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ENZYMATIC GLYCEROLYSIS OF VEGETABLE OILS AND ITS APPLICATION IN THE PRODUCTION OF HIGHLY UNSATURATED CHOCOLATE SPREADS

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1 INTRODUCTION

Consumption of food is an important issue in society's life because rather than being concerned about physical standards, health has become a conscious subject to avoid diseases. Currently, many foods contain fat, and although marketing has been in charge of creating a stigma around it, the truth is that fats are necessary for human health.

However, there are different types of fats unsaturated, saturated, and trans, which can be defined as good, not so bad, and bad respectively. Unsaturated fats are mainly of plant origin for instance vegetable oils (olive, canola, sunflower, soy, and corn oil), and help to lower LDL (low-density lipoprotein) cholesterols and increase HDL (high-density lipoprotein) which aids in removing LDL from the bloodstream leading to healthy cholesterol level. Saturated fats are predominantly of animal origin but there are vegetable exceptions as plant, cocoa, and coconut oils. Its special characteristic is the solid capacity at ambient temperatures that benefits be estimated in the food industry. On the other hand, they can raise blood cholesterol levels and provoke heart disease. Trans fats are frequently artificially derived by industrial hydrogenation of vegetable oils and are particularly linked with heart disease to raise levels of blood cholesterol (Gibson & Newsham 2018). Chocolate spread is a sweet chocolate-flavored suspension of solid particles whose ingredients are cocoa content, sugar content, and fat source. These components contain high levels of saturated fatty acids. This is the reason that removing and replacing trans fats is the main purpose of the product (Talbot 2011). Palm oil was the first option to substitute fatty acids, however, its nutritional value is questionable due to high levels of saturated fatty acids, and the environmental impact led to deforestation and habitat loss, which has increased together with the palm oil demand.

Another way to replace it is using other vegetable oils high in unsaturated fatty acids. However, they usually do not have favorable effects on the technological quality such as thermal loss, and organoleptic properties, but over time, investigations have been approached to develop an ideal vegetable oil modification through conventional techniques like hydrogenation, chemical interesterification, enzymatic interesterification, emulsification, oleogelation, and enzymatic glycerolysis (Soleimanian u. a. 2023). It consists of changing liquid oils into solid fat. The triacylglycerols (TAGs) convert into monoacylglycerols (MAGs) and diacylglycerols (DAGs). This process will increase the crystallization temperature of oils but keep the same fatty acid composition and provide the solidity required for food applications. Thereby, the innovation

purpose is to replace palm oil with highly unsaturated healthier oils but keep the same physical properties that palm oil in chocolate spread (Nicholson & Marangoni 2020).

2 OBJECTIVES

The main purpose of this thesis is to produce chocolate spread by substituting conventional fats with vegetable oils, particularly high in oleic sunflower oil, following an enzymatic glycerolysis reaction.

The study is structured into two main phases: enzymatic glycerolysis and chocolate spread formulation. Each phase will be comprehensively evaluated to assess various parameters indicative of product performance and characteristics.

The following steps will be undertaken: an analysis to determine the optimal duration for enzymatic glycerolysis, followed by the selection of the product exhibiting optimal levels of triacylglycerols and diacylglycerols for chocolate spread production.

Subsequently, the chocolate spread will undergo evaluation using texture analysis, differential scanning calorimetry, and sensory assessment.

Ultimately, this study aims to provide insights into the acceptability and behavior of the product.

3 LITERATURE OVERVIEW

3.1 Vegetable oils

In recent years, the use of vegetable oils has increased in the production of food, medical, and cosmetic products. Vegetable oils are obtained through methods based on mechanical processes such as pressing plant parts like seeds, the use of solvents like hexane, and some through the combination of both methods (Carbonell Verdú 2018).

Vegetable oils are composed of triglycerides, which are esters of fatty acids and glycerol (Agüero u. a. 2015). These oils are stable compounds but chemically deteriorate due to oxidation or hydrolysis (Carbonell Verdú 2018). The oxidation of polyunsaturated lipids leads to alterations known as rancidity, resulting in unpleasant flavors and odors (Carbonell Verdú 2018).

Among the most common, we can find palmitic, oleic, linoleic, linolenic, and stearic acids. Sunflower or soybean oils have high contents of polyunsaturated fatty acids such as linoleic and linolenic acids. In contrast, canola and palm oils are composed of large amounts of monounsaturated fatty acids like oleic acid (Carbonell Verdú 2018).

3.2 Types of oils

- Palm oil

Palm oil may be fractionated into two major fractions: a liquid oil (65–70%) palm olein and a solid fraction (30–35%) stearin. Palm oil is the second major edible oil used worldwide. Palm olein (PO), a liquid fraction obtained from the refining of palm oil, is rich in oleic acid (42.7–43.9%), beta-carotene, and vitamin E (tocopherols and tocotrienols)(Nicholson & Marangoni 2020).

It is used in a variety of food industry products, such as cooking oils, shortenings, and margarine, and it is also employed in the oleochemical industry as raw material for soap, candles, and lubricants manufacturing (Agüero u. a. 2015).

The physicochemical properties of palm oil include melting point, the profile of free fatty acids, moisture and volatile matter, and impurities that can be found in the oil, allowing for the determination of its potential percentage (Acuña Cristian u. a. 2019).

– Sunflower oil

Sunflower oil contains approximately 15% saturated, 85% unsaturated fatty acid and consists of 14–43% oleic and 44–75% linoleic acids in its unsaturated fatty acid content(Akkaya 2018).

It is obtained from the pressing of sunflower seeds and is marketed in three forms: the first one with a high content of polyunsaturated fatty acids, 75%; the second with 45% oleic acid, and a third form with 14% stearic acid (Agüero u. a. 2015).

The main properties of sunflower oil include the fatty acid profile highlighting a low content of saturated fatty acids, making it a healthy option, vitamin E content, high smoke point, as well as being neutral in taste and aroma (Naranjo u. a. 2015).

– Canola oil

Canola oil is considered a very healthy oil due to its fatty acid composition. It averages about 60% oleic acid (C18:1), 20% linoleic acid (C18:2), and 10% ALA (C18:3)(Barthet 2016).

It is extracted from the seeds of a plant belonging to the Brassicaceae family. It is recommended for improving omega-3 fatty acid levels (Agüero u. a. 2015).

The main properties that can be highlighted of canola oil are the fatty acid composition with low levels of saturated fats and high levels of monounsaturated and polyunsaturated fats, it contains phytosterols and antioxidants attributing benefits to cardiovascular health (Giacopini De Zambrano 2012).

– Soybean oil

Soybean oil contains 7–10% palmitic acid, 2–5% stearic acid, 1–3% arachidic acid, 22–30% oleic acid, 50–60% linoleic acid, and 5–9% linolenic acid. The fatty acid composition of soybean oil includes a high level of polyunsaturated fatty acids (Fan & Eskin, 2010)

It is obtained from pressing soybeans and is used in human and animal food. Although it has lower thermal stability compared to other oils, it is used for frying and cooking (Agüero u. a. 2015)

The properties that can be highlighted of soybean oil are the fatty acid composition where the presence of unsaturated fatty acids predominates. Among the chemical properties are the acid value, iodine value, peroxide value, and saponification value. Finally, a low moisture content is highlighted, indicating a lower probability of hydrolysis and therefore better oil quality (Lafont u. a. 2014).

3.3 Triacylglycerol

Triacylglycerol makes up the majority of fats and oils. It is made up of a glycerol molecule esterified to three fatty acids. The particular fatty acids esterified and the real position those fatty acids occupy define the physical characteristics of triacylglycerol. A simple triacylglycerol is a triacylglycerol that contains three identical fatty acids. The majority of the fat is composed of mixed triacylglycerols, which are triacylglycerols that include two or three distinct fatty acids (Lichtenstein 2013). According to their number of carbon chain lengths, the fatty acids and their corresponding triacylglycerols are classified as short fatty acids (SCFAs) with less than six carbons, medium-chain fatty acids (MCFAs) 8-12 carbon atoms, and long-chain fatty acids (LCFAs) 14 or more carbons(Ferreira & Tonetto 2017).

Table 1: Classification of Fatty acids

(Source: Ferreira & Tonetto 2017)

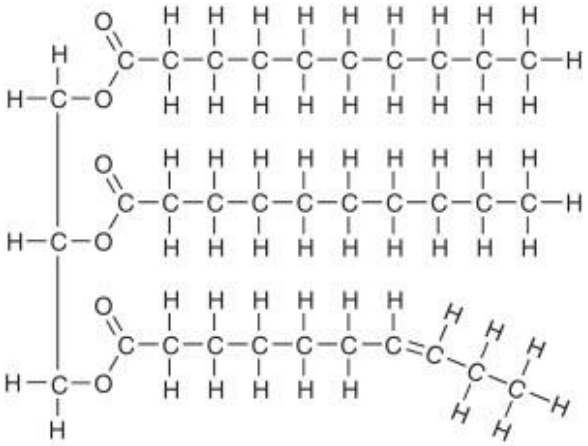
	Fatty Acids					
Classification	**SCFA C2-C6**	**MCFA C8-C12**	**LCFA C14-C22**			
Saturation degree	**Saturated**	**Saturated**	**Saturated**	**Monounsaturated Fatty Acids**	**Polyunsaturated Fatty Acids**	
					ω-6	**ω-3**
Types of Fatty Acids	Butyric acid 4:0	Lauric acid 12:0	Stearic acid 18:0	Oleic acid 18:1 (n-9)	Linoleic acid 18:2 (n-6)	Alpha-Linolenic acid 18:3 (n-3)
Examples of Fats and Oils that contain the Fatty Acids		Palm oil Coconut oil	Cocoa butter	Virgen olive oil High oleic sunflower oil	Soybean oil Sunflower oil	Fish oils Linseed oil

The classification according to the saturation degree

- Saturated Fatty Acids are chains that don't have double bonds. Straight molecules made of single bonds can solidify at normal temperatures(Ferreira & Tonetto 2017).
- Unsaturated Fatty Acids are chains with one or more double bonds. Because of the bend in the C chain caused by double bonds, the molecules pack more loosely and are liquid at ambient temperature. They are also classified into: Monounsaturated and Polyunsaturated Fatty Acids(Ferreira & Tonetto 2017).

The location and physical properties of the fatty acids esterified to glycerol define a triacylglycerol's melting point (their chain length, number, position, conformation of the double bonds, and stereochemical position)(Lichtenstein 2013)

Figure 1: Triacylglycerol molecule.
(Source: Lichtenstein ,2013)



The fatty acids make up about 90% of the molecular weight of triacylglycerol. Esterification of one or two fatty acids to glycerol is present in mono- and diglycerides, respectively. They are mostly byproducts of intracellular metabolism, circulatory clearance, or the breakdown of triacylglycerol. They are used in processed foods as emulsifiers(Lichtenstein 2013).

One of the most significant lipids in blood, triacylglycerides are deposited on the inside walls of arteries, primarily in the heart and brain. This results in atheromatous formations (plaques or atheromas) that diminish the interior arterial lumen, a condition known as atherosclerosis. These growths have the potential to obstruct the artery and result in ischemia or necrosis if they get big enough. illnesses that are indicative of cerebral or myocardial infarction (Ildefonso Arocha Rodulfo u. a. 2009).

3.4 Monoacylglycerides and Diacylglycerides

Monoacylglycerols (MAGs) and diacylglycerides (DAGs) are defined as esters synthesized from glycerol and fatty acid in conditions where pH commonly is greater than 7, the ram material where they are manufactured is through the plant or animal reaction. Since they provide various benefits, such as smooth ingredient mixing, prevention of separation, reduction of stickiness, control over crystallization, dispersion of ingredients, facilitation of product dissolution, and enhancement of overall product stability, mono and diglycerides are commonly used as food emulsifiers (Barfod & Sparsø 2007; The International Food Additives Council (IFAC) o. J.).

Besides, MAGs and DAGs are nonionic molecules with hydrophilic and hydrophobic portions. They have an ester linkage that though a covalent bond joins to glycerol. Diglycerides can be further classified as 1,2(2,3)-DAGs and 1,3-DAGs isomers, while Monoglycerides can be separated into 1(3)-MAGs and 2-MAGs isomers. The 1(3)-MAGs isomers make up the majority of MAGs; at low temperatures, they make up 92%–95% of the total, while at very high temperatures, they make up roughly 70%. In DAGs, 1,3-DAGs are specifically more thermodynamically stable for the steric impact of the structure (Zheng u. a. 2023).

3.5 Properties of Monoacylglycerides

Because MAGs have a hydrophilic head and a hydrophobic tail, they are good emulsifiers, especially when combined with water and oil. MAGs make for over 75% of all emulsifiers used in the food sector globally (Zheng u. a. 2023).

Omega-3 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are found in MAGs and can help prevent a variety of cardiovascular conditions. Because of their nutritional value, ability to regulate inflammation, metabolism of cholesterol, and brain activities, they are beneficial to human health(Zheng u. a. 2023).

3.6 Properties of Diacylglycerides

Esters of the trihydric alcohol glycerol in which two hydroxyl groups are esterified with long-chain fatty acids are known as diacylglycerols, or "diglycerides". DAGs perform a variety of functions, including stabilizing water-in-oil (W/O) and oil-in-water (O/W) emulsions, acting as possible antioxidants in O/W emulsions, and serving as an indicator of fresh oil quality (Zheng u. a. 2023)..

3.7 Enzymatic Glycerolysis

It is a method that changes the structure of liquid oils into solid fats. The process consists of converting native triacylglycerols (TAGs) to monoacylglycerols (MAGs) and diacylglycerols (DAGs) in the presence of glycerol and a catalyst (enzyme) (Soleimanian u. a. 2023). The process is realized through hydrolysis of the ester bond in the triglyceride, the next process is the esterification of free fatty acids and glycerol by 1,3-specific lipase(Diao u. a. 2017).

In the food field, the glycerolysis reaction is used to produce partial glycerides as emulsifiers. Partial glycerides (natural components of fats/oils) can crystallize at higher temperatures than TAGs, showing solid-like properties while improving the functionality of vegetable oils (Soleimanian u. a. 2023).

Likewise, glycerolysis products (GP) resemble intestinal digestion natural products and, thus, they are highly biocompatible and suitable as lipid delivery systems in formulations of functional foods and nutritional supplements of high bioavailability and bio-efficiency. Additionally, these enzymatic processes can be industrialized by keeping a clean environment and allowing self-emulsifying lipid mixtures (Bañares u. a. 2022) **.**

Additionally, Nicholson, R. A., & Marangoni, A. G. 2020 say DAG intake in place of TAGS has been shown to decrease low-density lipoprotein cholesterol and total cholesterol while increasing high-density lipoprotein cholesterol levels. It means the substitution TAG by DAG has health benefits because DAG mixtures have 60-70% of the 1,3 DAG isoform produced by acyl migration.

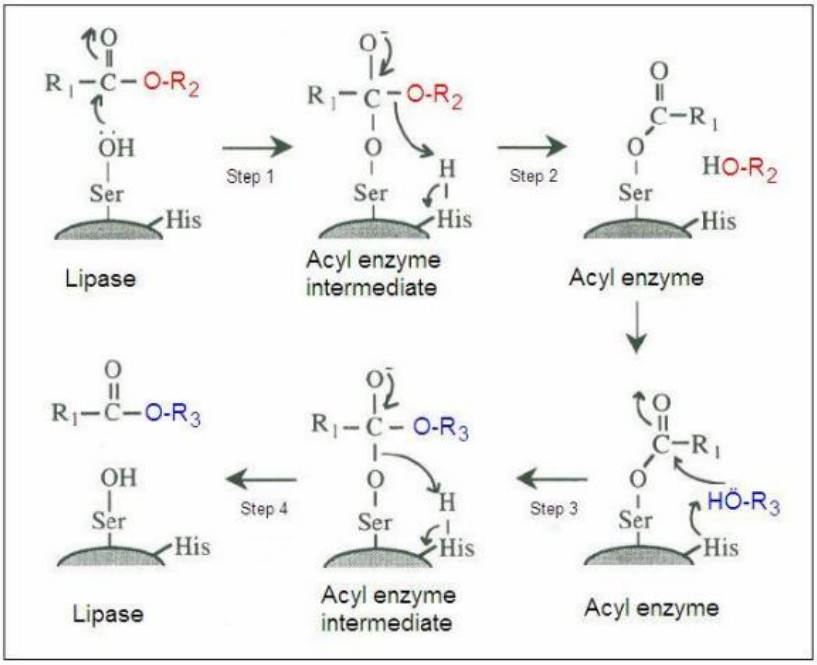
3.8 Lipases

Lipases are a group of enzymes that act as catalyzers on fats and oils for the release of free fatty acids, diacylglycerols, monoacylglycerols, and glycerol. Also, the lipases are involved in esterification, transesterification, and aminolysis (Singh u. a. 2013). The substrates that lipases need to start a reaction are acyl esters of cholesterol (cholesteryl esters), triacyl esters of glycerol (triacylglycerols), acyl esters of long-chain alcohols (wax esters), diacyl esters of glycerol (diacylglycerols), and monoacyl esters of glycerol(Brockman 2013). In general, hydrophobic substrates are preferred for these enzymes. However, full catalytic performance only is archived with the presence of water that allows the activation at the water/lipid interfaces (Singh u. a. 2013).

In enzymatic glycerolysis, lipase is a fundamental part of the process due to catalyzing the hydrolysis of ester in triglycerides for getting glycerol and fatty acids. Triglycerides consist of three fatty acids esterified with a glycerol backbone where lipase can break the ester bonds to produce glycerol and fatty acids (Bayly 2014).

One of the most important features of the enzymatic activity of lipases is their composition of protein structure that includes active site formed for the triad of amino acids: serine (Ser), histidine (His), and aspartic acid (Asp). The active site is covered by a polypeptide structure called a 'flap' or 'lid, the function of the covering is to prevent the active site from interacting with undesired substrates. On the other hand, with desired substrates, the covering moves away from the active site allowing the catalytic activity to start in the interface between the enzyme and its substrate.

Figure 2: Proposed mechanism for lipases catalytic action.
(Source: Damstrup ,2008)



Initially, an acyl-enzyme intermediate is produced when the hydroxyl oxygen (×) from the lipase's serine chain nucleophilically attacks a carbonyl carbon on the lipid substrate (R1-COO-R2). Next, a lipase histidine residue that is put close by receives a temporary transfer of hydrogen (H) from the serine hydroxyl group (Step 1). The acyl-group (R1-C=O-) of the intermediate that was created is then covalently ester linked to the lipase's serine group (termed acyl enzyme). Additionally, the

alcohol (HO-R2) that is released from the lipase is formed when the hydrogen from the histidine is transferred to the alcohol moiety of the substrate (-OR2) (Step 2). The "hydrolysis/alcoholysis process," as it is called once the product is liberated, consists of steps 1 and 2. (water or alcohol). Second, the lipase's carbonyl carbon linked to the serine is attacked nucleophilically by the hydroxyl oxygen (^) from water or another alcohol (HO-R3). Another "new" acyl enzyme intermediate is created as a result (Step 3). Once more, this intermediate rearranges, releasing a new ester (R1-COO-R3) (Step 4). After the ester-product is created, steps three and four are commonly referred to as "esterification" or "ester synthesis".(Damstrup 2008)

3.9 Reaction of Kinetic or mechanism in enzymatic glycerolysis

To understand the lipase-catalyzed reaction's mechanism is important to know the kinetic. A study by Choong u. a.(2018) where compared three models Ternary complex, ping-pong bi-bi, and complex ping-pong bi-bi using palm oil, and lipozyme TL IM , asserted that the complex ping-pong bi-bi model was the best model because it proved to have a better agreement between experimental data and model results. The mechanism of the model explains that diacylglycerol had produced become a substrate to the enzyme E forming a binary complex (E.DAG), had followed by the release of monoacylglycerol and modified enzyme complex (E.FFA)(Choong u. a. 2018).

Figure 3: Complex ping-pong bi-bi model.
(Source: Choong , 2018)

$$\mathrm{TAG} + \mathrm{E} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{E.TAG}$$

$$\mathrm{E.TAG} \xrightarrow{k_3} \mathrm{DAG} + \mathrm{E.FFA}$$

$$\mathrm{E.FFA} + \mathrm{G} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} (\mathrm{E.FFA}).\mathrm{G}$$

$$(\mathrm{E.FFA}).\mathrm{G} \xrightarrow{k_6} \mathrm{MAG} + \mathrm{E}$$

$$\mathrm{DAG} + \mathrm{E} \underset{k_8}{\overset{k_7}{\rightleftharpoons}} \mathrm{E.DAG}$$

$$\mathrm{E.DAG} \xrightarrow{k_9} \mathrm{MAG} + \mathrm{E.FFA}$$

3.10 Chocolate

Chocolate is the generic name for the homogenous products obtained from a mixture of cocoa derivatives (Theobroma cacao L.)(de Souza Correia Cozentino u. a. 2022). Also, it is a famous food that is formed by dispersed and continuous phases. The continuous phase is mostly composed of saturated fats (Tirgarian u. a. 2023a) and provides a crystalline network for the dispersion of solid particles (Fernandes Almeida et al., 2024; Li & Liu, 2023).For instance, cocoa butter plays an important role in chocolate's texture, gloss, snap thermal behavior, and bloom stability. The dispersed phase contains a mixture of sugar, cocoa powder, milk powder, and so on.

3.11 Chocolate spread

Chocolate spread is a water-in-oil emulsion formed for the mixture of dispersed (cocoa powder, milk powder, sugar, and flavoring agents) and continuous phase (solid fat). The product has the given characteristic of not solidifying during storage at room temperature when it is composed of an amount higher than 40% w/w of fats mixed with different dry ingredients (sugar, cocoa powder, milk powder, hazelnuts, and flavors) (Manzocco et al., 2014). This characteristic is linked to the performance of the lipid fraction, where chocolate spreads are required to be physically compatible with low-moisture foods, so fat reduction cannot be pursued by increasing moisture. On the other hand, another possibility to have a low-fat chocolate spread is taking the plasticizing properties of lipids with different crystallization levels, for instance, palm oils or another liquid oil (Manzocco u. a. 2014).

- Dispersed phase

It is also called the fat phase because is mostly composed of saturated fats, for example, cocoa butter, palm oil, and coconut oil. The fat is the one in charge of giving a creamy texture, glossy appearance, rich taste, and melt-in-mouth behavior (Selvasekaran & Chidambaram 2021). However, it can be harmful to health due to it is related to diseases such as obesity, diabetes, and cardiovascular diseases (Tirgarian u. a. 2023a).

- The chocolate spread in the current market

Chocolate creams are a staple in the daily diet of many people, primarily consumed at breakfast or as an ingredient in baking such as cakes and cookies (Tirgarian u. a. 2023b). In recent years, there has been a steady growth in the cocoa industry, leading to an increase in production. The chocolate

market in Europe is considered the largest in the world, accounting for 65% of global production and valued at 18.3 billion euros (Blanc u. a. 2022).

In recent years, the chocolate industry has undergone rapid evolution, introducing new product categories such as organic, gluten-free, and vegan chocolate, as well as various presentations and textures like chocolate spread. Chocolate consumption has shifted towards the domestic sphere, prompting chocolate companies to focus on online promotion and communication of health-related aspects and the benefits of chocolate on health (Blanc u. a. 2022).

4 MATERIALS AND METHODS

The methodology of the analysis is divided into two parts. First, enzymatic reaction where the used materials are high oleic sunflower oil, glycerol (99,97%), deionized water, and lipase Lipozyme® RM acquired from Novozym, this lipase originating from Aspergillus micro-organism.

Second, for making chocolate spread, the materials are the product of reaction instead of oil, sugar, hazelnut pasta, milk powder, soy flour, lecithin, and cocoa powder.

4.1 Enzymatic Glycerolysis

Enzymatic glycerolysis was conducted with high oleic sunflower oil, glycerol with 99,97% using Lipozyme® RM as the catalyst. The reaction was performed at 50 °C and 130 rpm 10h in the presence of a 1:1 (mol: mol) ratio of glycerol to triacylglycerol molecules(Rivero-Pino u. a. 2020). First, to mix glycerol and TAG (high oleic sunflower oil) in a beaker as the reaction vessel. Glycerol was added in function to the oil at a molar ratio of 1:1. The mass of glycerol was determined according to the Nicholson & Marangoni in 2020 investigation, who used an amount of 92 g/mol of molecular weight glycerol and used an equation to know TAG molecular weight MW=3 x (56 000/SV), where SV represents the saponification value of the oil (179-185). After, deionized water was added at 3,5 wt% relative to glycerol, and 2 wt% of lipases relative to the oil. Second, the beaker was lightly shaken to disperse the enzyme particles and after, the mixture was placed in the magnetic stirrer hotplate Heidolph MR 3002 with a 50°C temperature and 130 r.p.m speed. The samples of reaction were taken for times 30 min, 1 hour, 2 hours, 3 hours, 5 hours, 7 hours, and 10 hours. The samples were placed in the centrifugate MIKRO 220R setting up the time of 3 minutes and 4°C, where it helped to separate the mixture based on density differences by spinning them at high speeds. This causes heavier components such as lipases to move outward and settle at the bottom of the tube, while lighter components move towards the top making it easy to take out the sample.

4.1.1 TLC method

Thin Layer Chromatography or TLC is a chromatography technique that separates components in a mixture through two phases: a stationary phase, typically consisting of a thin layer of adsorbent material such as silica gel, and a mobile phase, which is a solvent that moves through the stationary phase via capillary action (Han u. a. 2023). The applied procedure was the modified method by Bakala-N'Goma et al. (2022).

First, 20 mg of each sample was mixed with isooctane on a microcentrifuge tube. After, they were placed in the tube vortex mixer per about 1 minute to dissolve the mixture.

Second, Standard and sample mixture solutions of enzymatic reaction were deposited on 20 cm x 20 cm silica gel 60 TLC plate with a glass substrate as stationary phase. A volume of 20 mL of each standard solution was applied on a plate and dried for 2 minutes. It was placed in a TLC chamber with n-heptane/diethyl ether/ formic acid (55:45:2) solution as the mobile phase. The plate was developed in a chromatographic chamber using 100 mL of mobile phase by ascending chromatography at room temperature (T = 20 °C). Later, the plate was taken out for 5 minutes and marked the point until the solvent up.

Third, the plate was placed into TLC chamber again until the mobile phase reached the marked point, and this one was taken out for 5 minutes. For dyeing, a solution of copper acetate and phosphoric acid was used, which was prepared by mixing a saturated solution of copper acetate with 85% phosphoric acid in a 1:1 volume ratio. After staining, the TLC plates were dried under a hood for 10 minutes and then heated in an oven at 180 °C for 15 minutes.

Finally, the stained lipids on the TLC plates were then evaluated using ImageJ software.

4.2 Chocolate Spread

The chocolate spread was prepared based on the method described by de Souza Correia Cozentino et al. (2022) with slight modifications. The spread was prepared in the machine Spectra 11– Stone Grinders using as ingredients: the product of the enzymatic glycerolysis 23,23%, sugar 33,10%, hazelnut pasta (12,96%), milk powder (11,85%), lecithin (0,37%), cocoa powder (11,84) and soy flour (6,66%).

Initially, sugar, cocoa, and milk powders were mixed mechanically with soy flour. Later, wet ingredients such as hazelnut pasta and lecithin were added. The product of the reaction was placed in the machine and subsequently rest ingredients were added together it. The mixture was processed in Spectra 11 Chocolate grinder for 8 hours.

4.2.1 Texture analysis

The texture analysis was done to measure the spreadability, this term is related to consistency and is defined as the force required to spread the product and make a thin and uniform layer(Acan, Kilicli, u. a. 2021). Firmness is the sensorial property that is implicit in the measurement because it is the force required to obtain its deformation or the measure of deformation under a given force(Acan, Kilicli, u. a. 2021).

Spreadability of samples was determined by the texture analyzer TA. HD Plus, Stable Micro System, equipped with Exponent software (Stable Micro Systems Ltd) and TTC Rig (HDP/SR) attachment. First, the calibration was done using an empty female cone to calibrate the machine. Samples were filled into a female cone (90° angle) to avoid bubble formation.

The male cone was driven through the sample at a speed of 3.0 mm/s until it reached 2 mm above the bottom or penetrated 23 mm of the female cone using the "confectionery: chocolate spreads" software. The measurements of hardness, represented by force expressed in N, and spreadability, represented by the area under the curve , were noted. (Bascuas u. a. 2021).

4.2.2 Differential scanning calorimetry (DSC)

Calorimetry is a technique for measuring the thermal properties of materials based on the relation between temperature and specific physical properties of substances (Gill u. a. 2010).

Differential scanning calorimeter (DSC) is a thermal analysis device measuring how the physical properties of a sample change, along with temperature against time. The mechanism of this apparatus work to measure the heat quantity that the sample absorbs or radiates during a temperature change based on the comparison between the sample and reference material (Gill u. a. 2010).

In the DSC experiment, energy is simultaneously added to a reference cell (which just contains the solvent) and a sample cell (which includes a solution containing the chemical of interest). Both cells' temperatures are gradually raised in the same way. The quantity of excess heat absorbed or released by the sample molecule is equal to the difference in the input energy needed to bring the temperature of the sample to that of the reference (Gill u. a. 2010).

The thermal behavior of chocolate spread was determined using differential scanning calorimetry (DSC 200 F3 Maia, NETZSCH). Before running tests, the DSC was calibrated. Approximately 10–15 mg of chocolate spreads were weighed and poured into sealed aluminum pans and the empty pan was used as the reference. The thermal behavior of the samples was examined at a heating rate of 10 °C/min in the temperature range of 20–200 °C and under a nitrogen atmosphere with a 50 ml/min flow rate (Tirgarian u. a. 2023a).

4.2.3 Sensory evaluation

- **Quantitative descriptive analysis**

For the quantitative descriptive analysis (QDA), we selected a panel of fifteen participants—comprising five males and ten females aged between 25 and 45. This sample size was determined based on the methodology employed by Behraad Tirgarian in his 2023 study, which also utilized a similar number of participants for the analysis. After developing fifteen sensory description terms the panel examined the samples. The sensory qualities were quantified using a 12-cm line scale that was anchored and ran from 0 at the left end to 10 at the right. Each participant in the test received 20 g of room-temperature chocolate spread with 3-digit randomized codes, water, and biscuits to help clear their palate in between samples. Three minutes were allowed between assessments.

- **Consumer test**

The attributes of appearance, aroma, flavor, and overall acceptability of the chocolates were determined by 15 different consumers (5 males and 10 females, aged between 20 and 45 years). The samples were served randomized with bread at room temperature where each person had 10 g of chocolate spread to evaluate. A 5-point hedonic scale (5 = like extremely to 1 = dislike extremely) was applied. The score was done through Google Forms to evaluate data.

Different volunteer consumers will determine the appearance, aroma, flavor, and overall acceptability of the chocolates using a hedonic scale where 5 will be like extremely and 1 will dislike extremely. The consumer can try 10 g chocolate spreads in a completely randomized order at room temperature. Later, they must fill a survey to identify their willingness to buy.

4.3 Statistical analysis

At least three duplicates of each test were run. INFOSTAT software, version 2020b, was used to do a one-way analysis of variance (ANOVA) on the data. Tukey's multiple comparison test was utilized to ascertain differences between treatments, and the difference between samples was accepted at a significance level of $p < 0.05$.

5 RESULTS

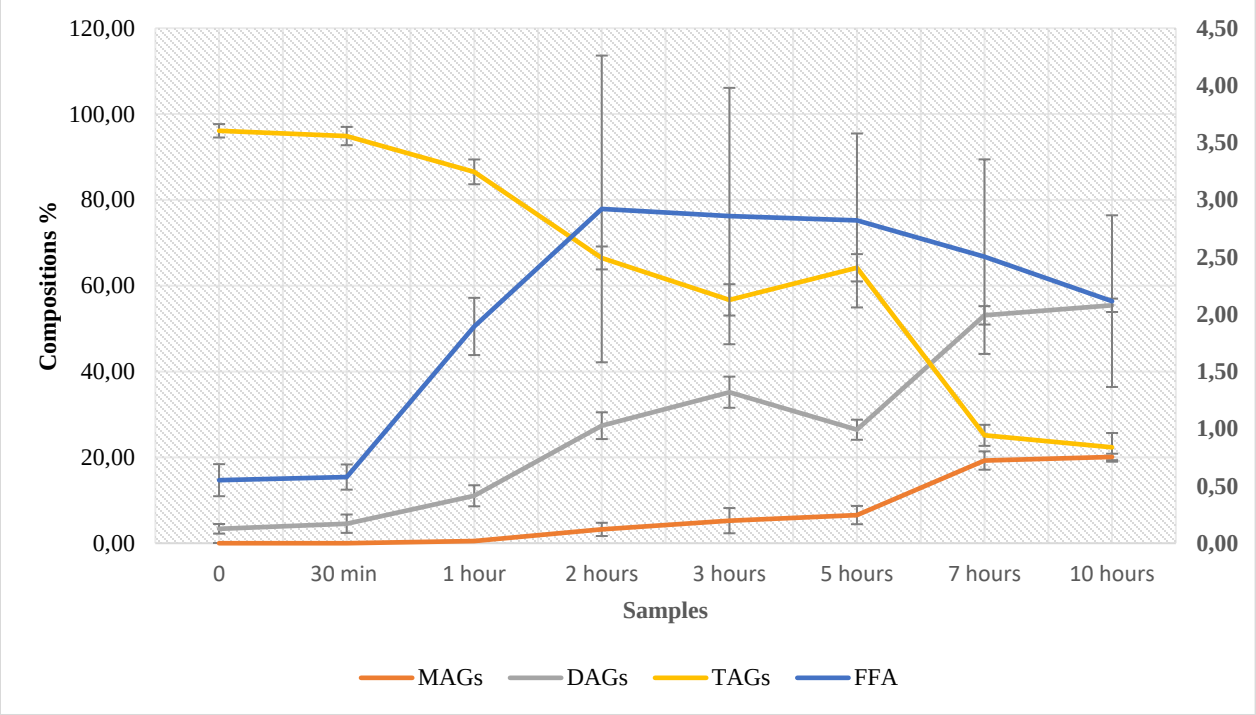
5.1 Enzymatic Glycerolysis

5.1.1 TLC

Table 2: Enzymatic glycerolysis reaction products after different reaction times
(Source: own work)

Time	FFA %	MAGs%	DAGs%	TAGs %
0 min	0,55	0,00	3,36	96,09
30 min	0,58	0,00	4,55	94,87
1 hour	1,89	0,53	11,05	86,53
2 hours	2,92	3,22	27,39	66,47
3 hours	2,86	5,26	35,19	56,70
5 hours	2,82	6,56	26,44	64,18
7 hours	2,50	19,26	53,10	25,14
10 hours	2,12	20,11	55,44	22,34

Figure 4: Relation of compositions of enzymatic glycerolysis reaction products and different reaction times
(Source: own work)



In a previous study by Nicholson and Marangoni 2020, the optimal glycerolysis reaction conditions were determined, so the reaction was done according to these instructions with lightly modifications. The maximum reaction time was 10 h with a 1:1 glycerol: TAG molar ratio, and 2 w/w% enzyme as the catalyst. Under these conditions, the reaction gave results of 20.11% monoacylglycerols (MAGs), 55.4% diacylglycerols (DAGs), 22.34% triacylglycerols (TAGs), and 2,12 % free fatty acids (FFA), which demonstrates that when time is increased during the reaction, it produces more amount of TAG and DAG.

Besides, the amount of DAG to be more than 50% agrees with an investigation by Zeng et al. 2009 found that the main product of glycerolysis was DAG at a low molar ratio of glycerol, however, this does not mean that a high glycerol amount can improve the amount of MAG because it will affect the polarity as well as the stability of the system affecting directly to the enzymatic stability and efficiency of the reaction.

Figure 4 shows the standard deviation of the samples, where the samples of free fatty acids have the highest deviation, which means that the data is more dispersed.

5.2 Chocolate Spread

5.2.1 Texture

The texture analysis is a crucial test for understanding the physical properties of chocolate spread, especially in terms of firmness and spreadability. These properties directly impact consumer experiences, such as how easy it is to spread the chocolate on bread or crackers, its smoothness on the palate, and its stability over time.

Chocolate spread at different temperatures was analyzed, including room and cold temperature (refrigerator temperature 3-5°C) because temperature influences rheological behavior.

Table 3: Results of Firmness and Spreadability
(Source: own work)

	Enzymatic Glycerolysis Sample		**Commercial**
	Room Temperature	**Cold Temperature**	**Room Temperature**
Firmness (g)	321,00	473,60	1397,67
Spreadability (g.s)	244,22	342,06	1154,68

Figure 5: Relation force and time of the Enzymatic Glycerolisis Sample at cold temperature

(Source: own work)

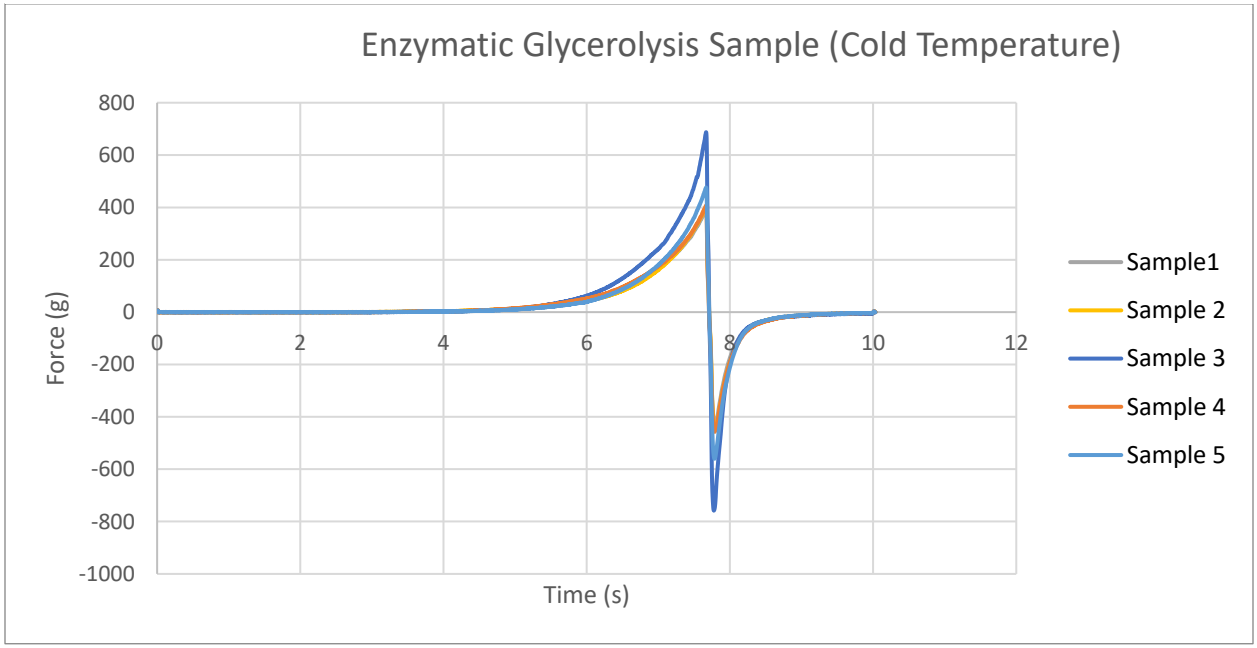


Figure 6: Relation force and time of the Enzymatic Glycerolisis Sample at room temperature

(Source: own work)

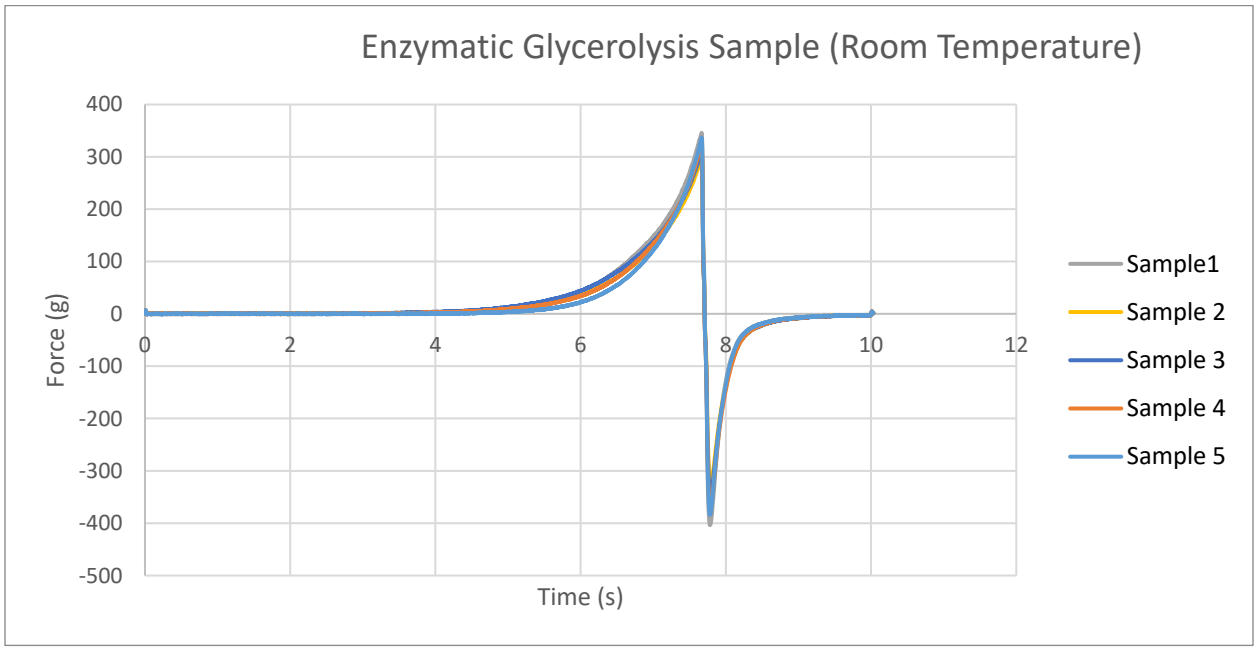
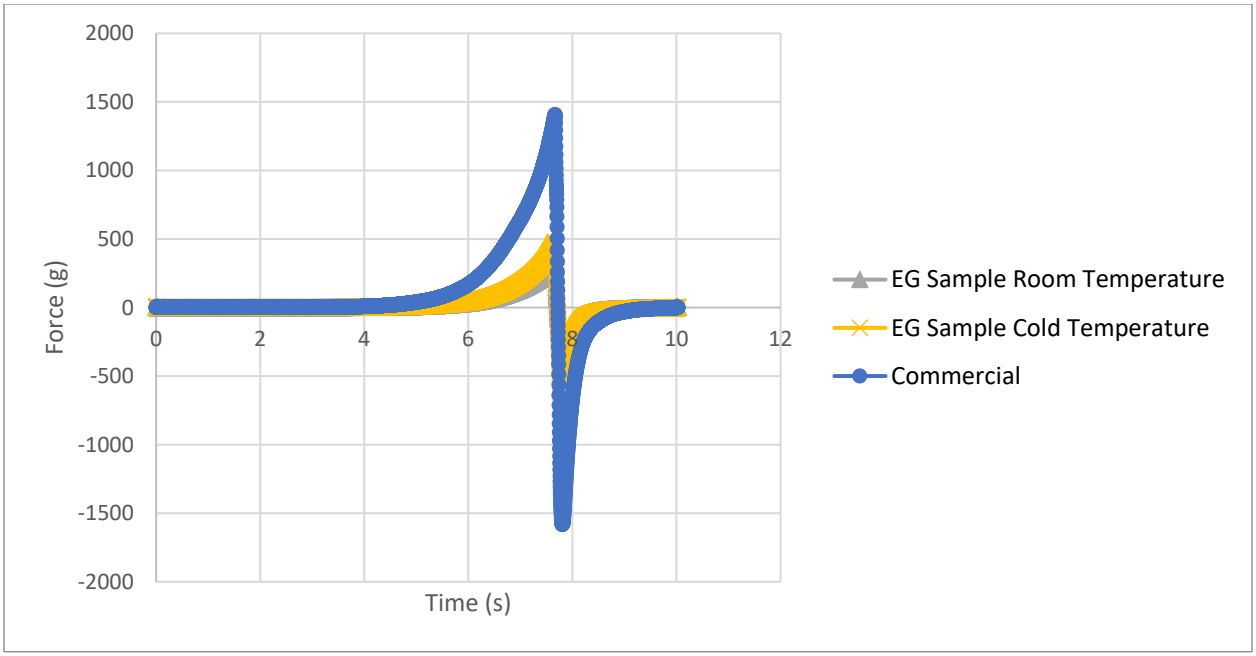


Figure 7: Comparison of Relation force and time between the Enzymatic Glycerolysis Sample at room and cold temperature and Commercial Chocolate Spread

(Source: own work)



One of the most crucial aspects of chocolate spreads' texture is their capacity to flow on a surface. Implicit information about the spread's hardness and spreadability reveals the maximum force needed for deformation and surface flow, respectively (Acan, Toker, u. a. 2021). Based on the results shown in Table 4, the chocolate spread at a cold temperature has similar properties to the room temperature, however, they have significant differences concerning the commercial chocolate spread. The results are in agreement with the sensory analysis where people assumed that chocolate spread with the product of the reaction is more glossy than commercial, which means that the sample is perceived as wet or oily so has a higher spread but less stability(Acan, Toker, u. a. 2021).

The firmness is directly relation to the particle size and the formulation, which means that the stronger the particle-particle interaction, the higher the hardness will be and it depends on particle size that it should be lower to create a stronger interaction so the particle size of the chocolate spread with the reaction product has a big particles that not allow to create the interaction (Acan, Toker, u. a. 2021). This occurs because the chocolate spread needs much finer particles to have a

smooth and creamy texture, but the Spectra 11 Stone Grinder is made primarily to grind cacao nibs into chocolate liquor, which usually results in very big particle sizes.

Figure 8: Statistical Results of Enzymatic Glycerolisis Sample at cold and room temperature and commercial chocolate spread

(Source: own work)

Firmness (g)

Variable	N	R^2	Adj R^2	CV
Firmness (g)	16	0.83	0.81	31.45

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model	3798354.10	2	1899177.05	32.10	<0.0001
Sample	3798354.10	2	1899177.05	32.10	<0.0001
Error	769139.33	13	59164.56		
Total	4567493.44	15			

Test:Tukey Alpha:=0.05 LSD:=394.75248

Error: 59164.5641 df: 13

Sample	Means	n	S.E.	
sampleroom1_	323.80	5	108.78	A
samplecold1_	473.60	5	108.78	A
nutella1	1397.67	6	99.30	B

Means with a common letter are not significantly different (p > 0.05)

Spreadability (g.s)

Variable	N	R^2	Adj R^2	CV
Spreadability (g.s)	16	0.80	0.77	37.69

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model	2813876.63	2	1406938.31	26.13	<0.0001
Sample	2813876.63	2	1406938.31	26.13	<0.0001
Error	699838.30	13	53833.72		
Total	3513714.92	15			

Test:Tukey Alpha:=0.05 LSD:=376.54875

Error: 53833.7150 df: 13

Sample	Means	n	S.E.	
sampleroom1_	242.47	5	103.76	A
samplecold1_	342.06	5	103.76	A
nutella1	1154.68	6	94.72	B

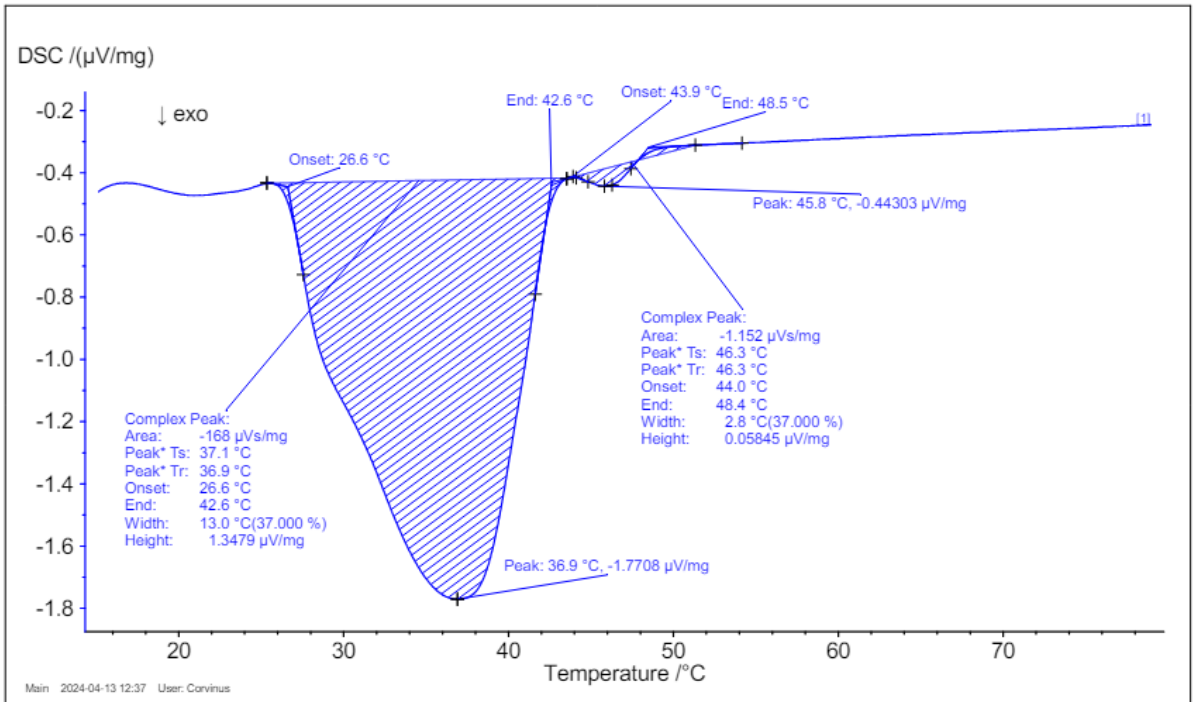
Means with a common letter are not significantly different (p > 0.05)

The statistical analysis showed that there is no significant difference between the repetition of samples, however, when we do a comparison of the commercial and the product reaction chocolate spread, they are significantly different from each other and room and cold temperatures of chocolate spread with the product of the reaction are in the same group, it means that they have similar data.

5.2.2 Thermal Behavior

Figure 9: Melting profile of chocolate spread prepared with the product of enzymatic glycerolysis

(Source: own work)



The chocolate spreads have special characteristics such as the capacity to be solid at room temperature (22–25 °C) and can become a smooth dense suspension at oral temperature (35–38 °C). This property is called liquefaction and is the process where the substance changes from a solid state to a liquid state, it is produced by an increase in temperature until reaches the fusion point, also during the process the molecules start to move with more thermal energy leading to debilitated intermolecular forces(de Souza Correia Cozentino u. a. 2022).

The DSC analysis of the chocolate spread with the product of the reaction and its thermogram is depicted in Fig, where a sharp endothermic peak was observed at 36,9 °C corresponding to the melting point, it compares with commercial chocolate spread that usually has a melting point of 37°C demonstrate that the chocolate spread had stability and can melt and the same time than the commercial (Fernandes Almeida u. a. 2024).

Cocoa butter is usually used to make chocolate spreads because It presents polymorphism and can crystallize into six polymorphic forms from inestable to stable. The polymorphic crystallization forms happen due to triglyceride compositions, where liquid fat converts into a solid as a result of fatty acid compositions(Furlán et al., 2017). Form VI (34–36 °C) is the most stable form, and the polymorphic form V (32–34 °C) is the most desirable, as it gives the chocolate the desired appearance and it melts just below body temperature (Furlán et al., 2017). Nevertheless, although chocolate spread with product of reaction is not placed in form V according to the melting point of 36,9°C, it is in form VI showing that it is stable.

5.2.3 Sensory Characteristics

In the present study, 15 sensory attributes were chosen for QDA analysis. The sensory lexicon was comprised of 2 aromatic, 4 basic flavors, 4 mouthfeel, and 5 appearance attributes (Fig. 10). These attributes were ticked off by the panelists according to their perception and criteria on the sensorial characteristics the chocolate spread had.

In the appearance attributes the ratings/grading were on spreadability, smoothness, viscosity, color, and brightness. The aromatics attributes taken into consideration were, cocoa and milk aroma. And for last the mouth attributes were the next attributes were decided: Sandiness, melting rate, no Mouth Coating and Aftertaste .

Figure 10: Sensory descriptive terms and characteristics.
(Source: own work)

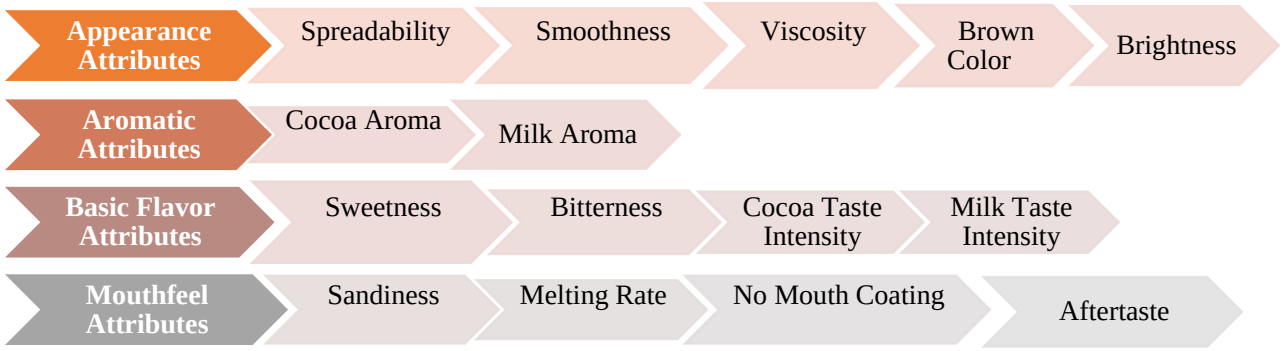


Table 4: Quantitative descriptive analysis results of chocolate spreads.
(Source: own work)

		Commercial	***New Product***
Appearance Attributes	**Spreadability**	6,225	9,125
	Smoothness	7,125	8,15
	Viscosity	8,0375	9,0625
	Brown Color	8,0125	11,0375
	Brightness	7,125	9,1125
Aromatic Attributes	**Cocoa Aroma**	3,0125	10,375
	Milk Aroma	9,025	7,05
Basic Flavor Attributes	**Sweetness**	11,04	7,00
	Bitterness	1,06	2,04
	Cocoa Taste Intensity	6,11	9,10
	Milk Taste Intensity	7,05	5,16
Mouthfeel Attributes	**Sandiness**	1,0375	7,1375
	Melting Rate	6,1875	5,125
	No Mouth Coating	3,0875	8,1125
	Aftertaste	4,0625	7,05

All the panelists sampled, analyzed and rated these parameters in a chocolate spread made by a commercial label and the new product to get the QDA results, the obtained average for each attribute is shown in table 4, subsequently, a radial diagram was created for the QDA analysis (Figure 11) were the results for the new product have higher scores in the spreadability smoothness

viscosity, color and brightness that corresponds to the appearance Were the panelists chose the new product against the commercial label.

When it comes to the aromatics the tendency for the cacao aroma in the new product presents again a higher score against the milk aroma from the commercial one and this can be explained through the fact of the new product having more cacao in its elaboration.

In the flavor attributes the new product has less sweetness and more bitterness in comparison to the commercial product nevertheless this last attribute does not show a big difference in the scores for both products, is important to mention that the score for cocoa taste intensity is higher for the new product, this is again related to the quantity of cocoa in its production

The mouth-feel attributes, the new product exhibits a high score for sandiness, the same is seem for the no mouth coating and aftertaste, the melting rate attribute is the only one showing a lower score in this parameter.

Figure 11: Sensory properties of chocolate spread samples.
(Source: own work)

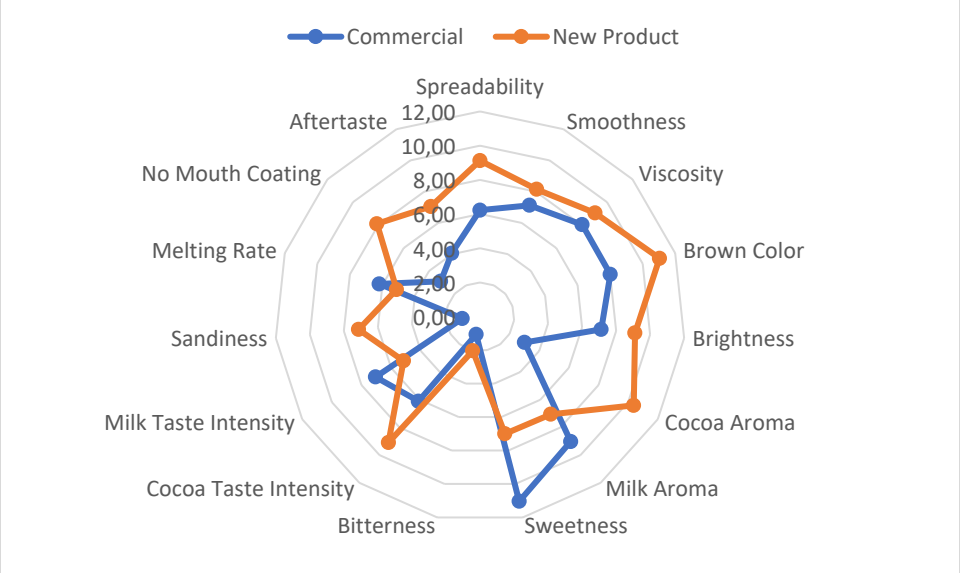


Figure 12: Results of acceptability test.
(Source: own work)

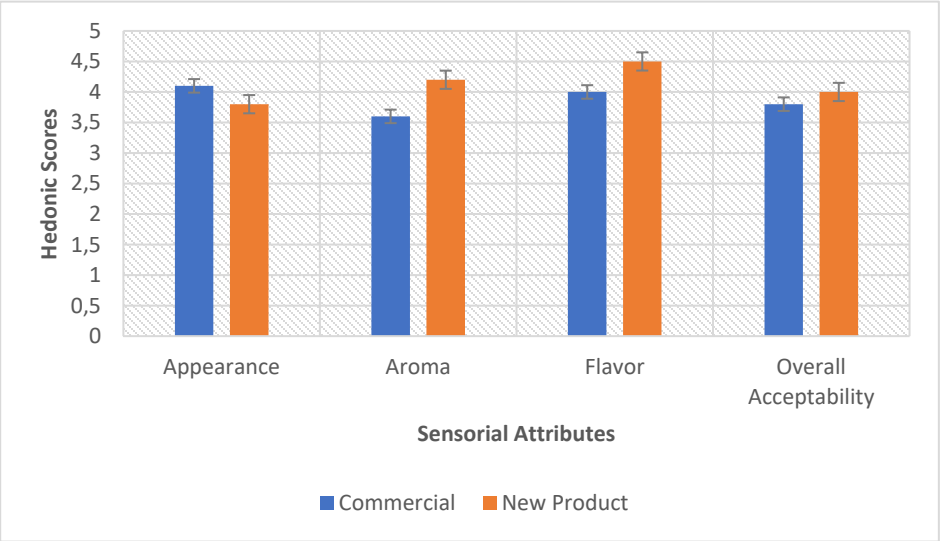
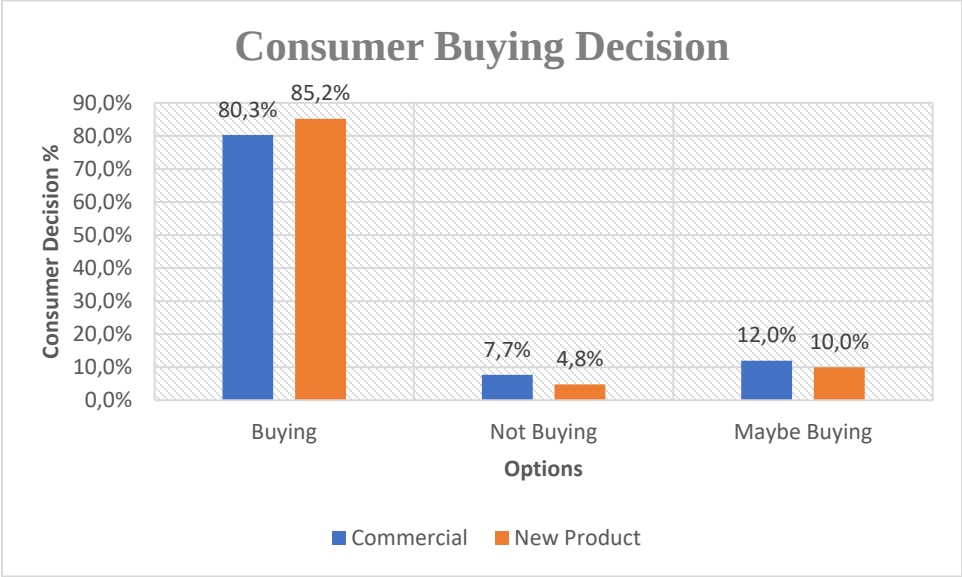


Figure 13: Consumer buying decision.
(Source: own work)



The results of the consumer test carried out by the 15 chosen consumers are presented in (figure 12 and 13), in the first one it can be seen that in the acceptability results where consumers evaluated the attributes of appearance, aroma, flavor, and overall acceptability The new product has a higher rating value in aroma, flavor, and overall acceptability over the commercial brand, which is

explained by the pleasant aroma and flavor of cocoa that consumers were able to experience when using a higher concentration of cocoa in When developing the new product, the appearance rating is a lower percentage than the commercial brand, but both maintain ratings close to 4 on the scale used. On the other hand, in the consumer buyer decision test, a higher percentage of the decision to purchase (buying) of more than 85% is observed by consumers for the new product compared to the commercial brand, which reflects good acceptance, there is a 10 % that maybe buying and only 4.8% that not buying the new product.

After thorough evaluation, consumers ultimately favored the chocolate spread derived from the reaction product due to its less pronounced sweetness and darker hue compared to the commercial variant. However, it was noted that the commercial spread boasted a superior texture, which aligns with the findings of texture analysis. Notably, samples of the chocolate spread with varying fat content exhibited noticeable particles, imparting an undesirable mouthfeel. Addressing this issue may require the utilization of a more suitable machine specifically tailored for chocolate spread production.

6 CONCLUSIONS

Based on the findings from Thin Layer Chromatography, the optimal duration for enzymatic glycerolysis was determined to be 10 hours. This duration yielded a higher concentration of monoacylglycerols and diacylglycerols, while reducing the levels of triacylglycerols. The resulting chocolate spread exhibited a favorable melting point of 36.9°C, indicating thermal stability. However, texture analysis revealed significant deviations from the commercial product, attributed to the presence of particles within the spread. To address this issue, the implementation of alternative machinery capable of mitigating particle formation may be necessary. By employing specialized equipment, such as a homogenizer or colloid mill, the particle-related texture discrepancies can potentially be resolved, ensuring consistency and enhancing the overall quality of the chocolate spread.

In addition to the before-mentioned analyses, sensory evaluation was conducted to assess consumer preferences. The results revealed a strong preference among participants for the chocolate spread derived from enzymatic glycerolysis. This preference was largely attributed to the perception that this variant contained less sugar compared to the commercial product. Furthermore, participants noted that the spread appeared to contain a higher proportion of chocolate, which is commonly associated with improved quality. In today's society, the association between reduced sugar content and health benefits, as well as the perception of higher quality associated with increased dark chocolate content, contributed to the favorable reception of the enzymatically derived chocolate spread.

SUMMARY

Chocolate spread traditionally contains high levels of saturated fatty acids, prompting efforts to remove and replace trans fats in modern formulations. Enzymatic glycerolysis, a process involving the conversion of native triacylglycerols (TAGs) into monoacylglycerols (MAGs) and diacylglycerols (DAGs) using glycerol and a catalyst, offers a promising solution. This reaction, conducted with high oleic sunflower oil as the triacylglycerol source, glycerol (99.97%), and Lipozyme® RM as the catalyst, was performed at 50°C and 130 rpm for 10 hours, maintaining a 1:1 (mol:mol) ratio of glycerol to triacylglycerol molecules.

The resulting product was analyzed using Thin Layer Chromatography (TLC), a chromatographic technique enabling the identification of sample components. Results from the TLC analysis after ten hours revealed 20.11% monoacylglycerols (MAGs), 55.4% diacylglycerols (DAGs), 22.34% triacylglycerols (TAGs), and 2.12% free fatty acids (FFA).

Using this enzymatically glycerolysed product, a chocolate spread was prepared using the Spectra 11– Stone Grinders machine, with ingredients comprising 23.23% of the enzymatically glycerolysed product, 33.10% sugar, 12.96% hazelnut paste, 11.85% milk powder, 0.37% lecithin, 11.84% cocoa powder, and 6.66% soy flour. Characteristics such as texture, thermal behavior, and sensory attributes were evaluated.

Results indicated a melting point of 36.9°C, indicating the stability of the mixture. However, firmness and spreadability were observed to be lower than commercial counterparts due to the presence of particles within the sample. Sensory analyses revealed favorable acceptance of the product, attributed to its reduced sugar content and increased cocoa content.

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LIST OF TABLES

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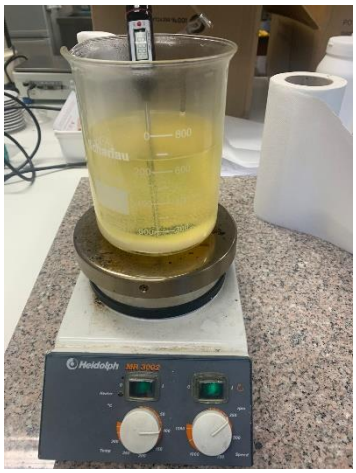
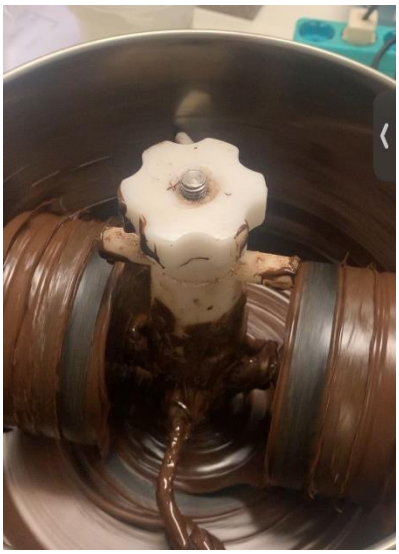
ANNEXES

Products of enzymatic glycerolysis according to different time



Sunflower oil during the enzymatic glycerolysis reaction



Process of Chocolate spread

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Year of publication:	2024
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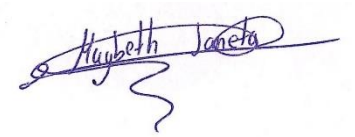

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