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Determination of Lipid Oxidation Level in Broiler Meat by Liquid Chromatography

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An assay was conducted for the determination of malondialdehyde (MDA) levels in broiler meat. The method involves extraction of tissues with trichloroacetic acid (TCA) and reaction of the TCA extract with 2,4-dinitrophenylhydrazine (DNPH). After separation of the MDA-DNPH complex using a solid-phase extraction C18 column, samples were eluted with 1 mL acetonitrile. Aliquots of 20 μL acetonitrile were analyzed by liquid chromatography on reversed-phase C18 column (3 μm) with UV detection. The products were eluted isocratically with the mobile phase containing acetonitrile–water–acetic acid (39 + 61 + 0.2, v/v/v). The retention time for MDA-DNPH was 6.5 min, and the detection limit was 3.5 μg/kg. Two extraction methods (cold and hot) were also used in the study. The results showed that hot extraction increased results about 55.8% and recovery from samples spiked with 116.6 μg/kg was lower (74.6%) in comparison with cold extraction (94.7%).

It is well known that lipid oxidation is one of the major causes of lipid-rich food deterioration. The oxidative deterioration of the polyunsaturated lipids of foods leads through formation of hydroperoxides to short-chain aldehydes, ketones, and other oxygenated compounds, which are considered to be responsible for development of rancidity in stored foods (1–3).

Quantification of hydroperoxides is difficult because of the unstable and reactive nature of these compounds (4, 5). Thus, malondialdehyde (MDA), a major degradation product of lipid oxidation, has attracted much attention as a marker for assessing the extent of lipid oxidation (6, 7). The compound is of particular concern as it has been shown to be mutagenic and carcinogenic (8, 9).

Nowadays, the most frequently used method for MDA determination in foods of animal origin is thiobarbituric value estimation (TBA test; 10). This test is simple and fast, and is based on the reaction of 1 molecule MDA with 2 molecules TBA (11). The color of the final complex is pink, and the absorbance of the complex is measured spectrophotometrically (12–15) or spectrofluorometrically (16, 17). However, the test is not only specific for MDA determination but also for other constituents of meat-like proteins, DNA, and ribose (10, 18), which produce the same color complex as the reaction between MDA and TBA. High temperatures used for TBA estimation, for example distillation, support oxidation and produce additional MDA and other TBA-reactive substances (TBARS). This process leads to overestimation of MDA content and decreases specificity of analysis. Most of the MDA occurring in food exists in bound form with –SH and/or –NH$_2$ groups (19–21); however, the free form of MDA occurs very rarely. Thus, acid must be added to the food to be analyzed in order to liberate the MDA.

Liquid chromatography (LC) has been used successfully to separate and quantitate the TBA–MDA complex (13, 22). The method is very sensitive and specific, but hot acid conditions are still necessary to liberate the MDA. Quantitation of MDA–TBA measured by LC gives a 2-fold higher value than those obtained from the MDA-2,4-dinitrophenyhydrazine (DNPH) analyzed by LC with UV detection (5).

Derivatization of MDA with 2,4-dinitrophenyhydrazine produces DNPH derivatives, which are rapid, simple, and quantitatively detected by UV detection. The other advantage is excellent elution and separation of DNPH derivatives on LC column (21, 23).

The purpose of our experiment was to develop and evaluate a convenient method for accurate MDA estimation in poultry meat after derivatization with DNPH. The influence of hot and cold extraction on the accuracy of MDA determination in food was also compared.

Experimental

Apparatus

(**a**) *Refrigerated centrifuge*.—Jouan (St. Herblain Cedex, France).

(**b**) *Microwave*.—DeLongi (DeLongi, Italy).

(**c**) *Homogenizer*.—CAV (Brno, The Czech Republic).

(**d**) *Water bath*.—Kavalier (Votice, The Czech Republic).

(**e**) *Solid-phase extraction (SPE) column*.—3 mL Supelcleen LC-18 column (Supelco, Bellefonte, PA).

(**f**) *LC column*.—3 μm, 125 × 3 mm Nucleosil C$_{18}$ reversed-phase (Mancherey Nagel, Düren, Germany).

Reagents

(**a**) *Solvents*.—Methanol, acetonitrile, hexane (Merck, Darmstadt, Germany).

(**b**) *Chemicals*.—Hydrochloric acid (HCl), trichloroacetic acid (TCA), acetic acid (Lachema CZ, Brno, The Czech Republic); DNPH (Fluka, Buchs, Switzerland); butylated hydroxytoluene (BHT) and ethylene diamine tetraacetic acid disodium salt (EDTA; Sigma, St. Louis, MO).

(**c**) *Water*.—Deionized.

(**d**) *LC mobile phase*.—Acetonitrile–water acetic acid (39 + 66 + 0.2, v/v/v) in an isocratic system.

(**e**) *Extraction solvent*.—TCA.

(**f**) *BHT solution*.—500 ppm in methanol (hot extraction).

(**g**) *BHT solution*.—0.8% in hexane (cold extraction).

(**h**) *EDTA solution*.—0.3% in water.

(**i**) *TCA solution*.—10% in water (hot extraction).

(**j**) *TCA solution*.—5% in water (cold extraction).

(**k**) *DNPH solution*.—31 mg DNPH in 10 mL 2M HCl.

Standard Solutions

(**a**) *MDA stock solution, 4.37 μg/mL*.—Prepared by acid hydrolysis of 10 μL 1,1,3,3-tetramethoxypropane (TMP; Sigma, Steinheim, Germany) in 10 mL 0.1M HCl in boiling water bath for 5 min. The solution was quickly cooled with tap water. MDA stock solution was prepared by pipeting 1.0 mL hydrolyzed acetal into 100 mL volumetric flask, diluted to volume with 0.1M HCl, and stored at 4°C. The solution remains stable for 1 week.

(**b**) *MDA working solutions*.—Resulting MDA stock solution (4.37 μg/mL) was further diluted with water to yield final concentrations of 4.37, 8.74, 17.5, 87.4, 437, and 1000 ng/mL. Fresh solutions were prepared daily.

Sample Preparation

The thighs of broilers obtained from Hydina ZK Košice (The Slovak Republic) were frozen at –21°C. After 3 months of storage at –21°C, the thighs were thawed in a microwave oven (900 W) for 4 min. Skins and bones were removed by hand and thighs were ground to 4.5 mm particle size.

Cold Sample Extraction (Procedure A)

The cold extraction of samples was performed according to Grau et al. (24) with some modifications. A ground sample of 1.5 g was weighed in a 50 mL centrifuge tube, and 1 mL 0.3% aqueous EDTA was added immediately. After gentle agitation, 5 mL 0.8% BHT in hexane was also added, and the tube was gently shaken again. Just before homogenization, 8 mL ice-cold 5% TCA was added to the tube and homogenization was carried out for 30 s at maximum speed. After centrifugation (5 min at 3500 × g, 4°C), the top hexane layer was discarded and the bottom layer was filtered through Whatman filter paper No. 4 into a 10 mL volumetric flask and diluted to volume with 5% TCA.

Hot Sample Extraction (Procedure B)

The hot extraction of samples was performed according to Draper et al. (13) with some modifications. A 1 g portion of the ground sample was weighed into the test tube and 0.5 mL 500 ppm BHT in methanol and 5 mL 10% TCA were added. After 30 min of incubation in a water bath (85°C), the samples were chilled and centrifuged at 3500 × g for 5 min. The supernatant was filtered through Whatman filter paper No. 4 into a 10 mL volumetric flask and diluted to volume with 10% TCA.

Derivatization

For derivatization, 100 μL DNPH reagent (3.13 μM) was added to 2 mL TCA extract (Procedure A or B) in a 12 mL test tube. Samples were mixed and incubated for 30 min at room temperature in the dark.

Solid-Phase Extraction

The 3 mL Supelcleen LC-18 columns were activated by washing with 2 mL acetonitrile and 2 mL water. The samples were slowly passed through the columns and washed with 2 mL water. MDA–DNPH complex was eluted with 1 mL acetonitrile.

Liquid Chromatographic Analysis

LC analysis of samples and standards was performed according to the conditions described by Pilz et al. (20) with slight modifications. A Hewlett-Packard Series 1050 liquid chromatograph equipped with quaternary autosampler, variable wavelength detector operated at 307 nm, and an integrator HP 339 6II was used. The column Nucleosil C18 reversed-phase (3 μm, 125 × 3 mm) was used. The flow rate was maintained at 1.0 mL/min. The operation was isocratic with mobile phase of acetonitrile–water–acetic acid (39 + 61 + 0.2, v/v). Both samples and standards were injected into the mobile phase. The volume injected was 20 μL. For determination of recoveries, MDA standards were added to the meat samples.

Calibration Curve

The MDA was quantified by using an external calibration curve in the concentration range from 5.8 to 1350 μg/kg. Each point of the calibration curve was an average value of 3 measurements.

Results and Discusssion

Sample Extraction Conditions

MDA amounts found in broiler chicken stored for 3 months at refrigeration conditions and thawed in a microwave oven are shown in Table 1. TCA was used for extraction of MDA from samples. The MDA amount derived by cold extraction with 5% TCA was 75 ± 6 μg/kg. Hot extraction of the samples with 10% TCA produced 135 ± 47 μg/kg MDA. Comparison of the results shows that MDA derived by hot extraction was about 55.8%, which is

Table 1. MDA concentration in poultry meat after different ways of extraction

Extraction procedure	Mean ± SD[a] MDA, μg/kg	RSD, %
Cold	75.0 ± 5.3	7.0
Hot	135.0 ± 17.0	12.6

[a] SD = Standard deviation.

higher than the amount obtained from cold extraction ($p < 0.05$).

Recovery

The recoveries were evaluated for meat samples fortified at different known concentration levels of MDA (58.3 and 116.6 μg/kg) and subsequently calculating the fraction of MDA recovered through the extraction procedure (Figure 1). Table 2 summarizes data on the recovery of MDA from the spiked samples. Recoveries derived from meat spiked with 58.3 μg/kg were lower (85.3% for cold extraction and 71.7% for hot extraction) than recoveries derived from meat spiked with 116.6 μg/kg MDA. Reproducibility of LC analysis of spiked meat samples analyzed by extraction methods derivatized by DNPH and determined by the relative standard deviation (RSD) for 116.6 μg/kg was 1.8% (cold extraction) and 6.5% (hot extraction). RSD for 58.3 ng/mL was 2.6% (cold extraction) and 7.6% (hot extraction). In both concentrations of MDA standard, hot extraction produced lower recovery. Recoveries after hot and cold extraction were significantly different ($p < 0.05$).

Liquid Chromatographic Analysis

The chromatography of standard, blank, and meat sample after MDA derivatization with DNPH is shown in Figure 2. The complex MDA–DNPH was detected with an isocratic

Figure 1. Comparison of LC recovery (μg/kg) of MDA after cold and hot extraction with addition of MDA standard, respectively. LC conditions: Nucleosil C18 reversed-phase (3 μm, 125 × 3 mm), acetonitrile–water–acetic acid (39 + 61 + 0.2), flow rate 1 mL/min, UV detection at 307 nm. Volume injected 20 μL.

gradient at 6.5 min. Mobile phase [acetonitrile–water–acetic acid (39 + 61 + 0.2, v/v/v)] was used for MDA–DNPH elution, and UV absorption for all the samples with maximum response was observed at 307 nm. At the optimized conditions, the peak MDA–DNPH was well separated from other peaks. The calibration curve showed the linear relationship between the MDA–DNPH area and the concentration of MDA in standard solutions in the range of 4.35–1000 ng/mL (r = 0.9989).

The detection limit of this method (20 μL injected) in pure MDA standard was 0.06 ng, which represents 2 μg/kg for broiler meat sample.

Poultry fat is formed by esters of higher unsaturated fatty acids with glycerol and by small quantities of phosphatides, sterols, and volatile free fatty acids. Poultry contains less fat than red meat. On average, broilers contain 3.5–5.0% fat (25). Poultry fat contains a higher portion of polyunsaturated fatty acids (PUFAs) than fats of other slaughtered animals. Because of the higher portion of PUFAs, poultry meat is a convenient material for MDA evaluation. Microwave heating has a serious influence on MDA concentrations. Halamíčková et al. (17) showed that microwave heating caused a 6× increase in mean MDA values in market broilers. Similarly, Wong et al. (26) recorded the same influence of microwave heating.

MDA in fresh or peroxidized biological samples results mainly from the oxidative degradation of PUFAs with more than 2 methylene-interrupted double bonds (18). The estimation of MDA from samples with a complex biological matrix is difficult. The method of MDA extraction from the sample has a serious influence on the result of MDA detection (6, 20, 24). In biological matrixes, MDA exists in both free and bound forms (-SH and -NH$_2$ groups of macromolecules such as proteins and nucleic acids). Draper et al. (13) concluded that higher values obtained with hot TCA provide further evidence that much MDA in animal tissues occurs in bound forms from which it must be released by hydrolysis requiring heat. Other workers (12, 27) found it difficult to hydrolyze all MDA bound to meat proteins without using strong acid conditions and heating.

These results were confirmed in our experiment that hot extraction produced a 55.8% higher amount of MDA than did cold extraction. We suggest that this significant difference is caused by overestimated production of MDA during fat decomposition at higher temperatures. Primarily, PUFAs in poultry are very sensitive to oxidation. High temperatures in combination with time (85°C, 30 min) especially cause their degradation. In our opinion, low temperatures and antioxidants must be used for stabilization of PUFAs during extraction and determination of MDA to avoid this situation. Higher temperature also causes change in raw meat samples, which become thermally treated during analysis and change character. Our opinion is in accordance with that of other authors (5, 6, 24) who indicated that higher temperature causes decomposition of PUFAs and hydroperoxides due to production of higher amounts of MDA as well as other aldehydes.

Table 2.   MDA recovery after different ways of extraction in poultry meat spiked with 58.3 and 116.6 μg/kg MDA standard (*n* = 6)

Extraction procedure	Amount added, μg/kg	Recovery, %	RSD, %
Cold	58.3	85.3	2.6
	116.6	94.7	1.8
Hot	58.3	71.7	7.5
	116.6	74.6	6.5

Strong acidic conditions without higher temperatures can lead to release of bound MDA from its protein bidding (20). Bird et al. (7) and Grau et al. (24) described same results. Ice-cold TCA (4°C) can be used as a protection against temperature increase during sample homogenization.

Good protection against autooxidation can be ensured when antioxidants such as BHT are added to the samples before the homogenization process (22, 24, 28). Addition of EDTA before blending in the presence of hexane had a more pronounced effect on the suppression of lipid oxidation (28). According to Grau et al. (24) EDTA is important mainly in thermal-treated products when metal ions are released. Bonding of metal ions with EDTA eliminates oxidation as well as additional MDA production.

The final concentration of MDA depends on the length and temperature of homogenization. The hexane layer is important to protect the sample against oxygen insertion and following oxidation, which can cause additional MDA production. Centrifugation and filtration are important steps to remove meat constituents (proteins), which can react with DNPH to form complexes; peaks produced by them interfere with the MDA–DNPH peak.

Cleaning of the derivatized samples on the C18 SPE cartridges before LC analysis removes all matter that can interfere during UV detection and increase MDA content. A similar effect was recorded elsewhere (6, 29). Application of SPE in the sample preparation is effective because of increasing the selectivity, column protection, and decreasing the analysis time in routine procedures (30).

Recovery of MDA, after addition of MDA standard (116.6 μg/kg) to the samples, was 94.7% for cold extraction. Pilz et al. (20) recorded 98% recovery of MDA in plasma with 12% perchloric acid. Lower recovery of MDA after hot extraction could be due to different conditions of extraction. We found that higher temperatures influenced the intensity of absorbance of MDA standard added to samples. Similarly, Cordis et al. (18) found that heating of pure MDA standard solutions for 30 min at 60°C in 0.5M NaOH caused total loss of absorbance at the specific wavelength of 307 nm.
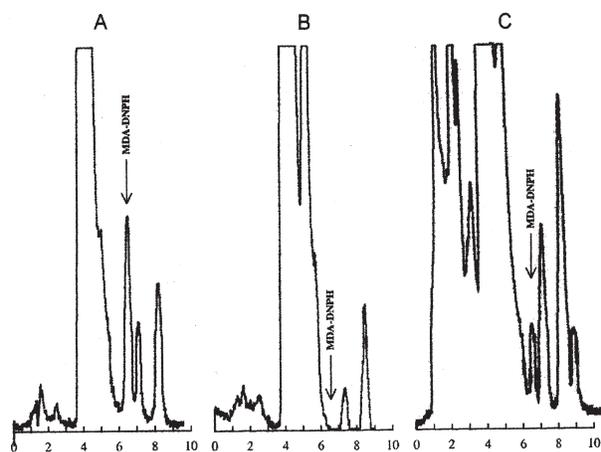
Conclusions

The LC method with cold extraction and derivatization of samples with DNPH is suitable and recommended for practical use of MDA determination. Elimination of higher temperatures during extraction and derivatization reduces the possibility of additional MDA production and increases the method specificity. Addition of BHT and EDTA before homogenization effectively protects samples against oxidation during homogenization. This LC method modification in combination with DNPH derivatization is a simple, accurate, and cost-effective way for determination of MDA, which is an important product of PUFA decomposition.

Figure 2.   (A) Chromatogram of MDA standard (MDA–DNPH complex), 87.4 ng/mL. (B) Chromatogram of reagent blank. (C) Chromatogram of sample (cold extraction, 73.8 μg/kg). LC conditions: Nucleosil C18 reversed-phase (3 μm, 125 × 3 mm), acetonitrile–water–acetic acid (39 + 61 + 0.2), flow rate 1 mL/min, UV detection at 307 nm. Volume injected 20 μL.

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