI/MS) owing to the strongly electronegative nature of the pentafluoro-

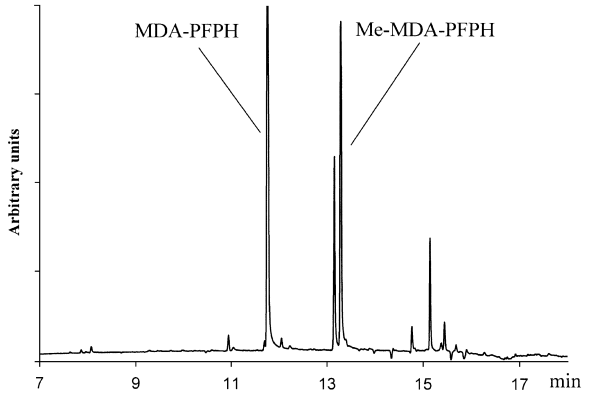

Fig. 4. Gas chromatograms of a derivatised standard solution of MDA and Me-MDA.

group of the reagent. MDA is known to form a cyclic derivative with PFPH [11]. In Fig. 4, a chromatogram of a derivatised standard mixture of MDA and Me-MDA is shown. The elution time is approximately 15 min. The injected amounts correspond to 180 pg MDA and 2.6 ng Me-MDA. The PFPH could only be obtained in technical quality. Therefore an abundance of impurities were seen in a blank derivatisation. Purification of the reagent would be necessary for the GC–ECD analysis of real samples.

3.4. GC–MS analysis

GC–MS experiments were performed in order to verify the identity of the derivatives formed by the reaction of MDA and Me-MDA with PFPH. EI mass spectra of PFPH–MDA and PFPH–Me-MDA are presented in Fig. 5. The EI mass spectra of the PFPH–MDA derivative have a very close resemblance to spectra earlier published in the literature [11,12]. Both MDA and Me-MDA form cyclic aromatic derivatives on reaction with PFPH, yielding stable molecular ions at $M^{+\cdot}=234$ and $M^{+\cdot}=248$, respectively. Upon loss of HCN, fragments are formed with m/z values of 207 for PFPH–MDA and 221 for PFPH–Me-MDA. The loss of a fluorine gives rise to fragments with m/z values of 215 and 229, respectively. Characteristic fragments for both the MDA and Me-MDA derivatives are seen at m/z 194, representing the penta-fluorophenyl group with a cyano-function attached, and 167, representing the penta-fluorophenyl group. The loss of NCH_2 from the Me-MDA derivative results in a fragment with an m/z ratio of 220.

An abundant fragment appears in the PFPH–Me-MDA spectra at m/z 247. A possible interpretation of this peak is that it represents a cyclic aromatic fragment formed by a rearrangement of the methyl group of Me-MDA into the ring with a consecutive loss of hydrogen. A loss of HCN from this rearranged molecular mass fragment would also give rise to a fragment at m/z 220.

3.5. Comparison of the presented techniques

Our ambition was to synthesise and evaluate a suitable internal standard for the determination of MDA by CZE with UV detection. To further investigate the versatility of the internal standard two other separation techniques, GC–ECD and HPLC-UV, have also been employed. It is apparent that both the HPLC and GC procedures presented require further refinements before being applicable for the analysis of real samples. In the case of HPLC the limit of detection would be improved through selection of a better detector. Further, the MDA peak shape can probably be improved by altering the mobile phase gradient. Regarding GC, the derivatisation procedure should be optimised in order to maximise the yields of the MDA– and the Me-MDA–PFPH derivatives, in particular the latter. As always, different techniques have different merits, in this case we find that the speed and the low sample volume requirements are advantages that speak in the favour of CZE. A further advantage is that CZE is a direct method of determination, in contrast to the derivatisation required by GC. When working with analytes in complex biological matrices, such as brain homogenates, the cheapness of changing separation capillaries for CZE, as compared to the relatively expensive columns for HPLC and GC, can be relevant. For validation purposes, the use of separation techniques with different separation mechanisms is valuable. Therefore, we think that the interchangeability between HPLC, GC and CZE that the use of Me-MDA as an internal standard enables, is an important feature for future comparative work.

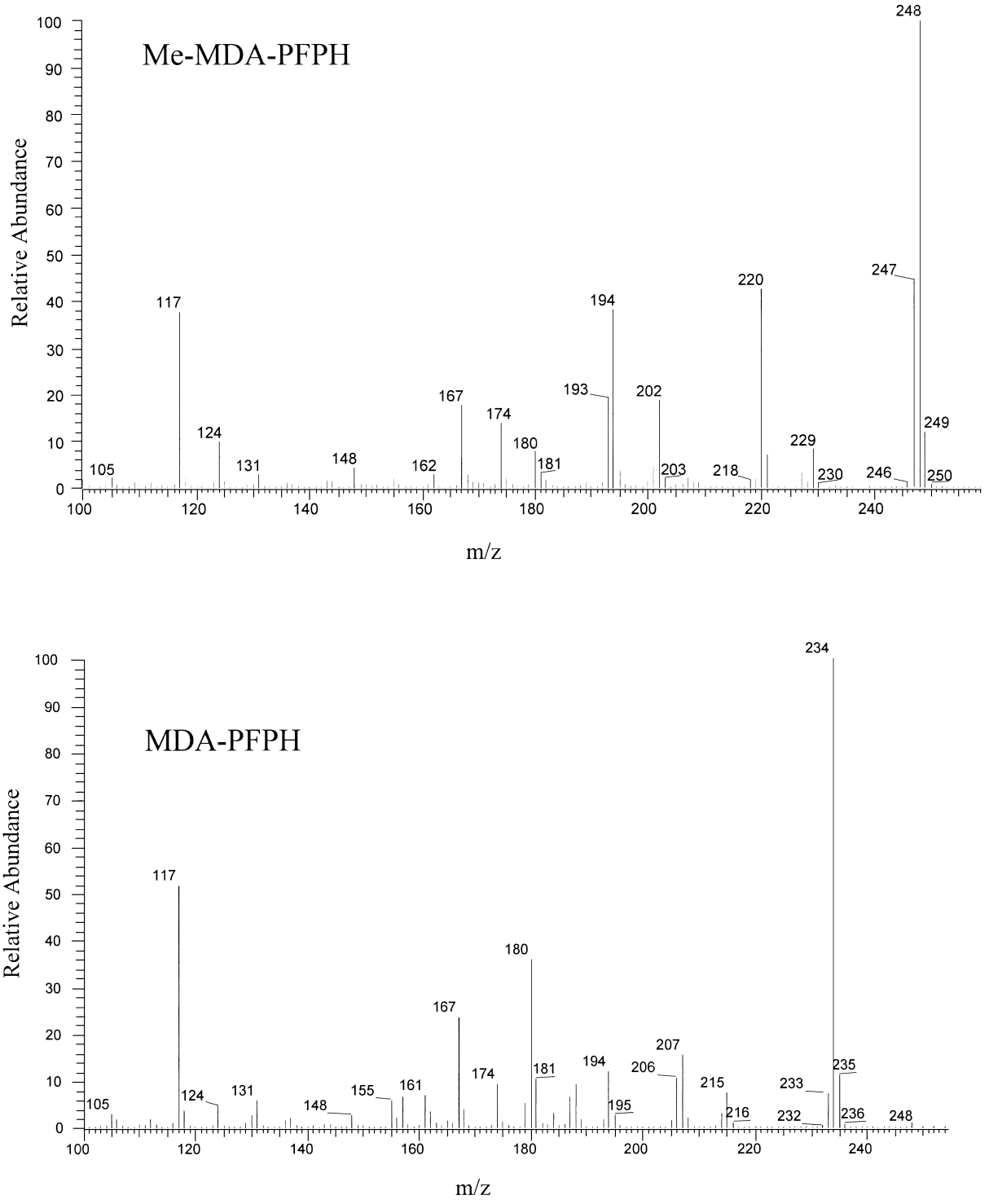

Fig. 5. Electron impact mass spectra of the Me-MDA–PFPH and MDA–PFPH derivatives.

4. Conclusions

In this paper, a fast, inexpensive and simple procedure for synthesising the sodium salt of Me-MDA is described. The purity and identity of the synthesised Me-MDA have been verified through various techniques. The versatility and usefulness of Me-MDA as an internal standard for the determination of MDA is demonstrated. The main focus is on CZE-UV, but HPLC-UV and GC–ECD have also been tested as analytical techniques, and are shown to be feasible. The use of Me-MDA as an internal standard provides an important means of validation in comparing the analytical methods available for MDA determination.

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