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Role of *Melissa officinalis* in cholesterol oxidation: antioxidant effect in model systems and application in beef patties

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ABSTRACT

Cholesterol oxidation products (COPs) constitute a known health risk factor. The antioxidant effect of a lyophilized aqueous *Melissa officinalis* extract against cholesterol degradation and COPs formation during a heating treatment was evaluated in a model system (180°C, 0-180 min) at a ratio 2 mg extract / 100 mg cholesterol. Furthermore, the plant extract was subsequently added to beef patties alone or incorporated within an oil-in-water olive oil emulsion to assess its effectiveness during cooking. Melisa extract protected cholesterol from thermal degradation in the model system, yielding higher remaining cholesterol and lower COPs values throughout the whole heating process. Maximum total COPs were achieved after 30 and 120 min heating for control and melisa-containing samples, respectively. In cooked beef patties, even though the olive oil emulsion was used as flavour-masking approach, melisa extract off-flavour limited the maximum dose which could be added. At these doses (65 µg/g and 150 µg/g without and with the emulsion, respectively), no additional protective effect of melisa over the use of the emulsion was found. Addition of natural extracts into functional foods should definitively take into account sensory aspects.

Keywords: cholesterol oxidation, antioxidant, model system, lemon balm, oddflavor, beef patties

Chemical compounds studied in this article: cholesterol (PubChem CID: 5997); rosmarinic acid (PubChem CID: 5281792); 7-ketocholesterol (PubChem CID: 91474); 7alpha-hydroxycholesterol (PubChem CID:107722); 7beta-hydroxycholesterol (PubChem CID: 473141); 5,6beta-epoxycholesterol (PubChem CID: 108109); 25-hydroxycholesterol (PubChem CID: 65094); cholestanetriol (PubChem CID: 53471228).

HIGHLIGHTS

1 - A lyophilized *Melissa officinalis* extract protected cholesterol from thermal degradation.

2 - Melisa extract inhibited COPs formation at 180 ºC up to 180 min.

3 - Melisa extract off-flavour restricted the viable dose to be added into beef patties.

4 - No protective effect was found in meat patties at sensory acceptable doses**.**

5 - An olive oil in water emulsion exerted antioxidant effect in beef patties

1. INTRODUCTION

Cholesterol Oxidation Products (COPs) have been related to several diseases (atherosclerosis, neurodegenerative diseases, mutagenic and carcinogenic effects, etc) (Otaegui-Arrazola, Menéndez-Carreño, Ansorena & Astiasarán, 2010; Biasi et al., 2013). They can be formed endogenously and also absorbed from the diet. As cholesterol is present in a variety of animal food samples, thermal oxidation, photooxidation and autooxidation can take place, compromising the safety of food. Thus, minimizing the formation of COPs leads to safer food.

The incorporation of antioxidants has been proposed as a good strategy for preventing sterol oxidation. Promising results have been reported in model systems (Xu, Guan, Sun & Chen, 2009; Yen, Inbaraj, Chien & Chen, 2010; Kmiecik, Korczak, Rudzinska, Kobus-Cisowska, Gramza-Michalowska & Hes, 2011), using direct application of antioxidants on cholesterol and also on fat matrices, such as triglycerides and lard. Butylhydroxytoluene, Conjugated Linoleic Acid, tocopherol, quercetin, green tea catechins and rosemary extracts, among others, are some of the tested antioxidants. A higher interest on natural antioxidants than on synthetic ones is nowadays increasing among industries and researchers, given their similar or even higher activity (Xu et al., 2009; Kmiecik et al., 2011) and their assumed safer and healthier properties.

Melissa officinalis is a medicinal plant, usually intaken as infusion, with a variety of beneficial effects, i.e. anti-depressive, anxiolytic, anti-tumoral, neurobiological and it has also been involved in the regulation of lipidaemic disorders and in the prevention of oxidative damage (Encalada et al., 2011; Fazli et al., 2012; Jun et al., 2012; Taiwo et al., 2012; López, Martin, Gomez-Serranillos, Carretero, Jager & Calvo, 2009). Its high

antioxidant capacity, due to the presence of phenolic compounds, mainly rosmarinic acid (Barros, Dueñas, Dias, Sousa, Santos-Buelga, 2013), has induced its addition, mainly as extracts, in foods to prevent lipid oxidation both for functional and technological purposes (Fazli et al., 2012, Petrovic at el., 2012, Berasategi et al., 2011; García-Iñiguez de Ciriano et al., 2010a; Poyato, Navarro-Blasco, Calvo, Cavero, Astiasarán & Ansorena, 2013). However, up to our knowledge, the potential inhibitory effect of this plant against cholesterol oxidation and formation of cholesterol oxidation products has not been evaluated yet.

When selecting a natural antioxidant and the concentration to be added to foodstuffs, sensory impact on the product (such as flavor or color) should be considered to achieve desired traits (Karre, López & Getty, 2013). These attributes are determinants of whether a consumer will purchase a specific type of meat or not (Goodson et al., 2002). When using melisa, sensory aspects have been evaluated on different meat derivatives, giving rise to products in which no sensory problems were noticed when using up to 686 µg /g in the case of dry fermented sausages (García-Iñiguez de Ciriano et al., 2010a) and up to 965 µg /g in the case of cooked pork sausages (Berasategi et al., 2011). It is worth noting that these are meat derivatives with a high content of sensory potent spices, that might mask its contribution to potential negative effects.

Considering all this, the aim of this study was to evaluate the antioxidant protective effect of a lyophylized aqueous *Melissa officinalis* extract against cholesterol degradation and cholesterol oxidation products formation in a model system. Once the effectiveness of melisa in the model system was probed, the application of this extract to

a food system (beef patties) was carried out in order to assess its effectiveness as antioxidant at doses that were sensory acceptable.

2. MATERIAL AND METHODS

2.1 Reagents

Cholesterol, 5α-cholestane, thiobarbituric acid, trolox, AAPH and fluorescein sodium salt were purchased from Sigma-Aldrich Chemical (Steinhei, Germany). 19-hydroxycholesterol was obtained from Steraloids (Wilton, NH, USA). Tri-sil reagent was obtained from Pierce (Rockford, IL, USA). Acetone, chloroform, ethyl acetate, methanol, hexane, 2-propanol, hydrochloric acid, cyclohexanone, trichloroacetic acid, potassium cloride, potassium hydroxide, anhydrous sodium sulphate and sodium phosphate were obtained from Panreac (Barcelona, Spain). Hexane for gas chromatography and dichloromethane for gas chromatography were from Merck (Whitehouse Station, NJ, USA). Strata NH_2 (55 µm, 70 A) 500 mg / 3 mL Solid Phase Extraction cartridges were obtained from Phenomenex (Torrance, USA). *Melissa officinalis* dried leaves were purchased from Plantaron S.L. (Barcelona, Spain). Beef meat was purchased in a minor local distributor, and showed *Ternera de Navarra* PGI (ES/PGI/0005/0130).

2.2 Preparation and characterisation of Melissa officinalis extract

Aqueous extract of *M. officinalis* was prepared as described in García-Íñiguez de Ciriano et al. (2010b). Briefly, 50 g of dried leaves were weighted and added to 500 mL of distilled water, preheated at 100 °C. The mixture was subjected to reflux during 30 min at the temperature above. Extraction process was repeated with 500 mL of distilled water and both extracts were joined, filtered and lyophilized. Determination of its rosmarinic acid content was performed by HPLC-UV as described in García-Íñiguez

de Ciriano et al. (2010b). Results were expressed as mg rosmarinic acid / g lyophilized melisa extract. Total Phenolic Content (TPC) was determined as described in Poyato et al. (2013). A 12-mg extract sample was solved in 10 mL water. Reagents were mixed: 237 µL distilled water, 3 µL sample solution, 15 µL of Folin-Ciocalteu´s reagent, and 45 µL of 20 % sodium carbonate anhydrous solution. After 2 hours in the dark, the absorbance was measured at 765 nm in a FLUOStar Omega spectrofluorometric analyzer (BMG Labtechnologies, Offenburg, Germany). TPC was expressed as µg gallic acid / mg sample (extract or oil).

2.3 Model system

2.3.1 Heating of samples

Thermo-oxidation of cholesterol was done at 180 ºC for various time durations: 0, 10, 20, 30, 60, 120 and 180 min. For the thermo-oxidation, 4 mL of cholesterol standard solution (5 mg/mL hexane) were added into open glass vials (15 x 100 mm). The solvent was evaporated under gentle nitrogen stream. Subsequently, the vials were placed open (allowing enough oxygen disposal) in the Tembloc (P Selecta, Spain) previously stabilized at 180°C. After the corresponding times, vials were removed from the Tembloc and placed in ice for 10 min. The residue was solved in 4 mL hexane and stored at -20 ºC until analysis. Same procedure was applied to the *Melissa officinalis*-containing samples with the following differences: 2 mL of standard solution (10 mg/mL cholesterol and 0.2 mg/mL melisa extract in a trichloromethane:methanol (2:1) mixture) were aliquoted, dried and heated as previously described. The experiment was performed in quadruplicate, being heating treatments done in four different days.

2.3.2 Cholesterol determination

50 µL were aliquoted from each sample (cholesterol or cholesterol+melisa) and 100 µL of the internal standard, 5α-cholestane (2 mg/mL, hexane:2-propanol, 3:2), were added. Chromatographic analysis, identification and quantification were performed according to Conchillo, Ansorena & Astiasarán (2005).

2.3.3 Cholesterol Oxidation Products determination

Firstly, 250 µL were aliquoted from each sample and 1 mL of 19-hydroxycholesterol (20 µg/mL, hexane:2-propanol, 3:2) as internal standard was added to the each aliquote. NH_2-SPE was used to separate COPs from non-polar and mid-polar products, as suggested by Rose-Sallin, Hugget, Bosset, Tabacchi and Fay (1995). COPs were finally eluted in acetone, which was further evaporated under a stream of nitrogen (35 ºC). Samples were then derivatized to trimethylsilyl (TMS) ethers. Chromatographic analysis, identification and quantification were performed according to the validated method of Menéndez-Carreño, García-Herreros, Astiasarán & Ansorena (2008). Seven different COPs were determined: 7α-hydroxycholesterol (7α-HC), 7β-hydroxycholesterol (7β-HC), 5,6β-cholesterol epoxide (5,6β-CE), 5,6α-cholesterol epoxide (5,6α-CE), 3,5,6-cholestanetriol (CT), 25-hydroxycholesterol (25-HC), 7-ketocholesterol (7-KC).

2.3.4 Antioxidant capacity along the heating process

Antioxidant capacity was assessed by means of the ORAC method, according to the procedure described in Ou et al., 2001, with slight modifications. Cholesterol and melisa extract containing sample was aliquoted (50 µL) and evaporated under a stream of nitrogen. Phosphate buffer (1 mL) and chloroform (300 µL) were added. Then, the samples were vortexed for 20 s and centrifuged at 4000 rpm for 10 min. A total of 0.5 mL of the aqueous layer were taken and kept in dark until analysis. A 0.5 M stock solution of

Trolox was prepared in 10 mM phosphate buffer, and divided into 1 mL aliquots, which were stored at -20 ºC until use. A new set stock Trolox vials were taken from the freezer daily for the preparation of the calibration curve and the quality controls (12.5 and 50 µM). The phosphate buffer solution was used as blank, to dissolve the Trolox quality controls and to prepare the samples. To conduct the ORAC assay, an aliquote of the sample (40 µL) and 120 µL of the fluorescein solution (132.5 nM) were added to the 96 well black plate. The microplate was equilibrated (5 min, 37 ºC), then the reaction was initiated by the addition of AAPH (40 µL, 300 mM); readings were obtained immediately, in a FLUOStar Omega spectrofluorometric analyzer (BMG Labtechnologies, Offenburg, Germany). The results were expressed as mg trolox equivalent / g sample.

2.3.5 Rosmarinic acid content along the heating process

Cholesterol and melisa containing sample was aliquoted (1 mL) and evaporated under a stream of nitrogen. Ultrapure water (1 mL) and hexane (1 mL) were added. Sample was vortexed for 20 s and centrifuged at 1300 g for 6 min. The upper layer was discarded and the process was repeated two more times. The aqueous layer was filtered through a 0.20 µL membrane filter (Millipore, USA) and analyzed using the chromatographic conditions described in García-Iñiguez de Ciriano et al. (2010b). Briefly, in a C18 column, and at a flow rate of 0.8 mL / min, a gradient of acidified water : acetonitrile was applied (starting at 90:10; changing to 70:30 during 20 min; and returning to 90:10 in 7 min). The profiles were recorded at 280 nm. The results were expressed as mg rosmarinic acid / g sample.

2.4 Food system

The experimental design applied to this part of the work is presented in Figure 1. Four different meat patties formulations were assessed, namely simple (S), simple+melisa (SM), emulsion-containing (E) and emulsion-containing+melisa (EM).

2.4.1 Total phenolic content in extra virgin olive oil

The procedure was the same as for TPC in the melisa extract but previous phenols extraction was performed, as described in Poyato et al. (2013).

2.4.2 Meat patties preparation

All the patties contained lean beef meat (*Ternera de Navarra* PGI, ES/PGI/0005/0130). Meat was conveniently double-minced and all patties weighed 80 g. "S" patties contained 79.2 g meat and 0.8 g common salt. For "SM" patties, salt was substituted with enriched salt (previously prepared by mixture and homogenization with the *Melissa officinalis* extract: 16 g salt + 64, 80, 104, 200, 600 or 800 mg melisa extract). Formulation of "E" patties consisted on 75.2 g of meat, 0.8 g salt and 4 g of an oil-in-water emulsion. To make the emulsion, 52.63 g extra virgin olive oil were slowly added to 42.1 g water (containing 5.3 g soya protein), while continuous homogenizing with an ultra-turrax. For "EM" patties, melisa extract (250, 300 or 400 mg) was added to the water phase of the emulsion before mixing with the oil.

Mixture of ingredients was compressed with a conventional burger maker until a compacted and homogenized patty was obtained (80 g, 8.6 cm diameter and 1.5 cm thickness).

2.4.3 Cooking procedure

For the different types of meat patties, four independent batches were prepared, each one containing 4 patties (two to keep raw and two for cooking). Patties were put in a

pre-heated oven at 185 ºC during 12 min, reaching 65 ºC of internal temperature. Just after the cooking process, they were cooled down for 10 min, weighted, minced, and stored at -20 ºC under vacuum until the analysis.

2.4.4 Melissa officinalis extract addition: sensory evaluation

The determination of the adequate quantity of melisa extract to be added to the meat patties (SM and EM) was done through sensory analysis with 9 semi-trained panellists, by means of a triangle sensory analysis on cooked samples. Panellists were trained by allowing them to taste beef patties in which different doses of melisa were added, in order to help in the identification of its taste. The comparisons made were: S vs SM and E vs EM. In each case, different amounts of *Melissa officinalis* extract were added to the respective type of patty (500, 375, 125, 65, 50 and 40 µg/g patty for SM and 200, 150 and 125 µg/g patty for EM). Additionally, differences between S and E were analyzed to take into account a potential effect of the emulsion, even without the extract, over the sensory evaluation of the product.

For every comparison, each panellist was presented with three samples, of which two were identical, and asked to indicate which one differed from the others. This process was repeated several times, once for each different concentration of extract tested. The number of correct answers for each type of comparison was determined. According to the ISO 4120:2004, for a 9 member-panel the difference between samples was significant if the number of correct answers was 6 ($p< 0.05$).

2.4.5 Moisture, fat and cholesterol content

AOAC official methods were used for moisture and total fat quantitative determination (AOAC, 2002a, b). The determination of cholesterol was similar to that of the model

system samples, but previous extraction was made according to Kovacs, Anderson and Ackman (1979).

2.4.6 TBARS determination

TBARS values were determined on previously extracted fat according to the method described by Poyato el al. (2013). The absorbance was measured at 532 nm in a FLUOStar Omega spectrofluorometric analyzer (BMG Labtechnologies, Offenburg, Germany).

2.4.7 Cholesterol Oxidation Products determination

Approximately 0.5g of the previously extracted fat (as reported by Folch, J., Lees, M., Stanley, G.H.S., 1957) was weighted in a flask containing 1M KOH in methanol and 1 mL 19-hydroxycholesterol (20 μg /mL in hexane:isopropanol 3:2) and kept at room temperature during 20 h to complete the cold saponification. The unsaponificable material was extracted with diethyl ether (3 x 10 mL). The whole organic extract was washed with water (3 x 5 mL) and filtered through anhydrous sodium sulphate. Then it was recovered in a round-bottom flask, and the solvent was evaporated under a stream of nitrogen. Purification by NH_2-SPE, derivatization to trimethylsilyl ethers and analysis by GC-MS were performed following the same procedure as in the model system (Rosesallin et al., 1995; Menéndez-Carreño et al., 2008).

2.5 Statistical analysis

For the statistical analysis of the data, Stata 12 program (SataCorp LP, Texas, U.S.A.) was used. Mean and standard deviation of data obtained from each replicate were calculated. For the evaluation of the significant differences of the amounts of cholesterol,

cholesterol oxides and TBARS along time and among different samples, one factor ANOVA with Bonferroni post hoc multiple comparisons ($p < 0.05$) was applied.

3. RESULTS AND DISCUSSION

3.1. Model system

3.1.1. Effect of Melissa officinalis extract on cholesterol degradation

Figure 2 shows the percentage of remaining cholesterol throughout the heating process of cholesterol heated with and without melisa (2 mg melisa / 100 mg cholesterol). A significant drop was noticed for samples without melisa extract (control) after 10 min of heating, when the percentage of remaining cholesterol was 66 %, whereas with melisa it remained on a 93 %. Degradation continued during 50 more minutes. As it has been previously found in studies dealing with neat cholesterol thermal stability (Barriuso, Otaegui-Arrazola, Menéndez-Carreño, Astiasarán & Ansorena, 2012; Ansorena, Barriuso, Cardenia, Astiasarán, Lercker & Rodríguez-Estrada, 2013), first stages of heating (10-20 min) were also critical at 180 °C. Throughout the whole process, the values were always lower ($p < 0.05$) for the control than for the treated samples, reaching 23 and 69 % at the end of heating (180 min), respectively. So it can be stated that *Melissa officinalis* extract, at the dose applied in this study, protected cholesterol from thermal degradation.

Known antioxidants such as green tea catequins and quercetin (200 ppm) have previously demonstrated their effectiveness during cholesterol heating at 180 ºC, where, after 30 min, around 60 and 95 % of initial cholesterol was found in control and antioxidant-treated samples (Xu et al., 2009). Yen et al. (2010), using 5 % Conjungated Linolenic Acid in cholesterol, also found a significant decrease in cholesterol degradation

(54 vs 67 %, for control and treatment). However, a study using rosemary extract showed no significant differences in campesterol degradation after 4h at 180 ºC, although differences among total sterol oxidation products content were detected (Kmiecik et al., 2011).

The cholesterol degradation curve presented a much higher slope for cholesterol alone than for melisa containing samples during the first 10 min, but very similar slopes could be observed thereafter for both samples. This could denote a high protective effect of the melisa during the first 10 min and slower protection thereafter. Accordingly, the antioxidant capacity values (ORAC) found for the model system that included the melisa extract were reduced after the first 10 min. Figure 3 shows that approximately half of the antioxidant capacity initially noticed in the model system was lost after 10 min heating, decreasing from 43.11 to 23.71 mg trolox / g sample.

The high content of phenolic compounds in the extract matrix (TPC was 356 µg gallic acid / mg extract) could explain its antioxidant effect. As the major antioxidative compound in this water melisa extract was rosmarinic acid (123 mg / g extract), monitorization of its remaining concentration during the heating process was also done (Figure 3). A similar decreasing curve as that of ORAC determination was observed, with a decrease of around 50 % after the first 10 min. Thus, a high correlation between the antioxidant capacity loss and rosmarinic acid loss was noticed (Pearson R = 0.9517). No antioxidant capacity was noticed when cholesterol was heated alone, except for minute 180, where 0.60 mg trolox / g sample was detected, meaning a 5.1 % of total ORAC value at this point. Therefore, the protective effect observed for melisa extract in the current study was mainly attributed to its high rosmarinic acid content, which is a

compound known by its antioxidant capacity (Erkan N., Ayranci, G., & Ayranci, E., 2008). Nevertheless, even if its contribution should be very important, other compounds found in the extract (showing peaks much smaller but not quantitated) could be also responsible for the antioxidant properties owing to synergistic effects, as it is stated in Miron et al. (2013).

3.1.2. Effect of Melissa officinalis extract on cholesterol oxidation products formation

COPs were progressively formed during heating until they achieved a maximum, and then their concentration started to decrease, following a different pattern depending on the type of COP and sample (Figure 4). Formation of COPs was quick and high in the control sample. At 10 min, 94 µg total COPs per mg initial cholesterol were formed in control sample, whereas practically no COPs were formed in the melisa-treated sample. This behavior is in accordance with data from cholesterol degradation, where the best antioxidant effectiveness was recorded during the first 10min of treatment.

Melisa-containing sample continued yielding COPs for a longer time, since the process was retarded in respect to the control. Consequently, the maximum COPs level was achieved at 30 and 120 min for control and treated samples, respectively, yielding 142.97 and 93.03 µg/mg in control and in melisa-treated samples. Similar times (10 and 20 min) were required in previous studies to reach maximum COPs levels in neat cholesterol samples at 180 ºC (Barriuso et al., 2012; Ansorena et al., 2013). Therefore, it can be stated that *Melissa officinalis* extracts inhibited cholesterol oxidation products formation by both delaying their appearance and decreasing their formation rate.

COPs formation has been previously reported to be depleted in presence of phenolic compounds such as green tea catequins and quercetin (Xu et al., 2009) from

around 12 % to less than 5 % respect to initial cholesterol content after 30min at 180 ºC. In the current study, at the same temperature-time conditions, similar reduction was found: from 31 to 11 % cholesterol oxidation. In general, better results have been observed for natural antioxidants than for synthetic ones regarding sterol oxidation products in model systems (Xu et al., 2009; Kmiecik et al., 2011).

Total COPs behavior was significantly affected by 7-ketocholesterol (Fig 4g), which was the most abundant COP among those analyzed. It was followed by epoxy and hydroxyl compounds, with triol at neglilible levels (Fig 4e), as expected, giving the lack of water in the medium (Lampi, Juntunen, Toivo & Piironen, 2002). 25-hydroxycholesterol only suffered a small increase (Fig 4f) which was also expected due to the sterol chain lower likelihood to oxidize in absence of enzymes.

3.2. Food system: meat patties

3.2.1. Incorporation of the Melissa officinalis extract into meat patties and sensory evaluation

The melisa extract dose used in the model system was 2 mg melisa / 100 mg cholesterol. To extrapolate this concentration to the food matrix (meat patty) it has to be considered that cholesterol is not the only lipid compound susceptible to oxidation in this foodstuff. Taking into account this fact, the concentration chosen was 2 mg melisa / 100 mg lipid fraction, which corresponded to 500 µg melisa /g meat patty. When the sensory evaluation was performed on these meat patties, an unpleasant taste was clearly detected by panellists. Therefore, sensory evaluation of meat patties samples containing decreasing levels of melisa extract (SM) was carried out until a non-detectable concentration of melisa was noticed. The comparison between the control patty (S) and

the different melisa-containing patties (SM) in the triangle sensory test (Table 1a) revealed that panellists were able to detect significant differences with doses over 65 µg/g patty.

In order to compare the antioxidant efficiency of melisa extract with that of a recognized potent antioxidant in meat patties (Rodríguez-Carpena, Morcuende & Estévez, 2012b), beef patties containing an extra virgin olive oil (E) were prepared. Besides, patties containing both extra virgin olive oil and melisa extract (EM) were also prepared to check possible additional or synergistic effects of melisa extract and olive oil. The tasty and flavorfull properties of olive oil would efficiently mask melisa oddflavor and would permit to enhance melisa dose in patties. Olive oil was applied through a oil-in-water emulsion, where melisa extract was solved within the water phase. This technology has been successfully applied previously by our group in other meat products (García-Íñiguez de Ciriano et al., 2010b; Berasategi et al., 2011) and it has also been used by other authors (López-López, Cofrades, Yakan, Solas & Jiménez-Colmenero, 2010) for improving the nutritional properties of the lipid fraction of new meat products formulations. In this case, the emulsion was a good system to include higher amounts of the antioxidant, since direct contact with taste buds and melisa is avoided.

It has to be pointed out that the percentage at which the emulsion was present in the formulation (5 %) did not modify the typical sensory properties of beef patties, as panelists were not able to discriminate between S and E samples ($p < 0.05$).

Then, increasing concentrations of melisa vehiculized through the emulsion (EM) were added and sensory tests were performed facing them to E samples (Table 1b). As it was hypothesided, results led to the conclusion that the level of undetectable melisa

extract was able to be increased up to 150 µg/g in emulsion-containing patties (EM), compared to 65 µg/g in patties where melisa was not vehiculized within an emulsion (SM).

Higher doses of *Melissa officinalis* extracts (965 and 686 µg/g) than those used in the current study have been previously added to meat products without noticing sensory problems - Berasategi et al. (2011) with Bologna-Type products and García-Iñiguez de Ciriano et al. (2010a) with fermented products-. Nevertheless, all these studies dealt with samples rich in aromas and flavours from garlic or red pepper, which can easily mask the sensory oddflavour melisa notes. This was not the case of our fresh beef patties (containing a quite simple formulation: beef meat, water, olive oil, soya protein and salt).

3.2.2. Effect of Melissa officinalis extract on lipid oxidation

Lipid oxidation in raw and cooked conditions was assessed for the following four types of patties: simple, without and with 65 µg/g melisa extract (S and SM) and emulsion-containing samples without and with 150 µg/g melisa extract (E and EM). Overall lipid oxidation results are shown in Figure 5 and cholesterol oxidation was monitored through COPs determination (Table 2). Raw samples did not show significant differences among the four types of patties, presenting mean values around 0.1 mg MDA / Kg and from 538 to 913 µg COPs / 100 g dry sample.

Cooking significantly increased the TBARS in simple patties but no efficient protection of the melisa extract was detected (S ≈ SM). The same behaviour was observed for COPs: their values significantly increased after cooking in all four types of patties, but similar values were reported for cooked simple patties with and without melisa extract addition (S ≈ SM).

Comparison between simple patties and those that incorporated the olive oil emulsion allowed us to conclude that the emulsion protected from lipid oxidation, probably due to the high phenolic content of olive oil (143 ppm gallic acid). Potential antioxidant properties of soy protein contained in the emulsion (Bloukas, Paneras & Fournitzis, 1997) could also be behind this behavior.

When the higher dose of melisa (150 ppm) was used within the olive oil emulsion, no additional protective effect of melisa was observed over that of the olive oil neither for TBARS nor for COPs values (0.18 vs 0.18 mg MDA / Kg and 1030 vs 972 µg COPs / 100 g dry sample for E vs EM, respectively).

The concentration was as high as the sensory quality allowed guaranteeing good flavour, so it had to be concluded that melisa extract was not efficient in these conditions. These unfavorable results were probably caused by the low dose of melisa used in the current experiment: sensory requirements have forced to decrease the concentration of melisa extract below the level at which antioxidant effects can be observed in the meat system. Besides, rosmarinic acid could have been disappeared during cooking, decreasing the extract antioxidant capacity, as it occurs in the model system (Figure 3).

A number of studies have reported more successful results in different meat patties enriched with high-phenolic extracts. Rodríguez-Caprena, Morcuende, Petron & Estévez (2012a); Sampaio, Saldanha, Soares & Torres (2012) and Duthie, Campbell, Bestwick, Stephen & Russel (2013) obtained from 20 to 85 % of reduction on TBARS values, and detected up to 1.8 mg MDA / Kg. In chicken and pork patties, Mariutti, Nogueira & Bragagnolo (2011); Rodríguez-Carpena et al. (2012a) and Karwoska et al. (2014) reported reductions from 2 to 7-fold as a consequence of antioxidant enrichment

(from sage, mustard and avocado), and the COPs contents ranged between 90 and 1350 µg/100g, probably due to the higher doses applied. However, not all of them assessed sensory evaluation of the products, which is critical for acceptability. Karwowska and Dolatowski (2014), using pork meat added with mustard seed, did not find any protection against MDA formation at the end of the cooking procedure (as in the current study), but only after 12 days of storage.

In the current study, *Melissa officinalis* was much more effective in the model system than in meat patties. This is frequently reported in comparative studies: green tea catequins, tocopherol and quercetin were much more effective when cholesterol was heated alone compared to when the experiment was made within lard (Xu et al., 2009). This lower efficiency found in food-like systems can be related to the presence of other surrounding lipids, which can act as protective factors themselves (Yen et al., 2010; Yen, Lu, Inbaraj & Chen, 2011; Ansorena et al., 2013).

In conclusion, *Melissa officinalis* aqueous extract protected cholesterol from oxidation in the model system, but no protective effect was found in meat patties at sensory-acceptable doses. Therefore, attention should be paid to sensory considerations in the evaluation of natural extracts as a source of bioactive compounds in foods. Besides, new technologies for the incorporation of these possible ingredients should be developed, such as the use of encapsulated structures or gelled emulsions.

4. ACKNOWLEDGEMENTS

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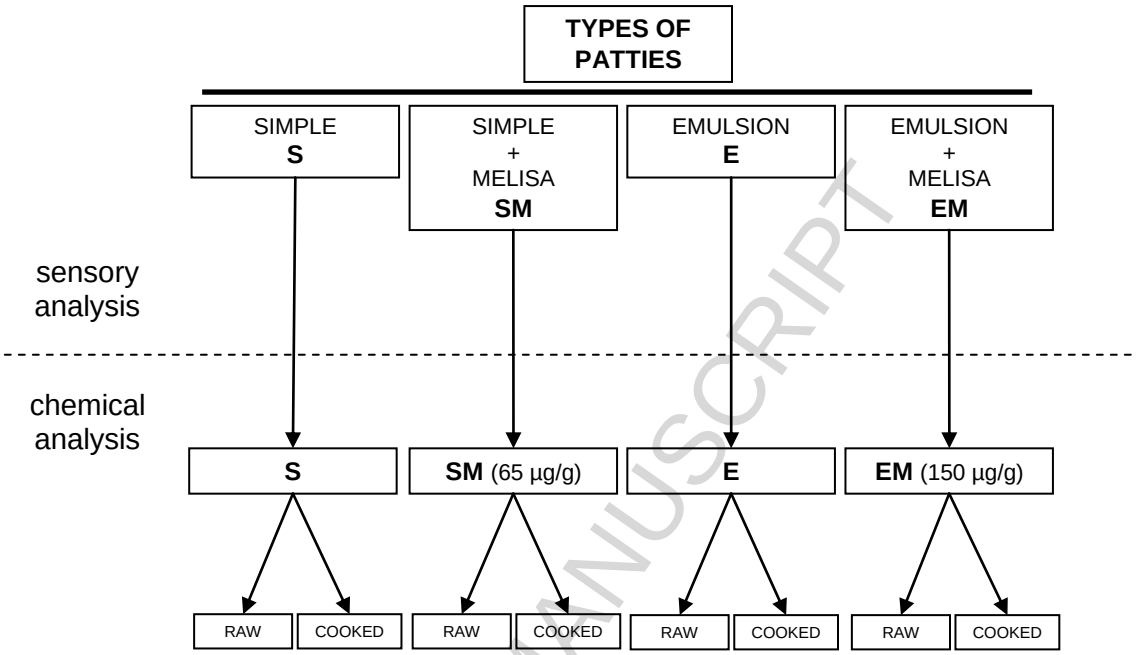

Figure 1. Experimental design for beef patties study.

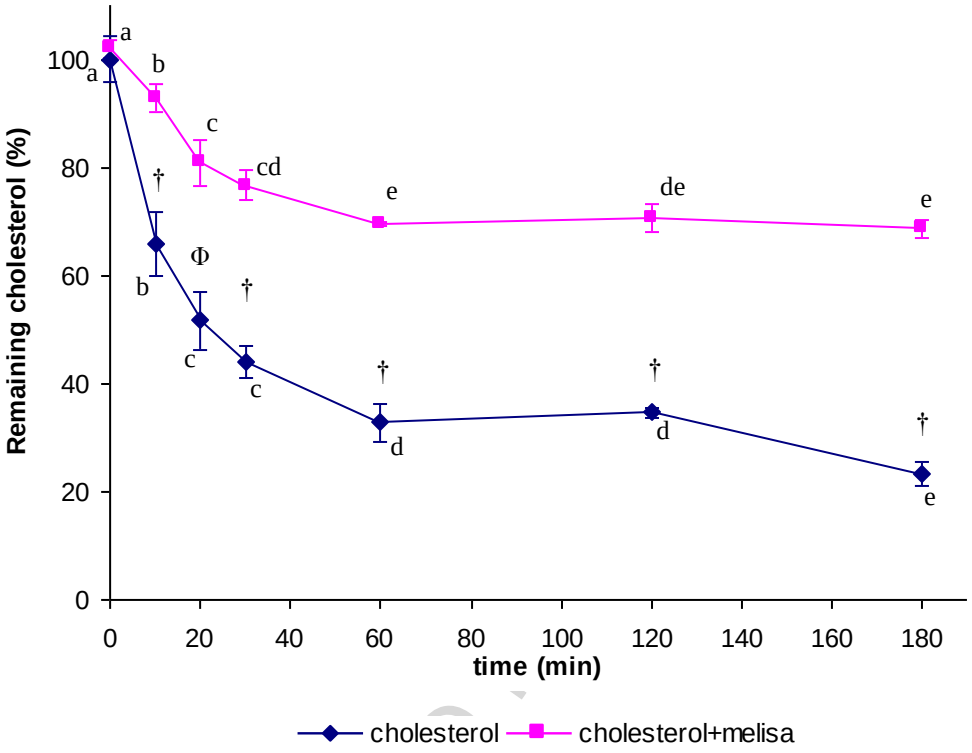

Figure 2. Remaining cholesterol (%) during the heating process in model system. Different letters for each sample denote statistical differences along time ($p<0.05$). At every time of analysis, Student t test compared both types of samples (* $p<0.05$; Φ $p<0.01$; † $p<0.001$).

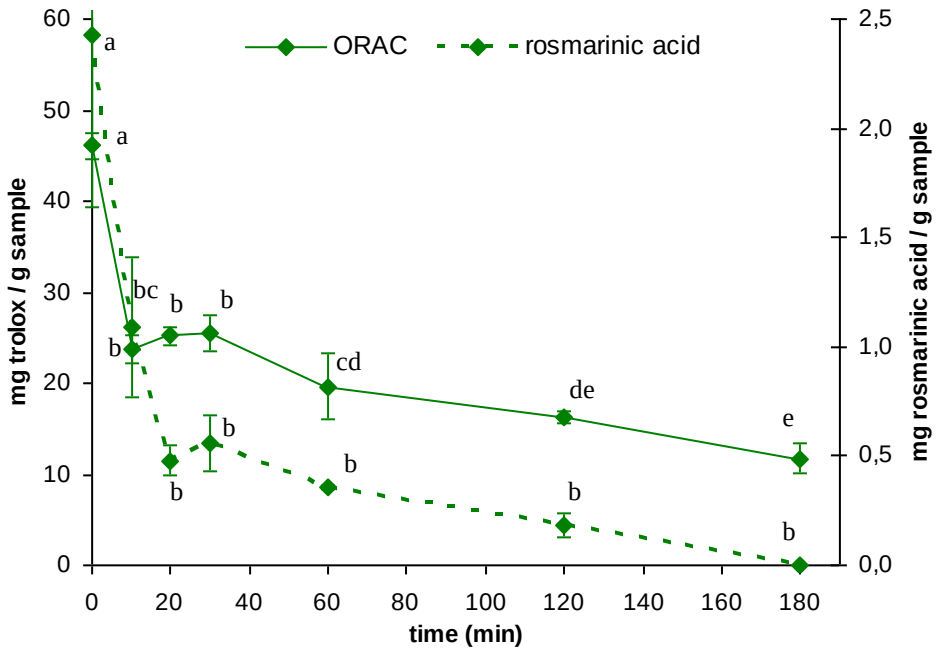

Figure 3. Antioxidant capacity (ORAC determination) and rosmarinic acid content during the heating process in the model system (cholesterol + melisa sample). Different letters for each sample denote statistical differences along time ($p<0.05$).

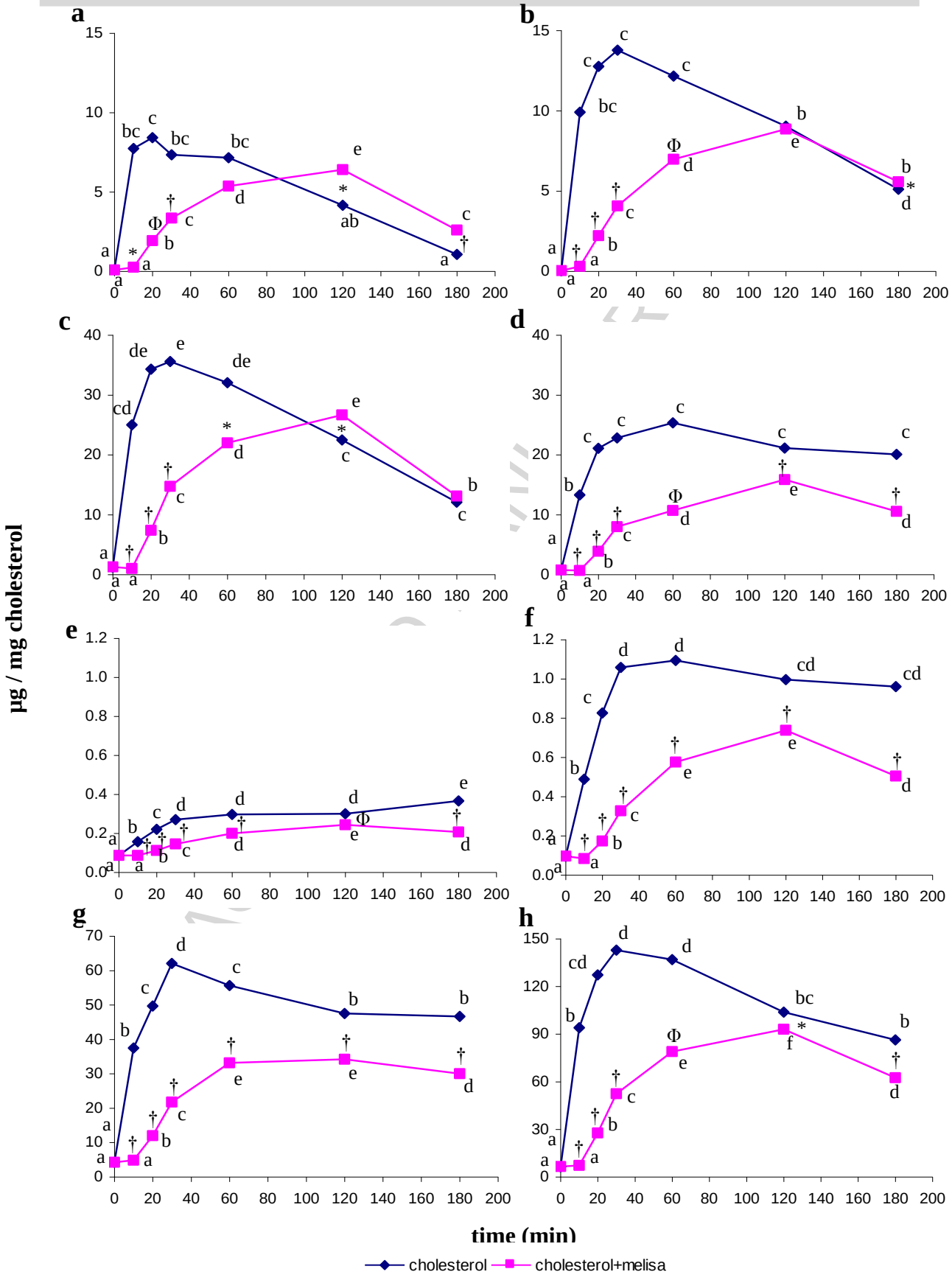

Figure 4. COPs in model system during the heating process a) 7α-HC, b) 7β-HC, c) 5,6β-EC, d) 5,6α-EC, e) CT, f) 25-HC, g) 7-KC, h) total COPs. Different letters for each sample denote statistical differences along time ($p<0.05$). At every time of analysis, Student t test compared both types of samples (* $p<0.05$; Φ $p<0.01$; † $p<0.001$).

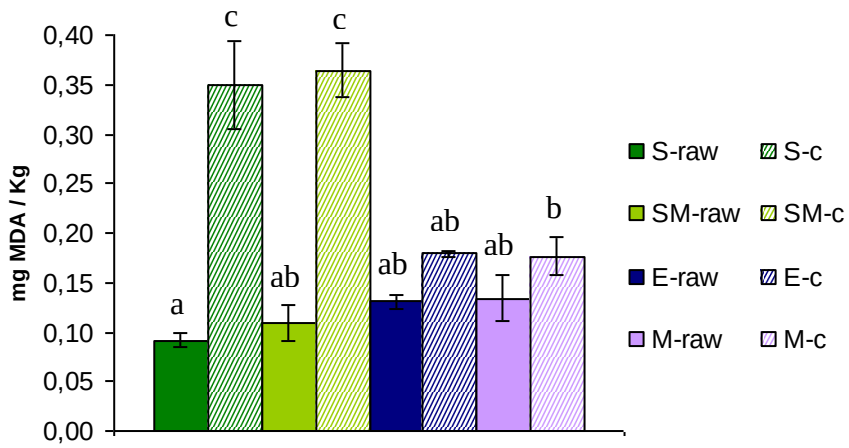

Figure 5. TBARS of beef patties (S, SM, E and EM). For each type of patty, the filled bar indicates raw sample and the striped bar indicates cooked sample. Different letters denote significant differences among samples ($p<0.05$).

Table 1. Scores of triangle sensory analysis. Comparisons between a) S and SM patties; b) E and EM patties

a

	S vs SM					
	500 µg/g	375 µg/g	125 µg/g	65 µg/g	50 µg/g	40 µg/g
Correct replies	9***	9***	7*	4ns	2ns	0
Incorrect replies	0	0	2	5	7	9

b

	E vs EM		
	200 µg/g	150 µg/g	125 µg/g
Correct replies	9*	4ns	1ns
Incorrect replies	7	11	6

For n=9, the difference between samples was significant if the number of correct answers was 6 (* = $p<0.05$), 7 (** = $p<0.01$), 8 (*** = $p<0.001$).ns: not significant

Table 2. COPs concentration (µg / 100 g dry sample) in raw and cooked beef patties (S, SM, E and EM). Different letters denote significant differences among samples ($p<0.05$).

	S-raw	S-c	SM-raw	SM-c	E-raw	E-c	EM-raw	EM-c
7α-HC	76.26 a	155.21 b	72.84 a	168.06 b	55.24 a	69.26 a	52.66 a	62.21 a
7β-HC	113.81 a	234.76 b	106.17 a	242.25 b	80.83 a	113.25 a	83.96 a	105.89 a
5,6β-EC	286.57 ab	529.20 c	260.49 a	533.72 c	144.91 a	370.90 a	240.51 a	351.49 bc
5,6α-EC	55.21 ab	57.69 a	56.79 a	62.60 a	20.53 c	64.57 b	37.30 b	64.59 a
CT	22.55 a	21.76 a	29.63 ab	13.43 a	33.73 b	20.63 a	19.61 a	24.84 a
25-HC	11.38 a	13.01 b	11.73 a	9.94 a	15.54 c	12.84 b	12.84 b	14.11c
7-KC	347.79 ab	761.47 d	335.80 ab	700.05 d	187.43 a	378.54 a	270.87 a	349.25 bc
Total COPs	913.58 ab	1773.10 d	873.46 ab	1716.46 d	538.22 a	1030.01 c	717.75 a	972.39 bc