Volatile profile of human milk subjected to high-pressure thermal processing

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Abstract

High pressure thermal (HPT) treatments consist of a combination of high pressure (500 - 900 MPa) and temperature (70 - 120°C) over a short holding time. The rapid temperature increase during compression and temperature decrease in the product upon decompression could help to reduce the hardness of thermal effects encountered in conventional thermal technologies. A synergistic effect of combined pressure and elevated temperature eliminates a wide range of spores, assuring microbiological high quality products, an important concern in human milk banks. The objective of this study was to evaluate the effect of HPT processing (300, 600 and 900 MPa combined with 50, 65 and 80 °C) on the profile of volatile compounds in breast milk. A total of 50 volatiles, belonging to 6 different chemical groups, were detected in milk samples. In general, HPT processing increased aldehydes, ketones, furans and pyrans, as well as alcohols, however, it did not modify the levels of carboxylic acids, and reduced the content of aliphatic hydrocarbons present in the non-treated human milk samples. HPT processing enhanced the total area content of volatile compounds derived from Maillard and lipid oxidation reactions, these changes being subjected to the intensity of pressure and temperature applied. Given that the levels of volatiles were significantly modified after the application of HPT processing, we can conclude that the range of the intensity of the treatments selected were not adequate to preserve the original profile of volatile compounds in breast milk.

Keywords: high-pressure thermal processing, human milk, volatile compound, aroma.

Abbreviations

Arbitrary area units: AAU

Gas chromatography: GC

High Pressure Processing: HPP

- 54 High Pressure Thermal: HPT
- 55 **Holder Pasteurization: HoP**
- 56 Mass spectrometry: MS
- 57 Ultra-high-temperature processed: UHT
- 58 Solid-phase microextraction technique: SPME
- 59

1. Introduction

Breastfeeding is strongly encouraged since it improves host defenses, digestion and absorption of nutrients, gastrointestinal function, and neurodevelopment in infants. The unique composition of human milk confers specific protection against many pathogenic bacteria and viruses by virtue of its content of immune cells, cytokines and proteins like immunoglobulins, mainly secretory IgA, lactoferrin, lysozyme and lactoperoxidase (Ballard & Morrow, 2013). Therefore, donor milk stored in human milk banks may be an adequate substitute when the mother's own milk is unavailable for several reasons.

The main objective of human milk banks is to ensure nutritional and biological quality and safety of human milk donated. Generally, human milk banks apply low-temperature/long-time thermal pasteurization, a process in which donor milk is heated at 62.5 °C for 30 min, also known as Holder Pasteurization (HoP). This treatment eliminates potential pathogenic microorganisms and hence assures the microbial safety of milk donated. However, it is detrimental to the bioactivity of human milk. Indeed, pasteurization causes the loss of variable amounts of IgA, IgM, IgG, lactoferrin, some vitamins, and other milk components (Henderson et al., 1998). Likewise, sterilization processing is not appropriate to preserve the quality of milk, in terms of its functional value, since it involves exposing food to a temperature generally exceeding 100 °C.

~~Nowadays, the interest in High Pressure Processing (HPP) as an innovative preservation technique is growing.~~ The High Pressure Processing (HPP) is a technique that can be applied to solid and liquid foods (usually 400–800 MPa; for short-term treatments 5-10 min) to provide microbiologically safe, nutritionally intact, and organoleptically high-quality products (Viazis, Farkas, & Jaykus, 2008). Although HPP inactivates pathogenic microorganisms, microbial spores cannot be inactivated by high pressure alone (Cheftel, 1995). At this respect, a more recent development in HPP is the use of moderate-high

process temperatures (High Pressure Thermal; HPT) as this technology was found to allow more efficient spore inactivation. HPT processing involves simultaneous application of high pressure (100-900 MPa) and heating (50-120 °C) for a short holding time. A synergistic effect of combined pressure and elevated temperature has been reported for a range of microbial spores, leading to inactivation of these spores at temperatures under pressure significantly lower than those required at atmospheric pressure conditions (Bull, Olivier, van Diepenbeek, Kormelink, & Chapman, 2009). Likewise, treatments at 50 °C of 400 MPa for 6 min and 500 MPa for 1 min, allow 6 log reductions of *Listeria monocytogenes* in ultra-high-temperature processed (UHT) whole milk (Chen & Hoover, 2003). Moreover, uniform compression during pressurization and adiabatic cooling upon decompression helps to reduce the negative effects of the conventional thermal treatment. Thus, it has been reported that HPT treatments provide better color, flavor and aroma retention compared to traditional thermally treated products (Gupta, Balasubramaniam, Schwartz, & Francis, 2010).

When tasting fresh breast milk, retronasal aroma is described by the respective mother as predominantly sweet, fatty, and soy bean-like, with slight buttery and cooked milk-like impressions (Spitzer & Buettner, 2010). Human milk has a characteristic and unique composition of volatile compounds which provide such odors. The character of the resulting aroma depends on a huge range of complicated mixtures of several compounds such as fatty acids, terpenoid substances, as well as saturated and unsaturated aldehydes and ketones, among others (Buettner, 2007; Spitzer & Buettner, 2009). Since, neonates are able to recognize specific smells with high reliability and to be greatly receptive for flavor learning (Mennella, Jagnow, & Beauchamp, 2001), the preservation of the original volatile compounds in human milk is a crucial concern.

The analysis of the changes in the volatile compounds of breast milk could serve as a

general method to assess the effect of novel technologies, since modifications of these compounds could be the result of the development of multiples chemical reactions (oxidation, Maillard reaction, polymerization...) that takes place during milk processing.

Previous studies evaluated the effect of HPP on the volatile profile of human milk (Contador, Delgado, García-Parra, Garrido, & Ramírez, 2015). However, taking into account that there is no study about the effects of HPT processing on the volatile profile of human milk, the goal of this study was to evaluate the changes in the volatile compounds present in human milk after the application of different HPT treatments.

2. Material and methods

2.1. Human milk collection

Mature breast milk samples were collected from volunteer healthy mothers (n = 6; between 32 and 36 years old and between 3 and 26 months from parturition) from Maire Association (Association of Breastfeeding support of Badajoz, Spain) in July 2012. Each mother donated between 100 and 200 mL of milk that was collected during the previous day of the assay. Milk was kept in the refrigerator until the experiments were carried out during the next morning.

Before processing, all milk donations were mixed in sterile conditions (under a laminar flow cabinet; Telstar AV-100[®]) reaching a final volume of 700-800 mL. Milk was mixed for a better evaluation of the effect of the processing. Milk aliquots (10 mL) were vacuum packed in polyethylene bags (9.3 mL O₂/m²/24 h at 0 °C) for HPT processing. Control samples were also vacuum-packed in plastic bags. All bags have the same dimensions 3.5 x 1.5 cm. Milk samples were protected from the light by using aluminium foil during

sample manipulation. The time between mixing, packaging, processing and freezing the samples was less than 3 h. After processing, samples (processed and unprocessed samples) were stored at -80°C (all at once) until analysis.

2.2 High pressure thermal (HPT) processing

A multi-vessel Resato unit (FPU-100-50, serial no. 14685/42798. Roden, Netherland) was used for the application of the HPT processing; per vessel, one bag was processed at the time. Before the application of the treatments, the bags were inserted 1 min to equilibrate the sample temperature to the initial working temperature. Vessel volume was approximately 50cc (dimensions were approximately 3 cm of diameter and 8 cm of height). The machine uses ethylene glycol as pressure-transmitting medium, and was equipped with thermostatic jacket for temperature control. Initial temperature of glycol was 50, 65 and 80°C and the pressures applied were 300, 600, 900 MPa for 1 min. This holding time was chosen because it was short enough to maintain a quasi-constant temperature of processing. This short holding time avoided the loss of heat during processing. Pressure build-up rate was 10 MPa sec^{-1} . Temperature of milk during HPT treatment could not be measured during processing. We have measured the temperature changes of the pressurization medium (the thermocouple was in the middle of the cylinder vessel), so temperature of milk bags should be lower. However due to the low amount of milk in each bag, differences of temperature between the milk and the pressurization medium should not be very different.

The increases of pressure and temperature during the treatments applied are shown in Fig. 1. The multi-vessel Resato unit registered the temperature of all vessels during the whole process. These data were extrapolated into an excel file. Graphics show the temperature of the pressurisation media and the pressure measured in the vessels were stable during the treatments applied to human milk. After processing, the bags containing the milk were

submerged in cold water to rapidly reduce the breast milk temperature.

Four bags per treatment were processed, analyzed and compared with five control samples.

So a total of 41 samples were studied.

2.3. Analysis of the volatile compounds in human milk

The identification and quantification of such compounds was effectuated by gas chromatography (GC) coupled to mass spectrometry (MS), with a prior step involving the isolation of the volatile fraction by solid-phase microextraction technique (SPME).

For the extraction, 8 g of human milk were placed in a 50 mL vial. The sample was stirred at 35 °C for 30 min to accelerate equilibrium of headspace volatile compounds between the milk matrix and the headspace. After that, volatile compounds were extracted by placing a 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane SPME fibre (Supelco, Bellefonte, PA) into the vial and exposing it to the headspace at 35 °C for 30 min. After extraction, samples were directly desorbed into the injection port of the GC, which was at 270 °C. The analyses of volatile compounds were performed on a Varian CP-3800 GC gas chromatograph coupled to a Varian Saturn 2200 MS (Varian Inc., Palo Alto, CA). Volatile compounds were separated using a capillary column (HP-5; 50 m x 0.32 mm ID x 1.05 µm film thickness; Agilent, Santa Clara, CA). The carrier gas was helium with a flow of 1 mL/min. The temperature programmed was isothermal at 40 °C for 10 min, and then it was raised at 5 °C/min to 240 °C and held for 11 min. The GC-MS transferline temperature was at 280 °C. The MS operated in electron impact mode with electron impact energy of 70 eV, and collected data at a rate of 0.7 scans/s over a range of m/z 40-650. The compounds were identified by comparison with commercial reference compounds provided by Sigma-Aldrich (St. Louis, MO) and by comparison of their mass spectra with those contained in

the NIST and Varian libraries. Volatile compounds were classified according to their most probable origin: lipid oxidation (L), Maillard reaction (M), and other reactions/sources (O). Due to the difficulties to classify all compounds, volatiles with unknown origin were marked as follows (-).

2.4. Statistical analysis

Data are expressed as mean values (n=4 for processed milk and n=5 for control milk) and standard error of the mean (SEM) of the numbers of determinations carried out. To compare the differences in the volatile profile of human milk after processing, statistical significance ($p < 0.05$) was calculated by one-way analysis of variance (ANOVA) followed by *post hoc* Tukey's test. All these statistical treatments were performed using the SPSS program for Windows, version 17.0 (SPSS Inc., Chicago, IL).

3. Results and Discussion

The purpose of this study was to evaluate the changes in the volatile compounds present in human milk after the application of different HPT treatments. For commercial sterilization of low-acid foods, such as milk, 12 log reductions of proteolytic *Clostridium botulinum* type A spores is required. A thermal treatment with a process value F₀ of 3 min has been adopted as the minimum standard for a sterilization process. However, at industrial level generally an F₀ of 5 min is applied. Until today, the limit to reach a sterilization effect is not clear. Vervoort et al., (2012) established that an initial temperature of 90 °C was required to result in a maximum temperature of 124.8 °C after pressure build-up till 700 MPa. According to those authors, these conditions applied during a holding time of 3 min should correspond to a F₀-value of at least 5 min. In the current paper, lower temperatures and shorter times than those required for sterilization were reached and they would be equivalent to a severe pasteurization treatment (Vervoort et al., 2012).

Table 1 presents the volatile compounds (represented by arbitrary area units (AAU) $\times 10^3$) detected in the headspace of fresh (untreated samples) and processed (samples subjected to different HPT treatments) human milk samples. A total of 50 volatile compounds detected in human milk were classified by chemical families: 20 hydrocarbons (aliphatic and aromatic), 5 carboxylic acids, 8 aldehydes, 5 ketones, 7 furans and pyrans, 4 alcohols, and other volatile compound which cannot be included in the previous chemical groups (1 volatile compound).

The application of HPT processing caused statistical significant differences ($p < 0.05$) in the concentration of numerous volatile compounds with respect to their corresponding initial values. In fact, 31 out of 50 volatile compounds suffered significant variations after the application of HPT technology. The application of any HPT treatment caused significant reductions in the AAU of aliphatic hydrocarbons while, in the rest of chemical groups, the variations observed after HPT processing were increased with respect to the control values. The most remarkable augmentations in AAU volatiles were detected in furans and pyrans, alcohols, and aldehydes groups, these increases being even higher than 100-fold (300 and 600 MPa combined with 80 °C), 60-fold (300 MPa combined with 50 and 80 °C), and 30-fold (300 MPa combined with 80 °C) over their respective control values (Table 1). It is important to highlight that these changes were dependent on the combination of pressure intensity and temperature assayed.

3. 1. Hydrocarbons

Hydrocarbons were the most abundant group detected in the headspace of milk samples. In fact, a total of 13 aliphatic and 7 aromatic hydrocarbons were found.

The origin of hydrocarbons is not well understood. In fact several hydrocarbons could not be classified according to their origin. In our study, some of the hydrocarbons detected have their origin in lipid oxidation reactions, such as 2,3,4-trimethyl pentane, 3-ethyl-2-

methy-pentane, octane, decane or 2-pentyl-furan, whereas others, such as the cyclopentanes found in the present study, are derived from Maillard reactions. In addition, we have identified volatiles that are derived from specific sources such as the diet (d-limonene), from thermal degradation of β -carotene (toluene and p-xylene). Based on the diversity of volatiles identified, it is difficult to find a general rule to explain the variations in the hydrocarbons levels occurred after the application of HPT technique.

Aliphatic hydrocarbons usually do not exhibit odors of interest or well-defined character. Nonetheless, certain cycloalkanes have been associated with typical and pleasant notes, such as fruity, green and floral. Aromatic hydrocarbons possess alternating double and single bonds between carbon atoms. The term 'aromatic' was assigned before the physical mechanism determining aromaticity was discovered, and was derived from the fact that many of these compounds have a sweet scent.

3.1.1. Aliphatic hydrocarbons

Aliphatic hydrocarbons were subgrouped in alkanes (8 compounds), cycloalkanes (4 compounds) and terpenes (1 compound).

In general, alkanes were modified after HPT processing. The levels of five of these volatiles (2,3,4-trimethyl-pentane; 3-ethyl-2-methyl-pentane; 2,2,4-trimethyl-hexane; 2,4-dimethyl-hexane; tetradecane) were significantly reduced ($p < 0.05$) after the application of any pressure (300, 600, 900 MPa) and any temperature (50, 65, 80 °C), with respect to their initial levels. However, octane was the unique alkane whose level was significantly increased ($p < 0.05$) after the application of HPT processing at 900 MPa and 65 °C, with respect to its initial value. Among compounds belonging to this group, decane was the unique volatile whose AAU were not modified after the applications of HPT treatments. This hydrocarbon produced a gasoline-like odor, and consequently, increases in this alkane would be associated with unpleasant scents in human milk.

Related to cycloalkanes, the application of HPT processing in human milk samples caused relevant reductions in the levels of these compounds with respect to those found in untreated samples. Some of these reductions were highly noticeable ($p < 0.05$) after the application of HPT processing at 80 °C, particularly in 1,2,4-trimethyl-(1 α , 2 α , 4 β)-cyclopentane and methyl-cyclooctane.

The compound D-limonene, which is characterized for producing pleasant citrus fragrance, was the unique terpene found in the human milk samples studied. The initial levels of this volatile compound suffered variations depending on the processing conditions like the majority of aliphatic hydrocarbons. In fact, its initial levels were significantly reduced ($p < 0.05$) after the application of any pressure assayed (300, 600, 900 MPa) at 50 and 80 °C. Contarini, Povolo, Leardi, and Toppino (1997) observed that D-limonene concentration changes in cow milk depended on the intensity of the thermal treatment applied. Based on these results, we can assume that processing influences, at least in part, the concentration of limonene.

Numerous authors have reported the presence of aliphatic hydrocarbons in fresh and processed milk, such as pasteurized or fermented milk (Solano-López, Ji, & Álvarez, 2006; Imhof & Bosset, 1994; Toso, Procida, & Stefanon, 2002), after the application of different preservation techniques. According to the literature, aliphatic hydrocarbons have their origin in the diet; however, processing could affect the initial levels of these compounds (Pereda, Jaramillo, Quevedo, Ferragut, Guamis, & Trujillo, 2008). In our study the initial levels of numerous alkanes suffered a significant reduction ($p < 0.05$) after the HPT treatment, suggesting that treatment conditions and in particular, the combination of pressure and temperature, may modify the alkanes content in human milk.

3.1.2. Aromatic hydrocarbons

Seven types of aromatic hydrocarbons were isolated in human milk samples. In this

chemical group, significant changes ($p < 0.05$) in the concentration of several hydrocarbons were detected due to the application of different treatment conditions.

Toluene is the most cited aromatic hydrocarbon in literature. It belongs to the mineral odor class, is classified as a compound with paint-like odor and may be originated from the breakdown of β -carotene (Contarini et al., 1997). Previous studies have also detected the presence of this compound in milk (Contarini et al., 1997; Valero, Villamiel, Miralles, Sanz, & Martínez-Castro, 2001; Mounchili et al., 2005). Its levels remained almost constant with respect to the initial values when the HPT treatments were applied. Nevertheless, it is important to highlight that the application of 300 MPa at 50 °C importantly reduced the initial levels of toluene, since this compound was not detected in these treated samples. Similar effects were observed in the concentration of **isopropenylphenol and 2,4-cyclopentadien-1-ylidenemethyl-benzene**. In both cases the application of any pressure (300, 600, 900 MPa) combined with any temperature (50, 65, 80 °C) dramatically diminished ($p < 0.05$) the initial levels of these compounds.

The combination of pressure and temperature also affected the levels of **2-pentylfuran and 1,2,3-benzenetriol**. Despite the fact that both compounds were not detected in control milk samples, the application of different HPT treatments caused the appearance of these aromatic hydrocarbons in the treated milk. It is important to highlight that **2-pentylfuran** is derived from oxidation of unsaturated fatty acids (Ramadan & Elsanhoty, 2012). In fact, it may be produced from oxidation of linoleic acid by both lipoxygenase and oxygen radicals. Consequently, its augmentation may be related with increases in oxidative processes occurred after the application of HPT processing. Moreover, fatty acids derived from volatiles have been considered to contribute to unpleasant notes (Dobson, Griffiths, Davies, & McNicol, 2004) and, hence, enhancements in this compound could produce negative scent in the human milk.

3.2. Carboxylic acids

Five carboxylic acids were isolated in the headspace of control and processed human milk. Octanoic and decanoic acids were the most abundant in control milk. Both compounds produce odors belonging to the dairy class (cheese, sweat, fat, rancid-like odors). Thus, Mounchili et al. (2005) connected the burnt milk odor with the presence of octanoic and n-decanoic acids. The levels of decanoic acid were not affected by HPT processing, however, treatments at 300 MPa/50 °C and 900 MPa/80 °C caused a significant decrease ($p < 0.05$) in octanoic acid levels present in treated milk samples with respect to control values. Nonetheless, the concentration of dodecanoic acid was enhanced ($p < 0.05$) at 300 MPa and 50 °C.

It has been reported that increases in free fatty acids represent the potential initial stage of a subsequent fat oxidation (Belitz, Grosch, & Schieberle, 2004). Moreover, high levels of short- and medium-chain fatty acids (C4:0–C14:0) are associated to the appearance of rancid odours (they are frequent in low quality milk). In our study, in general, the levels of carboxylic acids were not significantly modified after the HPT processing and, consequently, we suppose that this chemical group would not cause a negative impact in the final aroma of milk. Nonetheless, further studies (using sensory methods) would be necessary.

3.3. Aldehydes

Aldehyde compounds were importantly increased after the HPT treatments. In fact, they were the most abundant chemical group (in terms of AAU) found in processed human milk. This is an important concern since high levels of aldehydes in milk has been commonly associated with oxidized off-flavor or oxidative rancidity in this matrix. Based on the origin, we have identified different aldehydes in human milk: (1) aldehydes which are produced from lipid oxidation, and (2) aldehydes derived from the Maillard reaction. The

latter are usually more complex aldehydes, such as 2-furaldehyde, 2,5-furancarboxaldehyde, 5-(hydroxymethyl)-2-furancarboxaldehyde, while aldehydes originated from lipid oxidation are lineal aldehydes, such as hexanal, heptanal, 2-heptenal, octanal and 2-decenal (Namiki, 1988; Friedman, 1996; Elisia & Kitts, 2011).

Literature often points to hexanal, a decomposition product of linoleic acid, as a reliable indicator of lipid oxidation in human milk (Elisia & Kitts, 2011) that plays an important role in sensory off-flavors. In addition, pentanal represents another peroxidation product derived from linoleic acid that is characterized by its acrid-pungent odor-like. In our study, the initial levels of both hexanal and pentanal compounds were remarkably enhanced after the application of 600 and 900 MPa at 50, 65 and 80 °C. Moreover, Elisia and Kitts (2011) studied the levels of hexanal in human milk since this compound is used as indicator of the lipid oxidation extent in several foods. They found a relationship between hexanal content and the presence of antioxidants (such as tocopherol and ascorbic acid) in milk. The chromatographic signal matching octanal was not resolved correctly due to its overlapping with another compound, 2H-pyran-2,6(3H)-dione. For this reason, we decided to quantify both compounds together as octanal + 2H-pyran-2,6(3H)-dione. The initial levels of these two compounds, together with the initial levels of Z-2-heptenal, were significantly ($p < 0.05$) elevated after the application of 300, 600 and 900 MPa, although different increases were found depending on the temperature used during the process. By contrast, the concentration of 2-furaldehyde and 2,5-furancarboxaldehyde were significantly ($p < 0.05$) modified only after the application of 300 MPa at 80 °C. The absence of 5-(hydroxymethyl)-2-furancarboxaldehyde in control samples contrasted with the high levels found after HPT processing. Although no statistical differences were found among control and treated samples, this compound mainly contributed to the total area of aldehydes obtained after processing.

The current mechanisms of formation of aldehydes under high pressure are not known. It was hypothesized that the elevated content of aldehydes in HPT-treated milk could be caused by the fracture of fat globules (Fitz, Kerler, & Weenen, 1999) where lipids were immersed as a result of pressure intensity.

In general terms, 7 out of 8 aldehydes identified in our study were augmented after the application of HPT processing with respect to its content in non-treated milk, this increase being depending on the aldehyde identified. At this respect, Vázquez-Landaverde, Torres, and Qian (2006) have reported that HPP (480-620 MPa at 25 and 60 °C) caused minimum changes of volatile compounds in milk, whereas high-pressure at high temperature promoted the formation of aldehydes. Accordingly, we have also previously demonstrated that, in general, the application of HPP (400-600 MPa for 3 min) at low temperature produced minimum changes in the profile of volatile compounds of human milk (Contador et al., 2015), whereas the combination of high-pressure with high-temperature (HPT processing) increased the content of volatiles, especially aldehydes, as the current results show.

3.4. Ketones

Ketones are abundant constituents of most dairy products. As a consequence of their typical odors (green and grass-like notes) and low perception thresholds, they contribute remarkably to the aroma of products that contain them.

Our results showed that the combination of pressure and temperature produced different variations in the concentrations of the ketones detected. Only the levels of 3-heptanone were not affected by HPT treatment. In fact, the concentration of 1,2-cyclopentanedione was significantly ($p < 0.05$) increased after the application of 300 MPa at 50 °C. Similarly, the ketone 6-methyl-2-heptanone was not detected in control samples, whereas the combination of 600 MPa with 65 °C and 80 °C, and 900 MPa with 50 °C, 65 °C and 80 °C

caused a significant ($p < 0.05$) increase in this compound. In contrast, the level of 3-nonanone was remarkably decreased after the application of all treatments, with respect to the control values.

Although these compounds are naturally present in raw milk, most ketones can be formed during heat treatment by β -oxidation of saturated fatty acids followed by decarboxilation (Nawar, 2000) or by decarboxilation of β -ketoacids present in milk fat (Calvo & de la Hoz, 1992). Our results showed that not only temperature, but also pressure played an important role in the formation and/or degradation of ketones, since the combination with different temperatures produced diverse effects. At this respect, numerous authors have also reported that high pressure enhances oxidation of free fatty acids leading to the formation of ketone volatile compounds such as 2-heptanone (Oey, Lille, Van Loey, & Hendrickx, 2008; Van der Plancken et al., 2012).

3.5. Furans and pyrans as main products of Maillard reaction.

Historically, furan and its derivatives have been recognized as contributors to the sensory properties of a wide variety of foods. In the literature, many ways leading to the formation of furan have been reported, the major precursors being sugars (alone or in combination with amino acids), ascorbic acid, and unsaturated fatty acids followed by amino acids and carotenoids (Limacher, Kerler, Davidek, Schmalzried, & Blank, 2008; Owczarek-Fendor et al. 2011; 2012).

In the present study, seven types of furans and pyrans were detected. The chromatographic signals corresponding to the compounds dihydro-4-hydroxy-2(3H)-furanone and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one were not resolved correctly, since they overlapped with each other and they were quantified together (Table 1). In general, the application of any HPT treatment caused an increase in the furans and pyrans present in human milk, with respect to their corresponding control samples. In fact, the total area of

treated milk samples increased their content in furans and pyrans more than 100-fold over control. This augmentation is mainly due to the fact that 5 out of 7 of these compounds were not detected in control milk samples. It may be explained because volatile sulfur compounds present low threshold limit values and high reactivity, which turned them into a big challenge to detect and identify in food matrices.

The original content of furans and pyrans present in human milk, and foodstuffs in general, do not exert negative effects on their final quality. It is well known that these compounds are important end-products of Maillard reaction with strong caramel-like odors. Therefore, reductions in their original concentration might result in processed products with a different and/or unacceptable aroma, taste or color of food. Likewise, an abrupt increase in furan concentration may cause negative effects on foodstuffs. HPP have demonstrated to be effective on the preservation of bioactive compounds on human milk (Contador, Delgado-Adámez, Delgado, Cava, & Ramírez, 2013), however the results derived from the application of combined pressure and temperature (HPT processing) on the furan concentration in human milk suggest that processing conditions chosen were not adequate to preserved the quality of milk. Nevertheless, recent findings have proven that the application of HPT at high temperatures (above 80 °C) is effective in reducing furan formation in vegetable-based matrixes compared to commercial thermal treatments of sterilization. (Palmer, Grauwet, Kebede, Hendrickx, & Van Loey, 2014; Sevenich et al., 2015).

According to our findings, the application of HPT processing, particularly, the treatments at 80 °C, combined with any pressure, produced a significant ($p < 0.05$) increase on the concentration of certain furans and pyrans with respect to untreated milk, which may not produce positive sensorial acceptance. It is well known that the increase of temperature as one of the promoters of Maillard reactions which agrees with the high temperatures

reached during processing (90-110 °C) at HPT treatments at 80 °C (initial temperature). However, curiously the less intense treatment applied (300 MPa at 50 °C) also favored the increase of furans and pyrans. Probably, changes in the chemical configurations of these compounds under those conditions could be behind these increases.

3.6. Alcohols

The presence of alcohols in control milk was very low. In fact, only one type of alcohol (2-ethyl-1-pentanol) was detected. Curiously, the concentration of this alcohol was significantly ($p < 0.05$) decreased after the application of any HPT treatment assayed (300, 600, 900 MPa in combination with 50, 65 and 80 °C). By contrast, the presence of three alcohols: 3-furanmethanol, 1-hepten-3-ol and 1,2,3-Propanetriol,1-acetate was only observed after the application of HPT treatments, being absent in control milk samples. Among these compounds, the initial concentration of 1-hepten-3-ol was significantly ($p < 0.05$) rose after the application of 600 and 900 MPa at 50, 65 and 80 °C. In general, these volatiles are associated with green and beany aromas. Related to their origin, the vast majority of alcohols detected are originated from lipid oxidation (with the exception of 3-furanmethanol, that is derived from Maillard reaction), so their increase after such treatments would be in line with the high levels of other lipid-derived compounds such as aldehydes in processed milk.

3.7. Others

A chlorocarbon, namely tetrachloroethylene, also known as perchloroethylene or 'perc', was detected in the human milk samples studied. The concentration found in control samples was significantly ($p < 0.05$) diminished after the application of HPT treatment. Due to its lipophilicity, 'perc' has been found in biologic fluids such as human breast milk (Schreiber, 1997). Based on our results, the HPT processing would be an efficient tool to reduce the levels of this volatile compound in human milk, although this assumption would

need to be confirmed with specific studies.

3.8. Origin of volatile compounds after HPT processing

Maillard reaction and lipid oxidation are probably the two most important chemical reactions occurring in foods during processing and storage. However, these pathways are not independent and each of them influences the development of the other. Furthermore, the carbonyl compounds produced in the lipid oxidation pathway will compete with carbohydrate-derived carbonyls for amino compounds to produce carbonyl-amine reaction products with either beneficial or harmful properties. These reactions are the final step of the oxidation pathway when lipid oxidation occurs in the presence of amino compounds (Zamora & Hidalgo, 2011). As previous studies have reported, lipid oxidation and Maillard reaction are the main chemical reactions that induced volatile changes after the application of HPP in human milk (Contador et al., 2015). At this respect, Contador et al. (2015) have demonstrated that the application of HPP at 600 MPa for 6 min importantly enhanced the formation of volatiles, particularly, aldehydes, furans and pyrans, which are associated with an increase in lipid oxidation and with the degradation of sugars and amino acids via Maillard reaction. This increase was even higher than after the application of HoP. Similarly, in the present study, significant increases were detected in the levels of volatile compounds derived from Maillard and lipid oxidation reactions after HPT processing, these changes being subjected to the intensity of pressure and temperature applied (Fig. 2). The global evaluation of the changes of volatile compounds according to their origin indicates that the application of the most intense treatments conditions did not increase the level of compounds from Maillard and lipid oxidation reaction. In fact, treatments at 300 MPa and 50 and 80 °C showed the highest levels of these volatile compounds and concretely milk processed at 300 MPa and 80 °C showed the highest levels of volatiles from Maillard reaction. Specific changes that occur at that pressure conditions could

explain these results.

Delgado et al. (2013) concluded that not only volatiles, but also macronutrients and/or biological molecules with important physiological roles were affected by the intensity of pressure and temperature utilized in a HPT processing. According to the aforementioned study, only the treatments at 300 MPa and 50 °C could be suitable for the preservation of essential nutrients and immunological components of breast milk. However, although initial temperature of processing was moderate (50 °C) and despite of the fact that the holding time is shorter in HPT processing compared to HoP (30 min), the increase of temperature due to the adiabatic heating reached ~67 °C, temperature being even higher than 62.5 °C applied in HoP. The increase of temperature during processing could be responsible of the degradation effect of treatments.

4. Conclusions

In general, the application of HPT processing at 300, 600, 900 MPa combined with 50, 65, 80 °C in human milk, enhanced the levels of derived compounds from lipid oxidation and Maillard reaction such as aldehydes, ketones, furans and pyrans, and alcohols. In contrast, it reduced the content of aliphatic hydrocarbons and did not affect the levels of carboxylic acids. Negative odor attributes in human milk may be derived from these variations. Taking into account that changes in the volatile profile reflects modifications of the general composition of milk, we could assume that the HPT conditions utilized in the present study may not be adequate to process human milk. Nevertheless, we consider particularly relevant to assay different combinations of pressures and temperatures at less intense conditions to conclude the efficacy of the HPT technology to preserve milk quality in human milk banks.

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6. Conflict of interest

There are no conflicts of interest to disclose.

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Figure 2

Figure captions

Figure 1. Recorded pressure and temperature during the high pressure-high temperature processing applied in breast milk at 300 MPa (A), 600 MPa (B), and 900 MPa (C). T1: initial temperature of treatment at 50 °C; T2: initial temperature of treatment at 65 °C; T3: initial temperature at 80 °C.

Figure 2. Sum of the total content of volatile compounds (AAU x 10³) detected in unprocessed (control) human milk and processed samples by HPT technique (300, 600, 900 MPa with 50, 65, 80 °C) based on their origin.

Table 1. Mean and standard error of the mean of volatile compounds (AAU x 10³) isolated in breast milk processed by different high pressure-thermal treatments (300MPa/50 °C; 300MPa/65 °C; 300 MPa/80 °C; 600 MPa/50 °C; 600MPa/65 °C; 600MPa/80 °C; 900MPa/50 °C; 900 MPa/65 °C; 900MPa/80 °C).

	RT (min) ^x	Id Method ^y *	Control	300 MPa			600 MPa			900 MPa			SEM	p- value	Origin ^z
				50 °C	65 °C	80 °C	50 °C	65 °C	80 °C	50 °C	65 °C	80 °C			
ALIPHATIC HYDROCARBONS															
Alkanes															
2,3,4-Trimethyl-pentane	17.3	RF	302 ^a	0 ^b	74 ^b	0 ^b	52 ^b	58 ^b	0 ^b	66 ^b	0 ^b	0 ^b	19.1	0.001	L
3-Ethyl-2-methyl-pentane	17.6	TI	453 ^a	0 ^b	60 ^b	0 ^b	0 ^b	120 ^b	0 ^b	133 ^b	128 ^b	0 ^b	28.3	0.001	L
2,2,4-Trimethyl-hexane	19.0	TI	302 ^a	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	17.9	0.001	-
Octane	19.9	RF	121 ^{bc}	0 ^c	36 ^c	29 ^c	87 ^c	268 ^{ab}	58 ^c	282 ^{ab}	299 ^a	173 ^{abc}	20.0	0.001	L
2,4-Dimethyl-hexane	21.0	TI	156 ^a	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	10.8	0.001	-
Decane	28.6	RF	390	533	475	410	469	401	485	348	557	416	29.4	0.902	L
Dodecane	35.3	RF	848 ^a	179 ^b	277 ^b	175 ^b	49 ^b	190 ^b	320 ^{ab}	241 ^b	375 ^{ab}	329 ^{ab}	50.3	0.003	-
Tetradecane	41.1	RF	133 ^a	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	7.0	0.001	-
Subtotal			2705	712	922	614	657	1037	863	1070	1359	918			
Cycloalkanes															
1,2,4-trimethyl- (1α,2α,4β)-cyclopentane	19.4	TI	1665 ^a	375 ^b	314 ^b	0 ^b	525 ^b	555 ^b	0 ^b	438 ^b	802 ^b	0 ^b	97.1	0.001	M

1,2,4-trimethyl-(1 α ,2 β ,4 α)-cyclopentane	24.1	TI	238	35	93	20	112	106	48	80	145	75	16.6	0.069	M
(1,2-dimethylpropyl)-cyclopropane	26.4	TI	93	35	56	16	40	59	12	49	65	33	6.5	0.127	-
Methyl-cyclooctane	30.1	TI	163 ^a	151 ^a	102 ^{ab}	38 ^{bc}	49 ^{bc}	39 ^{bc}	0 ^c	26 ^{bc}	45 ^{bc}	0 ^c	10.9	0.001	-
<i>Subtotal</i>			2159	596	565	74	726	759	60	593	1057	108			
<i>Terpene</i>															
D-limonene	30.0	RF	728 ^a	466 ^{bcde}	632 ^{ab}	293 ^{ef}	415 ^{cde}	607 ^{abc}	207 ^f	497 ^{bcd}	605 ^{abc}	356 ^{def}	28.2	0.001	O
<i>Subtotal</i>			728	466	632	293	415	607	207	497	605	356			
AROMATIC HYDROCARBONS															
Toluene	18.4	RF	149 ^{abc}	0 ^d	74 ^{cd}	95 ^{bc}	160 ^{abc}	205 ^a	131 ^{abc}	173 ^{ab}	207 ^a	122 ^{abc}	11.0	0.001	O
p-Xylene	23.5	RF	491 ^a	350 ^{ab}	273 ^{ab}	69 ^b	295 ^{ab}	294 ^{ab}	124 ^{ab}	342 ^{ab}	443 ^{ab}	218 ^{ab}	30.9	0.031	O
2-Pentyl-furan	28.4	TI	0 ^f	0 ^f	60 ^{ef}	89 ^{def}	244 ^{cde}	510 ^{ab}	289 ^{bcd}	395 ^{abc}	574 ^a	303 ^{bcd}	34.4	0.001	L
Hexylbenzene	37.6	RF	49	110	83	121	140	83	100	103	98	95	6.6	0.103	O
p-Isopropenylphenol	38.6	TI	127 ^a	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	6.6	0.001	-
1,2,3-Benzenetriol	40.9	TI	0 ^b	1867 ^a	393 ^{ab}	1338 ^{ab}	787 ^{ab}	443 ^{ab}	1183 ^{ab}	633 ^{ab}	585 ^{ab}	792 ^{ab}	125.0	0.007	-
(2,4-Cyclopentadien-1-ylidenemethyl)-benzene	41.4	TI	84 ^a	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	4.5	0.001	-
<i>Subtotal</i>			900	2327	883	1712	1626	1535	1827	1646	1907	1530			
CARBOXYLIC ACIDS															
1,3-Butadiene-1-carboxylic acid	25.9	TI	0	8781	403	1484	793	375	918	415	904	838	768.9	0.332	L
Octanoic acid	34.2	RF	1663 ^a	0 ^b	766 ^{ab}	1089 ^a	935 ^a	1071 ^a	865 ^a	1059 ^a	879 ^a	0 ^b	102.8	0.001	L

2-Hydroxy-2-methyl-(S)-butanedioic acid	39.7	TI	0	4099	2227	5222	3820	2020	6640	2535	3173	4320	492.2	0.087	L
n-Decanoic acid	40.0	RF	1664	2765	1208	2232	1624	1281	1702	979	1156	1073	141.0	0.093	L
Dodecanoic acid	45.2	RF	920 ^b	10882 ^a	3461 ^{ab}	10590 ^{ab}	6314 ^{ab}	3153 ^{ab}	7723 ^{ab}	2881 ^{ab}	3543 ^{ab}	3916 ^b	764.8	0.014	L
<i>Subtotal</i>			<i>4247</i>	<i>26527</i>	<i>8065</i>	<i>20617</i>	<i>13486</i>	<i>7900</i>	<i>17848</i>	<i>7869</i>	<i>9655</i>	<i>10147</i>			
ALDEHYDES															
Hexanal	20.1	RF	282 ^e	90 ^e	550 ^e	2760 ^{de}	6251 ^{bcd}	6628 ^{bc}	4295 ^{cd}	12208 ^a	9721 ^{ab}	4681 ^{cd}	662.4	0.001	L
2-Furaldehyde	21.8	RF	32 ^b	2020 ^{ab}	592 ^{ab}	3467 ^a	830 ^{ab}	408 ^{ab}	2020 ^{ab}	638 ^{ab}	1297 ^{ab}	2127 ^{ab}	246.1	0.029	M
Heptanal	24.8	RF	157 ^{de}	27 ^e	102 ^e	403 ^{cd}	465 ^{bc}	906 ^a	697 ^{ab}	941 ^a	988 ^a	718 ^{ab}	57.5	0.001	L
(Z)-2-Heptenal	27.1	TI	25 ^e	7 ^e	52 ^e	320 ^d	630 ^c	1236 ^a	927 ^b	986 ^{ab}	1147 ^{ab}	605 ^c	73.9	0.001	L
Octanal + 2H-pyran-2,6(3H)-dione	28.8	RF	325 ^c	947 ^{abc}	420 ^{bc}	1152 ^{ab}	802 ^{abc}	1003 ^{abc}	1202 ^{ab}	1289 ^a	1217 ^{ab}	1004 ^{abc}	70.8	0.003	L
2,5-Furandicarboxaldehyde	31.6	TI	0 ^b	504 ^{ab}	302 ^{ab}	628 ^a	274 ^{ab}	245 ^{ab}	217 ^{ab}	306 ^{ab}	492 ^{ab}	510 ^{ab}	44.0	0.026	M
5-(Hydroxymethyl)-2-furancarboxaldehyde	36.4	RF	0	15728	2829	18528	4899	2572	6977	3213	8437	9291	1417.7	0.028	M
(Z)-2-Decenal	37.4	TI	92 ^d	378 ^{bcd}	191 ^{cd}	603 ^{abcd}	486 ^{abcd}	516 ^{abcd}	1023 ^{ab}	826 ^{abc}	863 ^{abc}	1135 ^a	68.3	0.001	L
<i>Subtotal</i>			<i>913</i>	<i>19701</i>	<i>5038</i>	<i>27861</i>	<i>14637</i>	<i>13514</i>	<i>17358</i>	<i>20407</i>	<i>24162</i>	<i>20071</i>			
KETONES															
3-Heptanone	24.2	RF	127	35	49	44	54	57	47	70	52	64	7.3	0.120	O
2-Heptanone	24.3	RF	34 ^{abc}	0 ^c	31 ^{abc}	18 ^{bc}	16 ^{bc}	38 ^{abc}	50 ^{ab}	64 ^{ab}	68 ^a	43 ^{abc}	4.4	0.029	L
1,2-Cyclopentanedione	26.1	TI	57 ^b	3588 ^a	707 ^{ab}	2297 ^{ab}	1832 ^{ab}	718 ^{ab}	1827 ^{ab}	579 ^b	1061 ^{ab}	1672 ^{ab}	234.1	0.016	-

6-Methyl-2-heptanone	27.0	TI	0 ^d	0 ^d	0 ^d	2 ^{cd}	5 ^{cd}	16 ^{bc}	27 ^{ab}	39 ^a	27 ^{ab}	37 ^a	2.6	0.001	-
3-Nonanone	31.8	TI	141 ^a	96 ^b	0 ^c	0 ^c	41 ^c	0 ^c	0 ^c	0 ^c	0 ^c	0 ^c	8.6	0.001	-
<i>Subtotal</i>			<i>359</i>	<i>3719</i>	<i>787</i>	<i>2361</i>	<i>1948</i>	<i>829</i>	<i>1951</i>	<i>752</i>	<i>1208</i>	<i>1816</i>			

FURANS AND PYRANS

2(5H)-Furanone	25.6	RF	84	1935	432	1729	976	417	1208	395	696	916	1442.3	0.033	M
Dihydro-4-hydroxy-2(3H)-furanone+ 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	34.1	TI	0	1540	2811	5709	3619	2664	6216	4083	5277	5910	580.4	0.147	M
3,5-Dihydroxy-2-methyl-4H-pyran-4-one	35.4	TI	0 ^b	1432 ^{ab}	1042 ^{ab}	2731 ^a	1737 ^{ab}	732 ^{ab}	1987 ^{ab}	1351 ^{ab}	1889 ^{ab}	1834 ^{ab}	177.0	0.008	M
Dihydro-3-(thioacetyl)-2(3H)-furanone	38.9	TI	52	2197	612	2293	865	615	1032	813	795	974	175.8	0.058	M
Furfuryl formate	25.0	TI	0	451	85	486	136	69	127	97	117	146	41.7	0.109	M
Maltol	33.1	TI	0 ^b	1554 ^a	532 ^{ab}	1787 ^a	1003 ^{ab}	641 ^{ab}	1633 ^a	1349 ^{ab}	694 ^{ab}	1522 ^a	125.2	0.002	M
Maltol propionate	31.9	TI	0	966	1513	1863	1145	1477	2197	2164	1381	2025	188.7	0.138	M
<i>Subtotal</i>			<i>136</i>	<i>10075</i>	<i>7027</i>	<i>16598</i>	<i>9481</i>	<i>6615</i>	<i>14400</i>	<i>10252</i>	<i>10849</i>	<i>13327</i>			

ALCOHOLS

2-Ethyl-1-pentanol	18.5	TI	62 ^a	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	3.4	0.001	L
3-Furanmethanol	21.1	TI	0	761	395	737	429	230	465	157	457	634	67.9	0.125	M
1-Hepten-3-ol	27.9	TI	0 ^c	0 ^c	32 ^c	194 ^c	526 ^b	1145 ^a	673 ^b	778 ^b	1094 ^a	587 ^b	67.6	0.001	L
1,2,3-Propanetriol, 1-acetate	36.9	TI	0	2993	324	3180	740	357	1810	500	681	1593	287.3	0.072	L

<i>Subtotal</i>			62	3754	751	4111	1695	1732	2948	1435	2232	2814		
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OTHERS

Tetrachloroethylene	20.7	TI	153 ^a	70 ^b	72 ^b	39 ^b	45 ^b	64 ^b	30 ^b	64 ^b	73 ^b	45 ^b	6.5	0.001	-
<i>Subtotal</i>			153	70	72	39	45	64	30	64	73	45			

^xRetention time ^yMethod of identification: RF, mass spectrum and retention time identical with a reference compound; TI, tentative identification by mass spectrum; SEM, standard error media. a, b, c: Different letters in the same row indicate significant statistical differences (Tukey's test, P < 0.05). ^zOrigin L: compound derived from lipid oxidation; M: compound derived from Maillard reaction; O: compound derived from other reactions/sources; -: compound derived from unknown source. Each value is expressed as mean of four (processed milk) and five (control milk) repetitions.