THE INFLUENCE OF EXPERIMENTAL CONDITIONS ON POLYPHENOL CONTENT DETERMINATION IN COCOA PRODUCTS

Chiş Adriana*, Purcărea Cornelia*, Borbély Mária, Balog Olivia*****

* University of Oradea, Faculty of Environmental Protection, 26 Gen. Magheru Bd., zip code 410048, Romania, e-mail: andichis@yahoo.com
** University of Debrecen, Centre for Agricultural and Applied Economic Sciences, Faculty of Agricultural and Food Sciences and Environmental Sciences, Central Laboratory, Hungary
*** Sugar Oradea S.A, 3 Borsului Drive, zip code 410605, Oradea, Romania

Abstract

This paper presents the results of the determination of polyphenol content in cocoa and cocoa products using Folin-Ciocâlteu assay in order to establish the best experimental variant in terms of sample amount (50µl and 100µl) and time of reaction (30, 60 90 and 120 minutes) necessary. Gallic acid was the used standard. The experiments show that the doubling of the volume of the sample leads to the decrease of the reaction time to one third. The tested samples shown that the polyphenol content decreases in the order cocoa > dark chocolate > Nesquik.

Key words: polyphenols, cocoa products, Folin-Ciocalteu assay.

INTRODUCTION

In the last decade people became more and more aware about the fact that food quality is very important for life quality. So, beyond the energetic contribution and main constituents i.e. proteins, lipids and sugars, minor constituents of food began to be screened in order to identify their role on human health. The substances having antioxidant role have been found to be particularly important and became an active area of scientific interest.

Among the most common food sources in terms of polyphenols can be named vegetables (Al-Juhaimi and Ghafoor, 2011, Ting et al., 2007, Vinson et al, 1998) but also fruits like berries, cherries, grapes, pears and plums, strawberries, raspberries and others (Pantelides et al., 2007, Sochor et al, 2010). Polyphenols can be obtained by drinking red wine (Stratil et al, 2008), coffee and chocolate (Wollgast, 2004, Kroyer and Molnar, 2009, Jonfia-Essien et al., 2008), green tea (Anesini et al, 2008), olive oil (Carrasco-Pancorbo A, 2005).

In food science an antioxidant is defined as a substance that significantly decreases or prevents the adverse effects of reactive species, such as reactive oxygen and nitrogen species (ROS/RNS), on normal physiological function in humans (Halliwell et al. 1995; Huang et al. 2005)

when present at low concentrations compared to those of an oxidizable substrate.

There is not a single assay to determine the antioxidant content or antioxidant activity. In view of the inactivation mechanism involved, the antioxidant capacity methods can be divided in two categories: hydrogen atom transfer reaction (HAT) and electron transfer (ET) reaction-based methods (Karadag et al. 2009). The differentiation between ET and HAT mechanism is not easy in complex samples as food, so interpretation can differ. Folin-Ciocâlteu assay was considered under the methods utilizing ET mechanism by Huang et al. (2005) and MacDonald-Wicks et al. (2006). But Prior et al. (2005) classified it under the methods utilizing both ET and HAT mechanisms.

For the experiments presented in this paper we used the Folin-Ciocâlteu assay. F-C is a complex reagent based on phosphomolybdic and phosphotungstic acid, the exact chemical composition being unknown. The phenolic compounds transfer electrons to molybdenum, forming blue complexes that can be monitored spectrophotometrically at 750–765 nm (Singleton, 1965). The phenolic compounds react with the reagent only under basic conditions (MacDonald-Wicks et al. 2006), sodium carbonate solution being used in order to rich pH 10.

This method is used to quantify polyphenolic compounds in many vegetal origin foods such are leafy vegetables and spices (Al-Juhaimi Fahad and Kashif Ghafoor, 2011), wine (Stratil et al, 2008), cocoa (Wollgast, 2004, Kroyer and Molnar, 2011, Jonfia-Essien et al., 2008), tomatoes (Moigrădean et al, 2007), sour cherry (Filimon et al, 2011) and animal origin such is honey and bee products. (Maghitaş et al, 2009). In the specific case of cocoa and cocoa products scientific literature presents different techniques for this assay, regarding the phenolic extraction and the determination as such of the phenols. The differences refers mainly to the volume of samples (phenolic extract) from 50 µl (Wollgast, 2004) to 250 µl (Jonfia-Essien et.al., 2008) but to the reaction time too, from 30 minutes (Wollgast, 2004) to 120 minutes (Tabernero et al., 2006, Belščak et al., 2009).

The aim of our experiments was to determinate the best experimental conditions for the quantification of phenolic compounds in cocoa and cocoa products using a variant of Folin-Ciocâlteu assay which will be exposed on Methods section.

MATERIALS AND METHODS

Materials

The tested materials consist in cocoa and cocoa based products, three samples of each:

1. M1 - Chocolate with a cocoa content of 85%, net weight 80 g. According to the label, the product has the following ingredients: cocoa, cocoa butter, cocoa powder, sugar, emulsifier: soy lecithin, flavouring: natural extract vanilla.
2. M2 - Cocoa powder, Bio product of controlled biological agriculture, net weight 125 g. According to the label, the product has the following ingredients: cocoa powder.
3. M3 - Nesquik instant cocoa with vitamins, net weight 15 g and According to the label, the product has the following ingredients: sugar, low fat cocoa 18%, emulsifier: soy lecithin, minerals: magnesium carbonate, calcium carbonate, salt, vitamin C, nicotiamide (niacin), vitamin E, vitamin B1, vitamin B6, folic acid, pantothenate, calcium (pantothenic acid), cinnamon, flavouring: natural vanilla.

All the used reagents were p.a. grade: Folin Ciocâlteu reagent and hexane from Merck Germany, methanol from Scharlau Germany, Gallic acid from Roth Germany and sodium carbonate from Chemopar Romania The laboratory devices were: ultrasonic bath Elma S 100H – Elmasonic, centrifuge Universal 320 - Hettik Germany, rotary evaporator IKA RV 10 digital and spectrophotometer UVMini-1240 (Shimatzu).

Methods

The Folin-Ciocalteu assay was used in order to determine the polyphenolic content of the tested samples. Due to the fat content of the samples the determination has several stages, as follows:

1. Degreasing 1 g sample with 10 ml hexane in an ultrasonic bath followed by centrifugation 10 minutes at 3000 rot/min and decantation (Wollgast, 2004).
2. Extraction of the polyphenols with a mixed solvent of water: methanol (80:20, v/v) in an ultrasonic water bath, the solvent being subsequently removed using a rotary evaporator at 50^0 C (Kroyer and Molnar, 2011).
3. Determination of polyphenols: 1.5 ml Folin-Ciocalteu reagent (diluted 1:10 with distilled water), 1.5 ml 7.5% sodium carbonate and 50 µl (A variant) or 100 µl (B variant) polyphenolic extract from cocoa based products was mixed in a test tube. Then the mixture was well stirred with a vortex and allowed to stand for

30 (60, 90, 120) minutes at room temperature. Then the absorbance was measured at 765 nm against a blank containing water instated of the sample.

The calibration curve was made with gallic acid as standard of phenols, stock solution concentration being 1g/l. Dilution of the stock solution was made with distilled water from 50 to 600 mg/ l.

RESULTS AND DISCUSSIONS

The calibration curves obtained by reading the absorbance at 30, 60, 90 and 120 minutes using 50 µl standard (A variant) show, as expected, linear correlation but with different correlation factor (R^2) respectively 0.7983 (30 min), 0.8661 (60 min), 0.9521 (90 min), 0.9666 (120 min). So for the calculation we used both the calibration curve obtained after 90 min and 120 min. The results are shown in table 1.

Table 1

Polyphenol content, sample volume 50 µl

Sample	90 min		120 min	
	mg GAE/g	∓SD	mg GAE/g	∓SD
1M	40.52	1.201	37.58	1.186
2M	92.96	8.780	87.87	7.993
3M	-25.09	25.811	-29.99	20.776

The experimental values for dark chocolate are closed to those obtained by Belščak et al., 2009 (22 - 32 mg/g GAE) but higher then those reported by Tabernero et al. 2006 (18,2 mg/g GAE) or Kroyer and Molnar , 2011 (8 mg/g GAE). As for cocoa, the variability of reported values is much higher, from 22 mg/g GAE at Kroyer and Molnar to 80 mg/g GAE at Tabernero et al. 2006 and Jonfia-Essien et al. 2008, witch are in the same area with those we calculated in the present work. The values obtained for Nesquik (3M) are not reliable so they cannot be compared with others from cocoa products. The problem could be due to the complex content of the products which do not allow a proper extraction of phenolic compounds in order to be quantified.

The use of 100µl sample (B variant) also shows linear correlation at 30, 60, 90 and 120 minutes, but with correlation factor (R^2), respectively 0.9476 (30 min), 0.9439 (60 min), 0.9497 (90 min), 0.9227 (120 min). The correlation is practically equal from 30 to 90 minutes, decreasing only at 120 minutes. The concentration values for the tested samples are shown in table 2. The calculation was done at 30 minutes, 60 minutes and 90 minutes. We also performed calculations for 120 minutes to compare their.

The content of polyphenols calculated at 30, 60 and 90 minutes are in the same value area as those obtained in A variant for cocoa and dark chocolate. For Nesquik the results are not reliable, nor ware they reliable in the A variant.

Table 2

Polyphenol content, sample volume 100 µl (B)

	Sample		
	1M	2M	3M
30 min			
mg GAE/g	48.60	105.96	-78.84
∓SD	5.576	9.133	77.345
60 min			
mg GAE/g	54.93	109.09	-50.48
∓SD	12.420	11.342	55.070
90 min			
mg GAE/g	39.87	99.42	-35.78
∓SD	16.040	13.795	40.098
120 min			
mg GAE/g	25.56	40.40	-44.80
∓SD	3.685	8.565	60.169

As the aim is to find the best experimental condition we compared the obtained results by statistical analysis. To this end the t-test was used. The calculation was made only for chocolate (1M) and cocoa (2M) because the values obtained for Nesquik (3M) were irrelevant in both variants, as it can be seen in table 1 and table 2. The comparison refers to A variant and B variant results at different experimental laps of time. On the other hand we compared the results of the two experimental variant applied. Table 3 presented the statistical results for A and B variants separately and table 4 the statistical results for A variant compared to B variant.

Table 3

Statistical analysis – t Test - A variant (50µl) and B variant (100 µl)

A variant		
1M - 90	1M - 120	ns
2M - 90	2M - 120	ns
B variant		
1M - 30	1M - 60	ns
	1M - 90	ns
	1M - 120	***
2M - 30	2M - 60	ns
	2M - 90	ns
	2M - 120	**

Legend:

ns Non-significant (p>0.05);
** Distinctly significant (p<0.01);
*** Very significant (p<0.001)

Table 4

Statistical analysis – t Test - A variant (50µl) versus B variant (100 µl)

A	**B**			
	1M - 30	1M - 60	1M - 90	1M - 120
1M – 90	ns	ns	ns	**
1M - 120	ns	ns	ns	**
	2M - 30	2M - 60	2M - 90	2M - 120
2M - 90	ns	ns	ns	*
2M - 120	ns	ns	ns	*

Legend:
ns Non-significant (p>0.05);
* Significant (p<0.05)
** Distinctly significant (p<0.01);

CONCLUSIONS

The determination of phenolic compounds by Folin Ciocâlteu assay in cocoa and cocoa products leads us to some conclusion.

The method can be applied in different experimental condition without affecting the results in some limits regarding the amount of sample and the period of reaction. So using 100 µl of sample (B variant) one can reach the results in only 30 minutes, towards the use of 50 µl (A variant) when we need at least 90 minutes for the determination of the same amount of phenolic compounds. This assertion proved to be valid for cocoa and rich cocoa products as dark chocolate (85% cocoa), but not for pour cocoa products (18%) as Nesquik. The complex content of this products leads to the conclusion that a different procedure must be applied for proper extraction of phenolic compounds.

After more then 90 minutes in the case of using 100 µl of sample, the coloured complex become unstable, it decomposes both for cocoa and dark chocolate.

As a general conclusion, for the particular tested products, depending on the availability of sample and time, the experiments show that increasing twice the sample volume lead to decreasing the reaction time at one third. Further experiments on different quality of cocoa and different chocolate types are needed in order to extend those conclusions.

The content of polyphenols in the tested samples decreases in the order cocoa > dark chocolate > Nesquik.

REFERENCES

1. Al-Juhaimi, F and K Ghafoor, 2011, Total Phenols and Antioxidant Activities in Leaf and Steam Extracts from Coriander, Mint and Parsley Grown in Saudi Arabia, pak. J. Bot., 43(4): pp 2235-2237
2. Anesini, C, G.E Ferraro, R Filip, T, 2008,otal Polyphenol Content and Antioxidant Capacity of Commercially Available Tea (Camellia sinensis) in Argentina, J. Agric. Food Chem., 56, pp 9225–9229
3. Belščak, A, D. Komes, D Horzic, K Kovačevič, K Ganic, D Karlovic, 2009, Comparative study of commercially available cocoa products in terms of their bioactive composition, Food Research International 42, pp 707–716
4. Carrasco-Pancorbo A, L, Cerretani,A. Bendini, G. Segura-Carretero, A. Del Carlo, T. Gallina-Toschi, 2005, Evaluation of the Antioxidant Capacity of Individual Phenolic Compounds in Virgin Olive Oil, J. Agric. Food Chem, 2005, 53, pp 8918-8925
5. Filimon R.V., D.Beceanu, M. Niculaua, C. Arion, 2011, Study on the Anthocyanin Content of Some Sour Cherry Varieties Grown in Iaşi Area, Romania, Cercetări Agronomice î n Moldova, Vol XLIV, No1(145): pp 81-91
6. Halliwell B, MA Murcia, S Chirico, OI Aruoma, 1995, Free radicals and antioxidants in food and in vivo: what they do and how they work. Crit Rev Food Sci Nutr pp 35:7
7. Huang D, B Ou, RL Prior, 2005, The chemistry behind antioxidant capacity assays. J Agric Food Chem 53: pp1841-1847
8. Jonfia-Essien W.A., G. West, PG. Alderson, G. Tucker, 2008, Phenolic content and antioxidant capacity of hybrid variety cocoa beans, Food Chemistry, 108: pp 1155–1159
9. Karadag Ayse, B. Ozcelik, S. Saner, 2009, Review of Methods to Determine Antioxidant Capacities, Food Anal. Methods2: pp 41–60
10. Kroyer G and T Molnar, 2011, Bioactive ingredients in Cocoa and Chocolate Products and their Health Promoting Properties, P2, Poster Session: Chemistry, Biochemistry and Composition, 1st International Congress on Cocoa, Coffee and Tea, Novara, Italy
11. MacDonald-Wicks LK, LG. Wood, ML. Garg, 2006, Methodology for the determination of biological antioxidant capacity in vitro: a review. J Sci Food Agric 86(13):pp 2046-2050
12. Mărghitaş l. Al**,** D. Dezmirean**,** Cristina Pocol, Marioara Ilea, Otilia Bobis, I. Gergen, 2010, The Developement of a Biochemical Profile of Acacia Honey by Identifying Biochemical determinantsnof its Quality, Not. Bot. Hort. Agrobot. Cluj 38(2) Special Issue, pp 84-90
13. Moigrădean, D, M Poiană, IGogoaşă, M Hărmănescu, I Gergen, A Lăzureanu, 2007, The Correlations Between Total Antioxidant Capacity and Total Polyphenols Content Established for Tomatoes, Lucrări Stiinłifice Medicină Veterinară VoL. XL, TIMISOARA, pp 486-489
14. Pantelidis, G.E., M. Vasilakakis, G.A. Manganaris, Gr. Diamantidis, 2007, Antioxidant capacity, phenol, anthocyanin and ascorbic acid contents in raspberries, blackberries, red currants, gooseberries and Cornelian cherries, Food Chemistry 102, pp 777–783

15. Prior RL, X Wu, K Schaich, 2005, Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem 53(8):pp 3101–3113.
16. Singleton V.L. and J.A Rossi, 1965, Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture 16, pp. 144-158
17. Sochor J, O Zitka, H Skutkova, D Pavlik, P Babula, B Krska, A Horna, A Vojtech, I Provaznik, R Kizek, 2010, Content of Phenolic Compounds and Antioxidant Capacity in Fruits of Apricot Genotypes, Molecules, 15, pp 6285-6305;
18. Stratil, P, V Vlastimil Kubáň, J Fojtová, 2008, Comparison of the Phenolic Content and Total Antioxidant activity in Wines as Determined by Spectrophotometric Methods, Czech J. Food Sci. Vol. 26, No. 4: pp 242–253
19. Tabernero Maria, J Serrano, F Saura-Calixto, 2006, The antioxidant capacity of cocoa products: contribution to the Spanish diet, International Journal of Food Science and Technology, 41 (Supplement 1), pp 28–32
20. Ting, S, JR. Powers, J Tang, 2007, Evaluation of the antioxidant activity of asparagus, broccoli and their juices, Food Chemistry, 105, pp 101–106
21. Vinson, J.A., Y Hao, X Su, L Zubik L., 1998, Phenol Antioxidant Quantity and Quality in Foods: Vegetables. Journal of Agricultural and Food Chemistry 46, pp. 3630-3634.
22. Wollgast, Ian, The contents and effects of polyphenols in chocolate, Gießen, 2004, Dissertation for obtaining the degree of doctor at the faculty of Agricultural and Nutritional Sciences, Home Economics, and Environmental Management at the University of Gießen, Germanygeb.uni-giessen.de/geb/volltexte/2005/2239/.../WollgastJan-2005-06-10.pd.