EXTRACTION AND CHARACTERIZATION OF FREE NON-POLAR LIPID FRACTION OF CHOCOLATE USING A RAPID ANALYTICAL PROCEDURE

Daniele Naviglio
Department of Chemical Sciences, University of Naples Federico II,
Monte S. Angelo Complex, Naples, Italy
Lydia Ferrara
Department of Pharmacy, University of Naples Federico II, Naples, Italy
Monica Gallo
Department of Molecular Medicine and Medical Biotechnology,
University of Naples Federico II, Naples, Italy

Abstract

In this work has been developed a new analytical procedure to extract the non-polar component of the lipid fraction of the chocolate, in a simple, rapid and economical way and its subsequent analysis by using a gas chromatograph equipped with flame ionization detector (GC-FID). The interest to isolate the non-polar lipid fraction containing triglycerides, waxes, sterol esters and minor components, such as sterols and liposoluble vitamins from the polar fraction could be of three types: analytical, nutritional and commercial. In analytical scope, the quality parameters of the lipid component of chocolate concern the non-polar component. Moreover, the chemical-physical parameters such as the melting point, refractive index, color, etc. were relative to a mixture of fatty substances was not well defined. From the nutritional point of view, the non-polar lipid fraction is the one that gives the largest contribution to the calorific value of the chocolate and then the separation of the two fractions could be useful to better assess the total caloric intake. For economic reasons, not the least important was considering the possibility of fraudulent additions of polar lipid components to replace the non-polar component more valuable, this alteration was not detectable only by applying the official method. The results obtained by the proposed procedure, combined with those obtained with the official method allowed to obtain more complete information on the composition of the lipid component of chocolate. Consequently, the ultimate goal could be reported this information on the nutrition labels of chocolate, for greater product transparency and greater consumer protection.

Keywords: Chocolate, lipid fraction, polar fraction, non-polar fraction,GC method, FID detector

Introduction

Chocolate is a food that can be used either as a finished product or as an ingredient in different types of sweets and ice cream. In the world, the countries that produce cocoa are distributed on three of the five continents such as Africa, Asia and America (Binam and others 2008; Lima and others 2011). The production of cocoa are all of the equatorial belt of the planet, given the climatic requirements of the plant and involving about 45 different countries, with a production of around 4 million and 250 thousand tons of cocoa (Wood and Lass. 2008). Its consumption is more and more increasing in industrialized countries due to its hedonistic character. Italy is the fifth largest country in Europe in the production and

consumption of chocolate (Turrini and others 2001; Fold N. 2001). The cocoa beans are compounds for the 50-57% from fat and these lipids are for the most part, saturated with a 35% stearic acid and 25% palmitic acid. Nevertheless, a good portion of lipids also comes by the acid oleic (35%) a monounsaturated fatty acid found mainly in olive oil and is known to have positive effects on the cardiovascular system. Moreover, its main fat, stearic acid, is absorbed in a very moderate body and in part (about 15%) is transformed in the liver in oleic acid. The dark chocolate is a food that does not influence the level of cholesterol in the blood (Hannum and Erdman 2000). In fact, despite its content of sugar, dark chocolate has in any case a relatively low glycemic index, half less than white bread and similar to that of orange juice. Conversely, the situation is different in the case of other types of chocolate (milk, with hazelnuts, almonds, etc.), in which a more or less important part of the content of lipids also comes from the fat of the milk or other ingredients. For this reason, the high content of lipids and sugars make the chocolate caloric food to be consumed in moderation. The interest about the beneficial effects of chocolate obviously does not come by its content in lipids and sugars, but rather by its abundance of polyphenols. In fact, 50 g of dark chocolate contain twice of polyphenols of a glass of red wine and the same amount of a cup of green tea infusion left in long. The main polyphenols in cocoa are the same as those found in large quantities in green tea (catechins) polymers formed by these molecules, proanthocyanidins, may constitute 12-48% by weight of cocoa beans. Because proanthocyanidins are powerful antioxidants, it is not surprising that cocoa possesses similar properties (Steinberg and others 2003; Kris-Etherton and Keen 2002; Taubert and others 2003). Indeed, calculations carried out so far indicate that chocolate, particularly dark chocolate, possesses antioxidant activity out of the ordinary: a cup of hot chocolate generates antioxidant activity equal to five times that of a cup of black tea three times that a cup of green tea and two to a glass of red wine. Consequently, this high content of polyphenols is proposed as the main responsible of the positive impact on the health of the chocolate and from the nutritional point of view are very interesting studies on the bioavailability of polyphenols (Gallo and others 2013). Moreover, polyphenols that have been shown to reduce the risk factors for diabetes type II and cardiovascular diseases, are recently suggested as complementary agents in the management of obesity through several mechanisms such as decreasing fat absorption and/or fat synthesis. In particular, in a recent work the dark chocolate, a high source of polyphenols, and flavanols in particular, has received attention for its possible role in modulating obesity because of its potential effect on fat and carbohydrate metabolism, as well as on satiety (Farhat and others, 2014).

On the other hand, the lipid component, although present in a minor amount compared to carbohydrates, is the component that gives the energy contribution greater average 300 Kcal against 200 Kcal of carbohydrates for every 100 grams of product (Afoakwa and others, 2007). The lipid component assumes an important role as regards the structure of chocolate giving the correct consistency and allowing processing in many different confectionery products. The official method allows to extract the total lipid component that can be subjected to subsequent characterization analysis of the fat, but turns out to be long and laborious (Adamson and others,1999). In general, the analytical determinations regarding the genuineness of the fat, aimed at exploring the categories of compounds belonging to the free non-polar lipid component, which owns triglycerides, waxes, esters of sterols and minor components that are included in this fraction for their lipophilicity, such as sterols, carotenoids, liposoluble vitamins etc. The fat extracted by the official method is analyzed as such without any prior separation.

The nutritional label of chocolate reports the result as a percentage of fat obtained using the official method. In our opinion, this information is not complete if you do not discriminate between the polar and non-polar fraction. For nutritional purposes, the free and

non-polar lipid component of the fat in chocolate is the one that gives the largest energy contribution to the total fat and therefore it is desirable that the nutrition label of chocolate showing with more detail, not only the weight of the lipid content total, but also the distribution of the fat in its two main components. Finally, for commercial reasons, the differentiation between the two components could be useful to detect possible fraudulent additions fat of polar nature to replace the non-polar component most prized and expensive.

Materials and Methods

Reagents and equipment

All reagents were of analytical purity degree: trichloroacetic acid (Carlo Erba, Milan, Italy), n-hexane (Fluka, Buchs, Switzerland), n-pentane (Fluka, Buchs, Switzerland), 1-pentanol (Fluka, Buchs, Switzerland). Sodium ter-pentoxide(Sigma Aldrich)

The benchtop centrifuge was a PK 131 model (ALC International, Milan, Italy), the rotary evaporator was a R-210 (BUCHI, Italy), to bring dry was used the nitrogen cylinder. Chromatographic separation for triglyceride analysis was performed using a gas chromatograph Autosystem XL (Perkin Elmer, Norwalk, CT, USA) equipped with PSS injector and FID detector and connected to the data acquisition system Turbochrom version 4.1.

Chromatographic separation for fatty acids was performed using a gas chromatograph DANI 8521-a (DANI, Monza, Italy) equipped with a PTV injector and FID detector and connected with an integrator HP mod. 8890 A (Hewlett Packard, Palo Alto, CA, USA

Chromatographic conditions of triglyceride analysis

Capillary column: 65% phenyl methyl silicone stationary phase HT (TG), RTX 65-TG (Restek, Bellefonte,CA,USA), l = 30 m, id = 0.25 mm; ft 0.25 □□

Programmed injector: 70 °C for 12 sec, increase of 999 °C/min up to 370 °C, hold for 5 min.

The temperature was programmed: 250 °C for 2 min., increase of 5 °C/min. up to 360 °C, hold for 5 min.

Detector temperature: 370°C. Flow rate: 1.5 mL/min. Split: 1:80

It was used the system reset of the drift (background) due to the temperature rise by calibrating with three acquisitions of the base line signal in order to allow an integration of the peaks of the triglycerides more simple.

Procedure: 50 mg of fat were weighed in a test tube and add 1 mL of n-hexane; stir until complete dissolution of the fat; inject 0.5 mL of solution into the gas chromatograph. Integrating triglycerides grouped by the total number of carbon atoms

Fatty acids analysis

Capillary column: stationary phase 90% bis-cyanopropyl fenilsilicone FAME (Restek, Bellefonte, CA, USA), l = 50 m, id = 0.25 mm, ft = 0.25 □□

Programmed injector: 50°C for 15 sec., Increase of 999°C/min. up to 270 °C for 3 min.

The temperature was programmed: 70°C for 2 min; increase 8°C/min. up to 250°C, hold for 3 min.

Detector temperature: 270°C. Flow rate 2 mL/min. Split: 1:80

Procedure: 50 mg of fat were weighed in a centrifuge tube, add 1 mL of n-pentane and stirring until complete dissolution of the fat, add 200 mL of 2 N sodium ter- pentoxide in pentanol and stir for two minutes, add 400 mL of 1 N hydrochloric acid and shake for thirty seconds; centrifuge at 2000 rpm for 1 minute; inject 0.5 mL of the upper organic phase.

Samples

The chocolate samples were purchased in the local market and were known brands. The types of chocolate analyzed were: extra bitter chocolate, dark chocolate and milk chocolate.

Quantitative analysis of non-polar lipid fraction of chocolate

Procedure: 10 grams of chocolate finely ground (C) were weighed into a 50 mL centrifuge tube; add 15 mL of trichloroacetic acid at 12% (w/v) and stir until the fluidization of the chocolate; add 10 mL of n-hexane and shake vigorously for 3 minutes and centrifuge at 8000 rpm for 5 minutes. Withdraw the supernatant (hexane phase) and transfer to a 50 mL separatory funnel and repeat the extraction with two other fractions of n-hexane and collect the separating funnel and wash the hexane fraction separated with two fractions of 10 mL of distilled water, transfer the hexane fraction in a volumetric flask and dry in rotary evaporator, remove the last traces of solvent under a stream of nitrogen. Weigh the sample to the technical balance by subtracting the tare to obtain the weight of the fat extracted (L).

The determination of lipids was carried out using a simple formula:

Lipids (%) = L * 100/C

Results and Discussion

Precision and accuracy of the method proposed

The proposed method was first evaluated for reproducibility: the analysis of samples of the three types of chocolate was repeated 5 times and the results obtained are reported in Table 1. The reproducibility of the proposed method was excellent for the three types of chocolate analyzed and the standard deviation from the average result was a maximum of 1.2%.

Table 1. Reproducibility of the proposed method

Sample	Extra chocolate	Dark chocolate	Milk chocolate
1	28.4%	24.0%	27.3%
2	28.2%	23.8%	27.4%
3	28.1%	24.1%	27.1%
4	28.3%	24.4%	27.4%
5	28.0%	24.2%	27.0%
Average	28.2%	24.1%	27.2%
Waste max %	0.7	1.2	0.7

In order to assess the completeness of the recovery of fat, three portions of chocolate from the three types of chocolate above were extracted respectively with two, three and four fractions of n-hexane. In Table 2 are reported the results of recoveries for the three types of chocolate in function of fractions of hexane added. The results show that the recovery can be considered quantity already from the extraction carried out with three fractions of hexane. While with two extractions the fat loss is about 5%, the difference between three and four fractions allows to recover about 1% remaining making complete the extraction.

Table 2. Fat recovery in function of the number of washings with n-hexane

Fraction number	Extra chocolate	Dark chocolate	Milk chocolate
2	95.4%	96.3%	94.1%
3	99.2%	99.4%	99.1%
4	99.9%	100.1%	99.8%

To evaluate the accuracy of the proposed method the three chocolate samples were subjected to analysis of fat by the official method (Adamson 1999). Analysis were repeated three times and the average result was compared with the average result obtained for the reproducibility tests reported in Table 1. Table 3 shows the percentage difference of three

samples compared to the value obtained with the official method, in perfect agreement with the quantity of fatty substance reported on the label for each sample.

Table 3. Accuracy of the proposed method

Sample	Proposed method	Official method	Label	Waste %)
Extra chocolate	28.2 ± 0.2	31.3 ± 0.2	31.3	9.9
Dark chocolate	24.1 ± 0.3	27.0 ± 0.2	27.0	10.7
Milk chocolate	27.2 ± 0.2	29.5 ± 0.2	29.5	7.8

The difference between the quantity of fat obtained with the proposed method compared to the official method represents the polar fraction of free lipids, such as phospholipids and glycolipids. To obtain a confirmation of what was assumed, the fat recovered by extraction with the official method was subjected to fractionation on TLC using a preparative silica gel plate and eluting with hexane and ethyl ether 4:1 (v/v) (Fuchs and others 2011). The bands corresponding to the polar lipid fraction was recovered, purified and analyzed for each type of chocolate. The results were corresponding to the difference percentage shown in Table 3 within the limits of experimental errors.

Analysis of free non-polar lipid fraction

The fat extracted with the proposed method was subjected to qualitative analysis of triglycerides and fatty acids. Figure 1 is shows the gas chromatograms of comparison between triglycerides obtained for the fat extracted from dark chocolate with the official method and that obtained by using the proposed method.

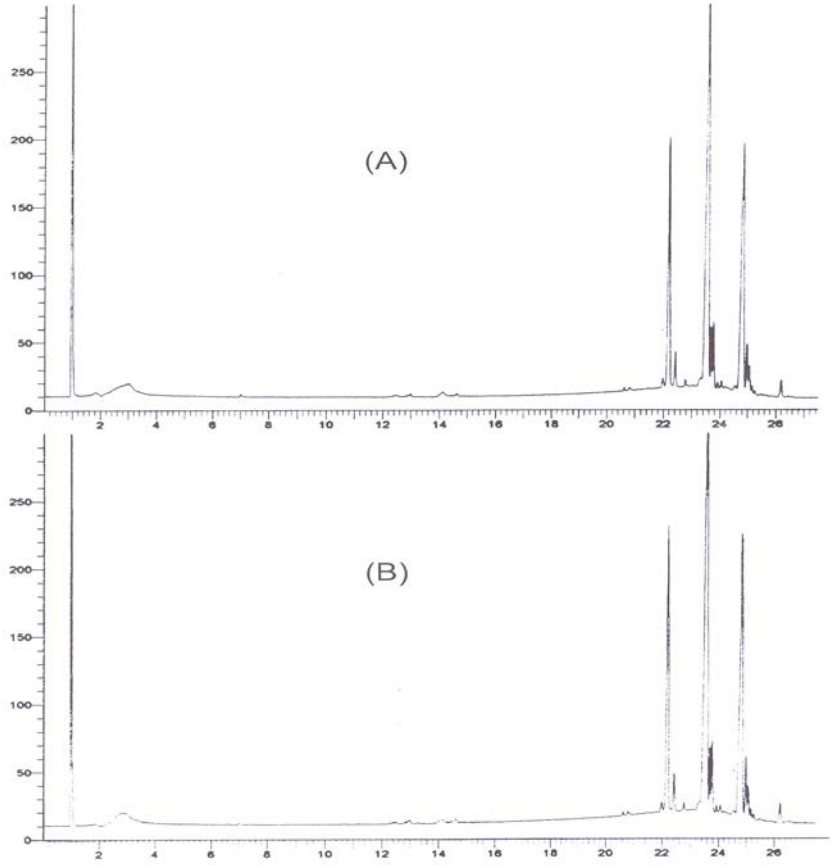

Figure 1. Gas chromatograms of triglycerides obtained by analyzing the fat separated from the same sample of dark chocolate with the official method (A) and with the proposed method (B).

Table 4. Distribution of triglycerides grouped by the same number of carbon atoms in the fat extracted with the official method (A) and in the fat extracted with the proposed method (B)

(A)

Peak #	Time [min]	Area [uV*sec]	Height [uV]	Area [%]	Norm. Area [%]	BL	Area/Height [sec]
1	6.403	980.70	230.10	0.02	0.00	*BB	4.2621
2	6.608	3571.49	729.75	0.07	0.00	*BB	4.8941
3	7.001	9846.20	2173.04	0.19	0.00	*BB	4.5311
4	12.988	49802.78	2455.80	0.95	0.00	*BB	20.2797
5	14.116	75278.09	3560.24	1.43	0.00	*BB	21.1441
6	20.620	7676.81	2736.61	0.15	0.00	*BB	2.8052
7	20.795	10618.90	2673.08	0.20	0.00	*BB	3.9725
8	22.211	1065016.05	191885.29	20.28	0.00	*BB	5.5503
9	23.583	2461684.50	309441.82	46.87	0.00	*BB	7.9552
10	24.835	1505787.71	189426.62	28.67	0.00	*BB	7.9492
11	26.187	62030.37	12672.39	1.18	0.00	*BB	4.8949
		5252293.60	717984.74	100.00	0.00		

(B)

Peak #	Time [min]	Area [uV*sec]	Height [uV]	Area [%]	Norm. Area [%]	BL	Area/Height [sec]
1	6.671	1495.00	314.97	0.02	0.00	*BB	4.7465
2	7.061	3279.50	697.55	0.05	0.00	*BB	4.7015
3	10.702	3841.50	737.97	0.06	0.00	*BB	5.2055
4	11.234	3952.00	513.65	0.06	0.00	*BB	7.6940
5	13.022	68971.15	3529.88	0.99	0.00	*BB	19.5393
6	14.159	103617.80	4117.39	1.49	0.00	*BB	25.1659
7	20.654	26868.00	3977.43	0.39	0.00	*BB	6.7551
8	22.264	1415505.00	223430.18	20.37	0.00	*BB	6.3353
9	23.628	3252221.00	344247.78	46.79	0.00	*BB	9.4473
10	24.893	1986755.00	236970.95	28.59	0.00	*BB	8.3840
11	26.246	83742.50	15959.55	1.20	0.00	*BB	5.2472
		6950248.45	834497.30	100.00	0.00		

In Table 4 are reported the results of the integrations of triglycerides grouped by number of total carbon atoms. As you can see the percentages of clusters obtained for the triglyceride fat derived according to the official method are the same as those obtained for the fat extracted according to the procedure proposed within the limits of experimental error

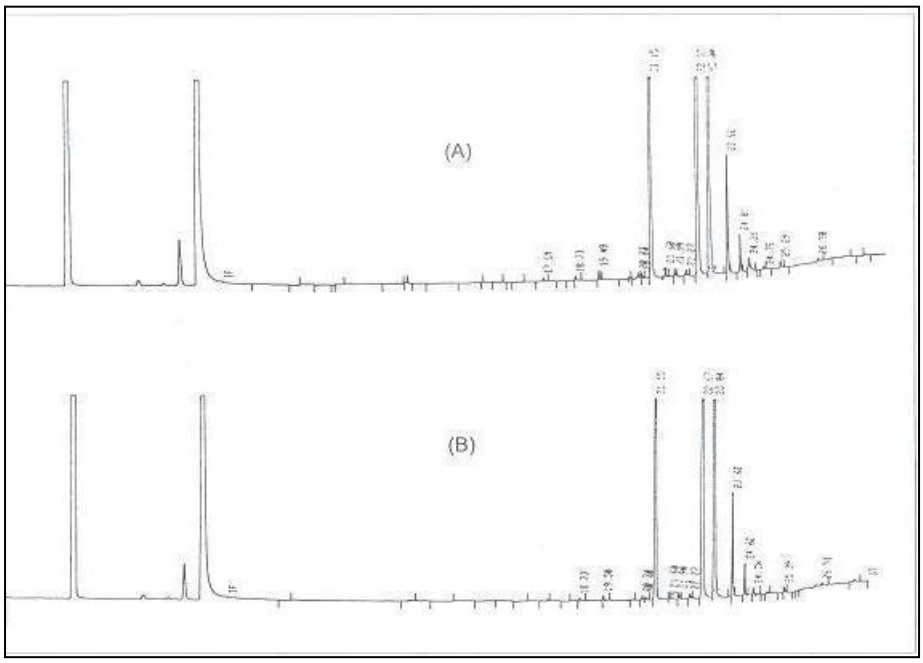

Figure 2. Gas chromatograms pentylic esters of fatty acids obtained by analyzing the fat separated from the same sample of dark chocolate with the official method (A) and with the proposed method (B).

Figure 2 shows the gas chromatograms of pentylic esters of fatty acids obtained as reported in a previous work (Nota and others 1998). The replacement of the alcohol with a longer chain as reagent for the transesterification has no contraindication in the analysis itself; gas chromatographic conditions were identical and the only variable modified was the increase of 20°C in the room both in the initial isotherm that in the final one and consequently also the injector was increased to 20 °C.

In Table 5 are reported the results of the integrations of the fatty acids of the free non-polar lipid fraction of dark chocolate extracted with the official method and with the method proposed. The results are in agreement with each other. The same comparison was also made for the extra dark chocolate and milk chocolate; also for these other two matrices the gas chromatographic analysis of the fat fraction extracted with the two methods leads to the same results, both in terms of triglycerides that of fatty acids. The results obtained from the qualitative analysis of the free non-polar lipid component of the fat of chocolate using the official method and the proposed method indicate that the fat is not in any way compromised by contact with trichloroacetic acid in the proposed procedure

Table 5 . Distribution of fatty acids in the fat of dark chocolate extract with the official method and the proposed method

Fatty acids	Proposed method		Official method	
	Retention time	Area %	Retention time	Area %
Palmitic acid	21.15	26.3	21.15	26.0
Stearic acid	22.67	34.3	22.67	35.8
Oleic acid	23.04	34.2	23.04	33.7
Linoleic acid	23.62	3.0	23.62	3.2
Linolenic acid	24.01	1.0	24.01	1.0
Other		1.2		0.3

Effect of trichloroacetic acid in separation of free non-polar lipid fraction

The trichloroacetic acid possesses a complexing ability and dehydrating against polar molecules. It combines the characteristic acidity of an organic complexing action due to the trichloroacetate ion size.

The removal of water molecules from the medium allowed to polar substances to join and separate in the solid form. The importance of this action is highlighted by the fact that if the trichloroacetic acid is replaced by trifluoroacetic acid, the latter did not lead to the same results. Additionally, the use of a mineral acid showed that chocolate cold was not immediately fluidized and consequently the addition of n-hexane did not elicit the desired effect of the separation of the free fraction non-polar. Conversely, the addition of trichloroacetic acid to the chocolate had an immediate effect fluidizing with release of fat; in fact, the centrifugation of chocolate dissolved in n-hexane led to the separation of fat as a separate phase in the supernatant; therefore, the addition of hexane was used to help the quantitative recovery of fat as well as to wash the solid matrix.

Conclusion

Currently, the extraction of total fat for the analysis of the lipid component of chocolate is made with the official method (Adamson 1999). This method is used by manufacturers of chocolate to determine the lipid content of labeling. It is possible to subject the extracted lipid fraction to fractionation on TLC to differentiate the non-polar lipid component free from that polar, however, this procedure is long and laborious. Using the method proposed in this work it was possible to obtain not only the non-polar lipid fraction separated from the rest of the matrix, but also the free fraction polar for difference with the value obtained with the official method. In this way, this procedure allowed to obtain information on the content of fat in the chocolate more complete and, also, could be used by manufacturers in order to appear on labeling the fat content of the chocolate in two separate

components. Consequently, the quantification of the non-polar lipid fraction of the chocolate was important from the standpoint of economic and nutritional, in addition, the separation upstream from the matrix of chocolate resulted also important from the analytical point of view.

References:

Adamson GE, Lazarus SA, Mitchell AE, Prior RL, Cao G, Jacobs PH, Kremers BG, Hammerstone JF, Rucker RB,, Ritter KA, Schmitz HH. 1999. HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. J. Agric. Food Chem., 47, 4184-4188

Afoakwa EO, Paterson A, Fowler M. 2007. Factors influencing rheological and textural qualities in chocolate – a review. Trends in Food Science & Technology 18(6): 290-298.

Binam JN, Gockowski J, Nkamleu GB. 2008. Technical efficiency and productivity potential of cocoa farmers in west african countries. The Developing Economies 46(3):242-263.

Farhat G, Drummond S, Fyfe L, Al-Dujaili EA. 2014. Dark Chocolate: An Obesity Paradox or a Culprit for Weight Gain? Phytotherapy Research 28(6):791-797.

Fold N. 2001. Restructuring of the European chocolate industry and its impact on cocoa production in West Africa. Journal of Economic Geography 1:405-420.

Fuchs B, Süß R, Teuber K, Eibisch M, Schiller J. Lipid analysis by thin-layer chromatography-A review of the current state. Journal of Chromatography A 2011, 1218(19), 2754-2774.

Gallo M, Vinci G, Graziani G, De Simone C, Ferranti P 2013 The interaction of cocoa polyphenols with milk proteins. Food Research International 54(1):406-415.

Hannum SM, Erdman Jr JW. 2000. Emerging Health Benefits from Cocoa and Chocolate. Journal of Medicinal Food 3(2): 73-75.

Kris-Etherton PM, Keen CL. 2002. Evidence that the antioxidant flavonoids in tea and cocoa are beneficial for cardiovascular health. Curr Opin Lipidol 13:41-49.

Lima LJR, Almeida MH, Nout MJR, Zwietering MH. 2011. Theobroma cacao L., "the food of the gods": quality determinants of commercial cocoa beans, with particular reference to the impact of the fermentation. Critical Reviews in Food Science and Nutrition 51(8):731-761.

Nota G, Naviglio D, Romano R, Sabia V, Spagna Musso S. 1998. Evaluation and improvement of transesterification methods of triglycerides. Anal. Lett. 31(14): 2499-2512.

Steinberg FM, Bearden MM, Keen CL. 2003. Cocoa and chocolate flavonoids: implications for cardiovascular health. J Am Diet Assoc. 103:215-223.

Taubert D, Berkels R, Roesen R, Klaus W. 2003. Chocolate and blood pressure in elderly individuals with isolated systolic hypertension. J Am Med Assoc 290:1029-1030.

Turrini A, Saba A, Perrone D, Cialfa E, D'Amicis A. 2001.Original Communication Food consumption patterns in Italy: the INN-CA Study 1994-1996. European Journal of Clinical Nutrition 55:571-588.

Wood GAR, Lass RA. 2008. Cocoa. Tropical agriculture series. Editors: John Wiley & Sons.