Versatility of Folin-Ciocalteu Method Applied on Honey Determination of Total Phenols

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The main benefits of honey regarding human health issue from its minor components mainly because of their antioxidant properties. So far the studies emphasise the phenolic compounds beneficial effect related to the antioxidant activity of honey. The aim of this study was to verify the influence of temperature and reaction time on honey total phenol content (TPC) value determined using Folin-Ciocalteu assay. Twenty samples of floral and honeydew honey were tested for total phenol content in four experimental variants: A –at 20°C for 2 h, B - 20°C for 1 h, C - 40°C for 20 min and D - 4°C for 30 min . The comparison of A variant, the most spread one, to the others shows that the reduction of time from 2 h to 1 h leads to non-significant differences while the rise of temperature, even for short laps of time, reveals significant differences between the recorded TPC values.

Keywords: honey, phenols, Folin-Ciocalteu, time, temperature

Honey is an animal origin food, but has very different characteristics among this category of food. So, the protein content is low, under 0.5% [1], but the carbohydrate content is very high, over 60% according to European [2] and Romanian regulations [3]. For this reason honey is used as a healthy natural sweetener no matter its origin - floral or honeydew. But the main benefits of honey regarding human health are due to its minor components as vitamins, phenolic acids, flavonoids and enzymes. So far the studies emphasise the phenolic compounds beneficial effect related to the antioxidant activity of honey [4, 5]. The antioxidative potential of honey from different botanical and geographical origin was reviewed including a large database for Total Phenolic Content (TPC) [6].

The Folin-Ciocalteu spectrophotometric assay[7] is now widely spread for the quantification of TPC in different vegetal origin foods as fruits [8-10],medicinal plants and spices [11, 12], tomatoes[13] or in wine[14, 15].In our knowledge, the method was first used for honey in 2005 [16]. Since then, most researchers including the Romanian ones [17- 20] used the same experimental conditions for the reaction mixture regarding temperature (20°C) and waiting time (2 h) until the absorbance of the blue phosphomolibdenic complex is registered.

Despite the fact that Folin-Ciocâlteu method is applied on a large scale, the comparison of the reported values for the same matrix, namely honey, is not always easy to approach because of several experimental approaches.

Besides previously named the basic method, other variants regarding temperature and waiting time, were applied for TPC determination in honey. Hence 60 min [21], respectively 90 min [22] as waiting time at 20°C were used in experiments. The time is even shorter referred to 20 min in another study [23]. There are different experiments conducted at such short period of time [24-26], but they cannot be compared to the others because they are performed without using sodium carbonate. Other researchers tried to reduce time and rise temperature in the same time using for example 15 min reaction time at 45°C [27] or 15 min at 50°C [28].

The goal of our study was (i) to verify the influence of temperature and reaction time on honey total phenol content value determined using Folin-Ciocalteu assay and (ii) to verify if the botanical origin of honey has influence in this issue.

Experimental part

Materials and methods

The material consists in twenty honey samples from Bihor County, presented in table 1. The samples code is the same all over the paper content. There are seven floral types (thirteen samples), six honeydew honey types (fir and honeydew) and one mixed honey. The samples were provided in glass jar of 200 – 400g, were kept in the dark at room temperature bellow 25°C until analysed. Crystallized honey was liquefied by gentle warming at 40°C in a thermostatic bath.

Honey sample: a quantity exactly weighted as close as possible to five grams was diluted in a beaker with approximate 25 mL of distillate water and then transferred quantitatively in a 50 mL flask. Honey solutions were filtered prior to use. From each sample four test tubes were prepared as follows. Over five hundred µL of filtered solution 2.5 mL of Folin-Ciocalteu reagent 0.2N was added and then strongly mixed by vortex. After 5 min, 2 mL of 7.5% sodium carbonate solution was added and again mixed by vortex.

Four experimental variants were tested:

A – Samples were allowed to stay at 20°C for 2 h

B - Samples were allowed to stay at 20°C for 1 h

C - Samples were allowed to stay at 40°C for 20 min

D - Samples were allowed to stay at 40°C for 30 min

For C and D variants, the test tubes were quickly cooled after removing from the thermostatic bath.

Two series of the tested samples were tested, each of them in duplicate.

Galic acid was used as standard from 0 to 250 mg/L. The same procedure regarding time and temperature as for the samples was applied on the test tubes containing the dilutions of the standard (variants A, B, C and D).

The absorbance was read using the same type of plastic cuvette, 1 cm pathway, at 760nm.

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Sample code	Type of honey	Botanical origin	Year	Provenance
Floral				
R1	Rape	*Brassica SPP*	2012	Oradea market
R2			2013	
FIS1		*Helianthus annuus*L.	2014	
FIS2	Sunflower		2014	
SM14	Acacia	*Robiniapseudacacia*	2013	
SB14			2014	Directly from beekeepers, Bihor County
IN13	Heather	*Calluna vulgaris*	2013	
IN14			2014	
Cs	Fruit Tree (Cherry)	*Prunusavium*	2012	
TB14	Lime	*Tilia*spp.	2014	
T14			2014	
PFM	Polifloral	*Mixed (mountain area)*	2012	
PFC		*Mixed (plain area)*	2013	
Mixed				
M/Z	Honeydew/Raspberry	*Unknown/Rubusidaeus*	2013	Beiuş market
Honeydew				
B12	Fir	*Abies*	2012	
B13			2013	Directly from beekeepers, Bihor County
M12			2012	
M13			2013	
M14	Honeydew	*Unknown*	2014	
MAp14			2014	Apicola,Oradea

Table 1
ANALYSED HONEY SAMPLES

Variant	A – 2h, 20°C		B – 1h, 20°C		C- 20', 40°C		D- 30', 40°C	
Floral								
Type	Mean ±SD		Mean ±SD		Mean ±SD		Mean ±SD	
	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum
R1	39.289±0.185		39. 379 ±1.825		46.599±2.805		47.126±0.245	
	39.069	39.474	49.182	42.705	49.182	42.705	46.851	47.428
R2	62.758± 0.189		62.205±0.862		64.760±1.321		69.243±1.952	
	62.552	62.948	61.280	63..228	63.232	66.071	66.662	71.212
FLS2	40.434±0.0.481		41.096±0.589		40.796±0.627		39.778±0.997	
	40.016	40.995	40.604	41.678	40.263	41.678	38.647	41.071
FLS1	48.027±1.347		47.638±2.152		51.967±2.326		51.885±1.822	
	46.886	49.640	45.555	50.656	50.007	55.348	50.114	54.343
TB14	36.341±1.848		37.607±2.306		42.563±3.859		39.568±1.400	
	34.632	38.678	35.579	39.689	36.975	45.317	37.809	40.729
T14	34.557±0.346		35.463±0.422		40.241±0.973		38.894±2.894	
	34.371	35.062	35.214	36.013	39.502	41.660	36.754	41.268
SM14	43.659±0.417		44.145±0.668		43.307±2.449		51.002±1.079	
	43.218	44.024	43.189	44.685	40.107	45.997	49.656	52.298
SB14	53.824±1.302		56.538±0.867		57.379±1.785		57.647±1.023	
	52.473	55.595	55.720	57.422	55.441	59.681	55.72	59.877
IN13	134.613±4.028		143.723±3.113		144.550±0.441		151.014±3.193	
	132.186	140.639	139.118	145.980	144.120	144.939	147.717	154.101
IN14	115.711±1.454		117.423±0.955		124.366±3.664		127.524±0.914	
	114.402	117.383	116.001	118.016	121.433	128.824	126.452	128.647
Cs	99.249±2.297		106.323±3.104		107.292±1.884		110.738±0.589	
	95.897	101.052	103.235	110.287	105.120	109.720	109.939	111.354
PFC	66.817±2.232		67.919±2.352		70.112±2.593		71.750±2.750	
	64.051	69.440	65.361	70.372	67.787	73.422	68.412	75.241
PFM	129.090±0.647		130.312±1.131		133.367±2.932		133.577±1.055	
	128.193	129.631	128.775	131.466	130.151	136.800	132.4567	134.973
Mixed								
M/Z	185.754±3.020		186.396±3.097		192.609±2.203		186.829± 0.408	
	182.091	189.885	182.418	189.445	190.268	195.046	186.461	187.483
Honeydew								
B12	168.185±0.783		167.331±1.977		171.524±0.214		175.209±0.418	
	166.961	169.002	165.175	169.434	171.331	171.758	174.720	175.720
B13	193.143±1.220		193.127±1.416		199.808±0.321		200.631±0.927	
	191.958	194.847	191.394	194.861	199.405	200.187	199.470	201.678
M12	136.716±1.026		134.410±1.225		139.751±1.751		144.468±0.188	
	135.784	138.137	131.979	132.808	138.222	141.859	144.320	144.720
M13	178.055±2.506		176.945±3.721		174.925±3.000		181.251±3.878	
	175.908	181.667	174.147	182.320	171.567	178.803	177.537	183.980
M14	146.524±1.635		147.095±1.582		154.862±0.488		154.146±1.197	
	144.863	148.580	146.313	147.713	153.938	155.833	153.320	155.920
MAp14	161.991±1.189		162.299 ±0.996		165.936±2.277		170.160±3.065	
	160.727	163.480	161.317	163.677	163.077	168.557	167.242	173.856

Table 2
TOTAL PHENOL CONTENT, MG GAE / 100G HONEY

Reagents and devices

All used reagents were analytical graded: Folin-Ciocalteu Reagent 2N from MERCK Germany, sodium carbonate from Chimopar Romania, Gallic acid from ROTH Germany. The used devices were vortex -HETTIK Germany, spectrophotometer UVMini-1240 –Shimadzu and thermostatic bath –Julabo.

Table 3

STATISTICAL ANALYSIS, VARIANTS COMPARISON

Variant / Statistical significance	B		C		D	
	Nr samples	% Samples	Nr samples	% Samples	Nr samples	% Samples
ns	16(10F;6H)	80	4(3F;1H)	20	3(1F;2H)	15
x	4(3F;1H)	20	6(4F;2H)	30	5(5F)	25
xx	-	-	7(6F;1H)	35	3(3F;1H)	15
xxx	-	-	3(3H)	15	9(5F;4H)	45

p>0.05= non-significant; p<0.05= * significant; p<0.01=** distinctly significant; p<0.001=*** very significant in comparison with A control variant

Table 4

"B" VARIANT COMPARED TO "A" VARIANT

Statistical significance	Botanical origin (a)				TFC content, GAE/100g (b)			
	Floral		Honeydew		< 100 mg		> 100 mg	
	Nr samples	% samples	Nr samples	% samples	Nr samples	% samples	Nr samples	% samples
ns	10	77	6	85.7	8	88.9	8	72.7
x	3	23	1	14.3	1 (F)	11.1	3 (2F;1H)	27.3
Total	13	100	7	100	9	100	11	100

Results and discussions

The calibration curves for the four experimental variants has a very good linearity, R^2 being over 0.99 in all cases. The reported results were obtained in different experimental sessions, each time the calibration curves were re-done in all four variants. We give one of the experimental situations as an example: A: y = 0.0097x + 0.1109, R2 = 0.9998; B: y = 0.0102x + 0.0625, R2 = 0.9945; C: y = 0.01x + 0.0694, R2 = 0.9989; D: y = 0.0099x + 0.0768, R2 = 0.9986. The results are presented in table 2 as mean .... standard deviation, minimum and maximum experimental values for every type and sample of tested honey. The codes for the tested samples are the same as presented in table 1. Looking to all experimental variants, the experimental values cover a large area as follows: nine samples (monofloral and a polyfloral one) presents TPC under 100 mg GAE/100g and eleven samples (cherry, heather, polyfloral, mixed and honeydew) between 100 and 200 mg GAE/100g. These values comply with those reported for Romanian honey [17, 19, 29] regarding sunflower, lime, acacia and honeydew. For heather much lower values (max 56,7 mg GAE/100g) were found by [20] but in Poland values in the same area as ours were reported, up to 189 mg GAE/100g [30].

Considering the A variant, the most used as control, we proceeded to compare it with the other ones applied. Mixed honey (M/Z) was included in honeydew honey category because it's TPC=185.0831mgGAE/100gindicates that honeydew is predominant. The results of statistical analysis (t-test) on the experimental values are synthesised in table 3.

As a general comment, only for two samples FLS2 and M13, there were non-significant differences between all applied variants. Regarding all tested samples, compared to the A variant, the B variant shows non-significant differences for 80% of the tested samples unlike C and D variants for which the percentage fall down to 20% and 15%, respectively. For the C variant the samples showing distinctly significant differences prevailed while for the D variant those showing very significant differences prevailed.

A detail analysis of the B variant is presented in table 4.

In the limit that the number of samples is not the same, it seems that the reaction time has more influence on floral honey that in honeydew honey (*a* column of table 4). The *b* column emphasizes this opinion even for floral honey with high TPC, over 100 mg GAE/100g (Cs and PFM). The situation can be explained by the fact that honeydew honey has lower sugar content than floral honey [2, 3, 21].

Conclusions

In the terms of the present experiment, the influence of reaction conditions on TPC determination in honey leads to several conclusions.

Temperature – the rise of temperature from 20 to 40°C (A variant versus C and D) lead to significant differences between the recorded TPC values, no matter the origin of honey.

Reaction time – the reduction of time from 2 to 1 h (A variant versus B) at 20^0C lead to non-significant between the recorded TPC values; the influence of botanical origin of honey in this issue is not yet evident, so further experiments are needed in order to clarify this aspect.

Temperature and time, combined – the reduction of time (C variant versus D) do not improve the correlation to the basic variant A.

Based on the present experiments results, the influence of reaction conditions on the determination of honey total phenol content by Folin- Ciocâlteu assay shows that for honey this method leads to reliable results after only one hour of reaction at room temperature. Hence reliable results can be reached in a shorter period of time. The attempt of more shortening the time combined with higher temperature affect the results.

The present experiment emphasises the requirement for the standardisation of Folin-Ciocâlteu assay in order to achieve reliable comparison of honey TPC, no matter its geographical or botanical origin.

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