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# Application of Differential Scanning Calorimetry and Thermogravimetry for Thermal Analysis of Dark Chocolates

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**Abstract:** Dark chocolate is a confectionery product traditionally made from cocoa beans, sugar, and vanilla essence. The aim of the study was to investigate the thermal properties of dark chocolates and fats extracted from these chocolates using thermal methods of food analysis, such as differential scanning calorimetry (DSC) and thermogravimetry (TG). The profile of fatty acids in the fat extracted from the chocolates was also determined. The presence of three fatty acids (palmitic P, stearic S, and oleic O) constituting triacylglycerols—SOS, POP, POS, POO, and SOO—was observed in all the samples. The presence of linoleic acid (L) was also found, which forms triacylglycerols such as PLP and PLS. The researched chocolates were characterized by a diverse composition of fatty acids. In all the obtained DSC melting curves of fats, the presence of endothermic peaks was observed. The peaks, appearing at negative temperatures, may be caused by the transition of low-melting triacylglycerols. The differences between the melting curves for the obtained dark chocolate fats may have resulted from the presence of less stable polymorphic forms of cocoa butter. Based on the shape of the TG and DTG curves, it could be possible to indicate the adulteration of chocolates.

**Keywords:** dark chocolate; differential scanning calorimetry; thermogravimetry



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## 1. Introduction

Consumers' positive opinion about a food product is a very important factor determining product acceptance and stimulating consumers to choose the product again in the future. The unique sensory properties and health benefits of dark chocolate contribute to the increased consumption of one of the most popular delicacies in the world [1–4].

Chocolate is a confectionery product, which has been studied for many years. What is characteristic for chocolate is that it is solid at ambient temperature (around 25 °C), and it melts at oral temperature (37 °C) forming a smooth suspension of solid particles in cocoa butter [5]. Chocolate belongs to premium snack food with good digestibility and quick metabolism. Sensory attributes comprise a snap (brittleness) when the chocolate is broken, a shiny gloss on the surface, and a smoothness that becomes obvious only when the chocolate liquefies in the oral saliva [6].

Dark chocolate is a product traditionally made from cocoa beans, sugar, and vanilla essence. However, in industrial-scale production, instead of cocoa beans, their processing products like cocoa mass, butter, and powder are used. As sweetener, usually sucrose from sugar beet or cane is used. For a better texture of chocolate, manufacturers apply emulsifiers

such as lecithin and/or polyglycerol polyricinoleate (PGPR) [7,8]. Dark chocolate quality depends on the percentage of cocoa. Only dark chocolate with a high content of cocoa, flavonoids, and theobromine and a low content of sugar would have health-promoting effects [9–12], including the prevention of cardiovascular disease. Similarly, cocoa induces positive effects on blood pressure, insulin resistance, and vascular function [13]. There is a continuous need for the chocolate industry to develop new methods for improvements in technology and formulas to fulfill the needs and satisfaction of customers [14]. However, the process of chocolate production is complex and associated with specific interactions of the ingredients, such as cocoa, cocoa butter, and sugar in the formation of an emulsion and their production stages such as mixing, refining, conching, tempering, molding, and packaging [15,16]. With the tendency to chocolate production "bean to bar" and "tree to bar", the consumer market's trend is to value cocoa due to its well-known sensory and bioactive properties. A growing group of consumers are interested in chocolates made with fewer added ingredients. These trends are meant to highlight the variety of styles and flavors of chocolate produced in small, artisan workshops [17,18]. Dishonest producers can reap large financial benefits by producing chocolate with fewer cocoa solids than declared in the product composition. The sensory, technological, and phytochemical properties of raw cocoa are conducive to chocolate adulteration [18,19]. Currently, a very important issue for the food industry is the confirmation of the authenticity of the composition of chocolates. Identifying discrepancies between the information on product labels and their actual composition intensifies the search for new research techniques and fuller use of analyses already introduced to the industry [20]. There are not many analytical techniques used to determine the cocoa solids content in chocolate [21].

The chocolate consumption rate continues to grow around the world year after year. According to the consumption statistics, Switzerland was the leading country in chocolate consumption. Austria was ranked second after Switzerland in per capita consumption of chocolate. Germany, Ireland, Great Britain, Sweden, Estonia, Norway, and Poland are popular examples of chocolate consumption in the world [22]. Chocolate consumption in Poland in 2022 amounted to 5.9 kg per person [23]. The health benefits of chocolate consumption and its delicacy are of great importance to consumers. Cocoa beans (*Theobroma cacao*) contain 50–57% of fat, predominantly known as cocoa butter [24]. One of the principal components of dark chocolate is cocoa butter. The major constituents of cocoa butter are oleic acid (33%), palmitic acid (25%), and stearic acid (33%) [25,26]. The manufacturing of chocolate is a multistep process. A large quantity of cocoa's valuable nutrients are lost during the long chocolate production process [27].

CB is the main component of the crystallized fat matrix in which the solid particles constituting the composition of chocolate are dispersed [28]. The availability of CB in world markets is insufficient in relation to the demand for this raw material [29]. Attempts are being made to replace CB with other fats, e.g., oils, vegetable fats (palm oil), or CB equivalents or substitutes [28,30,31]. The extraordinary chemical profile of CB endows it with its distinctive texture, snap, melting, gloss, and sensory properties. The majority of CB triacylglycerols (TAGs) is covered by symmetrical monounsaturated TAGs, such as POP, POSt, and StOSt (where *P* = palmitic acid, St = stearic acid, and *O* = oleic acid). In these TAGs, the symmetrical arrangement of fatty acids (FAs), with unsaturated FA in position 2 and saturated FAs in the sn-1,3 positions, provides the final desirable characteristics [32,33]. The unique physical characteristics make CB a highly desirable fat. The change in ecological and environmental conditions has a huge impact on the lower supply and high price of cocoa [34]. There is a constant search for cocoa butter substitutes [35].

Adding peanuts, eggs, or cheaper CB substitutes is used by dishonest manufacturers to adulterate chocolate [36]. The current European legislation (2000/36/CE), which regulates the production and placing on the market of chocolate, allows the addition of cocoa butter equivalents (CBEs) up to 5% of the total weight. CB is a very expensive fat, and various attempts are being made to replace it with other substances. Fats used as substitutes or equivalents of CB are characterized by properties similar to it, and detecting the addition of

these substances in chocolate is extremely difficult. Currently, the European directive does not propose a recognized and approved method for detecting and quantifying prohibited amounts of CB equivalents in chocolate. Creating or improving methods for detecting CB adulterations is extremely important. The use of chromatographic methods that allow for the analysis of fatty acids found in triacylglycerols is highly insufficient [37–39].

To determine the thermal stability of materials, the composition of complex mixtures, and the kinetics of their decomposition, it is useful to apply one of the thermal analysis methods of thermogravimetry (TGA) and derivative thermogravimetry (DTG). Using these methods, it is possible to control the quality of fuels, ceramics, polymers, absorbents, pharmaceutical products, and food.

Industrial processing methods such as drying or roasting can cause changes in the physical and chemical properties of food. Using TGA, it is possible to detect these changes [40,41].

The aim of the study was to investigate the thermal properties of dark chocolates and fats extracted from these chocolates using thermal methods of food analysis, such as differential scanning calorimetry and thermogravimetry. The composition of fatty acids in the fat extracted from the chocolates was also determined.

The problem in chocolate production is not only the adulteration of the content or quality of cocoa butter, but also the amount of cocoa liquor in dark chocolates. The use of the TGA technique allows for the quick and accurate detection of cocoa liquor in chocolate. Studies have already been conducted to accurately determine the content of cocoa liquor in chocolate using the TG apparatus. The research carried out in this article constitutes the first stage in analysing the amount of cocoa liquor in dark chocolates.

## 2. Materials and Methods

### 2.1. Material

The material for the research consisted of nine types of dark chocolate, purchased in local markets in Warsaw (Poland), and fat extracted from them. All information about the chocolates' composition was given according to the manufacturer's declaration (Table 1).

**Table 1.** Declarations of chocolate manufacturers present on the packaging regarding ingredients.

| Number of Chocolate | Cocoa Liquor Content (%) | Mass of a Bar (g) | Emulsifiers | Other Fats/Additives | Fat Content (g/100 g) | Sugar Content (g/100 g) |
|---|---|---|---|---|---|---|
| 1 | 45 | 90 | Soy lecithin, polyglycerol polyricinoleate | Palm, Shea | 30 | 49 |
| 2 | 65 | 80 | Soy lecithin | Whole milk powder | 39.5 | 32 |
| 3 | 60 | 100 | Soy lecithin | - | 32 | 37 |
| 4 | 45 | 100 | Soy lecithin | Palm, Shea | 28 | 49 |
| 5 | 40 | 90 | Soy lecithin | Palm, Shea | 27 | 54 |
| 6 | 60 | 90 | Soy lecithin | - | 32 | 30 |
| 7 | 70 | 100 | Soy lecithin | - | 39 | 30 |
| 8 | 52 | 100 | Soy lecithin | - | 32 | 49 |
| 9 | 74 | 100 | Soy lecithin | - | 40.5 | 27.1 |

### 2.2. Extraction of the Lipid Fraction

The samples of crushed chocolate were homogenized with chloroform/methanol (2/1). Next, the homogenates were washed with 0.9% NaCl solution. After vortexing for some seconds, the mixtures were centrifuged at low speed 24,270× $g$ RCF (relative centrifugal force) to separate the two phases. The time of the centrifugation was 15 min,

and the temperature was 4 °C. After centrifugation and syphoning of the upper phase, the lower chloroform phase containing lipids was evaporated under vacuum in a rotary evaporator [5,42].

Extraction of the lipid fraction from the chocolate was performed in triplicate.

### 2.3. Fatty Acid Composition/GC Analysis

The fatty acid composition/GC analysis was carried out according to the ISO method [43] and the procedure provided by Wirkowska et al. [44] and Bryś et al. [45].

The determination of fatty acid composition was carried out by gas chromatographic (GC) analysis of fatty acid methyl esters prepared according to the ISO method [43]. A YL6100 GC chromatograph equipped with a flame ionization detector, and a BPX-70 capillary column was used. The oven temperature was programmed as follows: 60 °C for 5 min, then increased by 10 °C $min^{-1}$ to 180 °C; from 180 to 230 °C, it was increased by 3 °C $min^{-1}$ and then kept at 230 °C for 15 min. Nitrogen flowing with the rate of 1 mL $min^{-1}$ was used as the carrier gas. The results were expressed as relative percentages of each fatty acid.

The samples were performed in triplicate.

### 2.4. Distribution of Fatty Acids in the sn-2 and sn-1,3 Positions of Triacylglycerols

The TAG's positions of fats samples were analysed according to Brzezińska et al. [46].

To determine the positional distribution of fatty acids in the sn-2 and sn-1,3 positions of TAG, 20 mg of purified pancreatic lipase, 1 mL of Tris buffer (pH 8.0), 0.25 mL of bile salts (0.05%), and 0.1 mL of calcium chloride (2.2%) were added to 50 mL centrifuge tubes and vortexed with 0.1 g of the fat sample. The mixture was incubated at 40 °C for 5 min, and then 1 mL of 6 mol $L^{-1}$ HCl and 1 mL of diethyl ether were added, and the mixture was centrifuged. The diethyl ether layer was collected and evaporated under nitrogen gas to obtain 200 µL volume and then placed on a silica gel TLC plate and developed with hexane/diethyl ether (50:50, $v/v$) acidified with 1 mL of acetic acid. The 2-MAG band was scraped off, extracted twice with 1 mL of diethyl ether, and centrifuged. The ether layers were collected and entirely evaporated under nitrogen, and then the sample was methylated according to the ISO method [43].

The samples were performed in triplicate.

### 2.5. DSC Measurements of Melting Characteristics of Fats Extracted from Chocolates

The DSC measurements were conducted according to Wirkowska et al. [44] and Tapia-Ledesma [47] with the use of DSC Q200 (TA Instruments, New Castle, DE, USA). Hermetically sealed aluminium pans were used. The nitrogen flow rate was 50 mL/min. The sample quantities were 3–4 mg. After removing the fats thermal memory, the melting characteristics of the sample were determined in the temperature range from −80 to 80 °C. The sample was heated at a rate of 10 °C/min.

The samples were performed in quadruplicate.

### 2.6. Thermogravimetry Analysis for Chocolates and Fats Extracted from Chocolates

The thermogravimetric study was performed according to Materazzi et al. [40] and Dolatowska-Żebrowska et al. [48]. The equipment used was Discovery TGA (TA Instruments, New Castle, DE, USA). The type of crucibles were platinum containers. The nitrogen flow rate was −25 mL/min. The sample quantities were 7–8 mg. The thermal analysis TG of the samples was determined in the temperature range of 50 to 700 °C. The samples of the chocolates and fats extracted from the chocolates were heated at a rate of 10 °C/min.

The samples were performed in quadruplicate.

### 2.7. Chemicals

The chemicals used in the extraction of the lipid fraction from the chocolates were the following:

Chloroform—Merck Life Science Ltd., Poznań, Poland; analytical standards.
Methanol—Merck Life Science Ltd., Poznań, Poland; analytical standards.
NaCl—Merck Life Science Ltd., Poznań, Poland; analytical standards.

The chemicals used in the distribution of the fatty acids in the sn-2 and sn-1,3 Positions of the triacylglycerols were the following:

Purified Pancreatic Lipase—Merck Life Science Ltd., Poznań, Poland; analytical standards.
Tris Buffer—Merck Life Science Ltd., Poznań, Poland; analytical standards.
Bile Salts—Merck Life Science Ltd., Poznań, Poland; analytical standards.
Calcium Chloride—Merck Life Science Ltd., Poznań, Poland; analytical standards.
HCl—Merck Life Science Ltd., Poznań, Poland; analytical standards.
Diethyl Ether—Merck Life Science Ltd., Poznań, Poland; analytical standards.
Hexane—Merck Life Science Ltd., Poznań, Poland; analytical standards.
Acetic Acid—Merck Life Science Ltd., Poznań, Poland; analytical standards.

### 2.8. Statistical Analysis

The entire statistical analysis was performed according to Dolatowska-Żebrowska et al. [48]. The principal component analysis (PCA) plots, VIP values, and dendogram were obtained using the STATISTICA 13 PL (StatSoft, Krakow, Poland) and Excel 2010 (Microsoft) computer programs.

## 3. Results

### 3.1. Analysis of Fatty Acid Composition Extracted from Dark Chocolates

Fats are characterized by a specific composition of fatty acids in triacylglycerol molecules. The basic factors that determine the composition of fatty acids are the type of fat and its origin. The composition of fatty acids in the dark chocolate mass was determined using gas chromatography.

The fats found in dark chocolate are characterized by a high content of SFA—Saturated Fatty Acids, a lower content of MUFA—Monousaturated Fatty Acids, and a much lower content of PUFA—Polyunsaturated Fatty Acids. Depending on the type of chocolate, the SFA content in 100 g of the product is considered to be 61%, and in the case of dark chocolate it may reach up to 67%; PUFA reaches up to 4% and MUFA from 30% to 35% [49,50]. In the analyzed fats extracted from the dark chocolates, the content of saturated fatty acids ranged from 61.36% to 63.54%, the content of MUFA fatty acids from 32.71% to 34.38%, and the PUFA content from 3.75% to 4.81%. The obtained results were comparable to those obtained in the study conducted by Kunachowicz et al. [49]. The PUFA content was higher in all the tested dark chocolates.

The highest content of saturated fatty acids was found in dark chocolate 7, which also had the lowest content of MUFA acids (Figure 1). From a health point of view, due to the high content of saturated fatty acids, it is chocolate that we should not consume in large quantities. Dark chocolate 2 was characterized by the highest content of unsaturated fatty acids, in which the sum of MUFA and PUFA acids was approximately 38.63%. Unsaturated fatty acids are beneficial to the human body because they take part in the synthesis of eicosanoids, build blocks of cells, and are responsible for the proper transport of lipids in the blood [51].

Statistical analysis showed that the researched fats extracted from the dark chocolate did not significantly statistically differ in terms of the content of SFA and MUFA acids. However, there were significant differences in the PUFA content (Figure 1). Chocolates 1, 4, 7, and 9 contained the lowest quantities of polyunsaturated fatty acids and did not significantly statistically differ from each other in terms of their content. The next group showing statistical similarities in PUFA content included chocolates 2, 8, and 9, of which

chocolate 9 did not significantly statistically differ from the first group, and chocolates 2 and 9 were similar to chocolate 3. The lowest PUFA content was found in chocolates 5 and 6, and slightly more of these acids were present in chocolate 3 (Figure 1).

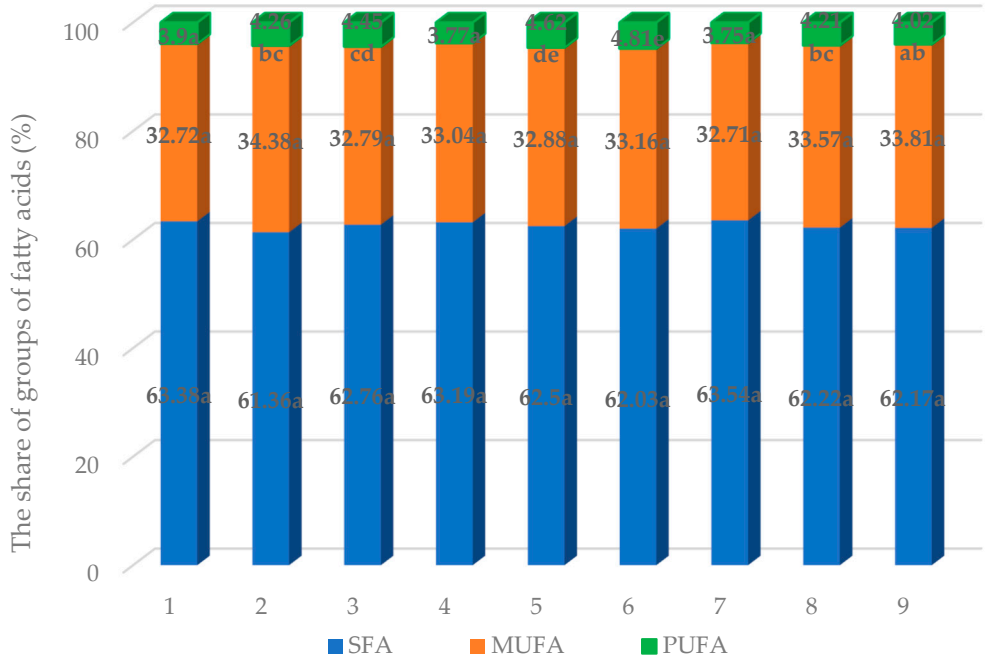


**Figure 1.** Comparison of PUFA, MUFA, and SFA content in fats extracted from dark chocolate. The letters a, b, c, d, e represent homogeneous groups.

The obtained results from gas chromatography analysis are presented in Figures 2–4. A characteristic feature of cocoa butter is the high content of saturated fatty acids, mainly stearic (C18:0) and palmitic (C16:0), and monounsaturated oleic acid (C18:1). In research conducted by Jia et al. [52], it was found that the content of these fatty acids should be in the range of 30.6–39.2%, 21.7–27.0% and 29.4–35.4%, respectively, and linoleic acid should be present in an amount of about 3%. Research conducted by Kowalska et al. [53] indicates that the percentage of these fatty acids in dark chocolates was 34.4%, 26.2%, and 37.3%, respectively, and 2.1% for linoleic acid.

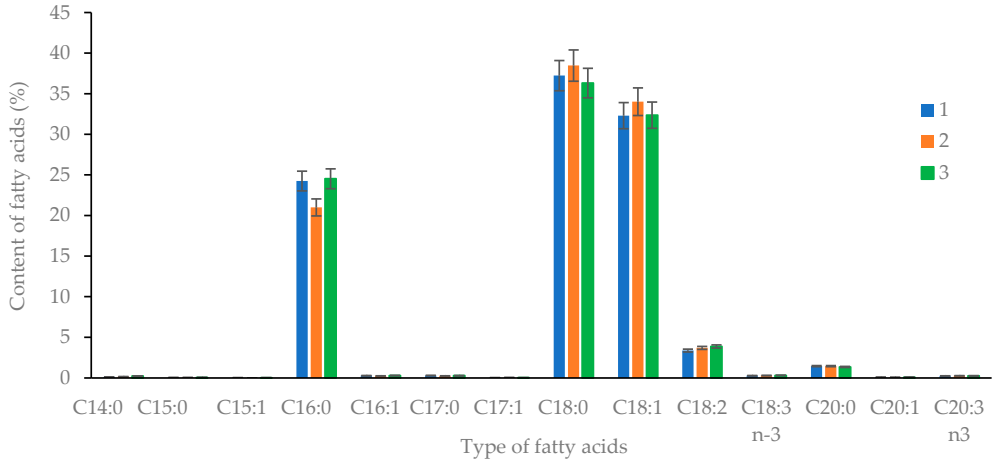


**Figure 2.** Fatty acid composition of fat extracted from chocolate 1, 2, 3. Values represent means ± standard deviations.

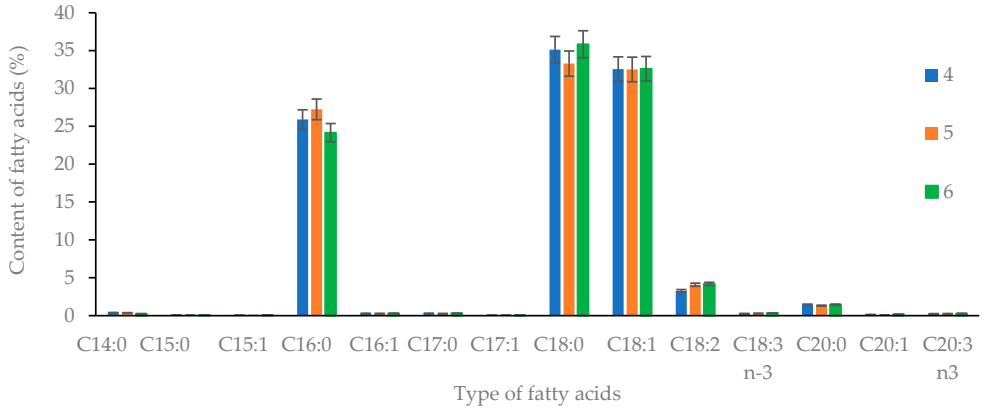


**Figure 3.** Fatty acid composition of fat extracted from chocolate 4, 5, 6. Values represent means ± standard deviations.

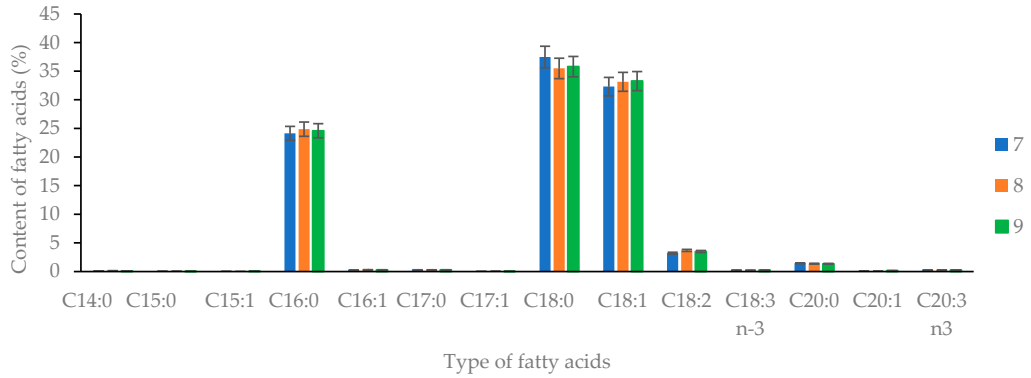


**Figure 4.** Fatty acid composition of fat extracted from chocolate 7, 8, 9. Values represent means ± standard deviations.

The conducted research showed that the fat extracted from chocolate 2 had the highest stearic acid content, which amounted to 38.49% (Figure 2). The manufacturer declares 65% of cocoa liquor on the label. In the fat extracted from chocolate 7 (Figure 4), compared to chocolate 2, the content of this acid (18:0) was 1% lower, despite the fact that chocolate 7 contained 5% more cocoa liquor. In the study conducted by Kowalska and Łata [54], chocolate containing 70% cocoa mass had a lower stearic acid content—35.41%.

Two organic dark chocolates, 8 and 9 (Figure 4), were similar in terms of stearic acid content: Its amount was 35.49% and 38.80%, respectively. Chocolates 3 and 6 were also characterized by a similar level of stearic acid content—36.31% and 35.86%, respectively.

Chocolates that contained the lowest declared amounts of cocoa mass: 1 (min. 45%), 4 (min. 45%), and 5 (min. 40%), were characterized by the following stearic acid content: 37.24%, 33.29%, and 35.11%, respectively (Figures 2 and 3).

In terms of content, the second fatty acid found in the analyzed dark chocolates was palmitic acid (16:0). In the research conducted by Kowalska et al. [53], the content of palmitic acid should be approximately 26.20%. In chocolates with a declared amount of cocoa liquor above 70%, i.e., 7 and 9, lower palmitic acid content was recorded compared to natural cocoa fats, and they amounted to—24.15% and 24.60%. Despite the high content of cocoa liquor, 65% in chocolate 2, the content of palmitic acid was the lowest—21%; the highest amount of this acid (16:0) was present in chocolate 5, which contained minimum 40% cocoa mass. The share of dominant and typical fatty acids was similar to the study conducted by Kruszewski and Obiedziński [55]. The analyzed dark chocolates were characterized by a high content of monounsaturated fatty acids. Among all the researched chocolates, dark chocolate fat 2 had the highest oleic acid (18:1) content (34.03%). This was a lower value than that obtained in the study by Kowalska et al. [53]. The fat of Chocolate 1 was

characterized by the lowest content of this acid—32.30%. The presence of polyunsaturated fatty acids represented by linoleic acid, found in all the researched chocolates, was also noted. The greatest amount of this acid (18:2) was present in the fact of chocolate 6—4.19%. Its presence in dark chocolate could be due to the presence of soy and soy lecithin [56]. These ingredients have been declared in the composition of all chocolates (Table 1).

Moreover, the fats from the tested chocolates contained myristic acid (C14:0) at a level below 0.4%, the highest in the case of chocolate 1 (0.39%), and its presence is characteristic for milk fat [54].

### *3.2. Analysis of the Distribution of Fatty Acids Between the sn-2 and sn-1,3 Positions of Triacylglycerols*

The composition of fatty acids has a large impact on the properties of fat. However, the distribution of fatty acids at individual positions of triacylglycerols also has an influence on the properties of fats. According to Kruszewski and Obiedziński [57], vegetable fats are characterized by the presence of fatty acids in amounts ranging from 5 to 15, while in animal fats their number ranges from 15 to even several hundred different fatty acids. Each acid residue can occupy different positions, i.e., internal (sn-2) or external (sn-1,3). The huge diversity of arrangement in triacylglycerol molecules is caused by the large quantity of fatty acids occurring in nature and the possibilities of their combinations in the arrangement in triacylglycerols. The arrangement of acyl groups in the triacylglycerol molecule is of key importance in technological processes. Differences in fat structures may have an influence on the kind of products produced in the human body as a result of hydrolysis, and thus on their digestibility and bioavailability [58]. Pancreatic lipase, which is responsible for the hydrolysis of ester bonds of triacylglycerols, found in the body, detaches fatty acids that are only located in the external positions. At a later stage, these acids, already as monoacylglycerols, are absorbed into the bloodstream unchanged. Fatty acids that have been released from external items can react with food ingredients. The distribution of fatty acids in triacylglycerols is important because it has an influence on the digestibility of fat and the absorption of other food ingredients [59].

Table 2 presents the composition of fatty acids in the external and internal positions of triacylglycerols of the researched dark chocolates. The obtained results are similar to those reported in the study conducted by Kruszewski and Obiedziński [55].

**Table 2.** Composition of fatty acids in the internal and external positions of triacylglycerols (TAG) in chocolates and the share of individual acids in the internal position (sn-2).

| Chocolate Number | Type of Fatty Acid | Composition of Fatty Acids in TAG [%] | The Composition of a Fatty Acid in Position [%] | | The Share of a Fatty Acid in Position sn-2 [%] |
|---|---|---|---|---|---|
| | | | sn-2 | sn-1,3 | |
| 1. | C16:0 | 24.3 | 11.9 | 30.4 | 16.3 |
| | C18:0 | 37.2 | 17.2 | 47.2 | 15.4 |
| | C18:1c | 32.3 | 63.0 | 17.0 | 65.0 |
| | C18:2c | 3.4 | 5.6 | 2.3 | 55.4 |
| 2. | C16:0 | 21.0 | 15.5 | 23.8 | 24.6 |
| | C18:0 | 38.5 | 21.7 | 46.9 | 18.8 |
| | C18:1c | 34.0 | 55.3 | 23.4 | 54.2 |
| | C18:2c | 3.7 | 5.1 | 3.0 | 45.9 |
| 3. | C16:0 | 24.5 | 10.0 | 31.8 | 13.5 |
| | C18:0 | 36.3 | 13.4 | 47.8 | 12.3 |
| | C18:1c | 32.4 | 67.6 | 14.7 | 69.7 |
| | C18:2c | 3.9 | 6.7 | 2.5 | 57.5 |

**Table 2.** *Cont.*

| Chocolate Number | Type of Fatty Acid | Composition of Fatty Acids in TAG [%] | The Composition of a Fatty Acid in Position [%] | | The Share of a Fatty Acid in Position sn-2 [%] |
|---|---|---|---|---|---|
| | | | sn-2 | sn-1,3 | |
| 4. | C16:0 | 25.9 | 19.7 | 29.0 | 25.3 |
| | C18:0 | 35.1 | 24.1 | 40.6 | 22.9 |
| | C18:1c | 32.5 | 48.9 | 24.4 | 50.0 |
| | C18:2c | 3.3 | 4.3 | 2.8 | 43.8 |
| 5. | C16:0 | 27.3 | 19.6 | 31.1 | 24.0 |
| | C18:0 | 33.3 | 25.4 | 37.2 | 25.4 |
| | C18:1c | 32.5 | 47.4 | 25.1 | 48.6 |
| | C18:2c | 4.1 | 4.6 | 3.8 | 37.7 |
| 6. | C16:0 | 24.2 | 10.6 | 30.9 | 14.7 |
| | C18:0 | 35.9 | 13.3 | 47.1 | 12.4 |
| | C18:1c | 32.6 | 68.0 | 14.9 | 69.5 |
| | C18:2c | 4.2 | 6.0 | 3.3 | 47.9 |
| 7. | C16:0 | 24.1 | 9.8 | 31.3 | 13.6 |
| | C18:0 | 37.5 | 13.0 | 49.7 | 11.6 |
| | C18:1c | 32.3 | 69.5 | 13.7 | 71.6 |
| | C18:2c | 3.2 | 5.6 | 2.0 | 58.0 |
| 8. | C16:0 | 24.9 | 12.3 | 31.2 | 16.4 |
| | C18:0 | 35.5 | 16.9 | 44.8 | 15.8 |
| | C18:1c | 33.2 | 63.6 | 17.9 | 64.0 |
| | C18:2c | 3.7 | 5.5 | 2.8 | 49.7 |
| 9. | C16:0 | 24.6 | 13.9 | 30.0 | 18.8 |
| | C18:0 | 35.8 | 17.9 | 44.7 | 16.7 |
| | C18:1c | 33.3 | 61.0 | 19.4 | 61.1 |
| | C18:2c | 3.5 | 5.9 | 2.3 | 56.3 |

In chocolate 1 (Table 2), oleic acid was most abundant in the sn-2 position, 63%, and its share in this position was 65%. Second in order in the sn-2 position was stearic acid in the amount of 17.2%, and it was mostly located in the external TAG positions. Palmitic and linoleic acids were present in the sn-2 position in amounts of 11.9% and 5.6%, respectively, of which palmitic acid accounted for 16.3% and linoleic acid 55.4%. Apart from stearic acid, oleic and palmitic acid were the most abundant in the internal TAG position of chocolate 2 (Table 2). Their share at the sn-2 position was also high, being 24.6% for palmitic acid and 54.2% for oleic acid. Linoleic acid occupied an internal position—its share was approximately 46%. The share of saturated stearic acid in the sn-2 position was 18.8%, so it was mainly located in the external TAG positions. The total content of this acid (18:0) in TAG was 38.5%. Saturated stearic acid constituted as much as 36.3% in the researched chocolate 3, but its share in the sn-2 position was only 12.3% and it was located mainly in the external TAG positions (Table 2). Oleic and linoleic acids were most abundant in the sn-2 TAG position. The share of oleic acid in the middle TAG position was 69.7%, so it was mainly located in the sn-2 TAG position, as well as linoleic acid—57.5%. In chocolates 4 and 5, the obtained values were very similar (Table 2). In both cases, in the largest amounts were stearic acid with a 24% share in the sn-2 position—it occupied mainly external TAG positions. The largest share in sn-2 positions was attributed to oleic acid,

which amounted to 50% in chocolate 4 and 48.6% in chocolate 5. Palmitic and linoleic acids occurred in the sn-2 position in amounts of approximately 19.7% and approximately 4.4%, respectively, of which palmitic acid accounted for approximately 24.5% and linoleic acid accounted for 37.7–43.8%. In chocolate 6, oleic acid was present in the highest amount in the sn-2 position and amounted to 68%, and its share in this position was 69.5%; it occurred mainly in the internal TAG positions (Table 2). The second largest content of fatty acid in the internal position was linoleic acid at 47.9%. Palmitic and stearic acids in the sn-2 position were present in amounts of 10.6% and 13.3%, respectively, of which the palmitic acid share accounted for 14.7% and stearic acid 12.4%. In chocolate 7, oleic acid was the most abundant in the middle TAG position, 69.5%, with a share of 71.6% (Table 2). Apart from oleic acid, the second acid in the sn-2 position was linoleic acid with a share of 58%. Stearic acid occupied mainly external positions—its share was close to 50%. The occurrence of saturated palmitic acid (16:00) in TAG was 24.1%, and its share in the internal position was 13.6%, so it was mainly located in the external positions of triacylglycerols. In the case of organic dark chocolates 8 and 9, differing in cocoa mass content, similar results were obtained (Table 2). In both cases, the highest content of saturated stearic acid was determined, with a share in sn-2 positions of approximately 16%, so it occupied mainly external TAG positions. The largest share in sn-2 positions was attributed to oleic acid, which was—64% in chocolate 8, and—61.1% in chocolate 9. Polyunsaturated linoleic acid was present in triacylglycerols in an amount of approximately 3.6%, and in both chocolates it occupied the internal position of the triacylglycerols. Similar quantities of palmitic acid (16:0) were also obtained in the sn-2 position, at the level of approximately 13%, and the share of this acid in the internal position was below 19%, which proved its presence in the external TAG positions. Similar results were obtained by Kruszewski and Obiedziński [57].

Research on cocoa fat substitutes showed that mixtures with a higher content of saturated fatty acids in the sn-1 and sn-2 positions, e.g., SSO, SPO, PPO, were characterized by a higher crystallization temperature [30]. This type of triacylglycerols also influenced the melting temperature and increased the chance of the formation of metastable β′ crystals during the crystallization process, which nevertheless transformed into more stable β crystals after a week of storage at 20 °C [30].

### *3.3. The Melting Profiles of Fat Extracted from Chocolates*

Chocolate is a suspension of cocoa, sugar, or milk powder particles in the continuous fat phase, which is cocoa butter. Cocoa butter is characterized by complex polymorphism, in which crystals can occur in six different polymorphic forms (I–VI). In order for the obtained product to be of high quality, cocoa butter must be crystallized mainly in the V form, because it is the most stable—obtained in the process of tempering chocolate. Differential scanning calorimetry (DSC) provides information about the "quality" of chocolate, which is influenced by processing methods such as the tempering and cooling of the product. Information obtained from thermal analyses (DSC) provide important data on the properties of chocolate, including melting and crystallization temperatures [60].

Research conducted by Beckett [61] proves that the melting curve of cocoa fat is characterized by peaks at maximum temperatures of 17.14 °C and 20.41 °C. The first crystalline form of cocoa fat melts in the temperature range of 16–18 °C, and the second crystalline form in the range of 22–24 °C. For deodorized cocoa butter, the first endothermic peak occurs at a temperature of about 18.46 °C, and the second peak at a temperature of about 20.54 °C. The appearance of the first peak may result from the melting of crystalline form I of cocoa butter, and subsequent, more pronounced peaks result from the melting of crystalline form II. The presence of double or several peaks in melting curves is most often the result of the presence of polymorphic structures of chocolate [62]. The appearance of more than one peak results in the existence of different crystal structures within the same product [63]. The melting temperatures of individual fat fractions, as well as the course of the melting curve, depend, among other things, on the composition of fatty acids, their distribution in the positions of triacylglycerols, polymorphic form, and the measurement

conditions, e.g., the rate of heating/cooling of the sample. The intensity of melting or crystallization peaks increases with increasing heating/cooling rates [64].

In Figure 5a, melting curves obtained for fats extracted from chocolates with low cocoa liquor content: 1—min. 45%, 5—min. 40% and 4–45% did not differ in the shapes of the obtained peaks. The researched fats reached their first peaks at the following temperatures: 19.21 °C for fat extracted from chocolate 4, 18.90 °C for fat extracted from chocolate 5, and 20.05 °C for fat extracted from chocolate 1. Small peaks can also be observed appearing at temperatures of around −10 °C. The appearance of these peaks on the melting curve could be the result of the presence of different types of fat in the researched fat: milk fat or vegetable fat declared by the producers on the packaging (Table 1). Research on the crystallization and melting curves of cocoa butter with the addition of palm oil showed that as the amount of added palm oil increased, the shape of the curve changed—the peak intensity decreased and the breadth increased, which increased the temperature range of the phase transition [64]. The addition of milk fat causes a lower melting temperature, slower solidification, and a softer chocolate texture. Liang and Hartel [65] also proved that the addition of various milk powders influences chocolate processing processes and its physical and organoleptic properties.

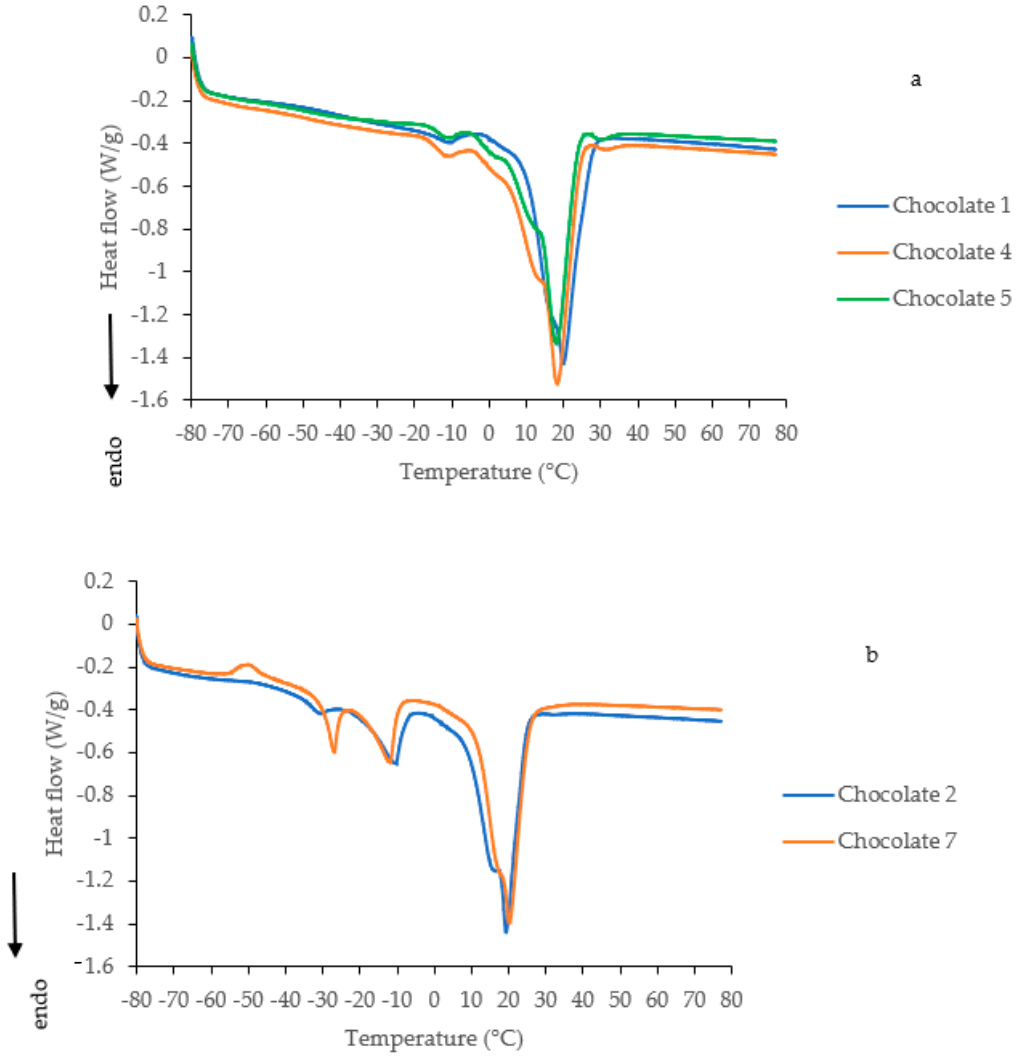


**Figure 5.** *Cont.*

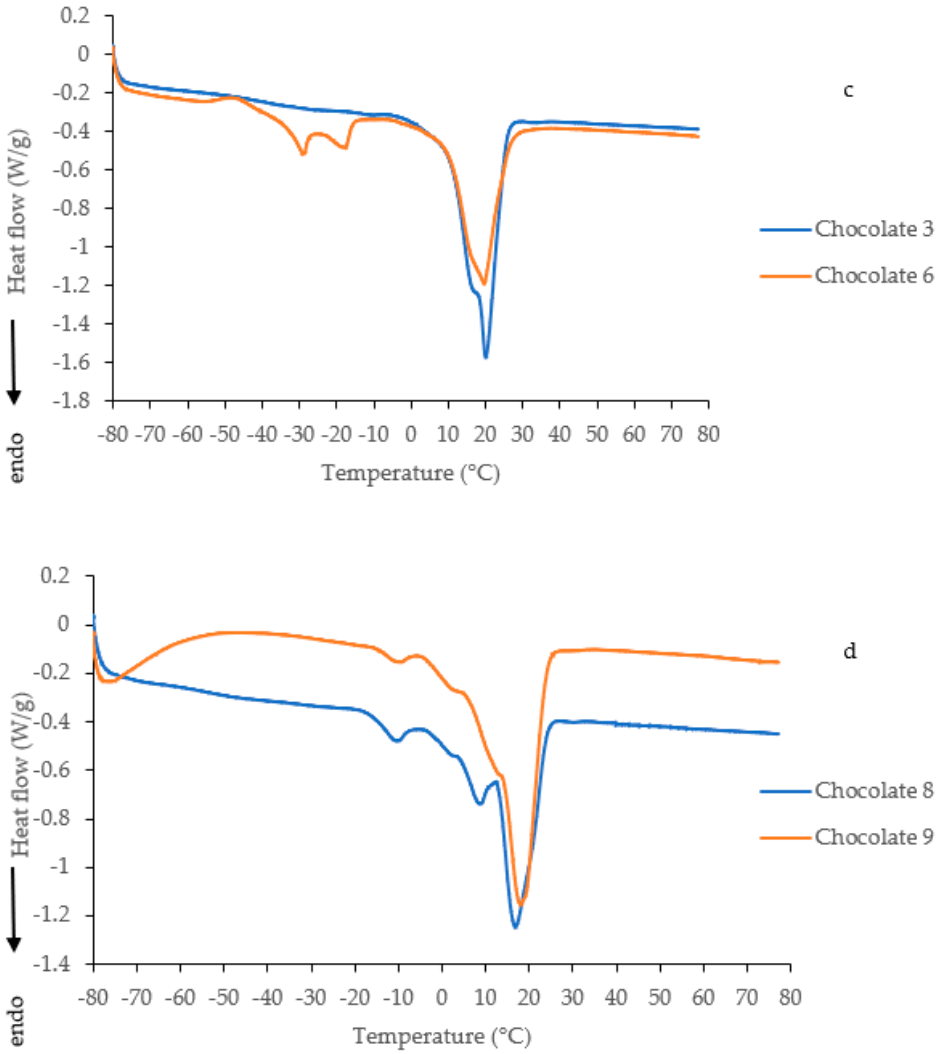


**Figure 5.** Melting characteristics of fat extracted from chocolates 1, 4, 5 (**a**); from chocolates 2, 7 (**b**); from chocolates 3, 6 (**c**) and from chocolates 8, 9 (**d**).

In the case of the melting curves of fats extracted from chocolate 2 and 7 presented in Figure 5b, differences in shape can be observed. Four peaks were present in the fat melting curve of chocolate 2. The first one occurred at a temperature of −30.02 °C, the second one at −13.48 °C, the next one at 17.11 °C, and the most intense last peak at a temperature of 20.56 °C. The melting curve obtained for chocolate fat 7 was also characterized by the presence of four peaks. The first peak occurred at −27.18 °C, the next at −12.42 °C, and the next at 18.25 °C. The most intense peak was observed at a temperature of 21.36 °C. The appearance of peaks very close to each other in the case of chocolate 2 at temperatures of 17.66 °C and 20.48 °C and of chocolate 7 at 18.25 °C and 21.36 °C could be the result of the presence of other types of fats in the researched fats, or it may indicate on the melting of the II and III polymorphic forms of cocoa butter. In studies by Afoakwa et al. [51], they found that the occurrence of the first peaks may be caused by the addition of milk fat.

In Figure 5c, the melting curves of fats extracted from chocolate 3 and 6 are shown. The courses of both curves at positive temperatures are characterized by a similar shape, while at negative temperatures, changes can be observed in the case of chocolate 6. There were two peaks present at temperatures −30.53 °C and −18.42 °C. The most intense peak for chocolate 6 occurred at a temperature of 20.12 °C. The melting curve of chocolate fat 3 was characterized by the occurrence of only one peak at a temperature of 20.91 °C. Comparing the melting curves of chocolates, it can be concluded that in the case of chocolate 3, melting of one polymorphic form of fat probably occurred.

Figure 5d shows the melting curves of fats extracted from dark chocolates 8 and 9 with cocoa liquor content of 52% and 74%. The curves differed significantly in the shapes of the peaks. The DSC curve of chocolate 9 initially had a completely different shape than the other melting curves. Its course may be the result of the presence of large amounts of unsaturated fatty acids in the fat being researched. Moreover, it was characterized by two peaks—the first at a temperature of 17.31 °C, the second most intense at 20.05 °C. The melting curve of fat extracted from chocolate 8 was characterized by the occurrence of two peaks, the first at a temperature of −10.12 °C and the most intense at 19.37 °C.

In the study conducted by Ostrowska-Ligęza et al. [5], the melting of cocoa butter was accompanied by two endothermic peaks: a smaller one at a maximum of 17.14 °C and a larger one at a maximum temperature of 20.41 °C. The first peak characterized the melting range of the first polymorphic form, and the second peak represented the second polymorphic form of cocoa butter. In turn, the DSC melting curves of fats extracted from milk chocolate had a more complex course. Low-melting fractions (with a high content of unsaturated fatty acids) melted already at the maximum peak temperature of −9.47 °C. The second peak, the most pronounced, reached a maximum temperature of approximately 17.01 °C and was probably the result of the overlap of two peaks originating from milk fat and cocoa butter, respectively. The third peak, the least pronounced, at a maximum temperature of approximately 30.24 °C, characterized high-melting fractions originating from milk fat [5].

The melting temperature of fat extracted from chocolate may also be influenced by cocoa butter equivalents (vegetable oils, for example palm, shea, or coconut) added by producers in small quantities, mainly to reduce production costs, and not always disclosed on the labels [61].

The values of melting enthalpy ($\Delta H_{melt}$) of all the fats extracted from the chocolates are presented in Table 3.

**Table 3.** Mean values of melting enthalpy of fats extracted from chocolates.

| Fat Extracted from Chocolate | Enthalpy [J/g] |
|---|---|
| 1 | 73.64 ± 0.59 $^{a}$ |
| 2 | 64.38 ± 0.44 $^{b}$ |
| 3 | 54.74 ± 0.19 $^{c}$ |
| 4 | 74.16 ± 0.69 $^{a}$ |
| 5 | 66.86 ± 0.54 $^{b}$ |
| 6 | 55.00 ± 0.65 $^{c}$ |
| 7 | 69.86 ± 0.09 $^{ab}$ |
| 8 | 64.50 ± 0.78 $^{b}$ |
| 9 | 75.39 ± 0.82 $^{a}$ |

Values represent means ± standard deviations. Means with equal superscripts in each group for the column are not significantly different ($p > 0.05$) by the Tukey's test.

The melting enthalpy of cocoa butter was determined using DSC and reached the value of 78.98 ± 0.06 J/g [31]. The composition of blends 55/45 CB/CNO (coconut oil) and 65/35 CB/SIO (sacha inchi oil) were prepared. The addition of CNO to cocoa butter slightly increased the enthalpy value to 79.17 ± 1.95 J/g, while the addition of SIO decreased the value of this parameter to 58.35 ± 1.14 J/g. The differences between the thermal properties of CB, CNO, SIO, and blends of their components are mainly due to the different molecular entities contained in each of them [31,66]. Chocolates 1, 4, and 9 were characterized by high values of melting enthalpy (Table 1). This may indicate a high content of pure CB in those chocolates. Sathivel et al. [67] investigated changes in melting points, enthalpy, and specific heat capacity of catfish visceral oil at each step of the purification process. Enthalpy of saturated fatty acids increased with increasing chain length, and the enthalpy

of unsaturated fatty acids generally decreased with increasing numbers of double bonds. Higher content of unsaturated fatty acids in triacylglycerols in chocolate fat causes an increase in the melting enthalpy of this fat. Large quantities of polyunsaturated fatty acids were observed in the fat of chocolates 3, 5, and 6 in Table 1. The enthalpies of melting transformations of these fats were characterized by lower values (Table 3).

### *3.4. Thermogravimetric Characterization of Chocolates*

Thermogravimetry is considered a quick and sensitive method used to determine the composition of dark chocolate, confirm the content of cocoa liquor declared by the manufacturer, or check the production process [40]. From the shape of the TGA curves, it is possible to draw conclusions about mass changes (mass losses) during thermal transitions and indicate the temperature ranges in which these transitions occur. However, it is not always possible to indicate the type of thermal process that is taking place, especially if a given transformation is not accompanied by a mass change. The amount of mass loss depends, among other things, on the stoichiometry of the reactions occurring during heating, on the type of gas flowing through the sample, and on the heating rate of the sample. In the case of food samples containing fats, the best resolution is obtained at a heating rate of 10 °C/min [40,68]. Performing thermogravimetric analyses in a nitrogen atmosphere allows a better understanding of the qualitative composition of the sample, especially in the case of mixtures [40].

Thermogravimetric curves and their first derivatives for all dark chocolates obtained in nitrogen flow are presented in Figures 6 and 7. Three stages of sample mass changes were observed in the thermogravimetric curves in the temperature ranges 170–250 °C, 250–340 °C, and 340–700 °C.

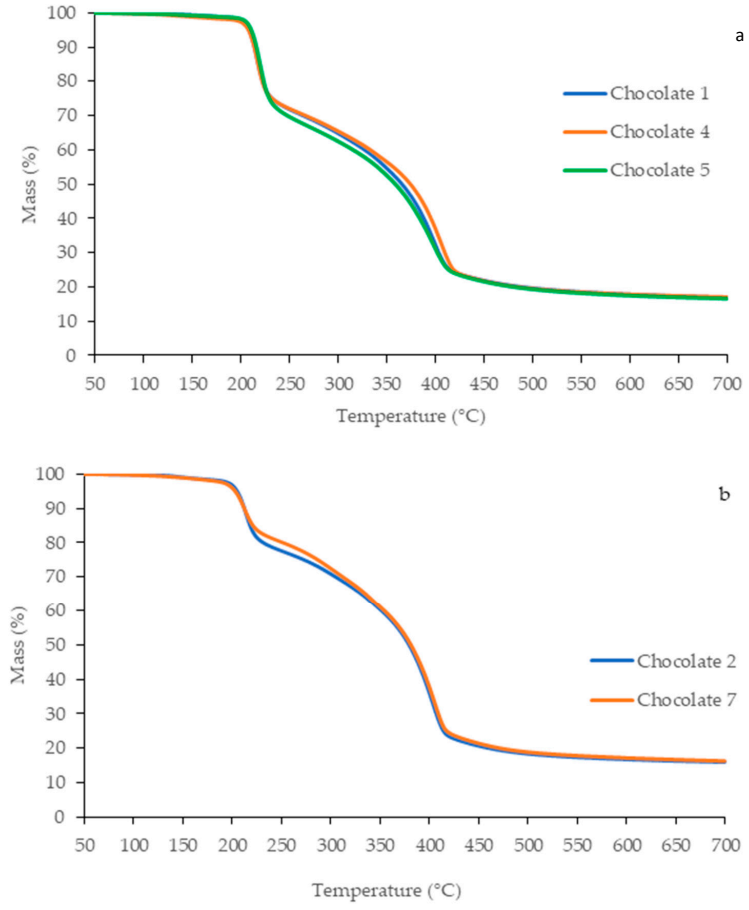


**Figure 6.** *Cont.*

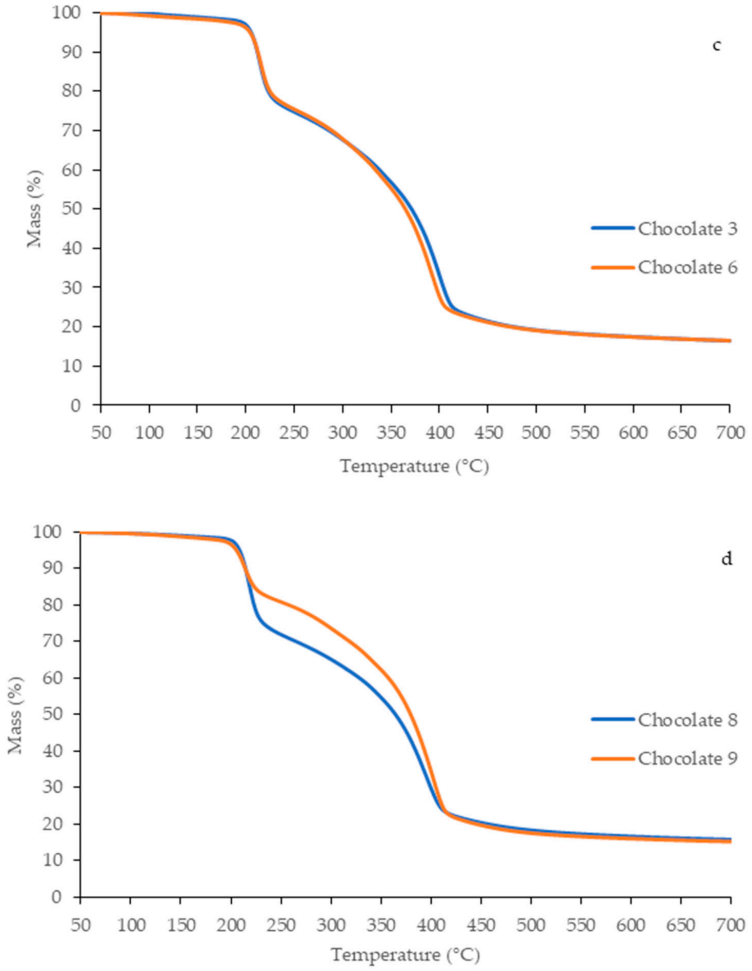


**Figure 6.** TGA curves in nitrogen of chocolates 1, 4, 5 (**a**); chocolates 2, 7 (**b**); chocolates 3, 6 (**c**); and chocolates 8, 9 (**d**).

In the case of the chocolates with the lowest cocoa liquor content (40–45%), almost identical TG and DTG curves were reported (Figures 6a and 7a). In all three chocolates (1, 4, and 5), a significant part of the composition was sugar, which presence was correlated with mass loss from 26% (chocolate 1 and 4) to 30% for chocolate 5. The maximum temperature of peak for all three chocolates was approximately 218.87 °C (Figure 7a). Such a high sugar content in the researched chocolates caused rapid mass loss of the samples under the influence of high temperature. The sections responsible for transitions related to cocoa liquor content were characteristic for this type of ingredient in the temperature range of 335–363 °C. The mass loss caused by the decomposition of this ingredient reached 7.84% for chocolate 1, 6.17% for chocolate 4, and 4.64% for chocolate 5. The peaks showing fat distribution were not very distinct, so it can be concluded that fat other than cocoa butter was added to the tested chocolates [55]. The maximum temperature peaks ranged from 397.36 °C (chocolate 5) to 406.51 °C (chocolate 4) for fat decomposition.

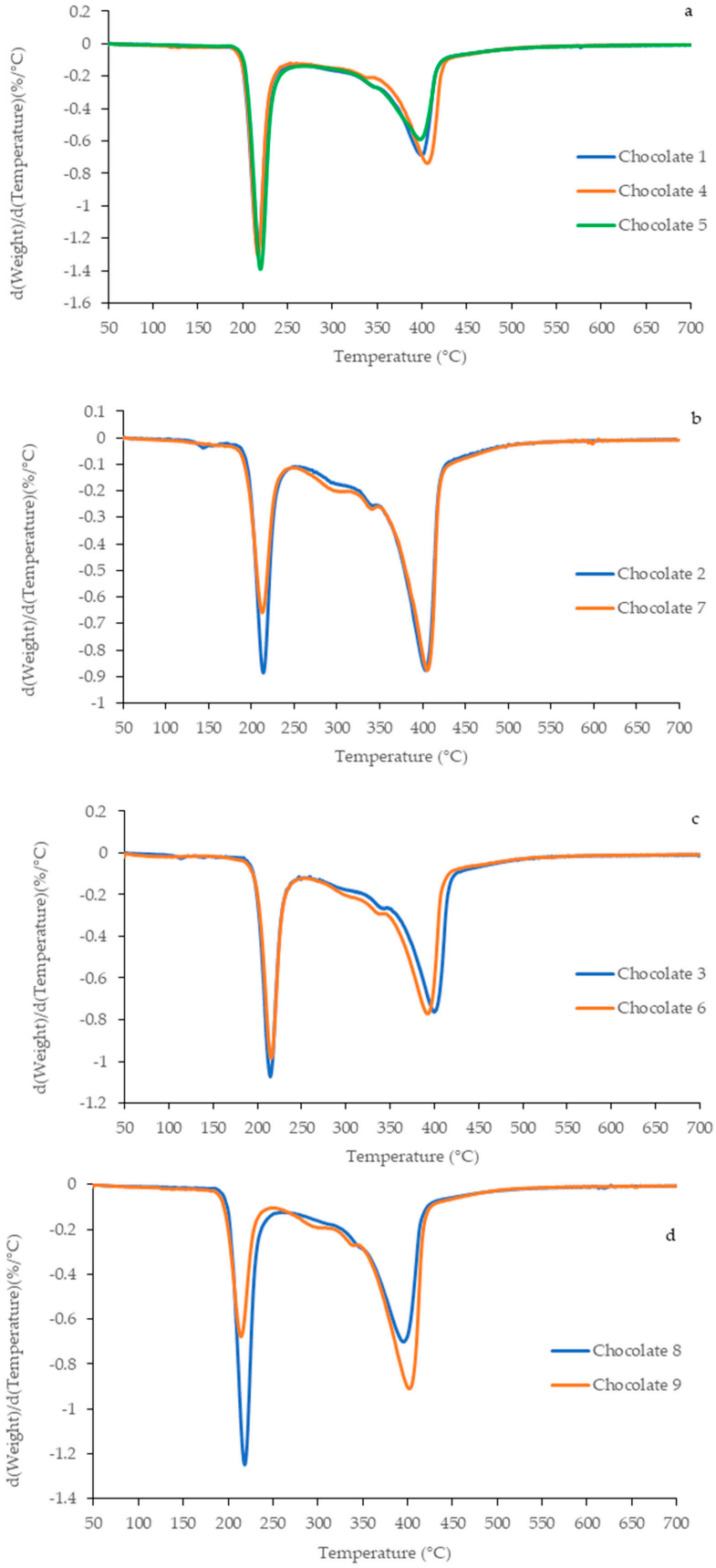


**Figure 7.** DTG curves in nitrogen of chocolates 1, 4, 5 (**a**); chocolates 2, 7 (**b**); chocolates 3, 6 (**c**); and chocolates 8, 9 (**d**).

Based on the obtained TG and DTG curves of chocolates 2 and 7, it can be concluded that these chocolates were different in the content of the main ingredients (Figures 6b and 7b). The peak that reached the maximum temperature value at approximately 214.84 °C for chocolate 2 indicated a much higher sugar content. A 22.94% mass loss caused by the degradation of this ingredient was observed. The characteristic, distinct shape of the peak indicated the presence of sucrose. The temperature range of 263–355 °C indicated the presence of cocoa liquor for both chocolates. For chocolate 7, unevenness of the curve in this range indicated a higher content of cocoa liquor. The mass loss for chocolate 2 reached 23.01%, and the mass loss for chocolate 7 reached 25.88% (Figures 6b and 7b). In the case of chocolate 2, fat decomposition occurred in the temperature range of 350–460 °C, while in chocolate 7, fat decomposition occurred in the temperature range of 300–470 °C. The peaks reached a maximum at the same temperature around 407.55 °C. The area of the peak responsible for fat distribution was larger in chocolate 7. Confirmation of the correctness of the analysis can be compared with the results obtained in the study conducted by Ostrowska-Ligęza et al. [68]. TG analysis covered chocolates and chocolate masses from various stages of production; the temperature range responsible for the transformation of cocoa liquor was from 214 to 348 °C, and the peak of cocoa butter decomposition was characterized by a distinct course in the range of temperatures from 387 to 391 °C.

Chocolates 3 and 6 were characterized by a similar composition declared on the packaging by the producers. Confirmation can be found in the obtained TG and DTG curves of the tested chocolates. In Figures 6c and 7c, it can be found that the amount of sugar in the researched chocolates was bigger than the amount of fat. The amount of cocoa liquor in the chocolates was very similar. The peaks responsible for fat decomposition were of not-so-very-expressive shapes, as in the case of cocoa butter, so it can be concluded that other fats were added to the chocolates. The TG and DTG curves obtained for chocolates 3 and 6 were characterized by a similar shape and course to those obtained in the research conducted by Materazzi et al. [40].

The TG and DTG curves clearly showed the differences between organic chocolates from the same manufacturer, differing in cocoa liquor content—52% and 74%. Chocolate 8, with 52% cocoa liquor, was characterized by a larger amount of added sugar, probably sucrose—a distinct peak was observed in the temperature range 190–260 °C. The mass loss responsible for the presence of cocoa liquor in both organic chocolates reached 14.33% for chocolate 8 and 20.83% for chocolate 9 (Figures 6d and 7d). The DTG curve of chocolate 9 indicated a higher content of cocoa liquor and cocoa butter. The mass loss responsible for the presence of fat was 39.89% for chocolate 8 and 55.31% for chocolate 9. However, the DTG curve of chocolate 8 indicated a larger amount of sugar with a mass loss of 27.26% compared to 17.82% for chocolate 9.

In the case of mixtures, mass loss may be accompanied by various thermal processes. At lower temperatures, the most common loss of water present in the sample in many forms is observed, as well as the evaporation of molten substances, often combined with their decomposition. At higher temperatures, thermal destruction occurs and, in the presence of oxygen, combustion of the organic residue of the tested substances. In a nitrogen atmosphere, these processes occur at a different pace. Nitrogen is less dense than oxygen, so it penetrates the sample more easily and speeds up the process of removing moisture. On the other hand, nitrogen is an inert gas, so the gaseous decomposition products released from the sample interact less with nitrogen than with oxygen, which is why decomposition in anaerobic conditions takes longer than decomposition in the presence of oxygen [40].

### *3.5. Thermogravimetric Characterization of Fat Extracted from Chocolates*

The thermal stability of any fat depends on the composition of the triacylglycerols, the distribution of fatty acids in the triacylglycerols, and the method of extracting the fat from the organic material. The thermal stability of fat may also be influenced by the conditions of fat processing and storage, as temperature changes accompanying fats during food production influence the formation of specific polymorphic forms, the diversity of which is large in the case of cocoa butter [61]. Chocolate is a food product with a high fat content. The properties of fat influence the sensory characteristics of chocolate, such as fragility, melting range in the mouth, and viscosity. Due to the wide use of chocolate for culinary purposes, the stability of chocolate during baking is also important. For analyses performed in a nitrogen atmosphere, the TG and DTG curves for fats extracted from chocolate did not show significant differences in the temperature ranges representing fat decomposition (Figures 8 and 9).

All the fats began to decompose in the temperature range of 275–290 °C and ended in the temperature range of 470–495 °C. Unlike the others, the fat extracted from chocolates 2 and 7 showed a slight mass loss of 3.18% and 8.67%, respectively, at lower temperatures, in the range of 214–275 °C (Figures 8b and 9b). This could have been the result of thermal decomposition of the anti-caking agent added to the cocoa powders to facilitate dosing. For all the fats extracted from the chocolates, the maximum transformation temperatures reached values ranging from 400 to 413 °C (Figures 8 and 9). The shape and course of the obtained peaks were similar despite the manufacturers' declarations about the addition of fats other than cocoa butter (Table 1). Ostrowska-Ligęza et al. [68] examined the thermal stability of ingredients used to produce chocolate and obtained a narrower range of cocoa fat degradation temperatures of 310–440 °C when measured in a nitrogen atmosphere.

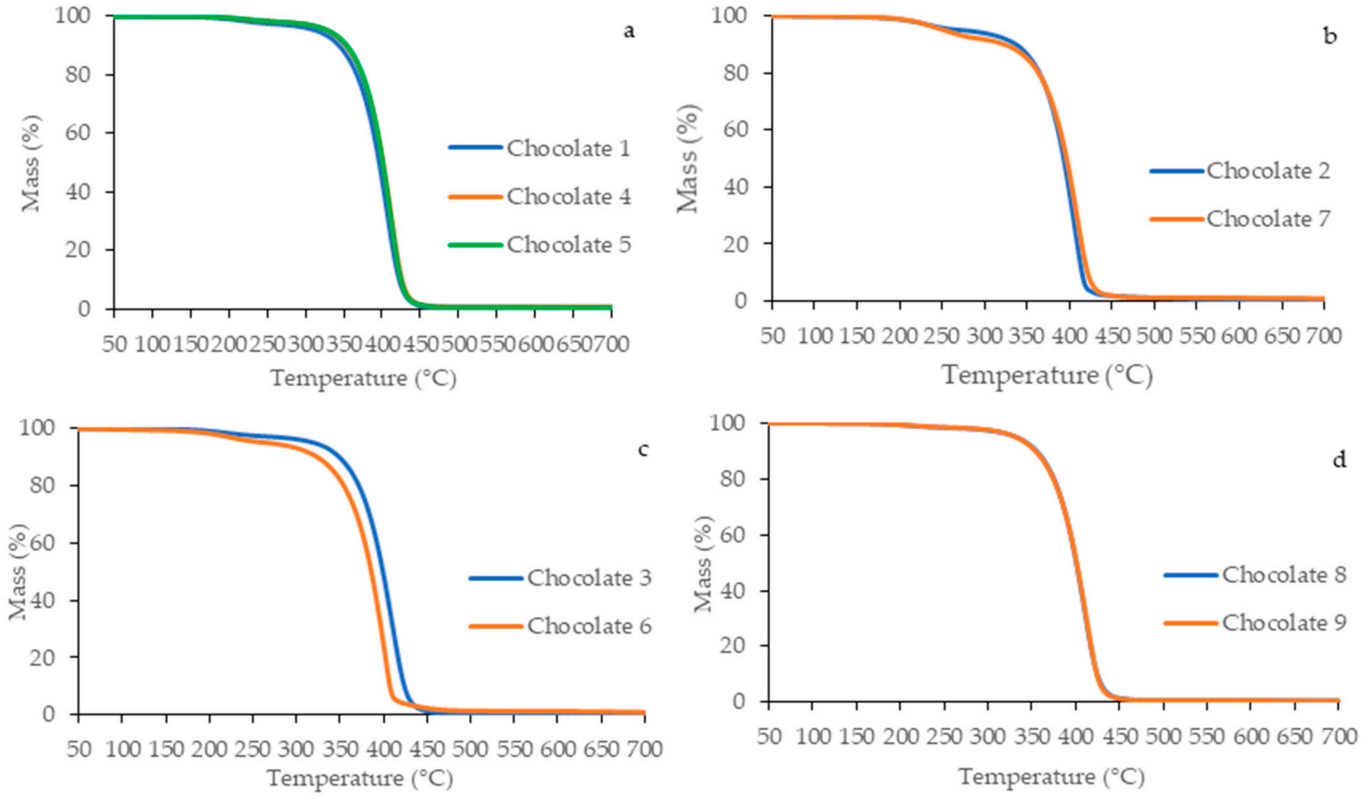


**Figure 8.** TGA curves in nitrogen of fat extracted from chocolates 1, 4, 5 (**a**); chocolates 2, 7 (**b**); chocolates 3, 6 (**c**); and chocolates 8, 9 (**d**).

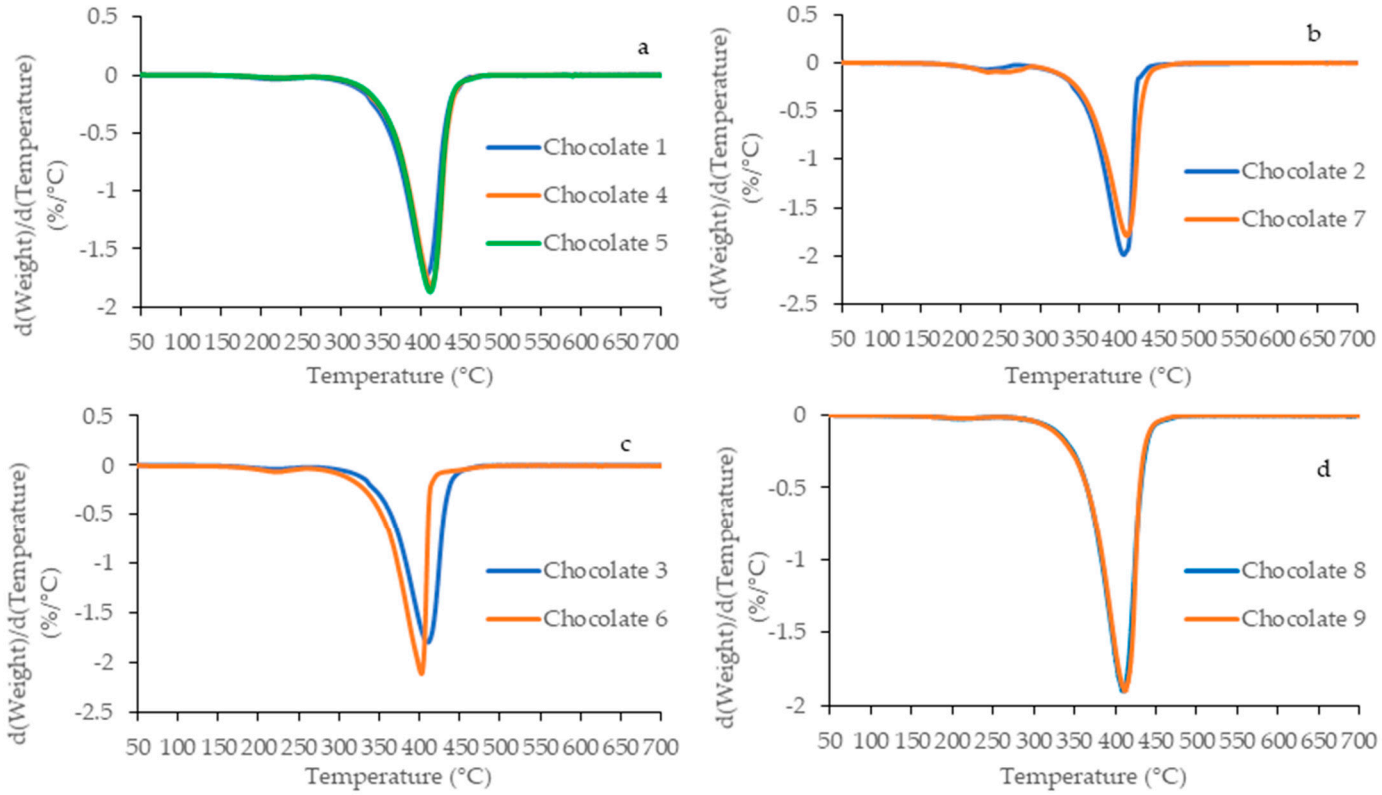


**Figure 9.** DTG curves in nitrogen of fat extracted from chocolates 1, 4, 5 (**a**); chocolates 2, 7 (**b**); chocolates 3, 6 (**c**); and chocolates 8, 9 (**d**).

Results from the GC associated with fatty acid profiles and thermogravimetry analysis were used to perform either cluster analysis or PCA. The results presented in Figure 10 show the chocolates clusters based on the similarity of their fatty acid profiles. PCA analysis confirmed that the most important fatty acids in chocolate are palmitic, stearic, oleic, and linoleic acids. The largest cluster consisted of chocolates 3, 4, 6, 8, and 9, which were characterized by a higher amount of oleic acid and a lower content of palmitic acid. Chocolates from the second cluster (1, 7) were characterized by the lowest content of oleic and linoleic acids and a high content of stearic acid. Both of these chocolates showed the greatest degree of similarity in the remaining acids. Chocolates 2 and 5 contained the highest levels of oleic and stearic acids (chocolate 2) and palmitic acid (chocolate 5). However, taking into consideration the results of statistical analyses regarding thermogravimetric parameters, a completely different way of grouping the chocolate samples can be seen (Figure 11). The first cluster (chocolates 1, 5, and 8) consisted of chocolates with a high sugar content and a low fat content. There was one noticeable outlier (chocolate 4) in the score plot, most notably due to its composition. The second cluster included chocolates 2, 3, 6, 7, and 9, which were characterized by a higher fat content, including liquid cocoa (more than 60%), and a lower sugar content. In the division, the most important factors, taking into account, were the values characterizing the second peak (the moment of its appearance and the end of its duration); this peak is responsible for the fats present in the chocolate. Another important factor in this division was the duration of the third interval responsible for the cocoa liquor.

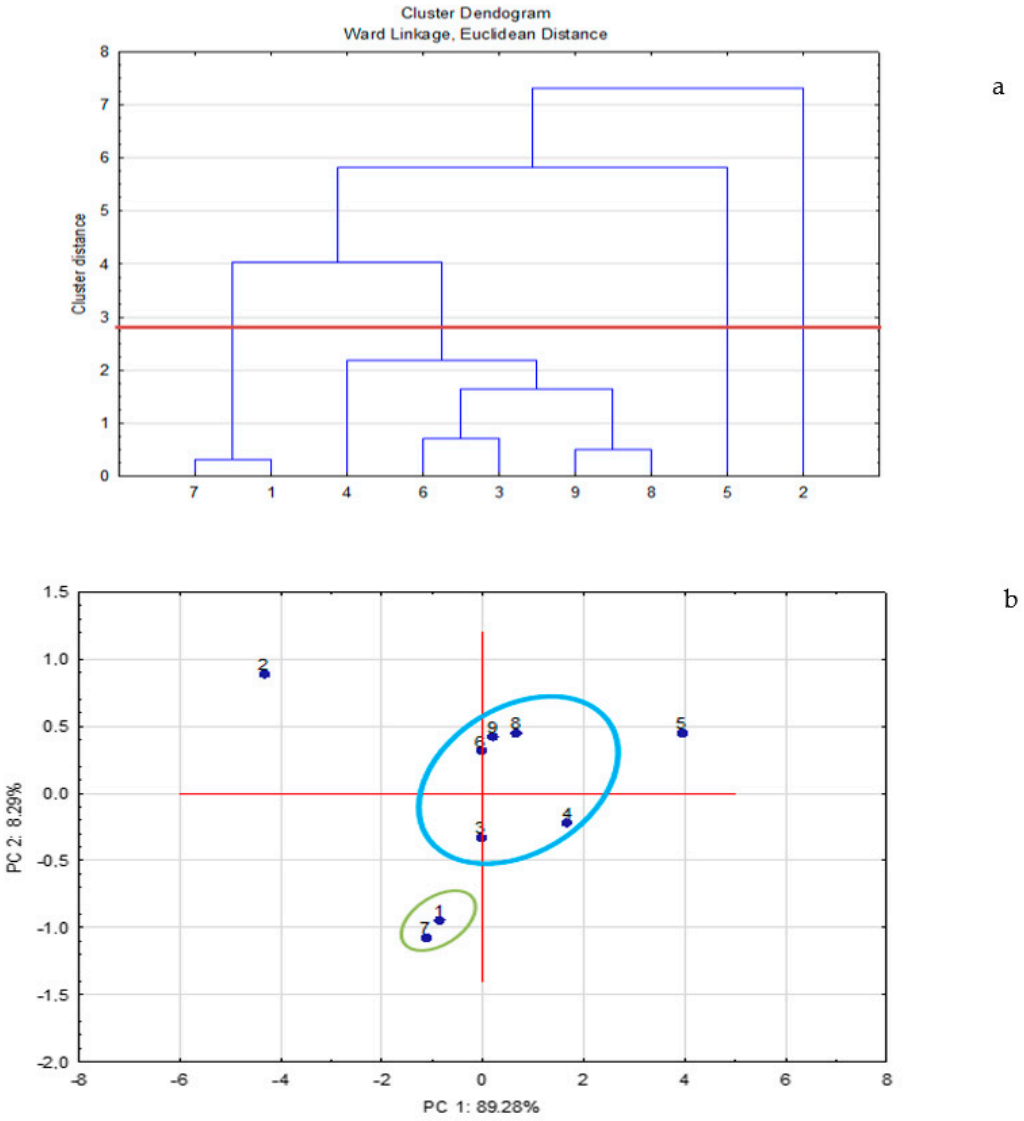


**Figure 10.** Dendogram (**a**) and PCA (**b**) based on the chemical profiles of fatty acids.

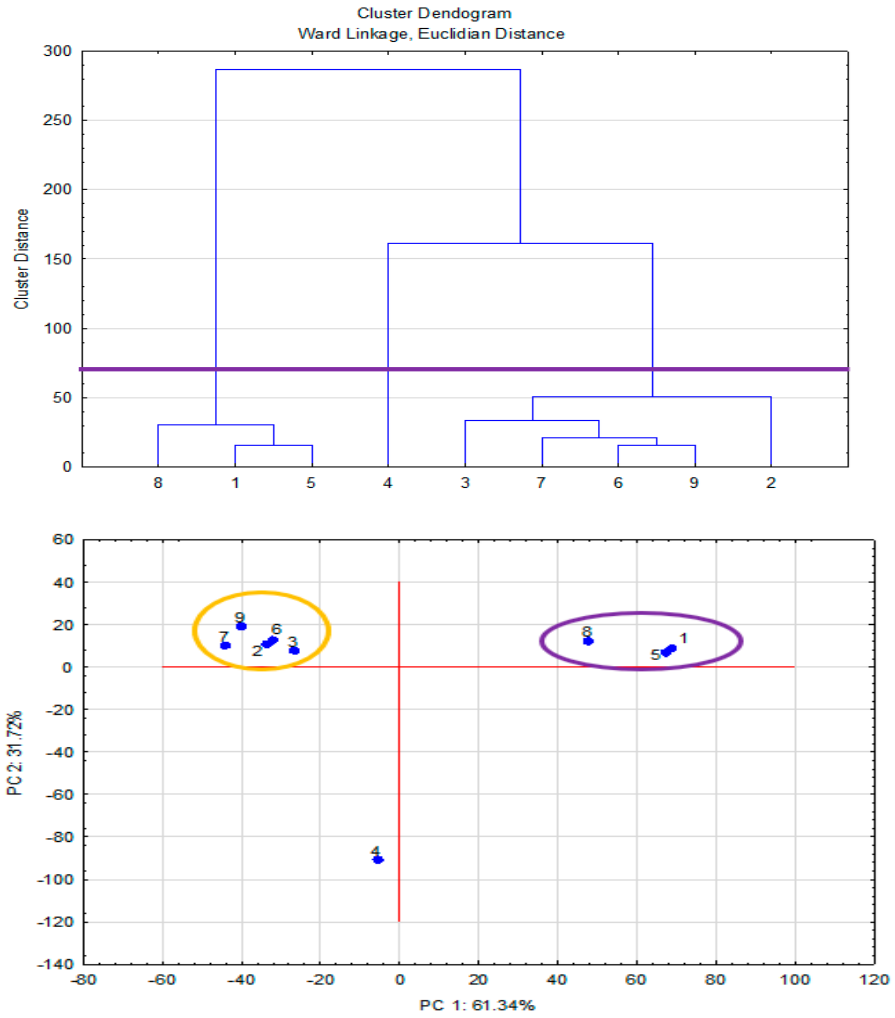


**Figure 11.** Dendrogram (**a**) and PCA (**b**) based on the TG values.

## 4. Conclusions

The presence of three fatty acids (palmitic P, stearic S, and oleic O) constituting triacylglycerols SOS, POP, POS, POO, and SOO was observed in all the samples. The presence of linoleic acid (L) was also found, which formed triacylglycerols such as PLP and PLS. The researched chocolates were characterized by a diverse composition of fatty acids. Chocolates in which the addition of fat other than cocoa butter was declared achieved results similar to other products in terms of fatty acid profiles. All the chocolates contained linoleic acid. Saturated fatty acids were also detected: myristic, pentadecanoic, margaric, monounsaturated: cis-10-pentadecanoic, palmitoleic, margaroleic, eicosenic, and polyunsaturated fatty acids: $\alpha$-linolenic and dihomo-$\gamma$-linolenic at levels below 1%, and arachidic acid, the content of which did not exceed 1.5%. This allowed us to confirm that the products were not adulterated with the addition of vegetable fat that had not been declared on the packaging. A high content of polyunsaturated fatty acids in the internal sn-2 position of TAG was recorded in the researched chocolates. This arrangement of fatty acids helps to slow down the oxidation process.

All the obtained DSC melting curves of the fats were characterized by the presence of two endothermic peaks. Additional peaks appeared in the case of chocolates 2, 6, 7, and 8. These peaks, appearing at negative temperatures, may be caused by the melting of low-melting triacylglycerols (with a high content of unsaturated fatty acids). The differences between the melting curves for the obtained dark chocolate fats may have resulted from the presence of less-stable polymorphic forms of cocoa butter.

Based on the shape of the TG and DTG curves, it could be possible to indicate the adulteration of chocolates. The obtained results allowed us to indicate the differences between the content of the cocoa butter, sugar, and cocoa liquor in the studied chocolate samples.

**Author Contributions:** Conceptualization, E.O.-L.; methodology, E.O.-L., I.P., R.B. and A.G.; investigation, M.W.-W., A.G., I.P. and E.G.; formal analysis, E.O.-L., A.G., M.W.-W., I.P., R.B. and A.S.; writing—original draft preparation, E.O.-L. and A.G.; writing—review and editing, E.O.-L., A.G. and M.W.-W. All authors have read and agreed to the published version of the manuscript.

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