



Original research article



In house validation for the direct determination of 5-hydroxymethyl-2-furfural (HMF) in “*dulce de leche*”

Josefina Barrera^a, Franco Pedreschi^a, Juan Pablo Gómez^a, Rommy N. Zúñiga^b,
María Salomé Mariotti-Celis^{c,d,*}

^a Departamento de Ingeniería Química y Bioprocesos, Pontificia Universidad Católica de Chile, P.O. Box 306, Santiago 6904411, Chile

^b Department of Biotechnology, Universidad Tecnológica Metropolitana, Las Palmeras 3360, Santiago, Chile

^c Universidad Finis Terrae, Santiago, Chile

^d Programa Institucional de Fomento a la Investigación, Desarrollo e Innovación, Universidad Tecnológica Metropolitana, Ignacio Valdivieso 2409, Santiago, Chile

ARTICLE INFO

Keywords:

5-hydroxymethyl-2-furfural
HMF
Validation
HPLC-DAD
“*dulce de leche*”
Dairy product
Food analysis
Food composition

ABSTRACT

In this work an HPLC-DAD determination method for free and total HMF in “*dulce de leche*” was developed and validated. The separation was carried out using a mobile phase of acetonitrile-water (4.5:95.5, v/v) on Acclaim™120 C18 (4.6 × 150 mm, 5 μm), at 30 °C and a flow rate of 1 mL/min. The detection was done at 284 nm. The method showed good selectivity and linearity (determination coefficient over 0.999). Low LOD and LOQ values were obtained (19.51 μg/L and 65.05 μg/L respectively). Relative standard deviation (RSD) for repeatability and intermediate precision was <4.12 % and <5.98 % respectively. Recovery rates were between 89.04 % and 91.89 %. In addition, the HMF contents in commercial “*dulce de leche*” from Chile was reported, ranging from 2.33 to 10.31 mg/kg for free HMF and 314.61–464.98 mg/kg for total HMF.

1. Introduction

“*Dulce de leche*” is a dairy product widely consumed in Latin American countries as spread on bread, filling for crepes, cookies and cakes, or as topping for ice cream and fruits, traditionally made from the heat concentration of whole milk and saccharose (Malec et al., 2005). “*Dulce de leche*” is characterized by being a thick, creamy product of brown color (Penci and Marín, 2016). These sensory characteristics are developed during the thermal treatment, mainly due to the Maillard Reaction (MR), which is also responsible by the formation of undesirable compounds such as 5-hydroxymethyl-2-furfural (HMF) (Francisquini et al., 2018; Pavlovic et al., 1994).

HMF is a furanic compound formed as an intermediate of the MR or by direct dehydration of saccharides in acidic conditions (Capuano and Fogliano, 2011). Among the factors that affect HMF formation are: temperature, pH, water activity, cation presence and type of saccharide (Capuano and Fogliano, 2011; Lund and Ray, 2017; Morales, 2009). HMF is practically absent in fresh food, but its content rises during storage or after severe heat treatment, making it one of the most used markers of thermal treatment in foods (De Andrade et al., 2016; Francisquini et al., 2018). Different studies have found carcinogenic and

mutagenic activity of HMF both *in vitro* and in animal assays (Bruce et al., 1993; Kitts et al., 2012; Nishi et al., 1989; Surh et al., 1994). Additionally, it is also cytotoxic and irritating to eyes, upper respiratory tract, skin and mucous membranes (Anese and Suman, 2013; Capuano and Fogliano, 2011; Matsushita et al., 2012; Morales, 2009; National Toxicology Program, 2010; Shapla et al., 2018). Furthermore, HMF can be converted in 5-sulfoxymethylfurfural (SMF) by species that express sulfotransferase, such as humans and some animals, increasing its mutagenic potential through the capacity of forming DNA-SMF adducts (Capuano and Fogliano, 2011; Fels-klerx et al., 2014; Pastoriza de la Cueva et al., 2016). However, other studies have detected a wide range positive effects of HMF on human health including antioxidant, anti-allergen, anti-sickling and anti-inflammatory capacities (Shapla et al., 2018). Even though toxicological studies are not conclusive, the potential risks associated with dietary intake of HMF makes essential to carry out studies on the distribution of this contaminant in foods (De Andrade et al., 2016).

Previous studies have analyzed the occurrence of HMF in “*dulce de leche*” by means of the traditional spectrophotometric analysis elaborated by Keeney and Bassette (1959) for dairy products (Francisquini et al., 2016, 2018; Pavlovic et al., 1994). Keeney and Bassette (1959)

* Corresponding author at: Universidad Finis Terrae, Santiago, Chile.

E-mail address: mmariotti@uft.cl (M.S. Mariotti-Celis).

proposed the differentiation between free HMF and total HMF (free HMF plus potential HMF derived from other MR intermediates). However this technique lacks specificity to HMF, generating highly variable results due to the colorimetric response of compounds other than HMF to the reactant (TBA), leading to 70 % of the measured compounds to be interferents (Albalá-Hurtado et al., 1997; Chávez-Servin et al., 2005; Morales, 2009).

On the other hand, liquid chromatography has proven to be an accurate and reliable technique for the measurement of furanic compounds in foods, including specific methods developed for determination of free and total HMF in dairy products (Chávez-Servin et al., 2005; Morales, 2009).

In this sense, the evaluation of the performance development parameters of an analytical method is needed to ensure that the reported contents of a specific quantified compound are real. An alternative widely used to accomplish this purpose is the in-house validation of the methodology. This systematic process considers the determination of different statistic parameters which allow to assess the precision and accuracy of the determinations.

Regarding to this issue, most of the HMF chromatographic methodologies have been developed and validated for powder milk and similar food matrixes which contain lower level of saccharose than “dulce de leche”. Although the use of these methods for HMF quantification in “dulce de leche” is of course a more specific alternative compared to spectrophotometric methods, the differences in the chemical composition of analysed matrixes could affect the accuracy of the determinations. Therefore, the development of specific analytical methodologies for this food matrix is mandatory to obtain reliable results.

The aim of this work was to conduct an in-house validation study of the determination of total and free HMF contents on “dulce de leche” by a HPLC-DAD method and establish their contents on commercially available “dulce de leche” on Chilean market.

2. Materials and methods

Free and total HMF contents were measured by Chávez-Servin et al. (2005) method with slight modifications. The procedure was as follows.

2.1. Chemical and materials

Analytical grade reagent standards and solvents were used in chemical analyses. HMF standard ($\geq 99.0\%$), oxalic acid dihydrate ($\geq 99.0\%$), trichloroacetic acid (TCA) ($\geq 99.5\%$) and solvents (distilled water and acetonitrile ($\geq 99.9\%$)) were purchased from Millipore Sigma (Burlington, MA, USA).

2.2. Samples

For the study three “dulce de leche” products produced in 2018 were analyzed. The products corresponded to the traditional formulation of the three mayor Chilean manufacturers (Oficina de Estudios y Políticas Agrarias Ministerio de Agricultura Gobierno de Chile, 2019). Three samples of each product produced in 2019 were acquired from different supermarkets in Chile. Each sample was analyzed in triplicate.

2.3. Instrument and chromatographic conditions

The HPLC-DAD system corresponded to Thermo Scientific Dionex Ultimate 3000 (Massachusetts, USA) equipped with a reverse phase Acclaim™ 120 C18 column ($5\ \mu\text{m}\ 120\ \text{\AA}\ 4.6 \times 150\ \text{mm}$).

Separation was performed isocratically at $30\ ^\circ\text{C}$ using a mixture of acetonitrile-water (4.5:95.5, v/v) as mobile phase and a flow rate of 1 mL/min. Injection volume was of 20 μL . Detection was made at 284 nm.

2.4. Samples preparation

Total HMF content: 0.8 g of “dulce de leche” was mixed with 4 mL of 0.2 N oxalic acid (freshly prepared) in sealed tubes. The tube was heated in a bath water system at $100\ ^\circ\text{C}$ for 25 min, and then left to cool at room temperature. 1.5 mL of 40 % (w/v) TCA solution was added and the mixture was stirred thoroughly for 2 min. It was then centrifugated at 4000 rpm for 15 min. The supernatant was passed through a paper filter and collected in a 10 mL volumetric flask. 4 mL of 4% (w/v) TCA was added to the solid residue, this was then mixed thoroughly for 3 min and centrifugated again. The supernatant was filtered and added to the volumetric flask, and the solid phase was discarded. The solution in the volumetric flask was made up to 10 mL with 4% (w/v) TCA. The mixture was filtered through a 0.22 μm nylon filter before HPLC analysis. 100 μL of the obtained solution were poured on a 2 mL HPLC vial, and 1.4 mL of 4% (w/v) TCA were added. After that, the samples were injected.

Free HMF content: the samples were prepared as described above but the water bath heating was omitted. The last dilution was omitted too, solution obtained after filtrating through 0.22 μm nylon filter was directly injected in the HPLC.

Each sample was injected by triplicate in the HPLC.

Free and total HMF were calculated with the following equations:

$$\text{Free HMF} \left[\frac{\mu\text{g}}{\text{kg}} \right] = \frac{\text{HPLC measured HMF} \left[\frac{\mu\text{g}}{\text{L}} \right]}{\frac{\text{“dulce de leche” sample [kg]}}{0.01 [\text{L}]}}$$

$$\text{Total HMF} \left[\frac{\mu\text{g}}{\text{kg}} \right] = \frac{\text{HPLC measured HMF} \left[\frac{\mu\text{g}}{\text{L}} \right]}{\frac{\text{“dulce de leche” sample [kg]}}{0.01 [\text{L}]}} * \frac{1.5 [\text{mL}]}{0.1 [\text{mL}]}$$

2.5. Validation study

The validation method evaluated selectivity, linearity, detection limit (LOD), quantification limit (LOQ), precision and accuracy (De Andrade et al., 2016).

2.5.1. Selectivity

HMF standard solution (500 $\mu\text{g}/\text{L}$), free and total HMF from samples prepared as described above were used to evaluate the selectivity. This parameter was analyzed by comparison of retention times and HMF peaks in the chromatograms of the standard solution and food samples.

2.5.2. Calibration and linearity

Calibration curve was constructed in a range from 50–5000 $\mu\text{g}/\text{L}$ from a stock solution of HMF (1000 mg/L). Three standard solutions were prepared for each point of the calibration curve and these were injected in triplicate. Linearity of the method was evaluated by applying a linear regression analysis at 95 % confidence level. The significance of the calibration curve equation coefficients was tested by *t*-test at 95 % confidence level (De Andrade et al., 2016). All statistical analyses were carried out using the statistical software Statgraphics Centurion XVI, version 16.103, software (StatPoint Inc., Warrenton, VA, USA).

2.5.3. Detection and quantification limits

Limits of detection (LOD) and quantification (LOQ) were calculated based on $(3 \times \text{SD})/m$ and $(10 \times \text{SD})/m$ respectively, where *m* is the calibration curve slope and SD is the standard deviation on the intercept of the same (De Andrade et al., 2016).

2.5.4. Precision

Precision was evaluated by repeatability, intermediate and by Hor-Rat value (AOAC International, 2012). In repeatability tests, three samples of each method (free and total HMF contents) were prepared as described above and analyzed on the same day. Relative standard deviations (RSDs) were calculated from the data and used as a repeatability

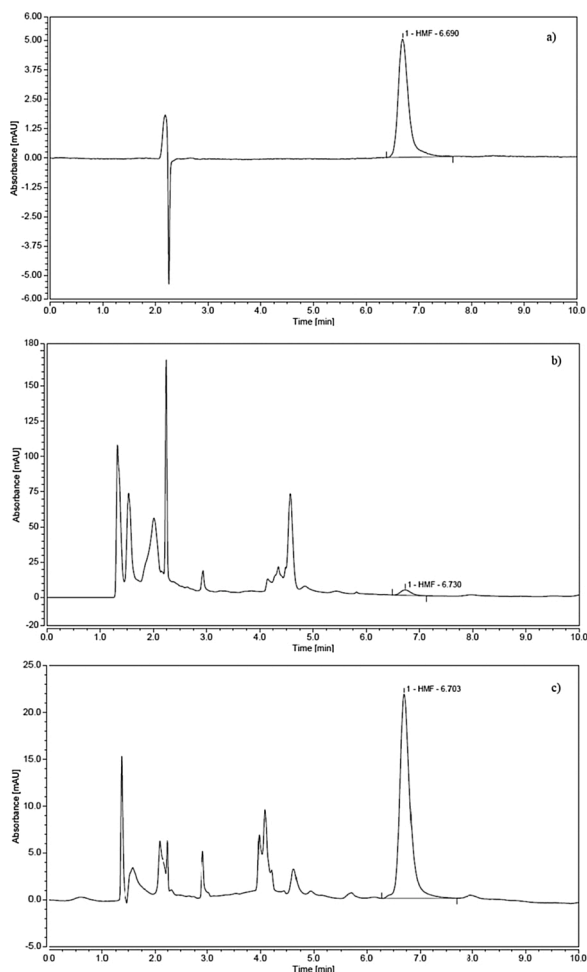


Fig. 1. Chromatograms of a) HMF standard solution (500 µg/L), b) free HMF on “dulce de leche”, c) total HMF on “dulce de leche”.

estimate. For intermediate precision test, the same procedure used on repeatability tests was carried out on five consecutive days. RSDs data was calculated and used as intermediate precision estimates. HorRat values were obtained using an analysis of variance (ANOVA) at 95 % confidence level, to verify the suitability of intermediate precision estimates for the method.

2.5.5. Accuracy

The accuracy was assessed through recovery tests. Sample solutions for free and total HMF were prepared as above and spiked with HMF standard solution, 1.4 g/L for free HMF and 0.093 g/L for total HMF samples. Analyses were performed in triplicate and recovery percentages (%) were calculated as the *amount of HMF recovered/amount of HMF added* x 100, this value was used as accuracy estimates.

2.6. Application in commercial products

To verify the applicability of the developed method the free and total HMF contents on different commercial “dulce de leche” samples found in Chilean markets were determined. Each food sample was analyzed in triplicate, and HMF content data were expressed as means and standard deviations.

Table 1

Precision (repeatability and intermediate precision) and accuracy (recovery) tests results and intralaboratory HorRat values.

Precision and accuracy parameters	Free HMF	Total HMF
Repeatability [RSD%]	4.12	1.99
Intermediate precision [RSD%]	5.98	3.62
HorRat _(r)	1.00	0.91
Recovery [%]	89.04	91.89

AOAC (2012) expected values: repeatability [RSD%] = ≤11 (≥ 1 mg/kg), ≤7.3 (≥ 10 mg/kg), ≤5.3 (≥ 100 mg/kg); predicted relative standard deviation for intralaboratory intermediate precision [PRSDr%] = 8 (≥ 1 mg/kg), 5,5 (≥ 10 mg/kg), 4 (≥ 100 mg/kg); recovery [%] = 80–110 (≥ 1 mg/kg), 90–107 (≥ 100 mg/kg).

3. Results and discussion

3.1. Validation of the proposed method

3.1.1. Selectivity

The selectivity allows to verify if the method is selective to the analyte of interest in complex samples containing other substances that can interfere in the analysis (Rao, 2018).

To evaluate the selectivity of the proposed method, the retention times of HMF in chromatograms of standards and samples were compared. Similar retention times were observed in the chromatograms (Fig. 1). Other peaks of unknown compounds can be observed on the chromatogram, but as none co-elute with the HMF peaks, they do not interfere with the analysis. Therefore, the proposed method presents an adequate selectivity for free and total HMF analysis on “dulce de leche”.

3.1.2. Linearity, LOD and LOQ

Under the chromatographic conditions tested, a linear relationship was verified on the calibration curves constructed with aqueous solutions of standard HMF in the range 50–5000 µg/L. Linear regression analysis probed an acceptable linearity ($R^2 = 0.999$), as did the high $F_{reg} = 138,900.76$ ($p=0.000$) at a 95 % confidence level. The significance of the analytical curve coefficients (intercept and slope) determined by t -test showed that both coefficients must be inserted in the model with 95 % confidence level, $p = 0.014$ and $p = 0.000$ respectively. Thus, it was proved that in the content range studied the analytical curve presents linearity and can be described by the following equation:

$$HMF \left[\frac{\mu g}{L} \right] = 376.34 \left[\frac{\mu g}{L * mAU * min} \right] * area [mAU * min] + 17.822 \left[\frac{\mu g}{L} \right]$$

Detection (LOD) and quantification (LOQ) limits were 19.51 µg/L and 65.05 µg/L. Limit of detection was lower than the obtained by Chávez-Servin et al. (2005) (25.05 µg/L) and Morales et al., 2000 (25.22 µg/L) on milks, and (De Andrade et al., 2016) (90.00 µg/L) on syrups. The obtained LOQ was higher than the obtained by Chávez-Servin et al. (2005) (39.24 µg/L) on milk and lower than De Andrade et al. (2016) (260.00 µg/L) result on syrups demonstrating the suitability of the method for determining low contents of HMF in “dulce de leche”.

3.1.3. Precision and accuracy

Precision results were calculated in terms of repeatability and intermediate precision estimates evaluated by corresponding RSD values. Results for both tests were appropriate, since these were smaller than the recommended by the AOAC International (2012), though higher repeatability and intermediate precision values were obtained compared to those obtained by Chávez-Servin et al. (2005) (1.05 % and 2.68 %, respectively) on milk. In comparison to De Andrade et al. (2016) results on syrups, the repeatability (0.57–1.95 %) was lower than those obtained in this study, while the intermediate precision result (0.83–6.43 %) were higher depending on the type of syrups Likewise, accuracy was acceptable according to the recovery test results and the AOAC International (2012) guidelines. Recoveries were lower than

Table 2

Free and total HMF concentrations in Chilean “dulce de leche”.

Sample	Free HMF [µg/g]	Total HMF [µg/g]
A1	3.01 ± 0.07	457.40 ± 9.79
A2	2.33 ± 0.05	448.19 ± 21.32
A3	2.56 ± 0.11	464.98 ± 4.17
B1	6.86 ± 0.13	314.61 ± 27.25
B2	10.31 ± 0.42	390.09 ± 1.39
B3	9.94 ± 0.21	354.64 ± 7.59
C1	3.94 ± 0.13	395.11 ± 15.49
C2	4.89 ± 0.17	405.11 ± 14.70
C3	4.47 ± 0.04	382.33 ± 6.98

those reported by Chávez-Servin et al. (2005) (96.32 %) on milk and De Andrade et al. (2016) (100–105 %) on syrups. Acceptable HorRat_(r) for intralaboratory studies were obtained, considering the acceptable values range from 0.3 to 1.3 according to AOAC International (2012). Table 1 presents the precision and accuracy tests results and the HorRat values. These results suggest that the chromatographic method is adequate in terms of precision and accuracy for the determination of free and total HMF contents in “dulce de leche”.

3.2. Application in commercial samples

The method was applied on three commercial products, three samples of each product were purchased and analyzed in triplicate. Samples were coded with letters for the manufacturer and a number for the sample. Free and total HMF contents were expressed as mean and standard deviations (Table 2).

Free HMF contents ranges from 2.33 to 10.31 mg/kg, whereas total HMF contents vary from 314.61–464.98 mg/kg. Difference in HMF contents among the samples can be explained considering factors that benefit the formation of HMF in food matrixes, such as thermal processing, composition and storage times, along with variability due to batch processes in which this products is frequently manufactured (Capuano and Fogliano, 2011; Lund and Ray, 2017; McSweeney and Fox, 2009; Morales, 2009). Among from others, there is also possibility of “dulce de leche” raw materials such as milk contain HMF because they are submitted to high temperature processing (UHT) during their elaboration.

HMF contents observed were significantly lower than those reported in literature carried out using the Keeney and Bassette method (0.11 and 16.44–118.93 g/kg for free HMF; 1.44 and 231.83–688.76 g/kg for total HMF) (Francisquini et al., 2016, 2018). This is in concordance with the low specificity of the method and the interference that other compounds may cause during the colorimetric procedure (Chávez-Servin et al., 2005; Morales, 2009; Morales and Jiménez-Pérez, 1998).

4. Conclusions

A quick method for determination of free and total HMF contents in “dulce de leche” was successfully developed and validated. The method showed good analytical performance (good selectivity, linearity, precision, accuracy and low limits of detection and quantification).

The content of HMF in commercial “dulce de leche” from Chile was reported as follows: 2.33–10.31 mg/kg for free HMF and 314.61–464.98 mg/kg total for HMF. These results are significantly lower than those reported in literature by spectrophotometric measurements, suggesting the presence of many interferent compounds in this complex dairy matrix when colorimetric methods are used. The chromatographic method proposed offers substantial advantages by being a quick and direct method, but also for preventing the interference of other compounds.

Although reported studies are not conclusive on whether HMF exposure represents a potential health risk for humans, a modified

theoretical added maximum daily intake (mTAMDI) for HMF was estimated on 1600 µg/person/day (Morales et al., 2009). Considering the recommended portion of “dulce de leche” as 30 g, the free HMF intake results in a significant percentage of the mTAMDI. In this sense, further studies should be conducted on this spread like product to mitigate the formation of HMF and prevent the potential health risks associated with this compound.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgements

Initiative funded by the Initiation R&D Program, year 2016, code L2-16-02, Universidad Tecnológica Metropolitana, Chile. FONDECYT grants N°1190080 and N° 1150146, ANID, Chile.

References

- Albalá-Hurtado, S., Veciana-Nogués, 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. <https://doi.org/10.1021/jf960770n>.
- Anese, M., Suman, M., 2013. Mitigation strategies of furan and 5-hydroxymethylfurfural in food. *Food Res. Int.* 51 (1), 257–264. <https://doi.org/10.1016/j.foodres.2012.12.024>.
- AOAC International, 2012. Guidelines for standard method performance requirements (Appendix F). *AOAC Off. Methods Analysis* 1–17.
- Bruce, W.R., Archer, M.C., Corpet, D.E., Medline, A., Minkin, S., Stamp, D., Zhang, X.M., 1993. Diet, aberrant crypt foci and colorectal cancer. *Mutat. Res.* 290 (1), 111–118. [https://doi.org/10.1016/0027-5107\(93\)90038-H](https://doi.org/10.1016/0027-5107(93)90038-H).
- Capuano, E., Fogliano, V., 2011. Acrylamide and 5-hydroxymethylfurfural (HMF): a review on metabolism, toxicity, occurrence in food and mitigation strategies. *Lwt - Food Sci. Technol.* 44 (4), 793–810. <https://doi.org/10.1016/j.lwt.2010.11.002>.
- Chávez-Servin, J.L., Castellote, A.I., López-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. <https://doi.org/10.1016/j.chroma.2005.04.046>.
- De Andrade, J.K., Komatsu, E., Perreault, H., Torres, Y.R., Da Rosa, M.R., Felsner, M.L., 2016. In house validation from direct determination of 5-hydroxymethyl-2-furfural (HMF) in Brazilian corn and cane syrups samples by HPLC-UV. *Food Chem.* 190, 481–486. <https://doi.org/10.1016/j.foodchem.2015.05.131>.
- Fels-klerx, Van Der, H.J., Capuano, E., Nguyen, H.T., Mogol, B.A., Kocada, T., Göncüoğlu, G., Gökmen, V., 2014. Acrylamide and 5-hydroxymethylfurfural formation during baking of biscuits: NaCl and temperature – time pro fit le effects and kinetics. *Food Res. Int.* 57, 210–217. <https://doi.org/10.1016/j.foodres.2014.01.039>.
- Francisquini, J.d'Almeida, de Oliveira, L.N., Pereira, J.P.F., Stephani, R., Perrone, I.T., da Silva, P.H.F., 2016. Avaliação da intensidade da reação de Maillard, de atributos físico-químicos e análise de textura em doce de leite. *Rev. Ceres* 63 (5), 589–596. <https://doi.org/10.1590/0034-737X201663050001>.
- Francisquini, J.d'Almeida, Neves, L.N., Torres, J.K., Carvalho, A.F., Perrone, I.T., da Silva, P.H.F., 2018. Physico-chemical and compositional analyses and 5-hydroxymethylfurfural concentration as indicators of thermal treatment intensity in experimental dulce de leche. *J. Dairy Res.* 85 (04), 476–481. <https://doi.org/10.1017/S0022029918000353>.
- 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. [https://doi.org/10.3168/jds.S0022-0302\(59\)90678-2](https://doi.org/10.3168/jds.S0022-0302(59)90678-2).
- Kitts, D.D., Chen, X.M., Jing, H., 2012. Demonstration of antioxidant and anti-inflammatory bioactivities from sugar-amino acid maillard reaction products. *J. Agric. Food Chem.* 60 (27), 6718–6727. <https://doi.org/10.1021/jf2044636>.
- Lund, M.N., Ray, C.A., 2017. Control of maillard reactions in foods: strategies and chemical mechanisms. *J. Agric. Food Chem.* 65, 4537–4552. <https://doi.org/10.1021/acs.jafc.7b00882>.
- 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. <https://doi.org/10.1111/j.1471-0307.2005.00202.x>.
- Matsushita, K., Ishii, Y., Kijima, A., Jin, M., Takasu, S., Kuroda, K., 2012. Reporter gene mutation in the livers of gpt delta mice treated with 5- (hydroxymethyl) -2-furfural. a contaminant of various foods 37 (5), 1077–1082.
- McSweeney, P.L.H., Fox, P.F., 2009. *Advanced dairy chemistry*. Adv. Dairy Chem. 3 <https://doi.org/10.1007/978-0-387-84865-5>.
- Morales, F.J., 2009. Hydroxymethylfurfural (HMF) and related compounds. *Process-Induced Food Toxicants: Occurrence, Formation, Mitigation, and Health Risks*, pp. 135–174.
- Morales, F.J., Jiménez-Pérez, S., 1998. Study of hydroxymethylfurfural formation from acid degradation of the amadori product in milk-resembling systems. *J. Agric. Food Chem.* 46 (10), 3885–3890. <https://doi.org/10.1021/jf980299t>.

- Morales, F.J., Romero, C., Jiménez-Pérez, S., 2000. Characterization of industrial processed milk by analysis of heat-induced changes. *Int. J. Food Sci. Technol.* 35 (2), 193–200. <https://doi.org/10.1046/j.1365-2621.2000.00334.x>.
- Morales, F.J., Martín, S., Açar, O.C., Arribas-Lorenzo, G., Gökmen, 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>.
- National Toxicology Program, 2010. *Toxicology and Carcinogenesis Studies of 5-Hydroxymethyl-2-Furfural*, 67. National Institutes of Health, p. 184.
- Nishi, Y., Miyakawa, Y., Kato, K., 1989. Chromosome aberrations induced by pyrolysates of carbohydrates in Chinese hamster V79 cells. *Mutat. Res. Lett.* 227 (2), 117–123. [https://doi.org/10.1016/0165-7992\(89\)90007-9](https://doi.org/10.1016/0165-7992(89)90007-9).
- Oficina de Estudios y Políticas Agrarias Ministerio de Agricultura Gobierno de Chile, 2019. *Boletín De La Leche*. Retrieved from. www.odepa.gob.cl.
- Pastoriza de la Cueva, S., Álvarez, J., Végvári, Á., Montilla-Gómez, J., Cruz-Lopez, O., Delgado-Andrade, C., Rufián-Henares, J.A., 2016. Relationship between HMF intake and SMF formation in vivo: an animal and human study. *Mol. Nutr. Food Res.* 61 (3), 1–24. <https://doi.org/10.1002/mnfr.201600773>.
- Pavlovic, S., Santos, R.C., Glória, M.B.A., 1994. Maillard reaction during the processing of 'Doce de leite'. *J. Sci. Food Agric.* 66 (2), 129–132. <https://doi.org/10.1002/jsfa.2740660204>.
- 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. <https://doi.org/10.1016/B978-0-12-384947-2.00700-5>.
- 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>.
- Shapla, U.M., Solayman, M., Alam, N., Khalil, M.I., Gan, S.H., 2018. 5-Hydroxymethylfurfural (HMF) levels in honey and other food products: effects on bees and human health. *Chem. Cent. J.* 12 (1), 1–18. <https://doi.org/10.1186/s13065-018-0408-3>.
- Surh, Y.J., Liem, A., Miller, J.A., Tannenbaum, S.R., 1994. 5-sulfoxymethylfurfural As a possible ultimate mutagenic and carcinogenic metabolite of the maillard reaction product, 5-Hydroxymethylfurfural. *Carcinogenesis* 15 (10), 2375–2377. <https://doi.org/10.1093/carcin/15.10.2375>.