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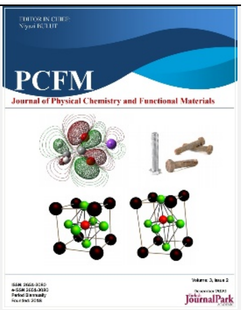

A Novel HPLC Method for Rapid Determination of HMF in *Dulce de Leche*: A Potential Tool for Quality Control

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ABSTRACT

A validated isocratic method utilizing reverse-phase high-performance liquid chromatography (RP-HPLC) was developed for the quantification of hydroxymethylfurfural (HMF) in five distinct commercially available Dulce de Leche samples that are sold in Turkish markets. The X-Terra RP-18 column, which has dimensions of 150 × 4.60 mm i.d. and a particle size of 5 µm, was selected for the stationary phase. The separation process was conducted isocratically by utilizing a methanol-water mixture (10:90, v/v) containing 1% TCA as the mobile phase. The separation was carried out at a temperature of 25 oC and a flow rate of 0.5 mL/min. The detection was performed at a wavelength of 284 nm. The methodology exhibited favorable selectivity and linearity, as evidenced by a correlation coefficient (r) exceeding 0.999. The obtained values for the limit of detection (LOD) and limit of quantification (LOQ) were 0.0019 µg/mL and 0.0059 µg/mL, respectively. Additionally, the recovery rate was determined to be 95.45%. Furthermore, it has been reported that the levels of HMF in commercially available Dulce de Leche originating from Turkey vary between 50.55 and 90.18 mg/kg for the total HMF content..

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Introduction

In the context of an increasingly interconnected global society, consumer expectations are undergoing transformation in response to the advancements observed within the food business. The prominence of food options that lack microbiological risks, possess extended shelf life, exhibit aesthetic appeal, are organically produced, and are free from additives has increased. One regularly employed strategy in the preservation of food is heat treatments. During these operations, distinct reactions take place, leading to variations between protein and sugar groups [1,2].

The caramelization reaction is influenced by factors such as pH, salt concentration, and sucrose concentration, resulting in the production of caramel color. The occurrence of a desired reaction in certain food products is sought after, however, when this reaction becomes unmanageable, it becomes undesired due to the resulting bitter and burnt taste [3].

During heat treatment applications in foods, the presence of flavor and aroma, as well as colorants, is influenced by various factors such as pH, type of reactants, temperature, and water activity. Additionally, the Maillard reaction gives rise to several products including hydroxymethyl furfural (HMF), furfural, melanoids, and acrylamide (Fig. 1) [4].

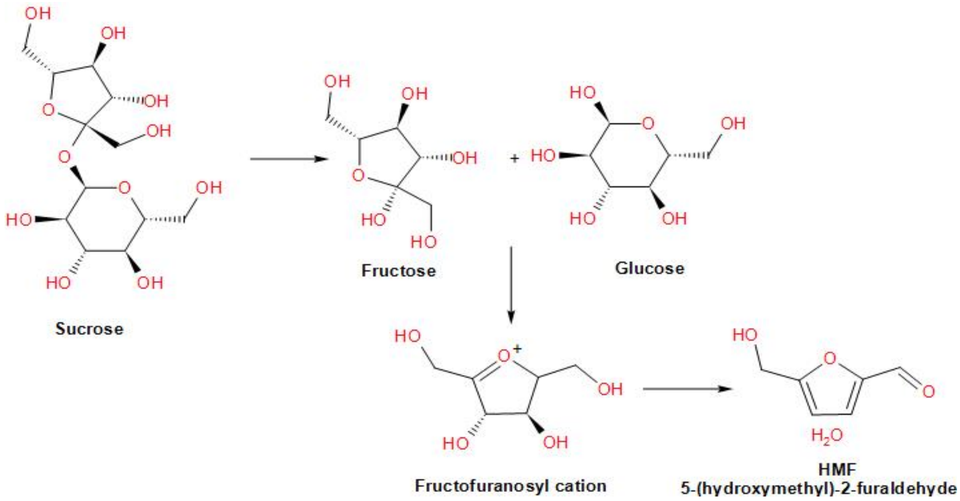


Fig. 1. Decomposition of sugars into HMF at elevated temperatures – Maillard Reaction

One of the indicated products is HMF [5, 6, 7]. This chemical is present at relatively low amounts in heat-treated foods. The levels of individual HMF in daily ingestion can exhibit significant changes depending on the quantity consumed. According to estimates, individuals following a dietary regimen can potentially get roughly 5-10 mg of HMF per day from the digestion of food [8]. The presence of HMF is typically little in unprocessed foods [9]; however, its concentration tends to rise upon exposure to heat treatment. Furthermore, the freshness of food is a crucial determinant of its quality [10, 11]. The level of HMF in various food products is contingent upon the degree and caliber of thermal processing. The item mentioned previously serves as an indicator for the purpose of establishing [12]. The synthesis of HMF is mostly attributed to the Maillard reaction, followed by the subsequent processes of browning and caramelization [13].

According to Ulbricht (2014), the administration of high concentrations of HMF not only leads to its harmful effects but also induces irritation in many areas such as the eyes, upper respiratory tract, skin, and mucous membranes [14]. Moreover, a multitude of studies have demonstrated its capacity to impede cellular proliferation, exert cytotoxicity, and facilitate the genesis and progression of tumors [15, 16, 17]. In previous research with mice, it has been documented that the application of HMF at doses ranging from 1260 to 3150 mg results in the formation of skin lesions and an elevated incidence of skin tumors. Additionally, a dosage of 200 mg per kilogram of body weight has been found to induce the development of liver tumors [18].

"Dulce de leche," a dairy product, is extensively consumed in Latin American nations. It is commonly used as a spread on toast, a filling for crepes, pastries, and cakes, or as a topping for ice cream and fruits. The classic method of preparing Dulce de Leche involves the heat concentration of whole milk and saccharose [19]. The confection known as "Dulce de Leche" is distinguished by its dense and velvety consistency, as well as its rich brown hue [20]. Prior research has examined the presence of HMF in "Dulce de Leche" using the conventional spectrophotometric measurement method developed by Keeney and Bassette specifically for dairy products [21-23]. In their seminal work, Keeney and Bassette (1959) introduced a conceptual distinction between two forms of HMF: free HMF and total HMF. Free HMF refers to the HMF that exists independently, while total HMF include both free HMF and potential HMF that can be produced from other intermediates of the Maillard reaction. Nevertheless, this particular methodology suffers from a lack of sensitivity towards HMF, resulting in a wide range of outcomes due to the colorimetric reaction of other compounds with the reactant. Consequently, around 70% of the molecules detected are considered to be interfering substances [24-26]. In contrast, liquid chromatography has demonstrated its efficacy as a precise and dependable approach for quantifying furanic chemicals in food samples. Notably, researchers have devised specialized methodologies for assessing the levels of free and total HMF in dairy products [27, 28].

In relation to this matter, the majority of HMF chromatographic techniques have been formulated and verified for powdered milk and analogous food matrices that possess a lower saccharose content compared to Dulce de Leche. The utilization of these techniques for quantifying HMF in Dulce de Leche is a more specialized option in contrast to spectrophotometric approaches. However, it is important to consider that variations in the chemical composition of the analyzed matrices may impact the precision of the measurements. Hence, it is imperative to create precise analytical procedures tailored to this particular food matrix in order to ensure the accuracy and dependability of the obtained results. The objective of this study was to determine the levels of HMF in Dulce de Leche using a high-performance liquid chromatography with diode array detection (HPLC-DAD) method, as well as to establish the HMF contents in commercially available Dulce de Leche products in the Turkish market.

Experimental

Chemicals and Material

The standard of HMF (≥ 99.0 %) was acquired from Sigma-Aldrich, a chemical supplier based in St. Louis, Missouri, USA. Sodium chloride, trichloroacetic acid (TCA), oxalic acid and sodium hydroxide were procured from Sigma-Aldrich. HPLC-grade acetonitrile (ACN), methanol (MeOH) and diethyl ether were procured from Merck, Darmstadt, Germany. The acquisition of ultrapure water, characterized by a conductivity level below 0.05 µS/cm, was accomplished using a Milli-Q system manufactured by Millipore, located in Bedford, MA, USA. The HMF standard was prepared using methanol as the solvent. All solutions were shielded from exposure to light and stored at a temperature of 4°C for short-term consumption on a daily basis, and at a temperature of -20°C for long-term usage spanning multiple days.

Apparatus and Chromatographic conditions

The liquid chromatography (LC) analysis was conducted using an Agilent 1260 series high-performance liquid chromatography (HPLC) system equipped with a ternary solvent pump, online degasser, automatic injection system, column heater, and multi-wavelength detector. The detection of ultraviolet (UV) radiation was conducted at a wavelength of 284 nm. The analyses were performed at a flow rate of 0.5 mL.min^{-1}. For the stationary phase, an X-Terra RP-18 column with dimensions of 150 × 4.60 mm i.d. and a particle size of 5 µm was chosen. The analyses were performed at a temperature of 25°C. Separation was performed isocratically using a mixture of methanol-water (10:90, *v/v*) with 1% TCA as mobile phase and injection volume was of 20 µL.

Samples

Commercial Dulce de Leche products were obtained from local markets and they were selected considering the five commercial brands (coded sample 1-5 in this study) with the highest consumption in Turkey. Each sample was analyzed in triplicate.

Preparation of *Dulce de Leche* for HPLC Analysis

The procedure that was used according to the methodology of Barrera et al. (2021) subjected to some modifications and it was carried out on numerous commercial Dulce de Leche samples [29]. The preceding items were acquired from Turkish markets. A mass of 1 gram of Dulce de Leche was measured and subsequently mixed with 10 mL of water to achieve dilution. A volume of 3 mL of the solution under consideration was extracted, followed by the addition and integration of 1.5 mL of a 0.3 M oxalic acid solution. Following a 20-minute waiting period, a volume of 1.5 mL of trichloroacetic acid (TCA) with a concentration of 40% (w/v) was subjected to filtration using an extra reduction filter paper. A volume of 2 mL of the filtrate was used for extraction, followed by the addition of 1 mL of saturated sodium chloride (NaCl) solution. The solution was subjected to three extractions using diethyl ether in a ratio of 20:10:10. The organic phase was collected and subjected to nitrogen gas drying at a temperature of 40 °C. Following this, 1 mL of methanol and 1 mL of a 1 M NaOH solution were added to the organic phase. The resulting mixture was then filtered through a membrane filter and subsequently subjected to analysis.

Results And Discussion

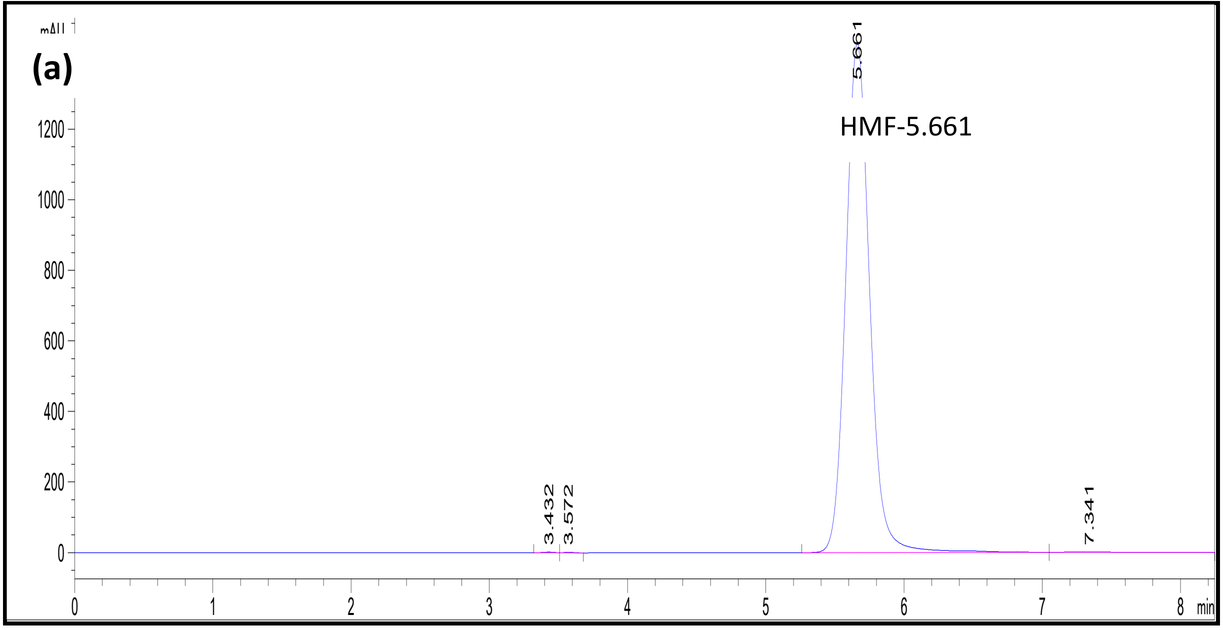
HMF is an intermediate product in the Maillard reaction and is also formed from the degradation of sugars at high temperatures [6, 30]. In this paper, HMF contents of five Dulce de Leche sample were determined by RP-HPLC method. A set of HMF standards were tested to determine the validation parameters (linearity, range, detection limit, and quantitation limit) [31]. The linearity was calculated by plotting the peak area versus concentration of compounds. Five standard solutions were prepared for calibration of compounds. Each solution was injected in duplicate. The calibration curves were obtained by linear least squares regression. The validation data are reported in Table 1. The method exhibited good linearity based on a correlation coefficient >0.999 for all compounds. The LOD and LOQ were calculated as $3.3\ s\cdot m^{-1}$ and $10\ s\cdot m^{-1}$, respectively, where s is the standard deviation of the response and m is the slope of the corresponding calibration curve [32, 33].

Table 1. Statistical evaluation of the calibration data of HMF by HPLC

	HMF
Linearity range (µg mL^{-1})	1-50.0
Slope	337.88
Intercept	436.21
Correlation coefficient (*r*)	0.999
Detection limit (LOD) (µg.mL^{-1})	0.0019
Quantitation limit (LOQ) (µg.mL^{-1})	0.0059

The concept of selectivity is employed to assess the ability of a method to specifically detect and quantify the analyte of interest in complicated sample matrices that may contain interfering chemicals [34].

In order to assess the selectivity of the proposed approach, a comparison was made between the retention periods of HMF in chromatograms of both standards and samples. The chromatograms displayed comparable retention times (Figure 2). Additional peaks of unidentified chemicals are shown on the chromatogram; however, as they do not co-elute with the peak of HMF, they do not have any impact on the study. Hence, the approach described demonstrates sufficient selectivity for the detection of HMF in Dulce de Leche.



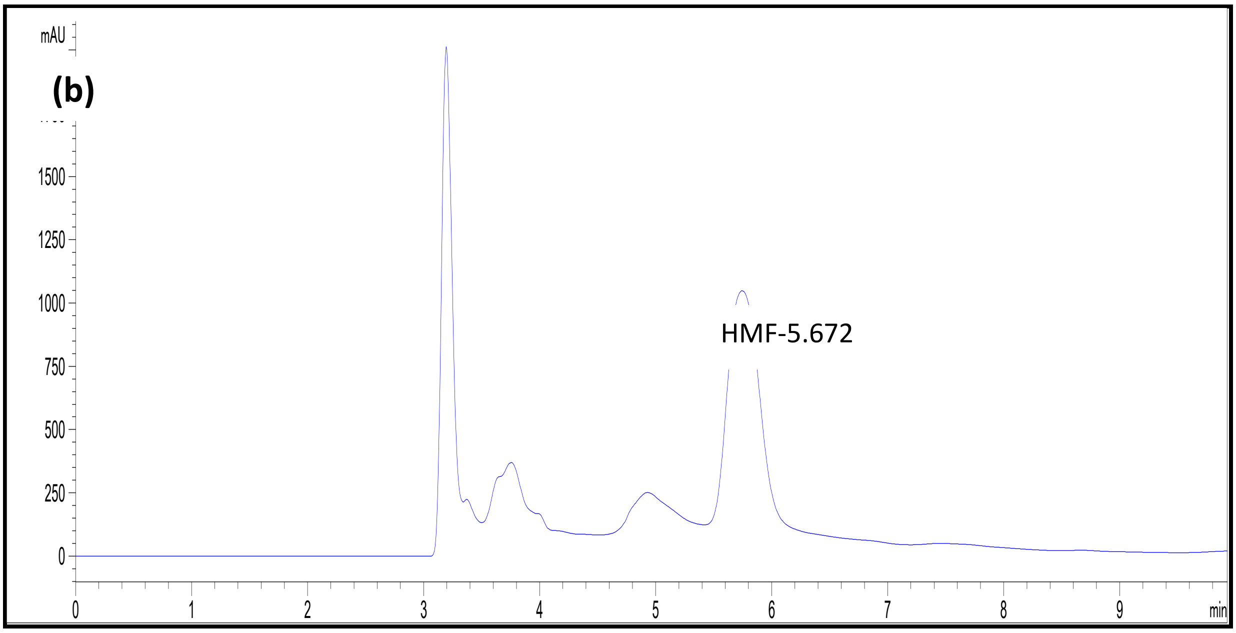


Fig. 2. Chromatograms of (a) HMF standard solution (50 µg/mL) obtained in 10% (*v/v*) MeOH-water mixture with 1%TCA, (b) total HMF on Dulce de Leche.

The assessment of accuracy was conducted by means of recovery tests. The preparation of sample solutions for HMF followed the previously described process, and these solutions were subsequently enriched with an HMF standard solution. The experiments were conducted in triplicate, and the recovery percentages were determined by dividing the amount of HMF recovered by the amount of HMF added and multiplying by 100. These recovery percentages were utilized as estimations of accuracy. The recovery test results were found as 95.45% and were higher than reported by Barrera et al. 2021 [29].

The methodology was implemented on a total of five commercially available products, with three samples obtained for each product. Each sample was subjected to triplicate analysis. The samples were assigned alphanumeric codes, with letters representing the company and numbers indicating the specific sample. The contents of HMF were presented in terms of the mean and standard deviations, as shown in Table 2.

Table 2. HMF content in Dulce de Leche samples.

Sample	HMF (mg.kg^{-1})
Sample 1	72.89 ± 0.12
Sample 2	50.55 ± 0.15
Sample 3	85.15 ±0.16
Sample 4	90.18 ±0.09
Sample 5	62.48 ±0.11

HMF contents ranges from 50.55 to 90.18 mg kg^{-1}. The observed values remained within a comparable range to those of commercial products, which aligns with the findings published by Gaze et al. (2015) and Francisquini et al. (2019) [35, 36]. The variation in HMF contents observed among the samples can be attributed to several factors that promote the formation of HMF in food matrices. These factors include thermal processing, composition and storage times. Additionally, the variability can be attributed to batch processes commonly employed during the manufacturing of these products [37].

In addition to other factors, it is worth considering the potential presence of HMF in the raw materials used for the production of Dulce de Leche specifically milk, due to the high temperature processing (UHT) involved in its preparation.

Conclusions

In conclusion, this study presents a novel and efficient High-Performance Liquid Chromatography (HPLC) method for the rapid determination of HMF in Dulce de Leche, a popular dairy product with a high risk of HMF formation during its manufacturing and storage processes. The proposed HPLC method offers several significant advantages for the assessment of HMF content in Dulce de Leche and has the potential to become a valuable tool for quality control in the dairy industry.

The results of our research have shown that the HPLC method is highly sensitive, specific, and reproducible in quantifying HMF levels in Dulce de Leche samples. This method allows for the accurate detection and quantification of HMF even at low concentrations, which is crucial for monitoring product quality and ensuring compliance with regulatory standards. The wide linear range, low detection limit, and excellent precision of the method make it suitable for a wide range of applications within the dairy industry.

Furthermore, this novel HPLC method can be readily integrated into routine quality control processes in dairy manufacturing facilities. It provides a rapid analysis of HMF levels, allowing for real-time monitoring of product quality and the early detection of any deviations from the desired specifications. This is essential for preventing the formation of excessive HMF, which can negatively impact the flavor, color, and safety of Dulce de Leche products.

The method's robustness and reliability were confirmed through a series of validation studies, including accuracy, precision, linearity, and recovery tests, which demonstrated its suitability for routine analytical use. The applicability of this method extends beyond quality control, as it can also serve as a valuable tool for research and development, facilitating the optimization of manufacturing processes and the development of new product formulations.

While the existing studies do not provide definitive evidence about the potential health risks associated with human exposure to HMF, Morales et al. (2009) have proposed a modified theoretical added maximum daily intake (mTAMDI) for HMF at a level of 1600 μg/person/day [37]. The HMF concentrations in our study varied from 50.55 to 90.18 mg kg^{-1}, indicating that the items available in Turkish markets have a significant amount of HMF content. When taking into account the suggested serving size of 30 g for Dulce de Leche, the consumption of HMF contributes a notable proportion to the maximum tolerable daily intake (mTAMDI). The state should establish and enforce restrictions on HMF levels in foods to ensure food safety and mitigate potential health hazards.

In summary, the innovative HPLC method presented in this study has the potential to greatly benefit the Dulce de Leche industry by enhancing the quality control processes and ensuring the production of safe and high-quality products. Its ability to provide rapid and precise HMF determination makes it an invaluable asset for both manufacturers and researchers in the field of dairy products. Future studies may further explore the application of this method in different dairy products and investigate its potential in assessing the impact of various processing conditions on HMF formation in Dulce de Leche. Overall, the development of such analytical tools is a testament to the continuous efforts to improve the quality and safety of dairy products.

References

[1] Husoy, T., Haugen, M., Murkovic, M., Jöbstl, D., Stølen, L. H., Bjellaas, T., 2008. Dietary exposure to 5-hydroxymethylfurfural from Norwegian food and correlations with urine metabolites of short-term exposure. Food and Chem. Tox. 46(12): 3697–3702.

[2] Ferrer, E., Alegria, A., Farre, R., Abellan, P., Romero, F., 2002. High-performance liquid chromatographic determination of furfural compounds in infant formulas. Change during heat treatment and storage. J. Chrom. A 947 (1): 85–95.

[3] Quintas, M.A.C., Branda, T.R.S., Silva, C.L.M. 2007. Modelling color changes during the caramelisation reaction. J. Food Eng. 83: 483–491.

[4] Hidalgo, F.J., Zamora, R., 2000. The role of lipids in nonenzymatic browning. Grasas y Aceites 51: 35– 49.

[5] Rufian-Henares, J.A., Delgado-Andrade, C., Morales, F.J., 2009. Assessing the Maillard reaction development during the toasting process of common flours employed by the cereal products industry. Food Chemistry 114: 93–99.

[6] Imperiale, S., Morozova, K., Ferrentino, G., Alam, M. R., & Scampicchio, M. 2022. Fast Detection of 5-Hydroxymethylfurfural in Dulce de Leche by SPE-LC–MS. Food Analytical Methods, 15(1), 1-9.

[7] Ramirez-Jimenez, A., Villanova, B.G. and Hernandez, E.G., 2000. Hydroxymethylfurfural and methylfurfural content of selected bakery products. Food Res. Int. pp. 833–838.

[8] Arribas-Lorenzo, G., Morales, F.J., 2010. "Estimation of dietary intake of 5- hydroxymethylfurfural and related substances from coffee to Spanish population". Food and Chem. Tox. 48 (2): 644–9.

[9] Babsky, N.E., Toribio, J.L., Lozano, J.E., 1986. Influence of storage on the composition of clarified apple juice concentrate. J. Food Sci. 51: 564–567.

[10] Fallico, B., Arena, E., Zappala, M., 2003. Roasting of hazelnuts. Role of oil in colour development and hydroxymethylfurfural formation. Food Chem. 81(4): 569–573.

[11] Teixidio, E., Nunez, O., Santos, F.J., Galceran, M.T., 2011. 5-Hydroxymethylfurfural content in foodstuffs

determined by micellar electrokinetic chromatography. Food Chem. 126: 1902–1908.

[12] Baldwin, I.T., Staszak-Kozinski L., Davidson R., 1994. Up in smoke: I. Smoke-derived germination cues for postfire annual, Nicotiana attenuata torr. ex. Watson. J. Chem. Eco. 20: 2345– 2371.

[13] Bozkurt, H., Gögüs, F., Eren, S. 1999. Nonenzymatic browning reactions in boiled grape juice and its models during storage. Food Chem. 64: 89–93.

[14] Ulbricht, R.J., Northup, S.J., Thomas, J.A., 1984. A Rewiev of 5-hydroxymethylfurfural (HMF) in parenteral solution. Fundamental and Applied Toxicology 4: 843–853.

[15] Janzowski, C., Glaab, V., Samımı, E., Schlatter, J., Eisebbrand, G. 2000. 5-Hydroxymethylfurfural: assessment of mutagenicity, DNA-damaging potential and reactivity towards cellular glutation. Food and Chem. Tox. 38: 801–809.

[16] Archer, M.C., Bruce, W.R., Chan, C.C., Carpet, D.E., Medline, A., Roncucci, L., Stamps, D., Zhang, X.M., 1992. Aberrant crypt foci and microadenoma as markers for colon cancer. Enviromental Health Perspectives 98: 195–197.

[17] Zhang, X.M., Chan C.C., Stamp D., Minkin S., Archer M.C., Bruce W.R., 1993. Initiation and promotion of colonic aberrant crypt foci in rats by 5- hydroxymethyl-2-furaldehyde in thermolyzed sucrose. Carcinogenesis 14: 773–775.

[18] Miyakawa, Y., Nishi Y., Kato K., Sato H., Takahashi M., Hayashi Y., 1991. Initiating activity of eight pyrolysates of carbohydrates in a two stage mouse skin tumorigenesis model. Carcinogenesis 12: 1169–1173.

[19] Malec, L.S., Llosa, R.A., Naranjo, G.B., Vigo, M.S., 2005. Loss of available lysine during processing of different dulce de leche formulations. Int. J. Dairy Technol. 58 (3), 164–168.

[20] Penci, M.C., Marín, M.A., 2016. Dulce De Leche: Technology, Quality, and Consumer Aspects of the Traditional Milk Caramel of South America. In Traditional Foods, pp. 123–136.

[21] Keeney, M., Bassette, R., 1959. Detection of intermediate compounds in the early stages of browning reaction in milk products. J. Dairy Sci. 42 (6), 945–960.

[22] Francisquini, J.d'Almeida, de Oliveira, L.N., Pereira, J.P.F., Stephani, R., Perrone, I.T., da Silva, P.H.F., 2016. Avaliaçao da intensidade da reaçao de Maillard, de atributos físico-químicos e analise de textura em doce de leite. Rev. Ceres 63 (5), 589–596.

[23] Pavlovic, S., Santos, R.C., Gloria, M.B.A., 1994. Maillard reaction during the processing of 'Doce de leite'. J. Sci. Food Agric. 66 (2), 129–132.

[24] Albala-Hurtado, S., Veciana-Nogues, M.T., Izquierdo-Pulido, M., Vidal-Carou, M.C., 1997. Determination of free and total furfural compounds in infant milk formulas by high-performance liquid chromatography. J. Agric. Food Chem. 45 (6), 2128–2133.

[25] Chavez-Servin, J.L., Castellote, A.I., Lopez-Sabater, M.C., 2005. Analysis of potential and free furfural compounds in milk-based formulae by high-performance liquid chromatography. Evolution during storage. J. Chromatogr. A 1076, 133–140.

[26] Morales FJ, van Boekel M.A.J.S. 1998. A study on advanced Maillard reaction in heated casein/sugar solutions: colour formation. Int. Dairy J., 8:907–915.

[27] Chavez-Servin, J.L., Castellote, A.I., Lopez-Sabater, M.C., 2005. Analysis of potential and free furfural compounds in milk-based formulae by high-performance liquid chromatography. Evolution during storage. J. Chromatogr. A 1076, 133–140.

[28] Morales, F.J., 2009. Hydroxymethylfurfural (HMF) and related compounds. Process Induced Food Toxicants: Occurrence, Formation, Mitigation, and Health Risks, pp. 135–174.

[29]. Barrera, J., Pedreschi, F., Gómez, J. P., Zúñiga, R. N., & Mariotti-Celis, M. S. 2021. In house validation for the direct determination of 5-hydroxymethyl-2-furfural (HMF) in "Dulce de Leche". J. Food Comp. Anal., 95, p. 103665.

[30]. Morales FJ, Romero C, Jimenez-Perez S. 1997. Chromatographic determination of bound hydroxymethylfurfural as an index of milk protein glycosylation. J. Agric. Food Chem., 45:1570-1573.

[31]. ICH, ICH, Topic Q2A Validation of Analytical Procedures Methodology, Geneva, Switzerland, 2000.

[32]. C. M. Riley and T. W. Rosanske, Development and Validation of Analytical Methods, Elsevier, New York, NY, USA, 1996.

[33]. M. E. Swartz and I. S. Krull, Analytical Method Development and Validation, Marcel Dekker Inc., New York, NY, USA, 1997.

[34]. Rao, T.N., 2018. Validation of analytical methods. Validation of Analytical Methods – a Sampling of Current Approaches (Vol. 55, pp. 600A-608A). InTech. https://doi.org/10.5772/intechopen.72087.

[35]. Gaze L, Costa M, Monteiro M, Lavorato J, Conte C, Raices R, Cruz A, Freitas M. 2015. Dulce de Leche, a typical product of Latin America: characterisation by physicochemical, optical and instrumental methods. Food Chem. 169:471–477. doi:10.1016/j.foodchem.2014.08.017.

[36]. Francisquini J, Rocha J, Martins E, Stephani R, Fonseca Da Silva P, Renhe I, Perrone ÍT, Fernandes De Carvalho A. 2019. 5-Hydroxymethylfurfural formation and color change in lactose-hydrolyzed Dulce de leche. J

Dairy Res. 86(4):477–482. doi:10.1017/S0022029919000815.

[37]. Morales, F.J., Martin, S., Açar, O.C., Arribas-Lorenzo, G., Gokmen, V., 2009. Antioxidant activity of cookies and its relationship with heat-processing contaminants: a risk/ benefit approach. Eur. Food Res. Technol. 228 (3), 345–354. https://doi.org/ 10.1007/s00217-008-0940-9.