THE INFLUENCE OF THE EXTRACTION SOLVENT ON THE POLYPHENOL CONTENT DETERMINATION IN COCOA PRODUCTS

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Abstract

This paper investigates the influence of the extraction solvent on the polyphenol determination in cocoa and cocoa products by Folin-Ciocâlteu assay using gallic acid as standard at 90 minutes reaction time. The experimental variants tested refer to extraction solvent (acetone and methanol) and sample or standard amount (50 µL- a variant and 100 µL – b variant). The results show that the solvent influences the determination of the polyphenol content only for dark chocolate in "a" variant, for all the others variant the differences were not significant. Using acetone instead of methanol does not improve the determination on low content cacao products such is Nesquik. The content of polyphenol was grater in cocoa that for dark chocolate.

Key words: methanol, acetone, polyphenols, cocoa products

INTRODUCTION

In order to preserve health, people all around the world are more and more interested in food quality. Nowadays this is related not only to the main constituents i.e. proteins, lipids and sugars, but also to the minor ones. Polyphenols are an important source of antioxidants in our diet so all the raw material for foods processing which contains this kind of component is valuable and subject to scientific interest.

The health benefit of cocoa induced by antioxidants was investigated regarding general effects (Abbe Maleyiki and Ismail, 2008) or on different specific directions like vascular health (Rimbach et al, 2009) or LDL-oxidative susceptibility (Wan et al, 2001).

A lot of vegetal origin foods have important antioxidant content. Saxeby, 2014, citing Perez-Jimenez et al, 2010, shows that among 100 investigated products, cacao is on the 4th place and chocolate on the 8th. That is really important because the other foods on the first places are spices so their consumption is lower. Scientific studies were conducted on a lot of those plant-origin antioxidants such as blackberries (Pantelides et al., 2007, Sochor et al, 2010), vegetables (Bahorun et al, 2004, Priecina and Karklina, 2013), spices (Terpinc et al, 2009, Drużyńska and Wojda, 2007, Cioroi and Dumitriu, 2009). Cocoa and cocoa based products were also investigated for determining their antioxidant activity by scientists all over the world: in Europe Wollgast, 2004, Kroyer and Molnar, 2011, Arlorio et al, 2008, Jonfia-Essien et al., 2008 Tabernero et al., 2006 in Asia Abbe Maleyki and Ismail, 2009, Lee et al, 2003 and Subhashini, 2010.

There are several tests for determining antioxidant content in food: ORAC (Antioxidant reaction with peroxyl radicals), FRAP (Antioxidant reaction with a Fe(III) complex), DPPH (Antioxidant reaction with an organic radical), ABTS (Antioxidant

reaction with an organic cation radical) or Total Polyphenol Content by Folin Ciocâlteu assay.(Karadag et al. 2009, Pisoschi and Negulescu, 2011)

In the present experiment we used Folin-Ciocâlteu assay which is widely used for polyphenol content determination in cocoa and cocoa-based products. Basically the phenolic compounds transfer electrons to molybdenum from this complex reagent based on phosphomolybdic and phosphotungstic acid only under basic conditions (MacDonald-Wicks et al. 2006). A blue complex is formed and its absorption is determined spectrophotometrically at 750–765 nm (Singleton, 1965).

As for cocoa and cocoa based products, scientific literature presents different techniques for this assay, regarding different aspects. The differences refer to the volume of samples (phenolic extract) from 50 µl (Wollgast, 2004) to 250 µl (Jonfia-Essien et.al., 2008) but also to the reaction time, from 30 minutes (Wollgast, 2004) to 120 minutes (Tabernero et al., 2006, Belščak et al., 2009 or extraction solvent acetone (Jonfia-Essien et.al., 2008, Wollgast, 2004) or methanol Serra Bonvehi, 1997, Adamson et al, 1999). The used standards are gallic acid or catechin.

Our previous experiments investigated the influence of the reaction time in relation to the sample amount (Chiș et al, 2013) using aqueous methanol extracts. The purpose of this experiment was to determine the influence of the extraction solvent, at a certain reaction time, in relation to the sample amount on the quantification of phenolic compounds in cocoa and cocoa based products using a variant of Folin-Ciocâlteu assay which will be exposed on Methods section

MATERIALS AND METHODS

Materials - The tested materials consist in black chocolate, with a cocoa content of 85% (code 1), cocoa powder, bio product of controlled biological agriculture (code 2) and Nesquik powder for children (code 3), three samples of each. Regarding the extraction solvent, the samples were coded 1M and 1A, 2M and 2A, respectively 3M and 3A.

The experiments were performed during 2013 in the Food control laboratory of The Environmental Protection Faculty

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

Methods - The polyphenolic content was determined using Folin-Ciocalteu assay, going through several steps, as follows:

1. Degreasing 1 g sample with 10 ml hexane twice, at 30^0C in an ultrasonic bath followed by 10 minutes centrifugation at 3000 rot/min and decantation (Wollgast, 2004).
2. Extraction of the polyphenols, two variants:

- with a mixed solvent of water: methanol (80:20, v/v) in an ultrasonic water bath, 10 minutes at 30^0C, the solvent being subsequently removed using a rotary evaporator at 50^0 C (Kroyer and Molnar, 2011)
- with a mixed solvent of acetone: water: HCl 1M (70:29.5:0,5 v/v/v) in an ultrasonic water bath, 10 minutes at 20^0C ,the solvent being subsequently removed using a rotary evaporator at 40^0 C (Wollgast, 2004, Garcia-Salas et al, 2010)

3. Determination of polyphenols: 1.5 ml Folin-Ciocalteu reagent 0,2N, 1.5 ml 7.5% sodium carbonate 7,5% and 50 µl (a variant) or 100 µL (b variant) of phenolic 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 in the same conditions with gallic acid as standard of phenols, stock solution concentration being 1g/l. Dilution of the stock solution was made with distilled water.

RESULTS AND DISCUSSIONS

All the calibration curves obtained with standard dilutions from 50 to 600 mg/L Gallic acid, using 50 µL or 100 µL standard show, as expected, linear correlation no meter the time of reaction, between 30 to 120 minutes. The correlation factor (R^2) values are shown in table 1 for all the experimental conditions tested in this experiment.

Table 1

R^2 value for Folin Ciocâlteu assay

Reaction time	Standard amount	
	50 µL	100 µL
30 minutes	0.7983	0.9476
60 minutes	0.8661	0.9439
90 minutes	0.9521	0.9497
120 minutes	0.9666	0.9227

The experimental variant using 50 µL shows the best correlation 90 minutes of reaction. When 100 µL of sample are used, there are no significant differences at 30, 60 or 90 min of reaction. So, in order to compare the results, we used the equation of the calibration curves at 90 minutes for both 50 and 100 µL variant. The polyphenol content calculated for the tested samples is shown in Table 2.

Table 2

Polyphenol content, mg GAE/g, mean ∓SD

Sample	Experimental variant	
	A	b
1A	79.92∓3.11	55.50∓5.98
1M	40.52∓1.201	39.87∓16.04
2A	122.83∓11.201	134.76∓9.833
2M	92.96∓8.78	99.42∓13.795
3A	- 25.29 ∓25.81	- 35.78∓ 40.09
3M	9.92∓5.22	58.06∓30.02

In all cases the acetonic extract lead to greater values for polyphenol content, both for cocoa and dark chocolate. The values obtained for Nesquik are not reliable nor for methanolic or acetonic extracts so can't they be compared with others referring to cocoa products. The complex content of the product do not allow a proper extraction of phenolic compounds in order to be quantified, using the present procedure.

Even for cocoa and dark chocolate the comparison with reported values is not easy because the applied experimental technique are not quite the same and the result expression is different as well as the standard used for calibration curve.

Yet the experimental values using methanol extracts for dark chocolate are close to those obtained by Belščak et al., 2009 (22 - 32 mg/g GAE), Vinson et al, 1999, (but

higher then those reported by Tabernero et al. 2006 (18,2 mg/g GAE), Kroyer and Molnar, 2011 (8 mg/g GAE) or Waterhouse et al 1996 (8,4 mg/g). As for cocoa, the variability of reported values is much higher, from 20-22 mg/g GAE at Waterhouse et al 1996 and Kroyer and Molnar to 58 mg/g at Serra Bonvehi et al, 1997 or 80 mg/g GAE at Tabernero et al. 2006 and Jonfia-Essien et al. 2008, which are in the same area with those we calculated in the present work. As for acetonic extracts, the reported values can not be compared due to their expression: mg/g as catechin or epicatechin, Wollgast, 2004 and Serra Bonvehi et al, 1997, mg/g total procyanidins (Adamson et al, 1999).

In order to compare the values obtained using different extraction solvents in different experimental variants, we used statistical analysis, the Student test (t value) for cocoa and dark chocolate for evident reasons. The calculation was made for cocoa (1) and chocolate (2) in acetonic extract (1A, 2A) and methanolic extract (1M, 2M) using 50 μL (a) and 100 μL (b) of extract.

Table 3 shows the results of comparison between acetonic and methanolic extract for the same product at the two applied experimental variants. At the same time we made a comparison for each tested product for the same extraction solvent in different experimental variants. As one can see, the differences between polyphenolic content of the tested samples are not significant with one exception, i.e. for cocoa in variant a.

Table 3

Statistical analysis – t Test results interpretation

Acetonic extract/versus methanolic extract			
Variant a		Variant b	
1Aa	*	1Ab	ns
1Ma		1Mb	
2Aa	ns	2Ab	ns
2Ma		2Mb	
Variant a versus variant b			
Acetonic extract		Methanolic extract	
1Aa	ns	1Ma	ns
1Ab		1Mb	
2Aa	ns	2Ma	ns
2Ab		2Mb	

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

CONCLUSIONS

The experiments performed in this study lead to some conclusions.

The content of polyphenols in the tested samples is greater in cocoa then in black chocolate. The determination in Nesquik by Folin – Ciocâlteu assay is not reliable no matter if the solvent is acetone or methanol. So, in this case the problem seems to be not the extraction step, but the separation of all constituents of the tested product, other techniques will be investigated and tested for this product.

For the determination of polyphenol content in cocoa by Folin-Ciocâlteu assay different organic solvent can be used if the reaction time and the volume of the sample are the same without significant changes in the results.

The type of solvent leads to significant differences only for dark chocolate when 50 μL of sample was used. This aspect could be due to the complex composition of

chocolate in respect to cocoa powder. Even for this product, doubling the amount of sample eliminate this effect.

Folin-Ciocâlteu assay is a widely and valuable test for determining polyphenol content in a large category of matrices. So, the standardisation of the method regarding all the steps for different categories of foods would be very useful in order to make proper appreciations and ranking, useful for a better diet.

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