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Máster Oficial en Calidad de Alimentos de Origen Animal

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Innovative generation of delivery systems containing bioactive compounds to finally be incorporated into food products.

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List of abbreviations

ANOVA = Analysis of variance

BSTFA = N-O-bis(trimethylsilyl) trifluoroacetamide

HHE = 4-hidroxy-2-hexenal

HNE = 4-hydroxy-2-nonenal

IS = Internal standard

LOD = Limit of detection

LOQ = Limit of quantification

PFBHA = O-(2,3,4,5,6-Pentafluorobenzyl)-hydroxylamine hydrochloride

PUFAs = Polyunsaturated fatty acids

TMCS = Trimethylchlorosilane

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Summary

The oxidation of polyunsaturated fatty acids present in food could produce 4-hydroxy-2-nonenal and 4-hydroxy-2-hexenal which are reactive hidroxyalkenals that affect the quality and the safety of products. These compounds, absorbed through the diet, have toxic pathways inducing several diseases such as Alzheimer's disease, cataract, diabetes, cancer and atherosclerosis. In the present study three fresh cheeses with modified fat were obtained by adding emulsions containing soybean oil and different essential oils. This emulsion is rich in polyunsaturated fatty acids and could release these oxidation products. It also includes antioxidant molecule present in the essential oil. These cheeses were analysed using a gas chromatography mass spectrometry to evaluate the presence of these compounds and alternatively the antioxidant effect of the emulsions. Measurements shows low quantity of HHE and HNE in all three cheeses. The reason could be attributed to a slow oxidation or to their high reactivity with matrix proteins. Sensorial analyses were carried out to evalue the effect of soybean oil and essential oil on fresh cheese production. Soybean oil add bitterness to the cheese but essential oil effect was well accepted.

Resumen

La oxidación de los ácidos grasos poliinsaturados puede producir 4-hydroxy-2-nonenal y 4-hydroxy-2-hexenal, hidroxyalkenales que afectan la calidad y la seguridad del producto. Estos compuestos, absorbidos por la dieta, tienen efecto toxico induciendo diversas enfermedades como Alzheimer, catarata, diabetes, cáncer and arteriosclerosis. En esto estudio tres diferentes quesos con grasa modificada fueron preparados añadiendo una emulsión que contiene aceite de soja y diferentes aceites esenciales. Esta emulsión es rica in grasa poliinsaturada y puede liberar estos productos de oxidación. También contiene moléculas antioxidantes presentes en los aceites esenciales. Estos quesos fueron analizados en cromatógrafo de gas con espectrometría de masa al fin de evaluar la presencia de estos compuestos y adicionalmente el efecto antioxidante de la emulsión. Las mediciones muestran baja cantidad de HHE y HNE en los tres quesos. La razón puede ser atribuida a una baja oxidación o alta reactividad con la matriz proteica. Un análisis sensorial fue hecho al fin de evaluar el efecto de los aceites de soja y de los aceites esenciales en la producción de queso. Aceite de soja añade amargor al queso, pero los aceites esenciales fueron bien aceptados.

Introduction

Cardiovascular diseases are the leading cause of death in whole Europe but 12 countries for men and 2 countries for women, accounting 37% of all deaths in the Europe (Wilkins et al., 2017). It is related to the change in lifestyle of the modern times, for example sedentarism, current diets and smoking. Cardiovascular diseases include numerous problems related to a process called atherosclerosis which is caused by fatty deposit that can clog arteries. These build-up deposits are called "plaques" made of cholesterol, fatty substances, cellular waste products, calcium and fibrin.

Cutting down on saturated fatty acids intake may lower risk of coronary heart disease. Swapping these foods for diets richer in unsaturated fats seems to boost heart health even further (Mozaffarian et al., 2010). Foods with a high percentage of saturated fats are the ones derived from animals, for example meat and dairy products. Nowadays, consumers are more sensible about natural and healthy food products and they demand animal products with better nutritional properties (Doménech-Asensi et al., 2013). Indeed the substitution of animal fat by vegetal fat could be a good strategy for improving the nutritional quality of an animal product, as it reduces the level of saturated while increasing the level of polyunsaturated fatty acids (Beiloune et al., 2014). These products, with a healthier fat composition, have been developed using emulsion based systems that could be water-oil or gel based (Berasategi et al., 2011).

Dairy products contain milk and milk derived foods that are daily consumed due to habits, ease of use and great taste. However, reducing only the fat composition of a cheese results in a more compact casein network that hold less water with the consequence of a tougher texture (Banks et al., 2004). Thus, to maintain textural characteristics, fat substitutes or fat mimics can be used to increase moisture level (Johnson et al., 2009). Concretely, in cheese products, water-dispersible fat replacer works by mechanically entrap water, giving better rheological characteristics but it may not be efficient in carrying flavour (Romeih et al., 2002). Replace milk fat with emulsified vegetable oil could be an option to obtain a cheese with a better texture, flavour and a healthier fat balance (Yu and Hammond, 2000). However, the chemical nature of unsaturated fats makes them very oxidable.

The oxidation of unsaturated lipids even leads to rancid odours and flavours, which decreases the quality of the products (Álvarez et al., 2011). Some compounds like 4-hidroxy-2-hexenal (HHE) and 4-hidroxy-2-nonenal (HNE) are produced from the oxidation of polyunsaturated fatty acids (PUFAs), concretely from omega-3 and omega-6, respectively. These hidroxyalkenals can be absorbed through the diet and have toxic pathways such as the induction of caspase enzymes, the laddering of genomic DNA, the release of cytochrome c from mitochondria, with the eventual outcome of cell death through both apoptosis and necrosis depending on concentration. Furthermore HNE has been linked in the pathology of several diseases such as Alzheimer's disease, cataract, diabetes, cancer and even atherosclerosis (Negre-Salvayre et al., 2010). To prevent or reduce lipid oxidation, antioxidants are used as food additives. In recent years much attention has been focused on natural antioxidants effect on lipids. For example, cinnamon essential oil contains eugenol, a strong antioxidant with phenolic structure which has been proven to have powerful antioxidant effect (Farag et al., 1989). These oils could even contain compounds which can extend food lifespan. Spice essential oils, owning potentially useful molecules, were studied for their medicinal, culinary and antimicrobial properties but little is known about their antioxidant properties (Özcan & Arslan, 2011).

The subject of this study was to produce a new fresh cheese with a modified fat composition, low saturated fat and high unsaturated fat percentage, which could have a healthier perception by the consumer. An emulsion containing soy oil, which is vegetable oil rich in polyunsaturated fats, was added to the vat milk before cheese production. To prevent lipid oxidation, two essential oils, oregano and cinnamon, were blended individually in each emulsion in order to evaluate their antioxidant effect. In this research the development and evolution of 4-hydroxy-2-nonenal and 4-hydroxy-2-hexenal in fresh cheese with modified fat composition during its shelf life were evaluated. Sensorial tests were also carried out to evaluate the effect of the soybean oil emulsions with oregano and cinnamon added to fresh cheese. Tests of the fresh cheeses with induced oxidation were also made to better understand the antioxidative effect of essential oils.

Materials and Methods

Supplies and Reagents

HNE and HHE standards were purchased from Cayman Chemical (Ann Arbor, MI, USA) and both were ≥ 98 % purity according to the certification provided by the supplier. HNE *d11* and HHE *d5* dimethyl acetals, used as internal standards (IS), were purchased from C/D/N Isotopes Inc (Pointe-Claire, Quebec, Canada). Sodium caseinate at 88% of proteins was purchased from Epi Ingrédients (Ancenis, Pays de la Loire, France). O-(2,3,4,5,6-Pentafluorobenzyl)-hydroxylamine hydrochloride (PFBHA) and N-O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) with trimethylchlorosilane (TMCS) were obtained by Sigma-Aldrich Quimica SL (Madrid, Spain). Rennet and Calcium Chloride were purchased from Laboratorios Arroyo (Santander, Spain). Deionized water (Milli-Q) of 18.0 MΩ cm^{-1} resistivity was used throughout the experiments and all the solvents used were of HPCL grade.

Emulsions preparation

Sodium caseinate was hydrated one day before of every cheese production in distillate water. Protein was kept overnight at 4°C by agitation with a mechanical blender until complete hydration. A concrete amount of sodium caseinate was used in order to achieve 5 % of protein in final emulsion. Then, coarse emulsions were treated manually with an immersion blender to break lumps up. Before mixing, protein dispersions were equilibrated at 20°C. Emulsions were obtained by adding, after complete hydration of the proteins, soy oil and essential oils, and mixed for 5 minutes. They were homogenized in a homogenizer at 250bar before being poured into the vats already filled with the milk.

Cheese making

Milk received from Can Badò farm (La Roca del Vallés, Barcelona, Spain) was pasteurized at 72°C for 15s and skimmed. It was placed in a multi-vat and cheese making conditions were set up. Emulsions were added to each milk vat and mixed with the milk until 2,5% fat in milk was obtained. When milk temperature reached 32°C, rennet and calcium chloride were added in 0,05% v/w. After 30min of renneting, curds were cut into small grains to remove serum. Curds

were poured into moulds and overturned two times to remove the most of serum. Three types of cheese were produced: a control cheese, an oregano cheese and a cinnamon cheese. Cheeses were stored under a temperature of 4°C for 10 days.

Internal standards and external curve preparation

In a vial, 100µl of 50ppm 4-hydroxy-2-nonenal *d11* solution and 100µl of 50ppm 4-hydroxy-2-hexenal *d5* solution were mixed and dried under nitrogen. 0,5ml of HCl 20mM were added to the vial and vortexed. After 1h of incubation at 4°C internal standard was ready to use. 40µl of IS was added to all samples, corresponding to 0,4µg. An external calibration curve was built using fixed quantity of the compounds, composed of 0,05µg, 0,1 µg, 0,3 µg, 0,5 µg, 0,8 µg, 1 µg 1,5 µg and 2 µg points. This calibration was performed on samples containing cheese to avoid matrix effect. Limit of detection (LOD) and limit of quantification (LOQ), of both HHE and HNE, were calculated using 46 blank data measures of 2 different productions. LOD equals to 3 × (SD of intercept / Slope), while LOQ equals to 10 × (SD of intercept / Slope).

Extraction techniques

To proceed to the extraction of samples, 1g of cheese was weighted in a 15ml falcon tube, added with 1,5ml of Milli-Q water, 3ml of acetonitrile, the internal standard previously activated and the compounds in the case of calibration curve samples. The mixture was vortexed for 1min in order to mix well the compounds. To separate water 0,3g of NaCl and 1g of $MgSO_4$ were added and the mixture was handshaked to shatter possible agglomeration of salts. Then, samples were centrifuged at 9000rpm for 10min at 4°C. The top layer was collected in a 15ml falcon tube and evaporated under nitrogen with 40°C heat block until dryness. When samples were totally dry, 1ml of Millipore water and 1ml of a solution containing 4mg of PFBHA/1ml of methanol were added before incubation for 1h at 40°C in a water bath. To extract the compounds 2ml of pentane were added and the sample was centrifuged using the same conditions as set up before. The top layer was taken and decanted into a glass Pasteur, prepared with a cotton pug inserted and filled with Na_2SO_4 to a flask. To recuperate as much as possible of the compounds, the extraction was done two times. The collected pentane was evaporated under nitrogen with heat block at 30°C. Every flask was rinsed many times with small amounts of pentane and transferred into an insert

vial. The sample was dried under nitrogen and 20µl of BSTFA+TMCS and 80µ of pyridine were added to derivatize the compounds.

Instrumental analysis

The analysis was performed in an Agilent 7890A gas chromatograph equipped with a 5975C Mass Spectrometer. The vial with 100µl of solution was inserted into the automatic syringe that transfered 1µl of the sample directly into the injector operating in the split less mode at 200 °C. The separation was carried out in an Agilent HP-5 (Crosslinked 5% PH ME Siloxane) 30 m, 0.32 mm, 0.25 µm capillary column. Helium was used as carrier gas at a constant flow of 0.8 mL min−1 and the oven temperature was programmed from 50 (held for 1 min) to 150 °C at a rate of 10 °C min−1, from 150 to 200 °C at a rate of 3 °C min−1and finally up to 240 °C at a rate of 40 °C min−1. The detection conditions were the following: capillary direct interface temperature, 240 °C; ionization energy, 70 eV; operating in selective ion mode; selected ions monitored, m/z 200, m/z 205, m/z 242, m/z 253, and m/z 352; scan rate 3.64 cycles/s. In order to obtain the full mass spectra of the HNE, HHE, and their deuterated isotopic oximes, the scan analysis was performed between m/z 50 and m/z 400.

Induced oxidation

An induced oxidation was carried out to evaluate if the protocol used could detect the compounds. It was used also to evaluate the antioxidant effect of essential oils. It was accomplished by using ultraviolet light for 2h on a 10g sample placed in a petri dish. Measurements were obtained for duplicate. No curve calibration was made because quantification of the compounds was not required for this evaluation.

Sensorial analysis

A sensory assessment was carried out at day 2 in 3 independent cheese productions. Attributes like smell, sweetness, bitterness, freshness, flavour and aftertaste were evaluated. Samples were presented and identified with a three digits' number, randomly chosen. Panellist carried out an evaluation of the smell in which panellists could choose, for single cheese, one or more options from tree different category (herbal, lactic and spicy). Control cheese was the first to be presented and then oregano cheese and cinnamon cheese equally distributed in second and third place.

Between samples, panelists rinsed their mouth with mineral water. Panellist marked responses on 5-point scale: excessively low, low, balanced, high, excessively high. Numerical values were set from 1 of the lowest estimation to 5 of the highest estimation in order to have numeric figures and run statistical analysis. Some other questions were added to acquire more feedback from panellists, like asking for general observations, how they would eat these cheeses, if they are habitual fresh cheese consumers and if they would buy the cheeses. For every production, 10 panellists familiarized with this kind of food carried out the test obtaining a total of 30 series of data.

Statistical analysis

Analysis of variance (ANOVA) was carried out by using the Statgraphics Centurion XVII.II software. Significant differences ($p < 0.05$) among samples, production and day of analysis were detected using Fisher's Least Significant Difference Test. Expressed values are means and standard deviation from duplicate measurements of two independent cheese productions.

Results and Discussion

Instrumental analysis protocol adaptation

For HHE and HNE analysis, we used a protocol described by Papastergiadis et al., (2014$_a$) adapted to laboratory equipment (Universitat autònoma de Barcelona, Facultat de Veterinaria). Several changes in protocol needed to be done in order to adapt the analysis method to our sample and facilities.

For example, during first production, the external curve calibration was structured on 5 points, from 0,3µg to 2µg of HHE and HNE. Later, it was lowered by 2 points due to the low concentrations found in samples. The final calibration curve was structured on 0,05µg, 0,1µg, 0,3µg, 0,5µg, 0,8µg and 1µg points.

For IS, 0,4µg of deuterated isotopic oximes were added instead of 0,2µg to a better performance of chromatogram output and obtain improved peaks resolution. By having both internal and

external calibration curve, compounds quantification was ensured to be achieved. Finally, calculation was carried out using the IS area and concentration, which is a robust method.

Centrifugation was extended to 9000 rpm at 4C° for 10 min to obtain a better separation of water solution from acetonitrile solution. All drying stages were made under nitrogen because of the quicker results instead of rotary evaporator processes, slower and laborious. In instrumental analysis the temperature of 250C° set up on chromatograph was lowered to 240C° in order to respect maximum column temperature.

Instrumental analysis

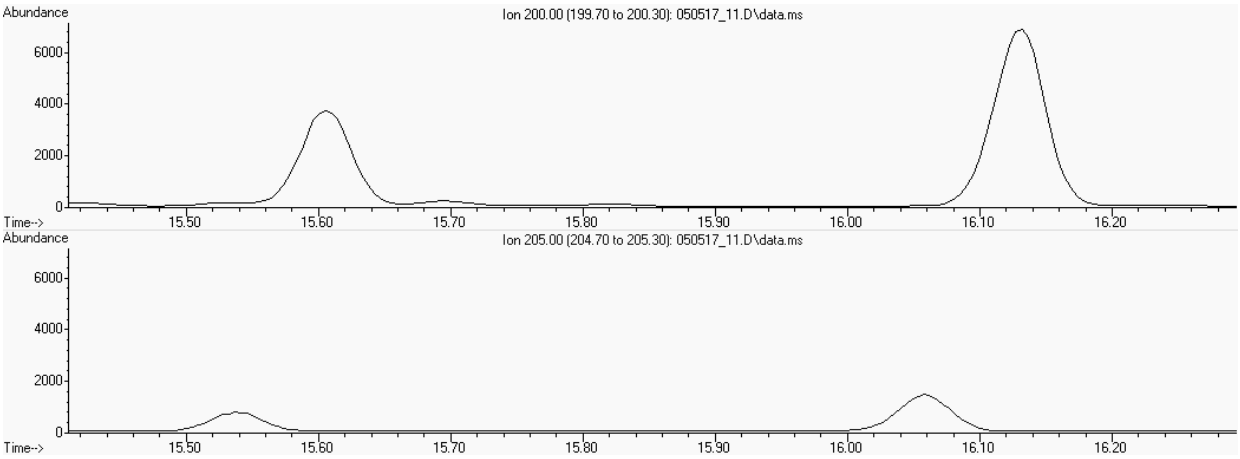


Figure 1. Typical chromatograms of HHE m/z 200 and HHE-d5 m/z 205

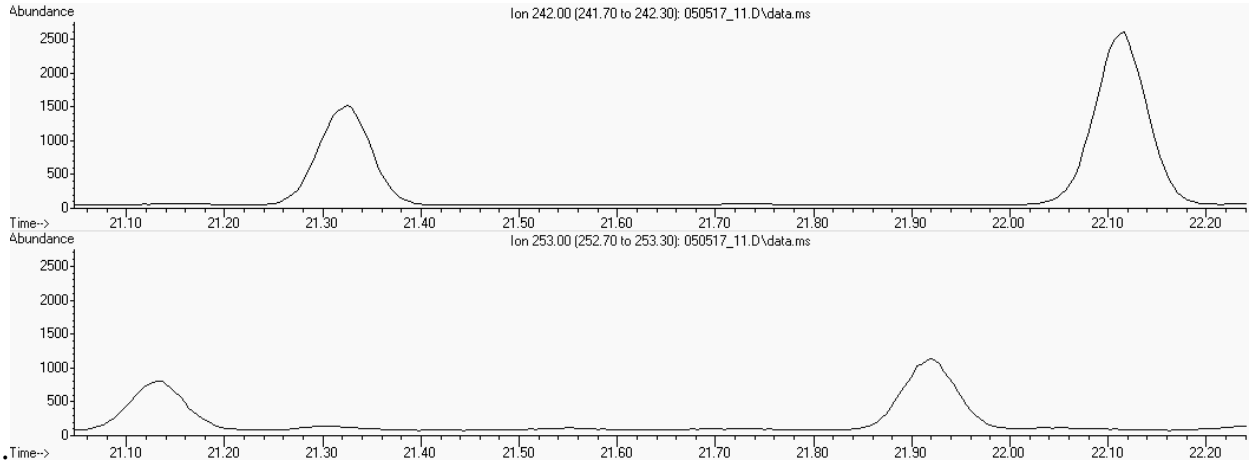


Figure 2. Typical chromatograms of HNE m/z 242 and HHE-d5 m/z 253

Figure 1 and 2 shows typical chromatograms for HHE, HNE and both IS, respectively.

The selected monitored ions to proceed to compounds identification were 200 and 242 for HHE and HNE, respectively. The use of IS (HHE *d5* and HNE *d11)* also helped to identify peaks of monitored compounds since they eluted some seconds before, thus having a slightly lower retention time. HHE, HNE and their IS were each visible in two peaks due to different stereotypes formed during derivatization. The reaction between these analytes and PBFA produce oximes which can be detected by mass spectrometer. For monitoring them, m/z 352 was chosen as an ion shared by all these oximes and detect if derivatization was performed properly (LaFond et al., 2011). Typical retention times for the two peaks were 15,60\16,15 for HHE, 15,55\16,08 for HHE *d5*, 21,36/22,15 for HNE and 21,16/21,96 for HNE *d11*.

Table 1. Limit of detection and limit of quantification (µg/g).

	HHE	HNE
LOD	0,079	0,38
LOQ	0,264	1,268

Table 1 shows LOD and LOQ of HHE and HNE. LOD is the lowest concentration of analyte which can be revealed in experimental method conditions. LOQ is the concentration limit up to which it is possible to obtain instrumental measurements with partial error. It was calculated on 46 blank data measures derived from 2 different productions. LOD and LOQ resulted higher than the most of the measures of HHE and especially of HNE.

Table 2 and table 3 show mean value and standard deviation of HHE and HNE identified in 3 different samples which are control cheese, oregano cheese and cinnamon cheese. Another variable is day of analysis, structured on 3 days which are day 1, day 5 and day 10 according to typical shelf life of fresh cheese.

Table 2. Mean value ± standard deviation (µg/g of sample) of HHE quantity identified in different samples (control, oregano and cinnamon) during cheese shelf life at day 1, day 5 and day 10.

Sample	Day 1	Day 5	Day 10
Control	0,354±0,275[a]	<LOD	<LOQ
Oregano	0,353±0,274[a]	<LOD	<LOQ
Cinnamon	0,355±0,276[a]	<LOD	<LOQ

Means within the same column followed by different superscript are significantly different.

The quantity of HHE is affected by the day of analysis with a highest value at day 1 and the lowest on day 5. Differences between samples and days could not be evaluated because all values were lower than LOD. Moreover, the type of samples appeared to be uninfluential to HHE and HNE concentration. Most of HHE values resulted lower than LOD or LOQ but a clearly evolution of this compound during the 10 days of storage could be seen. Highest value illustrated at day 1 going to the lowest at day 5 and turning up at day 10. HHE seems to modify the protein amino acid residues forming protein-HHE bounds (Shibata et al., 2004). This shows that HHE could react with these components of the cheese, during the first 5 days of storage being reduced its detection/concentration. On the other hand, an increment of production or reduction of the reactivity is shown during last 5 days.

Table 3. Mean value ± standard deviation (µg/g of sample) of HNE quantity identified in different samples (control, oregano and cinnamon) during cheese shelf life at day 1, day 5 and day 10.

Sample	Day 1	Day 5	Day 10
Control	<LOD	<LOD	<LOD
Oregano	<LOD	<LOD	<LOD
Cinnamon	<LOD	<LOD	<LOD

Means within the same column followed by different superscript are significantly different.

All the values obtained for HNE were lower than LOD. Other research works studying milk products obtained HNE concentrations lower than LOD (Papastergiadis et al., 2014$_b$). In this case

it was expected to find higher values of HNE because of the fat composition rich in omega-6 of soybean oil. It has been demonstrated that HNE react with amino acids such as lysine, glutamine, histidine to produce Michael additions (Zhao et al., 2012). These adducts are very stable C-C bonds and because of that they can be considered as irreversible reactions. In these terms, a very high reactivity of HNE or a low oxidation of the unsaturated fat could be proposed based on results obtained.

Table 4 shows the antioxidant effect evaluated on oregano essential oil and on cinnamon essential oil cheeses respect of control cheese. This evaluation was made in duplicate and area values instead of concentration of compounds (µg/g) were measured. In this case the objective was to estimate only the antioxidant effect and not the amount of compounds produced as result of secondary lipid oxidation, such as HHE and HNE concentrations. Therefore, forced oxidation conditions were applied to all samples and development of HHE and HNE was assessed following the same protocol of extraction as in previous experimental cheeses.

Induced oxidation

Table 4. Mean value ± standard deviation of duplicate measured areas of induced oxidated samples by ultraviolet light for 2h.

	HHE	HNE
Control	9880±1214 [a]	46709,5±4896 [a]
Oregano	3709±648 [b]	12125±1303 [b]
Cinnamon	1375,5±375,5 [c]	7116±3262 [c]

Means within the same column followed by different superscript are significantly different.

Low detected values of HHE and HNE, could suggest that at the consumption point of the modified lipid profile fresh cheese, no large oxidation has taken place. An accentuated oxidation may occur after long storage time but in these conditions microbiology counts probably would not permit to consume the product. That is the reason because induced oxidation could show whether the essential oils were affecting whether not cheese samples analyzed.

As expected, HNE values were higher than HHE because of the soybean oil composition rich in omega-6. In both oregano and especially in cinnamon cheeses, a significant effect in lowering oxidation can be seen. Essential oils have shown antioxidant properties in retarding lipid oxidation in oils and fatty foods due to the presence of hydroxy groups in phenolic compounds

(Asensio et Al., 2011). It was found that oregano essential oil is rich in thymol and carvacrol which have an antioxidant effect (Julianoet Al., 2000). Likewise, cinnamon essential oil contains eugenol, a compound which also has been proven to have powerful antioxidant effect (Farag et al., 1989).

Sensorial analysis

Table 5. Mean value ± standard deviation of sensorial data about smell, flavour, global aftertaste, sweetness, bitterness and freshness respect to samples (control, oregano and cinnamon).

	Smell	Flavour	Aftertaste	Sweetness	Bitterness	Freshness
Control	2,666±0,970^{b}	2,843±0,919^{b}	3,093±0,856^{c}	2,777±1,007^{a}	2,875±1,099^{b}	3,187±0,820^{a}
Oregano	3,6±1,042^{a}	3,968±0,932^{a}	4,093±0,892^{a}	2,5±0,949^{a}	2,968±0,966^{a}	3,468±1,015^{a}
Cinnamon	3,272±1,128^{a}	3,562±0,877^{a}	3,593±0,874^{b}	2,916±0,913^{a}	2,406±1,073^{ab}	3,218±0,832^{a}

Means within the same column followed by different superscript are significantly different.

Table 5 shows mean value and standard deviation of sensorial data obtained from testing analysis and modified to a numeric value to allow statistical calculation. As it was expected, significant differences were found in smell, aroma and global smell-taste persistence of cinnamon and oregano cheeses respect to control cheese. Oregano cheese has very durable smell-taste persistence. There was no statistical difference in sweetness and freshness acceptability. Control cheese had a higher bitterness value, compared to other samples, probably due to the absence of essential oil displaying higher effect of soy oil. More than 60% of panellists were habitual fresh cheese consumers. They appreciated most cinnamon cheese respect to the others. This means that panelists perceived more acceptable the scent of cinnamon cheese respect to the scent of oregano and control cheeses. Similar results were reported in other works where great acceptability in food added with cinnamon and oregano was observed (Ayala-Zavala et al., 2013; Rodriguez-Garcia et al., 2016). Panellists observed a soft texture of the cheeses and suggested to eat them in salads or with bread.

Conclusions

After evaluation of results obtained in this study, several conclusions could be drawn.

A method to identify and quantify compounds resulting from secondary lipid oxidation such as HHE and HNE was optimized and carried out successfully in CIRTTA facilities. Laboratory protocol was adapted and optimized, as well as chromatographic method and detection by mass spectrometry.

This study has shown a low presence of the monitored compounds (HHE and HNE) in modified lipid profile fresh cheese during their shelf-life. Furthermore, statistical analysis could not reveal any significant difference between the samples.

No effect of essential oils (oregano and cinnamon) on lipid oxidation of samples could be evaluated based on the lower amounts of HHE and HNE found on cheeses. This fact does not mean that essential oils have no effect on oxidation. The reason of lower levels of HHE and HNE concentrations could be attributed to whether a slow oxidation or whether to the high reactivity with matrix proteins of these compounds.

Induced oxidation experiment carried out on all cheeses showed a great level of oxidation in all samples, being higher in control cheese. Also from this experiment, could be concluded that essential oils have a strong antioxidant effect, especially the cinnamon oil, since values obtained for the monitored compounds in this sample showed the lowest level of concentration.

Regarding to sensorial tests, results obtained in this study have shown that soybean oil affect cheeses increasing bitterness sensation, specially the control cheese. Essential oils, though they have strong smell, had better acceptance by panellists.

Modified lipid profile fresh cheese is a feasible market product regarding to its inclusion of vegetal soybean oil since its shelf-life it is not enough to allow lipid oxidation development. The addition of essential oils like oregano and cinnamon could improve the sensory profile of these cheeses offering new serving suggestions like cheese for salads or aperitifs.

Annex

Annex 1: Cheese testing sheet

NOMBRE:

TIPO DE QUESO: QUESO FRESCO

CARACTERISTICA DEL QUESO	**Excesivamente baja**	**Baja**	**Equilibrado**	**Alta**	**Excesivamente alta**
INTENSIDAD DEL OLOR					
SENSACION DE DULZOR					
SENSACION DE AMARGOR					
INTENSIDAD DE FRESCOR					
INTENSIDAD DEL AROMA					
PERSISTENCIA GLOBAL OLFATO-GUSTATIVA					
EVALUACION DEL OLOR (puedes elegir más de una opción por queso)	**HERBAL**		**LACTICA**		**ESPECIES**

¿Eres consumidor habitual de Queso Fresco?

SI	**NO**

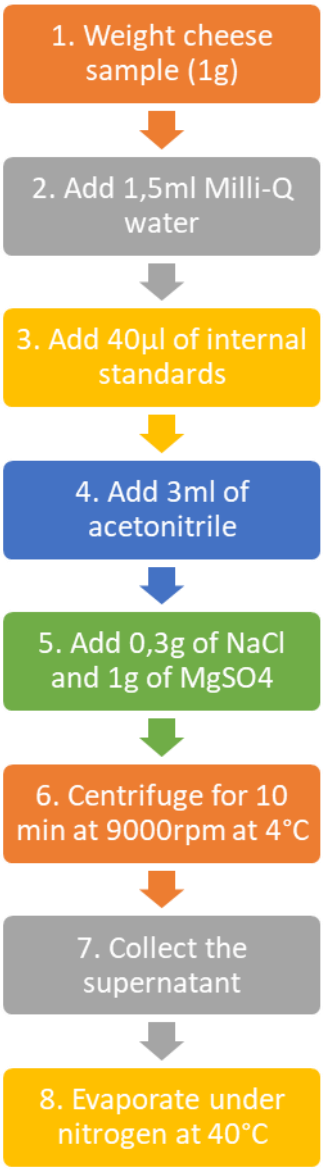
¿Te ha gustado el Queso?

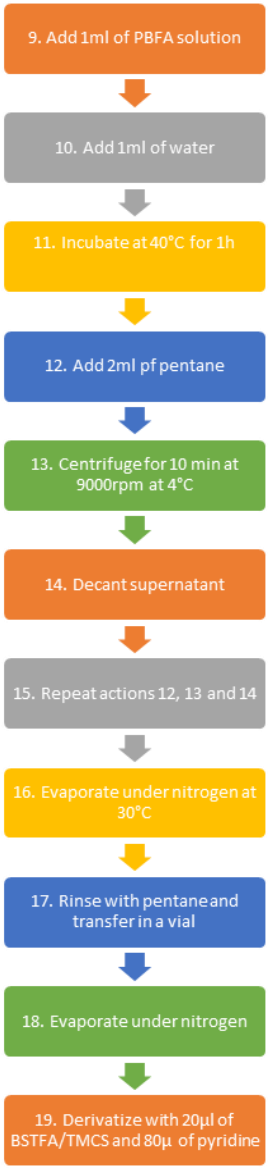
¿Come te comerías este Queso?

	SI	**NO**
125		
748		
999		

Observaciones:

Annex 2: HHE and HNE extraction technique and Derivatization Procedure models





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