THE INFLUENCE OF EXTRACTION SOLVENT ON FLAVONOIDES DETERMINATION IN COCOA AND COCOA PRODUCTS

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Abstract

This paper presents the results of the determination of flavonoids content in cocoa and cocoa products using the colorimetric assay with aluminium chloride solution in order to establish the best experimental variant in terms of solvent used for extraction. In the same time the stability of the pink complex at different reaction time (10, 30 and 60 minutes) was verified. Quercetine was the used standard. The experiments show that the acetonic extract shows higher amount of flavonoides in chocolate and cocoa, the difference being more significant for chocolate (32,7%) than for cocoa (5,5%). The tested samples shown that the flavonoids content decreases in the order cocoa > dark chocolate > Nesquik

Keywords: flavonoides, solvent, cocoa, dark chocolate

INTRODUCTION

Scientists and common people are more and more aware that of the food quality has a major contribute to human health so, nutrition concepts are progressing from "adequate nutrition" to "optimal nutrition" (Bellisle et al., 1998). A diet rich in vegetables and fruits proves to be very important in cancer prevention and cardiovascular diseases (Kris-Etherton et al., 2002; Temple and Gladwin, 2003). The protective effects seems to be mediated through different mechanisms, but more credible is the protection against ROS (radical oxygen species) through antioxidant micronutrients such as vitamins C, beta-carotene or non-nutrient phytochemicals, such as phytoestrogens, other carotenoids than beta-carotene and polyphenols. (Wollgast, 2004). American Dietetic Association considers that specific substances in foods may have a beneficial role in health as part of a varied diet (Bloch and Thomson, 1995). They are known as phytochemicals (Newmark, 1996), "chemo-preventers" (Zumbé, 1998) or "secondary plant products" (Watzl and Leitzmann, 1995)

Polyphenols are the most representative group of phytochemicals which can be found in vegetal row material. It is about fruits, especially the red ones (Pantelides et al., 2007, Sochor et al, 2010), vegetables ((Al-

Juhaimi and Ghafoor, 2011, Ting et al., 2007), cocoa and green tea (Anesini et al, 2008). Polyphenols can be found also in the related food products as red wine (Stratil et al, 2008), chocolate (Wollgast, 2004, Kroyer and Molnar, 2009, Jonfia-Essien et al., 2008), olive oil (Carrasco-Pancorbo A, 2005). Based on their structure, as chemical compounds, polyphenols can be divided into different classes. Flavonoids, are the most important single group of polyphenols and them selves, can be further divided into 13 classes with more than 5000 compounds (Bravo, 1998 cited by Wollgast, 2004) Their common structure consists of two aromatic rings linked through three carbons (C6-C3-C6) that usually form an oxygenated heterocycle (diphenylpropanes).

Flavonoids have been associaded with a lot of beneficial effect on human health: reduction in the risk of cardiovascular disease, protection of LDL cholesterol oxidation, some cancer prevention effects (Bhagwat et al., 2013).

Among other vegetal origin products, cocoa is a valuable source of antioxidants in human food by itself and as a source of a lot of cocoa products: chocolate, cocoa drinks, sweets, cookies, ice cream, etc. The determination of antioxidants in cocoa is relatively recent, results was reported by different scientists starting with Waterhouse et al., 1996 and Vinson et al. 1999. The scientist preoccupation on this field spread all over the world in the last five years: in Europe Jonfia-Essien et al., 2008, Kroyer and Molnar, 2009, Belščak et al, 2009, in Asia Subshasini et al, 2010, Othman et al, 2010, in Africa Oboh and Omoregie, 2011, in South America Pimentel et al, 2010.

The aim of this paper is to investigate the influence of the solvent used for flavonoids extraction and to verify the stability of the pink colorimetric compound formed with aluminum chloride.

MATERIALS AND METHODS

Materials

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

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: methanol from Scharlau Germany, acetone and hexane from Merck Germany, quercetine and $AlCl_3 \cdot 6H2O$ from Roth. 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, vortex Reax top Heindolph.

Methods

We used the method reported by Bahorun et al, 2004 for vegetables and adapted for cocoa and cocoa product by Kroyer and Molnar, 2011 with some minor modifications. The determination of flavonoids is based on the formation of chelatic colorimetrable compounds when flavones and flavanoles react with aluminium chloride.

Due to the fat content of the samples the determination had several stages, as follows:

1. Degreasing 1 g sample with 10 ml hexane in an ultrasonic bath (Wollgast, 2004) followed by centrifugation 10 minutes at 3000 rot/min and decantation.
2. Extraction of the flavonoids:

- with a mixed solvent (M) of water: methanol (80:20, v/v), 10 minutes at 20^0C, in an ultrasonic water bath, the solvent being subsequently removed using a rotary evaporator at 50^0 C, after filtration.
- with a mixed solvent (A) of acetone: water, acetic acid, v/v/v) for 10 minutes la 30^0C, in an ultrasonic water bath, the solvent being subsequently removed using a rotary evaporator at 40^0 C, after filtration (Bahorun et al, 2004 with some modifications).

3. A volume of 1.5 ml of each extract was added to an equal volume of a solution of 2% $AlCl_3 \cdot 6H2O$ in methanol and thoroughly mixed. The mixture was shaken in a vortex and the absorbance was read at 368 nm. Results were expressed in mg quercetin/ g sample.

Quercetine solutions in methanol was used at 0; 5; 10; 25; 40 mg/l concentration. The absorbance was determined at different laps of time (10, 30 and 60 minutes) for checking the colorimetrable compound stability.

RESULTS AND DISCUSSIONS

The calibration curves obtained by reading the absorbance at 10, 30 and 60 minutes show, as expected, linear correlation with correlation factor (R^2) practically identical and very close to 1, respectively 0.9999 (10 minutes), 0,9992 (30 minutes) and 0,9998 at 60 minutes. The average slope values (Fm) were 258.58□SD1.295, 256.82□SD3.806 and 246.98□SD3.650.

So we used for the calculation of the flavonoid content the absorbance obtained after 10 minutes, regression equation y = 0.0036x + 0.0189 (R^2 = 0.9999) and Fm value at 10 minutes because the low values of standard deviation allow it. The results are shown in table 1 and 2 for both calculation techniques.

Table 1

Calculation of the flavonoid content, methanolic extract

Sample	Regression ecuation		Fm		t - test
	mg QE/g	□SD	mg QE/g	□SD	
1A	3.05	0.536	3.75	0.307	ns
2A	5.31	0.717	5.63	0.878	ns
3A	0.47	0.376	1.25	0.422	**

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

Table 2

Calculation of the flavonoid content, acetonic extract

Sample	Regression ecuation		Fm		t - test
	mg QE/g	□SD	mg QE/g	□SD	
1M	2.05	0.380	2,97	0.430	*
2M	5.02	0.371	5.25	0.336	ns
3M	Solution Milky		Solution milky		-

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

The results shows that the flavonoid content is 32,7% higher in the acetonic extract than in methanolic extract -ciocolate and 5.5% - for cocoa in regression calculation, respectively 20,8% and 6,7% in Fm calculation. No comparison can be made for Nesquik because the methanolic extract does not allow the absorbance reading, being milky. The difference in terms of flavonoid content is bigger for chocolate than for cocoa.

For the acetonic extract, the statistic analysis (t-test) had shown no significant differences between the two calculation ways but for methanolic extract, that was the case only for cocoa, while for chocolate the differences ware significant.

As for the values themselves, the comparison with literature data is not very evident, because the expressions of the results, the used standard or the analytical methods are different. For example Bercsak et al, 2009 determined flavonoides content by calculation as the difference between total phenol and non-flavonoid content was expressed in mg GAE/g, Pimentel et al 2010 expressed the results in μmol CAT/g and Subhashini et al, 2010 in mg/serve. However for chocolate the results are closed to those reported by Kroyer and Molnar, 2010 (4 mg QE/g) and for cocoa they are lower (8.5 mg QE/g), but very close to those reported by Oboh and Omoregie, 2011 (5 mg QE/g).

CONCLUSIONS

The determination of flavonoids by aluminium chloride assay in cocoa and cocoa products leads us to some conclusions:

- The extraction of flavonoids by acetone leads to higher contents that using methanol;
- The fact that the difference in terms of flavonoid content is bigger for chocolate than for cocoa seems to indicate that the composition of the tested sample is very important in this determination;
- The lower absorbencies obtained for methanolic extract – chocolate doesn't allow proper calculation on the calibration curve 0- 40 mg/l; so, a new one for concentration 0 -10 mg/l is necessary for accurate calculations;
- The content of flavonoids in the tested samples decreases in the order cocoa > dark chocolate > Nesquik;
- The time of reaction between 10 and 60 minutes has no influence on the stability of the colorimetric compound.

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