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Evaluation of theobromine content and the relationship between cocoa percentages in dark chocolates

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Submission Date: July 4th, 2023; **Acceptance Date:** October 23rd, 2023; **Publication Date:** October 24th, 2023

Please cite this article as: Eren F. H., Kabaran S. Evaluation of theobromine content and the relationship between cocoa percentages in dark chocolates. *Functional Foods in Health and Disease* 2023; 13(10): 520 of 532. DOI: https://www.doi.org/10.31989/ffhd.v13i10.1141

ABSTRACT

Background*:* There are no specific regulations governing the labeling of dark chocolate or its bioactive component quantities.. The main alkaloid of the cocoa bean is theobromine. Chocolate is by far the main source of theobromine in the Western diet. To assess their effects on health, it is essential to understand the ingredients and composition of biologically active substances in chocolates available in stores.

Objective*:* The present study aims to analyze the theobromine contents of commercial chocolates and evaluates the association between the labeled cocoa mass percentage (%), theobromine contents and evaluated non-fat cocoa solid (NFCS) parameters of dark chocolates.

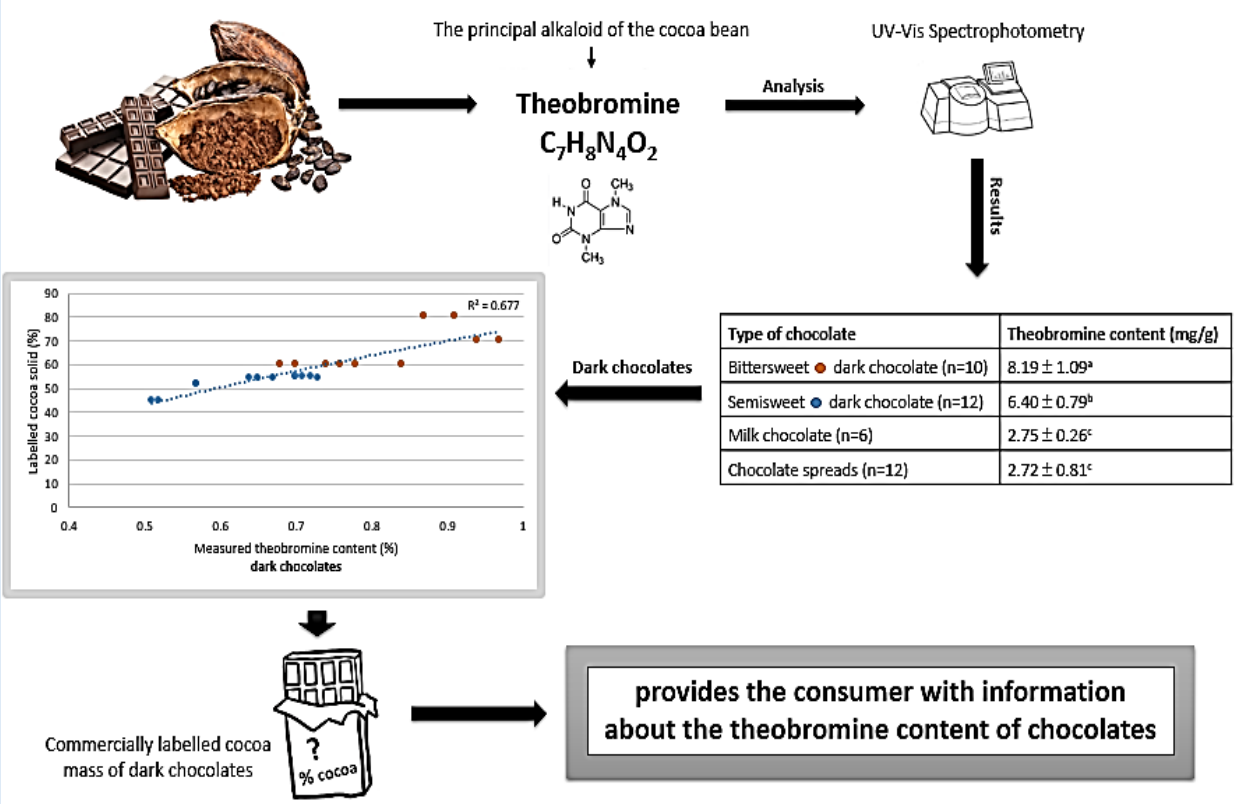
Materials and Methods*:* Several types of chocolates (bittersweet dark [60-80% cacao], semisweet dark [45-50% cacao], milk chocolate, and chocolate spreads) were analyzed using UV-Vis spectrophotometry with duplicate measurements.. Samples (n=20) typically 6 months apart from production dates have been selected.

Results: Overall range for theobromine content varied from 1.9 to 9.6 mg/g. Of all, bittersweet dark chocolate had the highest (8.1±1.01 mg/g) concentration of theobromine in comparison to semisweet dark chocolate (6.4±0.79 mg/g). Milk-chocolate (2.7±0.26 mg/g) and chocolate-spreads (2.7±0.81 mg/g) had the lowest concentration of theobromine. A strong correlation between theobromine content and declared cocoa solid % was found in both of the dark chocolate

categories (r = 0.523, p = 0.081 and r = 0.771, p = 0.009 for semisweet and bittersweet dark chocolate, respectively). NFCS indicated a correlation between the labeled cocoa solid % (R^2 = 0.766) and the calculated cocoa solid % (R^2 = 0.803) in dark chocolates. A high correlation has also been determined between the labeled cocoa solid percentage and the calculated cacao solid percentage in dark chocolates (R^2 = 0.902).

Conclusion*:* The labeled content of the cocoa mass of dark chocolates could be a preliminary information for the consumer about theobromine capacity.

Keywords*:* Theobromine, chocolate, dark chocolate, cocoa-percentage, UV-Vis spectrophotometry



INTRODUCTION

Products containing cocoa and chocolate represent a multi-million industry in both Europe and UK. The chocolate quantity used in Europe is higher than rest of the world. The cocoa levels used in chocolate-containing products are generally used in order to identify the quality of the same and methods for accurately quantifying those levels have been investigated for more than a century [1]. European countries and the US have informative laws and regulations in effect about the composition of chocolate products [2]. As for Turkey, labelling and contents of the chocolate containing products are regulated by Turkish Food Codex Regulation No 2017/29, which has been enacted as per European

Parliament's Directive 2000/36/EC [3]. These regulatory arrangements define a framework of assigned descriptions for the products containing chocolate involving minimum requirements for solid cocoa levels to be present in them [1]. Henceforth chocolate is designated as "the products made from cocoa derivatives and sugars and containing minimum 35% dry cocoa solids in total, of which at least 18% is cocoa butter and further containing minimum 14% dry non-fat cocoa body" [3]. The foregoing definition emphasizes the fact that the cocoa body may encompass cocoa liquor and/or pulverized cocoa and cocoa butter [4]. Cocoa liquor and pulverized cocoa are identifiable by high levels of methylxanthine alkaloid substances, that contribute to typical bitterness and palatability of a chocolate product [5-6]. Methylxanthines are psychopharmacologically active molecules [7-8]. The figures determined for methylxanthine compounds in cocoa products indicate that chocolate products' potential physiological effects are mainly contributed by theobromine (3,7-dimethylxanthine), with very negligible effects of caffeine (1,3,7-trimethylxanthine) [9]. Although caffeine stimulates the central nervous system five times more than theobromine, theobromine has a longer life and is found in greater quantities in chocolate [10].

Theobromine is the major alkaloid of the cocoa bean [10]. Chocolate is by far the main source of xanthine theobromine in the Western diet [11]. Theobromine may have a role in lowering plasma glucose and also shows an antitussive and broncho-dilating effect, a diuretic action, and a possible role in the reduction of angiogenesis in tumor growth [9, 12]. In addition, theobromine is responsible for preventing the development of cardiovascular diseases (CVD), is beneficial for hypertension arteriosclerosis, angina pectoris, and HDL-cholesterol increasing effect, and is also involved in several other biological functions such as stimulating the central nervous system (including mood changes) and smooth muscles [11. 13, 14]. Therefore, theobromine represents one of the most attractive molecules in cocoa.

The crucial determinant making the chocolate bars on the market distinguishable between, is the content of cocoa mass, which have to be stated by the manufacturer on the packaging label [4]. In Brazil, many common chocolate bars on the shelves claim to have a high percentage of cocoa, however it is determined that approximately 35% of the chocolates produced by known companies are actually chocolate candies which have high amounts of sugar and fat. Since it is not mandatory by law to clarify the amount of cocoa in the product within that country, it is confirmed that there is no supervision to guarantee the information on the label [2]. The highest cocoa content is determined in bitter chocolate bars which is generally more than 75%, meanwhile less cocoa content is available in confectionary chocolate bars (30–70%), and as for milk chocolate bars, it defined as solely 25%, in Polish markets [4]. In US markets, dark chocolate is a group of chocolates that consist of semisweet and bittersweet chocolates. Semisweet chocolate usually contains 35–45% cacao, making it sweeter. In contrast, bittersweet chocolate typically contains at least 50% cacao.Despite the foregoing is generally accepted norm, there are no legal regulations in effect to make the chocolate bars distinguishable between dark, bittersweet and semisweet [4, 15]. Furthermore, there are no definite regulations ruling the labeling of dark chocolate or of its bioactive component amounts. To determine possible health effects, it is important to understand the composition and contents of biologically active compounds in commercially available chocolates [7].

For chemical analysis, Ultraviolet-visible (UV-Vis) spectrophotometric assays are the most current methods to estimate the theobromine content in foods, including cocoa, chocolate, and chocolate products [16]. Direct measurement of alkaloid, theobromine, and/or

caffeine content enables the determination of total non-fat cocoa solids (NFCS). Theobromine levels might be used to calculate NFCS, and, in turn, calculated NFCS could be used as a marker to determine the presence of total phenolic content in chocolate. Increasingly, a high % NFCS is being used commercially to reflect chocolate quality [1]. Theoretically, the concentration of NFCS were highest in dark chocolates. Similarly, the highest theobromine content in dark chocolates was informed compared to milk and white chocolates due to a higher cocoa mass content [17].

So far, there is no detailed report on the theobromine contents of Turkish-manufactured chocolates and chocolate spreads undertaken. The present study aimed to analyze the quality/properties of chocolates, especially determining the theobromine levels in various chocolates and chocolate spread types. Since the physiological and health-related effects of cocoa are of importance to consumers and there is still a lack of knowledge on the relationship between the properties of commercially available dark chocolate bars and information on cocoa mass content that is readily accessible to the consumer. The second aim of the study was to understand more clearly the link between the labeled cocoa mass percent and theobromine contents, and evaluated NFCS parameters of commercially available dark chocolates.

MATERIALS AND METHODS

Samples: Several types of chocolates, dark chocolates of bittersweet dark (n=5), semisweet dark (n=6), milk-chocolate (n=3), and chocolate-spreads (n=6) were analysed using ultraviolet-visible spectrophotometer (T80 UV/VIS PG Instruments Ltd, UK) by duplicate measurements. Samples (n=20) of the same brand, from different production dates (typically 6 months apart) have been selected for each chocolate sample, therefore a total of 40 samples were analyzed to determine theobromine content. The percentage of cocoa solids of the dark chocolate samples was declared on the package (front-of-pack) by producers and ranged between 45-80% (45-50% and 60-80% for semisweet and bittersweet dark chocolates, respectively). National (n=4) and international brands (n=2 were randomly bought from local markets in Ankara, Turkey between 2020-2021. Due to the absence of added cocoa mass, white chocolate is not selected in this study. Producers that were included in the study were: Torku, Sarelle, Nutella (Ferrero Spa), Ülker, Eti and Nestle. Samples were coded based on descriptor name of chocolate types' abreviation and coding bears no relation to the order in above list. Samples from all chocolate types were prepared using the same method. Each chocolate bar (approximately 2 g) was shredded into small pieces and kept at -20°C prior to analysis.

Analysis of theobromine: The determination of theobromine were done according to the method described by TS 7800, Turkish Standard Institute, Ankara [18].

Device and materials: UV spectrophotometer; with 1cm light path, which can make reading in 250 nm and higher wavelength; Volumetric flask; 100 mL; Layered filter paper (large pores); Erlenmeyer flask; 300 mL; Pipette; 0,5 mL, 4,0 mL, 5,0 mL, 10,0 mL; Boiling stone.

Reagents: Sodium bicarbonate ($NaHCO_3$), hydrochloric (HCl) acid solution (10%) (37% Emsure® Merck, Germany) and lead (II) oxide (PbO) were obtained from Merck, Darmstadt, Germany.

Lead acetate solution: 115 gr lead acetate $Pb(CH_2COO)_2.3H_2O$ (Merck, Darmstadt, Germany) is dissolved in 350 mL boiling water and 60 gr lead oxide is added while slowly mixed. Solution of lead oxide (23%) is provided as much as possible and the solution is filtered

(Whatman No. 1) when still hot. Filter is washed with boiling water and the filtrate is cooled to 20°C, then completed to 500 mL.

Process: Extraction and rinsing: 3 gr is weighed from the sample and put into a 300 mL erlenmeyer flask, a few boiling stones are put in and mass of the erlenmeyer is found, 96 mL water is added and heated until boiling while shaking intermittently and kept in slight boiling condition. Erlenmeyer is taken from bunsen burner and 4 mL lead acetate solution promptly continuously shaking. Erlenmeyer is cooled, external surface is dried, again weighed and water is added until the liquid mass becomes 101 gr (4 mL lead acetate solution is about 5 gr), thoroughly mixed and filtered through layered filter paper. First 10 mL of the filtrate is disposed. 0,5 gr solid sodium bicarbonate is added to the obtained approximately 50 mL filtrate which must be clear or slightly cloudy and its rather well mixing and settlement of lead carbonate is waited. It is again filtered through layered filter paper and first 10 mL of the filtrate is disposed. 5 mL – 10 mL of the obtained clear filtrate is taken and put into small 100 mL volumetric flask. It is mixed after adding 0,5 mL %10 hydrochloric acid solution and filled to the level line of the volumetric flask.

Spectrophotometric reading: Absorbance of the prepared solution is read against water, in 1 cm quartz basin and with UV spectrophotometer at 306 nm (D_1). Then, starting from 260 nm, maximum absorbance value (D_2) and wavelength at which this value is read (approximately 272 nm) is read.

Calculation: Theobromine content in the sample is found by the following equation [19].

Theobromine (%) = $(17,7\ (A\_2 - A\_1\ ))/mxV$

A_1: Absorbance read at 306 nm, A_2: Absorbance at the wavelength where highest absorbance is read, m: Amount of sample (g), V: Amount of filtrate (ml), T: Theobromine amount (%).

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on signal to noise ratios (S/N) of 3 and 10, respectively. For theobromine, the LOD was 0.73 mg/g and the LQD was 2.42 mg/g. Theobromine standard curves had a mean CV of 4.39 % (n=2). Theobromine contents of the samples were experessed as mg/g sample.

Estimated calculations: The level of non-fat cocoa solids (NFCS) in the sample were found by the following equation: NFCS (%) = % theobromine (w/w) × 38.0 [20]. Also, the calculation of cacao percentage was an estimate obtained by summing together the percent fat on the label and calculated nonfat cocoa solid of each sample [21].

Statistical analysis: Results are expressed as mean values with the corresponding standard deviation. Once the normality of the distribution was verified, the data were submitted to analysis of variance (ANOVA) and Tukey test with significance levels of 5%, considering the type of chocolate. In order to demonstrate correlation evidence among the data, Pearson's correlation coefficient was employed. All statistical analyses were carried out by means of IBM SPSS v. 24.0 software (SPSS Inc., Chicago,IL, USA).

RESULTS AND DISCUSSION

Analysis of theobromine content in chocolates and chocolate spreads: The analyzed theobromine content results are shown in Table 1. The percentage values ranged from 0.19 ± 0.01 to 0.96 ± 0.02 in all chocolate samples. The observed percentages coincides with the percentage ranges reported by some earlier studies [7, 21]. In contrast, various types of chocolate made in Serbia showed theobromine contents in a higher range between

0.5 to 2.2 g/100g [10]. Furthermore, artisan and commercial chocolates from Brazil revealed theobromine levels ranging from 0.36 ± 0.01 to 4.41 g/100g [2]. Above all, chocolate formulation (52% and 70%) from the two regions of Peru showed differences in terms of theobromine contents. The samples from Quillabamba (0.187 and 0.209 g/100 g, respectively) differ from those of Bagua (0.488 and 0.652 g/100 g) mainly by having a higher fat and lower theobromine content [22]. The amount of theobromine in chocolates can vary depending on the extraction method [2], the source of the bean (cocoa genotype) [17], the time of the fermentation or alkalization processing conditions chosen [6, 9] and the chocolate-making process [23]. Unfermented West African (Forastero) cocoa species, known as higher-quality chocolate products, generally contain the highest amount of theobromine [6, 12]. According to a project undertaken by the UK Food Standards Agency, theobromine levels in cocoa were generally lower than results from previous surveys. The reasons for the reduction could contain such factors or any combination thereof as the varying cocoa varieties being cultivated, higher use of those hybrid cocoa varieties which are hardy and giving high yields, as well as climate changes and cocoa growing methods [1]. For the measured samples, Langer et al. (2011) determined that the typical theobromine levels in chocolate brands seem to have been constant for more than a decade, Miller et al. (2009) affirmed that chocolate producer typically blend various cacao bean types to make sure that their product are uniform [24]. Additionally, the concentration of theobromine in chocolate depends on the percentage of cocoa solids used [10]. Due to these factors, a wide range of results was found among the reported theobromine results of chocolates.

Table 1. Labelled (cocoa solid and fat percent) data, analyzed theobromine levels (percent mean content ± standard deviation (SD) (n=2 sample, duplicate)) and calculated NFC and cacao percentages in chocolate spreads (CS), milk (MC), semisweet dark (SSD) and bittersweet dark (BSD) commercial chocolates.

Sample	Labelled cocoa solid (%)	Labelled fat content (%)	Measured theobromine (%) (g/ 100g) ± SD	Calculated NFCS (%)	Calculated cacao (%)
CS01	7	33.6	0.40 ± 01	15.20	48.80
CS02	7	33.5	0.19 ± 01	7.03	40.53
CS03	7.4	30.9	0.22 ± 01	8.36	39.26
CS04	8	31.0	0.23 ± 00	8.74	39.74
CS05	8	30.2	0.25 ± 04	9.31	39.51
CS06	14	33.6	0.36 ± 01	13.49	47.09
MC07	29	32.0	0.26 ± 04	9.69	41.69
MC08	30	30.8	0.27 ± 01	10.26	41.06
MC09	32	34.5	0.30 ± 00	11.4	45.9
SDC10	45	34.0	0.52 ± 0.01	19.57	53.57
SDC11	52	34.0	0.57 ± 0.00	21.66	55.66
SDC12	54	32.0	0.66 ± 0.01	25.08	57.08
SDC13	54	35.7	0.69 ± 0.05	26.03	61.73

Sample	Labelled cocoa solid (%)	Labelled fat content (%)	Measured theobromine (%) (g/ 100g) ± SD	Calculated NFCS (%)	Calculated cacao (%)
SDC14	55	32.0	0.71 ± 0.01	26.98	58.98
SDC15	55	37.0	0.71 ± 0.01	26.79	63.79
BDC16	60	38.0	0.72 ± 0.02	27.36	65.36
BDC17	60	37.2	0.72 ± 0.04	27.36	64.56
BDC18	60	32.0	0.82 ± 0.03	30.78	62.78
BDC19	70	36.0	0.96 ± 0.02	36.29	72.29
BDC20	80	42.0	0.89 ± 0.02	33.82	75.82

Determination of theobromine content in different types of chocolates: In producing the different types of chocolates, chocolate brands may have varying levels of cocoa butter, cocoa powder, cocoa liquor, milk powder, and sugar in their products [17]. An assessment of theobromine content among chocolate spreads, milk, semisweet dark, and bittersweet dark chocolate samples is shown in Table 2. Among the tested commercially available chocolates, bittersweet dark chocolates had the highest (8.1 ± 1.01 mg/g) concentration of theobromine in comparison to semisweet dark chocolates (6.4 ± 0.79 mg/g), also milk-chocolate (2.7 ± 0.26 mg/g) and chocolate spreads (2.7 ± 0.81 mg/g) had the lowest concentration of theobromine. Of all chocolate samples, the highest content of theobromine was found in chocolate groups with the highest declared content of cocoa solids. Considerably lower levels of theobromine were found in milk and chocolate spreads compared to those of dark chocolates. The theobromine evaluations reported by Zoumas et al. (1980) [24], Belsack et al. (2009) [5], Meng et al. (2009) [17], Bobadilla and Roja (2018) [10] and Marcucci et al. (2021) [2] were consistent with the results in the present paper where Ramli et al. (2001) [25] determined no statically significant difference in the theobromine values between dark and milk chocolates. Furthermore, the levels of theobromine detected for the dark chocolates (both semisweet and bittersweet) being investigated, correspond well to the values regulated by the Ministry of Agriculture, Fisheries, and Food (1998), where the dark chocolates are suggested to contain 237-519 mg per 50 g portion [26]. On the other hand, a comparison of the study results with the recent literature data [7, 27] on milk chocolates reported slightly higher theobromine levels (230-300 mg/100 g vs. 117-196 mg/100 g). The said difference was explained in the above mentioned papers by the fact that the products, containing methylxanthine such as milk chocolates, and chocolate chips may not be precisely determined because scrutinized chocolates may include minute amounts of solid cocoa liquor/mass, which may result in an underestimation of their theobromine levels [17].

Table 2. Theobromine levels (mg/g) in different types of commercial chocolates and chocolate spreads.

Type of chocolate	Theobromine content (mg/g)
Bittersweet dark chocolate (n=10)	8.19 ± 1.09[a]
Semisweet dark chocolate (n=12)	6.40 ± 0.79[b]
Milk chocolate (n=6)	2.75 ± 0.26[c]
Chocolate spreads (n=12)	2.72 ± 0.81[c]

Values are shown as means ± standard deviation of duplicates. Means followed by the different letter within each colum are significantly different at $p < 0.05$ (estimated by analysis of variance (ANOVA) followed by Tukey's test).

Relationship between theobromine and declared cocoa content in different types of chocolates: One quality parameter which is known most is the cocoa solids percentage stated on the label for consumers; and this information represents the ratio of the chocolate in the product that is derived from the cocoa beans [21]. Therefore, the aggregate quantity of the cocoa liquor, pulverized cocoa, and cocoa butter, including any extra cocoa butter that may have been put in the chocolate batch during production, represents the total solid cocoa percentage. Besides, the chocolate producers provide a minimum, not an exact, estimation of cocoa mass the content in their chocolate products. In addition, these products may contain atypical components, such as milk powder in bitter chocolate bars and plant fats (palm, shea) in larger quantities than cocoa butter in dark chocolates [4]. Since the theobromine content mainly depends on cocoa content in chocolates, it was fascinating to test the correlation between actually measured theobromine levels and the cocoa levels declared on the labels of chocolate brands (Table 3). The correlation between theobromine and declared percentage of cocoa solid content was found to be strong in both of the dark chocolate categories, where as the bittersweet chocolates particularly indicated a significant correlation ($r = 0.523$, $p = 0.081$ and $r = 0.771$, $p = 0.009$ for semisweet and bittersweet dark chocolate, respectively) (Table 3). Moreover, despite the differences in theobromine content in samples from the different chocolate categories, a significant positive correlation was noticed between theobromine values of all commercially found dark chocolates and declared cocoa contents ($r = 0.823$, $p = 0.000$) (Table 3). Additionally, the obtained results confirmed that dark chocolate samples with the highest theobromine contents were those typically with the highest declared cocoa mass content ($R^2 = 0.677$) (Figure 1). This result demonstrated that declared cocoa content could indicate the theobromine capacity of dark chocolates sold in Turkey. This is critical to consumers since the data for the cocoa amount is the exclusively readily apparent info on the front-of-pack label of end products.

Table 3. Correlation between measured theobromine content (%) and declared cocoa solids (%) in diferent types of commercial chocolates and chocolate spreads.

Type of chocolate	Pearson's r	p-value
Chocolate spreads (n=12)	0.420	0.175
Milk chocolates (n=6)	0.769	0.074
Semisweet dark chocolates (n=12)	0.523	0.081
Bittersweet dark chocolates (n=10)	0.719	0.019*
Dark chocolates (n=22)	0.823	0,000*

*Correlation significant at 5% level of significance.

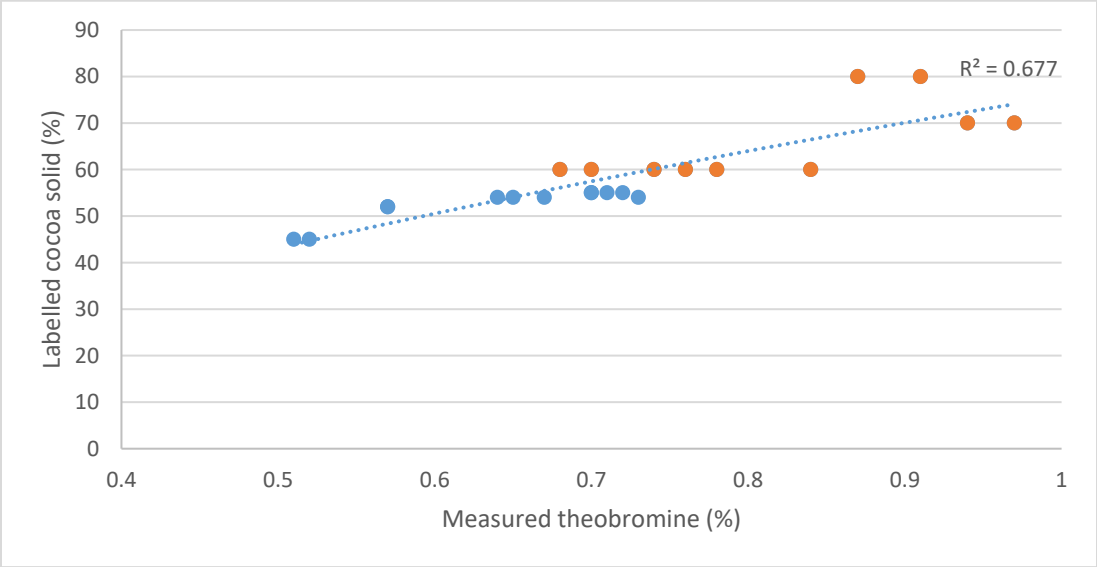


Figure 1. Relationship of measured theobromine content and declared cocoa solids within a sample set of semisweet (blue-circle) dark chocolate and bittersweet (red-circle) dark chocolate samples.

Estimated calculations of NFCS in dark chocolates: The quantity found by multiplying conversion factor by the percentage of theobromine in the samples, designates the dry fat-free cocoa solids (NFCS) in the chocolate [1]. NFCS appears to be related to non-fat cocoa particles. Besides, its linear relationship with theobromine is referred to for using it as an indicator of adulteration [21]. The percentage of cocoa solids stated on the product label and the estimated percent cocoa solids computed through the addition of the percent fat indicated on the label as well as calculated non-fat cocoa solids (NFCS) percentage are given in Table 1. In dark chocolates, NFCS showed a good correlation coefficient with the labeled cocoa solid percentage (%) (R^2 = 0.766); interestingly, with the calculated cocoa solid %, a strict linear correlation was observed as well (R^2 = 0.803) (Figure 2a and 2b). Likewise, in dark chocolates, a high correlation was found between the labeled cocoa solid % and the calculated cacao solid % (R^2 = 0.902) (data not shown). Richards and Wailes (2012) point out that differences in conversion factors to calculate the NFCS could be expected, due to the types of samples were not described clearly (i.e., cocoa, cocoa powder, cocoa liquor) in studies. Consequently, the cocoa level calculation in the checked samples of produced chocolate was usually in a better concurrence where total alkaloids were measured instead of only theobromine alone. A factor of 36.1 (Fat free dry cocoa (g/100g) = total alkaloids (g/100g) x 36.1) is suggested [1]. Additionally, depending on the chocolate type (or the cocoa product), the theobromine to caffeine ratio can differ significantly [9]. As consequence, it is hypothesized that since theobromine and caffeine are always present together in cocoa products, any behavioral influences following the chocolate consumption must depend on the quantity and bioavailability of both of methylxanthine derivatives' synergistic effect [17, 28]. Thus, the lack of a quantitative analysis of caffeine content and not knowing the total alkaloids present in chocolate samples could be the major limitation of the present study and warrants further determination.

Figure 2 (a).

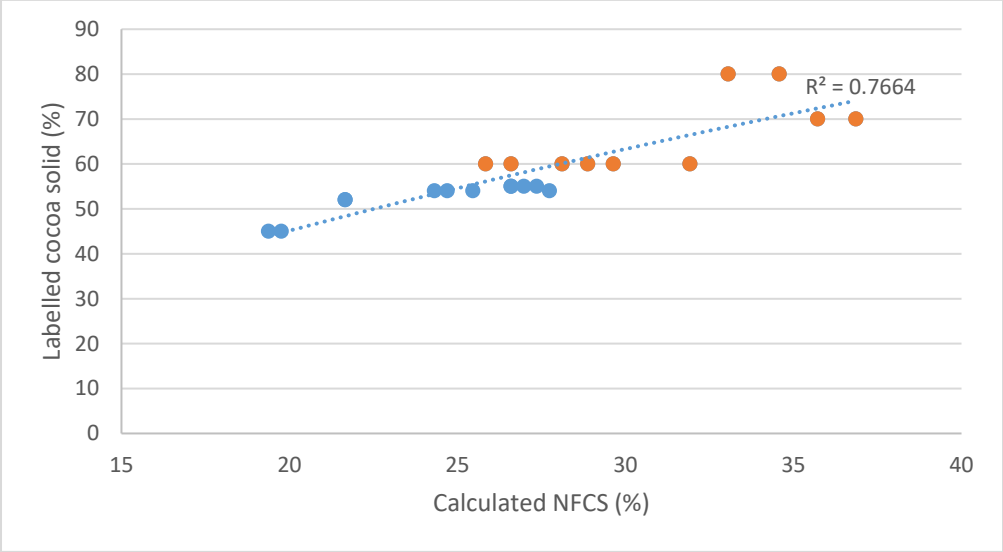


Figure 2 (b).

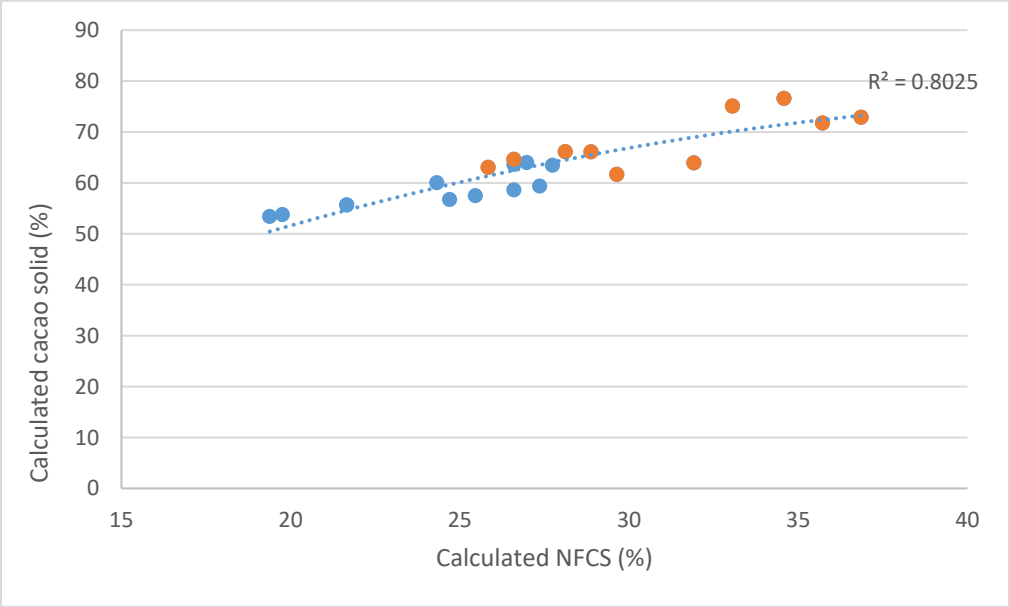


Figure 2. Relationship of **(a)** calculated non-fat cocoa solids (NFCS) and labeled cocoa solids. **(b)** calculated non-fat cocoa solids (NFCS) and calculated cocoa solids within a sample set of semisweet (blue-circle) dark chocolate and bittersweet (red-circle) dark chocolate samples.

UV-Vis spectrophotometric analysis of theobromine: Most of the studies have focused on chromatographic techniques to analyze the methylxanthine levels in cocoa and cocoa products [29]. However, since UV-Vis spectrophotometric assay can apply greater accuracy, precision and repeatability from small samples by using a relatively simple and inexpensive procedure, this method is preferred [30, 31]. However, due to the high spectral overlap of methylxanthines and the matrix interference, spectrophotometric methods have been unsuccessfully applied. To avoid interferences, an extractive-

spectrophotometric procedure of pre-treated samples has been developed. While this procedure showed improved analytical results, it had several preparation steps, and methylxanthine determination was performed in separate stages. Similarly, a spectrophotometric method that employs the first and second derivatives of the absorbance spectra has been claimed to determine methylxanthines in a multitude of food items. Nonetheless, in order to apply the suggested methodology, total matrix removal was suggested [32]. Consequently, employing more precise and accurate analytical methods to determine and quantify methylxanthine substances in chocolate products are particularly important. For this reason, various reversed-phase high-performance liquid chromatography (RP-HPLC), coupled to a diode-array detector (DAD), and mass spectrometry (MS) or tandem MS (MS/MS) have been suggested. Due to the difficulty of cocoa extracts and the absence of commercial standards, a sample purification step employing solid-phase extraction (SPE) has been recommended as well. The coupling of these analytical methodologies with multivariate analysis is also stated to enhance the classification and differentiation of these substances in cocoa and cocoa containing food products [16].

Fifty g of dark chocolate or 30 g of cocoa powder have sufficient theobromine content of 700 mg to produce neurophysiological effects [9-10, 28]. In a two-center, double-blind, randomized, placebo-controlled, full factorial parallel study, 850 mg of theobromine produced beneficial effects on blood lipids [11]. Furthermore, in a dose-range study, with 250, 500, and 1000 mg doses of theobromine, heart rate and alertness increased were no effect on either systolic or diastolic blood pressure observed, as well as the high doses of 1000 mg caused side effects [33]. The human diet includes a very small amount of cocoa products which results in a low concentration of methylxanthine and therefore almost no risk to human health [8]. However, according to our results, to deliver 700-850 mg of theobromine, approximately 100 g of dark chocolate or 300 g of milk chocolate is required. Nevertheless, this amount of chocolate also provides 540-1620 kcal energy with a relatively high content of sugar, around 30-50% w/w and 20-60 g saturated fat, respectively. In this sense, theobromine in chocolate has to be considered in moderation of optimal chocolate intake for health reasons. Therefore, another routine to increase dietary theobromine intake or the development of novel drugs should be recommended.

CONCLUSION

Grasping theobromine levels contained in chocolate products will enable better comprehension of the efficacy in the trials which can be monitored in order to better scrutinize food function and to allow for development of more targeted therapeutics. Following the foregoing, analyzing theobromine will give us an exhaustive approach to comprehend and use the potential of chocolate as a functional food for individualized nutritional cures and enhancing well-being in general. Theobromine level was high in dark chocolate samples, possibly thanks to the higher percentage of the cacao mass used as declared on the product label. It can be deduced that the cocoa mass percentage of dark chocolate bars as stated on the front-of-pack label could be preliminary information for the consumer on theobromine levels they are ingesting. Doing so, the cocoa quantities appearing on the product label, could entail greater access to the information for functional food market as well as acceptance and trustworthiness of functionality claims in order to grant legitimacy to the role of theobromine in chocolate for enhancing individual health. In addition to the foregoing, the impact of theobromine on human health and its possible synergy with other methylxanthine compounds in chocolate must

be clarified. Further studies which could help cover or update food composition databases must also be performed on total methylxanhine analysis of chocolate from different brands, varieties, matrices and on the detection techniques.

Abbreviations: UV-Vis: Ultraviolet-visible, NFCS: Non-fat cocoa solids, CS: chocolate spreads, MC: Milk chocolate, SSD: Semisweet dark chocolate, BSD: Bittersweet dark chocolate.

Authors Contribution: Concept and design: FHE, SK. Analysis and interpretation: FHE, SK. Data collection: FHE, SK. Writing the article: FHE, SK. Critical revision of the article: FHE, SK. Final approval of article: all authors. Statical analysis: all authors. Obtained funding: FHE, SK. Overall responsibilty: FHE

Competing Interests: Authors declare no conflict of interest.

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