TOWARDS THE DEVELOPMENT OF HIGH QUALITY UHT HYDROLYZED-LACTOSE MILK

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Towards the development of high quality UHT hydrolyzed-lactose milk

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Summary and aim of the PhD project

Lactose is present at high concentration in mammals' milk and it is metabolized by β-1,4-galactosidase (lactase) that splits the molecule into galactose and glucose, which are then absorbed in the small intestine. Lactose intolerance is a worldwide issue with both nutritional and economic implications. In traditional dairy consuming countries, the consumer perception about lactose intolerance is mounting; it is estimated that 70% of world population has non-persistence lactase. Lactase is always present in the newborn, but its activity naturally decreases after weaning. Undigested lactose is fermented in the gut increasing the abdominal pressure and intestinal transit causing unpleasant symptoms such as abdominal pain and diarrhea. Low Lactose (LL) or lactose free (LF) products represent the simplest solution to deal with the issue keeping the nutrients intake related to milk and dairy products consumption but eliminating intestinal discomfort. This product category will reach a turnover of €9 billion by 2022 and today represent the fastest growing market in the dairy segment. The most popular methodology applied to produce LL and LF products is through lactose hydrolysis by addition of free soluble lactase, which lowers the content of lactose in the milk from 4.5-5.0% down to <0.01%. The availability of several commercial lactase preparations (LPs) favored the development of different technological approaches to manufacture ultra-high temperature hydrolyzed-lactose milk (UHLM). On the other hand, depending on the production system employed, manufacturing poses several technological problems in relation to the stability upon production and shelf-life of the milk, with potential economic implications as well. Furthermore, the commercial LPs intended for UHLM production contain enzymatic side activities that, if not properly controlled and mitigated, can change the physicochemical and sensory properties of the final product during shelf-life, which may end up in consumers rejection. In particular, off-flavor formation is the major factor limiting the shelf-life of UHLM. UHLM with unaltered sensory characteristics during storage can be produced as long as the chemical nature of off-flavor formation is well understood. Therefore, there is a clear need to identify those molecules responsible for the off-flavor in UHLM in relation both to the processing technology applied and the sensory characteristics of the final product. In this context, the aim of this PhD project was to unravel the chemical nature of the changes in the flavor profile of UHLM occurring during shelf-life. In particular, the project attempted to explore the effect of different production and storage conditions on the formation and release of volatile compounds in different production and storage stages, elucidating the relationship between process technology and the variables

implicated in the definition of the final product quality. Utmost attention was given to Maillard reaction and its secondary pathways because, upon lactose hydrolysis and secondary proteolysis from the lactase, the reaction is facilitated causing unwanted modifications of the UHLM sensory properties. Priority was given to the UHLM produced by the "in batch" system, one of the two production technologies involving free soluble lactase currently available for dairy producers. The production system involves the thermal inactivation of the lactase after lactose hydrolysis which, according to the literature, may alleviate the proteolytic and arylsulfatase side effects of the commercial LPs. This aspect would be particularly beneficial in comparison to the other production technology available (the so-called "in pack" system): in this case, the lactase is added to the milk after thermal sterilization. This strategy allows the mitigation of Maillard reaction (MR) upon processing as the reducing sugars generated by lactose hydrolysis are not exposed to high temperature. On the other hand, the LP remains active throughout the product shelf-life and its side activity may alter the milk components until consumption. Starting from this hypothesis, these specific goals have been addressed along the PhD project:

1. Mapping the volatile organic compounds (VOCs) profile of UHLMs throughout the product lifecycle, both during production and shelf-life.
2. Development and fine-tuning of analytical methodologies for studying the chemical parameters associated with quality changes in UHLM (e.g. GC-MS, PTR-MS, LC-MS).
3. Identifying specific VOCs formed *via* Maillard reaction, elucidating the pathways of formation in relation to different lactases employed for production.
4. Defining how the flavor profile is correlated to the sensory characteristics of UHLM during shelf-life, explaining the chemical nature of the changes limiting the shelf-life of UHLM.
5. Proposing effective strategies to mitigate those reactions limiting the shelf-life of UHLM by looking at the response of the product to different storage temperatures.

List of Publications

Research article 1 (published)	Bottiroli, R., Aprea, E., Betta, E., Fogliano, V., & Gasperi, F. (2020). Application of headspace solid-phase micro-extraction gas chromatography for the assessment of the volatiles profiles of ultra-high temperature hydrolyzed-lactose milk during production and storage. *International Dairy Journal*, 104715.
Short communication (published)	Bottiroli, R., Zhang, C., Aprea, E., Fogliano, V., Hettinga, K., & Gasperi, F. (2020). Short-time freezing does not alter the sensory properties and the physical stability of ultra-high-temperature hydrolyzed-lactose milk. *Journal of Dairy Science, 103*(10), 8822-8828
Research article 2 (published)	Bottiroli, R., Troise, A.D., Aprea, E., Fogliano, V., Vitaglione P., & Gasperi, F. (2020). Chemical and sensory changes during shelf-life of UHT hydrolyzed-lactose milk produced by "in batch" system employing different commercial lactase preparations. *Food Research International, 109552.*
Research article 3 (published)	Bottiroli, R., Pedrotti, M., Aprea, E., Biasioli, F., Fogliano, V., & Gasperi, F. (2020). Application of PTR-TOF-MS for the quality assessment of lactose-free milk: effect of storage time and employment of different lactase preparations. *Journal of Mass Spectrometry,* e4505 .
Research article 4 (published)	Bottiroli, R., Troise, A. D., Aprea, E., Fogliano, V., Gasperi, F., & Vitaglione, P. (2021). Understanding the effect of storage temperature on the quality of semi-skimmed UHT hydrolyzed-lactose milk: an insight on release of free amino acids, formation of volatiles organic compounds and browning. *Food Research International*, *141*, 110120

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Chapter 1: General Introduction

Overall milk composition

Milk is a white colloid, composed by 87% of water, containing dissolved carbohydrates, fat and protein aggregated with minerals (Jost, 2000). The milk composition is designed, by nature, to provide a complete nutritional intake to newborns. Milk is an important source of both macronutrients (proteins, lipids and carbohydrates) and micronutrients (vitamins and minerals) and, for this reason, the mankind has exploited the one produced by other mammalian species as a primary source of nutritional intake since more than 8000 years (Goulding, Fox, & O'Mahony, 2020). Today, dairy products are a staple food in the diet of many people, with more than 6 billion worldwide consuming milk and milk products on a regular basis (FAO, 2018). Cow milk is the most consumed milk type, accounting for 81% of the world milk production, so it was chosen to carry on the PhD. Cow milk is followed in term of volumes by buffalo (15%). Goat, sheep and camel instead make altogether around the 4% of the worldwide production (OECD/Food and Agriculture Organization of the United Nations, 2019).

Proteins, probably the most valuable components of cow milk from an industrial viewpoint, account for the 3.0–3.5% of the total milk composition and are divided into two major groups: casein (CN) and whey protein (WP) (Goulding et al., 2020). As an ingredient, milk proteins encompass versatile functionality (e.g. viscosity enhancer, gelation, foaming, emulsification, oil- and water-binding capacity). For this reason, they found broad application in several food products both dairy-based (e.g. cheese, yoghurt, ice cream etc.) and non-dairy-based (e.g. meats, desserts and flavours) (Carr & Golding, 2016). CNs are the most abundant milk protein fraction (80% w/w) and can be distinguished in αS1-CN, αS2- CN, β- CN and k-CN which are present in the milk in a ratio of 4:1:4:1 (Y. Liu & Guo, 2008). In the bovine milk, CNs have relatively large particle size (80–300 μm) and are organized in micelles bounded with calcium phosphate. The α-CNs and β-CNs are present in the inner part of the structure, whereas the k-CNs surround the micelles surface acting as protectors (Sinaga, Bansal, & Bhandari, 2017). Hydrogen bonds, hydrophobic and electrostatic interactions stabilize the whole structure rendering CN micelles stable at standard milk conditions (Dalgleish & Corredig, 2012). The presence of the mineral makes the micelles capable of delivering high intakes of calcium and protein upon milk consumption. From an industrial perspective, CN-

based ingredients, such as milk protein concentrate and micellar CN concentrate, are produced by physical separation of the CN from the rest of the milk components (Carr & Golding, 2016). The remaining 20% of milk proteins are WP, with β-lactoglobulin (β-LG) (50%) and α-lactalbumin (α-LA) (20%) being the two most abundant (Jansson, 2014). Native WPs have a globular conformation which render them soluble over a wide range of pHs. Upon processing however, the proteins unfold due to different factors (e.g. heat, alcohol etc.), affecting WP structure and functionality (Morr, 1975). WPs have been considered for a very long time just a waste product of cheese and casein manufacturing but, thanks to important developments in the processing technology, today they represent a healthy ingredient broadly employed in infant formulas, sports nutrition and dietetic food products (Bansal & Bhandari, 2016). Release of free amino acids and peptides from milk protein due to proteolysis is often encountered in long-lasting dairy products, best of all UHT milk. Proteolysis of milk protein in UHT milk is caused by indigenous milk proteases (e.g. plasmin) and bacterial proteases that survive upon the thermal treatment (N. Datta & Deeth, 2001). If not properly controlled and mitigated, proteolysis during long-term storage can have detrimental effects on the quality of the milk including age gelation and bitterness (N. Datta & Deeth, 2001; Nivedita Datta, Elliott, Perkins, & Deeth, 2002). In dairy products involving the employment of β-1,4-galactosidase, proteolysis is enhanced by the presence of proteolytic side activity in the commercial preparation.

Lipids are present in milk mostly in the form of triglycerides (98%). Triglycerides define the physicochemical properties of milk fat as well as the functionality upon processing (Vyssotski, Bloor, Lagutin, Wong, & Williams, 2015). The triglycerides are non-polar molecules which, merging to each other's, form the core of the milk fat globules. These globules are present in milk in the form of an emulsion stabilized by the milk fat globule membrane (MFGM), which contains glycoproteins, phospholipids and enzymes (Truong, Palmer, Bansal, & Bhandari, 2016; Walstra, 1999). More than 400 fatty acids, both saturated and unsaturated, have been recognized in milk: butyric (butanoic; C4:0), caproic (hexanoic acid; C6:0), caprylic (octanoic acid; C8:0) and capric (decanoic; C10:0) acids are the ones distinguishing milk fats from other foods (Penfield & Campbell, 1990). Additional lipids present in milk are phospholipids, sterols, carotenoids and fat-soluble vitamins. Besides proteolysis, also the fat fraction of milk is prone to alteration in dairies with prolonged shelf-life, due to oxidative and thermal degradation. Lipid oxidation lowers the nutritional value of the milk and has also a strong negative impact on its sensory properties (Ajmal, Nadeem, Imran, & Junaid, 2018). The

reaction arises from the long-chain unsaturated fatty acids present in the milk resulting in the development of off-flavors (Havemose, Weisbjerg, Bredie, Poulsen, & Nielsen, 2006). The oxidation products of the reaction (e.g. hydroperoxides) are extremely reactive and convert rapidly to aldehydes, hydrocarbons, alcohols, ketones potentially responsible for off-flavors (Walsh, Duncan, Potts, & Gallagher, 2015).

With regards to the carbohydrate fraction, lactose is the main component of bovine milk (4.8%) that represents its exclusive dietary source (Ingram, Mulcare, Itan, Thomas, & Swallow, 2009). Glucose and galactose are also present but in trace amounts (Sundekilde, Larsen, & Bertram, 2013). Lactose itself found broad application in the food, feed and pharma industry. Moreover, it can also serve as precursor for lactose-derived bioactive ingredients (Schaafsma, 2008). For example, it is processed as an ingredient in food infant formulas. Moreover, it is the starting material for the production of lactulose, lactitol, and bioactive oligosaccharides, which are used as prebiotic promoting the proliferation of health-positive bacteria in the gastrointestinal tract (Panesar & Kumari, 2011; Yang & Silva, 1995). Lactulose in particular gathered recent popularity for its prebiotic effect, resulting in several applications ranging from functional food ingredients to medicine (Vera, Guerrero, Aburto, Cordova, & Illanes, 2020). In the food industry, lactose is employed for adding flavors and colors to bakery products, wafer and seasonings. Its particular crystallization properties render lactose an important ingredient also for the confectionery industry. Furthermore, lactose found application in the beer production as taste and flavor enhancer, because it is not fermented by beer yeasts (Zadow, 1984). Eventually, reactions involving modifications of milk proteins, lipid and carbohydrates are key factors to monitor, and the relative effects on the quality and characteristics of lactose free milk products is discussed throughout this PhD thesis.

Lactose intolerance

Lactose digestion by the human body is achieved by a specific enzyme: the β-1,4-galactosidase, also known as lactase-phlorizin hydrolase or, more simply, lactase. The enzyme is produced at the level of the small intestine, specifically on the surface of the "brush border" of the epithelial cells (Fox & McSweeney, 1998). The enzyme splits the lactose into glucose and galactose ensuring their absorption in the blood. The lactase is produced by a gene called LCT, which after several mutations regulates the ability of individuals to assimilate lactose. Without proper LCT gene mutations, lactose cannot be digested and absorbed (Peter J. T. Dekker, Koenders, & Bruins, 2019). This mechanism is always present in infants, but it naturally decreases with age, resulting in a programmed reduction of lactase activity and an estimated 70% worldwide population affected by primary lactase deficiency or lactose intolerance (Corgneau et al., 2017). Historically, although the first cases of lactose intolerance were discovered 400 years BCE, the syndrome has been scientifically taken into consideration only over the last 50 years (Fassio, Facioni, & Guagnini, 2018). If not properly hydrolyzed into glucose and galactose, lactose passes through the gastrointestinal tract, it is metabolized by the colon microflora producing metabolites (e.g. methane, carbon dioxide and hydrogen) which likely cause diarrhea, stomach pain, flatulence and constipation (Corgneau et al., 2017; Leonardi, Gerbault, Thomas, & Burger, 2012).

The severity of primary lactose deficiency varies according to several factors, including ethnicity and dairy consumption. The severity of the symptoms differs among individuals and depends on the amount of lactose consumed, the degree of lactase deficiency, and the form in which lactose is assimilated in the diet (Heyman, 2006). In populations having ancient milking traditions and characterized by high consumption of dairy foods (e.g. northern Europeans and Caucasians), low incidence of primary lactase deficiency was registered (Wiley, 2007). A positive mutation of the genes codifying for lactase persistence may explain the correlation. On the other hand, these selective mutations are not common in other ethnicity arising the preponderance of primary lactase deficiency up to 50% to 80% among Hispanic people, 60% to 80% in black and Jewish people, and almost 100% in Asian and American Indian people (Heyman, 2006).

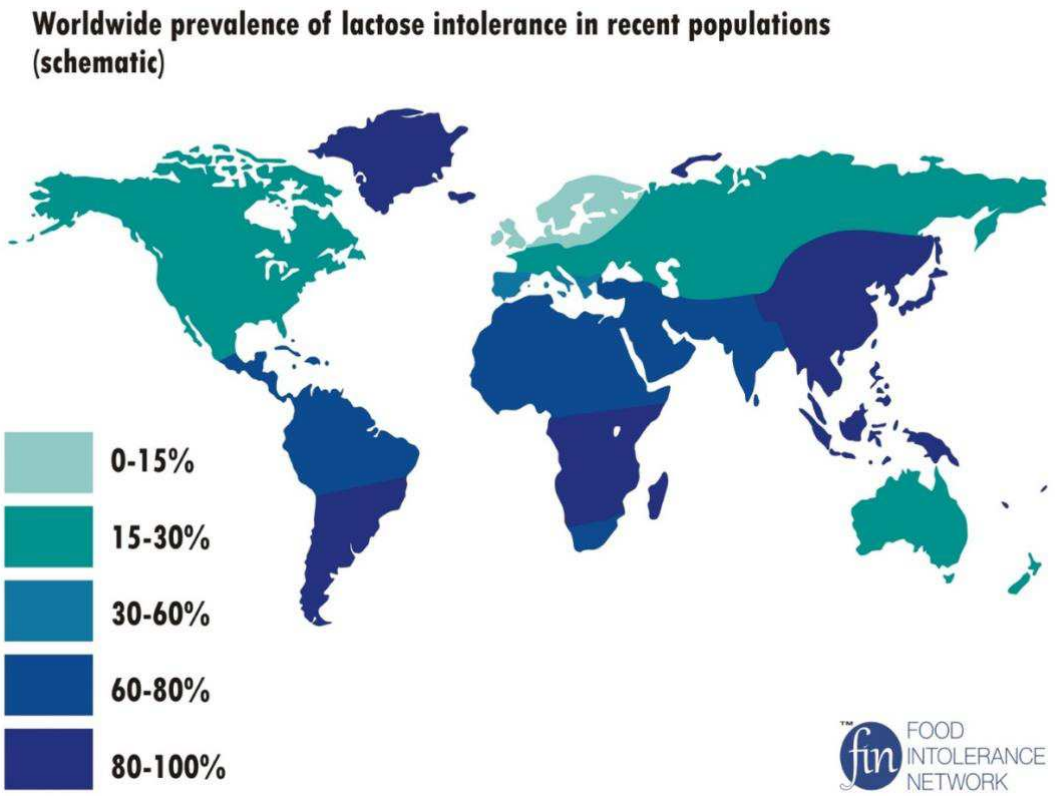

Figure 1. Lactose intolerance worldwide (Food Intolerance Network).

In Italy, an article published by Cavalli-Sforza and coworkers (1987) reported regional differences in the number of people affected by lactose intolerance: 52% in the north, 19% in central Italy and 41% in the south. Interestingly, Neapolitans were one of the segments suffering the most from lactose intolerance. This study demonstrated that sensitivity towards lactose malabsorption differ also at regional level, especially in populations having complex genetic history, like Italians.

The lactose-free milk market

Avoidance of lactose-containing foods is the main therapy to cope with the symptoms of lactose intolerance. On the other hand, generic symptoms associated with this discomfort influence also consumers who are not lactose intolerant to avoid regular milk products. For example, consumers considering themselves to be lactose intolerants do not eat cheese although it is well known that the lactose content of these products is minimized during production and ageing (Jelen & Tossavainen, 2003). In Europe and the US this increasing number of self-diagnosed lactose intolerant is influencing the dairy market remarkably (Astley, 2012). For such a reason, the dairy industry is continuously chasing the development of new types of low lactose (LL) lactose-free (LF) products (Trani et al., 2017), which today represent staple products in the diet of many consumers. A recent overview of the market developments in the LF dairy sector was depicted by DSM Food Specialties, a Dutch multinational company specialized in B2B ingredients commercialization (Peter J. T. Dekker et al., 2019).

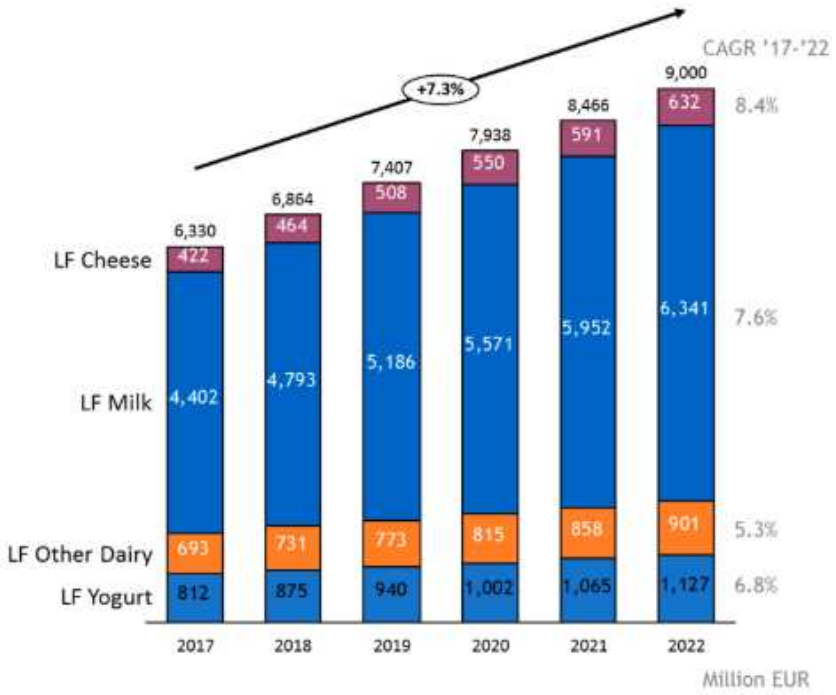

Figure 2. Estimation of the lactose-free (LF) dairy market (2017–2022). The total annual revenue (million EUR) is indicated. According to the authors, data displayed in the were elaborated from the Euromonitor analysis (Peter J. T. Dekker et al., 2019).

According to the report, LF is the dairy segment growing the most with a total of €9 billion revenues expected by 2022. LF dairy products are taking up the dairy market with sales growing almost 4 times faster than regular dairies (7.3% versus 2.3% of annual growth respectively). As it can be noticed in **Figure 2**, two-thirds of revenues comes from milk, whereas the steepest growth is foreseen for LF cheese. Dekker and coworkers (2019) reported also that the success of the LF dairy market is demonstrated by the increasing number of LF new products launched in the recent past spreading in different product categories. Historically, ultra-high-temperature hydrolyzed lactose milk (UHLM) was the first LF product available on the market. It was launched in Italy in the 70s by the Centrale del Latte di Milano (Vera et al., 2020; Zolnere & Ciprovica, 2017). Today the demand for this type of product is constant in the Western world but it has huge opportunities of growth in Asian countries. The request for dairy products in that part of the world rocketed over the past few years (Fuller, Huang, Ma, & Rozelle, 2006) and, with the majority of the population lactose intolerant, UHLM is the most suitable product to fill the demand (Jansson, 2014). This recent diffusion of exported UHLM to Asia is calling manufacturers to develop products lasting longer because, at the moment, UHLM is sold with a shelf-life of 3-4 months, way shorter than regular UHT milk expiring after 9-12 months at room temperature.

Production of ultra-high temperature (UHT) hydrolyzed-lactose milk

Heat treatment

Heat treatment extends the shelf-life of milk by reducing the microbial load and inactivating enzymes present in the raw milk. Depending on the combination of heating time and temperature supplied, products with different shelf-life length are achieved (Jansson, 2014). In this frame, milk thermal processing is distinguished based on heat severity (e.g. mild-pasteurization, pasteurization, extended shelf-life ultra-high temperature etc.) (Deeth & Lewis, 2017). With pasteurization, milk is heated at least at 71.7°C for 15 s, resulting in a product lasting in 5-20 days at refrigerated conditions, with a reduced number of microorganism and a partial inactivation of the native enzymes (Tossavainen, 2008). Longer shelf-life can be obtained with extended shelf-life (ESL) treatment by exposing the milk at 130-145 °C for less than one second. The ESL heated milk was found very similar in terms of taste and color to the pasteurized milk, but with a longer shelf-life up to one month under refrigeration (Jansson, 2014). ESL is particularly appealing in those areas where consumers require milk with long shelf-life, although off-flavor related to the severity of the heat treatment (e.g. "cooked" flavor in UHT milk) may end up in consumer rejection (Rysstad & Kolstad, 2006). Pasteurization and ESL treatment effectively eliminate pathogens, but they are not sufficient to inactivate the heat-resistant spores in milk, reaching the so-called "commercial sterilization". Commercial milk sterilization is achieved by UHT treatment, which extends the commercial shelf-life of the milk at room temperature up to 6-9 months. Pathogens, enzymes and spores present in the milk are inactivated by exposing the milk to elevate temperature (138-145°C) for a very short period of time (1-10 s) (Zabbia, Buys, & de Kock, 2012). In combination with filling into aseptic packaging, the final product is maintained sterile throughout the shelf-life (Valero, Villamiel, Miralles, Sanz, & Martínez-Castro, 2001). It is important to mention that, despite the extensive heat load provided during the UHT treatment, some bacteria will still remain alive in the final product as the inactivation rate is logarithmic. The major goal of the UHT process is achieving at 12-log reduction of *Cl. Botulinum,* the most heat-resistant pathogen spore detectable in food (Deeth & Lewis, 2017). Two major UHT treatments (direct and indirect) are distinguished based on the way how heat is supplied. In both cases, a pre-heating step at 80-90°C is performed to maintain the protein

soluble minimizing their unfolding and deposit (Nivedita Datta et al., 2002). In direct UHT, sterilization occurs by injection or infusion of superheated steam which transfers its latent heat of evaporation to the milk (Deeth & Lewis, 2017). In indirect UHT plants instead, the heat is transferred to the milk from a heating medium through a stainless steel wall (Jelen, 1982). The evolution of the time-temperature profile during both indirect and direct UHT processing is illustrated in **Figure 3.**

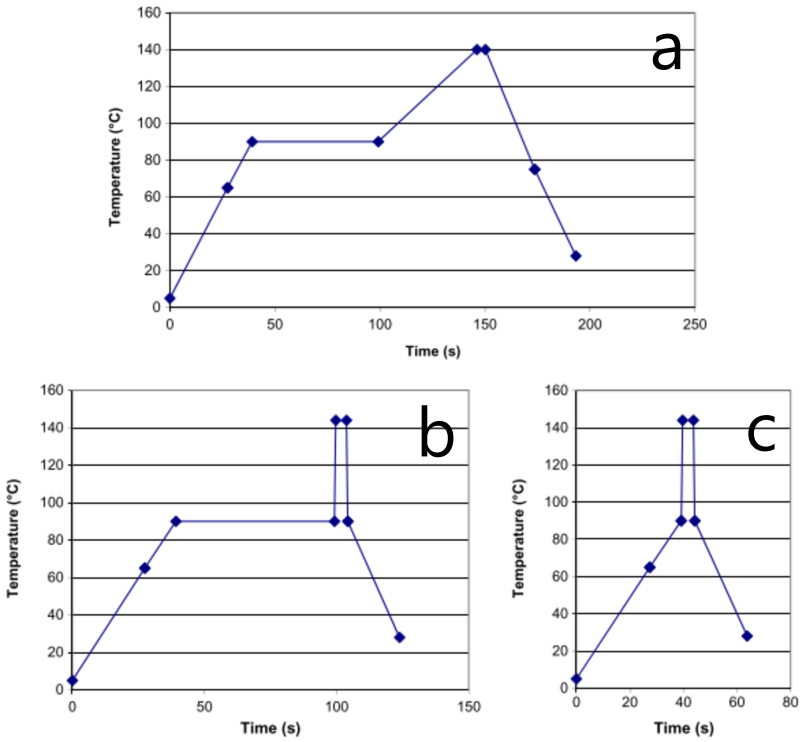

Figure 3. Temperature-time profiles for (**a**) an indirect UHT plant with a 60s preheat hold time, (**b**) a direct UHT plant with a 60s preheat hold time, and (**c**) a direct UHT plant with no preheat hold time (Deeth, 2020).

In direct UHT processing, heating and cooling are very rapid and occur in a very short period of time. It can be deduced that the process is more gentle compared to indirect UHT, although slightly higher temperatures are reached during manufacturing (Jensen et al., 2015). At the top of that, less oxygen is dissolved in the milk during direct UHT processing protecting the product from oxidative reactions (Nivedita Datta et al., 2002). Despite these advantages, indirect UHT processing remains the most applied heat treatment process by the dairy industries due to the lower cost compared to direct heating (Jensen et al., 2015). With this type of treatment, the more intense heating received by the milk is translated into the formation of heat-induced off-flavor considered unpleasant by many consumers (Nivedita

Datta et al., 2002). Recently, a Danish group included in its research activities also the investigation of different heat processing methods on UHLM (Jansson, Clausen, et al., 2014; Jansson, Jensen, Sundekilde, et al., 2014; Jensen et al., 2015). These studies revelead that the two heat treatments (direct and indirect) did not significantly affect the flavor profile of UHLM. Other factors (e.g. composition of reducing sugar) had a greater impact on those reactions underpinning physicochemical and sensory changes. According to their results, the comparison between direct and indirect UHT treatment UHLM was left out from the PhD project, whereas more emphasis was given to the processing and storage parameters.

Hydrolysis of the lactose

Among the available options, the enzymatic hydrolysis of lactose adding free soluble β-1,4-galactosidase (lactase) to the milk is the process most largely applied by the dairy industries (Jelen & Tossavainen, 2003). A graphical representation of the conversion of lactose into glucose and galactose by treatment with lactase is given in **Figure 4.**

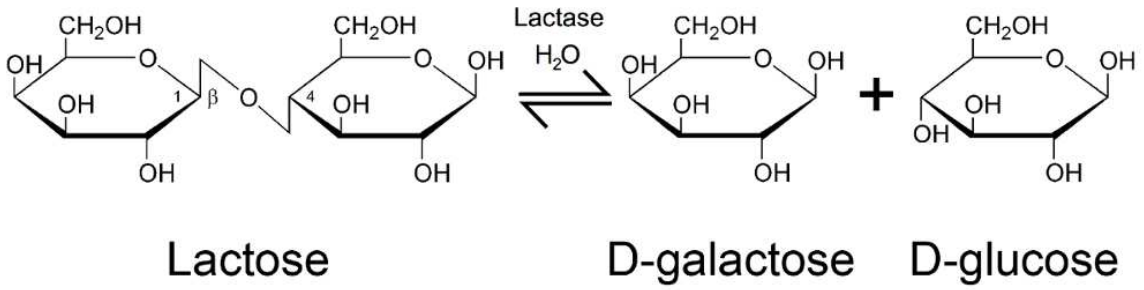

Figure 4. Enzymatic conversion of lactose into β-D-galactose and β-D-glucose by treatment with β-1,4-galactosidase (Leksmono et al., 2018).

The process is relatively simple even at large production scale, but many factors influence the performance of the reaction such as the initial lactose concentration, the pH, the combination of temperature and time during the reaction, the activity of the enzymatic preparation and the operating cost (Harju, Kallioinen, & Tossavainen, 2012). An example was given by Harju and coworkers (2012): maximum performance of lactose conversion is achieved by working at a temperature close to the optimum of the lactase (35-45°C) reducing production costs remarkably. At the same time, extensive microbial growth can occur in the milk at these conditions, so the risk of quality losses of the final product is relatively high.

Alternative processing methods

Although the majority of dairy manufacturers rely on lactose hydrolysis for the production of UHLM, other processing methodologies have been proposed and, sometimes, employed over the years (Jelen & Tossavainen, 2003; Mahoney, 1997; Zadow, 1984). The review published by Harju et al. (2012) gives a frame about the alternative processes for performing lactose hydrolysis at industrial level which, in this section, are summarized in **Table 1.**

Table 1. Overview of process alternative to addition of free soluble lactase to perform conversion of lactose at industrial level (Harju et al., 2012).

Process type	Process description	Current technological status
Acid hydrolysis	The process exploits the combination of acids and high temperature for cleaving the β-1,4 glycosidic bond. The pH is adjusted to 1.2 and the temperature is set to 150°C for a short period of time. At the end of the reaction, the hydrolyzed product is brown and additional processes (neutralization, demineralization and decolorization) are required before use. The process works only for milk streams free from protein (e.g. ultra-filtered milk permeates).	Poorly adopted on industrial scale because of the corrosiveness of the applied conditions, resulting also in waste management problems. Moreover, the costs required for the neutralization of the brown mixture upon acid hydrolysis are high.
Membrane reactors	The process is made up by reactors recovering the enzyme lactase from the reaction mixture through membrane filtration. The permeate, which contains the hydrolyzed lactose, is then re-introduced into the milk. Hydrolysis is usually carried out on a protein-free stream (e.g. milk or whey permeate upon ultra-filtration).	Complicated industrialization of the process due to its complexity, with microbial spoilage as major critical factor. In comparison to the commercial soluble lactases, these reactors require also substantial investments for the membranes.
Immobilized systems	In this process, the lactase is entrapped or absorbed through a carrier and, retaining its enzymatic activity, allows a continuous reuse of the stream. Various carriers have been tested for immobilized systems including ion exchange resin, PVC silica, active carbon, porous glass, acrylic beads, cellulose triacetate and adsorption resin. Fungal lactases are employed for this application with best activity retention of the enzyme achieved at pH 6.8 (50%).	The process is economically feasible; therefore, it has potential for industrial application. Moreover, the lifetime of an immobilized system is relatively long (thousands of hours), so costs reduction compared to soluble lactase can be achieved. The main limitation is the control of the microbial stability of the product over time at the applied processing conditions (pH=6.8). Currently, only one application of immobilized system for commercial purpose was undertaken for lactose-free milk.
Crude Cellular extract (CCE)	This process aims to the production of the lactase in the form of a crude cellular extract from *Lactobacillus delbrueckii ssp. bulgaricus*. The process involves the growth culture and the subsequent disruption of the cells by high pressure homogenization. The obtained product can be employed as such for lactose-free milk production.	The major limitation of the use of CCE for lactose-free milk production is the taste/flavor given by the ingredient to the milk. Authors indeed suggested the use of CEE for flavored product like yoghurt drinks.

Commercial lactase preparations

The first commercial lactase preparation (LP) intended for hydrolyzed-lactose milk was released in the 1970s and since then it represents one of the major enzymatic preparation employed in the food sector (DiCosimo, McAuliffe, Poulose, & Bohlmann, 2013; Harju et al., 2012). The lactase catalyzes one of two different reaction pathways depending on the conditions: at low lactose concentrations (~10% w/w) and high water activity, like the ones encountered in milk, trans-galactosylation is suppressed and lactose hydrolysis is predominant (Vera et al., 2020). Less conventional applications of LP have been reviewed in a recent article by Vera and coworkers (2020) and are based on tuning the trans-galactosylation and trans-glycosylation activity at the expenses of hydrolysis: this occurs, for example, when high concentration of substrate, organic solvents, ionic liquids or media with low water activity are employed in the reaction (Vera et al., 2020). At these conditions, production of galacto- oligosaccharides (GOSs) occurs (P. J.T. Dekker & Daamen, 2011). GOSs are well-known prebiotics that benefit the intestinal microbiota thanks to their bifidogenic effect resembling the oligosaccharides present in human milk (Vera et al., 2020). Research associated the health-promoting effects of GOSs to an improved immune system, better vitamin synthesis, lower blood cholesterol and risk of colon cancer (Roberfroid et al., 2010). Unconventional applications of LP include synthesis of other functional ingredients such as lactulose, lactosucrose, alkyl-glycosides and gal-polyols (Vera et al., 2020).

Several sources of lactase are available including vegetables, animals, microorganisms and fungi (Park, Santi, & Pastore, 1979). Two types of LP are available for industrial application: acid and neutral, with the latter suitable for neutral food products like milk. Neutral LPs are extracted from two different yeast species, namely *Kluyveromyces lactis* and *Kluyveromyces marxianus* (P. J.T. Dekker & Daamen, 2011). Examples of commercial LPs currently available in the European market are Maxilact® (DSM Food Specialties; Heerlen, the Netherlands), Godo YNL2® (Dupont; Wilmington, DE, USA), HA-LACTASE® (Chr. Hansen; Øresund, Denmark) and Lactozyme® Pure (Novozymes; Bagsværd, Denmark).

The milk after treatment with LP shows higher sweetness, because the glucose and galactose are sweeter than lactose, as well as a higher freezing point (from 0.454 to 0.650 °C according to Troise et al., 2016). Interestingly, the liking of the higher sweetness upon lactose hydrolysis varies among populations with different traditional consumption of dairy products and, as a consequence, different levels of lactose intolerance. Latins and Asians, which are populations with ground-level tradition of milk consumption, consider the extra sweetness of LF milk

positively, whereas consumers with a long-established dairy consumption (e.g. Northern Europeans) prefer taste and flavor closer to regular milk (Peter J. T. Dekker et al., 2019). For this reason, some manufacturers introduced micro and/or ultrafiltration in these countries to reduce the lactose content and modulate the sweetness of the final product (Troise et al., 2016). The increased sweetness provided by lactose hydrolysis is also an opportunity to produce dairy products with less sugar added: according to Zadow (1984) a sweetness corresponding to the addition of 2% sucrose can be achieved by hydrolysis of 70% of lactose contained in the milk. Apart from filtration, an alternative method to decrease the sweetness of UHLM was proposed by Flynn and coworkers (1994), who patented the addition of potassium chloride. The invention relies on the addition of the salt in a concentration range of 0.075-0.200% (w/w), which can take place at any stage of the production process or even after packaging for consumer use.

The "in batch" and "in pack" production system

Two industrial production systems are currently available for producing UHLM through lactose hydrolysis with LP: the "in batch" (also referred to pre-hydrolysis) and the "in pack" (also referred to post-hydrolysis) system. An overview of the two processes is given in **Figure 5**.

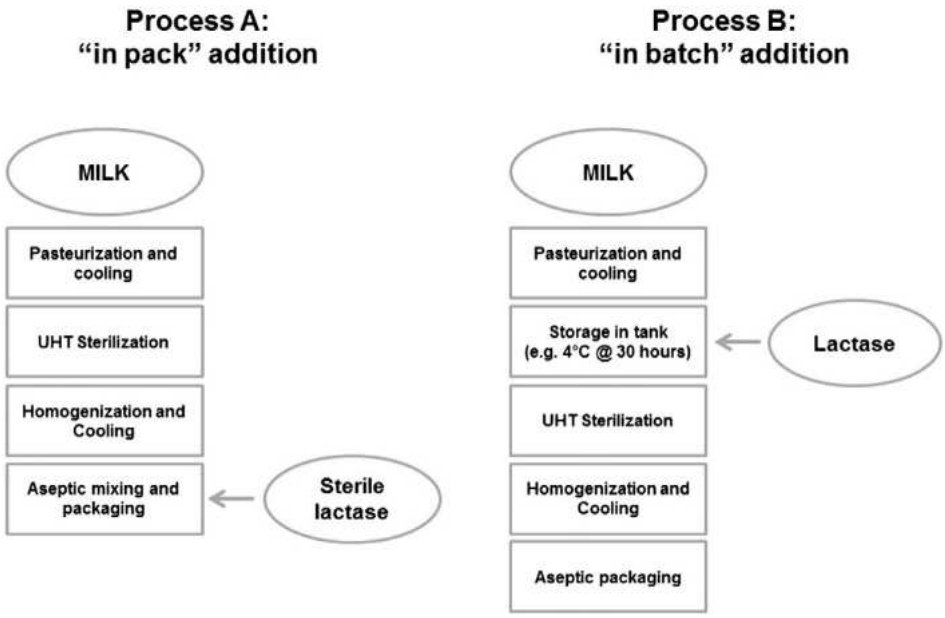

Figure 5. Overview of the production system currently available for UHLM manufacturing. The process **A** summarizes the "in pack" addition, in which lactase remains active throughout storage. The process **B** summarizes the "in batch" system, in which lactase activity is inactivated through UHT treatment (Troise et al., 2016).

The "in batch" process was the first one applied on industrial scale level (Vera et al., 2020). The initial popularity of the technology was determined by the fact that the same facilities used for regular UHT milk production are used. "In batch" production begins with the addition of the LP in a tank containing either raw or pasteurized milk. Lactose hydrolysis is then performed batch-wise until the desired concentration of residual lactose (<0.01%) is reached. The reaction occurs at refrigerated conditions (4-8°C) to prevent microbial contamination (Zadow, 1984), lasting in 24-48 hours depending on the LP dosage. The milk is then supplied to UHT processing and, after homogenization and cooling, it is packed aseptically. The "in pack" production system was developed later on as a cost-saving solution against some drawbacks related to "in batch" manufacturing. For example, milk producers employing the "in batch" system must reach the residual lactose target in a limited time-frame implying

higher dosage of LP (Peter J. T. Dekker et al., 2019). Moreover, dairy companies struggle in aligning the milk holding step of the "in batch" production with the daily milk processing designated for other productions.

Thanks to the introduction of aseptic packaging technology, sterile LP can be added directly into each milk package after the milk is sterilized (Dahlqvist, Asp, Burvall, & Rausing, 1977). "In-pack" LP can be either purchased already sterile and added into each milk package using specific dosing equipment or it can be sterilized in-house by filtration (Peter J. T. Dekker et al., 2019). The filtration step renders these LPs more expensive compared to the ones intended for "in batch" production (Jansson, 2014). On the other hand some experts believe that, as a lower quantity of the LP is employed and a longer time for lactose hydrolysis is given (~3-5 days), the overall cost of "in pack" production are lower compared to "in batch" production (Troise et al., 2016). Some authors also recommended the "in pack" system from a quality perspective, believing that lactose hydrolysis after the UHT treatment may avoid progress of Maillard reactions (MR) during storage (Mendoza, Olano, & Villamiel, 2005). These hypotheses have not been fully demonstrated yet. Spontaneous lactase inactivation due to lactose depletion (<0.01%) is expected in "in pack" UHLM but, differently from "in batch" UHLM, the LP remain active until the product is consumed, with the risk of alterations due to the presence of enzymatic side activity. The truth is that UHLM produced by "in batch" and "in pack" systems have many common features and, depending on the situation, can have both advantages and disadvantages (**Table 2**).

Table 2. Overview of the main advantages and disadvantages of the production process currently applied for the production of UHLM, namely "in batch" (pre-hydrolysis) and "in pack" (post-hydrolysis) systems.

Production system	Advantages	Disadvantages
"in batch"	• Production implementable on the same production line intended for UHT milk • Absence of residual lactase activity in the final product • Low sensitivity to enzymatic side activity of the lactase preparation • High process control and low technical expertise required	• High enzyme dosage • Discontinuous process • Exposure of free glucose and galactose to high temperature
"in pack"	• Low enzyme dosage • Continuous high-throughput process	• Presence of residual lactase activity in the final product • High sensitivity to enzymatic side activity of the lactase • Lack of process control and high technical expertise required

Modifications of UHT hydrolyzed-milk characteristics upon production and storage

Besides the commercial benefits of an extended shelf-life at room temperature, the heat treatment modifies the milk upon processing which may result in altered nutritional value, sensory properties and especially flavor (Jansson et al., 2019; Sunds, Rauh, Sørensen, & Larsen, 2018). Depending on the type and the severity of the heat treatment, various modifications can pop up in milk after UHT processing: some of those are barely detectable after production but are the trigger point of more relevant changes during shelf-life, whereas others (e.g. whey protein denaturation) are remarkable only once the milk has been thermally treated but have little impact during storage (Deeth & Lewis, 2017). Differently from regular UHT milk, the severity of the heat treatment applied during production plays a secondary role in the definition of the UHLM physicochemical characteristics and quality. The introduction of soluble lactase into the milk modifies the sugar formulation of the product which, under certain conditions, results in a different chemical and sensory profiles during storage (Jansson, 2014). Modifications occurring during the shelf-life are therefore way more relevant and the key target of research efforts for UHLM.

Maillard reaction

The major reaction at the basis of the modifications of UHLM upon production and storage is Maillard reaction (MR). The reaction occurs between a reducing sugar and an amino group, taking the name from the French chemist, Louis-Camille Maillard, who in 1912 discovered it. MR soon gathered an outstanding popularity in food science, especially because of the myriad of compounds formed along its progress which define the characteristic color, taste and aroma of many food products. With regards to milk however, its occurrence is highly undesirable because it denotes product modifications disliked by milk consumers. MR reduces the nutritional value of milk as well: for example protein glycation reduces the bio-accessibility of lysine lowering the overall protein digestibility (Sunds, 2016; van Boekel, 1998). Furthermore, in specific pathologies like diabetes, advanced products (AGEs) formed via MR were reported to cause adverse effects (Poulsen et al., 2013). The complexity of the array of reactions involved in MR makes its theoretical explanation challenging, so three stages (initial, advanced and final) were traditionally distinguished only for the sake of simplifying its interpretation (Nursten, 2005).

An extensive explanation of MR in milk was given by van Boekel (1998) and was then elaborated further by many authors over the years. The initial stages of MR involve the condensation of the carbonyl group from the reducing sugars with an amino group. In milk it is mostly the ε-amino group of the lysine residues bonded to milk proteins, with the one present on the casein micelles (k-CN especially) more reactive compared to the ones in the serum phase (van Boekel, 1998). Additional amino acid residues (e.g. indolyl-group of tryptophan; guanidino-group of arginine etc.) can take part to the MR as well, but not as reactively as lysine side chains (O'Brien, 2009). At this stage, color and flavor alteration are barely noticed, whereas the major implication for milk nutritional quality is the loss of available lysine (van Boekel, 1998). The condensation leads to the formation of a glycosylamine named Schiff base (Troise, Fiore, Roviello, Monti, & Fogliano, 2015). This molecule is highly reactive and undergoes a rapid rearrangement into Amadori products (APs), specifically ε-lactulosyllysine from lactose, ε-fructoselysine from glucose and ε-tagatoselysine from galactose (Evangelisti, Calcagno, Nardi, & Zunin, 1999; Mendoza et al., 2005). In general, APs are quantified indirectly by measuring the furosine content of the milk, which is obtained by a combination of acid hydrolysis and heating (Tossavainen & Kallioinen, 2008). Furosine is an artificial amino acid, not natively present in the milk, but still considered an index for heat treatment intensity in dairy products.

The advanced stages of MR involve the breakdown of APs. Two reaction pathways are possible, depending on milk pH: in acidic environment (pH<7) APs are converted via the 3-deoxyosone pathway, whereas at alkaline conditions the 1-deoxyosone-pathway via the 2,3 enolization takes over (van Boekel, 1998). The neutral pH of milk favors the 3-deoxyosone pathway leading to the formation furfural or hydroxymethylfurfural (HMF) depending if the reducing sugars involved in the reaction are pentoses or hexoses respectively (Martins, Jongen, & van Boekel, 2000). At this stage, flavor formation becomes more substantial as a consequence of Strecker degradation (SD) in which amino acids are deaminated and decarboxylated upon reaction with the dicarbonyls formed *via* MR (van Boekel, 2006). The reaction leads to the formation of flavor-active Strecker aldehydes causing off-flavors in milk (Jansson et al., 2017). As the reaction proceeds towards the end stages, brown pigments called melanoidins are also formed. Despite the growing research interest in studying their presence in food, melanoidins detection is challenging due to their complex formation and structure (Rodríguez et al., 2019). Milk turns brown slightly after heating but not as much as

during storage, especially if the storage temperature is higher than room temperature and in the case of UHLM (Deeth & Lewis, 2017).

Several studies demonstrated that MR occurs more intensively in UHLM compared to regular UHT milk, suggesting a higher vulnerability of the final product (Evangelisti et al., 1999; Jansson, Clausen, et al., 2014; Jansson, Jensen, Eggers, et al., 2014; Jensen et al., 2015; Mendoza et al., 2005; Messia, Candigliota, & Marconi, 2007; Milkovska-Stamenova & Hoffmann, 2017; Naranjo, Gonzales, Leiva, & Malec, 2013; Tossavainen & Kallioinen, 2007). As a result, UHLM is more prone to changes in its chemical and sensory properties, imposing dairy producers to commercialize the product with a shelf-life of 90-120 days, way lower than the one of regular UHT milk. Messia et al. (2007) associated this effect to a higher furosine content in the milk subjected to lactose hydrolysis compared to the regular UHT milk. The research was in line with the research article previously published by Mendoza et al. (2005). According to Evangelisti et al. (1999), stronger development of MR upon lactose hydrolysis was also demonstrated by higher levels of blocked lysine immediately after production. Thus, one of the main issues with UHLM is that, upon lactose hydrolysis, the molarity of reducing sugar in the milk is doubled (Tossavainen & Kallioinen, 2007). Moreover, the nature of the reducing sugars have an important influence on MR and the ones obtained from the lactose hydrolysis (glucose and galactose) are characterized by a higher tendency of react with protein (Milkovska-Stamenova & Hoffmann, 2017; Naranjo et al., 2013).

Enzymatic side activity of the lactase preparations

The different formulation in reducing sugars obtained after lactose hydrolysis is not the only aspect worrying dairy producers when dealing with UHLM: alteration of flavor and taste of the product may also arise from the enzymatic side activity present in the commercial LPs. The isolation of the lactase from microorganisms is achieved through several separation methods, but complete purification is difficult to achieve so secondary enzymatic activity remains in the commercial preparations (Zhao et al., 2019). Arylsulfatase and proteolytic side activity were found in the commercial preparations intended for UHLM production (Nielsen et al., 2018; Stressler, Leisibach, Lutz-Wahl, Kuhn, & Fischer, 2016). These LPs are available on the market in different purity, which defines the cost of the preparations as well as the risk level of altering the product during shelf-life. In particular, the proteolytic side activity of LP has gathered particular attention among researchers. These secondary proteases degrade milk protein favouring the release of free amino acids and peptides which, through the ongoing MR, result in the development of off-flavours during storage of UHLM. With more substrate available, the proteolytic side activity of LPs facilitates the progress of MR in the milk resulting in a higher risk of alteration of the overall flavor (Nielsen et al., 2017). Flavor-active VOCs are generated from Strecker degradation taking place at intermediate stages of MR (van Boekel, 2006). Strecker degradation was defined by Rizzi (1999) as "*the major pathway for conversion of amino acids into structurally related aldehydes of significant flavor value*". Strecker degradation starts when amino acids and either α-dicarbonyl or APs react together following deamination and decarboxylation (Cremer, Vollenbroeker, & Eichner, 2000; Hofmann & Schieberle, 2000), as depicted in **Figure 6.** The reaction leads to the formation of the so-called Strecker aldehydes that, if further reacting, generate secondary products which are flavor-active too. An overview of the flavors associated with specific product of Strecker degradation is given in **Table 3**.

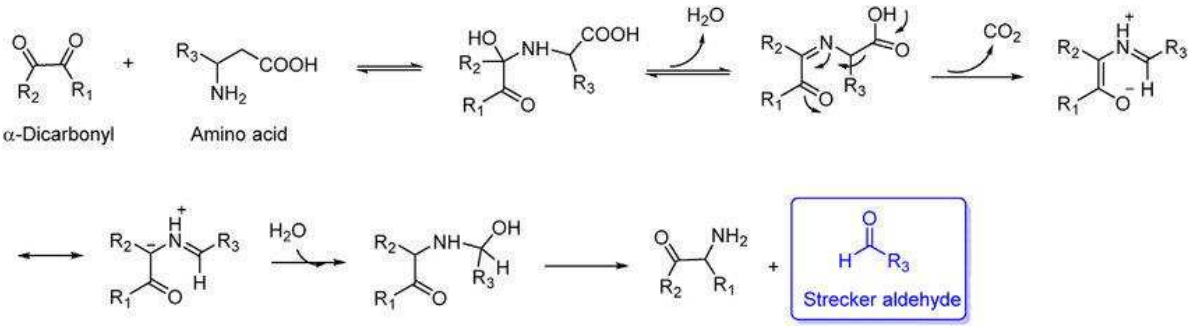

Figure 6. Overview of the mechanism of flavor-active aldehydes formation through Strecker degradation (Ho, Zheng, & Li, 2015).

Table 3. Summary of the compounds formed *via* Strecker degradation including both Strecker aldehydes and secondary products, together with the amino acid precursors and the associated flavors.

Amino acids	Strecker aldehydes	Associated flavor	Secondary products	Associated flavor
Ala[a]	Acetaldehyde[a]	Pungent[d]; Fruity[d]; Green apple[d]	2-Methylfuran[a]; 2-Pentyfuran[a]	Chocolate[g]; Nutty[h]
Cys[a]	Acetaldehyde[a]	Pungent[d]; Fruity[d]; Green apple[d]	Hydrogen sulfide[a]; Methanethiol[a]	Sulfur[b]; Gasoline[b]; Garlic[b]
Gly[a]	Formaldehyde[a]	Pungent[e]		
Ile[a]	2-Methylbutanal[a]	Malty[c]		
Leu[a]	3-Methylbutanal[a]	Green[f]; Malty[f]		
Met[a]	Methional[a]	Boiled potato[f]	Dimethyldisulfide[a]; Dimethyltrisulfide[a]	Onion[b]; Cabbage[b]
Phe[a]	Phenilacetaldehyde[a]		Benzaldehyde[a]	Almond[b]; Burnt sugar[b]
Ser[a]	2-Hydroxyethanal[a]			
Thr[a]	Lactaldehyde[a]		2-Methylfuran[a]	Chocolate[g]
Tyr[a]	2-(p-Hydroxyphenyl)ethanal[a]			
Val	2-Methylpropanal[a]	Pungent[g]		

[a] Jansson (2014); [b] www.flavornet.org; [c] Jensen et al. (2015); [d] Van Aardt et al. (2001); [e]Nowshad et al. (2018); [f] Friedrich and Acree (1998); [g] Kim et al. (2019); [h] Liu et al. (2017)

Besides the proteolytic side activity, LPs produced from *K. lactis* can also contain arylsulfatase side activity (Stressler et al., 2016). Arylsulfatases catalyzes the release of phenols and inorganic sulfate through hydrolysis of the arylsulfates ester bonds (Kim et al., 2004). First evidence of arylsulfatase activity in the commercial LPs arises when it was observed that milk produced with lactase having low proteolytic activity was still characterized by off-flavors. Stressler et al. (2016) described the off-flavor as an unpleasant "cowshed-like" odor and associated it to the release of p-cresol from alkylphenol sulfuric esters natively present in the milk. p-Cresol is characterized by a very low sensory threshold (1 ppb in air and 2 ppb in water according to Kim Ha & Lindsay, 1991), so it is directly linked to the sensory perception of milk. It is important to mention that the response of UHLM to the LP enzymatic side activity is largely influenced by the processing technology employed for manufacturing (Troise et al., 2016). So far, this aspect was reviewed only for the proteolytic side activity of LPs. When the lactose hydrolysis is performed after the UHT treatment, namely following the "in pack" production system, the release of free amino acids and peptides due to secondary proteases continues until consumption. The result is a product more prone to the release of flavor-active Strecker aldehydes during shelf-life (Zhang, 2020). When the lactase is added "in batch"

instead, these defects might be modulated by thermal inactivation of the LP (Peter J. T. Dekker et al., 2019; O. Tossavainen & Kallioinen, 2007), although the literature on this aspect is still limited, at least it was when this PhD project started.

Impact on the milk sensory properties

As a result of the combined effect of lactose hydrolysis and enzymatic side activity of the LP, the flavor profile and the sensory properties of UHLM are negatively affected during shelf-life. The development of UHLM with sensory characteristics resembling regular UHT milk is a major priority for dairy manufactures. On the other hand, when milk undergoes thermal sterilization and storage, cooked and stale flavor appear as a result of several chemical reactions (Jensen et al., 2015). In general, an increase in methyl ketones and aldehydes have been associated with the sensory defects occurring in regular UHT milk during storage (Jensen et al., 2015). These VOCs are originated from chemical modification occurring along MR, protein oxidation and lipid thermal degradation and, due to the higher sensitivity towards these phenomena, are expected to occur more intensively in UHLM. A limited number of research articles have considered the sensory properties of hydrolyzed-lactose milk during shelf-life (Adhikari, Dooley, Chambers IV, & Bhumiratana, 2010; Jensen et al., 2015; Nielsen et al., 2017; Troise et al., 2016). In this context, an overview was given by Adhikari et al. (2010), who assessed the descriptive sensory characteristics and liking of different lactose-free milk available in the U.S. market. The results highlighted a significant difference between regular milk and lactose-free milk due to a more intense sweet taste, chalkiness, cooked and processed flavor in the latter. Nevertheless, the study did not take into consideration the evolution of the sensory properties during shelf-life which, as described in other research articles, it is of utmost importance (Jansson, Jensen, Sundekilde, et al., 2014; Jensen et al., 2015; Tossavainen, 2008). According to Jensen et al. (2015), UHLM was characterized by a higher degree of stale flavor and bitter taste after 3 months of storage. Bitter taste in UHLM is associated with proteolytic side activity of the LPs too, as some peptides may be perceived as bitter upon consumption. Casein, in particular, contains high levels of hydrophobic amino acids, which are highly bitter and may be released in UHLM as a results of proteolysis, causing bitterness in product (Jansson, Clausen, et al., 2014)

Overview of the analytical methods employed for milk flavor analysis

Flavor is one of the most important sensory characteristics of food and volatiles organic compounds (VOCs) are responsible for its perception (Biasioli, Gasperi, Yeretzian, & Märk, 2011). VOCs assessment is relevant for several reason: getting a fingerprint of the aroma profile of a food product, identifying key compounds in defining specific aromas (Eugenio Aprea et al., 2011), keeping track of the formation of flavor molecules during both industrial and natural processing (Biasioli et al., 2011), spotting off-flavor development for quality control purposes (Pedrotti et al., 2018), correlating the flavor molecules with the sensory properties of a food and getting an insight on the chemical reaction underpinning food perception (Nielsen et al., 2017). Normally, a myriad of compounds reacts with the human olfactory system resulting in the definition of the food flavor (Jeleń, 2006). In the case of milk, the active volatile molecules define the flavor, with those above the sensory threshold contributing the most. On the other hand, also the effect of the VOCs below the sensory threshold is not negligible because, with more than 400 VOCs identified in milk so far (Jensen et al., 2015), the mixture can enhance the perception (Jeon, Thomas, & Reineccius, 1978). In order to be appreciated by consumers, milk should have a clean and sweet taste, with absence of odor and aftertaste, giving a smooth and refreshing mouthfeel (Francis et al., 2005). Therefore, the analysis of the volatile organic compounds (VOCs) is an approach of utmost importance for assessing the milk flavor throughout production and shelf-life. The VOCs profile can reveal chemical modifications in the milk before the concentration of the developed compounds reach the human perception threshold, with the risk of altering the final product.

Headspace Solid Phase Micro-Extraction Gas Chromatography-Mass Spectrometry (HS-SPME/GC-MS)

Various analytical techniques have been considered to assess the volatiles profiling of dairy products. Gas chromatography–mass spectrometry (GC-MS) is the most popular due to its relative high precision and sensitivity when coupled with a pre-concentration step (Cappellin et al., 2013). Among the available options, headspace solid phase micro-extraction (HS-SPME) was demonstrated as powerful tool to determine the off-flavors in processed milk (Marsili & Miller, 2002) and, today, represent a reference extraction method to couple with GC-MS. Application of HS-SPME/GC-MS in the dairy sector includes fermented milk, cheese and butter (Aprea et al., 2016; Condurso, Verzera, Romeo, Ziino, & Conte, 2008; Contarini, Povolo, Leardi, & Toppino, 1997; Endrizzi et al., 2012). HS-SPME is a solvent-less method which, by combining sampling and pre-concentration in one step, allows to reduce the time of analysis and the sample handling in comparison with other extraction techniques (González-Córdova & Vallejo-Cordoba, 2001; Vazquez-Landaverde, Velazquez, Torres, & Qian, 2005). Moreover, artifacts formation are minimized (Vazquez-Landaverde et al., 2005).

The technique was developed in the 1990 and represented a breakthrough innovation compared to the traditional static techniques previously employed for headspace analysis (Povolo & Contarini, 2003). An overview of the HS-SPME/GC-MS analysis is given in **Figure 7.** The analysis starts by sampling the food product in vials. An internal standard is often added ensuring good reproducibility of the results. The choice of the internal standard is a critical step for the success of the analysis. According to Kaseleht (2012), the standard must be a pure and chemically inert compound, having unique resolution from the other eluted peaks. Next, the fiber is exposed to the headspace of the sample and the VOCs are re-partitioned between the sample, the headspace and the fiber. The chemical mechanism behind the re-partition of the VOCs in such a multiphase system was reviewed by Kaseleht (2012). The concentration, the ratio and the number of extracted compounds depend on several parameters such as the extraction temperature, the extraction time, the volume of samples in the vial, the composition of the analyzed food sample (e.g. pH, salt content), the characteristics of the fiber and the affinity with the volatiles under investigation.

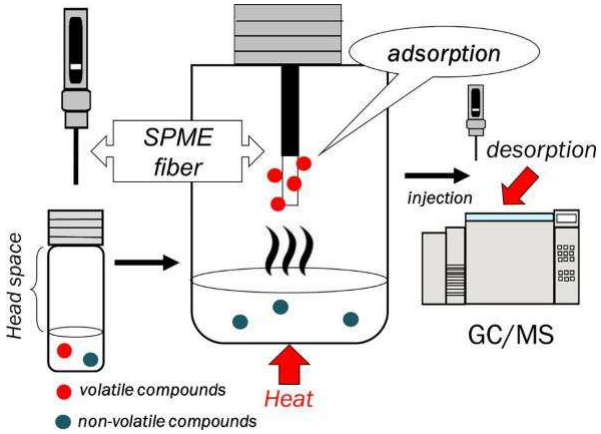

Figure 7. Sketch representing the principles behind the analysis of the volatiles compounds through HS-SPME/GC-MS (Saito et al., 2019).

After sampling, the analytes attached to the SPME fiber are desorbed in the injector port of the GC instrument through heating (Werkhoff, Brennecke, Bretschneider, & Bertram., 2001). The application of GC allows the separation of analytes extracted by the fiber through a capillary column with high power of resolution (Hung, Lee, Yang, & Lee, 2014). Next, molecules are fragmented in the MS and the obtained mass spectra are used for compounds identification. MS-based techniques have been historically used to detect VOCs after GC separation. The research article of Vazquez-Landaverde and coworkers (2005) is a golden standard concerning the application of HS-SPME/GC-MS to study the VOCs profile of milk. In particular, the authors successfully employed response surface methodology (RSM) to find the best SPME conditions for studying the formation of thermally derived compounds in milk. Such approach was taken as an inspiration in this thesis and was proposed again in Chapter 2 for a comprehensive evaluation of the UHLM VOCs profile, with emphasis on those compounds important from a sensory quality perspective. Other authors also compared the performance of SPME with other analytical techniques, such as Purge-and-Trap (Contarini & Povolo, 2002; Povolo & Contarini, 2003). According to the authors, despite the different performances of the two techniques, more sensitive measurements in a shorter period of time are obtained by SPME, concluding that this technique was more suitable for application in the dairy sector. Thus, other extraction methods were not mentioned in this thesis which will focus on the application HS-SPME/GC-MS for the detection of VOCs of UHLM.

Proton Transfer Reaction Mass Spectrometry (PTR-MS)

From the previous paragraph, it is clear that GC-MS is the reference method for VOCs assessment in food. Nevertheless, the technique presents some limitations. First of all, GC-MS is still considered a time-consuming procedure (Biasioli et al., 2011). Furthermore, when HS-SPME and GC-MS are coupled, the obtained VOCs profile, in terms of proportion among the compounds, is somehow different from the original profile of the food product, as different extraction conditions tune different proportions of molecules in the headspace (Jeleń, 2006) and the different affinities between fiber coating material and the single volatile compounds further enhance this effect. Therefore, there is a growing interest in MS-based techniques allowing rapid quantitative analysis, without the need of over-manipulating the samples (Majchrzak et al., 2018). Techniques avoiding chromatographic separation such as direct injection mass spectrometry (DIMS) methods gathered particular interest. Proton transfer reaction-mass spectrometry (PTR-MS) was reported as a successful DIMS methodology for rapid, noninvasive, sensitive assessment of VOCs in food science (Biasioli et al., 2011; Cappellin et al., 2013). The instrument exploits the ionization of VOCs by protons transfer coming from protonated water (H_3O^+).

Figure 8 illustrates a schematic overview of PTR-TOF-MS instrument employed in this thesis project. Four main section can be seen: the ion source, (where protonated water is supplied), the drift tube (where the VOCs are injected in continuous flow and a mass analyzer (where protonated VOCs are finally detected). When PTR is coupled with a quadrupole detector, resolution of molecules (no isobars separations) is limited (Fabris et al., 2010). Better resolution and detection was achieved when the instrument was coupled with a time-of-flight (TOF) mass spectrometer (Soukoulis et al., 2010). A rapid fingerprint of the whole VOCs profile of the sample (often referred to "volatilome") is then obtained, including also data detected at extremely low concentrations in the order of pptV/ppbV (Capozzi et al., 2020).

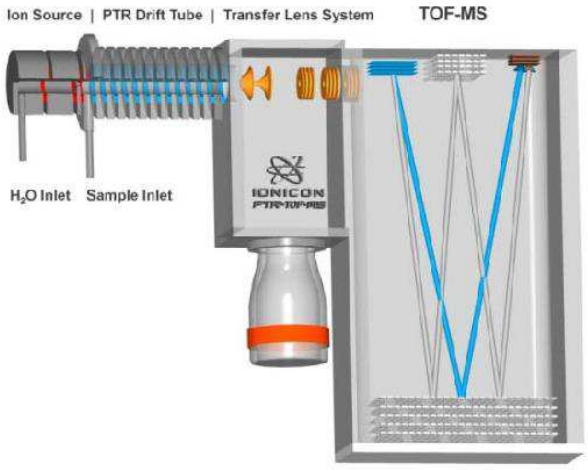

Figure 8. Schematic representation of a PTR-TOF-MS similar to the one used along the PhD project (Jordan et al., 2009)

In the dairy sector, PTR-MS has been applied to grana-type cheeses (Eugenio Aprea et al., 2007; Boscaini, Van Ruth, Biasioli, Gasperi, & Märk, 2003), butter (van Ruth et al., 2008), and yoghurt (Soukoulis et al., 2010) demonstrating good performance of the instrument for the quality assessment purposes. With regards to milk, limited information is available. Liu et al. (2018) studied the VOCs profile of different commercial milk as a function of the season (winter and summer) and the production system (organic, conventional and pasture) for traceability purposes. In that case, the instrument was not only capable of discriminating the milk based on their whole VOCs profile, but captured some molecules (e.g. terpenes) as marker of milk authenticity. A recent paper published Capozzi et al. (2020) further illustrated the good performance of PTR-MS in the dairy sector, specifically on Mascarpone cheese. Both regular and lactose-free Mascarpone samples were considered and a distinctive VOCs profile was measured, which provided relevant information on the product quality. In this frame, the suitability of PTR-MS in realistic scenarios dealing with UHLM quality have not been questioned yet and it was considered during this thesis project.

Project outline

The PhD thesis focuses on the preservation of the UHLM sensory quality during shelf-life, with emphasis on flavor. In particular, the core of the research is comprehending the response of milk to different processing practices and storage conditions. By studying the interplay of these factors, the chemical reactions unraveling sensory changes and off-flavor formation were clearly identified and can be considered by dairy R&D teams for designing UHLM with high-quality standards during shelf-life. In the next chapter (**Chapter 2**), the application of HS-SPME/GC-MS to study the VOCs profile of UHLM during industrial production and storage is discussed. This study was particularly important because it provided an identikit of the molecules responsible for the UHLM flavor together with the best experimental conditions for assessing VOCs though SPME. **Chapter 3** was dedicated to the optimization of the milk sensory analysis. The chapter deals with the changes in UHLM after short-time freezing with the final goal of understanding if such treatment can be introduced to align the sensory panel sessions. In **Chapter 4** UHLM was studied in a realistic industrial scenario: the knowledge gathered in the previous chapters was applied to study the chemical and sensory changes in the product during shelf-life. The experiments evaluated the product response to different LPs employed during "in batch" production in terms of proteolysis, off-flavours development and changes in the sensory properties. Results showed an implication of flavour. **Chapter 5** was dedicated to evaluate whether flavours could be also estimated by rapid fingerprinting with direct injection methods. Accordingly, PTR-ToF-MS was used to evaluate the same samples set of UHLM, introducing also an insight on the batch-to-batch milk variability. At this point, the phenomena underpinning modifications in UHLM during shelf-life were clearly identified and explained. **Chapter 6** filled the remaining gaps by studying the behaviour of UHLM in response to different storage temperatures. Finally, **Chapter 7**, provides a general discussion of all studies and reflects on potentials and pitfalls related to the production of UHLM. A brief consideration on the analytical techniques applied throughout the study, the way forward for future research and main conclusions are also given.

References

Adhikari, K., Dooley, L. M., Chambers IV, E., & Bhumiratana, N. (2010). Sensory characteristics of commercial lactose-free milks manufactured in the United States. *LWT - Food Science and Technology*, *43*(1), 113–118. https://doi.org/10.1016/j.lwt.2009.06.017

Ajmal, M., Nadeem, M., Imran, M., & Junaid, M. (2018). Lipid compositional changes and oxidation status of ultra-high temperature treated Milk. *Lipids in Health and Disease*, *17*(1), 1–11. https://doi.org/10.1186/s12944-018-0869-3

Aprea, E., Romanzin, A., Corazzin, M., Favotto, S., Betta, E., Gasperi, F., & Bovolenta, S. (2016). Effects of grazing cow diet on volatile compounds as well as physicochemical and sensory characteristics of 12-month-ripened Montasio cheese. *Journal of Dairy Science*, *99*(8), 6180–6190. https://doi.org/10.3168/JDS.2016-10929

Aprea, Eugenio, Biasioli, F., Gasperi, F., Mott, D., Marini, F., & Märk, T. D. (2007). Assessment of Trentingrana cheese ageing by proton transfer reaction-mass spectrometry and chemometrics. *International Dairy Journal*, *17*(3), 226–234. https://doi.org/10.1016/j.idairyj.2006.02.008

Aprea, Eugenio, Gika, H., Carlin, S., Theodoridis, G., Vrhovsek, U., & Mattivi, F. (2011). Metabolite profiling on apple volatile content based on solid phase microextraction and gas-chromatography time of flight mass spectrometry. *Journal of Chromatography A*, *1218*(28), 4517–4524. https://doi.org/10.1016/j.chroma.2011.05.019

Astley, M. (n.d.). Self-diagnosed lactose intolerance driving lactose-free dairy sales. *Dairy Reporter*.

Bansal, N., & Bhandari, B. (2016). Functional Milk Proteins: Production and Utilization---Whey-Based Ingredients. In P. L. H. McSweeney & J. A. O'Mahony (Eds.), *Advanced Dairy Chemistry: Volume 1B: Proteins: Applied Aspects* (pp. 67–98). New York, NY: Springer New York. https://doi.org/10.1007/978-1-4939-2800-2_3

Biasioli, F., Gasperi, F., Yeretzian, C., & Märk, T. D. (2011, July). PTR-MS monitoring of VOCs and BVOCs in food science and technology. *TrAC - Trends in Analytical Chemistry*. https://doi.org/10.1016/j.trac.2011.03.009

Boscaini, E., Van Ruth, S., Biasioli, F., Gasperi, F., & Märk, T. D. (2003). Gas chromatography-olfactometry (GC-O) and proton transfer reaction-mass spectrometry (PTR-MS) analysis of the flavor profile of Grana Padano, Parmigiano Reggiano, and Grana Trentino cheeses. *Journal of Agricultural and Food Chemistry*, *51*(7), 1782–1790. https://doi.org/10.1021/jf020922g

Capozzi, V., Lonzarich, V., Khomenko, I., Cappellin, L., Navarini, L., & Biasioli, F. (2020). Unveiling the Molecular Basis of Mascarpone Cheese Aroma: VOCs analysis by SPME-GC/MS and PTR-ToF-MS. *Molecules*, *25*(5), 1242.

Cappellin, L., Loreto, F., Aprea, E., Romano, A., Sánchez del Pulgar, J., Gasperi, F., & Biasioli, F. (2013). PTR-MS in Italy: A multipurpose sensor with applications in environmental, agri-food and health science. *Sensors (Switzerland)*, *13*(9), 11923–11955. https://doi.org/10.3390/s130911923

Carr, A., & Golding, M. (2016). Functional Milk Proteins Production and Utilization: Casein-Based Ingredients. In P. L. H. McSweeney & J. A. O'Mahony (Eds.), *Advanced Dairy Chemistry: Volume 1B: Proteins: Applied Aspects* (pp. 35–66). New York, NY: Springer New York. https://doi.org/10.1007/978-1-4939-2800-2_2

Cavalli-Sforza, L. T., Strata, A., Barone, A., & Cucurachi, L. (1987). Primary adult lactose malabsorption in Italy: Regional differences in prevalence and relationship to lactose intolerance and milk consumption. *American Journal of Clinical Nutrition*, *45*(4), 748–754. https://doi.org/10.1093/ajcn/45.4.748

Condurso, C., Verzera, A., Romeo, V., Ziino, M., & Conte, F. (2008). Solid-phase microextraction and gas chromatography mass spectrometry analysis of dairy product volatiles for the determination of shelf-life. *International Dairy Journal*, *18*(8), 819–825. https://doi.org/10.1016/j.idairyj.2007.12.005

Contarini, G., & Povolo, M. (2002). Volatile fraction of milk: Comparison between, purge and trap and solid phase microextraction techniques. *Journal of Agricultural and Food Chemistry*, *50*(25), 7350–7355. https://doi.org/10.1021/jf025713a

Contarini, G., Povolo, M., Leardi, R., & Toppino, P. M. (1997). Influence of Heat Treatment on the Volatile Compounds of Milk. *Journal of Agricultural and Food Chemistry*, *45*(3), 3171–3177. Retrieved from https://pubs.acs.org/sharingguidelines

Corgneau, M., Scher, J., Ritie-Pertusa, L., Le, D. t. l., Petit, J., Nikolova, Y., … Gaiani, C. (2017). Recent advances on lactose intolerance: Tolerance thresholds and currently available answers. *Critical Reviews in Food Science and Nutrition*, *57*(15), 3344–3356. https://doi.org/10.1080/10408398.2015.1123671

Cremer, D. R., Vollenbroeker, M., & Eichner, K. (2000). Investigation of the formation of Strecker aldehydes from the reaction of Amadori rearrangement products with of-amino acids in low moisture model systems. *European Food Research and Technology*, *211*(6), 400–403. https://doi.org/10.1007/s002170000196

Dahlqvist, A., Asp, N.-G., Burvall, A., & Rausing, H. (1977). Hydrolysis of lactose in milk and whey with minute amounts of lactase. *Journal of Dairy Research*, *44*(3), 541–548. https://doi.org/10.1017/S0022029900020495

Dalgleish, D. G., & Corredig, M. (2012). The Structure of the Casein Micelle of Milk and Its Changes During Processing. *Annual Review of Food Science and Technology*, *3*(1), 449–467. https://doi.org/10.1146/annurev-food-022811-101214

Datta, N., & Deeth, H. C. (2001). Age gelation of UHT milk - A review. *Food and Bioproducts Processing: Transactions of the Institution of of Chemical Engineers, Part C*, *79*(4), 197–210. https://doi.org/10.1205/096030801753252261

Datta, Nivedita, Elliott, A. J., Perkins, M. L., & Deeth, H. C. (2002). Ultra-high-temperature (UHT) treatment of milk: Comparison of direct and indirect modes of heating. *Australian Journal of Dairy Technology*, *57*(3), 211–227. Retrieved from https://search.proquest.com/docview/220297257?accountid=12834

Deeth, H. C. (2020). *The effect of UHT processing and storage on milk proteins*. *Milk Proteins* (3rd ed.). Elsevier Inc. https://doi.org/10.1016/b978-0-12-815251-5.00010-4

Deeth, H. C., & Lewis, M. J. (2017). *High temperature processing of milk and milk products*. *John Wiley & Sons.* https://doi.org/10.1002/9781118460467.ch6

Dekker, P. J.T., & Daamen, C. B. G. (2011). Enzymes Exogenous To Milk In Dairy Technology: β-d-Galactosidase. *Encyclopedia of Dairy Sciences: Second Edition*, 276–283. https://doi.org/10.1016/B978-0-12-374407-4.00152-7

Dekker, Peter J. T., Koenders, D., & Bruins, M. J. (2019). Lactose-Free Dairy Products: Market Developments, Production, Nutrition and Health Benefits. *Nutrients*, *11*(3), 551. https://doi.org/10.3390/nu11030551

DiCosimo, R., McAuliffe, J., Poulose, A. J., & Bohlmann, G. (2013). Industrial use of immobilized enzymes. *Chemical Society Reviews*, *42*(15), 6437–6474.

Endrizzi, I., Fabris, A., Biasioli, F., Aprea, E., Franciosi, E., Poznanski, E., … Gasperi, F. (2012). The effect of milk collection and storage conditions on the final quality of Trentingrana cheese: Sensory and instrumental evaluation. *International Dairy Journal*, *23*(2), 105–114. https://doi.org/10.1016/j.idairyj.2011.10.004

Evangelisti, F., Calcagno, C., Nardi, S., & Zunin, P. (1999). Deterioration of protein fraction by Maillard reaction in dietetic milks. *Journal of Dairy Research*, *66*(2), 237–243. https://doi.org/10.1017/S0022029999003453

Fabris, A., Biasioli, F., Granitto, P. M., Aprea, E., Cappellin, L., Schuhfried, E., … Endrizzi, I. (2010).

PTR-TOF-MS and data-mining methods for rapid characterisation of agro-industrial samples: Influence of milk storage conditions on the volatile compounds profile of Trentingrana cheese. *Journal of Mass Spectrometry*, *45*(9), 1065–1074. https://doi.org/10.1002/jms.1797

FAO. (2018). *Dairy Market Review*.

Fassio, F., Facioni, M., & Guagnini, F. (2018). Lactose Maldigestion, Malabsorption, and Intolerance: A Comprehensive Review with a Focus on Current Management and Future Perspectives. *Nutrients*, *10*(11), 1599. https://doi.org/10.3390/nu10111599

Flynn, R. G., Bakal, A. I., & Snyder, M. A. (1994). *US5334399*.

Fox, Patrick F., & McSweeney, P. L. H. (1998). *Dairy chemistry and biochemistry*. New York, USA: Thomson Science.

Francis, L. L., Chambers, D. H., Kong, S. H., Milliken, G. A., Jeon, I. J., & Schmidt., K. A. (2005). Serving Temperature Effects on Milk Flavor, Milk Aftertaste, and Volatile Compound Quantification in Nonfat and Whole Milk. *Journal of Food Science*, *70*(7), s413–s418. Retrieved from www.ift.org

Friedrich, J. E., & Acree, T. E. (1998). Gas Chromatography Olfactometry (GC/O) of Dairy Products. *International Dairy Journal*, *8*(3), 235–241. https://doi.org/https://doi.org/10.1016/S0958-6946(98)80002-2

Fuller, F., Huang, J., Ma, H., & Rozelle, S. (2006). Got milk? The rapid rise of China's dairy sector and its future prospects. *Food Policy*, *31*(3 SPEC. ISS.), 201–215. https://doi.org/10.1016/j.foodpol.2006.03.002

González-Córdova, A. F., & Vallejo-Cordoba, B. (2001). Quantitative determination of short-chain free fatty acids in milk using solid-phase microextraction and gas chromatography. *Journal of Agricultural and Food Chemistry*, *49*(10), 4603–4608. https://doi.org/10.1021/jf010108d

Goulding, D. A., Fox, P. F., & O'Mahony, J. A. (2020). *Milk proteins: An overview*. *Milk Proteins*. https://doi.org/10.1016/b978-0-12-815251-5.00002-5

Harju, M., Kallioinen, H., & Tossavainen, O. (2012, February). Lactose hydrolysis and other conversions in dairy products: Technological aspects. *International Dairy Journal*. https://doi.org/10.1016/j.idairyj.2011.09.011

Havemose, M. S., Weisbjerg, M. R., Bredie, W. L. P., Poulsen, H. D., & Nielsen, J. H. (2006). Oxidative Stability of Milk Influenced by Fatty Acids, Antioxidants, and Copper Derived from Feed. *Journal of Dairy Science*, *89*(6), 1970–1980.

https://doi.org/https://doi.org/10.3168/jds.S0022-0302(06)72264-0

Heyman, M. B. (2006). Lactose intolerance in infants, children, and adolescents. *Pediatrics*, *118*(3), 1279–1286. https://doi.org/10.1542/peds.2006-1721

Ho, C.-T., Zheng, X., & Li, S. (2015). Tea aroma formation. *Food Science and Human Wellness*, *4*. https://doi.org/10.1016/j.fshw.2015.04.001

Hofmann, T., & Schieberle, P. (2000). Formation of aroma-active Strecker-aldehydes by a direct oxidative degradation of Amadori compounds. *Journal of Agricultural and Food Chemistry*, *48*(9), 4301–4305. https://doi.org/10.1021/jf000076e

Hung, C. H., Lee, C. Y., Yang, C. L., & Lee, M. R. (2014). Classification and differentiation of agarwoods by using non-targeted HS-SPME-GC/MS and multivariate analysis. *Analytical Methods*, *6*(18), 7449–7456. https://doi.org/10.1039/c4ay01151a

Ingram, C. J. E., Mulcare, C. A., Itan, Y., Thomas, M. G., & Swallow, D. M. (2009). Lactose digestion and the evolutionary genetics of lactase persistence. *Human Genetics*. https://doi.org/10.1007/s00439-008-0593-6

Jansson, T. (2014). Chemical changes and off-flavor development in lactose- hydrolyzed UHT milk during storage. *Aarhus University, Department of Food Science.*, 1–192.

Jansson, T., Clausen, M. R., Sundekilde, U. K., Eggers, N., Nyegaard, S., Larsen, L. B., … Bertram, H. C. (2014). Lactose-hydrolyzed milk is more prone to chemical changes during storage than conventional ultra-high-temperature (UHT) milk. *Journal of Agricultural and Food Chemistry*, *62*(31), 7886–7896. https://doi.org/10.1021/jf501671z

Jansson, T., Jensen, H. B., Sundekilde, U. K., Clausen, M. R., Eggers, N., Larsen, L. B., … Bertram, H. C. (2014). Chemical and proteolysis-derived changes during long-term storage of lactose-hydrolyzed ultrahigh-temperature (UHT) milk. *Journal of Agricultural and Food Chemistry*, *62*(46), 11270–11278. https://doi.org/10.1021/jf504104q

Jansson, T., Jensen, S., Eggers, N., Clausen, M. R., Larsen, L. B., Ray, C., … Bertram, H. C. (2014). Volatile component profiles of conventional and lactose-hydrolyzed UHT milk - A dynamic headspace gas chromatography-mass spectrometry study. *Dairy Science and Technology*, *94*(4), 311–325. https://doi.org/10.1007/s13594-014-0164-7

Jansson, T., Rauh, V., Danielsen, B. P., Poojary, M. M., Waehrens, S. S., Bredie, W. L. P., … Lund, M. N. (2017). Green Tea Polyphenols Decrease Strecker Aldehydes and Bind to Proteins in Lactose-Hydrolyzed UHT Milk. *Journal of Agricultural and Food Chemistry*, *65*(48), 10550–10561. https://doi.org/10.1021/acs.jafc.7b04137

Jansson, T., Waehrens, S. S., Rauh, V., Danielsen, B. P., Sørensen, J., Bredie, W. L. P., … Lund, M.

N. (2019). Effect of green tea catechins on physical stability and sensory quality of lactose-reduced UHT milk during storage for one year. *International Dairy Journal*, *95*, 25–34. https://doi.org/10.1016/j.idairyj.2019.03.007

Jeleń, H. H. (2006). Solid-phase microextraction in the analysis of food taints and off-flavors. *Journal of Chromatographic Science*, *44*(7), 399–415. https://doi.org/10.1093/chromsci/44.7.399

Jelen, P. (1982). Experience with direct and indirect UHT processing of milk-a Canadian viewpoint. *Journal of Food Protection*, *45*(9), 878–883.

Jelen, P., & Tossavainen, O. (2003). Low lactose and lactose-free milk and dairy products - Prospects, technologies and applications. *Australian Journal of Dairy Technology*, *58*(2), 161–165. https://doi.org/http://www.scopus.com/inward/record.url?eid=2-s2.0-0141595866&partnerID=tZOtx3y1

Jensen, S., Jansson, T., Eggers, N., Clausen, M. R., Larsen, L. B., Jensen, H. B., … Bertram, H. C. (2015). Storage-induced changes in the sensory characteristics and volatiles of conventional and lactose-hydrolyzed UHT processed milk. *European Food Research and Technology*, *240*(6), 1247–1257. https://doi.org/10.1007/s00217-015-2427-9

Jeon, I. J., Thomas, E. L., & Reineccius, G. A. (1978). Production of volatile flavor compounds in ultrahigh-temperature processed milk during aseptic storage. *Journal of Agricultural and Food Chemistry*, *26*(5), 1183–1188. https://doi.org/10.1021/jf60219a039

Jordan, A., Haidacher, S., Hanel, G., Hartungen, E., Herbig, J., Märk, L., … Märk, T. (2009). An online ultra-high sensitivity Proton-transfer-reaction mass-spectrometer combined with switchable reagent ion capability (PTR + SRI – MS). *International Journal of Mass Spectrometry - INT J MASS SPECTROM*, *286*, 32–38. https://doi.org/10.1016/j.ijms.2009.06.006

Jost, R. (2000). *Milk and dairy products*. (Ullmann's Encyclopedia of Industrial Chemistry, Ed.).

Kaseleht, K. (2012). *Identification of Aroma Compounds in Food Using SPME-GC/MS and GC-Olfactometry*. Tallinn university of technology.

Kim Ha, J., & Lindsay, R. C. (1991). Contributions of Cow, Sheep, and Goat Milks to Characterizing Branched-Chain Fatty Acid and Phenolic Flavors in Varietal Cheeses[1]. *Journal of Dairy Science*, *74*(10), 3267–3274. https://doi.org/10.3168/jds.S0022-0302(91)78512-3

Kim, J.-H., Byun, D.-S., Godber, J. S., Choi, J.-S., Choi, W.-C., & Kim, H.-R. (2004). Purification and characterization of arylsulfatase from Sphingomonas sp. AS6330. *Applied*

Microbiology and Biotechnology, *63*(5), 553–559. https://doi.org/10.1007/s00253-003-1463-8

Kim, S., Chen, J., Cheng, T., Gindulyte, A., He, J., He, S., … Bolton, E. E. (2019). PubChem 2019 update: improved access to chemical data. *Nucleic Acids Research*, *47*(D1), D1102–D1109. https://doi.org/https://doi.org/10.1093/nar/gky1033

Leksmono, C. S., Manzoni, C., Tomkins, J. E., Lucchesi, W., Cottrell, G., & Lewis, P. A. (2018). Measuring lactase enzymatic activity in the teaching lab. *Journal of Visualized Experiments*, *2018*(138), 1–5. https://doi.org/10.3791/54377

Leonardi, M., Gerbault, P., Thomas, M. G., & Burger, J. (2012, February). The evolution of lactase persistence in Europe. A synthesis of archaeological and genetic evidence. *International Dairy Journal*. https://doi.org/10.1016/j.idairyj.2011.10.010

Liu, M., Liu, J., He, C., Song, H., Liu, Y., Zhang, Y., … Su, X. (2017). Characterization and comparison of key aroma-active compounds of cocoa liquors from five different areas. *International Journal of Food Properties*, *20*(10), 2396–2408. https://doi.org/10.1080/10942912.2016.1238929

Liu, N., Koot, A., Hettinga, K., de Jong, J., & van Ruth, S. M. (2018). Portraying and tracing the impact of different production systems on the volatile organic compound composition of milk by PTR-(Quad)MS and PTR-(ToF)MS. *Food Chemistry*, *239*, 201–207. https://doi.org/10.1016/j.foodchem.2017.06.099

Liu, Y., & Guo, R. (2008). pH-dependent structures and properties of casein micelles. *Biophysical Chemistry*, *136*(2–3), 67–73. https://doi.org/10.1016/j.bpc.2008.03.012

Mahoney, R. R. (1997). Lactose: Enzymatic Modification. In P F Fox (Ed.), *Advanced Dairy Chemistry Volume 3: Lactose, water, salts and vitamins* (pp. 77–125). Boston, MA: Springer US. https://doi.org/10.1007/978-1-4757-4409-5_3

Majchrzak, T., Wojnowski, W., Lubinska-Szczygeł, M., Różańska, A., Namieśnik, J., & Dymerski, T. (2018). PTR-MS and GC-MS as complementary techniques for analysis of volatiles: A tutorial review. *Analytica Chimica Acta*, *1035*, 1–13. https://doi.org/https://doi.org/10.1016/j.aca.2018.06.056

Marsili, R. T., & Miller, N. (2002). SPME-GC/MS testing for monitoring off-flavors in processed milk: Application of odor wheel plots. *ACS Symposium Series*, *836*, 80–94. https://doi.org/10.1021/bk-2003-0836.ch006

Martins, S. I. F. S., Jongen, W. M. F., & van Boekel, M. A. J. S. (2000). A review of Maillard reaction in food and implications to kinetic modelling. *Trends in Food Science & Technology*,

11(9), 364–373. https://doi.org/https://doi.org/10.1016/S0924-2244(01)00022-X

Mendoza, M. R., Olano, A., & Villamiel, M. (2005). Chemical indicators of heat treatment in fortified and special milks. *Journal of Agricultural and Food Chemistry*, *53*(8), 2995–2999. https://doi.org/10.1021/jf040406l

Messia, M. C., Candigliota, T., & Marconi, E. (2007). Assessment of quality and technological characterization of lactose-hydrolyzed milk. *Food Chemistry*, *104*(3), 910–917. https://doi.org/10.1016/j.foodchem.2006.12.045

Milkovska-Stamenova, S., & Hoffmann, R. (2017). Influence of storage and heating on protein glycation levels of processed lactose-free and regular bovine milk products. *Food Chemistry*, *221*, 489–495. https://doi.org/10.1016/j.foodchem.2016.10.092

Morr, C. V. (1975). Chemistry of Milk Proteins in Food Processing. *Journal of Dairy Science*, *58*(7), 977–984. https://doi.org/10.3168/jds.S0022-0302(75)84669-8

Naranjo, G. B., Gonzales, A. S. P., Leiva, G. E., & Malec, L. S. (2013). The kinetics of Maillard reaction in lactose-hydrolysed milk powder and related systems containing carbohydrate mixtures. *Food Chemistry*, *141*(4), 3790–3795. https://doi.org/10.1016/j.foodchem.2013.06.093

Nielsen, S. D., Jansson, T., Le, T. T., Jensen, S., Eggers, N., Rauh, V., … Larsen, L. B. (2017). Correlation between sensory properties and peptides derived from hydrolysed-lactose UHT milk during storage. *International Dairy Journal*, *68*, 23–31. https://doi.org/10.1016/j.idairyj.2016.12.013

Nielsen, S. D., Zhao, D., Le, T. T., Rauh, V., Sørensen, J., Andersen, H. J., & Larsen, L. B. (2018). Proteolytic side-activity of lactase preparations. *International Dairy Journal*, *78*, 159–168. https://doi.org/10.1016/j.idairyj.2017.12.001

Nowshad, F., Islam, M. N., & Khan, M. S. (2018). Concentration and formation behavior of naturally occurring formaldehyde in foods. *Agriculture & Food Security*, *7*(1), 17. https://doi.org/10.1186/s40066-018-0166-4

Nursten, H. E. (2005). *The Maillard reaction: chemistry, biochemistry, and implications.* Royal Society of Chemistry. Retrieved from www.themanager.org

O'Brien, J. (2009). Non-Enzymatic Degradation Pathways of Lactose and Their Significance in Dairy Products. In P. McSweeney & P. F. Fox (Eds.), *Advanced Dairy Chemistry: Volume 3: Lactose, Water, Salts and Minor Constituents* (pp. 231–294). New York, NY: Springer New York. https://doi.org/10.1007/978-0-387-84865-5_7

OECD/Food and Agriculture Organization of the United Nations. (2019). Dairy and dairy

products. *OECDFAO Agricultural Outlook 2019-2028*.

Panesar, P. S., & Kumari, S. (2011). Lactulose: Production, purification and potential applications. *Biotechnology Advances*, *29*(6), 940–948. https://doi.org/https://doi.org/10.1016/j.biotechadv.2011.08.008

Park, Y. K., Santi, M. S. S., & Pastore, G. M. (1979). Production and characterization of β-galactosidase from Aspergillus Oryzae. *Journal of Food Science*, *44*(1), 100–103. https://doi.org/10.1111/j.1365-2621.1979.tb10016.x

Pedrotti, M., Khomenko, I., Cappellin, L., Fontana, M., Somenzi, M., Falchero, L., … Biasioli, F. (2018). Rapid and noninvasive quality control of anhydrous milk fat by PTR-MS: The effect of storage time and packaging. *Journal of Mass Spectrometry*, *53*(9), 753–762. https://doi.org/10.1002/jms.4204

Penfield, M. P., & Campbell, A. M. (1990). Milk and milk products. In M. P. Penfield & A. M. B. T.-E. F. S. (Third E. Campbell (Eds.), *Experimental Food Science (Third Edition)* (pp. 162–183). San Diego: Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-157920-3.50012-0

Poulsen, M. W., Hedegaard, R. V, Andersen, J. M., de Courten, B., Bügel, S., Nielsen, J., … Dragsted, L. O. (2013). Advanced glycation endproducts in food and their effects on health. *Food and Chemical Toxicology*, *60*, 10–37. https://doi.org/https://doi.org/10.1016/j.fct.2013.06.052

Povolo, M., & Contarini, G. (2003). *Comparison of solid-phase microextraction and purge-and-trap methods for the analysis of the volatile fraction of butter*. *Journal of Chromatography A* (Vol. 985). Retrieved from www.elsevier.com/locate/chroma

Rizzi, G. P. (1999). The Strecker Degradation and Its Contribution to Food Flavor. *Flavor Chemistry*, (1952), 335–343. https://doi.org/10.1007/978-1-4615-4693-1_28

Roberfroid, M., Gibson, G. R., Hoyles, L., McCartney, A. L., Rastall, R., Rowland, I., … Meheust, A. (2010). Prebiotic effects: metabolic and health benefits. *British Journal of Nutrition*, *104*(S2), S1–S63. https://doi.org/DOI: 10.1017/S0007114510003363

Rodríguez, A., Lema, P., Bessio, M. I., Moyna, G., Panizzolo, L. A., & Ferreira, F. (2019). Isolation and Characterization of Melanoidins from Dulce de Leche, A Confectionary Dairy Product. *Molecules*, *24*(22), 4163. https://doi.org/10.1007/BF01263547

Rysstad, G., & Kolstad, J. (2006). Extended shelf life milk - Advances in technology. *International Journal of Dairy Technology*, *59*(2), 85–96. https://doi.org/10.1111/j.1471-0307.2006.00247.x

Saito, K., Kaneko, S., Furuya, Y., Asada, Y., Ito, R., Sugie, K., … Yanagawa, Y. (2019). Confirmation of synthetic cannabinoids in herb and blood by HS-SPME-GC/MS. *Forensic Chemistry*, *13*, 100156. https://doi.org/https://doi.org/10.1016/j.forc.2019.100156

Schaafsma, G. (2008). Lactose and lactose derivatives as bioactive ingredients in human nutrition. *International Dairy Journal*, *18*(5), 458–465. https://doi.org/10.1016/J.IDAIRYJ.2007.11.013

Sinaga, H., Bansal, N., & Bhandari, B. (2017). Effects of milk pH alteration on casein micelle size and gelation properties of milk. *International Journal of Food Properties*, *20*(1), 179–197. https://doi.org/10.1080/10942912.2016.1152480

Soukoulis, C., Aprea, E., Biasioli, F., Cappellin, L., Schuhfried, E., Märk, T. D., & Gasperi, F. (2010). Proton transfer reaction time-of-flight mass spectrometry monitoring of the evolution of volatile compounds during lactic acid fermentation of milk. *Rapid Communications in Mass Spectrometry*, *24*(14), 2127–2134. https://doi.org/10.1002/rcm.4617

Stressler, T., Leisibach, D., Lutz-Wahl, S., Kuhn, A., & Fischer, L. (2016). Homologous expression and biochemical characterization of the arylsulfatase from Kluyveromyces lactis and its relevance in milk processing. *Applied Microbiology and Biotechnology*, *100*(12), 5401–5414. https://doi.org/10.1007/s00253-016-7366-2

Sundekilde, U., Larsen, L., & Bertram, H. (2013). NMR-Based Milk Metabolomics. *Metabolites*, *3*(2), 204–222. https://doi.org/10.3390/metabo3020204

Sunds, A. V. (2016). *Evaluation of accelerated shelf life testing of UHT milk*.

Sunds, A. V., Rauh, V. M., Sørensen, J., & Larsen, L. B. (2018). Maillard reaction progress in UHT milk during storage at different temperature levels and cycles. *International Dairy Journal*. https://doi.org/10.1016/j.idairyj.2017.08.008

Tossavainen, O., & Kallioinen, H. (2007). Proteolytic changes in lactose hydrolysed UHT milks during storage. *Milchwissenschaft*, *62*(4), 410–415. Retrieved from http://cat.inist.fr/?aModele=afficheN&cpsidt=19123002

Tossavainen, Olli. (2008). *Heat-induced changes in lactose hydrolysed milks*. Retrieved from http://lib.tkk.fi/Diss/2008/isbn9789512293995/

Tossavainen, Olli, & Kallioinen, H. (2008). Effect of lactose hydrolysis on furosine and available lysine in UHT skim milk. *Milchwissenschaft*, *63*(1), 22–26.

Trani, A., Gambacorta, G., Loizzo, P., Cassone, A., Fasciano, C., Zambrini, A. V., & Faccia, M. (2017). Comparison of HPLC-RI, LC/MS-MS and enzymatic assays for the analysis of residual lactose in lactose-free milk. *Food Chemistry*, *233*, 385–390.

https://doi.org/10.1016/j.foodchem.2017.04.134

Troise, A. D., Bandini, E., De Donno, R., Meijer, G., Trezzi, M., & Fogliano, V. (2016). The quality of low lactose milk is affected by the side proteolytic activity of the lactase used in the production process. *Food Research International*, *89*, 514–525. https://doi.org/10.1016/j.foodres.2016.08.021

Troise, A. D., Fiore, A., Roviello, G., Monti, S. M., & Fogliano, V. (2015). Simultaneous quantification of amino acids and Amadori products in foods through ion-pairing liquid chromatography-high-resolution mass spectrometry. *Amino Acids*, *47*(1), 111–124. https://doi.org/10.1007/s00726-014-1845-5

Truong, T., Palmer, M., Bansal, N., & Bhandari, B. (2016). An Overview of Milk Fat Globules. In *Effect of Milk Fat Globule Size on the Physical Functionality of Dairy Products* (pp. 5–9). Cham: Springer International Publishing. https://doi.org/10.1007/978-3-319-23877-7_2

Valero, E., Villamiel, M., Miralles, B., Sanz, J., & Martínez-Castro, I. (2001). Changes in flavour and volatile components during storage of whole and skimmed UHT milk. *Food Chemistry*, *72*(1), 51–58. https://doi.org/10.1016/S0308-8146(00)00203-X

Van Aardt, M., Duncan, S. E., Bourne, D., Marcy, J. E., Long, T. E., Hackney, C. R., & Heisey, C. (2001). Flavor threshold for acetaldehyde in milk, chocolate milk, and spring water using solid phase microextraction gas chromatography for quantification. *Journal of Agricultural and Food Chemistry*, *49*(3), 1377–1381. https://doi.org/10.1021/jf001069t

van Boekel, M. A. J. S. (1998). Effect of heating on Maillard reactions in milk. *Food Chemistry*, *62*(4), 403–414.

van Boekel, M. A. J. S. (2006). Formation of flavour compounds in the Maillard reaction. *Biotechnology Advances*, *24*(2), 230–233. https://doi.org/10.1016/J.BIOTECHADV.2005.11.004

van Ruth, S. M., Koot, A., Akkermans, W., Araghipour, N., Rozijn, M., Baltussen, M., … Frankhuizen, R. (2008). Butter and butter oil classification by PTR-MS. *European Food Research and Technology*, *227*(1), 307–317. https://doi.org/10.1007/s00217-007-0724-7

Vazquez-Landaverde, P. A., Velazquez, G., Torres, J. A., & Qian, M. C. (2005). Quantitative determination of thermally derived off-flavor compounds in milk using solid-phase microextraction and gas chromatography. *Journal of Dairy Science*, *88*(11), 3764–3772. https://doi.org/10.3168/jds.S0022-0302(05)73062-9

Vera, C., Guerrero, C., Aburto, C., Cordova, A., & Illanes, A. (2020). Conventional and non-conventional applications of β-galactosidases. *Biochimica et Biophysica Acta - Proteins*

and Proteomics, *1868*(1), 140271. https://doi.org/10.1016/j.bbapap.2019.140271

Vyssotski, M., Bloor, S. J., Lagutin, K., Wong, H., & Williams, D. B. G. (2015). Efficient Separation and Analysis of Triacylglycerols: Quantitation of β-Palmitate (OPO) in Oils and Infant Formulas. *Journal of Agricultural and Food Chemistry*, *63*(26), 5985–5992. https://doi.org/10.1021/acs.jafc.5b01835

Walsh, A. M., Duncan, S. E., Potts, H., & Gallagher, D. L. (2015). Comparing quality and emotional responses as related to acceptability of light-induced oxidation flavor in milk. *Food Research International*, *76*(P2), 293–300. https://doi.org/10.1016/j.foodres.2015.02.027

Walstra, P. (1999). *Dairy technology: principles of milk properties and processes*. CRC Press.

Werkhoff, P., Brennecke, S., Bretschneider, W., & Bertram., H. (2001). Modern methods for isolating and quantifying volatile flavor and fragrance compounds. In *Flavor, Fragrance, and Odor Analysis, volume 115*. CRC Press.

Wiley, A. S. (2007). Lactose Intolerance. *Pediatric Clinical Advisor*, 331–331. https://doi.org/10.1016/B978-032303506-4.10186-5

Yang, S. T., & Silva, E. M. (1995). Novel Products and New Technologies for Use of a Familiar Carbohydrate, Milk Lactose. *Journal of Dairy Science*, *78*(11), 2541–2562. https://doi.org/10.3168/jds.S0022-0302(95)76884-9

Zabbia, A., Buys, E. M., & de Kock, H. L. (2012). Undesirable Sulphur and carbonyl flavor compounds in UHT milk: A review. *Critical Reviews in Food Science and Nutrition*. https://doi.org/10.1080/10408398.2010.487166

Zadow, J. G. (1984). Lactose: Properties and Uses. *Journal of Dairy Science*, *67*(11), 2654–2679. https://doi.org/10.3168/jds.S0022-0302(84)81625-2

Zhang, W. (2020). *Mechanism of Maillard Reactions in Lactose-hydrolyzed UHT Milk and Strategy of Inhibition by Galacto-Oligosaccharide*.

Zhao, D., Le, T. T., Larsen, L. B., Nian, Y., Wang, C., Li, C., & Zhou, G. (2019). Interplay between Residual Protease Activity in Commercial Lactases and the Subsequent Digestibility of β -Casein in a Model System. *Molecules*, *24*(16), 2876.

Zolnere, K., & Ciprovica, I. (2017). The comparison of commercially available ß-Galactosidases for dairy industry: Review. *Research for Rural Development*, *1*, 215–222. https://doi.org/10.22616/rrd.23.2017.032

Chapter 2: Application of Headspace Solid-Phase Micro-extraction Gas Chromatography for the assessment of the volatiles profiles of ultra-high temperature hydrolyzed-lactose milk during production and storage

In this chapter, the main aim was gathering as many information as possible concerning the VOCs characterizing UHLM during production and shelf-life. Headspace Solid-Phase Micro-extraction (HS SPME) coupled with gas chromatography-mass spectrometry (GC-MS) is a valuable technique to estimate volatile organic compounds (VOCs) of dairy products. As explained in the introduction, this task is particularly delicate when approaching UHLM because of the higher sensitivity towards physiochemical changes than regular UHT milk. Specific VOCs can potentially compromise the flavour and, consequently, the sensory perception of the product during shelf-life so mapping the phenomena underpinning these changes is of utmost importance. In this context, the best experimental conditions for the extraction of VOCs by the SPME fibre were set and applied to study UHLM during industrial production and commercial shelf-life. The aim was demonstrating the broad versatility of the technique as well as collecting valuable information for planning the next studies. This chapter can be seen as a reference guideline for readers interested in the VOCs profile of UHLM throughout the product lifecycle.

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Abstract

UHT hydrolyzed-lactose milk (HLM) is prone to chemical changes arising off-flavors in the product. For better investigation of this aspect, HS-SPME/GC-MS was applied to monitor the volatiles profile of HLM during production and shelf-life. Best conditions for the volatiles extraction were explored, focusing on those compounds affecting the milk aroma. The study about HLM production was the first investigating industrial scale manufacturing under controlled conditions, allowing direct associations between specific reactions and changes it the milk volatiles. The effect of the UHT treatment on milk flavor was shown, while the lactase, potentially containing undesirable proteases and odors, did not alter the milk "volatilome" after addition. Commercial samples exhibited different trends in the volatiles along the shelf-life due to differences in production systems, lactase purity and packaging. Merging all results, HS-SPME/GC-MS successfully mapped the phenomena causing modifications in the volatiles profile of UHT HLM at each step of the product life-cycle.

Introduction

Lactose is the most abundant carbohydrate found in milk (Ingram, Mulcare, Itan, Thomas, & Swallow, 2009) and its proper digestion depends on the presence of lactase (β-1,4-galactosidase) in the human gut. If not hydrolyzed into glucose and galactose, lactose passes through the gastrointestinal tract inducing the production of metabolites (e.g. methane, carbon dioxide and hydrogen) which likely cause diarrhea, stomach pain, flatulence and constipation (Corgneau et al., 2017; Leonardi, Gerbault, Thomas, & Burger, 2012). As a result, dairy industries increased the launch of low lactose milk (LLMs) products, as they represent a simple and handy solution against lactose malabsorption (Troise et al., 2016).

Addition of free soluble lactase in the milk is the most popular process applied in industry for the production of hydrolyzed lactose milk (HLM) (Jelen & Tossavainen, 2003). Alternative processes include acid hydrolysis, membrane reactors, immobilized systems for enzymatic hydrolysis, and cellular extract from lactobacilli (Harju, Kallioinen, & Tossavainen, 2012). The addition of free soluble lactase into the milk can be performed at refrigerated conditions before the ultra-high temperature (UHT) treatment, namely following the "in batch" production system (Troise et al., 2016). The process ascertains that the lactase is inactivated before the product is sold (Dekker, Koenders, & Bruins, 2019). On the other hand, it exposes glucose and galactose, which are more sensitive to Maillard reaction than lactose (Evangelisti, Calcagno, Nardi, & Zunin, 1999), to high temperature. Besides, a more versatile technology in which the lactase is added in each milk package aseptically after the thermal treatment ("in pack" production system) have been recently developed (Harju et al., 2012; Troise et al., 2016). This allows the employment of lower concentrations of lactase, reducing the overall costs of production (Troise et al., 2016). UHT HLM produced by "in batch" and "in pack" technology have many features in common, but differ mainly by the presence of active lactase during the shelf-life of the latter. This aspect is of particular interest because, when the lactase is purified from microorganisms, some secondary proteases are conveyed into the final preparation resulting in commercial lactases having different purity (Nielsen et al., 2018). This proteolytic side activity favor the release of free amino acids and peptides in the milk, which may push the proceeding of the Maillard reaction and the development of off-flavors during storage (Jansson, Jensen, Sundekilde, et al., 2014; Troise et al., 2016). When the lactase is added "in batch", these defects can be modulated as proteolytic side activity is partially or totally annulled by the UHT treatment (Dekker et al., 2019; Tossavainen & Kallioinen, 2007). Differently, when the lactase is added "in pack", no thermal inactivation

takes place, proteolysis and off-flavor development can therefore proceed and, if not properly controlled, the final product quality can perish faster (Nielsen et al., 2018; Troise et al., 2016).

For the assessment of chemical modifications that may occur during the shelf-life of milk, analysis of the volatiles organic compounds (VOCs) profile is an attractive approach. VOCs can reveal chemical modifications at early stages before the concentration of the developed compounds reach the human perception threshold, thus tainting the product. Among the available analytical techniques, headspace solid phase micro-extraction (HS-SPME) coupled to GC-MS has been applied for detecting the volatile profiles of many dairy products such as fermented milk, cheese and butter (Aprea et al., 2016; Condurso, Verzera, Romeo, Ziino, & Conte, 2008; Endrizzi et al., 2012; Povolo & Contarini, 2003). HS-SPME is a simple solvent-free method, which is less time-consuming and requires less sample handling step compared to other extraction techniques applied in food science (González-Córdova & Vallejo-Cordoba, 2001; Vazquez-Landaverde, Velazquez, Torres, & Qian, 2005). In this frame, the aim of the present work was to set the optimal extraction parameters for a HS-SPME/GC-MS methodology and apply it to study the volatile profiles of HLM. The optimized method was applied in two case studies, namely the formation of VOCs during production of UHT HLM by "in batch" system and the monitoring of the VOCs evolution in different commercial UHT HLMs during storage. To the best of our knowledge, this is the first work in which a HS-SPME/GC-MS methodology is optimized and applied for the assessment of the VOCs profiles of HLMs along production and during storage.

Material and Methods

Chemicals

Sodium chloride, 4-methyl-2-pentanone (internal standard with purity ≥ 99%), 2-methylbutanal (purity ≥ 95%) and 3-methylbutanal (purity ≥ 97%) were purchased from Sigma-Aldrich (Steinheim, Germany).

Ultra-high temperature (UHT) hydrolyzed-lactose milk samples

For the validation of the optimized HS-SPME conditions, the VOCs profiles of different UHT HLM samples were analyzed. An overview of the milk samples is reported in **Table 1**. Samples produced by "in batch" technology were collected at the following production step: before addition of the lactase (preH), at the end of the lactose hydrolysis (postH) and after the direct UHT processing (postUHT). Production occurred according to the details reported by Troise and coworkers (2016) for the "in batch" production system. Samples were taken from three consecutive batches of production, all performed under the same operating conditions, as an estimation of the initial milk variability. The same commercial lactase preparation was used for production: according to the technical sheet provided by the supplier, the lactase was extracted from *Kluyveromyces lactis* and had an average enzymatic activity ≥ 5000 NLU/*g*. For the shelf-life simulation study, three different semi-skimmed UHT HLM (HLM1, HLM2, HLM3), were purchased from supermarkets in February 2018. A 4-month (120 days) storage was then performed, keeping the samples in a climate chamber at room temperature (20°C) under controlled conditions. Samples were taken out from the climate chamber every 30 days and stored at -80°C until analysis. The time zero of the study was established within one week after the production date. At the moment of the purchase however, the production date of HLM3 was different compared to HLM1 and HLM2 and it was not possible to sample HLM3 from the beginning of the shelf-life. In order to deal with this difference, sampling of HLM3 started after 30 days of storage. The commercial UHT HLMs differed in terms of packaging and system of production used by producers (**Table 1**).

Table 1. Overview of the milk samples analyzed for the validation of the developed HS-SPME/GC-MS methodology.

Case studies	Samples acronym	Samples description[c]	Process of production	Packaging	Lactose content (g/100 mL milk)
I[a]	preH	Pasteurized milk sampled before lactose hydrolysis	In-batch	-	nd
I	postH	Pasteurized HLM sampled after lactose hydrolysis	In-batch	-	<0,1
I	postUHT	UHT HLM sampled after heat sterilization	In-batch	-	<0,1
II[b]	HLM1	Commercial UHT HLM	In-batch	Paperboard carton	<0,1
II	HLM2	Commercial UHT HLM	In-batch	HDPE	<0,1
II	HLM3	Commercial UHT HLM	In-pack	Paperboard carton	<0,1

[a] The heading refers to the study in which VOCs were evaluated at different steps during the "in batch" production system.
[b] The heading refers to the study in which VOCs were evaluated in three different commercial samples (HLM1, HLM2, and HLM3) stored at 20°C for 120 days.
[c] All the HLM samples analyzed were semi-skimmed

Selection of SPME coating phase and optimization of the extraction parameters

Based on literature and previous experience (Eugenio Aprea, Gasperi, Betta, Sani, & Cantini, 2018) a three-phasic (DVB-Carboxen-PDMS) SPME fiber was selected to grant the widest coverage of analytes. A 2^3 full factorial design with one centered point was applied to investigate the effect of sample size (5 to 10 mL), extraction time (30 to 90 min) and addition of salts (0% to 30% w/w of NaCl) on the amount of VOCs extracted by the SPME fiber. Full factorial design is an orthogonal experimental design method in which all the factors are combined simultaneously (Aprea et al., 2011). A central point is often included in the design to represent the average between the low and high level of the combined factors. In our case, the central point was: 7.5 mL of sample size, 60 min of extraction time, 15% w/w of NaCl. Temperature of the incubation and extraction were fixed at 40°C as a compromise to favor the volatilization of the VOCs without inducing chemical modification of the milk during the analysis. For the optimization of the procedure, we considered as target the total area of the identified integrated peaks. The sample used during the optimization steps was a commercial UHT HLM having 66 days of shelf-life.

Sample analysis

Once the optimal conditions for the HS-SPME were defined, the VOCs profiles of different UHT HLM during "in batch" production and storage was determined. The analysis was performed according to Bergamaschi et al. (2015). Measurements were performed using a 20 mL glass vials with 4-methyl-2-pentanone used as internal standard. For the aim of the present research absolute quantification by calibration curves was not necessary thus the internal standard was used also to normalise the data (semi-quantitative results). Frozen HLM samples were thawed at room temperature. Volatile compounds were first extracted at 40 °C with the 2 cm DVB-Carboxen–PDMS SPME fiber and then desorbed at 250°C in the injector port of a GC interfaced with a mass detector operating in an electron ionization mode (internal ionization source; 70 eV) with a scan range from m/z 33 to 300 (GC Clarus 500, PerkinElmer, Norwalk, CT). Procedure phases were automatically managed using an auto-sampling system (CTC combiPAL, CTC Analysis AG, Zwingen, Switzerland). Separation was achieved on a HP-Innowax fused-silica capillary column (30 m, 0.32 mm inner diameter, 0.5 µm film thickness; Agilent Technologies, Palo Alto, CA). The temperature program was set progressively as follow: 40 °C for 3 min, 180 °C for 6 min at 4 °C min^{-1}, 220 °C for 3 min

at 3 °C min^{-1}. Helium (flow rate equal to 2 ml/min) was chosen as carrier gas. The transfer line temperature was kept at 220 °C. Linear retention indices (LRI) were calculated under the same chromatographic conditions, injecting C7-C30 n-alkane series (Supelco, Bellefonte, PA). Compounds were identified using the mass spectra matching the NIST-2014/Wiley 7.0 libraries and comparing the calculated LRI with those available from literature.

Data analysis

For optimizing the HS-SPME conditions, the total peak area of the identified and verified volatile compounds was considered. Differences in the volatile profiles were investigated by analysis of the variance (ANOVA), and Tukey post-hoc test. A $P \leq 0.05$ was chosen as threshold for significant differences. Principal Component Analysis (PCA) was performed, on the log-transformed GC data scaled to unit variance, in order to explore the spatial distribution of the samples in both case studies. Statistical analysis of the results was performed with the software package STATISTICA 13.3 (StatSoft, Inc., Tulsa, OK, USA) and the R packages (version 3.3.3) FactoMineR and factoextra.

Results and Discussions

Optimization of Headspace Solid-Phase Microextraction (HS-SPME) conditions

The effect of each tested parameter by means of the 2^3 full factorial design was reported in the form of a Pareto chart in **Figure 1a**, whereas **Figure 1b** profiles the surface plot of the outcome as function of extraction time and sample size. In the Pareto chart, the impact of the investigated variables on a response is displayed in the form of a bar chart. The lengths of the bars are proportional to the relative standardized effect. If a bar exceed the vertical line (corresponding to the 95% confidence interval) the effect can be considered statistically significant. From **Figure 1a**, addition of 30% w/w NaCl was the most significant factor and affected negatively the HS-SPME performance. The combination of this parameter with the sample size (volume of milk in 20 mL vials) was also significant, even though at much lower extent.

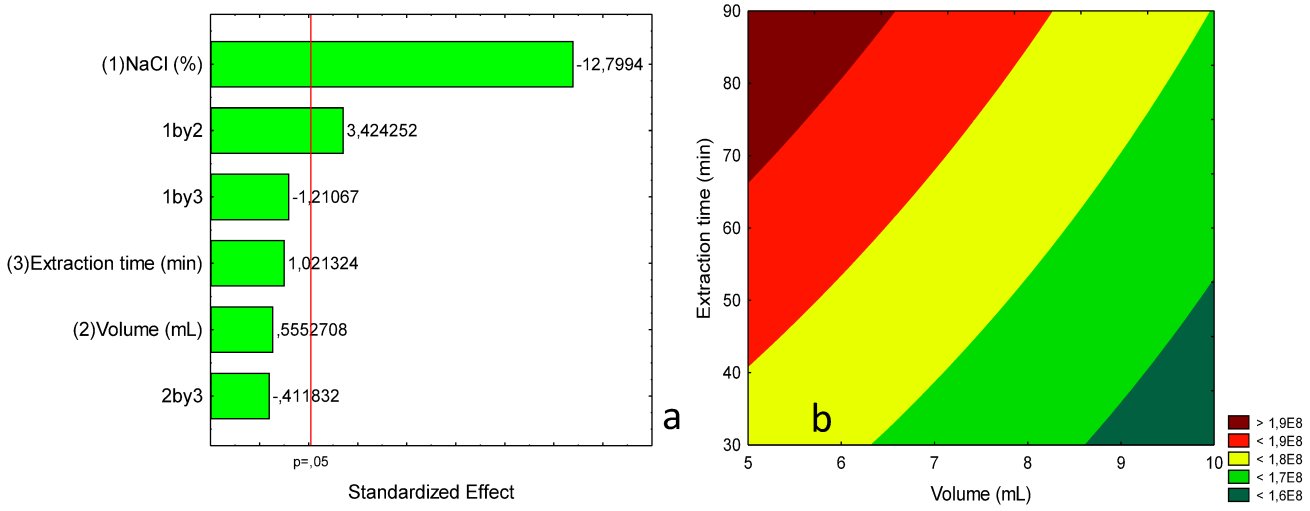


Figure 1. (a) Standardized Pareto chart. (1): addition of NaCl; (2): sample size; (3): extraction time; 1by2, 1by3, 2by3 indicate combinations of the parameters. **(b)** Surface plot for total peak area as function of extraction time and sample volume. NaCl (%) was set as 0 % w/w.

In gas chromatography, salting out effect is widely exploited to improve the extraction efficiency in the headspace (Yang & Peppard, 1994). Addition of monovalent and bivalent salts increases the ionic strength of the solution with a consequent decrease of solubility of those non-polar compounds, which tend to be pushed towards the headspace (Fiorini, Pacetti, Gabbianelli, Gabrielli, & Ballini, 2015). The salting out effect has been previously employed for better extraction of volatiles from milk (González-Córdova & Vallejo-Cordoba,

2001; Marsili, 1999). González-Córdova & Vallejo-Cordoba (2001) reported higher levels of acids extraction depending on the solubility of the compounds. The finding was in line with our results: when 90 min of extraction time was applied, the addition of 30% w/w NaCl in the samples ended up in an increase of octanoic acid detection up to 92%. Also decanoic acid extraction increased but at lower extent, due to its relative lower solubility in water compared to octanoic acid (**Supplementary Figure 1**). Nevertheless, VOCs with different polarity behaved differently in response to the HS-SPME conditions, aspect particularly relevant when performing VOCs profiling analysis. For examples, in our study the addition of salts suppressed the extraction of some compounds of interest for UHT HLMs, such as ketones and DMDS.

Methyl ketones and DMDS give a strong contribution to the overall sensory profile of UHT milk (Valero, Villamiel, Miralles, Sanz, & Martínez-Castro, 2001) and, therefore, an adequate sensitivity of the extraction step is necessary. In particular, DMDS underlies secondary pathways of Maillard reaction, which occur more extensively in UHT HLM than regular UHT milk. Thus, for further investigation of the optimum, salt was removed from the experimental design before constructing the surface plot (**Figure 1b**). Our approach differed from some previous publications substantially by the way how the impact of the VOCs on the milk aroma was considered. Authors working on similar products (e.g. regular pasteurized and UHT milk) preferred an optimization of the SPME performance focused only on a sole class of molecules related to a specific sensory defect. It is an example the work published by González-Córdova & Vallejo-Cordoba (2001) where only short-chain fatty acids extraction was considered. In UHT HLM sensory defects can arise from a multitude of factors, which make it reasonable to extend the investigation of the optimum to a broader range of VOCs. A similar strategy was employed by Vazquez-Landaverde and coworkers (2005) for raw, pasteurized and UHT milk, although the addition of NaCl was not considered in the optimization step of the SPME fiber. In **Figure 1b** The best conditions for the extraction by the HS-SPME fiber are displayed by the dark red region of the plot, which corresponds to a total area count of the peaks higher than 1.9×10^8. Optimal conditions for each class of compounds (ketones, aldehydes, organic acids, phenolic compounds, aromatic compounds, sulfurs and lactones) are reported in **Supplementary Table 2**. All these conditions should be considered valid solely in the tested range when performing a semi-quantitative profile analysis of the VOCs in HLM. Removing salt from the design resulted also in an absence of significant factors in the ranges we considered here, allowing a flexible rearrangement of the conditions. Accordingly, 60 min of extraction time was chosen as best compromise to

align the SPME method with the GC-MS run and to shorten the total time of analysis. Next, the selected conditions (sample size of 5 mL; extraction time of 60 min; 0% w/w NaCl) were applied to evaluate the formation of VOCs in different step of "in batch" production as well as to spot different VOCs profiles in commercial UHT HLMs stored at 20°C for 120 days.

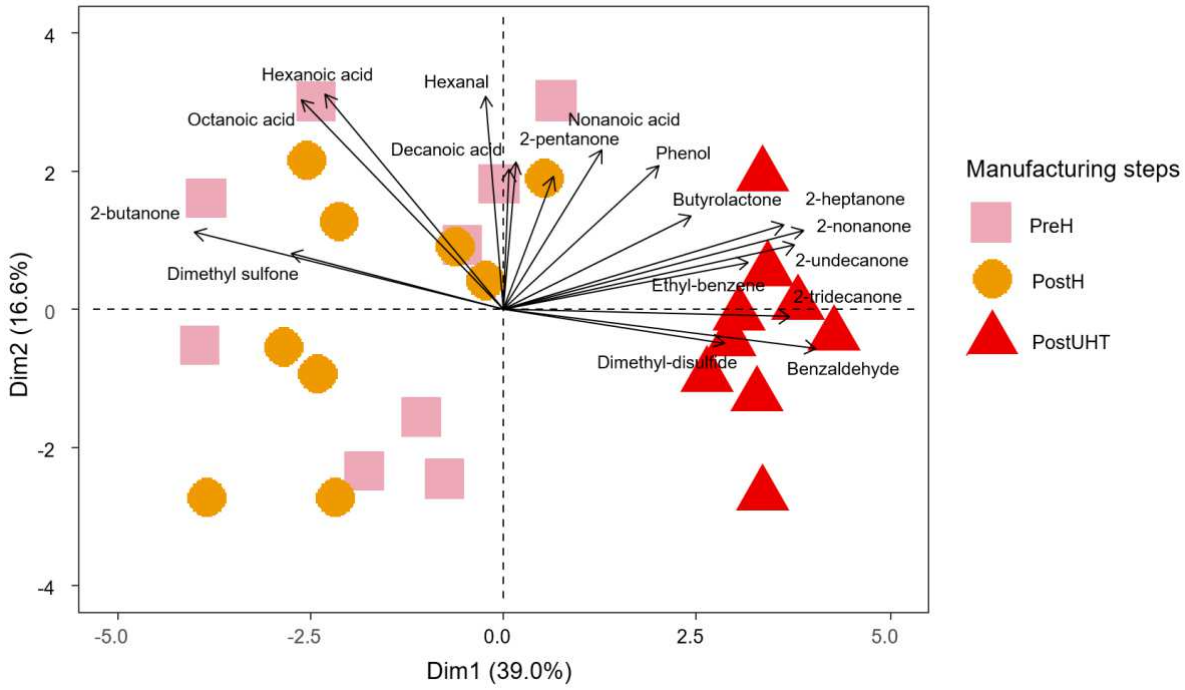


Figure 2. Principal Component Analysis (PCA) bi-plot for the 1st and 2nd component (Dim1 and Dim2), which describe respectively the 39.0% and 16.6% of the total variance. The displayed points represent the three production replicates of HLM samples taken during "in batch" production (preH: before lactase addition; postH: after lactose hydrolysis; postUHT: after the UHT treatment).

Volatiles profile of UHT HLM during "in batch" production system

A total of 17 compounds were identified and semi-quantified from the headspace of the HLM samples taken during "in batch" production (**Table 2**). Principal Component Analysis (PCA) was applied to reveal pattern in the dataset. In **Figure 2**, the bi-plot of the 1st and the 2nd principal component (Dim1 and Dim2, accounting respectively for 39.0% and 16.6% of the total variance) are plotted. The postUHT samples were characterized by positive values for Dim1, which clearly separate them from the rest of the samples. According to the loadings, differences in methyl-ketones concentrations were responsible for the separation of the postUHTs. Such indication was further validated by the analysis of the variance, where most of the methyl-ketones were significantly higher in the postUHTs. An opposite trend

was shown by 2-butanone, which registered lower values after the UHT sterilization. Explanations of the observation might come from the high volatility of the compound resulting in its stripping during degassing of the UHT process (Contarini et al., 1997). Methyl-ketones are a thermal-induced class of compounds, whose formation is related to both β-oxidation of fatty acids and decarboxylation of β-keto acids (Jansson, Clausen, et al., 2014). In this context, our study gave further confirmation about the increase of methyl-ketones in milk upon heating, previously reported by Contarini et al. (1997). Furthermore, focusing on the preH and postH samples, absence of significant differences in the VOCs profiles was reported. Accordingly, VOCs were not produced in significant amounts when lactase was added into the milk and during the hydrolysis of lactose that, according to the "in batch" production protocol, occurs at refrigerated conditions and lasts approximately in one day (Dekker et al., 2019; Troise et al., 2016).

Table 2. Volatile compound profile (µg IS/L) of preH, postH and postUHT in the three consecutive replicates of production from which the samples were collected.

Volatile compound	RI [1]	PreH [3]			PostH			PostUHT		
		(Mean	*±*	*St. Dev)*	*(Mean*	*±*	*St. Dev)*	*(Mean*	*±*	*St. Dev)*
2-butanone [2]	909	13.44[a]	±	4.74	12.92[a]	±	1.96	1.28[b]	±	0.36
2-pentanone	987	0.79	±	0.14	0.68	±	0.11	0.78	±	0.13
Dimethyl-disulfide	1085		nd			nd		0.41[b]	±	0.49
Hexanal	1099	8.76	±	7.66	3.94	±	2.64	3.55	±	3.13
Ethyl-benzene	1131	0.18[a]	±	0.07	0.11[a]	±	0.03	2.02[b]	±	1.33
2-heptanone	1193	4.69[a.b]	±	2.72	4.16[a]	±	2.63	7.32[b]	±	1.78
2-nonanone	1397	1.09[a]	±	0.66	1.18[a]	±	0.83	2.11[b]	±	0.18
Benzaldehyde	1533		nd			nd		0.23[b]	±	0.04
2-undecanone	1607	0.26[a]	±	0.14	0.25[a]	±	0.15	0.43[b]	±	0.11
Butyrolactone	1637	0.15	±	0.03	0.14	±	0.03	0.16	±	0.02
2-tridecanone	1819	0.02[a]	±	0.02	0.02[a]	±	0.02	0.09[b]	±	0.03
Hexanoic acid	1900	1.12[a]	±	0.40	1.06[a]	±	0.39	0.63[b]	±	0.22
Dimethyl sulfone	1910	2.96[a]	±	0.64	3.02[a]	±	1.66	1.57[b]	±	0.34
Phenol	2020	0.24	±	0.05	0.23	±	0.06	0.26	±	0.04
Octanoic acid	2144	1.75[a]	±	0.68	1.75[a]	±	0.44	1.08[b]	±	0.33
Nonanoic acid	2215	0.18	±	0.22	0.19	±	0.16	0.36	±	0.40
Decanoic acid	2307	0.89	±	0.28	0.92	±	0.33	1.03	±	0.37

[a-b] Means within a row with different superscript differ significanlty ($P < 0.05$)
[1] Linear retention index.
[2] Compounds tentatively identified matching the NIST-2014/Wiley 7.0 libraries.
[3] The headings indicate the production steps at which the milk was sampled along the "in batch" production system, namely before lactose hydrolysis (preH), after lactose hydrolysis (postH) and after UHT treatment (postUHT).

Volatiles profile during storage of commercial HLMs at 20°C

HS-SPME/GC-MS analysis was further conducted on three different commercial samples stored for 120 days at 20°C and sampled every 30 days. In total, 28 VOCs compounds were identified. The range of concentration (expressed as equivalent of internal standard) was between <0.01 and 14.85 µg/L milk and the profiles were similar to those previously determined by other authors working on the topic (Jansson, Jensen, Eggers, et al., 2014; Troise et al., 2016). Principal Component Analysis (PCA) was again performed. The first three principal components (PC1, PC2 and PC3) explained the 51.50% of the total variance and the best visualization was achieved plotting the PC1 and the PC2 (**Figure 3)**. Separation of the three hydrolyzed-lactose milks was observed (**Figure 3b**) and different concentrations of ketones, aldehydes and organic acids seemed to be the main responsible for the separation (**Figure 3a**). HLM3 was well described by 2-pentanone, 2-heptanone, 2-nonanone, 2-undecanone, 2-tridecanone, benzaldehyde and hexanal, whereas 2-methylbutanal, butanoic acid, hexanoic acid, octanoic acid, decanoic acid, ethyl-benzene and 2-propanone differentiated HLM2 from the other two commercial milk. The different technology employed for the manufacturing ("in batch" for HLM1 and HLM2, "in pack" for HLM3) can partially explain the results.

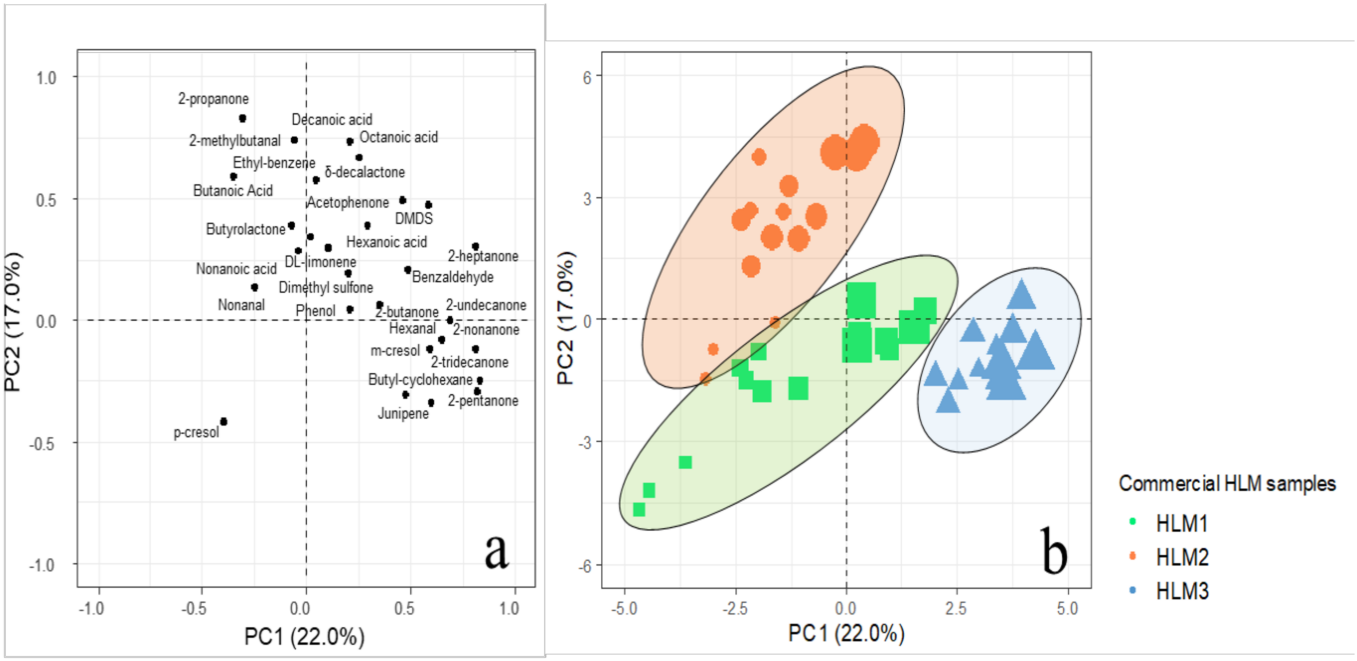


Figure 3. (a) Principal Component Analysis (PCA) loading plot for the 1st and 2nd component, which describe respectively the 22.0% and 17.0% of the total variance. **(b)** Corresponding score plot. The displayed points represent three measurements performed on the commercial samples (HLM1, HLM2, HLM3) during storage at 20°C. The progressive increase in dots size indicates the proceeding of the storage (0, 30, 60, 90, 120 days).

Some information about the analyzed UHT HLMs were however unavaiable. For example, we did not know the type and the purity of the lactase preparation used by suppliers. Bioscience companies sell lactases having different purity, intended as level of residual proteolytic activity and (in some cases) residual arysulfatase activity in the enzymatic preparation. Lactase purity was previously reviewed as crucial for defining the quality and the stability of UHT HLM (Nielsen et al., 2018; Troise et al., 2016). In particular, Troise and coworkers (2016) emphatized the importance of this aspect when performing "in pack" production. Although a direct comparison of the "in batch" and "in pack" production system has not been published yet, researchers believe that the quality of "in batch" UHT HLM is poorly dependent from the purity of lactase. Interestingly, HLM1 and HLM2 were clearly separated in **Figure 3b**, although both were produced by "in batch" system. In this case, the different packaging material (paperboard carton for HLM1 and HDPE for HLM2) may have contributed to the pattern. Overall, the different VOCs profiles of HLM1, HLM2 and HLM3 showed by the explorative PCA were the results of several variables (e.g. production system, lactase purity and packaging). Nevertheless, we can conclude that the chosen SPME conditions were appropriate for a sensitive detection of the VOCs independently from the sources of the variation.

Table 3. Volatile compound profile (µg IS/L) of HLM1. HLM2 and HLM3 as a function of storage time at 20°C.

Volatile compounds	RI[1]	HLM1					Trend[5]	HLM2					Trend	HLM3					Trend
		0[4]	30	60	90	120		0	30	60	90	120		0	30	60	90	120	
Ketones																			
2-propanone[2]	822	nd[3]	nd	nd	nd	nd		nd^{a}	5.29^{b}	7.23^{c}	3.82^{d}	3.94^{d}	↑	nm	nd	nd	nd	nd	
2-butanone	909	3.78^{a}	nd^{b}	1.86^{c}	4.77^{d}	3.92^{a}		2.84^{a}	2.36^{a}	1.09^{b}	3.26^{a}	2.95^{a}		nm	6.01^{a}	4.74^{a,b}	3.70^{b}	5.74^{a}	
2-pentanone	987	2.99	2.39	2.88	2.45	3.27		0.47^{a}	1.34^{b}	1.33^{b}	1.72^{b}	2.95^{c}	↑	nm	2.33^{a}	2.89^{a}	5.87^{b}	7.85^{c}	↑
2-heptanone	1193	2.44^{a}	5.65^{b}	7.63^{b}	7.84^{b}	10.14^{c}	↑	3.38^{a}	6.93^{b}	7.11^{b}	8.57^{b}	9.61^{b}	↑	nm	8.07^{a}	11.05^{b}	13.61^{b,c}	14.85^{c}	↑
2-nonanone	1397	1.17	1.09	1.67	2.45	2.17		1.10	1.14	1.13	1.73	1.49		nm	1.85	2.10	2.60	2.35	
2-undecanone	1607	0.22^{a}	0.23^{a}	0.33^{a}	0.38^{b}	0.33^{a}		0.25^{a}	0.25^{a}	0.26^{a}	0.39^{b}	0.31^{a}		nm	0.37	0.34	1.25	0.42	
Acetophenone	1660	nd^{a}	0.23^{b}	nd^{a}	nd^{a}	0.61^{c}	↑	nd^{a}	0.37^{b}	nd^{a}	nd^{a}	0.16^{b}		nm	nd^{a}	nd^{a}	0.74^{b}	nd^{a}	
2-tridecanone	1819	nd	nd	nd	nd	nd		nd	nd	nd	nd	nd		nm	0.10^{a}	0.14^{a}	1.07^{b}	0.57^{b}	↑
Aldehydes																			
2-methylbutanal	914	nd	nd	nd	nd	nd		nd^{a}	0.16^{b}	0.17^{b}	0.20^{b}	0.22^{b}	↑	nm	nd^{a}	nd^{a}	nd^{a}	0.15^{b}	↑
Hexanal	1099	1.10	1.83	0.89	1.44	2.54		1.49	1.63	0.98	0.99	1.73		nm	1.70	2.30	2.66	3.27	
Nonanal	1403	0.88	0.94	1.25	0.71	0.70		0.67	0.82	0.98	1.21	1.13		nm	nd^{a}	1.42^{b}	1.44^{b}	1.59^{b}	↑
Benzaldehyde	1533	0.46^{a}	0.90^{a}	1.05^{a}	1.44^{b}	1.04^{a}		0.65	1.23	0.61	1.19	0.98		nm	0.94	1.16	1.13	1.28	
Sulfides																			
Dimethyldisulfide	1085	nd	0.02	0.02	0.04	0.04		0.01	0.02	0.01	0.04	0.04		nm	0.03	0.03	0.02	0.03	
Dimethylsulfone	1910	0.96^{a}	0.60^{a}	0.90^{a}	1.46^{b}	0.87^{a}		1.41	0.86	1.92	0.84	1.89		nm	2.29	1.34	0.99	2.18	

[a-d] Means within within each commercial UHT HLM samples with different superscript differ significanlty ($P < 0.05$).
[1] Linear retention index.
[2] Compounds tentatively identified matching the NIST-2014/Wiley 7.0 libraries.
[3] Values reported as mean of three measurements (n=3); nd=not detected; nm=not measured.
[4] Days of storage at 20°C in climate chamber under controlled conditions.
[5] Trends as significance ($P<0.05$) between either 0 days (for HLM1, HLM2) or 30 days (for HLM3) and 120 days of storage; ↑= increase, ↓ = decrease.

Table 3. (continued)

Volatile compounds	RI[1]	HLM1					Trend[5]	HLM2					Trend	HLM3					Trend
		0[4]	30	60	90	120		0	30	60	90	120		0	30	60	90	120	
Carboxylic acids																			
Butanoic acid[2]	1689	nd[3]	nd	nd	nd	nd		nd[a]	0.21[a]	0.60[b]	0.19[a]	nd[a]		nm	nd	nd	nd	nd	
Hexanoic acid	1900	0.35	0.34	0.87	0.72	0.35		0.72	0.92	1.27	0.64	0.78		nm	0.80	1.00	1.90	0.51	
Octanoic acid	2144	0.69[a]	0.93[a,b]	0.85[a]	1.75[b]	0.98[a,b]	↑	1.38	2.10	1.64	1.39	1.55		nm	1.12	1.50	1.85	1.04	
Nonanoic acid	2215	0.69	0.96	1.09	1.20	0.92		1.57	1.41	0.48	0.83	1.30		nm	0.93	0.85	0.98	0.49	
Decanoic acid	2307	0.41[a]	0.63[a,b]	0.79[b]	0.77[b]	1.21[c]	↑	0.68	0.98	1.10	1.04	2.09		nm	0.86	0.74	0.65	0.86	
Others																			
Butyl-cyclohexane	1081	nd	nd	nd	nd	nd		nd	nd	nd	nd	nd		nm	0.72	0.79	0.91	0.93	
Ethyl-benzene	1136	nd	nd	nd	nd	nd		nd[a]	nd[a]	nd[a]	nd[a]	0.90[b]	↑	nm	nd	nd	nd	nd	
DL-limonene	1210	nd	nd	nd	4.46	0.26		nd[a]	nd[a]	nd[a]	nd[a]	2.42[b]	↑	nm	nd	nd	nd	nd	
Junipene	1568	nd[a]	0.22[b]	0.23[b]	0.25[b]	0.23[b]		nd	nd	nd	nd	nd		nm	0.91[a]	nd[b]	0.69[a]	0.93[a]	
Butyrolactone	1637	0.33[a]	1.41[b]	0.39[a]	0.42[a]	0.88[a]		0.40[a]	1.89[b]	0.53[a]	0.39[a]	0.68[a]		nm	0.63	0.42	0.99	0.33	
Phenol	2020	0.21	0.17	0.22	0.23	0.18		0.19	0.23	0.20	0.17	0.22		nm	0.30[a]	0.26[a]	0.15[b]	0.23[a]	
p-Cresol	2095	0.10	0.06	0.12	0.11	0.08		0.09[a]	0.08[a]	nd[b]	nd[b]	nd[b]	↓	nm	nd[a]	0.12[b]	nd[a]	nd[a]	
m-Cresol	2104	0.47	0.29	0.47	0.50	0.39		0.37	0.40	0.42	0.41	0.45		nm	0.60	0.55	0.45	0.56	
δ-Decalactone	2199	nd[a]	0.15[b]	0.12[b]	0.09[b]	0.10[b]		0.10[a]	0.23[b]	0.13[a]	0.07[a]	0.08[a]		nm	0.24[a]	0.18[a]	0.13[a]	0.05[b]	↓

[a-d] Means within within each commercial UHT HLM samples with different superscript differ significanlty ($P < 0.05$).
[1] Linear retention index.
[2] Compounds tentatively identified matching the NIST-2014/Wiley 7.0 libraries.
[3] Values reported as mean of three measurements (n=3); nd=not detected; nm=not measured.
[4] Days of storage at 20°C in climate chamber under controlled conditions.
[5] Trends as significance ($P<0.05$) between either 0 days (for HLM1, HLM2) or 30 days (for HLM3) and 120 days of storage; ↑= increase, ↓ = decrease

Table 3 reports the quantification of the identified VOCs: results are semi-quantitative and must be considered to highlight trends (increase/decrease) in the volatile profiles. The results showed temporal changes of some VOCs during storage (**Figure 4)**. As previously reported for UHT milk, ketones were the most abundant class in terms of number of identified compounds (Dursun, Güler, & Şekerli, 2017). The increase of methyl-ketones in UHT HLM was previously associated with "cooked taste" and "stale flavor" (Troise et al., 2016). In our study, a significant increase of methyl-ketones concentration over time was registered in line with previous literature (Jansson, Clausen, et al., 2014). 2-Heptanone was present at the highest concentrations, ranging from 9.61 µg IS/L to 14.85 µg IS/L at the end of the storage depending on the sample. Moreover, the results showed different concentrations of methyl-ketones in the three commercial samples at the different time of measurement. Remarkably, HLM3 (the sample produced by "in pack" production system) was the commercial hydrolyzed-lactose milk significantly richer in 2-butanone (**Figure 4a**), 2-pentanone (**Figure 4b**) and 2-heptanone (**Figure 4c**) after 120 days of storage.

Lactones tended to decrease during storage, for all three samples. The results of the ANOVA showed a significant temporal decrease of δ-decalactone between 30 and 120 days of storage in HLM3. Reasonably, the formation of lactones is considered an index of the heating applied during processing, and the decrease of δ-decalactone during storage was expected because of the polymerization during milk storage (Loney & Bassette, 1971). Some aldehydes increased during storage, while others did not. The concentration of benzaldehyde remained constant in all the three samples, in agreement with previous reports (Jansson, Jensen, Sundekilde, et al., 2014; Troise et al., 2016). 2-Methylbutanal followed particular trends depending on the commercial HLM sample. It is important to mention that, with the polar phase we used for the chromatographic separation, 2-methylbutanal and 3-methylbutanal peaks are not completely resolved (their retention indices are 914±8 and 918±7 respectively according to MS-NIST library). Consequently, external standards were injected to distinguish the two peaks. This analysis confirmed the presence of solely the 2-methylbutanal. As illustrated in **Figure 4d**, in HLM1 2-methylbutanal was not detected throughout the storage. Differently, it apperead in HLM3 after 120 days, while its tendency in HLM2 was to progressively increase over time. While the trend of the 2-methylbutanal in HLM3 (produced by "in pack" system and packed in paperboard carton) was previously reported (Troise et al., 2016) on other commercial samples

manufactured with the same method, the presence of 2-methylbutanal already after 30 days of storage in HLM2 (produced by "in batch" system and packed in HDPE) was difficult to interpret. A possible explanation could be the milk variability existing between the tested commercial samples. Dimethyl disulfide (DMDS) was detected in low concentration and at comparable level in all the three HLMs. The absence of significant changes in concentration of dimethyl sulfone, which can be originated from the oxidation of dimethyl disulfide, confirmed the results (Al-Attabi, D'Arcy, & Deeth, 2009). The results are in agreement with Jansson et al. (2017). Volatile sulfur compounds contribute to cooked/sulfurous flavor of UHT milk but their estimation is extremely challenging due to low concentration and high volatility (Al-attabi et al., 2014). The three mentioned aldehydes (benzaldehyde, 2-methylbutanal and DMDS) are compounds formed by the Strecker degradation, a reaction pathway previously associated to the proteolytic side activity of the lactase in the milk during storage (Jansson et al., 2019; Troise et al., 2016). As Maillard reaction creates the precursors (Rizzi, 1999), the detection of these compounds might indicate its progress in the analyzed samples. Benzaldehyde is formed by oxidation of phenylacetaldehyde (a Strecker aldehyde coming from phenylalanine), 2-methylbutanal is a degradation product of isoleucine, whereas DMDS is produced from methionine (Al-attabi et al., 2014; Fong & Yaylayan, 2008; Jansson et al., 2017). In this context, the different evolution of in 2-methylbutanal in the commercial HLMs during storage further indicated that the applied HS SPME methodology was capable to unreveal reaction pathways important for the definition of the final sensory quality of hydrolyzed-lactose milk.

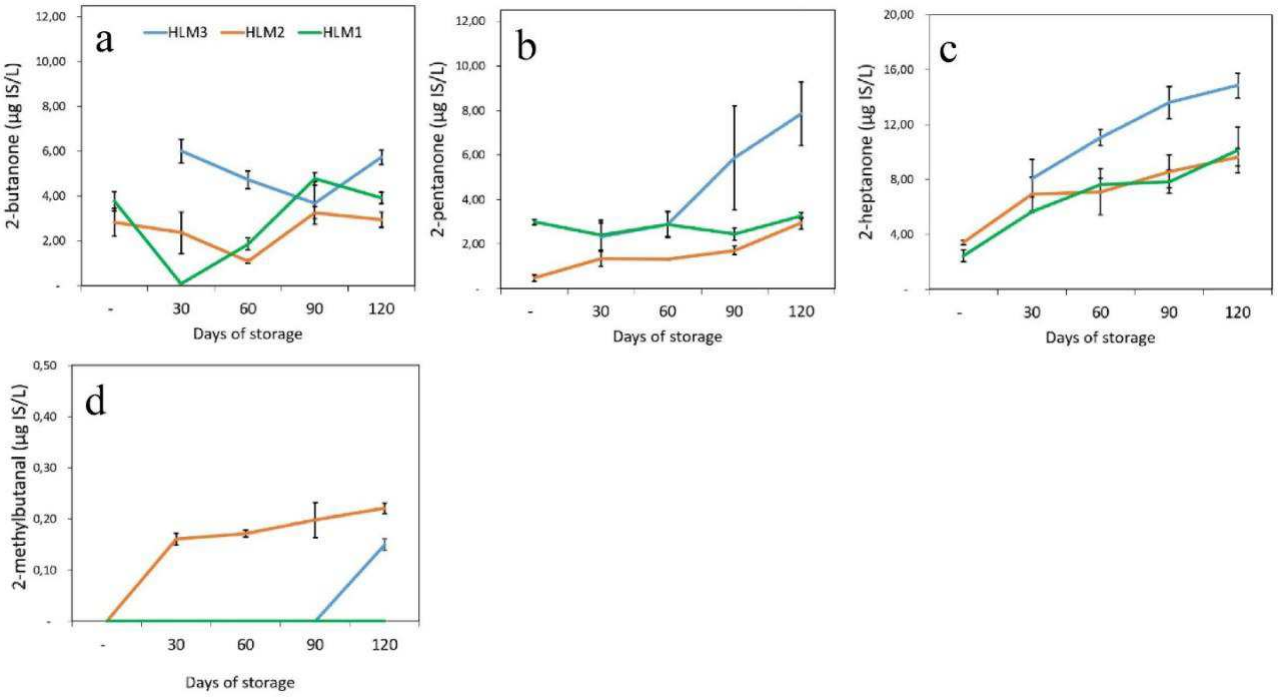


Figure 4. HS SPME/GC-MS results of **(a)** 2-butanone, **(b)** 2-pentanone, **(c)** 2-heptanone, **(d)** 2-methylbutanal in the three commercial UHT HLM analyzed (HLM1, HLM2, HLM3) during storage at 20°C for 120 days.

Conclusions

The present study supported the application of the HS-SPME/GC-MS technique as handy and efficient tool for extracting volatiles from HLMs in different scenarios. Applying a 2^3 full factorial design, optimized SPME conditions were selected and successfully applied to explore possible differences in the volatile profiles of three commercial UHT HLM, as well as among three typical steps of HLM manufacturing. Since UHT HLM manufactured for this study were produced under controlled processing conditions the observed differences can be attributed to specific factors. HLMs were discriminated based on their relative VOCs content both during production and storage. Hydrolysis of lactose during "in batch" production did not affect the product profile significantly, while most of the volatiles were formed after UHT treatment. This result is of particular interest if we think that backside enzymatic activity may not be the only unwanted characteristic of the commercial lactase. Lactase preparations contain also different levels of volatiles compounds (unpublished data). Interestingly, the results showed that when lactase was added into the milk tank, any volatile possibly present in the preparation was covered by the "volatilome" of the milk, probably due to a dilution effect. Because of a lack of technical information about the lactase preparation employed, the discussion of this particular aspect is however limited. Instead, the sources determining the differentiation of the three commercial HLMs over storage were several. Looking at the intensity and the temporal evolution of specific compounds, stronger development of VOCs occurred in HLM3, which was produced by the "in pack" system. Nevertheless, additional variables (e.g. lactase purity, packaging, batch variability) may have participated to the encountered results and deserves more attention in future studies. Overall, HS-SPME provided excellent extraction performance. Semi-quantitative analysis highlighted methyl-ketones and Strecker aldehydes as markers differentiating the commercial UHT HLM samples. Eventually, following the changes in the VOCs profile of UHT HLM during production and shelf-life was proved useful to investigate the reaction pathways underlining the final product quality.

References

Al-attabi, Z., Bruce, D., Deeth, H., Al-attabi, Z., Bruce, D., & Deeth, H. (2014). Volatile sulfur compounds in pasteurised and UHT milk during storage. *Dairy Science and Technology*, *94*, 241–253. https://doi.org/10.1007/s13594-013-0157-y

Al-Attabi, Z., D'Arcy, B. R., & Deeth, H. C. (2009). Volatile sulphur compounds in UHT milk. *Critical Reviews in Food Science and Nutrition*, *49*(1), 28–47. https://doi.org/10.1080/10408390701764187

Aprea, E., Romanzin, A., Corazzin, M., Favotto, S., Betta, E., Gasperi, F., & Bovolenta, S. (2016). Effects of grazing cow diet on volatile compounds as well as physicochemical and sensory characteristics of 12-month-ripened Montasio cheese. *Journal of Dairy Science*, *99*(8), 6180–6190. https://doi.org/10.3168/JDS.2016-10929

Aprea, Eugenio, Gasperi, F., Betta, E., Sani, G., & Cantini, C. (2018). Variability in volatile compounds from lipoxygenase pathway in extra virgin olive oils from tuscan olive germoplasm by quantitative SPME/GC-MS. *Journal of Mass Spectrometry*, *53*(9), 824–832. https://doi.org/10.1002/jms.4274

Aprea, Eugenio, Gika, H., Carlin, S., Theodoridis, G., Vrhovsek, U., & Mattivi, F. (2011). Metabolite profiling on apple volatile content based on solid phase microextraction and gas-chromatography time of flight mass spectrometry. *Journal of Chromatography A*, *1218*(28), 4517–4524. https://doi.org/10.1016/j.chroma.2011.05.019

Bergamaschi, M., Aprea, E., Betta, E., Biasioli, F., Cipolat-Gotet, C., Cecchinato, A., … Gasperi, F. (2015). Effects of dairy system, herd within dairy system, and individual cow characteristics on the volatile organic compound profile of ripened model cheeses. *Journal of Dairy Science*, *98*(4), 2183–2196. https://doi.org/10.3168/jds.2014-8807

Condurso, C., Verzera, A., Romeo, V., Ziino, M., & Conte, F. (2008). Solid-phase microextraction and gas chromatography mass spectrometry analysis of dairy product volatiles for the determination of shelf-life. *International Dairy Journal*, *18*(8), 819–825. https://doi.org/10.1016/j.idairyj.2007.12.005

Contarini, G., Povolo, M., Leardi, R., Toppino, P. M., Lattiero-caseario, I. S., Lombardo, V., & Salerno, V. B. (1997). Influence of Heat Treatment on the Volatile Compounds of Milk. *Journal of Agricultural and Food Chemistry*, *45*(8), 3171–3177. https://doi.org/10.1021/jf960849s

Corgneau, M., Scher, J., Ritie-Pertusa, L., Le, D. t. l., Petit, J., Nikolova, Y., … Gaiani, C. (2017). Recent advances on lactose intolerance: Tolerance thresholds and currently available answers. *Critical Reviews in Food Science and Nutrition*, *57*(15), 3344–3356. https://doi.org/10.1080/10408398.2015.1123671

Dekker, P. J. T., Koenders, D., & Bruins, M. J. (2019). Lactose-Free Dairy Products: Market Developments, Production, Nutrition and Health Benefits. *Nutrients*, *11*(3), 551. https://doi.org/10.3390/nu11030551

Dursun, A., Güler, Z., & Şekerli, Y. E. (2017). Characterization of volatile compounds and organic acids in ultra-high-temperature milk packaged in tetra brik cartons. *International Journal of Food Properties*, *2912*. https://doi.org/10.1080/10942912.2016.1213280

Endrizzi, I., Fabris, A., Biasioli, F., Aprea, E., Franciosi, E., Poznanski, E., … Gasperi, F. (2012). The effect of milk collection and storage conditions on the final quality of Trentingrana cheese: Sensory and instrumental evaluation. *International Dairy Journal*, *23*(2), 105–114. https://doi.org/10.1016/j.idairyj.2011.10.004

Evangelisti, F., Calcagno, C., Nardi, S., & Zunin, P. (1999). Deterioration of protein fraction by Maillard reaction in dietetic milks. *Journal of Dairy Research*, *66*(2), 237–243. https://doi.org/10.1017/S0022029999003453

Fiorini, D., Pacetti, D., Gabbianelli, R., Gabrielli, S., & Ballini, R. (2015). A salting out system for improving the efficiency of the headspace solid-phase microextraction of short and medium chain free fatty acids. *Journal of Chromatography A*, *1409*, 282–287. https://doi.org/10.1016/j.chroma.2015.07.051

Fong, L. C., & Yaylayan, V. A. (2008). Model studies on the oxygen-induced formation of benzaldehyde from phenylacetaldehyde using pyrolysis GC-MS and FTIR. *Journal of Agricultural and Food Chemistry*, *56*(22), 10697–10704. https://doi.org/10.1021/jf8022468

González-Córdova, A. F., & Vallejo-Cordoba, B. (2001). Quantitative determination of short-chain free fatty acids in milk using solid-phase microextraction and gas chromatography. *Journal of Agricultural and Food Chemistry*, *49*(10), 4603–4608. https://doi.org/10.1021/jf010108d

Harju, M., Kallioinen, H., & Tossavainen, O. (2012, February). Lactose hydrolysis and other conversions in dairy products: Technological aspects. *International Dairy Journal*. https://doi.org/10.1016/j.idairyj.2011.09.011

Ingram, C. J. E., Mulcare, C. A., Itan, Y., Thomas, M. G., & Swallow, D. M. (2009). Lactose digestion and the evolutionary genetics of lactase persistence. *Human Genetics*. https://doi.org/10.1007/s00439-008-0593-6

Jansson, T., Clausen, M. R., Sundekilde, U. K., Eggers, N., Nyegaard, S., Larsen, L. B., … Bertram, H. C. (2014). Lactose-hydrolyzed milk is more prone to chemical changes during storage than conventional ultra-high-temperature (UHT) milk. *Journal of Agricultural and Food Chemistry*, *62*(31), 7886–7896. https://doi.org/10.1021/jf501671z

Jansson, T., Jensen, H. B., Sundekilde, U. K., Clausen, M. R., Eggers, N., Larsen, L. B., … Bertram, H. C. (2014). Chemical and proteolysis-derived changes during long-term storage of lactose-hydrolyzed ultrahigh-temperature (UHT) milk. *Journal of Agricultural and Food Chemistry*, *62*(46), 11270–11278. https://doi.org/10.1021/jf504104q

Jansson, T., Jensen, S., Eggers, N., Clausen, M. R., Larsen, L. B., Ray, C., … Bertram, H. C. (2014). Volatile component profiles of conventional and lactose-hydrolyzed UHT milk - A dynamic headspace gas chromatography-mass spectrometry study. *Dairy Science and Technology*, *94*(4), 311–325. https://doi.org/10.1007/s13594-014-0164-7

Jansson, T., Rauh, V., Danielsen, B. P., Poojary, M. M., Waehrens, S. S., Bredie, W. L. P., … Lund, M. N. (2017). Green Tea Polyphenols Decrease Strecker Aldehydes and Bind to Proteins in Lactose-Hydrolyzed UHT Milk. *Journal of Agricultural and Food Chemistry*, *65*(48), 10550–10561. https://doi.org/10.1021/acs.jafc.7b04137

Jansson, T., Waehrens, S. S., Rauh, V., Danielsen, B. P., Sørensen, J., Bredie, W. L. P., … Lund, M. N. (2019). Effect of green tea catechins on physical stability and sensory quality of lactose-reduced UHT milk during storage for one year. *International Dairy Journal*, *95*, 25–34. https://doi.org/10.1016/j.idairyj.2019.03.007

Jelen, P., & Tossavainen, O. (2003). Low lactose and lactose-free milk and dairy products - Prospects, technologies and applications. *Australian Journal of Dairy Technology*, *58*(2), 161–165. https://doi.org/http://www.scopus.com/inward/record.url?eid=2-s2.0-0141595866&partnerID=tZOtx3y1

Leonardi, M., Gerbault, P., Thomas, M. G., & Burger, J. (2012, February). The evolution of lactase persistence in Europe. A synthesis of archaeological and genetic evidence. *International Dairy Journal*. https://doi.org/10.1016/j.idairyj.2011.10.010

Loney, B. E., & Bassette, R. (1971). Changes in Free Fatty Acids and Lactones in Sterile

Concentrated Milk During Storage. *Journal of Dairy Science*, *54*(3), 343–348. https://doi.org/10.3168/jds.S0022-0302(71)85841-1

Marsili, R. T. (1999). SPME - MS - MVA as an Electronic Nose for the Study of Off-Flavors in Milk. *Journal of Agricultural and Food Chemistry*, 648–654. https://doi.org/10.1021/jf9807925

Nielsen, S. D., Zhao, D., Le, T. T., Rauh, V., Sørensen, J., Andersen, H. J., & Larsen, L. B. (2018). Proteolytic side-activity of lactase preparations. *International Dairy Journal*, *78*, 159–168. https://doi.org/10.1016/j.idairyj.2017.12.001

Povolo, M., & Contarini, G. (2003). *Comparison of solid-phase microextraction and purge-and-trap methods for the analysis of the volatile fraction of butter*. *Journal of Chromatography A* (Vol. 985). Retrieved from www.elsevier.com/locate/chroma

Rizzi, G. P. (1999). The Strecker degradation and its contibution to food flavour. *Flavor Chemistry*, 335–342.

Tossavainen, O., & Kallioinen, H. (2007). Proteolytic changes in lactose hydrolysed UHT milks during storage. *Milchwissenschaft*, *62*(4), 410–415. Retrieved from http://cat.inist.fr/?aModele=afficheN&cpsidt=19123002

Troise, A. D., Bandini, E., De Donno, R., Meijer, G., Trezzi, M., & Fogliano, V. (2016). The quality of low lactose milk is affected by the side proteolytic activity of the lactase used in the production process. *Food Research International*, *89*, 514–525. https://doi.org/10.1016/j.foodres.2016.08.021

Valero, E., Villamiel, M., Miralles, B., Sanz, J., & Martínez-Castro, I. (2001). Changes in flavour and volatile components during storage of whole and skimmed UHT milk. *Food Chemistry*, *72*(1), 51–58. https://doi.org/10.1016/S0308-8146(00)00203-X

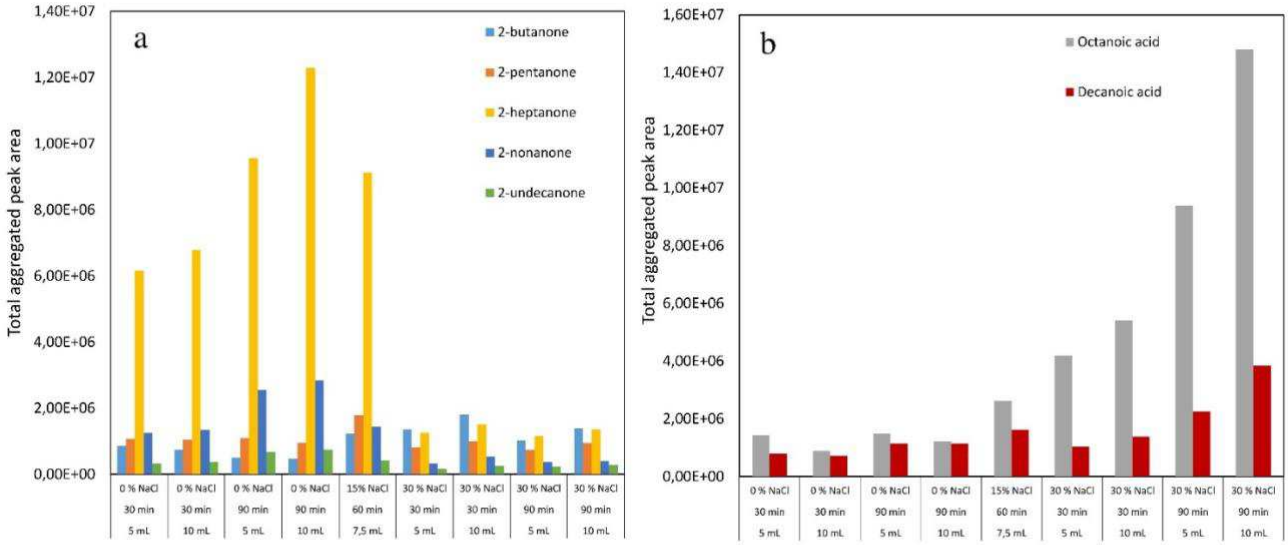
Vazquez-Landaverde, P. A., Velazquez, G., Torres, J. A., & Qian, M. C. (2005). Quantitative determination of thermally derived off-flavor compounds in milk using solid-phase microextraction and gas chromatography. *Journal of Dairy Science*, *88*(11), 3764–3772. https://doi.org/10.3168/jds.S0022-0302(05)73062-9

Yang, X., & Peppard, T. (1994). Solid-Phase Microextraction for Flavor Analysis. *Journal of Agricultural and Food Chemistry*, 1925–1930. https://doi.org/10.1021/jf00045a018

Supplementary material

Supplementary Table 1. Inter-day and intra-day repeatability of the developed HS SPME/GC-MS methodology. Coefficients of variation (CV) are expressed as percentage (%) and were calculated dividing the standard deviation by the mean of each peak quantified as µg of IS/L milk.

Volatile compounds	CV (%) inter-day	CV (%) intra-day
2-butanone	8%	5%
2-methylbutanal	7%	16%
2-pentanone	2%	5%
Dimethyldisulfide	47%	48%
Hexanal	4%	20%
Ethyll-benzene	29%	8%
2-heptanone	6%	3%
DL-limonene	19%	16%
2-nonanone	5%	2%
Nonanal	12%	13%
Benzaldehyde	37%	9%
Junipene	42%	18%
2-undecanone	8%	7%
Butyrolactone	34%	10%
Aceto-phenone	28%	12%
2-tridecanone	15%	11%
Dimethyl-sulfone	39%	54%
Hexanoic acid	24%	31%
p-cresol	17%	15%
m-cresol	17%	9%
Octanoic acid	21%	20%
δ-decalactone	15%	16%
Decanoic acid	20%	24%



Supplementary Figure 1. Effect of NaCl addition (0-30% w/w), sample size (5-10 mL) and extraction time (30-90 min) on the extraction performance of **(a)** methyl-ketones and **(b)** octanoic and decanoic acid by the SPME fiber.

Supplementary Table 2. Best conditions for the extraction by the HS-SPME fiber reported both for each class of compounds and for each VOCs detected during the optimization of the extraction parameters. The proposed conditions should be considered the tested range only when a semi-quantification of the VOCs profile in the HLM headspace is performed.

Ketones	**2-butanone**	**2-pentanone**	**2-heptanone**	**2-nonanone**	**2-undecanone**
NaCl = 0 %	NaCl = 0 %	NaCl = 15 %	NaCl = 0 %	NaCl = 0 %	NaCl = 0 %
Volume = 10 mL	Volume = 10 mL	Volume = 7,5 mL	Volume = 10 mL	Volume = 10 mL	Volume = 10 mL
Ext. Time = 90 min	Ext. Time = 90 min	Ext. Time = 60 min	Ext. Time = 90 min	Ext. Time = 90 min	Ext. Time = 90 min
Aldehydes	**Hexanal**	**Nonanal**	**Decanal**	**Benzaldehyde**	
NaCl = 30 %	NaCl = 0 %	NaCl = 0 - 30 %	NaCl = 30 %	NaCl = 0-30 mL	
Volume = 10 mL	Volume = 5 mL	Volume = 10 mL	Volume = 10 mL	Volume = 10 mL	
Ext. Time = 30 min	Ext. Time = 90 min	Ext. Time = 90 min	Ext. Time = 30 min	Ext. Time = 30-90 min	
Organic acids	**Butanoic acid**	**Octanoic acid**	**Decanoic acid**		
NaCl = 30 %	NaCl = 30 %	NaCl = 30 %	NaCl = 30 %		
Volume = 10 mL	Volume = 10 mL	Volume = 10 mL	Volume = 10 mL		
Ext. Time = 90 min	Ext. Time = 90 min	Ext. Time = 90 min	Ext. Time = 90 min		
Phenolic compounds	**Phenol**	**p-cresol**	**m-Cresol**		
NaCl = 0-30 %	NaCl = 0-30 %	NaCl = 0-30 %	NaCl = 0-30 %		
Volume = 10 mL	Volume = 10 mL	Volume = 10 mL	Volume = 10 mL		
Ext. Time = 30 min	Ext. Time = 30 min	Ext. Time = 30 min	Ext. Time = 30 min		
Aromatic compounds	**Ethylbenzene**	**Xylene**			
NaCl = 0 %	NaCl = 0 %	NaCl = 0 %			
Volume = 5 mL	Volume = 5 mL	Volume = 5-10 mL			
Ext. Time = 90 min	Ext. Time = 90 min	Ext. Time = 90 min			
Sulfurs	**Dimethyldisulfide**	**Dymethyl-sulfone**			
NaCl = 30 %	NaCl = 0 %	NaCl = 30 %			
Volume = 5 mL	Volume = 10 mL	Volume = 10 mL			
Ext. Time = 30 min	Ext. Time = 30 min	Ext. Time = 90 min			
Lactones	**Butyrolactone**	**δ-decalactone**			
NaCl = 0-30 %	NaCl = 0-30 %	NaCl = 0-30 %			
Volume = 10 mL	Volume = 10 mL	Volume = 10 mL			
Ext. Time = 30 min	Ext. Time = 30 min	Ext. Time = 30 min			

Chapter 3: Short-time freezing does not alter the sensory properties and the physical stability of ultra-high-temperature hydrolyzed-lactose milk.

While in the previous chapter the best condition for the HS-SPME/GC-MS methodology were spotted and validated for UHLM, this section was dedicated to optimize the sensory sessions. The sensory panel of a well-known Italian dairy company (made up by people working in the R&D team of the company) was employed. Arranging the sensory tests according to the schedule of each team member is a major challenge. For this reason, freezing the milk for a short period of time was proposed as a solution. From a preliminary literature review, skepticisms around milk freezing prior to sensory analysis arose because, when milk is frozen, several reactions occur at different stages of the freezing process. Most research was focused on the changes after prolonged frozen storage, while there is a lot of confusions concerning the response of milk to short-time freezing. In this context, the researcher decided, as a side project, to investigate the stability of milk when frozen and thawed within 72 hours, filling the gap of knowledge on a phenomenon particularly relevant in dairy science

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Abstract

In this study, the effect of milk freezing was studied, focusing on the changes in 1% and 3% fat ultra-high temperature hydrolyzed-lactose milk (UHLM) after slow-rate freezing (-20°C) and fast-rate freezing (-80°C) for 72 hours. Changes on the sensory properties were first assessed by discriminant analysis (triangle test), and then by volatiles organic compounds (VOCs) analysis and color. The milk emulsion stability was characterized by optical centrifugation, particle size analysis, and confocal microscopy. The sensory panel was not able to discriminate the milk subjected to freezing from the control (72 hours at 20°C). The VOCs and color analysis demonstrated that both freezing rates did not cause any significant changes in the milk aroma and color characteristics. The results of physical properties confirmed that short-time freezing did not lead to a distinct destabilization, except for a slight increase in the mean particle diameter at -80°C. Taking all the results together, UHLM was not significantly altered during the operation of freezing/thawing and, therefore, short-time freezing at both -20°C and -80°C can be used for milk storage without altering the product.

Introduction

Freezing the milk is a common practice in the dairy sector, especially in situations where the milk supply is limited, unstable or affected by the season (Fava, Külkamp-Guerreiro, & Pinto, 2014). For example, freezing was introduced by farmers in Wisconsin to reach sufficient quantities of ovine milk before further processing (Wendorff et al., 2001). Another example is the ewe milk from Greece, which is mostly produced in spring, frozen and used for yoghurt production in summer/autumn (Katsiari, Voutsinas, & Kondyli, 2002). Furthermore, freezing can be applied for quality control (QC) activities in order to have a constant reference milk to verify if the freshly produced one meet certain quality standards. From a research perspective, freezing the milk is also particularly relevant to increase the flexibility and reproducibility of the experiments: different milk samples can be stored until analysis, so measurements can be run all together at once along the same sample set. Nevertheless, researchers and experts in dairy industry expressed skepticism about this practice, considering the possible adverse effects of freezing on milk stability (Needs, 1992).

When milk is frozen, several reactions occur and it is important to distinguish those linked to the final freezing temperature from the ones related to the length of the frozen storage (Muir, 1984). Two major types of instability can emerge in milk upon freezing: fat separation, occurring when the milk undergoes the freezing process and protein flocculation, which depends instead on the final storage temperature and length of the frozen storage. Literature lacks up-to-date data on the stability of these two milk components against freezing, especially considering the physicochemical nature of the modifications. As a matter of fact, the latest published articles dealt only with dairy products after prolonged frozen storage (De la Fuente, Requena, & Juàrez, 1997; Katsiari et al., 2002; Tejada, Sánchez, Gómez, Vioque, & Fernández-Salguero, 2002; R. H. Zhang, Mustafa, Ng-Kwai-Hang, & Zhao, 2006), so a clear gap of knowledge on the response of milk to short-time freezing is evident. (Muir, 1984).

In raw milk, lipids are present in the form of an emulsion stabilized by a milk fat globule membrane (MFGM), which is made up of protein and phospholipids (Truong, Palmer, Bansal, & Bhandari, 2016). The freezing process damages the MFGM, resulting in fat separation (Muir, 1984). The phenomenon is influenced by the cooling rate: it is generally believed that slow-rate freezing is more likely to cause separation of the fat globules (Webb & Hall, 1935). Eventually, these modifications of the fat fraction may end up in the development of oxidized off-flavor (R.

H. Zhang et al., 2006). The issue is minimized by homogenization, because of the higher stability the fat droplets with reduced size (Babcock, Roerig, Stabile, Dunlap, & Randall, 1946; Herrera & Hartel, 2000).

With regards to protein flocculation induced by freezing, caseins are the most sensitive fraction but require weeks at frozen conditions before becoming insoluble (Webb & Hall, 1935; Yamauchi, Chen, & Tsugo, 1967). The aim of this study was to clarify the relationship between freezing and milk stability, by estimating the effect of a 72 hours slow-rate (-20°C) and fast-rate (-80°C) freezing on parameters commonly defining the sensory quality and physical properties of the milk. Limited freezing time was chosen in order to leave out any effects related to the length of the frozen storage, focusing just on phenomena happening during freezing and thawing.

Materials and Methods

Hydrolyzed-lactose ultra-high-temperature milk (UHLM) was chosen for the experiment. As the fat fraction is prone to freezing-induced changes, both low fat (1% fat) and whole fat (3% fat) UHLM from the same brand were considered. Three different commercial lots were analyzed in consideration of milk batch variability. High-density-polyethylene (HDPE) bottles (1 L) were stored at 20°C (control), -20°C (slow-rate freezing) and -80°C (fast-rate freezing). The freezing step lasted in 72 hours. Straight after, all the samples were placed at 4°C for another 72 hours for complete thawing. We opted for UHT milk because it is shelf stable, so we could expect no changes in the characteristics of the control sample during the 72 hours at 20°C, making the comparison of the frozen and unfrozen milk more realistic. Furthermore, we decided to test a lactose-free milk, because of the emerging interest in this product category. In particular, many researchers pointed out higher instability of UHLM compared to regular UHT milk (Jansson, Clausen, et al., 2014; Jansson, Jensen, Sundekilde, et al., 2014; Troise et al., 2016). On the other hand, lactose hydrolysis may also prevent protein destabilization when milk is frozen (Koschak, Fennema, Amudson, & Lee, 1981).

Changes in the UHLM sensory quality were evaluated focusing on sensory properties, volatiles organic compounds (VOCs) and color. Triangle test (ISO, 2008) was chosen as overall discriminant method for determining whether perceptible sensory differences exist among UHLMs stored at different temperatures. The panel (27% female and 73% male; age range 23-62 years, mean age=32.6, SD=11.1) consisted of employees and students at the Edmund Mach Foundation (San Michele all'Adige, Italy) and University of Trento (Trento, Italy) with previous record of participation in sensory tests. 49 and 36 panelists took part in the two evaluation sessions respectively. The 91% of the judges had previous experience and training in discriminant analysis at the moment of the tests. Furthermore, the 28% of them had also long-standing experience with descriptive tests. In each of these sessions three consecutive triangle tests were carried out to compare the 3 UHLMs by pairs: +20°C versus -80°C; +20°C versus -20°C , -80°C versus -20°C. Tests were replicated in two separate sessions, one for each fat content and in each session the 3 triangle tests were presented to panelists in a randomized order. For each storage treatment, UHLMs coming from the three different commercial lots were mixed together. Then, 20 mL of milk was poured into transparent plastic-covered cups coded with a 3-digit random number and presented to the panelists in sensory booths under

red light. The temperature of the samples was stabilized at 15°C before serving. VOCs were analyzed by headspace solid-phase microextraction gas-chromatography-mass spectrometry (HS SPME/GC-MS). The procedure was previously optimized (Bottiroli et al., 2020). A three-phasic (DVB-Carboxen-PDMS) SPME fiber was used for the extraction step. VOCs were desorbed at 250 °C in the injector port of a GC Clarus 500 (PerkinElmer, Norwalk, CT, USA) interfaced with a mass detector operating in an electron ionisation mode (internal ionisation source; 70 eV) with a scan range from 33 to 300 m/z. A HP-Innowax fused-silica capillary column (30 m, 0.32 mm inner diameter, 0.5 µm film thickness; Agilent Technologies, Palo Alto, CA, USA) was used for the separation. Results were semi-quantitative highlighting trends in the VOCs data. Color changes were evaluated with a colorimeter (Minolta CM-3500d, Japan). The results were registered by the instrument in the CIELAB color system: L* (black: L* = 0 and white: L*=100), a* (red-green: negative a* = greenness and positive a* = redness), b* (yellow-blue: negative b* = blueness and positive b* = yellowness). The instrument was internally calibrated with distilled water and an opaque material provided by the manufacturer. Measurements were performed in triplicate using quartz cells with a 1 cm optical path.

Changes in the UHLM physical stability was characterized by optical centrifugation, particle size analysis, and confocal laser scanning microscopy (CLMS). LUMiFuge particle separation analyzer (L.U.M.GmbH, Berlin, Germany) was used. Sample preparation and LUMiFuge settings followed the method reported by Zhang and coworkers (2020). Prior to analysis, the stored UHLMs were diluted 10 times with simulated milk ultrafiltrate (SMUF, pH 6.8) to reduce the turbidity of low fat and full-fat UHT milk, in order to facilitate the optical measurement. The samples in LUMiFuge were centrifuged at 2300 × g for 43 min at room temperature, and the NIR transmission at a wavelength of 865 nm was measured every 10s. The integrated transmission percentage against time, which is referred to as the "instability index", was used to quantify the emulsion stability of the samples. The instability index was automatically integrated with the LUMiFuge software SEPView 6.3. Higher instability index values indicate lower emulsion stability.

The mean particle diameter and the distribution of particle sizes in UHLMs stored at different temperatures were determined in triplicates by laser light diffraction (Mastersizer 3000, Malvern Instruments, Ltd, Malvern, UK). Samples diluted 50 times with MilliQ water were directly injected into the dispersion cell (containing MilliQ water) under agitation at 1500 rpm. The refractive

indexes used in this study were 1.45 for the particles and 1.33 for the continuous phase (MilliQ water), respectively (C. Zhang et al., 2020). The particle absorption index was set as 0.002.

CLSM analyses were performed on UHLMs stored at different temperatures to investigate the distribution of lipid and protein, as previously described by Zhang and coworkers (2020).

The software package STATISTICA 13.3 (StatSoft, Inc., Tulsa, OK, USA) and RStudio (version 3.3.3) were used for the statistical analysis of the data. Triangle test outputs were collected and analyzed using the FIZZ 2.46A software (Biosystemes, Couternon, France) and interpreted based on the ratio between the total number of responses and the minimum number of correct answers required to define an existing significant difference among the products (based on binomial distribution with P=1/3).

Explorative multivariate analysis of the VOCs profiles was achieved by Principal Component Analysis (PCA). Variables were previously mean centered and scaled to unit variance, after log-transformation. Analysis of variance and Tukey post-hoc tests were further applied to assess differences in VOCs ($P \leq 0.05$), instability index ($P \leq 0.01$) and mean sizes ($P \leq 0.01$) between the samples stored at different temperatures. Color data were instead analyzed by the non-parametric Kruskal-Wallis test.

Results and Discussions

Results of the triangle tests showed that no overall difference among the UHLMs was perceived by the panel for both low fat (1% fat) and whole fat (3% fat) milk (Table 1). Results were further confirmed by the similarity test: 5 out of the 6 triangle tests highlighted a similarity, showing that the difference between the frozen UHLMs and the control was too small to be detectable.

Table 1. Results of the triangle tests performed to verify the effect of slow-rate (-20°C) and fast-rate freezing (-80°C) for 72 hours on the overall sensory perception of UHLM.

Treatment comparison[1]	Fat content	Total responses	Correct responses[2]	Difference test[3]	Similarity test[4]
+20°C vs -80°C	1%	49	13	0.8788	0.0002
	3%	36	11	0.6967	0.0002
+20°C vs -20°C	1%	49	11	0.9653	0.0005
	3%	36	11	0.8103	<0.0001
-80°C vs -20°C	1%	49	18	0.3565	0.1460
	3%	36	13	0.4225	0.0022

[1] UHLM samples stored for 72 hours at 20°C, -80°C and -20°C respectively; [2] Correct responses are intended as number of panelists which correctly identified the odd sample in each triangle test; [3] The column reported the p-value related to the error of type I (α) calculated by the difference test ($P < 0.05$) with FIZZ software (Biosystemes, Couternon, France); [4] The column reported the p-value related to the error of type II (β) calculated by the similarity test ($P < 0.05$) with FIZZ software (Biosystemes, Couternon, France). The Pd chosen was 37.5%.

Triangle tests have been previously used by researchers to study the sensory perceived differences in milk due to packaging (Smet et al., 2009), proteolysis (Santos, Ma, Caplan, & Barbano, 2003) and heat treatment (Pagliarini, Vernile, & Peri, 1990) but not to study the effect of short-time freezing on milk. Our results clearly demonstrated that the short freezing/thawing treatment did not alter the sensory properties; this evidence was further confirmed by the VOCs and color analysis. In total, 25 VOCs matched the mass spectra reported in the NIST/Wiley libraries, ranging between <0.10 and 15.95 µg/L milk. The values, expressed as equivalent of internal standard, are summarized in Table 2. The detected VOCs were already reported in other published studies on regular and hydrolyzed-lactose UHT milk (Bottiroli et al., 2020; Jansson, Jensen, Eggers, et al., 2014; Troise et al., 2016). Visualization of the VOCs data was given by Principal Component Analysis (PCA).

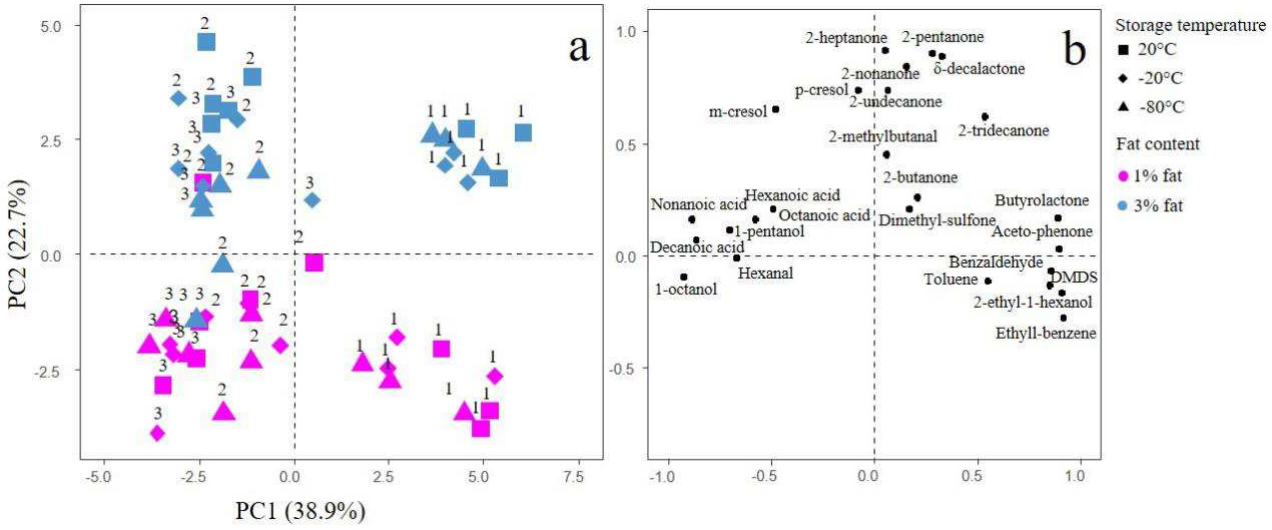


Figure 1a. Score plot of the Principal Component Analysis (PCA) for the 1st (PC1) and 2nd (PC2) principal component, accounting respectively for the 38.9% and 22.7% of the total variance. The numbers 1, 2 and 3 indicates the commercial lots of each type of UHLM samples (1% and 3% fat). **Figure 1b**. Corresponding loading plot.

Figure 1 illustrates the score (**Figure 1a**) and loading plot (**Figure 1b**) of the 1st and 2nd principal components, with an explained variance of 38.9% and 22.7% respectively. At a first glance, no changes due to freezing were detectable. The result was confirmed by the analysis of the variance: none of the VOCs registered significant differences due to the applied storage treatments. Slow-rate freezing performed as good as fast-rate freezing, although previously considered more harmful for the milk stability (Webb & Hall, 1935). Also, the instability of milk fat upon freezing, mentioned in earlier research (Muir, 1984; Webb & Hall, 1935), did not emerge as we could not detected particular trends in the VOCs linked to fat oxidation. The spatial distribution of the PCA was primarily defined by the different fat content and the batch variability existing among the analyzed UHLM samples. A similar result was obtained from the color analysis. Overall, the combined results of the triangle tests, VOCs profiling and color analysis distinctly indicated that the sensory quality of low fat and whole fat UHLM is not altered by short-time freezing.

Table 2. Volatile compound profile (µg IS/L) of the analyzed UHLMs. For each storage treatment, results are reported as the mean of three measurements performed on three different lots of production.

		1% fat			3% fat		
Volatile compounds	RT[2]	20°C[3]	-20°C	-80°C	20°C	-20°C	-80°C
Ketones							
2-butanone[1]	2.67	1.05	0.93	1.02	1.10	0.97	0.92
2-pentanone	4.05	1.70[a]	1.55[a]	1.54[a]	3.80[b]	3.37[b]	3.17[b]
2-heptanone	9.78	7.31[a]	6.57[a]	6.65[a]	15.95[b]	13.71[b]	13.75[b]
2-nonanone	16.68	1.42[a]	1.31[a]	1.41[a]	2.62[b]	2.28[b]	2.24[b]
2-undecanone	23.20	0.43[a]	0.44[a]	0.43[a]	0.65[b]	0.59[b]	0.57[b]
Aceto-phenone	24.70	0.26	0.31	0.20	0.29	0.23	0.24
2-tridecanone	29.14	0.09[a]	0.08[a]	0.08[a]	0.11[b]	0.11[b]	0.10[b]
Aldehydes							
2-methylbutanal	2.82	0.17[a]	0.16 [a]	0.16 [a]	0.22[b]	0.19[b]	0.19[b]
Hexanal	6.61	5.43	6.90	6.50	5.58	6.25	6.10
Benzaldehyde	20.91	1.49	1.49	1.33	1.48	1.24	1.27
Organic acids							
Hexanoic acid	30.84	2.92	2.33	1.35	2.14	2.39	1.54
Octanoic acid	36.21	1.62	1.58	1.40	1.63	1.52	1.22
Nonanoic acid	38.79	0.89	0.89	0.97	1.00	0.96	0.87
Decanoic acid	41.99	1.48	1.43	1.47	1.61	1.29	1.33
Sulfur compounds							
Dimethyl disulfide	6.33	0.37	0.37	0.34	0.34	0.29	0.29
Dimethyl sulfone	31.51	2.66	2.24	1.70	2.06	2.58	1.60
Alcohols							
1-pentanol	12.15	0.10	0.11	0.12	0.10	0.11	0.09
2-ethyl-1-hexanol	19.98	0.39	0.42	0.46	0.37	0.27	0.31
1-octanol	22.08	0.78	0.92	0.97	0.77	0.73	0.74
Lactones							
Butyrolactone	24.03	0.25	0.28	0.22	0.30	0.27	0.26
δ-decalactone	38.59	0.16[a]	0.16[a]	0.15[a]	0.39[b]	0.38[b]	0.36[b]
Others							
Toluene	5.46	0.75	0.70	0.72	0.77	0.71	0.73
Ethyll-benzene	7.87	2.58	2.26	1.70	0.93	1.05	1.04
p-cresol	36.12	0.07[a]	0.07[a]	0.07[a]	0.08[b]	0.08[b]	0.07[b]
m-cresol	36.31	0.35	0.34	0.33	0.39	0.39	0.35

[a-b] Means within a row with different superscript differ significanlty ($P < 0.05$).
[1] Compounds tentatively identified matching the NIST-2014/Wiley 7.0 libraries.
[2] Retention time expressed in minutes.
[3] All the three storage treatment (20°C, -20°C, -80°C) lasted in 72 hours.

Besides this aspect, physical stability is a major concern for the dairy industry, especially because the demixing of non-skimmed UHT milk is difficult to judge visually due to the high turbidity (C. Zhang et al., 2020). Therefore, LUMiFuge was used to assess the stability behavior by recording the migration of particles under centrifugal forces. For both low fat and whole fat milk, no differences were observed in the instability index between the frozen UHLMs and the ones stored at room temperature. Such finding suggests that freezing at slow-rate (-20°C) and fast-rate (-80°C) for 72 hours have no negative impact on the sedimentation/creaming stability of UHLM. On the other hand, the instability indices of 1% fat UHLM were higher than for 3% fat UHLM (**Supplementary Figure 1**), confirming the inverse relationship between the fat globule destabilization and the fat content of the milk (Lerche, Piesendel, & Senge, 2003). Effect of short-time freezing on the particle size was also investigated by means of laser light scattering (**Figure 2**). The distributions of casein micelles overlapped with the fat globules, forming a pseudo-monomodal size distribution for the control UHLMs with 1% and 3% fat content. This pseudo-monomodal size distribution centered at approximately 0.2 µm, which is similar to the normal full-fat UHT milk (C. Zhang et al., 2020). To further probe changes in size, we compared the surface-weighted mean particle diameter D[3,2], which is sensitive to the presence of fine particulates, and the volume-weighted mean particle diameter D[4,3], which is sensitive to the presence of large particulates in the size distribution. For 1% fat UHLM, freezing had no significant impact on the size distribution and the mean diameter. In 3% fat UHLM some tiny peaks in the size range over 1 µm appeared in the frozen samples, especially in those subjected to fast-rate freezing, as illustrated in the insert of **Figure 2b.** This indicates the formation of some larger aggregates. Accordingly, fast-rate freezing (-80°C) led to a slight increase in both D[3,2] and D[4,3] compared to the control milk and the slow-rate frozen one ($P \leq 0.01$). Because the effect appeared only in UHLM with 3% fat, these peaks were probably the result of fat destabilization. The effect is related to an increase in ice crystal size resulting in less distance between the fat globules (Yanniotis, 2008). Hypothetically, at -20°C part of the water was still unfrozen, whereas at -80°C all water was present in the form of ice crystals, causing the partial coalescence of the fats. The emulsion stability and the extent of droplets aggregation were also visualized by CLSM. The CLSM pictures of UHLMs stored at all three temperatures were identical. Both the casein micelles and the fat globules were homogeneously distributed and had uniform

size. No obvious aggregation was observed in the frozen samples and, overall, it cannot be concluded that freezing led to a profound effect on the microstructure and stability of UHLM.

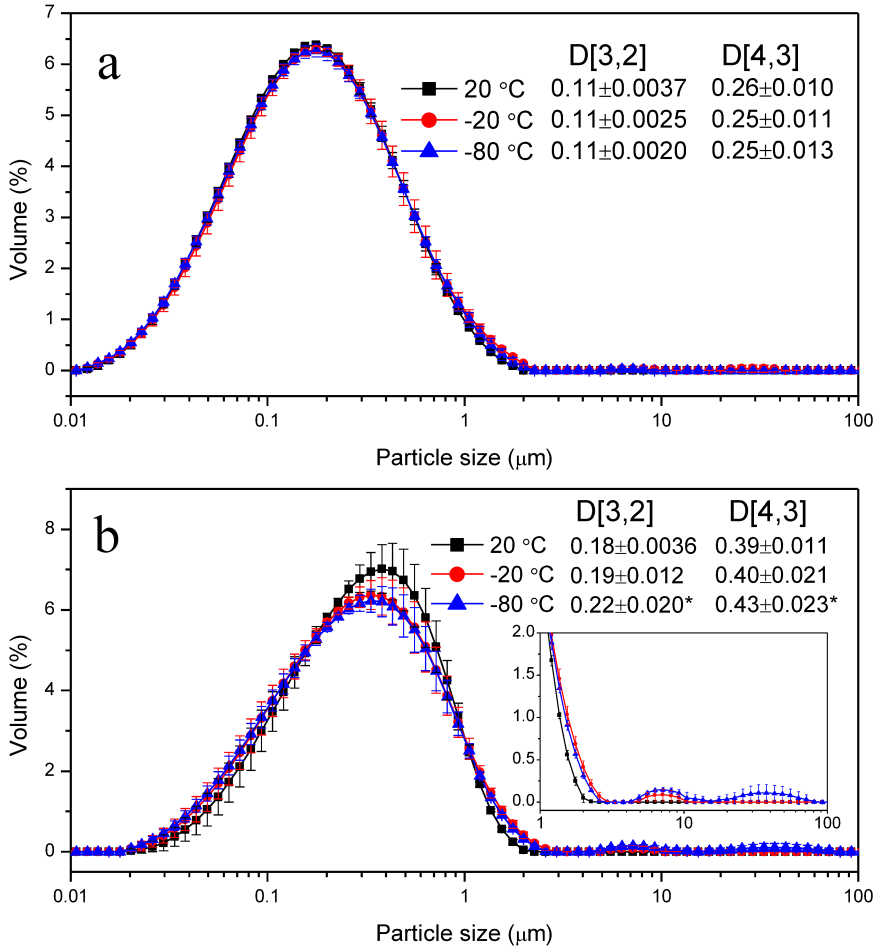


Figure 2. Volume based particle size distribution, surface-weighted mean particle diameter D[3,2], and the volume-weighted mean particle diameter D[4,3] in UHLMs with 1% fat **(a)** and 3% fat **(b)** stored at 20°C, -20°C, -80°C for 72 hours. The insert in Figure 2b shows the size distribution higher than 1 μm on an expanded scale. Data are presented as mean values of samples from three lots in triplicates, error bars presented SD, * indicates *P<0.01*.

Taking all the results on the physical properties together, freezing for 72 hours did not lead to destablization of UHLM. It should only be noted that in the fast-rate frozen samples, a slight tendency of aggregation was observed. Thus, freezing at -80°C did not provide any benefits. As previously shown, milk proteins and fats may modify upon freezing. For the fat fraction, if destabilization arise, it is expected as soon as the milk undergoes the freezing. In this context,

the final freezing temperature does not play a crucial role (Muir, 1984), but it is the physical state of the fat at the beginning of the freezing that matters. Key phenomena in this respect are the crystallization of milk fat and the size of the fat crystals (Jonkman, Walstra, Van Boekel, & Cebula, 1999). For example, when the freezing starts at refrigerated conditions, some crystals already exist and may grow excessively in size.

The effect can pierce the fat globules and cause destabilization. In our study, the freezing started at room temperature and we can suppose that only small crystals were formed, contributing to the stability of the fat globules. Differently, destabilization of milk protein is influenced by other factors such as temperature, length of frozen storage, pre-treatments on the milk (e.g. concentration and heating) and modifications of the milk composition (Koschak et al., 1981). From our results, milk protein did not destabilize upon freezing, as the aggregates bigger than 1 µm observed by the Mastersizer were related to fat destabilization. The protein fraction of the UHLM did not seem altered by the tested conditions, which is in line with the literature coupling the modifications of the protein to longer periods of frozen storage (Muir, 1984; Webb & Hall, 1935). Koschak et al. (1981) reported that also lactose hydrolysis may prevent protein destabilization when milk is frozen. The soluble sugars, whose content is increased by the conversion of lactose, delay milk protein precipitation maintaining their stability for longer periods during frozen storage (Wells & Leeder, 1963). In our experiment, we can assume that the lactose hydrolysis played a role in keeping milk proteins stable, although such assumption will require further validation.

Conclusions

In conclusion, our research determined the stability of UHLM frozen for 72 hours in terms of both sensory and physicochemical properties. According to the available literature, we initially expected that possible changes might occur in the fat fraction. Nevertheless, fat destabilization did not appear for both low fat and whole fat milk. Homogenization was in all probability crucial in maintaining the fat fraction stable. Moreover, the velocity of freezing was not critical: similar results were obtained when the milk was exposed to slow-rate (-20°C) and fast-rate freezing (-80°C). In conclusion, UHLM can be considered insensitive to alterations occurring during short freezing/thawing. This suggests addressing future research towards the phenomena occurring during frozen storage by a more comprehensive approach.

References

Babcock, C. J., Roerig, R. N., Stabile, J. N., Dunlap, W. A., & Randall, R. (1946). Frozen Homogenized Milk. 1. Effect of Freezing and Storage Temperature on the Physical Characteristics of Homogenized Milk. *Journal of Dairy Science*, *29*(10), 699–706. https://doi.org/10.3168/jds.S0022-0302(46)92527-1

Bottiroli, R., Aprea, E., Betta, E., Fogliano, V., & Gasperi, F. (2020). Application of headspace solid-phase micro-extraction gas chromatography for the assessment of the volatiles profiles of ultra-high temperature hydrolysed-lactose milk during production and storage. *International Dairy Journal*, 104715. https://doi.org/10.1016/j.idairyj.2020.104715

De la Fuente, A. M., Requena, T., & Juàrez, M. (1997). Salt Balance in Ewe's and Goat's Milk during Storage at Chilling and Freezing Temperatures. *Journal of Agricultural and Food Chemistry*, *45*(1), 82–88. https://doi.org/10.1021/jf960388a

Fava, L. W., Külkamp-Guerreiro, I. C., & Pinto, A. T. (2014). Evaluation of physico-chemical characteristics of fresh, refrigerated and frozen Lacaune ewes' milk. *Arquivo Brasileiro de Medicina Veterinaria e Zootecnia*, *66*(6), 1924–1930. https://doi.org/10.1590/1678-7675

Herrera, M. L., & Hartel, R. W. (2000). Effect of processing conditions on physical properties of a milk fat model system: Microstructure. *Journal of the American Oil Chemists' Society*, *77*(11), 1197–1205. https://doi.org/10.1007/s11746-000-0186-2

ISO. (2008). ISO. *Sensory Analysis, Methodology, Triangle Test ISO Standard 4120*, *Genève*(Switzerland:), ISO.

Jansson, T., Clausen, M. R., Sundekilde, U. K., Eggers, N., Nyegaard, S., Larsen, L. B., … Bertram, H. C. (2014). Lactose-hydrolyzed milk is more prone to chemical changes during storage than conventional ultra-high-temperature (UHT) milk. *Journal of Agricultural and Food Chemistry*, *62*(31), 7886–7896. https://doi.org/10.1021/jf501671z

Jansson, T., Jensen, H. B., Sundekilde, U. K., Clausen, M. R., Eggers, N., Larsen, L. B., … Bertram, H. C. (2014). Chemical and proteolysis-derived changes during long-term storage of lactose-hydrolyzed ultrahigh-temperature (UHT) milk. *Journal of Agricultural and Food Chemistry*, *62*(46), 11270–11278. https://doi.org/10.1021/jf504104q

Jansson, T., Jensen, S., Eggers, N., Clausen, M. R., Larsen, L. B., Ray, C., … Bertram, H. C. (2014). Volatile component profiles of conventional and lactose-hydrolyzed UHT milk - A dynamic headspace gas chromatography-mass spectrometry study. *Dairy Science and Technology*,

94(4), 311–325. https://doi.org/10.1007/s13594-014-0164-7

Jonkman, M. J., Walstra, P., Van Boekel, M. A. J. S., & Cebula, D. J. (1999). Behaviour of casein micelles at conditions comparable to those in frozen yoghurt. *International Dairy Journal*, *9*(3–6), 385–386. https://doi.org/10.1016/S0958-6946(99)00099-0

Katsiari, M. C., Voutsinas, L. P., & Kondyli, E. (2002). Manufacture of yoghurt from stored frozen sheep's milk. *Food Chemistry*, *77*(4), 413–420. https://doi.org/10.1016/S0308-8146(01)00367-3

Koschak, M. S., Fennema, O., Amudson, C. H., & Lee, J. Y. (1981). Protein Stability of Frozen Milk as Influenced by Storage Temperature and Ultrafiltration. *Journal of Food Science*, *46*(4), 1211–1217. https://doi.org/10.1111/j.1365-2621.1981.tb03025.x

Lerche, D., Piesendel, L., & Senge, B. (2003). Characterization of food quality and structural stability by analytical centrifugation. *3rd International Symposium on Food Rheology and Structure*, *c*, 149–153.

Muir, D. D. (1984). Reviews of the progress of Dairy Science: Frozen concentrated milk. *Journal of Dairy Research*, *51*, 649–664.

Needs, E. C. (1992). Effects of long–term deep-freeze storage on the condition of the fat in raw sheep's milk. *Journal of Dairy Research*, *59*(1), 49–55. https://doi.org/10.1017/S0022029900030235

Pagliarini, E., Vernile, M., & Peri, C. (1990). Kinetic Study on Color Changes in Milk Due to Heat. *Journal of Food Science*, *55*(6), 1766–1767. https://doi.org/https://doi.org/10.1111/j.1365-2621.1990.tb03625.x

Santos, M. V., Ma, Y., Caplan, Z., & Barbano, D. M. (2003). Sensory threshold of off-flavors caused by proteolysis and lipolysis in milk. *Journal of Dairy Science*, *86*(5), 1601–1607. https://doi.org/10.3168/jds.S0022-0302(03)73745-X

Smet, K., De Block, J., De Campeneere, S., De Brabander, D., Herman, L., Raes, K., … Coudijzer, K. (2009). Oxidative stability of UHT milk as influenced by fatty acid composition and packaging. *International Dairy Journal*, *19*(6–7), 372–379. https://doi.org/10.1016/j.idairyj.2009.02.006

Tejada, L., Sánchez, E., Gómez, R., Vioque, M., & Fernández-Salguero, J. (2002). Effect of freezing and frozen storage on chemical and microbiological characteristics in sheep milk cheese. *Journal of Food Science*, *67*(1), 126–129. https://doi.org/10.1111/j.1365-

2621.2002.tb11371.x

Troise, A. D., Bandini, E., De Donno, R., Meijer, G., Trezzi, M., & Fogliano, V. (2016). The quality of low lactose milk is affected by the side proteolytic activity of the lactase used in the production process. *Food Research International*, *89*, 514–525. https://doi.org/10.1016/j.foodres.2016.08.021

Truong, T., Palmer, M., Bansal, N., & Bhandari, B. (2016). An Overview of Milk Fat Globules. In *Effect of Milk Fat Globule Size on the Physical Functionality of Dairy Products* (pp. 5–9). Cham: Springer International Publishing. https://doi.org/10.1007/978-3-319-23877-7_2

Webb, B. H., & Hall, S. A. (1935). Some Physical Effects of Freezing upon Milk and Cream. *Journal of Dairy Science*, *18*(5), 275–286. https://doi.org/10.3168/jds.S0022-0302(35)93146-0

Wells, P. R., & Leeder, J. G. (1963). Changes Related to Casein Precipitation in Frozen Concentrated Milk. *Journal of Dairy Science*, *46*(8), 789–798. https://doi.org/10.3168/jds.S0022-0302(63)89150-X

Wendorff, W. L., Rylqh, Z., Plon, V., Iur, Z. D. V, Dw, H. Q., Shulrg, P. R. V., … Xsshu, W. K. H. (2001). Freezing Qualities of Raw Ovine Milk for Further Processing. *Journal of Dairy Science*, *84*, E74–E78. https://doi.org/10.3168/jds.S0022-0302(01)70200-7

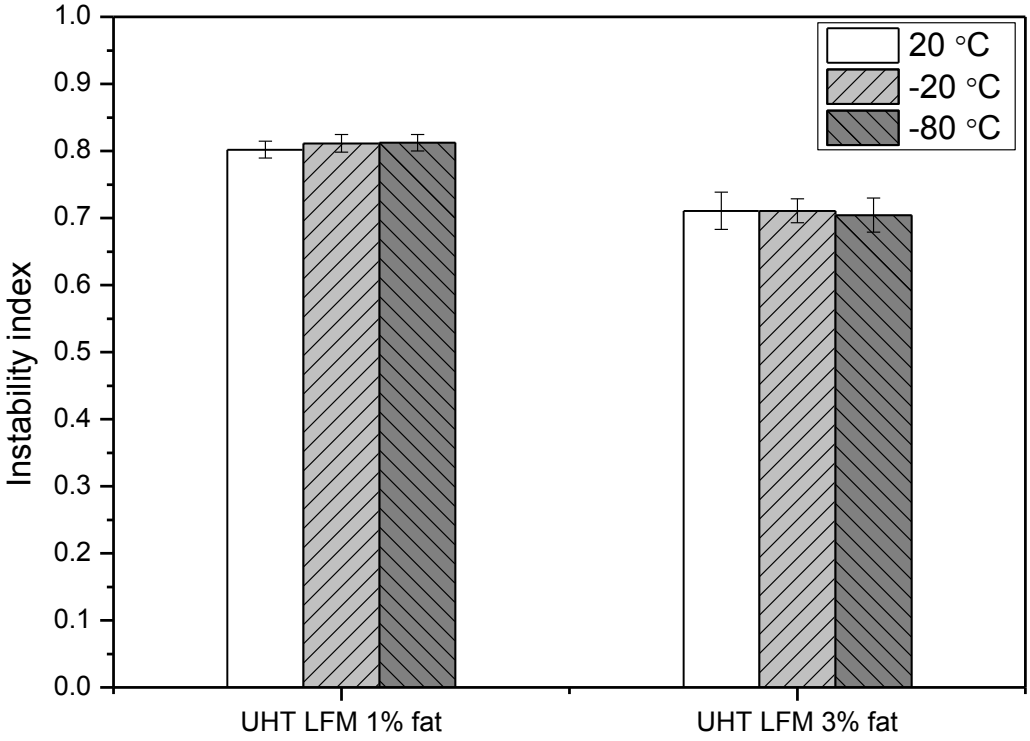
Yamauchi, K., Chen, C. M., & Tsugo, T. (1967). Studies on destabilization of caseinate complex during frozen storage of milk. *Agricultural and Biological Chemistry*, *31*(5), 581–599. https://doi.org/10.1080/00021369.1967.10858844

Yanniotis, S. (2008). Cooling and freezing. *Food Engineering Series*, 193–214. https://doi.org/10.1007/978-0-387-73514-6_15

Zhang, C., Bijl, E., Muis, K. E., & Hettinga, K. (2020). Stability of fat globules in UHT milk during proteolysis by the AprX protease from Pseudomonas fluorescens and by plasmin. *Journal of Dairy Science*, *103*(1), 179–190. https://doi.org/10.3168/jds.2019-17150

Zhang, R. H., Mustafa, A. F., Ng-Kwai-Hang, K. F., & Zhao, X. (2006). Effects of freezing on composition and fatty acid profiles of sheep milk and cheese. *Small Ruminant Research*, *64*(3), 203–210. https://doi.org/10.1016/j.smallrumres.2005.04.025

Supplementary Material



Supplementary Fig. 1. Instability index obtained by Lumifuge of UHLMs (with 1% and 3% fat) stored at 20°C, -20°C, -80°C for 72 hours. All the samples were 10-fold diluted to facilitate the optical measurement. Data are presented as mean ± SD value of samples from three lots.

Chapter 4: Chemical and sensory changes during shelf-life of UHT hydrolyzed-lactose milk produced by "in batch" system employing different commercial lactase preparations.

This chapter represents the core of the whole PhD thesis and deals directly with an R&D issue typical of real industrial scenarios: the choice the lactase preparation (LP) for UHLM manufacturing. Using the correct lactase is crucial due to unwanted effects possibly arising from the enzymatic side activity of the LPs. If not properly controlled, these secondary proteolytic and arylsulfatase activities may alter the quality of the final product determining consumer rejection and, in some case, a recall of the products from the market. It is for example what happened at the end of the 2017 to a well-known Italian dairy company, which withdrawn several commercial lots of lactose-free milk from supermarkets due to the presence of clumps and instability upon cooking (https://ilfattoalimentare.it/granarolo-richiamo-latte-accadi.html). Therefore, the consequences of choosing the wrong LP for UHLM production should never be underestimated. In the study proposed in this chapter different commercial LPs has been tested for the production of UHLM through "in batch" system, whose details are reported in the Introduction (Chapter 1). As the literature lacks information on this particular processing technology, the results of the study completed the knowledge on the chemical change happening in "in batch" UHLM during shelf-life, revealing modifications of the UHLM quality and flavor.

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Abstract

Manufacturing shelf-stable Ultra-high temperature hydrolyzed-lactose milk (UHLM) is a challenge for dairy producers, as the product undergoes chemical changes during storage due to both reducing sugars reactivity and proteolysis arising from the impurity of the lactase preparations. In the present study, the "in batch" production system, which includes the addition of the lactase before the thermal treatment, was demonstrated a valuable alternative to the more popular "in pack" system, where lactase is added directly into each milk package after thermal sterilization. The features of the technology were investigated by monitoring the changes in free amino acids, volatile organic compounds, color and sensory properties of UHLMs produced with three different lactase preparations (LPs), up to 120 days at 20°C. Upon UHT processing, the proteolytic side activity of lactases was minimized, so minimum breakdown of milk protein was achieved. The release of free amino acids was dependent on the lactase purity only in the early production phases, whereas it did not change over time. The Strecker aldehydes benzaldehyde and 2-methylbutanal resulted as effective markers to correlate with the initial lactase purity during storage. Color and sensory slightly changed during storage but were poorly correlated with the different lactases resembling to phenomena typical of milk aging. This latter result suggested that production costs may be lowered by opting for less-purified lactases when considering the "in batch" technology supporting the application of this production system for the design of UHLM with high-quality standards and low risk of alterations during shelf-life.

Introduction

Many consumers avoid lactose-containing products as a consequence of discomfort related to lactose digestion (Sahin, Hamamci, & Garayev, 2016). Upon weaning, the activity of intestinal lactase significantly decreases, hampering lactose hydrolysis in subjects with low levels of lactase persistence (Itan, Jones, Ingram, Swallow, & Thomas, 2010). With an estimated 70% world population suffering of lactase deficiency, low-lactose (LLM) and lactose-free (LFM) milk products are the most convenient solution to face lactose intolerance (Deng, Misselwitz, Dai, & Fox, 2015).Manufacturing of LLM and LFM is based on the hydrolysis of lactose into glucose and galactose by addition of soluble lactase (Jelen & Tossavainen, 2003). Since the two monosaccharides increase the perceived sweetness of the milk, filtration is sometimes introduced to reduce the concentration of lactose prior to the enzymatic conversion (Harju, Kallioinen, & Tossavainen, 2012). At the same time, lactose hydrolysis doubles the reducing sugar molarity in the milk favoring Maillard reaction (MR) (Tossavainen & Kallioinen, 2007). In the case of severe thermal treatment, such as those occurring in Ultra High Temperature (UHT) processing, chemical modifications on proteins occur and advanced Maillard reaction products (MRPs) are formed (Troise et al., 2016). The proteolytic side activity of the lactase preparation (LP) increases the amount of free amino group in the milk and consequently the formation rate of volatiles MRPs (Jansson, Clausen, et al., 2014a). Therefore, MR proceeds more extensively in UHT hydrolyzed-lactose milk (UHLM) compared to regular UHT milk (Jansson, Clausen, et al., 2014). This higher sensitivity towards non-enzymatic browning is the main reason why producers have shortened the commercial shelf-life of the product down to 90-120 days.

This is a big limiting factor for European manufacturers to export their UHT hydrolyzed-lactose milk (UHLM) overseas. The request for dairy products in Asian countries is increasing dramatically (Fuller, Huang, Ma, & Rozelle, 2006) and, with a majority of the population lactose intolerant, UHLM is a profitable opportunity to fill the demand (Jansson, 2014). On the other hand, temperature fluctuations during transportation as well as quarantine periods during customs checks are frequent when products are traded to a different country. In this harsh environment, MR proceeds extensively in UHLM increasing the risk of quality losses before the product reaches the final destination. Developing UHLM with shelf-life close to regular UHT milk (9-12 months) will facilitate the distribution (Nielsen et al., 2017), but answers to the critical factors limiting the UHLM shelf-life are also related to a deep understanding of the technology

employed for production and the associated chemical modifications. Two main production systems, commonly called "in batch" and "in pack", are currently available for UHLM production with soluble LP, as recently outlined by Dekker and coworkers (2019). The "in batch" production system starts with the addition of the LP in tank and the incubation of the milk at refrigerated conditions. The enzyme is then inactivated by UHT treatment before bottling. In the "in pack" process, LP is instead added directly into each milk package, after the UHT treatment. As lactose hydrolysis occurs after packaging, the production system reduces the overall cost of production by lowering the amount of enzyme required (Troise et al., 2016). The aseptic packaging technology facilitated the diffusion of the "in pack" system among producers as demonstrated by the number of research articles published on the milk produced following this procedure (Jansson, Clausen, et al., 2014; Jansson, Jensen, Sundekilde, et al., 2014; Jensen et al., 2015; Zhao et al., 2019). Nevertheless, this production system has also negative aspects to consider. For example, LP is not inactivated so its proteolytic side activity can cause unpleasant quality defects in the milk during shelf-life (Jansson, Jensen, Sundekilde, et al., 2014). The quality of UHLM produced by "in pack" system has been largely investigated focusing on lysine blockage, peptide release, formation of unwanted volatile organic compounds (VOCs) and sensory properties (Jansson, Clausen, et al., 2014; Jansson, Jensen, Sundekilde, et al., 2014; Jensen et al., 2015; Nielsen et al., 2017, 2018; Troise et al., 2016). Nielsen and coworkers (2018) paralleled the quality of the "in pack" UHLM to the initial proteolytic activity of the commercial LP, hence the modifications occurring during the shelf-life are the key target of research efforts. In this frame, although authors prioritized the employment of the "in pack" system to control MR in UHLM (Mendoza, Olano, & Villamiel, 2005), production by "in batch" system is a valid alternative to minimize the defects due to excessive proteolysis and MRPs formation in the final product. Partial survival of the LP proteases after UHT treatment was previously reported (Tossavainen & Kallioinen, 2007). As a matter of fact, the literature lacks detailed studies concerning the effect of different LPs on the quality of UHLM obtained by this particular production system. Thermally-induced modifications occurring in the milk added with LP before UHT treatment are still unclear and the effects on final product quality still unexplored. Accordingly, the purpose of this study was to investigate the impact of different commercial LPs on the proteolysis, the volatile organic compounds (VOCs) evolution, the color alteration and the changes in sensory perception in UHLM produced by the "in batch" production system during shelf-life.

Material and Methods

Chemicals

Acetonitrile and water for liquid chromatography high resolution mass spectrometry (LC-HMRS) analysis were obtained from Merck (Darmstadt, Germany). Formic acid, the analytical standards [4,4,5,5-d_4]-L-lysine hydrochloride (d_4-Lys), L-Lysine-6-^{13}C dihydrochloride (13C-Lys), 4-methyl-2-pentanone (purity ≥ 99%) and the 20 L-amino acids analytical standards were purchased from Sigma-Aldrich (St. Louis, MO). O-phthaldialdehyde (OPA) and dithiothreithol for the OPA method were purchased from Sigma-Aldrich (St. Louis, MO). Disodiumtetraborate-Decahydrate and sodiumdodecylsulfate were obtained from Merck (Darmstadt, Germany). Nilac low-heat skim milk powder was acquired from NIZO Food Research (Ede, The Netherlands).

Milk samples production and shelf-life study

Three commercial LPs (LP1, LP2 and LP3) were tested in the experiment. The three LPs were obtained in the form of a liquid with a shelf-life of 24 months each at refrigeration conditions. All the tested LPs were purified from *Kluyveromyces lactis* and had an average enzymatic activity reported on the technical sheet higher than 5000 NUL/g. LP3 was an extended purified version of the enzymatic preparation LP2. Manufacturing of UHLM occurred at industrial level following the "in batch" operating conditions described by Troise et al. (2016). Samples were produced starting from semi-skimmed pasteurized milk. Production was repeated three times for each LP tested, starting from milk always having the same composition, in order to include an estimation of the batch-to-batch milk variability. Each LP was added to the milk to reach an approximate enzymatic concentration of 3000 NUL/L milk. Lactose hydrolysis was performed in tank at refrigerated conditions and took approximately 25-35 hours for all the lots of production. Lactose was monitored according to the method previously described by our group (Troise et al., 2016) and, at the end of the hydrolysis step, the residual lactose content was <0.1g/100 mL milk. Milk was then supplied to direct UHT processing and, after homogenization and cooling, it was packed aseptically. The same operating conditions were applied to each industrial production. At the end of each production batch, the time 0 of analysis was taken, whereas the rest of the samples were placed into a climate chamber set at 20°C. The shelf-life study lasted in 120 days with samples collected every 30 days and stored at -80°C until analysis.

Free amino group

The proteolytic activity was determined with Nilac low-heat skim milk powder as substrates. The newly-formed amino groups were detected after derivatization with OPA. Reconstituted milk (25 g/L) was pre-incubated at 37 °C for 5 min. The enzymatic reaction was started by adding 0.2 mL enzyme solution to 1 mL reconstituted milk, and terminated by adding OPA reagent. One hundred mL of OPA reagent contained 3.18 g sodium tetraborate, 0.088 g dithiothreitol, 0.1 g sodium dodecyl sulphate and 0.08 g OPA (dissolved in 3 mL ethanol). Four hundreds µL of appropriately diluted sample was mixed with 3 mL OPA reagent, meanwhile, the duration of enzymatic reaction (from adding enzyme solution to substrate till adding OPA reagent) was recorded. The absorbance at 340 nm was read exactly after 2 min using a spectrophotometer (Cary 50 Bio, Varian). The measurement was performed in triplicate. L-Serine in deionized water (12.5-150 mg L^{-1}) was used as a standard. One katal of proteolytic activity was defined as the amount which breaks a mole of peptide bonds per second.

Free amino acids

Free amino acids were analyzed according to Troise and coworkers (2018), with some modifications. An aliquot of milk samples (0.1 mL) was spiked with d4-Lys then mixed with 0.1 ml of acetonitrile and centrifuged at 4 °C, for 15 min at 21100 *x g*. Samples were diluted ten times in a mixture of acetonitrile and water 50:50, then filtered by using cellulose filters (RC, 0.22 µm Sartorius, Gottingen, Germany). Before injection, samples were spiked again with 13C-lysine and the chromatographic separation of amino acids was achieved by 0.1% formic acid in acetonitrile (solvent A), 0.1% formic acid in water (solvent B) as LC mobile phases. The following linear gradient of solvent B (min/%B): (0/5), (1.50/5), (8/90), (10/90) was used, the flow rate was set to 300 µL/min and the injection volume was 5 µL. Chromatographic separation of amino acids was completed through a thermostated (30 °C) sulfobetaine modified zwitterionic HILIC (Syncronys HILIC 3.0 µm, 50 x 2.1 mm, Thermo Fisher). The Accela 1250 U-HPLC system (Thermo Fisher Scientific, Bremen, Germany) was interfaced to an Exactive Orbitrap HRMS (Thermo Fisher Scientific, Bremen, Germany) and the analytes were detected through a heated electrospray interface (HESI-II) operating in positive mode. Molecular ions $[M+H]^+$ listed in **Table 1** were scanned in the *m/z* range of 60–400, while the resolving power was set to 50000 full width at half maximum (FWHM, *m/z* 200) resulting in a scan time of 1 s with a maximum injection time

of 100 ms. The interface parameters were as follows: spray voltage 4.5 kV, capillary voltage 20.5 V, skimmer voltage 16 V, tube lens voltage 110 V, capillary temperature 295 °C, heater temperature 300 °C, sheath gas flow 42 and auxiliary gas flow 6 arbitrary units. Calibration of the instrument and analytical performances were monitored according to previous papers from our group. The exact mass of 13C-Lys ($[M+H]^+$ 148.11616) was used as a reference to maximize instrumental response after each injection while deuterated lysine was used to monitor recovery upon each extraction.

Table 1: Liquid chromatography high-resolution mass spectrometry (LC-HRMS) performances of reference calibration curves. RT (retention time, min); TM (theoretical mass, $[M+H]^+$); EM (experimental mass, $[M+H]^+$); error (ppm), IS (internal standard).

Type	Amino acids $[M+H]^+$	Rt (min)	TM	EM	Error (ppm)
Aliphatic	Alanine	4.48	90.05496	90.05518	-2.44
	Valine	4.47	118.08626	118.08622	0.34
	Leucine	3.00	132.10191	132.10143	3.63
	Isoleucine	3.23	132.10191	132.10182	0.68
Aromatic	Phenylalanine	3.83	166.08626	166.08589	2.23
	Tryptophan	4.13	205.09715	205.09697	0.88
	Tyrosine	4.44	182.08117	182.08169	-2.86
	Methionine	4.19	150.05833	150.05818	1.00
	Serine	5.02	106.04987	106.04951	3.39
	Threonine	4.83	120.06552	120.06559	-0.58
	Asparagine	5.08	133.06077	133.06098	-1.58
	Glutamine	1.94	147.07642	147.07651	-0.61
Acidic	Aspartic acid	5.69	134.04478	134.04457	1.57
	Glutamic acid	5.11	148.06043	148.06004	2.63
Basic	Arginine	4.67	175.11895	175.11888	0.40
	Histidine	4.65	156.07675	156.07677	-0.13
	Lysine	4.71	147.11280	147.11273	0.48
Unique	Proline	4.68	116.07061	116.07071	-0.86
	Glycine	4.79	76.03930	76.03922	1.05
	Hydroxyproline	4.99	132.06551	132.06547	0.30
IS	^{13}C-Lysine	4.71	148.11616	148.11599	1.15
	d4-Lysine	4.71	151.13791	151.13761	1.98

Volatiles organic compounds

Analysis of the volatiles organic compounds (VOCs) was performed by SPME GC-MS according to Bottiroli, Aprea, Betta, Fogliano, & Gasperi (2020) with minor modifications. Five mL of sample were placed into 20 mL glass vials for gas-chromatography (Supelco, Bellefonte, PA,) and 4-methyl-2-pentanone was chosen as internal standard. Volatiles compounds were extracted with a 2 cm DVB-Carboxen–PDMS SPME fiber at 40 °C for 60 min. Desorption occurred at 250°C in the injector port of a GC Clarus 500 (PerkinElmer, Norwalk, CT) interfaced with a mass detector operating in an electron ionization mode (internal ionization source: 70 eV; scan range: m/z 33-300). An auto-sampling system (CTC combiPAL, CTC Analysis AG, Zwingen, Switzerland) automatically managed the procedure. Characteristics of the HP-Innowax fused-silica capillary column (Agilent Technologies, Palo Alto, CA) used for the separation are described as follow: 30 m, 0.32 mm inner diameter, 0.5 µm film thickness. The oven temperature steps of the GC consisted in 40 °C for 3 min, 180 °C for 6 min at 4 °C/min and 220 °C for 3 min at 3 °C/min. The gas carrier was helium with a flow rate set at 2 mL/min. Compound identification was achieved by match of the observed mass spectra with the ones available from the NIST-2014/Wiley 7.0 libraries. Linear retention indices (LRI) were also calculated by the analysis of a homologous series of n-alkanes C7-C30 (Supelco, Bellefonte, PA) applying the same chromatography conditions. Results of the applied methodology were semi-quantitative. The same GC methodology was also applied to estimate the initial VOCs profile of the tested LPs, which were diluted with deminarelized until a similar enzymatic concentration to the UHLMs was reached.

Color analysis

A colorimeter (CM-3500d, Minolta, Japan) was used to measure the color of the UHLM samples at the different stages of shelf-life. The instrument registered the light transmittance of the sample and expressed the results in the CIELAB color system. Internal calibration was performed with distilled water and an opaque material provided by the manufacturer. UHLM samples were poured in quartz cells having 1 cm of optical path and measurements were carried out at room temperature. Three repetition were performed for each sample.

Sensory evaluation

Sensory evaluation of UHLM samples was carried out by descriptive analysis, whose procedure is described elsewhere (Troise et al., 2016). A trained panel was used for the sensory evaluation of the samples. The panel was composed by professionals employed in the dairy sector with a long-standing experience on sensory evaluation of milk. Each product was evaluated twice along the shelf-life, namely at 30 and 120 days of storage. For each tasting session, 16-21 judges participated to the sensory test and were asked to describe the UHLM samples in term of the following attributes: white color, overall flavor, mouthfeel, sweetness, cooked flavor, milky flavor, stale flavor, irregular flavor. The intensity of each attribute were rated on a 9-point category scale (1=attribute not detected and 9=attribute extremely strong). In each tasting session, samples were labelled with three-digit random numbers and a blind replicate was introduced to keep track of the panel performances. Samples were presented to the judges in a balanced order.

Statistical analysis

Principal Component Analysis (PCA) was performed on free amino acids profiles to explore spatial distribution of the UHLM samples at the different stages of shelf-life. Partial Least Square Discriminant Analysis (PLS-DA) was also employed on the same dataset to sharpen the separation between the samples and identify those variables responsible for the classification. Before applying the two mentioned statistical techniques, free amino acids profile data were log-transformed and scaled to unit variance. Differences in the VOCs profiles, sensory properties and color due to the storage time and the LP employed were investigated by analysis of the variance. When coming across a significant difference, Tukey post-hoc test was applied. Correlations among sensory and instrumental variables were investigated by Multiple Factorial Analysis (MFA). The analysis was run on the mean values of free amino acids, VOCs, color and sensory data at 30 and 120 days of shelf-life. Storage time and type of LP were supplied to the MFA as supplementary data, whereas sensory and instrumental measurements were considered as active. The number of factors to consider was chosen according to the Kaiser's criterion (eigenvalue > 1), as previously done by Boris et al. (2018). Univariate correlation analysis (based on Pearson regression coefficient) was also performed in support to the MFA for a better interpretation of the linear relationships among the variables. RStudio (version 3.3.3) and

STATISTICA 13.3 (StatSoft, Inc., Tulsa, OK) were used for both univariate and multivariate data analysis.

Result and Discussion

Proteolytic side activity and release of free amino acids

The proteolytic side activity of each LP was estimated by o-phthalaldehyde (OPA) method to get insights on their initial purity. **Figure 1** illustrates the results of the analysis and pointed out a significant difference in the proteolytic activity of the commercial LPs.

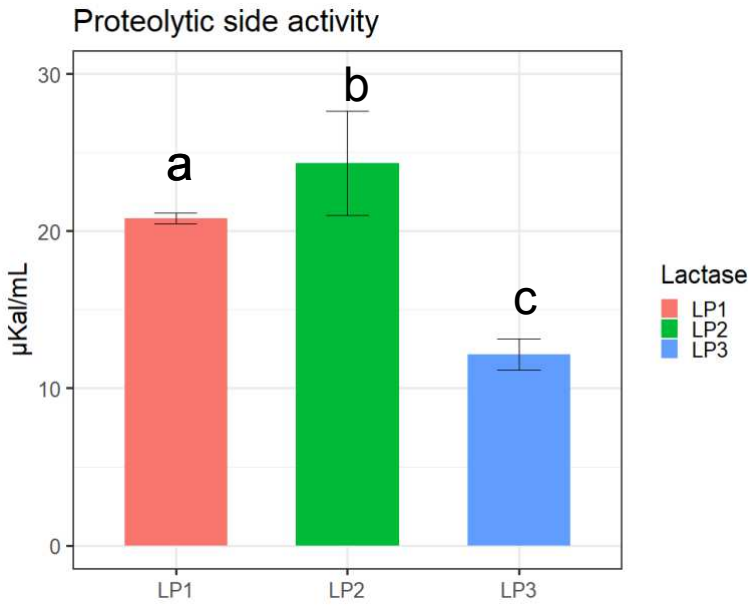


Figure 1. Proteolytic side activity of the tested commercial LPs estimated by o-phthalaldehyde (OPA) method. Nilac low-heat skim milk powder was used as substrate.

LP2 was characterized by the highest proteolytic activity and, as a consequence, it can be considered the least purified lactase according to the OPA method. Its level of proteolytic activity (µKal/mL) was significantly higher than LP1 (P=0.0218) and LP3 (P=0.0002). At the same time, the proteolytic side activity of LP3 was significantly lower than LP1 (P=0.0002), meaning that this enzymatic preparation was the one containing the lowest level of proteolytic side activity. The results confirmed that the lactases intended for UHLM production are available with various degree of purity in the market (Nielsen et al., 2018). Different separation methods are usually applied for their isolation from microorganisms (e.g. centrifugation and column chromatography), but apparently secondary proteases are not entirely removed (Zhao et al., 2019) and promote proteolysis in UHLM. To investigate the ability of the "in batch" production of minimizing unwanted proteolytic activity, LC-HRMS was applied to monitor the release of

free amino acids during storage. Following the HILIC zwitterionic separation, Cys was not detected whereas Ile and Leu were quantified as one single peak (Ile/Leu) because the separation of the two isomers was not achieved in milk samples, but only in calibration curve trials. Time course evolution of each amino acid (**Supplementary Figure 1**) showed that the concentration of Ile/Leu, Trp, Val, Phe, Tyr, Arg, His, Pro, Gln, ProOH, Glu and Asp was constant throughout the shelf-life of UHLM. These results confirmed that the milk produced by "in batch" system is poorly affected the proteolytic side activity of the LP, probably because these proteases are inactivated by the UHT treatment (Dekker et al., 2019; Troise et al., 2016).

Explorative Principal Component Analysis (PCA) was performed for dimension reduction to visualize possible patterns in the samples based on their free amino acids profile. A tendency of separation was shown by the bi-plot of the first two principal components (**Supplementary Figure 2**), but the best visualization of this trend was given by plotting the first (accounting for 26.1% of the explained variance) and the fifth principal components (7.3% of the explained variance) (**Figure 2**). This plot revealed that the tested LP contributed to a specific free amino acids profile in the milk and that this behavior was poorly related to the storage time. Apparently, the differences in free amino acids were registered already at the beginning of the shelf-life study, namely at 0 days of storage. Proteolysis occurred at different extent before the UHT treatment, likely during the 25-30 hours of incubation adopted to achieve the desired level of lactose hydrolysis. According to the "in batch" production system, lactose hydrolysis is carried out at refrigerated temperature (Harju et al., 2012), although this conditions is far from the optimal temperature of the lactase (35-65°C according to Mahoney, 1997). Therefore, a holding time is necessary to complete the batch hydrolysis implying higher dosages of LP (Tossavainen & Kallioinen, 2007). This explains the different release of free amino acids in the UHLM observed before shelf-life. The assumption is reasonable because when the lactase is not thermally inactivated, a typical feature of the "in pack" system, the action of the LP proteolytic side activity causes temporal changes in the free amino acids profile (Jansson, Jensen, Sundekilde, et al., 2014; Nielsen et al., 2017; Troise et al., 2016). Moreover, the relatively low percentage of variance explained by the PCA model may suggest that, in realistic industrial settings, the breakdown of the milk protein is limited when UHLM is produced "in batch" due to the destructive effect of the UHT treatment on the proteolytic side activity of the LP.

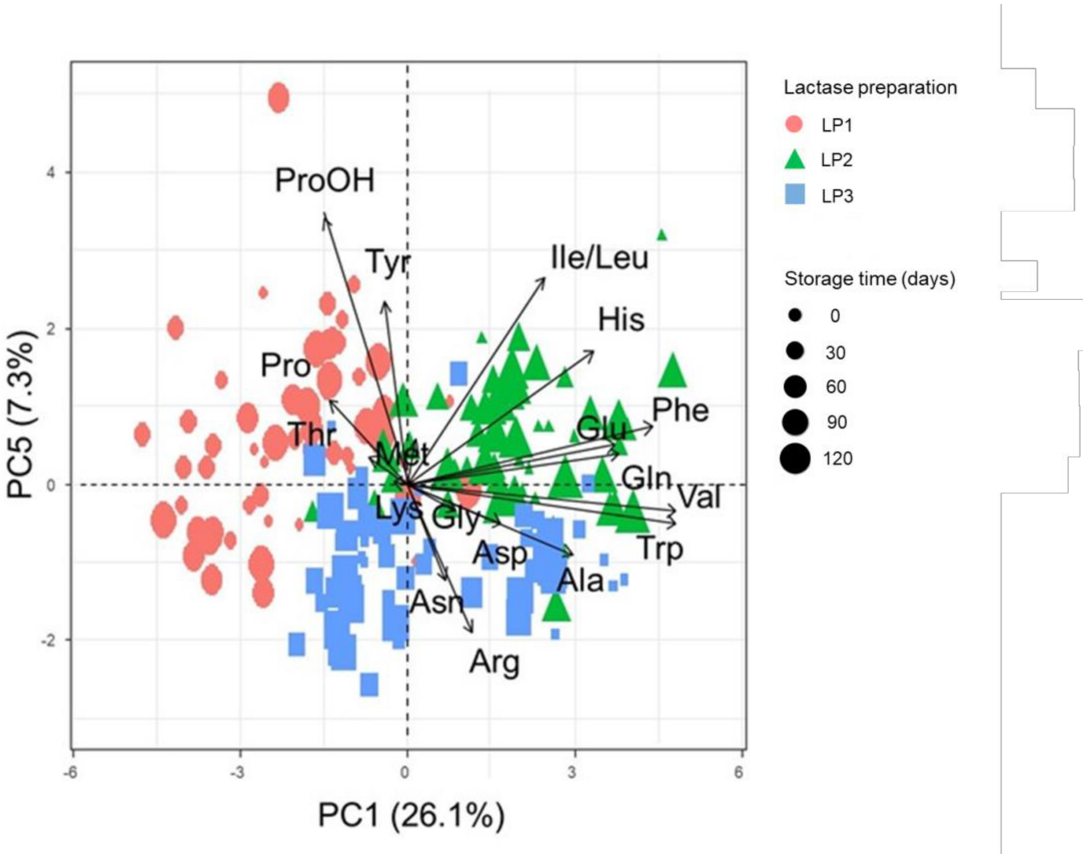


Figure 2. Principal Component Analysis (PCA) bi-plot of the first (PC1) and the fifth (PC5) principal components performed on the free amino acids data of the UHLM samples produced by "in batch" system with LP1 (red), LP2 (green), LP3 (blue) during storage at 20°C for 120 days. The explained variance for each component is also reported. The proceeding of the storage time is represented by an increasing size of the dots. Black arrows indicated the corresponding weights of the loadings on the spatial distribution of the UHLM samples.

In order to better identify the variables responsible for the observed trends, PLS-DA was applied. This model relies on the rotation of the PCA components to maximize the separation among the groups of observation (Aprea et al., 2011). The score plot of the PLS-DA model is given in **Figure 3a**. The robustness of the model was confirmed by the low balanced error rate obtained (BER = 0.03125) as well as by a correct responses in prediction higher than 90% for all the cases (**Table 2**).

Table 2. Confusion matrix generated by the PLS-DA model for the different UHLM produced by "in batch" system using three different LP. The last column reports the percentage of correct classifications based on the maximum prediction distance. Training set (2/3 of the dataset) and test set (1/3 of the dataset) were generated in random manner to compute the model.

Lactase preparations	Predicted as LP1	Predicted as LP2	Predicted as LP3	Total	% correct
LP1	20	0	0	20	100%
LP2	0	21	0	21	100%
LP3	1	2	29	32	91%

Next, the impact of each amino acid on the classification was investigated by calculating the variable importance in prediction value (VIP): those higher than 1 (**Figure 3b**) were considered the most relevant for explaining the separation (Aprea et al., 2011). In total, 6 out of the 18 quantified amino acids demonstrated a VIP>1, with Ile/Leu and ProOH registering the highest values (VIP equal to 2.41 and 1.69 respectively). Large amounts of hydroxyproline (ProOH) and proline (Pro) are present in collagen and milk protein (Wu et al., 2011), specifically hydroxyproline is not detected in milk background in free form and its concentration may arise as a consequence of protein hydrolysis or free proline hydroxylation (Abernethy & Higgs, 2013). The fact that the levels of ProOH in the analyzed samples were 6-7 times lower than Pro confirmed the assumption. ProOH separated UHLM with LP1 from LP2 and LP3, while Ile/Leu, Glu, Trp and Val separated the UHLM with LP2 from the rest of the samples. The same pattern was visualized by the loadings of the PCA described in **Figure 2**. All together the results demonstrated higher concentration of free amino acids in UHLM containing LP2, namely the lactase characterized by the highest level of proteolytic side activity according to the OPA results (**Figure 1**). Differently, milk with LP3 was the more purified lactase and it was slightly affected by the release of free amino acids.

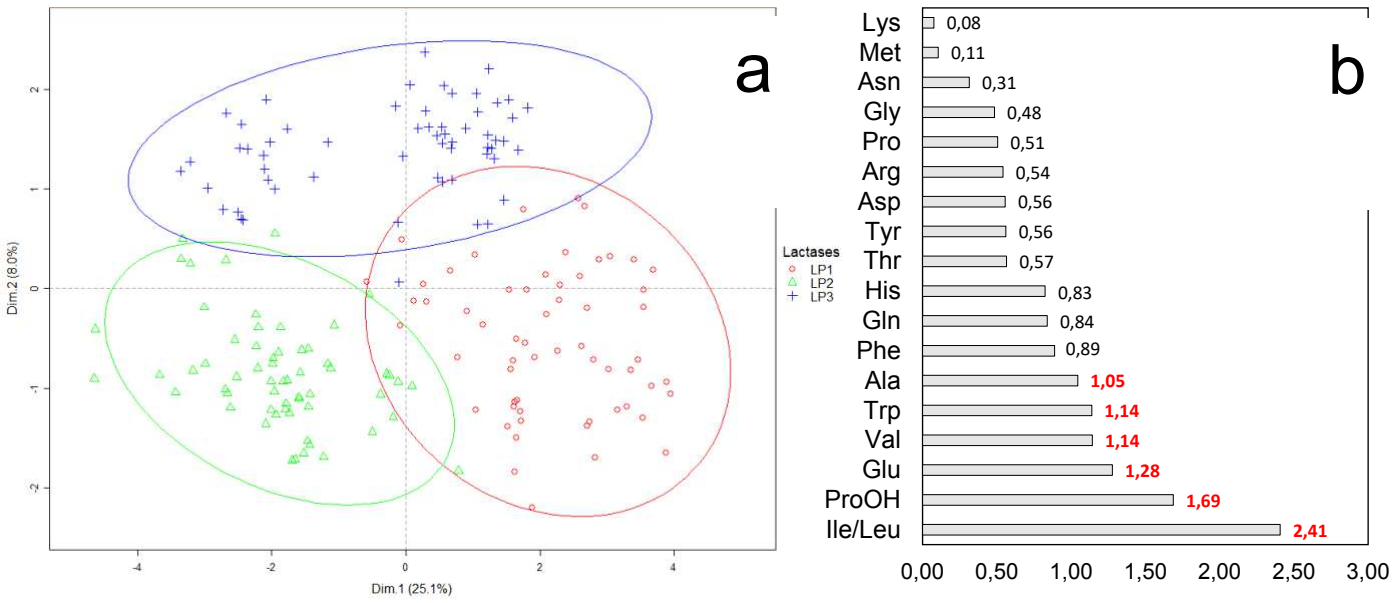


Figure 3a. Partial Least Square Regression Discriminant Analysis (PLS-DA) score plot performed on the free amino acids data showing classification of samples according to the added LP. The model was validated by a 7-fold cross-validation permuted for 100 times. Classification performances were assessed focusing on the balanced error rate (BER) and on the confusion matrix according to Rohart et al. (2017). Their estimation was carried out dividing the data in training (2/3 of the dataset) and test set (1/3 of the dataset) in random manner. **Figure 3b.** Variable importance in projection (VIP) scores of each variable used in the PLS-DA model.

According to Jansson, Jensen, Sundekilde, et al. (2014) and Nielsen et al. (2018), proteolysis in UHLM targets the intact *β-casein (β-CN)* and α_{s1}-casein (α_{s1}-CN), denoting a specificity of the secondary proteases to particular protein structures. Nielsen et al. (2018) in particular, reported that degradation of *β-CN* and α_{s1}-CN vary depending on the LP purity and is related to a mutual presence of exopeptidases and endopeptidases in the preparations, which release C-terminal amino acids residues (Ile-Ile-Val, Ile-Val and Trp) in the milk during storage. Our results indicated higher levels of free Ile/Leu, Val and Trp in the UHLM produced with LP2. Therefore, we might consider the release of free amino acids in UHLM as an interplay between the different degree of proteolytic side activity of the LP and the specificity of these proteases towards particular sites of *β-CN* and α_{s1}-CN. On the other hand, we cannot exclude that also other types of protein are degraded by the proteolytic side lactase activity of LP. Overall, the results

demonstrated that the release of free amino acids in UHLM produced by "in batch" depends on the initial proteolytic side activity of the LP, at least until the UHT treatment is applied.

Volatiles organic compounds profile

Table 3 summarizes the VOCs measured by HS SPME GC-MS. A total of 22 VOCs matched the spectra of the NIST/Wiley libraries, with several compounds already reported in other published articles on both regular and hydrolyzed-lactose UHT milk (Jansson, Jensen, Eggers, et al., 2014c; Jeon, Thomas, & Reineccius, 1978; Rysstad, Ebbesen, & Eggestad, 1998). The calculated LRIs for each VOC were similar to the one previously reported by our group (Bottiroli, Aprea, et al., 2020; Bottiroli, Pedrotti, et al., 2020), as well as to the ones avaiable from the NIST library.

Table 3. List of the volatile compounds (VOCs) identified and quantified in the headspace of the UHLM produced by "in batch" system using the three different commercial LP during storage at 20°C for 120 days by SPME GC-MS. The concentrations expressed as μg/L of internal standard (4-methyl-2-pentanone) for each VOCs analyzed in UHLM sample are available in the Appendix (**Supplementary Table 1**).

Compound name[a]	RT[b]	LRI[c]	ReferenceLRI[d]
2-Butanone	2.66	909	907 ± 11
2-Methylbutanal	2.83	918	914 ± 8
2-Pentanone	4.01	985	981 ± 11
Toluene	5.49	1051	1042 ± 11
Hexanal	6.62	1099	1083 ± 8
Ethyll-benzene	7.90	1137	1129 ± 8
2-Heptanone	9.70	1191	1182 ± 8
2-Nonanone	16.61	1395	1390 ± 7
Nonanal	16.77	1400	1391 ± 8
Benzaldehyde	20.92	1531	1520 ± 14
1-Octanol	22.07	1576	1557 ± 8
2-Undecanone	23.16	1605	1598 ± 6
γ-Butyrolactone	24.07	1636	1632 ± 15
Aceto-phenone	24.74	1660	1647 ± 13
2-Tridecanone	29.16	1818	1809 ± 6
Hexanoic acid	31.06	1890	1846 ± 12
Dimethyl-sulfone	31.56	1909	1903 ± 9
p-Cresol	36.18	2095	2080 ± 12
m-Cresol	36.36	2103	2091 ± 18
Octanoic acid	36.40	2104	2060 ± 15
δ-Decalactone	38.62	2198	2194 ± 15
Decanoic acid	42.25	2295	2267 ± 14

[a] Compound identification by match with the NIST-2014/Wiley 7.0 libraries.
[b] Retention time (min)
[c] Linear retention index
[d] Reference linear retention index reported in the NIST-2014 library

Statistically significant differences were found in the VOCs profile of the UHLM produced with the different LPs. **Figure 4** shows that 2-methylbutanal (**Figure 4a**) and benzaldehyde (**Figure 4c**) exhibited different temporal evolution depending on the LP employed. Both volatile compounds are mainly formed in UHT milk from the intermediate stages of MR through Strecker degradation (van Boekel, 2006). The reaction arises when the amino acids react either

with α-dicarbonyl compounds or Amadori products by deamination and decarboxylation (Cremer, Vollenbroeker, & Eichner, 2000; Hofmann & Schieberle, 2000).

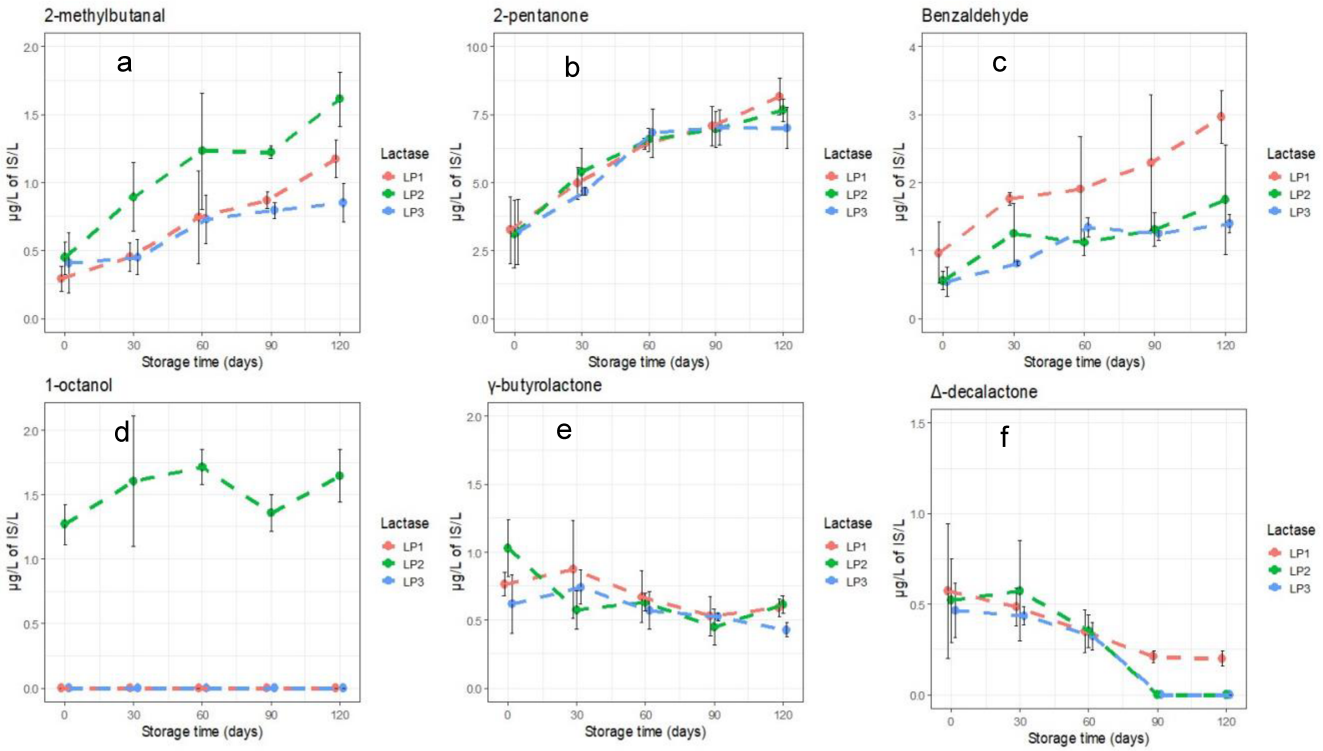


Figure 4. Concentration of 2-methylbutanal, 2-pentanone, benzaldehyde, 1-octanol, γ-butyrolactone and δ-decalactone detected by HS SPME GC-MS in the UHLMs produced with LP1, LP2, LP3 during storage at 20°C for 120 days. The data are the mean values of the three replicates of production (n=3).

The aldehyde 2-methylbutanal is formed *via* the degradation of α-amino acids initiated by carbonyl compounds and, alternatively, by branching-off of Ile/Leu Amadori compounds (Cremer et al., 2000; Jansson, Clausen, et al., 2014). Benzaldehyde is also formed *via* Strecker degradation of Phe and subsequent oxidation of phenylacetaldeyde (Fong & Yaylayan, 2008). Overall, specific trends in the release of Strecker aldehydes were shown in relation to the initial purity of the tested LP. The formation rate of 2-methybutanal was higher in the UHLM containing LP1 and LP2 compared to LP3.

The highest concentration of this compound was reached at the end of the shelf-life study by the UHLM with LP2. Interestingly, the release of 2-methylbutanal was proportional to the initial

higher levels of free Ile/Leu in this product as well as to the lower purity of LP2. Similarly, benzaldehyde remarkably increased in UHLM produced with LP1 and the concentration was significantly higher after 120 days of storage compared to the UHLM with LP3. In this case, a match with the trend of Phe was not found, as the initial concentration of the free amino acid in the UHLMs did not differ based on the tested LP. In combination with the free amino acids profiles, the measurement of 2-methylbutanal and benzaldehyde are therefore important data to understand the pathway of off-flavor formation in UHLM depending on the LP purity. The free amino acids released by the proteolytic side activity of LP fuel the on-going MR and, together with the reducing sugar from lactose hydrolysis, result in the formation of flavor-active Strecker aldehyde responsible for changes in the UHLM flavor (Nielsen et al., 2017).

Mitigation strategies for undesired flavor development have been recently outlined by Jansson and colleagues (2019): addition of green tea extract (GTE) favored a reduction in the concentration of Strecker aldehydes in UHLM, with concomitant decrease in the mushroom flavor of the product. According to Nielsen et al. (2017), Strecker aldehydes contribute to the development of stale flavor in UHLM during storage. Strecker-derived volatiles compounds can therefore be considered the bridge to correlate the initial level of proteolytic side activity of the LP with the proceeding of MR and the off-flavor development in UHLM. In our case, the combined measurements of VOCs and free amino acids demonstrated that, despite the thermal inactivation of the LP during "in batch" production, MR proceeded in UHLM at different extent based on the LP employed. Besides the proteolytic activity, the arylsulfatase activity coming from the LP may confer "cowshed-like" off-flavor to the milk during storage (Stressler et al., 2018). This is related to the formation of p-cresol from the sulphonated-cresol naturally present in milk (Dekker et al., 2019). In our study, p-cresol was detected but its concentration did not differ significantly in the UHLMs based on the LP and was constant throughout the storage, indicating a poor contribution of the arylsulfatase activity to the final milk flavor.

Figure 4 shows the trends over time of other VOCs highlighting those compounds that increased significantly during shelf-life, such as 2-pentanone (**Figure 4b**). Some other methyl-ketones followed a similar trend but the batch-to-batch milk variability hindered the possibility of spotting significant variations. Methyl-ketones are thermally-induced compounds in milk. Their increase upon UHT processing is broadly discussed in literature, as the class is correlated to the severity of the heat treatment applied (Contarini & Povolo, 2002; Contarini, Povolo,

Leardi, & Toppino, 1997; Valero, Villamiel, Miralles, Sanz, & Martínez-Castro, 2001; Vazquez-Landaverde, Velazquez, Torres, & Qian, 2005).

In contrast with the increase in methyl-ketones, a decrease in lactones (**Figure 4e**, **Figure 4f**) was observed during storage. Nevertheless, the trends highlighted by methyl-ketones and lactones are typical of UHT milk aging and did not end up in a differentiation of the UHLM based on the LP. The data of 1-octanol in UHLM (**Figure 4d**) were particularly interesting: this compound was detected only in the UHLM produced with LP2 and remained constant throughout its shelf-life. Literature lacks of information regarding detection of 1-octanol in milk. Thus, it was reasonable to measure the volatile profiles of the three LP as such (**Figure 5**) in order to verify how 1-octanol ended up in the UHLM with LP2. As expected, the peak of 1-octanol (RT: 22.06) was detected only in LP2. Thus, the higher level of proteolytic side activity was not the only factor differentiating LP2 from the other tested lactases. This latter result indicated that the initial VOCs profile of the LP may be an additional parameter for further investigation of the differences among UHLM produced with different lactase. To the best of our knowledge, this was the first time this aspect was taken into consideration.

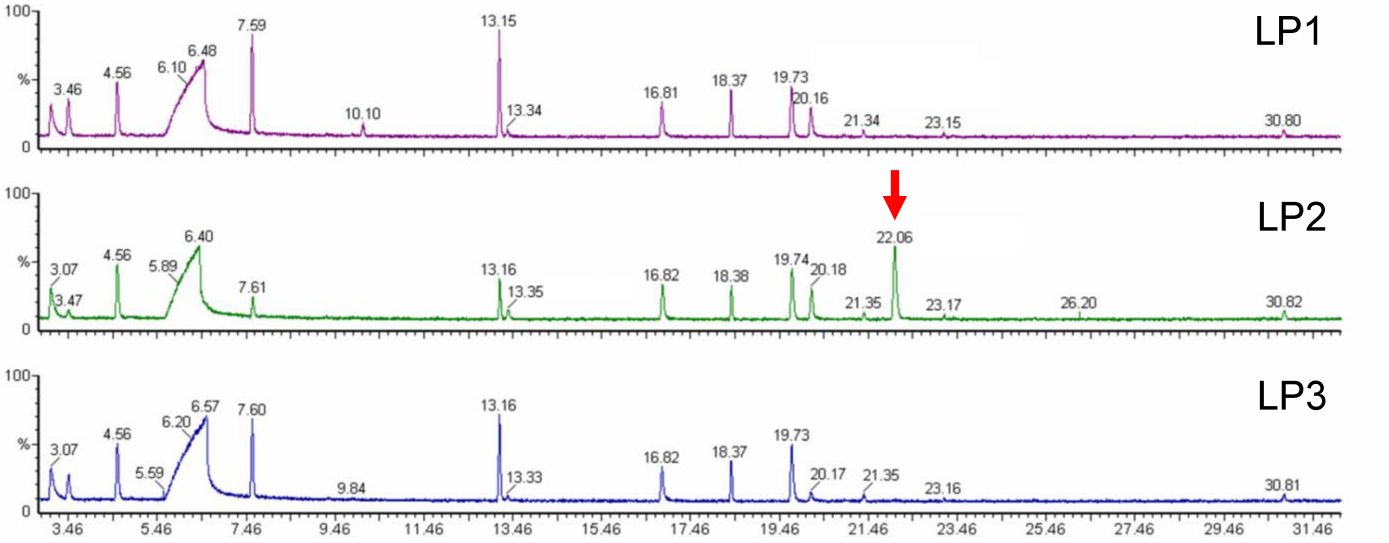


Fig 5. GC-MS chromatograms coming from the analysis of the three different LP studied. *Purple*: LP1; *Green*: LP2; *Blue*: LP3. Chromatograms are reported as total ion current (TIC). LP were diluted with demineralized water to a concentration comparable to their level in the UHLM samples. The arrow indicates the peak associated with 1-octanol (RT: 22.06).

Color, sensory changes and correlation with small molecules analysis

The changes in the color of the analyzed UHLM are reported in the Appendix (**Supplementary Figure 3**). The color of the milk did not differ as function of the LP employed for production. L* (black: L* = 0 and white: L*=100) and b* (yellow-blue: negative b* = blueness and positive b* = yellowness) remained constant, while a* (red-green: negative a* = greenness and positive a* = redness) increased significantly during storage. This results are in line with a recent study published by Jansson et al. (2019). The increase in red color denotes non-enzymatic browning undergoing in the milk during storage (Bosch, Alegría, Farré, & Clemente, 2007).

Sensory profiling of UHLMs was determined at 30 and 120 days of storage at 20°C. UHLMs were not perceived as different based on the tested LP. Furthermore, descriptors associated with a negative impact on the milk sensory properties (cooked flavor, stale flavor and irregular flavor) did not registered a significant increase during the shelf-life. Thus, all the milk samples did not develop significant defects during storage, although the positive attributes white color and milky flavor significantly decreased after prolonged shelf-life. The absence of differences

among the sensory profile of UHLM produced with the different lactases is an important result at industrial level, because the retail price of LP is related to the relative amount of proteolytic side activity present in the preparation, with more purified lactases costing the most (Mittal, Newell, Hourigan, & Zadow, 1991). Our results suggested that, because the quality of "in batch" UHLM is poorly correlated to the initial LP purity, production costs may be lowered by using less purified lactase. Indications on this aspect are however limited by the number of LPs we tested, so a higher number of commercial options should be evaluated in the future to validate the hypothesis.

Sensory attributes and analytes were then correlated by Multiple Factorial Analysis (MFA) to explore associations among the observations. **Figure 6** illustrates the relative loading plot of the MFA analysis that coherently interpreted the sensory and instrumental data (RVs > 0.69). Three factors, namely Dim1 (24.78%), Dim2 (12.75%) and Dim3 (12.51%), were considered significant and explained 50.04% of the total variance. Significant contribution to Dim1 was given by the sensory (29.77%), color (29.58%) and VOCs (26.48%) data, whereas only color (38.22%) and VOCs (32.42%) had a relevant impact on Dim2. Interestingly, free amino acids was the group of variables providing the highest contribution to Dim3 (64.25%). The release of free amino acids in UHLM, which we associated to an effect of the tested LP, was confirmed as a separated phenomenon from the trends exhibited by the other groups of variables, which we associated to an effect of the storage time. Because the trained panel did not recognized any effects of the different LPs on the sensory quality of UHLM, it was reasonable to use the combination of Dim1 and Dim2 to describe the correlations of the sensory properties with the instrumental parameters.

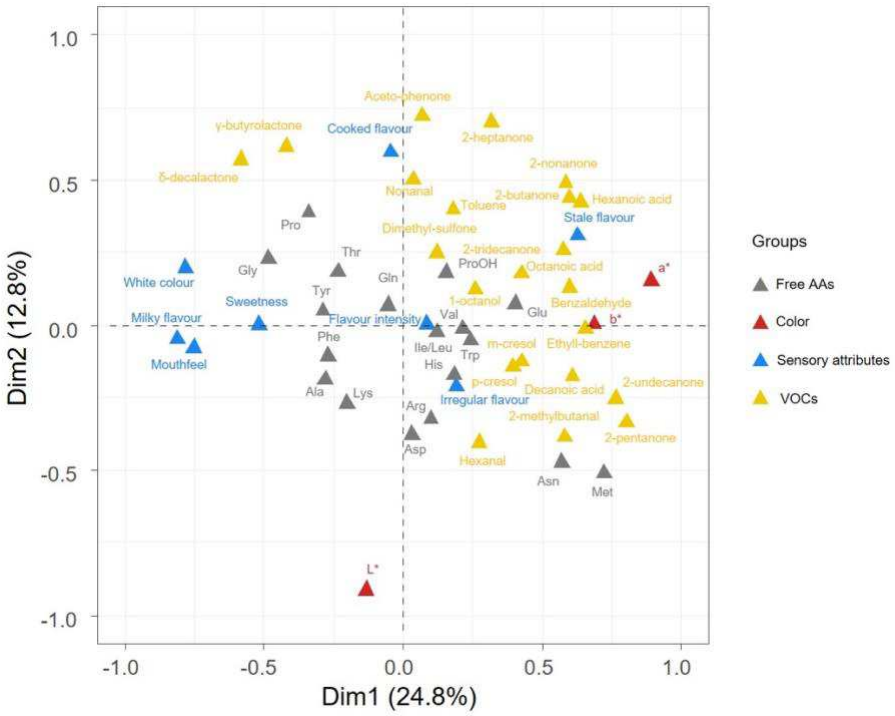


Figure 6. Multiple factor analysis (MFA) loading plot of the quantitative variable projected on Dim1 and Dim 2 (24.8% and 12.8% of the total variance respectively). The analysis was performed on the mean values of the free amino acids(grey), VOCs (yellow), color (red) and sensory (blue) data extrapolated from the analysis of the UHLM samples produced with LP1, LP2 and LP3 at 30 and 120 days of shelf-life.

Dim1 reflected the effects of the storage time on the sensory and chemical properties of UHLM. White color, milky flavor and mouthfeel gave strong negative contributions to the dimension and were inversely correlated to stale flavor, some VOCs and the a* and b* color parameters. Looking at the Pearson correlation coefficient (r), white color registered the highest negative relationship with a* (r = -0.83), which was at the same time positively correlated to methyl ketones (r > 0.65 for 2-butanone, 2-pentanone, 2-nonanone and 2-undecanone) and, at lower degree, to 2-methylbutanal (r=0.49) and benzaldehyde (r = 0.55). MR underlies the results: the reaction is facilitated in UHLM due to the higher reactivity of the formed glucose and galactose (Naranjo, Gonzales, Leiva, & Malec, 2013), Strecker aldehydes are formed at the intermediate stages (van Boekel, 2006) and its proceeding eventually ends up in the formation of brown pigments and polymers as melanoidins (van Boekel, 2001). Formation of brown pigments, already estimated by Sunds and coworkers (2018) by the CIELAB system, explained the

significant increase of a* (+a*= redness, -a* = greenness) registered in our experiment. The Strecker aldehydes themselves could also have contributed to such change in color, as a result of condensation (Morales & van Boekel, 1998), even if the major contribution of Strecker degradation is given to the off-flavors. Indeed, stale flavor, one of the most encountered sensory defects in UHT milk, correlated positively with cooked flavor (r = 0.55) and negatively with milky flavor (r = -0.60) and mouthfeel (r = -0.52) indicating its increase during storage at the expenses of those sensory attributes highly desirable in milk. Stale flavor is associated to an increase in methyl ketones and aldehydes during the shelf-life for both UHLM and regular UHT milk (Jensen et al., 2015; Perkins, Elliott, D'Arcy, & Deeth, 2005). In our study, positive Pearson coefficients were found between stale flavor and 2-butanone (r = 0.48), hexanoic acid (r = 0.57) and octanoic acid (r = 0.49). While 2-butanone was already pointed out as good predictor of the flavor in UHLM (Jensen et al., 2015), the correlation with the organic acids indicated that the sensory attribute was considered by the panel probably also as "rancid" and/or "oxidized". Overall, the MFA unveiled the relationship among sensory and instrumental observations in UHLM produced by "in batch" technology. The model gave clear insights on the impact that LP and storage time provided to the UHLM characteristics, emphasizing the role of the latter in the definition of the final product quality.

Conclusion

Commercial LPs can have different degrees of proteolytic activity. Our study provided evidence about the importance of choosing the proper lactase preparation in combination with the production process in order to produce high quality UHLM. Specifically, we demonstrated that during "in batch" production, the lactase purity does not play a crucial role, as most of the proteolytic activity is inactivated by the thermal treatment. However, the pool of free amino acids produced during the "in batch" hydrolysis is a reservoir of reactive molecules which allow the formation of volatiles during shelf-life, at different extent depending on the purity of the LP. This did not end up in a differentiation of the milk color and sensory properties. The increased diffusion of UHLM in Asia is calling the manufacturing of products with a longer shelf-life. Currently the shelf-life of UHLM is limited: understanding the mechanisms of milk aging is the prerequisite to develop strategies prolonging it up to 9-12 months, as for conventional UHT milk.

References

Abernethy, G., & Higgs, K. (2013). Rapid detection of economic adulterants in fresh milk by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, *1288*, 10–20. https://doi.org/10.1016/j.chroma.2013.02.022

Aprea, E., Gika, H., Carlin, S., Theodoridis, G., Vrhovsek, U., & Mattivi, F. (2011). Metabolite profiling on apple volatile content based on solid phase microextraction and gas-chromatography time of flight mass spectrometry. *Journal of Chromatography A*, *1218*(28), 4517–4524. https://doi.org/10.1016/j.chroma.2011.05.019

Boris, S., Martin, K., Matej, P., Anna, R., Jana, M., Bohuslava, T., & Eva, D. (2018). Application of multiple factor analysis for the descriptive sensory evaluation and instrumental measurements of bryndza cheese as affected by vacuum packaging. *International Journal of Food Properties*, *21*(1), 1508–1522. https://doi.org/10.1080/10942912.2018.1494194

Bosch, L., Alegría, A., Farré, R., & Clemente, G. (2007). Fluorescence and color as markers for the Maillard reaction in milk-cereal based infant foods during storage. *Food Chemistry*, *105*(3), 1135–1143. https://doi.org/10.1016/j.foodchem.2007.02.016

Bottiroli, R., Aprea, E., Betta, E., Fogliano, V., & Gasperi, F. (2020). Application of headspace solid-phase micro-extraction gas chromatography for the assessment of the volatiles profiles of ultra-high temperature hydrolysed-lactose milk during production and storage. *International Dairy Journal*, 104715. https://doi.org/10.1016/j.idairyj.2020.104715

Bottiroli, R., Pedrotti, M., Aprea, E., Biasioli, F., Fogliano, V., & Gasperi, F. (2020). Application of PTR-TOF-MS for the quality assessment of lactose-free milk : Effect of storage time and employment of different lactase preparations. *Journal of Mass Spectrometry*, (February), 1–9. https://doi.org/10.1002/jms.4505

Contarini, G., & Povolo, M. (2002). Volatile fraction of milk: Comparison between, purge and trap and solid phase microextraction techniques. *Journal of Agricultural and Food Chemistry*, *50*(25), 7350–7355. https://doi.org/10.1021/jf025713a

Contarini, G., Povolo, M., Leardi, R., & Toppino, P. M. (1997). Influence of Heat Treatment on the Volatile Compounds of Milk. *Journal of Agricultural and Food Chemistry*, *45*(3), 3171–3177. Retrieved from https://pubs.acs.org/sharingguidelines

Cremer, D. R., Vollenbroeker, M., & Eichner, K. (2000). Investigation of the formation of Strecker aldehydes from the reaction of Amadori rearrangement products with of-amino acids in

low moisture model systems. *European Food Research and Technology*, *211*(6), 400–403. https://doi.org/10.1007/s002170000196

Dekker, P. J. T., Koenders, D., & Bruins, M. J. (2019). Lactose-Free Dairy Products: Market Developments, Production, Nutrition and Health Benefits. *Nutrients*, *11*(3), 551. https://doi.org/10.3390/nu11030551

Deng, Y., Misselwitz, B., Dai, N., & Fox, M. (2015). Lactose intolerance in adults: Biological mechanism and dietary management. *Nutrients*, *7*(9), 8020–8035. https://doi.org/10.3390/nu7095380

Fong, L. C., & Yaylayan, V. A. (2008). Model studies on the oxygen-induced formation of benzaldehyde from phenylacetaldehyde using pyrolysis GC-MS and FTIR. *Journal of Agricultural and Food Chemistry*, *56*(22), 10697–10704. https://doi.org/10.1021/jf8022468

Fuller, F., Huang, J., Ma, H., & Rozelle, S. (2006). Got milk? The rapid rise of China's dairy sector and its future prospects. *Food Policy*, *31*(3 SPEC. ISS.), 201–215. https://doi.org/10.1016/j.foodpol.2006.03.002

Harju, M., Kallioinen, H., & Tossavainen, O. (2012, February). Lactose hydrolysis and other conversions in dairy products: Technological aspects. *International Dairy Journal*. https://doi.org/10.1016/j.idairyj.2011.09.011

Hofmann, T., & Schieberle, P. (2000). Formation of aroma-active Strecker-aldehydes by a direct oxidative degradation of Amadori compounds. *Journal of Agricultural and Food Chemistry*, *48*(9), 4301–4305. https://doi.org/10.1021/jf000076e

Itan, Y., Jones, B. L., Ingram, C. J., Swallow, D. M., & Thomas, M. G. (2010). *A worldwide correlation of lactase persistence phenotype and genotypes*. Retrieved from http://www.biomedcentral.com/1471-2148/10/36

Jansson, T. (2014). Chemical changes and off-flavor development in lactose- hydrolyzed UHT milk during storage. *Aarhus University, Department of Food Science.*, 1–192.

Jansson, T., Clausen, M. R., Sundekilde, U. K., Eggers, N., Nyegaard, S., Larsen, L. B., … Bertram, H. C. (2014). Lactose-hydrolyzed milk is more prone to chemical changes during storage than conventional ultra-high-temperature (UHT) milk. *Journal of Agricultural and Food Chemistry*, *62*(31), 7886–7896. https://doi.org/10.1021/jf501671z

Jansson, T., Jensen, H. B., Sundekilde, U. K., Clausen, M. R., Eggers, N., Larsen, L. B., … Bertram, H. C. (2014). Chemical and proteolysis-derived changes during long-term storage of lactose-

hydrolyzed ultrahigh-temperature (UHT) milk. *Journal of Agricultural and Food Chemistry*, *62*(46), 11270–11278. https://doi.org/10.1021/jf504104q

Jansson, T., Jensen, S., Eggers, N., Clausen, M. R., Larsen, L. B., Ray, C., … Bertram, H. C. (2014). Volatile component profiles of conventional and lactose-hydrolyzed UHT milk - A dynamic headspace gas chromatography-mass spectrometry study. *Dairy Science and Technology*, *94*(4), 311–325. https://doi.org/10.1007/s13594-014-0164-7

Jansson, T., Waehrens, S. S., Rauh, V., Danielsen, B. P., Sørensen, J., Bredie, W. L. P., … Lund, M. N. (2019). Effect of green tea catechins on physical stability and sensory quality of lactose-reduced UHT milk during storage for one year. *International Dairy Journal*, *95*, 25–34. https://doi.org/10.1016/j.idairyj.2019.03.007

Jelen, P., & Tossavainen, O. (2003). Low lactose and lactose-free milk and dairy products - Prospects, technologies and applications. *Australian Journal of Dairy Technology*, *58*(2), 161–165. https://doi.org/http://www.scopus.com/inward/record.url?eid=2-s2.0-0141595866&partnerID=tZOtx3y1

Jensen, S., Jansson, T., Eggers, N., Clausen, M. R., Larsen, L. B., Jensen, H. B., … Bertram, H. C. (2015). Storage-induced changes in the sensory characteristics and volatiles of conventional and lactose-hydrolyzed UHT processed milk. *European Food Research and Technology*, *240*(6), 1247–1257. https://doi.org/10.1007/s00217-015-2427-9

Jeon, I. J., Thomas, E. L., & Reineccius, G. A. (1978). Production of volatile flavor compounds in ultrahigh-temperature processed milk during aseptic storage. *Journal of Agricultural and Food Chemistry*, *26*(5), 1183–1188. https://doi.org/10.1021/jf60219a039

Mahoney, R. R. (1997). Lactose: Enzymatic Modification. In P. F. Fox (Ed.), *Advanced Dairy Chemistry Volume 3: Lactose, water, salts and vitamins* (pp. 77–125). Boston, MA: Springer US. https://doi.org/10.1007/978-1-4757-4409-5_3

Mendoza, M. R., Olano, A., & Villamiel, M. (2005). Chemical indicators of heat treatment in fortified and special milks. *Journal of Agricultural and Food Chemistry*, *53*(8), 2995–2999. https://doi.org/10.1021/jf040406l

Mittal, S., Newell, G., Hourigan, J., & Zadow, J. (1991). The effect of protease contamination in lactase on the flavour of lactose-hydrolyzed milks. *Australian Journal of Dairy Technology*, *46*(1), 46–47.

Morales, F. J., & Van Boekel, M. A. J. S. (1998). A study on advanced Maillard reaction in heated

casein/sugar solutions: Colour formation. *International Dairy Journal*, *8*(10–11), 907–915. https://doi.org/10.1016/S0958-6946(99)00014-X

Naranjo, G. B., Gonzales, A. S. P., Leiva, G. E., & Malec, L. S. (2013). The kinetics of Maillard reaction in lactose-hydrolysed milk powder and related systems containing carbohydrate mixtures. *Food Chemistry*, *141*(4), 3790–3795. https://doi.org/10.1016/j.foodchem.2013.06.093

Nielsen, S. D., Jansson, T., Le, T. T., Jensen, S., Eggers, N., Rauh, V., … Larsen, L. B. (2017). Correlation between sensory properties and peptides derived from hydrolysed-lactose UHT milk during storage. *International Dairy Journal*, *68*, 23–31. https://doi.org/10.1016/j.idairyj.2016.12.013

Nielsen, S. D., Zhao, D., Le, T. T., Rauh, V., Sørensen, J., Andersen, H. J., & Larsen, L. B. (2018). Proteolytic side-activity of lactase preparations. *International Dairy Journal*, *78*, 159–168. https://doi.org/10.1016/j.idairyj.2017.12.001

Perkins, M. L., Elliott, A. J., D'Arcy, B. R., & Deeth, H. C. (2005). Stale flavour volatiles in Australian commercial UHT milk during storage. *Australian Journal of Dairy Technology*, *60*(3), 231–237.

Rysstad, G., Ebbesen, A., & Eggestad, J. (2009). Sensory and chemical quality of UHT-milk stored in paperboard cartons with different oxygen and light barriers. *Food Additives & Contaminants*, *15*(1), 112–122.

Sahin, S., Hamamci, H., & Garayev, S. (2016). Rheological properties of lactose-free dairy desserts. *Food Science and Technology International*, *22*(7), 609–620. https://doi.org/10.1177/1082013216636262

Stressler, T., Reichenberger, K., Glück, C., Leptihn, S., Pfannstiel, J., Swietalski, P., … Fischer, L. (2018). A natural variant of arylsulfatase from Kluyveromyces lactis shows no formylglycine modification and has no enzyme activity. *Applied Microbiology and Biotechnology*, *102*(6), 2709–2721. https://doi.org/10.1007/s00253-018-8828-5

Sunds, A. V., Rauh, V. M., Sørensen, J., & Larsen, L. B. (2018). Maillard reaction progress in UHT milk during storage at different temperature levels and cycles. *International Dairy Journal*. https://doi.org/10.1016/j.idairyj.2017.08.008

Tossavainen, O., & Kallioinen, H. (2007). Proteolytic changes in lactose hydrolysed UHT milks during storage. *Milchwissenschaft*, *62*(4), 410–415. Retrieved from

http://cat.inist.fr/?aModele=afficheN&cpsidt=19123002

Troise, A. D., Bandini, E., De Donno, R., Meijer, G., Trezzi, M., & Fogliano, V. (2016). The quality of low lactose milk is affected by the side proteolytic activity of the lactase used in the production process. *Food Research International*, *89*, 514–525. https://doi.org/10.1016/j.foodres.2016.08.021

Troise, A. D., Wiltafsky, M., Fogliano, V., & Vitaglione, P. (2018). The quantification of free Amadori compounds and amino acids allows to model the bound Maillard reaction products formation in soybean products. *Food Chemistry*, *247*, 29–38. https://doi.org/10.1016/J.FOODCHEM.2017.12.019

Valero, E., Villamiel, M., Miralles, B., Sanz, J., & Martínez-Castro, I. (2001). Changes in flavour and volatile components during storage of whole and skimmed UHT milk. *Food Chemistry*, *72*(1), 51–58. https://doi.org/10.1016/S0308-8146(00)00203-X

van Boekel, M. A. J. S. (2006). Formation of flavour compounds in the Maillard reaction. *Biotechnology Advances*, *24*(2), 230–233. https://doi.org/10.1016/J.BIOTECHADV.2005.11.004

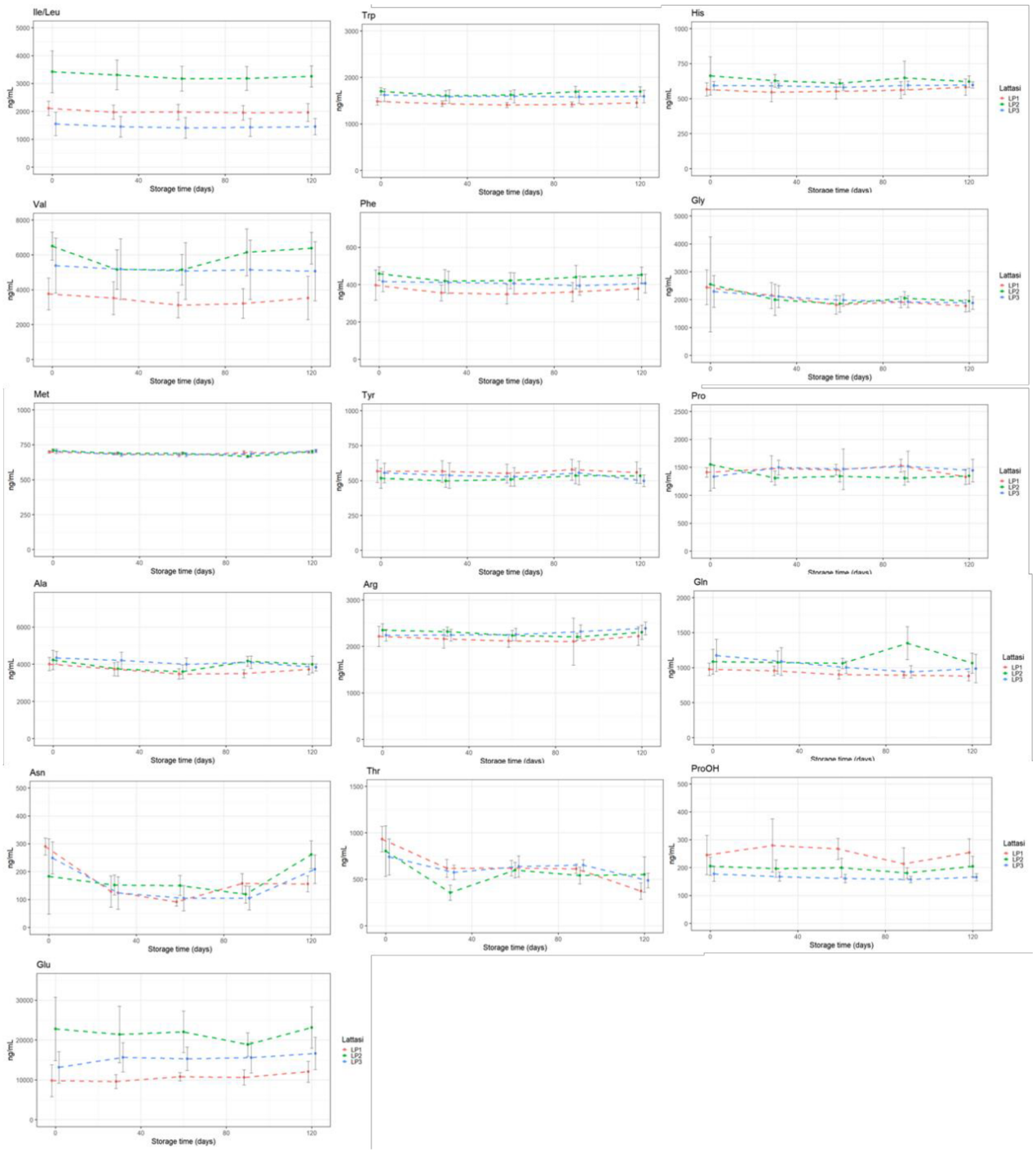
Van Boekel, M. A. J. S. (2001). Kinetic aspects of the Maillard reaction: A critical review. *Nahrung - Food*, *45*(3), 150–159. https://doi.org/10.1002/1521-3803(20010601)45:3<150::AID-FOOD150>3.0.CO;2-9

Vazquez-Landaverde, P. A., Velazquez, G., Torres, J. A., & Qian, M. C. (2005). Quantitative determination of thermally derived off-flavor compounds in milk using solid-phase microextraction and gas chromatography. *Journal of Dairy Science*, *88*(11), 3764–3772. https://doi.org/10.3168/jds.S0022-0302(05)73062-9

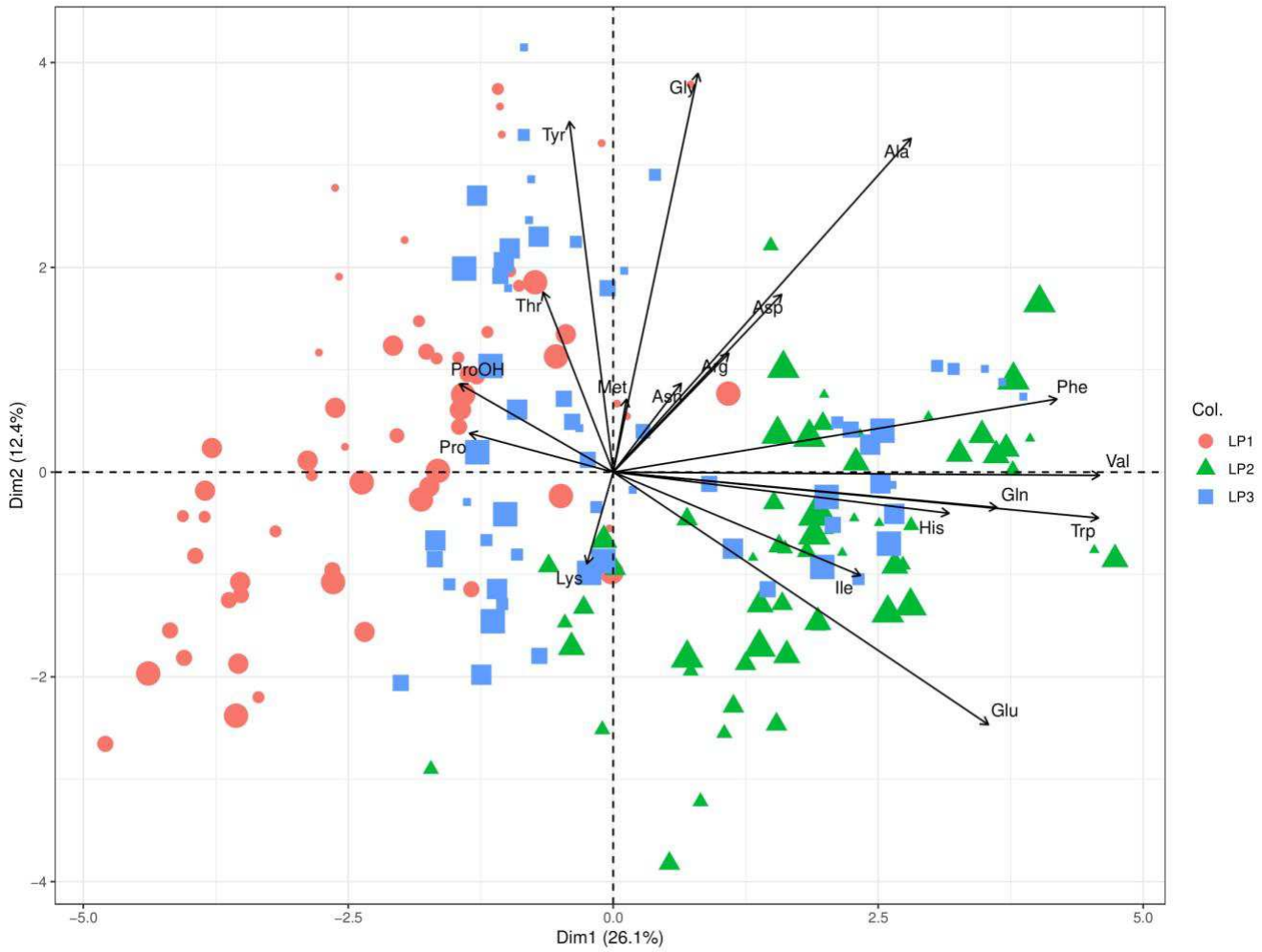
Wu, G., Bazer, F. W., Burghardt, R. C., Johnson, G. A., Kim, S. W., Knabe, D. A., … Spencer, T. E. (2011, April). Proline and hydroxyproline metabolism: Implications for animal and human nutrition. *Amino Acids*. https://doi.org/10.1007/s00726-010-0715-z

Zhao, D., Le, T. T., Larsen, L. B., Nian, Y., Wang, C., Li, C., & Zhou, G. (2019). Interplay between Residual Protease Activity in Commercial Lactases and the Subsequent Digestibility of β - Casein in a Model System. *Molecules*, *24*(16), 2876.

Supplementary material



Supplementary Figure 1. Line plots displaying the concentration of the free AAs detected by LC-HRMS in the UHLM produced using three different LP (LP1, LP2 and LP3) during storage at 20°C for 120 days. The data are the mean values of four instrumental replicates coming from the three different replicates of production for each lactase preparations (n=12).



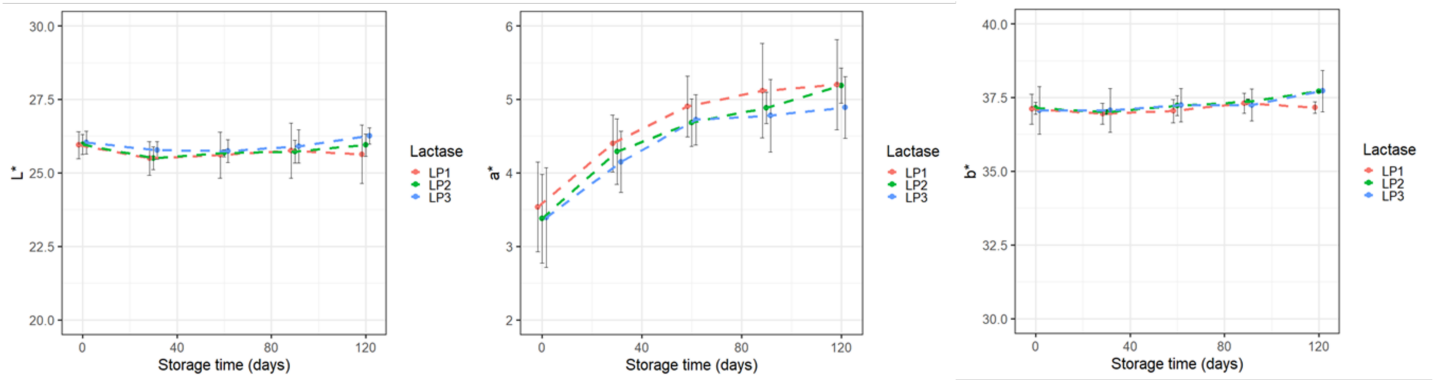
Supplementary Figure 2. Principal Component Analysis (PCA) bi-plot of the first (PC1) and the second (PC2) principal components performed on the free AAs data of the UHLM samples produced by "in batch" system with LP1 (red), LP2 (green), LP3 (blue) during storage at 20°C for 120 days. The proceeding of the storage time is represented by an increasing size of the dots. The explained variance for each component is also reported. Black arrows indicated the corresponding weights of the loadings on the spatial distribution of the UHLM samples.

Supplementary Table 1. Volatile organic compound (VOCs) profile of the UHLM samples produced with LP1, LP2 and LP3 during shelf-life for 120 days at 20°C. Results are expressed as µg/L of the internal standard 4-methyl-2-pentanone.

VOCs	LP1					T[a]	LP2					T	LP3					T
	0	30	60	90	120		0	30	60	90	120		0	30	60	90	120	
2-butanone	3,89	4,52	4,83	5,02	5,51		3,36	4,66	4,63	4,44	4,76		3,25	3,79	4,31	4,40	4,19	
2-methylbutanal	0,29	0,45	0,74	0,87	1,17	↑	0,44[a]	0,89[a]	1,23[a]	1,22[a]	1,61[b]	↑	0,41	0,45	0,73	0,79	0,85	↑
2-pentanone	3,25	4,96	6,42	7,09	8,17	↑	3,09	5,39	6,57	6,95	7,66	↑	3,18	4,68	6,82	7,03	7,00	↑
Toluene	0,51	0,77	0,83	0,79	0,91		0,97	1,62	1,36	1,01	0,91		0,45	0,95	0,86	0,81	0,96	
Hexanal	6,48	4,63	6,30	5,72	6,51		5,43	10,71	11,81	11,22	9,76		8,15	6,87	10,68	9,23	9,83	
Ethyll-benzene	0,98	0,68	1,27	1,71	2,22		2,34	0,76	2,90	2,07	1,69		0,76	0,72	2,27	1,76	1,92	
2-heptanone	11,43	15,10	15,89	12,44	16,38		6,71	15,78	20,71	12,63	16,45		9,84	14,88	14,49	12,91	10,75	
2-nonanone	4,02	4,23	4,79	4,97	5,75		3,40	3,92	4,51	4,23	4,70		3,94	4,00	4,71	4,45	3,87	
Nonanal	0,80	0,51	0,80	0,69	1,05		0,44	0,75	1,27	0,66	nd	↓	0,66	0,40	0,95	nd	nd	↓
Benzaldehyde	0,96	1,76	1,90	2,29	2,96	↑	0,55	1,24	1,12	1,31	1,75	↑	0,54	0,81	1,34	1,25	1,39	
1-octanol	nd	nd	nd	nd	nd		1,27	1,61	1,71	1,36	1,65		nd	nd	nd	nd	nd	
2-undecanone	0,97	0,65	0,78	0,82	0,96		0,63	0,69	0,71	0,81	0,88		0,72	0,61	0,77	0,85	0,79	
γ-butyrolactone	0,76	0,87	0,67	0,53	0,59		1,03	0,58	0,63	0,45	0,62	↓	0,62	0,74	0,57	0,52	0,43	
Aceto-phenone	0,46	0,80	0,51	0,83	0,89		0,60	0,47	0,73	0,52	0,54		0,46	0,65	0,22	0,18	0,20	
2-tridecanone	0,31	0,35	0,40	0,44	0,55		0,30	0,37	0,32	0,33	0,31		0,33	0,26	0,39	0,33	0,39	
Dimethyl-sulfone	2,37	1,31	1,62	1,24	2,19		1,10	2,13	2,53	2,03	2,30		1,99	1,93	2,96	1,98	0,89	
Hexanoic acid	4,27	2,17	2,29	2,50	4,47		2,16	2,71	2,88	2,12	3,07		2,83	1,81	5,17	2,87	1,83	
p-cresol	0,28	0,27	0,30	0,31	0,36		0,27	0,29	0,32	0,26	0,30		0,26	0,22	0,34	0,29	0,27	
m-cresol	1,03	1,02	1,00	1,05	1,18		0,94	1,06	1,22	0,92	1,08		0,90	0,85	1,21	1,07	1,00	
Octanoic acid	4,09	3,27	3,69	3,66	6,33		2,62	3,75	4,13	3,43	4,20		4,06	2,78	4,98	3,62	2,78	
δ-decalactone	0,57	0,48	0,35	0,21	0,20		0,52	0,57	0,35	nd	nd	↓	0,47	0,43	0,32	nd	nd	
Decanoic acid	4,87	4,69	4,96	5,91	7,59		2,73	4,60	6,26	4,42	5,66		4,15	3,47	5,37	4,57	5,17	

[a] T = trends of the VOCs within the same UHLM sample type between 0 days and 120 days of shelf-life at 20°C reported as results of the one-way ANOVA. (↑= significant increase, ↓ = significant decrease).

[b] Values reported as mean of three different production replicates (n=3) for each UHLM produced with a different LP. (nd=not detected)



Supplementary Figure 3. Changes in color expressed in the CIELAB system of the UHLM produced using the three different lactase preparations (LP1, LP2, LP3) during storage at 20°C for 120 days. The description of the color dimensions detected by the instrument comes from Sunds et al. (2018): L* (black: L* = 0 and white: L*=100), a* (red-green: negative a* = greenness and positive a* = redness), b* (yellow-blue: negative b* = blueness and positive b* = yellowness).

Chapter 5: Application of PTR-ToF-MS for the quality assessment of lactose-free milk: effect of storage time and employment of different lactase preparations.

Proton Transfer Reaction-Mass Spectrometry (PTR-MS) is a rapid methodology for the volatiles organic compounds (VOCs) assessment, pointed out as valid alternative to other more time-consuming techniques (e.g. gas chromatography). In this chapter, the potential of this technique was studies in the same industrial scenario described in Chapter 4: UHLM produced "in batch" with different lactase preparations were stored at 20°C for 150 days and then analysed. The instrument was coupled with a time-of-flight (ToF) mass spectrometer for better resolution and detection of the mass peaks. The optimized HS-SPME/GC-MS techniques discussed in Chapter 1 was also employed to support the identification of the mass peaks, allowing a clear portray of the "volatilome" of UHLM.

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Abstract

Lactose-free dairy products undergo several chemical modifications during shelf life due to the reactivity of glucose and galactose produced by the lactose enzymatic hydrolysis. In this study, proton transfer reaction-mass spectrometry (PTR-MS), coupled with a time-of-flight (ToF) mass analyzer, was applied to get an insight on the phenomena occurring during the shelf-life of ultra-high-temperature (UHT) lactose-free milk (LFM). UHT LFMs produced by three different commercial lactase preparations were evaluated during storage at 20°C over a 150 days period, sampling the milk every 30 days. Production was repeated three times, on three consecutive weeks, in order to take milk variability into consideration. Principal Component Analysis applied to the whole "volatilome" data demonstrated the capability of PTR-ToF-MS in detecting the milk batch-to-batch variability: freshly produced milk samples were distinguished based on the week of production at the beginning of shelf-life. Additionally, a clear evolution of the volatiles organic compounds (VOCs) profiling during storage was highlighted. Further statistical analysis confirmed VOCs temporal evolution, mostly due to changes in methyl ketones concentration. Differences caused by the commercial lactases did not emerged, except for benzaldehyde. All together data demonstrated PTR-ToF-MS analysis as a valuable and rapid method for the detection of changes in the VOCs profiling of ultra-high-temperature lactose-free milk.

Introduction

Complications related to lactose malabsorption have gathered the attention of both scientists and consumers in the recent past (Szilagyi & Ishayek, 2018). The increasing demand of healthier foods may push the preference for lactose-free milk (LFM) over conventional milk even among consumers which are not lactose-intolerant (Nielsen et al., 2018). In this scenario, LFM have experienced a valuable increase in market shares (Jelen & Tossavainen, 2003) and today represents a staple products in the diet of many consumers.

Milk sensory quality is driven by absence of odor and aftertaste, yet rejection might arise due unpleasant reaction occurring during shelf-life (Francis et al., 2005). Focusing on LFM, manufacturing is not trivial and it poses additional challenges. For example, even though heating the milk at ultra-high-temperature (UHT) guarantees a prolonged shelf-life (Valero, Villamiel, Miralles, Sanz, & Martínez-Castro, 2001), the presence of free glucose and galactose produced by lactose hydrolysis renders the product susceptible to Maillard reaction (Jansson et al., 2017). Moreover, most UHT LFMs are produced by adding free soluble β-1,4-galactosidase (lactase) to the milk (Jelen & Tossavainen, 2003). The commercially available options were found to contain arylsulfatase and proteolytic side activities which can potentially modify the sensory quality of the milk during shelf-life (Nielsen et al., 2018; Troise et al., 2016). In a study conducted by Stressler and co-workers (2016) for example, arylsulfatases from the β-1,4-galactosidase altered the odor of milk imparting a defect recognized as "cowshed-like" (Stressler, Leisibach, Lutz-Wahl, Kuhn, & Fischer, 2016). Differently, proteolysis leads to the release of peptides and free amino acids in the milk. The phenomena may confer bitterness and astringency to the product (Santos, Ma, Caplan, & Barbano, 2003), as well as provide further substrate for the formation of volatiles and non-volatile compounds by Maillard reaction (Jensen et al., 2015).

Therefore, the importance of volatiles organic compounds (VOCs) in defining the quality of UHT LFM during shelf-life is unquestionable. VOCs are responsible for the complexity of food flavor and aroma, as their release occur at each step of production, storage and consumption (Biasioli, Gasperi, Yeretzian, & Märk, 2011). In this frame, it appears relevant to develop methodologies that allow a fast and reliable analysis of VOCs to monitor the quality of food products (Biasioli et al., 2011). Proton transfer reaction-mass spectrometry (PTR-MS) was reported as a successful methodology for rapid, non-invasive, sensitive assessment of VOCs in food science (Biasioli et al., 2011; Cappellin et al., 2013). The instrument exploits the ionization of VOCs by proton transfer from H_3O^+ and registers a mass spectrum in which the

measured signal and the mass/charge ratio (m/z) of the ions are plotted together (Ellis & Mayhew, 2013). In the dairy sector, applicability of PTR-MS has been previously verified on various products such as grana-type cheeses (Aprea et al., 2007; Boscaini, Van Ruth, Biasioli, Gasperi, & Märk, 2003), butter (van Ruth et al., 2008) and yoghurt (Soukoulis et al., 2010). When PTR is coupled with a quadrupole detector, distinction of molecules by PTR-MS is limited (Fabris et al., 2010). Better resolution and detection were achieved when the instrument was coupled with a time-of-flight (ToF) mass spectrometer (Soukoulis et al., 2010).

Different approaches has been previously applied to evaluate the VOCs profiling of UHT LFM during shelf-life. For example, dynamic headspace (DHS) GC-MS was applied by Jansson, and co-workers (2014) to investigate the differences in VOCs evolution between UHT LFM and conventional UHT milk over a 9-month storage. Fifteen VOCs were quantified using external standards and a sharper increase in concentration was registered by the lactose-free. Together with other analysis, the study demonstrated stronger occurrence of Maillard reaction in LFM. As a solution, the same research group published an article proposing the addition of green tea extract (GTE) to UHT LFM to inhibit the proceeding of the reaction (Jansson et al., 2017). They studied the volatiles profile of UHT LFM added with GTE again by DHS GC-MS and a significant decrease of specific VOCs was demonstrated. Troise and co-workers (2016) applied solid-phase micro extraction (SPME) GC-MS for the evaluation of the VOCs in commercial UHT LFM samples (Troise et al., 2016). In that case, external standards were injected and the analysis was limited to specific VOCs. The results shown different temporal trend in the evolution of the compounds depending on the commercial samples. A different degree of proteolytic activity in the lactases used by producers was suggested as main responsible for such variations.

From the studies mentioned above it was clear that UHT LFMs is highly sensitive to changes in the "volatilome" during shelf-life. The production process, the lactase preparations and storage conditions play a crucial role in the definition of the final product quality. In this frame, the present study aims to investigate the application of PTR-ToF-MS to characterize how the VOCs profile of UHT LFM evolves during shelf-life at 20°C. By pairing PTR-ToF-MS with an auto-sampling system, we attempt a rapid characterization of the milk thanks to the high speed of the analysis, which allowed us to measure one sample per minute. To our knowledge, despite relevant published literature on the VOCs formation in UHT LFM during shelf-life, this is the first time in which the task is performed by the rapid PTR-MS technique.

Materials and Methods

Chemical

4-methyl-2-pentanone (purity ≥ 99%) used as internal standard for gas chromatography mass spectrometry (GC-MS) analysis was purchased from Sigma-Aldrich (Steinheim, Germany).

Preparation of the lactose-free milk samples

Manufacturing of UHT LFM was carried out industrially on three consecutive weeks to include the milk batch-to-batch variability in the experimental design. A schematic drawing of each week of production is illustrate in **Figure 1**.

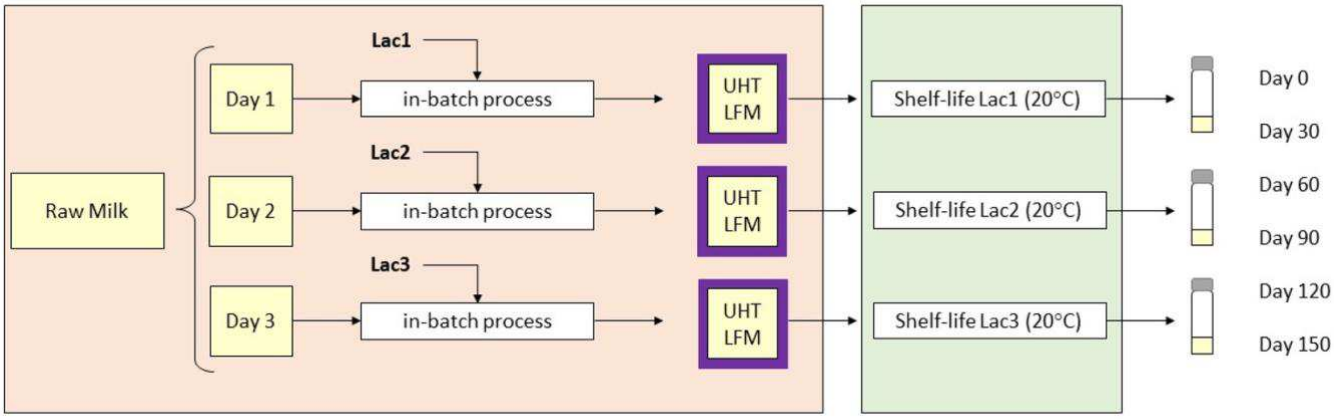


Figure 1. Simplified representation of the experimental design. UHT LFM was produced adding the three different commercial lactases (Lac1, Lac2, Lac3) prior to UHT treatment. Manufacturing of the three LFM occurred on three consecutive days within the same week of production. The experiment was replicated three times (namely on three different weeks of production) in the same manner in order to have three production replicates for each UHT LFM and take the milk batch-to-batch variability into consideration. All the LFM samples were stored at 20°C under controlled conditions for the shelf-life study. As soon as after production (0 days of storage), sampling was then performed every 30 days until 150 days of storage.

Three different commercial lactase preparations were purchased and employed for the experiment (Lac1, Lac2 and Lac3). Semi-skimmed milk was firstly pasteurized and employed for the production of the samples. The commercial lactase was added to the milk and lactose conversion lasted for about 25-35 hours at refrigerated conditions. UHT treatment was then applied and the milk was packed aseptically.

A 150-days shelf-life simulation was performed, storing the samples in a climate chamber at 20°C under controlled conditions. Samples were collected at time 0 and every 30 days and stored at -80°C until the analysis was performed. A 5 mL of each UHT LFM was placed into 20 mL glass vials (Supelco, Bellefonte, PA, USA). Prior to analysis, samples were thawed at room temperature until thoroughly defrosted. Empty vials were used as blanks while the repeated measurement of a reference milk was used as quality control.

GC-MS measurements

In concomitance with the PTR-ToF-MS measurements, to identify the compounds responsible for the mass peaks observed, headspace solid phase microextraction (HS-SPME) GC-MS analysis has been carried out. The analysis was carried out both at the beginning and the end of the shelf-life study (namely at 0 and 150 days of storage) on the UHT LFMs from the first week production. Measurements were based on the methodology already applied by our research group in previous studies (Bergamaschi et al., 2015). VOCs extraction was performed at 40°C for 60 min with a 2 cm DVB-Carboxen–PDMS SPME fiber. Volatiles compounds were desorbed at 250°C in the injector port of a GC interfaced with a mass detector operating in an electron ionization mode (70 eV). Mass scan ranged from m/z 33 to 300 (GC Clarus 500, PerkinElmer, Norwalk, CT). An auto-sampling system (CTC combiPAL, CTC Analysis AG, Zwingen, Switzerland) was used to manage the measurement in an automatic manner. A HP-Innowax fused-silica capillary column (30 m, 0.32 mm inner diameter, 0.5 μm film thickness; Agilent Technologies, Palo Alto, CA) was used for the separation of the compounds. Linear retention indices (RIs) were calculated and, using the NIST-2014/Wiley 7.0 libraries, an identification of the VOCs was provided.

PTR-ToF-MS measurements

The set-up of the measuring conditions was based on PTR-MS procedures described elsewhere (Pedrotti et al., 2018). Briefly, a PTR-ToF-MS 8000 (Ionicon Analytik GmbH, Innsbruck, Austria) operating in V mode (standard configuration of the instrument) was used

for measuring the samples headspace. The following ionization conditions were set in the drift tube: extraction voltage of 24.3 V, drift voltage of 628 V, drift temperature of 110°C, and drift pressure 2.78 mbar corresponding to an E/ N value of 128 Townsend (1 Td = 10^{-21} V·m^2). The mass resolution (m/Δm) was higher than 3800.

All measurements were automatically performed using an auto-sampling system (Gerstel GmbH, Mulheim am Ruhr, Germany) connected to the PTR-MS inlet, namely a PEEK capillary tube (inner diameter, 0.40 mm), heated at 110°C. Vials containing the milk samples were incubated at 50°C for 30 minutes prior to analysis. Three instrumental replicates were measured for each HLM UHT sample. Each vials was measured for 60 seconds (flow rate of 35 sccm) with an acquisition rate of one spectrum per second (m/z range: 21 – 300).

Peak selection and data handling

Results of the extracted m/z were expressed with three decimal places. The procedure of dead time correction, peak extraction and internal calibration of the data was based on a previous articles published by Cappellin and co-workers (2010)**.** The following mass peaks were used for internal calibration: 21.0221 (H_3O^+), 29.9974 (NO^+), and 203.9430 (1,3-diiodobenzene fragment). The latter was continuously injected as internal reference throughout the PTR-ToF-MS analysis using the PerMaSCal device (Ionicon, Innsbruck, Austria). The formula proposed by Lindinger et al. (1998) was applied by assuming a constant reaction rate coefficient of k_R = 2 x 10^{-9} cm^3/s to express the results in absolute concentrations (ppbV, part per billion by volume). This approximation lead to a systematic error that in most cases was below 30% for all the compounds (Cappellin et al., 2012). Mass spectra signals were averaged over 30 spectra.

Statistical analysis of the results

^{13}C isotopologues and interference masses were removed from the dataset. Mass peaks detected in the samples were compared with the blanks via Student t-test applying Bonferroni correction in order to select those mass peaks significantly higher than blank. On this reduced dataset, Principal Component Analysis (PCA) was performed after log-transformation and Pareto scaling to explore pattern in the data. For data exploration, replicates of production were kept separated to visualize the different milk batches. Loadings considered relevant in defining the trends of the PCA, namely the ones with values ≥ 0.15 and ≤ -0.15 on the 1st component and those ≥ 0.10 and ≤ -0.10 on the 2nd

component, were further considered in order to reduce the dimensionality of the dataset. Selected mass peaks on which a tentative identification was attempted were reported in a summary table (Table 2). The table was also implemented with those compounds discarded by the described dataset reduction procedure but revealed by the GC analysis.

Mean ± standard deviation of the 3 instrumental replicates coming from the 3 different batches of production (n=9) was further considered for each selected peak mass. 2-ways ANOVA with Tukey post-hoc was applied when necessary to investigate the evolution of the selected VOCs in the UHT LFM samples. A $\alpha \leq 0.05$ was chosen as threshold for significant differences. Statistical analysis were performed using the software package STATISTICA 13.3 (StatSoft, Inc., Tulsa, OK, USA) and the R packages FactoMineR and factoextra.

Results and Discussions

VOCs fingerprinting by PTR-ToF-MS

In total 368 mass peaks were extracted from the PTR-ToF-MS spectra and 268 mass peaks were found significantly higher than blanks. Principal Component Analysis was performed to explore the spatial distribution of the UHT LFM samples as function of the considered mass peaks. Pareto scaling was applied to adjust different fold changes among the variables and penalize the ones with large variation in the measurements, most likely associated to noise (van den Berg, Hoefsloot, Westerhuis, Smilde, & van der Werf, 2006). **Figure 2** shows the score plot of the 1st and 2nd principal component (PC1 and PC2), explaining respectively 43.81% and 10.90% of the total variance. The analysis distinguished the UHT LFM as function of the batch of production. Moreover, the effect of the storage time was also highlighted. At 0 days of storage, the three different replicates (weeks) of production were somehow separated. The effect seemed to be explained by the combination of PC1 and PC2. Batch-to-batch milk variability appeared not relevant at intermediate and final stages of shelf-life. Batch-to-batch variability in UHT lactose-free milk was already reported by Jansson and co-workers (2014). In that case, variations emerged due to initial differences in ketones concentration. Many factors can render the VOCs profile of milk variable among different batches of production. For example, changes in composition of the pasture fed to cows can convey different aromas to the milk (Rapisarda et al., 2013). From the results, we can conclude that the applied PTR-ToF-MS methodology was appropriate to discriminate UHT LFMs coming from different batches of milk. Nevertheless, from the explorative analysis the three commercial lactases (Lac1, Lac2 and Lac3) did not lead to a clear distinction of the samples, suggesting a similar VOCs profiling among the products during shelf-life.

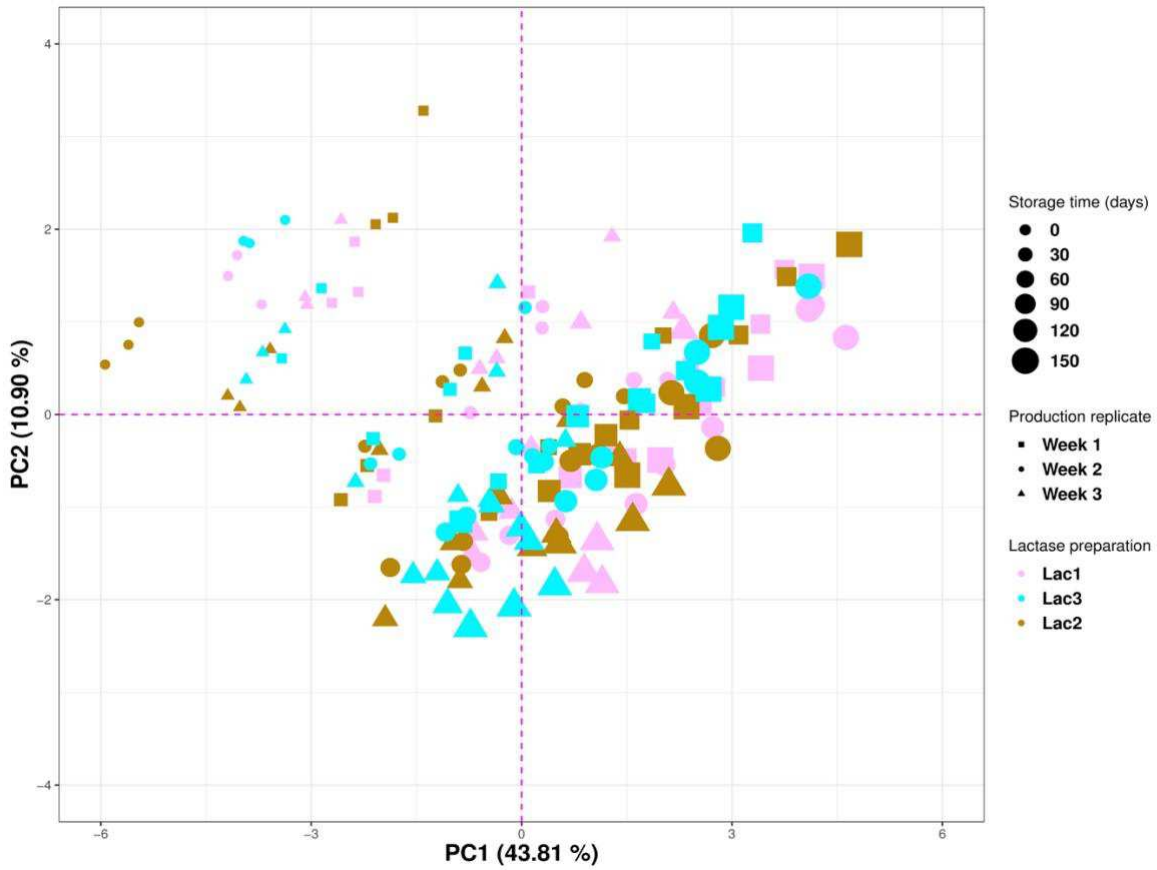


Figure 2. Principal Component Analysis (PCA) score plot of the 1st (PC1) and 2nd (PC2) component, which describe respectively the 43.81% and 10.90% of the total variance. Analysis was performed on the Pareto scaled and mean-centered data including all the time points.

Mass peak identification by HS-SPME GC-MS

HS-SPME GC-MS analysis was carried out in support to the tentative identification of the mass peaks detected by PTR-ToF-MS. A total of 24 VOCs were identified in the headspace of the UHT LFMs (Table 1). The profile was in line with the literature available on the topic (Jansson, Jensen, et al., 2014; Troise et al., 2016). The compounds detected by HS-SPME GC-MS with higher frequency were ketones. The class is considered a good indicator of deterioration of the milk upon heating, as their formation goes hand to hand with the severity of the treatment (Contarini & Povolo, 2002). Ketones were followed by aldehydes in terms of incidence. Even in this case, all the 4 identified aldehydes were reported in UHT lactose-free milk by other authors (Jansson, Jensen, et al., 2014). Besides the detection in UHT milk straight after production, aldehydes and methyl-ketones are important VOCs to monitor because of their contribuion to the stale and oxidize flavour of the milk during

storage (Perkins, D'Arcy, Lisle, & Deeth, 2005). Mass peaks considered relevant for the trends showed in the PCA (**Figure 2**) were investigated in more details and those tentatively identified are summarized in Table 2. The identity of 11 VOCs was confirmed by the results of HS-SPME GC-MS analysis. Additionally, the table was also implemented with the mass peaks whose identification was attempted on the basis of relevant literature, the chemical formula and the fragmentation pattern (Aprea et al., 2015; Pedrotti et al., 2018; Zardin, Tyapkova, Buettner, & Beauchamp, 2014). This led to a total 18 VOCs associated to a mass peak identified in the UHT LFMs headspace.

Mass peak m/z = 73.064 (associated to 2-butanone/butanal) was the reported compound which registered the highest abundancy (ppbV) at the end of the shelf-life period. It was followed by m/z = 69.070, m/z = 63.026 and m/z = 49.011. These masses were associated respectively to isoprene, dimethyl sulfide (DMS) and methanethiol (MeSH). Some compounds were present at higher concentration but, as the trend shown by the PCA was not considered relevant and a match with the GC-MS results was not found, it was chosen to leave them out from the summary table. Mass peak m/z = 45.033 is a clear example. It was associated to acetaldehyde, a well-known index of light oxidation in dairy products (Van Aardt et al., 2001). Its concentration in the headspace ranged between 3.58 ppbV and 77.08 ppbV throughout the storage of the UHT LFMs. However, the impact on the PCA (**Figure 2**) was tiny, so it was decide to exclude it from further statistical considerations.

Table 1. Volatiles compounds identified by HS-SPME GC-MS in the headspace on UHT LFM produced along the first replicate (week) of production both at the beginning and the end of the shelf-life study (namely at 0 and 150 days respectively).

Volatile compounds	Rt[a]	RI[b]
2-Butanone[c]	2.67	909
2-Methylbutanal	2.86	920
2-Pentanone	4.02	985
Toluene	5.47	1050
Dimethyldisulfide	6.35	1087
Hexanal	6.62	1099
Ethyll-benzene	7.91	1138
2-Heptanone	9.66	1190
Xylene	9.73	1192
2-Nonanone	16.56	1394
Nonanal	16.69	1398
Benzaldehyde	20.91	1531
1-Octanol	22.23	1574
2-Undecanone	23.13	1604
γ-Butyrolactone	24.05	1636
Aceto-phenone	24.77	1660
2-Tridecanone	29.12	1816
Dimethyl-sulfone	31.55	1908
Hexanoic acid	31.10	1891
p-Cresol	36.18	2095
m-Cresol	36.36	2102
Octanoic acid	36.42	2105
δ-Decalactone	38.64	2199
Decanoic acid	42.24	2295

[a] Retention time (min)

[b] Linear retention index

[c] Compounds tentatively identified matching the NIST-2014/Wiley 7.0 libraries

Table 2. Tentatively identified mass peaks in the UHT LFM samples over a 150-days storage at 20°C. Values are reported in ppbV as mean of three measurements from the three different production replicates (n=9).

Measured m/z	Tentative Identification	GC-MS	Lac3						Lac2						Lac1					
			-	30	60	90	120	150	-	30	60	90	120	150	-	30	60	90	120	150
43.054	Alkyl fragment		0.36	0.36	0.38	0.37	0.36	0.39	0.34	0.34	0.37	0.33	0.34	0.37	0.39	0.40	0.42	0.39	0.41	0.43
49.011	Methanethiol (MeSH)		3.64	2.24	1.53	1.74	1.40	2.10	3.39	2.16	1.85	1.68	1.77	1.90	3.08	2.74	1.97	1.75	1.89	1.87
63.026	Dimethyl sulfide (DMS)		0.43	0.87	1.28	1.45	1.52	2.39	0.42	0.86	1.50	1.61	1.83	2.32	0.46	1.08	1.52	1.71	2.19	2.50
69.070	Isoprene		0.44	1.65	2.74	2.73	2.72	3.39	0.47	1.76	3.16	2.87	2.99	3.61	0.51	1.87	3.30	3.08	3.59	3.65
73.064	2-Butanone; butanal	X	10.32	8.15	8.74	10.84	9.18	9.68	9.91	8.28	8.62	9.33	7.78	8.67	6.84	5.45	6.85	8.89	7.26	7.89
87.044	2,3-Butanedione; γ-Butyrolactone	X	0.24	0.47	0.59	0.62	0.62	0.80	0.23	0.47	0.65	0.60	0.66	0.77	0.24	0.53	0.66	0.67	0.75	0.86
87.081	2-Pentanone; 2/3-Methylbutanal; Pentanal	X	0.48	0.80	1.01	1.05	1.09	1.39	0.40	0.73	0.97	0.94	1.03	1.23	0.43	0.83	1.03	1.03	1.18	1.40
89.060	Butanoic Acid; Acetoin	X	0.37	0.40	0.38	0.37	0.36	0.36	0.43	0.44	0.42	0.38	0.40	0.41	0.48	0.43	0.40	0.39	0.43	0.43
93.066	Toluene	X	0.10	0.10	0.11	0.10	0.09	0.10	0.09	0.10	0.09	0.09	0.10	0.11	0.09	0.09	0.10	0.09	0.10	0.10
97.062	2-Ethyl furan		0.03	0.04	0.04	0.04	0.04	0.05	0.03	0.04	0.05	0.05	0.05	0.05	0.03	0.04	0.05	0.05	0.05	0.05
97.101	Heptanal fragment		0.07	0.09	0.09	0.09	0.10	0.11	0.06	0.08	0.10	0.09	0.10	0.11	0.07	0.09	0.10	0.10	0.11	0.12
101.096	Hexanal	X	0.09	0.14	0.16	0.14	0.15	0.17	0.08	0.13	0.16	0.15	0.15	0.17	0.09	0.15	0.18	0.16	0.17	0.19
107.049	Benzaldehyde	X	0.06	0.11	0.13	0.15	0.14	0.18	0.05	0.11	0.15	0.16	0.18	0.21	0.06	0.12	0.15	0.18	0.20	0.24
115.112	2-Heptanone	X	0.60	0.77	0.93	0.96	1.00	1.22	0.55	0.77	0.98	0.96	1.07	1.22	0.58	0.87	1.01	1.04	1.18	1.35
117.091	Hexanoic acid	X	0.08	0.07	0.07	0.08	0.07	0.08	0.07	0.06	0.07	0.07	0.07	0.08	0.08	0.07	0.07	0.07	0.07	0.10
129.128	2-Octanone; octanal		0.03	0.03	0.04	0.03	0.04	0.04	0.02	0.03	0.04	0.03	0.04	0.04	0.03	0.03	0.04	0.04	0.04	0.04
143.145	2-Nonanone; nonanal	X	0.12	0.13	0.15	0.15	0.16	0.18	0.12	0.12	0.15	0.15	0.16	0.18	0.13	0.14	0.15	0.16	0.18	0.19
145.123	Octanoic acid	X	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.02

Effect of storage time on the VOCs profile

Two-ways ANOVA and Tukey HSD were performed to investigate the effect of storage time and commercial lactases on the reported mass peaks. The analysis highlighted the presence of significant differences among the UHT LFMs due to storage time. In fact, most of the reported mass peaks were found at significantly higher concentrations at the end of the storage (150 days of storage) compared to the freshly produced samples (0 days of storage). Mass peaks tentatively associated to methyl ketones registered a significant increase during storage for all the UHT LFMs produced with the three commercial lactases. All the methyl-ketones detected by PTR-ToF-MS were also confirmed by the HS-SPME GC-MS analysis, with the exception of the mass peaks m/z = 129.128, associated to 2-octanone/octanal. It is not the first time that this VOC is reported in UHT milk (Calvo & de La Hoz, 1992). However, in our study the compound was detected at very low levels (< 0.05 ppbV). 2-pentanone (m/z = 87.081) and 2-heptanone (m/z = 115.112) were the methyl-ketones that experienced the most remarkable increase during shelf-life. In the freshly produced samples, 2-pentanone was present in the range of 0.40-0.48 ppbV and increased up to 1.23-1.40 ppbV depending of the lactase preparation employed (Lac1, Lac2, Lac3). 2-heptanone followed a similar trend: it increased progressively from 0.55-0.60 ppbV and it reached 1.22-1.35 ppbV after 150 days of storage. Significant increase of methyl ketones during shelf-life was already described both for conventional and UHT lactose-free milk. Jansson et al. (2014) reported higher rates of increase of methyl ketones in the latter. Methyl ketones are formed upon heating either by the decarboxylation of β-keto fatty acids or the β-oxidation and decarboxylation of free saturated fatty acids (Deeth & Lewis, 2017). Jensen et al. (2015) estimated the class as good predictor for the development of "stale flavor" during shelf-life of lactose-free milk.

Even though an overall increase in VOCs in UHT LFM was registered during storage, some mass peaks followed a different trend. Mass peak m/z = 49.011, tentatively identified as methanethiol (MeSH) decrease during storage, although the drop was not statistically significant when biological variability of the milk was considered. Methanethiol is formed from methional and contributes, together with other volatiles sulphur compounds, to the "cooked flavor" of pasteurized and UHT milk (de Wit & Nieuwenhuijse, 2008). Presence of free methional in the milk could therefore justify detection of MeSH in our experiment. Temporal decrease of MeSH is reasonable because it reflects the proceeding of oxidation, which might lead to the formation of DMS and H_2S (de Wit & Nieuwenhuijse, 2008).

Protein-bound methionine is also the precursor of DMS (m/z = 63.026) so its significant increase might be also associated with the decrease of MeSH during storage. Thus, the result indicated possible degradation of methionine during storage of UHT LFM, already reported in the literature and potentially imputable to the proteolytic side activity originally present in the commercial lactase preparations (Troise et al., 2016).

Effect of the different types of commercial lactase preparations on the VOCs profile

Quality losses in UHT lactose-free milk has been associated with presence of proteolytic and arylsulfatase activities in the commercially available lactase preparations. Thus, besides storage time, the effect of different commercial lactase preparations (Lac1, Lac2, Lac3) on the VOCs profiling of UHT LFM was also investigated. Overall, almost all the mass peaks did not change significantly at each storage time based on the different lactases employed. At a first glance, the results might indicate that the tested commercial lactases were similar and led to an almost indistinguishable VOCs profile in the products. Alternatively, the manufacturing process might explain the similarity in VOCs profiling among the samples. The applied manufacturing process, so-called "in batch", relies on the inactivation of the lactase by heating the milk at ultra-high-temperature (UHT) before packaging and storage (Dekker, Koenders, & Bruins, 2019; Troise et al., 2016). With regards to the proteolytic side activity of lactase, the literature lacks information concerning the relative stability upon heating. A review published by Dekker and co-workers (2019) reported that, when lactose hydrolysis is performed before the thermal treatment, the proteolytic side activity of the lactase is inactivated (Dekker et al., 2019). The finding is in line with our results and the similar VOCs profiles detected by PTR-ToF-MS analysis might be a consequence of the applied process for UHT LFM manufacturing. Nevertheless, the analysis of the variance revealed the presence of significant differences for the mass peak m/z = 107.049. Basically, its concentration over time increased for all the UHT LFM samples following different trends (**Figure 3**). This means that for m/z = 107.049, tentatively identified as benzaldehyde, the temporal evolution was dependent on the lactase preparations employed. UHT LFM produced with Lac1 and Lac2 were significantly higher in benzaldehyde both after 60 and 120 days of storage. At the end of the shelf-life (150 days), Lac1 was the commercial lactase preparation causing the highest release of benzaldehyde in the UHT LFM headspace.

The concentration was significantly higher than samples produced with Lac3, but not in comparison to Lac2. Benzaldehyde can be considered a thermally induced compound in milk (Potineni & Peterson, 2005). The formation may occur alongside Maillard reaction starting from phenylalanine (Jansson et al., 2017). The mechanism involves the conversion of the amino acid to phenylacetaldehyde via Strecker degradation and subsequent oxidation to benzaldehyde (Fong & Yaylayan, 2008). Following this pathway, our results showed different extent of benzaldehyde formations possibly due to different degrees of proteolytic side activity in the tested lactases. A study published by Troise and co-workers in 2016 already reported changes of phenylalanine and benzaldehyde in a commercial UHT lactose-free milk produced by "in-batch" technology. In that case, release of phenylalanine was minimum and seemed not related to an increase of benzaldehyde during shelf-life. On the other hand, only one commercial milk was evaluated and the lactase preparation employed by producers was unknown. Thus, further research on the link between phenylalanine and benzaldehyde is required. Eventually, the significant increase of benzaldehyde over time might suggest the heat stability of some proteolytic side activity natively present in the commercial lactase, another aspect which should be further investigated.

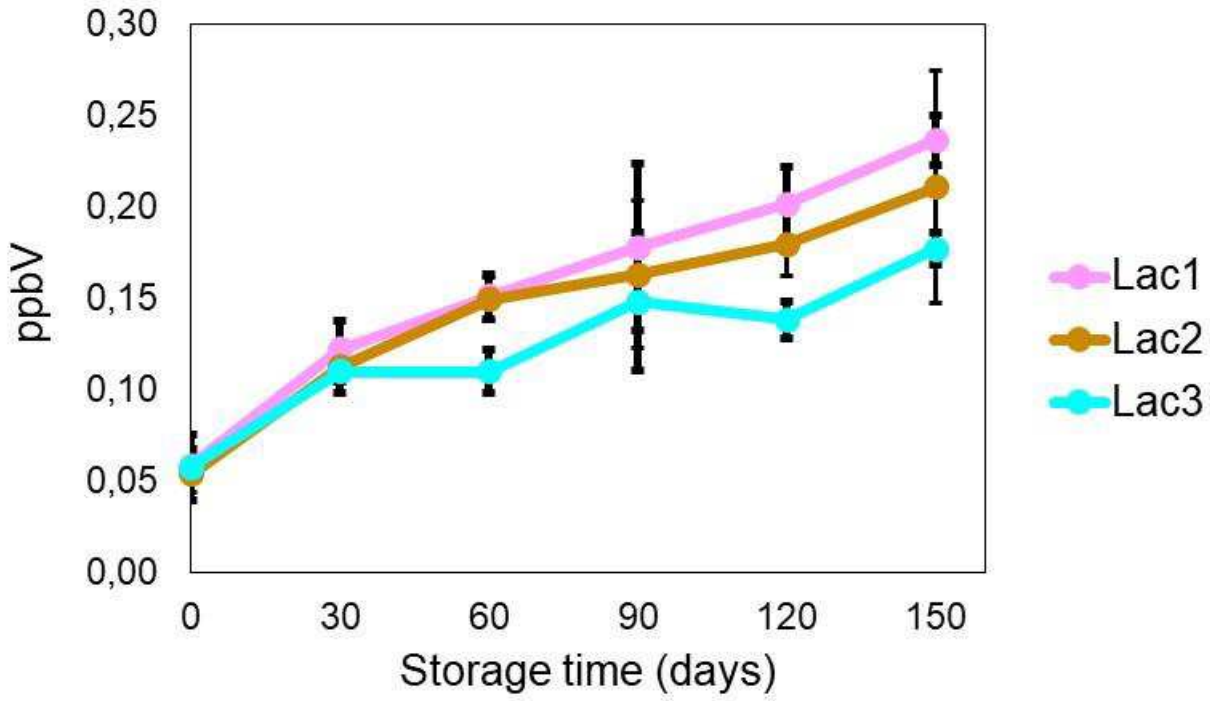


Figure 3. Time evolution (day 0-150) during storage at 20°C of the mass peak m/z 107.048, tentatively identified as benzaldehyde, of UHT LFMs produced with three different commercial lactase preparations (Lac1, Lac2, Lac3).

Conclusion

In the present study, changes in the VOCs profile of ultra-high-temperature lactose-free milk (UHT LFM) during storage at ambient temperature were evaluated by PTR-ToF-MS coupled with a multipurpose autosampler. Applying PTR-ToF-MS, we significantly diminish the time of analysis. This allowed the design of a more complex experiment, in which several variables were simultaneously assessed. For example, the inclusion of different milk batches gave a realistic interpretation of industrial variability and allowed to experiment the response of PTR-ToF-MS in a complex lifelike situation. Batch-to-batch variability of the milk was highlighted by Principal Component Analysis (PCA), which also denoted a temporal evolution of the VOCs profiles of the UHT LFMs during storage at 20°C. Different VOCs profiling induced by the different commercial lactases employed did not emerge for most of the identified mass peaks. Possibly, the UHT treatment, which in this case occurred after lactose hydrolysis, inactivated most of the side activity of the lactase preparations. However, the different evolution of m/z 107.049 (benzaldehyde) during storage might be associated with the lactase preparations employed: phenylalanine was pointed out as possible precursor of benzaldehyde formation and it can derive from the proteolytic side activity originally present in the lactases. Therefore, the study suggested benzaldehyde as possible marker to monitor in UHT LFM if the aim is to attempt a discrimination based on the lactase preparations used. However, understanding whether the slight variations found in the study can affect the final quality of the products is still uncertain.

References

Aprea, E., Biasioli, F., Gasperi, F., Mott, D., Marini, F., & Märk, T. D. (2007). Assessment of Trentingrana cheese ageing by proton transfer reaction-mass spectrometry and chemometrics. *International Dairy Journal*, *17*(3), 226–234. https://doi.org/10.1016/j.idairyj.2006.02.008

Aprea, E., Romano, A., Betta, E., Biasioli, F., Cappellin, L., Fanti, M., & Gasperi, F. (2015). Volatile compound changes during shelf life of dried Boletus edulis: Comparison between SPME-GC-MS and PTR-ToF-MS analysis. *Journal of Mass Spectrometry*, *50*(1), 56–64. https://doi.org/10.1002/jms.3469

Bergamaschi, M., Aprea, E., Betta, E., Biasioli, F., Cipolat-Gotet, C., Cecchinato, A., … Gasperi, F. (2015). Effects of dairy system, herd within dairy system, and individual cow characteristics on the volatile organic compound profile of ripened model cheeses. *Journal of Dairy Science*, *98*(4), 2183–2196. https://doi.org/10.3168/jds.2014-8807

Biasioli, F., Gasperi, F., Yeretzian, C., & Märk, T. D. (2011, July). PTR-MS monitoring of VOCs and BVOCs in food science and technology. *TrAC - Trends in Analytical Chemistry*. https://doi.org/10.1016/j.trac.2011.03.009

Boscaini, E., Van Ruth, S., Biasioli, F., Gasperi, F., & Märk, T. D. (2003). Gas chromatography-olfactometry (GC-O) and proton transfer reaction-mass spectrometry (PTR-MS) analysis of the flavor profile of Grana Padano, Parmigiano Reggiano, and Grana Trentino cheeses. *Journal of Agricultural and Food Chemistry*, *51*(7), 1782–1790. https://doi.org/10.1021/jf020922g

Calvo, M. M., & de La Hoz, L. (1992). Flavour of Heated Milks. A review. *International Dairy Journal*, *2*(2), 69–81.

Cappellin, L., Biasioli, F., Fabris, A., Schuhfried, E., Soukoulis, C., Märk, T. D., & Gasperi, F. (2010). Improved mass accuracy in PTR-TOF-MS: Another step towards better compound identification in PTR-MS. *International Journal of Mass Spectrometry*, *290*(1), 60–63. https://doi.org/10.1016/J.IJMS.2009.11.007

Cappellin, L., Karl, T., Probst, M., Ismailova, O., Winkler, P. M., Soukoulis, C., … Biasioli, F. (2012). On quantitative determination of volatile organic compound concentrations using proton transfer reaction time-of-flight mass spectrometry. *Environmental Science and Technology*, *46*(4), 2283–2290. https://doi.org/10.1021/es203985t

Cappellin, L., Loreto, F., Aprea, E., Romano, A., Sánchez del Pulgar, J., Gasperi, F., & Biasioli, F. (2013). PTR-MS in Italy: A multipurpose sensor with applications in environmental, agri-food and health science. *Sensors (Switzerland)*, *13*(9), 11923–11955. https://doi.org/10.3390/s130911923

Contarini, G., & Povolo, M. (2002). Volatile fraction of milk: Comparison between, purge and trap and solid phase microextraction techniques. *Journal of Agricultural and Food Chemistry*, *50*(25), 7350–7355. https://doi.org/10.1021/jf025713a

de Wit, R., & Nieuwenhuijse, H. (2008). Kinetic modelling of the formation of sulphur-containing flavour components during heat-treatment of milk. *International Dairy Journal*, *18*(5), 539–547. https://doi.org/10.1016/j.idairyj.2007.11.011

Deeth, H. C., & Lewis, M. J. (2017). *High temperature processing of milk and milk products*. *John Wiley & Sons.* https://doi.org/10.1002/9781118460467.ch6

Dekker, P. J. T., Koenders, D., & Bruins, M. J. (2019). Lactose-Free Dairy Products: Market Developments, Production, Nutrition and Health Benefits. *Nutrients*, *11*(3), 551. https://doi.org/10.3390/nu11030551

Ellis, A. M., & Mayhew, C. A. (2013). *Proton transfer reaction mass spectrometry: principles and applications*. *John Wiley & Sons.* Elsevier Ltd. https://doi.org/10.1016/B978-0-08-101009-9.00010-1

Fabris, A., Biasioli, F., Granitto, P. M., Aprea, E., Cappellin, L., Schuhfried, E., … Endrizzi, I. (2010). PTR-TOF-MS and data-mining methods for rapid characterisation of agro-industrial samples: Influence of milk storage conditions on the volatile compounds profile of Trentingrana cheese. *Journal of Mass Spectrometry*, *45*(9), 1065–1074. https://doi.org/10.1002/jms.1797

Fong, L. C., & Yaylayan, V. A. (2008). Model studies on the oxygen-induced formation of benzaldehyde from phenylacetaldehyde using pyrolysis GC-MS and FTIR. *Journal of Agricultural and Food Chemistry*, *56*(22), 10697–10704. https://doi.org/10.1021/jf8022468

Francis, L. L., Chambers, D. H., Kong, S. H., Milliken, G. A., Jeon, I. J., & Schmidt., K. A. (2005). Serving Temperature Effects on Milk Flavor, Milk Aftertaste, and Volatile Compound Quantification in Nonfat and Whole Milk. *Journal of Food Science*, *70*(7), s413–s418. Retrieved from www.ift.org

Jansson, T., Clausen, M. R., Sundekilde, U. K., Eggers, N., Nyegaard, S., Larsen, L. B., … Bertram, H. C. (2014). Lactose-hydrolyzed milk is more prone to chemical changes during storage than conventional ultra-high-temperature (UHT) milk. *Journal of Agricultural and Food Chemistry*, *62*(31), 7886–7896. https://doi.org/10.1021/jf501671z

Jansson, T., Jensen, S., Eggers, N., Clausen, M. R., Larsen, L. B., Ray, C., … Bertram, H. C. (2014). Volatile component profiles of conventional and lactose-hydrolyzed UHT milk - A dynamic headspace gas chromatography-mass spectrometry study. *Dairy Science and Technology*, *94*(4), 311–325. https://doi.org/10.1007/s13594-014-0164-7

Jansson, T., Rauh, V., Danielsen, B. P., Poojary, M. M., Waehrens, S. S., Bredie, W. L. P., … Lund, M. N. (2017). Green Tea Polyphenols Decrease Strecker Aldehydes and Bind to Proteins in Lactose-Hydrolyzed UHT Milk. *Journal of Agricultural and Food Chemistry*, *65*(48), 10550–10561. https://doi.org/10.1021/acs.jafc.7b04137

Jelen, P., & Tossavainen, O. (2003). Low lactose and lactose-free milk and dairy products - Prospects, technologies and applications. *Australian Journal of Dairy Technology*, *58*(2), 161–165. https://doi.org/http://www.scopus.com/inward/record.url?eid=2-s2.0-0141595866&partnerID=tZOtx3y1

Jensen, S., Jansson, T., Eggers, N., Clausen, M. R., Larsen, L. B., Jensen, H. B., … Bertram, H. C. (2015). Storage-induced changes in the sensory characteristics and volatiles of conventional and lactose-hydrolyzed UHT processed milk. *European Food Research and Technology*, *240*(6), 1247–1257. https://doi.org/10.1007/s00217-015-2427-9

Lindinger, W., Hansel, A., & Jordan, A. (1998). On-line monitoring of volatile organic compounds at pptv levels by means of Proton-Transfer-Reaction Mass Spectrometry (PTR-MS) Medical applications, food control and environmental research. *International Journal of Mass Spectrometry and Ion Processes*, *173*(3), 191–241.

Nielsen, S. D., Zhao, D., Le, T. T., Rauh, V., Sørensen, J., Andersen, H. J., & Larsen, L. B. (2018). Proteolytic side-activity of lactase preparations. *International Dairy Journal*, *78*, 159–168. https://doi.org/10.1016/j.idairyj.2017.12.001

Pedrotti, M., Khomenko, I., Cappellin, L., Fontana, M., Somenzi, M., Falchero, L., … Biasioli, F. (2018). Rapid and noninvasive quality control of anhydrous milk fat by PTR-MS: The effect of storage time and packaging. *Journal of Mass Spectrometry*, *53*(9), 753–762. https://doi.org/10.1002/jms.4204

Perkins, M. L., D'Arcy, B. R., Lisle, A. T., & Deeth, H. C. (2005). Solid phase microextraction of stale flavour volatiles from the headspace of UHT milk. *Journal of the Science of Food and Agriculture*, *85*(14), 2421–2428. https://doi.org/10.1002/jsfa.2243

Potineni, R. V., & Peterson, D. G. (2005). Influence of thermal processing conditions on flavor stability in fluid milk: Benzaldehyde. *Journal of Dairy Science*, *88*(1), 1–6. https://doi.org/10.3168/jds.S0022-0302(05)72655-2

Rapisarda, T., Pasta, C., Belvedere, G., Schadt, I., La Terra, F., Licitra, G., & Carpino, S. (2013). Variability of volatile profiles in milk from the PDO Ragusano cheese production zone. *Dairy Science and Technology*, *93*(2), 117–134. https://doi.org/10.1007/s13594-012-0104-3

Santos, M. V., Ma, Y., Caplan, Z., & Barbano, D. M. (2003). Sensory threshold of off-flavors caused by proteolysis and lipolysis in milk. *Journal of Dairy Science*, *86*(5), 1601–1607. https://doi.org/10.3168/jds.S0022-0302(03)73745-X

Soukoulis, C., Aprea, E., Biasioli, F., Cappellin, L., Schuhfried, E., Märk, T. D., & Gasperi, F. (2010). Proton transfer reaction time-of-flight mass spectrometry monitoring of the evolution of volatile compounds during lactic acid fermentation of milk. *Rapid Communications in Mass Spectrometry*, *24*(14), 2127–2134. https://doi.org/10.1002/rcm.4617

Stressler, T., Leisibach, D., Lutz-Wahl, S., Kuhn, A., & Fischer, L. (2016). Homologous expression and biochemical characterization of the arylsulfatase from Kluyveromyces lactis and its relevance in milk processing. *Applied Microbiology and Biotechnology*, *100*(12), 5401–5414. https://doi.org/10.1007/s00253-016-7366-2

Szilagyi, A., & Ishayek, N. (2018). Lactose Intolerance, Dairy Avoidance, and Treatment Options. *Nutrients*, *10*(12), 1994. https://doi.org/10.3390/nu10121994

Troise, A. D., Bandini, E., De Donno, R., Meijer, G., Trezzi, M., & Fogliano, V. (2016). The quality of low lactose milk is affected by the side proteolytic activity of the lactase used in the production process. *Food Research International*, *89*, 514–525. https://doi.org/10.1016/j.foodres.2016.08.021

Valero, E., Villamiel, M., Miralles, B., Sanz, J., & Martínez-Castro, I. (2001). Changes in flavour and volatile components during storage of whole and skimmed UHT milk. *Food Chemistry*, *72*(1), 51–58. https://doi.org/10.1016/S0308-8146(00)00203-X

Van Aardt, M., Duncan, S. E., Bourne, D., Marcy, J. E., Long, T. E., Hackney, C. R., & Heisey, C. (2001). Flavor threshold for acetaldehyde in milk, chocolate milk, and spring water using solid phase microextraction gas chromatography for quantification. *Journal of Agricultural and Food Chemistry*, *49*(3), 1377–1381. https://doi.org/10.1021/jf001069t

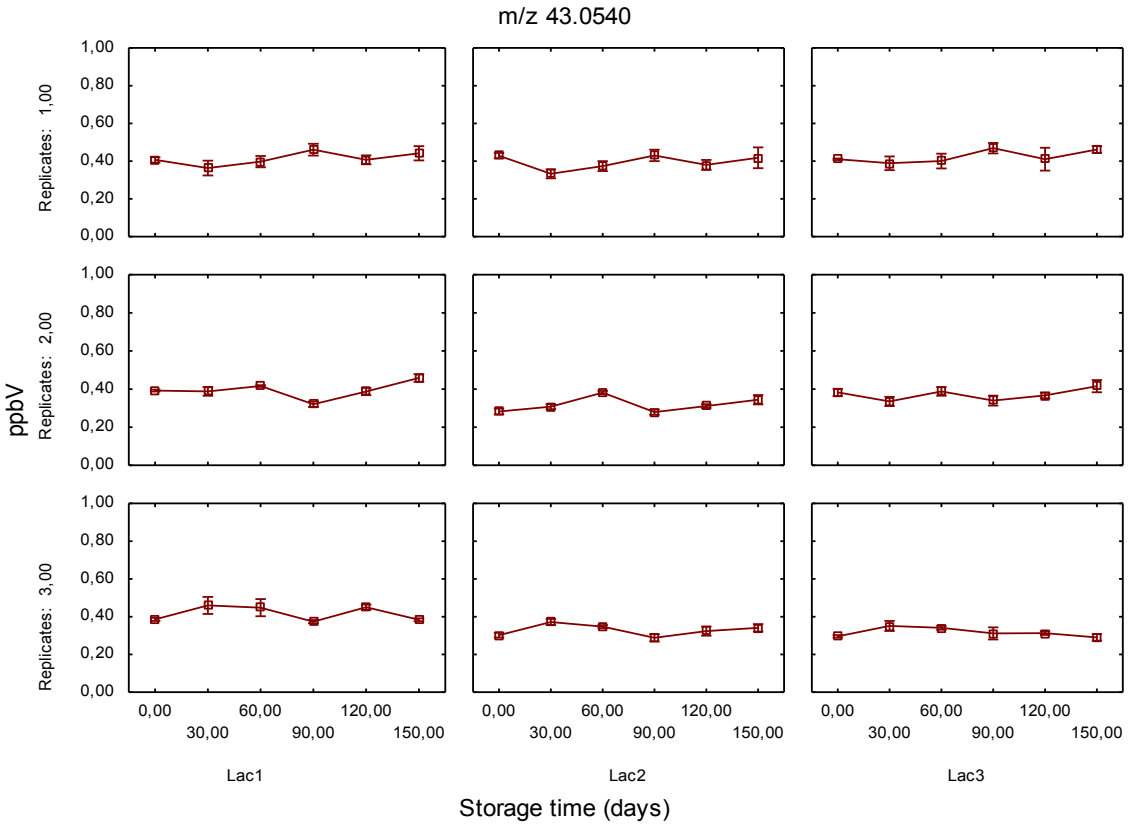
van den Berg, R. A., Hoefsloot, H. C. J., Westerhuis, J. A., Smilde, A. K., & van der Werf, M. J. (2006). Centering, scaling, and transformations: Improving the biological information content of metabolomics data. *BMC Genomics*, *7*. https://doi.org/10.1186/1471-2164-7-142

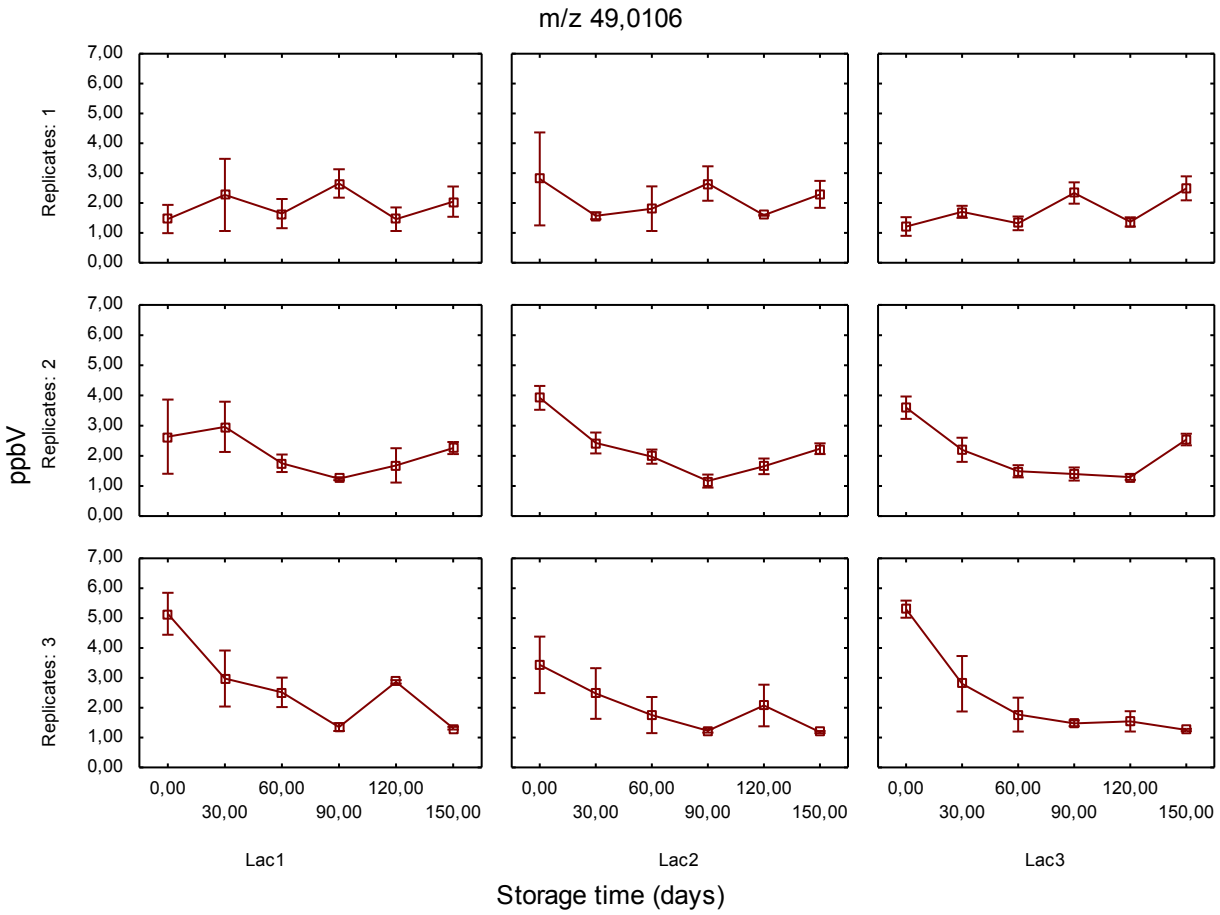
van Ruth, S. M., Koot, A., Akkermans, W., Araghipour, N., Rozijn, M., Baltussen, M., … Frankhuizen, R. (2008). Butter and butter oil classification by PTR-MS. *European Food Research and Technology*, *227*(1), 307–317. https://doi.org/10.1007/s00217-007-0724-7

Zardin, E., Tyapkova, O., Buettner, A., & Beauchamp, J. (2014). Performance assessment of proton-transfer-reaction time-of-flight mass spectrometry (PTR-TOF-MS) for analysis of isobaric compounds in food-flavour applications. *LWT - Food Science and Technology*, *56*(1), 153–160. https://doi.org/10.1016/j.lwt.2013.10.041

Supplementary materials

All the mass peaks reported in Table 2 and discussed thoroughout the main body of the manuscript were plotted. Lactase preparations and replicates of production were kept separated in order to allow a better visualization of the trends. Each point displayed represent the mean value of three different instrumental replicates (n = 3).

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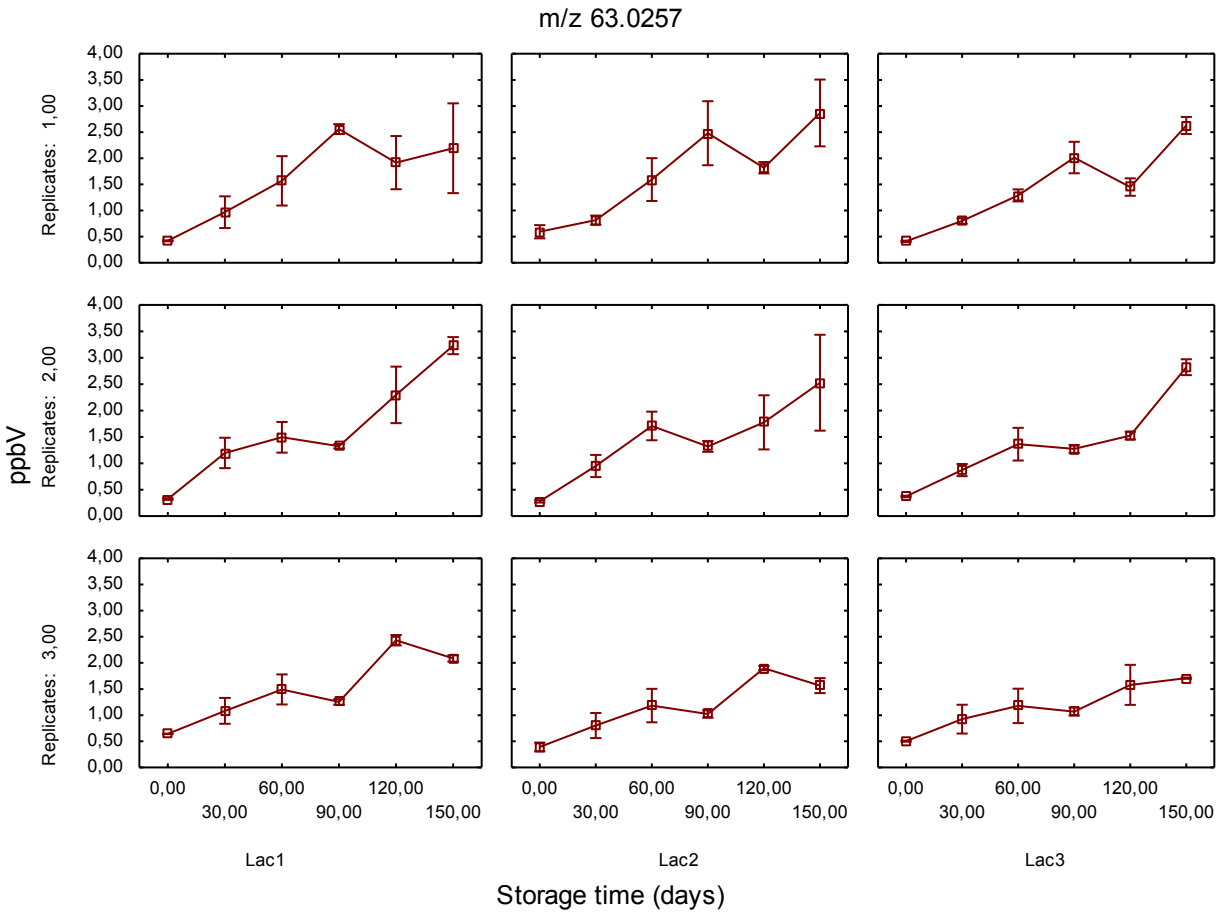
0,00
0,00
30,00
60,00
90,00
120,00
150,00
Lac1
Lac2
Lac3
Storage time (days)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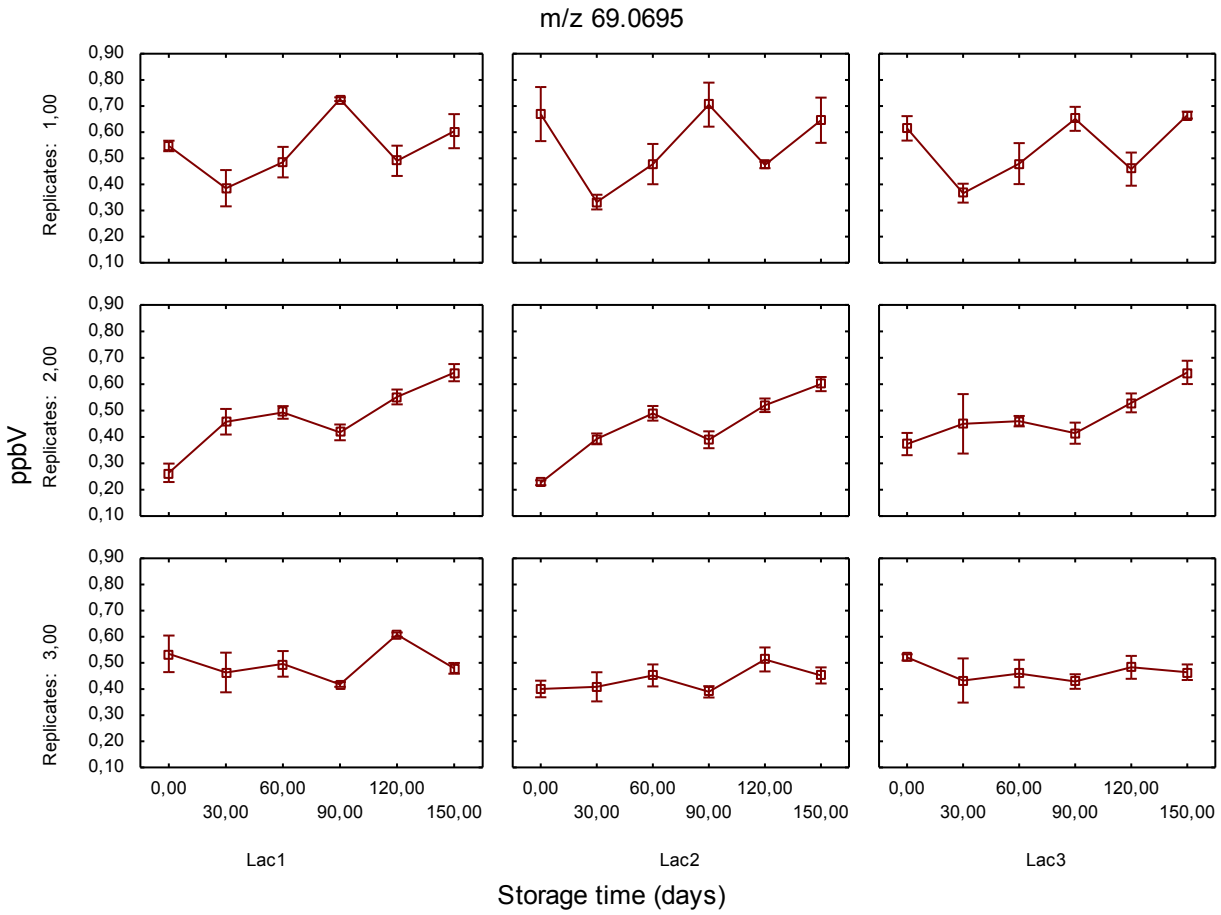
Lac1
Lac2
Lac3
Storage time (days)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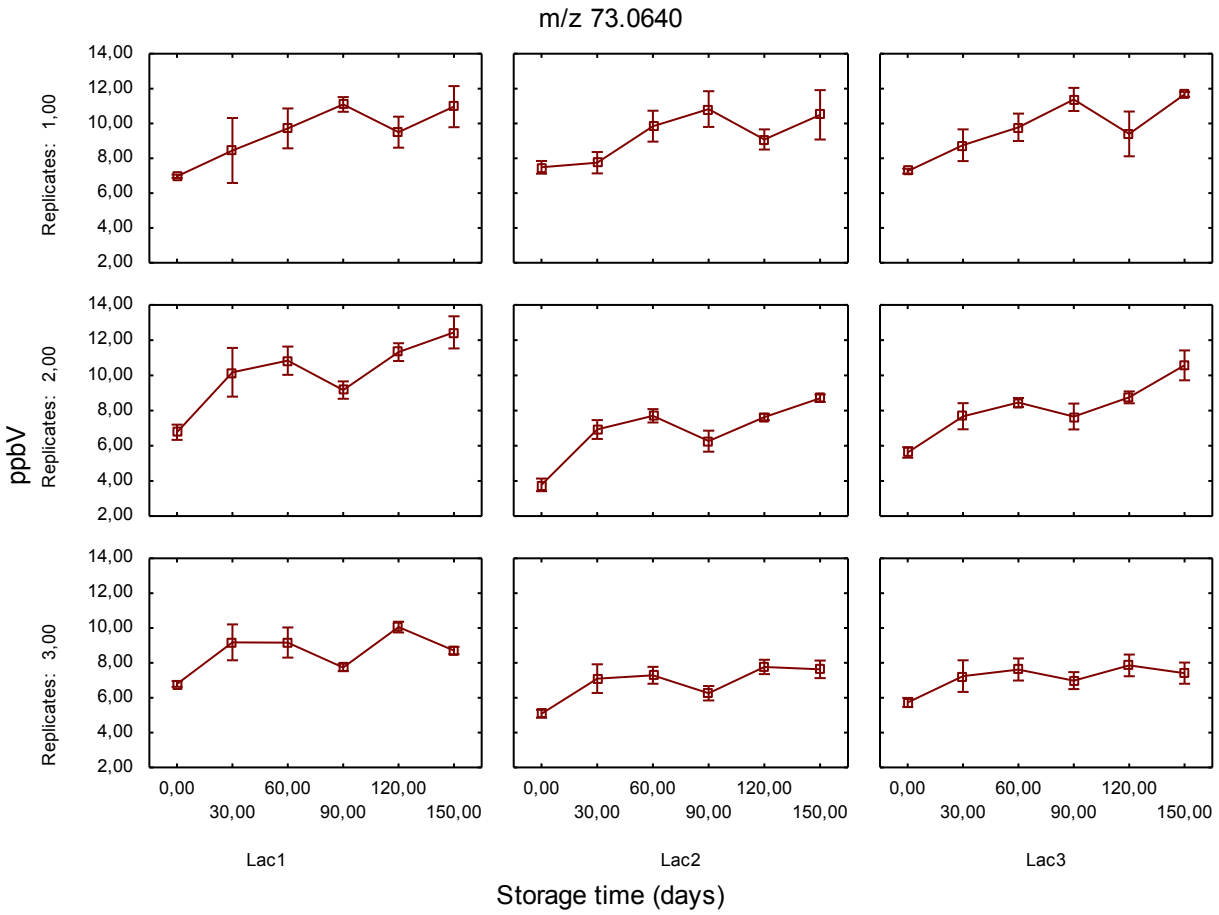
150,00
Lac1
Lac2
Lac3
Storage time (days)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30,00
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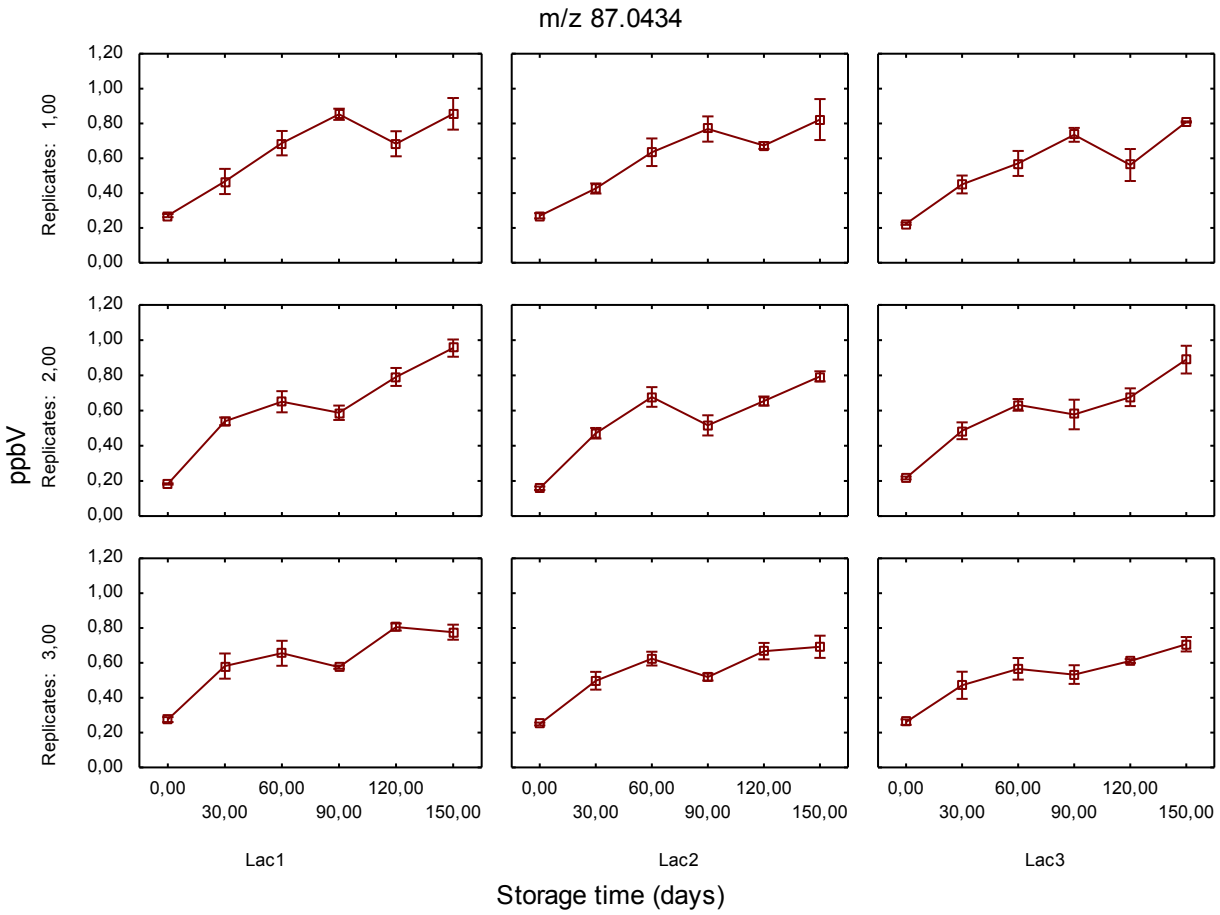
120,00
150,00
Lac1
Lac2
Lac3
Storage time (days)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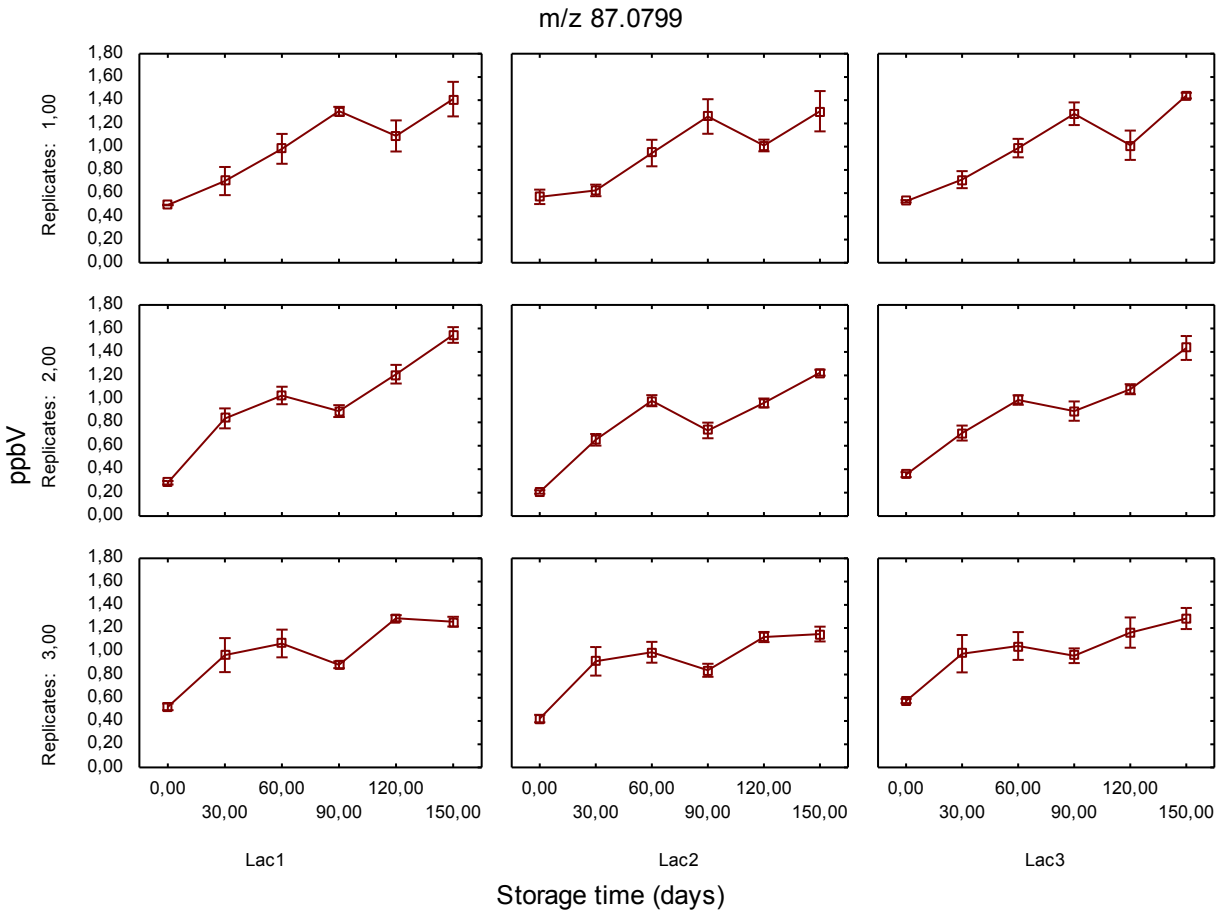
120,00
150,00
Lac1
Lac2
Lac3
Storage time (days)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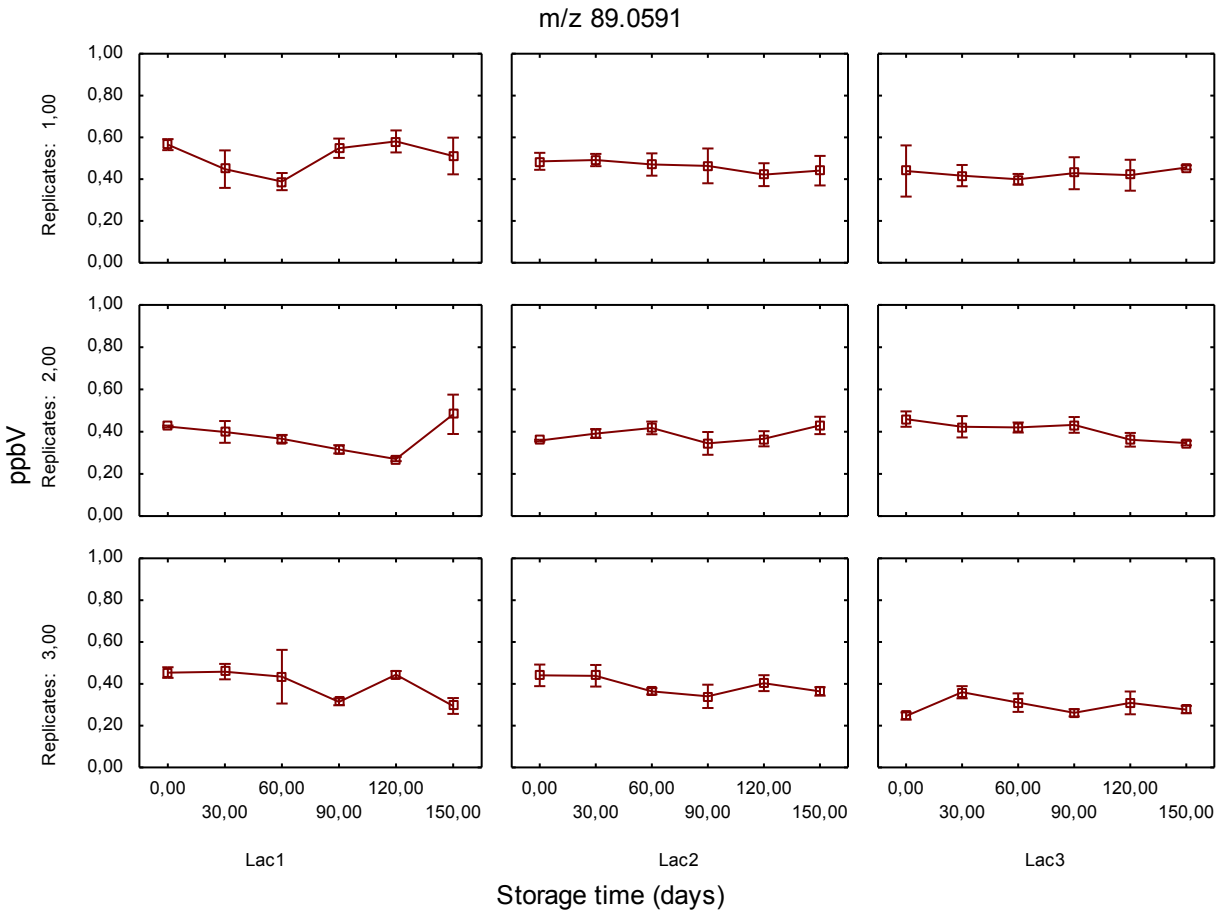
90,00
120,00
150,00
Lac1
Lac2
Lac3
Storage time (days)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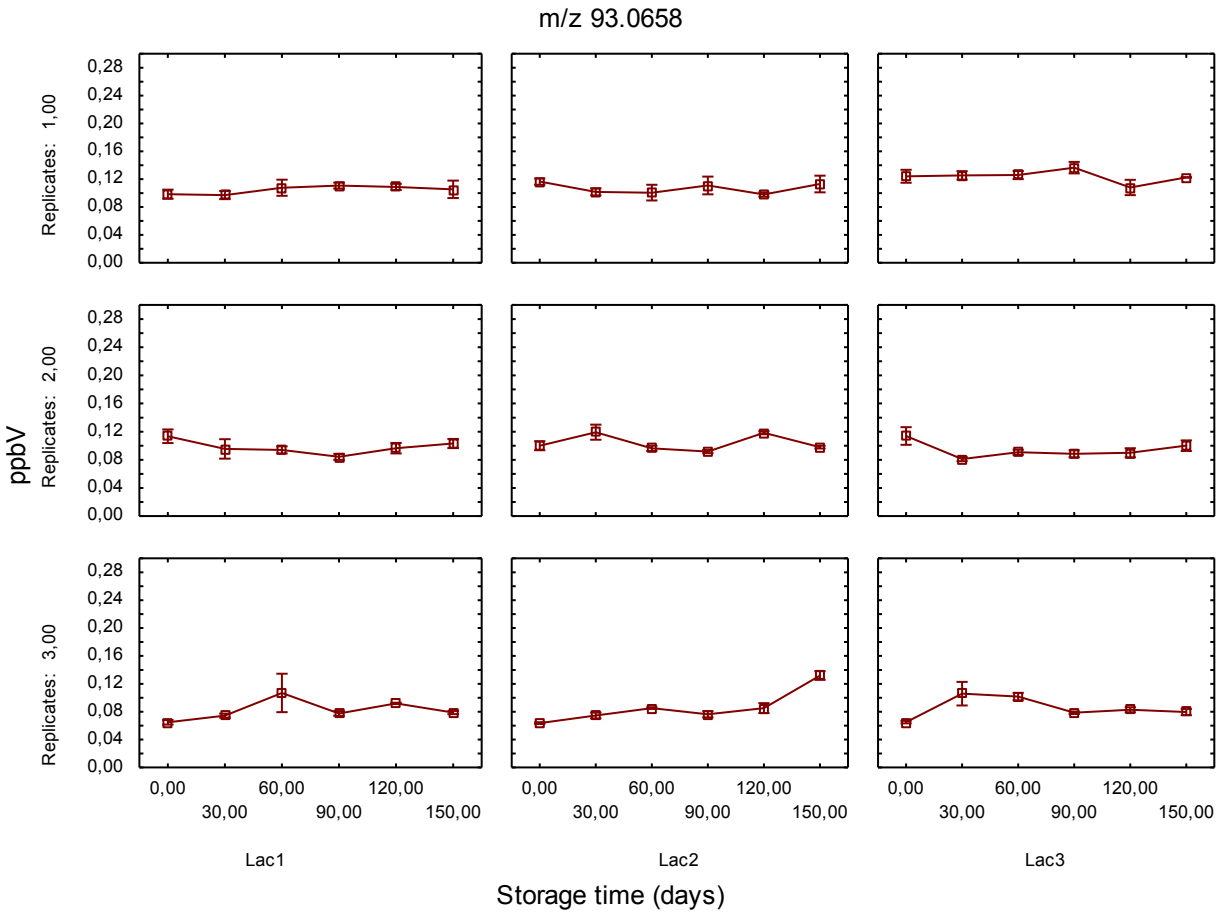
120,00
150,00
Lac1
Lac2
Lac3
Storage time (days)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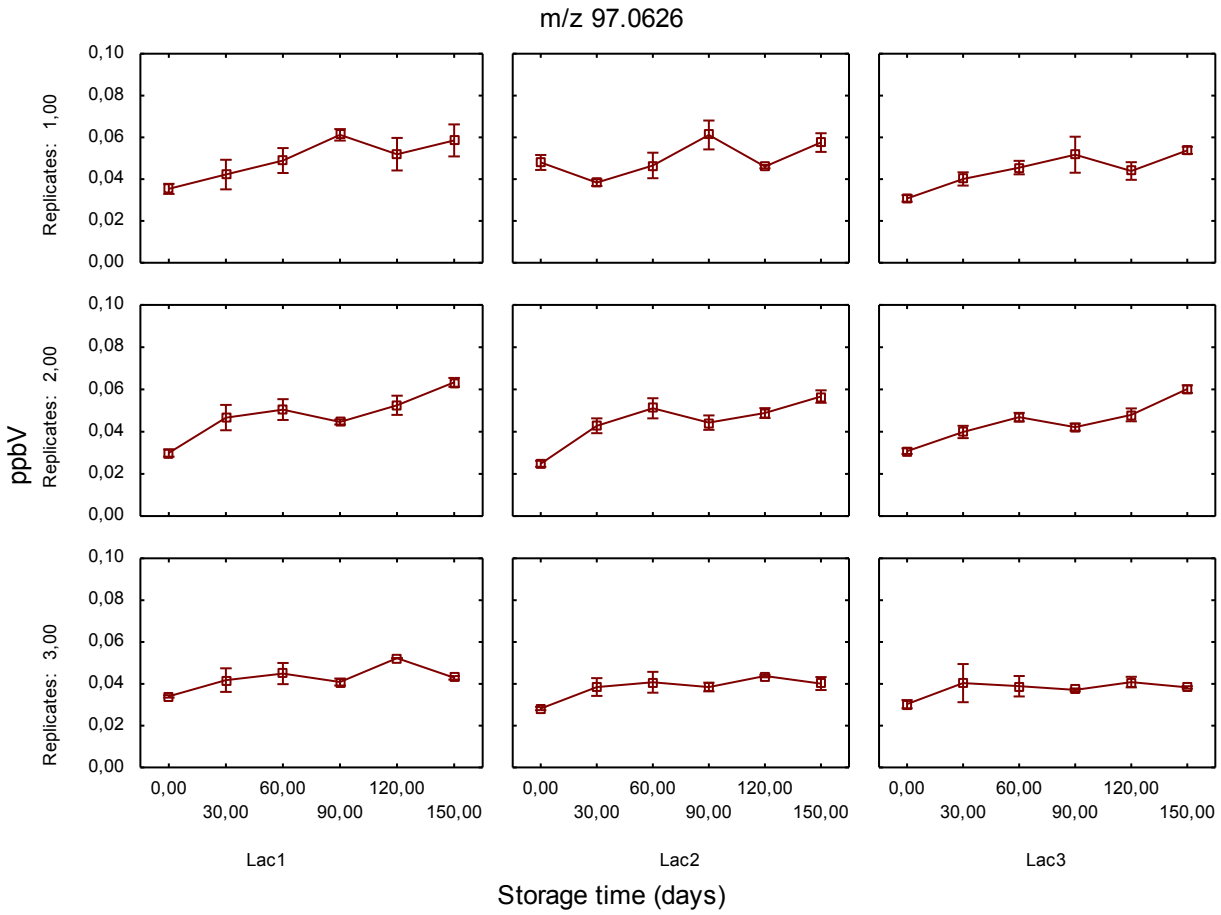
60,00
90,00
120,00
150,00
Lac1
Lac2
Lac3
Storage time (days)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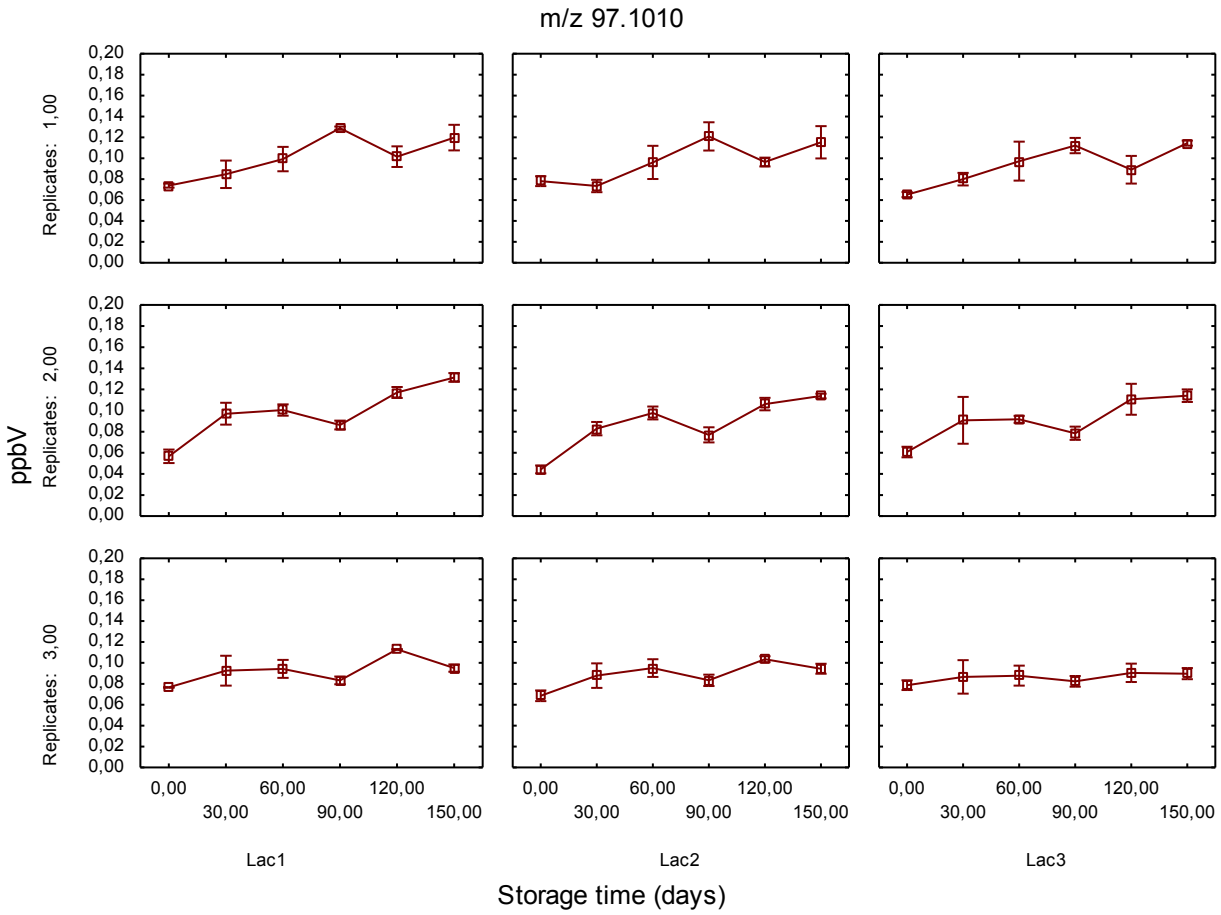
Lac1
Lac2
Lac3
Storage time (days)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Lac1
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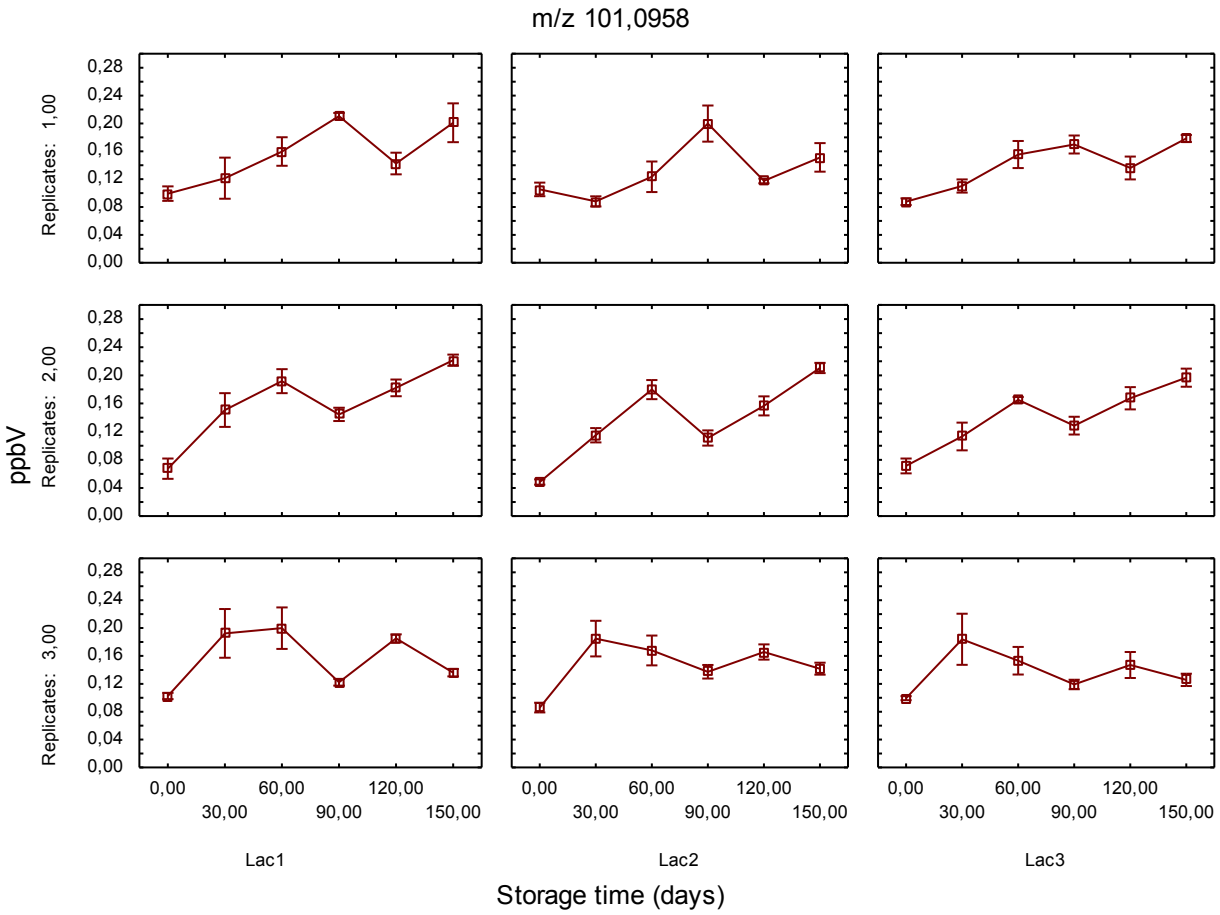
Lac3
Storage time (days)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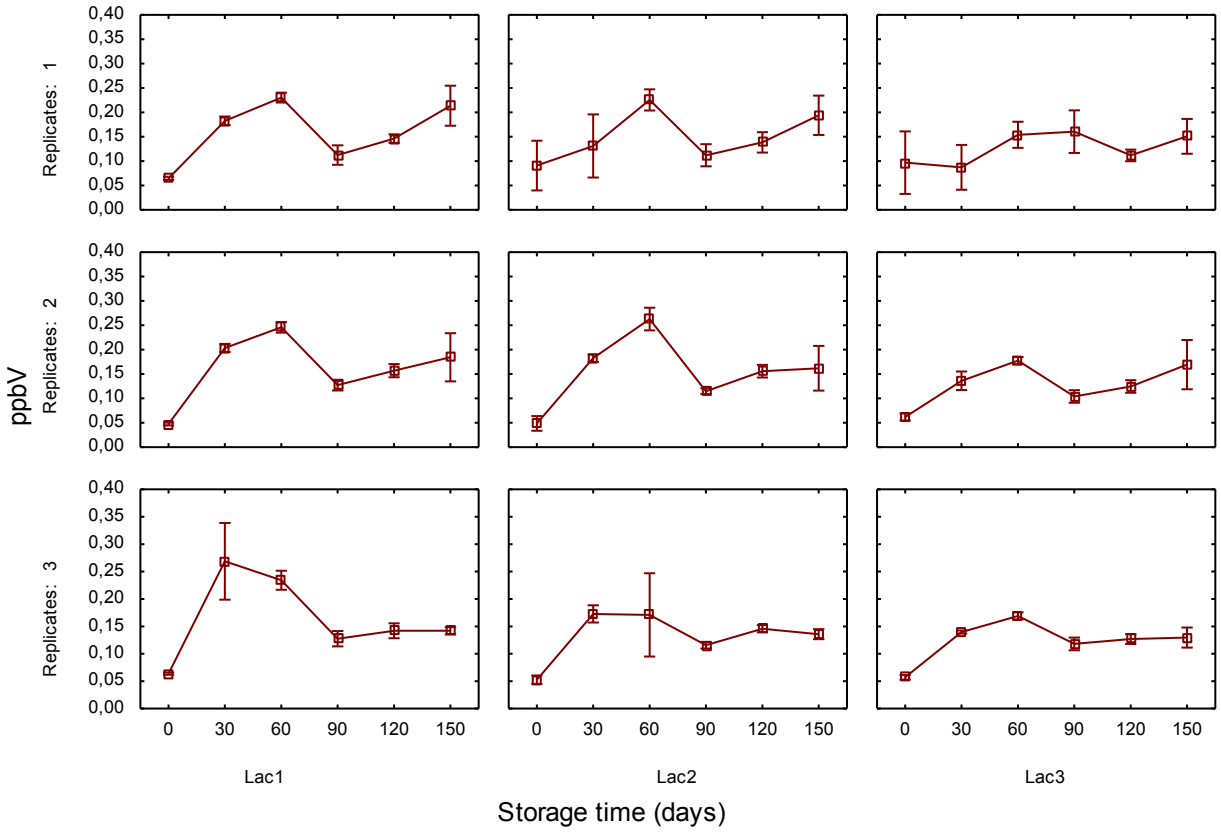
Lac1
Lac2
Lac3
Storage time (days)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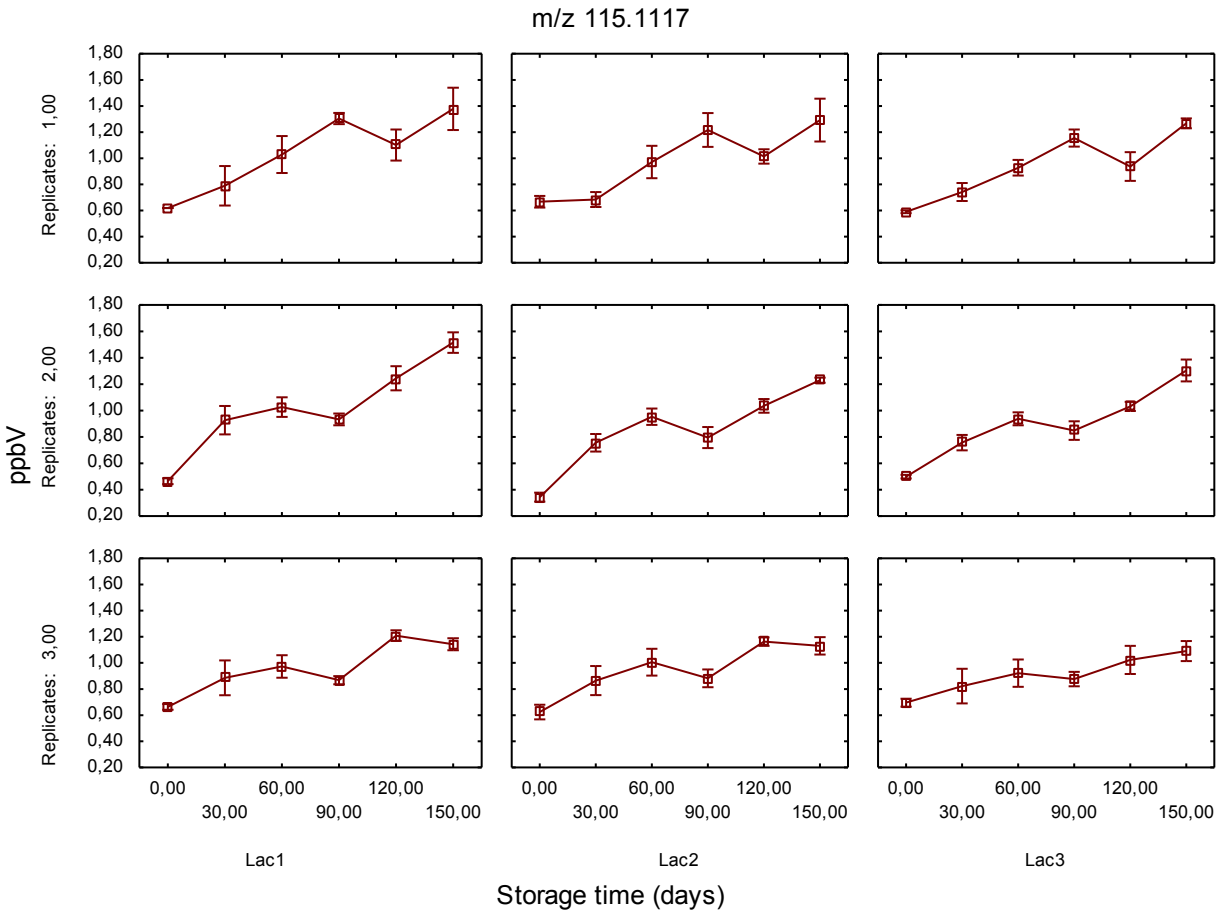
Lac2
Lac3
Storage time (days)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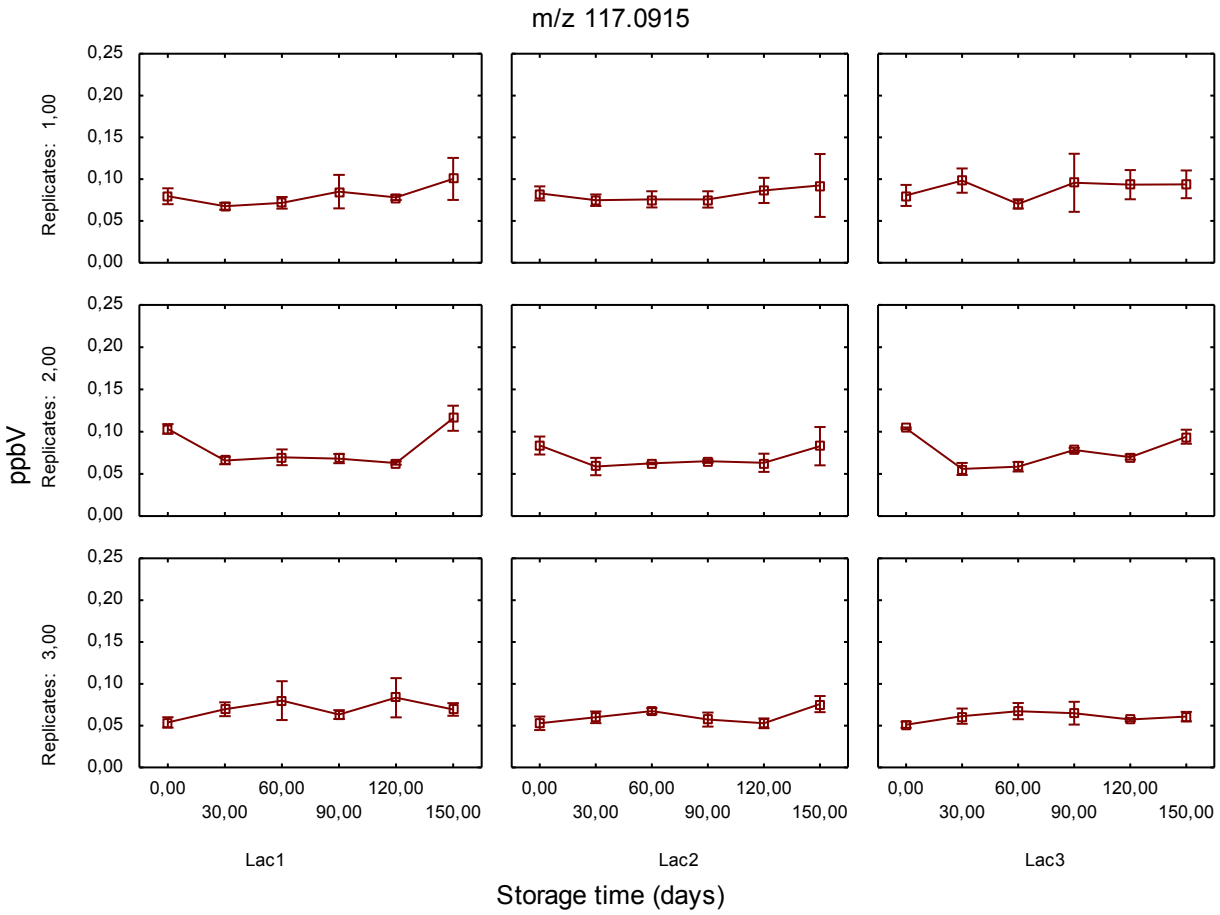
Lac1
Lac2
Lac3
Storage time (days)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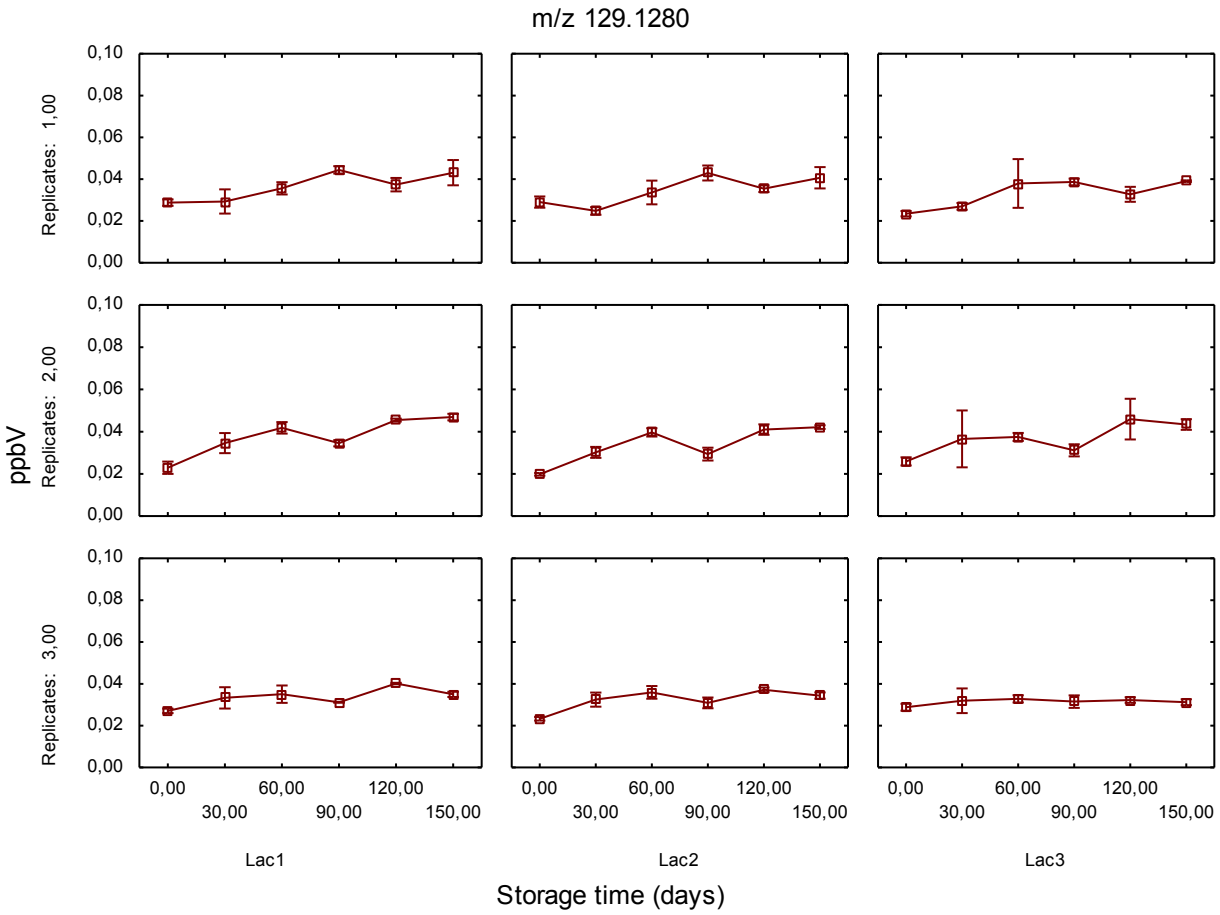
120,00
150,00
Lac1
Lac2
Lac3
Storage time (days)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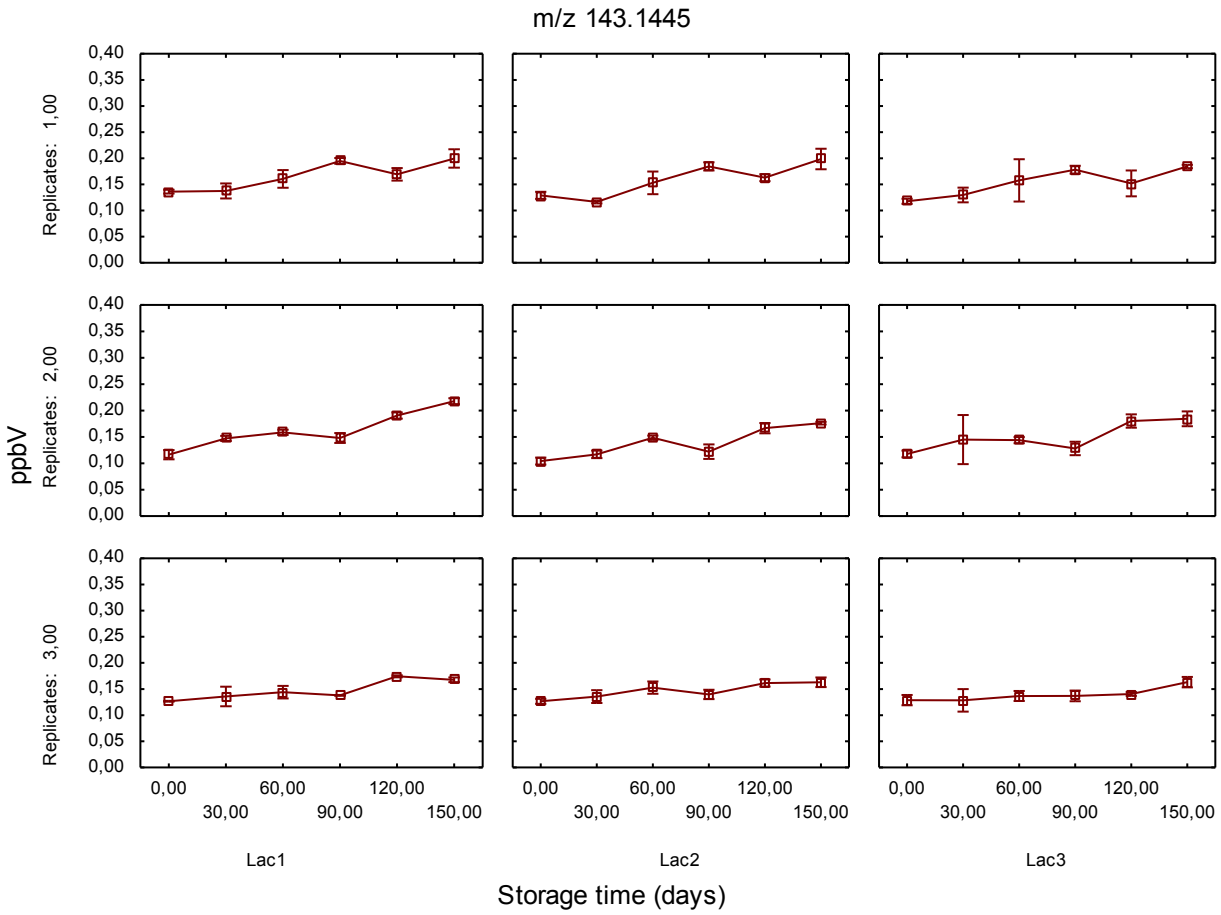
Lac3
Storage time (days)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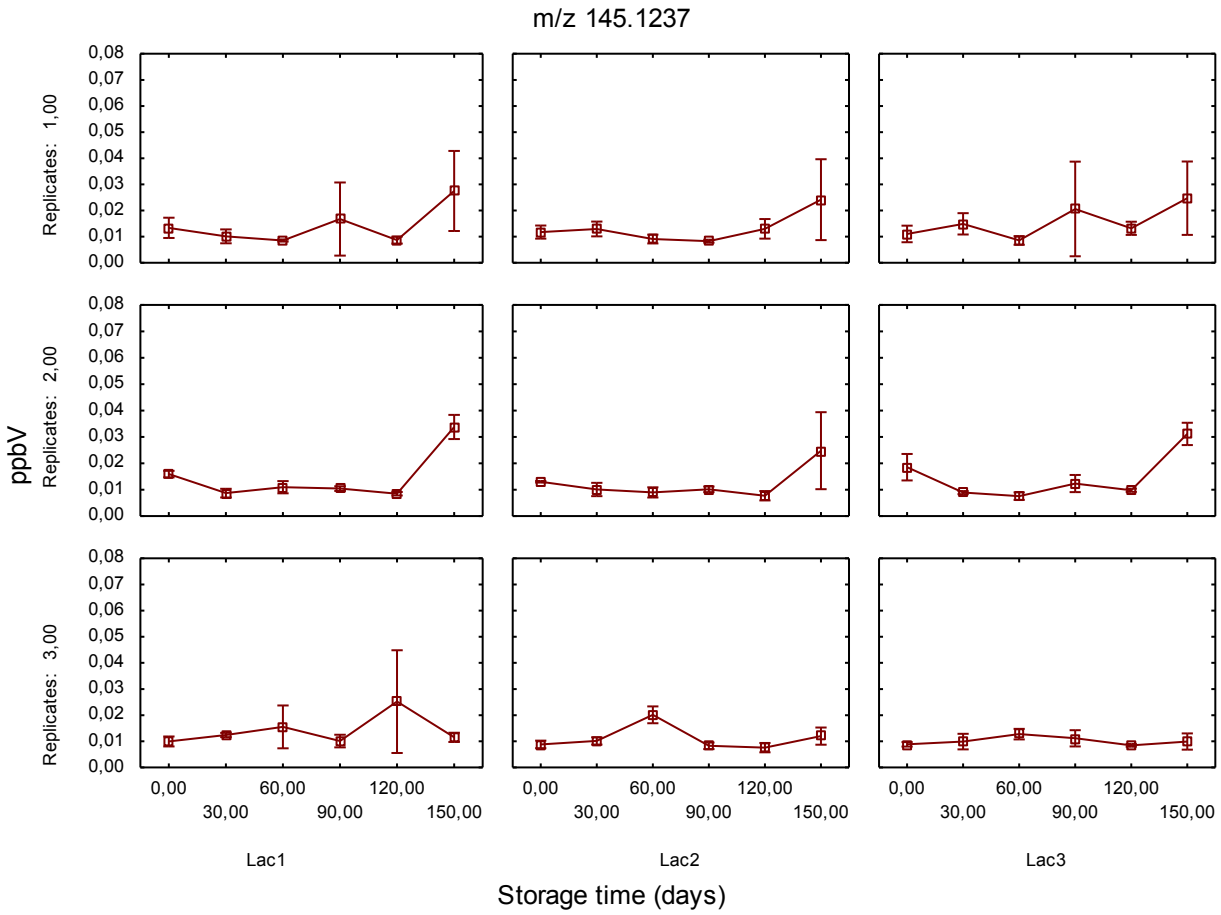
Storage time (days)



Supplementary Figure 1. Temporal trend of the selected mass peaks reported in Table 2, separated for each replicate of production and lactase preparation tested.

Chapter 6: Understanding the effect of storage temperature on the quality of semi-skimmed UHT hydrolyzed-lactose milk: an insight on release of free amino acids, formation of volatiles organic compounds and browning

From the previous chapters, it is now clear that although UHLM is a highly profitable market optimal production is a challenge because of the possible development of off-flavors and sensory defects during shelf-life. In Chapter 4 it was highlighted that the different lactase preparations can modulate quality losses in UHLM during storage at room temperature. This time we followed up the research investigating the response of UHLM, again produced by "in batch" system, to different storage temperatures. The commercial shelf-life of UHLM (3-4 months) is much shorter than regular UHT milk (9-12 months) and it is not suitable for export at the moment: temperature fluctuations along shipping may result in a decay of the product quality, so strategies for extending the shelf-life to values close to regular UHT milk are requested. Accordingly, in this chapter we addressed the issue storing UHLM at 4°C, 20°C, 30°C, 40°C and analyzing the release of free amino acids, the development of volatiles organic compounds and the changes in the milk color over time for 120 days.

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Abstract

Proteolytic side activity of the lactase preparations (LPs) intended for ultra-high temperature hydrolyzed-lactose milk (UHLM) production induces changes in the product quality during shelf-life. The problem is particularly relevant when the enzyme is added aseptically in the packaging ("in pack" process), while the negative quality effects can be mitigated following the "in batch" process adding the LP before thermal sterilization. In this study, we monitored the quality over time of UHLM produced "in batch" and stored at 4, 20, 30 and 40°C focusing on proteolysis, volatiles organic compounds (VOCs) formation and color changes. The goal was to identify the key reactions and compounds relevant for the product quality. An increase in storage temperature determined significant changes in the free amino acids profile increasing Strecker aldehydes and methyl ketones formation. At 30 and 40°C, Maillard reaction and lipid oxidation ended up in a modification of the milk color, whereas at 4 and 20°C no significant alteration was observed. Altogether, the results suggested a coordinate involvement of Maillard reaction, protein and lipid oxidation to milk browning and off-flavors formation in UHLM.

Introduction

In the current market, the high demand of long-lasting dairy products with stable quality is satisfied by heat treatment (Sunds, Rauh, Sørensen, & Larsen, 2018). In particular, ultra-high temperature (UHT) treatment extends the commercial shelf-life of the milk at room temperature up to 6-9 months, by exposing the product to elevate temperature for very short time (Zabbia, Buys, & de Kock, 2012). This is accomplished by aseptic packaging, which maintains the product sterile throughout the shelf-life once the heat load inactivates the pathogens present in the milk (Valero, Villamiel, Miralles, Sanz, & Martínez-Castro, 2001).

Europe is currently the largest market for regular UHT milk, with an estimate of 7 out of 10 people consuming the product regularly (Bimbo, Bonanno, Liu, & Viscecchia, 2016). On the other hand, the milk market globalization and the price war started by food retailers is pushing European manufacturers to expand their product portfolio (Bimbo et al., 2016), rendering also the export an appealing opportunity to increase profit margins. In this context, launches of hydrolyzed-lactose milk (HLM) products rocketed in the recent past, riding the wave of the increasing awareness towards lactose intolerance (Nielsen et al., 2017). HML has a lower shelf-life than regular UHT milk and this is hampering the export in Asia, where lactose intolerance has high prevalence and the market is expanding. According to Dekker et al. (2019) fluid milk is the product category growing the most and UHT hydrolyzed-lactose milk (UHLM) leads the positive trend.

Manufacturing of UHLM is challenging and operating conditions should be carefully monitored to maintain high quality standards (Bottiroli, Pedrotti, et al., 2020). In general, reactions occurring during UHT processing may alter the sensory profile (especially the flavor) of UHT milk, jeopardizing its acceptability (Jansson et al., 2019; Sunds et al., 2018). Maillard reaction (MR), lipid oxidation and protein denaturation, oxidation and hydrolysis are the key reactions and they tend to proceed faster in UHLM compared to regular UHT milk (Jansson et al., 2014, 2017; Messia, Candigliota, & Marconi, 2007; Olli Tossavainen & Kallioinen, 2008). The higher reactivity of glucose and galactose compared to lactose, as well as the proteolytic side activity of the commercial lactase preparations (LPs), promote MR resulting in products with a shorter commercial shelf-life of 90-120 days at room temperature (Milkovska-Stamenova & Hoffmann, 2017; Naranjo, Gonzales, Leiva, & Malec, 2013; Nielsen et al., 2018). Chemical modifications influence physicochemical parameters of milk and in the case of lactose-hydrolyzed milk, glycation alters protein structures and conformation, thus modifying also hydrophobicity and aggregation with consequences on

viscosity and gel formation (Hannß, Hubbe, & Henle, 2018). The MR is triggered by the reaction between reducing sugars and protein. In UHLM, the proteolytic side activity of the commercial lactase enhances the release of free amino acids by increasing the substrate available for the propagation of MR (Jansson et al., 2014; O. Tossavainen & Kallioinen, 2007; Troise et al., 2016). Besides browning and loss of nutritional value, flavor compounds are generated *via* MR (van Boekel, 2006) and are considered the major limiting factors affecting the sensory quality of both regular UHT milk and UHLM (Jansson et al., 2019; Sunds et al., 2018).

The development of UHLM with sensory characteristics resembling to regular UHT milk is a major priority for dairy manufactures, but a limited number of research articles have been published on the topic (Adhikari, Dooley, Chambers IV, & Bhumiratana, 2010; Jensen et al., 2015; Nielsen et al., 2017; Troise et al., 2016). In order to be appreciated by consumers, milk should provide a refreshing mouthfeel without any odors and aftertaste (Francis et al., 2005). Sensory properties of UHLMs tend to differ from regular UHT milk in terms of sweetness, cooked flavor, processed flavor and chalkiness, all more intense in the former (Adhikari et al., 2010). Jensen et al. (2015) studied the off-flavors formation in UHLM during storage in relation to the sensory profile and reported that the formation of specific volatiles organic compounds (VOCs) following Maillard pathways underpins the development of stale flavor. An increase in the concentration of methyl ketones, aldehydes and organic acids during storage appeared as major responsible for the development of this sensory defect in UHLM (Bottiroli, Troise, et al., 2020; Jensen et al., 2015). Our group estimated these VOCs in UHLM both by HS SPME GC-MS and PTR-TOF-MS, outlining that off-flavors are created mostly alongside Strecker degradation (Bottiroli, Aprea, Betta, Fogliano, & Gasperi, 2020; Bottiroli, Pedrotti, et al., 2020).

Along with the availability and chemical nature of the substrate, temperature, time, pH and water content are other key factors that supervise the formation of VOCs *via* MR (van Boekel, 2006). The propagation of the MR towards advanced stages is modulated by the storage time and temperature which, if not properly controlled, can lead to changes in color, nutritional value and flavor of the milk (van Boekel, 1998). Therefore, understanding the influence of the storage conditions on milk aging is necessary to develop strategies aimed to extend the shelf-life of UHLM, minimizing sensory defects. The aim of this study was to investigate the effect of different storage temperatures (4, 20, 30 and 40°C) on chemical modifications of UHLM considering the proteolysis, the VOCs evolution and the color development, focusing on parameters linked to MR and product quality.

Materials and Methods

Chemical Reagents

Acetonitrile and water were purchased from Merck (Darmstadt, Germany). Formic acid, the analytical standards [4,4,5,5-d_4]-L-lysine hydrochloride (d_4-lysine), L-lysine-6-^{13}C dihydrochloride (^{13}C-lysine), the 20 L-amino acids analytical standards for the LC-HMRS and the 4-methyl-2-pentanone (purity ≥ 99%) for the GC-MS analysis were obtained from Sigma-Aldrich (St. Louis, MO).

UHT Hydrolyzed-Lactose Milk Samples

Milk was manufactured at industrial scale following the "in batch" production system as described in a previous work (Bottiroli, Troise, et al., 2020). The "in batch" UHLM is produced by adding the LP intended for lactose hydrolysis in a tank before UHT sterilization. In this way, the desired level of lactose hydrolysis is achieved before packaging and the LP remains inactivated throughout the product shelf-life. The starting milk was semi-skimmed and its proximate composition is reported in **Supplementary Table A.1**. Production occurred at industrial scale and it was repeated three times, on three consecutive weeks, taking into account the batch variability. Lactose conversion was performed at refrigerated conditions until a final lactose concentration of <0.1 g/L was obtained. Residual lactose content was monitored according to Troise et al. (2016). The commercial LP used for production was extracted from *Kluyveromyces lactis* and had an enzymatic activity higher than 5000 NUL/g. Direct UHT treatment was then applied and the obtained UHLM were packed aseptically (1L bottle). After production, UHLM bottles were stored in different climate chambers at the following temperatures: 4°C, 20°C, 30°C, 40°C. Sampling started after 30 days of storage and was carried on until 120 days, based on the best-before date of each milk package. UHLM samples were collected every 30 days and kept at -80°C until analyses.

Liquid Chromatography–High-Resolution Mass Spectrometry (LC-HRMS)

Free amino acids were analyzed according to the procedure previously reported by our group (Troise, Wiltafsky, Fogliano, & Vitaglione, 2018). Briefly, 0.1 mL of acetonitrile were added to milk samples (0.1 mL), then spiked with d_4-lysine; after protein precipitation, samples were centrifuged at 4 °C, for 15 min at 21100 *x g*. Samples were diluted ten times in a mixture of acetonitrile and water 50:50 and filtered by using cellulose filters (RC, 0.22 µm Sartorius, Gottingen, Germany). Chromatographic separation of amino acids was achieved at 30 °C by using a Syncronys HILIC (3.0 µm, 50 x 2.1 mm, Thermo Fisher, Bremen, Germany). Mobile phases were 0.1% formic acid in acetonitrile (solvent A), 0.1% formic acid in water (solvent B) and the following linear gradient of solvent B (min/%B): (0/5), (1.50/5), (8/90), (10/90) was used, the flow rate was set to 300 µL/min and the injection volume was 5 µL. The Accela 1250 U-HPLC system (Thermo Fisher Scientific, Bremen, Germany) was interfaced to an Exactive Orbitrap HRMS (Thermo Fisher Scientific, Bremen, Germany) and the analytes were detected through a heated electrospray interface (HESI-II) operating in positive mode. Interface parameters and analytical performances were set and evaluated according to a previous work (Bottiroli, Troise, et al., 2020). Samples were spiked with ^{13}C-lysine before each injection and its exact mass ($[M+H]^+$,148.11616) was used as a reference to maximize response and to check instrumental resolution and performance.

Headspace Solid-Phase Micro-Extraction Gas Chromatography Mass Spectrometry (HS SPME GC-MS)

The HS-SPME GC-MS analysis was conducted according to a previous research article (Bottiroli, Aprea, et al., 2020). For each UHLM sample, 5 mL were collected in 20 mL vials (Supelco, Bellefonte, PA). 4-Methyl-2-pentanone was added as internal standard (IS). A 2 cm DVB-Carboxen–PDMS SPME fiber heated at 40 °C for 60 min was used for the VOCs collection. VOCs were desorbed in the injector port of a GC Clarus 500 (PerkinElmer, Norwalk, CT) at 250°C. The GC was interfaced with a mass detector operating in an electron ionization mode (internal ionization source: 70 eV; scan range: m/z 33-300). Measurements were operated in automatic by an auto-sampler (CTC combiPAL, CTC Analysis AG, Zwingen, Switzerland). The separation of the injected mix of VOCs was obtained using a HP-Innowax fused-silica capillary column (Agilent Technologies, Palo Alto, CA) having the following characteristics: 30 m length, 0.32 mm inner diameter, 0.5 µm film thickness.

The oven temperature was set with different consecutive steps, namely 40 °C for 3 min, 180 °C for 6 min at 4 °C min^{-1} and 220 °C for 3 min at 3 °C min^{-1}. Helium was used as gas carrier (flow rate set at 2 mL/min). Compounds extracted from the UHLM headspace were identified using the mass spectra matching the NIST-2014/Wiley 7.0 libraries and comparing the calculated LRI with those available from literature. Semi-quantitative data were calculated as µg/L of internal standard 4-methyl-2-pentanone.

Color analysis

The color was measured with a CM-3500d (Minolta, Japan). The instrument was calibrated with distilled water and the opaque material provided by the manufacturer. Samples were measured at room temperature using a quartz cell with 1 cm of optical path. Results were expressed using the CIELAB color system with L* (lightness; range: 0-100, 0 = black and 100 = white), a* (−a*: green color; +a* red color), and b* (−b*blue color; +b* yellow color). Results were averaged on three readings taken on each UHLM sample.

Statistical Data Analysis

All the statistical analyses were performed using RStudio (RStudio Team 2018. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA) and STATISTICA 13.3 (StatSoft, Inc., Tulsa, OK). Patterns in the free amino acids profile were explored by Principal Component Analysis (PCA) performing a log-transformation and a scaling to unit variance on all the variables prior to the analysis. The effect of the storage time and temperature on each detected free amino acids, VOCs and color parameter was further investigated by the analysis of variance and Tukey post-hoc test. A confidence interval ($P \leq 0.05$) was chosen as threshold for significance. The relationships among the three datasets, that we considered an approximation of the three different stages of MR (initial: free amino acids profile by LC-HRMS; intermediate: VOCs profile by GC-MS; final: color by CIELab system) were studied by Multiple Factorial Analysis (MFA). These three datasets were considered active data, whereas storage time and temperature were included in the model as supplementary. To further support the relationships pointed out by the multivariate analysis, Pearson regression coefficients (r) were calculated for direct paired comparison of the variables.

Result and Discussion

Free Amino Acids Profile and Initial Stages of Maillard Reaction

The concentration of free amino acids in the UHLMs stored at different temperatures ranged from 0.154 to 33.304 μg/mL**.** Acidic (glutamic acid, aspartic acid) and aliphatic (alanine, valine, isoleucine/leucine) amino acids were the two classes of amino acids exhibiting the highest levels in their free form during the shelf-life. The highest median value was observed for acidic amino acids due to the huge contribution of free glutamic acid, which was by far the amino acid present in the highest amounts (concentration range: 8.016 – 33.304 μg/mL). Among the aliphatic amino acids, comparable levels of alanine (concentration range: 2.185-4.884 μg/mL) and isoleucine/leucine (concentration range: 1.895-3.707 μg/mL) were encountered, whereas valine registered higher values (concentration range: 2.965-8.581 μg/mL). An explorative snapshot of the influence of the two independent factors on the free amino acids content was obtained through Principal Component Analysis (PCA), whose score and loading plots are reported in **Figure 1**. The first two scores of the UHLM samples showed a spatial distribution of the tested milk depending on the applied storage temperature. The factor appeared more important for distinguishing the free amino acids profiles of UHLMs compared to the storage time, that did not show any particular trend. All samples stored at 4°C (30-120 days) were discriminated based on their free amino acids profiles from the milk stored at the higher temperatures. According to the loadings, an overall higher concentration of free amino acids was responsible for the separation from the rest of the samples.

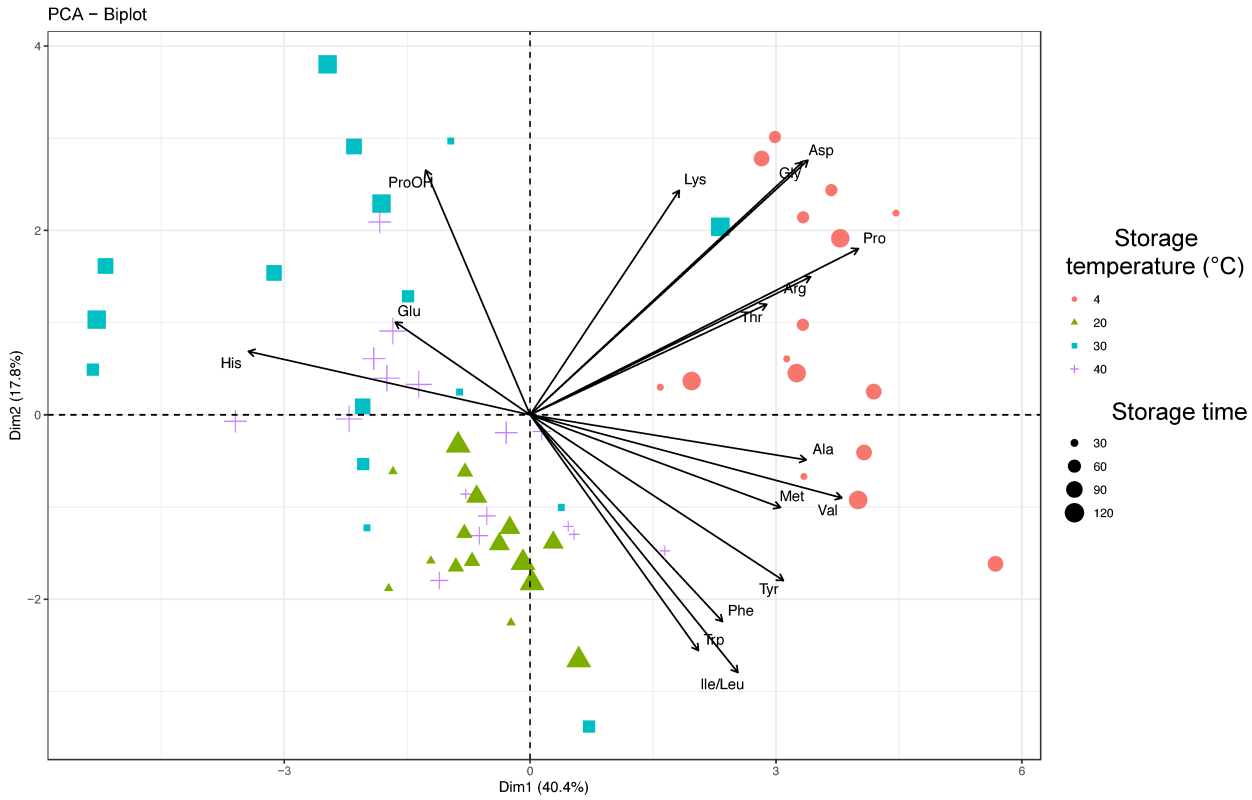


Figure 1. Principal Component Analysis (PCA) bi-plot of the first (PC1; 41.60% of the total variance) and the second (PC2; 16.90% of the total variance) principal components performed on the free amino acids data of the UHLMs stored at different temperatures (the 4 related series are represented by different symbols and colors) for 120 days. Storage time (30-120 days, sampling every 30 days) is represented by an increasing size of the points. Black arrows indicated the corresponding weights of the loadings on the spatial distribution of the UHLM samples. Prior to analysis, data were log-transformed and scaled to unit variance, whereas outliers were removed.

From the bi-plot, a higher concentration of free hydroxyproline, histidine and glutamic acid characterized the UHLMs stored at temperature higher than conventional 20°C. From the time course evolution of the free amino acids (**Supplementary Fig. A.1),** a change in the trends of these three amino acids can be observed: from being relatively constant over time when milk was stored at 4°C and 20°C, the three amino acids shifted towards an upward trend when the storage temperature was increased. This pattern was particularly remarkable at 30°C. The occurrence of free hydroxyproline in the milk is determined by free proline hydroxylation, but the reaction kinetics at the tested temperatures are unknown (Abernethy & Higgs, 2013). The trend of free glutamic acid instead may confirm the specificity of the proteolytic side activity of the LP towards specific amino residues as well as a positive

correlation with the temperature. In a previous work, we showed that the type of amino acids released during "in batch" production does not depend only on the degree of lactase purity but also on the specificity of the proteolytic activity, as different free amino acids profiles were obtained in UHLMs treated with different lactases (Bottiroli, Troise, et al., 2020). In particular, UHLM produced with the same lactase used in this study caused a significant release of glutamic acid in milk. Although in this case glutamic acid was the only amino acid showing this behavior, the result may indicate that, at storage temperature higher than 20°C, release of free amino acids can still occur in the milk. Previous data taken at 20°C are on the other hand not in agreement (Bottiroli, Troise, et al., 2020). The fact that glutamic acid is not converted through Strecker degradation may explain also the opposite trend showed at 40°C by those amino acids involved in the reaction (e.g. isoleucine/leucine, methionine, phenylalanine). At the same time, we cannot exclude that also other types of proteases (e.g. heat stable proteases deriving from the raw milk) did not contributed to the pattern.

The 2-ways ANOVA ($P \leq 0.05$) tested the effect of storage temperature and time on the free amino acids profile of each milk batch and further demonstrated the trend shown in the PCA. A significant effect of the storage time was highlighted only for phenylalanine, tyrosine, lysine and glutamic acid: UHLM was poorly affected by temporal changes in the free amino acid profile. These findings add valuable information to the chemical nature of the modification occurring in UHLM produced by "in batch" system (Bottiroli, Troise, et al., 2020). UHLM undergoes proteolysis that, releasing free amino groups, increases the MR rate (Jansson et al., 2017). This drawback is minimized during "in batch" production because the UHT treatment inactivates the secondary proteases of the LP. On the other hand, free amino acids accumulate during the "in batch" hydrolysis raising the formation of volatiles MRPs during the shelf-life at room temperature (Bottiroli, Troise, et al., 2020). In this study, this reservoir of free amino acids generated during "in batch" production was converted at higher rates when the milk was stored at temperature higher than 4°C. Therefore, fluctuations of the temperature during warehousing, shipment and commercialization can trigger directly MR, as a lag-phase of proteolysis does not occur, so quality defects linked to unwanted color and flavor can occur in a relative short time forcing to reduce the best before consumption date.

Volatiles Compounds and Intermediate Stages of Maillard Reaction

A total of 24 compounds were identified in the headspace of the samples, in line with other VOCs profiles reported for UHLM (Bottiroli, Aprea, et al., 2020; Troise et al., 2016); as a consequence of the high temperature used for milk storage (40°C), the chromatographic profile was characterized by the presence of new peaks, such as furfural, a compound having furan as constituent unit. A summary of the identified VOCs is given in **Table 1**, whereas the complete VOCs profile of the analyzed UHLMs is reported in the Supplementary section (**Supplementary Table B.1-2**). Furfural concentration increased linearly over the storage at 40°C, whereas it was not detected in the milk stored at the other temperature (**Figure 2d**). Furfural was previously detected in UHLM by Jansson et al. (2014) who hypothesized that its release is favored at pH<7 alongside Amadori or 3-deoxyglucosone compounds formation (O'Mahony, Drapala, Mulcahy, & Mulvihill, 2017). Furfural formation is associated with the severity of the heat treatment supplied during milk sterilization. Previous research proved that, by increasing storage temperature, furfural is formed in parallel with formic and acetic acids along MR; as a consequence, the pH of the product tends to decrease during storage (Brands & Van Boekel, 2001; van Boekel, 1998). Therefore, based on this assumption, our results indicate a positive correlation of high storage temperature and furfural formation, so milk sterilization should not be considered the major cause of furfural formation when the UHLM is stored at temperatures higher than the conventional 20°C, but it promotes the formation/accumulation of triggering agents that boost the reaction when milk is stored at 40°C.

Table 1. Overview of the volatile compounds (VOCs) , calculated linear retention index (LRI) and reference linear retention index (RLRI) considered in the study by SPME GC–MS. The concentration range for each storage temperature is also reported. The complete VOCs profile of the analyzed UHLMs, with the concentrations expressed as µg/L of internal standard (4-methyl-2-pentanone) for each VOCs, is reported in the Supplementary section of the manuscript.

Compound name[a]	LRI[b]	RLRI[c]	4°C[d]	20°C	30°C	40°C
2-Butanone	909	907 ± 11	2.89 - 7.54	3.37 - 5.73	3.88 - 6.87	4.76 - 12.10
2-Methylbutanal	918	914 ± 8	0.28 - 1.01	0.63 - 1.78	0.65 - 3.01	1.53 - 18.18
2-Pentanone	985	981 ± 11	3.24 - 8.01	4.52 - 8.03	6.19 - 15.70	12.45 - 42.88
Toluene	1051	1042 ± 11	0.45 - 4.89	0.57 - 1.59	0.50 - 2.06	0.29 - 1.00
Dimethyl disulfide	1099	1083 ± 8	0.11 - 0.51	0.40 - 0.90	0.24 - 1.05	0.43 - 6.15
Hexanal	1137	1129 ± 8	3.04 - 11.8	7.52 - 14.25	3.59 - 13.63	3.43 - 13.51
2-Heptanone	1191	1182 ± 8	8.63 - 29.03	8.70 - 27.00	17.95 - 56.07	27.50 - 105.53
2-Nonanone	1395	1390 ± 7	2.79 - 6.52	3.12 - 5.83	5.48 - 29.00	1.010 - 65.56
Nonanal	1400	1391 ± 8	0.35 - 2.23	nd - 1.66	1.11 - 3.23	1.81 - 7.30
Furfural	1476	1461 ± 11	nd	nd	nd	nd - 2.48
Benzaldehyde	1531	1520 ± 14	1.11 - 3.09	0.84 - 2.68	2.83 - 15.24	4.66 - 31.82
1-Octanol	1576	1557 ± 8	1.08 - 5.05	1.21 - 1.83	1.16 - 4.02	0.92 - 7.95
2-Undecanone	1605	1598 ± 6	0.73 - 1.96	0.63 - 0.92	1.51 - 8.29	3.11 - 13.05
Butyrolactone	1636	1632 ± 15	0.58 - 1.25	0.29 - 0.70	0.57 - 2.44	0.75 - 1.89
Aceto-phenone	1660	1647 ± 13	nd - 0.37	nd - 0.84	0.26 - 1.57	0.31 - 0.87
2-Tridecanone	1818	1809 ± 6	0.21 - 0.76	0.06 - 0.47	0.30 - 2.11	0.6 - 3.10
Dimethyl sulfone	1890	1846 ± 12	1.80 - 6.43	1.48 - 2.87	0.97 - 6.47	1.24 - 8.85
Hexanoic acid	1909	1903 ± 9	2.05 - 8.48	1.44 - 4.36	1.45 - 9.87	2.92 - 46.07
p-Cresol	2095	2080 ± 12	0.15 - 0.44	0.22 - 0.37	0.21 - 1.86	0.25 - 0.88
m-Cresol	2103	2091 ± 18	0.63 - 1.71	0.87 - 1.30	0.77 - 6.45	0.94 - 3.52
Octanoic acid	2104	2060 ± 15	2.25 - 9.63	2.70 - 4.88	1.77 - 15.86	3.93 - 31.21
Delta-decalactone	2198	2194 ± 15	0.30 - 0.81	nd - 0.46	nd - 0.38	nd - 0.40
Decanoic acid	2295	2267 ± 14	2.95 - 8.34	3.27 - 9.98	3.37 - 24.50	4.59 - 26.05

[a] Compound identification by match with the NIST-2014/Wiley 7.0 libraries.
[b] Calculated Linear Retention Index
[c] Reference Linear Retention Index reported in the NIST-2014 library
[d] Concentration range (µg/L of internal standard) detected for each storage temperature during the UHLM shelf-life (120 days with sampling every 30 days).

Along with furfural, Strecker degradation products were detected in the UHLM samples. Strecker degradation is considered as the major contributor to the development of stale off-flavor in UHLM (Nielsen et al., 2017). Specifically, 2-methylbutanal increased significantly at 20°C, 30°C and 40°C, whereas DMDS and benzaldehyde did at 30°C and 40°C (**Figure 2a-c**). In all the mentioned cases, the compounds followed a linear growth, and 40°C was the temperature most sensitive to Strecker degradation, which takes place when amino acids react with α-dicarbonyl compounds (Hofmann & Schieberle, 2000). Alternatively, before α-dicarbonyls formation, Amadori products (APs) can also react forming Strecker aldehydes through oxidative deamination and the consequent decarboxylation of the amino acids (Cremer, Vollenbroeker, & Eichner, 2000). Products of Strecker degradation accumulate faster in UHLM than conventional UHT milk, due to the proteolytic activity of lactases employed for UHLM manufacturing, which are sold in different purities (Jansson et al., 2014; Nielsen et al., 2018).

The pool of free amino acids previously mentioned upon "in batch" production provides the substrate for Strecker degradation (Bottiroli, Troise, et al., 2020). The trends of 2-methylbutanal, DMDS and benzaldehyde demonstrated that, at storage temperature of 30°C and 40°C, intermediate stages of MR are facilitated. In particular, at these temperatures, the reactivity of the free amino acids is higher and their conversion *via* Strecker degradation faster, explaining the progressive lower levels of free amino acids encountered when the storage temperature was higher than 4°C.

Thermally-induced oxidation of lipids is also relevant when dealing with milk aging and can be estimated through the concentration of methyl ketones in the headspace. During "in batch" production, methyl-ketones are significantly formed when the milk undergoes thermal sterilization after lactose hydrolysis (Bottiroli, Aprea, et al., 2020). In this study, methyl ketones were the most frequent class of VOCs detected, in accordance with previous data on UHT milk (Contarini & Povolo, 2002; Contarini, Povolo, Leardi, & Toppino, 1997; Vazquez-Landaverde, Velazquez, Torres, & Qian, 2005). The higher incidence of even-carbon-numbered fatty acids in milk justifies the detection of methyl ketones having an odd number of carbons (Perkins, Elliott, D'Arcy, & Deeth, 2005). The level of methyl ketones in UHLM increased as both storage time and temperature increased, confirming the temperature dependence of their formation: 2-pentanone and 2-heptanone were the most abundant VOCs, reaching maximum values of 37.4±5.58 μg/L and 95.99±8.85 μg/L respectively in the milks stored at 30°C and 40°C (**Figure 2e-f**). Already after 30 days at 40°C, their concentration was significantly higher than the maximum reached at all the other

storage temperatures (*P≤0.05*). The trend shown by 2-heptanone was particularly interesting: after 90 days at 40°C, its concentration dropped to values close to the ones registered in UHLM at end of the shelf-life at 30°C. Lipid oxidation and its interplay with Maillard reaction and/or protein oxidation are other routes influencing UHLM sensory quality (Jensen et al., 2015). Methyl ketones are also positively correlated to molecules (e.g. protein bound lysine Amadori compounds and lactulose) describing heat milk damaging, and therefore, they can be considered an alternative way for evaluating the quality status of heated milk (Perkins et al., 2005). Both Strecker aldehydes and methyl ketones exhibited the same trend: the temperature step between 30°C and 40°C was crucial for their formation and an acceleration of the reaction kinetics can be hypothesized.

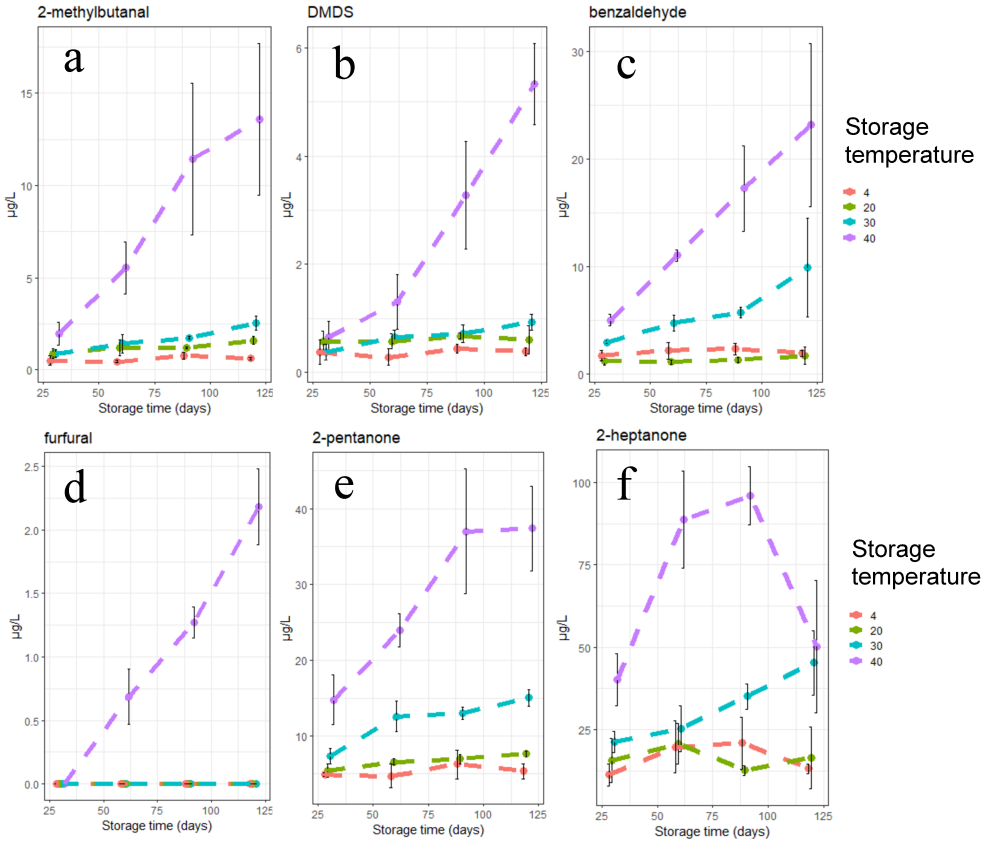


Figure 2. Concentration of 2-methylbutanal (**a**), dimethyl disulfide (**b**), benzaldehyde (**c**), furfural (**d**), 2-pentanone (**e**), 2-heptanone (**f**) detected the UHLMs stored at different temperatures (4°C, 20°C, 30°C, 40°C) for 120 days. The data are the mean values of the three replicates of production (n=3).

Color Changes and Final Stages of Maillard Reaction

Changes in milk color occurs at the end of MR, when compounds called melanoidins and other colored pigments with a molecular weight below 500 g/mol are formed (Rizzi, 1997; van Boekel, 1998). Color measurements is a valid rapid alternative for approximating the color of UHT milk in relation to the formation of melanonids and other colored compounds (Sunds et al., 2018). The results are reported in **Figure 3**. Significant changes in L*, a*, b* during storage were registered only when milk was stored at 30°C and 40°C, suggesting that milk browning does not occur substantially at 4°C and 20°C during the 120-days storage considered in this study. Above 30°C differences in color were already evident after 30 days, whereas at lower temperature the color was basically unaltered. This confirmed the heat load subjected to the milk during storage as major factor affecting milk browning, in a more relevant manner than the severity of UHT treatment itself (Deeth & Lewis, 2017).

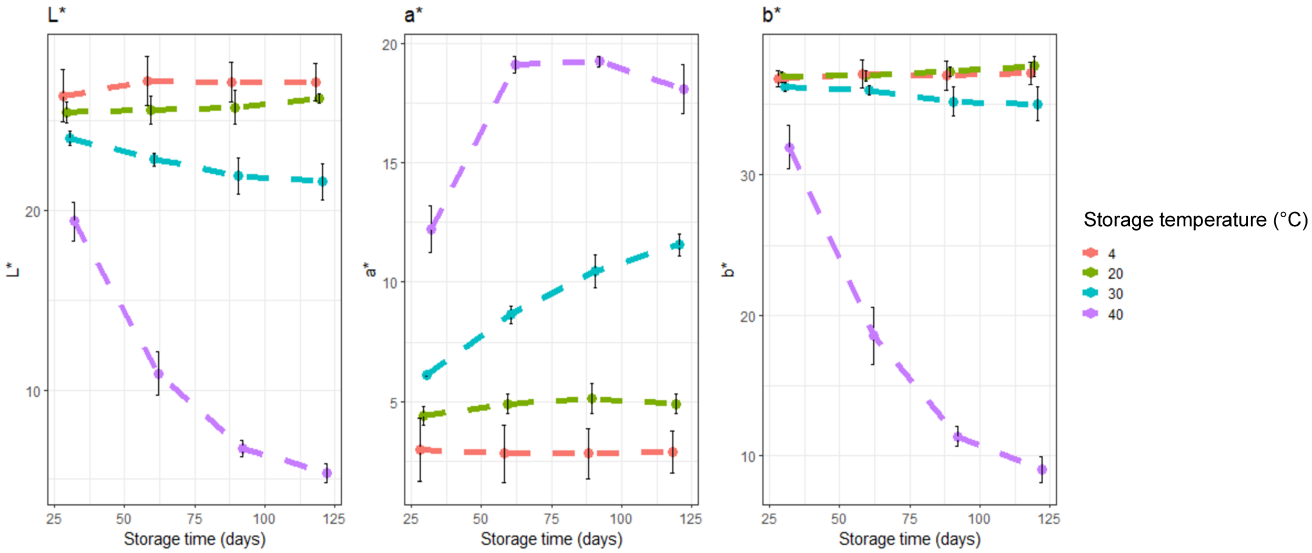


Figure 3. Changes in the CIELab parameters L*, a*, and b* in UHLM during storage at 4°C, 20°C, 30°C, 40°C. The data are the mean values of three measurement from the three replicates of production (n=9).

The parameter L* and b* significantly dropped by increasing the storage temperature, whereas the opposite was observed for the parameter a*. Interestingly, at 40°C the temporal evolution of L*, a* and b* experienced a slowdown after 60 days of storage. Such change in time and temperature profiles was particularly evident for the parameter a*. This indicates

that, at high storage temperature, formation of colored pigments may reach a plateau in milk. In UHLM, the higher reactivity towards MR of glucose and galactose, obtained upon lactose hydrolysis, renders the product more susceptible to browning (Deeth, 2020). This occurs presumably at the end of MR (van Boekel, 1998). In the case of UHLM, precursors depletion may have limited the formation of colored pigments and melanoidins, in contrast with what reported by Sunds et al. (2018) for regular UHT milk, as a consequence of the higher reactivity of glucose and galactose toward non-enzymatic browning (Milkovska-Stamenova & Hoffmann, 2017).

Comprehensive Effect of Different Storage Temperature to UHT Hydrolyzed-Lactose Milk Quality

The effect of storage time and temperature on the parameters defining the UHLM quality were the result of the interplay among MR, protein and lipid oxidation. Interrelated pathways can contribute to the formation of several molecules, denoting that an estimation of MRPs through free amino acids, VOCs linked to Strecker degradation and color changes was not sufficient. MR kinetics is intricate and several interconnected pathways can simultaneously occur (Deeth & Lewis, 2017). Indeed, at certain conditions, Strecker aldehydes can condensate with themselves, with sugar fragments and with furfurals, forming brown pigments and affecting the color as well (Morales & Van Boekel, 1998). Intermediates from lipid oxidation can also participate to MR resulting in polymerization and contribution to non-enzymatic browning (Zamora & Hidalgo, 2005). This inter-correlation between MR and lipid oxidation might explain the downtrend of 2-heptanone, in relation to the levelling-off of the a* colorimetric parameter after 90 days of storage in the UHLM maintained at 40°C. This is a clear situation where methyl-ketones participated to MR and to milk browning.

In this kind of scenarios, where relationships among different sets of variables are proposed, Multiple Factoria Analysis (MFA) is a valuable tool for data discussion. It is important to highlight that the systematization of the MR in three stage disentangles the intricate network and rationalizes the significance of the compounds formed or degraded, but this does not entirely match the complexity of the MR pathways (Hodge, 1953). The resulting loading plot is reported in **Figure 4**. The first two dimensions of the MFA accounted for 58.4% of the total variance (Dim1: 41.7%; Dim2: 16.7%). Each dataset gave a different contribution to the two dimensions: for Dim1, color (41.79%) and VOCs data (37.90%) had a relevant impact,

whereas only the free amino acids profile (72.09%) was significant for the definition of Dim2. This confirms that the breakdown of milk protein in UHLM produced through "in batch" system is a secondary effect in comparison to the changes linked to the VOCs and color (Bottiroli, Troise, et al., 2020). Moreover, the effects of storage temperature overwhelmed the one of storage time on both dimensions, as already hypothesized looking at the PCA in **Figure 1**. The increase in the storage temperature was the principal cause of UHLM modification for all the tested parameters, so utmost attention on temperature fluctuations at each step of the product life-cycle is requested. The different profiles of MR in the milk due to different storage temperature was nicely depicted on Dim1: most free amino acids, present at higher concentrations in UHLM kept at 4°C for 120 days, gave a negative contribution to the dimension and were counterpoised to a positive contribution of the VOCs, whose maximum formation occurred at 40°C. Thus, the route of MR towards advanced stages is modulated by the storage temperature which influence the rate at which specific VOCs (e.g. 2-methylbutanal, DMDS benzaldehyde) are formed from the pool of free amino acids generated upon "in batch" production. Other reaction pathways overlapped MR when the storage temperature was higher than the conventional 20°C. Indeed products of Strecker degradation were positively correlated to 2-butanone ($r>0.69$), 2-pentanone ($r > 0.89$), 2-heptanone ($r > 0.50$), 2-nonanone ($r > 0.78$), 2-undecanone ($r > 0.83$) and 2-tridecanone ($r > 0.80$) indicating a possible coordination of fatty acids oxidation and MR. All the Pearson coefficient calculated between Strecker degradation products and methyl-ketones were statistically significant (*$P \leq 0.05$).* Volatiles MRPs and methyl-ketones were associated to milk browning represented by an increase of a* (red/green, $r>0.65$) and a simultaneous decrease of L* (lightness, $r>-0.76$) and b* (yellow/blue, $r>-0.76$). Even in this case the correlations were statistically significant (*$P \leq 0.05$).* MR and fat oxidation follow similar reaction pathways that can interact with each other and share common intermediates (Zamora & Hidalgo, 2005), so a mutual participation to the milk browning is plausible. In our study, the relationship between MR and lipid oxidation emerged only when the UHLM was stored for 120 days at 30°C and, especially, 40°C. Therefore, as long as UHLM is maintained around room temperature throughout shelf-life, the two reactions are monitored without risks of color and flavor alteration. Contrarily, fluctuations towards higher storage temperature concomitantly trigger MR and lipid or fatty acids oxidation with the formation of a myriad of compounds potentially detrimental for the sensory quality of the product. In this frame, the MFA was demonstrated as a useful tool for mapping the chemical reaction underpinning

the response of UHLM produced by "in batch" system to different storage temperature during shelf-life.

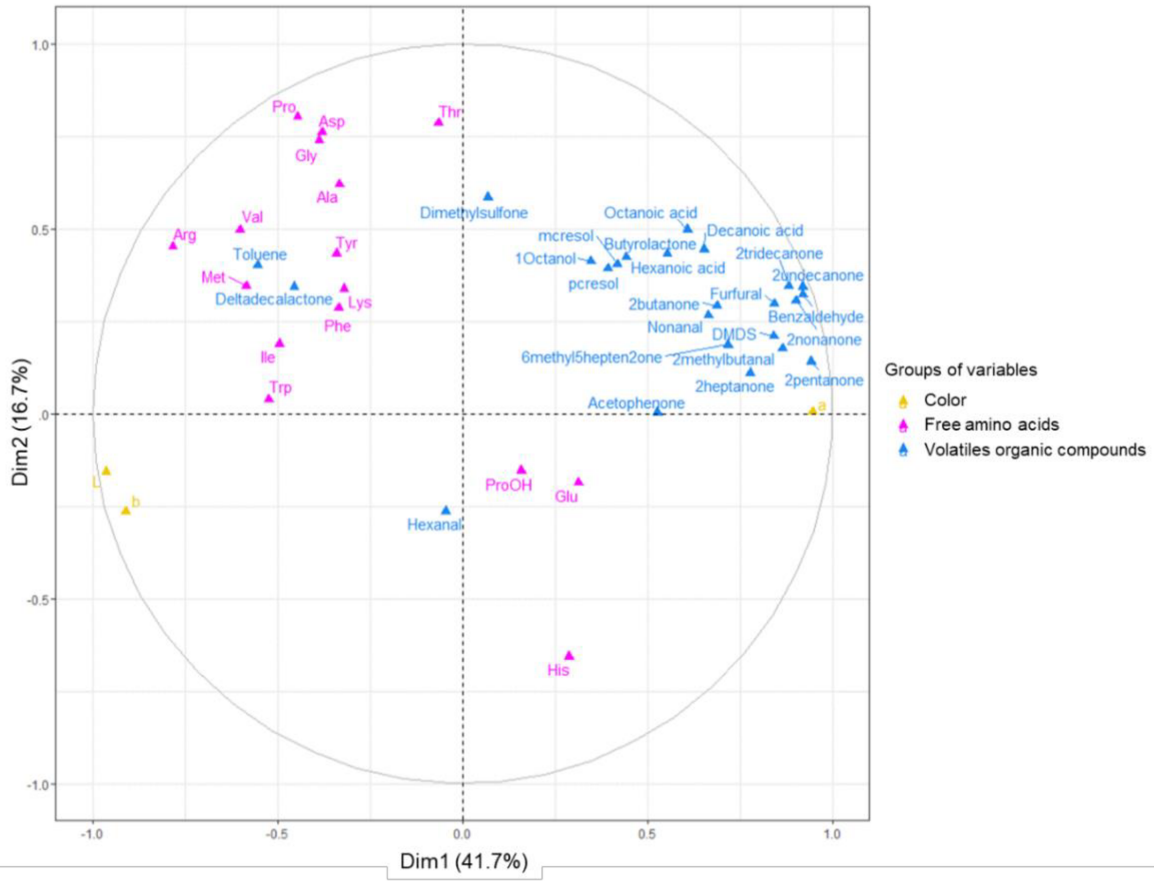


Figure 4. Loading plot of the multiple factor analysis (MFA) with Dim1 and Dim 2 explaining 41.7% and 16.7% of the total variance respectively. The analysis was performed on the mean values of the free amino acids (magenta), VOCs (blue), color (yellow) data obtained from the analysis of the UHLM samples stored at 4°C, 20°C, 30°C, 40°C for 30, 60, 90 and 120 days.

Conclusion

The severity of thermal treatment, the consequent array of reactions and the chemical nature of precursors render the design of shelf-life stable UHLM a challenge for dairy producers. The study of quality parameters cannot be confined in one single reaction, but a multi-faceted approach is required to depict a cause-effect relationship between the technology used and desired outcomes as prolonged shelf-life, control of off-flavors formation and unaltered milk color. We outlined how the control of temperature during storage is a crucial parameter to tune the formation of methyl-ketones and VOCs related to off-flavors in UHLM. Developing UHLM with shelf-life close to regular UHT milk would facilitate the distribution and minimize the financial losses of both producers and retailers (Nielsen et al., 2017). Furthermore, we introduced the possibility that lipid oxidation with formation of ketones, protein oxidation with the formation of methionine oxidation products and MR *via* Strecker degradation (2-methylbutanal and phenylacetaldehyde) can be characterized by interconnected reaction pathways with implications on color development. In this respect, ad hoc purification and mass spectrometry techniques can provide further evidence on the control of color and flavor development in dairy-based products and in particular to lactose-hydrolyzed milks.

References

Abernethy, G., & Higgs, K. (2013). Rapid detection of economic adulterants in fresh milk by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, *1288*, 10–20. https://doi.org/10.1016/j.chroma.2013.02.022

Adhikari, K., Dooley, L. M., Chambers IV, E., & Bhumiratana, N. (2010). Sensory characteristics of commercial lactose-free milks manufactured in the United States. *LWT - Food Science and Technology*, *43*(1), 113–118. https://doi.org/10.1016/j.lwt.2009.06.017

Bimbo, F., Bonanno, A., Liu, X., & Viscecchia, R. (2016). Hedonic analysis of the price of UHT-treated milk in Italy. *Journal of Dairy Science*, *99*(2), 1095–1102. https://doi.org/10.3168/jds.2015-10018

Bottiroli, R., Aprea, E., Betta, E., Fogliano, V., & Gasperi, F. (2020). Application of headspace solid-phase micro-extraction gas chromatography for the assessment of the volatiles profiles of ultra-high temperature hydrolysed-lactose milk during production and storage. *International Dairy Journal*, 104715. https://doi.org/10.1016/j.idairyj.2020.104715

Bottiroli, R., Pedrotti, M., Aprea, E., Biasioli, F., Fogliano, V., & Gasperi, F. (2020). Application of PTR-TOF-MS for the quality assessment of lactose-free milk : Effect of storage time and employment of different lactase preparations. *Journal of Mass Spectrometry*, (February), 1–9. https://doi.org/10.1002/jms.4505

Bottiroli, R., Troise, A. D., Aprea, E., Fogliano, V., Vitaglione, P., & Gasperi, F. (2020). Chemical and sensory changes during shelf-life of UHT hydrolyzed-lactose milk produced by "in batch" system employing different commercial lactase preparations. *Food Research International*, *136*(July), 109552. https://doi.org/10.1016/j.foodres.2020.109552

Brands, C. M. J., & Van Boekel, M. A. J. S. (2001). Reactions of monosaccharides during heating of sugar - Casein systems: Building of a reaction network model. *Journal of Agricultural and Food Chemistry*, *49*(10), 4667–4675. https://doi.org/10.1021/jf001430b

Contarini, G., & Povolo, M. (2002). Volatile fraction of milk: Comparison between, purge and trap and solid phase microextraction techniques. *Journal of Agricultural and Food Chemistry*, *50*(25), 7350–7355. https://doi.org/10.1021/jf025713a

Contarini, G., Povolo, M., Leardi, R., & Toppino, P. M. (1997). Influence of Heat Treatment on the Volatile Compounds of Milk. *Journal of Agricultural and Food Chemistry*, *45*(3), 3171–3177. Retrieved from https://pubs.acs.org/sharingguidelines

Cremer, D. R., Vollenbroeker, M., & Eichner, K. (2000). Investigation of the formation of

Strecker aldehydes from the reaction of Amadori rearrangement products with of-amino acids in low moisture model systems. *European Food Research and Technology*, *211*(6), 400–403. https://doi.org/10.1007/s002170000196

Deeth, H. C. (2020). *The effect of UHT processing and storage on milk proteins*. *Milk Proteins* (3rd ed.). Elsevier Inc. https://doi.org/10.1016/b978-0-12-815251-5.00010-4

Deeth, H. C., & Lewis, M. J. (2017). *High temperature processing of milk and milk products*. *John Wiley & Sons.* https://doi.org/10.1002/9781118460467.ch6

Francis, L. L., Chambers, D. H., Kong, S. H., Milliken, G. A., Jeon, I. J., & Schmidt., K. A. (2005). Serving Temperature Effects on Milk Flavor, Milk Aftertaste, and Volatile Compound Quantification in Nonfat and Whole Milk. *Journal of Food Science*, *70*(7), s413–s418. Retrieved from www.ift.org

Hannß, M., Hubbe, N., & Henle, T. (2018). Acid-Induced Gelation of Caseins Glycated with Lactose: Impact of Maillard Reaction-Based Glycoconjugation and Protein Cross-Linking. *Journal of Agricultural and Food Chemistry*, *66*(43), 11477–11485. https://doi.org/10.1021/acs.jafc.8b04176

Hodge, J. E. (1953). Dehydrated foods, Chemistry of Browning Reactions in Model Systems. *Journal of Agricultural and Food Chemistry*, *1*(15), 928–943. https://doi.org/10.1021/jf60015a004

Hofmann, T., & Schieberle, P. (2000). Formation of aroma-active Strecker-aldehydes by a direct oxidative degradation of Amadori compounds. *Journal of Agricultural and Food Chemistry*, *48*(9), 4301–4305. https://doi.org/10.1021/jf000076e

Jansson, T., Clausen, M. R., Sundekilde, U. K., Eggers, N., Nyegaard, S., Larsen, L. B., … Bertram, H. C. (2014). Lactose-hydrolyzed milk is more prone to chemical changes during storage than conventional ultra-high-temperature (UHT) milk. *Journal of Agricultural and Food Chemistry*, *62*(31), 7886–7896. https://doi.org/10.1021/jf501671z

Jansson, T., Rauh, V., Danielsen, B. P., Poojary, M. M., Waehrens, S. S., Bredie, W. L. P., … Lund, M. N. (2017). Green Tea Polyphenols Decrease Strecker Aldehydes and Bind to Proteins in Lactose-Hydrolyzed UHT Milk. *Journal of Agricultural and Food Chemistry*, *65*(48), 10550–10561. https://doi.org/10.1021/acs.jafc.7b04137

Jansson, T., Waehrens, S. S., Rauh, V., Danielsen, B. P., Sørensen, J., Bredie, W. L. P., … Lund, M. N. (2019). Effect of green tea catechins on physical stability and sensory quality of lactose-reduced UHT milk during storage for one year. *International Dairy Journal*, *95*, 25–34. https://doi.org/10.1016/j.idairyj.2019.03.007

Jensen, S., Jansson, T., Eggers, N., Clausen, M. R., Larsen, L. B., Jensen, H. B., … Bertram, H. C.

(2015). Storage-induced changes in the sensory characteristics and volatiles of conventional and lactose-hydrolyzed UHT processed milk. *European Food Research and Technology*, *240*(6), 1247–1257. https://doi.org/10.1007/s00217-015-2427-9

Messia, M. C., Candigliota, T., & Marconi, E. (2007). Assessment of quality and technological characterization of lactose-hydrolyzed milk. *Food Chemistry*, *104*(3), 910–917. https://doi.org/10.1016/j.foodchem.2006.12.045

Milkovska-Stamenova, S., & Hoffmann, R. (2017). Influence of storage and heating on protein glycation levels of processed lactose-free and regular bovine milk products. *Food Chemistry*, *221*, 489–495. https://doi.org/10.1016/j.foodchem.2016.10.092

Morales, F. J., & Van Boekel, M. A. J. S. (1998). A study on advanced Maillard reaction in heated casein/sugar solutions: Colour formation. *International Dairy Journal*, *8*(10–11), 907–915. https://doi.org/10.1016/S0958-6946(99)00014-X

Naranjo, G. B., Gonzales, A. S. P., Leiva, G. E., & Malec, L. S. (2013). The kinetics of Maillard reaction in lactose-hydrolysed milk powder and related systems containing carbohydrate mixtures. *Food Chemistry*, *141*(4), 3790–3795. https://doi.org/10.1016/j.foodchem.2013.06.093

Nielsen, S. D., Jansson, T., Le, T. T., Jensen, S., Eggers, N., Rauh, V., … Larsen, L. B. (2017). Correlation between sensory properties and peptides derived from hydrolysed-lactose UHT milk during storage. *International Dairy Journal*, *68*, 23–31. https://doi.org/10.1016/j.idairyj.2016.12.013

Nielsen, S. D., Zhao, D., Le, T. T., Rauh, V., Sørensen, J., Andersen, H. J., & Larsen, L. B. (2018). Proteolytic side-activity of lactase preparations. *International Dairy Journal*, *78*, 159–168. https://doi.org/10.1016/j.idairyj.2017.12.001

O'Mahony, J. A., Drapala, K. P., Mulcahy, E. M., & Mulvihill, D. M. (2017). Controlled glycation of milk proteins and peptides: Functional properties. *International Dairy Journal*, *67*, 16–34. https://doi.org/10.1016/j.idairyj.2016.09.012

Perkins, M. L., Elliott, A. J., D'Arcy, B. R., & Deeth, H. C. (2005). Stale flavour volatiles in Australian commercial UHT milk during storage. *Australian Journal of Dairy Technology*, *60*(3), 231–237.

Rizzi, G. P. (1997). Chemical structure of colored Maillard reaction products. *Food Reviews International*, *13*(1), 1–28. https://doi.org/10.1080/87559129709541096

Sunds, A. V., Rauh, V. M., Sørensen, J., & Larsen, L. B. (2018). Maillard reaction progress in UHT milk during storage at different temperature levels and cycles. *International Dairy Journal*. https://doi.org/10.1016/j.idairyj.2017.08.008

Tossavainen, O., & Kallioinen, H. (2007). Proteolytic changes in lactose hydrolysed UHT milks during storage. *Milchwissenschaft*, *62*(4), 410–415. Retrieved from http://cat.inist.fr/?aModele=afficheN&cpsidt=19123002

Tossavainen, Olli, & Kallioinen, H. (2008). Effect of lactose hydrolysis on furosine and available lysine in UHT skim milk. *Milchwissenschaft*, *63*(1), 22–26.

Troise, A. D., Bandini, E., De Donno, R., Meijer, G., Trezzi, M., & Fogliano, V. (2016). The quality of low lactose milk is affected by the side proteolytic activity of the lactase used in the production process. *Food Research International*, *89*, 514–525. https://doi.org/10.1016/j.foodres.2016.08.021

Troise, A. D., Wiltafsky, M., Fogliano, V., & Vitaglione, P. (2018). The quantification of free Amadori compounds and amino acids allows to model the bound Maillard reaction products formation in soybean products. *Food Chemistry*, *247*, 29–38. https://doi.org/10.1016/J.FOODCHEM.2017.12.019

Valero, E., Villamiel, M., Miralles, B., Sanz, J., & Martínez-Castro, I. (2001). Changes in flavour and volatile components during storage of whole and skimmed UHT milk. *Food Chemistry*, *72*(1), 51–58. https://doi.org/10.1016/S0308-8146(00)00203-X

van Boekel, M. A. J. S. (1998). Effect of heating on Maillard reactions in milk. *Food Chemistry*, *62*(4), 403–414.

van Boekel, M. A. J. S. (2006). Formation of flavour compounds in the Maillard reaction. *Biotechnology Advances*, *24*(2), 230–233. https://doi.org/10.1016/J.BIOTECHADV.2005.11.004

Vazquez-Landaverde, P. A., Velazquez, G., Torres, J. A., & Qian, M. C. (2005). Quantitative determination of thermally derived off-flavor compounds in milk using solid-phase microextraction and gas chromatography. *Journal of Dairy Science*, *88*(11), 3764–3772. https://doi.org/10.3168/jds.S0022-0302(05)73062-9

Zabbia, A., Buys, E. M., & de Kock, H. L. (2012). Undesirable Sulphur and carbonyl flavor compounds in UHT milk: A review. *Critical Reviews in Food Science and Nutrition*. https://doi.org/10.1080/10408398.2010.487166

Zamora, R., & Hidalgo, F. J. (2005). Coordinate contribution of lipid oxidation and Maillard reaction to the nonenzymatic food browning. *Critical Reviews in Food Science and Nutrition*, *45*(1), 49–59. https://doi.org/10.1080/10408690590900117

Supplementary material

Supplementary Table A.1. Nutritional label of the UHLM industrially produced and tested in the study, reported as mean value for 100 mL of product. Analysis for each mentioned component has been performed by the industrial partner through internal protocols.

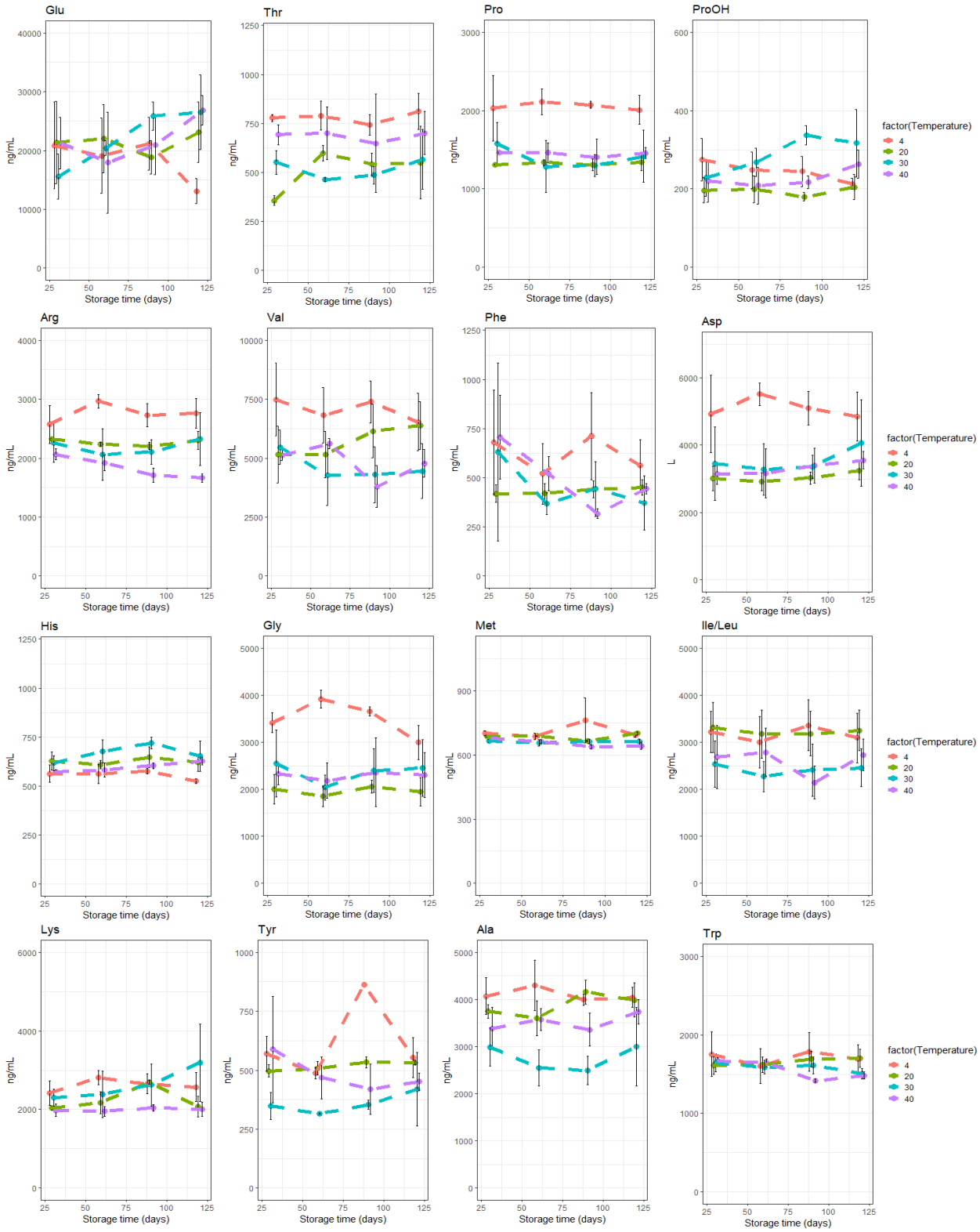
Milk component	g/100 mL
LIPIDS	0.8-0.9
CARBOHYDRATES	4.8-5.0 g
Sugars	4.8-5.0 g
Glucose	~2.5 g
Galactose	~2.5 g
Lactose	<0.1 g
PROTEIN	2.8-3.1 g

Supplementary Table B.1. Volatile compound profiles of UHLM samples stored at 4°C and 20°C for 120 days (sampled every 30 days: d30, d60, d90, d120). Reported values are the mean of three different production replicates (n=3)

		4°C				20°C			
Volatile compounds	LRI	d30	d60	d90	d120	d30	d60	d90	d120
2-Butanone	909	4.78±0.67	4.66±1.73	5.92±1.97	4.98±1.26	4.66±1.18	4.63±0.64	4.44±0.94	4.76±0.84
2-Methylbutanal	918	0.51±0.27	0.45±0.07	0.79±0.19	0.61±0.05	0.89±0.25	1.23±0.42	1.22±0.04	1.61±0.2
2-Pentanone	985	4.94±0.24	4.7±1.54	6.24±1.87	5.34±1.01	5.38±0.86	6.57±0.43	6.95±0.64	7.66±0.42
Toluene	1051	1.27±0.74	1.92±0.32	3.12±1.33	3.44±1.27	1.62±1.19	1.36±0.33	1±0.51	0.9±0.28
Dimethyl disulfide	1099	0.37±0.23	0.27±0.15	0.43±0.07	0.38±0.07	0.57±0.18	0.57±0.13	0.66±0.05	0.59±0.26
Hexanal	1137	8.18±3.74	5.63±3.38	8.9±0.83	7.19±1.94	10.71±3.02	11.8±3.5	11.22±2.65	9.76±1.56
2-Heptanone	1191	11.28±3.32	19.81±7.99	20.93±7.84	13.21±1.43	15.77±6.67	20.71±6.18	12.63±1.55	16.44±9.41
2-Nonanone	1395	3.6±0.69	4.32±1.95	4.38±0.22	3.67±0.43	3.91±0.85	4.51±0.32	4.22±0.36	4.69±1.21
Nonanal	1400	1±0.24	1.02±0.34	1.71±0.51	0.95±0.65	0.74±0.35	1.27±0.34	0.65±0.17	nd
Furfural	1476	nd	nd	nd	nd	nd	nd	nd	nd
Benzaldehyde	1531	1.7±0.51	2.19±0.77	2.36±0.53	1.92±0.27	1.24±0.44	1.11±0.19	1.3±0.24	1.74±0.8
1-Octanol	1576	1.11±0.04	2.44±2.24	1.51±0.15	1.17±0.06	1.6±0.5	1.71±0.13	1.35±0.14	1.64±0.2
2-Undecanone	1605	0.94±0.2	1.32±0.55	1.4±0.27	1.12±0.12	0.68±0.05	0.71±0.04	0.8±0.05	0.87±0.04
Butyrolactone	1636	0.72±0.16	1.04±0.33	0.72±0.06	0.75±0.05	0.57±0.14	0.63±0.06	0.44±0.13	0.61±0.06
Aceto-phenone	1660	0.15±0.13	0.31±0.06	0.23±0.03	0.26±0.05	0.46±0.41	0.73±0.12	0.52±0.03	0.54±0.47
2-Tridecanone	1818	0.37±0.2	0.4±0.3	0.4±0.1	0.33±0.11	0.37±0.09	0.32±0.03	0.32±0.09	0.3±0.21
Dimethyl sulfone	1890	0.63±0.36	1.49±0.86	0.79±0.46	1.98±1.14	0.4±0.23	0.31±0.18	0.53±0.3	0.3±0.17
Hexanoic acid	1909	0.45±0.26	3.34±1.92	2.32±1.34	0.69±0.4	1.37±0.79	0.41±0.23	0.29±0.16	1.28±0.74
p-Cresol	2095	0.03±0.01	0.15±0.09	0.04±0.02	0.02±0.01	0.08±0.04	0.04±0.02	0.03±0.02	0.02±0.01
m-Cresol	2103	0.12±0.07	0.6±0.35	0.11±0.06	0.05±0.03	0.13±0.07	0.13±0.07	0.04±0.02	0.09±0.05
Octanoic acid	2104	1.08±0.62	3.72±2.14	1.94±1.12	0.54±0.31	1.41±0.81	0.65±0.37	0.73±0.42	0.39±0.22
Delta-decalactone	2198	0.06±0.03	0.27±0.16	0.12±0.07	0.03±0.01	0.27±0.15	0.09±0.05	nd	nd
Decanoic acid	2295	1.14±0.66	2.8±1.62	2.23±1.29	0.32±0.18	0.75±0.43	3.29±1.9	1.35±0.78	0.7±0.4

Supplementary Table B.2. Volatile compound profiles of UHLM samples stored at 30°C and 40°C for 120 days (sampled every 30 days: d30, d60, d90, d120). Reported values are the mean of three different production replicates (n=3)

		30°C				40°C			
VOCs	RT	d30	d60	d90	d120	d30	d60	d90	d120
2-Butanone	909	4.81±0.94	5.6±1.78	4.69±0.43	4.83±0.32	5.31±0.77	6.11±0.15	9.88±2.01	10.14±2.36
2-Methylbutanal	918	0.89±0.22	1.44±0.5	1.75±0.07	2.55±0.39	1.96±0.62	5.53±1.41	11.43±4.11	13.58±4.12
2-Pentanone	985	7.35±1.04	12.57±1.98	12.99±0.83	15±1.05	14.77±3.28	23.97±2.18	37.02±8.28	37.4±5.58
Toluene	1051	1.46±0.84	1.42±0.81	0.98±0.2	1.19±0.33	0.72±0.38	0.47±0.3	0.45±0.12	0.54±0.02
Dimethyl disulfide	1099	0.36±0.14	0.65±0.12	0.71±0.16	0.92±0.15	0.64±0.3	1.29±0.5	3.27±0.99	5.33±0.75
Hexanal	1137	8.56±5.02	10.15±0.91	6.16±1.93	8.4±2.04	8.59±6.95	7.68±5.15	8.44±0.94	8.29±4.47
2-Heptanone	1191	21.28±3.19	25.4±6.92	35.1±3.97	45.25±9.88	40.21±7.94	88.74±14.66	95.99±8.85	61.56±4.53
2-Nonanone	1395	6.11±0.75	9.55±1.52	11.65±0.54	19.5±8.23	7.21±8.77	24.68±2.25	31.72±2.01	42±20.43
Nonanal	1400	1.34±0.07	1.72±0.65	1.66±0.31	2.16±1.06	2.4±0.18	4.99±2.31	3.58±1.55	2.8±1.53
Furfural	1476	nd	nd	nd	nd	nd	0.68±0.21	1.27±0.12	2.18±0.3
Benzaldehyde	1531	2.98±0.14	4.74±0.71	5.7±0.49	9.93±4.59	5.01±0.51	11.03±0.54	17.25±3.96	23.16±7.56
1-Octanol	1576	1.25±0.1	1.69±0.67	1.43±0.22	2.3±1.48	1.5±0.11	1.63±0.14	1.63±0.53	3.31±4.01
2-Undecanone	1605	1.63±0.11	2.48±0.32	2.73±0.63	5.14±2.77	3.16±0.08	6.09±1.07	8.14±1.19	9.93±2.7
Butyrolactone	1636	0.84±0.35	0.69±0.05	0.91±0.17	1.37±0.92	0.83±0.12	1.45±0.4	1.15±0.43	1.04±0.29
Aceto-phenone	1660	0.35±0.13	0.49±0.1	0.55±0.07	0.83±0.63	0.38±0.1	0.66±0.1	0.61±0.1	0.79±0.13
2-Tridecanone	1818	0.39±0.14	0.53±0.04	0.67±0.25	1.35±0.65	0.83±0.33	1.2±0.22	1.71±0.62	2.32±0.67
Dimethyl sulfone	1890	1.3±0.75	3.88±2.74	0.87±0.5	1.2±0.69	0.01±0.01	1.38±0.79	2.34±1.65	4.27±2.46
Hexanoic acid	1909	1.2±0.69	3.81±2.69	0.69±0.39	3.98±2.29	0.38±0.27	0.65±0.37	4.6±3.25	22.7±13.1
p-Cresol	2095	0.02±0.01	0.09±0.06	0.08±0.04	0.78±0.45	0.05±0.03	0.05±0.03	0.05±0.03	0.16±0.09
m-Cresol	2103	0.05±0.03	0.25±0.18	0.28±0.16	2.68±1.55	0.2±0.14	0.18±0.1	0.1±0.07	0.81±0.46
Octanoic acid	2104	1.81±1.05	3.73±2.64	0.67±0.39	6.71±3.87	0.63±0.45	2.23±1.29	4.51±3.19	12.88±7.43
Delta-decalactone	2198	0.08±0.04	0.13±0.09	nd	nd	0.06±0.04	0.11±0.06	0.16±0.11	nd
Decanoic acid	2295	1.47±0.85	2.63±1.86	1.67±0.96	10.05±5.8	1.4±0.99	2.46±1.42	4.7±3.32	7.6±4.38



Supplementary Fig. A.1. Time course evolution of the free AAs quantified by LC-HRMS in the UHLMs stored at different temperatures (4°C, 20°C, 30°C, 40°C) for 120 days. Line graphs represent the mean for the three replicates of "in batch" production performed. The following amino acids were not detected: Cys, Ser, Asn and Gln. The peak named Ile/Leu in the data refers to the sum of isoleucine and leucine, because the separation of the two isomers was not achieved throughout the analysis

Chapter 6: additional hints

Effect of the storage temperature on hydrolyzed-lactose milk: insight on flavors profile by PTR-ToF-MS

In this additional section of Chapter 6, the potential of PTR-ToF-MS was further investigated monitoring the UHLM flavor during shelf-life at different temperatures. The same UHLM samples discussed in Chapter 6, stored under the same conditions (4°C, 20°C, 30°C, 40°C for 120 days with sampling every 30 days), were analyzed. Measurements and data handling was performed according to the procedure already explained in Chapter 5 (Bottiroli et al., 2020). Also in this case, 5 mL of each UHLM sample were collected in 20 mL vials (Supelco, Bellefonte, PA). A PTR-ToF-MS 8000 (Ionicon Analytik GmbH, Innsbruck, Austria) operating in V mode was employed. **Table 1** summarizes the instrument settings of the drift tube. An auto-sampler (Gerstel GmbH, Mulheim am Ruhr, Germany) was connected to the PTR inlet heated at 110°C (PEEK capillary tube; inner diameter: 0.40 mm) and managed all the measurements automatically. Before measurement, milk samples were incubated at 50°C for 30 minutes. Three replicates for each UHLM were performed, measuring each samples for 60 seconds (flow rate of 35 sccm; acquisition rate of one spectrum per second). Mass peaks were detected in the range of m/z 20 to 300. Results (m/z) were extrapolated according to Cappellin et al. (2010) and were expressed with three decimal places. Internal calibration was ensured by injection of m/z 21.0221 (H3O+), 29.9974 (NO+), and 203.9430 (1,3-diiodobenzene fragment). The latter was continuously injected through the PerMaSCal device (Ionicon, Innsbruck, Austria). Final results were expressed as ppbV. Statistical analysis of the spectra was done using RStudio (version 3.3.3) and STATISTICA 13.3 (StatSoft, Inc., Tulsa, OK). The statistical approach followed was similar to Chapter 5 (Bottiroli et al., 2020). First of all, 13C isotopologues and interference masses were removed from the dataset. Milk samples and the blanks were compared *via* Student t-test and those mass peaks significantly higher in the

milk were further considered. Patterns in this reduced dataset was initially explored by Principal Component Analysis (PCA), pre-treating the data by log-transformation and Pareto scaling. Mass peak for further discussions were selected looking at the loading plot: those higher than 0.25 and lower than -0.25 on the first two principal components were considered relevant in the definition the data pattern. A tentative identification of the remaining mass peaks was attempted and the results of the GC-MS identification from Chapter 6 were used in support to the PTR-ToF-MS analysis. Eventually, the effect of the storage time and temperature on each tentatively identified mass peak was investigated by the analysis of variance and Tukey post-hoc test (when necessary) with a $P \leq 0.05$ set as threshold for significance.

Table 1. Settings of the PTR-TOF-MS instrument for the analysis of the UHLMs stored for 120 days at different temperature

Instrument parameter	Setting
Extraction voltage	24.3 V
Drift voltage	628 V
Drift temperature	110°C
Drift pressure	2.78 mbar*
Mass resolution	>3800 m/Δm

*The value corresponds to an E/N value of 128 Townsend (01 Td = 10−21 Vm2)

From this experiment, 357 mass peaks were extracted from the headspace of the UHLM samples, 320 of them significantly higher than the blanks. On this smaller dataset, PCA was applied for data exploration. First (PC1, 44.6% of the total variance) and second (PC2, 26.9% of the variance) principal components gave the best visualization of the trends in the data. The score plot of the PCA is showed in **Figure 1** and highlighted the tendency of the UHLM samples to group as function of the storage temperature. The effect was particularly relevant at higher temperatures: while UHLMs stored at 4°C and 20°C were poorly separated from each other, storing the milk at 30°C and 40°C lead to a clear distinction in the UHLM "volatilome". The effect of the storage time (displayed in **Figure 1** by an increase in dots size) did not appear, while the effect of the storage temperature was much stronger.

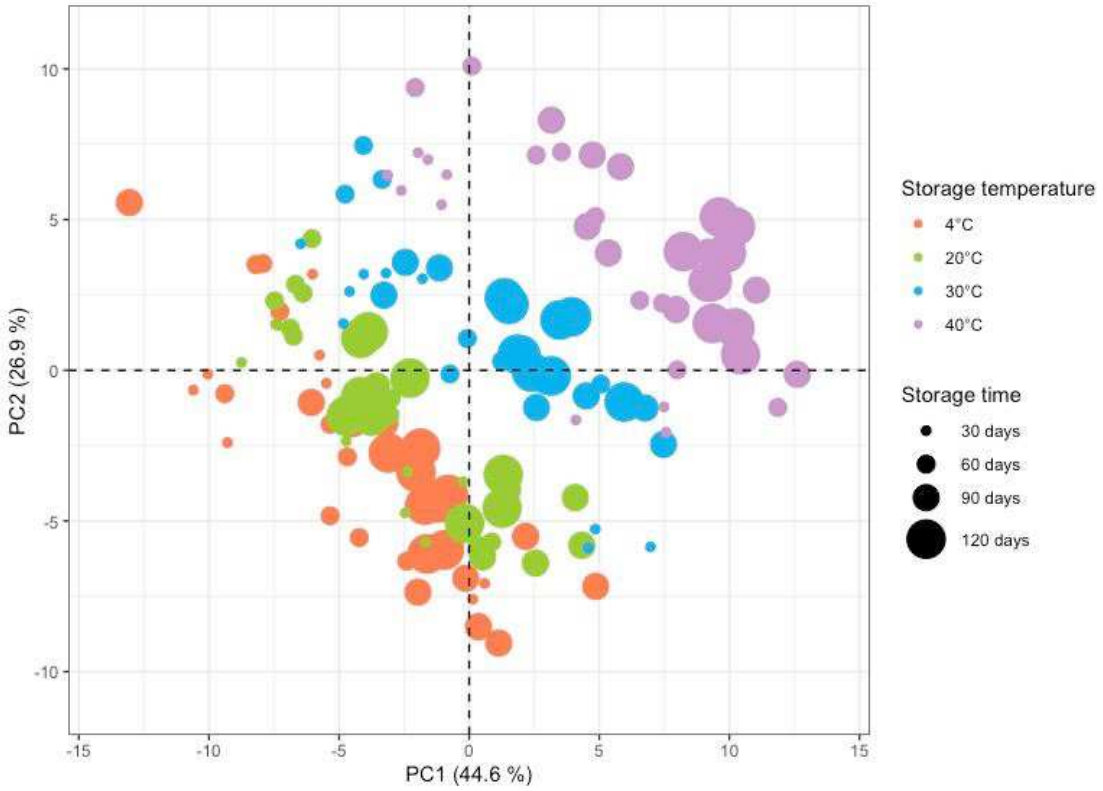


Figure 1. Score plot of the principal component analysis (PCA) performed on the results of the PTR-ToF-MS. The first two principal components (PC1 and PC2 describing 44.6% and 29.9% of the total variance respectively) gave the best visualization of the data. Analysis was performed on the log-transformed data after Pareto scaling. The progressive increase in dots size indicates the proceeding of the storage (0, 30, 60, 90, 120 days), whereas the different colors represent the differed storage temperature tested in the study (4°C, 20°C, 30°C, 40°C).

A sort of temporal evolution was observed only at 40°C highlighting the relationship between the temperature and the kinetics of reactions occurring in milk. From these results, PTR-ToF-MS analysis was appropriate to elucidate the effect of storage temperature on the VOCs profile of UHLM. Nevertheless, the effect of storage time poorly emerged. For further investigate this aspect, we considered in more details the mass peaks having a strong contribution on the PCA, namely those having loadings either higher than 0.25 or lower than -0.25 on PC1 and PC2. The identification of 31 of these mass peaks was achieved based on the formula, the fragmentation pattern, and the literature (Aprea et al., 2015; Bottiroli et al., 2020; Pedrotti et al., 2018; Zardin, Tyapkova, Buettner, & Beauchamp, 2014). The GC analysis confirmed the identity of 13 VOCs **(Table 2)** . In comparison to the VOCs profiles reported in

Chapter 5, this time new compounds were identified, such as 2-ethylfuran (m/z 97.063) and 2-pentylfuran (m/z139.114). The two compounds were also previously reported for UHLM (Jansson et al., 2014, 2019). Because furans are thermally-derived compounds (Limacher, Kerler, Davidek, Schmalzried, & Blank, 2008), the PTR-ToF-MS analysis clearly elucidate that new compounds are formed in UHLM as function of the temperature supplied during storage. Furan denotes progress of MR in food resulting in sweet, burnt, pungent and caramel-like (van Boekel, 2006). Furan detection is challenging due to the compound instability and the release is tiny amounts (Maga & Katz, 1979). In this experiment however, good performance of the PTR-ToF-MS methodology in detecting this class of compounds was observed. **Figure 2** shows the trends of m/z 97.063 (**Figure 2a**) and m/z 139.114 (**Figure 2b**) in the UHLM during the shelf-life study. As expected, the release of 2-ethylfuran and 2-pentylfuran was proportional to the storage temperature, with highest concentrations released during storage at 40°C. The increase of 2-ethyl-furan in UHLM during shelf-life was significant only at 40°C. 2-Pentylfuran instead registered a significant increase at all tested temperature, more remarkably at 30°C and 40°C.

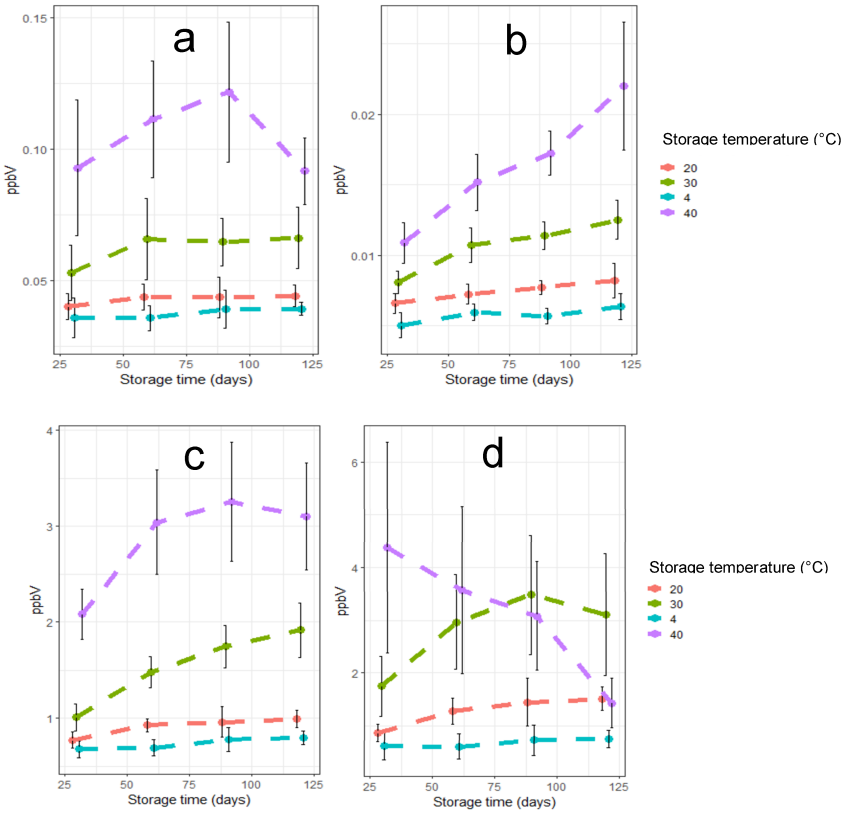


Figure 2. Concentration (ppbV) of m/z 97.063 (a), m/z 139.114 (b), m/z 115.111 (c) and m/z 63.026 (d) in the UHLM samples analyzed by PTR-TOF-MS and stored for 120 days at 4°C, 20°C, 30°C and 40°C.

Results of the analysis of the variance demonstrated that m/z 97.063 and m/z 139.114 were not the only two masses whose release was significantly affected by the storage time and temperature. **Table 2** showed that higher the temperature at which UHLM was stored, greater the number of VOCs registering significant temporal trends. This result confirmed that storage temperature tunes the release of VOCs in UHLM, confirming what described by the PCA (**Figure 1**). When UHLM was stored at 4°C for example, only the 23% of the tentative identified masses changed significantly over time. Oppositely, 84% of the reported masses registered significant differences in UHLMs during storage 40°C. Chemical reaction rates in milk increase at higher storage temperature resulting in stronger development of volatile compounds over time (Rerkrai, Jeon, & Bassette, 1987). Gaucher and coworkers (2008) reported the same effect on the physical stability of UHT milk concluding that age gelation at 40°C was facilitated compared to 4°C and 20°C. The mass peaks associated to methyl-

ketones were particularly relevant to describe the pattern. The class is linked to development of stale, cooked and oxidized flavor in UHT milk (Contarini, Povolo, Leardi, & Toppino, 1997; Perkins, D'Arcy, Lisle, & Deeth, 2005). The formation relies on the thermal modification of the fat fraction, mostly dependent on the heat load supplied during milk sterilization (Contarini & Povolo, 2002). Methyl ketones increase linearly at all the tested temperature at different rates. In UHLMs stored at 40°C the concentration of the mass peak associated to methyl ketones (m/z 73.064: 2-butanone ; m/z 87.043: 2-pentanone ; m/z 115.111: 2-heptanone; m/z 129.128: 2-octanone; m/z 143.145: 2-nonanone) was already higher after 30 days of storage compared to the milk kept at the other temperatures. At 40°C these values were higher than the maximum concentration of methyl-ketones registered throughout the storage at 20°C. This indicates that 30 days at 40°C causes more changes in the UHLM VOCs profile than the ones expected over the whole commercial shelf-life of the product, namely 120 days.

Table 2. Tentative identification and results of the analysis of variance on the mass peaks detected in the headspace of the UHLMs stored for 120 days at 4 different temperatures (4°C, 20°C, 30°C, 40°C) considered significant in explaining the trends shown in the PCA.

m/z	Tentative ID	4°C[a]	20°C	30°C	40°C	GC
34.995	Hydrogen sulfide					
49.011	Methanethiol					
63.026	Dimethyl sulfide		* [b]	*	*	
63.043	1,2-Ethanediol					
67.054	1,3-Cyclopentadiene; 2-Pentenal					
68.050	Pyrrole					
69.033	Furan					
69.070	Isoprene; 3-Hexen-2-ol			*	*	
71.050	2-Butenal; But-3-en-2-one; 2,3-Dihydrofuran					
73.064	2-Methylpropanal; Butanal; 2-Butanone			*	*	√[c]
78.967	Dimethyl disulfide (fragment)					
83.050	2-Methylfuran; 3-Methylfuran					
85.028	2-Furanone		*			
85.064	Hexanol		*		*	
87.043	Butyrolactone; 2,3-Butanedione					
87.080	2/3-Methylbutanal; Pentanal; 2-Pentanone	*	*	*	*	√
91.069	1,2-Butanediol					
95.015	Dimethyl sulfone				*	√
97.024	2-furfural					
97.063	2-Ethyl furan; 2,5-Dimethylfuran				*	
97.101	Heptanal (fragment)			*	*	
99.113	2-Hexenal					
101.059	2,3-Pentanedione; y-Valerolactone; 1,5-Pentanedial		*	*	*	
101.096	Hexanal				*	√
105.057	Methyl-lactate					
107.049	Benzaldehyde		*	*	*	√
115.111	2-Heptanone; Heptanal	*	*	*	*	√
117.092	Hexanoic acid	*				√
125.059	Guaiacol; 2-Acetyl-5-methylfuran					
129.128	2-Octanone; Octanal			*	*	
139.114	2-Pentylfuran	*	*	*	*	
143.145	2-Nonanone; Nonanal			*	*	√
149.107	Methyl-chiavicol; Ethenyl-ethyl-methylpyrazine					
159.135	Nonanoic acid				*	√
171.175	2-Undecanone			*	*	√
173.154	Decanoic acid		*	*	*	√

[a] The heading indicates the temperature at which the shelf-life of 120 days (with milk sampling every 30 days) was performed; [b] The symbol indicates the presence of significant differences in the temporal trend of the mass peak at the given temperature ($P \leq 0.05$); [c] The symbol indicates a match between the tentative identification of the mass peak and the results of the GC-MS analysis

Interestingly, the mass peak associated with dimethyl sulfide (DMS) follow different trends depending on the storage temperature considered. UHLM stored at 4°C, 20°C and 30°C experienced a significant increase in its concentration throughout shelf-life, whereas at 40°C the compound follow a downward trend. This indicates that an increase in the storage temperature to 40°C did not only accelerate the flavor formation, but potentially changed the kinetics of the involved reactions. DMS contributes to the cooked/sulfurous flavor of milk subjected to heat treatment (de Wit & Nieuwenhuijse, 2008). The compound arise in the product due to Maillard reaction: firstly methional is formed via Strecker degradation of methionine and then it is oxidized to DMS (Jo, Benoist, Barbano, & Drake, 2018). The significant increase of the DSM concentration in the UHLM stored at 20°C was in discordance with previous work dealing with regular UHT milk (Al-attabi et al., 2014). In that case, a very high concentration of DSM was detected in the beginning, but then it progressively decrease during storage at 22-23°C due to oxidation. The different sensitivity towards MR between regular UHT milk and UHLM may explain the divergence in the results: UHLM is more prone to Strecker degradation, so the formation of DMS via this pathways may have taken over its oxidation in the 4-30°C temperature range. At higher storage temperature, the oxidation of DMS was instead facilitated resulting in its drop during the shelf-life. Overall, the output of the PTR-ToF-MS analysis is clear and strengthen the conclusions emerged in Chapter 5 and 6: PTR-ToF-MS demonstrate high performance in monitoring the quality of UHLM during shelf-life and high storage temperature changes the rate and kinetics of those reaction involved in off-flavor formation.

References

Al-attabi, Z., Bruce, D., Deeth, H., Al-attabi, Z., Bruce, D., & Deeth, H. (2014). Volatile sulfur compounds in pasteurised and UHT milk during storage. *Dairy Science and Technology*, *94*, 241–253. https://doi.org/10.1007/s13594-013-0157-y

Aprea, E., Romano, A., Betta, E., Biasioli, F., Cappellin, L., Fanti, M., & Gasperi, F. (2015). Volatile compound changes during shelf life of dried Boletus edulis: Comparison between SPME-GC-MS and PTR-ToF-MS analysis. *Journal of Mass Spectrometry*, *50*(1), 56–64. https://doi.org/10.1002/jms.3469

Bottiroli, R., Pedrotti, M., Aprea, E., Biasioli, F., Fogliano, V., & Gasperi, F. (2020). Application of PTR-TOF-MS for the quality assessment of lactose-free milk : Effect of storage time and employment of different lactase preparations. *Journal of Mass Spectrometry*, (February), 1–9. https://doi.org/10.1002/jms.4505

Cappellin, L., Biasioli, F., Fabris, A., Schuhfried, E., Soukoulis, C., Märk, T. D., & Gasperi, F. (2010). Improved mass accuracy in PTR-TOF-MS: Another step towards better compound identification in PTR-MS. *International Journal of Mass Spectrometry*, *290*(1), 60–63. https://doi.org/10.1016/J.IJMS.2009.11.007

Contarini, G., & Povolo, M. (2002). Volatile fraction of milk: Comparison between, purge and trap and solid phase microextraction techniques. *Journal of Agricultural and Food Chemistry*, *50*(25), 7350–7355. https://doi.org/10.1021/jf025713a

Contarini, G., Povolo, M., Leardi, R., & Toppino, P. M. (1997). Influence of Heat Treatment on the Volatile Compounds of Milk. *Journal of Agricultural and Food Chemistry*, *45*(3), 3171–3177. Retrieved from https://pubs.acs.org/sharingguidelines

de Wit, R., & Nieuwenhuijse, H. (2008). Kinetic modelling of the formation of sulphur-containing flavour components during heat-treatment of milk. *International Dairy Journal*, *18*(5), 539–547. https://doi.org/10.1016/j.idairyj.2007.11.011

Gaucher, I., Mollé, D., Gagnaire, V., & Gaucheron, F. (2008). Effects of storage temperature on physico-chemical characteristics of semi-skimmed UHT milk. *Food Hydrocolloids*, *22*(1), 130–143. https://doi.org/10.1016/j.foodhyd.2007.04.007

Jansson, T., Clausen, M. R., Sundekilde, U. K., Eggers, N., Nyegaard, S., Larsen, L. B., … Bertram, H. C. (2014). Lactose-hydrolyzed milk is more prone to chemical changes during storage than conventional ultra-high-temperature (UHT) milk. *Journal of Agricultural and Food*

Chemistry, *62*(31), 7886–7896. https://doi.org/10.1021/jf501671z

Jansson, T., Waehrens, S. S., Rauh, V., Danielsen, B. P., Sørensen, J., Bredie, W. L. P., … Lund, M. N. (2019). Effect of green tea catechins on physical stability and sensory quality of lactose-reduced UHT milk during storage for one year. *International Dairy Journal*, *95*, 25–34. https://doi.org/10.1016/j.idairyj.2019.03.007

Jo, Y., Benoist, D. M., Barbano, D. M., & Drake, M. A. (2018). Flavor and flavor chemistry differences among milks processed by high-temperature, short-time pasteurization or ultra-pasteurization. *Journal of Dairy Science*, *101*(5), 3812–3828. https://doi.org/10.3168/JDS.2017-14071

Limacher, A., Kerler, J., Davidek, T., Schmalzried, F., & Blank, I. (2008). Formation of furan and methylfuran by maillard-type reactions in model systems and food. *Journal of Agricultural and Food Chemistry*, *56*(10), 3639–3647. https://doi.org/10.1021/jf800268t

Maga, J. A., & Katz, I. (1979). Furans in foods. *Critical Reviews in Food Science and Nutrition*, *11*(4), 355–400. https://doi.org/10.1080/10408397909527268

Pedrotti, M., Khomenko, I., Cappellin, L., Fontana, M., Somenzi, M., Falchero, L., … Biasioli, F. (2018). Rapid and noninvasive quality control of anhydrous milk fat by PTR-MS: The effect of storage time and packaging. *Journal of Mass Spectrometry*, *53*(9), 753–762. https://doi.org/10.1002/jms.4204

Perkins, M. L., D'Arcy, B. R., Lisle, A. T., & Deeth, H. C. (2005). Solid phase microextraction of stale flavour volatiles from the headspace of UHT milk. *Journal of the Science of Food and Agriculture*, *85*(14), 2421–2428. https://doi.org/10.1002/jsfa.2243

Rerkrai, S., Jeon, I. J., & Bassette, R. (1987). Effect of Various Direct Ultra-High Temperature Heat Treatments on Flavor of Commercially Prepared Milks. *Journal of Dairy Science*, *70*(10), 2046–2054. https://doi.org/10.3168/jds.S0022-0302(87)80252-7

van Boekel, M. A. J. S. (2006). Formation of flavour compounds in the Maillard reaction. *Biotechnology Advances*, *24*(2), 230–233. https://doi.org/10.1016/J.BIOTECHADV.2005.11.004

Zardin, E., Tyapkova, O., Buettner, A., & Beauchamp, J. (2014). Performance assessment of proton-transfer-reaction time-of-flight mass spectrometry (PTR-TOF-MS) for analysis of isobaric compounds in food-flavour applications. *LWT - Food Science and Technology*, *56*(1), 153–160. https://doi.org/10.1016/j.lwt.2013.10.041

Chapter 7: General Discussion and Conclusions

Discussion of the chemical and sensory changes in UHT hydrolyzed-lactose milk during production and storage

Nowadays UHLM represents a staple product for many consumers, including those who can digest lactose. Consumers appreciate milk with a refreshing clean taste, without off-flavors and weird aftertaste (Francis et al., 2005). This PhD project exhibits that delivering a product with such sensory characteristics is an arduous task to achieve for dairy producers. The flavor of UHLM is complex, as different VOCs can arise at different stages of production and shelf-life (Chapter 2). Before manufacturing, the milk flavor can change due to seasonality, geographical origin, cow's diet and farming system (Rapisarda et al., 2013). All these factors define the so-called batch-to-batch milk variability that is showed as a relevant parameter distinguishing milk upon supply. However, according to this thesis results, during manufacturing, the variability among different milk batches tends to disappear and the UHT treatment gives the major contribution to the flavor. Literature showed that the severity of the heat treatment has greater influence on the VOCs development in UHLM compared to regular UHT milk. Lactose hydrolysis doubles the molarity of reducing sugar (Tossavainen & Kallioinen, 2007) and the obtained glucose and galactose exhibit more reactivity towards protein glycation (Milkovska-Stamenova & Hoffmann, 2017; Naranjo, Gonzales, Leiva, & Malec, 2013). On the other hand, looking at the results of the first case study described in Chapter 2, differences in the milk flavor before and after UHLM manufacturing emerged from modifications in the fat fraction. Methyl-ketones, which are formed upon β-oxidation of fatty acids and decarboxylation of β-keto acids, increased in the milk upon heating. Therefore, from a flavor perspective, the impact of MR and UHLM processing (e.g. production technology and enzymatic side activity of LP) is not evident straight after production but becomes relevant during shelf-life. Indeed, specific MR volatiles compounds were found in UHLMs both commercial (Chapter 2) and industrially produced (Chapter 4) during storage at 20°C, following

different trends. From the results discussed in these two chapters, it is clear that the central hub of flavor modification in UHLM is Strecker degradation which occurs at intermediate stages of MR (van Boekel, 2006). The extent of the reaction is the results of an interplay among several variables but, looking at the results of Chapter 4, it can be satisfactorily stated that the proteolytic side activity of the commercial LPs gives the major contribution. Increased levels of free amino acids were observed in the milk treated with LPs with high proteolytic activity. Specific trends in the release of 2-methylbutanal and benzaldehyde were shown in relation to the initial lactase purity. A higher release of the free amino acid isoleucine/leucine in the milk, due to a higher proteolytic activity of the LP, contributed positively to the release of 2-methylbutanal in the product headspace. Without an extensive proteolysis, Strecker degradation would be discouraged, as demonstrated by the lower concentration of 2-methylbutanal in the milk produced with a highly pure lactase. Studies performed in this PhD project demonstrated that a sound understanding of MR in milk derives from a deep knowledge on the chemical nature of the individual free amino acids present in the product. The combined measurements of the free amino acids and VOCs provided insights on the flavor-contributing pathways of MR. Therefore, LC/HRMS methodology applied in Chapter 4 and 6 for free amino acids quantification expressed excellent performance when applied to UHLM. Beside an accurate quantification of the free amino acids, the proposed methodology was already demonstrated suitable for the simoultaneous quantification of the corresponding APs, approach that might be taken into consideration for future research (Troise, Fiore, Roviello, Monti, & Fogliano, 2015; Troise, Wiltafsky, Fogliano, & Vitaglione, 2018). The proteolytic side activity of the LP is represented by exopeptidases and endopeptidases that hydrolyze β-casein (β-CN) and αs1-casein (αs1-CN) (Jansson et al., 2014; Nielsen et al., 2018). Intriguingly, our experiments further showed that these peptidases tend to cleave specific amino acids residues of both β-CN and αs1-CN, as a different free amino acids profiling was obtained when the milk was treated with different LPs. This indicates that a pattern underlining protein degradation exists and it might be predicted in relation to the type of enzymes and peptidic bonds involved. In this context, it is important to consider that not all the free amino acids are converted *via* Strecker degradation, so a mere quantification of the proteases might not be sufficient to understand the response in terms of off-flavor development during storage. Proteolysis lowers the milk quality

considerably when the free amino acids leading to Strecker degradation are released by the LP proteolytic activity. The release of other types of free amino acid residues should not be underestimated: beside providing further substrate for MR, previous research articles demonstrated that bitterness may arise in UHLM due to the release of bitter tasting peptides (Nielsen et al., 2017). Troise et al. (2016) suggested that changes occurring in UHLM should be contextualized to the technology employed for production. Therefore, this PhD thesis was extensively dedicated to the features of "in batch" production. The production system was demonstrated suitable to neutralize the proteolytic side effects of the LPs, mitigating the formation of MR volatiles compounds during storage (Chapter 4). At the end of their commercial shelf-life, all the tested UHLMs did not develop remarkable sensory defects indicating that the "in batch" process maintained the quality of the product acceptable longer than expected. Furthermore, the potentially negative effects of the LPs did not emerge at sensory level, because the UHT sterilization inactivated the proteolytic side activity before the product storage started. According to this thesis, dairy producers may consider to extend the commercial shelf-life of UHLM beyond 120 days as long as the LP side activity is properly inactivated following "in batch" production. Inactivation of the LP before the product is sold can be an advantage according to Dekker and coworkers (2019), especially for commercialization in those nations having particular regulations and labelling restrictions. Sensory analysis revealed that stale flavor, associated to consumer rejection in UHLM, was not significantly developed over the 120-days storage at 20°C. Thus, despite different trends among the UHLMs produced with different LPs, Strecker degradation was slackened and those off-flavoring molecules contributing to stale flavor (e.g. 2-methylbutanal) were not developed up to concentration detectable by the panelists. The price of LP is related to its relative purity, so cost-saving strategies can be implemented following the "in batch" process by opting for less purified lactases (Mittal, Newell, Hourigan, & Zadow, 1991). Novel LPs improving production performance may find their space "in batch" too. Maxilact® Smart (DSM, Heerlen, The Netherlands) is an example. The lactase is capable of converting lactose into glucose and galactose faster enabling a more efficient production and higher throughput (Dekker et al., 2019). All these considerations raise the appeal of the "in batch" technology for industrial production of high quality UHLM. On the other hand, complications may arise when UHLM is kept at temperatures higher

than conventional, as illustrated in Chapter 6. At 30 and 40°C storage the propagation of the MR was boosted. Despite the "in batch" process provides some kind of protection from MR and off-flavor development, an increase in storage temperature causes a cascade of reactions diminishing the quality of UHLM. An interplay was triggered among lipid oxidation (with formation of methyl ketones), protein oxidation (with the formation of methionine oxidation products) and MR (*via* Strecker degradation). In particular, Strecker aldehydes and further secondary products may generate off-flavors at high storage temperature, so sensory defects can still pop up. Strategies for mitigating MR and off-flavors in UHLM might be required to keep the sensory quality stable, prolonging the shelf-life and reducing the financial risk when milk is subjected to temperature fluctuations during warehousing and shipment, such as those during exportx. An article published by Jansson et al. (2017) proposed the addition of green tea extract (GTE), exploiting the capacity of the epicatechins of trapping α-dicarbonyls and free amino acids rendering them unavailable for Strecker degradation. This strategy was successfully applied to UHLM during storage at room temperature: the concentration of Strecker aldehydes was reduced and, consequently, also the stale flavor of the product, associated with a mushroom-like flavor by the researchers, was lowered significantly. Another research article proposed the application of FAD-fructosamine oxidase I (Faox I) to control MRPs formation (Troise, Buonanno, Fiore, Monti, & Fogliano, 2016). The enzyme promotes the deglycation of APs through oxidation leading to the formation of deoxyglucosone and amino acids (Takahashi, Pischetsrieder, & Monnier, 1997). Following this mechanism, the level of protein-bound MRPs (e.g. protein-bound HMF, CML etc.) formed in UHLM was reduced significanlty and MR was kept under control (Troise, Buonanno, et al., 2016). GTE and Faox I target specific MR stages, so the mitigating of MR is expected even when UHLM is stored at temperatures higher than usual. Moreover, preliminary results published on the application of these two additives in UHLM seem promising (Jansson et al., 2017; Troise, Buonanno, et al., 2016). Nevertheless, in the opinion of the researcher, the main limitation of these approaches is defining the product from a legal and commercial perspective, because, with the addition of GTE or Faox I, the product cannot probably be intended as milk anymore but, most likely, as milk-based drink.

Considerations on the methodologies applied for milk volatiles assessment

Along this PhD project, two MS-based techniques were applied to assess the flavor profile of the several UHLM samples proposed in the various chapters. A HS-SPME/GC-MS technique was optimized specifically for UHLM and applied to study the UHLM flavor in all its complexity. Beside, PTR-ToF-MS was tested as a less-time consuming technique for VOCs estimation in UHLM. First of all, the applied PTR-ToF-MS methodology measured accurately the entire VOCs profile of a great deal of UHLM samples, within a very short period of time. Coupling the instrument with a GC autosampler (Gerstel, Germany) with temperature controlled trays improved the performance of analysis even further, rendering the system fully automated. This fast and efficient way of measuring UHLM samples allowed an increase in the sample size of the experiments, so the effect of more independent variables was catched by the analysis in comparison with HS-SPME/GC-MS. For example, in Chapter 5 the batch-to-batch flavor variability of UHLM manufactured at industrial level was fully depicted by PTR-ToF-MS. In this sense, PTR-ToF-MS coupled with an autosampler should find valid applications in industrial QC activities involving an elevated number of milk samples.
However, although researchers agreed on the good analytical performance of PTR-MS, disputes still arise concerning the best approach for performing data analysis (Esslinger, Riedl, & Fauhl-Hassek, 2014). When dealing with PTR-MS data, the way how the mass peaks are extracted, selected and statistically considered has a huge impact on the interpretation of final results. In the PTR-MS experiments proposed in this PhD thesis (Chapter 5 and 6), data extraction was performed following a validated procedure (Cappellin et al., 2011; Pedrotti et al., 2018), whereas for the peak selection a novel approach based on the spatial distribution of the scores and loadings from the explorative PCA was used. Those mass peaks which described better the principal components, characterized by a loading score higher than a certain threshold, were gathered in summary tables used for statistical analysis. Following this procedure, the patterns of the PTR-MS data were strongly dependent on the method chosen for data scaling. Scaling to unit variance represents a golden standard to treat VOCs data before multivariate analysis. On the other hand, in food products like milk, VOCs are released at very low concentrations sometimes close to the detection limit of the

instrument. This causes a larger variations in the measurements and, when scaling to unit variance is applied, those signals are less variable because far from the detection limit might be penalized when looking at them at multivariate level. For this reason, Pareto scaling was applied to adjust different fold changes among the PTR-MS variables and the uncertanty associated to the noise was reduced. This indicates that for a successful application of the PTR-MS in industrial QC programs research efforts should be addressed on designing a standard strategy that can be applied by the operators without particular difficulties and with consistent reliability. Moreover, precision of VOCs identification is limited with PTR-MS (Biasioli, Gasperi, Yeretzian, & Märk, 2011). The analytes are not fully fragmented during analysis so the tentative identification of the VOCs is based primarily on the protonated molecule mass (Majchrzak et al., 2018). For this reason, part of the sample set analyzed by PTR-ToF-MS in Chapter 5 and 6 was assessed in parallel by HS-SPME/GC-MS, providing a clearer identity to the VOCs present in the UHLM headspace. The performance of the applied HS-SPME/GC-MS methodology was demonstrated excellent throughtout the PhD project. The DVB-Carboxen-PDMS fiber guaranteed a broad coverage of analytes belonging to different classes. The extraction time chosen (60 min) was also appropriate because, beside an accurate detection of the different VOCs, the SPME method was efficiently aligned with the GC-MS run speeding up the analysis. Compared to the PTR-ToF-MS, HS-SPME/GC-MS worked better for the detection of Strecker degradation products, which potentially cause stale flavor in UHLM. For example, by HS-SPME/GC-MS the 2-methylbutanal was correctly identified and distinguished from its isomer 3-methylbutanal despite the poor resolution laying between the two compounds. 2-Methylbutanal was linked to m/z 87.044 when measured by PTR, but that signal was also associated to other VOCs (e.g. 2-3-butandione, 2-pentanone, γ-butyrolactone and pentanal), rendering the trend of the compound difficult to follow during shelf-life. At the same time, aldehydes undergo different fragmentation patterns when transit through the drift tube of the instrument. In the case of 2-methylbutanal, H_2O is lost upon fragmentation and the protonated mass 69^+ is formed (Fall, Karl, Jordan, & Lindinger, 2001). This fragmentation pattern is common also to many other compounds, which compromise the distinction of 2-methylbutanal from the rest of the mass peaks.

Therefore, although the application of PTR-ToF-MS was demonstrated beneficial in some ways, for a deep insight on the chemical nature of the off-flavor development in UHLM, it is advised to monitor the Strecker degradation products with HS-SPME/GC-MS. Overall, following the time course evolution of the VOCs by PTR-ToF-MS basing the identification of the compounds by GC-MS the disadvantages of both techniques were compensated and provided a reliable and complete estimation of the VOCs profile of UHLM at all the stages of production and storage.

Recommendations for the future and main conclusions

From the studies performed, milk batch variability emerged. Thus, the quality of the raw milk should be a priority for dairy producers to obtain UHLM with constant characteristics and harmonize the variations among batches of production. For example, raw milk should be transported and stored at low temperature for very short time in order to minimize the modifications occurring at the protein and lipid fraction before production. Furthermore, future research activity should always take the batch-to-batch milk variability into account; wrongful consideration may be drawn otherwise. Consider 3-5 batches for consistent results at industrial level.

The "in batch" system is advised for the production of shelf-life stable UHLM, with an acceptable sensory profile. The thermal inactivation of the LP before shelf-life stabilized the product quality, so recalls due to unwanted enzymatic activity are unlikely. Opting for this technology may lower the final product costs too, if a less-purified lactase is applied for production. This aspect requires further investigation: a larger number of commercial LPs should be screened based on their purity and impact of the UHLM quality, whereas a detailed cost analysis of the actual impact of the production system on the overall profit margin should be considered as well. On the other hand, UHLM produced by "in batch" technology is not totally insensitive to chemical changes during shelf-life. This PhD thesis revealed that a pool of amino acids is generated upon lactose hydrolysis triggering Strecker degradation. Strecker degradation products should be investigated further in the future in order to define their specific contribution to the UHLM flavor. Beside "in batch" production, UHLM can also be produced "in pack", adding the LP once the milk already underwent thermal sterilization. At the moment, there are no studies comparing the "in batch" and "in pack" UHLM production, so future research efforts should be addressed in this direction. Designing such experiment is very complicated however, because the two technologies rely on two totally different principles and equipment. First of all, LPs intended for "in pack" production are obtained by filtration of the unsterilized LPs applied for "in batch" production. The resulting preparations have a lower enzymatic activity, making it difficult to obtain an "in pack" and "in batch" UHLM with comparable enzymatic concentration and activity. Secondly, the "in pack" production requires a specific dosing equipment to inject the sterile lactase into each milk package (e.g. the Flexdos® system from Tetrapak). This renders the simulation of the process at small

scale barely possible. From the experience of the researcher, it is not advisable to use conventional milk processing facilities to study the features of "in pack" UHLM on laboratory scale because adding the lactase preparation after UHT sterilization renders the product extremely prone to microbial contamination. Therefore, it makes more sense to compare the "in batch" and "in pack" production directly on the industrial production lines.

The PhD project portrayed also the sensitivity of UHLM to different temperatures during storage. It is important that the product does not exceed a certain level of Maillard reaction and lipid oxidation to avoid quality and nutritional losses. This goal is however difficult to achieve when dealing with UHLM intended for export: fluctuations of the temperature during warehousing, shipment and commercialization can accelerate unwanted color and flavor development reducing the best before consumption date. For this reason, dairy producers should start investing in data-driven tools for monitoring the status of the UHLM lots at each stage of the supply chain. The gathered data can be used as a basis for predictive modelling. The response of UHLM to temperature fluctuations is the result of a complex array of reactions which should be tackled with multi-response modelling to depict a cause-effect relationship among the different stages of MR and lipid oxidation. With multi-response modelling all the measured parameters, representing early-, intermediate- and end-stages of the reactions, can be considered together in simple kinetics predicting a specific response. In this frame, it is crucial to choose the response best representing the end of UHLM shelf-life: after all, which is that parameter that, above certain levels, causes the rejection of UHLM among consumers at first?

References

Biasioli, F., Gasperi, F., Yeretzian, C., & Märk, T. D. (2011, July). PTR-MS monitoring of VOCs and BVOCs in food science and technology. *TrAC - Trends in Analytical Chemistry*. https://doi.org/10.1016/j.trac.2011.03.009

Cappellin, L., Biasioli, F., Granitto, P. M., Schuhfried, E., Soukoulis, C., Costa, F., … Gasperi, F. (2011). On data analysis in PTR-TOF-MS: From raw spectra to data mining. *Sensors and Actuators, B: Chemical*, *155*(1), 183–190. https://doi.org/10.1016/j.snb.2010.11.044

Dekker, P. J. T., Koenders, D., & Bruins, M. J. (2019). Lactose-Free Dairy Products: Market Developments, Production, Nutrition and Health Benefits. *Nutrients*, *11*(3), 551. https://doi.org/10.3390/nu11030551

Esslinger, S., Riedl, J., & Fauhl-Hassek, C. (2014). Potential and limitations of non-targeted fingerprinting for authentication of food in official control. *Food Research International*, *60*(June 2014), 189–204. https://doi.org/10.1016/j.foodres.2013.10.015

Fall, R., Karl, T., Jordan, A., & Lindinger, W. (2001). Biogenic C5 VOCs: release from leaves after freeze–thaw wounding and occurrence in air at a high mountain observatory. *Atmospheric Environment*, *35*(22), 3905–3916. https://doi.org/https://doi.org/10.1016/S1352-2310(01)00141-8

Francis, L. L., Chambers, D. H., Kong, S. H., Milliken, G. A., Jeon, I. J., & Schmidt., K. A. (2005). Serving Temperature Effects on Milk Flavor, Milk Aftertaste, and Volatile Compound Quantification in Nonfat and Whole Milk. *Journal of Food Science*, *70*(7), s413–s418. Retrieved from www.ift.org

Jansson, T., Jensen, H. B., Sundekilde, U. K., Clausen, M. R., Eggers, N., Larsen, L. B., … Bertram, H. C. (2014). Chemical and proteolysis-derived changes during long-term storage of lactose-hydrolyzed ultrahigh-temperature (UHT) milk. *Journal of Agricultural and Food Chemistry*, *62*(46), 11270–11278. https://doi.org/10.1021/jf504104q

Jansson, T., Rauh, V., Danielsen, B. P., Poojary, M. M., Waehrens, S. S., Bredie, W. L. P., … Lund, M. N. (2017). Green Tea Polyphenols Decrease Strecker Aldehydes and Bind to Proteins in Lactose-Hydrolyzed UHT Milk. *Journal of Agricultural and Food Chemistry*, *65*(48), 10550–10561. https://doi.org/10.1021/acs.jafc.7b04137

Majchrzak, T., Wojnowski, W., Lubinska-Szczygeł, M., Różańska, A., Namieśnik, J., & Dymerski, T. (2018). PTR-MS and GC-MS as complementary techniques for analysis of volatiles: A tutorial review. *Analytica Chimica Acta*, *1035*, 1–13.

https://doi.org/https://doi.org/10.1016/j.aca.2018.06.056
Milkovska-Stamenova, S., & Hoffmann, R. (2017). Influence of storage and heating on protein glycation levels of processed lactose-free and regular bovine milk products. *Food Chemistry*, *221*, 489–495. https://doi.org/10.1016/j.foodchem.2016.10.092
Mittal, S., Newell, G., Hourigan, J., & Zadow, J. (1991). The effect of protease contamination in lactase on the flavour of lactose-hydrolyzed milks. *Australian Journal of Dairy Technology*, *46*(1), 46–47.
Naranjo, G. B., Gonzales, A. S. P., Leiva, G. E., & Malec, L. S. (2013). The kinetics of Maillard reaction in lactose-hydrolysed milk powder and related systems containing carbohydrate mixtures. *Food Chemistry*, *141*(4), 3790–3795. https://doi.org/10.1016/j.foodchem.2013.06.093
Nielsen, S. D., Jansson, T., Le, T. T., Jensen, S., Eggers, N., Rauh, V., … Larsen, L. B. (2017). Correlation between sensory properties and peptides derived from hydrolysed-lactose UHT milk during storage. *International Dairy Journal*, *68*, 23–31. https://doi.org/10.1016/j.idairyj.2016.12.013
Nielsen, S. D., Zhao, D., Le, T. T., Rauh, V., Sørensen, J., Andersen, H. J., & Larsen, L. B. (2018). Proteolytic side-activity of lactase preparations. *International Dairy Journal*, *78*, 159–168. https://doi.org/10.1016/j.idairyj.2017.12.001
Pedrotti, M., Khomenko, I., Cappellin, L., Fontana, M., Somenzi, M., Falchero, L., … Biasioli, F. (2018). Rapid and noninvasive quality control of anhydrous milk fat by PTR-MS: The effect of storage time and packaging. *Journal of Mass Spectrometry*, *53*(9), 753–762. https://doi.org/10.1002/jms.4204
Rapisarda, T., Pasta, C., Belvedere, G., Schadt, I., La Terra, F., Licitra, G., & Carpino, S. (2013). Variability of volatile profiles in milk from the PDO Ragusano cheese production zone. *Dairy Science and Technology*, *93*(2), 117–134. https://doi.org/10.1007/s13594-012-0104-3
Takahashi, M., Pischetsrieder, M., & Monnier, V. M. (1997). Isolation, purification, and characterization of amadoriase isoenzymes (fructosyl amine-oxygen oxidoreductase EC 1.5.3) from Aspergillus sp. *The Journal of Biological Chemistry*, *272*(6), 3437–3443. https://doi.org/10.1074/jbc.272.6.3437
Tossavainen, O., & Kallioinen, H. (2007). Proteolytic changes in lactose hydrolysed UHT milks during storage. *Milchwissenschaft*, *62*(4), 410–415. Retrieved from http://cat.inist.fr/?aModele=afficheN&cpsidt=19123002

Troise, A. D., Bandini, E., De Donno, R., Meijer, G., Trezzi, M., & Fogliano, V. (2016). The quality of low lactose milk is affected by the side proteolytic activity of the lactase used in the production process. *Food Research International*, *89*, 514–525. https://doi.org/10.1016/j.foodres.2016.08.021

Troise, A. D., Buonanno, M., Fiore, A., Monti, S. M., & Fogliano, V. (2016). Evolution of protein bound Maillard reaction end-products and free Amadori compounds in low lactose milk in presence of fructosamine oxidase i. *Food Chemistry*, *212*, 722–729. https://doi.org/10.1016/j.foodchem.2016.06.037

Troise, A. D., Fiore, A., Roviello, G., Monti, S. M., & Fogliano, V. (2015). Simultaneous quantification of amino acids and Amadori products in foods through ion-pairing liquid chromatography-high-resolution mass spectrometry. *Amino Acids*, *47*(1), 111–124. https://doi.org/10.1007/s00726-014-1845-5

Troise, A. D., Wiltafsky, M., Fogliano, V., & Vitaglione, P. (2018). The quantification of free Amadori compounds and amino acids allows to model the bound Maillard reaction products formation in soybean products. *Food Chemistry*, *247*, 29–38. https://doi.org/10.1016/J.FOODCHEM.2017.12.019

van Boekel, M. A. J. S. (2006). Formation of flavour compounds in the Maillard reaction. *Biotechnology Advances*, *24*(2), 230–233. https://doi.org/10.1016/J.BIOTECHADV.2005.11.004

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"Ti avverto. Guarda ogni strada attentamente e deliberatamente. Mettila alla prova tutte le volte che lo ritieni necessario. Quindi poni a te stesso, e a te stesso soltanto, una domanda: "Questa strada ha un cuore?" Se lo ha la strada è buona. Se non lo ha non serve a niente. Entrambe le strade non portano da alcuna parte, ma una ha un cuore e l'altra no. Una porta un viaggio lieto; finché la segui sei una sola cosa con essa. L'altra ti farà maledire la tua vita. Una ti rende forte; l'altra ti indebolisce".

(Carlos Castaneda – Gli insegnamenti di Don Juan)"

Una strada è solo una strada, siamo noi a dargli valore. Grazie quindi a tutti coloro che hanno lasciato un segno in questo mio strano percorso che, negli ultimi 3 anni, mi ha portato a zonzo tra Campania, Trentino Alto-Adige e Olanda. Che possiate tutti voi vivere una vita piena di "cuore", gioendo per i vostri successi e piangendo altrettanto per gli insuccessi, sentendo la vita scorrere ogni giorno nelle vostre vene.

Cheers