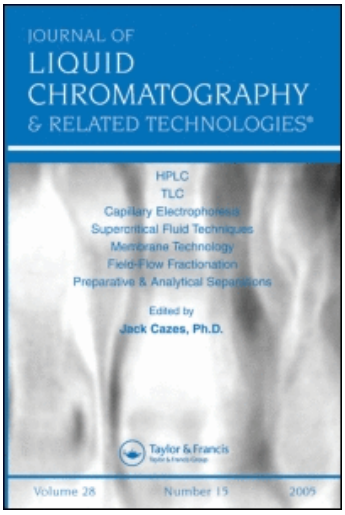

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Analysis of Malonyldialdehyde Using Ion Exclusion Chromatography

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ABSTRACT

Malonyldialdehyde (MDA) plays an important role as a marker of the cascade of free radical reactions with lipids. It is also responsible for their toxicity. In this paper, preliminary results of the analysis of MDA by means of ion exclusion chromatography (IEC) is presented. Two methods based on IEC with photometric detection are discussed. The first one is based on the derivatization of MDA with thiobarbituric acid (TBA). In the second direct separation is obtained using ion-pairing reagent added to the mobile phase. Better separation is obtained for the second method although, for the first one, lower detection limit is achieved.

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It is shown that MDA peak broadening is strongly affected by column temperature, which is probably caused by kinetic effects.

Key Words: Malonyldialdehyde; Ion exclusion chromatography.

INTRODUCTION

Free radicals interact with all cell components and compounds they are built of. Lipids are most strongly affected by free radicals, since the number of other radicals and stable lipid hydrogen peroxides,[1] which are usually more reactive and toxic than substrates, are formed as a product of reaction with radicals. Lipid radicals are also formed by non-metal enzymatic reactions. This metabolically uncoupled lipid peroxidation plays a crucial role in the degeneration associated with aging, and in the pathogenesis of a number of diseases.[2,3]

Degradation of cell membranes during reaction of free radicals with polyunsaturated fatty acids (PUFA) is observed. This process yields lipid hydroperoxides, aldehydes, and ketones, which are relatively stable and cytotoxic.[1] They interact with the cell and membrane components, showing strong chemical affinity. When formed in blood (endothelium, fibroblasts, heart muscle, etc.), they are responsible for arteriosclerosis. Additionally, they affect the change of electric charge, hydrophobicity, and "liquidity" of membranes, decreasing their mobility. New electric charges on the membrane surface indicate that new carboxylic groups are formed during peroxidation. The membrane becomes more hydrophilic, which results in a change in its structure (with respect to transport and receptors).

Estimation of oxygen radical damage in biological systems is usually done by the determination of malonyldialdehyde (MDA).[4,5] The thiobarbituric acid (TBA) test is most frequently used. This assay has been favored because of its simplicity and sensitivity.[6] It is still in use despite the fact that the TBA assay is intrinsically non-specific, and is generally poor when applied to biological samples. Positive response is obtained with sugars, some amino, and bile acids, alkenals, alkadienals, etc. Certain improvement can be obtained using RP-HPLC separation followed by fluorometric[7,8] or photometric detection at 535 nm.[9–13] In this case, TBA influences both, the retention (separation) as well as detection conditions. However, this method requires a derivatization step. Additionally, the results are questionable because identical adducts can be produced from different substances. Similar method is based on the derivatization with 2,4-dinitrophenylhydrazine[14–16] or diaminonaphthalene[17] followed by photometric detection at 310 nm. Simplification of this method (no derivatization required and easy

sample preparation) was achieved by application of the reversed phase ion pair chromatography.[18,19] The detection, accomplished by monitoring absorbance at 267 nm, enabled analysis of MDA in the presence of two antioxidants, namely ascorbic acid (AA) and uric acids (UA). Unfortunately, very small and broad peaks of MDA are obtained with this method.

Ion exclusion chromatography (IEC) is a technique widely applied to separate ionic from non-ionic compounds, as well as mixtures of weak acids (or bases).[20] The characteristic feature of IEC is the same sign of the electric charge of the dissociated functional groups of the ion-exchange resin and analyzed ionic compounds. It follows that samples of negatively charged ions, e.g., dissociated acidic compounds, are separated on cation-exchange resins with anionic functional groups. Usually, these are sulfonic acid groups. Similarly, samples containing positively charged species (bases) are separated on the anion-exchange resin containing cationic functional groups. Usually, these are tetraalkylammonium groups. The same columns can be used in both IEC and in ion-exchange chromatography, although in the latter case, real ion-exchange reactions are not involved. The usual supports are based on the macro-porous styrene and divinylbenzene copolymers. For some compounds, the observed retention is stronger than that provided by ion exclusion mechanism. This can be explained by hydrophobic as well as π-electron interaction of analyte with the resin network.[20] Ion interaction reagents used in this technique strongly influence retention because of their interaction with the resin functional groups, as well as the solute ions.[21]

The aim of the paper was to elaborate on a new, simple chromatographic method of the MDA analysis. Because MDA is a medium strength acid ($pK_a \approx 4.5$), it seems that IEC should be the preferable technique.[20]

EXPERIMENTAL

Apparatus

The chromatographic system consisted of P-580-A-LPG degasser and pump, STH-585 column oven, UVD 170S four channel photometric detector (Gynkotek, Germering, Germany), 2097 injector (Rheodyne, USA), and ion exclusion column-strong cation exchanger based on PS/DVB [TSK-GEL SCX (H^+)] 5 μm, 300 × 7.8 mm^2 I.D., > 4.2 meq/g, (TosoHaas, Japan). The above system was controlled under ChromLeon (Gynkotek, Germering, Germany) software, installed on IBM Pentium compatible computer (Simens, Germany). Incubation was performed using TB-9414 (JWE-electronic, Warsaw, Poland) thermoblock.

Materials

Acetonitrile (ACN), sulfuric acid (SA), and UA were purchased from E. Merck (Darmstadt, Germany), AA from Avocado (Heysham, England), and malonyldialdehyde *bis*(dimethyl acetal) from Sigma (St. Louis, USA). All other reagents, being of analytical reagent grade, were used without further purification. The Milli-Q (Millipore, Bedford, USA) water system was used to prepare all solutions. The mobile phases were filtered through a Millipore 0.22 membrane filter and degassed in an ultrasonic bath prior to use.

Procedure

The source of MDA was malonyldialdehyde *bis*(dimethyl acetal). A 10 mM stock solution was prepared by hydrolysis of acetal, using 1% SA.[22] Reaction between TBA and MDA was carried out at pH 3, at 90–100°C for 10–15 min.[23]

The chromatographic experiments were performed with a flow rate 0.9 mL/min. The column was stabilized for 1 hr at the temperature of 20°C prior to the chromatographic measurements.

Solutions of 1 mM SA and 3 mM tetrabutylammonium bromide (TBABr) in ACN were used as mobile phases. Stock solutions (10 mM) of the solutes were prepared in Milli-Q water system and diluted to the required concentration before use. The solute solutions were injected into the chromatographic system with a 100 μL syringe (Hamilton, Reno, USA) through the injection port. The injected volumes were equal to 20 μL, and the signal output of the dual channel photometric detector working at 267 and 535 nm was continuously monitored on the computer.

RESULTS AND DISCUSSION

In the biomedical practice MDA is usually analyzed using the TBA assay.[6] It is based on the photometric measurement of the product of TBA and MDA reactions. As it is well known, the TBA test is intrinsically nonspecific.[6] Positive response is obtained with other compounds, especially those containing double bonds in their molecular structure. Ion exclusion chromatograms of the products of the reaction of TBA with MDA, obtained with photometric detection at 535 nm, are presented in Fig. 1. The appearance of a number of peaks, as well as their broadening, suggest no stoichiometric reaction between TBA and MDA. Certain improvement can be obtained by adding an ion interaction reagent to the mobile phase. In this case, the ion

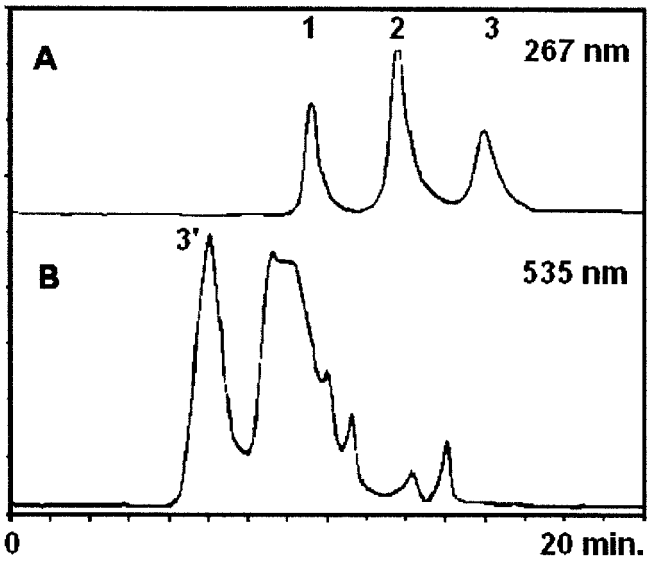

Figure 1. IEC chromatograms: (1) AA, (2) UA, (3) MDA, and (3′) MDA · TBA. Chromatographic conditions: column—300 × 7.8 mm^2 I.D., >4.2 meq/g, TSK-GEL SCH(H^+) (TosoHaas); detector UV-267/535. The mobile phase: (A) 1 mM H_2SO_4, 3 mM TBABr, ACN, temp. 40°C; (B) 1 mM H_2SO_4, ACN, temp. 20°C.

interaction reagent interacts with the analyte, as well as the functional group of the resin. It enables detection of MDA in the presence of AA and UA without derivatization steps. This can be accomplished by monitoring absorbance at 267 nm (cf. Fig. 1). However, very broad and small MDA peaks were obtained at room temperature. It was found that the broadening is strongly dependent on the temperature (cf. Figs. 2 and 3). It is not clear, at the moment, why temperature influences broadening of MDA peaks only. Probably, it is due to kinetic effects that occur in the sample. According to our knowledge, it is the first such observation of the influence of temperature on the separation performance in IEC. This, and the application of the described method in neurochemical investigations, will be subject of our next paper.

Performance Characteristic of the Proposed Method

Optimized chromatographic conditions were set and the following analytical characteristics were evaluated: precision and accuracy, limit of detection (LOD) and limit of quantification (LOQ), linearity.

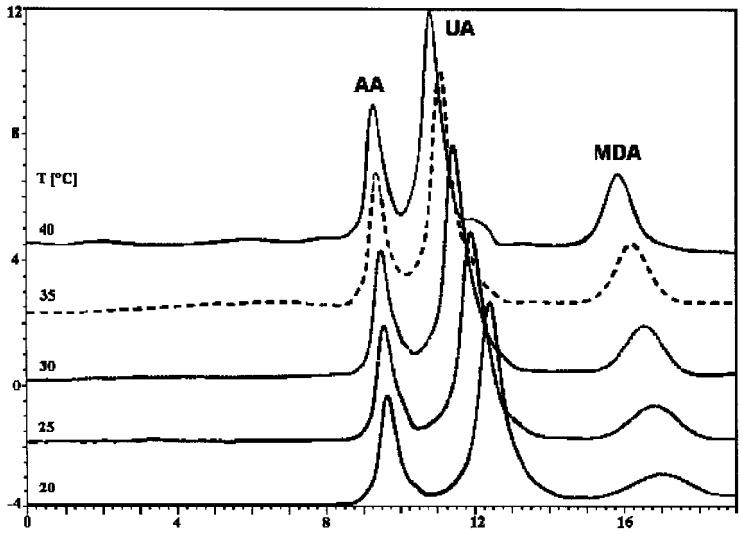

Figure 2. IEC chromatograms of AA, UA, and MDA obtained for different column temperatures. Chromatographic conditions: column 300 × 7.8 mm^2 I.D., TSK-GEL SCH(H^+) (TosoHaas); mobile phase: 1 mM H_2SO_4, 3 mM TBABr, ACN; detector UV-267 nm; temp. 20°C–40°C.

Precision and Accuracy

The repeatability of the method was verified by replicate injections ($n = 6$) of appropriately diluted (with 1% SA) stock standard solutions: 100 μM for MDA and 25 μM for MDA · TBA. The standard deviation (SD, in μM) and relative standard deviation (RSD, in %) are summarized below.

Sample	Found ± SD	RSD
100 μM MDA	104 ± 5	5
25 μM MDA · TBA	23 ± 2	8

LOD and LOQ

Working standard solutions in the concentration range of 1–100 μM were prepared by appropriate dilution of stock standard solutions with 1% SA.

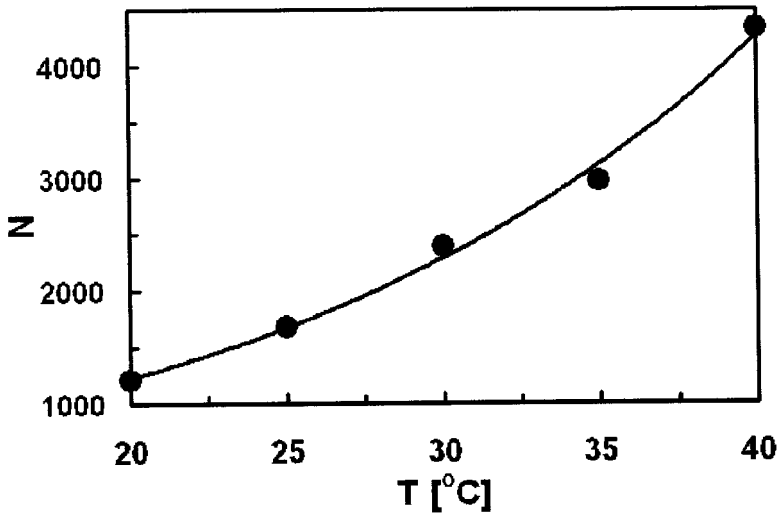

Figure 3. The number of theoretical plates (N) plotted against the temperature (T) for MDA. Chromatographic conditions as in Fig. 2.

LOD (in μM), the quantity yielding the detector response approximately three times the size of the background noise, and LOQ (in μM) are summarized below.

Sample	LOD	LOQ
MDA	8	25
MDA · TBA	1.2	3.8

Linearity

It was estimated that the linearity of the calibration curve extends over approximately four orders of magnitude in the concentration for MDA, approximately from 10 μM to 100 mM.

CONCLUSIONS

It was shown that IEC can be applied to the analysis of MDA. Two methods were compared. The first one was based on the derivatization of

MDA with thiobarbituric acid; and the second one was based on direct separation with ion interaction reagent added to the mobile phase. Better separation was obtained for the second method, although lower detection limit was achieved with the first one. Peak broadening was strongly affected by column temperature, which was probably caused by kinetic effects that occur in the sample.

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