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THE EFFECT OF ANTIOXIDANTS ON QUANTITATIVE CHANGES OF LYSINE AND METHIONINE IN LINOLEIC ACID EMULSIONS AT DIFFERENT pH CONDITIONS*

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ABSTRACT

Background. Plants are an important source of phenolic compounds. The antioxidant capacities of green tea, thyme and rosemary extracts that contain these compounds have been reported earlier. However, there is a lack of accessible information about their activity against lipid oxidation in emulsions and inhibit the interaction of lipid oxidation products with amino acids. Therefore, the influence of green tea, thyme and rosemary extracts and BHT (butylated hydroxytoluene) on quantitative changes in lysine and methionine in linoleic acid emulsions at a pH of isoelectric point and a pH lower than the isoelectric point of amino acids was investigated.

Material and methods. Total phenolic contents in plant extracts were determined spectrophotometrically by using Folin-Ciocalteu's reagent, and individual phenols by using HPLC. The level of oxidation of emulsion was determined using the measurement of peroxides and TBARS (thiobarbituric acid reactive substances). Methionine and lysine in the system were reacted with sodium nitroprusside and trinitrobenzenesulphonic acid respectively, and the absorbance of the complexes was measured.

Results. Extract of green tea had the highest total polyphenol content. The system containing antioxidants and amino acid protected linoleic acid more efficiently than by the addition of antioxidants only. Lysine and methionine losses in samples without the addition of antioxidants were lower in their isoelectric points than below these points. Antioxidants decrease the loss of amino acids. The protective properties of antioxidants towards methionine were higher in a pH of isoelectric point whereas towards lysine in pH below this point.

Conclusion. Green tea, thyme and rosemary extracts exhibit antioxidant activity in linoleic acid emulsions. Moreover, they can be utilized to inhibit quantitative changes in amino acids in lipid emulsions. However, the antioxidant efficiency of these extracts seems to depend on pH conditions. Further investigations should be carried out to clarify this issue.

Keywords: lipid oxidation, linoleic acid emulsion, green tea, rosemary and thyme extracts, lysine, methionine, environmental pH

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INTRODUCTION

Formed during fat oxidation, compounds such as free radicals, peroxides and secondary oxidation products may react with other food ingredients. In terms of technological and nutritional value, the interactions of these products with proteins and amino acids are particularly unfavorable. The interaction of lipid oxidation products and proteins is multidirectional in terms of both the chemical changes to which protein is subjected and the consequences of these changes (Pokorný et al., 2005). Chemical modifications of proteins, in general, can cause polymerization of molecules and a decrease in the content of amino acids as a result of oxidation, blocking, functional group transformations and the formation of additional crosslinks. The directions of these changes are quite conventional, since the oxidation reactions of amino acids may constitute the beginning of protein crosslinking, as well as a transformation in their functional groups. Protein crosslinking occurs as a result of two basic reactions – oxidation of free amino acid groups in the presence of hydroperoxides and nucleophilic addition in the presence of carbonyl compounds. Most sensitive to losses are sulphur-containing amino acids (methionine and cysteine) and lysine. Lysine and methionine degradation is particularly unfavorable because they are essential amino acids. As a result of such oxidations, sulfoxide and sulfone from methionine are formed (Davídek et al., 1983; Zhang et al., 2013). Blocking or conversion of functional groups relates to lysine. Hydroperoxides can react with the primary amine group of lysine and consequently imino groups are formed, following which the lysine residues are converted into Schiff bases in subsequent reactions. Similar transformations are observed for lysine residues in reactions with secondary aldehyde products of lipid oxidation (Davídek et al., 1983). Even a small amount of aldehydes can bind ε-amino groups of lysine, which results in the formation of colorless pyrrole compounds with Schiff base properties (Chelh et al., 2007). Imines formed in the reactions of aldehydes with lysine residues can be, as a result of the cyclization reaction, converted into pyrimidine rings, which results in a rise in the hydrophobicity of the protein and an increase in its susceptibility to crosslinking. As a result of the aldol condensation and pyrrole polymerization, brown-colored macromolecular compounds are formed. Chemically, these products are complexes of Schiff bases and Michael adducts and exhibit properties similar to melanoids formed in the classic Maillard reaction (Adams et al., 2009). These modifications occur with the formation of both covalent and electrostatic bonds. There is also a possibility of the non-covalent binding of a number of hydrogen bonds (Hidalgo and Zamora, 2002). The number of possible combinations of these interactions is very large.

The kinetics of amino acid-oxidized lipid interaction are determined by the presence of catalysts and inhibitors in the environment, pH and the presence of water. The intensity of these interactions is also affected by the reaction time and temperature (Hęś, 2017). A multitude of factors lie behind the still unknown dependencies that exist between these components of food. However, a study of these interactions appears to be necessary, given the need to provide high quality nutritional and health products. Due to the relatively small number of studies in the field of oxidized lipid-amino acid reaction products, it is important to determine to what extent the addition of antioxidants may lead to the blocking of the reactive sites in the polypeptide chain, reducing the process of oxidative metabolism and forming lipid oxidation products. Though widely used, synthetic antioxidants are highly-effective (e.g., BHA, BHT, TBHQ), and there is growing consumer demand for natural ingredients application in processed foods. This has motivated consumers to look for more 'label-friendly' antioxidant alternatives such as natural carotenoids, tocopherols and plant extracts; e.g. vegetable, tea, herbs and spice plants. The high phenolic compound content in these raw materials enables them to become a source of effective and safe natural antioxidant additives that reduce the number of lipid and protein oxidation products (Duthie et al., 2013; Shahidi and Zhong, 2010). These additives do indeed exhibit activity within the human body and shape the nutritional value of products (Hęś, 2017; Hęś and Gramza-Michałowska, 2016). Therefore, it seems particularly important to search for appropriate forms of applying them and assessing their effectiveness in products. The ability of an antioxidant to delay or inhibit lipid oxidation in emulsions will depend on its physical location in the system, as well as possible chemical interaction with other components, and also

on the system's physical conditions (Waraho et al., 2011). The objective of this research was to study the effect of antioxidants (green tea, rosemary and thyme extracts) on quantitative changes in lysine and methionine in linoleic acid emulsions at a pH of isoelectric point in the investigated amino acids and a pH lower than their isoelectric point.

MATERIAL AND METHODS

Chemicals

The following chemicals were used: Folin-Ciocalteu's reagent (FCR), Tween 20 (lauric acid, ≥40% (balance primarily myristic, palmitic, and stearic acids), 2-thiobarbituric acid, 2,4,6-trinitrobenzenesulphonic acid (picrylsulfonic acid, TNBS), sodium nitroprusside, acetonitrile, trifluoroacetic acid; phenolic acids: caffeic, *p*-coumaric, ferulic, protocatechuic, vanillic, rosmarinic, and gallic; flavonoids: (+)-catechin (C), (−)-epicatechin (EC), (+)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin gallate (EGCG), and (+)-gallocatechin gallate (GCG), rutin, quercetin, kaempferol, eriocitrin, narirutin, naringenin-7-O-glucoside, eriodictyol, luteolin, apigenin, and thymol, caffeine, linoleic acid (>99%), amino acids: DL-lysine monohydrochloride, DL-methionine, glycine were obtained from Sigma-Aldrich (Poznań, Poland); methanol, ethanol, diethyl ether, hydrochloric acid, sodium hydroxide, ammonium thiocyanate, trichloroacetic acid, sodium hydrogen carbonate, sodium carbonate were purchased from POCh (Gliwice, Poland). Butylated hydroxytoluene (BHT) was purchased from Merck (Germany). All chemicals and solvents used were of analytical grade.

Plant materials

Dried thyme (*Thymus vulgaris* L.) was purchased from a herb shop and green tea (*Cammelia sinensis* L.) from a specialist shop, which guaranteed the origin and the freshness of the raw materials; both plants originated from the 2006 crop. The rosemary extract, Oxy'Less. U, was purchased from Naturex (France).

Preparation of plant extracts

Thyme and tea extracts were prepared by mixing 100 g of dried material with 250 cm^3 of 80% ethanol, and triple-macerating overnight at room temperature. The supernatants collected were filtered and ethanol was removed at 50°C on a rotary evaporator (Buchi, Switzerland). All samples were frozen at –20°C for 24 hours and then freeze-dried (Alpha 1–4 LSC Freeze dryer, Christ, Germany) and stored at 4°C in a dark place (Gramza et al., 2006a).

Preparation of linoleic acid emulsion

The investigation of factors that could determine the degree of oxidative changes and quantitative changes in lysine and methionine was conducted in 10 mM emulsions of linoleic acid. Emulsions were prepared directly before measurements in the following buffers: phosphate buffer with pH of 5.74 (isoelectric point of methionine), phosphate-citrin buffer with pH of 4.20 and phosphate-borax buffer with pH of 9.60 (isoelectric point of lysine) using Tween 20. 0.5 cm^3 of Tween 20 was added to 5 cm^3 of the buffer and mixed for 5 min on a magnetic stirrer. Then, within 10 min 500 mg of linoleic acid was added and stirred for 2 min. In the next step 11 cm^3 of the buffer was added and stirred for 10 min. After the addition of 150 cm^3 of the buffer, the emulsion was stirred for 5 min and adjusted to a suitable pH. Implementation of a buffer system in the emulsions would make it easier to control the initial pH of the emulsions, as well as make the system more stable regarding changes in pH during the experiments.

Antioxidants (plant extracts, 0.005% relative to the emulsion), BHT (0.002% relative to the emulsion), amino acids (lysine or methionine, 0.15% relative to the emulsion), and combinations of additives – lysine with antioxidants and methionine with antioxidants – were added to the emulsion. The stability of both amino acids was tested at the isoelectric point and at a pH lower than the isoelectric point. The samples were incubated for 1–9 days at a temperature of 37°C, with no access to light. The following were determined at specific time intervals: peroxide content using the thiocyanate method, TBARS (2-thiobarbituric acid reactive substances) content and lysine and methionine content.

Determination of total phenolic compounds

The content of total phenolic compounds in the extract was estimated using the Folin-Ciocalteu's

reagent (FCR) (Horwitz, 1970). Aliquot of extract (0.2 cm^3) was added to 8.3 cm^3 of distilled water and 5 cm^3 of FCR. The mixture was mixed with 1 cm^3 of a saturated sodium carbonate solution. After incubation at room temperature for 30 min, the absorbance of the mixture was read at 750 nm (Specord 40). The results are expressed as mg of (+) catechin equivalents per gram of dry extract [mg CE/g dry extract] for green tea and as mg of gallic acid equivalents per gram of dry extract [mg GAE/g dry extract] for thyme and rosemary.

HPLC analysis of phenolic compounds

Separation and identification of phenolic acids and flavonoids present in the extracts was carried out using high-performance liquid chromatography (HPLC). In this study, we used a Waters (Milford, MA, USA) type 600 instrument, equipped with a photodiode-array 996 detector. For the separation we used the Waters Symmetry C_{18} (3.9×150 mm, 5 μm) analytical column. The mobile phase consisted of acetonitrile and 0.1% trifluoroacetic acid. The solvent flow was of a gradient character. The flow rate of the mobile phase was 1 cm^3/min. The injection volume of the sample was 0.02 cm^3.

The compounds being investigated were identified by comparing their retention times with the retention times of standards of phenolic acids and flavonoids used as external and internal standards. Additionally, the presence of these compounds in the extracts studied was confirmed on the basis of the absorption spectrum obtained using the photodiode-array detector.

The level of individual compounds in the extracts was determined on the basis of standard curves prepared at least twice for each compound under the same chromatographic conditions used for the separation of polyphenyl compounds in the extracts investigated. Unidentified flavonoids were determined using the standard curve of apigenin. Luteolin glycosides were calculated from the aglycone content after acidic hydrolysis.

The number of determinations for each extract was at least six (at least three different weighted portions of extracts, with two replicate injections on the chromatographic column). The results are presented in mg/g of the extract.

Determination of linoleic acid oxidation

Determination of peroxide content. The thiocyanate method consists in measuring the intensity of the colorimetric red complexes formed by ammonium thiocyanate iron (III) when oxidized by peroxides (Shantha and Decker, 1994). When more oxidization occurs, the intensity of the complexes is higher.

The tubes provided 0.05 cm^3 of the emulsion, 10 cm^3 of absolute ethanol, 0.05 cm^3 of 30% ammonium thiocyanate and 0.05 cm^3 of 0.02 M $FeSO_4 \times 7H_2O$ in 3.5% HCl. After exactly 3 minutes the absorbance of the red complex at a wavelength of 500 nm was measured against the blank. Peroxide content was calculated using a standard curve of appropriate dilutions of ferric-ammonium alum and expressed in mM/cm^3 of the emulsion, using the fact that 1 mole of iron is equivalent to 1 mole of peroxide.

Determination of TBARS content. This method is based on the colorimetric measurement of color complex formed by reacting the compounds with thiobarbituric acid in an acidic environment (Buege and Aust, 1978). Absorbance measurement was made at a wavelength of 532 nm. The intensity of the pink color is proportional to the degree of oxidation of fat.

The tubes provided 0.5 cm^3 of the emulsion, 2 cm^3 of phosphate buffer at pH 6 and 3 cm^3 of TBA reagent (solution of 15% trichloroacetic acid and 0.375% thiobarbituric acid in 0.25 M HCl). After mixing, the trial sample was placed for 15 min in a boiling water bath. In the next step it was cooled and then centrifuged for 10 min. After centrifugation, the absorbance was measured against the blank test.

Malondialdehyde content was calculated using a standard dimethylacethal. The results are expressed in nM malondialdehyde/cm^3 emulsion.

Determination of amino acid

Methionine content. The methionine in the linoleic acid emulsion was reacted with sodium nitroprusside as described by McCarthy and Sullivan (1941), and the absorbance of the complex was measured.

In a test-tube, 0.5 cm^3 of the emulsion containing methionine to be tested was introduced, to which 1.5 cm^3 bidistilled water, 0.5 cm^3 of 5 N NaOH, 0.5 cm^3 of 3% aqueous solution of glycine, and 0.1 cm^3 of

a 10% aqueous solution of sodium nitroprusside was added, with the emulsion being mixed after each addition. The tube was then placed in a water bath at a temperature of 40°C for 15 min. It was then cooled in ice water for 2 min, after which 1 cm^3 of 6 N HCl was added while the tube was being shaken. The tube was shaken well for 1 min and then cooled in water at room temperature for 15 min. The absorbance at 520 nm of the complex was measured. Pure DL-methionine was used as a standard. Methionine content was expressed as mg per 1 cm^3 of emulsion.

Lysine content. Lysine was determined by the method described by Hall et al. (1973). The free epsilon-amino groups in lysine were reacted with trinitrobenzenesulphonic acid and the absorbance of the ε-trinitrophenyllysine (ε-TNP-lysine) complex was measured. 0.5 cm^3 1 M $NaHCO_3$ and 1 cm^3 1% TNBS (2,4,6-trinitrobenzenesulphonic acid) was added to 0.5 mL of emulsion containing lysine. The reaction was allowed to proceed at 40°C for 75 min, at which time 3 cm^3 of 11 M HCl was added. The reaction mixture was placed at 100°C for 2 h. After the hydrolyzate had been allowed to cool to room temperature, 5 cm^3 of distilled water was added. Interfering substances, such as free picric acid, were removed twice by extraction with approximately 10 cm^3 of diethyl ether. Residual ether was eliminated from the aqueous phase by placing each tube in hot water for at least 5 min, and the absorbance of the remaining yellow aqueous solution of ε-TNP-lysine was measured at 415 nm. Pure DL-lysine monohydrochloride was used as a standard. Lysine content was expressed as mg per 1 cm^3 of emulsion.

Statistical analysis

Statistical analyses were performed with Statistica Version 10 (StatSoft). The data represent means ±SD of two experiments (two independent samples) and three independent measurements for each sample (n = 6). The effect of additives or storage time was analyzed separately. Analyses of variance (ANOVA) were applied as well as Tukey's test ($p \leq 0.05$). Correlations were calculated between the lipid oxidation products and the content of the available amino acids.

RESULTS

In the plant extracts the presence of individual phenolic compounds and total content of polyphenols were established. The study showed that extracts of green tea had the greatest total polyphenol content. Thyme and rosemary extracts contained 2.5- and 2.7-times lower amounts of polyphenols, respectively (Table 1). The results of the study on phenolic compound content in spices and green tea extracts are presented in Tables 2 and 3, respectively. Phenolic acids and flavonoids (primarily flavanons and flavons) were identified in the extracts of thyme and rosemary. In the rosemary extract flavonoid content amounted to 10.4 mg/g, whereas in the thyme extract it was 17.6 mg/g. Among the phenolic acids in both extracts, rosmarinic acid (59% of the determined phenolic compounds) was dominant. Of the terpenes in the thyme extract thymol was identified. In the green tea extract the level of selected catechins were analyzed; e.g. gallic and caffeic acid and the flavonols rutin, quercetin and myricetin. The dominant catechin in the extract was EGCG (206.5 mg/g), but no GC, EGC and C were detected.

Table 1. Total phenolic content of plant extracts

Sample	Total phenolic
Green tea, mg CE/g	430.96 ±9.26*
Thyme, mg GAE/g	174.32 ±5.82*
Rosemary, mg GAE/g	158.51 ±2.72*

The results are presented as means (n = 6) ±SD.
*Statistically significant for $p < 0.05$.

Oxidation changes in linoleic acid in emulsion systems at different pH conditions (9.6, 5.74, 4.2) were determined by analysing the level of primary (peroxides) and secondary (substances reacting with 2-thiobarbituric acid – TBARS) oxidation products. The major factors that influence the interaction between oxygen and lipids are antioxidants, prooxidants, reactive oxygen species (ROS), environmental conditions, and oxygen scavengers (Johnson and Decker, 2015). The addition of the synthetic antioxidant BHT

Table 2. Polyphenol content in ethanol extracts of spices

Phenolic substances mg/g dry extract	Rosemary	Thyme
Caffeic acid	0.17 ±0.02	–
p-coumaric acid	0.09 ±0.02	–
Ferulic acid	0.09 ±0.02	–
Vanillic acid	0.32 ±0.03	–
Protocatechuic acid	0.10 ±0.02	0.52 ±0.02
Rosmarinic acid	15.85 ±0.41	26.11 ±1.11
Eriocitrin	–	0.55 ±0.04
Narirutin	–	0.49 ±0.04
Naringenin-7-O-glucoside	–	0.74 ±0.04
Eriodictyol	–	0.69 ±0.04
Free luteolin	–	1.48 ±0.11
Glycosides form of luteolin*	–	4.55 ±0.22
Apigenin	0.59 ±0.03	0.99 ±0.06
Other flavonoids**	9.85 ±0.32	8.07 ±0.76
Thymol	–	7.08 ±0.40
Phenolic acids sum	16.62 ±0.51	26.63 ±1.11
Sum of all substances	27.06 ±0.84	51.27 ±2.81

*Determination after acid hydrolysis.
**Expressed as apigenin equivalent.
The results are presented as means (n = 6) ±SD.

Table 3. Polyphenol and caffeine content in ethanol extracts of green tea

Phenolic substances, mg/g dry extract	
GC	ND
EGC	ND
C	ND
EC	7.87 ±0.15
EGCG	206.47 ±4.80
GCG	14.34 ±0.32
ECG	55.24 ±1.36
Catechins sum	283.92 ±6.53
Gallic acid	0.66 ±0.04
Caffeic acid	ND
Rutin	3.51 ±0.09
Rutin derivative	3.09 ±0.36
Quercetin	0.02 ±0.01
Kaempferol	<0.01
Phenolic acids sum and flavonols	7.28 ±0.48
Caffeine	113.44 ±2.27
Sum without caffeine	291.20 ±6.93
Sum of all subastances	404.64 ±9.09

(+)-catechin (C), (−)-epicatechin (EC), (+)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin gallate (EGCG), and (+)-gallocatechin gallate (GCG).
ND – not detected; myricetin, apigenin, ellagic acid not found.
The results are presented as means (n = 6) ±SD.

to the emulsion in each pH range was characterized by the best protective action toward emulsified linoleic acid. It almost entirely inhibited the formation of both primary (Fig. 1) and secondary (Fig. 2) products of oxidation. The lowest limiting activity of linoleic acid oxidation product formation was characteristic of the plant extract, although this additive also significantly affected the inhibition of the oxidation process. The mixture of BHT and green tea extract with amino acids (lysine and methionine) exhibited very good antioxidant properties. The system containing a plant extract and amino acid protected emulsified linoleic acid from autoxidation more efficiently than by the addition of extracts only. The protective action of amino acid and plant extract mixtures was higher in the isoelectric points of the amino acids investigated than in a pH below these points. Only in the case of inhibiting the formation of peroxides was greater activity shown by a mixture of methionine and plant extracts in pH below the isoelectric point of the amino acid (pH 4.2). An important fact is that in an emulsion at pH 9.6 with different variants of additives, lower amounts of primary and secondary oxidation products were produced than in emulsions at pH 4.2 and 5.74. That indicates the effect of the pH of the environment on the process of emulsified linoleic acid oxidation.

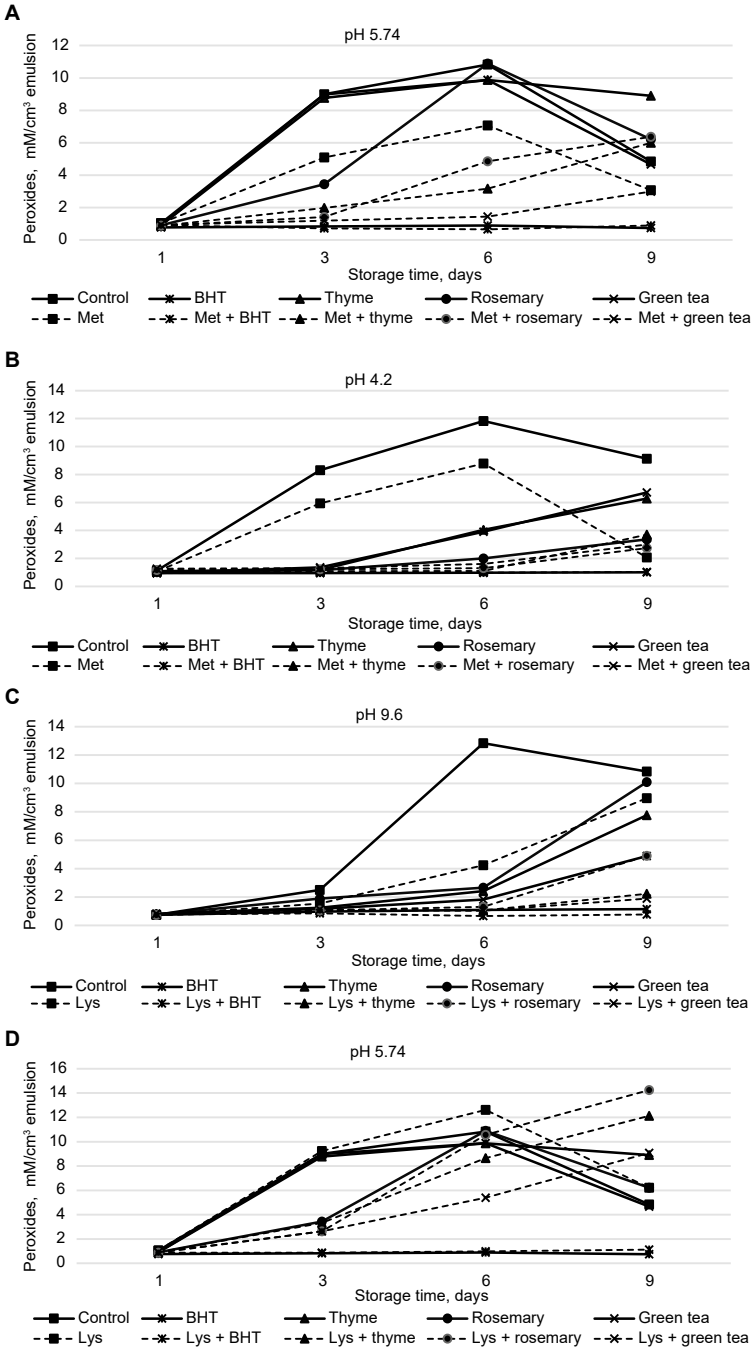

Fig. 1. The effect of additives on peroxides changes during linoleic acid emulsion incubation in different pH conditions, mM/cm^3 emulsion: A – pH of isoelectric point of methionine, B – pH below of isoelectric point of methionine, C – pH of isoelectric point of lysine, D – pH below of isoelectric point of lysine. Without addition: Control, Met – methionine, Lys – lysine. The results are presented as means (n = 6)

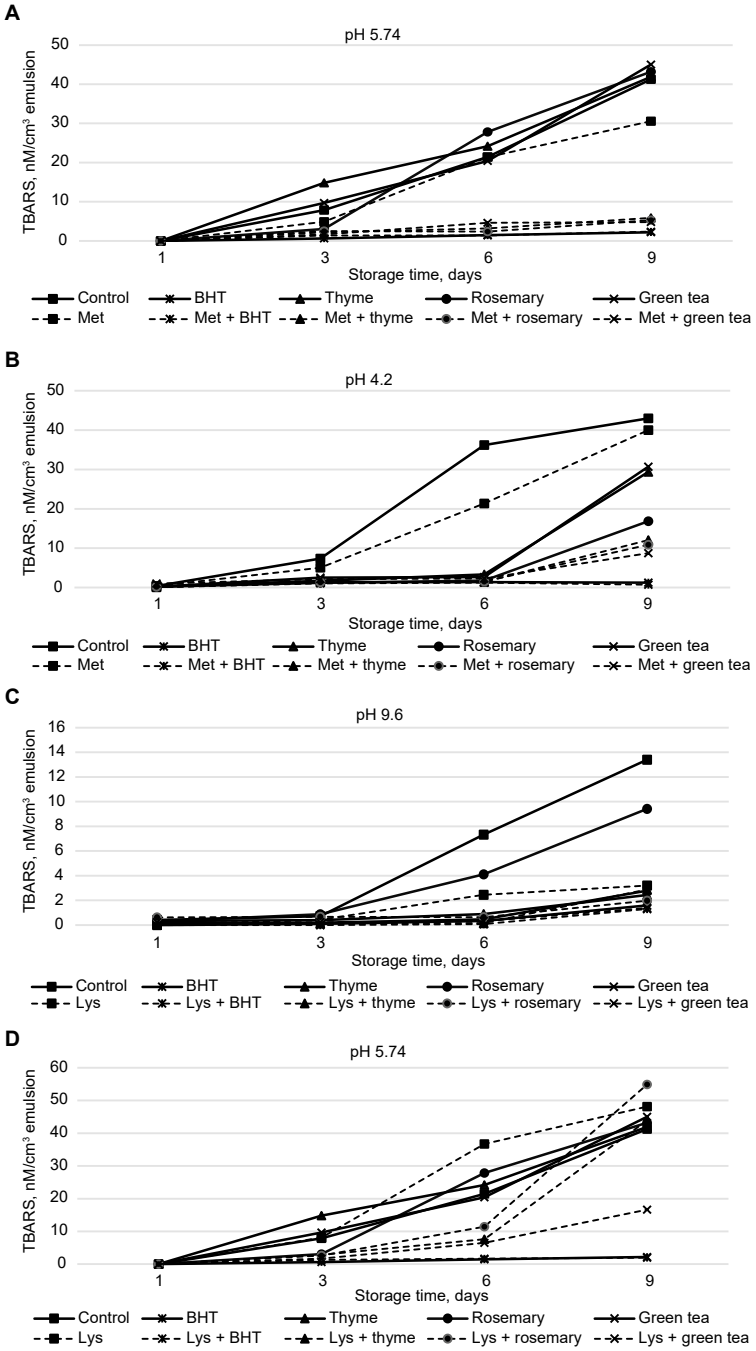

Fig. 2. The effect of additives on TBARS changes during linoleic acid emulsion incubation in different pH conditions, mM/cm³ emulsion: A – pH of isoelectric point of methionine, B – pH below of isoelectric point of methionine, C – pH of isoelectric point of lysine, D – pH below of isoelectric point of lysine. Without addition: Control, Met – methionine, Lys – lysine. The results are presented as means (n = 6)

The addition of only amino acids to the emulsion had a prooxidant or antioxidant effect, depending on the pH of the environment (Figs. 1 and 2).

During incubation of emulsion at pH 5.74 and 4.2, a decrease in the methionine content was observed (Fig. 3). Significantly lower ($p < 0.05$) amino acid content relative to the initial value was observed after 6 days of storing the emulsion at both pH regarding samples with the addition of methionine only (14.0%), and regarding samples with the addition of methionine and rosemary or green tea at pH 4.2 (18.1 and 15.3% losses, respectively). The highest amino acid content was observed in samples with the addition of methionine and BHT. Amino acid losses in the sample with the addition of BHT amounted only to 3.8% at pH 5.74 and 5.3% at pH 4.2. Among natural additives the best protective activity toward methionine was exhibited by thyme in both pH values (losses of 6.2% in emulsion at pH 5.74 and 8.5% at pH 4.2) and green tea and rosemary extracts at pH 5.74 (6.7 and 8.2% losses, respectively). After nine days of storing the emulsion at pH 5.74 (methionine isoelectric point) recorded a lower percent of amino acid losses relative to the initial value in samples with the addition of thyme, rosemary and BHT than the emulsion at pH 4.2. Methionine losses in the sample without the addition of antioxidants was 11.2% lower in the case of the emulsion at pH 5.74 than in the emulsion at pH 4.2. These findings were analyzed statistically for any correlation between lipid oxidation products and methionine content. A high negative correlation occurs between peroxides and TBARS and methionine content in samples

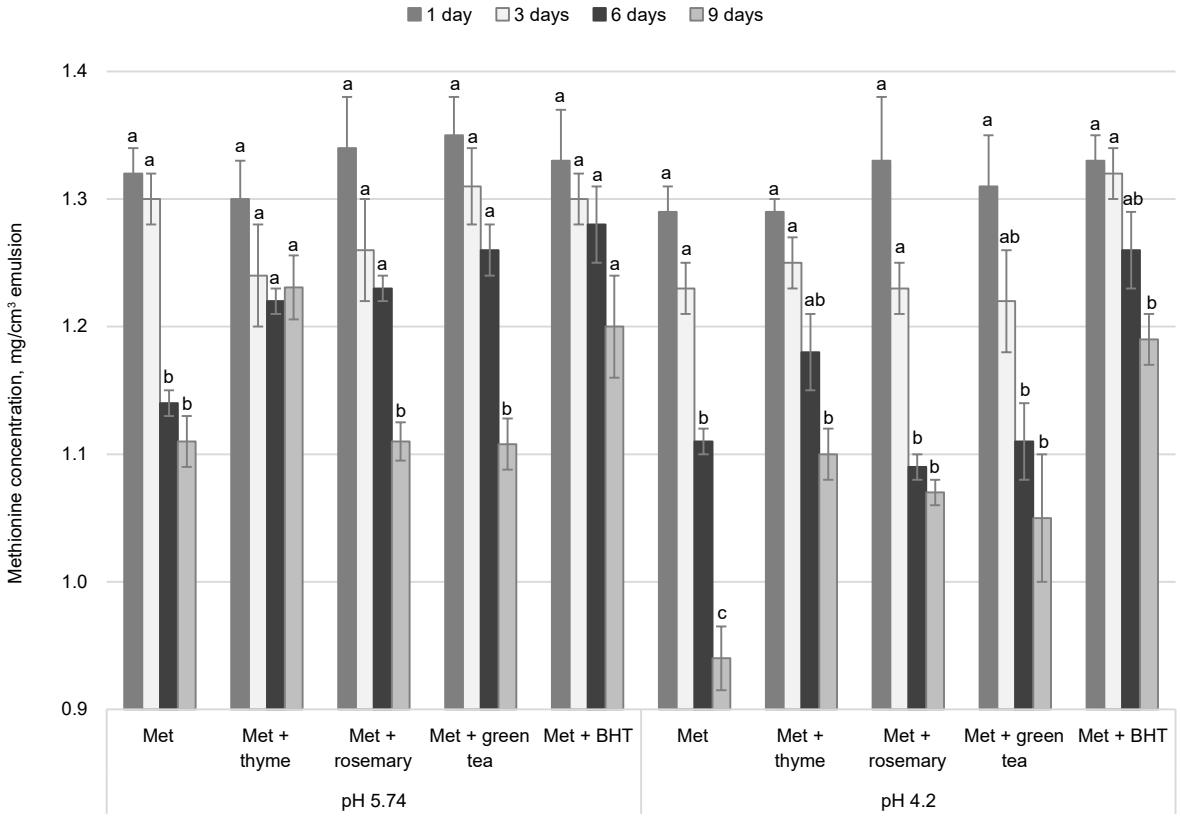

Fig. 3. The effect of additives on changes of methionine content during linoleic acid emulsion incubation in different pH conditions, mg/cm³ emulsion: 5.74 – pH of isoelectric point of methionine, 4.2 – pH below of isoelectric point of methionine, Met – methionine. Mean values (bars) with different letters are significantly different (one-way ANOVA, Tukey's test, $P = 0.05$)

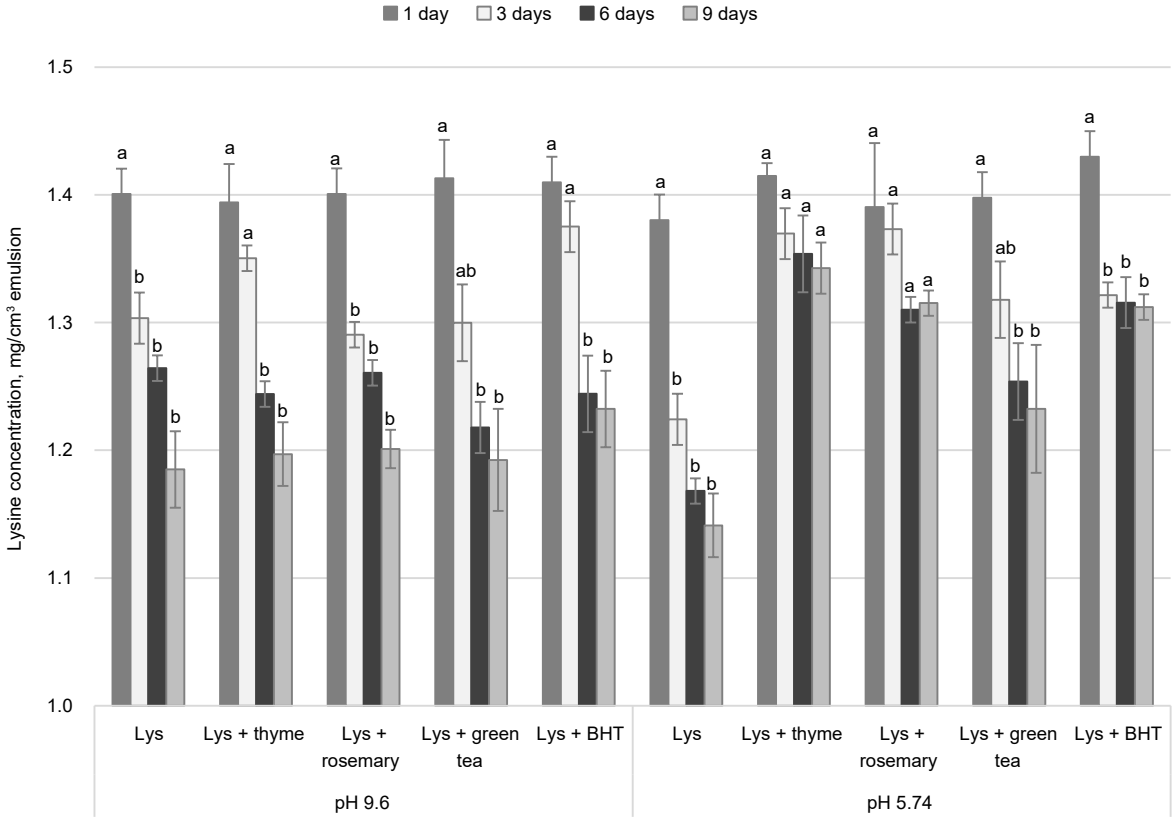

Fig. 4. The effect of additives on changes in lysine content during linoleic acid emulsion incubation in different pH conditions, mg/cm^3 emulsion: 9.6 – pH of isoelectric point of lysine, 5.74 – pH below of isoelectric point of lysine, Lys – lysine. Mean values (bars) with different letters are significantly different (one-way ANOVA, Tukey's test, $P = 0.05$)

without the addition of antioxidants (r = –0.83 and r = –0.98 at pH 5.74, respectively; r = –0.85 and r = –0.88 at pH 4.2, respectively). Changes in methionine content occurring in emulsions containing a mixture with tea extract and rosemary extract indicate high negative correlation with TBARS (r = –0.80 and r = –0.92, respectively). In the samples, in which methionine was introduced to the emulsion with the addition of rosemary, a total negative correlation with TBARS was determined. Lack of or low correlation dependence was determined in the case of the effect of peroxides on methionine content in the sample with the addition of BHT and tea extract (r = –0.01 and r = –0.24, respectively).

During incubation of the emulsion at pH 9.6 and pH 5.74, a decrease in lysine content was observed (Fig. 4). After three days of storing the emulsion at both pHs, a significant decrease in amino acid content was observed in the sample with the addition of the amino acid only, and with the addition of rosemary extract and BHT at pH 9.6 and pH 5.74, respectively. After six days of storing the emulsion at pH 9.6, it was determined that the amount of lysine was significantly lower when compared to the initial value in samples containing a mixture of amino acid with BHT, tea extract, thyme and rosemary extract, however in the emulsion at pH 5.74 in the sample with the addition of tea extract and BHT. In the final storage period of emulsion at pH 9.6 (9 days), differences in lysine content in samples with individual additives were statistically insignificant. In the case of emulsion at pH 5.74, the protective activity of BHT and thyme

and rosemary extracts toward the amino acid was observed. The lysine content in these samples was 14.97, 17.65 and 15.23% (respectively) higher compared to the sample with the addition of the amino acid only. The percentage decrease in lysine content in the final storage period was lower in the case of emulsion at pH 9.6 (lysine isoelectric point) than in the emulsion at pH 5.74 for samples containing amino acid only (15.4 and 17.3% losses, respectively). Correlation dependencies exhibited the low reactivity of peroxides and TBARS toward lysine. Existing dependence was detected only between amino acid content in the sample without other additives (emulsion at pH 5.74) and the sample containing tea extract (emulsion at pH 9.6) and peroxide content ($r = -0.82$ and $r = -0.81$, respectively). These correlation dependencies also confirmed secondary oxidation product reactivity toward lysine at pH 5.74 (sample containing only lysine, $r = -0.75$) and at pH 9.6 (sample containing lysine and BHT, $r = -0.66$).

DISCUSSION

Total polyphenol content in the plant extracts investigated is similar to the results presented in other publications. According to Gramza et al. (2005), green tea extracts are characterized by a polyphenol content from 300 to 800 mg CE/g (CE – (+)-catechin equivalents) of the extract, depending on the type of solvent used and its concentration. Friedman et al. (2008) determined individual catechin content in the leaves of green teas and found considerable diversity. The sum of catechins in the material fluctuated significantly (68.9–119.2 mg/g). Among them trace amounts of C and EGC were determined, the content of the remaining compounds was as follows: EC (6.4–12.3 mg/g), EGCG (49.5–75.3 mg/g), GCG (0.29–0.86 mg/g), ECG (11.4–28.8 mg/g), CG (1.18–2.52 mg/g). Similar results were obtained by Rusak et al. (2008), who analyzed the chemical composition of aqueous and ethanolic extracts of white and green tea. They found a significantly lower catechin content in white tea leaves; additionally, myricetin, kaempferol and quercetin were detected in trace amounts, higher in ethanolic than aqueous extracts. Differences between these results and those presented in the literature may come from the use of different extraction methods for phenolic compounds from plant material. Of considerable importance is the type of solvent used, its concentration, time and conditions of extraction and the differences in plant material. Gramza-Michałowska et al. (2008) evaluated the antioxidant activity of tea extracts (white, green, yellow, oolong and black) in linoleic acid emulsion. The results showed that highest activity, comparable to BHT and rosemary extract was evaluated in a sample with the addition of yellow tea ethanol extract. Huang et al. (1997) showed that the antioxidant's ability to inhibit lipid oxidation significantly depends on different factors, like its polarity, concentration, physical location and medium (e.g., pH, temperature, and ionic strength). The results of different studies have demonstrated that both non-polar and surface-active antioxidants (e.g., TBHQ, ascorbyl palmitate, BHT, α-tocopherol, carnosol) show higher efficiency in oil-in-water emulsions, since they remain in the oil droplets or accumulate at the oil-water interface, where the lipid oxidation process is most prevalent. It was found that polar antioxidants (e.g. ascorbic acid, carnosic acid, and rosmarinic acid) are the most effective in bulk oils, due to their accumulation at the air-oil interface or in reverse micelles within the oil where lipid oxidation reactions occur (Chaiyasit et al., 2007; Heins et al., 2007; McClements and Decker, 2000). The higher efficiency of nonpolar antioxidants in emulsions is a result of their concentration at the interface between the lipid and the polar phase, so that the antioxidant is ideally located to protect the lipids from radicals generated in the aqueous phase. Recent reports have shown that the formation of higher surface activity derivatives may lead to less effective antioxidant activity in oil-in-water emulsions. This indicates the importance of the other factors, e.g., changes in antioxidant structure affecting the stability of its derived radicals, interactions with metals or emulsifiers (Gordon, 2010).

The antioxidant efficiency to inhibit lipid oxidation depends greatly on pH. Many antioxidants have ionizable groups, which are strongly influenced by the pH of the surrounding aqueous phase. The antioxidant ionized form is much more polar than the non-ionized form and therefore has a greater affinity for aqueous solutions (Schwarz et al., 1996). Dawidowicz and Olszowy (2011) demonstrated that apart from diverse pH, BHT antioxidant activity is influenced by the type of metal ions and water content. In turn, Gramza et al.

(2006b) showed that antioxidative activity of tea extracts in methyl linoleate emulsion was not influenced by pH range. However, significant activity differences between samples at pH 3.6 and 9.0 have been noticed. Donnelly et al. (1998) reported that oxidation of a whey protein isolate-stabilized emulsion decreased with lower pH (3−7), but in a Tween 20 stabilized emulsion results showed a higher oxidation level with a decreasing pH. Mancuso et al. (1999) reported that low pH from 3.0 to 7.0 decreased lipid oxidation rates. However, Mei et al. (1998) found increased oxidation of sodium dodecyl sulfate-stabilized emulsions as pH decreased from 8.0 to 3.0, while oxidation of polyoxyethylene 10 lauryl ether and dodecyltrimethylammonium bromide emulsions remain unaffected. These results indicate that the effect of various pHs on lipid oxidation were contradictory. This phenomenon results from the different experimental plan adopted and the surfactant used.

The study conducted by Hęś et al. (2006) also showed that the addition of amino acids can influence emulsified fatty substrates in two ways. Antioxidant or prooxidant activity of lysine and methionine depended on such factors as the concentration and type of fatty substrate, the presence of water and catalysts, the pH of the environment or the structure of amino acid (zwitterion form or cation form) and the concentration of amino acid. It was demonstrated that lysine in its isoelectric point (pH 9.6, zwitterion form) always exhibited strong antioxidant properties. Instead, at a lower pH (cation form) it accelerated the formation of oxidation products. Methionine at its isoelectric point (pH 5.74) also exhibited higher antioxidant properties than in a pH below these points (pH 4.2). Methionine exhibited stronger antioxidant activity than lysine. Research by Filippenko and Gribova (2011) demonstrated that the emulsion of sunflower oil lysine hydrochloride was a more effective antioxidant than methionine. The antioxidant properties of the amino acid residues are associated with their chemical structures. The most reactive and susceptible to oxidation are –SH, $–NH_2$ and –OH groups and the heterocyclic rings. Sulfhydryl groups exhibit the ability to scavenge free radicals and decompose peroxides. The size and type of the heterocyclic ring also has an effect on the antioxidant activity of the amino acid: the larger and more heteroatomic the ring is, the better its antioxidant properties. The most active amino acids with antioxidant properties are histidine, lysine, as well as phenylalanine, tryptophan, proline, methionine, cysteine and cystine, which are essentially the same, and the losses of which, due to protein-lipid interactions, cause a decrease in the nutritional value and functional properties of the protein. Peptides with a high lysine and histidine content absorb more strongly at the interface of the emulsion droplets and chelate more transition metals; and thus exhibit good protective properties. Lysine inhibits fatty acid oxidation. Methionine also has proven antioxidant properties and is treated as an endogenous antioxidant; however, its effectiveness is greatly determined by the proximity of other amino acids (Levine et al., 1996). Some amino acids have been shown to act as pro-oxidants when they are present in relatively high concentrations (Mc-Clements and Decker, 2000). Hidalgo et al. (2003) reported that oxidized lipid/amine reaction products antioxidative activity relate to their ability to scavenge radical species. It was found that antioxidative activity increased with the polymerization suffered by the initially formed some of the monomer oxidized lipid/amine reaction products. However, the synergism observed between the antioxidative potentials of these compounds and those of both natural (Hidalgo et al., 2007) and synthetic antioxidants (Ahmad et al., 2008) are also noticeable. Findings reveal that a slight initial oxidation was observed to increase fatty acid stability in polyunsaturated fatty acid/protein mixtures (Alaiz et al., 2008).

The degree to which products of lipid oxidation affected amino acid content depended on the pH of the environment. In the final stage of incubation losses in the content of methionine in each samples and lysine in samples without the addition of antioxidants were lower in their isoelectric points than below these points. In turn, lysine losses in samples with the addition of antioxidants were higher in the isoelectric points. The destructive effect of the products of fatty acid oxidation on the content of amino acids was confirmed in a paper by Hęś et al. (2006), among other studies. Also, the different pH conditions of the emulsion systems investigated (zwitterion form and cation form) had an effect on lysine and methionine losses. There is a lack of data in the available literature on the reactivity of amino acids depending on the ion structure. This also applies

to the susceptibility of these amino acid forms to interactions with lipid oxidation products (e.g., hydroperoxides and carbonyl compounds). The nucleophilic addition reaction (the presence of keto and aldehyde compounds) encompasses a nucleophilic attack by the basic nitrogen compound (H_2N-G) on the carbon atom of the carbonyl group. Protonation of the oxygen atom of this group makes the carbon atom more susceptible to the nucleophilic attack and thus, if we consider a carbonyl compound, higher acidity of the environment will promote addition reaction. However, the nitrogen compound can also be a subject of protonation. Protonation of H_2N-G leads to the formation of $^{+}H_3N$-G ion, which does not contain a free electron pair and is not a nucleophile. Therefore, higher acidity of the environment should not promote addition. For this reason the conditions under which addition occurs at the slowest rate are the result of a compromise: the solution cannot be too acidic, so as not to provide too high an amount of protonic carbonyl compound, but it also cannot be too low in acidity for the free nitrogen compound concentration to be lowered (Hęś, 2017). This shows that the selection of the proper pH of the environment constitutes the basis for limiting the nucleophilic addition reaction. Introducing antioxidants to a model emulsion system significantly decreases losses of the amino acids investigated. In other studies it was demonstrated that antioxidants can limit amino acid losses in meat products (Hęś et al., 2007). However, a study conducted by Hęś et al. (2011) demonstrated that the addition of plant extracts (thyme, rosemary and green tea) and BHT to dried meat not only did not exhibit protective properties toward amino acids, but in some cases also increased their loss. Such discrepancies in results show the complexity of interactions between antioxidants, lipid oxidation products and amino acids.

CONCLUSIONS

During the study it was found that numerous factors had an effect on the process of emulsified linoleic acid oxidation, i.e. increased storage temperature, differing pH, free access to oxygen, addition of antioxidants and amino acids. By limiting the formation of products of emulsion oxidation, the antioxidants used exhibited protective properties towards lysine and methionine. The effect of products of lipid oxidation on the content of amino acid depended on the pH of the environment. Methionine and lysine losses in samples without the addition of antioxidants were lower in their isoelectric points than below these points. No evident effect of pH on changes in amino acid content in emulsion systems with the addition of antioxidants was observed. The protective properties of antioxidants towards methionine and lysine were higher in a pH of isoelectric point and in a pH lower than isoelectric point, respectively. These results justify the need for further study to explain the causes and consequences of oxidation reactions of lipids with amino acids and proteins. This is a very important aspect from both the technological and nutritional point of view, because the addition of natural and synthetic antioxidants may affect the preservation of higher nutritional value of protein in stored products. Polyphenols derived from plant species may exhibit a number of properties in various model systems. Enrichment of food products with polyphenols from thyme, rosemary, tea extracts etc. can influence their oxidative stability and nutritional value in a beneficial way.

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