Article

DETERMINATION OF MAILLARD REACTION MARKERS IN *DOCE DE LEITE* BY HPLC-PDA

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This study was carried out as a preliminary mapping of Maillard reaction (MR) compounds in *doce de leite* (DL), as possible indicators for the intermediate stage and chemical pathways in industrial processing. For that, the objective of the manuscript was to developed and validated an analytical method to simultaneously analyze four furfural compounds in DL: 5-hydroxymethylfurfural, 2-furaldehyde (F), 2-furyl-methyl ketone (FMC) and 5-methyl-2-furaldehyde (MF) by high performance liquid chromatography-photodiode-array detector (HPLC-PDA). The method fulfilled the acceptance criteria: selectivity, linearity (0.16-5 μg mL^{-1} for HMF, and 0.06-2 μg mL^{-1} for F, FMC and MF, $R^2 > 0.9974$), precision (CV = 1.33 to 2.12% for HMF; 1.57 to 4.34% for F; 0.84 to 1.40% for FMC and 1.40 to 4.18% for MF), accuracy (94.40 to 102.25% for HMF; 92.91 to 108.15% for F; 90.13 to 108.48% for FMC and 93.31 to 107.70% for MF), limit of detection of 0.041 μg mL^{-1} for HMF; 0.030 μg mL^{-1} for F; 0.042 μg mL^{-1} for FMC and MF, and limit of quantification of 0.125 μg mL^{-1} for HMF and F, 0.066 μg mL^{-1} for FMC and 0.1280 μg mL^{-1} for MF. Thus, it was concluded that an analytical method was developed and validated for quantifying HMF, F, FMC and MF in DL.

Keywords: validation; sugars; methodology; chromatography; furfurals.

INTRODUCTION

Monitoring the Maillard's reaction (MR) is important in several aspects for dairy production. It serves as a reference to control some nutritional changes, such as loss of available proteins, amino acids, and peptides. In addition, it can induce sensory changes in color, flavor, and texture. As there are dairy products in which the MR is not desirable, it is relevant to establish methods to measure its progress.[1-3]

As the MR is characterized as a cascade of reactions, there is also a huge set of possible marker products to monitor it. MR is commonly divided into three main steps, the initial one being the glycation of a free amino group with a reducing sugar to give an unstable *N*-glucosamine product that undergoes a rearrangement to more stable molecules such as Amadori products. The intermediate stage is profoundly dependent on pH conditions and can develop a large group of products. In summary, if the pH is greater than 7, the dominant products will be dicarbonyls and aldehydes, but if the pH is less than or equal to 7, it will generate furfural, in which small molecules will be responsible for releasing the aroma. These intermediate products go through a final step, in which dehydration and fragmentation give rise to a very reactive molecule that condense with other free amino groups by polymerization into melanoidins, that are brown compounds of high molecular weight responsible for browning.[4-6]

Consequently, to monitor the MR it is possible to focus on a specific molecule or family of molecules produced along it. Among the possibilities, furfurals are one of the largest groups of MR products which represents a good choice for monitoring MR in slight acidic foods.[7,8]

The extension of the MR can be monitored by the appearance of compounds, which makes it possible to evaluate the intensity of the thermal processing and the nutritional alterations related to it. These compounds include furosine, carboxymethyllysine and furfural derivatives.[9-11] Currently, people start consuming foods rich in MR at an earlier age. Advanced glycation end products can have a pathogenic effect when they reach high amounts in tissues and are associated with the development of chronic diseases. As for furfurals, HMF was considered mutagenic and genotoxic and F is toxic and can induce liver and skin cancer, considering high doses of these compounds.[12] The European Community and Codex Alimentarius prescribe an acceptable daily dose (ADI) of 0.5 mg kg^{-1} for furfurals.

Four compounds derived from furfural in processed foods are reported in the literature: 5-hydroxymethylfurfural (HMF), 2-furaldehyde (F), 2-furyl-methyl ketone (FMC) and 5-methyl-2-furaldehyde (MF), the structures of which are shown in Figure 1. F, FMC and MF are less common and can be formed through interconversion pathways from HMF in more advanced stages of MR.[13-15]

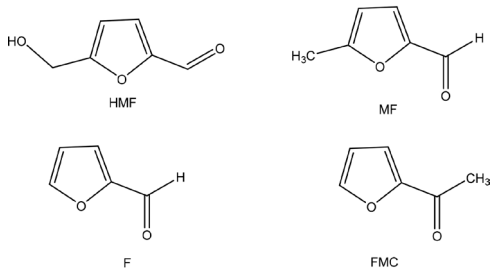

Figure 1. *Chemical structures of intermediate of Maillard reactions: 5-hydroxymethylfurfural (HMF), 5-methyl-2-furaldehyde (MF), 2-furaldehyde (F), 2-furyl-methyl ketone (FMC)*

Doce de leite (DL) is one of the dairy products that suffers the most from the MR, due to the severe heat treatment it undergoes during manufacture. DL is a dairy product originally from South America and is a unique culinary item in the countries that consume it due to its incomparable color, flavor and texture.[16,17] It is a food of great sensory acceptance and is defined as a product obtained from

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milk or reconstituted milk and added sucrose (partially replaced or not by monosaccharides and/or other disaccharides), with or without the addition of other food substances, obtained by concentration and the action of heat under normal conditions or reduced pressure.[18]

Brazil is a major producer of DL and, according to data from the Annual Industrial Survey (PIA) released by the Brazilian Institute of Geography and Statistics (IBGE), the production in Brazil is 345.19 million reais, which is equivalent to 0.82% of what is produced by the dairy sector in the country.[19,20] Recently, a study carried out by a Brazilian research group mapped all the factories in the country, from small, medium and large products, totaling 350 factories. Due to this plurality, the country has products with different characteristics, such as tone (for example, lighter in the south and darker in the central regions), texture and flavor.[21]

MR takes place during the thermal and evaporation production steps and determines not just the main flavor and color features, but also DL microstructure, hence its texture and rheology.[22,23] Only few research groups are dedicated to study the chemistry and technology of DL, mainly at the interface of academia with the industrial sector accounting for only few published scientific documents.

This study presents the development and validation of a methodology for extraction and simultaneously quantification of HMF, F, FMC and MF in DL by high performance liquid chromatography - photodiode-array detector (HPLC-PDA), enabling the evaluation of MR progress in these products.

EXPERIMENTAL

Chemicals

The analytical standard of HMF, F, FMC, MF and trichloroacetic acid (TCA) were obtained from Sigma-Aldrich (Burlington, USA), and acetonitrile from Merck (Burlington, USA). Water was purified using a Milli-Q system from Millipore (Burlington, USA).

Production of *doce de leite*

The DL was produced in a laboratory scale using a system that simulates industrial evaporation devices, developed, and implemented by the research group in which the project was carried out. The system consists of a Thermomix® TM5 benchtop evaporator (Vorwerk, Wuppertal, Germany) coupled to a load processor (Ramuza IDR 7.500, Santana de Parnaíba, Brazil) with 1 g precision, a PT-100 temperature sensor and a balance to monitor the mass loss of water. The time required for each evaporation was an average of 103 min and the temperature used was the "Varoma" option (temperature of the Thermomix® TM5 benchtop processor), which corresponds to 120 °C.[16]

Since the Brazilian regulation determines sucrose addition to a maximum of 30 kg 100 L^{-1} of milk,[18] 1500 g of whole milk, 300 g of sucrose (20% of the milk mass) and 1 g of sodium bicarbonate, were used to prepare the DL recipe, all the ingredients were obtained in the local stores in Juiz de Fora.[16]

Method development and validation

The development of the method for this analysis was adapted from the work of the authors Chávez-Servín *et al.*,[14] and Lund *et al.*,[8] who also investigated the presence of the analytes HMF, F, FMC and MF in dairy samples. Different concentrations of protein precipitating agents were tested to determine the best method, *i.e.*, the one that includes sample treatment without influencing the concentration of the analyte of interest. An analytical method was developed and validated for the determination of HMF, F, FMC and MF in DL. For this, the parameters of selectivity, linearity, precision (repeatability and intermediate precision), accuracy, limit of detection and quantification and recovery were evaluated.[24]

Chromatographic conditions

The separation and quantification of the analytes of interest were carried out using high-performance liquid chromatography (HPLC) system Waters, model 1252, detector UV-Vis (PAD - photodiode array detector) with a binary pump. The analysis was carried out in gradient elution mode, with a mobile phase composed of water and acetonitrile (ACN) in an initial ratio of (95.5:4.5) H_2O:ACN; 5 min/(80:20) H_2O:ACN; 9 min/(95.5:4.5) H_2O:ACN and 10 min/(95.5:4.5) H_2O:ACN, maintaining a constant flow rate of 1 mL min^{-1}. The chromatographic separation employed a Waters Spherisorb column (150 mm × 4.6 mm; particle size of 3 μm; ODS2), kept at 30 °C. The injection volume was 20 μL and HMF, F, FMC and MF were detected at 284 nm.

Selectivity

Selectivity was analyzed by comparing the equivalence of the retention time for HMF, F, MFC and MF as external standard and by the increasement of the peak area in fortified samples. Selectivity was also evaluated for the solutions used in sample preparation and mobile phase and was proven by the absence of analytical response at the retention time of HMF, F, FMC and MF.

Linear range

The analytical curves were constructed using five points, in triplicate. HMF calibration standard solutions were prepared from the HMF stock solution and diluted with a trichloroacetic acid solution at 4% m/v, to a concentration range from 0.15 to 5.00 μg mL^{-1} for HMF and 0.06 to 2.0 μg mL^{-1} for F, FMC and MF.

Limits of detection (LOD) and quantification (LOQ)

The LOD and LOQ values were calculated using the parameters of the analytical curves, such as the slope of the calibration curve (IC) and the standard deviation of the intercept with the Y-axis (σ), according to the Equations 1 and 2.[24]

$$LOD = (3.3\ \sigma)/IC \quad (1)$$

$$LOQ = (10\ \sigma)/IC \quad (2)$$

Precision and accuracy

The precision was expressed through the repeatability to evaluate the samples under the same operating conditions. For the study of repeatability, nine replicates were prepared using standards of HMF, F, MFC and MF. For HMF, concentrations of 0.40, 1.75, and 3.50 μg mL^{-1} were used, and for F, MFC and MF concentrations of 0.16, 0.70 and 1.4 μg mL^{-1} were used.

Analysis and recovery rate (percentage) of *doce de leite*

The recovery was expressed as the percentage ratio of the analyte of known concentration added to the sample and as the corresponding theoretical concentration, according to Equation 3. Aliquots of HMF, F, FMC and MF stock solutions (50 μg mL^{-1}) were added to 1 g of

DL samples to a final solution of 1% (m/v). Ultra-pure water was subsequently added up to a volume of 1.2 mL and mixed by vortexing for 20 s. Following that, 300 µL of 55% (m/v) TCA was added and mixed by vortexing, and centrifuged (2680 g, 10 min). After that, 0.5 mL of the supernatant was mixed by vortexing with 100 µL of ultra-pure water and 300 µL of 55% (m/v) TCA. The samples were centrifuged (2680 g, 20 min) and the supernatant were filtered (0.45 µm), for posterior HPLC-PDA analysis.

$$\text{Recovery (\%)} = \frac{\text{C(sample added)} - \text{C(sample)} \times 100}{\text{C(theoretical)}} \quad (3)$$

RESULTS AND DISCUSSION

Method validation

The Figure 2a shows a chromatogram obtained for simultaneous analysis of the four MR markers analyzed. The peaks were well defined and separated, which indicates that the elution of the mobile phase was efficient in obtaining a good resolution of the chromatogram.

Selectivity was the first parameter evaluated during method validation. Using the chromatograms of the DL MR standards (Figure 2a), control sample (Figure 2b) and diluent solutions (Figure 2c), it was possible to ensure that the response peak was exclusively for the compound of interest. In the control sample, the presence of the HMF marker was observed, so a recovery study was applied to assess the matrix effect, which should be considered whenever the analytical method developed aims to quantify components in a complex matrix, such as food.

Linearity was determined by injection of different concentrations from a standard solution and examination of the linear regression of the responses, which showed linear correlation coefficient R^2 (Table 1) and quadratic fit using analysis of variance (ANOVA). The homoscedasticity was demonstrated by statistical test, Cochran's C test (5% significance level) and the verification of the absence of outliers was done by the Grubbs test.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to Equations 1 and 2, determined by the method based on the response of three replicates of an analytical curve. The obtained values for both LOD and LOQ were low, ensuring that the method is not only capable of quantifying HMF, F, FMC and MF, but also to detect traces of this marker in the products under study (Table 1).

Precision represents a dispersion of results between independent assays, whether from the same sample, similar samples or standards, under defined conditions. Precision can be expressed by estimating the relative standard deviation (RSD), methods that quantify compounds in macro quantities require an RSD of 1 to 2%. In methods for trace or impurity analysis, RSDs of up to 20% are accepted, depending on the sample complexity.[25] Accuracy was in the range of 90 to 110% associated with the precision values, shown in Table 2.

The precision data are in accordance with acceptance criteria established by Regulatory Agencies, the data showed RSD less than 5%.

Samples results and recovery rate

Following the development of the method for the simultaneous analysis of the four MR markers by HPLC-PDA, the DL samples were subjected to the preparation that precedes the analysis to verify the presence of the analytes in the matrix. It was possible identified

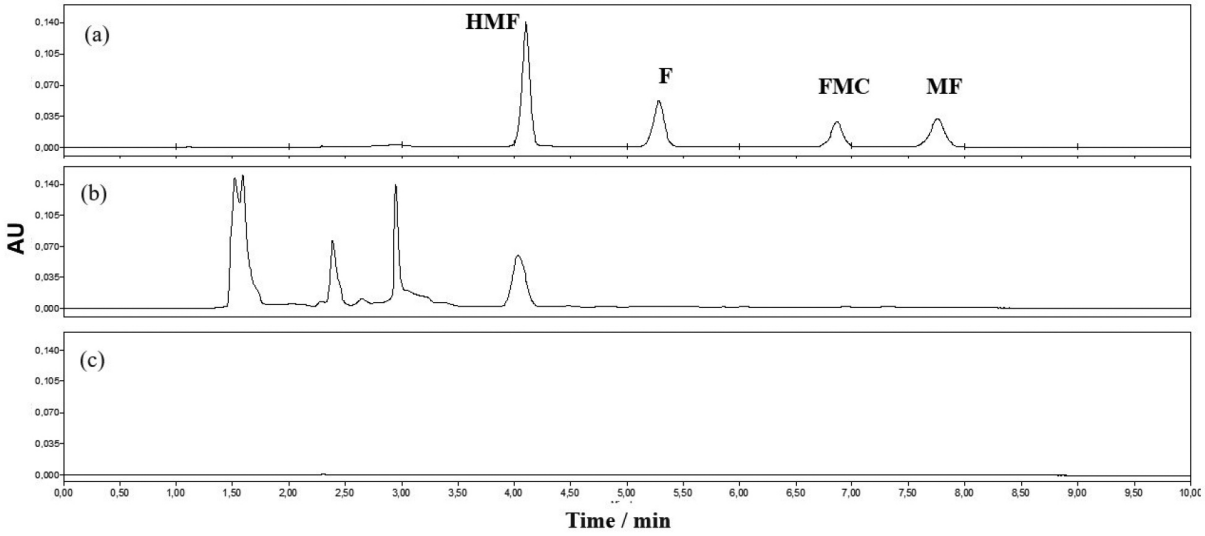

Figure 2. *HPLC-PDA chromatograms obtained for doce de leite Maillard´s reaction standard (a), control sample (b) and diluent solutions (c). HMF: 5-hydroxymethylfurfural, F: 2-furaldehyde, FMC: 2-furyl-methyl ketone, MF: 5-methyl-2-furaldehyde*

Table 1. Parameters determined from the analytical curve

Analyte linear range	Linear range / (µg mL^{-1})	Equation	R^2	LOD / (µg mL^{-1})	LOQ / (µg mL^{-1})
HMF	0.32-5.0	y = 168144x + 3562.9	0.9995	0.041	0.125
F	0.06-2.0	y = 214819x + 4015.7	0.9974	0.030	0.125
FMC	0.06-2.0	y = 125023x + 1310.5	0.9984	0.042	0.066
MF	0.06-2.0	y = 214819x + 4015.7	0.9984	0.042	0.128

R^2: coefficient of determination; LOD: limit of detection; LOQ: limit of quantification; HMF: 5-hydroxymethylfurfural; F: 2-furaldehyde; FMC: 2-furyl-methyl ketone; MF: 5-methyl-2-furaldehyde.

Table 2. Parameters determined from the precision and accuracy

Analyte	Concentration / (μg mL^{-1})	Rep. RSD / %	Rep. accuracy / %
HMF	0.40	2.12	98.09-102.84
	1.75	1.46	94.40-97.22
	3.50	1.33	95.11-97.77
F	0.16	4.34	92.91-101.57
	0.70	2.61	102.96-108.15
	1.40	1.57	100.73-103.81
FMC	0.16	1.26	107.07-108.48
	0.70	1.40	90.13-92.92
	1.40	0.84	92.83-94.48
MF	0.16	4.18	99.83-107.70
	0.70	1.70	93.39-94.91
	1.40	1.48	93.31-96.14

Rep. RSD: relative standard deviation for repeatability; Rep. accuracy: method accuracy by repeatability test; HMF: 5-hydroxymethylfurfural; F: 2-furaldehyde; FMC: 2-furyl-methyl ketone; MF: 5-methyl-2-furaldehyde.

and quantified HMF in the samples, F, FMC and MF were not detected. Other studies[8,14,26] have also failed to find FMC and MF in dairy samples. Er Demirhan *et al.*[27] determined HMF and F in foods intended for children, based on cereals and milk, and identified HMF and F in all samples, except for one in which no F was identified.

The literature reports that in the intermediate stage of MR, furfural derivatives are formed, with HMF being the most widely mentioned and quantified, followed by F. The formation of FMC and MF under MR conditions has not yet been well explored and discussed, especially for DL, but some authors[1-3] report that the appearance of these compounds may be associated with high heat loads on the product, *i.e.*, possibly a more severe heat treatment or prolonged storage time. It is known that these factors can extend the MR and could thus boost the formation of FMC and MF through conversions between the furfural derivatives already formed in the matrix.

Recovery is defined as the proportion of the amount of the substance of interest, present or added in the analytical portion of the material, that is extracted and can be quantified.[25] Exact amounts of the analytical HMF standard were added to the dairy products at three different concentrations within the linear range of the method.

The recovery rate gives us information on the percentage of the analyte that is retained in the sample or lost during the preparation method. This evaluation is necessary whenever the determination is made in complex matrices. According to Brazil,[24] a complex matrix is one that contains an indefinite number of unmonitored substances, which cannot be obtained without the presence of the analyte. Milk has components such as proteins, fats, salts and sugars that can interact with the analyte in such a way as to hinder its quantification and identification in small quantities, making it important to evaluate the recovery rate. The recovery rate was calculated according to Equation 3 and was carried out using the standard addition method, by fortifying the samples with known concentrations of 4 analytes. The recoveries ranged from 90 to 110% for all four MR compounds analyzed, indicating that the sample treatment was efficient in eliminating possible interferents, without compromising the identification of the analyte.

CONCLUSIONS

Considering the statistical results, the method developed and validated using HPLC-PDA proved to be suitable for quantifying HMF, F, FMC and MF in DL. The statistical treatment showed that the method can be considered precise, selective, linear over a wide working range and accurate with recovery greater than 90% for samples analyzed. The method can also be considered sensitive, with detection and quantification limits compatible with the analytical curve and the nature of the (DL) samples analyzed.

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