Modifying the health functionality of formulated chocolates

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Ultrasonic-Assisted Extraction (UAE)

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Abstract

The moderate consumption of dark chocolate, a rich source of flavonoid, has been associated with a lower risk of stroke and cardiovascular diseases. In the present study, an attempt has been made to formulate dark chocolate with the incorporation of isomalt, erythritol and xylitol for helping with blood sugar modulation. The effects of different concentrations of these polyols on physical and sensory parameters were studied using a simplex-lattice mixture design. The optimized formulation of sugar-free dark chocolate was found to be a combination of 5.16% isomalt, 20.99% erythritol and 3.85% xylitol with the desirability of 0.66, by maximizing plastic viscosity, yield stress and brightness. Furthermore, chocolates made with isomalt, xylitol, erythritol and the optimized formulation were incorporated with different concentrations of olive leaf extracts (10 g/kg, 15 g/kg, and 20 g/kg) to improve the anti-diabetic effect of chocolate. The effects of various olive leaf extract in the rheological, textural, melting, visual, and physicochemical properties of chocolate were investigated. Oleuropein content was found to be in the range of 0.24 – 0.52 mg/100 g of sample in oleuropein-enriched sucrose-free dark chocolates.

Résumé

La consommation modérée de chocolat noir, une source riche en flavonoïdes, a été associée à un risque plus faible d'accident vasculaire cérébral et de maladies cardiovasculaires. Dans cette étude, nous avons tenté de formuler du chocolat noir en incorporant de l'isomalt, de l'érythritol et du xylitol pour aider à la modulation de la glycémie. Les effets de différentes concentrations de ces polyols sur les paramètres physiques et sensoriels ont été étudiés en utilisant un plan expérimental en réseau simplex. La formulation optimisée du chocolat noir sans sucre s'est avérée être une combinaison de 5,16% d'isomalt, 20,99% d'érythritol et 3,85% de xylitol avec une désirabilité de 0,66, en maximisant la viscosité plastique, la contrainte de rendement et la brillance. En outre, différentes concentrations d'extraits de feuilles d'olivier (10 g/kg, 15 g/kg et 20 g/kg) ont été incorporées dans du chocolat fabriqué avec de l'isomalt, du xylitol, de l'érythritol ainsi que la formulation optimisée pour améliorer l'effet antidiabétique du chocolat. Les effets de divers extraits de feuilles d'olivier sur les propriétés rhéologiques, texturales, de fusion, visuelles et physicochimiques du chocolat ont été étudiés. La teneur en oleuropéine s'est avérée être comprise entre 0,24 et 0,52 mg/100 g d'échantillon dans les chocolats noirs sans sucrose enrichis en oleuropéine.

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Outline of the Research

Chocolate is one of the fastest-growing confectionery products in the world. It is prepared from the fruit of the tree *Thermobroma cacao.* The processing of dark chocolate using various ingredients (such as cocoa mass, cocoa butter, sugar, flavour, and lecithin) includes refining, conching, tempering and molding to attain the desired physical, sensory, and bioactive characteristics. Because of the consumers increasing concern about sucrose and the calorie intake from chocolate consumption, chocolate manufacturers are searching for alternative bulk sweeteners that can provide both physical bulk, sweetness, and mouthfeel similar to sucrose without the glycemic effect. One of the methods of providing this functionality to chocolate is by using polyols (such as isomalt, erythritol and xylitol) instead of sucrose. These polyols have lower caloric values compared to sucrose because of their incomplete digestion and poor absorption in the human body, however, the sweetness power of these polyols is less than that of sucrose. To overcome this problem of sweetness, intense sweeteners such as aspartame must be used along with polyols to reach the expected sweetness in chocolates. The overall strategic objective of this master research was to optimize sucrose-free dark chocolates using isomalt, erythritol and xylitol coupled with aspartame as an intense sweetener. Furthermore, developed sugar-free dark chocolates were subsequently formulated with olive leaf extracts (in different percentages on a weight-by-weight basis) to further provide enhanced antioxidant property (oleuropein) without the glycemic effect, and their physico-chemical properties were assessed to understand the formulation effects on the physical, bioactive, and sensory characteristics of the prepared chocolates.

The first chapter provides a short introduction to the global market of chocolates, different chocolate types available in the market and their processing methods. The second chapter provides

a review of the suitability and applicability of alternative bulk sweeteners, such as isomalt, erythritol and xylitol, in the production of sucrose-free chocolates. A critical review on the importance of oleuropein as a nutraceutical ingredient is highlighted in Chapter 3. Chapter 4 presents the optimization of a sucrose-free dark chocolate in a refiner using a simplex lattice mixture design. The physical, sensory, and bioactive characteristics of a sucrose-free dark chocolate enriched with olive leaf extracts (10 g kg^{-1}, 15 g kg^{-1} and 20 g kg^{-1}) are investigated in Chapter 5. Chapter 6 gives a general conclusion of this research and provides recommendations for further studies.

Chapter 1

Global market of cocoa and chocolate

1.1 History

Chocolate is made from the fruit of a tree called *Theobroma cacao*. Once a pod of cacao has been opened and the fermentation of the cacao beans has occurred then the common name used for the product is cocoa (for example- cocoa mass and cocoa butter). The eventful past of cocoa beans starts from a phase during which it was used as a currency by the civilizations of Aztecs and Mayas. It was first brought to Europe by Christopher Columbus and later was commercially exploited as a drink by Don Cortez (Minifie, 2012). This chocolate drink was then later sweetened with sugar by the Spaniards, and the popularity of this drink spread to Northern and Central Europe. The patent to manufacture liquid or box-form chocolate was granted to David Challiou by King Louis XIV of France in 1659, whereas the first engine-based cocoa grinder was invented and petitioned for patent by Walter Churchman to make chocolates in 1729 (Schmid, 2009). Some of the important dates in the history of chocolate and cocoa have been summarized in Table 1.1.

Table 1.1: Some important dates in the history of chocolate (Barry Callebaut, 2019, 2020; Beckett, 2011; Dumarche et al., 2009; Grivetti & Shapiro, 2009)

1519	Cortez discovered that Mexica/Aztec people in Latin America had been cultivated cacao for more than 3000 years
1528	Cortez introduced a drink made from cacao to Spain
1592	Medicinal uses of chocolate were identified by Agustin Farfan
1606	Introduction of a chocolate drink into Italy
1657	Introduction of chocolate beverage in London
1687	First mention of production of chocolate drink in Bayonne, France
1728	Chocolate manufacturing starts in Bristol, England by Walter Churchman
1756	First chocolate factory established in Germany
1765	First chocolate factory established in North America
1776	Invention of the hydraulic machine by Doret to make a paste from cacao seeds
1828	Caspar Van Houten patents the cocoa beans press
1831	Manufacturing of drinking chocolate and cocoa started by John Cadbury
1847	First chocolate candy bar was produced by Fry's factory
1875	Manufacturing of milk chocolate by Daniel Peter
1879	Invention of the conching machine for chocolate processing by Rudolphe Lindt
1930	Introduction of white chocolate by Nestle
2004	Barry Callebaut discovered 'ruby' cacao beans
2009	Barry Callebaut patents the invention relating to acidified cocoa nibs with a European patent
2017	Introduction of ruby chocolate by Barry Callebaut group

1.2 Global cocoa production and chocolate consumption

The important ingredient of chocolate, which provides its unique flavour and melting properties is cocoa. It is commercially grown within 20° north and south of the Equator (Beckett, 2011). According to the report published by the International Cocoa Organization (ICCO) in 2020, approximately 4.745 million tons of cocoa were produced globally during the year 2018/19 and was forecasted to be 4.824 million tons for the year 2019/20. The trend in the worldwide production of cocoa from 1980/81 to 2018/19 can be found in Fig 1.1. There are three main cocoa production regions, namely Africa, America and Asia/Oceania. Africa is the largest producer of

cocoa beans in the world amounting for approximately 3.624 million tons as compared to 0.838 million tons in America and 0.283 million tons in Asia/Oceania in the crop year 2018/19 (ICCO 2020) (Fig 1.2). Whereas, the largest by-country producer of cocoa beans is Ivory Coast (about 2.154 million tons in 2018/19), followed by Ghana, Ecuador, Cameroon, Nigeria, Indonesia and Brazil (ICCO 2020) (Fig 1.3).

There are historically three broad types of cocoa tree varieties in the world, which are Criollo, Trinitario and Forastero (ICCO 2021). Criollo variety is mostly found in South and Central America, and its beans are of higher quality. Whereas, the most widely cultivated sub-variety of Forastero, which is Amelonado, is mostly found in Brazil and West Africa. The hybrid of Criollo and Forastero is Trinitario which is grown in Trinidad, Venezuela, Ecuador, Samoa, Cameroon, Java, Sri Lanka and Papua New Guinea. However, when morpho-geographic and genomic similarities and differences are taken into consideration, recent findings have classified cocoa tree varieties into ten groups (ICCO 2021). These groups are Amelonado, Cacao Criollo, Cacao Guiana, Contamana, Curaray, Iquitos, Maranon, Nacional, Nanay, and Purus.

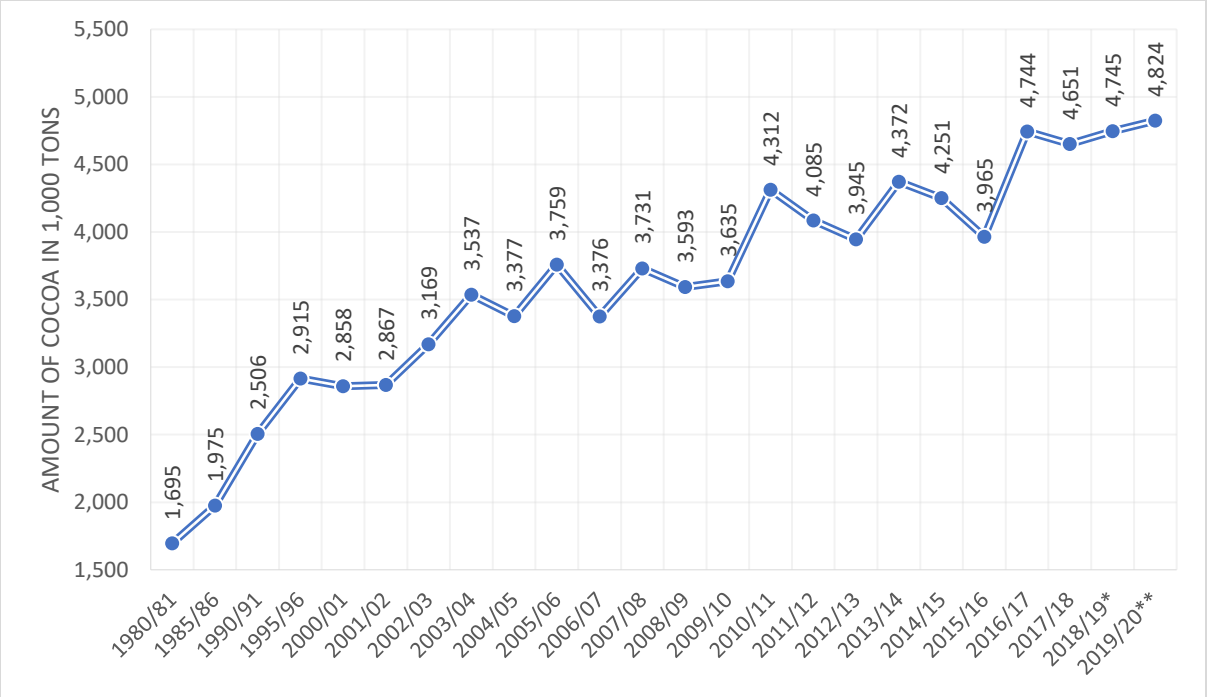

Figure 1.1: Timeline illustrating the trend in the worldwide cocoa production between 1980/81 and 2018/19, with a forecast for the year 2019/20 (ICCO, 2020)

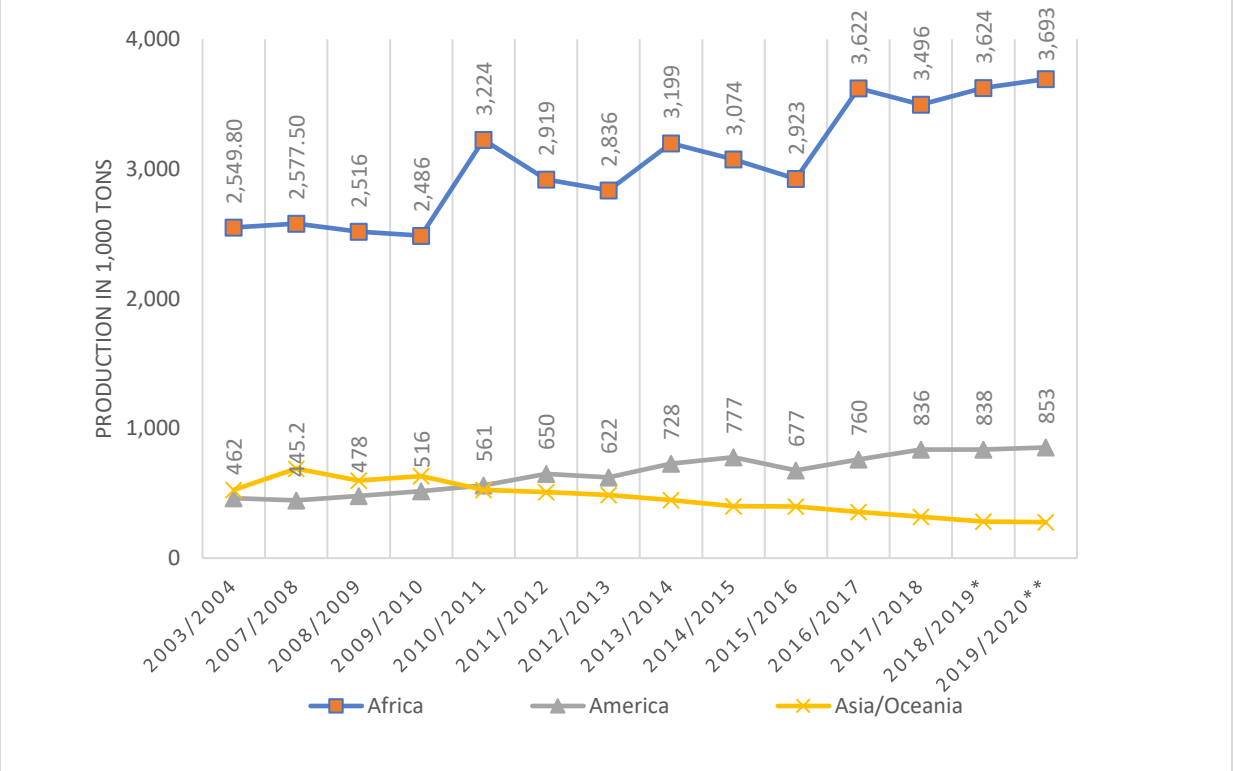

Figure 1.2: Timeline production of cocoa beans, by regions, between the year 2003/04 and the year 2018/19, providing a forecast for the year 2019/20 (ICCO, 2020)

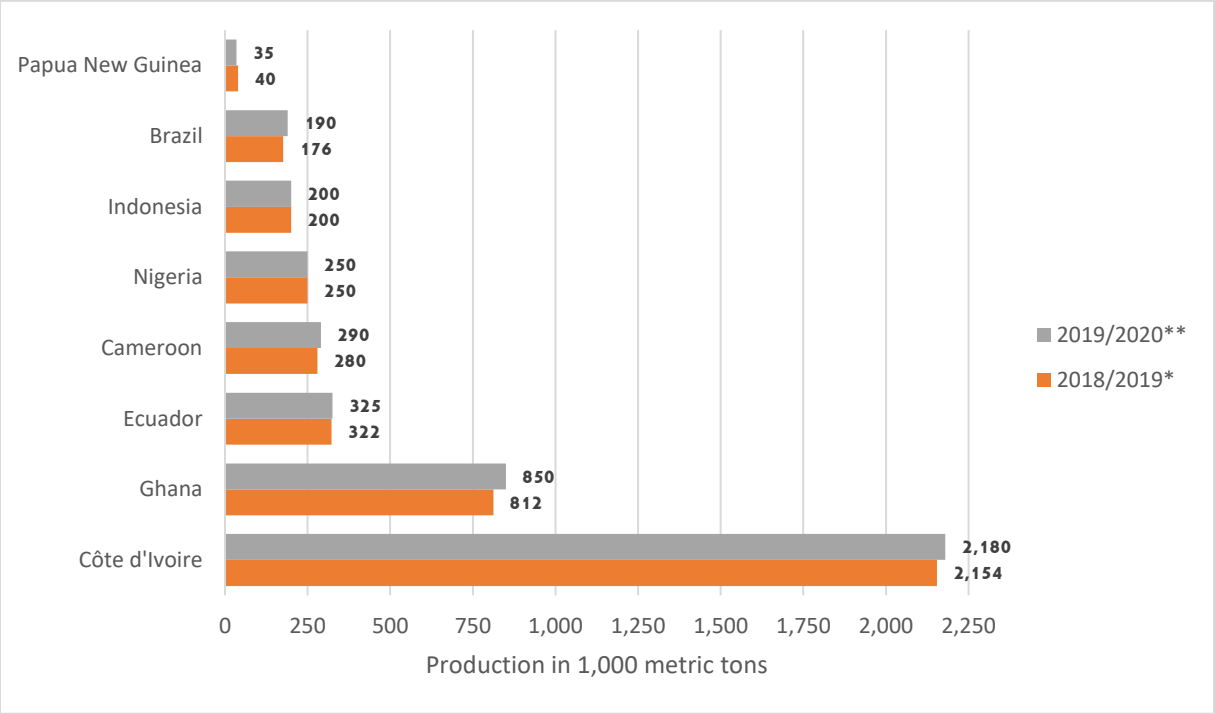

Figure 1.3: Global cocoa production, by country, in the crop years 2018/19 and 2019/20 (ICCO, 2020)

Cocoa is mostly consumed as chocolate-coated products (such as cookies, biscuits, cakes and ice-cream) and as chocolate confectionery. The chocolate confectionery, which accounted for 43 percent of all cocoa consumption in 2017, had a global retail sales value of around 98.2 billion U.S. dollars in 2016 (Candy Industry, 2017; Euromonitor, 2018). This worldwide market of chocolate confectionery is projected to expand by approximately 60 billion U.S. dollars between the year 2019 (around 130.56 billion U.S. dollars) and 2027 (around 187.1 billion U.S. dollars) (Statista, 2020). This growth of chocolate confectionery may be due to its market development in emerging markets such as China, India and Brazil (Barry Callebaut, 2019). In the year 2019, 33 percent of the global chocolate confectionery market (in volume) was in Western Europe, which is the largest share of this market worldwide (Fig 1.4) (Barry Callebaut, 2019). According to the data published by Lindt and Sprungli (2018), the leading country in the consumption of chocolate

per capita is Switzerland in 2017 (with approximately 9 kilos of chocolate consumption per capita). The per capita global consumption of chocolate in 2017, by leading country in terms of consumption, can be found in Fig 1.5.

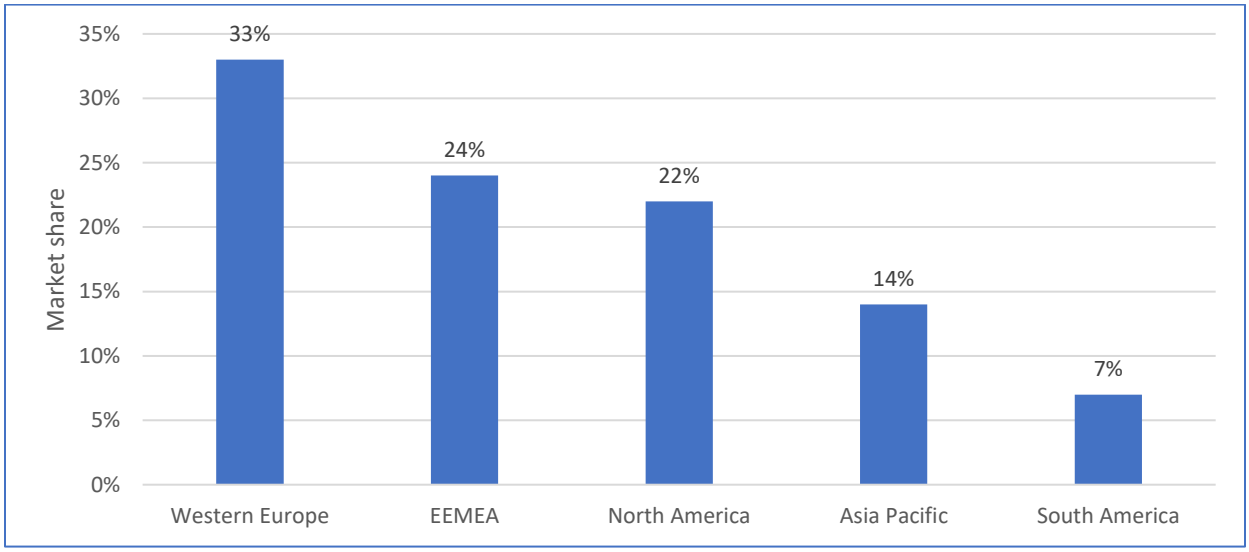

Figure 1.4: Market share of global chocolate confectionery, by region, in 2019 (EEMEA stands for Eastern Europe, Middle East and Africa) (Barry Callebaut, 2019)

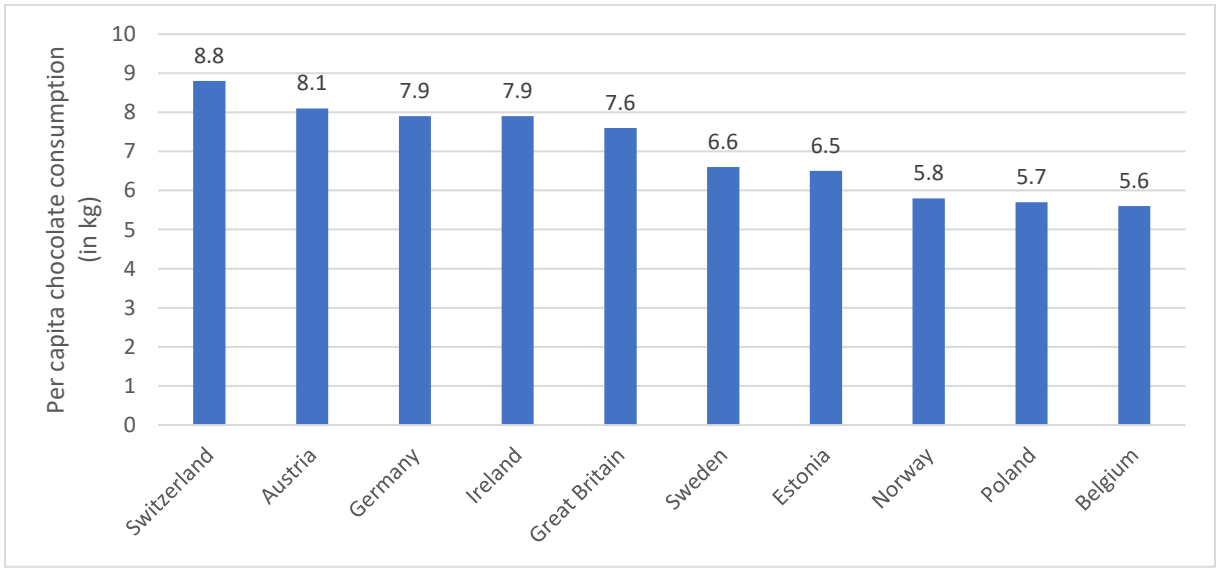

Figure 1.5: Chocolate consumption per capita, by European country, in 2017 (Lindt and Sprungli, 2018)

Increasing concerns of consumers towards the consumption of sucrose-made chocolate has given rise to sucrose-free chocolate (generally called sugar-free chocolate). This type of sugar-free chocolate uses alternative bulk sweeteners (such as polyols) to substitute sucrose. In a report published by 360 Research Reports in 2020, the worldwide market of sugar-free chocolate is projected to expand to 563 million U.S. dollars by the year 2026 from 408.3 million U.S. dollars in the year 2020 at a compound annual growth rate of 5.5% (360 Research Reports in 2020). In the same report, North America was reported to be the largest market, by region, of sugar-free chocolate with 40 percent of global sales. Moreover, the largest market of sugar-free chocolate, by chocolate types (dark and milk chocolates), was reported to be for dark chocolate with around 68 percent of the market share, in 2018 (360 Research Reports in 2020).

1.3 Types of chocolate

There are different types of chocolate which are classified based on the content of cocoa solids, cocoa butter and milk fat. The primary categories of chocolate are white, milk and dark chocolate (often includes sweet chocolate and bittersweet chocolate). The composition of white chocolate is cocoa butter, sugar and milk, whereas milk chocolate has cocoa butter, cocoa liquor, sugar and milk solids. Afoakwa, Paterson, Fowler, and Vieira (2008) described dark chocolate as coated particles of sucrose and cocoa solids in phospholipids, mainly in cocoa butter, with total solid content ranging between 65% and 75%. The composition of chocolate often also includes emulsifiers (such as polyglycerol polyricinoleate and lecithin) and flavouring (such as vanilla, spices, natural or artificial flavours, and salt).

Recently a different type of chocolate, called Ruby chocolate, was created and introduced to the public by Barry Callebaut in 2017. Ruby cacao beans are found in Brazil, Ecuador, and Ivory

Coast. Chocolate from this type of cacao beans, which is claimed to be the fourth type of chocolate by Barry Callebaut, has neither a milky, sweet or bitter taste, rather has a fruity berry-like taste and does not rely on additives for taste and appearance. The ingredients of Callebaut's ruby chocolate are sugar, cocoa butter, whole milk powder, unsweetened ruby chocolate, soy lecithin, citric acid and natural flavour. The exact processing step of ruby chocolate is yet to be known to the general public, however, the presence of citric acid as one of the ingredients may suggest that the key step in ruby chocolate production lies in acidification. According to the Barry Callebault patent registered to the European Patent Office in 2009 by Dumarche et al. (2009), cocoa nibs (unfermented or underfermented) when treated with an acidic aqueous solution (acid type = any food-grade mineral acid such as citric acid, lactic acid and phosphoric acid, concentration = 0.5 to 20% acid on weight by weight basis, time = up to about 24 hours, pH = less than 6 and temperature = less than or about 50^{o}C) produces red or purple nibs. These nibs when further processed are reported to produce red or purple coloured chocolate called ruby chocolate. The legal standards for chocolate types in the U.S. and Canada can be found in Tables 1.2 and 1.3 respectively.

Table 1.2: Standards regulated by the Food and Drug Administration (FDA), for dark, milk and white chocolate in the U.S. (FDA, 2019)

Product	Chocolate liquor (by weight)	Cocoa fat (by weight)	Sugar (by weight)	Milk solids (by weight)	Milk fat (by weight)
Sweet chocolate	≥ 15%	-	-	<12%	-
Bittersweet chocolate	≥ 35%	-	-	<12%	-
Milk chocolate	≥ 10%	-	-	≥ 12%	≥ 3.39%
White chocolate	-	≥ 20%	≤ 55%	≥ 14%	≥ 3.5%

Table 1.3: Standards for dark, milk and white chocolate in Canada (Food and Drug Regulations, 2020)

Product	Cocoa solids (by weight)	Cocoa butter (by weight)	Fat-free cocoa solids (by weight)	Milk solids (by weight)	Milk fat (by weight)
Sweet chocolate	≥ 31%	≥ 18%	≥ 12%	<12%	-
Bittersweet chocolate	≥ 35%	≥ 18%	≥ 14%	<5%	-
Milk chocolate	≥ 25%	≥ 15%	≥ 2.5%	≥ 12%	≥ 3.39%
White chocolate	-	≥ 20%	-	≥ 14%	≥ 3.5%

1.4 Chocolate processing

The three important ingredients which are required to make chocolate are sugar, cocoa butter and cocoa liquor. The common steps followed in the manufacturing of chocolate from chocolate liquor or paste are mixing, refining, conching and tempering. In the mixing process, chocolate ingredients (such as chocolate liquor, cocoa fat, sugar, vanilla and sometimes milk solids) are mixed to obtain a homogenous mixture. This homogenous mixture is then passed through stone rollers or roll refiners to reduce the particle size of the chocolate ingredients. The acceptable average particle size of chocolate depends on the market in which it is expected to be sold and the type of chocolate, however, in general most particles must be smaller than 40 microns (Beckett, 2011). The reduction of the particle size of ingredients is critical to obtain the desired textural and rheological properties of chocolate (Do, Hargreaves, Wolf, Hort, & Mitchell, 2007). Afoakwa, Paterson, and Fowler (2008) reported a significant decrease in viscosity, thixotropy and yield of dark chocolate with an increase of particle size. Moreover, they reported that an increase in lecithin and fat levels intensified the effect of particle sizes on the rheological characteristics of chocolate.

The refined chocolate is a thick paste that does not flow. To improve the viscosity and remove volatile acids and any remaining moisture in the chocolate paste, a conching process is applied.

This process, which is very complex and has yet to be fully understood, consists of mixing, shearing, and aeration of chocolate masses during heating at an appropriate temperature (Owusu, Petersen & Heimdal, 2012). The changes in viscosity and flavour during conching are affected by temperature (dark chocolate = 50 to 90^{o}C, milk chocolate = 40 to 70^{o}C and white chocolate = 40 to 50^{o}C), time (4 to 36 hours depending on chocolate types), type and action of conche for mixing, amount of emulsifier and fat addition, and exposure to air during the process (Beckett, 2011). These parameters vary depending on the types of chocolate and composition. For example, temperatures of 40 to 50 ^{o}C are recommended for white chocolate to avoid browning (since this problem limits the shelf life of the final product). Moreover, for chocolate processing with sucrose substitutes, such as polyols, lower conching temperature is recommended to avoid agglomeration and melting of particles. For instance, at relatively very low temperatures standard isomalt loses its crystallization that's why conching temperature for chocolate made with isomalt may not be more than 45 ^{o}C (Beckett, 2011).

The next step of chocolate processing is tempering, a thermal process of treating a chocolate mass to produce fractionated, homogeneously dispersed, and highly stable fat crystals (Beckett, 2011). During this process, stable cocoa butter crystals are formed (polymorphic form V) with a melting point of 32 to 34 ^{o}C. This stable polymorphic form of cocoa butter gives good contraction, shelf-life stability, snap and gloss to the chocolate (Greweling, 2007).

Chapter 2

Suitability and applicability of polyols in the industrial manufacture of sucrose-free chocolates

2.1 Introduction

In the conventional chocolate manufacturing industry, sucrose is used as a sweetening agent. However, due to increasing concern about the high-calorie content of sucrose-made chocolate, manufacturers are searching for alternative sweeteners which can provide both physical bulk and the sweetness of sucrose to chocolate with low calorie or limited glycemic effect. Many of the alternative sweeteners which provide considerable advantages over conventional sucrose (in the manufacturing of chocolate with desired properties) are polyols (sugar alcohols). The low sweetening power of polyols can be offset using intense sweeteners such as stevia and aspartame during chocolate processing. Health Canada has currently approved the use of 10 different types of sugar alcohols, which includes isomalt, erythritol, xylitol, maltitol, hydrogenated starch hydrolysates, lactitol, maltitol syrup, sorbitol, mannitol, and sorbitol syrup. Some sugar alcohols occur naturally in various fruits and vegetables (such as xylitol in mushrooms and erythritol in grapes) in a small amount. The large-scale production of these polyols for commercial use needs industrial processes to be applied. For example, the industrial production of xylitol has been done by chemical hydrogenation of D-xylose and biotechnological production by metabolically engineered yeasts (such as *Candida* and *Saccharomyces cerevisiae*) (Granström, Izumori, & Leisola, 2007). Similarly, the industrial production of erythritol is done by fermentation of glucose with an osmophile yeast whereas enzymatic conversion of sucrose followed by hydrogenation is used for standard isomalt (Kruger, 2009). The purpose of this chapter is to provide a short review on the suitability and applicability of polyols (with a particular focus on isomalt, erythritol and xylitol) as raw materials in sucrose-free chocolate production.

2.2 Physiological characteristics of polyols

Polyols are carbohydrates that are also known as polyalcohols, polyhydric alcohols or sugar alcohols due to their resemblance in part with sugar as well as with alcohol in terms of their chemical structure. The characteristic properties of polyols can be found in Table 2.1. These polyols are not metabolized by cariogenic bacteria in the mouth and thus do not produce acid and form dental plaque, leading to tooth-friendly chocolate (Health Canada, 2005). These sugar alcohols provide a very low glycemic effect because, once absorbed, the process through which their energy conversion occurs requires very little or no insulin, and thus are suitable chocolates for diabetic individuals (Health Canada, 2005). The glycemic response of polyols-made chocolate is generally lower than that of sucrose-made chocolate. This has been demonstrated when a glycemic response of milk chocolate made with sucrose was found to be 37 as compared to 19 for chocolate made with maltitol and 6 for chocolate made with lactitol /polydextrose (Kruger, 2009). The composition of milk chocolate was cocoa liquor (11%), cocoa butter (22%), full cream milk powder (20%), skimmed milk powder (1.48%), sugar or sweeteners (45% sucrose or 45% maltitol or 33% anhydrous lactitol with 11.85% polydextrose, 0.13% aspartame and 0.02% acesulfame K), lecithin (0.5%) and vanilla (0.02%) (Kruger, 2009). There is convincing evidence from scientific studies that low glycemic effect- diets, due to their low glycemic response, reduce the risk of coronary heart disease, improve glycemic control of type 1 and type 2 diabetic individuals, and play an important role in the prevention of obesity (Monro & Shaw, 2008).

Table 2.1: Characteristics of polyols (Kruger, 2009; Tiefenbacher, 2017)									
Substance	Chemical Abstracts Service (CAS) number	Status (food additive no. E in EU	Type of saccharide	Relative sweetness	Energy content (Kcal/g)	Water Solubility (g/100g, 20^{o}C)	Glycemic response vs glucose (=100)	Cooling effect	Melting point (^{0}C)
Sucrose	57-50-1	Food	disaccharide	1.0	4	66	65	No	190
Lactitol	585-86-4	E966	disaccharide	0.3 – 0.4	2.4 (EU); 2.0 (US)	58	2	Low	122
Maltitol	585-88-6	E965	disaccharide	0.75 – 0.9	2.4 (EU); 2.1 (US)	58	34	Low	150
Isomalt	64519-82-0	E953	disaccharide	0.45 - 0.5	2.4 (EU); 2.0 (US)	25	4.7	Low	145-150
Mannitol	69-65-8	E421	monosaccharide	0.5 - 0.6	2.4 (EU); 1.6 (US)	16	<5	Strong	165
Sorbitol	50-70-4	E420	monosaccharide	0.5 - 0.6	2.4 (EU); 2.6 (US)	69	<5	Strong	97
Xylitol	87-99-0	E967	monosaccharide	0.9	2.4 (EU, US)	63	8	Strong	94
Erythritol	149-32-6	E968	monosaccharide	0.6	0 (EU); 0.2 (US)	33	0	Strong	121

Despite these healthier properties, excessive consumption of sugar alcohols may cause gastrointestinal effects (such as laxative or gas producing effects) which is a limitation to chocolate production from polyols (Grembecka, 2015). This is due to the poor absorption of these substances in the gastrointestinal tract (with the notable exception of erythritol). The likelihood of such effects occurring is dose-dependent in healthy individuals, and malabsorption increases when these substances are consumed in combination (Lenhart & Chey, 2017). Nevertheless, the frequent consumption of polyols- and/or polydextrose-made chocolates may develop a tolerance and healthy individuals may increase the consumption without experiencing any side effects (Health Canada, 2005).

2.3 Cooling effect of polyols

A sensorial property of all polyols is their negative heat of solution (Fig 2.1) due to which a cooling sensation in the mouth occurs when consumed. The cooling effect depends on the solubility of polyols at mouth temperature, enthalpy of dissolution (or heat of solution), and the particle size (the finer the particles, the more rapid dissolving occurs, leading to a cooling effect). In comparison to sucrose, xylitol and erythritol have a high negative heat of solution and thus have strong cooling effects. That is why these sugar alcohols can be advantageous in the production of filled chocolates and mints (since cooling sensation is a desired characteristic in these products). Whereas this cooling sensation is not expected in traditional chocolates (such as dark chocolate, milk chocolate, or white chocolate) where its effects may need to be minimized or balanced by combining this type of sugar alcohols with other bulk sweeteners (such as polydextrose or inulin), which can offer a complementary positive enthalpy of solution (Kruger, 2009). This can be demonstrated by one of the active patents in which the cooling effect was reported to be reduced by co-crystallization of hydrogenated maltodextrin and polyols (erythritol and xylitol) (Cunningham, Kuenzle,

Stanizewski & Jamieson, 2005). However, this was argued in another patent saying that this observed reduced cooling effect must be considered as a diluted effect and provided another solution to reduce this effect (in particular for erythritol) by using fibres (specifically water-soluble dietary fibres) and/or sugar esters (Vercauteren, Ronny, Leontina & Marcel, 2008). This patent claimed that the ratio of fibres and/or sugar esters to erythritol from 1:5 to 1:300 (in weight-by-weight basis) will be able to reduce the cooling effect of erythritol in chocolate (where erythritol is present in 1 to 70% w/w of the total composition of chocolate). In the case of isomalt-containing chocolates, there are no unpleasant cooling effect in the mouth due to its low negative heat of solution (-9.4 cal/g) (McNutt & Sentko, 2003).

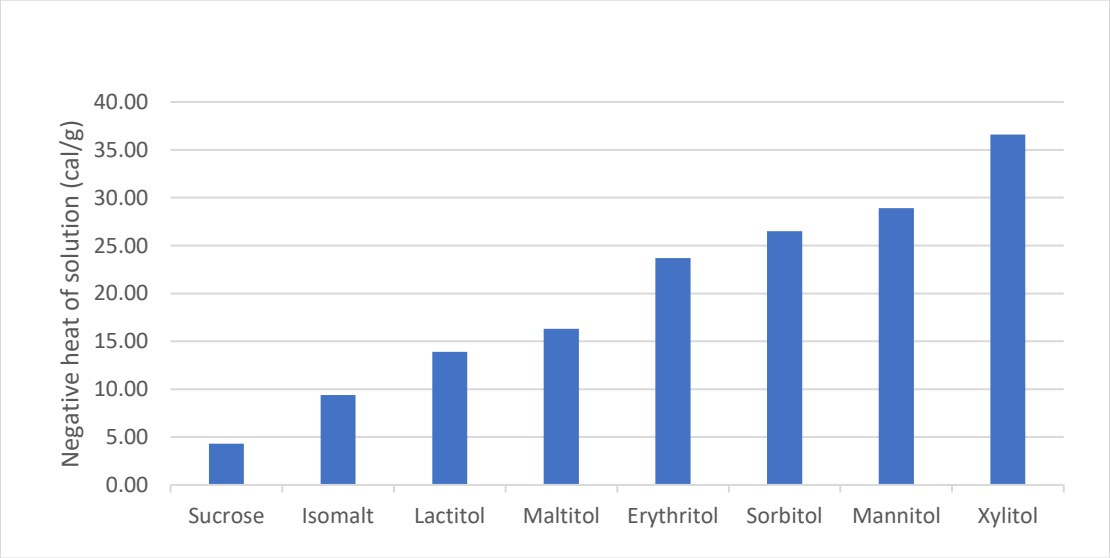

Figure 2.1: Heat of solution of polyols and sucrose at 25ºC (Zumbe, Lee, & Storey, 2001).

2.4 Conching temperatures of sugar-free chocolates with polyols

The properties (such as melting point and solubility) of bulk sweeteners play a very important role in the processing condition of sugar-free chocolates. The low melting point or high solubility of a polyol can result in the gritty agglomeration leading to undesirable changes in flow properties of

polyols-made chocolates at a higher conching temperature (Kruger, 2009; Zumbe et al., 2001). Moreover, the presence or absence of water of crystallization determines the maximum conching temperature for processing of chocolates. Polyols, such as standard isomalt, lose their water of crystallization at relatively low temperature, hence, in order to lower the risk of formation of undesirable changes in the flow properties of isomalt-made chocolate, the conching temperature in this respect may not be higher than 45^{o}C. There are different forms of isomalt for various specific applications in today's market. One of these forms of isomalt is isomalt LM, which has high-temperature stability and low hygroscopicity (less than 1% moisture content), for the production of sugar-free chocolate (Rad, Pirouzian, Konar, Toker, & Polat, 2019). This isomalt LM can be used for making plain dark chocolates at a conching temperature of up to 80^{o}C, whereas for milk chocolates up to 70^{o}C (Kruger, 2009). Similarly, erythritol can be conched up to 80^{o}C for plain dark chocolates, whereas up to 70^{o}C for milk chocolates. The maximum temperature of polyols-made chocolates with conventional conching can be found in Table 2.2.

Table 2.2: Maximum temperature of polyols-made chocolate masses with conventional conching (Kruger, 2009) ('+' sign implies 'presence' and '–' sign implies 'absence').

Polyols	Water of crystallization	Anhydrous	Maximum temperature (^{o}C)
Standard isomalt	+	+	40
Isomalt LM	-	+	80
Xylitol	-	+	50
Erythritol	-	+	80
Sorbitol	-	+	40
Maltitol	-	+	80
Lactitol anhydrous	-	+	80
Lactitol monohydrate	+	-	60

2.5 Conclusion

Advances in product development have led to the diversified utilization of polyols to replace higher calorie confectionery products by providing low calorie and complementing sugar's

functionality. There is still a problem of limited tolerance due to the gastrointestinal effects (such as laxative or gas producing effects) of polyols which needs to be completely addressed to provide a risk-free consumption of sugar-free chocolate made with these bulking sweeteners.

Chapter 3

Oleuropein: A potential functional food ingredient

Abstract

Olive, native to the Mediterranean region, is a promising and significant source of heath beneficial bioactive phenolic compounds such as oleuropein. The bitter nature of unprocessed and immature olives is due to the presence of oleuropein, which is also the main glycoside present in olives. Oleuropein has been found to show health beneficial effects in human such as cardio protection, anticancer, and antidiabetic which are mainly attributed to its anti-inflammatory and antioxidant activities. Thus, many scientific studies have been conducted with this polyphenol from olives over the past decades to provide evidence for its health benefits and to support the use of oleuropein and oleuropein-rich extracts as a functional food ingredient. Also, several studies have been performed to improve the extraction method of oleuropein and its yield from olives. The present review discusses the studies performed on the health benefits of oleuropein along with its occurrence, structure, extraction methods, biosynthesis, and toxicology.

Keywords: Oleuropein, Olives, Olive oil, Oleaceae

3.1 Introduction

Olive trees are an evergreen small tree belonging to the family Oleaceae and subfamily Oleoideae and are botanically known as *Olea europaea*. The characteristic phenolic compound present in olives, known to cause a bitter taste, is oleuropein (Shasha & Leibowitz, 1961). This polyphenol which is the most abundant in the leaves of olive is found to induce apoptosis in human breast cancer cell line (MCF-7) via a p-53 dependent pathway (Hassan et al., 2013),to improve the insulin sensitivity in overweight human with type-2 diabetic mellitus (De Bock et al., 2013), and to improve lipid metabolism to reduce body weight in high cholesterol diet-induced obesity rat (Hadrich, Mahmoudi, et al., 2016).

3.2 Occurrence

Oleuropein occurs in numerous species of the Oleaceae family such as *Fraxinus americana* (Sanz et al., 2012), *Syringa reticulata* (Bi et al., 2011), *Phyllirea latifolia* (Agati, Cerovic, Pinelli, & Tattini, 2011), *Ligustrum lucidum* (Guo et al., 2011), *Jasminum officinale* L.f. var. *grandiflorum* (Teerarak, Laosinwattana, & Charoenying, 2010),and *Chionanthus virginicus* (Boyer et al., 2005). It was reported to be present in the leaf (Le Tutour & Guedon, 1992), fruit (Servili, Baldioli, Selvaggini, Macchioni, & Montedoro, 1999), and in the root and stem (Baidez, Gomez, Del Rio, & Ortuno, 2006) of the olive tree. Ansari, Kazemipour, and Fathi (2011) reported the content of oleuropein to be ranging between 6.1 and 13.0 mg/g dry weight in the olive leaves obtained from Iran's different geographical regions. The content of oleuropein has been found to be higher during the first maturation stage of the fruit, which later decreased as fruit ripened (Guodong, Jianguo, Xiaoxia, & Ying, 2019). Guodong et al. (2019) also reported on average 6.32 times more oleuropein content in leaves than in fruit. The content of oleuropein is affected by cultivar, plant part, climate, harvesting season, olives maturation period and genetic factor (Ranalli et al., 2006),

as well as by extraction and pre-treatment methods (Abaza, Taamalli, Nsir, & Zarrouk, 2015; Ahmad-Qasem, Barrajon-Catalan, Micol, Mulet, & Garcia-Perez, 2013; Stamatopoulos, Katsoyannos, Chatzilazarou, & Konteles, 2012). Recently, Lama-Munoz et al. (2019) reported that the moisture content of olive leaves also plays a significant role in the content of oleuropein since it affects its extraction.

3.3 Structure

Oleuropein (Figure 3.1) was discovered in the fruit of *O. europaea L.* by Bourquelot and Vintilesco in 1908 (Garcia, Castillo, Lorente, Ortuno, & Del-Rio, 2000). It has three structural subunits namely hydroxytyrosol (4-(2-hydroxyethyl) benzene-1,2-diol), elenolic acid (a secoiridoid) and a glucose molecule (Omar, 2010). Oleuropein was reported to have a low energy conformation of 11.97 kcal/mol which was determined via semi-empirical and ab initio calculations and later confirmed by molecular dynamics simulations (Gikas, Bazoti, & Tsarbopoulos, 2007). In the same study, low energy conformation of oleuropein was reported to have a tight geometry where sugar moiety and hydroxytyrosol are in close proximity, and conformation stabilization occurs because of the three hydrogen bonds present (Gikas et al., 2007). However, Gikas et al. (2007) reported that their simulation was only performed in the gas phase (solvent devoid environment). Recently, Souilem et al. (2016) reported that the conformation of oleuropein strongly depends on its microenvironment and demonstrated the amphiphilic character of this polyphenolic compound using radial distribution function analysis in vacuum, water, and triolein-water systems.

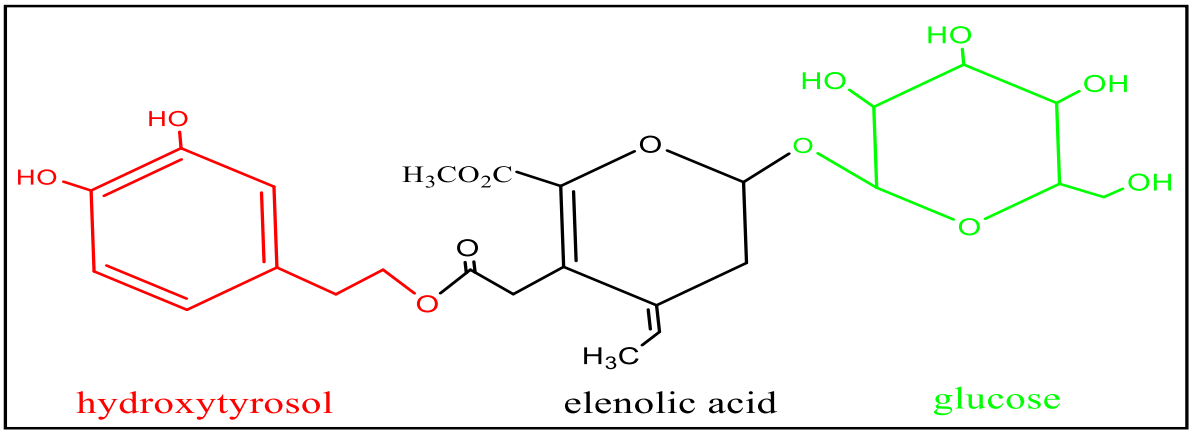

Figure 3.1: A structure of oleuropein.

3.4 Extraction method

Over the last few years, studies have focused on developing extraction methods of oleuropein and oleuropein-rich extract for commercial applications. The extraction of oleuropein from olive tree is usually carried out using traditional extraction method such as maceration, a solvent extraction method in which solvents such as methanol, ethanol, diethyl ether, acetone, and ethyl acetate are used (Abaza et al., 2015). Maceration is considered a favorable process since heat-sensitive compounds like oleuropein can be recovered at low temperature. This, however, requires very long extraction time, a large quantity of solvent, high energy consumption (during the recovery process) as well as provides low extraction yields. In order to minimize all these drawbacks, advanced oleuropein extraction methods were developed which include pressurized liquid extraction (Lama-Muñoz et al., 2019; Xynos et al., 2014), ultrasonic-assisted extraction (UAE) (Cifa, Skrt, Pittia, Di Mattia, & Poklar Ulrih, 2018; Japon-Lujan, Luque-Rodriguez, & De Castro, 2006a; Vural, Algan Cavuldak, Kenar, & Akay, 2019), ultrasonic-assisted and reduced-pressure extraction (URPE) (Xie, Huang, Zhang, You, & Zhang, 2015), microwave-assisted extraction (MAE) (Japón-Luján, Luque-Rodríguez, & De Castro, 2006b), microwave-assisted extraction and dispersive liquid-liquid microextraction (MAE-DLLME) (Habibi, Mohammadi, Farhoodi, & Jazaeri, 2018), superheated liquid extraction (Japon-Lujan & de Castro, 2006), salting-out-assisted cloud point

extraction (Stamatopoulos, Katsoyannos, & Chatzilazarou, 2014) and supercritical antisolvent extraction (Baldino, Della Porta, Osseo, Reverchon, & Adami, 2018).

When compared with conventional extraction method, UAE was reported to be an effective alternative as it offers improvements in oleuropein yield, lower solvent consumption, lower extraction time and is most importantly a low temperature extraction technique (Cifa et al., 2018), however in one comparative study led by Xie et al. (2015), it was pointed out that the highest oleuropein yield and shortest extraction time were achieved by URPE when compared with conventional solvent extraction (under atmospheric pressure), reduced pressure boiling extraction, and UAE. Similarly in another study, oleuropein yield was reported to be equal among MAE (3 minutes extraction time), supercritical carbon dioxide fluid extraction (2 hours of extraction time), and Soxhlet extraction (6 hours of extraction time) (Dang, Huang, Zhang, Feng, & Dun, 2006). Even after developing these advanced non-convectional and combined extraction techniques over the past years, there are still some limitations such as expensive equipment cost, costly solvents (while some solvents are either toxic or not environmentally friendly), and difficulties in continuous operation which need to be addressed for their further industrial application. Recently, Yasemi, Heydarinasab, Rahimi, and Ardjmand (2017) reported to have developed and optimized a green, inexpensive, and continuous method, called microchannel system, for extraction of oleuropein from olive leaves at the industrial scale. In this microchannel system, there are two micro syringe pumps of which one pump is used to mix olive leave powder and ethyl acetate whereas another one is filled with either ethanol, methanol, or ethyl acetate. These pumps divert feed in inlet stream of the T-shaped microchannel mixer which has a coil in the outlet stream for enhancing the residence time. At the end of extraction, aqueous phase is injected to HPLC system. This microchannel method has been reported to have a high extraction yield (96.29%) as compared

to maceration (around 40%) Soxhlet (62%), and ultrasound-assisted (81.29%) methods under the optimum conditions. The optimal conditions for ultrasound-assisted method were 80 kHz frequency, 100 W ultrasound power and temperature of 25^0C whereas for microchannel method the best condition for oleuropein extraction were temperature of 35^0C, pH of 2.23, volumetric flow rate ratio of 1.75 of two phases and contact time of 30 s of two phases. This microchannel method operates at low temperature and has a shorter extraction time, thus preventing thermal degradation of sensitive compounds while enhancing their bioavailability and their antioxidant activity.

The identification and quantification of oleuropein is generally performed using reversed phase high – performance liquid chromatography (HPLC) using gradient elution method mainly at 280 nm (Abaza et al., 2015). Oleuropein is detected at the wavelength of 280 nm because the absorption of oleuropein lies in the UV range with maximum wavelength at 280 nm. Another oleuropein quantification method, high-resolution gas chromatography (HRGS) was also reported, but it was found to have a longer analysis time than HPLC (Ranalli et al., 2009). Other oleuropein detections and isolation methods are TLC (Thin Layer Chromatography), HPLC-PDA (HPLC – photodiode array), and HPLC-MS techniques.

3.5 Biosynthesis

Damtoft, Franzyk, and Jensen (1993) proposed the biosynthesis pathway for oleuropein formation in the Oleaceae which is shown in Figure 3.2. At first, three molecules of acetyl coenzyme-A condense to produce HMG-CoA (ester β-hydroxy-β-methylglutaryl-coenzyme A), which in turn undergoes hydrolysis and enzymatic reduction to produce mevalonic acid (Dewick, 2002). This mevalonic acid further undergoes branching from secondary metabolism in the mevalonic acid pathway producing oleosides (Damtoft, Franzyk, & Jensen, 1992), through which oleuropein is derived (Damtoft et al., 1993).

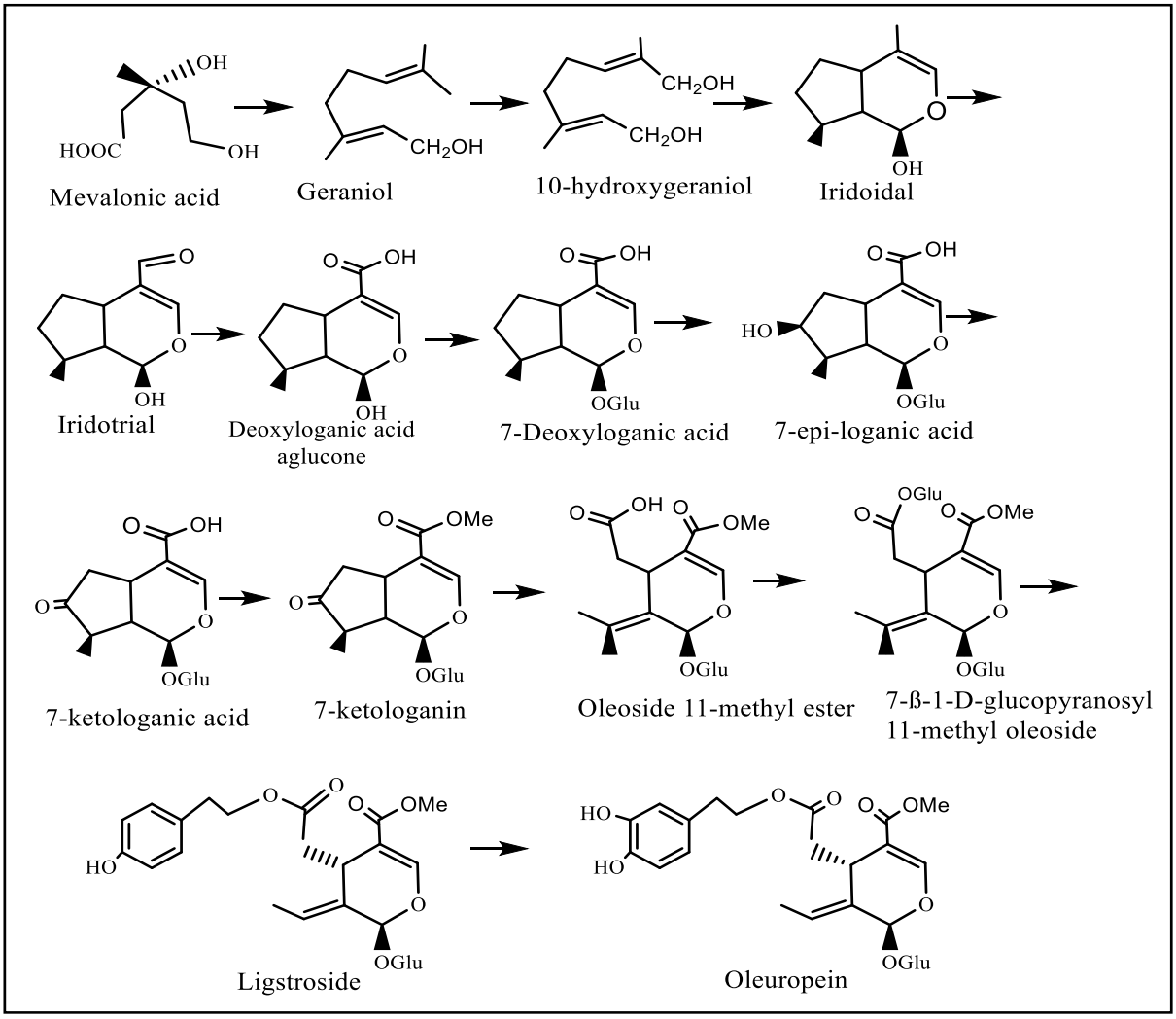

Figure 3.2: Biosynthesis pathway of oleuropein in Oleaceae (Damtoft et al., 1993).

3.6 Pharmacological activities

The main reported pharmacological activities of oleuropein are anticancer, antidiabetic, cardioprotective and anti-microbial. These health benefits are mainly attributed to the anti-inflammatory and antioxidant activities of oleuropein.

3.6.1 Antioxidant activity

For the proper physiological function of the human body, a balance is needed between free radicals (reactive oxygen species (ROS) and reactive nitrogen species (RNS)) and antioxidants. If the amount of free radicals surpasses the ability of the body to regulate them then the condition is

called oxidative stress (Sies, 1997). The hepatic oxidative stress markers are catalase (CAT), glutathione peroxidase (GPx), malondialdehyde (MDA), superoxide dismutase (SOD), and reduced glutathione (GSH). Oxidative stress was found to induce cancer, atherosclerosis, dementia, and diabetes (Liguori et al., 2018). The excess amount of oxidative stress can kill cells either by apoptosis or necrosis (Zamzami et al., 1995). The antioxidant compounds, both natural and synthetic, act as free radical scavengers. The commercially available synthetic antioxidant compounds such as butylated hydroxytoluene (BHT) and butylated hydroxyanisol (BHA) are cheap, highly stable, and effective, however, they are found to be carcinogenic (Ito, Fukushima, & Tsuda, 1985). Thus, the importance of natural antioxidant is increasing, and it has been reported that oleuropein provides higher antioxidant activity than BHA and BHT at the same concentration of 20 μg/mL (Gulcin, Elias, Gepdiremen, Taoubi, & Koksal, 2009). Moreover, in an in-vitro study of four commercially available natural products, it has been reported that the antioxidant activity of olive leaf extract is higher than that of lutein but lower than that of ellagic acid and sesamol when measured by DPPH (2,2-diphenylpicrylhydrazyl), FRAP (Ferric Reducing Antioxidant Power), ORAC (Oxygen Radical Absorbance Capacity) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay (Hayes, Allen, Brunton, O'Grady, & Kerry, 2011). The antioxidant activity of oleuropein is due to the existence of 1,2-dihydroxybenzene moiety in its structure (Vogel et al., 2015). Oleuropein prevents the formation of free radicals through its metal-chelating activities by catalyzing the production of free radicals (Andrikopoulos, Kaliora, Assimopoulou, & Papageorgiou, 2002) as well as through inhibition of inflammatory enzymes. The antioxidant activity of oleuropein was determined using FRAP, trolox equivalent antioxidant capacity assay (TEAC), radical scavenging activity assay (DPPH assay and $ABTS^{+}$ assay), and

superoxide anion assay (Ahmad-Qasem et al., 2013; AlShaal, Karabet, & Daghestani, 2019; Bi et al., 2011).

It has been reported that oleuropein and its derivative oleacin can suppress ROS production (O_2^-, and H_2O_2) and RNS production (NO, and $ONOO^-$) in concentration-dependent manner (1-50 μM) in in- vitro non-cellular systems (Czerwinska, Kiss, & Naruszewicz, 2012). In the same study, oleuropein was also reported to reduce neutrophils-released myeloperoxidase (an enzyme which can catalyze the formation of a highly reactive species called HOCl), and to reduce the production of ROS via the stimulation of polymorphonuclear neutrophils in a cellular system. Oleuropein was reported to enhance the therapeutic effect of human mesenchymal stem cells (MSC) against ischemic diseases by reducing the effect of H_2O_2-induced apoptosis via regulation of Bax (a pro-apoptotic marker) and Bcl-2 and Mcl-1 (both anti-apoptotic markers) and through the inhibition of H_2O_2-indcued autophagy via the modulation of autophagy-death signals like mTOR, ULK1, Beclin-1, AMPK, and LC3 in dose-dependent manner in an in- vitro study (Ji et al., 2018). In one of the in- vivo studies, oleuropein was shown to exhibit antidepressant effects in dose- and time-dependent manners in intraperitoneally reserpine-induced mice due to its antioxidant activity where it was reported to reduce MDA (a product of lipid peroxidation) and the NO levels in serum and brain, and increase serum and brain antioxidant capacity (Rabiei, Jahanbazi, Alibabaei, & Rafieian-Kopaei, 2018). Other studies also reported that oleuropein reduces both MDA and NO levels and increases CAT and GPx levels in colchicine-induced rats (10, 15, and 20 mg/kg dose of oleuropein for 10 days) (Pourkhodadad et al., 2016), and aged rats (50mg/kg dose of oleuropein for 6 months) (Sarbishegi, Mehraein, & Soleimani, 2014). Moreover, oleuropein was found to increase the level of antioxidants such as GSH, and SOD, decrease the level of liver enzymes such as alkaline phosphate (ALP), alanine transaminase (ALT), and aspartate transaminase (AST),

decrease in the tumor necrosis factor alpha (TNF-α) protein level, and significantly increase the expression and binding activity of Nrf2 and HO-1 hepatic gene in cyclophosphamide-induced male Wistar rats (30 mg/kg/day dose of oleuropein for 10 days) (Sherif, 2018) and carbon tetrachloride-induced mice (100 and 200 mg/kg/day dose of oleuropein for 3 days) (Domitrović et al., 2012). Recently, Bonechi et al. (2019) reported successfully developing a zwitter-ionic liposome-assisted drug delivery system loaded with oleuropein to increase the antioxidant activity in osteoarthritic pathologies and to overcome the side effects of classical drugs. They also reported this drug delivery system to be non-cytotoxic in nature at any concentration via a cytotoxicity assay. Additionally, in the same study, oleuropein-loaded liposomes were found to have better physical stability than hydroxytyrosol-loaded and tyrosyl-loaded liposomes because of more negative overall charge for oleuropein-loaded liposomes (encapsulation of oleuropein, hydroxytyrosol and tyrosyl change the net surface charge of a liposome), indicating a lower chance of aggregation of liposomes with time. Collectively, it can be said that oleuropein, with its antioxidant properties, has the potential to provide nutritional support to detoxify oxidative stress-inducing substances.

3.6.2 Anti-inflammatory activity

Inflammatory responses due to injury, toxic compounds and pathogens within organs may potentially lead to diseases or tissue damage. The damaged tissues at first activate inflammatory stimuli, such as TNF-α, interleukin – 1β (IL-1β) and interleukin-6 (IL-6). The activation of these stimuli further lead to the activation of inflammatory signaling pathways, such as toll like receptor / nuclear factor kappa-B (TLR / NF-κB), Janus kinase / signal transducers and activators of transcription (JAK / STAT), and mitogen-activated protein kinase (MAPK) pathways (Chen et al., 2018). These pathways then later release inflammatory mediators to promote inflammatory responses. TNF-α and IL-1β are also involved in the induction of inducible nitric oxide synthase

(iNOS) and cyclooxygenase (COX) – 2 at the site of injury. iNOS and COX – 2 have been reported to play a vital role in the development of inflammation – related diseases (Murakami & Ohigashi, 2007).

The protective effect of oleuropein or oleuropein-rich extract against certain cancers and cardiovascular diseases can, to some extent, be attributed to its anti-inflammatory effects. It was reported that oleuropein elicited anti-inflammatory effects by the modulation of inflammatory parameters, inhibition of proinflammatory cytokines biosynthesis, or lipoxygenase activity (De la Puerta, Gutierrez, & Hoult, 1999; Giamarellos-Bourboulis et al., 2006; Puel et al., 2006).

Puel et al. (2006) reported that bone – sparing effect of oleuropein on bone loss induced by talc granulomatosis in ovariectomized rats was due to its anti-inflammatory property, probably by modulating the inflammatory parameters. The modulation of inflammatory parameters was shown by the decrease in plasma fibrinogen concentration and spleen weight. In another study by Khalatbary and Zarrinjoei (2012), they reported that the intraperitoneal administration of oleuropein (immediately and an hour after spinal cord injury) significantly attenuated the expression of TNF-α and IL-1β, and as a result to the expression of iNOS and COX-2. Similarly, Ryu et al. (2015) demonstrated the anti – inflammatory effect of oleuropein (100 – 300 uM) by the inhibition of MAPK and TLR signaling pathways in lipopolysaccharide (LPS) – induced RAW 264.7 macrophage cells and a Zebrafish model. They reported that oleuropein reduces the production of LPS – induced NO by downregulating iNOS and COX – 2 in a dose – dependent manner. In similar dose – dependent fashion, they found that oleuropein suppresses the LPS – induced release of pro – inflammatory cytokines (IL-1β and IL-6) in enzyme immunoassay. However, in their study, they did not explore whether TNF-α and IL – 10 were also regulated by oleuropein. Authors of this study reported that inhibition of the TLR signaling pathway was due

to the interference of oleuropein with the inhibitory kappa B-α (IκB-α) phosphorylation, and consequently suppressing the NF-κB translocation into the nucleus. Moreover, they reported that the inhibition of MAPK signaling pathway was due to the significant decrease in the LPS – activated extracellular signal – regulated protein kinase (ERK) ½ and c – Jun N – terminal kinase (JUN). Giamarellos-Bourboulis et al. (2006) also reported that oleuropein prolonged survival in *Pseudomonas aeruginosa* – induced sepsis rabbits by inhibiting biosynthesis of TNF-α and IL-6. In the study performed by De la Puerta et al. (1999), they reported that oleuropein elicits anti – inflammatory effects by inhibiting the 5 – leukotriene (LO) pathway of arachidonate metabolism in the ionophore – activated leukocytes. This inhibition was thus further reported to reduce the generation of leukotriene B_4. Recently, in a study of the drug delivery system for oleuropein in inflammatory bowel diseases treatment, it was reported that oleuropein loaded in nanostructured lipid carriers was more efficient in reducing the secretion of TNF-α and IL – 6 compared to the traditional form of oleuropein (Huguet-Casquero, Xu, Gainza, Pedraz, & Beloqui, 2020).

3.6.3 Anticancer activity

Numerous experiments have confirmed the anticancer activities of oleuropein which can be observed in human cancer cell lines such as T-24 urinary bladder carcinoma (Goulas et al., 2009), HL60 and K562 leukemia tumor cells (Fabiani et al., 2011; Fulco, Fagan, & Phelan, 2019), MCF-7 and MDA-MB-231 breast adenocarcinoma (Bayat, Mansoori Derakhshan, Mansoori Derakhshan, Shekari Khaniani, & Alivand, 2019; Elamin et al., 2013; Han, Talorete, Yamada, & Isoda, 2009; L. Liu et al., 2019; Lu et al., 2019; Przychodzen et al., 2019; Sepporta et al., 2014), esophageal cancer (Zhang & Zhang, 2019), U-251 and A-172 glioma cells (M. Liu, Wang, Huang, Chen, & Li, 2016), A-549 and H1299 lung carcinoma cells (Antognelli, Frosini, Santolla, Peirce, & Talesa, 2019; Mao et al., 2012; Wang et al., 2019), HeLa human cervical carcinoma cells(Yao

et al., 2014), and HepG2 liver cancer cells (Yan, Chai, Cai, Miao, & Ma, 2015). In a study performed by L. Liu et al. (2019) to study the effects of oleuropein (in dose – and time-dependent manners) on human breast cancer cells (estrogen receptor-positive MCF-7 and estrogen receptor-negative MDA-MB-231), it has been found that oleuropein has a more pronounced effect on MDA-MB-231 cells compared with MCF-7 cells showing survival of a 4.3-fold difference between both cell lines. The higher efficacy of oleuropein against estrogen receptor-negative cells (ER- cells) than that on estrogen receptor-positive cells (ER+ cells) was also found by Elamin et al. (2013) with a 10-fold difference in survival between these two cancer cell lines at 200 μM oleuropein. The overexpression of p-glycoprotein in MCF-7 cells (Wosikowski et al., 1995) could be a reason behind low effectiveness of oleuropein in these cells. Liu et al. (2019) reported, using basal-like MDA-MB-231 cell line, that oleuropein acts as an anticancer agent by inducing cellular apoptosis via mitochondrial mechanisms, increasing the accumulation of ROS, and by suppressing the NF-kB signaling cascade activation. In this study, the cellular apoptosis effect of oleuropein through mitochondrial pathway in MDA-MB-231 cells is found to be mediated by an increase in the cleavage of PARP (poly (ADP-ribose) polymerase) and activity of caspase-3/7. In oleuropein-induced ER- SKBR-3 cell lines, Chimento et al. (2014) also reported that apoptosis occurs because of activation of GPER/GPR30-dependent associated with caspase 3/9 and increase in PARP-1. Liu et al. (2019) also reported that the excessive accumulation of a signaling molecule ROS in the presence of oleuropein leads to inhibition of PI3K/Akt pathway which in turn causes apoptosis in ER- cells. A similar result was reported in HepG2 human hepatocellular carcinoma cell lines treated with oleuropein (Yan et al., 2015). Moreover, it has been demonstrated that oleuropein is able to inhibit the activation of NF-kB signaling cascade in MDA-MB-231 cell lines via a suppression of phosphorylated IkBα protein expression in the cytoplasm and by negatively

regulating the upstream kinase of IkBα (Liu et al., 2019). For the first time, oleuropein is reported to induce cytostatic and cytotoxic effect in MCF-7 cell lines by the concentration dependent inhibition of non-receptor tyrosine phosphatase 1 (PTP1B) activity (Przychodzen et al., 2019). Furthermore, oleuropein-treated MCF-7 cells resulted in the inhibition of cell proliferation which was mediated through delay in cell cycle from G1 to S phase (Han et al., 2009), whereas the antiproliferative effect on oleuropein-treated pancreatic cancer cells (MIA PaCa -2) was mediated through a cell cycle arrest at G2-phase (Goldsmith et al., 2018). Few studies have been performed to examine the effects of oleuropein in tumor-grown animals (in vivo). When oleuropein was administered (at 125 mg/kg bodyweight for 35 days) in female nu/nu athymic mice injected with MCF-7 cells into their mammary fat pads, it was observed that oleuropein inhibited the growth of xenografted MCF-7 cells as well as their invasiveness into mouse lung (Sepporta et al., 2014). However, in another oleuropein administered (100µg or 0.3 mg/kg bodyweight for 5 days) study performed by Martinez-Martos et al. (2014), in male Wistar rats xenografted with C6 glioma cells into both dorsal flanks, reported no inhibition of the C6 glioma cells.

A novel nanocapsular system has been formulated by loading oleuropein in an in-vitro study on HCT-116 colon cancer cells. This nanocapsule was found to be 28 times more cytotoxic as compared to oleuropein alone (Al-Karaki, Awadallah, Saleh, & Nasr, 2019). In another study by Sherif and Al-Gayyar (2018), oleuropein was found to increase the antitumor activity of cisplatin, a standard antitumor drug against solid tumors, in HepG2 cells by affecting matrix metalloproteinase-7 (MPP-7) activity. Thus, this study concluded that oleuropein can help to improve the side effects of cisplatin by its treatment dose-reduction. This strong evidence, in both in- vitro and in-vivo studies, demonstrated that oleuropein has a potential to inhibit the development and proliferation of cancer cells, however, it is very difficult to apply the outcomes

of these studies to humans since the human body is very complex and much different when compared to cell models. Thus, clinical trials are further required to prove the anti-cancerous activity of oleuropein in human.

3.6.4 Anti-diabetic effect

An in- vitro study of oleuropein treated C2C12 muscle cells demonstrated that oleuropein (at 200-400 µM) when administered alone activates insulin-independent AMPK /ACC (adenosine monophosphate-activated protein kinase/Acetyl- CoA carboxylase) pathways (Hadrich et al., 2016), which is a common mechanism of action of anti-diabetic drugs (such as metformin and rosiglitazone) for treatment of T2DM (type-2-diabetes mellitus) (Fryer, Parbu-Patel, & Carling, 2002; Zhou et al., 2001). The activation of AMPK stimulates GLUT4 (Glucose transporter 4) translocation into the cell membrane which further improves insulin-stimulated glucose uptake (Zheng et al., 2001) whereas total GLUT4 protein level is up-regulated by oleuropein (Hadrich et al., 2016). Furthermore, phosphorylation and regulation of downstream targets such as ACC (acetyl-CoA carboxylase) in the body is a result of AMPK activation (Fryer & Carling, 2005). Hadrich et al. (2016) also reported that when oleuropein was mixed with insulin in C2C12 cells, it led to activation of insulin-dependent PI3 (Phosphatidylinositol 3-kinase)/Akt pathway which further stimulates translocation of GLUT4. In the same muscle cells type (C2C12), it has been demonstrated that lower concentration of oleuropein, such as 10 µM, is sufficient to enhance the uptake of glucose via the AMPK signaling pathway (Fujiwara et al., 2017).

Several in- vivo experiments have also been performed in both mice and rats over the last decade. For example, a streptozotocin diabetic-induced rat (60 mg/kg body weight), was intraperitoneally administered with oleuropein (3 mg/kg and 5 mg/kg for 56 days), showed a significant decrease in their blood glucose level (Sangi, Sulaiman, El-wahab, Ahmedani, & Ali, 2015). It was also

demonstrated that an ethanolic extract of olive leaves (EEOL) improved diabetes- caused adipokines changes by reducing leptin and increasing adiponectin level in rats with diabetes induced by a low dose of streptozotocin and high-fat diet (Guex et al., 2019).

Adiponectin, a key adipokine, was found to regulate glucose metabolism (Tsao, Lodish, & Fruebis, 2002) and an increase in its level can reduce blood glucose levels (Guimaraes, Sardinha, Mizurini, & Carmo, 2007). It has been reported that an olive-rich extract reduced pro-inflammatory cytokine such as IL-1, IL-2 IL-6, IL-17A, IL-1β, COX-2, CD (cluster of differentiation) 4+, CD8+, IFN (interferon) -γ, and TNF– α, but increased IL-10, an anti-inflammatory cytokine, and enhanced IRS-1 (insulin receptor substrate) expression in streptozotocin-induced diabetic rats and mice (Guex et al., 2019; YN Liu, Jung, Park, & Kin, 2014; Mohamed, Abdou, & Marzouk, 2018; Park, Jung, Yang, & Kim, 2013). In the type-2 diabetic animal model called Tsumura Suzuki Obese Diabetes (TSOD) mice, OPIACE (oleuropein-containing supplement with more than 35% in w/w basis) administration showed that in the long run it can improve hyperglycemia and impaired glucose tolerance (Murotomi et al., 2015). The anti-hyperglycemic effect of olive leaves extract from the Tunisian Gerboui variety was reported in alloxan-induced diabetic rats (intraperitoneal injection of 120 mg of alloxan monohydrate per kg rat body weight for 21 days and 100 mg of leave extract per kg alloxan body weight), with a significant decrease in blood glucose level and an increase in plasma insulin concentration and hepatic glycogen (Salah, Hafedh, & Manef, 2017). A similar result was also obtained by Qadir, Ali, and Qader (2016) in oleuropein-treated (5-20 mg/kg body weight) alloxan-induced (subcutaneous injection of 100 mg of alloxan monohydrate per kg body weight) type-1 diabetic rats.

The anti-hyperglycemic activity of oleuropein and oleuropein-rich extracts has also been confirmed in human (Carnevale et al., 2018; De Bock et al., 2013; Visen et al., 2009; Wainstein et

al., 2012) (Table 3.1). T2DM involves a defect in insulin sensitivity (Ferrannini, 1998; Gerich, 1998) which was found to be improved when middle-aged overweight humans were treated with oleuropein-rich extracts (De Bock et al., 2013). When oleuropein and oleuropein-rich extracts were administered in healthy humans, they were found to decrease postprandial glycemia (Carnevale et al., 2018; Visen et al., 2009), whereas when administered in T2DM patient, oleuropein-rich extract was found to normalize glucose homeostasis (Wainstein et al., 2012). Therefore, although further studies are needed in oleuropein-treated diabetic patient to clearly understand its anti-hyperglycemic mechanisms, oleuropein could be recommended for use as a food additive to help modulate glycemia in Type 2 diabetes consumers.

Table 3.1: Anti-diabetic activity of oleuropein and oleuropein-rich extracts in human.

No. of participants	Study design	Subject (Human condition)	Age (in years)	Oleuropein source and dose	Mechanism	Result	References
20	Randomized, Double-blind, Crossover, Placebo-controlled	Healthy	33.9 ± 6.9	20 mg of oleuropein	Downregulation of NOX2 activation	Lowers postprandial glycemia	(Carnevale et al., 2018)
46	Randomized, Double-blind, Crossover, Placebo-controlled	Overweight, Anti-hypertensive or Hypolipidemic administered	46.4 ± 5.5	4 capsules of olive leaf extract as a single dose/day (51.124 mg oleuropein in total)	Increase in concentration of IL6	Improvement in insulin sensitivity (irrespective of physical activity, dietary intake, BMI, or fat distribution in the subject)	(Carey et al., 2006; De Bock et al., 2013)
79	Randomized, Double-blind, Placebo-controlled	Type-2 diabetic mellitus	18 to 79	500 mg of an olive leaf extract from Barnea cultivar	Reduction of glycated hemoglobin (HbA1c)	Efficacy to control blood glucose level	(Wainstein et al., 2012)
20	No diet change, except consumption of extra virgin olive oil (EVOO) and 4-week daily intake of EVOO	Healthy	26 ± 2	50 mL EVOO	No mechanism explained	Lowers glycemia	(Oliveras-Lopez, Innocenti, Martin Bermudo, Lopez-Garcia de la Serrana, & Mulinacci, 2012)
16	Randomized, Double-blind, Crossover, Placebo-controlled	Healthy	20 to 55	2 capsules of *Fraxinus excelsior L.* seed extract as a single dose /day (1 g extract in total)	No mechanism explained	Lowers postprandial glycemia	(Visen et al., 2009)

3.6.5 Cardioprotective activity

The cardioprotective effect of oleuropein occurs due to its anti-apoptotic, antioxidant, anti-inflammatory, and vasodilatory properties. The mechanism for the cardioprotective effect of oleuropein in neonatal rat subjected to myocardial ischemia-reperfusion induced injury in an in-vitro study was demonstrated by Zhao et al. (2017). They highlighted that the protective effect of oleuropein was mediated by the reduction of intracellular ROS and inhibition of apoptosis in cardiomyocytes. They also for the first time suggested that the antiapoptotic effect of oleuropein was due to the activation of the reperfusion injury salvage kinase (RISK) pathway. In another in-vitro study, oleuropein (100 μM) was reported to demonstrate cardiovascular protection by inhibiting the proliferation of bovine vascular smooth muscle cell (SMC) (Abe et al., 2011). The inhibition of SMC proliferation by oleuropein occurred due to the blocking of cell cycle between G1 and S phase, which was suggested to be regulated by ERK ½.

In one of the in-vivo studies, administration of oleuropein (200 mg/kg/day; oral gavage method) in the ketamine-induced cardiotoxicity Wistar Albino rats (60 mg/kg ketamine) led to the reversion of the ketamine-induced oxidative stress and inflammation (Çömez et al., 2020). This protective effect of oleuropein on the heart was observed due to decrease in MDA, TNF-α, interleukin-6, COX-2, NF-κB, Troponin I, and creatin kinase myocardial band (CK-MB), and increase in glutathione (GSH), glutathione peroxidase (GSH-Px) and catalase (CAT). Similar result was reported by studies when ketamine-induced (60 mg/kg) and xylazine-induced (8 mg/kg) acute myocardial infraction in male Sprague-Dawley rats was pretreated with oleuropein (5, 10, 20, or 30 mg/kg/day) (Janahmadi, Nekooeian, Moaref, & Emamghoreishi, 2015, 2017). The treatment of chronic doxorubicin-induced (18 mg/kg) cardiomyopathy (DXR-CM) Wistar rats with oleuropein (1000 or 2000 mg/kg; intraperitoneally injection) was reported to prevent histopathological,

structural, and functional cardiac effects of doxorubicin toxicity (Andreadou et al., 2014). This preventive role of oleuropein in DXR-CM was found due to adenosine monophosphate- activated protein kinase (AMPK) activation and iNOS suppression. It was reported that the activation of AMPK leads to the inhibition of processes involved in ATP consumption (such as glycogen synthesis, protein synthesis and gluconeogenesis), which ultimately helps to restore amino acids level and protein biosynthesis. In another study, the treatment of acute doxorubicin- induced (20 mg/kg) cardiotoxicity in Wistar rats by oleuropein (100 or 200 mg/kg/body weight) demonstrated that it reduces the doxorubicin toxicity by the inhibition of lipid peroxidation products, reduction of iNOS, and decrease of oxidative stress in cardiomyocytes (Andreadou et al., 2007). In this study, authors reported no statistically significant difference between doses of 100 and 200 mg of oleuropein. They further enhanced their findings by demonstrating that oleuropein eliminates biomarkers (succinate and acetate) accumulation to restore disturbance of metabolic energy pathways induced due to doxorubicin treatment in myocardial tissues (Andreadou et al., 2009). These findings agree with the result of Nekooeian, Khalili, and Khosravi (2014) in which type 2 diabetic (60 mg/kg streptozotocin- induced; intraperitoneal injection) and hypertensive (60 mg/kg ketamine and 8 mg/kg xylazine – induced) male Sprague – Dawley rats, when treated with oleuropein (20, 40 or 60 mg/kg/day), showed the cardioprotective effects by increasing the indices of cardiac contraction and relaxation, and decreasing coronary resistance. Another study performed by Esmailidehaj et al. (2012) reported that a single dose of oleuropein (100 mg/kg; intraperitoneal administration) has anti-infarct, anti-stunning and anti-antiarrhythmic, but not preconditioning effects against ischemic-reperfusion injury in the heart of isolated Wistar rats. This cardioprotective effect, which lasted for 6 hours after oleuropein administration, was demonstrated by the reduction of infarct size, decrease in the magnitude of ischemic and

reperfusion arrhythmia and improvement in the cardiac dysfunction. Authors of this study did not observe two phases of cardioprotection (early and delayed phases) that can be found in preconditioning phenomenon, which was the evidence of the no preconditioning effect of oleuropein (Andreadou et al., 2006).

3.6.6 Antimicrobial effects

Oleuropein has been reported for its antibacterial activity against human pathogenic bacterial strains (both gram-positive and gram-negative) (Pereira et al., 2007), antiviral activity mainly against enveloped virus (Lee-Huang et al., 2007), as well as antimycoplasmal activity (Furneri, Marino, Saija, Uccella, & Bisignano, 2002) and antifungal activity (Zoric et al., 2016). It was reported than the antibacterial activity of oleuropein to be significantly more toxic for gram positive bacteria than for gram negative bacteria which could be due to the presence of glycosidic groups leading to the inability to penetrate the outer layer of the microorganism (Bisignano et al., 1999). Casas-Sanchez, Alsina, Herrlein, and Mestres (2007) have shown that oleuropein interacts with phosphatidylglycerol of the bacterial cell membrane causing change in the cytoplasmic membrane which further leads to the cell envelope disruption. Kiraz et al. (2016) recently investigated the effect of oleuropein (20 mg/kg/day for 30 days, intragastric gavage) on the enteric bacterial flora of male Wistar albino rats and demonstrated that oleuropein helps to decrease the total aerobic bacterial count. The authors pinpointed that the reduction in the enteric bacterial count by oleuropein might be beneficial for critically ill patients with potential risk of bacterial translocation, though further investigations need to be performed to come to this conclusion. In another study, oleuropein was reported to have a mild antimicrobial effect on oral pathogens with minimum inhibitory concentration (MIC) between 625 μ*g*/mL for *S. mutans, S. sobrinus, S. aureus, P. gingivalis and F. nucleatum*, and 1250 μ*g*/mL for *S. oralis, E. faecalis* and *P. micra*

(Karygianni et al., 2019). Oleuropein has been reported to be ineffective against *Moraxella catarrhalis* and *Haemophilus influenzae* (Bisignano et al., 1999), and *P. intermedia* (Karygianni et al., 2019).

In the food processing industry, the formation of a biofilm is a major microorganism management problem and many studies have reported that oleuropein and oleuropein-rich extracts have the potential to control biofilms formed by bacteria, although the involved mechanisms of antibacterial action have not been yet well understood. Oleuropein has been reported to improve the antimicrobial effect of peracetic acid (an active compound which is commonly found in commercial sanitizers) on biofilms formed by *L. monocytogenes* (Dominciano, Lee, Corassin, Martinis, & Oliveira, 2016) and *Staphylococcus aureus* and *Escherichia coli* cells (Dominciano et al., 2016). Edziri et al. (2019) demonstrated that oleuropein-rich methanolic extracts from Chetoui and Meski Tunisian cultivar inhibited the biofilm of *Pseudomonas aeruginosa, Staphylococcus aureus, Bacilus subtilis, Escherichia coli, Enterococcus faecalis* and *Candida albicans* with inhibition rates from 72 to 89.8% at the concentration of twice of their MIC values. Yanhong Liu, McKeever, and Malik (2017) also reported that an oleuropein-rich ethanolic extract inhibits the biofilm of *Listeria monocytogenes,* and *Salmonella enteritidis* with an inhibition rate up to 74% at the concentration of one-fourth of their MIC value. MIC values of oleuropein and oleuropein- rich extracts against microbial strains can be found in Table 3.2.

Oleuropein was reported to have a MIC value of 12.5 mg/mL against *Candida albicans* strains in an in-vitro study and was able to inhibit this fungus by targeting virulence factors which are associated with fungal infection establishment via the dose-dependent inhibition of shrimp alkaline phosphatase protein (SAP, a cellular enzyme related to pathogenicity of *Candida albicans*), reduction of total sterol content in the cell membrane and through the inhibition of filamentation

(Zoric et al., 2016). *Candida albicans* are reported to be responsible for oral diseases (Salvatori, Puri, Tati, & Edgerton, 2016).

Oleuropein was reported, in an in-vitro study, to inhibit mycoplasmas (*M. hominis, M. fermentans, M. pneumoniae and M. pirum)* at concentrations from 20 to 320 μg/mL (Furneri et al., 2002). Furneri et al. (2002) also reported that oleuropein shows anti-mycoplasmal activity because of the presence of O-diphenol system on its backbone structure, though the exact mechanism has not been discussed. Chemotherapeutic agents such as erythromycin and tetracyclines are effective against the infection caused by many mycoplasmas, however *Mycoplasma fermentans and* Mycoplasma hominis show resistance against these two antibiotics but were found to be inhibited in the presence of oleuropein (Furneri et al., 2002). There is little information available on the anti-mycoplasmal activity of oleuropein and thus it is very difficult to say that oleuropein will show this activity in either in-vivo studies or clinical trials.

Oleuropein obtained from the flowers of *Jasminum officinale* L. var. grandiflorum has been reported to effectively block the secretion of HBsAg (with IC_{50} = 23.2 μg/ml) in HepG2 2.2.15 cells (Hepatitis B virus-transfect human HepG2 cell line) in an in- vitro study in a dose-dependent manner (Zhao, Yin, & Dong, 2009). Zhao and coworkers in the same study also reported the reduction of viruses in the serum of hepatitis B virus-infected ducks when oleuropein (80 mg/kg) was intraperitoneally administered twice daily for 10 days. Oleuropein was also reported to have dose-dependent anti-HIV activity by the up-regulation of the expression of the IAP1 and IAP2s (apoptosis inhibitor proteins), IL-2 and IL-2Rα, and ornithine decarboxylase (ODC1) genes, and the downregulation of the expression of hedgehog receptor Ptc1 without any detectable toxicity (Lee-Huang, Zhang, Huang, Chang, & Huang, 2003). Furthermore, Bao et al. (2007) reported anti-HIV activity of oleuropein via its binding ability with the fusion protein gp41 and thus effectively

leading to the block of HIV-1 virus to enter human cells. Oleuropein-rich extract from leaves was demonstrated to have antiviral activity against herpes simplex type-1 virus (HSV-1) by interacting with the phospholipid bilayer of the virus envelope (Khattab, Hosny, Abdelkawy, Fahmy, & ElMenoufy, 2016). Oleuropein was also demonstrated to have antiviral activity against viral haemorrhagic septicaemia rhabdovirus (VHSR, a rhabdovirus which infects sea farmed fish and wild marine species) via its virucidal effect by inhibiting VHSR-induced cell to cell membrane fusion in uninfected cells (Micol et al., 2005). Oleuropein has also been claimed in a U.S. patent to have antiviral activities against hepatitis virus, herpes mononucleosis, rotovirus, canine parvovirus, bovine rhinovirus, and feline leukemia virus (Fredrickson, 2000). Moreover, oleuropein has been reported possibly to be the best binder to inhibit Ebola protein VP24 among oleuropein, kaempferol and quercetin (Plesko, Volk, Luksic, & Podlipnik, 2015), although more experimental studies are needed.

Table 3.2: Antimicrobial activity of oleuropein and oleuropein-rich extracts.

Microbial strains	Minimum inhibitory concentration of oleuropein (μg/mL)	References
Gram positive bacteria		
Listeria monocytogenes F2365	62600	(Yanhong Liu et al., 2017)
Listeria monocytogenes ATCC 7644	200	(Dominciano, Oliveira, Lee, & Corassin, 2016)
Staphylococcus aureus ATCC 25923	200	
Staphylococcus aureus	62.5 for ATCC 25923 strain, 62.5-125 for penicillin-susceptible strain, and 31.25-125 for penicillin-resistant strain	(Bisignano et al., 1999)
Staphylococcus aureus ATCC25923, MRSA^{112}, MRSA^{234}, MRSA^{675}	64 (leaves of Chetoui cultivar), 32 (leaves of Meski cultivar)	(Edziri et al., 2019)
Pseudomonas aeruginosa CI122 and CI311	64 (leaves of Chetoui cultivar and Meski cultivar)	
Bacillus cereus ATCC 11778	64 (leaves of Chetoui cultivar), 32 (leaves of Meski cultivar)	
Bacillus subtilis ATCC 14579	64 (leaves of Chetoui cultivar), 32 (leaves of Meski cultivar)	
Streptococcus mutans DSM 20523	625	(Karygianni et al., 2019)
Streptococcus sobrinus DSM 20381	625	
Streptococcus oralis ATCC 35037	1250	
Enterococcus faecalis ATCC 29212	1250	
Staphylococcus aureus ATCC 25923	625	
Parvimonas micra ATCC 23195	1250	
Listeria monocytogenes F2365	25000	(Yanhong Liu, McKeever, Suo, Jin, & Malik, 2018)
Gram negative bacteria		
Haemophilus influenzae	500 for ATCC 9006 strain, and >500 for clinical strain	(Bisignano et al., 1999)
Moraxella catarrhalis	>500 for both ATCC 8176 strain, and clinical strain	
Vibrio parahaemolyticus	62.5 for ATCC 17802 strain, and 125 for clinical strain	

Salmonella typhi	125 for ATCC 6539 strain, and 125-250 for clinical strain	
Salmonella enteritidis	62600	(Yanhong Liu et al., 2017)
Escherichia coli ATCC 25922	200	(Dominciano et al., 2016)
Escherichia coli ATCC 25922, CI423	64 (leaves of Chetoui cultivar and Meski cultivar)	(Edziri et al., 2019)
Klebsiella pneumoniae CI29	32 (leaves of Chetoui cultivar), 64 (leaves of Meski cultivar)	
Enterococcus faecalis ATCC 29212	32 (leaves of Chetoui cultivar), 64 (leaves of Meski cultivar)	
Enterococcus faecium CI234	32 (leaves of Chetoui cultivar), 64 (leaves of Meski cultivar)	
Escherichia coli ATCC 25922	1250	(Karygianni et al., 2019)
Porphyromonas gingivalis W381	625	
Fusobacterium nucleatum ATCC 25586	625	
Fungi		
Candida albicans DSM 1386	1250	(Karygianni et al., 2019)
Candida albicans	12500	(Zoric et al., 2016)
Candida albicans ATCC 90028	32 (leaves of Chetoui cultivar), 64 (leaves of Meski cultivar)	(Edziri et al., 2019)
Candida kreusei ATCC 6258	32 (leaves of Chetoui cultivar), 32 (leaves of Meski cultivar)	
Candida parapsilosis ATCC 22019	32 (leaves of Chetoui cultivar), 32 (leaves of Meski cultivar)	
Candida glabrata ATCC 90030	32 (leaves of Chetoui cultivar), 64 (leaves of Meski cultivar)	
Mycoplasmas		
Mycoplasma hominis	20	(Furneri et al., 2002)
Mycoplasma fermentans	20	
Mycoplasma pneumoniae	160	
Mycoplasma pirum	320	

3.7 Toxicology

There is not any official recommendation pertaining to the dose of oleuropein and / or oleuropein-rich extracts for humans. Studies on toxicological safety assessments of olive leaf extracts has shown a good safety profile. For example, the chronic toxicity of oleuropein-rich extracts (360 – 1000 mg/kg body weight/day for 90 days) from olive leaves did not result in any deaths or toxic symptoms in a repeated-dose oral toxicity study (Clewell et al., 2016). Recently, Guex et al. (2018) also reported similar results when an ethanolic extract of olive leaves was administered orally to Wistar rats in acute toxicity (2000 mg/kg) and sub-chronic toxicity (100-400 mg/kg for 28 days) studies. Simsek et al. (2018) study in rabbits also support these previous studies and reported that the oral administration of oleuropein – rich diet (20 mg/kg/day of oleuropein) did not produce any toxic effects on hepatic functions in a 15-day study. In healthy young adults, administration of dietary supplement with oleuropein-rich extracts (one capsule or one 5 mL liquid; greater than 95% of total phenolic content as oleuropein on both extracts; 3 times a day for 28 days) demonstrated no significant differences on oxidative markers, such as 8-isoprostan (marker for lipid peroxidization) and 8-oxo-2'-deoxyguanosine (marker for DNA oxidation), when compared with control (Kendall et al., 2009). Moreover, the oral administration of ethanolic extract of olive leaf (500 mg twice daily dose; 19.9 % oleuropein) to stage-1 hypertension human subjects in a clinical study demonstrated that oleuropein did not affect renal and liver functions as well as hematological parameters and electrolyte balance (Susalit et al., 2011). However, on the contrary to these studies, a 14-week study in mice has demonstrated that the administration of olive leaf extracts (0.5 % and 0.75 % of diet) can lead to cholestasis, hepatic fibrosis, and hyperplasia of bile ducts (Arantes-Rodrigues et al., 2011). Similarly, another study in Wistar rats reported hepatocellular and renal abnormalities when olive leaf extract was fed at a dose of 0.9% (Omer et

al., 2012). Therefore, further studies on toxicological safety assessments of oleuropein or oleuropein-rich extracts are needed so that this extract can be used for its pharmacological benefits without any toxic effects.

3.8 Conclusion

This review has summarized and discussed the occurrence, biosynthesis, extraction techniques and bioactivities of oleuropein. In the last few years, great interest has been raised in the role of oleuropein and oleuropein-rich extracts as a functional ingredient, and the scientific evidence pointed out in this review suggests the use of oleuropein for providing a variety of health-enhancing properties. However, if oleuropein and oleuropein-rich extracts are to be utilized for nutraceutical and functional food product development, a cost-effective and safe extraction method (using generally recognized as safe (GRAS) solvents) for oleuropein, additional in vivo studies to better understand the protective mechanism of action of oleuropein, as well as more clinical trials and toxicity study, are urgently needed.

Chapter 4

Optimization of sugar-free dark chocolate using simplex lattice mixture design

Abstract

The aim of this study was to optimize formulations of sugar-free dark chocolate using response surface methodology. Fourteen formulations with varying ranges of standard isomalt (0-30%), erythritol (0-30%) and xylitol (0-30%) were processed using a three-component constrained simplex lattice design. The effects of these different combinations of components on the physical (plastic viscosity, yield stress, flow behavior index, moisture content, and color) and sensory (appearance, mouthfeel, flavor, taste, after-taste, and overall acceptability) parameters of sugar-free dark chocolate were studied. The optimized formulation of sugar-free dark chocolate was found to be a combination of 5.16% isomalt, 20.99% erythritol and 3.85% xylitol with the desirability of 0.66, by maximizing plastic viscosity, yield stress and brightness.

Keywords: Polyols, Isomalt, Erythritol, Xylitol, Simplex lattice mixture design, Dark chocolate

4.1 Introduction

Chocolate is one of the fastest growing confectionery products with global retail sales showing a compound annual growth rate (CAGR) of 7.6% from 2011 to 2018 (approximately 45.6 billion U.S. dollars) and is projected to grow at a CAGR of around 5.2% during 2019 to 2024 (approximately 83.34 billion U.S. dollars) (IMARC Group, 2019). Because of the increasing concerns of consumers about sucrose and calorie intake from chocolate consumption, manufacturers are searching for alternative bulk sweeteners which can provide both the physical bulk and sweetness of sucrose to chocolate without the glycemic effect. One of the alternative sweeteners which provide considerable advantages over conventional sucrose (in the manufacturing of chocolate) with desired properties is polyols (sugar alcohols). These polyols provide functionality and bulk as of sucrose with tooth friendly, low glycemic and low-calorie benefits to chocolates (Health Canada, 2005).

Several studies have examined the utilization of polyols as bulking sweeteners in the effort of developing sugar-free chocolates such as the use of isomalt, maltitol and xylitol (Rad, Pirouzian, Toker, & Konar, 2019; Sokmen & Gunes, 2006), lactitol (de Melo, Bolini, & Efraim, 2009), maltitol and xylitol (Pirouzian, Peighambardoust, & Azadmard-Damirchi, 2017), and maltitol and tagatose (Son et al., 2018). In these studies, it was shown that polyols affect the physical (plastic viscosity, yield stress, and flow behavior index) and sensory (appearance, mouthfeel, flavor, taste, after-taste, and overall acceptability) properties of chocolate. From the available research, it is difficult to recommend a single polyol which can meet all requirements of chocolate application. Thus, the most logical method is to use a combination of polyols designed to meet functional properties and sensory attributes of chocolate. A combination of polyols containing chocolate are also found in the market. These considerations have created a basis for the extensive study on the

effects of addition of different polyols in combination in chocolate confectionery. The current study was purposed to identify the optimum concentrations of xylitol, erythritol and isomalt, based on rheological properties, in sugar-free dark chocolate formulations.

The simplex lattice mixture design is an optimization technique that helps to develop the best possible product by identifying the optimum concentration of ingredients used in chocolate formulation and has been successfully applied by many researchers (Abbasi & Farzanmehr, 2009; R. P. Aidoo, Afoakwa, & Dewettinck, 2014; Chu & Resurreccion, 2004; Rad, Pirouzian, Toker, et al., 2019). The simplex lattice design is a type of mixture design in which all the ingredients (which are to be optimized) must have the same range (which means sum of all ingredients is constant). Thus, in the present study, a simplex lattice mixture design was used to observe the individual or combination usage effects of isomalt, xylitol and erythritol on the moisture content, colour, rheological and sensory properties of sugar-free dark chocolate.

4.2 Materials and methods

4.2.1 Materials

Materials such as cocoa mass (Delicacies Valley, Canada), cocoa butter (Advantage Health Matters Inc., Canada), isomalt carbonated crystals (Poppingfun, USA), xylitol (Prescribed for Life, USA), erythritol (Prescribed for Life, USA), sugar (Redpath Sugar Limited, Canada), aspartame (Bulk Supplements, USA), vanilla powder (Nielsen-Massey Vanillas Inc., USA), sunflower lecithin (Now Foods, USA), were used in this study. Aspartame was used to adjust the sweetness for sugar-free dark chocolate containing polyols.

4.2.2 Preparation of dark chocolate samples

Sugar-free dark chocolates were produced according to the subsequent formulation: 50 % cocoa mass (total fat content of 54 % +/- 2%; blend of Trinitario and Amazonico beans), 19.65 % cocoa butter, 30% polyols, 0.31% sunflower lecithin, and 0.04 % vanilla powder. For control, polyol was replaced with sucrose in equal proportion to the mixture.

4.2.3 Chocolate processing

Sugar-free dark chocolates were produced in a refiner (3.6 kg capacity, Diamond Custom Machines Corporation, USA) and formulated from the simplex-lattice mixture design (Table 4.1). Chocolate with sucrose was also prepared as a control sample using the refiner. This chocolate refiner is equipped with a two-roller stone to crush and refine the chocolate ingredients, and two deflector blades to create better aeration and conching. The technical specifications of this chocolate refiner are 203 mm drum diameter, 140 mm drum height, 105 mm roller stone diameter, 35 mm roller stone width and with speed of 120-140 revolutions per minute. This chocolate refiner does not have a temperature controller, so an infrared thermometer gun (Fisher Scientific, USA) was used to measure the temperature whenever necessary during the processing stage.

In a first stage, the cocoa butter and cocoa mass were separately melted at a temperature below 55^0C in a double boiler (handheld infrared thermometer was used to measure the temperature). This melted cocoa butter was then added to the chocolate refiner to circulate in the system at 120 – 140 revolutions per minutes for 10 seconds. Afterwards, melted cocoa mass was added to a chocolate refiner and mixed homogeneously for 30 minutes. Meanwhile, to help ensure a cohesive mass in the chocolate refiner in the later stage of processing, additional heat was provided using a hair dryer (Braun, Ireland) by keeping the heat directed on the roller stones. The temperature of

the mixture in the chocolate refiner for all formulations was kept below 45^{o}C throughout the processing period since standard isomalt crystals lose their crystallization at relatively low temperature (Kruger, 2009). A temperature of around 40^{o}C was maintained by switching off the chocolate refiner for 5 minutes whenever the temperature of the mixture reached close to 45^{o}C. After 30 minutes of homogenization, one-third of the total polyol/s or sugar was gradually added to the mixture in the refiner. For the addition of another one-third of the total polyol/s or sugar and the remaining amount after that, a time gap of 20 minutes was kept. This strategy was followed to make sure that polyol/s are fully incorporated in the mixture and to evaporate any residual moisture. Polyol such as standard isomalt used in this processing has a high moisture content (2.73 ± 0.03) which may cause the sugar-fat mixture to seize and in addition damage the equipment. This strategy also helped to smoothly run the refiner because loading the refiner too quickly caused roller stones to stop and prevented the machine from spinning. Finally, after the addition of polyol/s or sugar, aspartame, vanilla powder, and sunflower lecithin were gradually added to the sugar-fat mixture in the refiner. The refining / conching time was 12 hours. Conching helps to develop the flavor and texture of chocolate (Prawira & Barringer, 2009). The conching time varies from study to study.

Table 4.1: Experimental design and mass fraction of three components in dark chocolate formulation (Formulation 10 is a center of the simplex lattice design whereas formulations 1, 2 and 5 are the vertexes of it. Following formulations are equivalent: formulation 1 = 14; 2 = 7; 5 = 6; 10 = 11. In the table, X_1 = isomalt, X_2 = erythritol and X_3 = xylitol).

Formulations	Level (Uncoded values)			Coded values (% of polyol in chocolate sample)		
	X_1 (%)	X_2 (%)	X_3 (%)	X_1	X_2	X_3
1	100	0	0	30	0	0
2	0	100	0	0	30	0
3	50	0	50	15	0	15
4	66.66	16.66	16.66	20	5	5
5	0	0	100	0	0	30
6	0	0	100	0	0	30
7	0	100	0	0	30	0
8	50	50	0	15	15	0
9	16.66	16.66	66.66	5	5	20
10	33.33	33.33	33.33	10	10	10
11	33.33	33.33	33.33	10	10	10
12	16.66	66.66	16.66	5	20	5
13	0	50	50	0	15	15
14	100	0	0	30	0	0

The tabling method was used for tempering chocolate using a marble slab as discussed by (Greweling, 2007) with some modifications. Sugar-free dark chocolate (one half to two thirds of the total chocolate mass) was poured onto a marble slap and agitated continuously to prevent it from solidifying, and to form the requisite cocoa butter seeds. In the end of this step, the temperature of the prepared chocolate mass was maintained at around 28^{o}C using a hair dryer. Afterwards, the agitated chocolate was mixed with the remaining chocolate mass (the remaining

one third) so that a uniform chocolate mixture was formed. The temperature of the chocolate mass at the end of this step was maintained at around 32^{o}C. Finally, the tempered chocolate mass was distributed into a silicone mold (each cavity in the mold measures 15 × 15 × 10 mm) and cooled down to 5^{o}C in a refrigerator. Then the solid chocolate was removed from the mold and stored in a cool, dark, and odorless place in an airtight polyethylene bag covered with aluminum foil at 5 +/- 2^{o}C until required for analysis.

4.2.4 Rheological measurements

The rheological property measurements of the chocolate samples were carried out at 40^{o}C using a controlled stress rheometer (AR2000, TA Instruments, New Castle, Delaware, USA) as suggested by International Confectionery Association (2000) and Rad, Pirouzian, Toker, et al. (2019). Chocolate samples were pre-melted at 45^{o}C for 1 hour prior to measurement. The melted chocolate samples were pre-sheared at 5 s^{-1} shear rates for 10 min. Then, shear stresses were measured as a function of shear rate from 2 to 50 s^{-1}. Each measurement took 180 s and 18 data points were collected. The collected data of each sample were fitted to mathematical models such as Casson, Herschel-Bulkley, Power law, and Bingham models. The two statistical indexes of coefficient of determination (R^2) and root mean square error (RMSE) were calculated to determine the best model to understand the rheological properties of chocolate samples. Using the best fitted model, yield stress (Pa), viscosity (Pa. s) and flow behaviour index were calculated. For each formulation, rheological evaluations were performed in triplicate and mean values were determined.

4.2.5 Moisture content

The moisture content of chocolate samples was determined according to the AOAC official method (1990). Approximately 2 g of prepared chocolate sample were dried until a constant weight in an aluminum dish in hot air oven at 100^{o}C. The loss in weight was reported as the moisture

content on wet basis. For each formulation, moisture content test was performed in triplicate and mean value was calculated.

4.2.6 Color measurement

The surface color of all formulations was measured using a chromameter (CR – 300X, Minolta Camera Co. Ltd., Japan). The SCE-mode was used with the color expressed in terms of the CIELAB system. The following CIELAB system color coordinates were determined: L* (lightness ranging from black (0) to white (100)), a* (green to red) and b* (blue to yellow). The chroma meter was calibrated with the standard white tile before performing measurements. For each formulation, color measurements were performed in triplicate.

4.2.7 Sensory evaluation

Twenty-eight volunteer untrained panelists (12 males, 14 females and 2 preferred not to specify) took part in the sensory analysis. This study took place remotely and data was collected using LimeSurvey software. Chocolate samples were available to pick up at the Macdonald Stewart Building, Macdonald Campus, McGill University, Canada on the 18th of September 2020 from 3 pm to 5 pm. Each participant wase provided with 14 sucrose-free dark chocolates and chocolate made with sucrose (each weighing approximately 3.3 g) in polyethylene bags and each sample was noted with a three-digit code. All participants were requested to assess the samples using a hedonic scale from 1 to 9 (where 1 = dislike extremely, 2 = dislike very much, 3 = dislike moderately, 4 = dislike slightly, 5 = neither like nor dislike, 6 = like slightly, 7 = like moderately, 8 = like very much, and 9 = like extremely; unless otherwise specified) based on appearance, mouthfeel, flavor, taste, after-taste, and overall acceptability of each sample. Furthermore, they were requested to rinse their mouth with tap water after evaluating each sample. Approval for the study was obtained

from the Research Ethics Board Office of McGill University, and the written consent was given by all panelists. All selected panelists agreed to

a. Be at least 18 years of age or older
b. Be in good health, in general, and specifically on the day of the sensory evaluation
c. Be available to provide their comments for sensory analysis
d. Have no allergies/intolerance against chocolate
e. Have no chocolate craving
f. Have normal perception abilities
g. Have no history of severe chronic medical disease including gastrointestinal problems, severe abdominal discomfort, and severe constipation.

4.2.8 Experimental design and Statistical analysis

The effects of polyol proportions on the rheological properties, moisture content, color and sensory parameters of dark chocolate were studied using the simplex-lattice mixture design as described by Karaman, Yilmaz, and Kayacier (2011). Design-Expert Version 13 Software (trial version) was used to develop this mixture design. This three-component constrained simplex lattice mixture design comprised isomalt (X_1), erythritol (X_2) and xylitol (X_3) as components. The component proportions were expressed as fractions of chocolate mixture with a sum ($X_1 + X_2 + X_3$) of 30. Following polynomial equation 1 of function X_i (the design expert software also used alphabet letters – A for isomalt, B for erythritol and C for xylitol) was fitted for each component assessed at each experimental point:

$$Y = \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 \quad (1)$$

where Y = estimated response; β_1, β_2, β_3, β_{12}, β_{13}, and β_{23} are constant coefficients for each linear and non-linear (interaction) term produced for the prediction models of processing components.

The levels and experimental design of these three components in terms of coded and uncoded as 14 combinations are presented in Table 4.1. The analysis was performed using uncoded units. The responses (yield, viscosity, flow behavior index, moisture content, color, appearance, mouthfeel, flavor, taste, after-taste, and overall acceptability) of these combinations were analyzed and the fitted models were subjected to analysis of variance (ANOVA) to determine significance ($P < 0.05$), lack of fit and determination coefficient (R^2). The best fit equations for all responses were obtained after removing the non-significant terms. Numerical optimization tool of the mixture design was used to search for a combination of component levels that simultaneously satisfy all the criteria placed on the responses and components.

Mathematical model fittings of rheological data obtained from rheometer were evaluated using statistical package SAS version 9.4 and TRIOS software (ARES-G2 platform). Moreover, plastic viscosity, flow behavior index, and yield stress from the best fitted mathematical model of each chocolate formulation were obtained using TRIOS software (ARES-G2 platform).

4.3 Results and Discussion

Mean values for the responses (Herschel-Bulkley plastic viscosity, Herschel-Bulkley yield stress, flow behavior index, moisture content, color, appearance, mouthfeel, flavor, taste, after-taste, and overall acceptability) were analyzed and can be found in Table 4.2 and Table 4.4. The experimental results obtained for the studied parameters were statistically analyzed by fitting the data to different models using Scheffe's test and the calculated regression coefficients can be found in Table 4.3 and Table 4.5. The best fitted model with a maximum R^2, p-value less than 0.05, and a non-significant lack of fit for residuals was selected. The selected models for each response were statistically significant (p-value < 0.05) illustrating a significant relationship between polyols (components) and the responses at the 95% confidence level. The non-significant lack of fit

confirms the applicability of the model to adequately describe the functional relationship between components and the responses.

4.3.1 Rheological properties

Rheological experiments showed that apparent viscosity of all chocolate samples (including the ones containing sucrose) decreased with increasing shear rate (data not shown). This exhibition of shear thinning behavior of chocolate samples agrees with previous studies on chocolate (Fernandes, Müller, & Sandoval, 2013; Pirouzian et al., 2017; Rad, Pirouzian, Toker, et al., 2019). This behaviour could be explained by the structural decomposition of the aggregated molecules due to continuous shearing and pressure force applied, and the increased alignment of the constituent molecules during processing (Alpaslan & Hayta, 2002; Fernandes et al., 2013; Izidoro, Scheer, Sierakowski, & Haminiuk, 2008; Rao & Tattiyakul, 1999).

In order to understand the effects of sugar substitutes on the rheological behavior of sugar-free dark chocolate, data obtained from the rheometer were fitted to non-Newtonian models, such as Power law model (equation 2), Herschel- Buckley model (equation 3), Casson fluid model (equation 4), and Bingham plastic model (equation 5), to describe the relationship between shear rate and shear stress.

$$\sigma = \kappa\gamma^{n} \quad (2)$$

$$\sigma = \kappa\gamma^{n} + \sigma_0 \quad (3)$$

$$(\sigma)^{0.5} = (\kappa_1)^{0.5}(\gamma)^{0.5} + (\sigma_0)^{0.5} \quad (4)$$

$$\sigma = \mu_{pl}(\gamma) + \sigma_0 \quad (5)$$

where σ = shear stress (Pa); n = flow behavior index (dimensionless); κ = consistency coefficient (Pa.s^n); γ = shear rate (s^{-1}); σ_0 = yield stress (Pa); κ_1 = plastic viscosity (Pa.s) for Casson model; μ_{pl} = plastic viscosity (Pa.s) for Bingham model.

Table 4.2: Mean and Standard deviation of quality parameters (or responses) (n = flow behavior index; control = chocolate made with sucrose).

Formula-tions	Herschel-Bulkley viscosity (Pa. s^n)	Herschel-Bulkley yield (Pa)	Flow behavior index	Moisture (%)	Brightness (L*)
1	1.43 ± 0.01	2.06 ± 0.18	0.93 ± 0.00	1.99 ± 0.02	26.38 ± 0.15
2	1.75 ± 0.19	7.12 ± 0.97	0.92 ± 0.02	1.08 ± 0.03	26.94 ± 0.21
3	1.91 ± 0.25	2.87 ± 0.53	0.86 ± 0.06	1.54 ± 0.08	24.10 ± 0.28
4	1.61 ± 0.33	2.31 ± 0.39	0.85 ± 0.04	1.28 ± 0.07	24.52 ± 0.68
5	2.46 ± 0.33	7.61 ± 0.79	0.89 ± 0.03	0.71 ± 0.05	22.50 ± 0.22
6	2.37 ± 0.65	7.90 ± 1.27	0.88 ± 0.06	0.73 ± 0.05	23.80 ± 0.11
7	1.72 ± 0.15	7.20 ± 0.70	0.92 ± 0.02	1.08 ± 0.02	26.74 ± 0.37
8	1.55 ± 0.06	2.59 ± 0.01	0.89 ± 0.01	1.24 ± 0.10	26.78 ± 0.42
9	1.29 ± 0.08	4.94 ± 0.03	0.93 ± 0.01	0.79 ± 0.04	26.20 ± 0.68
10	1.27 ± 0.04	5.13 ± 0.15	0.95 ± 0.01	0.91 ± 0.03	25.13 ± 0.74
11	1.48 ± 0.03	5.85 ± 0.16	0.95 ± 0.00	0.90 ± 0.04	24.04 ± 0.39
12	1.67 ± 0.03	7.74 ± 0.37	0.93 ± 0.00	0.82 ± 0.03	25.00 ± 0.30
13	1.53 ± 0.11	8.07 ± 0.21	0.95 ± 0.02	0.99 ± 0.10	23.61 ± 0.34
14	1.42 ± 0.02	2.19 ± 0.25	0.94 ± 0.00	1.98 ± 0.02	26.76 ± 0.44
Control	2.13 ± 0.11	8.80 ± 0.82	0.91 ± 0.01	1.01 ± 0.06	27.52 ± 0.45

Table 4.3: Regression models for physicochemical parameters of sugar-free dark chocolates.

Fitted model	Viscosity special quartic (P = 0.0003)	Yield special quartic (P < 0.0001)	Brightness Linear (P = 0.0066)
Lack of fit	P = 0.3177	P = 0.6324	P = 0.1184
R^2 (%)	0.9719	0.9948	0.5990
Adjusted R^2 (%)	0.9390	0.9887	0.5261
Adequate Precision	15.8099	30.4939	7.0003

Table 4.4: Mean rating of sensory attributes with its overall acceptability.

Formulation	Appearance	Mouthfeel	Flavor	Taste	After-taste	Overall acceptability
1	6.21 ± 1.69	5.36 ± 2.33	6.59 ± 1.72	6.37 ± 1.82	5.93 ± 2.18	5.93 ± 1.90
2	7.07 ± 1.30	6.57 ± 1.62	6.57 ± 1.57	6.01 ± 1.71	6.00 ± 2.02	6.54 ± 1.73
3	7.14 ± 1.30	6.04 ± 1.87	5.54 ± 2.06	6.30 ± 2.06	5.29 ± 2.23	5.61 ± 1.95
4	6.15 ± 1.77	5.11 ± 2.79	6.32 ± 1.54	6.21 ± 2.10	6.00 ± 1.92	5.96 ± 1.99
5	6.25 ± 1.78	6.18 ± 2.34	6.46 ± 1.77	6.68 ± 1.59	6.39 ± 2.02	6.50 ± 1.99
6	6.71 ± 1.41	6.96 ± 1.73	6.86 ± 1.67	6.96 ± 1.55	6.04 ± 1.95	6.75 ± 1.53
7	6.46 ± 1.75	5.71 ± 2.09	6.07 ± 1.90	5.93 ± 1.78	5.29 ± 2.11	5.86 ± 1.80
8	6.18 ± 1.66	5.21 ± 2.01	6.04 ± 1.64	5.82 ± 1.79	5.86 ± 1.98	5.61 ± 1.73
9	6.33 ± 1.49	5.11 ± 2.36	6.15 ± 1.56	6.48 ± 1.78	5.85 ± 1.83	6.00 ± 1.66
10	6.71 ± 1.67	5.30 ± 2.27	6.19 ± 1.85	5.82 ± 1.74	5.61 ± 1.91	5.82 ± 1.83
11	6.48 ± 1.89	5.37 ± 2.27	5.85 ± 1.66	5.67 ± 1.78	5.85 ± 1.92	5.74 ± 1.89
12	6.86 ± 1.51	5.18 ± 1.98	5.79 ± 1.89	5.64 ± 1.77	5.68 ± 1.70	5.64 ± 1.73
13	6.36 ± 1.68	6.25 ± 1.86	6.61 ± 1.45	6.63 ± 1.71	6.25 ± 2.03	6.46 ± 1.69
14	6.68 ± 1.49	5.25 ± 2.30	6.32 ± 1.91	6.07 ± 2.16	6.21 ± 1.83	6.18 ± 1.96
Control	6.44 ± 1.60	6.04 ± 1.75	6.51 ± 1.42	6.41 ± 1.31	5.07 ± 1.49	6.26 ± 1.35

Table 4.5: Regression models for sensory parameters of sugar-free dark chocolates.

Fitted model	Mouthfeel quadratic (P = 0.0300)	Flavor quadratic (P = 0.0246)	Taste Linear (P = 0.0103)	After-taste quadratic (P = 0.0291)	Overall acceptability quadratic (P = 0.0290)
Lack of fit	P = 0.4003	P = 0.5423	P = 0.0785	0.8870	0.6537
R^2 (%)	0.5667	0.5927	0.5645	0.5780	0.7402
Adjusted R^2 (%)	0.4368	0.4706	0.4853	0.4514	0.5779
Adequate Precision	6.1487	7.3601	7.0358	6.4828	6.0848

Table 4.6: Effects of experimental data obtained from sugar-free chocolates in rheological models in terms of R^2 and RMSE.

Formulations	Bingham model		Casson model		Power law model		Herschel-Buckley model	
	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE
1	0.999	0.299	0.999	0.127	0.999	0.201	0.999	0.121
2	0.997	1.144	0.999	0.461	0.998	0.685	0.999	0.279
3	0.995	0.383	0.998	0.195	0.999	0.167	0.999	0.137
4	0.998	0.668	0.999	0.330	0.999	0.357	0.999	0.257
5	0.999	0.683	0.999	0.318	0.998	1.001	0.999	0.199
6	0.998	0.689	0.999	0.364	0.997	1.003	0.999	0.193
7	0.999	0.415	0.999	0.352	0.997	0.960	0.999	0.170
8	0.999	0.444	0.999	0.160	0.999	0.387	0.999	0.152
9	0.999	0.299	0.999	0.275	0.997	0.68	0.999	0.153
10	0.999	0.226	0.999	0.304	0.997	0.723	0.999	0.117
11	0.999	0.295	0.999	0.379	0.999	0.848	0.999	0.195
12	0.999	0.385	0.999	0.429	0.996	1.050	0.999	0.207
13	0.999	0.297	0.999	0.500	0.995	1.144	0.999	0.182
14	0.999	0.346	0.999	0.216	0.999	0.397	0.999	0.215
Control	0.999	0.549	0.999	0.366	0.997	1.131	0.999	0.189

The obtained experimental data were fitted with these models and evaluated based on two statistical parameters namely, coefficient of determination (R^2) and root mean square error (RMSE) (Table 4.6). Based on the statistical calculations, Bingham, Casson and Herschel-Buckley models were found to be the best models based on the highest R^2 values. However, further

evaluation of RMSE revealed that the best model to describe the shear stress vs. shear rate relationship was the Herschel-Buckley model due to the highest r^2 and the lowest RMSE values. Therefore, all rheological parameters (plastic viscosity, flow behavior index, and yield stress) were determined using the Herschel-Buckley model. This model was also reported to describe the rheological behavior of dark chocolates made with isomalt, xylitol and maltitol by Sokmen and Gunes (2006), milk chocolates made with polydextrose by Shah, Jones, and Vasiljevic (2010), and milk chocolates made with isomalt, maltitol and inulin by Konar (2013). Nevertheless, the most recommended and the widely used model to describe the rheological behavior of chocolates by the International Confectionery Association (ICA) or previously the International Office of Cocoa, Chocolate and Sugar Confectionery (IOCCC) is the Casson model. This model, however, was reported not to provide the acceptable reproducibility (Servais, Ranc, & Roberts, 2003).

4.3.1.1 Plastic viscosity

The consistency coefficient (κ) or Herschel-Buckley plastic viscosity of sucrose-free dark chocolates were found between 1.25 to 2.46 $Pa.s^n$. The highest plastic viscosity was found in sugar-free dark chocolate made with xylitol (2.46 $Pa.s^n$), followed by sucrose-made chocolate (2.13 $Pa.s^n$). Whereas the lowest plastic viscosity was found in a chocolate made with 10% isomalt, 10% erythritol and 10% xylitol (1.27 $Pa.s^n$). The Casson plastic viscosity, when measured over shear rate from 5 to 60 s^{-1}, for dark chocolate has been previously reported between 2.1 and 3.9 Pa.s (Aeschlimann & Beckett, 2000). The plastic viscosity of xylitol-made chocolate and sucrose-made chocolate fell in this range; however, it was lower for all other individual and combination of polyols-made chocolate. The relationship between the ingredients (isomalt, erythritol and xylitol) and Herschel-Buckley plastic viscosity was a special quartic. The regression equation for

Herschel-Buckley plastic viscosity response (Y) in terms of L_Pseudo components, obtained from simplex-lattice mixture design, is presented in equation (6).

$$Y = 1.44\ A + 1.72\ B + 2.41\ C - 0.03\ AB + 0.07\ AC - 2.23\ BC + 28.24\ AB^2C - 50.26\ ABC^2 \quad (6)$$

Where, A = isomalt, B = erythritol and C = xylitol. The physical characteristics of polyols, particle size distribution, and moisture content may be the reason for the different plastic viscosity values among the samples (Afoakwa, Paterson, & Fowler, 2007; Gonçalves & Lannes, 2010). In the presence of moisture, the crystals of polyols dissolve and an increase in the interaction between sugar particles occurs (Saputro et al., 2017). This may lead to an increase in the internal friction and thus may result in higher viscosity. Among xylitol, isomalt and erythritol, xylitol has the highest hygroscopicity thus it will absorb moisture from the environment leading to a chocolate with a higher viscosity (Zumbe, Lee, & Storey, 2001). This is consistent with our result in which xylitol-made chocolate showed a highest viscosity, however it must be noted that the moisture content of this final chocolate sample was found to be very low as compared to isomalt- made chocolate. Furthermore, from the fitted quartic model (equation 6) and figure 4.1, we can conclude that xylitol produces chocolate with the highest viscosity since the constant coefficient for linear term of xylitol (2.41) is higher than for erythritol (1.72) and isomalt (1.44). In addition, since the constant coefficient for the interaction term of isomalt and xylitol (0.07) is positive, the mixture of isomalt and xylitol produces a higher viscosity chocolate than would be expected by averaging the viscosity of individual polyols-made chocolate. This is an example of synergistic blending effect. Moreover, standard isomalt used in this study for making chocolate had a moisture content of nearly 2.7% (moisture content was 0.35% for erythritol, 0.45% for xylitol, and 0.1% for white sugar); during conching, the water of crystallization inherently present in this polyol is released

and some moisture may be absorbed by xylitol due to its hygroscopic nature, which increases the viscosity of chocolate.

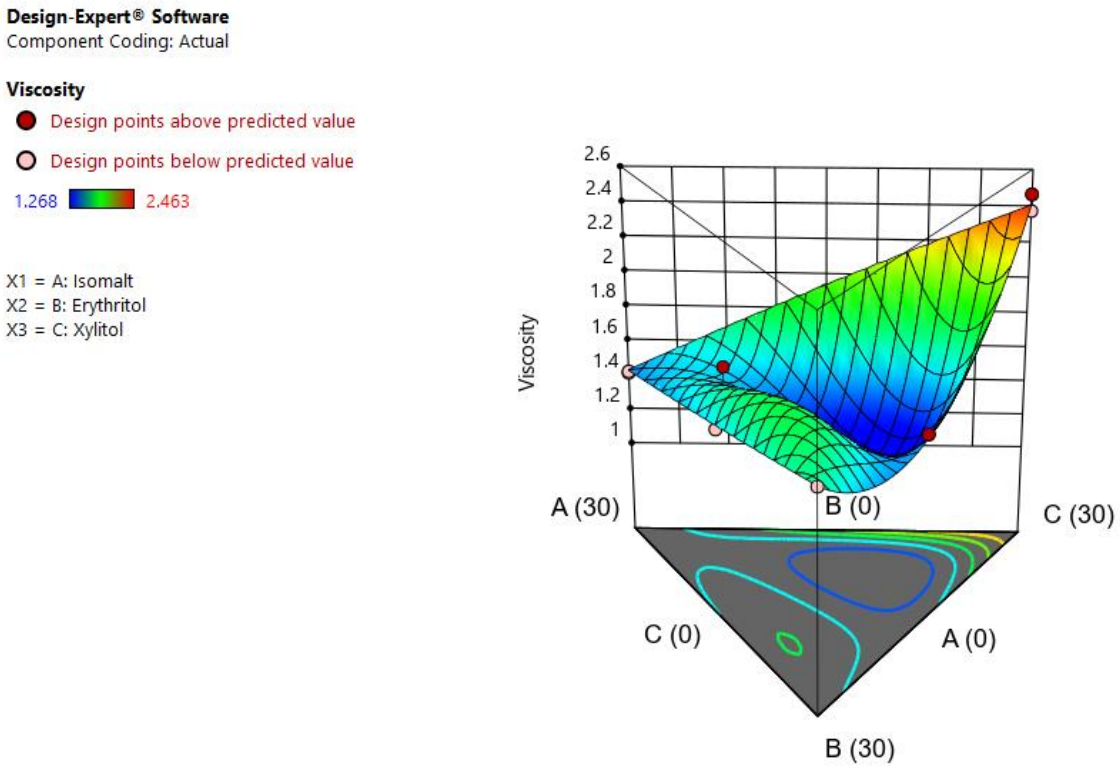

Figure 4.1: Estimated response surface plot indicating effect of isomalt (A), erythritol (B) and xylitol (C) on consistency coefficient (Herschel-Buckley plastic viscosity) of chocolate samples.

The inconsistency result (in literature) in the plastic viscosity of chocolate may be due to the fluctuations in conching temperature and time, as there is decrease in viscosity with increase in conching temperature (Vivar-Vera, Torrestiana-Sanchez, Monroy-Rivera, & Brito-De La Fuente, 2008). In a study performed by Olinger (1994), it has been reported that isomalt-made chocolate resulted in higher viscosity than xylitol, sucrose and maltitol after conching for 18 hours at 50^{o}C but when conched at 60^{o}C produced lower viscosity than xylitol. And, thus suggested to consider different conching temperatures to make chocolate with different polyols. Nevertheless, the processing method applied in our study was different (around 40^{o}C for 12 hours

refining/conching), this study found a higher viscosity in a xylitol-made chocolate than with isomalt-made chocolate. The higher viscosity of xylitol-made chocolate may also be due to the smaller particle size during processing. The smaller particle size results in larger surface area for a given amount of solids. As the surface area in contact with fat (continuous phase) increases, the internal friction increases as well, leading to the higher viscosity (Sokmen & Gunes, 2006). Moreover, the viscosity can double with the increase of solid content by a few percentages for a high solids content suspension (Colin Servais, Jones, & Roberts, 2002).

The flow behavior index of all chocolate samples was found between 0.85 and 0.95 (Table 4.2). These values lower than 1 indicate the pseudoplastic nature of chocolate, exhibiting shear thinning behaviour above the yield stress. This means the apparent viscosity of chocolate samples decreases as the shear rate increases. Overall, the chocolate samples made with the equal combination of all polyols were found to have higher flow behavior index. The different values of flow behavior index could be related to the strength of aggregated particle-particle network system of polyols-made chocolate blend during processing stages (Servais et al., 2003). Similar flow behavior index values of less than 1 (range from 0.71 to 0.84) were reported in chocolate made with xylitol and maltitol (in individual or in combination) (Pirouzian et al., 2017). However, in another study led by Sokmen and Gunes (2006), results presented an average flow behavior index of 1.006 for sucrose-made chocolate, 1.003 for maltitol-made chocolate, 1.011 for isomalt-made chocolate and 1.033 for xylitol-made chocolate indicating slight shear thickening behavior of their chocolate samples.

4.3.1.2 Yield stress

The Herschel-Buckley yield stress of sucrose-free dark chocolates were found between 2.06 and 8.07 Pa (Table 4.2). The highest yield stress was found in dark chocolate made with sucrose (8.80

± 0.82 Pa), as compared to polyol-made chocolates. Whereas the lowest yield stress was found in isomalt-made chocolate (2.06 ± 0.18 Pa). The previously reported Casson yield stress for dark chocolate was between 4 and 8 Pa (Aeschlimann & Beckett, 2000). All chocolate samples except samples containing higher amount of isomalt (formulations 1, 3, 4, 8 and 14), fell in this range. The relationship between the ingredients (isomalt, erythritol and xylitol) and the Herschel-Buckley yield stress was a special quartic. The regression equation for the Herschel-Buckley yield stress response (Y) in terms of L_Pseudo components, obtained from simplex-lattice mixture design, is presented in equation (7).

$$Y = 2.12\ A + 7.15\ B + 7.75\ C - 8.34\ AB - 8.41\ AC + 2.31\ BC + 220.27\ AB^2C - 113.09\ ABC^2 \quad (7)$$

Where, A = isomalt, B = erythritol and C = xylitol. From the fitted quartic model (equation 7) and figure 4.2, we can conclude that xylitol produces chocolate with the highest yield stress since the constant coefficient for the linear term of xylitol (7.75) is higher than for erythritol (7.15) and isomalt (2.12). In addition, since the constant coefficient for the interaction term of erythritol and xylitol (2.31) is positive, it is possible to assert that the yield stress of a chocolate can be increased by addition of erythritol and xylitol in combination, rather than only using individually. This can be seen in formulation 13 where the yield stress value was found to be 8.07 ± 0.21 Pa, as compared to 7.20 ± 0.70 Pa for erythritol-made chocolate and 7.61 ± 0.79 Pa for xylitol-made chocolate. The higher yield value of xylitol-made chocolate could be due to its spatial structure. Due to this structure, there are more active and free hydroxyl groups, which results in more interactions between the components present in the chocolate suspension (Furlan, Baracco, Zaritzky, & Campderrós, 2016; Rad, Pirouzian, Konar, Toker, & Polat, 2019). This phenomenon further leads to higher yield value and thus more force is needed for the flow of chocolate.

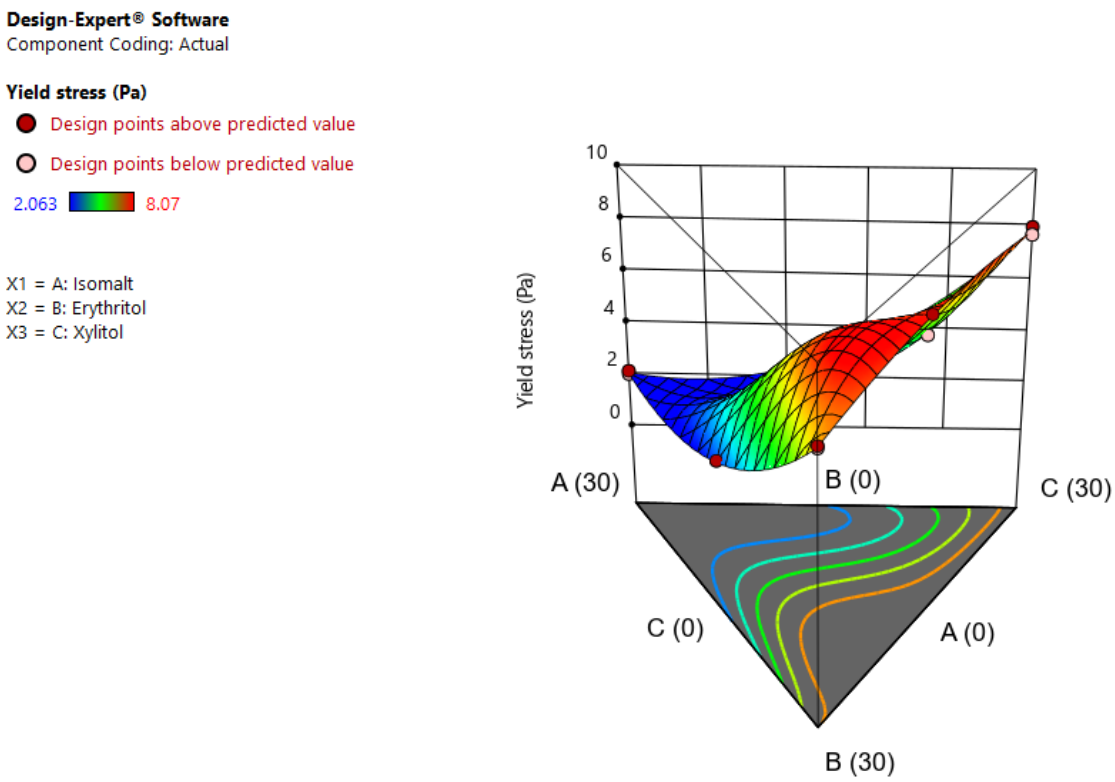

Figure 4.2: Estimated response surface plot indicating effect of isomalt (A), erythritol (B) and xylitol (C) on yield stress of chocolate samples.

The higher yield value of sucrose-made chocolate (control) than polyol-made chocolate (as observed in this study) was also reported by Konar (2013), where control samples (made with fine sugar) displayed higher yield stress (5.94 Pa) than maltitol- and isomalt- made chocolate (2.98 Pa and 0.27 Pa respectively) at a conching temperature of 50°C. However, in another study performed by Rad, Pirouzian, Toker, et al. (2019), xylitol displayed higher yield value (8.85 ± 0.21 Pa), followed by isomalt (3.02 ± 0.18 Pa) and maltitol (1.27 ± 0.08 Pa), than the control chocolate sample (0.88 ± 0.27). These differences may be related to the different processing applied to produce chocolate. Chocolate produced from a ball mill may display different rheological properties than from a refiner. Furthermore, in the study by Konar (2013), the Herschel-Bulkley model showed the best fitting for predicting rheology of prebiotic milk chocolate as opposed to the Casson model for dark chocolate as reported by Rad, Pirouzian, Toker, et al. (2019).

4.3.2 Moisture content

The effect of various combinations of polyols on the mean values of moisture content has been reported in Table 4.2. As it can be seen that the highest moisture content was found in the isomalt-made chocolate (1.99 ± 0.02% in wet basis), whereas the lowest was in xylitol-made chocolate (0.71 ± 0.05% in wet basis). Furthermore, sucrose-made chocolate (control) was found to have 1.01 ± 0.06% on a wet basis. Overall, all chocolate samples containing isomalt showed higher moisture content (often more than 1%). The high moisture content in chocolate made with isomalt could be due to the inefficiency of a refiner to remove moisture during the manufacturing process since isomalt used in this study had a moisture content of 2.73 ± 0.03% (in wet basis). Interestingly, the moisture content of most of the chocolate samples (excluding formulations 1, 3 and 14) were within the range of 0.5 to 1.5% as recommended to be an acceptable range by Afoakwa (2010) without any drastic effects on their rheological properties.

The assessment of moisture content showed that it was significantly ($P < 0.05$) affected by the proportion of isomalt, erythritol and xylitol and the fitted model was quadratic, however lack of fit was found to be significant ($P < 0.0001$) leading to the conclusion that this fitted regression model should not be used for prediction of moisture content (response). This could be due to the poor choice of experimental design (Stat-Ease). That is why no predicted model was reported for moisture content in this study.

4.3.3 Color

The key contributor to the appearance of chocolate is its color, and it influences consumers' perceptions. The important parameters that affect the color of chocolate are the composition and processing conditions applied during manufacturing. The effect of various combinations of polyols

on the color of chocolate has been reported in Table 4.2 and illustrated in Figure 4.3. It can be seen from the reported data that the brightness (L*) values range from 22.50 ± 0.22 to 26.94 ± 0.21 for polyols-made chocolate. Lower values for L* illustrate a darker appearance of the chocolate samples. The relationship between the ingredients (isomalt, erythritol and xylitol) and the color of the chocolate samples was linear. The regression equation for color response (Y) in terms of L_Pseudo components, obtained from the simplex-lattice mixture design, is presented in equation (8).

$$Y = 26.12\ A + 26.33\ B + 23.08\ C \quad (8)$$

Overall, polyols-made chocolates were found to have a darker appearance as compared to sucrose-made chocolate (L* = 27.52 ± 0.45). This finding agrees with previous study performed by Rad, Pirouzian, Toker, et al. (2019) in which milk chocolates made with maltitol, xylitol and isomalt (in individual or in combination) were found to have a darker chocolate appearance compared to control, regardless of levels of sugar substitutes. They argued that lighter color of sucrose-made chocolate compared to polyol-made chocolate could be linked to the increased light diffusing character of sucrose. Sucrose contains multi-dimensional crystals with sharp edges scattering the light in all direction from matrix, whereas sugar alcohols contain amorphous arrangement of crystals and thus minimum reflection of light occurs leading to a darker appearance in comparison with sucrose-made chocolate.

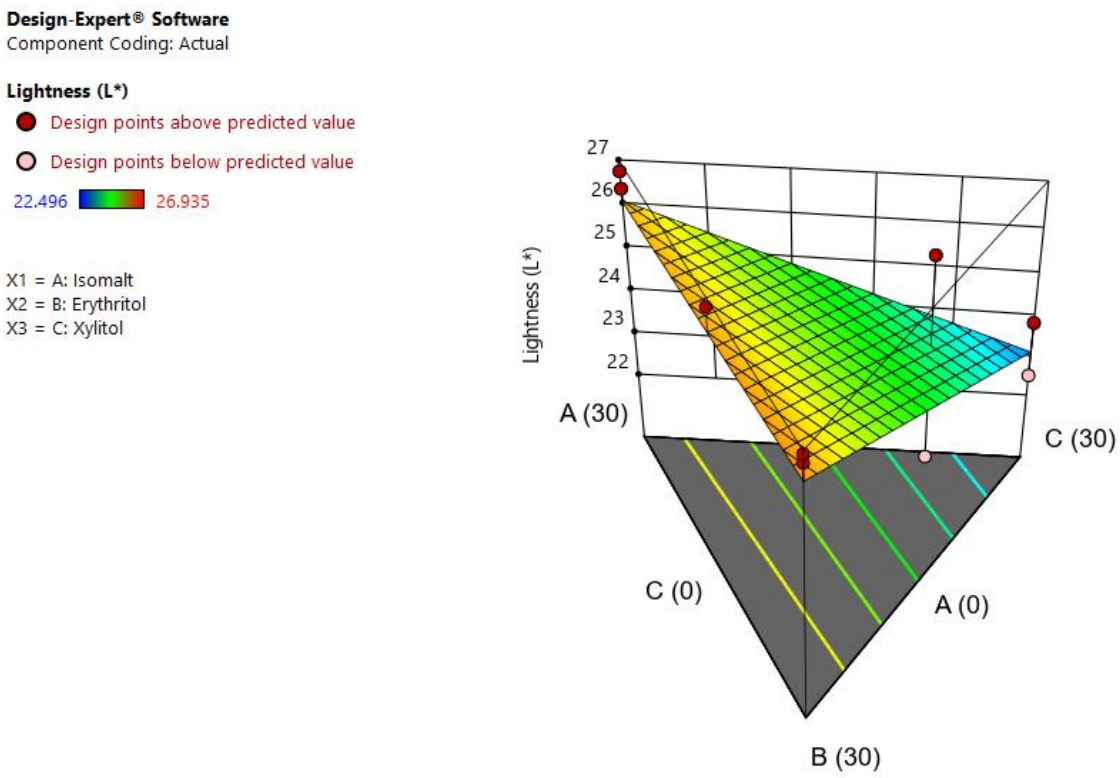

Figure 4.3: Estimated response surface plot indicating effect of isomalt (A), erythritol (B) and xylitol (C) on color of chocolate samples.

4.3.4 Sensory analysis

The mean rating test scores of the intensity of sensory characteristics of sucrose-free chocolate and control is presented in Table 4.4. Overall, xylitol-made chocolate received the best score in the case of all sensory parameters (6.75 ± 1.53). Whereas chocolates made with equal proportion of isomalt and xylitol (formulation 3) and isomalt and erythritol (formulation 8) received the lowest overall score (5.61 ± 1.95 and 5.61 ± 1.73 respectively). The estimated response surface plot indicating the effect of isomalt (A), erythritol (B) and xylitol (C) on the overall acceptability of chocolate samples is presented in Figure 4.4. The regression equation for sensory parameter responses (Y) in terms of L_Pseudo components, obtained from the simplex-lattice mixture design,

is presented in Table 4.7.

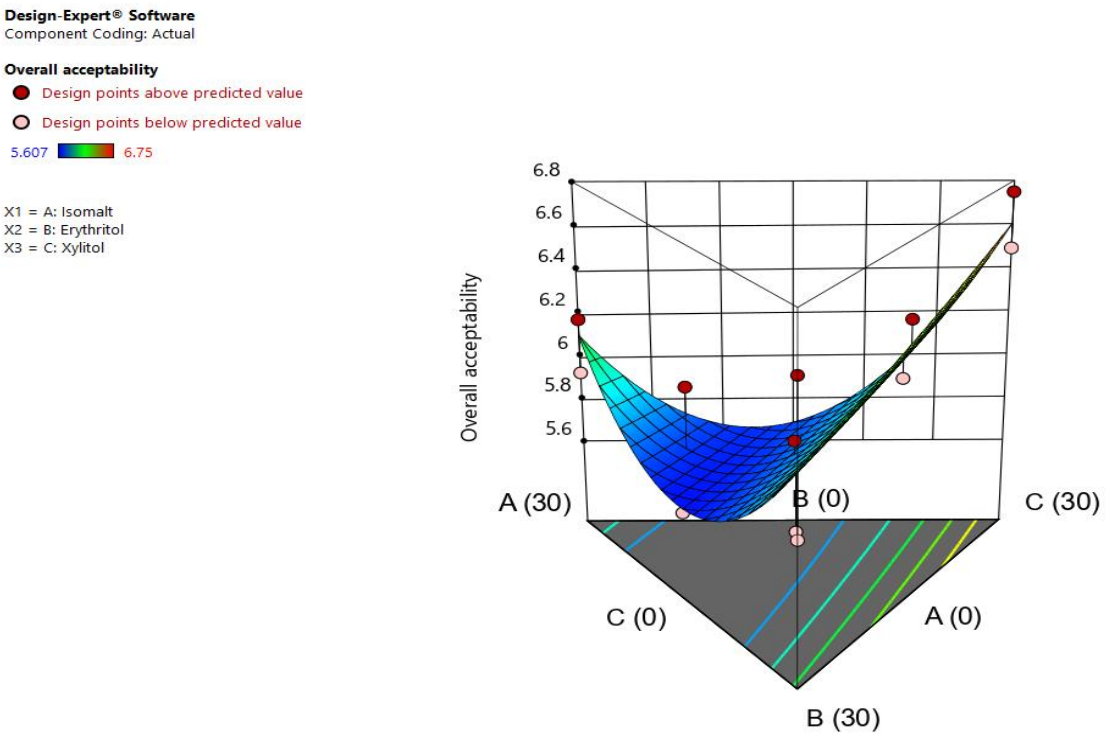

Figure 4.4: Estimated response surface plot indicating effect of isomalt (A), erythritol (B) and xylitol (C) on overall acceptability of chocolate samples.

Table 4.7: Final predicted equations for the experimental data of sugar-free dark chocolate formulations in terms of L_Pseudo components.

Sensory parameters (responses)	Predicted model type	Predicted model
Mouthfeel	Quadratic	5.29 A + 6.02 B + 6.28 C – 3.49 AB
Flavor	Quadratic	6.43 A + 6.18 B + 6.67 C – 3.72 AC
Taste	Linear	6.04 A + 5.79 B + 6.73 C
After-taste	Quadratic	6.11 A + 5.70 B + 6.28 C – 3.03 AC
Overall acceptability	Quadratic	6.10 A + 6.15 B + 6.61 C – 2.00 AB – 2.68 AC – 0.09 BC

The combination of xylitol and isomalt (in equal proportion) received a higher appearance score (7.14 ± 1.30) compared to other formulations and the control (6.44 ± 1.60). The appearance of the chocolate samples is important as it affects the perceived and expected sensory properties. In this

study, the fitted model for appearance (response) was found to be insignificant because the P value for the model was 0.4213 (which is greater than $P < 0.05$ required for model significance). Therefore, no predicted model has been reported for the appearance (response) of chocolate samples from the simplex-lattice mixture design. The higher appearance score of formulation 3 (xylitol and isomalt in equal proportion) could be related to the color and gloss of the chocolate samples. Briones, Aguilera, and Brown (2006) investigated the effect of surface topography (or surface roughness) of chocolate samples on its color and gloss and reported that the surface structure of chocolate plays a decisive role in its visual quality appearance.

The assessment of mouthfeel showed that it was significantly ($P < 0.05$) affected by the proportion of isomalt, erythritol and xylitol. The mouthfeel attributes of chocolate samples were rated from grainy (corresponding to 1 or dislike extremely) to smooth (corresponding to 9 or like extremely). The common mouthfeel perception when hard particles/granulations are present in chocolate is grittiness, whereas soft particles may not be noticeable during consumption (melting in the mouth). A mean score of 6.96 (Table 4.4), suggesting a very smooth mouthfeel, was found for the chocolate formulation that only had xylitol. Increasing the proportion of xylitol in formulations gave chocolate a smooth mouthfeel (Figure 4.5). On the other hand, increasing the proportion of isomalt and erythritol (in combination) produced chocolate with a grainy mouthfeel. In this study, it was found that there was a positive and significant correlation ($r = 0.733$) between viscosity and mouthfeel for the chocolate samples. This strong correlation agrees with previous studies (H. Aidoo, Sakyi-Dawson, Abbey, Tano-Debrah, & Saalia, 2012).

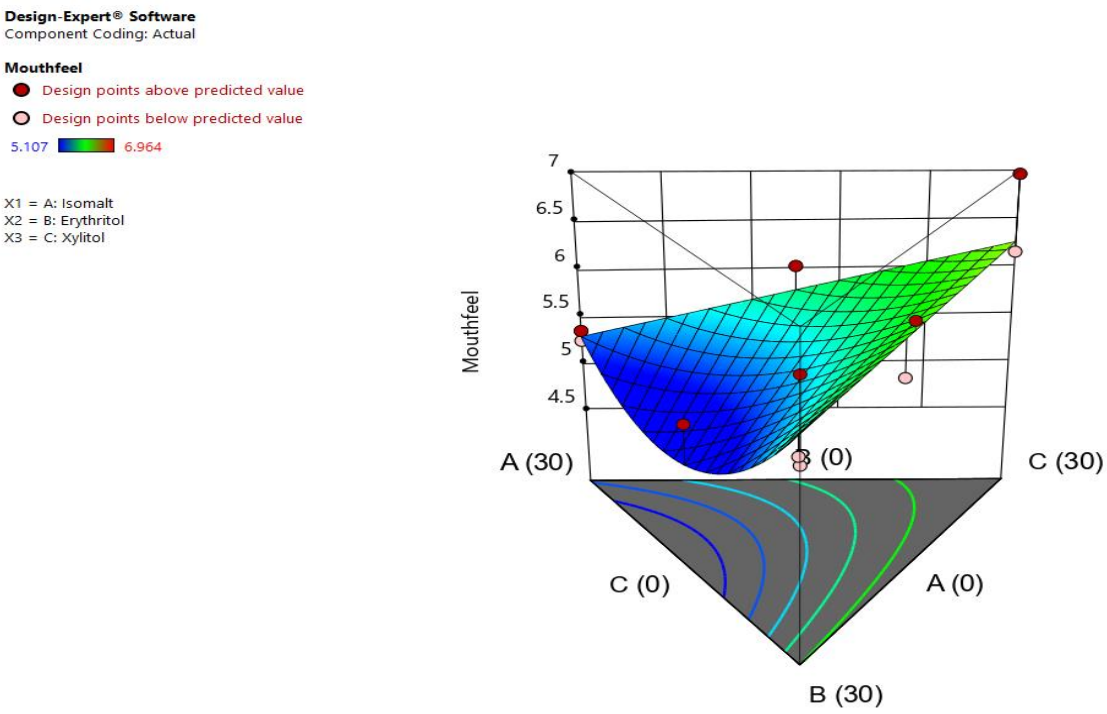

Figure 4.5: Estimated response surface plot indicating effect of isomalt (A), erythritol (B) and xylitol (C) on mouthfeel of chocolate samples.

The chocolate made with polyols was found to be liked only slightly with the mean flavor ratings ranging from 5.54 ± 2.06 to 6.86 ± 1.67 pointing out that sucrose-free dark chocolates were moderately flavored. The chocolate made with sucrose received a mean flavor rating of 6.51 ± 1.75. The flavor attribute of chocolate samples were rated from mild (corresponding to 1 or dislike extremely) to intense (corresponding to 9 or like extremely) chocolate flavor. In general, dark chocolate has an intense chocolate flavor due to presence of high concentrations of cocoa solids. Moreover, sweeteners play an important role in the release of flavor in chocolate which could be associated with their solubility (Zumbe et al., 2001). The solubility of isomalt, erythritol and xylitol is less than for sucrose at body temperature, and thus lower solubility of a sweetener leads to a lower facilitation of the perception of the flavor attribute in the mouth (Rad, Pirouzian, Toker, et al., 2019). In our study, 10 formulations (except formulations 1, 2, 6, and 13) were found to have

a lower mean flavor rating than sucrose-made chocolate. Furthermore, the assessment of chocolate flavor showed that it was significantly ($P < 0.05$) affected by the proportion of isomalt, erythritol and xylitol, even though the predictive model may not be very accurate (R^2 adjusted = 0.4706, R^2 predicted = 0.2267). Figure 4.6 shows the influence of isomalt, erythritol and xylitol on flavor of chocolate samples and illustrates that increasing the proportion of isomalt and xylitol leads to a decrease in chocolate flavor.

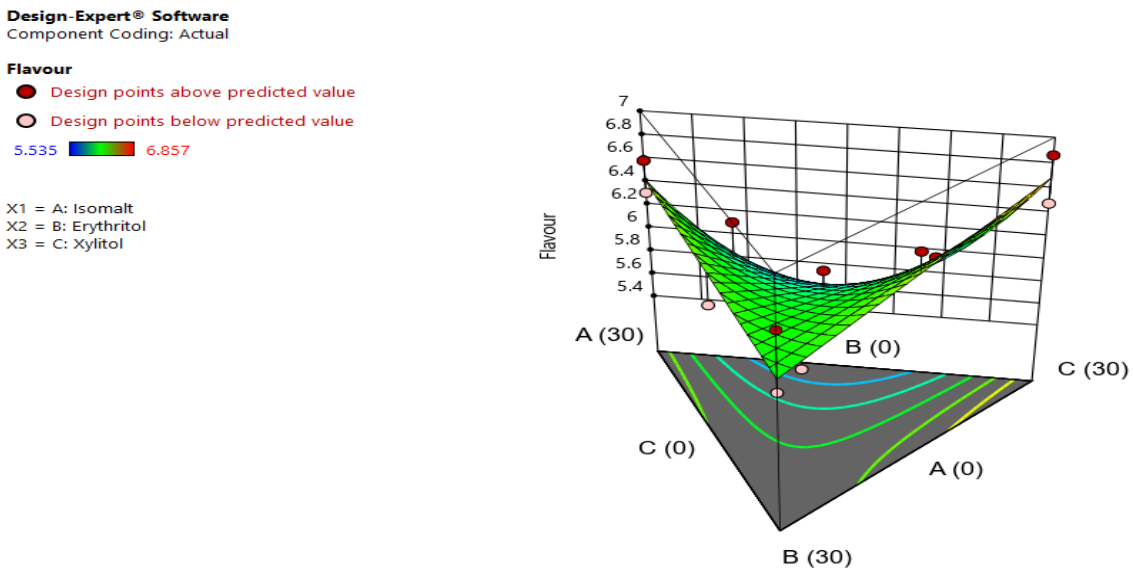

Figure 4.6: Estimated response surface plot indicating effect of isomalt (A), erythritol (B) and xylitol (C) on flavor of chocolate samples.

The taste attribute of dark chocolate samples was rated from very sweet (corresponding to 1 on the hedonic scale) to very bitter (corresponding to 9 on the hedonic scale). The most bitter chocolate had a mean score rating of 6.96 ± 1.55 and was produced from the chocolate sample containing the highest amount of xylitol. Whereas the least bitter chocolate (formulation 12) recorded a mean score rating of 5.64 ± 1.77 compared to the control of 6.41 ± 1.31. Moreover, the assessment of chocolate taste showed that it was significantly ($P < 0.05$) affected by the proportion of isomalt, erythritol and xylitol, however the predictive model may not be very accurate and applicable due

to relatively low probability of lack of fit (Table 4.5). The lingering flavor that persists after the consumption of food, known as 'aftertaste,' is another important attribute in determining the overall taste quality of chocolate. The aftertaste attribute was rated from none (corresponding to 1 on the hedonic scale) to waxy (corresponding to 9 on the hedonic scale). Good sucrose-made dark chocolate should have an aftertaste that is not oily, unpleasant, or overly bitter. Overall, xylitol-made chocolate had the highest mean score of 6.39 ± 2.02 compared to 5.07 ± 1.49 for the control. The high mean score rating of xylitol-made chocolate implied that it had more burnt and bitter aftertaste compared to other formulations and the control. Figures 4.7 and 4.8 show the influence of isomalt, erythritol and xylitol on taste and aftertaste of chocolate samples, respectively.

The bitter taste with sweet aftertaste (or bitter and astringent taste) of dark chocolate may be related to the presence of caffeine, catechin and theobromine in the cocoa solids (Drewnowski & Gomez-Carneros, 2000). In the present study, sucrose-free dark chocolates were produced by the addition of aspartame to accommodate the sweetness intensity of sucrose. The differences in taste or aftertaste perception among chocolate samples may have resulted from the characteristics of aspartame. This high potency sweetener has a sweet taste with bitter and off-flavor aftertaste (Nahon, Roozen, & de Graaf, 1998). In addition, strong and noticeable cooling effects of xylitol and erythritol due to their high negative heat of dissolution could have also played a role in the perception and sensory properties affecting the taste and aftertaste of sucrose-free chocolate (Zumbe et al., 2001).

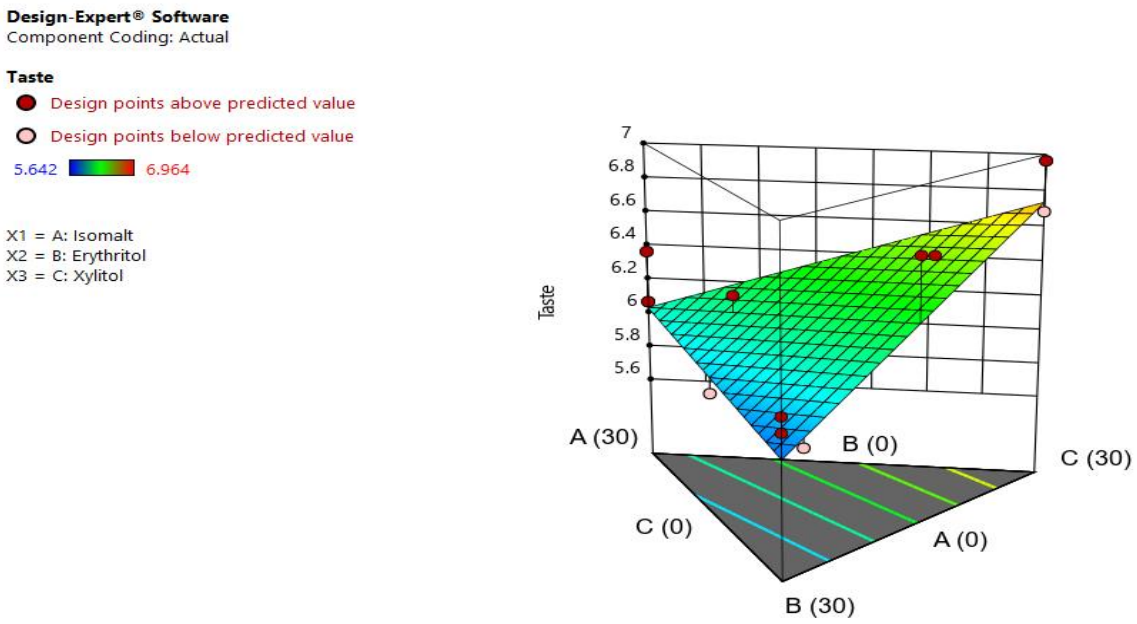

Figure 4.7: Estimated response surface plot indicating effect of isomalt (A), erythritol (B) and xylitol (C) on taste of chocolate samples.

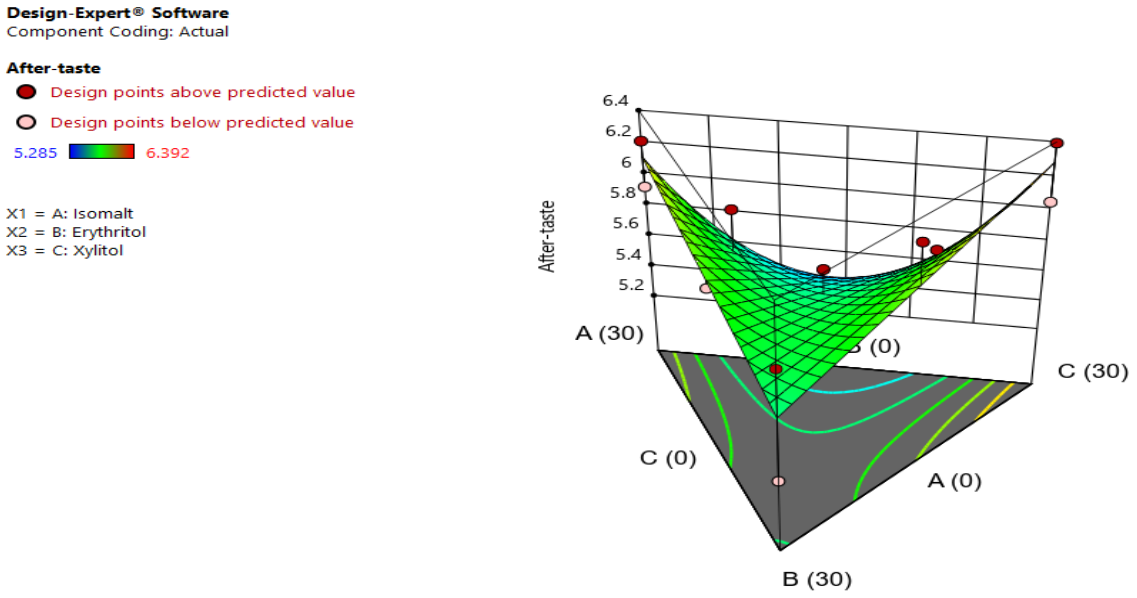

Figure 4.8: Estimated response surface plot indicating effect of isomalt (A), erythritol (B) and xylitol (C) on aftertaste of chocolate samples.

Moreover, high level of monosaccharides such as xylitol and erythritol may be linked to a 'burning scratchy' aftertaste in chocolate samples. This is possibly due to the higher osmotic pressure caused by the dissolution of these polyols in the mouth (Kruger, 2009). However, disaccharides such as isomalt and sucrose have mild sweetness with no aftertaste and cooling effect (Kruger, 2009).

4.4 Optimization of Chocolate Formulation

This stage determines the combination of experimental factors which simultaneously optimize multiple responses. A numerical optimization technique based on desirability function was applied to optimize the chocolate formulation. Taking chocolate characteristics into account and using the optimization tool to maximize plastic viscosity, yield stress and brightness of chocolate samples, a formulation consisting of 5.16% isomalt, 20.99% erythritol and 3.85% xylitol was selected as having the maximum desirability (desirability = 0.66). Based on optimization results, the optimum limits for plastic viscosity, yield stress and brightness were 1.77 $Pa.s^n$, 7.53 Pa and 25.88, respectively. The combination of variables (isomalt, erythritol and xylitol) which gives the overall optimum desirability are presented in Figure 4.9.

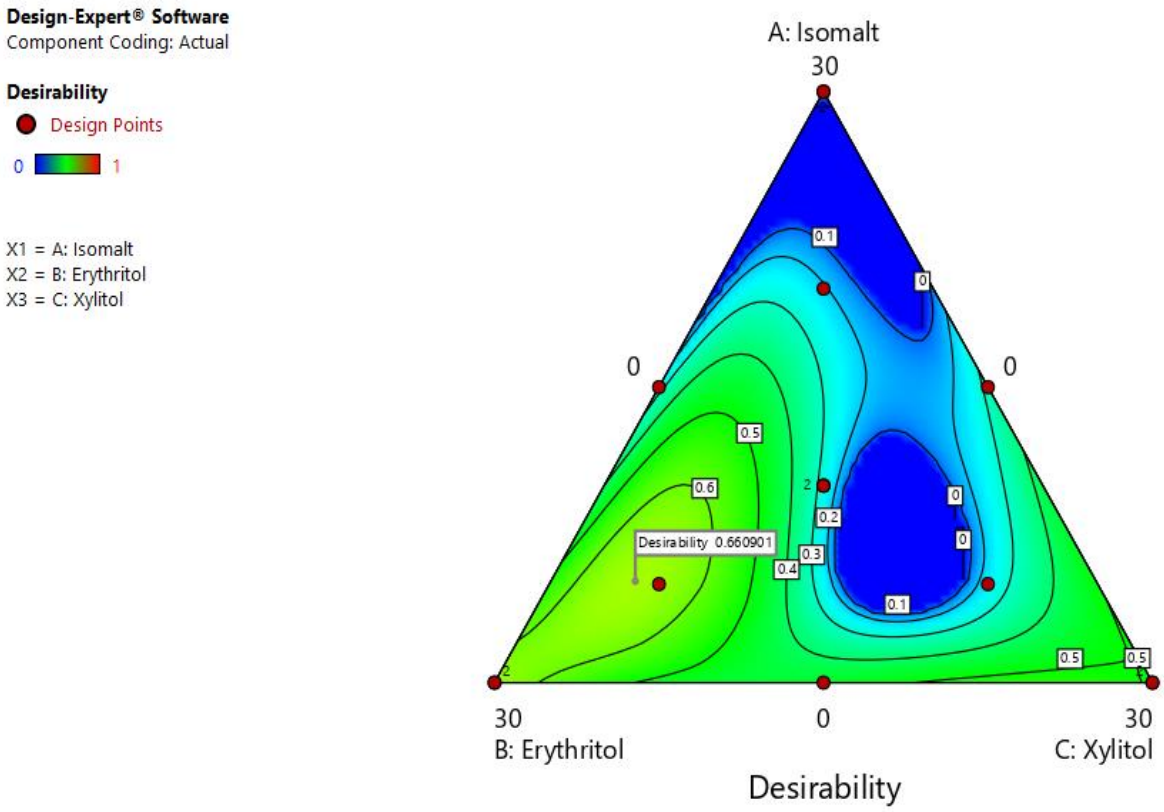

Figure 4.9: Contours of estimated response surface illustrating the point where optimum level was achieved (A = isomalt, B = erythritol and C = xylitol).

4.5 Conclusion

Polyol-made chocolate offers considerable advantages over conventional sucrose-made chocolate in terms of reduced calorie, reduced glycemic index and tooth friendly confectionery products. The substitution of sucrose by isomalt, erythritol and xylitol in dark chocolate has varied influences on its physical and sensory characteristics. In this study, the effect of these polyols is found to be dependent not only on the type of polyol used but also on the proportions present in the dark chocolate. Furthermore, the relationship between chocolate properties (physical and sensory) and polyols (isomalt, erythritol and xylitol) has been established. Thus, different combinations of isomalt, erythritol and xylitol could be used to improve the rheological and sensory parameters of sucrose-free chocolates produced from a chocolate refiner. The optimum concentrations of 5.16%

isomalt, 20.99% erythritol and 3.85% xylitol were found based on the studied quality parameters. Future work will focus on the application of isomalt having a lower residual moisture content instead of standard isomalt used in this study to produce a high-quality chocolate made with polyols.

Chapter 5

Modifying the health functionality of formulated chocolates with olive leaf extract

Abstract

This study determines the possibility of using olive leaf extract to incorporate oleuropein, a bioactive compound from *Olea europaea* L, in sucrose-free dark chocolate. Therefore, the effects of various olive leaf extract concentrations (10, 15 and 20 g/kg chocolate) on the rheological, textural, melting, visual, and physiochemical properties of sucrose-free dark chocolate (made with isomalt, erythritol and xylitol) were investigated. Overall, the addition of olive leaf extract caused no significant changes ($P > 0.05$) in the moisture content, color (L*), hardness and melting behavior of chocolate samples compared to control, with some exceptions. Furthermore, the oleuropein was found to be in the range of 0.24 – 0.52 mg/100 g of sample in oleuropein-enriched sucrose-free dark chocolates. In addition, there was a significant increase ($P < 0.05$) in the phenolic and flavanol contents of oleuropein-enriched chocolates with respect to control.

Keywords: Oleuropein, Olive leaves extract, Chocolate, Isomalt, Erythritol, Xylitol

5.1 Introduction

The prevalence of diabetes, one of the largest global public health concerns, has reached epidemic proportions globally. The number of people suffering from diabetes is progressively increasing and rose to 422 million in 2015 from 108 million in 1980 (World Health Organization, 2021). It was estimated that 451 million (age 18 to 99 years) people live with diabetes worldwide in the year 2017 and has been projected to increase to 693 million by 2045 (Cho et al., 2018). Most of the population with diabetes is considered to have type 2 diabetes mellitus (T2DM), and this type of diabetes is a major result of physical inactivity and being overweight (World Health Organization, 2021). Furthermore, hyperglycemia is a common effect of uncontrolled diabetes which may over time lead to long-term complications such as cardiovascular diseases and kidney failure. For this reason, eating a healthy diet, avoiding sugar, may help to delay or prevent the onset of T2DM.

Among food products, dark chocolate may be of particular interest because the antioxidants (largely flavonoids such as catechin and procyanidins) it contains can prevent the low-density lipoprotein cholesterol leading to cardiovascular protection (Afoakwa, 2010). Moreover, the use of polyols (such as isomalt, erythritol and xylitol) leads to the production of sugar-free chocolate with tooth-friendly, low glycemic and low-calorie health benefits (Aidoo, Depypere, Afoakwa, & Dewettinck, 2013). In addition, oleuropein-enriched sugar-free chocolate may allow people suffering from diabetes to eat chocolate and benefit from the combined potential synergistic effects of oleuropein and sucrose-free chocolate on cardiovascular and nervous systems without abrupt changes of blood glucose level. In a reported study, the consumption of oleuropein-enriched milk chocolate has showed a modest increase or no change of glycemia in healthy and T2DM subjects (Del Ben et al., 2020). This oleuropein-enriched milk chocolate was made by enriching 40 g chocolate with extra virgin olive oil (EVOO) as a source of oleuropein to achieve a final

concentration of 4 mg% oleuropein. Beyond the positive effects on insulin and glycemia levels of healthy and T2DM subjects, oleuropein has been shown to have anti-inflammatory, antioxidant, anti-angiogenic, anti-cancer, cardioprotective, antihypertensive, and neuroprotective functions (Sun, Frost, & Liu, 2017).

Keeping these health benefits of oleuropein-enriched sucrose-free chocolate in view, it is important to examine the effects of oleuropein as an ingredient on the quality properties of chocolate before using for the purpose of functional product development. The main aim of this study was to investigate the effect of the addition of oleuropein (10 g/kg, 15 g/kg, and 20 g/kg) on the rheological, textural, melting, visual, and physicochemical properties of sucrose-free dark chocolate (made with isomalt, erythritol and xylitol). In this study, olive leaf extract (OLE) was used as a source of oleuropein.

5.2 Materials and methods

5.2.1 Materials

Materials such as cocoa mass (Delicacies Valley, Canada), cocoa butter (Advantage Health Matters Inc., Canada), isomalt LM-PF (Beneo Palatinit, Germany), xylitol (Prescribed for Life, USA), erythritol (Prescribed for Life, USA), aspartame (Bulk Supplements, USA), vanilla powder (Nielsen-Massey Vanillas Inc., USA), sunflower lecithin (Now Foods, USA), and olive leaf extract (Olive Innovations, Tunisia) was used in this study. Aspartame was used to provide proper sweetness for sucrose-free dark chocolate.

5.2.2 Chemicals

Hexane, methanol (HPLC grade), L-ascorbic acid were obtained from Fisher Scientific (USA). Sodium nitrite, sodium hydroxide, folin-ciocalteu phenol reagent, phosphoric acid, 2,2-diphenyl-

1-picrylhydrazyl (DPPH), catechin hydrate, analytical standard oleuropein, gallic acid were purchased from Sigma-Aldrich (USA). Aluminum chloride was purchased from Fluka Analytical (USA). Sodium carbonate was obtained from Alfa Aesar (USA).

5.2.3 Preparation of dark chocolate samples

Sucrose-free dark chocolates were produced according to the subsequent formulation: 50 % cocoa mass (total fat content of 54 % +/- 2%; blend of Trinitario and Amazonico beans), 19.65 % cocoa butter, 30% polyols, 0.31% sunflower lecithin, 0.04 % vanilla powder, and olive leaf extract (10 g/kg, 15 g/kg, or 20 g/kg).

5.2.4 Chocolate processing

Four batches of 2 kg of sucrose-free dark chocolate was produced in a refiner (3.6 kg capacity, Diamond Custom Machines Corporation, USA). Each batch was for isomalt-made chocolate, erythritol-made chocolate, xylitol-made chocolate, and optimized polyols-made chocolate (Table 5.1). The optimized formulation of sucrose-free dark chocolate was a combination of 5.16% isomalt, 20.99% erythritol and 3.85% xylitol (details are presented in Chapter 4). This chocolate refiner did not have a temperature controller, so a handheld infrared thermometer (Fisher Scientific, USA) was used to measure the temperature whenever necessary during the processing stage.

Firstly, the cocoa butter and cocoa mass were separately melted at a temperature below 55°C in a double boiler (infrared thermometer was used to measure the temperature). This melted cocoa butter was then added to a chocolate refiner to circulate in the system for 10 seconds. Afterwards, melted cocoa mass was added to the refiner and mixed homogeneously for 30 minutes. Meanwhile,

to help ensure a cohesive mass in the chocolate refiner in later stage of processing, additional heat was provided using a hair dryer (Braun, Ireland) by keeping the heat on the roller stones.

Table 5.1: Composition of sucrose-free dark chocolate samples.

Oleuropein-enriched sucrose-free dark chocolate					
Batches	Isomalt (in % w/w)	Erythritol (in % w/w)	Xylitol (in % w/w)	Olive leaf extract (OLE) (in g/kg)	Chocolate samples
1	30	-	-	-	$I_{control}$
				10	I_{10}
				15	I_{15}
				20	I_{20}
2	-	30	-	-	$E_{control}$
				10	E_{10}
				15	E_{15}
				20	E_{20}
3	-	-	30	-	$X_{control}$
				10	X_{10}
				15	X_{15}
				20	X_{20}
4 (optimized)	5.16	20.99	3.85	-	$O_{control}$
				10	O_{10}
				15	O_{15}
				20	O_{20}

Then, polyol (isomalt, erythritol, xylitol) was gradually added to the refiner. Finally, after the addition of polyol, aspartame, vanilla powder, and sunflower lecithin were gradually added to the mixture in the refiner. This mixture was refined for 5.5 hours followed by conching during 6 hours in the refiner. 400 g portions of this chocolate mixture were then excluded, and each portion was enriched with three amounts (10, 15 and 20 g/kg) of olive leaf extract, which was dosed immediately before tempering. Control without the addition of olive leaf extract was also produced. OLE was sieved using 230 mesh (Thermo Fisher Scientific, US) before dosing in the chocolate (Figure 5.1).

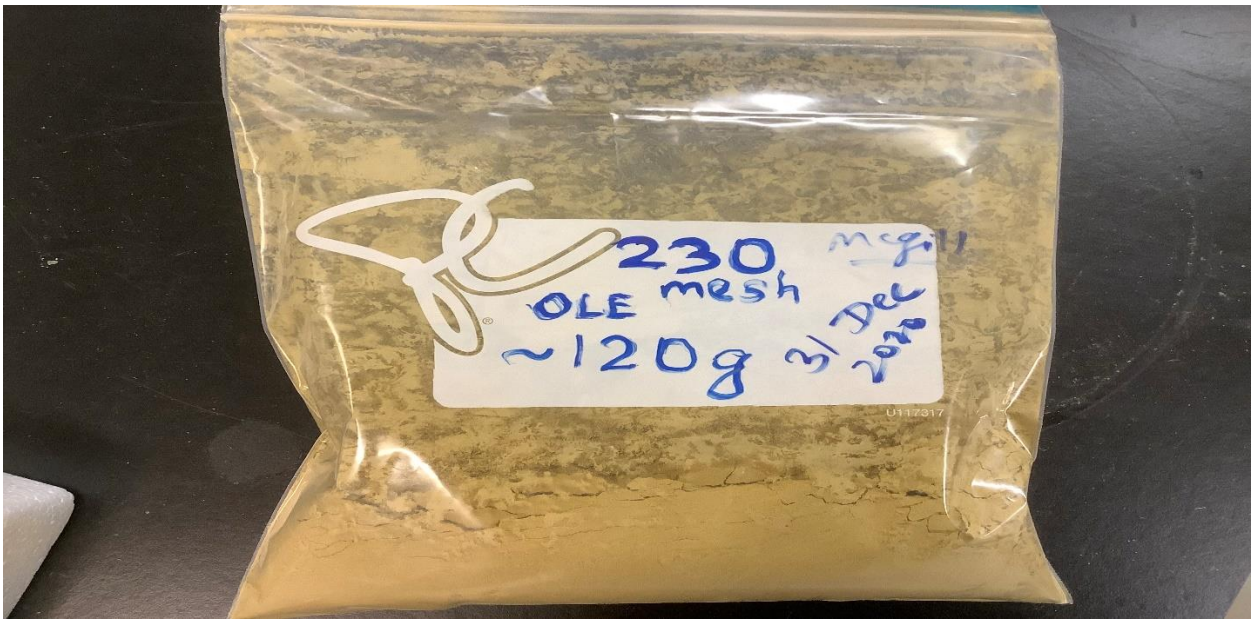

Figure 5.1: Olive leaf extract after sieving.

The tabling method was used for tempering chocolate using a marble slab as discussed by (Greweling, 2007) with some modifications. Sugar-free dark chocolate (one half to two thirds of the total chocolate mass) was poured onto a marble slab and agitated continuously to prevent it from solidifying, and to form the requisite cocoa butter seeds. In the end of this step, the temperature of the prepared chocolate mass was maintained at around 28^{o}C using a hair dryer. Afterwards, the agitated chocolate was mixed with the remaining chocolate mass (the remaining one third) so that a uniform chocolate mixture was formed. The temperature of the chocolate mass at the end of this step was maintained at around 32^{o}C (hair dryer was used to provide heat if necessary). Finally, the tempered chocolate mass was distributed into a silicone mold (each cavity in the mold measures 15 * 15 * 10 mm) and cooled down to 5^{o}C in a refrigerator for 40 minutes. Then the solid chocolate was removed from the mold and stored in a cool, dark, and odorless place in an airtight polyethylene bag covered with aluminum foil at 5 +/- 2^{o}C until required for analysis.

5.2.5 Rheological measurements

The method is as outlined in Section 4.2.4.

5.2.6 Moisture content

The method is as outlined in Section 4.2.5.

5.2.7 Color measurement

The surface color of all formulations were measured using a chromameter (CR – 300X, Minolta Camera Co. Ltd., Japan). The SCE-mode was used with the color expressed in terms of the CIELAB system. Following CIELAB system color coordinates were determined: L* (lightness ranging from black (0) to white (100)), a* (green to red) and b* (blue to yellow). The chroma meter was calibrated with the standard white tile before performing experiments. For each formulation, color measurement was performed in triplicate and mean value was determined. Chroma (C*), hue (h^o), whiteness index (WI) and color difference (ΔE) values were calculated using the equations 1, 2, 3 and 4, respectively.

$$C^* = (a^{*2} + b^{*2})^{0.5} \quad (1)$$

$$h^o = \arctan (b^*/ a^*) \quad (2)$$

$$WI = 100 – [(100 – L^*)^2 + a^{*2} + b^{*2}]^{0.5} \quad (3)$$

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{0.5} \quad (4)$$

Where, ΔL* = difference in the values of L* between control and oleuropein-enriched chocolate sample;

Δa* = difference in the values of a* between control and oleuropein-enriched chocolate sample;

Δb* = difference in the values of b* between control and oleuropein-enriched chocolate sample.

5.2.8 Melting properties

The melting properties of sucrose-free dark chocolate samples were measured using a Differential Scanning Calorimeter (DSC) (model DSC 2500, TA Instruments, New Castle, Delaware, USA) as discussed by Aidoo, Appah, Van Dewalle, Afoakwa, and Dewettinck (2017) with some modifications. Approximately 10 mg of chocolate samples were weighed into aluminum pans. The hermetically sealed pans were then heated from 10 to 65^{o}C at 3^{o}C/min in the DSC using an empty aluminum pan as reference. The onset temperature (T-onset), peak temperature (T-peak), endset temperature (T-end), and enthalpy (normalized) of melting (ΔH) were automatically calculated after integrating the melting peaks from the curve of heat flow (normalized; W/g) versus temperature (^{o}C) using TRIOS software. Mean values from duplicate measurements and standard deviations were recorded.

5.2.9 Hardness

The hardness of chocolate samples was measured with a texture analyzer (TA-HD plus model, Stable Micro Systems, UK) as discussed by Rad, Pirouzian, Toker, and Konar (2019) and Do, Hargreaves, Wolf, Hort, and Mitchell (2007). A stainless-steel puncture probe of 2 mm diameter (TA-52) was used to penetrate through the chocolate samples. Test settings were as follows: pretest speed of 1 mm/sec, test speed of 1 mm/sec over 4 mm distance, post-test speed of 1 mm/sec, and trigger force of 5 g. Hardness was measured as the maximum peak force in kilogram-force (kgf). Mean values from 5 replicate measurements and standard deviations were recorded.

5.2.10 Determination of bioactive parameters

5.2.10.1 Chocolate extract preparation

The preparation of chocolate extracts was performed according to the method by Belscak-Cvitanovic et al. (2012) with some modifications. Chocolate samples and cocoa mass were

manually grated. Approximately 2 g of chocolate sample or cocoa mass was extracted 3 times with 10 mL of n-hexane to remove its lipids. Afterwards, the defatted sample was air-dried for 24 hours to remove residual organic solvent (Adamson et al., 1999). The phenolic compounds were extracted 2 times from 2 g of defatted sample or 2 g of olive leaf extract with 10 mL aqueous methanol (70% v/v) for 30 min in an ultrasonic bath. After each extraction, mixture was centrifuged for 10 minutes at the speed of 3000 rpm and the supernatant was decanted. The supernatants were then combined and filtered using Whatman filter papers 1 (GE Healthcare Life Sciences, Canada) to remove the residual particles present in the mixture. This chocolate extract, cocoa mass extract or olive leaf extract solution were kept in a freezer at -18^{o}C. All samples were extracted in triplicate.

5.2.10.2 Determination of oleuropein content

To determine oleuropein of olive leaf extract and chocolate extract, reversed phase high-performance liquid chromatograph (HPLC) technique was applied as described by Lins, Pugine, Scatolini, and de Melo (2018) with some modifications. The Agilent HPLC system consisting of quaternary pumps, vacuum degasser, and a variable wavelength detector with Agilent chromatography software (ChemStation version 38) using a silica based C18 bonded phase column (250 mm × 4.6 mm ID, 5 μm particle size) and mobile phase containing a mixture of a gradient of water with 0.1% phosphoric acid and methanol (HPLC grade) in the ratio of 6:4. The flow rate was set at 1 mL/min and the injection volume of OLE solution or chocolate extract was 1 μL. The response of the peaks was detected at 280 nm using UV detector. The retention time was 9.8 minutes, and the total time was 30 minutes at 35^{o}C. All samples were analyzed in triplicate. The standard curve was developed by measuring the area for the peaks of 0.005, 0.01, 0.05, 0.1, 0.5 and 1 mg standard oleuropein/mL methanol. Methanol HPLC grade was used as a blank.

5.2.10.3 Determination of total phenol content

The total phenol content of olive leave extract and chocolate extract was determined using the Folin-Ciocalteu method as described by Lee, Kim, Lee, and Lee (2003) with some modifications. Briefly, 100 µL of test sample or standard was added to a 10 mL test tube containing 900 µL of dH_2O. Afterwards, 100 µL of Folin-Ciocalteu phenol reagent was added to the mixture and vortexed. After 5 min, 1 mL of a 7% Na_2CO_3 solution was added to the mixture. The solution was then immediately diluted to a volume of 2.5 mL with dH_2O and vortexed. This mixture was incubated for 90 min at room temperature and the absorbance relative to that of a blank solution at 750 nm was measured using Ultrospec Pro 2100 spectrophotometer (Biochrom, Cambridge, England). The blank solution consisted either of 100 µL of methanol (70% v/v) for test samples or 100 µL of dH_2O for gallic acid standard instead of test sample or standard, respectively. The standard curve was developed by measuring absorbance of 0.03125, 0.0625, 0.125, 0.25 and 0.5 mg gallic acid/mL dH_2O. The content of total phenolic is expressed as milligrams of gallic acid equivalents (GAE) per 100 g sample. All samples were analyzed in triplicates. The test samples in this study were OLE solution, chocolate, and cocoa mass extracts.

5.2.10.4 Determination of total flavonoid content

The total flavonoid content of olive leave extract and chocolate extract was determined using a colorimetric assay as described by Lee et al. (2003) with some modifications. Briefly, 1 mL of test sample or standard was added to a 25 mL test tube containing 4 mL of dH_2O. Afterwards at time zero, 0.3 mL of 5% $NaNO_2$ was added to the mixture and vortexed. At 5 minutes, 0.3 mL of 10% $AlCl_3$ was added to the mixture and vortexed. At 6 minutes, 2 mL of 1M NaOH was added to the mixture which was immediately followed by the addition of 2.4 mL of dH_2O. The absorbance of a mixture relative to that of a blank solution at 510 nm was measured using Ultrospec Pro 2100

spectrophotometer, upon the development of pink color. The blank solution consisted either of 100 μL of methanol (70% v/v) for test samples or 100 μL of dH_2O for (+)- catechin hydrate standard instead of test sample or standard, respectively. The standard curve was developed by measuring absorbance of 0.03125, 0.0625, 0.125, 0.25 and 0.5 mg (+)- catechin hydrate/mL dH_2O. The total flavonoid content of test samples are expressed in milligrams of catechin hydrate equivalents (CHE) per 100 g sample. All samples were analyzed in triplicates. The test samples in this study were OLE solution, chocolate extracts and cocoa mass extract.

5.2.11 Statistical analysis

The experiments were performed in triplicate and the obtained data were presented as means of replicate values ± standard deviation. The obtained results were statistically analyzed by one-factor analysis of variance (ANOVA) with subsequent comparisons by Tukey's test, with a significance level of 0.05 using SAS version 9.4 software.

5.3 Results and Discussion

5.3.1 Moisture content

The moisture content plays an important role in the shelf life, flow properties, and hardness of chocolate. The acceptable range, without drastic effects on rheological properties, of moisture content in chocolate as recommend by Afoakwa (2010) is between 0.5 to 1.5%. This range is determined by chocolate composition and processing method (Konar, 2013). Interestingly in this study, the moisture contents of all chocolate samples were in the range of 0.85 to 1.41% (in wet basis) (Table 5.2), which is within the acceptable range. In general, the addition of olive leaf extract at different concentrations led to an increase in the moisture content. This increase between the control samples and the different concentrations of olive leaf extract (10, 15 and 20 g/kg), however, was only found to be significant ($P < 0.05$) in isomalt-made chocolate samples. Furthermore, no

significant difference ($P > 0.05$) in the moisture content was observed between I_{10} and I_{15}, I_{10} and I_{20} and I_{15}, and I_{20} samples. Thus, it can be concluded that the addition of OLE does not significantly affect the moisture content of sucrose-free chocolate.

Table 5.2: Moisture content of oleuropein-enriched sucrose-free dark chocolates.

Samples	Moisture content (%, in wet basis)			
	Isomalt-made chocolate	Erythritol-made chocolate	Xylitol-made chocolate	Optimized chocolate
control	0.85 ± 0.02^{B}	1.29 ± 0.07^{aA}	0.92 ± 0.16^{aB}	1.21 ± 0.01^{aA}
10 g/kg OLE	0.91 ± 0.02^{aB}	1.31 ± 0.03^{aA}	0.92 ± 0.11^{aB}	1.25 ± 0.01^{aA}
15 g/kg OLE	0.91 ± 0.00^{aB}	1.40 ± 0.08^{aA}	0.93 ± 0.17^{aB}	1.24 ± 0.01^{aA}
20 g/kg OLE	0.90 ± 0.01^{aB}	1.41 ± 0.02^{aA}	0.91 ± 0.13^{aB}	1.23 ± 0.11^{aA}

[a]Means within the same column sharing the same superscript are not significantly different from each other (Tukey's test, $P < 0.05$). [A,B]Means within the same row that have no superscript in common are significantly different from each other (Tukey's test, $P < 0.05$).

5.3.2 Color

The key contributor to the appearance of chocolate is its color, and it influences the consumers perception. The important parameters that affect the color of chocolates are the composition and processing conditions applied during manufacturing. The effect of different concentration of olive leaf extract (10, 15 and 20 g/kg) on polyols-made chocolate has been reported in Table 5.3. With the use of OLE (in different concentrations) in sucrose-free dark chocolate samples, significant increase in the values of L* (26.14 ± 0.67 to 27.41 ± 0.42) for erythritol-made chocolate and L* (26.64 ± 0.51 to 30.41 ± 0.37) for xylitol-made chocolate were observed ($P < 0.05$). Higher values of L* illustrate a lighter appearance chocolate sample. The redness (+a*) associated with oleuropein-enriched chocolate samples did not show any significant changes with respect to their control samples, except in the case of O_{10} chocolate where it was found to have a significant increase in the values of a* (4.07 ± 0.33) compared to $O_{control}$ (3.65 ± 0.29) ($P < 0.05$). Furthermore, the yellowness (+b*) associated with oleuropein-enriched chocolate samples showed no significant changes ($P > 0.05$) in erythritol-made and optimized chocolate samples as opposed to

significant decrease ($P < 0.05$) in isomalt-made chocolate samples and increase ($P < 0.05$) in xylitol-made chocolate samples, compared to their respective control samples. Similarly, there was a significant increase in the values of h^o in xylitol-made and optimized chocolate samples (42.91 ± 1.99 to 51.80 ± 0.96 and 44.79 ± 3.34 to 49.27 ± 2.18, respectively) compared to a significant decrease in isomalt-made chocolate samples (32.18 ± 1.39 to 39.68 ± 1.01) by the addition of OLE (in different concentration). The addition of OLE (10, 15 and 20 g/kg) on the whiteness index (WI) of isomalt-made and optimized chocolate samples was found to have no significant effect, however there was a significant increase in the values of WI in erythritol- and xylitol-made chocolate samples. These changes in color quality characteristics of chocolate samples could be attributed to a consequence of color changes induced by inadequate conditions during tempering, molding, and storage (Papadakis, Abdul-Malek, Kamdem, & Yam, 2000). Furthermore, the changes in the values of L*, a*, b* and h^o in oleuropein-enriched chocolate samples could be associated with the particle size distribution of OLE in chocolate (Afoakwa, Paterson, & Fowler, 2008). In addition, the changes in color parameters may also be due to the characteristic yellow-brown color of the OLE used in this study (Figure 5.1).

Table 5.3: Color parameters of oleuropein-enriched sucrose-free dark chocolates.

	Color parameters						
Chocolate samples	L*	a*	b*	C*	h^{o}	WI	ΔE
Isomalt-made chocolate							
$I_{control}$	28.13±0.64^{a}	4.42±0.54^{a}	4.66±0.46	6.42±0.71^{a}	46.56±0.68	27.84±0.69^{a}	nd
I_{10}	27.43±0.35^{a}	4.40±0.17^{a}	2.77±0.14	5.20±0.18^{b}	32.18±1.39	27.24±0.36^{a}	2.02±0.57
I_{15}	27.82±0.61^{a}	4.37±0.24^{a}	3.63±0.31^{a}	5.69±0.38^{ab}	39.68±1.01^{a}	27.60±0.63^{a}	1.07±0.34
I_{20}	27.79±0.91^{a}	4.60±0.52^{a}	3.65±0.25^{a}	5.88±0.38^{ab}	38.53±4.18^{a}	27.55±0.91^{a}	1.08±0.35
Erythritol-made chocolate							
$E_{control}$	26.14±0.67^{b}	3.88±0.39^{a}	2.52±0.19^{a}	4.63±0.35^{a}	33.15±3.19^{a}	25.99±0.66^{b}	nd
E_{10}	27.41±0.42^{a}	4.15±0.10^{a}	2.92±0.28^{a}	5.08±0.13^{a}	35.07±2.99^{a}	27.24±0.43^{a}	1.37±0.38
E_{15}	26.78±0.16^{ab}	3.85±0.24^{a}	2.74±0.44^{a}	4.73±0.39^{a}	35.35±3.85^{a}	26.62±0.14^{ab}	0.68±0.58
E_{20}	26.89±0.15^{ab}	3.65±0.28^{a}	2.29±0.43^{a}	4.31±0.46^{a}	31.97±2.81^{a}	26.76±0.16^{ab}	0.82±0.58

^{a,b}Means within the same column of same type of polyol-made chocolate sharing the same superscript are not significantly different from each other (Tukey's test, $P < 0.05$). nd: not determined.

Table 5.3 (continued): Color parameters of oleuropein-enriched sucrose-free dark chocolates.

	Color parameters						
Chocolate samples	L*	a*	b*	C*	h^{o}	WI	ΔE
Xylitol-made chocolate							
$X_{control}$	26.66±0.66^{a}	3.74±0.02^{a}	3.80±0.32^{a}	5.34±0.24^{a}	45.39±2.26^{a}	26.47±0.66^{a}	nd
X_{10}	26.64±0.51^{a}	3.66±0.21^{a}	3.43±0.17^{a}	5.02±0.04^{a}	43.13±3.04^{a}	26.47±0.51^{a}	0.39±0.29
X_{15}	27.74±0.27^{a}	4.00±0.32^{a}	3.72±0.34^{a}	5.46±0.43^{a}	42.91±1.99^{a}	27.53±0.27^{a}	1.11±0.49
X_{20}	30.41±0.37	3.99±0.14^{a}	5.07±0.12	6.45±0.15	51.80±0.96	30.11±0.38	3.96±0.37
Optimized chocolate							
$O_{control}$	27.56±1.02^{a}	3.65±0.29^{ab}	3.22±0.18^{a}	4.86±0.34^{ab}	41.44±0.74^{b}	27.40±1.04^{a}	nd
O_{10}	28.24±1.09^{a}	4.07±0.33^{a}	4.06±0.60^{a}	5.76±0.61^{a}	44.79±3.34^{ab}	28.01±1.06^{a}	1.17±0.43
O_{15}	29.13±1.59^{a}	3.31±0.14^{b}	3.59±0.24^{a}	4.89±0.13^{ab}	47.26±2.84^{ab}	28.96±1.59^{a}	1.65±0.59
O_{20}	29.42±0.86^{a}	3.03±0.11^{b}	3.52±0.14^{a}	4.64±0.04^{b}	49.27±2.18^{a}	29.26±0.86^{a}	1.98±0.24

^{a,b}Means within the same column of same type of polyol-made chocolate sharing the same superscript are not significantly different from each other (Tukey's test, $P < 0.05$). nd: not determined.

It is necessary that the visual properties of oleuropein-enriched sucrose-free dark chocolate match the standard. If not, customer satisfaction may not be achieved. Because of this, identifying the color difference (ΔE) between a sample and the control plays an important role in the chocolate production process. In this study, there was an increase in ΔE values for xylitol-made and optimized chocolate samples with the increase in the concentration of OLE. However, the addition of OLE decreased the ΔE values in isomalt- and erythritol-made chocolate.

5.3.3 Rheological properties

Rheological experiments showed that apparent viscosity of all oleuropein-enriched chocolate samples (including controls) decreased with increasing shear rate (data not shown). This exhibition of shear thinning behavior of chocolate samples agrees with previous studies on chocolates (Fernandes, Müller, & Sandoval, 2013; Pirouzian, Peighambardoust, & Azadmard-Damirchi, 2017; Rad et al., 2019). This behaviour could be explained by the structural decomposition of the molecules due to continuous shearing and pressure force applied, and the increased alignment of the constituent molecules during the processing stage (Alpaslan & Hayta, 2002; Fernandes et al., 2013; Izidoro, Scheer, Sierakowski, & Haminiuk, 2008; Rao & Tattiyakul, 1999).

In order to understand the effects of OLE (10, 15 and 20 g/kg) on the rheological behavior of sucrose-free dark chocolate, data obtained from the rheometer were fitted to non-Newtonian models, such as Power law model (equation 5), Herschel- Buckley model (equation 6), Casson fluid model (equation 7), and Bingham plastic model (equation 8), to describe the relationship between shear rate and shear stress.

$$\sigma = \kappa\gamma^{n} \quad (5)$$

$$\sigma = \kappa\gamma^{n} + \sigma_0 \quad (6)$$

$$(\sigma)^{0.5} = (\kappa_1)^{0.5}(\gamma)^{0.5} + (\sigma_0)^{0.5} \quad (7)$$

$$\sigma = \mu_{pl}(\gamma) + \sigma_0 \quad (8)$$

where σ = shear stress (Pa); n = flow behavior index (dimensionless); κ = consistency coefficient (Pa.s^n); γ = shear rate (s^{-1}); σ_0 = yield stress (Pa); κ_1 = plastic viscosity (Pa.s) for Casson model; μ_{pl} = plastic viscosity (Pa.s) for Bingham model.

Table 5.4: Effects of experimental data obtained from sucrose-free dark chocolates in rheological models in terms of R^2 and RMSE.

Samples	Herschel-Buckley model		Power law model		Casson model		Bingham model	
	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE
$I_{control}$	0.9999	0.1736	0.9994	0.5020	0.9999	0.1966	0.9996	0.4187
I_{10}	0.9999	0.1967	0.9993	0.6379	0.9999	0.2830	0.9997	0.3743
I_{15}	0.9999	0.2503	0.9994	0.6763	0.9999	0.3081	0.9997	0.4213
I_{20}	0.9999	0.2131	0.9997	0.4760	0.9999	0.2737	0.9993	0.7670
$E_{control}$	0.9997	0.2588	0.9965	0.8055	0.9991	0.3934	0.9993	0.3482
E_{10}	0.9998	0.2402	0.9975	0.7665	0.9996	0.3199	0.9993	0.4319
E_{15}	0.9998	0.2188	0.9977	0.8171	0.9996	0.3112	0.9992	0.4683
E_{20}	0.9999	0.1412	0.9975	0.8898	0.9997	0.7522	0.9995	0.8995
$X_{control}$	0.9998	0.1696	0.9959	0.7906	0.9993	0.3210	0.9994	0.2948
X_{10}	0.9998	0.2044	0.9961	0.8619	0.9993	0.3632	0.9995	0.3172
X_{15}	0.9998	0.1798	0.9963	0.8661	0.9994	0.3564	0.9995	0.3143
X_{20}	0.9997s	0.2487	0.9962	0.8992	0.9993	0.3934	0.9994	0.3666
$O_{control}$	0.9999	0.2223	0.9979	0.8846	0.9996	0.3891	0.9997	0.3311
O_{10}	0.9999	0.2348	0.9982	0.9710	0.9997	0.4041	0.9996	0.4243
O_{15}	0.9999	0.2433	0.9985	0.9308	0.9998	0.3850	0.9992	0.6290
O_{20}	0.9999	0.2142	0.9983	1.0344	0.9997	0.4182	0.9997	0.4107

The obtained experimental data were fitted with these models and evaluated based on two statistical parameters namely, coefficient of determination (r^2) and root mean square error (RMSE) (Table 5.4). Based on the statistical calculations, Herschel-Buckley model was found to be the best model due to the highest R^2 and the lowest RMSE values to describe the shear stress vs. shear rate relationship. Therefore, all rheological parameters (plastic viscosity, flow behavior index, and yield stress) were determined using the Herschel-Buckley model. This model was also reported to describe the rheological behavior of dark chocolates made with isomalt, xylitol and maltitol by Sokmen and Gunes (2006), milk chocolates made with polydextrose by Shah, Jones, and Vasiljevic (2010) and milk chocolates made with isomalt, maltitol and inulin by Konar (2013). Nevertheless, the recommended and the widely used model to describe the rheological behavior of chocolates by International Confectionery Association (ICA) or previously International Office of Cocoa, Chocolate and Sugar Confectionery (IOCCC) is the Casson model. This model, however, was reported not to provide the acceptable reproducibility (Servais et al., 2003).

5.3.3.1 Plastic viscosity

The consistency coefficients (κ) or the Herschel-Buckley plastic viscosity of sucrose-free dark chocolates with different levels of OLE were determined (Table 5.5). The flow behavior of chocolate has a significant effect on the quality of the final product due to its effects on some important parameters such as mouthfeel, viscosity, and consistency (Glicerina & Romani, 2017). In this study, the consistency coefficient was determined between 2.05 ± 0.08 and 2.91 ± 0.15, 1.51 ± 0.19 and 1.69 ± 0.07, 1.25 ± 0.03 and 1.34 ± 0.09, and 1.98 ± 0.12 and 2.44 ± 0.62 $Pa.s^n$ for isomalt-, erythritol-, xylitol-, and optimized chocolate samples with different levels of OLE, respectively. Whereas the consistency coefficient for the controls made with isomalt, erythritol, xylitol, and optimized were found to be 1.84 ± 0.08, 1.22± 0.24, 1.15 ± 0.01, 1.61 ± 0.07 $Pa.s^n$,

respectively. With the use of OLE in sucrose-free dark chocolate samples, a significant increase ($P < 0.05$) in the plastic viscosity was observed. However, no significant changes ($P > 0.05$) in the plastic viscosity were found when the level of OLE was increased from 10 to 15 g/kg or 10 to 20 g/kg or 15 to 20 g/kg. The notable exception to this case was seen in isomalt-made dark chocolate where plastic viscosity of 20 g/kg OLE-enriched chocolate was found to be significantly different ($P < 0.05$) from 10 g/kg or 15 g/kg OLE-enriched samples. The Casson plastic viscosity, when measured over shear rate from 5 to 60 s^{-1}, for dark chocolate has been reported between 2.1 and 3.9 Pa.s (Aeschlimann & Beckett, 2000). Nevertheless, the plastic viscosity of only 15 and 20 g/kg OLE-enriched chocolate samples made with isomalt and optimized polyols fell in this range.

Table 5.5: Consistency coefficient of oleuropein-enriched sucrose-free dark chocolates.

Samples	Consistency coefficient ($Pa.s^n$)			
	Isomalt-made chocolate	Erythritol-made chocolate	Xylitol-made chocolate	Optimized chocolate
control	1.84 ± 0.08	1.22 ± 0.24^{b}	1.15 ± 0.01^{b}	1.61 ± 0.07^{b}
10 g/kg OLE	2.05 ± 0.08^{a}	1.51 ± 0.19^{ab}	1.25 ± 0.03^{ab}	1.98 ± 0.12^{ab}
15 g/kg OLE	2.22 ± 0.06^{a}	1.69 ± 0.07^{a}	1.29 ± 0.04^{a}	2.44 ± 0.62^{a}
20 g/kg OLE	2.91 ± 0.15	1.66 ± 0.09^{a}	1.34 ± 0.09^{a}	2.12 ± 0.05^{ab}

[a,b]Means within the same column sharing the same superscript are not significantly different from each other (Tukey's test, $P < 0.05$). n: flow behavior index.

The rheological properties are affected by the composition and the processing condition (during refining, conching, and tempering) applied for manufacturing chocolate (Schantz & Rohm, 2005; Vavreck, 2004). In this study, the reason behind the increase in the plastic viscosity may be associated with the higher moisture content in oleuropein-enriched sucrose-free dark chocolate samples compared to the control sample. In the presence of moisture, the crystals of polyols dissolve and an increase in the interaction between sugar particles occur (Saputro et al., 2017). This may lead to an increase in the internal friction and thus may result in higher viscosity.

5.3.3.2 Yield stress

The yield stresses of sucrose-free dark chocolates with different levels of OLE were determined (Table 5.6). In this study, the yield stress was observed between 2.91 ± 0.54 and 4.16 ± 0.29, 5.54 ± 0.40 and 6.53 ± 0.38, 6.50 ± 0.30 and 6.65 ± 0.93, and 6.53 ± 0.83 and 7.12 ± 0.07 Pa. for isomalt-, erythritol-, xylitol-, and optimized chocolate samples with different levels of OLE, respectively. Whereas the yield stress for the controls made with isomalt, erythritol, xylitol, and optimized were found to be 3.22 ± 0.06, 5.70 ± 0.65, 6.08 ± 0.42, and 6.07 ± 0.13 Pa, respectively. With the use of OLE, significant increase ($P < 0.05$) in the yield stress was observed in isomalt-made and optimized chocolate samples with the notable exception in I_{20} sample. However, no significant difference ($P > 0.05$) in the yield stress was found between oleuropein-enriched erythritol-made chocolate samples and $E_{control}$ sample, and oleuropein-enriched xylitol-made chocolate samples and $X_{control}$ sample. The Casson yield stress for dark chocolate has been reported between 4 and 8 Pa (Aeschlimann & Beckett, 2000). All chocolate samples except $I_{control}$, I_{10} and I_{20} fell within this range.

Table 5.6: Yield stress of oleuropein-enriched sucrose-free dark chocolates.

Samples	Yield stress (Pa)			
	Isomalt-made chocolate	Erythritol-made chocolate	Xylitol-made chocolate	Optimized chocolate
control	3.22 ± 0.06^{bc}	5.70 ± 0.65^{a}	6.08 ± 0.42^{a}	6.07 ± 0.13^{b}
10 g/kg OLE	3.96 ± 0.50^{ab}	5.54 ± 0.40^{a}	6.50 ± 0.30^{a}	6.69 ± 0.12^{ab}
15 g/kg OLE	4.16 ± 0.29^{a}	6.00 ± 0.49^{a}	6.54 ± 0.15^{a}	6.53 ± 0.83^{ab}
20 g/kg OLE	2.91 ± 0.54^{c}	6.53 ± 0.38^{a}	6.65 ± 0.93^{a}	7.12 ± 0.07^{a}

[a,b,c]Means within the same column sharing the same superscript are not significantly different from each other (Tukey's test, $P < 0.05$).

In this study, the increase in the yield stress may be associated with the particle size distribution (with specific surface area and mean particle size) of OLE in sucrose-free dark chocolate. This could be due to the greater particle-particle interactions in chocolate (Afoakwa et al., 2008). Furthermore, Prasad et al. (2003) reported that the yield value is affected largely by inter-particle

contacts and has a linear dependency on specific surface area. The yield stress is also related to shape retention, pattern holding, presence of air bubbles and inclined surface coating (Seguine, 1988).

5.3.3.3 Flow behavior index

The flow behavior index (n) plays a crucial role in determining the stability of chocolate samples. The flow behavior index of all chocolate samples was found between 0.90 and 0.95 (Table 5.7). Values lower than 1 indicate the pseudoplastic nature of chocolate, exhibiting shear thinning behaviour above the yield stress. This means that the apparent viscosity of chocolate samples decreases as shear rate increases. Overall, no significant difference ($P > 0.05$) was observed between the flow behavior index of oleuropein-enriched sucrose-free dark chocolate samples and the control.

Table 5.7: Flow behavior index of oleuropein-enriched sucrose-free dark chocolates.

Samples	Flow behavior index (dimensionless)			
	Isomalt-made chocolate	Erythritol-made chocolate	Xylitol-made chocolate	Optimized chocolate
control	0.93 ± 0.01^{ab}	0.94 ± 0.04^{a}	0.93 ± 0.01^{a}	0.95 ± 0.01^{a}
10 g/kg OLE	0.95 ± 0.02^{a}	0.91 ± 0.02^{a}	0.93 ± 0.00^{a}	0.94 ± 0.01^{a}
15 g/kg OLE	0.95 ± 0.01^{a}	0.91 ± 0.00^{a}	0.93 ± 0.00^{a}	0.91 ± 0.03^{a}
20 g/kg OLE	0.90 ± 0.01^{b}	0.93 ± 0.01^{a}	0.93 ± 0.01^{a}	0.95 ± 0.02^{a}

[a,b]Means within the same column sharing the same superscript are not significantly different from each other (Tukey's test, $P < 0.05$).

5.3.4 Hardness

The hardness of sucrose-free dark chocolates with different levels of OLE (10, 15 and 20 g/kg) was determined (Table 5.8). Overall, no significant difference ($P > 0.05$) was observed between the hardness of oleuropein-enriched chocolate and control samples. The notable exception to this behavior has been observed in the xylitol-made chocolate samples where the hardness of the X_{20} sample (11.55 ± 0.60 kgf) was found to be significantly different from $X_{control}$ (7.93 ± 0.52 kgf) and X_{10} (8.27 ± 0.57 kgf).

Table 5.8: Hardness of oleuropein-enriched sucrose-free dark chocolates.

Samples	Hardness (kgf)			
	Isomalt-made chocolate	Erythritol-made chocolate	Xylitol-made chocolate	Optimized chocolate
control	15.98 ± 1.60[a]	11.62 ± 1.42[a]	7.93 ± 0.52[b]	15.60 ± 2.50[a]
10 g/kg OLE	15.37 ± 2.61[a]	10.77 ± 0.42[a]	8.27 ± 0.57[b]	13.65 ± 1.60[a]
15 g/kg OLE	16.28 ± 1.37[a]	10.92 ± 0.69[a]	10.02 ± 1.31[ab]	12.53 ± 1.51[a]
20 g/kg OLE	17.75 ± 0.67[a]	9.66 ± 0.08[a]	11.55 ± 0.60[a]	11.27 ± 0.97[a]

[a,b]Means within the same column sharing the same superscript are not significantly different from each other (Tukey's test, $P < 0.05$). kgf: kilogram-force.

The hardness of the chocolate sample largely depends on the relative concentration of crystallized lipid phase, solid dispersed phase (such as cocoa solids and sugar crystals) and their interactions (Liang & Hartel, 2004). Furthermore, factors such as the type of bulk sweetener, tempering conditions, processing method and particle size distribution could have influenced the hardness of the chocolate samples (Barišić et al., 2021). An under-tempered chocolate has an increase in softness while an over-tempered chocolate has an increase in hardness, compared to properly tempered chocolate (Barišić et al., 2021; Ilmi, Praseptiangga, & Muhammad, 2017). Another possible reason behind the changes in hardness with the addition of different concentration of OLE in sucrose-free dark chocolates may be compactness. A high compactness due to the more evenly filling of the cavities and spaces in the chocolate sample increases its hardness (Praseptiangga, Invicta, & Khasanah, 2019).

5.3.5 Melting properties

The melting properties of chocolate are an important quality parameter to understand the in-mouth melting behaviour and other physical properties such as crystallization and hardness (Braipson-Danthine & Deroanne, 2004). The melting behavior (a curve of heat flux versus temperature) of polyols-made chocolate can be seen in Figure 5.2. From the curve, we can see that there are multiple peaks indicating inhomogeneous melting behavior of the chocolate samples. The onset temperature (T-onset), peak temperature (T-peak), endset temperature (T-end), and enthalpy of

melting (ΔH) of polyols-made chocolates are presented in Table 5.9. Overall, in all chocolate samples including controls, the onset temperature ranged from 15.02 to 15.29ºC, the peak temperature from 18.05 to 18.49ºC, the endset temperature from 24.64 to 26.83ºC, and the enthalpy of melting from 14.54 to 28.98 J/g. From these results, in general the addition of OLE (10, 15 and 20 g/kg) did not significantly ($P > 0.05$) affect the melting behavior of sucrose-free dark chocolate, with the notable exception in isomalt-made chocolate. The peak temperature between $I_{control}$ (18.42 ± 0.04ºC) and I_{10} samples (18.25 ± 0.05ºC) was found to be significantly different ($P < 0.05$) from each other. However, in line with this overall observation it must be noted that the low melting temperatures around 25ºC implied the formation of low-melting polymorphic forms of cocoa butter. The formation of polymorphic forms of cocoa butter depends on the processing conditions (Afoakwa, Paterson, & Fowler, 2007). The most desirable form of cocoa butter in a well-tempered chocolate is form V (which has a melting point of 34 - 35ºC) and gives a good snap, glossy appearance, and resistance to bloom (Beckett, 2019).

Table 5.9: Melting characteristics of oleuropein-enriched sucrose-free dark chocolates having onset temperature at around 15°C.

Chocolate samples	T-onset (°C)	T-peak (°C)	T-end (°C)	ΔH (J/g)
Isomalt-made chocolate				
$I_{control}$	15.26 ± 0.02	18.42 ± 0.04[a]	25.43 ± 0.03[a]	16.45 ± 0.21
I_{10}	15.02 ± 0.03[a]	18.25 ± 0.05[b]	24.80 ± 0.03[b]	19.92 ± 0.03
I_{15}	15.09 ± 0.02[a]	18.34 ± 0.03[ab]	25.23 ± 0.01[a]	25.30 ± 0.00
I_{20}	15.10 ± 0.02[a]	18.36 ± 0.04[ab]	24.89 ± 0.02[b]	22.84 ± 0.02
Erythritol-made chocolate				
$E_{control}$	15.22 ± 0.00[a]	18.07 ± 0.01[a]	24.74 ± 0.13[a]	14.54 ± 0.51[a]
E_{10}	15.19 ± 0.08[a]	18.08 ± 0.02[a]	24.64 ± 0.02[a]	15.15 ± 0.92[a]
E_{15}	15.15 ± 0.01[a]	18.06 ± 0.04[a]	24.66 ± 0.06[a]	16.47 ± 0.08[a]
E_{20}	15.20 ± 0.01[a]	18.16 ± 0.05[a]	24.87 ± 0.08[a]	15.17 ± 0.50[a]
Xylitol-made chocolate				
$X_{control}$	15.18 ± 0.02[a]	18.15 ± 0.01[a]	24.93 ± 0.01[a]	22.17 ± 0.17[a]
X_{10}	15.29 ± 0.09[a]	18.38 ± 0.13[a]	25.09 ± 0.08[a]	23.93 ± 2.64[a]
X_{15}	15.21 ± 0.01[a]	18.28 ± 0.33[a]	25.09 ± 0.01[a]	24.17 ± 1.63[a]
X_{20}	15.34 ± 0.01[a]	18.45 ± 0.14[a]	25.18 ±0. 14[a]	28.98 ± 4.81[a]
Optimized chocolate				
$O_{control}$	15.09 ± 0.04[a]	18.13 ± 0.03[a]	25.85 ± 0.54[a]	27.26 ± 0.63[ab]
O_{10}	15.02 ± 0.00[a]	18.13 ± 0.00[a]	26.55 ± 0.00[a]	27.17 ± 0.01[ab]
O_{15}	15.07 ± 0.02[a]	18.05 ± 0.11[a]	26.83 ± 0.09[a]	27.98 ± 0.00[a]
O_{20}	15.32 ± 0.38[a]	18.49 ± 0.56[a]	26.81 ± 0.75[a]	26.17 ± 0.00[b]

[a,b]Means within the same column of same type of polyol-made chocolate sharing the same superscript are not significantly different from each other (Tukey's test, $P < 0.05$).

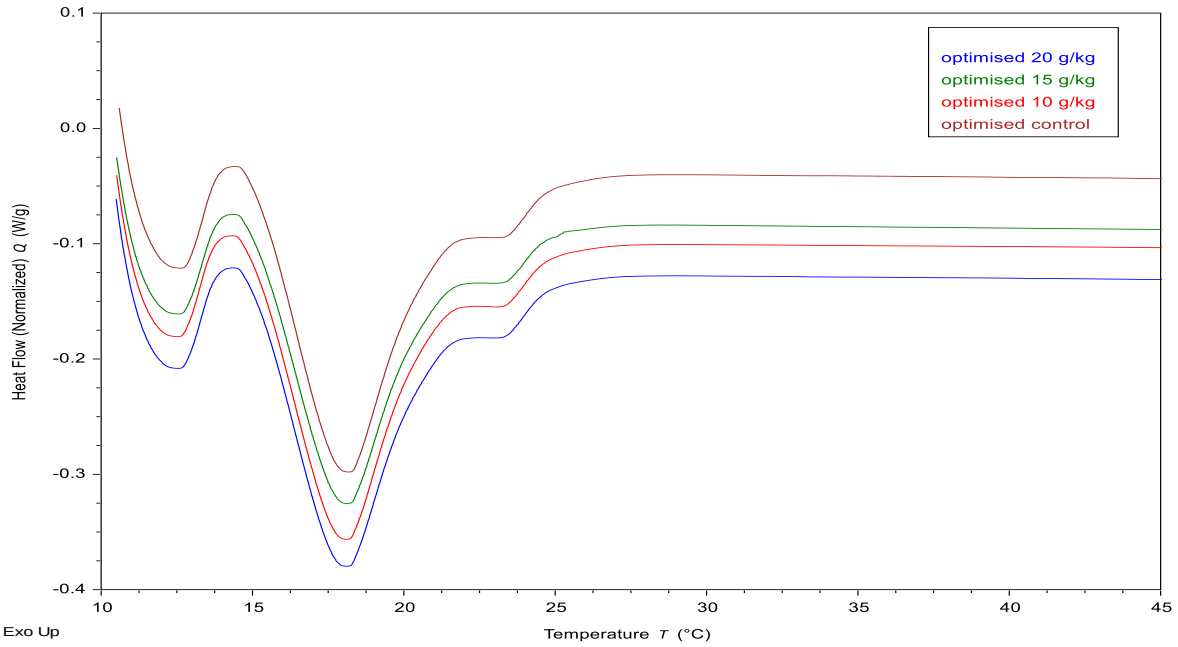

Figure 5.2a: Differential scanning calorimetry melting profiles of control and oleuropein-enriched optimized chocolates.

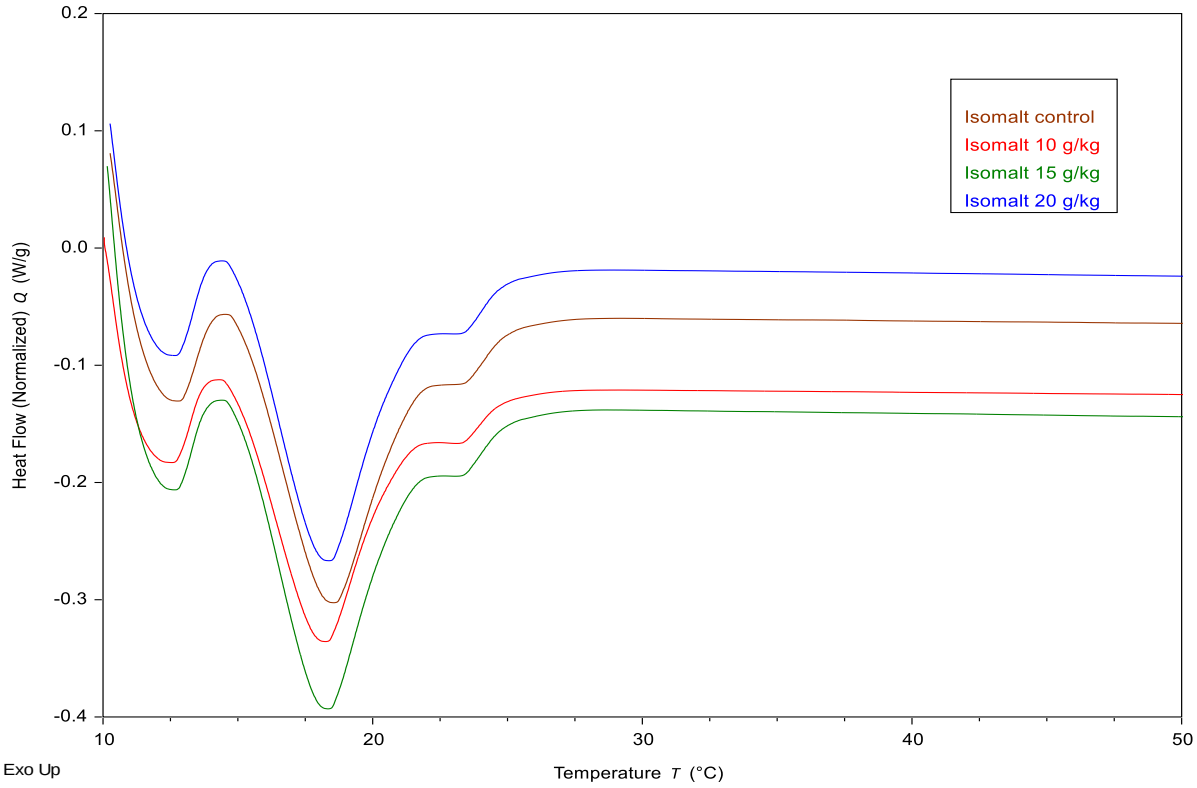

Figure 5.2b: Differential scanning calorimetry melting profiles of control and oleuropein-enriched isomalt-made chocolates.

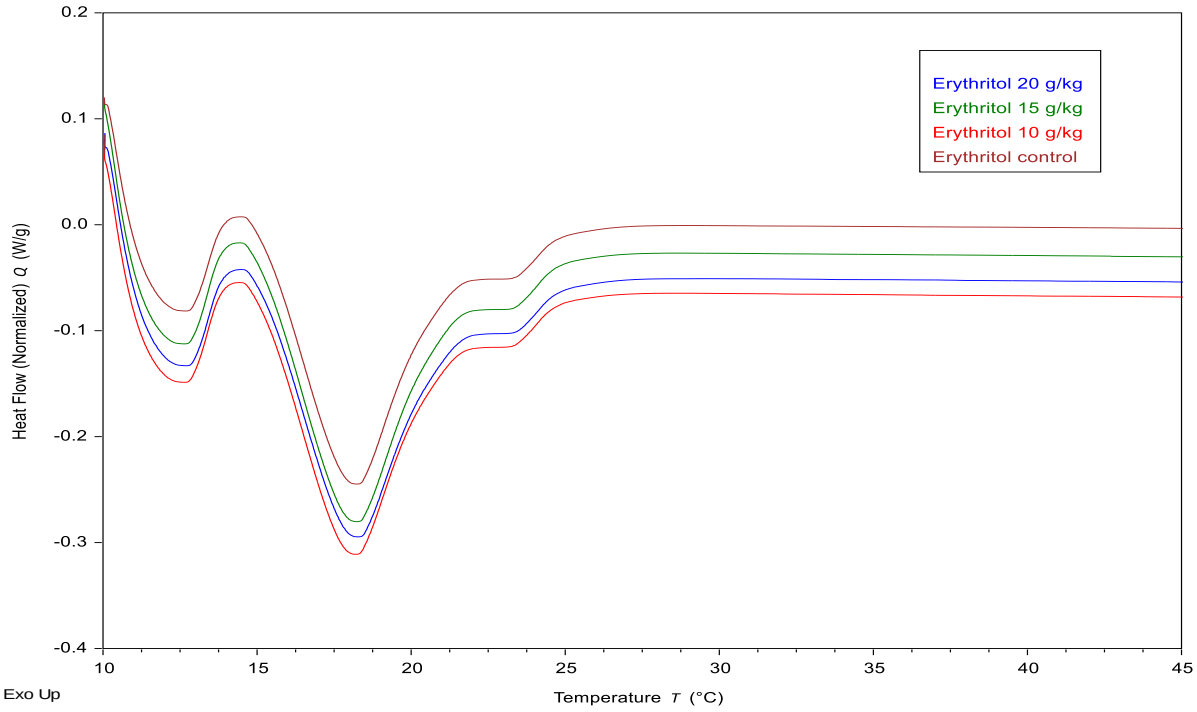

Figure 5.2c: Differential scanning calorimetry melting profiles of control and oleuropein-enriched erythritol-made chocolates.

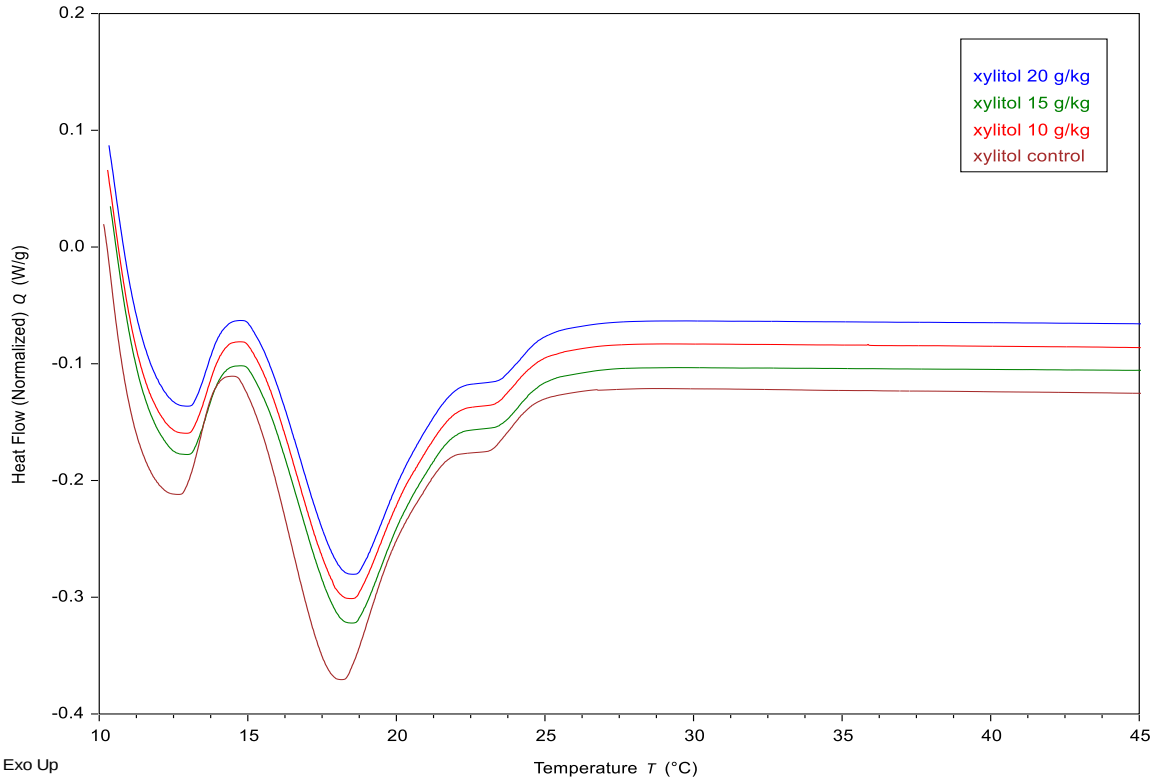

Figure 5.2d: Differential scanning calorimetry melting profiles of control and oleuropein-enriched xylitol-made chocolates.

5.3.6 Oleuropein content

The proportion of phenolic compounds in olive leaf extract depends on the interaction of cultivar type, geographical production zone, extraction process, climate, and the harvesting time, however, in most studies, oleuropein was found to be the main active phenolic compound (Omar, 2010). The suggested suitable procedure for oleuropein determination in complex matrices (such as pharmaceutical formulation) was reversed-phase chromatography. In this study, reversed phase HPLC technique was applied for the determination of oleuropein as described by Lins et al. (2018) with some modifications. The linearity of this applied method was evaluated in a concentration from 0.005 to 1 mg/mL by injecting the analytical standard oleuropein solutions at 280 nm. The chromatogram (x-axis = time (min) and y-axis = absorbance (mAU)) of the standard oleuropein solution is presented in Figure 5.3.

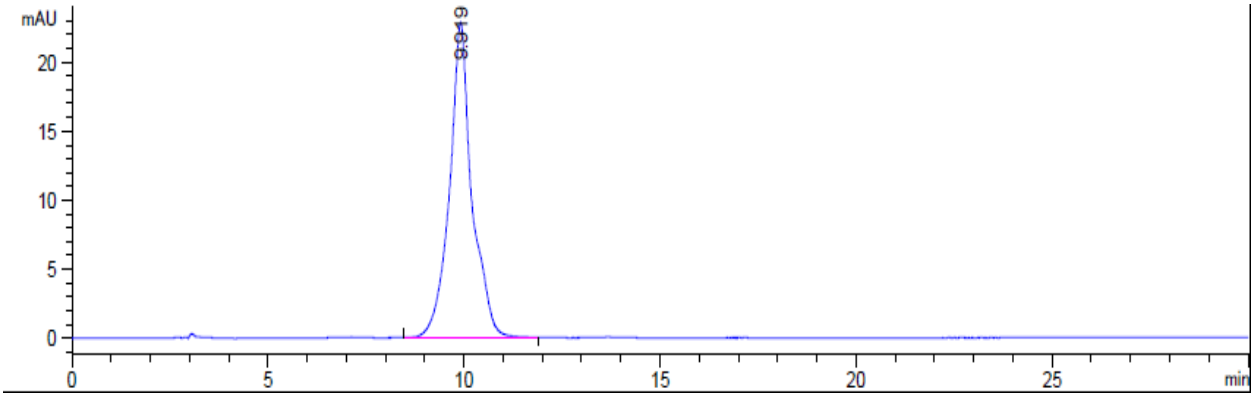

Figure 5.3: Chromatogram of oleuropein standard solution (0.5 mg/mL).

The standard calibration curve obtained under the chromatographic conditions of this study exhibited good linearity with a coefficient of determination (R^2) of 0.99. The regression equation obtained was Y = 1799.2X + 0.6608 (where X = concentration of oleuropein and Y = area (mAU * s)). The identification of oleuropein in the olive leaf extract used in this study was performed based on comparison of the chromatographic retention time and UV absorbance spectra obtained

from the chromatogram of standard oleuropein solutions (Figure 5.4). The retention time of oleuropein in this study was 9.85 ± 0.07 minutes.

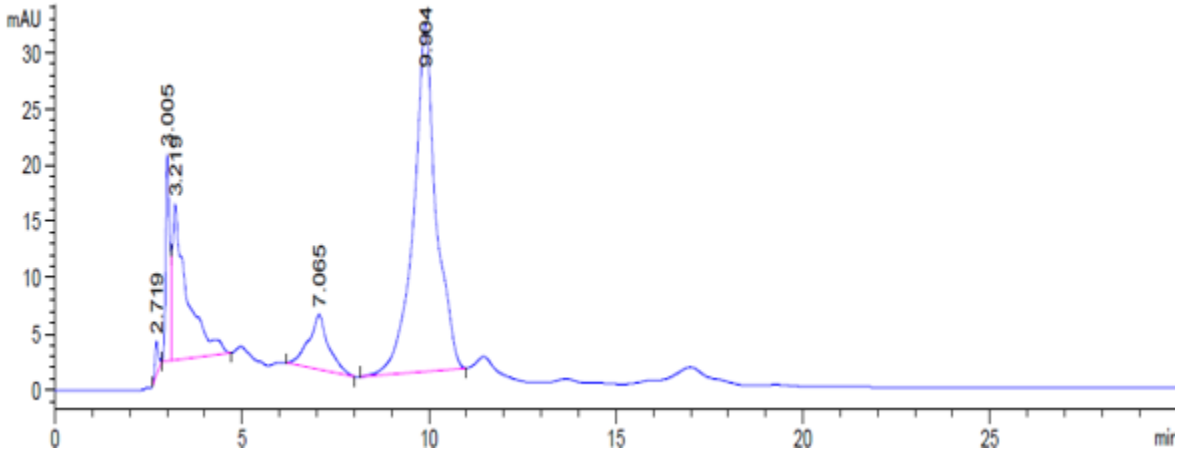

Figure 5.4: Chromatogram of Olive leaf extract used in this study (10 mg OLE/mL methanol).

In Figure 5.3, the area of the peak at retention time of 9.904 minutes was found to be 1289.47 mAU * s. Therefore, the oleuropein content in the olive leaf extract used in this study was 0.07 mg/mg. This concentration was found to be close to the oleuropein content reported by Jemai, Bouaziz, Fki, El Feki, and Sayadi (2008) in which it was 0.04 mg/mg of dry Chemlali olive leaves. Furthermore, a clear peak having a small area was obtained in oleuropein-enriched sucrose-free dark chocolate samples compared to the control in which there was no such peak at the chromatographic retention time = 9.8 minutes, in the UV absorbance spectra obtained from the chromatogram (Figure 5.5).

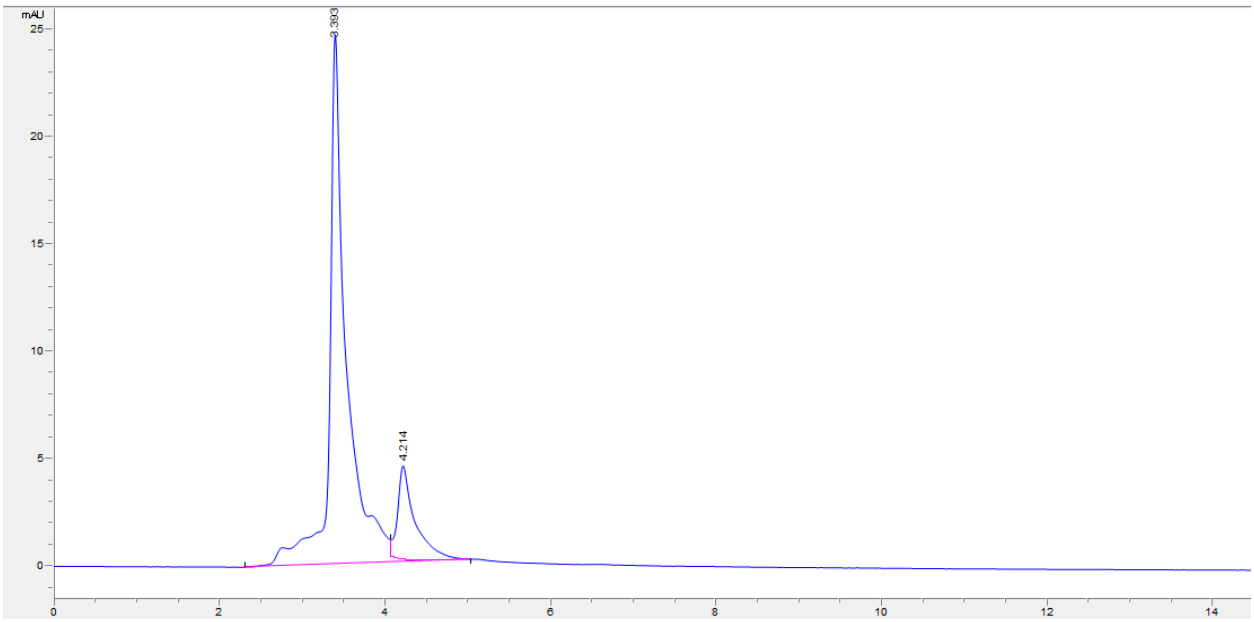

Figure 5.5a: Chromatogram of xylitol-made dark chocolate (control).

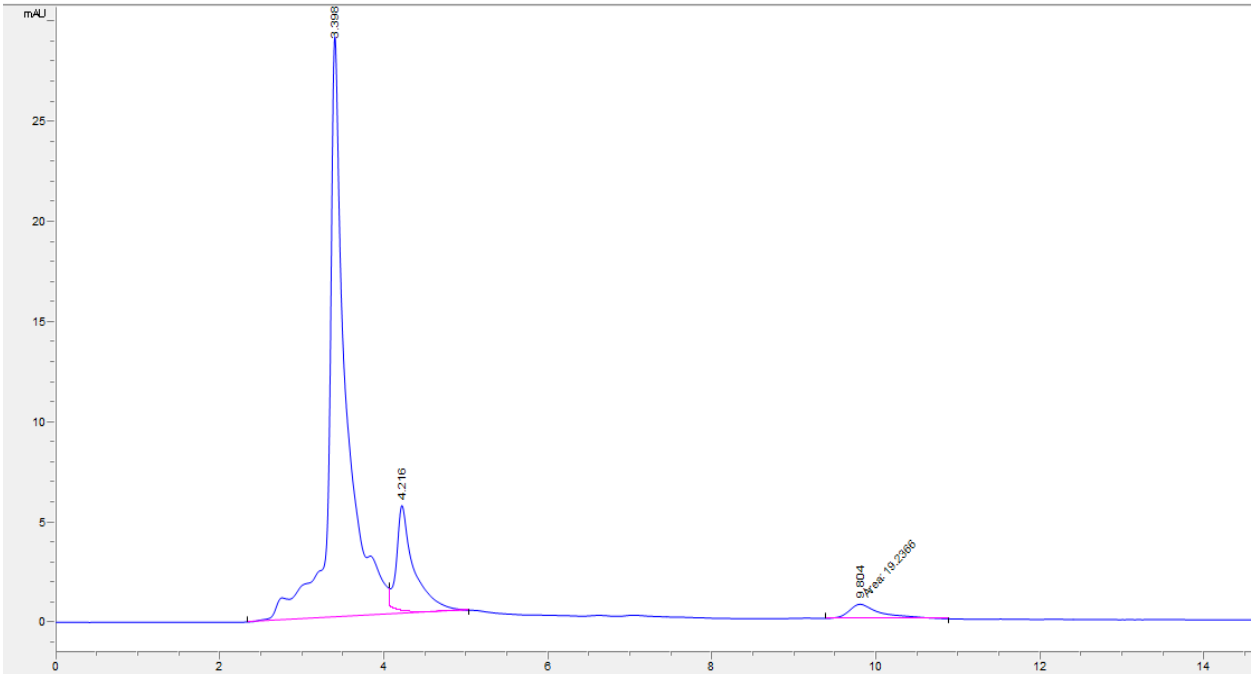

Figure 5.5b: Chromatogram of oleuropein-enriched xylitol-made dark chocolate (20 g OLE/kg chocolate).

The oleuropein content in oleuropein-enriched sucrose-free dark chocolate samples can be found in Table 5.10. Overall, there was a significant ($P < 0.05$) increase in the oleuropein content with the addition of OLE from 15 g/kg to 20 g/kg in the sucrose-free dark chocolate samples, with the

exception in optimized-chocolate samples. The oleuropein content in this study (0.24 – 0.52 mg/100 g of chocolate sample) was found to be less than in Del Ben et al. (2020) study where oleuropein-enriched chocolate made with sucrose (4 mg oleuropein in 40 g chocolate) showed a modest increase or no change of glycemia in patients with type 2 diabetes mellitus and healthy subjects.

Table 5.10: Oleuropein content of oleuropein-enriched sucrose-free dark chocolates.

Chocolate samples	Peak area (mAU * s) at retention time = 9.8 min	Oleuropein content (mg/g chocolate)
Isomalt-made chocolate		
$I_{control}$	nd	nd
I_{10}	9.72 ± 0.29	0.0025[a]
I_{15}	13.30 ± 0.76	0.0032[a]
I_{20}	17.27 ± 1.36	0.0046
Erythritol-made chocolate		
$E_{control}$	nd	nd
E_{10}	5.75 ± 0.79	n.d.
E_{15}	9.17 ± 0.52	0.0024
E_{20}	13.60 ± 0.90	0.0036
Xylitol-made chocolate		
$X_{control}$	nd	nd
X_{10}	7.21 ± 1.04	n.d.
X_{15}	12.97 ± 0.96	0.0034
X_{20}	19.24 ± 2.48	0.0052
Optimized chocolate		
$O_{control}$	nd	nd
O_{10}	6.38 ± 0.37	nd
O_{15}	9.72 ± 0.31	0.0025[a]
O_{20}	14.12 ± 1.61	0.0034[a]

[a]Means within the same column of same type of polyol-made chocolate sharing the same superscript are not significantly different from each other (Tukey's test, $P < 0.05$). nd: not determined due to absence of oleuropein or falling out of the range of oleuropein standard curve.

5.3.7 Bioactive parameters

The standard calibration curve for the phenolic content obtained from 0.03125, 0.0625, 0.125, 0.25 and 0.5 mg gallic acid/mL dH_2O exhibited a good linearity with the coefficient of determination (R^2) of 0.99. The regression equation obtained for the phenolic content was $y = 4.6827x + 0.0393$, where x = concentration (mg/mL) and y = absorbance. Similarly, the standard curve for the flavanol content also showed a good linearity with $R^2 = 0.99$. The regression equation obtained for the flavanol content was $y = 4.1509x + 0.0002$.

The phenolic content of cocoa mass and olive leaf extract used in this study was found to be 43.39 ± 7.23 and 286.94 ± 14.64 mg of gallic acid equivalent (GAE)/100 g of sample, respectively. Whereas the flavanol content of cocoa mass and olive leaf extract was 25.60 ± 4.31 and 291.02 ± 9.20 mg of catechin hydrate equivalent (CHE)/100 of sample, respectively. The phenolic content and the flavanol content of sucrose-free dark chocolate samples are presented in Table 5.11. In this study, sucrose-free dark chocolates with 50% cocoa mass were found to have a phenolic content between 11.97 ± 2.12 and 40.50 ± 1.15 mg GAE/100 g of chocolate sample, and a flavanol content between 7.90 ± 2.15 and 22.26 ± 3.21 mg CHE/100 g of chocolate sample, respectively.

From our study, we found that the phenolic content of control samples ranged between 11.97 ± 2.12 and 19.21 ± 3.00 mg GAE/100 g of chocolate sample. Similar, the flavanol contents of the control samples were found to be from 7.90 ± 2.15 to 11.58 ± 1.14 mg CHE/100 g of chocolate sample. Meng, Jalil, and Ismail (2009) reported the flavanol content of bitter dark chocolate to be 28.30 mg/100 g sample, however cocoa mass content was not specified in this study. In another study performed by Mikołajczak and Tańska (2020), they reported the free flavonoids content in the range of 29.01 – 89.55 mg/100 g sample in the bitter chocolate bars containing cocoa mass from 40 to 90%. Moreover, they further reported the free phenolic content to be between 242.38

and 703.13 mg/100 g of chocolate sample. Leite, Maciel, Opretzka, Soares, and Bispo (2013) studied the phenolic content in dark chocolate bars containing 70% cocoa mass and found to be from 91.1 to 154.6 mg/100 g of sample. Furthermore, the phenolic content of 46 plain dark chocolate bars containing 34 to 100% cocoa mass was found to be between 340 and 2340 mg/100 g sample (Cooper et al., 2008). These different results in the phenolic and flavanol contents of chocolate, which were higher than the result obtained from this study, suggest that the concentration of phenolic compounds depends on cultivar employed, climatic characteristics and processing techniques (such as alkalinization of cocoa powder and fermentation of cocoa beans) (Wollgast & Anklam, 2000).

Table 5.11: Bioactive parameters of sucrose-free dark chocolate, olive leaf extract and cocoa mass.

Samples	Phenolic content (mg GAE/100 g chocolate)	Flavanol content (mg CHE/100 g chocolate)
Isomalt-made chocolate		
$I_{control}$	19.21 ± 3.00	11.58 ± 1.14^{b}
I_{10}	33.01 ± 6.70^{a}	18.14 ± 3.22^{a}
I_{15}	31.54 ± 1.23^{a}	16.98 ± 1.42^{ab}
I_{20}	40.50 ± 1.15^{a}	22.26 ± 3.21^{a}
Erythritol-made chocolate		
$E_{control}$	14.54 ± 4.30^{a}	7.90 ± 2.15^{b}
E_{10}	18.09 ± 1.75^{a}	12.33 ± 3.00^{ab}
E_{15}	19.06 ± 2.10^{a}	14.45 ± 1.57^{a}
E_{20}	20.90 ± 2.13^{a}	17.22 ± 1.26^{a}
Xylitol-made chocolate		
$X_{control}$	11.97 ± 2.12^{b}	8.93 ± 1.72
X_{10}	21.90 ± 2.44^{ab}	13.90 ± 0.21^{a}
X_{15}	23.53 ± 7.25^{a}	14.88 ± 0.16^{a}
X_{20}	23.38 ± 2.34^{a}	17.12 ± 2.97^{a}
Optimized chocolate		
$O_{control}$	18.20 ± 1.11^{b}	10.04 ± 1.40^{a}
O_{10}	24.17 ± 4.58^{ab}	12.84 ± 1.14^{a}
O_{15}	24.89 ± 2.10^{ab}	16.81 ± 2.12
O_{20}	28.65 ± 2.86^{a}	22.26 ± 0.31

^{a,b}Means within the same column of same type of polyol-made chocolate sharing the same superscript are not significantly different from each other (Tukey's test, $P < 0.05$).

With the addition of olive leaf extract, there was a significant increase ($P < 0.05$) in the phenolic and flavanol contents of sucrose-free dark chocolate samples compared to control. The notable exception to this significant difference in the phenolic content was seen in erythritol-made chocolate samples. Furthermore, the increase in the concentration of olive leaf extract from 10, 15 to 20 g/kg chocolate did not significantly increase ($P > 0.05$) the phenolic and flavanol contents in oleuropein-enriched chocolate samples. One exception to this case can be found in polyols-optimized chocolate samples, where the flavanol content of O_{10} sample (12.84 ± 1.14 mg/100 g sample) was found to be significantly different ($P < 0.05$) from the O_{20} sample (22.26 ± 0.31 mg/100 g sample).

5.4 Conclusion

Various bioactive compounds have functional effects on chocolate. Oleuropein, a bioactive compound from *Olea europaea* L, is a significant food component with health benefits. It is therefore important to examine the effects of oleuropein as an ingredient on the quality properties of chocolate before using for the purpose of functional product development. With the addition of olive leaf extract (10, 15 and 20 g/kg chocolate), no significant changes ($P > 0.05$) were observed in the moisture content, color (L*), hardness and melting behavior of chocolate samples, compared to the control. Furthermore, the potential health benefits of sucrose-free dark chocolate were further improved with the incorporation of oleuropein.

Chapter 6

Overall Summary, Conclusions, Contribution to Knowledge and Future Work Recommendations

6.1 Overall Summary, Conclusions and Contribution to Knowledge

Being one of the most consumed food products in the world, the progressive growth of the global chocolate market including sucrose-free chocolate was discussed in Chapter 1. With the rising concerns regarding the consumption of sucrose, manufacturers are searching for alternative bulk sweeteners (such as polyols) for chocolate processing. Thus, there has been a need to understand the changes in texture or taste with sucrose-free chocolate (made with polyols) compared to traditional chocolate. Furthermore, manufacturers are looking for sucrose-free chocolate with an improved health profile than traditional chocolate. These concerns led to two main objectives in this thesis. One of those objectives was to manufacture chocolate with polyols and understand their effect on their physical and sensory properties. Another one was to improve the health profile of sucrose-free chocolate with oleuropein and investigate its effect as an ingredient on the quality properties (such as flow and melting behaviour) of sucrose-free chocolate. To fulfil these two objectives, isomalt, xylitol and erythritol were used for polyols, olive leaf extract for oleuropein, and dark chocolate for the chocolate type.

The literature review in Chapter 2 showed the suitability and the applicability of polyols (such as isomalt, xylitol and erythritol) in the processing of sucrose-free dark chocolate. This chapter further discussed the considerable advantages of polyols-made chocolate over conventional sucrose-made chocolate in terms of reduced-calorie, reduced glycemic index and tooth-friendly confectionery products. These reasons thus led to a study in which the relationship between chocolate properties (plastic viscosity, yield stress, flow behaviour index, moisture content, colour and sensory) and polyols (isomalt, erythritol and xylitol) were established in Chapter 4. Moreover,

from this study, 5.16% standard isomalt, 20.99% erythritol and 3.85% xylitol were found to be the optimum concentration of polyols for manufacturing 70% dark chocolate (containing 50% cocoa mass and 19.65% cocoa butter).

The importance of oleuropein as a nutraceutical ingredient was critically reviewed in Chapter 3. Keeping those health benefits (such as anti-inflammatory, antioxidant, anti-angiogenic, anti-cancer, cardioprotective, antihypertensive, and neuroprotective) of oleuropein in view, sucrose-free dark chocolates (made with isomalt, erythritol and xylitol) were enriched with this bioactive compound as discussed in Chapter 5. Furthermore, the effects of the addition of oleuropein on the rheological, textural, melting, visual, and physicochemical properties of sucrose-free dark chocolate were investigated. This investigation led to the conclusion that the addition of oleuropein does not significantly change ($P > 0.05$) the moisture content, color (L*), hardness and melting behaviour of sucrose-free dark chocolate compared to control, with exceptions in some chocolate samples. The source of oleuropein in this study was olive leaf extract, which had 0.07 mg/mg of the bioactive compound.

Overall, this thesis contributes to knowledge on sucrose-free dark chocolate by investigating the effect of different polyols as well as oleuropein on the physical and sensory properties of chocolate. The acquired knowledge would help in the development of a new functional product in the field of sucrose-free chocolate.

6.2 Future work recommendations

Following are the recommendations for future research based on the current study and understanding:

a. Explore the use of isomalt LM-PF instead of standard isomalt, for its lower moisture content. This will lead to chocolate production without any restriction on the conching process and may help to further improve the quality of sucrose-free chocolate.
b. Conduct a study on the effect of the particle size distribution of polyols or oleuropein on the physical and sensory properties of sucrose-free dark chocolate. This may help to better understand the effect of the addition of polyols and/or oleuropein on viscosity and yield stress of chocolate.
c. Explore the use of a pure source of oleuropein to enhance the health profile of chocolate, especially white and milk chocolate. In addition, conduct a study to understand the effect of the addition of pure oleuropein on rheological and sensory properties of chocolate.
d. Conduct a study to understand the effect of the dry conching process on moisture content and flavour development of sucrose-free chocolate. The optimum dry conching temperature and time would help to manufacture desired chocolate flow properties as with the rise of temperature, cohesive forces between particles start breaking leading to effective moisture removal. This moisture escape may take many of the unwanted flavour compounds with it. Furthermore, the optimum parameters of dry conching may help to minimize the risk of the formation of agglomerates.

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