Lipid oxidation in cured meat model systems containing either antioxidant or prooxidant: A comparative study on determination of malondialdehyde concentration by using conventional, test-kit and chromatographic assays

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Abstract

The impacts of curing plus antioxidant or prooxidant inclusion in model meat systems on malondialdehyde (MDA) levels detected by three different assays as spectrophotometry (M-1), test kit (M-2), and high-performance liquid chromatography (M-3) were explored. Four different treatments that included non-cured, cured, cured plus zeaxanthin-added, and cured plus ferric iron-added meat systems were produced. MDA levels during storage recorded by M-1 were higher than both M-2 (up to 29-fold) and M-3 (up to 53-fold). The effects of curing were visibly detected by all assays, whereas the impacts of antioxidant or prooxidant addition were much obvious when determined by M-2 and M-3. Despite the variations within numerical values, both M-1 and M-2 presented strong correlations with M-3. Overall, it was concluded that spectrophotometric assays could be reliable on condition that acceptable coefficients are utilized to avoid overestimation. Besides, the simultaneous improvement and simplification of chromatographic techniques as well as rapid test kits would pave the way for their widespread use.

1. Introduction

Autoxidation of lipids has long been known as the major chemical cause of quality deterioration in muscle foods that leads to negative alterations in color, texture, flavor, and nutritional value (Shah et al. 2014) along with the formation of toxic compounds associated with health risks (Falowo et al. 2014). Lipid peroxidation, an oxygen-induced and self-feeding radical-chain reaction, takes place in three phases known as initiation, propagation, and termination (Feiner 2006) where the interaction of alkyl and peroxy radicals formed during the first phases results in the generation of final non-radical products during termination phase (Falowo et al. 2014). Although hydroperoxides that are regarded as the first oxidation products are odorless and do not contribute to the aroma, these unstable compounds rapidly decompose and form a large number of secondary compounds (Domínguez et al. 2019). The most characteristic secondary products of the chain reactions are aldehydes, ketones, epoxides, hydroxy compounds, oligomers and polymers, in which malondialdehyde (MDA) is regarded as one of the main representative and abundant compounds among them (Barriuso et al. 2013; Jung et al. 2016), and thereby, the concentration of MDA is widely used to express the oxidation level of meat products.

The most common method to determine MDA concentration is based on the reaction of MDA with 2-thiobarbituric acid (TBA) that produces a pink-colored dimeric chromogen complex (Ghani et al. 2017; Bertolín et al. 2019) which is so-called TBA-reactive substances (TBARS) assay (Caprioli et al. 2011). Although plenty of modifications of this assay is still in use today, remarkable disadvantages are attributed to this technique such as the lack of specificity of TBA-MDA reaction and the interference of both TBA and MDA with different food constitutes that could lead to overestimation or erroneously low values (Caprioli et al. 2011; Díaz et al. 2014; Jung et al. 2016), as well as further oxidation risk derived from high temperatures, long incubation periods, and use of aggressive acids (Barriuso et al. 2013). From this point of view, the quantification of MDA by high-performance liquid chromatography (HPLC) as an advanced method is highly recommended to increase analytical sensitivity and specificity, offering a good solution to avoid such problems mentioned for conventional techniques (Karatas et al. 2002;

Mendes et al. 2009; Papastergiadis et al. 2012; Jung et al., 2016). Apart from the conventional spectrophotometry and chromatography, ready-to-use assay kits to determine MDA concentrations are commercially available for use in biological tissues and foods. Utilization of such rapid test kits is preferable in most cases since they offer good sensitivity and repeatability with allowing the analyzes to be carried out under standard conditions in different laboratories (Goodridge et al. 2003).

The utilization of natural antioxidants specifically from plant origin has become the center of attention in the last decades due to the growing concerns of consumers against synthetic antioxidants used in meat product formulations (Seo et al. 2021). Zeaxanthin (ZX), a natural carotenoid used as a colorant in various food products, is pointed out as one of the most effective compounds to enhance the oxidative stability of meat products (Mercadante et al. 2010). On the other side, prooxidant materials such as iron is regarded as the main catalyst that lead to the formation of reactive oxygenated free radicals (Bechaux et al. 2018). Up to date, to the best of our knowledge, no research has evaluated the application of different assays to determine MDA concentrations in cured meat systems formulated with either ZX or iron. Considering that curing process as a common application in the manufacture of meat products besides the incorporation of antioxidant and prooxidants could highly affect the rate of lipid oxidation in meat systems, the present work was carried out to examine the MDA concentrations in model meat systems by running different analytical procedures to get a better understanding of the impacts of those parameters and the interactions of the assays.

2. Materials And Methods

2.1. Materials

Twenty-four hours post-mortem beef as boneless rounds (*Biceps femoris*) that had a pH of 5.6 ± 0.03 and was consisted of 74.4 ± 1.04% moisture, 18.9 ± 0.95% protein, 5.8 ± 0.68% lipid, and 0.9 ± 0.07% ash was purchased from Migros Integrated Meat Processing Plant (Izmir, Türkiye) and it was then cold chain-transported to the laboratory. Curing additives (sodium chloride, sodium nitrite and sodium ascorbate) incorporated into model meat systems were supplied from Kimbiotek Chemicals Inc. (Istanbul, Türkiye). ZX (DSM OPTISHARP®, 5% CWS/S-TG) used as an antioxidant was kindly donated by ATS Advanced Technology Supplies Co. (Istanbul, Türkiye) which consisted of reddish-colored and free-flowing particles (beadlets) (< 0.5 mm) fully dispersible in water according to the specification of the manufacturer. Ferric iron (FI) as ferric chloride ($FeCl_3$) form (157740, reagent grade, 97%) included in the cured meat systems as a prooxidant was purchased from Sigma-Aldrich Chemie GmbH (Darmstadt, Germany).

2.1.1. Reagents and standards

Either ACS or HPLC grade chemicals (Sigma-Aldrich Chemie GmbH, Germany) for analysis were used without further purification. The major chemicals involved in the analysis were TBA (PubChem CID: 2723628), trichloroacetic acid (TCA, PubChem CID: 6421), butylated hydroxyanisole (BHA, PubChem CID: 8456), butylated hydroxytoluene (BHT, PubChem CID: 31404), 1,1,3,3-tetraethoxypropane (TEP, PubChem

CID: 67147), acetonitrile (PubChem CID: 6342), ethanol (PubChem CID: 702), dipotassium hydrogen phosphate (K_2HPO_4, PubChem CID: 24450), sodium hydroxide (NaOH, PubChem CID: 14798), hydrochloric acid (HCl, PubChem CID: 313) and ortho-phosphoric acid (H_3PO_4, PubChem CID: 1004). OXI-TEK TBARS Assay Kit (ALX-850-287-KI01, Enzo® Life Science Inc., New York, USA) was supplied from MedSanTek Laboratory Supplies Trade & Industry Ltd. (Istanbul, Türkiye). Ultrapure water was obtained from a Zeneer Power I Water Purification System (Human Corp., Songpa-Gu, Seoul, South Korea). All the other glassware and plasticware materials were purchased from ISOLAB Laborgeräte GmbH (Eschau, Germany).

2.2. Experimental design and preparation of cured meat model systems

Refrigerated meat was defatted and denerved before it was minced through a 3 mm plate using a meat grinder (Arnica, Türkiye). Table 1 presents the whole ingredients involved in four different formulations of model meat systems: Control (C) treatment was composed of none but meat and water, whereas CR treatment was the formulation that included curing ingredients as a cured control sample. The rest two treatments, CRA and CRP, were prepared by adding either antioxidant (ZX) or prooxidant (FI) to the cured meat systems, respectively.

Table 1. Formulations of cured meat model systems.

Treatments[1]	*Ingredients* (g)						
	Minced beef	Distilled water	*Curing agents*[2]			ZX	FI[3]
			Salt (NaCl)	Sodium nitrite ($NaNO_2$)	Sodium ascorbate ($C_6H_7NaO_6$)		
C	500.0	100.0	0.0	0.0	0.0	0.0	0.0
CR	500.0	100.0	9.0	0.09	0.15	0.0	0.0
CRA	500.0	100.0	9.0	0.09	0.15	6.0	0.0
CRP	500.0	100.0	9.0	0.09	0.15	0.0	30.0

[1]C: Non-cured control meat system, CR: Cured control meat system, CRA: Cured meat systems containing antioxidant, CRP: Cured meat systems containing prooxidant.

[2]Curing agents were consisted of 1.5% salt, 0.015% sodium nitrite, and 0.25% sodium ascorbate of the total amount of meat plus water.

[3]25 μM $FeCl_3$

Two different production methods previously utilized by He and Shahidi (1997) and Öztürk-Kerimoğlu (2021) were inspirational in the preparation of the cured meat models. Figure 1 illustrates the flow diagram of model beef system production. The minced meat was pre-mixed with distilled water (4°C) at 500 rpm for 3 min using a food processor (Vorwerk Thermomix TM5, Germany) which was the only stage for preparation of C treatment. The curing additives, dissolved in half of the water beforehand using a high-shear homogenizer (Ultra-Turrax® T25, Germany) at 4000 rpm for 1 min, were added to the processor for preparing CR treatment. ZX powder was mixed simultaneously with the curing agents in water (for CRA treatment) while FI liquid solution (25 μM $FeCl_3$) was directly introduced into the formulation along with the curing additives (for CRP treatment). The incorporation levels of ZX and FI were selected according to Mercadante et al. (2010) and Zhou and Elias (2012), respectively, to obtain the most visible impacts in the meat systems. Once the pre-mixing was completed, all the final mixtures were further homogenized at 1100 rpm for 3 min. The temperature of the batters did not exceed 10°C in any of the treatments throughout the production. Approximately 30 grams of batter at a time were stuffed into 50 mL polypropylene tubes, and they were then centrifuged (Nüve, NF 400, Türkiye) at 2500 ×g for 5 min in order to eliminate trapped air. The tubes were then placed in a water bath (Nüve, Türkiye) at 80°C, and they were subjected to heat treatment until the core temperature reached 72°C which was continuously controlled by using thermocouples that were penetrated in 3 different samples randomly selected. Afterward, the tubes were immediately transferred to an ice water bath, cooled to room temperature for 15 min, and then refrigerated at 4°C for 3 h. The samples were collected and analyzed to determine MDA concentration after 0, 3, 7, 12 and 15 days of cold storage.

2.5. Determination of MDA concentration of model meat systems by using different methods

2.5.1. Method-1 (M-1): Conventional spectrophotometric assay

Numerous analytical methods and their modifications are available in literature that target to measure the absorbance of the pink-colored complex formed by the interaction of MDA with TBA. Ghani et al. (2017) underlined that the reason for the current presence of a great number of different methods for TBARS determination is quite unclear. Therefore, a more recent and more common assay based on aqueous acid extraction described by Du and Ahn (2002) was selected as a representative method for the spectrophotometric determination. The analysis was applied with slight modifications as follows: Five grams of sample was homogenized in 15 mL of pure water using a high-shear homogenizer (HG-15D, Daihan Scientific, South Korea) at 6500 rpm for 1 min. Afterward, 1 mL of the homogenate was transferred to a test tube, 50 μL of 7.2% (w/v) BHA in EtOH and 2 mL of TBA/TCA stock solution (15 mM TBA/15% TCA, w/v) were added, and the mixture was vortexed (WiseMix VM-10, Daihan Scientific, South Korea) at 3300 rpm for 30 s. This mixture was then incubated in water bath at 100°C for 15 min, cooled in ice, re-vortexed, and finally centrifuged at 2600 ×g for 15 min. The absorbance of the resulting supernatant was measured at 531 nm against a blank sample prepared with 1 mL of water plus 2 mL of

TBA/TCA solution. TBARS value was expressed as mg MDA/kg sample that was calculated from the standard curve plotted by using 1×10^{-3} M TEP standard with ascending (0.36, 0.72, 1.44, 2.16, 2.77 and 3.6 ppm) concentrations.

2.5.2. Method-2 (M-2): Assay kit

The OXI-TEK TBARS Assay Kit, designed to provide a standardized and reproducible assay with consistent results according to the producer, was used to determine MDA concentrations of model meat systems. Samples were prepared according to Hopkins et al. (2014): One gram of sample was homogenized in 5 mL of normal saline solution. The application sheet of the manufacturer was followed for the analysis. Briefly, 100 µL of the homogenate was gently mixed with 100 µL of sodium dodecyl sulfate (SDS) solution and 2.5 mL of TBA/Buffer reagent that comprised acetic acid, sodium hydroxide and TBA. Each tube was covered with a glass marble and then incubated in a 95°C water bath (Wisd WSB-18, Daihan Scientific, South Korea) for 60 min. After cooling the tubes to room temperature in an ice bath for 10 min, the samples were centrifuged at 1000 ×g for 15 min. The absorbance of the supernatants was read at 532 nm. A series of 5 standards (0, 12.5, 25, 50 and 100 nmol/mL) using undiluted MDA standard (100 nmol/mL) was prepared according to the specification for constructing the calibration curve. MDA concentrations were calculated as mg malonaldehyde/kg sample.

2.5.3. Method-3 (M-3): Quantification of MDA by Reverse-Phase (RP)-HPLC

MDA concentrations of model meat systems were detected utilizing the method of Jung et al. (2016) with slight modifications. Previously, those researchers stated that direct quantification of MDA after MDA extraction with acetonitrile is more useful when compared with MDA-dinitrophenyl hydrazine (DNPH) separation assay for accurate measurement of MDA levels in processed meat products. Accordingly, this method was chosen as the representative chromatographic assay in the present study.

2.5.3.1. Preparation of MDA extracts and standards

Three grams of sample was extracted with 50 µL of 7.2% (w/v) BHT in ethanol and 6 mL of distilled water using a high-shear homogenizer (HG-15D, Daihan Scientific, South Korea) at 16000 rpm for 1 min. Then, 100 µL of 6 M NaOH solution was added into 500 mg of the homogenate for alkaline hydrolysis of protein-bound MDA. Thus, the final concentration was adjusted to be 1 M. The mixture was kept in water bath at 60°C for 45 min and then cooled to room temperature. After adding 1 mL of acetonitrile to the mixture, it was vortexed for 1 min and then centrifuged at 13000 ×g for 10 min (Pro-microcentrifuge Set, CF-10, Daihan Scientific, South Korea). The obtained supernatant was used as the MDA extract.

TEP solution, used as MDA standard, was diluted with 0.1 M HCl to 3.2 mM concentration. For hydrolysis, it was kept in the dark at room temperature for 2 h. This stock solution was diluted with distilled water to obtain solutions with concentrations of 0.1, 0.2, 0.4, 0.8 and 1.6 µM. All the MDA standards and extracts

were filtered through 0.2 µm polytetrafluoroethylene (PTFE) syringe filters. The filtrates were collected in vials prior to HPLC injection.

2.5.3.2. Chromatographic determination of MDA

MDA contents of the samples were analyzed with an Agilent 1200 LC system (Agilent, Santa Clara, CA, USA) equipped with an automatic injection unit and diode array detector (DAD). This LC system had the Chemstation software (Agilent, Santa Clara, CA, USA), which created a 3D data set of retention time, absorbance and wavelength. An RP-LC assay was performed using an Ascentis C18 (250 mm × 4.6 mm, 5-µm particles) column (Merck KGaA, Germany) with a column temperature of 35°C. A mobile phase of 30 mM K_2HPO_4 (pH adjusted to 6.2 with phosphoric acid) was used as an isocratic system with a flow rate of 1.2 mL/min. The injection volume was 50 µL and the detection wavelength was 425 nm. MDA peaks in the sample chromatograms were identified using the standard addition technique as well as matching the UV spectra and retention times of the samples with the standards. MDA contents in the samples were quantified by plotting a five-point regression curve for the MDA standard. The results were expressed as mg MDA/kg sample.

2.5.3.3. Validation, sensitivity and accuracy

For validation of the analytical method, linearity, limit of detection (LOD), limit of quantification (LOQ), and recovery were determined. Linearity was evaluated by calculating y-intercept, slope, and coefficient of determination (r^2) with the help of least squares regression. The sensitivity of the analytical method used was determined by the signal-to-noise ratio (S/N). The accuracy of the method was determined by adding the original contents of MDA defined in the samples to the sample extracts. It was performed by five replicate analyses for each sample and expressed as percentage recovery of the amount of added MDA.

2.6. Data analysis

The trial was conducted in triplicate (three independent batches) on sequential days and MDA analyses were performed in quadruplicate for each batch at each storage day. The statistical evaluation of the data was carried out with SPSS software package version 20.0 (SPSS Inc., Chicago, USA). Three-way analysis of variance (ANOVA) was carried out in order to expose the effects of independent variables “method type” (M1, M2 and M3), “treatments” (C, CR, CRA and CRP), and “storage time” (0, 3, 7, 12 and 15 days) on MDA concentrations (dependent variable) of the model meat samples as well as the interactions among them. The effect of the treatments and storage time on MDA levels of the samples was evaluated by two-way ANOVA. Statistically significant difference in cases where a factor effect was observed was defined by Post-hoc (Duncan) test at a 0.05 significance level. The strengths of association among MDA assays were determined by using simple bivariate correlation analysis. Pearson product-moment correlation coefficient was detected with two-tailed test of significance.

3. Results And Discussion

3.1. The effects of curing and inclusion of ZX and FI in model meat systems on MDA concentrations detected by different assays

Lipid oxidation in muscle foods is a complex phenomenon that takes place depending on numerous intrinsic and extrinsic factors. The oxidative stability of meat is related to the balance of antioxidant and prooxidants as well as the concentration and characteristics of oxidizable substrates such as polyunsaturated fatty acids (PUFAs), cholesterol, proteins, and pigments (Grotta et al. 2017). In addition, curing of meat has a big influence on lipid oxidation rate since sodium nitrite as the major curing agent not only indicates an antimicrobial effect specifically against *Clostridium botulinum* but also plays a strong antioxidant role (Bonifacie et al. 2021). Taking all these facts into consideration, in the current study we evaluated the effects of curing and introducing ZX and FI to the cured systems to better understand the changes in MDA concentration when detected by different assays.

3.1.1. General remarks on MDA concentrations of different meat systems and overestimation

The effects of different analytical methods, different formulations, storage time, and their interactions on MDA concentrations of the model meat system samples are presented in Table 2. As is seen from the table, all the factors analyzed were found to be significantly effective on MDA levels ($P < 0.05$). Relatedly, single (A, B, C) or several two-factor interactions (A × B, A × C, B × C) as well as three-factor interaction (A × B × C) all yielded significant variations on MDA concentrations. For this reason, they were then analyzed individually to better discuss the effects of treatments and storage time on MDA concentrations detected by different methods.

Table 2
Factorial ANOVA table indicating the statistical significance of the method types, treatments, storage time, and their interactions on MDA concentrations of model meat systems.

Source of variation[1]	Type III Sum of Squares (SS)	Degree of freedom (df)	Mean Squares (MS)	F	*P* value
A	11.64	2	5.82	14743.89	0.00
B	5.20	3	1.73	4391.90	0.00
C	1.30	4	0.33	824.80	0.00
A × B	7.74	6	1.29	3264.71	0.00
A × C	1.44	8	0.18	456.28	0.00
B × C	1.05	12	0.09	222.43	0.00
A × B × C	1.95	24	0.08	205.60	0.00
Error	0.071	180	0.00	-	-
Total	43.068	240	-	-	-

[1]A represents the methods (M1, M2 and M3) applied for the determination of MDA concentrations, B represents the treatments (C, CR, CRA and CRP), and C represents the storage time (0., 3., 7., 12. and 15. days).

MDA concentrations of the model meat systems recorded with different analytical methods M1, M2 and M3 during storage are presented in Fig. 2. Initial MDA concentrations recorded on Day-0 (immediately after production) ranged between 0.116–0.409, 0.013–0.082 and 0.002–0.018 mg MDA/kg sample whereas final concentrations recorded on Day-15 ranged between 0.243–1.935, 0.06–0.116 and 0.024–0.143 mg MDA/kg sample for M-1, M-2 and M-3, respectively. Those ranges pointed out that using the conventional method (M-1) to detect MDA content resulted in significant higher ranges of MDA compared with test kit (M-2) and chromatography (M-3) ($P < 0.05$). These remarkably high values recorded by M-1 were regardless of either the formulation or the storage time. As also mentioned in Section 1, the biggest disadvantage of the conventional TBARS analysis is that TBA, the main reagent in the assay, reacts not only with MDA but also with several other carbonyl compounds resulting from lipid peroxidation such as alkenals, alkadienals, ketones and other aldehydes (Díaz et al. 2014; Reitznerová et al. 2017). Furthermore, TBA may react with various other compounds such as carbohydrates, amino acids, fatty acids, nitrites and nitrates, pyridines, pigments, metal chelators and other additives present in the meat system (Mendes et al. 2009; Díaz et al. 2014) that could lead to the generation of yellow or orange colored complexes (Díaz et al. 2014). Those complexes form chromogens that absorb at similar wavelengths (530–535 nm) to the MDA-TBA complex which results in overestimation of the values (Bertolín et al. 2019). In addition, Mendes et al. (2009) emphasized that high temperatures (95–100°C) and strong acidic conditions (pH 1.5–3.5) which are required for MDA-TBA reaction could cause an

artifactual peroxidation of sample constituents. The major reason for higher MDA concentrations recorded in M-1 compared with M-2 and M-3 is thought to be the overestimation of the data due to the combined effects of all those emphasized facts. Previously, MDA levels in raw meat and processed meat products were evaluated by Bertolín et al. (2019). In their study, MDA concentrations were overestimated by using the spectrophotometric method when compared with the chromatographic methods, where specifically in processed meat products, the difference was between 54% and 2068%. Concordant data was also found by Papastergiadis et al. (2012) who reported that in processed meats and cooked fish, spectrophotometric measurements of MDA resulted in an overestimation up to a factor of more than 10 when compared with HPLC measurements. Specifically processed meat samples indicated those behavior when compared with uncooked meats that confirms the interactions of TBA with other additives and ingredients present in the formulation. In our study, on different storage days, the overestimation of MDA concentrations for the conventional method ranged between 2 to 53-fold when compared with the chromatographic method whereas this range differed from 2 to 29-fold between conventional and test kit methods. This overestimation of MDA could be decreased when test kits were used and, in this way, MDA levels obtained with M-2 were 0.4 to 8 folds higher than the MDA levels obtained with M-3. Despite of those reports, the trends of MDA change in different assays throughout the storage followed a similar pattern thus leading to high correlations among them. These correlations evaluated among different methodologies would be further discussed in the following sections.

3.1.2. Curing of model meat systems and changes in MDA levels

The expected impact of curing on retarding lipid oxidation was clearly followed by all analytic methods (Fig. 2): At all storage days, CR samples in which curing was included yielded significantly lower MDA concentrations compared with C samples in which no curing was included ($P < 0.05$). Values in C samples were almost 5 times higher than the cured samples after 3 days of storage. In the absence of nitrites, free iron is released and it promotes lipid oxidation via Fenton reaction in which oxygenated free radicals are formed by the reaction with lipoperoxides (Bonifacie et al. 2021). Nitrite, as a very typical agent in curing, has the ability to bind both heme and non-heme iron which prevents the further release of this catalytic iron and thereby acts against oxidation (Karwowska et al. 2020). In addition, ascorbic acid, a highly effective reducing agent that promotes curing reactions, also serves as an oxygen scavenger that improves antioxidant protection (Terns et al. 2011). Therefore, lower MDA concentrations of the cured samples most probably arise from the combined effects of nitrite and ascorbate. Our results were in agreement with Bonifacie et al. (2021) who reported that cured and cooked meat models containing sodium nitrite had much lower lipid oxidation than the meat models without nitrite, besides, no additional effect of ascorbates to retard oxidation was recorded. According to another approach, lower MDA concentrations of meat models containing nitrite could be because of the reaction of nitrites with MDA under acidic conditions causing the underestimation of MDA (Jung et al. 2016). Since strong acids like TCA and perchloric acid are used for extraction of MDA in standard spectrophotometric TBARS assays, this statement could be prevailing; however, in our study, lower MDA concentrations in meat systems with

nitrite were still observable in the chromatographic technique where acid extraction was not employed. Therefore, at this point, it is thought that the antioxidant effects of curing agents were the major reason for lower oxidation rather than interferences of the nitrite with MDA.

3.1.3. The effects of antioxidant addition on MDA concentrations

Even though the curing process seemed eminently effective to control the oxidation rate, introducing ZX to the cured meat models led to further decrements in MDA levels (Fig. 2): TBARS values detected by M-1 showed that the lowest MDA content on Day-12 and Day-15 belonged to CRA samples containing ZX ($P < 0.05$). This result indicated that ZX was effective to decrease the oxidation rate particularly on the last days of storage although it did not lead to any considerable changes on the earlier days. These results corroborate the findings of Sellimi et al. (2017) who recorded lower TBARS contents after 10 days of refrigerated storage in sausages containing fucoxanthin, which further provided evidence that carotenoids are potent antioxidants for ensuring lipid stability in meat systems. In this respect, Domínguez et al. (2019) stated that carotenoids act as important antioxidants since they scavenge proxy radicals that lead to the formation of a stabilized carbon-centered radical. Right along with the data from M-1, lower MDA levels in CRA samples were detected by M-2 and M-3. According to M-2 data, MDA concentrations of CRA samples were the lowest among other samples on Day-0, Day-3 and Day-15 ($P < 0.05$). Similarly, according to M-3, the lowest MDA concentrations belonged to CRA samples on Day-3, Day-12 and Day-15 ($P < 0.05$). Therefore, the last two methods (M-2 and M-3) revealed that the addition of ZX to cured meat model systems resulted in lower oxidation rates at both the beginning and the end of the storage.

3.1.4. The effects of prooxidant addition on MDA concentrations

It is well-known that the presence of iron plays a considerable role in triggering lipid oxidation chain reactions. Although catalysis of lipid oxidation was ascribed to myoglobin and other heme compounds previously, it was later understood that in cooked meats, non-heme iron was a more active catalyst of oxidation when compared to heme iron (Baron and Andersen, 2002). Gheisari et al. (2010) stressed that iron released from heme pigments during cooking of meat could lead to an increase in non-heme iron that is responsible for accelerating the oxidation rate. This data was a reason behind the selection of the prooxidant included in the cured meat models in the current study. Indeed, different methods selected to determine the oxidation rate resulted in pretty much similar trends (Fig. 2): For M-1, utilization of iron in ferric form ($FeCl_3$) gave a rise to MDA concentrations of cured meat models on most of the storage days. In this traditional method, CRP samples containing prooxidant had higher MDA concentrations than CRA samples containing antioxidant on Day-0, Day-12 and Day-15 ($P < 0.05$), which indicated that the impact of iron in triggering oxidation reactions was visible. On the side, C treatment without curing showed higher TBARS values than CRP treatments for all the storage days ($P < 0.05$). This was presumably due to the abnormal increase in oxidation without curing; thus, when curing was applied to the meat systems,

even if prooxidant is present the samples showed a lower oxidation trend than the samples in which curing was not involved. Indeed, as previously emphasized in Section 3.1.2, as the effective antioxidant, nitrite is offered to favor iron chelation and thereby stabilization of unsaturated lipids (Skibsted, 2011). Based on this fact, in the cured samples the probable effects of the prooxidant material might be suppressed.

Although the traditional method (M-1) showed a rising trend for oxidation by the action of prooxidant materials, this effect was much obvious when detected with other methods. For M-2, CRP samples had higher TBARS values than both CR and CRA samples on all the storage days ($P < 0.05$). Similarly, for M-3, CRP samples had higher MDA concentrations when compared with CR and CRA on Day-0, Day-3, Day-12 and Day-15 ($P < 0.05$). Those data pointed out that rapid kits and chromatographic methods could be more useful to better specify the effects of introducing pro- or antioxidative compounds on lipid oxidation rate of meat systems.

3.2. The effects of storage time on MDA concentrations of model meat systems evaluated by different methods

Storage time has an indisputable influence on the progress of lipid oxidation in muscle foods due to the possibility that radicals cause damage to lipids increases with time (Domínguez et al. 2019). As shown in Table 2, both storage time itself and its interactions with type of the method and the treatments were all significant on MDA contents of the model meat systems ($P < 0.05$). Evaluating the progress of different treatments during storage (Fig. 3), it was found that for M-1, C samples produced without curing had the maximum TBARS value at the end of the storage (Day-15) ($P < 0.05$). However, the maximum MDA content in C treatment belonged to Day-7 for M-2 and to Day-3 for M-3 ($P < 0.05$), which showed that the rising trend of oxidation was not stable throughout the storage. In CR samples in which curing was involved, a significant decrease in MDA level was recorded by M-1 and M-3 after 12 days of storage ($P < 0.05$). Similar to this, in CRA samples containing ZX, there was an increase in the oxidation rate in earlier stages of storage, however, after 7 days, MDA content of these samples showed a decreasing trend which was both detected by M-1 and M-3 ($P < 0.05$). The variations in MDA content during chilled storage could be due to the balance between the formation and destruction reactions according to Mendes et al. (2009). The initial increase of MDA concentrations highlights the proceeding of oxidation reactions, the intermediate declines might arise from the decomposition of MDA and/or reactions of MDA with other polymers such as proteins, and the thereafter increase in some of the samples could be related with a markedly high rate of MDA formation after several storage days (Mendes et al. 2009; Öztürk-Kerimoğlu et al. 2019).

On contrary to the other methods, the data obtained from M-2 indicated that there was a continuous increase in TBARS values of all cured meat models (CR, CRA and CRP) after 7 days of storage ($P < 0.05$). This data demonstrated that when compared with traditional and chromatographic assays, rapid test kits yielded a more different trend for oxidation rate during storage. Nevertheless, the results indicated that

MDA concentrations of all the meat model systems including control samples without curing did not exceed the threshold level (< 2 mg MDA/kg sample) suggested for bovine muscles (Campo et al. 2006). On the side, the relationship between the perception of rancidity and MDA levels should also be considered since it was previously reported that oxidized flavor could be detected from 0.6 to 2.0 mg MDA/kg in beef (Greene and Cumuze, 1981). Thus, rancid notes might be perceived in some of the samples that had TBARS values higher than 0.6 mg MDA/kg. A noticeable point is that the limit values for MDA could be modified based on the determination assay. For instance, Zhang et al. (2019) suggested that sensory quality of beef muscles was acceptable when their TBARS values reached up to 2.5 mg MDA/kg detected by test kits whilst TBARS values up to 10.0 mg MDA/kg detected by spectrophotometric assay could be sensorially tolerable. Those findings are remarkable in terms of the necessity of designating different limit values for MDA concentrations of meat and meat products which are determined by different assays.

3.3. Correlations evaluated between different MDA detection assays

As highlighted earlier in both Table 2 and Fig. 2, the results of this study indicated that the application of three different analytical methods used to determine MDA concentrations in model meat systems resulted in significant variations ($P < 0.05$). The interrelationships between the assays used in the present study were further evaluated with correlation analysis. The correlation matrices indicating those interrelationships on different storage days are presented in Fig. 4. At each storage day, at least one significant positive correlation ($r > 0.5$) was detected between the methods ($P < 0.05$). M-1 had a significant positive correlation with M-3 through the storage ($P < 0.05$) and specifically at the beginning and the end of this period. The strong correlations between M-1 and M-3 indicated that the traditional spectrophotometric method showed good compatibility with the chromatographic method even if the numerical MDA concentrations were different as mentioned in the previous sections. Since the TBA reagent is not used in the HPLC method, no condensing TBA compounds were present which might have interfered with other substances (Karatas et al. 2002). Therefore, MDA concentrations detected by the HPLC method were much lower than those detected by the traditional method. Despite this, the overall trends of both spectrophotometric and chromatographic methods were in a similar line thus presenting positive correlations. The interactions between classical spectrophotometric and chromatographic methods for MDA detection were formerly investigated by Reitznerová et al. (2017) in different meat products. Similar to our findings, they recorded an overestimation with spectrophotometry, however, the results of the traditional assay correlated in all meat samples with the results of the RP-HPLC method. For this reason, the authors suggested that traditional methods could be used to determine the lipid oxidation level if no legislative limit is stated. Nonetheless, in the case of implementation of oxidation limits, specific chromatographic methods would be necessary, and preferentially the specificity of MDA determination could be increased by utilizing correlation factors among different assays. On the other hand, those outcomes were contrary to that of Mendes et al. (2009) who reported that correlation coefficients between spectrophotometric and chromatographic MDA concentrations of fish samples were low although the coefficient between two different HPLC separation methods (MDA-TBA or MDA-DNPH)

was higher. Depending on their findings, the authors declared a greater uncertainty in the traditional method without HPLC separation. The conflicting results of those findings with our study are presumably due to the different separation techniques used in the HPLC method where MDA-protein interactions could possibly take place.

Data from the test kit assay (M-2) was also coherent with the HPLC method (M-3), where it yielded strong positive correlations with M-3 on most of the storage days (Day-0, Day-3, Day-7 and Day-12) ($P < 0.05$). Despite this, M-2 had much weaker correlations with M-1, specifically on Day-12 and Day-15. In accordance with this, Zhang et al. (2019) found no significant relationship between spectrophotometric and test kit assays of TBARS determination in beef muscles, which was probably due to the differences in the sample extraction conditions and the reagents used. On the side, our outputs revealed that the rapid test kit assay gave more consistent results with the chromatographic assay when compared with the traditional spectrophotometric assay. Those strong correlations evaluated between M-2 and M-3 were promising, and the results highlighted that test kits could be a good alternative to HPLC in case of simplification and enhancing their specificity for meat products since the overestimation of MDA was also lower than the traditional method.

3.4. Validation, sensitivity and accuracy of RP-HPLC assay

RP-HPLC method used in the present work showed high validation, sensitivity and accuracy: The calibration curve was found to be linear in the tested concentration ranges (0.0220–0.3525 ng/μL). The regression equation for MDA was y = 645.7832x + 498.1791 ($r^2 = 0.9927$). A correlation coefficient greater than 0.99 indicated that the method had a high degree of correlation and good linearity. LOD value was calculated as 3 times the S/N, and LOQ value was calculated as 10 times the S/N. As is known, the LOD value is the lowest analyte concentration that the instrument can detect but not quantify whereas the LOQ value is the lowest analyte concentration that the instrument can detect and quantify (Rao, 2018). The S/N ratio for the smallest standard concentration determined from the instrument was 2311.7. Accordingly, the LOD value for MDA was 2.86×10^{-5} mg/L, while the LOQ value was 9.53×10^{-5} mg/L. These data showed that the analytical method provided adequate sensitivity. The values of recovery were evaluated as 79.88% for C, 94.43% for CR, 100.99% for CRA, and 83.69% for CRP. All recovery percentages were in the range of 80–101%, showing the good accuracy of the analytical method. All those data pointed out that the quantification of MDA by chromatographic techniques offers high reliability to follow the oxidation degree of meat systems throughout storage.

5. Conclusions

The present work revealed that the HPLC method including the direct quantification of MDA could be applied to cured meat model systems, yielding fast and accurate results. Although chromatographic methods could be useful and more accurate, today spectrophotometric TBARS methods are still accepted and frequently applied to follow lipid oxidation in meat products. Besides, ready-to-use test kits present advantages due to their practical usage although they still do not offer high specificity for muscle foods.

Since both the traditional method and test kits gave good correlations with the chromatographic assay, it may be a suggestion to set constant coefficients between those methods to reflect the actual MDA values to avoid overestimation. At this juncture, standardization in the oxidation assays and detection of the limits for different meat products would be beneficial since lipid oxidation reactions affect multi-functional quality attributes throughout the production and storage of muscle foods.

Declarations

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Ethical Approval

This article does not contain any studies with human or animal subjects.

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Data Availability

The data supporting the discussions and conclusions are included within the article.

Consent to Participate

The authors agreed with the content and further publication of this manuscript.

Conflict of Interest

The authors declare that they have no significant competing financial interests or personal relationships for the present study.

Author Contribution

Dr. Ozturk Kerimoglu was responsible for conceptualization, methodology, formal analysis, investigation, resources, writing-original draft, writing-review&editing, visualization, and project administration. Dr. Nakilcioglu contributed to methodology, formal analysis, investigation, resources, validation, and writing-original draft, while Dr. Serdaroglu took part in resources and supervision. All authors reviewed the manuscript.

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Figures

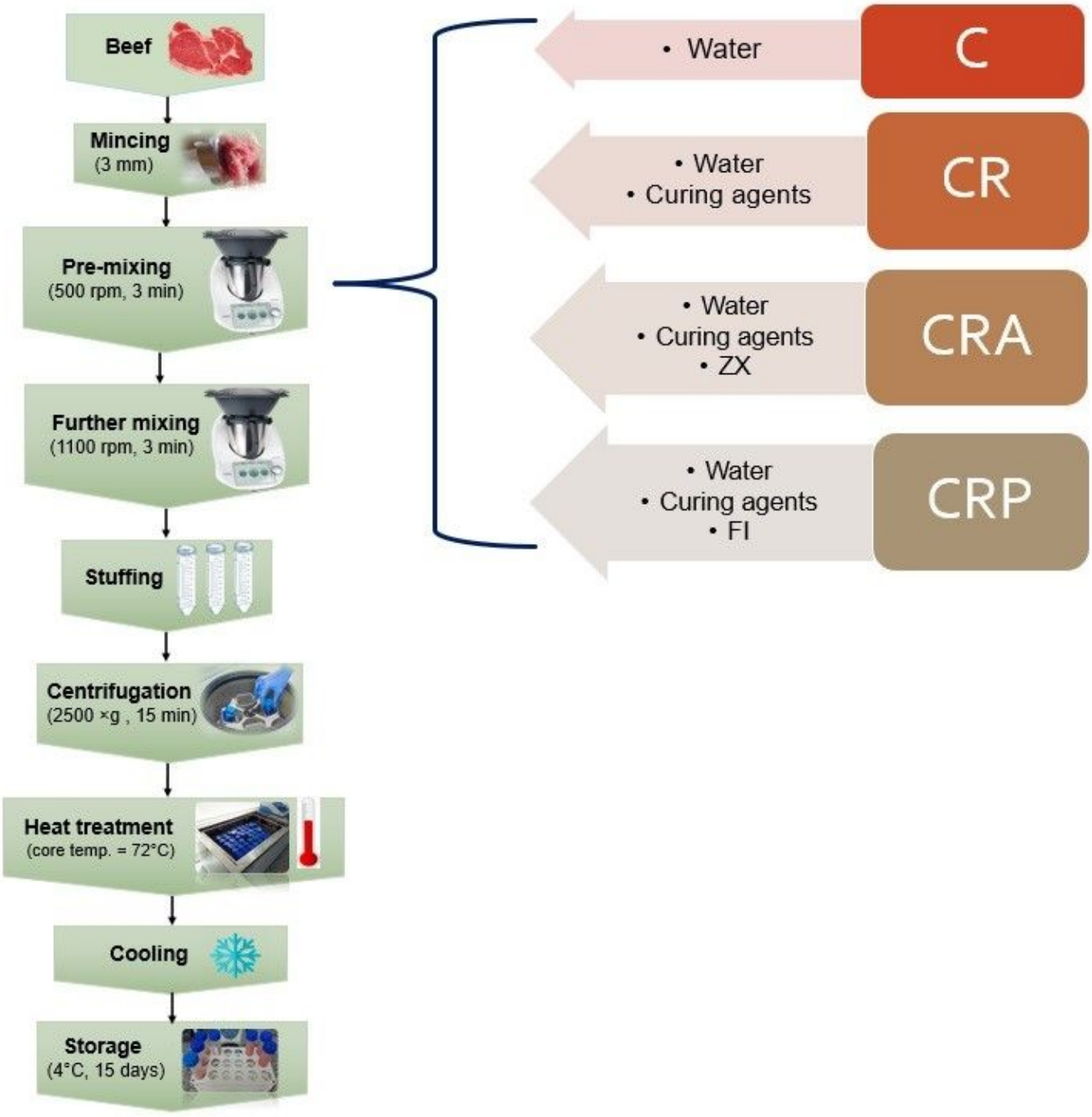

Figure 1

Flow diagram for production of model systems including the experimental design.

C: Non-cured meat model system (control), CR: Cured meat model system, CRA: Cured meat model system containing antioxidant, CRP: Cured meat model system containing prooxidant.

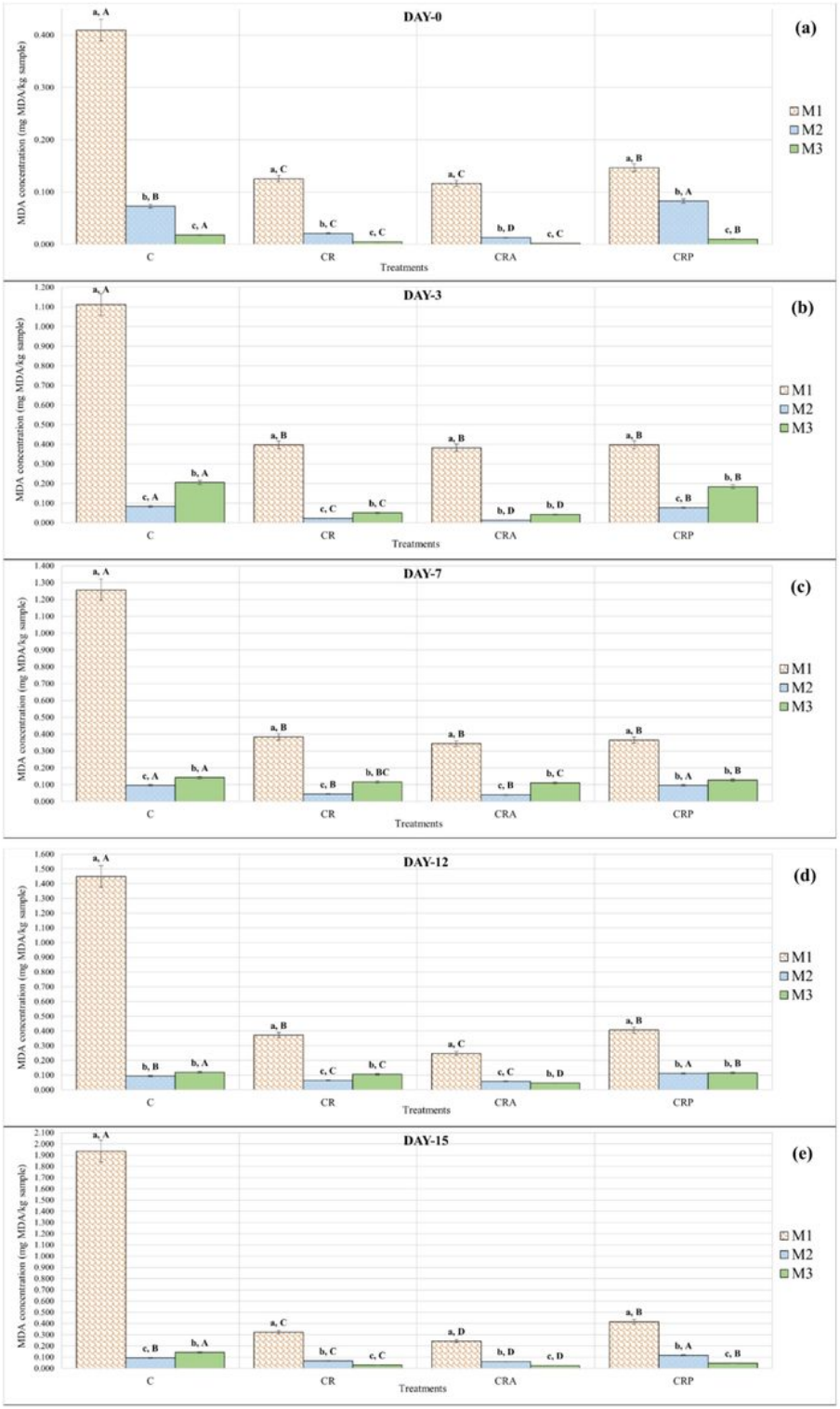

Figure 2

MDA concentrations of model meat systems determined by M-1: Conventional spectrophotometric assay, M-2: Assay kit, and M-3: RP-HPLC at Day-0 (a), Day-3 (b), Day-7 (c), Day-12 (d), and Day-15 (e). The treatments represent the formulations as follows: C: Non-cured meat model system (control), CR: Cured meat model system, CRA: Cured meat model system containing antioxidant, CRP: Cured meat model system containing prooxidant. Data were presented with standard error bars.

a, b, c, …: Means with different lowercase letters within the same treatment are significantly different *(P* < 0.05).

A, B, C, …: Means with different uppercase letters within the same analytical method are significantly different (*P* < 0.05).

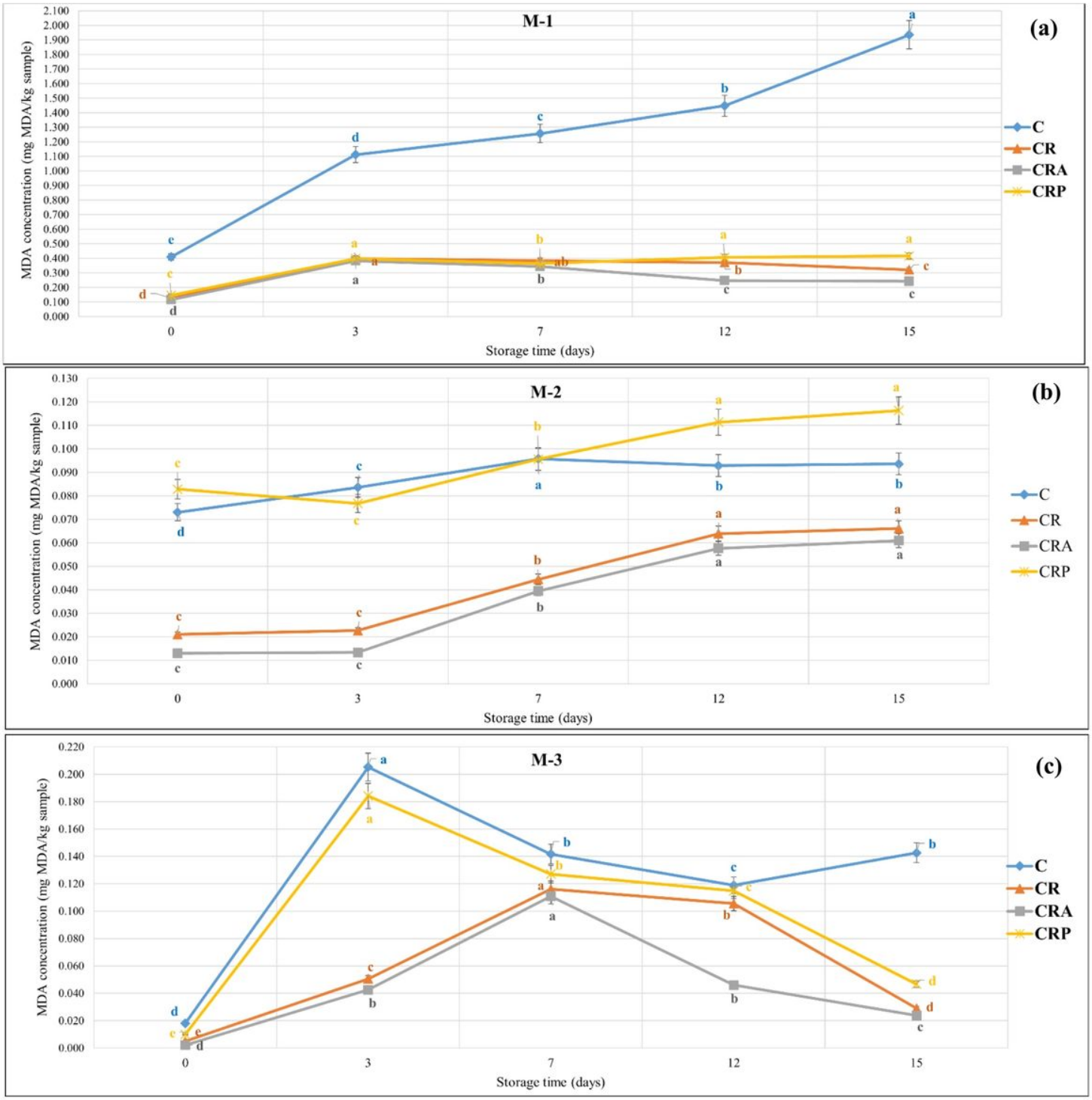

Figure 3

The changes in MDA concentrations of model meat systems during 15 days of storage. MDA levels were determined by M-1: Conventional spectrophotometric assay (a), M-2: Assay kit (b), and M-3: RP-HPLC (c). The treatments represent the formulations as follows: C: Non-cured meat model system (control), CR: Cured meat model system, CRA: Cured meat model system containing antioxidant, CRP: Cured meat model system containing prooxidant. Data were presented with standard error bars.

a, b, c, …: Means with different letters on different storage days are significantly different ($P < 0.05$).

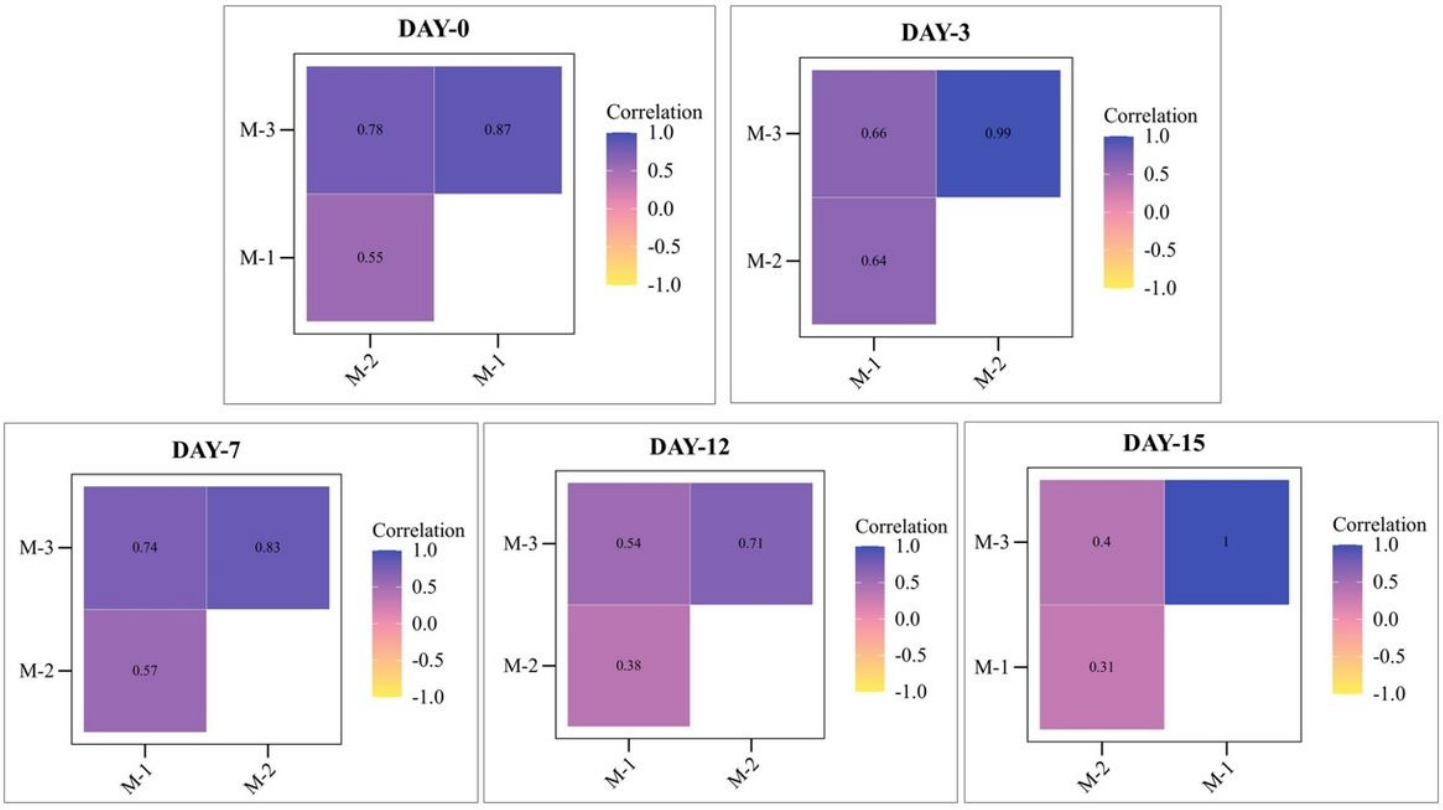

Figure 4

Correlation matrices for the interrelationships between different assays detected for different storage days where M1 is the conventional spectrophotometric assay, M2 is the TBARS assay kit and M3 is the RP-HPLC assay. The squares filled with blue indicate a positive correlation between two genes, while the squares filled with yellow indicate a negative correlation between two genes, and the number in each cell indicates the correlation coefficient (r).