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Determination of malondialdehyde (MDA) by high-performance liquid chromatography in serum and liver as a biomarker for oxidative stress Application to a rat model for hypercholesterolemia and evaluation of the effect of diets rich in phenolic antioxidants from fruits

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Abstract

A high-performance liquid chromatography (HPLC) method to determine malondialdehyde (MDA) as the 2,4-dinitrophenylhydrazine (DNPH) derivative was applied to biological samples (serum and liver homogenates). Since MDA is considered a presumptive biomarker for lipid peroxidation in live organisms, a model for nutritionally induced oxidative stress (hypercholesterolemic rats) was studied in comparison with normocholesterolemic animals. The effect of diet supplementation with fruits rich in antioxidant polyphenols was assessed. The proposed method showed to be precise and reproducible, as well as sensitive enough to reflect differences in the oxidative status in vivo. A significant decrease of serum and liver MDA concentrations in animals fed diets containing 0.3% of polyphenols from strawberry, cocoa or plum was observed in the normocholesterolemic groups. This reduction was especially noteworthy in the hypercholesterolemic animals, with increased MDA levels indicating enhanced lipid peroxidation in the controls, yet with values parallel to the normocholesterolemic groups in animals fed the polyphenol-rich diets. These results point out the beneficial effects of phenolic antioxidants from fruits in preventing oxidative damage in vivo.

Keywords: Malondialdehyde; HPLC; 2,4-Dinitrophenylhydrazine; Lipid peroxidation; Oxidative stress; Serum; Liver; Fruit antioxidant polyphenols

1. Introduction

Free radical mediated cytotoxicity and lipid peroxidation are associated with cell aging and chronic diseases such as cancer, atherosclerosis, inflammation, etc. [1–4]. Reactive oxygen species (ROS) can react with double bonds of polyunsaturated fatty acids (PUFAs) to yield lipid hydroperoxides. One of the major secondary oxidation products of peroxidized polyunsaturated fatty acids is malondialdehyde (MDA) [5,6], which has been inferred to have mutagenic and cytotoxic effects, and possibly to participate in the onset of atherosclerosis [7]. Since MDA has been found elevated in various diseases thought to be related to free radical damage, determination of this biomarker has been widely applied as the most common approach for the assessment of lipoperoxidation in biological and medical sciences [7,8]. MDA is also widely used in food sciences as an index of lipid oxidation and rancidity in foods and food products [9].

MDA is most frequently determined spectrophotometrically as thiobarbituric acid reactive substances (TBARS) after its reaction with thiobarbituric acid (TBA) at 100 °C in acidic media and measuring absorbance of the reaction mixture at 532 nm [8]. This is a simple and inexpensive method, yet highly inaccurate since TBA reacts not only with MDA but also with many other compounds (e.g. carbohydrates, pigments, amino acids, pyridins, etc.) [9,10], interfering in the TBA assay and resulting in considerable vari-

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ability in the results. On the other hand, its chromatographic approach measures the 2TBA–MDA adduct after separation by reversed-phase high-performance liquid chromatography (HPLC) with fluorescence [6,11–13] or UV–vis detection at 532 nm [7,14]. However, reaction of MDA with TBA still requires treatment at high temperatures (95–100 °C) for extended incubation times (up to 150 min) [6,11–14] in strong acidic conditions (pH 1.5–3.5), which may result in an artefactual peroxidation of sample constituents even in the presence of added antioxidants (i.e. BHT). In spite of this, TBARS is still widely used to determine MDA levels in human [15–19] and animal experiments [20–26], measuring MDA in biological samples such as plasma, or organs like liver, lung or kidney.

Derivatisation of MDA and other aldehydes with pentafluorophenylhydrazine and separation by GC–MS has been applied to foods as well as biological samples with good results [27–29], allowing the simultaneous determination of several aldehydes, although this method requires the use of expensive equipment. Alternatively, derivatisation of MDA with 2,4-dinitrophenylhydrazine (DNPH) and conversion into pyrazole and hydrazone derivatives has been found to allow specific estimation of this compound, especially if combined with its separation using HPLC [30,31], a technique now commonly available in research laboratories. This approach has been used to determine MDA levels in biological samples, such as rat and human plasma [5,31,32] or rat urine [30], although it has not been applied yet to tissue samples such as liver or lung.

In a previous work [33], we reported a methodology to determine MDA by HPLC as the 2,4-dinitrophenylhydrazone derivative in human hepatoma HepG2 cells in culture. This showed to be an accurate, sensitive and reproducible method that reflected the effect of induced oxidative stress on the levels of lipid peroxidation in cells, suggesting that it could be used as a reliable biomarker for cellular oxidative stress. The aim of this study, was to adapt this method to biological samples, such as serum and liver homogenates, and to test its feasibility as a biomarker for oxidative stress in vivo in a rat model for hypercholesterolemia.

2. Experimental

2.1. Materials

Acetonitrile, methanol, sodium hydroxide, sodium chloride, di-sodium hydrogen phosphate anhydrous, potassium di-hydrogen phosphate, as well as formic, hydrochloric, perchloric and sulphuric acids were acquired from Panreac (Barcelona, Spain). Trizma base, sucrose, DL-dithiotreitol (DTT), 2,4-dinitrophenylhydrazine and 1,1,3,3-tetraethoxypropane (TEP) were purchased from Sigma Chemical Co. (Madrid, Spain). All reagents were of analytical grade unless stated otherwise.

Table 1
Composition of the experimental diets (g/kg dry weight)[a]

	Control	Strawberry	Cocoa	Plum
Casein	140	140	140	140
Dextrose	155	155	155	155
Sucrose	100	0	100	0
Soya oil	40	40	40	40
BHQ[b]	0.008	0.008	0.008	0.008
Mineral mix	35	35	35	35
Vitamin mix	10	10	10	10
L-Cystine	1.8	1.8	1.8	1.8
Corn starch	465.692	460.692	350.692	460.692
Choline bitartrate	2.5	2.5	2.5	2.5
Cellulose	50	35	0	35
PP-rich ingredient	–	120	165	120
Total	1000.00	1000.00	1000.00	1000.00

[a] Hypercholesterolemic diets were supplemented with 20 g cholesterol and 4 g sodium cholate per kg of diet, at the expense of starch.
[b] *tert*-Butylhydroquinone.

2.2. Animal experiment

Male Wistar rats (8 weeks old) were obtained from the School of Medicine, Universidad Complutense (Madrid, Spain). They were placed individually in metabolic cages and housed in a room under controlled conditions of temperature (19–23 °C), humidity (50–60%) and light (12 h light/12 h dark cycles). Rats were randomly assigned to the different dietary groups (eight groups, eight animals per group). The composition of the diets is given in Table 1.

Normocholesterolemic diets were prepared from a Fiber Free AIN-93M Purified Rodent Diet (Panlab S.L., Barcelona, Spain), which provides the nutrients required by adult rats according to the National Research Council guidelines [34,35]. Hypercholesterolemic diets were prepared from the AIN-93M diet supplemented with 20 g of cholesterol and 4 g of sodium cholate per kilogram of the basal diet [36]. Cellulose (5%) was added to the basal fibre free diet and fed to the control groups. The experimental diets were prepared from the fibre free basal diet and contained 12% of freeze-dried strawberry or plum powder, or 16.5% of cocoa fibre. These powdered fruit supplements were added as a source of dietary fibre and polyphenols at the expense of starch. Diets were formulated to provide 3 g of polyphenols per kg of diets. Cellulose was added to the strawberry and plum diets to make up for a final 5% dietary fibre. Since both strawberry and plum powders were rich in soluble sugars, sucrose was not added to the corresponding diets (Table 1). Caloric content was similar in all four diets.

Animals were adapted to the powdered diets for four days prior to the experimental period that lasted three weeks. During this time, rats were fed the corresponding normo or hypercholesterolemic diets. Animals were given free access to food and water. Body weight and food intake were monitored daily throughout the experiment. After the experimental period, rats were sacrificed, troncal blood collected, and serum separated by centrifugation (3000 rpm, 10 min, 4 °C) and kept

frozen at −80 °C. Livers were collected, immediately frozen in liquid nitrogen and kept at −80 °C until further use.

All animal procedures were conducted in strict conformation with the NIH guidelines [35] for animal care and the study submitted to the approval of the Bioethics Committee of the CSIC.

2.3. Determination of MDA in biological samples

Liver homogenates were prepared by mixing one portion of 0.5 g of liver with 2.5 ml of 0.25 M Trizma base buffer pH 7.4 (containing 0.2 M sucrose and 5 mM DTT) and homogenized in a mechanically driven Teflon glass homogenizer (Polytron, Heidolph RZR 1, Germany). The homogenate was centrifuged at 10,000 × *g* in an automatic high-speed cold centrifuge for 30 min at 4 °C (Biofuge Primo R, Heraeus, Germany). The supernatant was collected and kept for estimation of malondialdehyde (MDA) by the method described in the present work. Serum samples were analysed directly.

An aliquot of 250 μl of sample (serum or liver homogenate) was placed in a 1.5-ml Eppendorf and 50 μl of 6 M NaOH were added. Alkaline hydrolysis of protein bound MDA was achieved by incubating this mixture in a 60 °C water bath for 30 min. Then, protein was precipitated with 125 μl of 35% (v/v) perchloric acid, and the mixture was centrifuged at 2800 × *g* for 10 min. A 250 μl volume of supernatant was transferred to an Eppendorf vial and mixed with 25 μl of DNPH prepared as a 5 mM solution in 2 M hydrochloric acid. Finally, this reaction mixture was incubated for 30 min at room temperature protected from light. An aliquot of 50 μl of this reaction mixture was injected onto an Agilent 1100 HPLC-DAD with a Nucleosil 100 RP-18 column (4.0 mm × 125 mm, 5-μm particle size, Agilent) preceded by a Lichrospher guard column (4.0 mm × 4.0 mm). Samples were isocratically eluted with a mixture of 0.2% (v/v) acetic acid in deionised water, and acetonitrile (62:38, v/v) at a flow rate of 0.6 ml/min at room temperature. Chromatograms were acquired at 310 nm.

To test whether biological samples undergo abiotic oxidation during sample treatment, with a concomitant artefactual increase of MDA, different concentrations of ascorbic acid (50–250 μM for serum samples, up to 2500 μM for liver homogenates) were added to the samples at the beginning of the procedure (i.e. before addition of NaOH).

Two calibration curves were prepared diluting a standard solution of MDA (prepared by hydrolysis of tetraethoxypropane in 1% sulphuric acid) in either phosphate buffer saline (PBS) or Trizma base buffer. In both cases, the standards were treated as the experimental samples (i.e. treatment with 6 M NaOH for 30 min at 60 °C followed by protein precipitation with 35% perchloric acid, and derivatisation with DNPH). The calibration curve in PBS was used to calculate MDA concentration in serum samples, while that in Trizma was used for the liver samples, which were homogenised in Trizma base buffer. Chromatographic parameters like linearity, limits of detection and quantification, reproducibility and recovery were calculated for both calibration curves.

Concentrations were expressed as nmol MDA per mg of protein in liver tissue or per ml of serum samples. Protein content in liver homogenates was estimated by the Bradford's method [37], using a Bio-Rad protein assay kit (500–0006, Bio-Rad Ltd., München, Germany).

2.4. Statistical analysis

Results are expressed as means ± standard deviation (S.D.) of eight animals. Samples were analysed in duplicate. To contrast groups, one way analysis of variance (ANOVA) followed by Duncan's multiple comparison tests was used. The method used to test the homogeneity of variances was the Cochran's test, and to discriminate among means the Fisher's least significant difference procedure was applied. The level of significance was $p < 0.05$. A Statgraphics Plus program version 2.1 (Statistical Graphics Corp. Rockville, MD) was used.

3. Results

3.1. Chromatographic determination of MDA in serum and liver samples

Fig. 1 shows the HPLC chromatogram at 310 nm of serum and liver homogenates after treatment for MDA determination. Peak 1 corresponds to the hydrazone derivative of MDA, which was confirmed by spiking samples with standard MDA before the treatment. Retention times varied slightly between runs due to the lack of thermostatisation of the column. Control references of serum and liver homogenates were prepared omitting the addition of the derivatising agent (DNPH). The chromatograms thus obtained were similar to those of the test samples, with no MDA-hydrazone (peak 1) detected (data not shown).

The analytical conditions applied (alkaline hydrolysis of protein bound MDA, protein precipitation and derivatisation of MDA with DNPH) yielded clear supernatants and thus no further extraction was necessary. A very good chromatographic peak resolution was obtained, with no interfering peaks, which allowed a straightforward determination of the MDA derivative. In the case of liver homogenates, a previous centrifugation before alkaline hydrolysis is essential to minimise variability of the results. Also, protection of samples from light is important during the derivatisation with DNPH and ulterior steps, since reductions of up to 18% of the initial MDA peak signal had been observed within 1 h due to the light sensitivity of the MDA hydrazone derivative [33].

Addition of ascorbic acid as an antioxidant did not result in variations in the MDA levels in biological samples. In liver homogenates, rich in cell membranes highly susceptible to oxidation and where MDA contents were relatively high, MDA values varied between 101 and 109% of those in

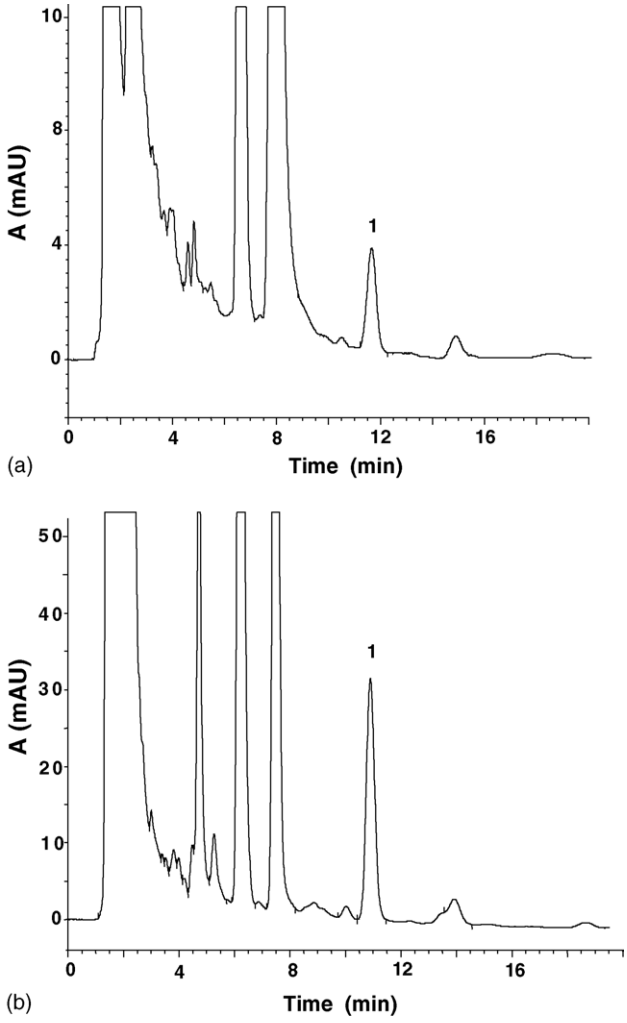

Fig. 1. HPLC chromatogram at 310 nm of biological samples: (a) serum and (b) liver. Peak 1 (retention time 11.1 min) corresponds to malondialdehyde as its 2,4-dinitrophenylhydrazone.

samples with no added antioxidant, even in the presence of ascorbic acid concentrations as high as 2.5 mM, which could have shown a pro-oxidative effect. Except during treatments with NaOH and DNPH derivatisation, samples were always maintained at low temperatures (during liver homogenization, centrifugation, etc.) and in the dark, which apparently does not enhance sample oxidation. Ascorbic acid was used instead of BHT based on previous observations by our group, where vitamin C showed to be a more effective antioxidant than BHT preventing oxidation processes during cell culture (unpublished results).

Table 3
Effect of the experimental diets on serum malondialdehyde (MDA) content (nmol/ml)[a,b]

Group	Normocholesterolemic rats	Hypercholesterolemic rats
Control	2.2 ± 0.1 b	2.8 ± 0.1 a
Strawberry	1.8 ± 0.1 c	2.3 ± 0.1 b
Cocoa	1.8 ± 0.1 c	2.0 ± 0.1 bc
Plum	1.7 ± 0.1 c	1.7 ± 0.2 c

[a] Values are mean ± S.D. of duplicate determinations of MDA in eight rats per group.
[b] Different (a, b, c) letters indicate statistically significant differences ($p < 0.05$).

Calibration curves prepared in PBS or Trizma buffer were used for determination of MDA concentrations in serum and liver samples, respectively. In both cases, a linear response of MDA in a range of concentrations from 0.20 to 20.00 nmol/ml was obtained (Table 2), being the chromatographic response of MDA in each vehicle very similar. Limits of detection (LOD) and quantification (LOQ) were slightly higher for the standards in PBS than for those in Trizma, although the reproducibility and the percentage of recovery were good in both cases. We used the two different calibration curves because liver samples were homogenised in Trizma base buffer; alternatively, they can be homogenised in PBS and the corresponding calibration curve used for calculations.

3.2. MDA in serum and liver of normo and hypercholesterolemic rats

The experimental diets given to rats were formulated to provide similar amounts of polyphenolic compounds and thus test the potential effect of diets enriched in phenolic antioxidants on MDA as a biomarker of oxidative damage to body lipids. An animal model of severe hypercholesterolemia was also used, since high levels of blood lipids would result in increased peroxidation of serum lipoproteins and cell membranes.

Diets were well tolerated by animals, with similar food intakes in all the experimental groups, although weight gain was higher in the animals fed on the hypercholesterolemic diets (data not shown), reflecting the higher caloric content of these diets. Hyperlipidemia was established in rats fed the cholesterol-rich diets as confirmed by the higher serum levels of cholesterol (both HDL- and LDL-cholesterol), triglycerides and fatty acids (data not shown).

Serum levels of MDA are shown in Table 3. Differences in the concentration of this aldehyde were observed not only between normo and hypercholesterolemic animals fed the

Table 2
Calibration values, limit of detection (LOD), limit of quantification (LOQ), recovery and coefficient of variation (C.V.) of the two MDA standard curves prepared in PBS and Trizma base buffer

	Slope	Intersect	R^2	LOD (nmol/ml)	LOQ (nmol/ml)	Recovery (%)	C.V. (%)
PBS	28.037	0.300	0.999	0.10	0.20	96.3–99.8	1.3–4.2
Trizma	23.885	−1.929	0.999	0.15	0.25	96.1–99.5	1.5–4.8

Table 4
Effect of the experimental diets on liver malondialdehyde (MDA) content (nmol/mg protein)[a,b]

Group	Normocholesterolemic rats	Hypercholesterolemic rats
Control	0.90 ± 0.09 b	1.20 ± 0.09 a
Strawberry	0.75 ± 0.09 bcd	0.57 ± 0.09 cd
Cocoa	0.78 ± 0.09 bcd	0.81 ± 0.10 bc
Plum	0.50 ± 0.11 d	0.54 ± 0.13 cd

[a] Values are mean ± S.D. of duplicate determinations of MDA in eight rats per group.
[b] Different (a, b, c, d) letters indicate statistically significant differences ($p < 0.05$).

same PP-rich diet, but also between groups submitted to the same alimentary pattern (i.e. normo or hypercholesterolemic) consuming different fruits as a source of antioxidant polyphenols. Except for the groups consuming diets with freeze-dried plums, serum levels of MDA were always significantly higher in the hypercholesterolemic rats in comparison with the normocholesterolemic animals. Therefore, high levels of plasma lipids as in the rat model for hypercholesterolemia lead to increased lipid peroxidation, suggesting a situation of oxidative stress.

Basal values of serum MDA in healthy adult Wistar rats consuming a polyphenol-free diet for three weeks were of 2.2 ± 0.1 nmol/ml (Table 3). This value was significantly reduced when 0.3% of phenolic antioxidants from different plant foods (strawberry, plum or cocoa) were included in the diet, with MDA values of approximately 1.7 nmol/ml. This suggests a reduction of lipoperoxidation associated to the consumption of fruit phenolics.

As in the normocholesterolemic animals, introduction of fruits rich in antioxidant polyphenols in the hypercholesterolemic diets resulted in a significant decrease of serum MDA compared to the rats fed the polyphenol-free control diet. This diminution was especially noteworthy in the cocoa and plum groups, where serum levels of MDA were similar to those in the respective normocholesterolemic groups. The strawberry diet, although less effective in decreasing MDA concentration, still reduced it to values similar to the control normocholesterolemic animals (Table 3).

When MDA was analysed in liver homogenates, differences between normo and hypercholesterolemic animals could also be observed. In the normocholesterolemic groups, a significant decrease of liver MDA was observed only in the animals fed the plum diet (Table 4). Although the values obtained in the strawberry and cocoa groups were lower than in the controls, these differences were not statistically significant. However, all three diets were effective in reducing MDA concentrations in hypercholesterolemic rats. In these animals, peroxidation of lipids in the liver was significantly increased as a consequence of the high cholesterol intake, as shown by the higher MDA levels in the hypercholesterolemic controls as compared to the normocholesterolemic ones. Nevertheless, inclusion of fruit phenolics in the diet decreased MDA concentration in liver homogenates to values similar to (cocoa and plum groups) or even lower (strawberry group) than those of their respective normocholesterolemic groups.

4. Discussion

Determination of serum MDA levels is still the most commonly applied assay for lipid peroxidation in biomedical sciences, since MDA is one of the major aldehydes formed after breakdown of lipid hydroperoxides. Therefore, it is considered a good biomarker of the involvement of free radical damage in pathologies associated to oxidative stress.

Although determination of MDA after reaction with thiobarbituric acid is considered to have important limitations, even in its chromatographic approach, it is still the method most widely used in research laboratories. However, Piltz et al. [5] reported an alternative methodology based on derivatisation of MDA with 2,4-dinitrophenylhydrazine, a method that allowed determination of free and bound MDA in plasma with good results. Recently, we reported a modification of this method and its application to estimate oxidative damage to cultured cells with excellent results in terms of precision, sensitivity and reproducibility [33]. In the present work, that method was applied to biological samples, both serum and liver, in an attempt to check its potential as a biomarker for oxidative stress in vivo. In this sense, to our knowledge this is the first time that MDA levels are determined in a solid tissue like the liver by a method different from the TBARS.

This method, based on HPLC determination of the MDA hydrazone, has proved to be precise and reproducible, as well as sensitive enough to reflect differences in the oxidative status in vivo, both in serum and in liver samples. A simple homogenization and centrifugation of solid samples is required, followed by release of protein-bound MDA by alkaline hydrolysis of supernatants or liquid samples like serum (or urine), precipitation of protein, and derivatisation of MDA without further extraction steps before direct injection onto the chromatographic system. Addition of an antioxidant like ascorbic acid does not significantly modify the results, suggesting that MDA concentrations are not artefactually increased due to oxidation during sample handling. A rapid separation by HPLC and monitorization of the well-resolved MDA peak by UV detection, with good recovery, reproducibility and sensitivity, makes this method an easy and suitable tool to quantify MDA in biological samples.

The values of serum MDA reported here are lower than those found in the literature for rat plasma, analysed as thiobarbituric acid reactive substances (TBARS), which ranged between 3 and 8 nmol/ml [23,25]; however, as mentioned before, compounds different from MDA would be measured as TBARS and thus artefactually increase MDA values. Data in the literature on MDA content in rat liver (0.5–0.7 nmol/mg of liver protein in control animals, analysed as TBARS) [21,24], were not very different from the values obtained here. Some other authors, however, report values as high as

200–300 nmol/g of rat liver [20,23], even up to 25 μmol/g of liver in mice [26].

Dietary antioxidants, including polyphenolic compounds, are considered beneficial because of their potential protective role in the pathogenesis of multiple diseases associated to oxidative stress such as cancer, inflammation or atherosclerosis. Flavonoids are a large group of polyphenolic compounds present in plant foods [38] that exhibit a wide range of biological activities [39]. They can inhibit lipid peroxidation, platelet aggregation, capillary permeability and the activity of enzyme systems including lipoxygenase and cycloxygenase, processes that are associated with cardiovascular disease [1–4,40]. In the present study, the effect caused by the introduction of fruit powders rich in antioxidant polyphenols of different nature (mainly flavonoids like anthocyanidins and flavonols in strawberry, flavanols and procyanidins in cocoa, as well as hydroxycinnamic acids in plums) proved to be very effective in reducing circulating levels of MDA not only in healthy animals fed normal diets, but also in hypercholesterolemic rats. In these animals, with an increased oxidative stress due to the high fat intake and in consequence showing higher concentrations of serum and liver MDA, the reduction of the concentration of this biomarker of lipid peroxidation was especially significant, paralleling values found in healthy animals. These results suggest a lower level of lipid peroxidation and free radical generation after the ingestion of diets rich in strawberry, cocoa and plum, which points out the beneficial effects of phenolic antioxidants in preventing oxidative damage in vivo. Since all diets were formulated to contain similar amounts of polyphenols (0.3%), the differences observed in the extent of MDA reduction should be ascribed to the different composition of the phenolic fraction, although the potential contribution of other non-phenolic antioxidants in fruits cannot be ruled out.

Some authors have also reported a positive effect of dietary polyphenols from different sources decreasing lipid peroxidation in rats subjected to oxidative stress. Thus, Yokozawa et al. [25] reported a significant increase of MDA in LDL particles, measured as TBARS, in rats fed hypercholesterolemic diets as compared to controls. These authors found a significant reduction of TBARS when animals were given increasing amounts of green tea polyphenols in the diet (up to 2.5%) for 5 weeks, although final values were always higher than in controls. Green tea polyphenols also decreased MDA levels (as TBARS) in the liver of rats subjected to exercise-induced oxidative stress as compared to animals that drank water instead of tea, although basal levels of resting animals were not achieved [24]. In rat aorta, a decrease of TBARS in hypercholesterolemic animals was also observed upon supplementation of the diet with 0.1% of wine proanthocyanidins, yet again values were higher than in control normocholesterolemic animals [22]. Wine proanthocyanidins failed to reduce serum MDA levels in this animal experiment.

From the results reported here it can be concluded that differences in the alimentary pattern, with diets modelling high fat intake and/or consumption of foods rich in antioxidant polyphenols like fruits or cocoa, result in measurable differences in the concentration of a biomarker of oxidative damage to lipids such as MDA. This effect was observed not only in serum, but also in a key organ like the liver, the major organ responsible for the metabolism of dietary antioxidants and also one of the main organs susceptible to oxidative damage by free radicals as responsible for detoxification of xenobiotics as well as due to its key role in lipid metabolism. Determination of MDA as a biomarker of lipid peroxidation reflects the effect of diet on the oxidative status in vivo. The method proposed here to measure MDA as the 2,4-dinitrophenylhydrazone by HPLC with UV detection is a reproducible, precise and sensitive method that can be easily applied to biological samples.

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