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Natalia Casas Mesa

Assessment of the influence of vanillin, vanilla extract and sodium metabisulfite on Maillard reaction in sweetened condensed milk and doce de leite

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Dissertation presented to the Graduate Program in Chemistry, Federal University of Juiz de Fora as a partial requirement to obtain the title of master's in chemistry. Concentration area: Physical Chemistry.

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I dedicate this work to my mother, godmother, and great-grandmother (*in memoriam*) who always gave me love and courage to achieve my dreams.

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RESUMO

A reação de Maillard (MR) é impulsionada pela glicação de proteínas com açúcares redutores disponíveis em diferentes matrizes alimentares, seguida por várias reações de oxidação-redução. Portanto, espera-se que moléculas com propriedades antiglicantes ou atividade antioxidante possam interferir no andamento dessa reação. A literatura relata que a vanilina, um aldeído sintetizado ou extraído das frutas de baunilha, é um aditivo com atividade antiglicante e, portanto, poderia modificar a evolução do MR. O objetivo deste estudo foi avaliar a influência na RM do extrato de baunilha (VE) e seu principal componente, a vanilina (V), em leite condensado (SCM) aquecido. O metabissulfito de sódio (SMB) foi usado como um agente retardador de RM positivo. O progresso da reação foi medido pelo perfil dos furfurais livres 5-hidroximetil-2-furaldeído (HMF), 2-furaldeído (F), 2-furil-metil cetona (FMK) e 5-metil-2-furaldeído (MF), pH e cor (Browning Index, BI). Amostras de SCM foram adicionadas com SMB, VE e V, separadamente, com concentrações de 0,05 a 1,0% (p/p) e armazenadas a 40°C por 21 dias, e comparadas com os perfis, de amostras apenas com SMB nestas concentrações e aquecidas a 121 °C/15 min. O perfil de concentração dos furfurais livres F, MF e FMK foi inferior ao limite de quantificação do método (0,125, 0,128 e 0,066 µg/mL, respectivamente), e apenas o HMF livre foi quantificável. O SCM apresentou pH de 6,3 ± 0,02, IB de 14,3 ± 0,7 e concentração de 63,2 ± 0,5 µmol HMF/kg. SMB foi adicionado a 1,0% (p/p) ao SCM, ou HMF diminuiu para 40,8 µmol HMF/kg. Observou-se também que o SCM, adicionando 0,05% e 1,0% (p/p) de SMB, mostrou apenas um leve aumento de 20,1 e 15,2 no BI após o aquecimento, respectivamente. Amostras de SCM com adição de 1,0% SMB, V e VE, atingiram 311,4 ± 47,7 µmol HMF/kg, 205,7 ± 41,0 µmol HMF/kg e 286,7 ± 97,9 µmol HMF/kg, respectivamente, e só as duas últimas amostras escureceram. Nas amostras com 0,05% e 1,0% (p/p), o BI aumentou para 35,0 e 83,0 para V e um pequeno aumento de 29,5 e 29,6 para VE, respectivamente. O SMB reduziu o pH inicial do SCM para 5,7 ± 0,1, mantendo-o constante, enquanto V e VE não alteraram o pH inicial. Testes com diferentes ácidos para obter um pH de 5,7 ± 0,1 como as amostras de SMB 1,0% (p/p) escureceram, sugerindo que o efeito retardador do RM devido ao SMB não é apenas consequência da diminuição do pH. Contrário do esperado, V favoreceu RM, apresentando um escurecimento das amostras. No entanto, o VE não favoreceu o RM, apresentando valores de HMF, pH e BI semelhantes ao controle SCM, tornando-se um aditivo natural alternativo aos aditivos artificiais.

Palavras-chave: Reação de Maillard. Furfurais. Browning index. Produtos lácteos.

ABSTRACT

The Maillard reaction (MR) is driven by the glycation of proteins with reducing sugars available in different food matrices, followed by various oxidation-reduction reactions. Therefore, it is expected that molecules with antiglycant properties or antioxidant activity could interfere with the progress of this reaction. The literature reports that vanillin, an aldehyde synthesized or extracted from two vanilla fruits is a common flavoring, with antiglycant activity and, therefore, could modify the evolution of MR. The objective of this study was to evaluate the influence of vanilla extract (VE) and its main component, vanillin (V), the RM into condensed milk (SCM) heated to force MR. Sodium metabisulfite (SMB) is used as a positive MR retarding agent. The progress of the reaction was measured by the profile of free furfurals 5-hydroxymethyl-2-furaldehyde (HMF), 2-furaldehyde (F), 2-furyl-methyl ketone (FMK), and 5-methyl-2-furaldehyde (MF), pH and color (Browning Index, BI). Samples of SCM were added with SMB, VE, and V, separately, with concentrations of 0.05 to 1.0% (w/w) and stored at 40°C for 21 days, and compared as profiles for the same parameters of samples only with SMB in these concentrations, heated at 121 °C/15 min. The concentration profile of two free furfurals F, MF, and FMK was lower than the quantification limit of the method (0.125, 0.128, and 0.066 µg/mL, respectively), and only free HMF was quantifiable. The SCM showed a pH of 6.3 ± 0.02, a BI of 14.3 ± 0.7, and a concentration of 63.2 ± 0.5 µmol HMF/kg. SMB was added at 1.0% (w/w) to the SCM, or HMF decreased to 40.8 µmol HMF/kg. It was also observed that our SCM, adding 0.05% and 1.0% (w/w) of SMB, would present only a slight increase of 20.1 and 15.2 of BI after heating, respectively. As SCM samples with the addition of 1.0% SMB, V and VE reached 311.4 ± 47.7 µmol HMF/kg, 205.7 ± 41.0 µmol HMF/kg, and 286.7 ± 97.9 µmol HMF /kg, respectively, being that the last two samples show darkening. In samples with 0.05% and 1.0% (p/p), we verified respectively an increase in BI to 35.0 and 83.0 for V and a smaller increase of 29.5 and 29.6 for VE. The SMB reduced the initial pH of the SCM to 5.7 ± 0.1, keeping it constant, while V and VE did not alter the initial pH and kept it constant. Tests using different acids to obtain a pH of 5.7 ± 0.1 as the samples of SMB 1.0% (w/w) showed darkening, suggesting that the retarding effect of RM due to SMB is not just a consequence of the diminution do pH. Contrary to what was expected, V favored RM, presenting a darkening of samples. However, VE did not favor RM, presenting HMF, pH, and BI values like the SCM control, becoming a promising natural additive and an alternative to artificial additives.

Keywords: Maillard reaction. Furfurals. Browning index. Dairy products.

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LIST OF ABREVIATIONS AND ACRONYMS

ACE	Acetic acid
AGEs	Advanced glycation end products
ASC	Ascorbic acid
BI	Browning Index
BSA	Bovine serum albumine
$CaHPO_4$	Calcium hydrogen phosphate
CAS	Sodium caseinate
DAD	Diode array detector
DL	Doce de leite
F	2-furaldehyde
FDA	Food and Drug Administration
FMK	2-furyl-methyl ketone
GAL	Gallic acid
HCl	Hydrochloric acid
HMF	5-hydroxymethyl-2-furaldehyde
HPLC	High performance liquid chromatography
LAC	Lactic acid
MF	5-methyl-2-furaldehyde
MR	Maillard reaction
MRP	Maillard reaction products
pH	Potential of hydrogen
SCM	Sweetened condensed milk
SMB	Sodium metabisulfite
TCA	Trichloroacetic acid
TBA	Thiobarbituric acid
UV vis	Ultraviolet-visible
V	Vanillin
VE	Vanilla extract

SUMMARY

1 INTRODUCTION

This document is the sum of the results obtained in the chemical and food technology project “Assessment of the influence of vanillin, vanilla extract and sodium metabisulfite on Maillard reaction in sweetened condensed milk and doce de leite”. The MR is one of the most important reactions in food chemistry, and its relevance to the research group QUIMTEC has been growing for its influence on physicochemical properties, color development of dairy products, and its occurrence due to the diverse thermal treatments used for processing and sterilization.

Dairy products are also extremely important for the food industry, and, regarding their composition, they are an ideal place for the occurrence of MR. Milk is intrinsically rich in proteins and lactose which is a reducing sugar, having there the reagents of MR. In addition, derived milk products could also include additional protein or reducing sugars in their formulas. Even though, the MR should be controlled to guarantee the physicochemical, sensorial, and quality properties in those dairy products that are expected to be white, for instance, in products like milk itself, SCM, some ice creams, creams, yogurts, and some milk drinks.

Considering that MR is driven by the glycation of proteins with the available reducing sugars in the food matrix, followed by many reactions of oxidation-reduction, is possible to purpose that molecules with antiglycation properties or antioxidant activity could interfere with the progress of this reaction (LUND, M. N.; RAY, 2017;YU et al., 2020). Commonly used in the food industry as flavoring, vanillin (V) is an aldehyde with antiglycant activity, and it can be synthesized or extracted from vanilla fruits. The literature report that V can also modify the progress of MR (SHYAMALA et al., 2007;SIDDIQUI et al., 2018;TAI et al., 2011). Sulfites are additives used in some foods for their antibrowning, antioxidant, and preservative properties, and many studies report sulfites as MR inhibitors (D’AMORE et al., 2020;FRIEDMAN; MOLNAR-PERL, 1990). They are not permitted for use in dairy products, but studying their role in MR inhibition may help to identify strategies and alternative additives for controlling it.

Therefore, this project had the purpose of assessing the influence of vanilla extract (VE) and its main component, V, into the progress of the MR. This was achieved by storing at high temperature SCM with the addition these potential MR modification agents, measuring the

progress of the reaction by chromatographical quantification of free furfural profile (5-hydroxymethyl-2-furaldehyde (HMF), 2-furaldehyde (F), 2-furyl-methyl ketone (FMK) and 5-methyl-2-furaldehyde (MF)), and by pH and color analyses Browning Index (BI), using the CIELab color space. This cooking process transforms technologically SCM into DL, a food product characterized by its brown color as consequence of the MR.

2 THEORETICAL REFERENCE

In the following sections, it will be summarized the scientific literature related to SCM and DL production, MR in dairy products, controlling strategies and alternatives to delay this reaction. Among the techniques described are high performance liquid chromatography (HPLC) and colorimetry.

Additionally, the study included SMB as an antioxidant and blenching food additive well-known for being a MR inhibitor, (WEDZICHA, 1992). For that reason, SMB was used as a positive control of MR inhibition to compare its influence with VE and V. The results of this study aimed to enhance the knowledge of MR development and inhibition in dairy food matrixes.

2.1 SWEETENED CONDENSED MILK AND DOCE DE LEITE

According to the Brazilian legislation, SCM is a product resulting from the partial dehydration of milk, concentrated milk, or reconstituted milk, with the addition of sugar, and may have its fat and protein contents adjusted solely to meet the characteristics of the product (SDA N° 47, 2018). Doce de leite (DL) is the product, with or without the addition of other food substances, obtained by concentration and action of heat at a normal or reduced pressure of reconstituted milk or milk, with or without the addition of milk solids and/or cream and added sucrose (partially replaced or not by monosaccharides and/or other disaccharides) (BRASIL, 1997).

The composition of these two dairy products is very close, the main difference between them is related to their production process. DL production involves a cooking to evaporate water from milk by transferring energy in form of heat, and leading to the development of MR, causing changes in color, texture, and flavor. For that reason, DL helps as model system of occurrence of MR from the heating SCM.

In other terms if SCM is subjected to a cooking process it starts to develop physicochemical characteristics of DL, which the most important is the color change and appearing of browning like the DL. Then, these two dairy-concentrated products, allow the study of the MR from food systems with the same composition if the cooking is done in closed

vessels. In this way, the influence of composition changes is standardized and the addition of any additive on SCM with the subsequent heat treatment allows to study the influence of that additive on the progress of MR regardless of composition changes.

2.2 MAILLARD REACTION IN DAIRY PRODUCTS

In early MR, a reducing sugar reacts with a free amino group, this is also known as glycation. The reducing sugar in milk is lactose, and one of its main amino acids is the lysine, an amino acid which has a free amino group in its side chain. Thus, in milk, the carbonyl group of lactose and the ε-amino acid residue of lysine can be condensed by glycation, and then, a water molecule is removed, the product is a Schiff's base, an unstable imine. Following, it undergoes the Amadori rearrangement to constitute an Amadori compound (lactulosyl-lysine) that has more stability (HELLWIG; HENLE, 2014;HODGE, 1955;PERRONE, A. et al., 2020).

At the intermediate stage, the pH conditions have a fundamental role because it will conduce to a predominate production of certain products; at pH lower than 7 the pathway of synthesis of furfural products such as HMF, F, FMK, MF, and at pH higher than 7 the pathway of formation of dicarbonyl products is preferred. Until this point, all products are colorless. The sugar residues suffer degradation or fragmentation giving colorless or yellowish compounds with strong near-UV adsorption. In this step, the flavor and aroma are developed, mainly because of Strecker degradation of amino acids, in which aldehydes, formic acid, acetic acid, and dicarbonyls are formed accompanied by an increase in the reducing power (STAROWICZ; ZIELIŃSKI, 2019;STRECKER, 1850;YAYLAYAN, 2003).

At the final stage of MR, intermediary products undergo further transformations up to the formation of advanced glycation end products also called “AGEs”. Condensations of the remanent free amino groups with the aldehydes and carbonyls formed in previous stages lead to the formation of high-molecular-weight colored nitrogenated end polymers, called melanoidins (HODGE, 1953; 1955). The expression “MRPs”, currently summarizes all MR products, including very reactive products like dicarbonyl compounds, and more stable products, such as HMF, which is commonly used in food industry as MR intermediary marker and indicator of heating treatment (FRANCISQUINI et al., 2018;JIA et al., 2023;PERRONE,

A. et al., 2020). In this study, the quantification of the furfurals HMF, F, FMK and MF were defined as the targeted MRP for the following MR progress.

2.3 KEY POINTS OF MAILLARD REACTION CONTROLLING

Considering the three main stages in which MR occurs, which involve oxidation-reduction reactions, this can suggest that the addition of an antioxidant may influence the MR rate and extent. Some previous studies of MR inhibition or control have assessed the addition of natural extracts and phytochemicals in model systems, and proved the inhibitory MR activity of some polyphenols, for example those from coffee peel (REBOLLO-HERNANZ et al., 2019) grapes extract (RAČKAUSKIENĖ et al., 2019) green tea, grapes, and cocoa extracts which are rich in epicatechin, a strong antioxidant that traps α-dicarbonyls (TOTLANI; PETERSON, 2005).

However, achieve the same results in real food matrices is a challenge due to their complexity, and for that, this study looks forward to evaluating an alternative to control or retard MR rates with the addition of antioxidant compounds in dairy products. Some studies have achieved good results in dairy food matrices (YU et al., 2020), demonstrating that resveratrol, an antioxidant found in grapes has a good inhibiting activity of MR reducing the concentration of dicarbonyl MR products in baked yogurt and baked milk. They also found a dosage dependence and without color and flavor changes.

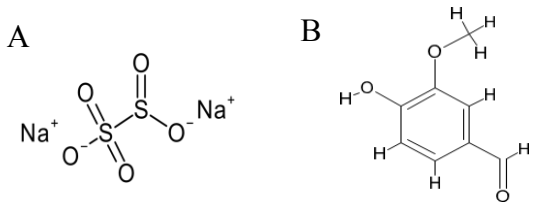
Methods to controlling MR as well as its understanding itself, imply considering multiple reaction steps. Some potential strategies to reduce reaction rates or even stopped it at some point had been purposed. Considering the initial step of glycation, it can be included an antiglycant or antioxidant agent able to trap the reducing sugars and free radicals at the initial stages of the reaction (CUI et al., 2020). Strategies for stopping or retard the intermediary stage are blocking carbonyl groups or degradation products of MR by the addition of nucleophiles (ZHU et al., 2021) and in the last stage, the breaking of cross-links between proteins and dicarbonyls would reduce the formation of AGEs (LUND, M. N.; RAY, 2017). Then, antioxidants with properties to capture free radicals, reduce oxidative stress and decreasing the production of reactive carbonyl and dicarbonyl groups could be potential agents to delay MR (YEH et al., 2017).

Polyphenolics are compounds with strong antioxidant properties, as consequence are ideal to act as MR retarder because they can react with amines forming benzoquinone imines and similar adducts or make them unavailable by reduction, delaying subsequent glycoxidation. Antioxidants that scavenge radicals can block hydroxyl and superoxide radicals, and this may avoid the formation of dicarbonyl compounds, in addition, some other antioxidants can chelate metal ions that promote oxidative reactions (REBOLLO-HERNANZ et al., 2019). Some natural antioxidants such as chlorogenic acid obtained from coffee (Rebollo-Hernanz et al., 2019), resveratrol from grapes (Krishnaswamy et al., 2013) may be potential MR inhibitory agents. VE is a great example of antioxidant additive, with the advantage of being a well-accepted ingredient in the food industry, as well as its main component V, which is an aldehyde with antiglycant properties (Shyamala et al., 2007).

2.4 POTENTIAL AGENTS OF MR MODIFICATION

As many MR stages are involved in redox or radical reactions, it is possible to suggest the alternative to add substances with antioxidant or antiglycant activities to stop or even delay its progress. The purposed substances assessed in this study were SMB, VE, and V. Their chemical structures are shown in figure 1.

Figure 1 – MR modification agents.



Font: elaborated by the author (2023)

Note: A. Sodium metabisulfite. B. Vanillin

2.4.1 Sodium metabisulfite

Belonging to the sulfite family, it is a food additive used due to its antioxidant, blenching, and preservative properties (D'AMORE et al., 2020;FRIEDMAN; MOLNAR-PERL, 1990) . It could be found in potato preparations, corn syrup, brewing, and wine processing. However, sulfites induce some health consequences, and some people are sensitive. They can react with

thiamine, making vitamin B unavailable, and for that, they are not recommended in foods rich in this vitamin, like in meat. In addition, sulfites have been banned by the Food and Drug Administration (FDA) for use on fresh fruits and vegetables that are meant to be consumed raw, defined that sulfite residues above 10 ppm are not allowed (D'AMORE et al., 2020). Then, is highlighted that its use for the present study was only as positive control of MR inhibition.

2.4.2 Vanilla extract

Commercial vanilla products are produced from cured, dried, and conditioned pods of fully ripe fruits of the Vanilla orchid genus(HAVKIN-FRENKEL; BELANGER, 2018).

2.4.3 Vanillin

Vanillin (4-hydroxy-3-methoxy benzaldehyde) is the principal compound in VE, an aromatic aldehyde responsible for its characteristic flavor. It is found (up to approximately 2.5% w/w) in vanilla beans, a plant native from Mexico and cultivated in Madagascar, Comoros, Indonesia, and Papuand New. Vanillin is known as a flavoring since 1816 (ZIEGLER, 2007), it is one of the most popularly used flavoring components extracted from the seedpods of *Vanilla planifolia* and is widely used in foods, beverages, cosmetics, and drugs. The concentrations used in food and beverage products range widely from 0.3 to 33 mM. VE has been reported to have a strong antioxidant activity and a strong antiglycant effect (LI; GRUN; FERNANDO, 2000;SHYAMALA et al., 2007;SIDDIQUI et al., 2018;TAI et al., 2011).

2.5 PARAMETERS TO MONITOR MAILLARD REACTION PROGRESS

The furfurals are stable intermediate products of the MR (SOLAYMAN; SHAPLA; KHALIL, 2023). It could be formed in dairy products during sterilization and storage. In systems with pH less than 7, as in most dairy products, the MR prefer the pathway of 1,2-enolization, and the main molecules formed are furfurals, like HMF. If the initial sugar is a hexose the dominant product will be HMF and if it is a pentose will be F. Other furfurals such as F, MF, FMK are formed at slightly higher pH and the advanced stage of MR or by inter-

conversion between them under more intense heating conditions and long-term storage (ERBERSDOBLER; SOMOZA, 2007;FERRER et al., 2000)

HMF has been used for many years as a marker for MR in dairy and other food products, and (CHÁVEZ-SERVÍN; CASTELLOTE; LÓPEZ-SABATER, 2005;FRANCISQUINI et al., 2018; 2019;NUNES et al., 2019;ZHANG et al., 2021) many studies had related that the HMF content in samples of milk, milk powder, fermented milk, and reconstituted milk significantly increases with the browning of these products and with the degree of thermal treatment and fermentation time.

The present study required a precise and accurate analytical technique to monitor MR progress. The intrinsic aromatic character of furfurals permits to quantify them, as they absorb ultraviolet radiation with a major UV-vis absorbance peak at 278 nm. It also can be quantified by spectrophotometric traditional methods. However, spectrophotometric methods are not very sensitive and despite being faster and less expensive, they can be influenced by factors such as time, temperature, presence of interferences and are not practical for analyze large number of samples.

In contrast, chromatographic methods also permit the separation of furfural compounds, after a proper sample preparation, they are introduced in a flowing mobile phase that passes a stationary phase which retains stronger or weaker the different compounds of the sample, according to their affinity and then those compounds are released separately in time with the mobile phase. In addition, high-performance liquid chromatography (HPLC) gives superior levels in terms of sensitivity and precision (MOLDOVEANU; DAVID, 2022).

Color is another key parameter to monitor MR, it can be measured using the CIELab system that expresses color as three coordinates: L* for luminosity (black = 0 to white = 100), a^* (green (-) to red (+)) and b^* (blue (-) to yellow (+). With this parameters is possible to determine the Browning Index, BI. This is a value in some way related to the darkness of samples and this gives an idea of how dark a sample is. BI measurements using the CIELab system to alanylze different foods, such as milk and bakery products, are used to provide data on a global response or change in color. In this study, the progress of MR on the systems with and without potential inhibitor agents, before and after heating, will be evaluated by

determining the BI using a colorimeter tristimulus according to the CIELab scale (MCLAREN; RIGG, 1976;MORALES; BOEKEL, VAN, 1998;OBÓN et al., 2009).

Finally, the pH change is other physicochemical parameter that could contribute to the control and study MR progress. The initial pH of a system gives an idea of how much MR would happen. If the pH is high, amino groups will be more available and MR is expected to occur with more probability than if the initial pH is lower because amino groups would be blocked to glycate. Over the MR, the pH also plays a direct role in conducting the reaction to one pathway or to another, because it always defines the availability and reactivity of amino groups and pKa equilibrium of molecules in the system. The acidification of the initial pH in comparison to the final, of a sample that suffered heat treatment or storage, is also related to MR. The MR leads to a higher reducing capacity by the increased number of hydroxyl groups provided by conjugations along MR (HWANG; SHUE; CHANG, 2001;NOOSHKAM; VARIDI; BASHASH, 2019;RAO et al., 2011). Then, the pH after heating will be a consequence of the level of MR developed.

3 OBJECTIVES

The main objective of this project was to evaluate the influence of VE and V in the Maillard reaction through the obtention of DL from the cooking of SCM with the addition of those additives.

3.1 SPECIFIC OBJECTIVES

Among the specific objectives of the work are:

a) to determines if the quantification of free furfurals Furfural (F), Methyl Furfural (MF), Furyl Methyl Ketone (FMK) and 5-Hydroxymethylfurfural (HMF), pH monitoring, and the Browning indexes are suitable parameters to monitor Maillard reaction (MR) in samples of sweetened condensed milk (SCM) with V and VE;
b) to evaluates and understand the inhibitory activity of sodium metabisulfite (SMB) as a positive retarding agent of Maillard reaction (MR) into sweetened condensed milk (SCM) and *doce de leite* (DL) based on the parameters of free furfurals, pH monitoring, and the Browning Index (BI);
c) to identifies the effect of Vanilla extract (VE) and Vanillin (V), on the Maillard reaction by the quantification of free furfurals, monitoring pH, and BI based on the comparison with these parameters obtained with the addition of sodium metabisulfite (SMB) as a positive control of MR inhibition;
d) to identifies the characteristics of possible alternative additives to sodium metabisulfite (SMB) based on the results obtained in the evaluation of its influence in MR and that obtained with vanilla extract (VE) and vanillin (V).

4 METHODOLOGIES

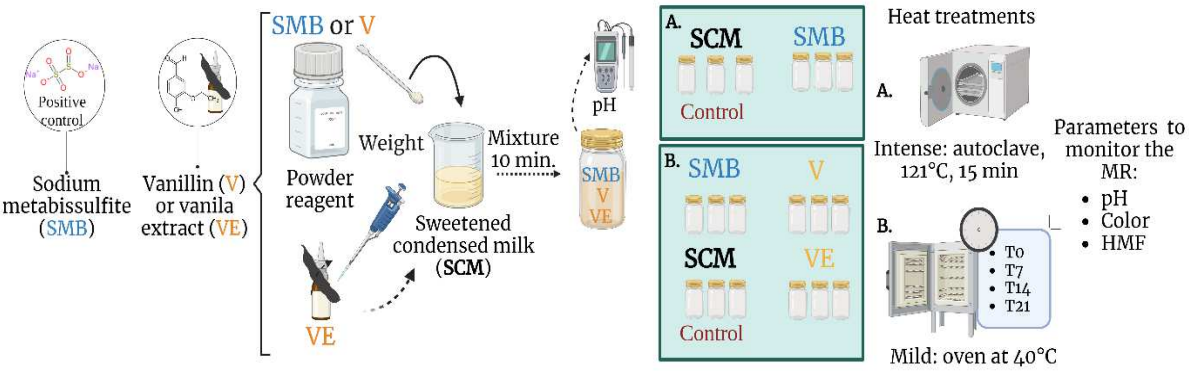
Here is the description of experimental methodologies used in this project.

4.1 SAMPLES AND REAGENTS

Samples of SCM with contents of 11.70 % (w/w) lactose, 35.25% (w/w) sucrose, 8.55% (w/w) glycose, for a total sugar content of 55.00% (w/w). A fat content of 4.00% (w/w), 7.05% (w/w) of milk proteins. SMB (PA, ACS reagent, 98,0%) and V (SIGMA *ReagentPlus*®, 99%), commercial hydroalcoholic vanilla natural extract (Burbon, gourmet, brand *Valeso*).

4.2 PRELIMINARY ANALYSIS

Figure 2 – Methodology of sample preparation.



Font: Made by the author (2023).
Note: Illustrations created with BioRender. SMB: sodium metabisulfite. VE: vanilla extract. V: vanillin. SCM: sweetened condensed milk. A. Samples submitted at 121°C for 15 min. B. Samples stored at 40 °C for 21 days.

4.2.1 Addition of sodium metabisulfite, vanilla extract, and vanillin and monitoring of MR

As it does not exist a standard or official method to determine the progress of the MR in foods, the first step of this study consists of some procedures to force the reaction to happen into samples of SCM with and without the addition of the MR modification agents. To guarantee the solubility of SMB and V on the SCM it was added concentrations of 1, 2, and 5 mg/g of SCM for SMB, and V. For VE it was added volumes of 0.1, 0.2, 0.5, and 1 mL to 10g of SCM. Mixtures were homogenized by stirring for 10 minutes and left to stand for two hours.

Phase separation and pH were monitored 24 hours later to assess compatibility of the agents with SCM. Heat treatments were assessed by heating control samples of SCM in an autoclave at 121°C for times from 5 to 45 minutes to stimulate the MR.

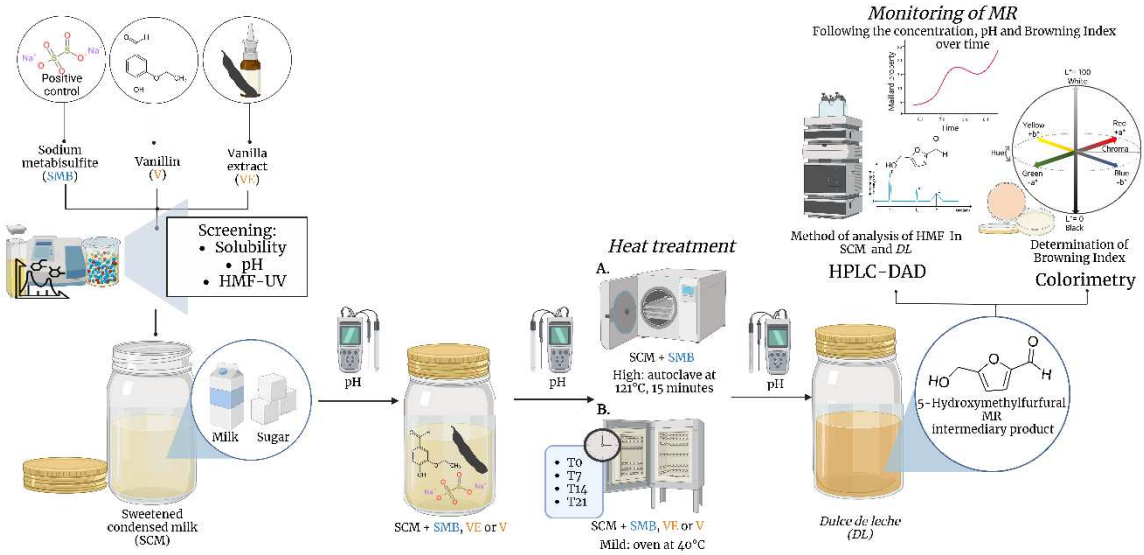
4.2.2 Screening of HMF quantification by UV-VIS

After the screening of the preliminary tests, samples of SCM were added with SMB, VE, and V at their best concentrations and times obtained from 0.05, to 1.00% (w/w) heating in the system A: in autoclave at 120°C and for 15 minutes and B in oven at 40°C for longer periods (Figure 2), for samples with SMB, V and VE, to verify the suitability of that conditions with the inclusion of the agents. Following, were measured the pH, color, and HMF-UV quantification to see if the systems had differentiated responses when the inhibitor concentrations and heating conditions changed. With these results was established the general methodology of this project that is summarized in Figure 3.

Samples were subjected to a thermal treatment in an autoclave at 120°C for 15 minutes. After heating, the HMF was extracted and quantified by UV-VIS spectrophotometric analysis at a wavelength of 443 nm (Shimadzu UV-1601 UV-VIS Spectrophotometer) based on the method of analysis purposed by (KEENEY; BASSETTE, 1959), with some modifications. Briefly, were prepared solutions of control SCM and with SMB, VE, and V at 5.0% (w/v) in deionized water. An aliquot of 5 mL of that solution was added with 2.5 mL of oxalic acid 0.30 mol L^{-1} and mixed. Then, 2.5 mL of 40.0% (w/v) trichloroacetic acid (TCA) was added to precipitate fat and proteins. Samples were separated by gravitational filtration and 4 mL of supernatant was mixed with 1 mL of 0.05 mol L^{-1} of thiobarbituric acid (TBA). Following that, samples were incubated in a thermostatic water bath at 40°C for 30 min for color development. Quantification was made at a wavelength of 443 nm. The HMF concentration (μmol L^{-1}) was calculated from a standard curve made up of this adduct formed from standard solutions from 2 to 60 μmol L^{-1} HMF.

To assess the progress of MR at 40° C, samples of 40 g of SCM added with standard SMB, V, and commercial VE, at concentrations from 0.05 to 1.0% (w/w) or (v/w) for VE, were placed into closed glass flasks and disposed into an oven (DeLeo, Equipment's laboratories) at 40°C. The monitoring of the MR progress parameters (free HMF content, pH, and BI) was made by removing the sample and inserting it again into the oven before each analysis.

Figure 3 – Methodology to monitor Maillard reaction.



Font: Made by the author (2023).
Note: Illustrations created with BioRender. SMB: sodium metabisulfite. VE: vanilla extract. V: vanillin. SCM: sweetened condensed milk. A. Samples submitted at 121°C for 15 min. B. Samples stored at 40° C for 21 days.

4.3 HPLC-DAD- FURFURAL ANALYSIS METHOD

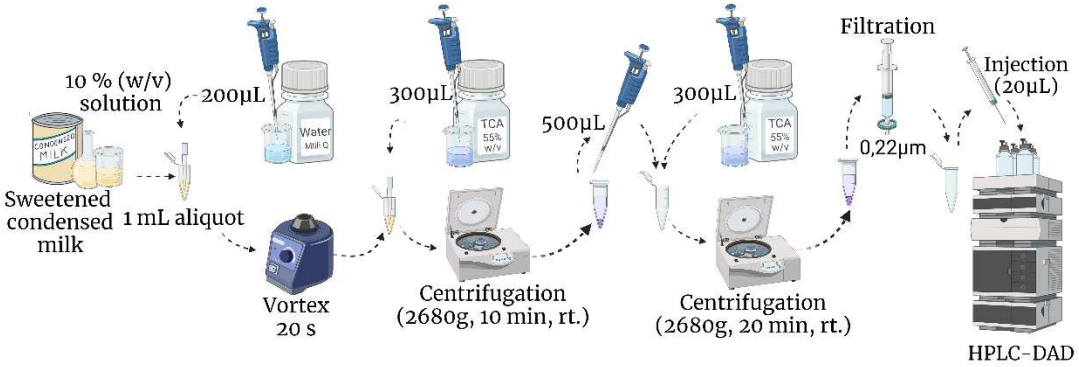
The concentration of furfurals in samples of SCM with the addition of potential MR mosidication agents (SMB, VE, and V), was measured under the better conditions determined on the preliminary tests but replacing the spectrophotometric analyses for the use of the high-performance liquid chromatographic method with diode-array detection (HPLC-DAD) method developed and validated by (PINTO, 2023).

4.3.1 Validation of sample preparation for HPLC-DAD analysis

Furfurals were extracted from SCM and DL from a novel sample preparation method developed in this study, adapted from previous reported analyses of free furfurals in infant formulas (CHÁVEZ-SERVÍN; CASTELLOTE; LÓPEZ-SABATER, 2005;LUND, P. et al., 2022), as it is summarized in Figure 4. Solutions of SCM or DL were prepared at a concentration of 10.0% (w/v) in ultra-pure water. An aliquot of 1 mL was subsequently diluted to a volume of 1.2 mL with ultra-pure water and mixed by vortexing for 20 seconds. Following that, 300 µL

of 55.0% (w/v) trichloroacetic acid (TCA) was added, mixed, and centrifuged (2680 *g*, 10 min, rt.). Then, 0.5 mL of the supernatant was mixed by vortexing with 100 µL of ultra-pure water and 300 µL of 55% (w/v) trichloroacetic acid (TCA). The samples were centrifuged (2680 *g*, 20 min, rt.) and the supernatant was filtered (0.22 µm), for posterior injection (20 µL).

Figure 4 - Methodology of sample preparation for analysis of furfurals by HPLC-DAD



Font: Made by the author (2023).
Note: Illustrations created with BioRender.

4.3.2 Furfural analysis

Furfurals were quantified according to the validated method of PINTO, (2023) using high-performance liquid chromatography (HPLC) in a system Waters equipment, model 1252, with detector UV-Vis (DAD) and equipped with a binary pump. The analysis was performed in isocratic elution mode, with a mobile phase composed of water and acetonitrile in the ratio (95.5:4.5), 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 3µm; ODS2), kept at 30°C. The injection volume was 20µL and furfurals were detected at a wavelength of 284 nm. Using standard furfural solutions of HMF, F, FMK, and MF to build calibration curves within the range of 0.16–5.0 µg/mL for quantitative determination of HMF and of 0.06–2.0 µg/mL for F, FMK, and MF.

4.3.3 Recovery rate

To determine recovery of furfurals in samples of SCM, it was fortified with HMF, F, FMK, and MF at the concentrations of 2.5 for HMF and 1.0 µg· ml^{-1} for F, FMK, and MF. Aliquots of the furfural stock solutions of 50 µg· ml^{-1} were added to the 10.0% (w/v) solution

of SCM. For quantification of free HMF 10.0% (w/v) solution of SCM or DL were mixed by vortexing with 200 μL of ultra-pure water, following the same process as described above in the 4.5.1 section, and in triplicate. Recovery was expressed as the percentage ratio of the analyte of known concentration added to the sample and as the corresponding theoretical concentration (n=3).

4.4 COLORIMETRY ANALYSIS AND BROWNING INDEX CALCULATION

Sample color was measured before and after inhibitor additions and heat treatments by a Chroma meter CR-400 model colorimeter (KONICA MINOLTA). BI were determined from CIELab parameters measurements of all samples with Equation 1 and 2, considering the values L*, a*, b* (MASKAN, 2001). All results are reported as an average (n=3).

$$BI = \frac{[100\ (x-0.31)]}{0.17} \quad (1)$$

$$x = \frac{(a*+1.75L*)}{(5.645L*+a*-3.012b*)} \quad (2)$$

4.5 pH CHANGE

pH measures were made using a pH portable electrode PG1400 GEHAKA® of the samples with and without addition of potential MR inhibitors, at room temperature in triplicate. Results were reported as the average of lectures (n=3).

4.5.1. Influence of the pH in the retarding effect of MR caused by sodium metabisulfite

To study the isolated effect of pH on the MR, samples of SCM were added with different acids and exposed under 40° C heating conditions at 40°C for up to 40 days without stirring (n=3). The initial pH was equilibrated to 5.8, using different acids: lactic, gallic, ascorbic, acetic, and hydrochloric, with SMB as positive control of MR inhibition including a SCM control with initial pH of 6.3 was also included. The MR development was also assessed by monitoring furfural content, pH, and BI changes before, including after heating for 20 and 40 days.

5 RESULTS

The results of all the experimental analyzes carried out in this project are shown below.

5.1 PRELIMINARY ANALYSIS

The results of screening tests are shown below.

5.1.1 Addition of sodium metabisulfite, vanilla extract, and vanillin and monitoring of MR

The increase of HMF concentration in heat-treated foods has been reported previously to be related to the severity of heating treatments, besides to other factors like the type of sugar and proteins, pH, and water activity in the food matrices (ZHANG et al., 2021). Therefore, for this study an intense heating treatment was proposed to force MR, like a heating process in an autoclave at 121°C for 15 minutes.

Figure 5 – Screening of browning in *doce de leite* (DL) and sweetened condensed milk (SCM) samples with SMB and VE after intense and 40 °C heating

Font: Made by the author (2023).
Notes: Samples of sweetened condensed milk and doce de leite with SMB (A), (C) and VE (B), (D) after intense heating at 121°C for 15 min and after mild heating at 40 °C, 48 hours, respectively.

In contrast, a thermal treatment under 40 °C conditions like the one applied in this study using an oven at 40 °C for longer periods, revealed to be suitable to undergo less intense browning and to guarantee that is a consequence of MR instead of by some grade of caramelization due to high temperatures (CORZO-MARTÍNEZ et al., 2012). Results also demonstrated suitability and kept the integrity of the food product; therefore, these two thermal systems allowed the study of the MR progress over time with the possibility to compare both systems' rates.

5.1.2 Screening of 5-Hydroxymethylfurfural (HMF) quantification by UV-VIS spectrophotometry

At intense heating of samples with SMB, good results were observed in which the samples showed different browning levels in the function of SMB concentration (Figure 5). Based on these results, was established the selected dose range of 0.05 to 1.00% (w or v/w) for the other MR modification agents, VE, and V in SCM. The last, because was identified that at a concentration of SMB, 1.00% (w/w) is possible to inhibit MR leading to a concentration of HMF and a color close to the initial SCM. Results of HMF content shown in Table 1, demonstrated to be a suitable thermal process to evidence fast MR development and obtain darker DL.

Table 1 - Screening of HMF concentration after heating samples with sodium metabisilfite and vanilla extract

	SCM + SMB		SCM + VE	
% (w or v/w)	Autoclave	Oven	Autoclave	Oven
	($\mu mol \cdot kg^{-1}$)			
0.00	263.90	258.04	273.64	55.33
0.10	152.17	47.17	267.20	49.39
0.50	111.65	51.52	274.39	54.32
1.00	68.76	63.66	308.86	54.32

Font: made by the author (2023)
Notes: Sweetened condensed milk (0.0%) before heating: 50.08 μmol HMF·kg^{-1}. Sodium metabisulfite (SMB) and Vanilla extract (VE). Oven: 40 °C/48 h. Autoclave: 121°C/15 min.

Though, V and VE can oxidate easily and it is favored at high temperatures (WEERAWATANAKORN et al., 2015). Then, preliminary assays heating samples with VE and V at 121°C did not reveal any difference in color among the samples after the heating as can be seen in figure 5, probably because these high temperatures can be too strong and cause a possible thermal inactivation or degradation of the active compounds. Previous studies have exposed V mixtures with Bovine Serum Albumin (BSA) and Sodium Caseinate (CAS) at 68°C to simulate minimum sterilization temperatures with good results but there are not reports of exposure to higher temperature conditions.

Considering that the objective of this study was to assess the antiglycant and antioxidant activities of VE and V, SMC samples were enriched with SMB and analyzed under two thermal conditions 121°C in autoclave and 40°C in oven, while samples of VE and V were only analyzed under lower heating, to avoid thermal degradation of the bioactive compounds. Mild heating using a warm storage of the samples allowed to observe slower development of the MR, and consequently to study the evolution of the reaction in more detail.

Table 2 - Screening of pH, and BI of samples heated with vanilla extract

	Autoclave				Oven			
% (w or v/w)	pH_0	pH_{MR}	ΔpH	BI	pH_0	pH_{MR}	ΔpH	BI
0.00*	6.32	5.52	-0.80	80.8	6.32	6.39	0.07	22.0
0.10	6.32	5.54	-0.78	80.2	6.30	6.35	0.05	22.8
0.50	6.30	5.54	-0.76	79.5	6.29	6.36	0.07	19.3
1.00	6.31	5.54	-0.77	79.5	6.18	6.25	0.07	23.1

Font: made by the author (2023)
Notes: sweetened condensed milk (0.0%) BI before heated 18.5. pH_{MR}: pH after heating; measured at 20.0 ± 0.5 °C. Browning Index (BI).

The results of monitoring HMF concentration, are presented below in the Graphic 1. After the addition of SMB, VE, V, and after the thermal treatment the results showed fluctuation in the HMF profile. This was expected, considering thar the pH of SCM was in average 6.3 ± 0.02, which favors the pathway of furfurals synthesis and enhance the suitability of using HMF as MR marker in dairy products.

Although it is not possible to describe the mechanisms of SMB interaction on MR based on the obtained results, it can be purposed the following hypotheses: SMB interact with the HMF content present in the samples of SCM since the moment of the addition. This interaction

with MR intermediary products, prevents in some way the formation of advanced MR products, like melanoidins, and consequently decreasing or avoiding the appearing of brownish color.

The role of SMB as a strong nucleophile, due to the polarizability of sulfur electrons and the availability of empty d orbitals to overlap, makes it a competitive nucleophile agent at the earliest and intermediary stages of MR, limiting the possibility of glycation and the same effect for the condensation of dicarbonyl intermediates, by the suppression of free-radical formation, localizing it into the sulfate structure and as consequence, it can interfere with the polymer formation of AGEs like melanoidins (SCOTTER; CASTLE, 2004).

5.2 HPLC-DAD FURFURAL ANALYSIS AND SAMPLE VALIDATION METHOD.

The increase in the content of furfurals due to the occurrence of MR under 40° C heating conditions was small, when compared with the content obtained after exposure at higher temperatures. For this reason, the present study required a precise and accurate analytical technique. Furfurals have an intrinsic aromatic character; they absorb ultraviolet radiation with a major UV-vis absorbance peak at 278 nm and can be quantified by spectrophotometric traditional methods. However, spectrophotometric methods are not very sensitive and despite being faster and less expensive, they are inferior to HPLC analysis methods in terms of sensitivity and precision.

Therefore, in this study was developed and validated the sample preparation method summarized in figure 4, after having tested three concentrations of trichloroacetic acid (TCA); 40, 55 and 70% (w/v) this reagent has the function of separating fat and proteins from the food matrix, leaving the furfurals in the aqueous phase of the soluble fraction of the sample. This step was crucial for the effectiveness of the analytical method and confirms the importance of TCA concentration for furfural extraction and analyses in dairy products. The concentration of 55% (w/v) was selected as the most adequate, because a higher concentration promoted liberation of furfurals linked to proteins, generating an overestimation of free furfurals, and a lower concentration gave not reproducible results.

Table 3 - Recovery of furfurals in SCM with the HPLC-DAD analytical method

Furfural	Concentration (µg /mL)	Recovery (%)
HMF	2.50	100.75 ± 2.39
F	1.00	102.61 ± 3.37
FMK	1.00	89.57 ± 6.47
MF	1.00	90.75 ± 2.54

Font: made by the author (2023).

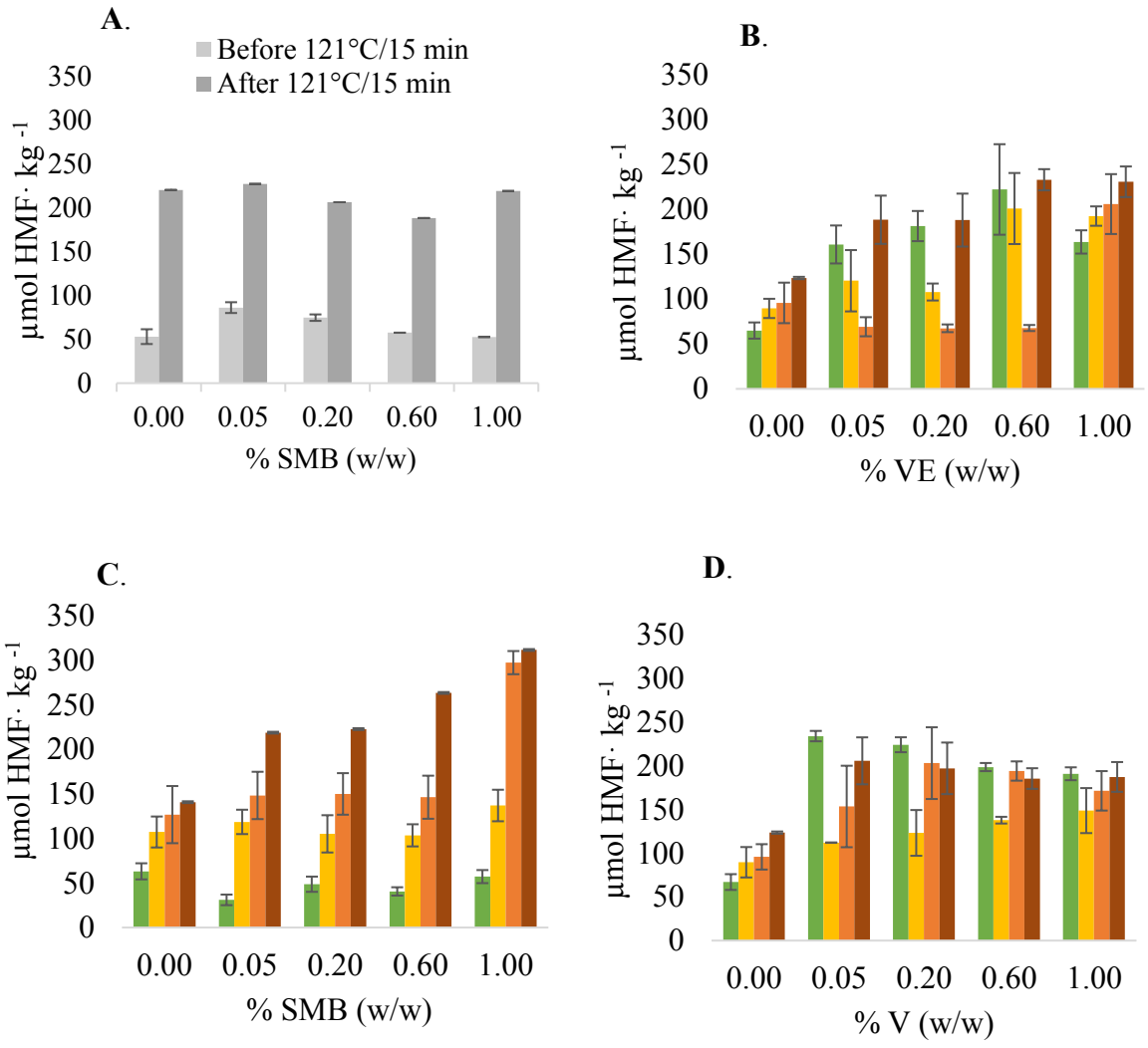
As shown in Table 3, though the recovery percentage obtained for all the furfurals was superior to 80%, for any sample in this study was possible to detect free FMK with this analytical method, and only few samples had detectable amounts of free F, and free MF, of which some were detected but not reproducible among repetitions and all of them had values lower than the quantification limits (LOQ), 0.125, 0,066 and 0.128 µmol·kg^{-1} SCM for F, FMK, or MF, respectively, as reported by (PINTO, 2023). The same result was obtained by them, in which only the content of HMF was quantifiable, reproducible, and comparable among different samples. Therefore, in this study only the HMF content behavior was reliable to monitor MR progress.

As consequence of these hypothesis, due to the trapping by SMB, the concentration of HMF decreases at the beginning but then the capture of HMF unbalances the reaction of furfural formation towards more formation of HMF. The observed increase of HMF concentration from 57.2 ± 7.4 for the control of SCM (without SMB) rising to 311.4 ± 47.4 µmol kg^{-1} for the sample added with 1.0% SMB after 21 days of heating. The addition of SMB did not stop or interfere irreversibly the development of MR at all, because HMF continued being formed along the time and with a slight concentration dependence of SMB.

As shown in Graphic 1, the HMF contents in samples with SMB were higher over those with V, and VE. Samples with VE showed a content of HMF like SMB samples at the end of the 21 days of heating, going from 65.0 ± 9.0 rising to 230.8 ± 20.7 µmol kg^{-1}, differentiated by a non-uniform tendence along the days and with slight change in color and BI values compared to the control (see BI from Graphic 3). Those results also contrast with the ones from V samples, in which HMF concentrations decreased from 205.7 ± 17.1 µmol kg^{-1} of the sample with V 0.05% to 187.3 ± 21.5 µmol kg^{-1} for the sample with V 1.00 % after 21 days.

That result can be explained because in samples with V the MR apparently was accelerated with faster darkening than the control sample, and this can be associated with faster consume of HMF to participate in the melanoidin formation, lowering its concentration. While samples with VE behave like samples with SMB, they change the profile of HMF formation and consumption, and consequently the melanoidin synthesis. This is supported by the lower values of BI for samples of SMB and VE compared to the BI for those with V (Graphic 3).

Graphic 1 - Profile of 5-hydroxymethylfurfural (HMF) of samples with sodium metabisulfite (SMB), Vanilla extract (VE), and vanillin (V)



Font: Made by the author (2023).
Note: HMF profile of samples with SMB after autoclave heat treatment (A). HMF profile of samples after oven heating at 40 °C with VE (B), SMB (C), and V (D). Before heating. After heating 7 days After heating 14 days. After heating 21 days. (n=3).

V is one of the most widely used and desirable aroma additives in beverages, foods, pharmaceuticals and daily chemicals. Some studies reported the interaction of V with proteins, like BSA. The interaction primarily occurs via the Schiff base formation, by spontaneus hydrogen bonding, and hydrophobic interaction. More precise studies of the binding parameters of V to milk proteins can be made by using techniques like isothermal titration calorimetry, molecular docking, fluorescence spectroscopy and secondary structural changes with circular dichroism. (SIDDIQUI et al., 2018). Other studies demonstrated that BSA interacted more with V than CAS, showing that hydrogen bonding appeared to be the major force for the interaction of V with CAS and for the V-BSA system the hydrophobic interaction between nonpolar-fragment of vanilllin and hydrophobic peptide chains of protein molecules (CHOBPATTANA; JEON; SMITH, 2000).

The intensity of the V flavor in a food system is affected greatly by other food components, especially proteins, with an increased interaction with temperature (CHOBPATTANA; JEON; SMITH, 2000). This suggests that V could disfavor MR competitively with reducing sugars, by reacting with available free amino groups from proteins, peptides, or amino acids in SCM. This was demonstrated in studies by Siddiqui et al., (2018), in which V binds spontaneously to BSA giving a complex BSA-V, which supports its antiglycation activity. Other studies have also demonstrated binding with caseins and high affinity with whey proteins (LI; GRUN; FERNANDO, 2000).

Although, for flavoring industries this is not desirable due to the decreasing of the intensity of flavor and aroma, the aim of the present study is to assess the hypothesis of a competence of V with reducing sugars taking its place by binding proteins decreasing the availability of the main reagents to retard MR.

5.3 COLORIMETRY ANALYSIS AND BROWNING INDEX DETERMINATION

However, in this study it was demonstrated that competitive antiglycation of V could not be enough to guarantee MR inhibition. Based on the results of HMF content, pH change and BI obtained in this study and compared to control SCM samples, (Graphics 1, 2 and 3). The values for the parameters of samples with V indicated a darkening even more intense than the obtained for the controls. It was also found a dose-dependent activity because values of HMF and BI values increased with the V concentration. Additionally, the final color of V samples after 21

days makes evident the formation of a different kind of melanoidin polymers with absorbance in different wavelengths giving DL more orange when compared to those the controls as shown in Figure 6.

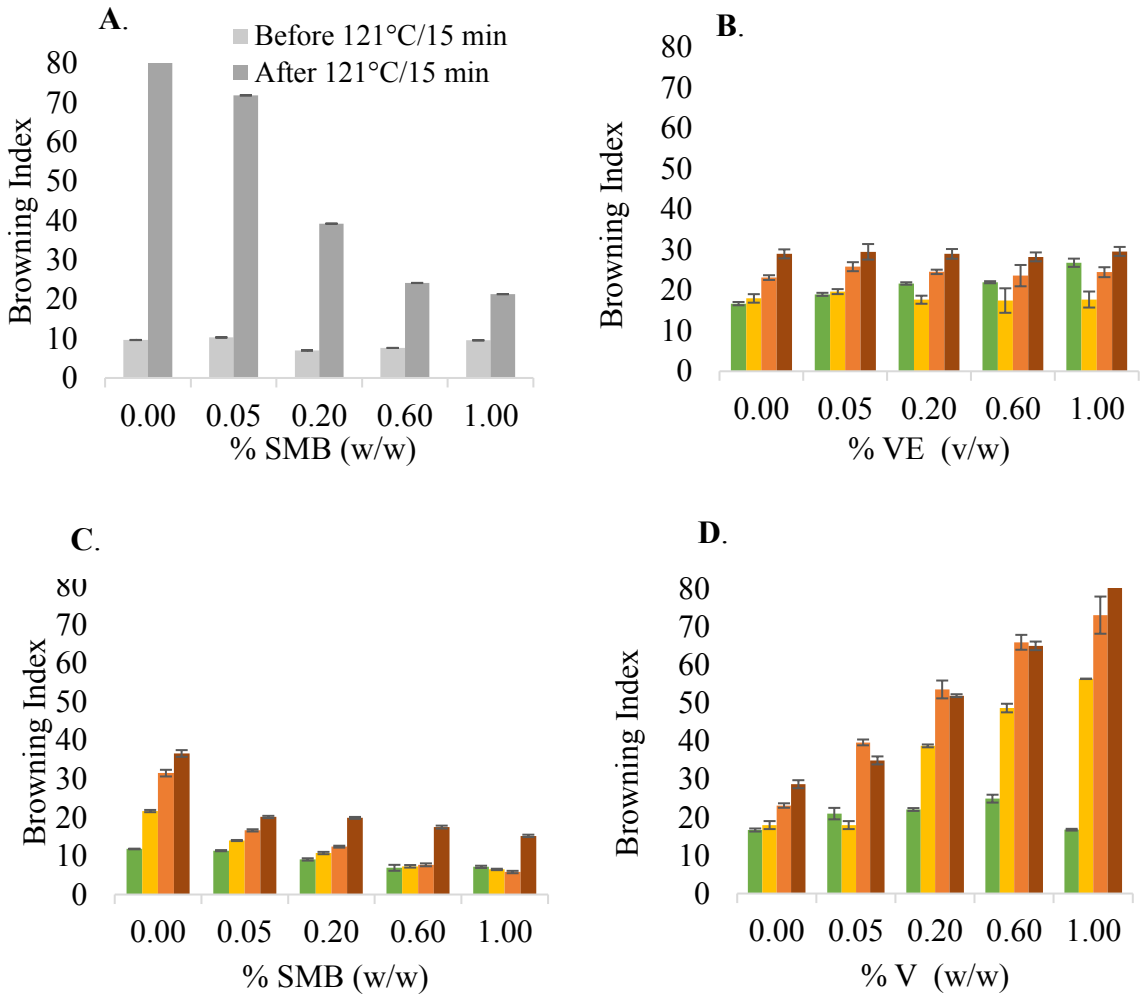
If V succeeded in inhibiting or retarding MR, the expected result would be finding lower levels of HMF and BI content over time and before heating treatments. The opposite was observed, and even darker DL were obtained at all the V concentrations used. The last result supposes that if the hypothesis of competition at early stages of MR is correct, and V takes place of the reducing sugar, (lactose, in the case of dairy products). Although V can act as a competitor, the formation of Schiff bases of proteins with V or its oxidation products could be favored, and these products undergo similar pathways of MR until the synthesis of colored molecules analogues to melanoidins. Then, V does not act as a full inhibitor of MR, but it seems to produce analogues of MR products, being a non-inhibitor competitor. This hypothesis needs to be deeply studied.

In terms of VE, it is not possible to attribute an antiglycation activity or even inhibitory activity of MR to a single compound of it, because it is made up from a complex mixture of biomolecules mainly polyphenolic compounds. In addition to V, which is the principal flavoring component of vanilla and can be found at levels ranging from 0.2 g/100 mL (0.2%) for a good quality extract to less than 0.02 g/100 mL (0.02%) for products with less quality (HAVKIN-FRENKEL; BELANGER, 2018), VE also contains vanillyl alcohol, 3,4-dihydroxybenzaldehyde, 4-hydroxybenzoic acid, vanillic acid, 4-hydroxybenzaldehyde, V, p-coumaric acid, ferulic acid, and piperonal (SCHIPILLITI; BONACCORSI; MONDELLO, 2017). Then, the possibility of having a great combination of polyphenolic compounds would cause higher inhibiting activity than the isolated V, as well as a synergism effect between all the active compounds.

It is important considering the stability and costs of natural extracts. These aspects represent a disadvantage for antioxidants or natural inhibition alternatives because they are expensive, thermolabile and their thermal degradation could leave to inconclusive results. It is important to consider which thermal conditions are needed to assess, produce, and store the food into which the extract or molecule is going to be include.

A transversal result obtained in this study is related to the importance of good storage practices of dairy products. It was possible to observe the effect of temperature on values of pH, HMF concentration and BI for control samples of SCM. As Graphic 1 shows, the content of HMF after an exposure at 40°C for 21 days, samples of SCM form approximately half of the content after heating a sample at intense conditions of 121°C for 15 minutes. The last, enhance the importance of maintaining adequate temperature conditions of storage for dairy products to guarantee their quality and sensory properties, because MR is highly dependent on the temperature. Therefore, the control of temperature, pH, water activity, other food processing and storage conditions would not be ignored as control points to regulate MR.

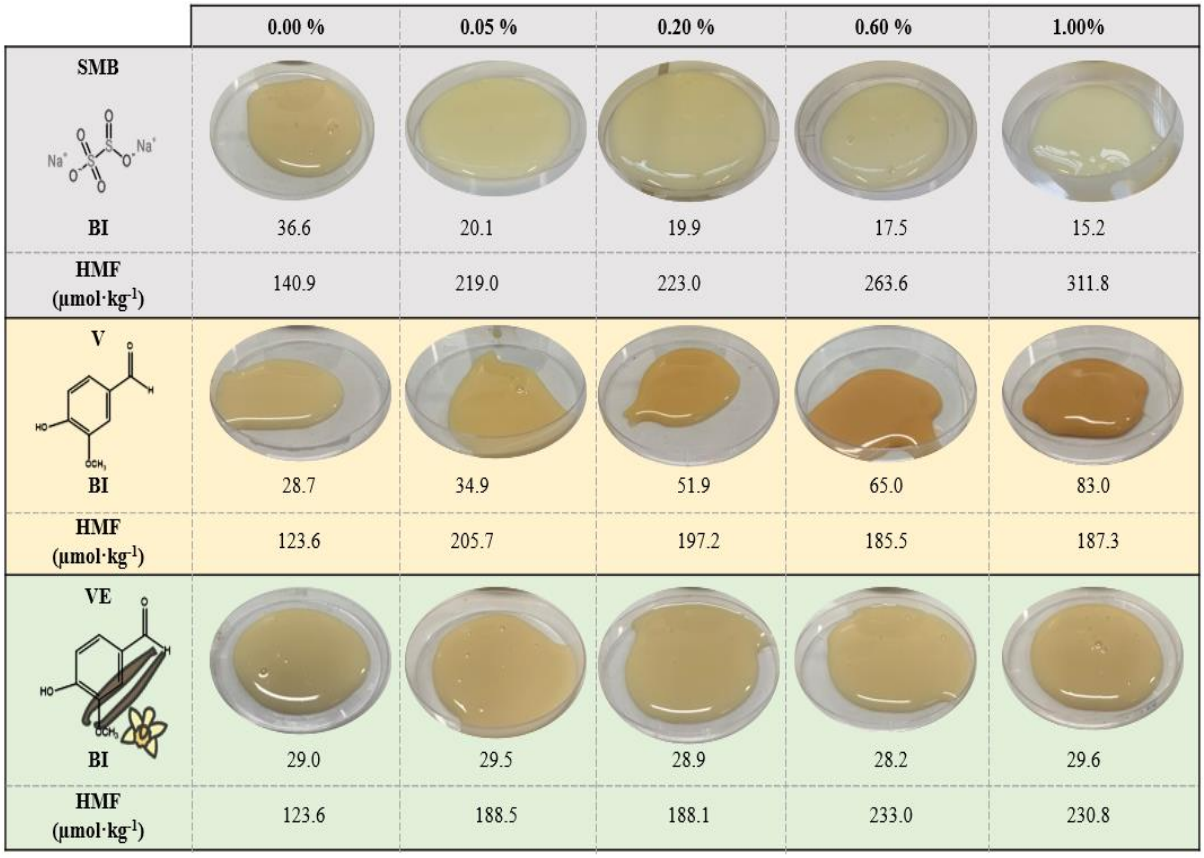
Graphic 2 - Browning Index (BI) profile of samples with sodium metabisulfite (SMB), Vanilla extract (VE), and vanillin (V)



Font: Made by the author (2023).
Note: Browning index profile of samples after autoclave heat treatment (A). BI profile of samples after oven heating at 40° C with VE (B), SMB (C), and V (D). Before heating. After heating 7 days After heating 14 days. After heating 21 days (n=3).

BI values presented greater differences among samples with SMB and V mixtures as it is shown in Graphic 3. Samples with SMB did not super a BI value of 20 in comparison to those added with V, where BI values rise to 83 for sample with 1.0% (w/w) V after 21 days at 40°C. The browning behavior of samples with V showed an increment with time, resulting in darker samples compared to the control of SCM, which would suggest an enhancement of MR when V is added.

Figure 6 – Samples obtained after heated with sodium metabisulfite (SMB), Vanilla extract (VE), and Vanillin (V) at 40°C for 21 days



Font: Made by the author (2023).

Along the experiments was identified no direct correlation between HMF and BI, as can be seen in the Figure 6, in which samples added with SMB and VE, in which presented the highest values of HMF and presented the lower values of BI. In contrast, samples added V presented the highest BI and the lowest values of HMF. Considering the Equation 1, this result can be explained considering that BI is defined from the color parameters a* and b*, but also

from the lightness value, L*. As consequence, samples with different lightness but equal color will have different BI values, as was observed in this study.

The colorimeter used for the analyses made in this study did not exclude the specular effect caused by lightness of the samples. For that, the results are influenced by the shining of the sample. For comparison of samples with characteristic or very different lightness an alternative is the use of a color equipment that exclude the specular effect or made conclusions directly from the color parameters a* and b*.

Samples enriched with SMB after 21 days of storage did not develop significant browning, as can be seen in Figure 6 and the low values of browning index (Graphic 3). Samples with SMB heated at 121°C for 15 min presented values of HMF between 188 and 227 $\mu mol \cdot kg^{-1}$, samples heated for 21 days at 40°C from 218 to 311.85 $\mu mol \cdot kg^{-1}$ and to the controls with 220.5 and 244.04 $\mu mol \cdot kg^{-1}$, with the difference that those heated at 40°C did not developed browning. Although the values of HMF for the same concentrations of samples with SMB were higher for those heated at 40°C the browning with intense heating at 121°C was higher.

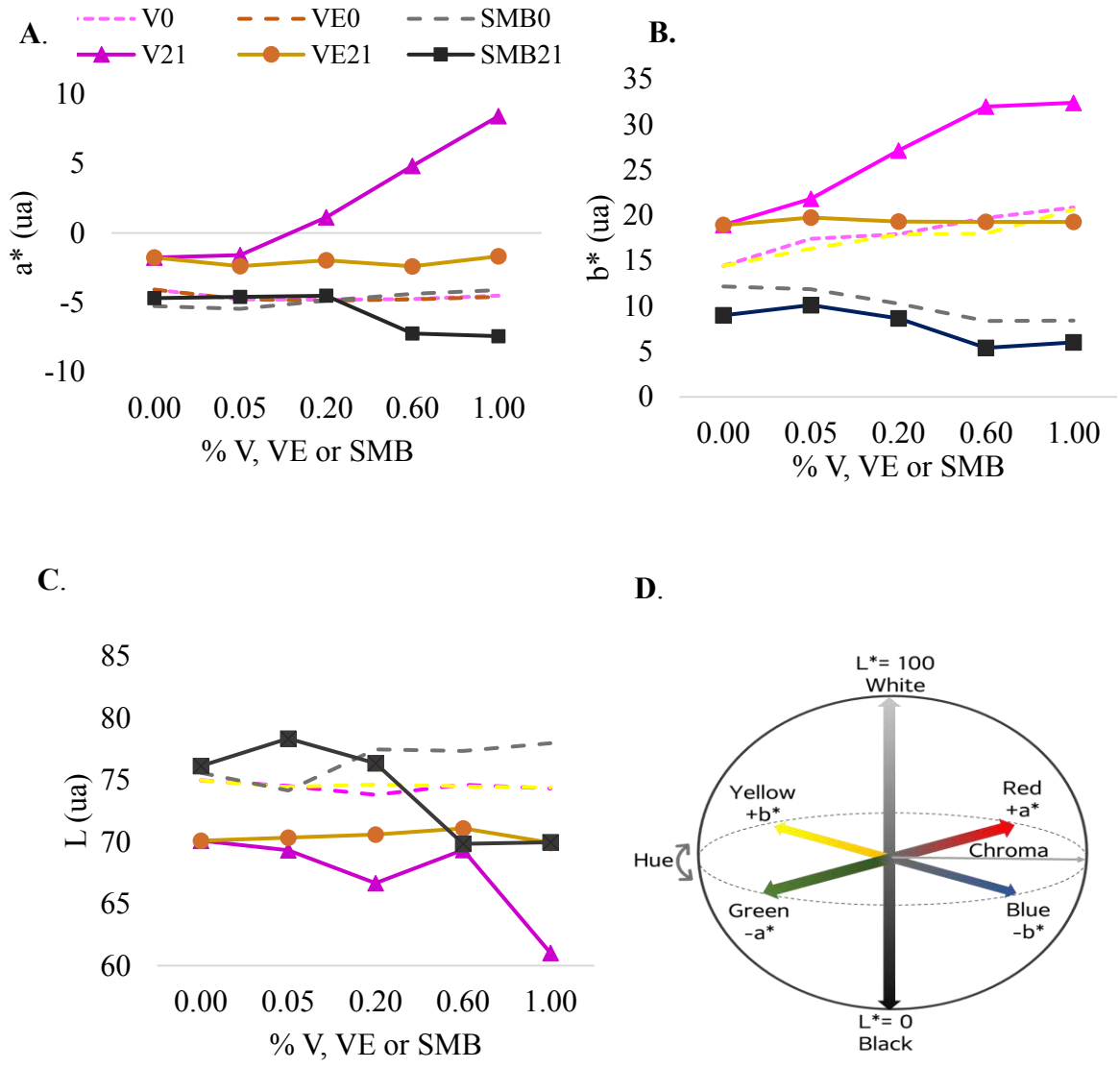
In the case of V, the MR products can convert into melanoidins to achieve the final brownish color which is evidenced by higher values of BI (Graphic 3). Another hypothesis to explain the role of SMB in MR would be that SMB can interact with proteins by favoring their cross-linking. Sulfites are known to cause protein precipitation (FRIEDMAN; MOLNAR-PERL, 1990) and many of these mechanism interactions are irreversible. Therefore, the presence of SMB can limit the availability of proteins to develop MR analogue to a sterically hindrance. This hypothesis needs to be deeply studied.

In the Graphic 4, are shown the changes in the parameters of lightness L*, a* and b* along the heating during 21 days for all samples, their combination helps to explain the color of the DL. The parameters a* and b* presented similar tendences, and for those with V was evidenced the highest values (Graphic 4, a and b), which means these samples tend to be more red and yellow, which is congruent with their characteristic orange color observed for these samples.

It was evidenced a decreasing of lightness as consequence of the heating process, from L* of 76.12 ± 1.13 to 70.10 ± 0.89 for the control of SCM. This fact is expected because lightness is also related to viscosity and is well known in the DL technology that heating

increases the product viscosity due to the denaturation and loss of solubility of milk proteins (PERRONE, Í. T. et al., 2012). Though, samples of V had the lowest values of lightness which decreased with V concentration reaching to 61.03 ± 0.76 for the sample with 1.00% of V. As lightness is related to the fat-protein tridimensional structure of the DL this could means V caused a differentiated structure of DL than the others.

Graphic 3 - Color attributes of samples after heated with sodium metabisulfite (SMB), Vanilla extract (VE), and Vanillin (V) at 40 °C for 21 days.



Font: Made by the author (2023).
Note: V0, VE0, SMB0: Samples before heating. V21, VE21, SMB21: samples after 21 of heating.

In contrast, samples with SMB had the lowest values for the parameters of color a* and b* which did not change significantly when compared to the values for the control of SCM and as consequence their color did not change, staying lighter. However, the decreasing of lightness showed dependence on the concentration of SMB. Lastly, samples with VE had the most uniform change behavior of the color parameters, when compared to control of SCM, keeping almost the same color after the heating and among the range of VE concentration assessed.

5.4 pH CHANGE

If the initial pH of the system after the addition of inhibitor is low, the more protonated amino groups are present and therefore, less reactive for glycation (MARTINS; JONGEN; BOEKEL, VAN, 2000). This equilibrium is very dependent on the pKa's of the amino groups involved, that for lysine pKa's involved at the pH range worked are 10.52 (pKa3) and 8.92 (pKa2) (ISOM et al., 2011), those would be the most involved amino group on the medium of SCM.

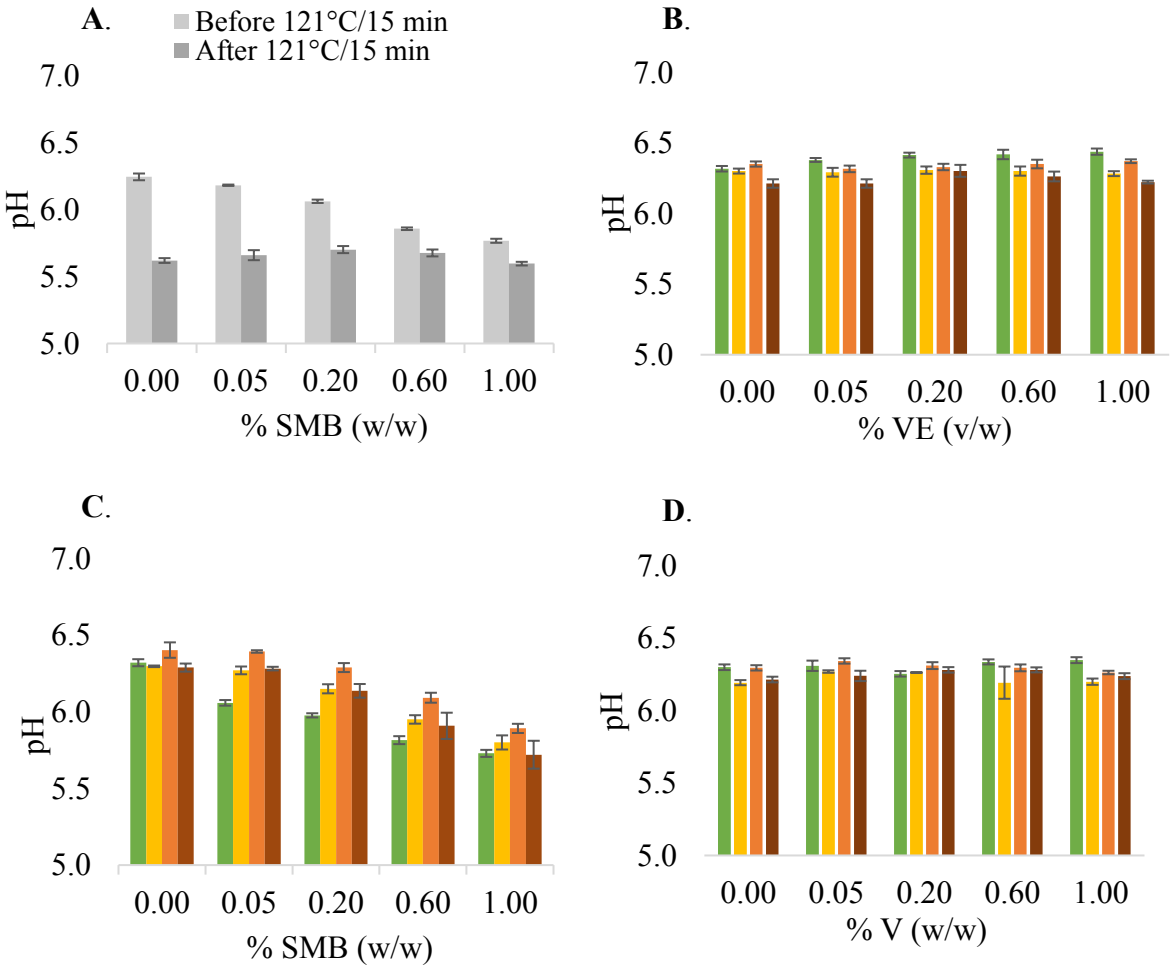
The Graphic 2 shows the pH profile along the time of samples of SCM with SMB, VE, and V. After the addition, the initial pH of the SCM was influenced by the nature and concentration of the substance added. SMB mixtures have a steeper negative slope at the range of evaluated concentrations and lower values over time. The pH for samples with SMB showed a fall with the increasing of its concentration and with the time, going from 6.31 ± 0.02 for the SCM control reaching to 5.72 ± 0.01 after 21 days for the sample with SMB 1,0% (w/w). The same result was obtained at intense and 40° C heating conditions (Graphic 2.a, and 2.b).

This drop of pH disfavors the availability of basic nitrogen from the amines that should participate in MR, limiting the development of browning, and although the MR could continue to occur, at these acidic conditions it will preferentially take the route of formation Schiff bases precursors of furfurals (MARTINS; JONGEN; BOEKEL, VAN, 2000).

In addition, the production of DL is intrinsically associated with an acidity development, from the milk to the final product. Heating treatment of milk induces changes in salt equilibrium because calcium phosphate is transferred from its soluble form to a colloidal state, liberating protons and decreasing the pH (Perrone, et.al, 2019). This effect was clearly observed in graphic 3.a for the control of SCM after intense heating.

However, previous studies of amino acid-sugar systems have demonstrated MR can also contribute to pH fall because some MRPs like carboxylic acids, aldehydes or intermediary products with multiple hydroxyl groups are formed by condensations and with strong reducing power (MARTINS; JONGEN; BOEKEL, VAN, 2000). This pH decrease can be buffered by the components of the food matrix like the remaining lysine residues of milk proteins. Meanwhile, in the absence of any buffering species, for example, at later stages of the reaction when these lysine residues have been consumed, the pH drop can be large and significant and the rates of further pathways with acid-catalyzed mechanisms like furfural synthesis will be increased (NEWTON et al., 2012). Then, MR itself may have a contribution in the typical lowering of pH in the production of *DL*.

Graphic 4 - pH profile of samples with sodium metabisulfite (SMB), Vanilla extract (VE), and vanillin (V)



Font: Made by the author (2023).
Note: pH profile of samples with SMB after autoclave heat treatment (A). pH profile of samples after oven heating at 40° C with VE (B), SMB (C), and V (D). Before heating. After heating 7 days After heating 14 days. After heating 21 days (n=3).

The pH fall for samples after 40° C heating was lower, even after 21 days. This corroborates the influence of temperature on the MR rates. However, unlike the SMB samples, VE, and V, did not significantly change the initial pH of the condensed milk. Samples with VE slightly increase the pH from 6.31 ± 0.02 for the SCM control reaching to 5.72 ± 0.01 after 21 days for the sample with SMB 1,0% (w/w).

When compares pH fall of the controls of SCM with the falling in samples with V, and VE, is possible to conclude the presence of them cause changes in the sample, favoring the route of MR. With merely a slight decreasing of pH for all the different concentrations tested and along the time for samples with VE, and V, pH did not change significantly respect to the control of SCM with the concentration and time.

5.4.1 Influence of the pH in the retarding effect of MR due to SMB

No browning was evidenced on SCM after heated at 1.00% (w/w) of SMB, the initial pH of SCM was 5.8, being a noticeable factor that disfavor MR. From the above, it was evaluated if the lowering of pH is the main factor to inhibit the melanoidin formation. So, it was evaluated if the adjustment of pH to 5.8 with different acids, could also slowdown the darkening of SCM.

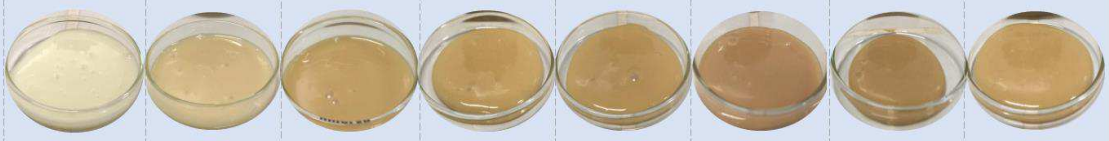
The drop of pH when SMB dissolves in SCM may be a consequence of the change in the ionic equilibria in the food matrix, releasing protons that can bind with the electronic pair of nitrogen from proteins, leaving it less reactive or available to glycate and delaying MR progress. However, other possibilities can exist, for instance, a chemical interaction of SMB with proteins and sugars, by blocking them occupying their reactive place or decreasing their affinities. It can also be a combination of all these factors.

In this study, the pH effect was studied individually establishing a constant pH of 5.8 based on the drop caused by SMB 1.0% but with five different acids, that were acetic, hydrochloric, lactic, gallic, and ascorbic acid. The study included a control of SCM at unaltered pH. The change of pH for the control of SCM showed a small increase after heating for 20 days, and then a decrease to reach a pH of 5.8 after 40 days. This developing of total acidity after thermal treatments of dairy products is known in DL production, due to lactose degradation, casein dephosphorylation and precipitation of calcium phosphate, but also, due to formation of

acids like formic, acetic, lactic, pyruvic, propionic, and butyric in the MR (CARNEIRO et al., 2021).

Results in Figure 6 suggest a buffer effect of SCM, avoiding the decrease of pH below 5.8, and the SMB seems to strength that effect along the time. All the acids, except ascorbic, showed the same behavior as the control of SCM, a small increasing of the pH after 20 days and then after 40 days a fall to the initial value of 5.8. However, considering that the pH of the samples with acids stayed almost constant, and at lower values than the SCM control, it was expected that they should be lighter than SCM and presented a color close to SMB samples, but all the acid samples darkened compared to the control and SMB 1.0% (w/w). This demonstrated that dropping of the pH due to the addition of SMB is not the only feature that makes SMB delay MR.

Figure 7 – Samples equilibrated with initial pH of 5.8 with different acids after heated for 40 days at 40 °C

Sample	SMB 1.0%	Control 25°C	Control 40°C	Lactic	Acetic	Gallic	Ascorbic	Hydrochloric
								
BI	3.6	25.3	40.9	42.3	46.0	46.4	49.9	50.4
HMF ($\mu mol \cdot kg^{-1}$)	212.5	120.1	205.0	239.0	182.7	223.2	124.8	176.2

Font: made by the author (2023).
Note: samples of SCM heated at 40°C for 40 days.

As in the previous tests with SMB, VE, and V, the free furfurals F, FMK, and MF, did not appear constantly, nor with a noticeable tendency among the samples, while HMF appeared in all of them with reproducibility and it was used as intermediary marker of the progress of MR.

The HMF behavior oscillated along the time for samples with the acids, compared to the profile of samples with SMB that increased with the time. Nevertheless, among them the behavior was similar, all of them increased the initial content of HMF with their solubilization, probably due to releasing part of the HMF linked to proteins. A lower increase was evidenced for the samples with ascorbic acid which with the higher pH obtained for its samples, revealed the presence of ascorbic acid induced alteration in MR routes, giving different colored final products.

After 20 days of heating, the HMF content grew constantly for the control and SMB samples (Graphic 5), with similar values for samples with SMB. While for the samples with acids the content of HMF decreased. Neither acid decreased the content of free HMF as SMB did before the heating, only the samples with hydrochloric and ascorbic acid ended with less content of HMF than at the beginning, with a noticeable steep for the ascorbic acid sample.

Then, after 40 days of heating, HMF increased for all the samples, and the sample with lactic acid presented the lower final content of HMF. This showed a nonlinear behavior of HMF formation in these food matrices that can be related to the complexity of dairy products, allowing huge possibilities of paths for MR occurrence with temporary favorable conditions.

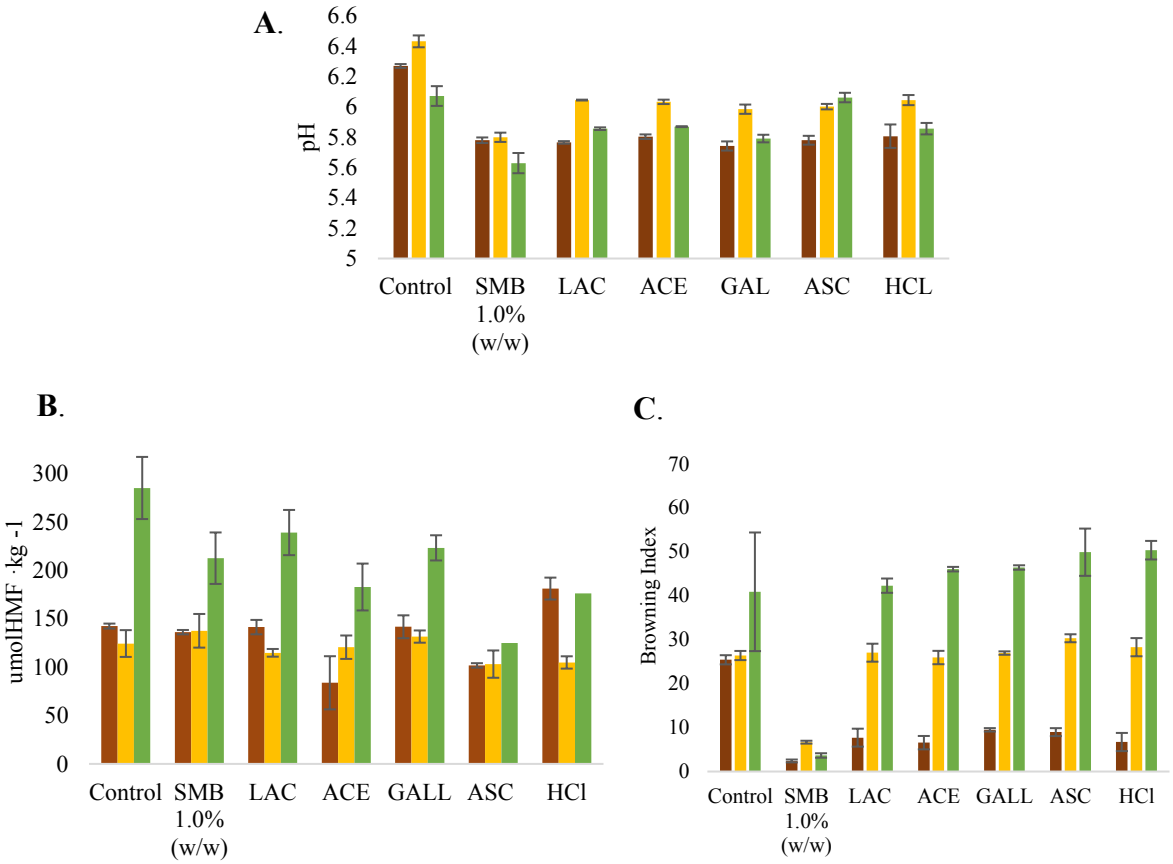
The behavior of color parameters obtained in the systems with VE did not change considerably in comparison to the control of SCM after heating, giving samples with low BI values and light darkening perceptible along the concentrations assessed. However, the content of HMF for those samples was higher than the obtained for controls and for samples with V. This behavior was also observed for samples with SMB, considering that VE would be a promissory alternative flavoring for foods which include vanilla flavor or aroma in which browning is undesirable, instead of the use of the synthetic V. Natural VE, despite being more expensive, demonstrated to be suitable for avoiding browning, in comparison with V additive.

Ascorbic and gallic acid samples showed evident darkening (Figure 6) but without an increasing of BI, in comparison with the samples with lactic, hydrochloric, and acetic acid. These both compounds are thermolabile and unstable under many conditions, then, their degradation products can interfere in the progress of MR, with the possibility of a boosting effect that causes the observed browning. The presence of these acids can cause that MR undergoes different routes, in which HMF is consumed to form another kind of melanoidins that need a higher proportion of HMF, and this can be supported considering the difference on the color of the DL obtained in comparison to the other acids (Figure 6).

It was also evidenced that samples with acetic acid had darker color than hydrochloric samples but also had lower values of BI. However, hydrochloric acid samples showed to be lighter than acetic ones. This result demonstrated that having higher values of BI are not necessarily associated with darker samples, and this could be understood if consider that the

equation of BI (Equation 1) in addition to the color parameters a* and b* also depends on the value of lightness L*. The applied method of analyses did not exclude the secular parameter, this means that lightness of the sample can influence the final BI value. Then, as SCM and DL are samples with characteristic lightness, is expected to generate BI scales that not necessarily correlates with their browning.

Graphic 5 – pH change, 5-hydroxymethylfurfural (HMF) and Browning Index (BI) profile of samples with initial pH of 5.8



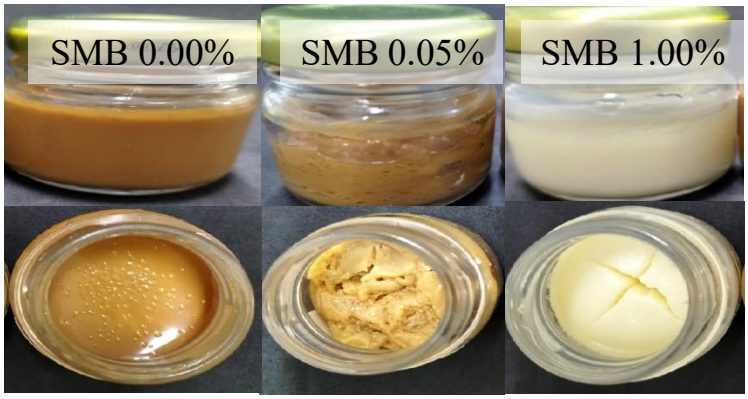
Font: Made by the author (2023).
Note:pH (A), HMF (B) and BI (C) profile of samples after 40° C heat treatment with SMB, lactic acid (LAC), gallic acid (GAL), hydrochloric acid (HCl), acetic acid (ACE) and ascorbic acid (ASC). Before heating. After heating 20 days. After heating 40 days (n=3).

The addition of acids decreased the initial BI value compared to the control, but none of them reach the steep lowering of BI caused by SMB addition. Considering that melanoidins are polymers made up from intermediary MRPs, like HMF, it is congruent to infer that part of the content of HMF is consumed to form them, giving darker DL in comparison with the other

samples. On the other hand, acetic and lactic acid samples ended with the higher values of HMF and at the same time with large values of BI.

After 20 days of heating, the BI of the control stayed almost constant while for all samples with acids it increased, contrary to the SMB sample. The sample of acetic acid was the one with a higher final BI value, but visually was not the darker one. Samples with lactic acid did not get as dark as the ascorbic and gallic ones, although they showed the higher value of HMF. This can be related since lactic acid and acetic acid are products of the MR in the intermediary stage mainly due to Strecker degradation. Then the increasing in concentration of those acids can alter the reaction equilibrium favoring towards the reactants and not towards the products, which in this case would be the melanoidins, and in consequence those samples were lighter.

Figure 8 – Appearance of samples with SMB



Font: made by the author (2023).

In terms of observable texture, samples with SMB from a concentration of 0.05% (w/w) had rougher texture than the respective controls (Figure 8). In the technology of DL production is well known the fact that in parallel to the MR, heating also causes a pH decrease due to changes in ionic force, favoring protein denaturation and crosslinking which increases the product firmness. The MR itself causes a loss in available lysine of 36.2% in SCM as reported before by Pizzoferrato et al., 1998, can modify the protein structure and functional properties, like protein bioavailability, solubility, foaming, emulsifying, and heating stability. Therefore, MR is also involved in the firmness development of the product.

All this ultimately change in global texture evidenced by the addition of SMB may be due to a favoring of these interactions because of the action of the SMB as a bridge between milk proteins. Whey proteins are the most available and more susceptible to thermal denaturation and precipitation in the heating process of dairy products (Perrone, et.al, 2019), their functional groups have a higher exposition and probability of reacting easily with SMB until its aggregation. In dairy products, β-Lactoglobuline drives the thermal behavior of whey proteins, acting as linker by forming disulfide bonds (SHIMAMURA; UKE, 2012), this may be favored in presence of SMB, and protagonists in that higher firmness development.

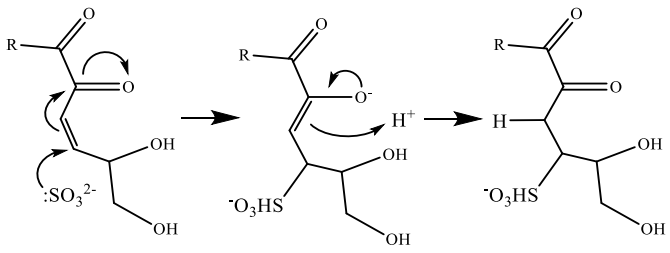
The above is contrasted with the fact that samples prepared with other acids, presented smoother consistencies when compared to SMB samples. Acids like acetic, hydrochloric, lactic, gallic and ascorbic are not as great protein linkers, like SMB is. Then, even lowering the pH heated samples did not develop the same firmness. All these hypotheses should be evaluated deeply with rheology analyses and protein interaction studies.

$$SO_2 \cdot H_2O \rightleftharpoons HSO_3^- + H^+ \quad (3)$$

$$HSO_3^- \rightleftharpoons SO_3^{2-} + H^+ \quad (4)$$

To sum up the role of SMB as MR inhibitor, the obtained results and the reported literature suggests that the drop of the sample pH is related to the fact that dissolution of sulfites releases protons (Equations 3 and 4) (WEDZICHA; BELLION; GODDARD, 1991), and the presence of sulfite species also can modify the salt balance and ionic strength of dairy matrices. In the presence of sulfite species, and the consequent releasing of protons, the dairy system will respond releasing the calcium hydrogen phosphate ($CaHPO_4$) associated to casein micelles, solubilizing, leaching out it from the micelle structure, leading them unstable and more available to aggregation (GAUCHERON, 2005).

In addition, sulfite ion is a great nucleophile that may bind irreversibly with electrophilic residues of the sample, like dicarbonyl groups (Equation 5). This should explain the interaction of SMB with the initial HMF of SCM, reducing its concentration, but it also demonstrated the possibility to interact with melanoidins, acting as a bleaching agent and decreasing the value of BI of SCM. These hypotheses also must be deeply studied by techniques of protein interactions.



(5)

The effect of SMB over the texture of DL, as Figure 8 shown, can be another feature that makes it a MR inhibitor. It evidently can affect the tridimensional structure of the food matrix, giving rough DL, and this can limit the lactose molecules mobility to react with proteins, delaying the occurrence of the reaction. This probably is related to an interaction of SMB with electropositive residues of proteins acting as a bridge favoring protein cross-linking, that in combination with the change in ionic strength due of altering the salt equilibria can favor micelle aggregations and formation of rigid dairy structures.

5.5 PROPERTIES OF AN IDEAL RETARDING AGENT OF MR

A suitable retarding agent of MR could be purposed based on the properties of SMB identified in this study. Considering the early stages of MR it should interact with reagents of the medium (reducing sugars and proteins/ amino acids or free amino groups) to dismiss their availability or the concentration of their reactive form, for example, protonating the free electronic pair of nitrogen or substituting the carbonyl of the reducing sugar, but after that they should not undergo similar pathways as the MR to the formation of colored products, like was evidenced for V.

Some other substances, such as phenolic acids, vitamins, aminoguanidine, thiols extracted from garlic or onion, and L-cysteine (L-cys) had demonstrated activity preventing MR by the removal of HMF, converting it into another adduct with less reactivity, then HMF is considered as a key product to inhibit the reaction (YANG et al., 2021).

For the intermediary stage of MR, trapping agents of radicals and intermediate MR products like furfurals and dicarbonyls could work with good results, this stage of MR is a strategic point of the reaction to avoid synthesis of melanoidins and formation of brown color.

For the purposes of the present study, the behavior of SMB and VE would be the most adequate. When compared V with VE samples, all the properties assessed of HMF content, pH change BI and color and lightness parameters demonstrated superiority on VE over V, giving DL with lighter color at the range of concentrations assessed.

It is important to consider the conditions of processing and the nature of the food matrix to select a possible additive to delay MR, at storage conditions where temperatures are not too drastic. Natural extracts may be a good alternative because conditions are favor to its antioxidant activities. However, if the additive needs to be subjected to drastic processing conditions together with the food product, is necessary to consider the thermal stability of the additive, to guarantee its activity and bioavailability. Then, the moment of addition among the processing steps, and the conditions of processing are also factors to consider for the selection of a possible candidate to retard MR in real food systems.

6 CONCLUSIONS

SMB should have many roles as retarding agent of MR in addition to the lowering of the initial pH of SCM, capturing HMF. The visual appearance of the DL obtained with SMB suggests it could also interact with proteins modifying the firmness of DL by limiting their mobility and availability to undergo MR, however, these hypotheses need to be studied deeply.

V showed a competitive-non inhibiting MR activity. The obtained parameters of color a* and b* suggested the production of colored analogs of melanoidins at higher rates than in control samples, boosting effect or catalyzing MR instead of inhibiting it.

VE would be a promissory alternative flavoring or aroma for foods which include vanilla in which browning is undesirable, instead of the use of the synthetic V. Natural VE, despite being more expensive, here demonstrated being suitable for avoiding browning, in comparison with the synthetical additive. The set of compounds that the extract has, could work together synergically to delay the MR.

6.1 FINAL CONSIDERATIONS

SCM and DL obtained by heating it, were suitable food systems to study the MR progress in real food matrices. The heating treatments elucidated the great influence of temperature in MR development on dairy products, at two comparable reaction rates, showing that after 21 days at 40 °C the content of HMF is almost half of the content obtained for heating 15 min at 121°C.

The BI demonstrated to have an important influence of the lightness value of samples of DL and SCM. This made that the correlation between darkness and BI of light samples were not direct for all the experiments. This should be considered when this parameter is pretended to be used working with shining samples, in these cases, the study of parameters of color a* and b* should be a better option.

Controlling MR does not necessarily means stop it, at the same level that SMB does. Ideally, food additives may not interact irreversibly with nutrients, like amino acids or proteins, because it reduces their availability and nutritional value into the food. Additionally, an ideal MR inhibitor may not decrease too much initial pH, because low pHs favor salts precipitation and micelle aggregation, in many dairy products this negatively alters their quality and stability. In this sense, it should be a good nucleophile, able to react faster than lysine with reducing sugars or with enough nucleophilicity to block carbonyl groups of intermediary and advanced MR products and not reduce the nutritional value of the product.

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