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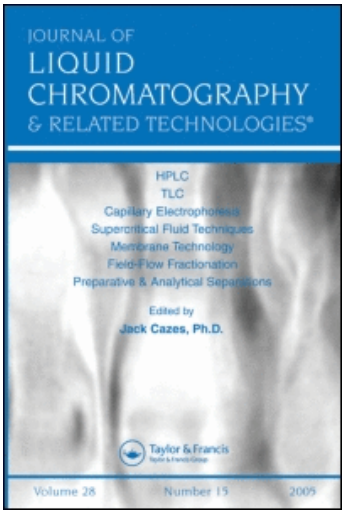

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M. Elisa Soares[a]; Márcia Carvalho[a]; Fernando Remião[a]; Félix Carvalho[a]; Maria de Lourdes Bastos[a]
[a] REQUIMTE, Toxicology Department, Faculty of Pharmacy, University of Porto, Porto, Portugal

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Implementation of HPLC Methodology for the Quantification of Malondialdehyde in Cell Suspensions and Liver

M. Elisa Soares, Márcia Carvalho, Fernando Remião, Félix Carvalho, and Maria de Lourdes Bastos*

REQUIMTE, Toxicology Department, Faculty of Pharmacy, University of Porto, Porto, Portugal

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ABSTRACT

Determination of malondialdehyde (MDA) is commonly used for the evaluation of lipid peroxidation in biological human samples, especially in plasma, serum, and urine, as a non-invasive biomarker of some oxidative-stress-related diseases. The objective of the present study was to implement a HPLC methodology for the quantification of MDA in cell suspensions and liver, after complexing this compound with thiobarbituric acid (TBA), giving rise to the formation of the complex MDA–$(TBA)_2$. The implemented methodology was validated and allowed a reliable separation and quantification of MDA, with high precision and recovery, and a low detection limit. The stability of MDA in the

*Correspondence: Maria de Lourdes Bastos, REQUIMTE, Toxicology Department, Faculty of Pharmacy, University of Porto, R. Aníbal Cunha, 164, 4099-030 Porto, Portugal; E-mail: mlbastos@ff.up.pt.

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biological samples, as well as of the MDA–$(TBA)_2$ complex, was also evaluated during storage at different temperatures and for different time periods. The data obtained from the stability studies concerning the MDA–$(TBA)_2$ complex prepared from hepatocyte suspensions and liver samples, indicated that the chromatographic analysis should be performed within the period of 4 hr when it is maintained at ambient temperature or at 40°C, and within the period of 48 hr if the temperatures of the storage are 4°C, −20°C, or −80°C. When the biological samples are conserved for posterior preparation of the complex, MDA can be measured in the cell suspensions conserved at any of these temperatures in the subsequent 24 hr, but liver samples must be conserved at 4°C or −20°C in order to obtain reliable results of MDA.

Key Words: Malondialdehyde; HPLC/UV; Cell suspensions; Liver.

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INTRODUCTION

Oxidative stress, an important mechanism of cell toxicity, can be elicited by several endogenous and exogenous processes because of its unequivocal characterization in biological samples. Oxygen radicals react with polyunsaturated fatty acid residues in phospholipids, resulting in lipid peroxidation, which, depending on the extension, can cause reversible or dramatic injury culminating in cell death.

One of the most abundant carbonyl products of lipid peroxidation is malondialdehyde (MDA) which is also generated as a side-product of prostaglandin biosynthesis.[1] Thus, the specific and accurate determination of MDA is crucial in research for evaluation of the cell lesions and, consequently, for establishing the mechanism of cell injury caused by xenobiotics. Moreover, the presence of MDA in biological systems is also very pernicious due to its reactivity and phenomenons like aging, mutagenesis, and carcinogenesis are attributed to this molecule.[1,2]

The most common methodologies referred in literature for indirect detection and quantification of lipid peroxidation, still consists of the spectrophotometric determination of MDA, after complexing with thiobarbituric acid (TBA), giving rise to the formation of the complex MDA–$(TBA)_2$.[3]

In recent literature citing this subject, several proposals of reverse-phase liquid chromatographic methods for the determination of MDA have been reported already. Generally, the authors argue in favor of these chromatographic methods, specially because they enable the individualization of the compound, free of endogenous interferences. These interferences, which can be relevant in biological materials, can give rise to overstated values of MDA when the spectrophotometric assays are applied. This fact may explain the

wide range of concentration levels of the compound in human serum that has been found by several authors.[4]

Some of the papers published in the last years involving HPLC methodologies, are concerned with the HPLC determination in serum or plasma blood samples of MDA in its free form,[5] as a complex with the thiobarbituric acid,[4,6] or after a derivatization step.[7] The HPLC determination of MDA in human urine after derivatization, for the characterization of lipid peroxidation by a non-invasive manner, was also recently published.[8] An on-line microdialysis system coupled with HPLC was also implemented for determination of TBA adduct of MDA, and applied to the monitorization of the lipid peroxidation in an in vitro study constituted by linoleic acid incubated with metal ions.[9]

The present study consisted of the adaptation and improvement of published RP-HPLC methods to measure the complex MDA–$(TBA)_2$ in the biological matrices, rat liver, and hepatocyte suspensions. Furthermore, we evaluated the stability of MDA in the biological samples during storage before analysis, as well as of the MDA–$(TBA)_2$ complex maintained at different temperatures, and for different periods of time.

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EXPERIMENTAL

Materials

1,1,3,3-Tetraethoxypropane (TEP), TBA, and butylated hydroxytoluene (BHT) were obtained from Sigma (St Louis, MO). All other chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany). Deionized suprapure water (Seral Water Purification Systems, Munich, Germany) was used for HPLC and to prepare all solutions and buffers.

To avoid contamination of the samples, all materials were immersed in freshly prepared 15% (v/v) pro-analysis HNO_3 during 24 hr, then rinsed thoroughly with deionized suprapure water.

High Performance Liquid Chromatography

The HPLC system consisted of a Hewlett Packard, HP 1100 Series Quaternary Pump, equipped with a manual injector and a variable wavelength detector. Data and chromatograms were processed by a HP Chemstation for HPLC.

The column was a Waters Spherisorb C18, 5 μm ODS2 (4.6 × 250 mm analytical cartridge), Waters Corporation.

The mobile phase consisted of methanol : ammonium acetate buffer 0.05 M, pH 5.5 (40 + 60). The separation was carried out isocratically at room temperature. The flow rate was 0.7 mL/min, the detector wavelength was set at 532 nm and the injection volume was 50 μL.

Standards and Reagent Solutions

The TEP standards were prepared daily in absolute ethanol 40% in deionized suprapure water. Working standard solutions 0.25, 0.5, 1.0, 2.0, and 5.0 μM were made by dilution from a 1000 μM stock solution. A 1.0% of trichloroacetic acid solution and 1% of TBA solution were prepared daily in deionized suprapure water. A 0.2% of BHT solution was prepared daily in 40% absolute ethanol in deionized suprapure water.

Phosphate buffer solution (pH 7.0) was prepared by dissolving 1.7025 g of KH_2PO_4 in 200 mL deionized suprapure water and 2.67 g of $Na_2HPO_4 \cdot H_2O$ in 300 mL deionized suprapure water, and mixing the two solutions. Triton X-100 (0.5 mL) was added to this phosphate buffer, the final concentration being 0.1%.

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Samples for Analysis and Pre-treatment

Samples of rat liver and suspensions of freshly isolated rat hepatocytes were used to improve HPLC published methods applied to human plasma determination of MDA, in order to adapt them to these complex biological matrices.

The pre-treatment of the biological samples in parallel with TEP standard solutions and formation of MDA–$(TBA)_2$ complex, is schematically presented in Fig. 1. Briefly, 250 μL of cell suspensions or TEP standards were added to 25 μL of 0.2% BHT in order to prevent further lipid peroxidation. Liver samples (0.25 g) were homogenized with 1 mL phosphate buffer and 25 μL of 0.2% BHT was added. The precipitation of the proteins was achieved by addition of 1 mL 1.0% TCA. The samples were then vortexed and centrifuged (10,000 rpm for 10 min, at 4°C). For the complexation reaction, 500 μL of 1% TBA was added to the supernatant layer and allowed to stand at 95°C during 45 min. After cooling at 4°C, the samples were vortexed, centrifuged, and 50 μL supernatant were directly injected onto the HPLC column.

Validation of the Method

Both in standard solutions and in the biological samples, several concentrations of TCA were tested in order to find the convenient pH that

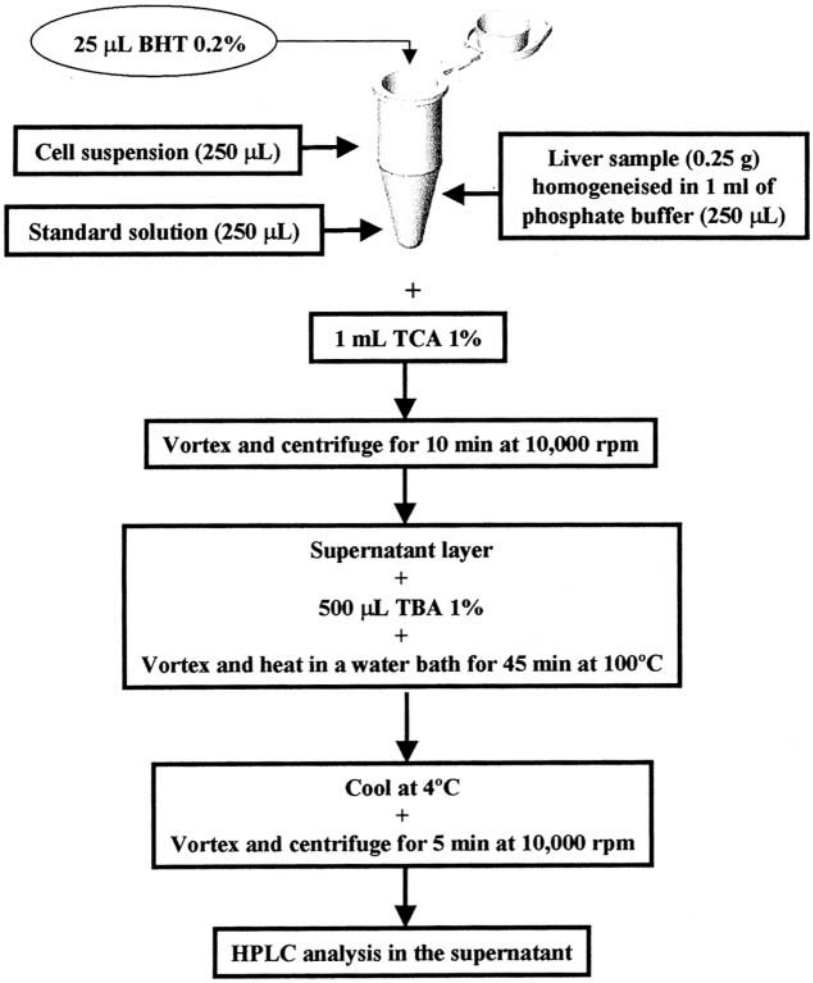

Figure 1. Scheme for preparation of the MDA–$(TBA)_2$ complex in standard solution and biological samples.

proportionates the complete precipitation of the proteins without altering the stability of the MDA–$(TBA)_2$ complex. The best results were obtained with the concentration of 1.0%, which corresponded to pH 2.1. Similar study was done for TBA. Three concentrations of TBA were tried, 0.1%, 0.5%, and 1%,verifying that 1% gave the most reproducible results for the formation of MDA–$(TBA)_2$ complex, both for cell suspensions and for liver samples.

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Under the described conditions, the detection limit of the complex MDA–$(TBA)_2$ was defined as three times of the background noise in the chromatogram baseline.

The precision of the analytical method was evaluated by analyzing 10 times the complex in the same supernatant obtained both from cell suspensions and liver samples. For evaluation of the precision of the overall procedure, 14 different final supernatants obtained from cell suspensions and liver samples containing the MDA–$(TBA)_2$ complex prepared as described above, were analyzed.

For the recovery studies, three different concentrations of TEP standard solutions were added to the cells and liver samples (6–8 portions for each concentration), submitted to the overall procedure, and the complex quantified by the established chromatographic conditions.

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RESULTS

The values obtained for the precision (relative standard deviation; RSD) of the analytical technique for quantification of the complex MDA–$(TBA)_2$ were 3.7% and 3.8% and for the overall procedure were 10.8% and 12.9% for cell suspension and liver samples, respectively (Table 1).

The results of the recovery study obtained by the standard additions method (0.5, 1.0, and 2.0 μM of TEP) were between 94% ± 3 and 99% ± 1 for the cell suspensions, and between 96% ± 2 and 97% ± 2 for the liver samples (Table 2).

The detection limit of the method was 0.028 μM and the linear range was set between 0.028 and 10.0 μM.

Considering 0.25 g of liver for quantification of MDA, and 2.525 mL the final supernatant volume, the limit of quantification was 0.28 μM. For 0.25 mL of cell suspension and 1.775 mL the final supernatant volume, the limit of quantification was 0.20 μM.

Results were calculated as μM MDA equivalent from the TEP standard calibration (1 : 1 conversion under acidic conditions).[6]

Table 1. Precision (%) of the analytical method and overall procedure for quantification of MDA–$(TBA)_2$ complex in biological samples.

Sample	Analytical method ($n = 10$)	Overall procedure ($n = 14$)
Cell suspension	3.7	10.8
Liver	3.8	12.9

Table 2. Statistical results for the recoveries of MDA–$(TBA)_2$ complex obtained after TEP standard additions.

	Parameter		
	Concentration (μM)	*n*	Recovery(%) ± SD
Cell suspension	0.5	8	94 ± 3
	1.0	8	95 ± 3
	2.0	6	99 ± 1
Liver	0.5	7	97 ± 2
	1.0	7	97 ± 2
	2.0	7	97 ± 2

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Data obtained from the stability study of the MDA–$(TBA)_2$ complex, both at different temperatures and during different periods of time, are summarized in Tables 3 and 4 and expressed as percentage of variation relative to time zero. The variations of the complex levels prepared from cell suspensions and preserved at ambient temperature and at 40°C during 5 hr were lower than 10.9%. The variations of the complex levels prepared from liver samples were lower than 13.8% during the first 4 hr; at 5 hr, the variations were 21.7% and 18.9% at ambient temperature and 40°C, respectively. When the MDA–$(TBA)_2$ complex was prepared from cell suspensions and conserved at 4°C, −20°C, and −80°C, the variations of levels were between 2.8% and 12.7%. For liver samples, the variations were between 8.3% and 14.3% during 48 hr at the tested temperatures; at 96 hr, the variations were higher than 22.0%.

Data obtained from the stability study of MDA present in the biological samples at time zero, and 24 hr after their collection and maintenance at different temperatures, are presented in Table 5. In cell suspensions, the variations

Table 3. Results of the stability studies (expressed as percentage of variation relative to time 0) of MDA–$(TBA)_2$ complex prepared from cell suspensions and liver samples at ambient temperature and at the temperature of 40°C ($n = 3$).

	Ambient temperature		40°C	
Time (hr)	Cells	Liver	Cells	Liver
1	1.2	3.4	10.9	5.0
2	9.5	13.8	0.0	9.3
3	7.1	11.2	10.3	2.6
4	9.8	21.7	9.5	18.9

Table 4. Stability study (expressed as percentage of variation relative to time 0) of the MDA–$(TBA)_2$ complex at different times after its formation (24, 48, and 96 hr) at several temperatures (4°C, −20°C, and −80°C) ($n = 10$).

	4°C		−20°C		−80°C	
Time (hr)	Cells	Liver	Cells	Liver	Cells	Liver
24	10.0	10.0	12.7	14.3	3.6	8.4
48	3.9	8.3	10.0	9.7	2.8	9.3
96	5.1	31.0	5.3	58.0	8.5	22.0

were 1.8%, 8.4%, and 15.3% at 4°C, −20°C, and −80°C, respectively. In the liver samples the variations of the MDA contents were 5.5%, 2.0%, and 45.7% at 4°C, −20°C, and −80°C, respectively.

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DISCUSSION

Determination of MDA is commonly used for the evaluation of lipid peroxidation in biological human samples, especially in plasma, serum, and urine as a non-invasive biomarker of some diseases. Blood levels of this parameter have been used by some authors to characterize several human pathologies like atherosclerosis,[10] glomerular disease,[6] and goiter.[5] For decades, a simple and reliable method for the evaluation of lipid peroxide level in serum or plasma by the use of TBA reaction was universally adopted. However, this method offers a rather low specificity. In the last years, some HPLC methods were implemented for the specific determination at low levels of the stable product of lipid peroxidation, the MDA, enabling the establishment of real normal blood human levels and, consequently, to correlate the elevated values with some diseases.

The characterization of lipid peroxidation is also very important in research studies, both in vivo and in vitro, in order to evaluate the injury

Table 5. Study of the stability of MDA in the biological samples (hepatocyte suspensions and liver) at different temperatures (4°C, −20°C, and −80°C) during 24 hr. Values are expressed as % of variation relative to time zero ($n = 4$).

	4°C	−20°C	−80°C
Cell suspension	1.8	8.4	15.3
Liver samples	5.5	2.0	45.7

evoked by reactive species and some xenobiotics at biomembrane levels. It would be rather complex to refer to the thousands of mechanistic research works published to date, that used the spectrophotometric methods for evaluating lipid peroxidation based on the complexation of TBA with MDA, albeit the recognition of the limitations of the method.

Taking as basis, the recent and above referred HPLC methods that selectively separate and quantify the MDA–$(TBA)_2$ complex in human blood samples, we improved and adapted them to other biological samples, namely rat liver and isolated hepatocyte suspensions. This last preparation is, nowadays, a common in vitro model for hepatotoxic and hepatoprotector studies.[11,12]

Concerning the pre-treatment of the biological samples, it consisted essentially of two steps: the simplification of the samples by adding the trichloroacetic acid for protein precipitation, and the subsequent formation of the MDA–$(TBA)_2$ complex. Immediately after collecting the samples, either cells or liver, the antioxidant BHT was added, as proposed by other authors,[6] to prevent additional formation of MDA at the drastic adopted conditions, namely the acid pH and the high temperature of incubation of the samples needed to proportionate the formation of the MDA–$(TBA)_2$ complex.

In parallel with the biological samples, a standard TEP solution was also submitted to all the pre-treatment steps. Since MDA is unstable, TEP was used because it is quantitatively converted to MDA during the pretreatment procedure, i.e., acidic pH and high temperature.[10]

The validation of the method was carried out by the determination of the precision, both of the analytical and of the overall procedures. As it is presented in Table 1, the obtained values, expressed in RSD%, were very good for the first (3.7% and 3.8% for the cell suspensions and liver, respectively) and very acceptable for the second (10.8% and 12.9% for the cell suspensions and liver, respectively). In the recovery studies performed by the standard additions method, the obtained values were always higher than 94%, for the two biological samples at all the added concentrations. The detection limit of the method, 0.028 μM, was low enough to quantify the levels considered normal in these biological samples and, consequently, to characterize the lipid peroxidation resulting from the injury elicited by reactive species or other adverse conditions. Comparing the limit of detection obtained for the present method with other HPLC methods applied to human fluid samples, it may be concluded that it is similar or even better.[6,8]

The mobile phase used in the chromatographic analysis, consisting of a mixture of methanol and acetate buffer operating in an isocratic mode, allowed the separation of the MDA–$(TBA)_2$ complex from endogenous compounds, as is shown in Fig. 2. The possibility of using acetate buffer is an advantage when compared with the phosphate buffer used by others,[4–6,9] especially in the interest of preserving the HPLC pumping system.

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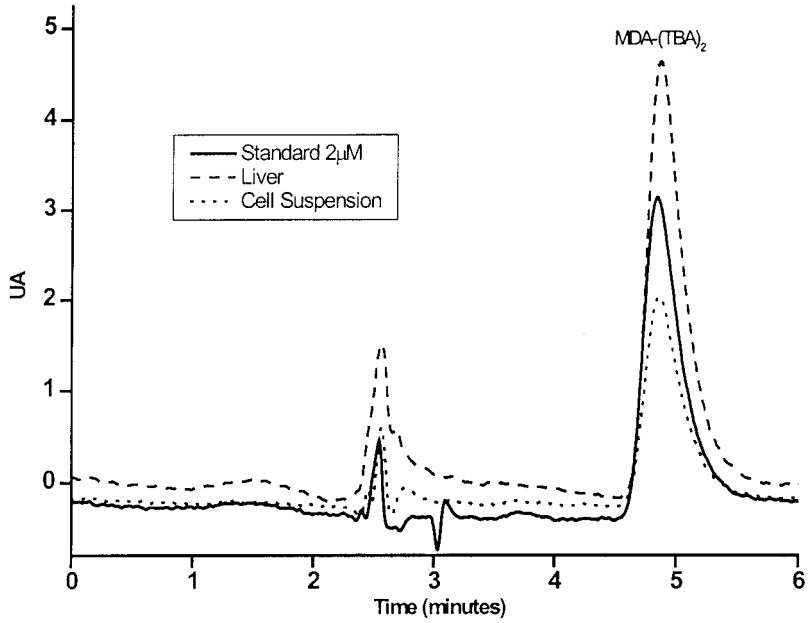

Figure 2. HPLC chromatograms of MDA–$(TBA)_2$ complex prepared from (——) TEP standard solution, (· · · · · ·) cell suspensions, and (----) liver samples.

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In research, it is common to collect, in the same experiment, several tens of biological samples for quantification of lipid peroxidation. This results in the impossibility of analyzing all of them immediately, which means that the conservation of the samples and quantification of MDA during several days is important in order to establish analysis protocols that provide accurate results for reliable interpretation. Also, for HPLC analysis, it is very comfortable to use an automatic sampler, enabling the sample analysis in the absence of an operator. Thus, it is imperative to study the stability of MDA in the biological samples before analysis, as well as the stability of the MDA–$(TBA)_2$ complex obtained from the biological and standard samples, at different temperatures and during several periods of time before chromatographic analysis.

To evaluate the stability of the MDA–$(TBA)_2$ complex at ambient temperature ($20 \pm 2^{\circ}C$) and at $40^{\circ}C$, standard samples, cell suspensions, and liver samples were submitted to the pre-treatment procedure, as schematized in Fig. 1, and the supernatant containing the MDA–$(TBA)_2$ complex maintained during 5 hr at those temperature conditions. The complex levels were quantified hourly by the established HPLC conditions against the standard curve. The variations of the complex contents along the time (1–5 hr) were

calculated and compared with the levels obtained at time zero. As is presented in Table 3, the complex levels variations were progressively higher along the time, but the variation is still acceptable after 5 hr in the cell samples conserved at the ambient temperature, and at 40°C (variations $<13.8\%$). For the liver samples, the variations of the levels of MDA–$(TBA)_2$ complex at the 5 hr period were too high for both temperatures, attaining levels that compromise the interpretation of data, and consequently, the quantification of the complex in liver must be measured within 4 hr after preparation. It is important to mention that the standard work solutions of MDA–$(TBA)_2$ complex must be prepared and preserved in parallel with the complex prepared from the biological samples, because degradation was also verified in standard solutions.

The stability of the MDA–$(TBA)_2$ complex prepared from the biological samples was also evaluated by its quantification at time 0 and after 24, 48, and 96 hr of storage at 4°C, −20°C, and −80°C. The variations were calculated as referred to above. As can be observed in Table 4, compared with time 0, the variations of the complex levels were still acceptable after 96 hr for the cell samples and after 48 hr for the liver samples, at all tested storage temperatures. The excessive positive variation values obtained at time 96 hr for the MDA–$(TBA)_2$ complex obtained from the liver samples (31.0%, 58.0%, and 22.0%), can be justified by the presence of lipids in the supernatant, and formation of MDA, which complexes with TBA present in excess, indicating that lipid peroxidation continues after 48 hr of storage of liver at any of the studied conditions.

Also performed, was the study of MDA stability in the cell suspensions and in liver samples by quantifying the MDA–$(TBA)_2$ complex formed immediately after the collection of the samples and 24 hr later, maintaining the samples at three different temperatures (4°C, −20°C, and −80°C). It was observed, that the MDA content in the biological samples was stable during 24 hr at the assayed temperatures (variations $<15\%$), except for the liver at −80°C in which the variation of the contents were considerably higher (45%), as shown in Table 5. The reason for this effect induced by freezing at very low temperatures is still unclear, but may be due to an upper cell disruption provoked by ice volume expansion, exposing cells to a more extensive lipid peroxidation during thawing. When comparing with the stability obtained for the MDA–$(TBA)_2$ complex, this study indicates that it is more correct to prepare the complex immediately after collecting the biological samples and preserve it at low temperatures until analysis, than conserve the biological samples for preparing the MDA–$(TBA)_2$ complex afterwards.

Taking both the data obtained from the stability studies concerning the MDA–$(TBA)_2$ complex prepared from hepatocyte suspensions and liver, we can conclude that the chromatographic analysis should be performed within the period of 4 hr when it is maintained at ambient temperature or at 40°C,

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and within the period of 48 hr if the temperatures of the storage are 4°C, −20°C, or −80°C. When the biological samples are conserved for posterior preparation of the complex, MDA can be measured in the cell suspensions conserved at any of these temperatures in the subsequent 24 hr, but liver samples must be conserved at 4°C or −20°C in order to obtain reliable results of MDA.

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