

THE EFFECT OF STORAGE TEMPERATURE AND THERMAL PROCESSING ON CATECHINS, PROCYANIDINS AND TOTAL FLAVONOID STABILITY IN COMMERCIALY AVAILABLE COCOA POWDERS

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Abstract. *Storage stability of catechin, epicatechin, procyanidins B1-B4 and total flavonoids in cocoa powder samples was studied over the temperature range 4-35 °C. Thermal stability of total flavonoids was studied over the temperature range 95-125 °C. Total flavonoids concentration decreased as a function of time and the degradation was accelerated at higher temperatures: a half-life ($t_{1/2}$) of total flavonoids was much shorter at room temperature than during cold storage. A first-order kinetic model fitted well to all the data. Temperature dependence of the degradation rate constants, described by the Arrhenius equation, was more pronounced during heating than during storage. In the temperature interval from 4 °C to 35 °C, the calculated activation energies (E_a) for catechin, epicatechin and procyanidins B1-B4 were 20.4 kJ/mol, 12.5 kJ/mol, 9.4 kJ/mol, 21.6 kJ/mol, 19.4 kJ/mol, 23.7 kJ/mol, respectively.*

Key words: *cocoa powder, catechins, procyanidins, total flavonoids, thermal processing, degradation kinetics*

1. INTRODUCTION

Chocolate and other cocoa-containing products are rich in polyphenolic compounds, especially in flavonoids. The main flavonoids in cocoa and chocolate are flavan-3-ols, catechin and epicatechin, as well as their various oligomers (proanthocyanidins), Figure 1 (Wollgast and Anklam, 2000a). The health benefits of chocolate and cocoa are frequently related to the antioxidant effects of these compounds (Wollgast and Anklam, 2000b; Weisburger, 2001; Ding et al., 2006; Cooper et al., 2008; Schroeter et al., 2006; Balzer et

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al., 2008) [2-7]. Furthermore, as summarized in the recent review by Mehrinfar and Frishman (2008), flavanols have cardioprotective properties.

Cocoa-related food products are usually thermally processed prior to consumption (Wollgast and Anklam, 2000a). Unfortunately, many flavonoid compounds (e.g. catechins and procyanidins) are heat-sensitive and can undergo chemical degradation during heat processing or even storage (Wollgast and Anklam, 2000a; Zhu et al., 1997; Yilmaz, 2006; Friedman et al., 2009; Bazinet et al., 2010; Ananingsih et al., 2013; Waterhouse and Laurie, 2006; García-Falcón et al., 2007; Castillo-Sánchez, 2008) [1, 10-17]. Unlike in the case of green and black tea (Zhu et al., 1997; Yilmaz, 2006; Friedman et al., 2009; Bazinet et al., 2010; Ananingsih et al., 2013) or red wine (Waterhouse and Laurie, 2006; García-Falcón et al., 2007; Castillo-Sánchez, 2008), there are only limited data regarding storage/temperature induced changes in the flavonoid profile of cocoa powder and cocoa products (Hurst et al., 2009). To accurately predict these changes, kinetic parameters regarding (thermal) degradation of flavonoids are needed. For that reason, we present herein the results of a kinetic study of the effects of elevated temperature on catechin, procyanidin B1-B4 and total flavonoid stability in commercially available cocoa powders used in foodstuff.

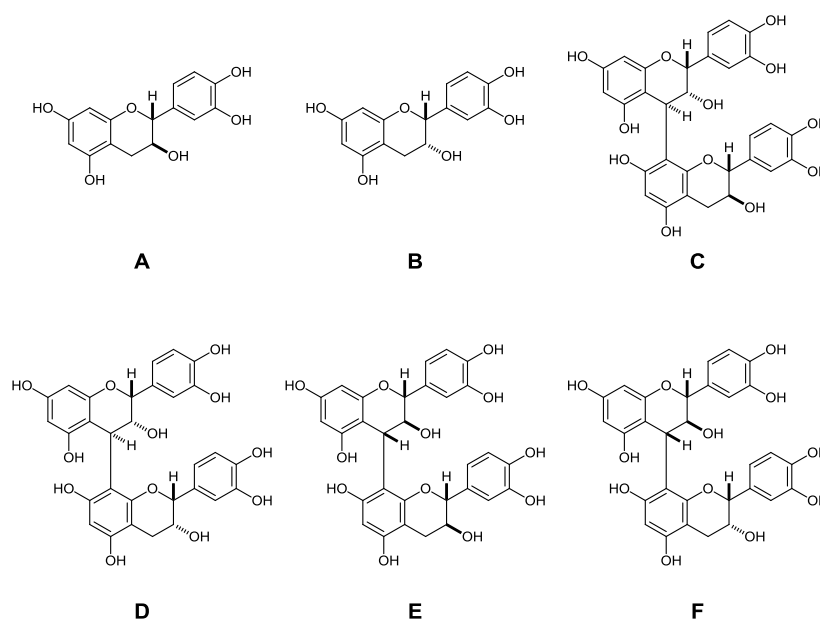


Fig. 1 Structures of flavonoid compounds found in cocoa beans and related foodstuff: **A**-catechin, **B**-epicatechin, **C**-procyanidin B1, **D**-procyanidin B2, **E**-procyanidin B3 and **F**-procyanidin B4

2. MATERIALS AND METHODS

2.1. Chemicals

(+)-Catechin, (-)-epicatechin, procyanidin B1, B2 and B3 were purchased from Sigma Aldrich (Steineheim, Germany). Sodium hydroxide, sodium nitrite, aluminum chloride hexahydrate, hexane, acetic acid, formic acid, acetonitrile and acetone were purchased from Merck® (Darmstadt, Germany). Purified water (18 MΩcm), prepared by a MicroMed purification system (TKA Wasseraufbereitungssysteme GmbH, Niederelbert, Germany), was used to prepare all samples and standards.

2.2. Instruments

An Agilent 8453 UV/Vis spectrophotometer (Agilent Technologies, Santa Clara, California, USA) was used for absorbance measurements and spectra recording, using optical cuvette of 1 cm optical path. A model 1200 (Agilent Technologies, Santa Clara, California, USA) was used for HPLC analysis. The analytical column was C₁₈ Zorbax Eclipse XDB-C18, 5µm, 4.6×150 mm (Agilent Technologies, Santa Clara, California, USA).

2.3. Cocoa samples

Eight commercially available cocoa powder products were purchased from the local supermarkets (Niš, Serbia). Samples were stored in the darkness at three temperatures (4° C, 22 °C and 35 °C) and their catechin, procyanidin B1-B4 and total flavonoid contents were determined at chosen time intervals during storage of 30 days (catechins and procyanidins) and 45 days (total flavonoids).

2.4. Preparation of cocoa powder samples for further analysis

The cocoa samples were prepared for analysis by the slightly modified procedure of Adamson et al., 1997. Ten grams of cocoa powder was defatted 3-5 times with 10 mL of hexane, and the residue was dried under a gentle nitrogen stream. The defatted material (1 g) was then extracted three times with 10 mL of acetone-water-acetic acid (70:29.5:0.5 v/v/v) by centrifugation at 3000 rpm for 10 min. The supernatant was decanted to a clean 50 mL volumetric flask and diluted with water to mark.

2.5. Heating procedures

Metal tubes were filled with cocoa powder samples and tightly closed without headspace. These tubes were thermostated (an oil-bath) for 25, 50, 70 or 100 min, at 95±1, 100±1 110±1 and 125±1 °C. After that, samples were cooled in ice water to stop further thermal degradation and immediately analyzed.

2.6. Total flavonoid content (TF)

The total flavonoid content was assayed using the slightly modified aluminum chloride spectrophotometric method, described by Zhishen et al. (1999) and Yang et al. (2004). Reaction mixture was prepared by mixing 0.25 mL of an appropriate sample, 3 mL of deionized water and 0.3 mL of 5% NaNO₂. After incubation at room temperature for 5 min, 1.5 mL 2% aluminum chloride hexahydrate (AlCl₃·6H₂O) was added. Again, the

flask was kept at room temperature for 5 min and then 2 mL of 1 mol/L sodium hydroxide (NaOH) was added.

Table 1 Storage-induced changes in catechin and procyanidines B1-B4 contents (c_{sr})^a

Epicatechin				Catechin			
Storage temperature (°C)	Day	$c_{sr} \pm SD^a$ (mg/g)	RSD (%)	Storage temperature (°C)	Day	$c_{sr} \pm SD^a$ (mg/g)	RSD (%)
4	7	0.286 ± 0.004	1.40	4	7	0.132 ± 0.004	3.03
	15	0.261 ± 0.004	1.53		15	0.126 ± 0.002	1.59
	30	0.230 ± 0.003	1.30		30	0.113 ± 0.002	1.77
22	7	0.276 ± 0.004	1.45	22	7	0.119 ± 0.002	1.68
	15	0.240 ± 0.003	1.25		15	0.110 ± 0.002	1.82
	30	0.201 ± 0.002	0.99		30	0.095 ± 0.001	1.05
35	7	0.255 ± 0.004	1.57	35	7	0.116 ± 0.002	1.72
	15	0.226 ± 0.003	1.33		15	0.103 ± 0.002	1.94
	30	0.183 ± 0.002	1.09		30	0.083 ± 0.001	1.20
Procyanidin B1				Procyanidin B2			
Storage temperature (°C)	Day	$c_{sr} \pm SD^a$ (mg/g)	RSD (%)	Storage temperature (°C)	Day	$c_{sr} \pm SD^a$ (mg/g)	RSD (%)
4	7	0.038 ± 0.002	5.26	4	7	0.043 ± 0.001	4.30
	15	0.032 ± 0.001	3.12		15	0.037 ± 0.001	2.70
	30	0.022 ± 0.001	4.54		30	0.031 ± 0.001	3.23
22	7	0.036 ± 0.001	2.78	22	7	0.042 ± 0.001	2.38
	15	0.028 ± 0.001	3.57		15	0.032 ± 0.001	3.12
	30	0.021 ± 0.001	4.76		30	0.024 ± 0.001	4.17
35	7	0.033 ± 0.001	3.03	35	7	0.040 ± 0.001	2.50
	15	0.024 ± 0.001	4.17		15	0.027 ± 0.001	3.70
	30	0.0152 ± 0.0004	2.63		30	0.0151 ± 0.0004	2.65
Procyanidin B3				Procyanidin B4			
Storage temperature (°C)	Day	$c_{sr} \pm SD^a$ (mg/g)	RSD (%)	Storage temperature (°C)	Day	$c_{sr} \pm SD^a$ (mg/g)	RSD (%)
4	7	0.0135 ± 0.0003	2.22	4	7	0.0105 ± 0.0002	0.98
	15	0.0129 ± 0.0002	1.55		15	0.0080 ± 0.0002	2.5
	30	0.0106 ± 0.0002	1.89		30	0.0070 ± 0.0001	1.43
22	7	0.0133 ± 0.0002	1.50	22	7	0.0084 ± 0.0002	2.38
	15	0.0121 ± 0.0002	1.65		15	0.0068 ± 0.0001	1.47
	30	0.0096 ± 0.0001	1.04		30	0.0048 ± 0.0001	2.08
35	7	0.0130 ± 0.0002	1.54	35	7	0.0081 ± 0.0002	2.47
	15	0.0110 ± 0.0002	1.82		15	0.0057 ± 0.0001	1.75
	30	0.0069 ± 0.0001	1.45		30	0.0026 ± 0.0001	3.85

^a C_{sr} is an average for 8 samples.

The flask was filled up to 10 mL with deionised water. The absorbance of the reaction mixture was measured against the prepared reagent blank at 510 nm. Catechin was chosen as a standard and the results expressed as milligram catechin equivalents per gram of sample (mg CE/g). The levels of total flavonoid contents were determined in triplicate.

2.7. HPLC analysis of catechins and procyanidins

An Agilent chromatograph equipped with an autosampler and photodiode-array and fluorescence detectors (1200 Series) was used for the HPLC analysis. Separation was performed with a Zorbax Eclipse C₁₈ (XDB-C18, 5µm, 4.6x150 mm) column kept at 25 °C, at a flow rate of 0.8 mL/min, and an injection volume of 20 µL. Catechins were monitored using UV detector at 289 nm. Procyanidins were determined using fluorescence detector. The excitation and emission wavelengths were 275 and 322 nm, respectively. The binary mobile phase consisted of solvent A (5% formic acid in water) and solvent B (80% acetonitrile and 5% formic acid in water). The 45 min gradient programme (Gu et al., 2006) was slightly modified as follows: 0-10 min 0% B; 10-28 min 0-25% B; 28-30 25% B; 30-35 min 25-50% B; 35 to 40 min 50-80% B; 40-45 min 80-0% B; followed by 10 min of re-equilibration of the column before the next run. The individual catechins and procyanidins were separated within 50 min. Identification was carried out by comparing the retention times and spectral data with those of standards or with data (procyanidin B4) reported in the literature (Porter et al., 1991; Adamson et al., 1999). Quantitative determination of individual phenolic compounds in the samples was calculated using calibration lines.

3. RESULTS AND DISCUSSION

Temperature/storage induced changes in the total flavonoid content of commercially available cocoa powder samples was studied by UV/VIS and HPLS analyses. The changes in the content of several individual flavonoids (epicatechin, catechin and procyanidin B1-B4, Fig. 1) were also assessed (Figs 2-5). The obtained results are summarized in Tables 1 and 2. The absolute amount of epicatechin was higher than that of catechin in all tested samples. The most abundant procyanidin was B2, followed by B1, B3 and B4. As clearly visible from the data given in Table 1, the contents of catechin and procyanidin decreased during storage in time and temperature-dependent manner.

The linear regression analysis confirmed that degradation of individual flavonoids in the cocoa powder samples followed a first-order reaction (Figures 2 and 3). In this case, a first-order reaction can generally be expressed using Equation (1) (Kirca and Cemeroglu, 2003; Harbourne et al., 2008).

$$c = c_0 \cdot \exp(-k \cdot t) \quad (1)$$

Here, c_0 (mg/g) represents initial flavonoid concentration in cocoa sample, t is treatment time (min), c stands for flavonoid concentration after treatment time t , while k is the first-order degradation rate constant (min^{-1} or h^{-1} or day^{-1}).

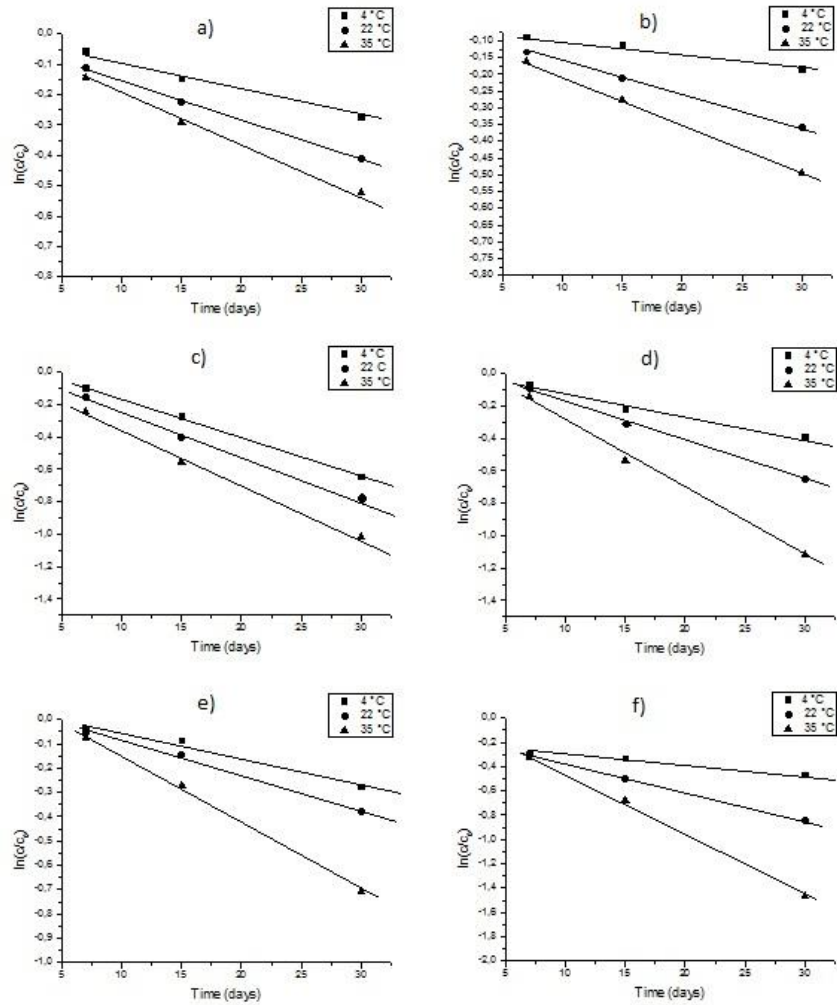


Fig. 2 Degradation of catechins and procyanidins in cocoa powder samples during storage: a) epicatechin; b) catechin; c) procyanidin B1; d) procyanidin B2; e) procyanidin B3; f) procyanidin B4

The half-lives ($t_{1/2}$) of the flavonoids were calculated by the Equation (2) and the temperature-dependence degradation rate constant was represented by the Arrhenius equation (Equation (3)).

$$t_{1/2} = -\ln(0.5)/k = 0.693/k \quad (2)$$

$$k = k_0 \times e^{-E_a/RT} \quad (3)$$

Here, k_0 is the frequency factor (per min), E_a is the activation energy (kJ/mol), R is the universal gas constant (8.314 J/molK) and T is the absolute temperature (K).

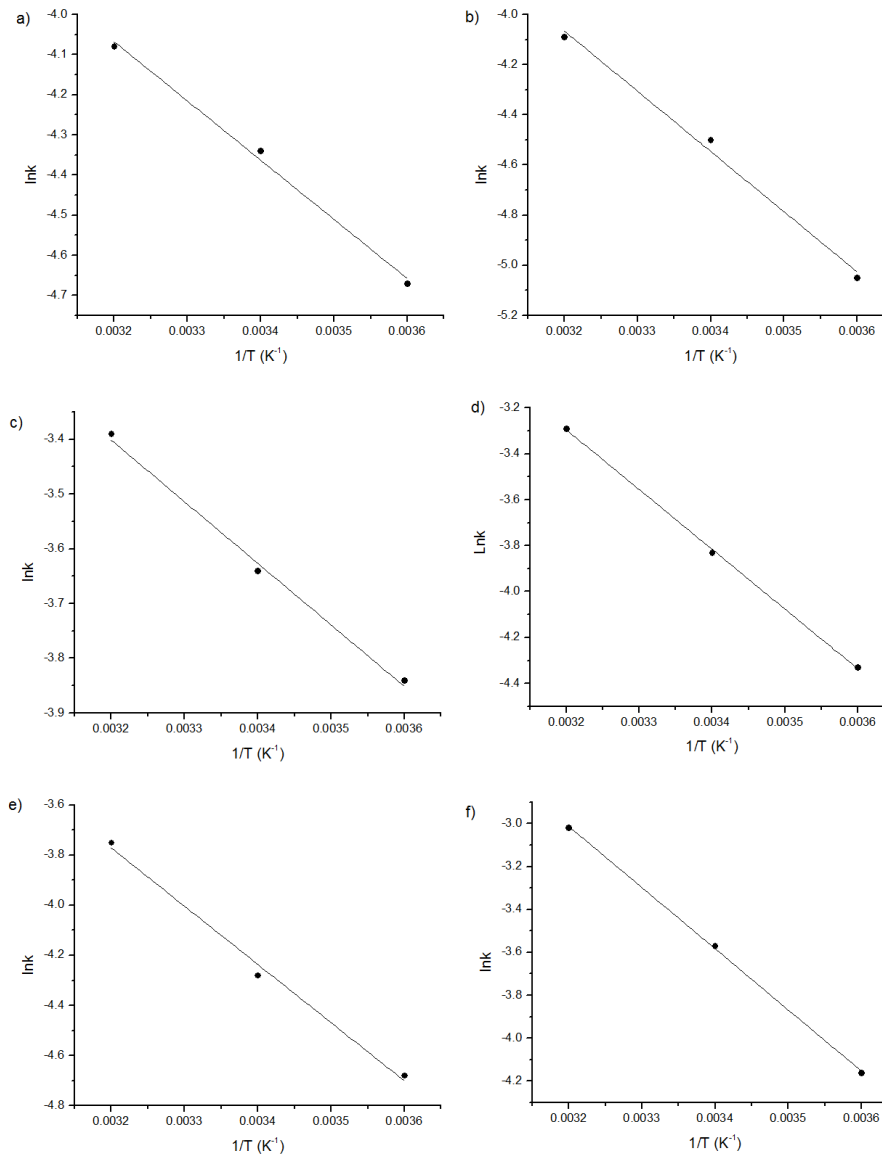


Fig. 3 Arrhenius plots for degradation of: a) epicatechin; b) catechin; c) procyanidin B1; d) procyanidin B2; e) procyanidin B3; f) procyanidin B4 in cocoa powder samples during storage.

Table 2 Storage/temperature-induced changes in total flavonoid content (c_{sr})^a of cocoa powder

Storage temperature (°C)	Time (days)	$c_{sr} \pm SD$ (mg/g)	RSD (%)	Heating temperature (°C)	Time (min)	$c_{sr} \pm SD$ (mg/g)	RSD (%)
4	7	14.0 ± 0.3	2.14	95	25	12.3 ± 0.1	0.82
	15	13.8 ± 0.3	2.17		50	11.6 ± 0.2	1.72
	30	13.5 ± 0.2	1.48		70	10.7 ± 0.1	0.99
	45	13.0 ± 0.2	1.54		100	10.1 ± 0.1	0.99
22	7	13.7 ± 0.3	2.19	100	25	11.9 ± 0.2	1.68
	15	12.8 ± 0.2	1.56		50	10.2 ± 0.1	0.98
	30	11.5 ± 0.2	1.74		70	9.6 ± 0.1	1.04
	45	10.3 ± 0.2	1.94		100	8.1 ± 0.1	1.23
35	7	13.2 ± 0.3	2.27	110	10	11.5 ± 0.2	1.73
	15	11.7 ± 0.2	1.71		20	10.9 ± 0.2	1.83
	30	9.8 ± 0.2	2.04		30	9.9 ± 0.1	1.01
	45	7.9 ± 0.1	1.27		40	8.9 ± 0.1	1.12
				125	5	11.4 ± 0.4	3.51
					10	10.5 ± 0.3	2.86
					15	9.5 ± 0.2	2.11
					20	8.0 ± 0.2	2.51

^a C_{sr} is an average for 8 samples; initial flavonoid concentration was $c_0 = 14.2 \pm 0.3$ mg/g.

Table 3 The effect of storage and heating temperature on the k , $t_{1/2}$ and E_a values of flavonoid degradation (total flavonoids) in cocoa powder samples

Temperature (°C)	$k \times 10^3$ (day ⁻¹)	R ²	$t_{1/2}$ (day)
4	1.9 ± 0.1	0.9905	365
22	6.9 ± 0.3	0.9961	100
35	13.2 ± 0.4	0.9971	53
Temperature (°C)	$k \times 10^3$ (min ⁻¹)	R ²	$t_{1/2}$ (h)
95	3.4 ± 0.1	0.9899	3.4
100	5.6 ± 0.2	0.9945	2.1
110	11.8 ± 0.4	0.9959	1.0
125	29.1 ± 0.7	0.9847	0.4

The effect of storage temperature/heating temperature on the flavonoids degradation (total flavonoids) in the cocoa powder samples is given in Table 2. The kinetic parameters of flavonoids degradation during storage are shown in Table 3. Temperature significantly affected the degradation rate of flavonoids. During storage, degradation was fastest at 35 °C, and the slowest at 4 °C. The $t_{1/2}$ values varied from 365 to 53 h for the studied cocoa powder samples at 4, 22 and 35 °C (Figure 4).

Storage at 4 °C gave over a three-fold increase in the half-lives compared to the room temperature (Table 3). After 45 days storage at 4 °C the cocoa powder sample still contained over 91% and at room temperature over 72% of total flavonoid contents. Storing temperature has been previously shown to affect the stability of anthocyanins in different juices (Buckow et al., 2010; Gancel et al., 2011) and catechins in tea drinks (Bazinet et al., 2010). The effect of storage temperature on the individual flavonoids degradation rate constants is shown in Figure 3. The temperature-dependent rate constant

obeyed the Arrhenius relationship (Equation (3)). The values of the activation energy for catechin, epicatechin and procyanidins B1, B2, B3 and B4 were 20.4 kJ/mol, 12.5 kJ/mol, 9.4 kJ/mol, 21.6 kJ/mol, 19.4 kJ/mol, 23.7 kJ/mol, respectively.

The results of the kinetic study of the high-temperature induced degradation of flavonoids are shown in Table 3 and Figure 5. Heating temperature had a strong influence on the degradation of flavonoids. Again, the data plotted as $\ln(c/c_0)$ against time can be fitted by linear curves and thus a first-order kinetic model can be applied. It is clear that degradation increased with increased heating temperature and time. The high R^2 -values confirm this finding (Table 3). The percentage of decomposition of flavonoids in the first 20-25 min strongly depends on the treatment temperature and was ranged from 13% at 95 °C to 44% at 125 °C. The $t_{1/2}$ values varied from 3.4 to 0.4 h for cocoa powder samples at 95, 100, 110 and 125 °C.

The obtained half-lives of the degradation of flavonoids imply that degradation of catechins and procyanidins is more susceptible to temperature elevation during heating than during storage. The same findings are also found for anthocyanins (Patras et al., 2010). Also, our finding is in agreement with the results of the previous studies where it was found that magnitude and duration of heating had a strong influence on polyphenols stability in foodstuffs (Patras et al., 2010; Wollgast and Anklam, 2000a).

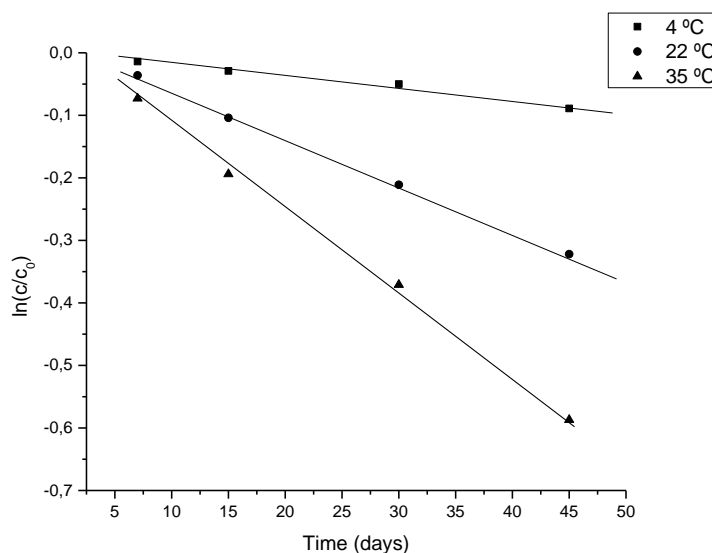


Fig. 4 Degradation of total flavonoids in cocoa powder samples during storage (different temperature protocols)

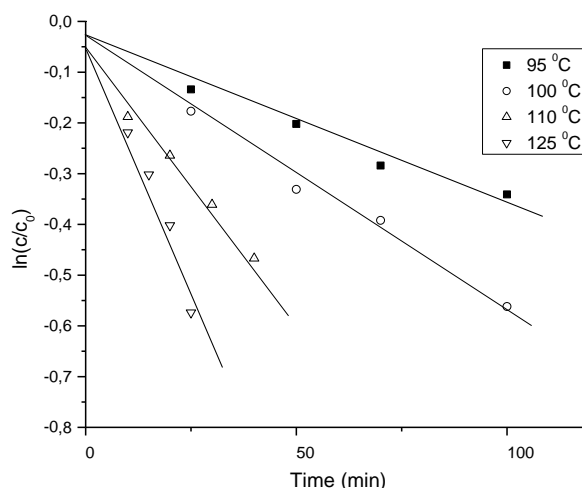


Fig. 5 Degradation of total flavonoids in cocoa powder samples during heating

4. CONCLUSIONS

In this study, degradation of flavonoids in cocoa powder samples during storage and heating was investigated. The present data show that degradation follows first-order reaction kinetics. The variation in the degradation rate constants with temperature obeyed the Arrhenius relationship. During heating, the cocoa powder flavonoids degraded more quickly with an increase in temperature. Thus, a higher stability of flavonoids during the processing of cocoa powder into a cocoa drink could be achieved by employing short-time heating at lower temperatures.

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UTICAJ TEMPERATURE ČUVANJA I ZAGREVANJA NA SADRŽAJ KATEHINA, PROCIJANIDINA I UKUPNIH FLAVONOIDA U KOMERCIJALNIM UZORCIMA KAKAOA U PRAHU

Utica temperature čuvanja na stabilnost katehina, epikatehina, procijanidina B1-B4 i ukupnih flavonoida u uzorcima kakaoa u prahu praćen je u temperaturnom opsegu od 4 °C do 35 °C. Uticaj zagrevanja na sadržaj ukupnih flavonoida je praćen u temperaturnom opsegu od 95 °C do 125 °C. U toku skladištenja uzoraka je došlo do smanjenja u sadržaju ukupnih flavonoida. Pomenuta promena je bila izraženija na višim temperaturama: vreme polu-života ($t_{1/2}$) ukupnih flavonoida u ispitivanom uzorku kakaoa u prahu bilo je kraće kada je on čuvan na sobnoj temperaturi nego kada je čuvan u frižideru. Promene u sadržaju flavonoida u ispitivanim uzorcima prate kinetiku prvog reda. Konstante brzine degradacije, izračunate pomoću Arenijusove jednačine, veće su tokom zagrevanja nego tokom čuvanja ispitivanih uzoraka na nižim temperaturama. Izračunate energije aktivacije (E_a) za katehin, epikatehin i procijanidine B1, B2, B3 i B4 u temperaturnom intervalu od 4 °C do 35 °C bile su: 20,4 kJ/mol, 12,5 kJ/mol, 9,4 kJ/mol, 21,6 kJ/mol, 19,4 kJ/mol i 23,7 kJ/mol.

Ključne reči: kakao prah, katehin, procijanidini, ukupni flavonoidi, termička obrada, degradaciona kinetika