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Cholesterol oxides in chicken liver pâté supplemented with coenzyme Q_{10} and ascorbic acid*

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A b s t r a c t: Oxidation is a major reason for deterioration of fat and fat-containing meat-based foods because of its negative effects on sensory quality, nutritional value and as well may be responsible for the production of toxic compounds. The formation of secondary oxidation products of cholesterol (COPs) in meats is of concern, as they are known to be absorbed from dietary sources and display a wide range of potentially harmful effects, such as cytotoxycity, mutagenicity and cancerogenicity. Meat processing facilities introduced antioxidant additives and technique to prevent oxidative damages.

The main purpose of this study was to determine whether supplemental addition of coenzyme Q_{10} and ascorbic acid, used individually or in combination, could prevent oxidative damages in chicken liver pâté, which reflects in reduced formation of cholesterol oxidation products (COPs) and in well-preserved sensorial quality. Three separate groups of chicken liver pâtés were manufactured: control (C) without added antioxidants, group Q_{10}, supplemented with coenzyme Q_{10} (0.2 g/kg) and group Q_{10}AA, with added Q_{10} (0.2 g/kg) and ascorbic acid (2 g/kg). All products were pasteurized (82 °C). Four COPs, including 7α-, 7β-, 20- and 25-HC, were found. COP radical scavengers' function of coenzyme Q_{10} alone was not statistically confirmed (C = 3.26 mg/kg vs. Q_{10} = 2.86 mg/kg). The most efficient was combination Q_{10}AA, where formation of COPs was below the limit of detection. Supplement of coenzyme Q_{10} affected cholesterol levels in pâté after pasteurization.

***Key words**: meat product, pâté, coenzyme Q_{10}, ascorbic acid, cholesterol oxides.*

Introduction

Oxidation is a major reason for deterioration of fat and fat-containing meat-based foods because of its negative effects on sensory quality, nutritional value and as well may be responsible for the production of toxic compounds (*Ghiretti et al.,* 1997). The formation of secondary oxidation products of cholesterol (COPs) in meats is of concern, as they are known to be absorbed from dietary sources (*Linseisen et al.,* 1998) and display a wide range of potentially harmful effects, such as cytotoxycity, mutagenicity and cancerogenicity. Meat processing facilities introduced antioxidant additives and technique (vacuum processing, vacuum packaging, etc) to prevent oxidative damages.

Possible cholesterol oxidation can be managed by the addition of different antioxidants to meat/pâté-batter. The antioxidants tested in this study were chosen from those already known for their antioxidant properties - ascorbic acid (*Grau et al.,* 2001) and coenzyme Q_{10} (*Lambelet et al.,* 1992). Especially interesting is coenzyme Q_{10}, known as important lipid soluble antioxidant that protects cells from damage caused by the action of free radicals (*Brea-Calvo et al.,* 2006). Furthermore, the aim of the present work was to determine the effect of supplemented antioxidants against oxidation of cholesterol constituents in pasteurized meat product – pâté and, from nutritional point of view, to assess this product which may have beneficial effects on health.

Material and methods

Material

Mechanically separated chicken meat and chicken liver were purchased in local company. Chicken liver pâté contained 50% mechanically separated chicken meat, 15% sunflower oil, 15% chicken liver and 20% water. Additives added were as follow: nitrite salt (1.4%), Na-caseinate (1.8%), powdered milk (1.0%), and spices.

Preparation of three groups of pâté regarding to amounts of antioxidants added was as follows: control group (C) was made without antioxidants; to 1 kg of homogeneous mass 35 g of sunflower oil

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was added, mixed for 2 minutes, filled into eight glass jars and sealed with screw caps. Second group (Q_{10}) was made following the procedure described, except coenzyme Q_{10} (Sigma, C9538), (0.2 g/kg of pâté batter) was previously dissolved into sunflower oil. In the third group (Q_{10}AA), besides coenzyme Q_{10} dissolved in oil (0.2 g/kg of pâté batter), we also added ascorbic acid (Riedel-de Haën, 33034), (2 g/kg of pâté batter). Thermal treatment was as follows: four glass jars from each group were pasteurized (up to internal temperature of 80 °C). Pasteurized pâtés were cooled slowly and kept for 24 hours in the refrigerator (4 °C), when sensory and chemical analyses were carried out. Chicken liver pâté was made in triplicate.

Methods

pH was determined directly using a spear combined glass-gel electrode (type 03, Testo pH electrode) with a thermometer (type T, Testo penetration temperature probe) connected to a pH meter (Testo 230, Testo). pH was measured in the centre of each pâté, twice.

Minolta CR 200b colorimeter (Illuminant C, 0° viewing angle) was used to determine the CIE L^* (lightness), a^* (±, red to green) and b^* (±, yellow to blue) values of the pâté samples. A white ceramic tile with a specification of $Y = 93.8$, $x = 0.3134$, $y = 0.3208$ was used to standardise the colorimeter. The CIE L^*, a^*, b^* colour values were measured at four different locations on the surface of each pâté.

For the purpose of evaluating sensory qualities, a panel composed of four qualified and experienced panellists in the field of meat products was appointed, while sensory properties of coded samples were determined in a standard sensory laboratory. The same panel evaluated all samples during one session. The panel assessed the samples (50 g placed in white china plates), separately one by one in groups that consisted of 5 samples.

For the purpose of evaluation, the panel applied the analytical-descriptive test (*Golob et al.,* 2005), on the basis of a preliminary testing. The analysis was performed by scoring sensory properties by assigning a non-structured scale from 1 to 7 points, where higher score means more expressed property. An exception was the texture, which was evaluated by scoring on a scale 1-4-7. The score of 4 points was considered optimal, scores of 4.5 or more indicated firm and those of 3.5 points or less indicated tender texture. Sensory descriptors of pâté are the following: colour hue (from white to pink colour), colour intensity (from pale to dark colour), texture (from firm, hard to tender), odour (odour characteristic), flavour (flavour characteristic), and acidity (intensity).

The analytical method for the determination of coenzyme Q_{10} in pâté samples (rich in fat) was developed and validated in our laboratory. The characteristics of this method are innovative sample preparation (using classical extraction and novel solid phase extraction) and LC-MS quantification in ESI+ mode (*Lušnic,* 2008).

Homogenized samples (3.000 ± 0.001 g) were weighted in centrifuge tubes and dissolved in 15 mL water at a temperature of 40 °C, then well shaken (5 min) and transferred to an ultrasonic bath (Bransonic 3510E-DTH, Branson, Germany) for 15 min at 40 °C. Extraction was performed twice with 25 mL diethyl ether (Merck, 1.00921), by vortexing for 5 min, ultrasonication for 15 min, and centrifugation for 10 min (Eppendorf, Centrifuge 5810 at 1750×*g*), each time. The combined extracts were concentrated and dried under a steam of dry nitrogen. Solid-phase extraction followed, using a Supelclean™ ENVI™ Florisil® column (Supelco, 57053).

HPLC analyses were performed on an Agilent 1100 system. The analytical column was a Gemini C18 (150 mm × 2 mm, particle size 3 μm) from Phenomenex (Torrance, CA, USA; 00F-4439-B0). Coenzyme Q_{10} was determined through its retention time and the m/z ([M+H] = 864.4) of the coenzyme Q_{10} standard (Sigma, C9538). The flow rate of the mobile phase of acetonitrile (Merck, 1.00030): 2-propanol (Merck, 1.00998), (60/40, v/v) was 0.25 mL min^{-1} at 25 °C. Injection volume was15 μL.

The mass-selective detector (Micromass Quattro micro® API, Waters, USA) was equipped with electrospray ionisation (ESI) probe, cone voltage of 63 V was used, a source temperature of 120 °C, and a capillary voltage of 4.0 kV for positive ionisation of the analytes (ESI+ mode). The dessolvation gas (nitrogen) was heated to 350 °C, flow was set to 400 L h^{-1}. The cone gas flow was 50 L h^{-1} (nitrogen). The detection (m/z = 864.4 [M+H]) was performed in selected ion recording (SIR) mode. The processing of the data was carried out using the quantify function of the MassLynxTM V4.0 (2004) software.

The cholesterol and cholesterol oxide content in the pâté was determined according to a modified method of *Ubhayasekeraa et. al,* (2004), followed by gas chromatography (GC) analysis. The data are expressed as mg/kg pâté.

Homogenized samples (2.000 ± 0.001 g) were weighted in a 100 mL screw-ca ped Erlenmeyer flasks, to which was added 100 μL (2.30 mg/g hexane) solution of 5α-cholestan-3η-ol (Merck, 8.41513) as internal standard. Three mL dichloromethane (Merck, 1.06044) and 7 mL 1.0 mol L^{-1} KOH

(Merck, 1.05033) in ethanol (96%; Merck, 1.00971) were added. This mixture was vortexed (IKA, RT10 Power) and left overnight (18-20 h) in the dark at room temperature. The saponificated solution was transferred into 50-mL centrifuge tubes, and 10 mL diethyl ether (Merck, 1.00921) and 10 mL water were added. The samples were vortexed for 5 min, ultrasonicated for 15 min, and centrifuged for 10 min (1750×*g*; Centrifuge 5810, Eppendorf). An aliquot of the diethyl ether extract (5 mL) was dried under vacuum by using a nitrogen flush. The sample was prepared for solid phase extraction.

The solid phase extraction applied a Strata Si-1 column (Phenomenex; 8B-S012-EAK), pre-conditioned with 3 mL hexane (Merck, 1.04371) and equilibrated with a 2 mL mixture of hexane (Merck, 1.04371): diethyl ether (Merck, 1.00921), (3/1, v/v). The loaded sample was dissolved in 2 mL of hexane: diethyl ether mixture. The Strata Si-1 cartridges were washed with hexane (3 mL) and 3 mL hexane: diethyl ether mixture. The analytes were eluted with 2 mL of 2-propanol (Merck, 1.00998): acetonitrile (Merck, 1.00030) (2/3, v/v) mixure.

Prior to GC analysis all samples were derivatised to TMS-ethers. After evaporating the solvent under nitrogen stream, 100 μL of Tri-Sil reagent (Pierce, IL, USA) was added and the tubes were kept at 60 °C for 45 min. The solvent was evaporated under a stream of nitrogen and the TMS-ether derivatives were dissolved in 100 μL of hexane. The samples were sonicated in an ultrasonic bath for 1 min, centrifuged for 3 min and then analysed by GC.

The content of COPs was determined by GC on an Agilent Technologies 6890 gas chromatograph with a flame ionisation detector and a 25 m × 0.2 mm × 0.33 μm DB-5MS capillary column (Agilent Technologies; Cat.No. 128-5522). Separation and detection were performed under the following conditions: initial column temperature 150 °C (hold 4 min), gradient 4 °C/min to 180 °C (hold 5 min), gradient 3 °C/min to 240 °C (2 min hold); injector temperature 250 °C; detector temperature 280 °C; injector: split: splitless, 1:30 split ratio; injection volume 1μL; carrier gas 2.3 mL/min He; make-up gas (N_2), flow 45 mL/min; detector gases: 40 mL/min H_2 and 450 mL/min synthetic air (21% O_2). The cholesterol and COPs were determined according to their retention times in comparison to standards (cholesterol, Sigma, C-8667; 7α-hydroxycholesterol, Steraloids Inc., C6420-008; 7β-hydroxycholesterol, Sigma, H-6891; 20α-hydroxycholesterol, Sigma, H6378-5MG; 22 (S)-hydroxycholesterol, Sigma, H5884-5MG; and 25-hydroxycholesterol, Sigma, H-1015).

The experimental data were evaluated statistically using the SAS/STAT programme (*SAS Software,* 1999). The basic statistical parameters were calculated by the MEANS procedure and the data were tested for normal distribution and analysed according to a GLM (General Linear Model) procedure.

Statistical model 1 was used for analysing the data for pHs and $L^*a^*b^*$ colour, sensory properties, contents of cholesterol, cholesterol oxides and coenzyme Q_{10}. Model 1 is represented as:

$$y_{ijk} = \mu + G_i + R_j + e_{ijk}$$

were y is the observed parameter, μ – the general mean, G_i – the effect of coenzyme Q_{10} and ascorbic acids (C – control group, Q_{10} – coenzyme Q_{10} addition and Q_{10}AA – coenzyme Q_{10} and ascorbic acid addition), R_j – the effect of production repetition (l to 3), and e – a residual random term with variance σ_{2e}. The means for the experimental groups were obtained by using the Duncan procedure, and they were compared at a 5% probability level.

Results and discussion

As the consumption of meat products increases, it has become important to look for the possible formation of potentially harmful substances, such as COPs, during their production, as well as to evaluate the impact of added antioxidants on COPs formation, pH value, colour values and sensory quality of these products.

General pH values in pâté (Tables 1) fall in a normal range for products of such a type. A decrease of approximately 0.2 pH units has been observed in group Q_{10}AA. pH values may vary due to presence or absence of ascorbic acid in pâté as expected.

The instrumental measurement of surface colour (Table 1) has shown a significant difference between the control group and those supplemented with antioxidants, for all three $L^*a^*b^*$ values. The colour of the group with added ascorbic acid was noticeably redder than other ones. It is known that reducing agents, such as ascorbic acid and its salts, are used to accelerate curing, create uniform colour, while the remainder of them acts as antioxidants (*Honikel,* 2008).

Generally speaking sensory evaluated pâté colour hue has been slightly improved by adding coenzyme Q_{10} and ascorbic acid in comparison to control one. No differences in texture were observed, which was generally evaluated by panellists as slightly to tender. Odour of all experimental pâté groups was like in control group. As for flavour, it can be said that Q_{10}AA group was slightly worse then in control and Q_{10} groups. Except in control group, acidity was observed in experimental paté groups.

Table 1. Effects of coenzyme Q_{10} and ascorbic acid supplement on pH values, colour values (L^*, a^* and b^*), sensory properties, content of cholesterol, cholesterol oxides (COPs) and coenzyme Q_{10} in chicken liver pâtés thermally treated to internal temperature of 82 °C (Model 1, Duncan test, $\alpha = 0.05$).
Tabela 1. Uticaj dodataka koenzima Q_{10} i askorbinske kiseline na pH vrednost, vrednosti boje (L^*, a^* i b^*), senzorna svojstva, sadržaj holesterola, oksida holesterola (COP) i koenzima Q_{10} u pilećoj jetrenoj pašteti termički obrađenoj do unutranje temperature 82°C (Model 1, Duncan test, $\alpha = 0,05$).

Parameter /Group	C	Q_{10}	Q_{10}AA
pH value (n = 3)	6.4 ± 0.0^{a}	6.4 ± 0.0^{a}	6.2 ± 0.0^{b}
Colour values (n = 12):			
L^*	49.3 ± 1.4^{a}	48.4 ± 1.0^{b}	49.5 ± 0.7^{a}
a^*	6.3 ± 0.7^{b}	6.2 ± 0.8^{b}	6.9 ± 0.6^{a}
b^*	9.5 ± 0.3^{c}	9.8 ± 0.6^{b}	10.6 ± 0.3^{a}
Sensory properties (score) (n = 12):			
Colour hue (1-7)	5.8 ± 0.2^{b}	5.8 ± 0.3^{b}	6.1 ± 0.2^{a}
Colour intensity (1-7)	6.0 ± 0.3^{a}	6.2 ± 0.3^{a}	6.2 ± 0.3^{a}
Texture (1-4-7)	3.3 ± 0.3^{a}	3.3 ± 0.3^{a}	3.4 ± 0.4^{a}
Smell (1-7)	5.8 ± 0.2^{a}	5.9 ± 0.3^{a}	5.9 ± 0.3^{a}
Flavour (1-7)	5.9 ± 0.3^{a}	5.9 ± 0.3^{a}	5.7 ± 0.2^{b}
Acidity (1-7)	1.0 ± 0.1^{b}	1.3 ± 0.4^{ab}	1.5 ± 0.4^{a}
Cholesterol oxides (mg/kg) (n = 6):			
7α-hydroxycholesterol	0.56 ± 0.5^{a}	0.64 ± 0.6^{a}	$< 0.01^{b}$
7β-hydroxycholesterol	1.09 ± 0.3^{a}	1.21 ± 0.6^{a}	$< 0.01^{b}$
20-hydroxycholesterol	0.79 ± 0.5^{a}	0.63 ± 0.5^{a}	$< 0.01^{b}$
25-hydroxycholesterol	0.81 ± 0.6^{a}	0.38 ± 0.4^{b}	$< 0.01^{c}$
ΣCOP	3.26 ± 1.2^{a}	2.86 ± 2.0^{a}	$< 0.01^{b}$
Cholesterol (mg/kg) (n = 6)	612 ± 12^{a}	591 ± 7^{b}	602 ± 3^{ab}
Coenzyme Q_{10} (mg/kg) (n = 6)	37 ± 2^{c}	117 ± 62^{b}	219 ± 35^{a}

C – Control group. Q_{10} – Addition of coenzyme Q_{10}. Q_{10}AA – Addition of coenzyme Q_{10} and ascorbic acid. n − Number of observations within group. Means with a different superscript within rows (a, b, c) differ significantly ($p \leq 0.05$). < 0.01 below limit of detection./
C – kontrolna grupa. Q_{10} – Dodatak koenzima Q_{10}. Q_{10}AA – Dodatak koenzima Q_{10} i askorbinske kiseline. n − Broj ispitivanih uzoraka po grupi. Srednje vrednosti sa različitim oznakama u superskriptu (a, b, c) se značajno razlikuju ($p \leq 0, 05$). < 0,01 ispod limita detekcije.

According to literature data several COPs, such as CT (cholestanetriol), α-CE (cholesterol-5α,6α-epoxide), β-CE (cholesterol-5β,6β-epoxide), 7α-HC (7α-hydroxycholesterol), 7β-HC (7β-hydroxycholesterol), 7-K (7-ketocholesterol) and 25-HC (25-hydroxycholesterol), among others, have been found in fresh and processed food of animal origin (*Paniangvait et al.* 1995*; Grau et al.,* 2001*; Lee et al.,* 2001). In addition to the possible formation of those oxidized derivatives, components such as free lipids and cholesterol affect their sensory quality during thermal processing of meat products.

In our experiments several COPs, such as 7α-HC, 7β-HC, 20-HC (20-hydroxycholesterol) and 25-HC, have been found in pâtés (Table 1). The cholesterol oxides determined in pasteurized pâté has shown a significant ($p < 0.05$) decrease of all four oxides in Q_{10}AA group in comparison to the control group. Formation of COPs in group with Q_{10} was observed in the same extent as in control group, with exception of 25-HC (Q_{10}), where lower quantities were determined in comparison to the control group.

The significant effect of antioxidant supplement on COPs formation in chicken liver pâté is based on individual or cooperative function of coenzyme Q_{10} and ascorbic acid. Coenzyme Q_{10}, lipoidal substance, has been demonstrated to act as radical

scavenger (*Navas et al.,* 2007). In this study, COP radical scavengers' function of coenzyme Q_{10} in pasteurized pâté limited formation of COPs with no great significance (C group 3.26 mg/kg *vs.* Q_{10} group 2.86 mg/kg).

As it was said, the radical-scavening antioxidant functions were not only individual, but also cooperative and sometimes synergistic with other antioxidants. In this study the most effective combination in inhibition of COP has been proved to be coenzyme Q_{10} with ascorbic acid. It was found that combination of coenzyme Q_{10} with ascorbic acid decreases formation of COPs below limit of detection (C group 3.26 mg/kg *vs.* Q_{10}AA group < 0.01 mg/kg). It has been reported that ascorbic acid does not spare ubiquinol, suggesting that ascorbic acid does reduce ubiquinol semiquinone efficiently. *Niki* (1997) observed that ascorbic acid did not spare ubiquinol, nor did ubiquinol spare ascorbic acid, therefore they must function independently.

It should be noted here that coenzyme Q_{10} in oxidized form (ubiquinon) was added in pâté-batter. Obviously, very efficient reductive mechanism in pâté matrix keeps coenzyme Q_{10} in active form (ubiquinol).

We were aware that raw meat products may contain some COPs, as demonstrated by *Rodriguez-Estrada et al.,* 1997, but purpose of this study was to evaluate decreasing in COP after supplement of different antioxidants in pâté, after thermal treatment. *Vicente et al.* (2007) assumed that the conversion of cholesterol to COPs is temperature dependent. In fact, up to 120 °C, no, or very little conversion is expected. This ascertainment is in slight disagreement with our findings, where in all control samples (without added antioxidants) thermally treated to internal temperature 82 °C (pasteurized) a noticeable amount of COPs was found (3.26 mg/kg).

Supplement of coenzyme Q_{10} and ascorbic acid in pâté-batter affected cholesterol level in pâté after pasteurization. Of all analyzed groups, the highest cholesterol levels were found in the control and Q_{10}AA groups (612 mg/kg and 602 mg/kg, respectively) and the lowest in the Q_{10} group (591 mg/kg). Aberrations in cholesterol content in groups supplemented with antioxidants compared to control group are difficult to explain, presumably coenzyme Q_{10} alone more successfully lowered cholesterol level in samples.

In this study control groups of pasteurized pâtés contained 37 mg/kg coenzyme Q_{10} (Table 1). After supplement of coenzyme Q_{10} in pâté-batter (0.2 g/kg) about 50% of it broke during thermal treatment. The highest content of coenzyme Q_{10} remained in groups supplemented with ascorbic acid (219 mg/kg). The acceptable daily intake (ADI) of coenzyme Q_{10} is 12 mg/kg/day; calculated from the no-observed-adverse-effect level (NOAEL) is 720 mg/day for a person weighing 60 kg (*Hidaka et al.,* 2008). Risk assessment for coenzyme Q_{10}, based on various clinical trial data, indicates that the observed safety level (OSL) for coenzyme Q_{10} is 1200 mg/day/person (*Hathcock et al.,* 2006*; Hidaka et al.,* 2008*)*. Therefore the addition of coenzyme Q_{10} in pâté was below acceptable daily intake and was in accordance with the recommendations.

Conclusion

Modern consumers require natural, fresh and nutritional food which may have beneficial effects on health. This is a reason why the oxidation of cholesterol in meat and meat products has received considerable attention. One of the possible ways to prevent occurence of COPs in meat products is simultaneous use of antioxidants. From this study it can be concluded that the most effective in inhibition of COPs has been proved to be the combination of coenzyme Q_{10} with ascorbic acid. This combination decreases formation of COPs below the limit of detection. It should be noted that the supplement of coenzyme Q_{10} in pâté-batter also lowered cholesterol level in pâté after pasteurization. Furthermore, meat products supplemented with coenzyme Q_{10} can be the source of coenzyme Q_{10} in daily diet.

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Oksidi holesterola u pilećoj jetrenoj pašteti sa dodatkom koenzima Q_{10} i askorbinske kiseline

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R e z i m e: Cilj naših istraživanja bio je da se utvrdi uticaj dodatka koenzima Q_{10} i askorbinske kiseline, pojedinačno ili zajedno, na sprečevanje oksidativnog kvara u pilećoj jetrenoj pašteti, koji može da se odrazi u smanjenom formiranju produkata oksidacije holesterola i očuvanju senzorskog kvaliteta paštete. Formirane su tri grupe pilećih jetrenih pašteta: kontrolna grupa (C), bez dodatka antioksidanata; grupa (Q_{10}), sa dodatkom koenzima Q_{10} (0,2 g/kg) i grupa (Q_{10}AA) sa dodatkom iste količine Q_{10} (0,2 g/kg) i askorbinske kiseline (2 g/kg). Svi proizvodi bili su pasterizovani (82 °C). Dokazano je prisustvo četiri proizvoda oksidacije holesterola, 7α-, 7β-, 20- i 25-HC. Funkcija koenzima Q_{10} kao odstranjivača radikala oksida holesterola u ovoj studiji nije bila statistički potvrđena (C = 3,26 mg/kg vs. Q_{10} = 2,86 mg/kg). Najefikasnija je bila kombinacija Q_{10}AA, gde je formiranje proizvoda oksidacije holesterola ispod granice detekcije. Dodatak koenzima Q_{10} je uticao na sniženje sadržaja holesterola u pašteti nakon pasterizacije.

***Ključne reči**: pileća jetrena pašteta, koenzim Q_{10}, askorbinska kiselina, oksidi holesterola.*

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