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The simple and sensitive measurement of malondialdehyde in selected specimens of biological origin and some feed by reversed phase high performance liquid chromatography

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ABSTRACT

A method for the determination of malondialdehyde (MDA) concentrations in specimens of animal tissues and feed has been developed using high performance liquid chromatography. The MDA concentration in acidified urine samples was determined after its conversion with 2,4-dinitrophenylhydrazine (DNPH) to a hydrazone (MDA-DNPH). Samples of blood plasma, muscle, liver and feed were prepared by saponification followed by derivatisation with DNPH to MDA-DNPH. The MDA concentration in chicken and hen feed samples was analysed after saponification and derivatisation followed by extractions with hexane. The free MDA in plasma samples was determined after deproteinization followed by derivatisation of MDA with DNPH. The chromatographic separation of MDA-DNPH samples was conducted using Phenomenex C_{18}-columns (Synergi 2.5 μm, Hydro-RP, 100 Å, the length of 100 mm) with an inner diameter of 2 or 3 mm. MDA in processed biological samples was analysed using a linear gradient of acetonitrile in water, and the photodiode detector was set to 307 or 303 nm for detection. The current method that was utilised was based on the high-efficient derivatisation of MDA and was more sensitive compared to previously used methods. The selective and sensitive photodetection of the column effluent was found to be suitable for the routine analysis of MDA in urine, plasma, muscles and liver of animals and some feed samples. Because urine or blood plasma samples can be derivatised in a simple manner, the proposed method can also be suitable for the routine, non-invasive evaluation of oxidative stress in animals and humans.

1. Introduction

An imbalance between oxidative stress and antioxidant capacity has been proposed to play a crucial role in the development and progression of chronic diseases in living organisms, especially in the elderly [1–5]. Oxidative stress is caused by reactive oxygen compounds, which damage lipids and biomolecules such as proteins or cellular RNA. Recent studies have shown that lipid oxidation contributes to the development of atherosclerosis, cancer, cardiovascular and liver diseases as well as the ageing process [3–7]. Malondialdehyde (MDA) and other carbonyl compounds are naturally occurring byproducts of lipid peroxidation and prostaglandin biosynthesis [2]. The longevity and very high reactivity of MDA and other carbonyl-containing species allow for the occurrence of reactions inside and outside cells of living organisms. MDA is a highly toxic molecule and is able to disturb many physiological processes in animals and humans. Therefore, MDA should be considered to be more important than a mere lipid peroxidation byproduct. Moreover, levels of MDA in living organisms have been found to be significantly modified in many pathological situations (e.g., gastric, lung or breast cancer and atherosclerotic or cardiovascular diseases) [2,8–10].

For analytical applications it is better to derivatize MDA with reagents possessing high molar absorptivity at longer UV wavelengths (>254 nm). The high molar absorptivity and the close proximity of the absorbance maximum to ~300 nm make a MDA derivative almost ideally suited for detection using a diode array system. Moreover, the derivatised MDA is substantially retained on the C_{18} columns [11] compared with underivatised MDA [12]; derivatised MDA is distinct from other endogenous substances in assayed biological samples or from background interference. Indeed, a MDA derivative is monitored at a unique wavelength (~300 nm), thus, other endogenous components present in biological samples did not interfere with a MDA derivative; these components are transparent at longer UV wavelengths (i.e., >254 nm). Therefore, it was important to develop a sensitive and

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selective method to detect MDA based on a derivatisation with a reagent possessing high molar absorptivity at longer UV wavelengths. One of the first methods applied to measure MDA, which is still used today, is based on MDA's derivatisation with thiobarbituric acid (TBA) [2,13]. Unfortunately, the TBA assay is non-specific for MDA because TBA is very reactive towards other compounds (nucleic acids, amino acids, proteins and phospholipids) and various species derived from oxidation [14–16]. To overcome the biases from the derivatisation of MDA with TBA, new derivatising reagents were tested. Major improvements were found in the selectivity measurements of MDA in biological samples by derivatising MDA with 2,4-dinitrophenylhydrazine (DNPH) to its respective hydrazone (MDA-DNPH) [11,17–19]. Therefore, the aim of the present study was to improve on a procedure involving the derivatisation of MDA with DNPH and subsequently to optimise the analysis using reversed-phase high performance liquid chromatography (RP-HPLC) with a photodiode array detector (DAD). It was expected that the improved method would assure a simple, selective and sensitive measurement of MDA in muscle, blood plasma and urine samples of examined animals.

2. Experimental

2.1. Chemicals

All reagents were of analytical grade, and the organic solvents were HPLC grade. Acetonitrile, methanol and hexanes were purchased from Lab-Scan (Eire); 2,6-di-*tert*-butyl-*p*-cresol (BHT), 25% aqueous 1,5-pentanedialdehyde (PDA) solution (as an internal standard), 2,4-dinitrophenylhydrazine (DNPH, containing *ca.* 30% water), 1,1,3,3-tetramethoxypropane (TMP) (99%) and trichloroacetic acid (TCA) were supplied by Sigma (St Louis, MO, USA). HPLC-grade water was used for the preparation of a mobile phase, and chemical reagents were prepared using an Elix^{TM} water purification system (Millipore, Toronto, Canada).

Fresh meat and liver samples were homogenized at speed 20,000 rpm using a tissue homogentor (type M-20, KIKA-Werke, GMBH & C.KC, Germany).

2.2. Standard and derivatisation agent preparation

To prepare a MDA standard solution, 20 ml of 0.1 M HCl was added to 34 μl of TMP. The resulting solution was incubated at 40 °C for 1 h to hydrolyse TMP into MDA (the final concentration ~0.01 M). The concentration of MDA in the standard solution was determined by measuring its absorbance at 245 nm (ε = 13,700) [12,20]. This stock-standard solution contained approximately 1.986 μg/ml of MDA. MDA standard solutions were stored at 1–2 °C in dark place and were freshly prepared on a weekly basis.

The derivatising agent (*ca.* 3.875 mM) was prepared by dissolving 5 mg of DNPH in 5 ml of 4 M HCl. The DNPH solution was stored at approximately −10 °C in the dark. Before use, the DNPH solution was vigorously shaken for 5–10 min in the dark. The derivatising agent solution was freshly prepared on a weekly basis.

The internal standard (IS) solution of PDA was prepared on the day of the experiment by diluting 100 μl of a 25% aqueous PDA solution with 0.1 M HCl up to 5 ml in a volumetric flask. A volume of 50 μl of this IS solution was added to each assay and used to quantify the volume of the organic phase removed after the derivatisation/extraction procedure.

2.3. Biological sample preparation

The preparation of blood plasma, muscle and liver samples consisted of gentle alkaline saponification and derivatisation. The method of measuring MDA in hen and chicken feed samples was based gentle alkaline saponification and derivatisation followed by extractions with hexane. Preparation of urine samples involved only acidification and the derivatisation step.

2.3.1. Preparation and derivatisation of MDA in muscle and liver samples

Homogenized muscles of pigs, chicken and turkeys (95–105 mg) or homogenized pig liver samples (115–120 mg) were saponified with 1 ml of 1 M KOH and 10 μl of 0.02 M BHT in methanol. The mixture was placed in a sealed tube and then in a water bath at 60 °C for 1 h with continuous shaking in the dark. At the end of this procedure, the resulting solution was allowed to cool down and was then acidified with concentrated HCl to approximately pH 2. Then the acidified hydrolysate solution was centrifuged at 15,000 × *g* for 5 min at ~5 °C. To 200 μl of the supernatant, 300 μl of 0.1 M HCl and 50 μl of the DNPH solution were added. The resulting mixture was vigorously mixed and kept at 50 °C for 1 h in the dark. At the end of the derivatisation procedure, if necessary, the sample was allowed to cool down and centrifuged at 15,000 × *g* for 5 min at ~5 °C. The clear solution was transferred to a vial and then 10–45 μl of a resulting solution was injected onto the column for chromatographic analysis.

2.3.2. Determination of free MDA in plasma samples

A blood plasma sample (200 μl) from rats, goats or sheep was cooled down to 1–2 °C and then mixed with 100 μl of a precipitation agent (i.e., acetonitrile or a 20% cooled solution of TCA water, approximately −5 °C). After 10 min of centrifugation at 10,000 × *g*, 200 μl of the supernatant was transferred to a reacti-vial. Next, 20 μl of a DNPH solution was added to the mixture and briefly mixed; the reacti-vial was incubated at 50 °C for 1 h in the dark. The resulting solution was pipetted into a vial for elution. Injection volumes were 40–65 μl.

2.3.3. Determination of total MDA in blood plasma samples

Determination of total (i.e., protein bound and free) MDA in rat, goat or sheep plasma samples was carried out in 2 ml capacity pyrex glass bottles with PTFE-lined screw-on lids. A plasma sample (200 μl) was saponified with 50 μl of 6 M NaOH. The mixture was placed in a sealed tube and then in a water bath at 60 °C for 30 min in the dark [18]. At the end of alkaline saponification, the resulting solution was allowed to cool down to 1–2 °C and was then mixed with 250 μl of a 20% cooled solution of TCA water (approximately −5 °C). After 10 min of centrifugation at 10,000 × *g*, the resulting supernatant (~450 μl) was neutralized to pH 6–7 with 5 μl of 6 M NaOH and then acidified with 10 μl of 1 M HCl to approximately pH 2; if necessary, the processed plasma sample was centrifuged at 15,000 × *g* for 5 min at ~5 °C. Next, to 200 μl of the resulting solution, 300 μl of 1 M HCl and 50 μl of a DNPH solution were added. The solution was vigorously mixed and kept at 50 °C for 1 h in the dark. The resulting solution was pipetted into a vial for elution. Injection volumes were 35–45 μl.

2.3.4. Preparation and derivatisation of MDA in chicken and hen feed samples

Fine powdered chicken and hen feed samples (101–103 mg) were saponified with 1 ml of 1 M KOH and 10 μl of 0.02 M BHT in methanol. The mixture was placed in a sealed tube and then in a water bath at 60 °C for 1 h with continuous shaking in the dark. At the end of this procedure, the resulting solution was allowed to cool down and was then acidified with concentrated HCl to approximately pH 2. PDA (50 μl, as the internal standard) was added to the acidified hydrolysate solution followed by centrifugation at 15,000 × *g* for 5 min at ~5 °C. The DNPH solution (100 μl) and 1 ml of hexanes were added to 1 ml of the supernatant in a reacti-vial. The resulting mixture was vigorously mixed and kept

at 50 °C for 1 h with continuous shaking in the dark. At the end of the derivatisation procedure, the sample was allowed to cool down and centrifuged at 10,000 × *g* for 5 min at ~5 °C. The upper organic phase was transferred to a vial. To avoid any loss of the MDA-DNPH adduct, two successive extractions with hexane (2 × 1 ml) were carried out. All the upper organic layers were combined, and the hexane was removed under a stream of argon at ~30 °C. The residue was dissolved in 200 μl of a 50% (v/v) acetonitrile–water solution. The solution was transferred to a vial, and then 10–50 μl was injected onto the column for analysis.

2.3.5. Preparation and derivatisation of MDA in urine samples and standard solutions of MDA and PDA

To a solution containing 380 μl of ovine, pig or goat urine, 20 μl of concentrated HCl was added. The resulting solution was vigorously agitated for 5 min using a vortex mixer. Next, after 10 min of centrifuging at 15,000 × *g* at the room temperature, the supernatant was transferred to a reacti-vial. Then, 40 μl of DNPH solution was added, and the resulting solution was incubated at 50 °C for 1 h with continuous shaking in the dark. At the end of the incubation, the resulting solution was pipetted into a vial, and then 40–50 μl were injected onto the column for analysis.

The derivatising procedure for the standards of MDA or PDA (as IS) was the same as for the derivatisation of the biological samples. Briefly, to 50 μl of standard solutions of MDA or PDA in 0.1 M HCl, 150 μl 0.1 M HCl and 20 μl of DNPH solution was added, and the resulting solution was incubated at 50 °C for 1 h in the dark. 1–50 μl of derivatised standard solutions of MDA or PDA were injected onto the column for analysis.

Biological materials assayed were replaced by 0.1 M HCl for the blank samples.

2.4. Chromatographic equipment, conditions and gradient composition

A high performance liquid chromatography (HPLC) system (SHIMADZU, Japan), incorporating two LC-20ADXP liquid chromatographs ($UFLC_{XR}$), a SIL-20AC_{XR} auto-sampler ($LFLC_{XR}$), a CBM-20A communications bus module (UFLC), a CTO-20A column oven, a DGU-20A_5 degasser and a SPD photodiode array detector (DAD) was used in this study. A sensitive DAD was equipped with a flow cell of volume 10 μl. The columns utilised were either a Phenomenex C_{18}-column (Synergi 2.5 μm, Hydro-RP, 100 Å, 100 mm × 2 mm) or a Phenomenex C_{18}-column (Synergi 2.5 μm, Hydro-RP, 100 Å, 100 mm × 3 mm). The autosampler thermostat was set to 4 °C. The DAD was operated in the UV range of 195–420 nm. A guard column, Phenomenex C_{18} (5 mm × 2 mm), was placed in front of the analytical columns for protection. A column heater maintained the temperature at 40 °C. The samples were analysed using a linear binary gradient of acetonitrile in water (Table 1). Solvent A consisted of water–acetonitrile (95:5, v/v) and solvent B of acetonitrile.

The MDA-DNPH peak in biological samples was identified on the absorption spectra and by comparing retention times of processed MDA standards that were injected separately and by adding MDA standard solutions to processed biological samples. For determination of MDA levels in processed muscle, liver, urine, plasma and feed samples, the calibration equation was used (Table 2). For determination of MDA levels in processed chicken and hen feed samples, the PDA solution (as IS) was used to quantify the volume of the hexane phase removed after the derivatisation and extraction procedure. The yield of extractions was determined from IS of processed feed samples and IS of processed standard solutions of IS (i.e., as described in Section 2.3.5).

The limit of detection (LOD) was calculated at a signal-to-noise ratio of 3, while the limit of quantification (LOQ) was defined as 10

Table 1
Binary gradient programs used for the analysis of MDA-DNPH in assayed samples.[a]

Time (min)	Flow-rate (ml/min)	Composition (%)	
		Solvent A	Solvent B
Used column: Phenomenex C_{18}-column (Synergi 2.5 μm, Hydro-RP, 100 Å, 100 mm × 2 mm)[b]			
0	0.5	90.0	10.0
13.0	0.5	68.5	31.5
14.0	0.5	5.0	95.0
14.5	1.0	5.0	95.0
17.0[a]	1.2	2.0	98.0
Used column: Phenomenex C_{18}-column (Synergi 2.5 μm, Hydro-RP, 100 Å, 100 mm × 3 mm)[c]			
0	1.10	90.0	10.0
12.0	1.10	90.0	10.0
13.0	1.10	68.5	31.5
14.0	1.20	65.0	35.0
23.0	1.24	64.0	36.0
23.5	1.30	1.0	99.0
26.0[d]	1.4	1.0	99.0

[a] After 17 min, the column was re-equilibrated for 5 min in 90% solvent A (water–acetonitrile, 95:5) and 10% solvent B (acetonitrile) at a flow-rate of 0.5 ml/min; the maximum system pressure was 58 MPa.
[b] The first binary gradient program and the column used for analysis of MDA-DNPH adduct in urine, a pig liver, goat blood plasma samples and thigh muscles of pigs and turkeys. UV detection at 307 nm.
[c] The second binary gradient program and the column used for analysis of MDA-DNPH in chicken and hen feed samples. UV detection at 303 nm.
[d] After 26 min, the column was re-equilibrated for 6 min in 90% solvent A and 10% solvent B at a flow-rate of 1.15 ml/min; the maximum system pressure was 47 MPa.

Table 2
Dependence of the peak area (S_n) corresponding to the derivatised MDA in goat urine samples and the processed MDA standard upon the derivatisation method (i.e., the new and original derivatisation method).[a] The calculated limits of detection (LOD) and quantification (LOQ) derived from the determination of MDA in the processed MDA standard and biological samples[b] with the applied new derivatisation method are also displayed (UV detection at 307 nm).

Item	New derivatisation method	Original derivatisation method [11]
Goat urine sample		
S_n	33,187	3241[c]
LOD	0.037 ng	0.687 ng
LOQ	0.123 ng	2.294 ng
MDA standard		
S_n	15,754,429	790,464
LOD	0.017 ng	0.386 ng
LOQ	0.057 ng	1.285 ng
Linear regression line standard of MDA, correlation coefficient (r)		
y[d] = $4.831 \times 10^{-5} S_n - 0.85$; $r = 0.9995$; SES[e] = 3.9×10^{-7}; SEI[f] = 0.89		

[a] The derivatisation procedures: 0.457 μg of MDA in 100 μl of 0.1 M HCl was added the volume of 10 μl of a solution containing 1 mg (the new derivatisation method) or 0.08 mg (the original derivatisation method) of DNPH in 1 ml of 4 M HCl. The resulting mixture was mixed and reacted at 50 °C (the new derivatisation method) or 37 °C (the original derivatisation method) for 1 h with shaking in the dark. The volume of the DAD flow cell was 10 μl. Column used: Phenomenex C_{18}-column (Synergi 2.5 μm, Hydro-RP, 100 Å, 100 mm × 2 mm).
[b] The LOD and LOQ values for MDA of processed biological samples: pig thigh muscles: 0.019 and 0.063 ng; pig liver samples: 0.043 and 0.143 ng; turkey chest muscles: 0.020 and 0.067 ng. The LOD and LOQ for the total MDA of goat blood plasma: 0.018 and 0.060 ng, respectively.
[c] The injection volume: 50 μl.
[d] S_n, y are the peak areas and MDA amounts (ng) in processed MDA standards injected onto the HPLC column, respectively. The linear regression line was determined using the least-squares method. This regression equation was determined from two repetitions. The number of MDA standards analysed: 5; individual concentrations of MDA in standard solutions: 0 (a blank sample), 10.2, 20.4, 51.0, 102.1 and 153.4 ng MDA. Applied new derivatisation procedure of MDA standards is described in Section 2.3.5.
[e] The standard error in slope.
[f] The standard error in the intercept.

times the average noise level [21,22]. The average noise level was calculated from heights of noises on both sides of a MDA-DNPH peak in three MDA standards and assayed biological samples (from three injections) [22].

2.5. Analysis of the purity of the MDA-DNPH peak in processed biological samples

The purity of the MDA-DNPH peak was determined using our previously described method [22]. Briefly, the purity ($P_{\%}$) of the MDA-DNPH peak in assayed samples was assessed by determining the relationships between the monitoring wavelength (λ_{nm}) and the ratios of the MDA-DNPH peak areas (S_{sample}) in the biological samples (R_{sample}) and comparing them to the MDA-DNPH peak area ($S_{standard}$) in the processed MDA standards ($R_{standard}$), i.e., $P_{\%} = (R_{sample}/R_{standard}) \times 100\%$. The values of $R_{standard}$ and R_{sample} were calculated using the relationship between the MDA-DNPH peak area in standards and biological samples ($S_{max\text{-}standard}$ and $S_{max\text{-}sample}$, respectively) monitored at the maximum absorbance wavelength of the MDA-DNPH adduct (i.e., at 307 or 303 nm), and the MDA-DNPH peak areas in the standard ($S_{standard}$) and biological samples (S_{sample}) were obtained at wavelengths ranging from 200 to 360 nm (i.e., $R_{standard} = S_{standard}/S_{max\text{-}standard}$; $R_{sample} = S_{sample}/S_{max\text{-}sample}$).

Moreover, the purity of the MDA-DNPH peak in standards and all assayed biological samples was determined using SHIMADZU LC workstation "LCsolution" software (SHIMADZU 2008; Japan). Similarly, the purity of DNPH derivatives of PDA in standards and hen or chicken feed samples was determined using our previously described method [22] as well as SHIMADZU LC workstation "LCsolution" software (SHIMADZU 2008; Japan).

3. Results and discussion

3.1. Method development, matrix effects and interferences

To avoid the problems caused by the overlap of the MDA-DNPH peak and endogenous species present in the assayed biological materials, MDA was derivatised prior to RP-HPLC separation to obtain a chromophore species with an aromatic ring of high molar absorption at longer UV wavelengths. Fortunately, DNPH converted MDA to a derivative with two wide UV bands in the spectral ranges 200–250 nm and 260–350 nm; these bands were visible because of the presence of the aromatic ring and lone electron pairs. High molar absorptivity and the occurrence of the absorption maxima close to 215 ± 1 nm and 305 ± 3 nm make this derivative almost ideally suited for analysis using liquid chromatography with UV detection. Moreover, derivatised MDA was substantially retained on the C_{18}-column packed with a strongly hydrophobic bonded phase. The MDA-DNPH adduct peak was eluted at 9.9 ± 0.1 min using both binary gradient elution programs (Table 1).

It was determined that derivatisation of MDA in standards and assayed biological materials with a solution containing 1 mg of DNPH in 1 ml of 4 M HCl (the new derivatisation method) at a higher incubation temperature (i.e., 50 °C) permitted a higher yield of the MDA-DNPH adduct (Table 2) compared to the previous derivatisation method [11].

As can be seen from results summarised in Table 2, the LOD and LOQ values for MDA of urine samples and MDA standards were lower for our new derivatisation method than for the original derivatisation method [11]. Moreover, the LOD and LOQ values for MDA of muscle, liver and plasma samples were similar to the LOD and LOQ for MDA of urine samples when our new derivatisation method was used.

The new derivatisation method assured a satisfactory peak shape for the MDA-DNPH adduct, which was close to symmetrical and exhibited excellent baseline stability (Fig. 1). Conversely, a higher concentration of DNPH in 4 M HCl, especially above 2 mg DNPH per 1 ml of 4 M HCl, resulted in the formation of an unsymmetrical MDA-DNPH peak and higher noise on both sides of the MDA-DNPH peak. Moreover, higher concentrations of DNPH in 4 M HCl caused a relatively small increase (approximately 11%) in the formation yield of the MDA-DNPH adduct in processed samples compared with the derivatising reagent that contained 1 mg of DNPH in 1 ml of 4 M HCl.

As can be seen in Fig. 1A, the optimised processing procedure and the binary linear gradient system (Table 1) was composed of water in acetonitrile, which provided a wide range of solvent strengths and an excellent baseline stability. The MDA-DNPH adduct can be well separated using Phenomenex C_{18}-columns (Synergi 2.5 μm, Hydro-RP, 100 Å, a length of 100 mm) with inner diameters of 2 or 3 mm and a selective photodiode detector at longer UV wavelengths (i.e., above 300 nm). On the other hand, monitoring at short UV wavelengths (~215 nm) resulted in higher background fluctuation and noise interference, especially for biological materials, compared with a more selective detection at 307 or 303 nm. Moreover, in biological samples, the close presence of the peaks of unidentified species could seriously interfere with the accurate and precise integration of the MDA-DNPH peak while using the detection at short wavelengths (i.e., below 220 nm). As expected, the peak of the MDA-DNPH adduct was absent in the blank sample when the proposed gradient elution program and photodiode detection was set to a UV range from 200 to 350 nm. Typical chromatograms showing elution profiles of DNPH derivatives of MDA and other peaks corresponding to unidentified endogenous components, which are normally present in assayed biological samples, are shown in Fig. 1B–F.

Fig. 1B shows the typical chromatograms at 307 nm for free MDA in goat plasma samples after protein precipitation with a 20% solution of TCA water followed by derivatisation with DNPH. Protein precipitation with acetonitrile followed by derivatisation of plasma MDA with DNPH did not results in variation in the MDA concentration in goat, sheep or rats of plasma in comparison with precipitation with TCA followed by derivatisation with DNPH. Thus, the present study documented that acetonitrile or TCA can be used for determination of free MDA in blood plasma. Moreover, experiments showed that our chromatographic method offered satisfactory linearity of free MDA quantification in goat blood plasma ($r = 0.9998$; the range of the content of MDA in processed five plasma samples: 0.23–1.89 ng).

Saponification in 1 M KOH or 6 M NaOH solutions made it possible to remove MDA from muscle, liver, blood plasma and feed samples. Indeed, a significant amount of MDA can be bound to matrices in biomolecules; therefore, strongly basic sample *pre*-treatments can be used to hydrolyse the bound fraction of the molecule allowing a quantification of total MDA (i.e., protein bound and free MDA) in muscle, liver, blood plasma and selected feed samples [2,17,18]. As is shown in Fig. 1, the concentration of free MDA in plasma (Fig. 1B) is considerably lower than the concentration of total MDA in plasma (Fig. 1C). Indeed, the total MDA is the sum of protein bound MDA and free MDA.

Typical chromatograms at 307 nm for processed pig thigh muscles and pig liver samples are shown in Fig. 1D and E. Detailed analysis of chromatograms revealed that the analytical conditions used (alkaline saponification of protein bound MDA [17,18]), the homogenate centrifugation and derivatisation yielded clear reaction solutions and therefore no further extractions were necessary [18]. The employment of the first elution program and a Phenomenex C_{18}-column with an inner diameter of 2 mm (Table 1) and photodiode detection at the maximum absorbance wavelength of

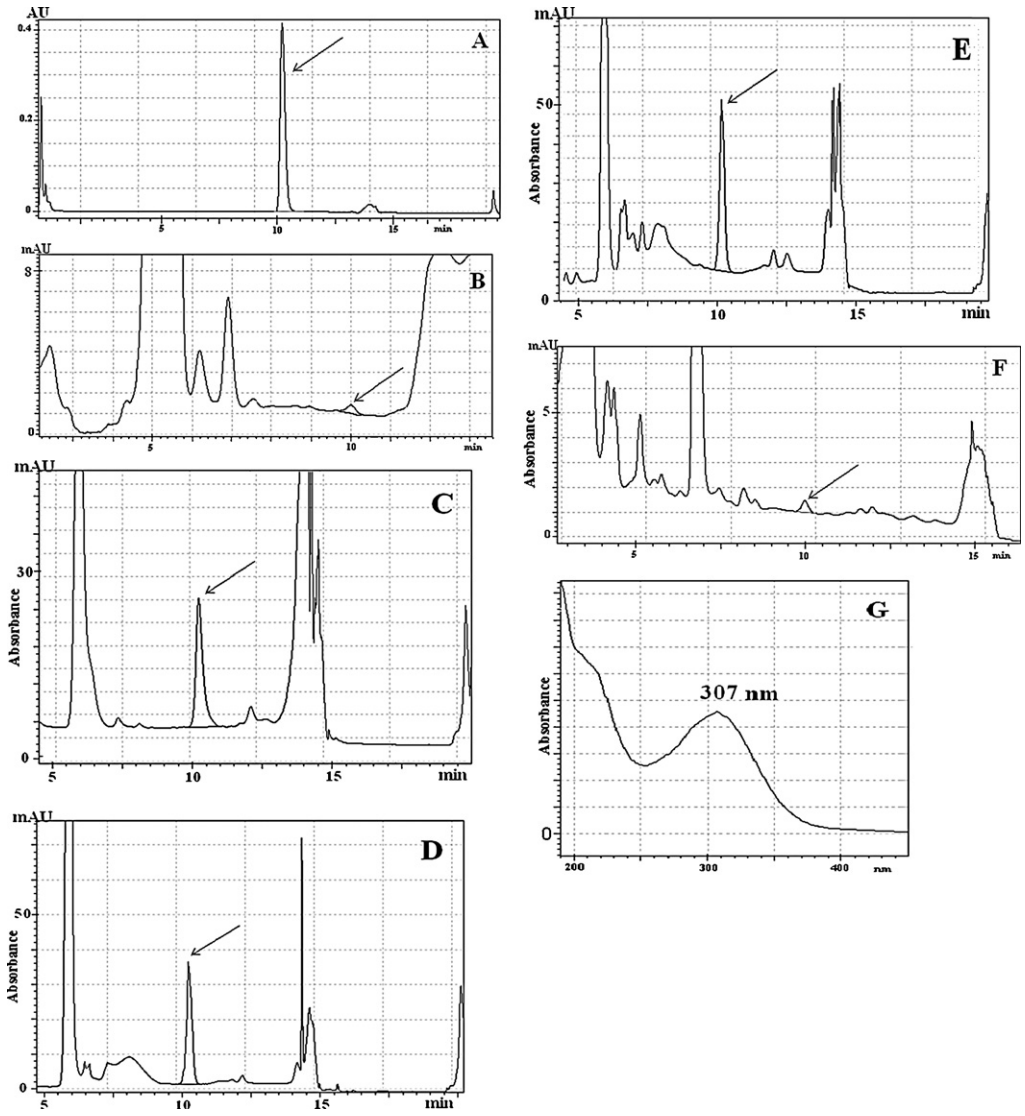

Fig. 1. Typical chromatograms for (A) an MDA standard solution (0.435 ng/ml; injection volume: 10 μl), (B) the free MDA in goat blood plasma, (C) the total MDA in goat blood plasma, (D) pig femoral muscles, (E) the pig liver, (F) ovine urine, (G) typical UV spectrum of derivatised MDA standard. AU – the absorbance unit. Arrows indicate the elution time for the MDA-DNPH adduct. All samples were immediately analysed after processing. Injection volumes of processed biological samples were 35–50 μl.

the MDA-DNPH adduct (i.e., at 307 nm; Fig. 1G) allows the very good chromatographic separation of a MDA-DNPH adduct from other endogenous species present in plasma, muscles and liver samples.

Fig. 1F shows the chromatogram of processed ovine urine samples; ovine or goat urine samples were derivatised with DNPH in acidic pH (final concentration of HCl in the assay ~0.55 M). Therefore, the procedure used in this paper determines the total amount of MDA present in urine [11]. Similarly, Exley et al. successfully applied derivatisation of MDA with DNPH in acidic pH together with RP-HPLC equipped with DAD to quantify the total amount of MDA in urine samples [11].

Fig. 2A and B shows the chromatograms at 303 and 361 nm of hen feed samples after alkaline saponification and derivatisation followed by extractions with hexane. To avoid the problems caused by the overlapping of many peaks corresponding to endogenous components present in chicken and hen feed samples, three extractions with hexane were conducted. Indeed, due to complexity of a matrix of processed feed samples, the MDA-DNPH adduct must be extracted. The extraction of the MDA-DNPH adduct with hexane significantly reduces background fluctuations on both sides of the MDA-DNPH peak (Fig. 2A) compared the processed feed samples without extractions. Really, matrix effects in the processed hen feed samples without extractions with hexane were responsible for the very poor purity of the MDA-DNPH peak ($P_{\%} < 45\%$) in the shorter ($\lambda < 270$ nm) and longer ($\lambda > 330$ nm) UV ranges.

An IS solution was added after the acidification of the feed hydrolysates to quantify the volume of the hexane phase removed after the derivatisation and extraction procedure (Fig. 2B). PDA was used as the IS in chicken and hen feed samples; PDA was not found in all processed feed samples. The PDA-DNPH adduct peak was eluted at 20.9 ± 0.1 min using a Phenomenex C_{18}-column (Synergi 2.5 μm, Hydro-RP, 100 Å, 100 mm × 3 mm) and the second binary elution program (Table 1). Moreover, no interfering species were found using our chromatographic method and photodiode detection at the maximum absorbance wavelength of the MDA-DNPH adduct (i.e., at 303 nm; Fig. 2A) and the PDA-DNPH adduct (IS) (i.e., 361 nm; Fig. 2B and C).

Purities of MDA-DNPH and PDA-DNPH peaks in hen feed samples are shown in Table 3, as the noise interferences and background fluctuations in hen feed samples were higher than in chicken feed samples. Indeed, the hen feed samples were stored at 5–6 °C for 6 months, while the chicken feed samples were stored at 5–6 °C

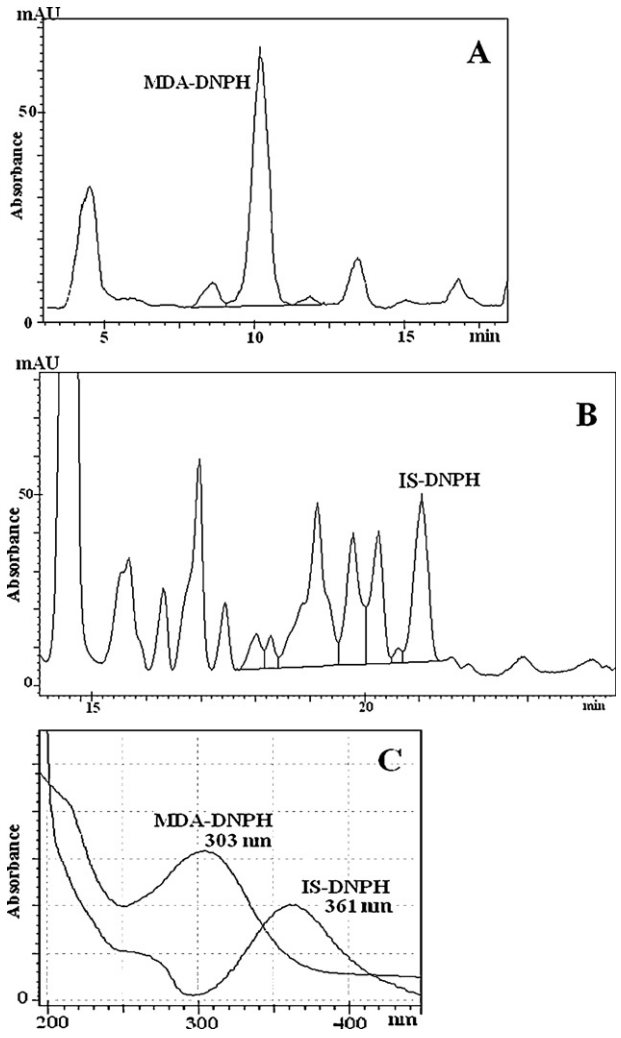

Fig. 2. Typical chromatograms for the feed sample spiked with 1,5-pentanedialdehyde (PDA) as the internal standard (IS). A hen feed sample: UV detection at 303 nm (A) and 361 nm (B). MDA indicates the elution time for the MDA-DNPH adduct. (C) Typical UV spectra of the MDA-DNPH and IS-DNPH adducts and their absorbance maxima. Feed samples were immediately analysed after processing using Phenomenex C_{18}-column (Synergi 2.5 μm, Hydro-RP, 100 Å, 100 mm × 3 mm). Injection volumes of processed samples were 40 μl.

Table 3
The purity ($P_{\%}$) of the MDA-DNPH peak and PDA-DNPH peak in hen feed samples[a] calculated from the relationships between the monitored wavelength and the ratios of the MDA-DNPH or PDA-DNPH peak areas in feed samples to the ratios the MDA-DNPH or PDA-DNPH peak areas in processed standards of MDA or PDA (IS), respectively [22].

Monitoring wavelength (nm)	Purity of the MDA-DNPH peak[b] (%)	Monitoring wavelength (nm)	Purity of the PDA-DNPH peak (%)
200	93.57	–	–
205	93.76	210	11.28
210	94.70	220	14.48
215	94.94	230	3.55
225	92.79	240	13.10
235	87.87	250	73.60
245	84.64	260	98.42
255	85.98	270	98.18
265	87.61	280	99.85
275	90.15	290	96.19
280	91.73	300	94.90
285	93.73	310	98.62
290	96.56	320	96.76
293	98.13	330	94.35
296	99.10	340	96.80
298	99.70	345	95.81
300	99.92	350	94.53
301	99.90	355	98.61
302	99.95	359	96.82
λ_{max}: 303[c]	100.00	λ_{max}: 361[d]	100.00
304	99.92	363	96.45
305	100.00	368	96.74
306	99.93	372	99.73
308	99.77	380	94.60
310	99.47	390	94.40
313	99.04	400	94.21
316	98.56	410	96.82
321	97.36	420	98.16
335	88.15	430	98.02
345	71.52	440	98.75
355	31.61	450	96.42
360	4.13	–	–

[a] The hen feed samples were stored at 5–6 °C for 6 months.
[b] The MDA concentration in a processed hen feed sample: 0.41 μg MDA/g of hen feed; the injection volume: 40 μl; the second binary program was used for the analyses of MDA-DNPH and PDA-DNPH in all feed samples. The LOD and LOQ for MDA of processed hen feed samples: 0.317 and 1.057 ng, respectively.
[c] The absorption maximum of the MDA-DNPH adduct in feed samples (i.e., at 303 nm).
[d] The absorption maximum of the PDA-DNPH adduct in feed samples (i.e., at 361 nm).

for 1 week. The results summarised in Table 3 indicate that the MDA-DNPH peak for the hen feed samples was pure and free from the influence of closely located signals of unidentified species at wavelengths ranging from 200 to 335 nm. Difficulties in the accurate integration of the MDA-DNPH peak in hen feed samples in the longer (λ > 335 nm) UV ranges were responsible for the poor purity of the MDA-DNPH peak. Indeed, the purity of the MDA-DNPH peak in hen or chicken feed samples assessed using LC workstation "LCsolution" software (SHIMADZU) was also satisfactory as the peak purity index was of 1.000000 (i.e., the MDA-DNPH peak is without impurities). There was no disturbing spectral noises on both sides of the PDA-DNPH peak in the assayed chicken and hen feed samples stored at 4 °C in the autosampler for 48 h. Also, there was no loss of PDA-DNPH adduct concentration in the processed feed samples kept in the autosampler at 4 °C for 48 h.

The results summarised in Table 3 indicate that the purity of the PDA-DNPH peak in hen feed samples determined using our method [22] was satisfactory in the UV wavelength range from 260 to 450 nm (97 ± 3%). Similarly, the purity of the PDA-DNPH peak in all assayed feed samples assessed using LC workstation "LCsolution" software (SHIMADZU) was satisfactory as the peak purity index was of 1.000000 (i.e., the PDA-DNPH peak is without impurities). In addition, there were no noticeable changes in the purity of the IS peak in all assayed feed samples kept in the autosampler at 4 °C for 48 h.

Surprisingly, the addition of the IS solution to the assayed feed samples before alkaline hydrolysis led to a nearly complete decomposition of PDA (i.e., IS) in the processed hen and chicken feed samples. PDA can easily polymerise by the aldol condensation reaction, which yields alpha, beta-unsaturated polyglutaraldehyde; this reaction can especially occur at alkaline pH values [23]. Therefore, the PDA peak was not detectable in the processed feed samples when the DPA solution was added before alkaline hydrolysis.

Concentrations of MDA in chicken and hen feed samples were significantly higher compared to concentrations of MDA in urine and especially in blood plasma samples (Tables 3–5). Therefore, the Phenomenex C_{18}-column with an inner diameter of 3 mm (Synergi 2.5 μm, Hydro-RP, 100 Å, 100 mm × 3 mm (Table 1)) should preferentially be used for the elution of the MDA-DNPH adduct. This C_{18}-column assured a lower chromatographic system pressure and a satisfactory measurement of sensitivity for the detection of the MDA-DNPH adduct in all assayed feed samples. Indeed, amounts

Table 4
The purity ($P_{\%}$) of the MDA-DNPH peak in assayed biological samples calculated from the relationships between the monitored wavelength and the ratios of the MDA-DNPH peak areas in biological samples to the ratios the MDA-DNPH peak areas in processed MDA standards [22].

Monitoring wavelength (nm)	Purity of the MDA-DNPH peak (%)						
	Pig thigh muscles	Pig liver	Turkey thigh muscles	Hen chest muscles	Total MDA in goat plasma[a]	Free MDA in goat plasma[b]	Ovine urine[c]
200	93.79	82.79	73.57	89.76	95.99	2.04	97.23
205	96.84	88.88	80.03	92.50	91.05	1.23	97.60
210	95.16	89.79	81.31	90.83	89.14	4.66	97.33
215	96.58	92.47	83.99	90.26	91.50	21.79	96.66
225	98.56	95.42	87.24	92.61	96.44	20.52	95.83
235	97.80	95.92	93.15	91.79	97.98	26.93	94.73
245	97.92	96.86	95.48	91.91	96.28	25.78	94.06
255	98.85	97.97	96.83	92.80	95.27	28.94	93.60
265	99.29	98.12	97.24	94.08	95.30	31.05	94.53
275	98.28	97.17	96.21	94.45	96.73	39.31	96.44
280	97.81	96.82	95.88	94.71	97.96	50.79	97.07
285	97.62	96.79	95.94	95.41	99.26	58.42	98.37
290	97.84	97.19	96.45	96.51	99.85	74.82	99.04
293	98.20	97.63	97.00	97.31	99.42	79.05	99.34
296	98.74	98.22	98.36	98.14	99.37	86.91	99.72
298	99.07	99.00	98.77	98.61	99.40	98.32	99.82
301	99.43	99.39	99.40	99.11	99.56	95.24	99.89
303	99.67	99.64	99.67	99.41	99.66	98.97	99.91
305	99.77	99.75	99.79	99.54	99.72	99.62	99.95
λ_{max}: 307[d]	100.00	100.00	100.00	100.00	100.00	100.00	100.00
309	99.79	99.78	99.77	99.76	99.85	94.85	99.93
311	99.71	99.72	99.70	99.73	99.94	95.01	99.91
314	99.51	99.36	99.49	99.55	99.58	93.28	99.79
316	99.24	99.25	99.22	99.34	99.47	88.51	99.37
318	98.99	99.04	99.01	99.20	99.22	86.34	99.58
320	98.70	98.77	98.60	99.07	98.76	70.38	99.68
321	98.48	98.62	98.51	99.17	98.21	73.96	99.75
325	98.28	98.52	98.53	99.78	97.19	68.67	99.18
335	97.92	98.69	99.63	97.08	92.08	23.54	96.26
345	95.82	97.34	99.39	93.82	83.78	12.83	92.85
355	92.12	95.50	96.47	88.12	68.92	14.39	88.63
360	89.68	94.74	93.34	83.59	58.58	–[e]	86.49

[a] The injection volume: 45 μl.
[b] The free MDA concentration: 12.6 ng MDA/ml of goat plasma; the injection volume: 50 μl.
[c] For 35 days a male Polish Merino lamb with a final body weight of 30.6 kg was fed *ad libitum* the standard concentrate-hay diet enriched in 5% linseed oil. The injection volume of urine samples: 40 μl.
[d] The wavelength for absorbance maximum of the MDA-DNPH adduct in muscles, liver, plasma and urine samples (the first binary program was used for the analyses of MDA-DNPH in assayed samples). Injection volumes of muscle and liver samples: 35 μl.
[e] The MDA-DNPH peak – not detected.

of MDA in feed samples were ~200 times greater than the LOQ for the processed MDA standards (Table 2).

3.2. Reliability of the HPLC method

The main analytical problem in the present work was to obtain the highest yield of derivatised MDA in all assayed samples and accomplish the extraction of the MDA-DNPH adduct formed in processed muscle, liver and feed samples. Based on the systematic optimisation of the conditions of the derivatisation reaction, the yield of the MDA derivative was found to increase with the increasing temperature of the derivatisation of MDA with DNPH. The highest values of the areas of MDA-DNPH peaks were observed when the derivatisation was performed at 50 °C for 1 h and was very similar at 60 °C. Moreover, at temperatures higher than 50 °C, background levels increased on both sides of the MDA-DNPH adduct, especially in processed samples of blood, urine, muscle and feed. The experiments indicated that the areas of the MDA-DNPH peaks were practically equal if processed MDA standards or assayed biological samples were protected from light and stored for 92 h in the autosampler at 4 °C. Considering the above results, it was determined that the derivatisation performed at 50 °C proved to be a suitable processing procedure for the routine chromatographic determination of MDA in the liver, meat, feed, blood plasma and urine samples.

Another important analytical problem addressed in the present work was to develop a suitable separation technique for the MDA-DNPH adduct to avoid the interference of endogenous substances present in assayed biological materials. Therefore, efficiency of our proposed chromatographic separations was assessed by examining the purity of the MDA-DNPH peak in assayed biological samples. The results summarised in Table 4 indicate that the MDA-DNPH peak for the liver, muscle and urine samples was pure and free from the influence of closely located signals of unidentified species

Table 5
Recoveries (R, %, mean ± SD)[a] of the MDA standard added to processed biological samples.

Assayed sample	Number of replicates	Added MDA standard (ng)	R (%)
Ovine urine[b]	5	4.31	97.8 ± 3.4
Goat plasma[b,c]	6	70.90	98.7 ± 4.7
Pig thigh muscles[b]	3	35.45	96.4 ± 2.9
	7	70.90	97.3 ± 2.1

[a] Recovery was calculated as: $R\,(\%) = ({}^{1}S_n - {}^{0}S_n) \times 100\%/S$, where ${}^{0}S_n$ and ${}^{1}S_n$ are measurement before and after addition of the MDA standard and S is the amount of added MDA standard.
[b] The concentration of MDA in ovine urine, goat plasma and pig thigh muscle samples: 4.5 ng/ml, 57.3 ng/ml and 1.0 μg/g, respectively. Injection volumes of urine, plasma and muscle samples: 40, 45 and 35 μl, respectively.
[c] The total MDA analysis in goat blood plasma samples.

Table 6
Intra ([intra]C.V., %) and inter ([inter]C.V., %) coefficients of variations derived from the determination of MDA in a MDA standard[a] and biological samples.

Assayed sample	[intra]C.V. (%)[b]	[inter]C.V. (%)[c]
MDA standard	0.87[b]	1.27[b]
Ovine urine[d]	2.09[b]	2.84[b]
Hen feed[d]	1.63[b]	3.47[b]
Free MDA in ovine plasma[e]	2.91[b]	3.57[b]
Free MDA in goat plasma[e]	3.15[b]	3.94[b]
Total MDA in goat plasma[e]	1.72[c]	2.05[c]
Pig thigh muscles[f]	1.39[c]	1.89[c]

[a] The concentration of MDA – 4.57 μg/ml; injection volumes: 20 μl.
[b] [intra]C.V. – values based on three samples, each with three or four injections; [inter]C.V. – values based on three samples repeated three times (processing and injection).
[c] [intra]C.V. – values based on four samples, each with three or four injections; [inter]C.V. – values based on three samples repeated four times (processing and injection).
[d] Injection volumes: 40 μl.
[e] Injection volumes: 45 μl.
[f] Injection volumes: 35 μl.

at wavelengths ranging from 200 to 360 nm. Similarly, when the total MDA was analysed in blood plasma, the purity of the MDA-DNPH peak was satisfactory in the UV wavelength range from 200 to 345 nm. Moreover, the purity of the MDA-DNPH peak in these processed biological samples assessed using LC workstation "LCsolution" software (SHIMADZU) was satisfactory as the peak purity index was of 1.000000 (i.e., the MDA-DNPH peak is without impurities).

Conversely, when using the shorter or longer wavelength ranges, the background fluctuation or the close presence of some species' peaks interfered with the accuracy of the free-MDA-DNPH peak in blood samples. Indeed, difficulties in the accurate integration of the small free-MDA-DNPH peak in plasma samples in the shorter ($\lambda < 293$ nm) and longer ($\lambda > 318$ nm) UV ranges were responsible for the poor purity of the free-MDA-DNPH peak. Therefore in this case, the purity ($P_{\%}$) of this peak was significantly less than 100%.

The reliability of the current method was assessed by the addition of an MDA standard solution to the processed ovine urine, goat plasma and pig thigh muscle samples. The results summarised in Table 5 indicate that the recoveries (R, %) of added MDA standards to the processed urine, muscle and plasma samples were satisfactory for detection at 307 nm. The stability of the MDA-DNPH adduct in these biological samples was tested by reinjecting the processed spiked samples 24 h later after they had been stored in the dark at 4 °C. There were no noticeable changes in the peak areas in all spiked samples, which indicates that the MDA-DNPH adduct is stable and background fluctuations and noise interferences are invariable for 24 h when spiked biological samples stored in dark at 4 °C for 24 h.

Reproducibility of the present method was also assessed by performing replicate injections of processed MDA standards, urine, hen feed, plasma and pig thigh muscle samples (the intra-assay coefficients of variations; [intra]C.V., %). Moreover, the inter-assay coefficients of variations ([inter]C.V., %) were calculated by using replicate processing aliquots of an MDA standard and these biological samples (Table 6). Low values of the intra- and inter-assay C.V. as well as the LOD and LOQ values (Table 2) for MDA of biological samples demonstrated the satisfactory precision and sensitivity of the proposed procedure, regardless of the use of an IS in assayed feed samples.

4. Conclusion

In this paper, the improved method for the quantification of MDA as hydrazone in biological materials is presented based on the optimised derivatisation reaction using high performance liquid chromatography and a sensitive photodiode detector equipped with 10 μl of a flow cell. The current method allowed a precise, accurate and selective determination of MDA in the liver, muscles, blood plasma and urine of animals as well as in selected feed samples. Because MDA of urine or blood plasma samples were simply derivatised, the proposed method can be routinely used as a non-invasive evaluation of oxidative stress in animals and humans. The current method was based on the use of widely available C_{18}-columns, the high-efficient derivatisation of MDA and the selective and sensitive photodetection of the column effluent; this method can be suitable for routine analysis of MDA in tissues of animals and selected feed samples. The proposed method was more sensitive compared to previously used methods.

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