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Effect of Storage Temperature on the Decay of Catechins and Procyanidins in Dark Chocolate

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Abstract

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The storage stability of catechins, procyanidins, and total flavonoids in dark chocolate was investigated. The obtained results showed that the degradation of flavonoids followed first-order reaction kinetics. Temperature-dependent degradation was modelled on the Arrhenius equation. The activation energy for the degradation of dark chocolate flavonoids during storage was 61.2 kJ/mol. During storage, flavonoids degraded more rapidly at 35°C ($k = 7.8 \times 10^{-3}$/day) than at 22°C ($k = 5.4 \times 10^{-3}$/day) and 4°C ($k = 2.2 \times 10^{-3}$/day).

Keywords: degradation kinetics; HPLC; polyphenols; total flavonoids

Cocoa powder and baking chocolates are the cocoa products with the highest amount of non-fat cocoa solids, followed by dark chocolate which contains around 20–29.5% non-fat solids and is derived from cocoa liquor (Miller *et al.* 2008). In recent years, several studies have documented the beneficial effects of dark chocolate on human health. Dark chocolate has been reported to have a protective effect against chronic illnesses, especially cardiovascular diseases (Kuebler *et al.* 2016; Montagnana *et al.* 2017). It has also been proven that the polyphenols from dark chocolate have a protective effect on the function of erythrocytes (Radosinska *et al.* 2017). This has generally been attributed to chocolate's high level of antioxidant flavonoids, particularly catechins, epicatechins and procyanidins. The major catechin-type flavonoids in dark chocolate are catechin and epicatechin, which belong to a group of functional compounds included in the flavan-3-ols (Yilmaz 2006). Procyanidins are present in chocolate in mixtures consisting of dimers, trimers, tetramers and polymers of up to 10 units (Counet & Collin 2003). Dimers contribute most to the total procyanidin content of chocolate (Wollgast & Anklam 2000). Chocolate contains larger amounts of procyanidins per unit weight (3.8–4.9 mg/g) than foods such as red wine (close to 0.2 mg/g), cranberry juice (0.1 mg/g), and apple (0.5–1 mg/g) (Hammerstone *et al.* 2000). In addition, as precursors flavonoids contribute to flavour formation in cocoa and chocolate (Afoakwa 2008). The flavonoid content of chocolate differs substantially from those in raw cocoa beans, cocoa powder or cocoa liquor, and content and concentration depend on cocoa bean variety, processing and storage (Wollgast & Anklam 2000). Several studies have provided evidence of polyphenol degradation during the processing and storage of cocoa beans, and of cocoa powder (Kyi *et al.* 2005; Andres-Lacueva

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et al. 2008; Mazor Jolić *et al.* 2011; Ioannou *et al.* 2012). Moreover, in these previous studies, different heating methods (drying, pressured-steam heating, roasting, heating by autoclave) were used and their effects were analysed. On the other hand, however, no studies have evaluated the effect of storage conditions on the flavonoid content and antioxidant activity of dark chocolate. For this reason, the objective of this study was to investigate the degradation kinetics of individual catechins, procyanidins, and total flavonoids during the storage of dark chocolate at different temperatures. The antioxidant activity was also investigated, under the same conditions. For commercial applications of dark chocolate polyphenols as a functional ingredient, it is important to understand the stability of dark chocolate constituents.

MATERIAL AND METHODS

Chemicals. (+)-catechin, (–)-epicatechin, procyanidin B1, B2, B3, and 2,2-diphenyl-1-picrylhidrazyl (DPPH) were purchased from Sigma Aldrich (Germany). Sodium hydroxide, sodium nitrite, aluminium chloride hexahydrate, hexane, acetic acid, formic acid, acetonitrile, and acetone were purchased from Merck® (Germany). Purified water (18 MΩ/cm), prepared by a MicroMed purification system (TKA Wasseraufbereitungssysteme, Germany), was used to prepare all samples and standards.

Instruments. An Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, USA) was used for absorbance measurements and spectra recording, using optical cuvettes with 1-cm optical pathlength. An Agilent 1200 (Agilent Technologies, USA) was used for HPLC analysis. The analytical column was C_{18} Zorbax Eclipse XDB-C18, 5 µm, 4.6 × 150 mm (Agilent Technologies, USA).

Samples. Dark chocolate (70% cocoa) was purchased from a local market as a quintuple. All samples were stored in the dark at three different temperatures (4, 22, and 35°C). Their individual catechin, procyanidin and total flavonoid contents, as well as antioxidant activities, were determined at chosen intervals (day 7, 15, 30, and 45) for up to 45 days. The typical retail storage duration is one year, but according to Beckett (2008) and Andrae-Nightingale *et al.* (2009), if a chocolate holds its integrity for 6–12 weeks, then the product can be predicted to maintain its integrity for 12 months.

Preparation of dark chocolate samples. The dark chocolate samples were prepared for analysis using a slightly modified procedure of Adamson *et al.* (1999). Each chocolate sample (100 g) was individually ground (except for chocolate at 35°C that was already melted) to obtain a homogeneous material. Ten grams of each ground/melted chocolate sample was defatted 3–5 times with 10 ml of hexane, and the residue was dried under a gentle nitrogen stream. Then, the defatted material (1 g) was 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 minutes. The supernatant was decanted into a clean 50-ml volumetric flask and diluted with water until the flask was full.

Total flavonoid content (TF). The content of total flavonoids was evaluated using the slightly modified aluminium chloride spectrophotometric method described by Zhishen *et al.* (1999) and Yang *et al.* (2004). The reaction mixture was prepared by mixing 0.25 ml of sample, 3 ml of deionized water and 0.3 ml of 5% $NaNO_2$. After incubation at room temperature for 5 min, 1.5 ml 2% aluminium chloride hexahydrate ($AlCl_3{\cdot}6H_2O$) 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. The flask was filled up to 10 ml with deionised water. The absorbance of the reaction mixture was measured against a prepared reagent blank at 510 nm. Catechin was chosen as a standard and the results are expressed as milligram catechin equivalents per gram of sample (mg CE/g). The levels of total flavonoids were determined in triplicate.

Antioxidant activity analysis. The DPPH radical scavenging capacity of each sample was determined according to the method described by Brand-Williams *et al.* (1995). The final results were exxpressed as micromoles of Trolox equivalents (TE) per gram of samples (µmol TE/g).

Analysis of catechins and procyanidins using HPLC. An Agilent chromatograph equipped with autosampler and photodiode-array and fluorescence detector (1200 Series) was used for the HPLC analysis. The separation was performed with a Zorbax Eclipse C_{18} (XDB-C18, 5 µm, 4.6 × 150 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 the UV detector at 289 nm. Procyanidins were determined using the 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

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45-min gradient programme (Porter *et al.* 1991) was slightly modified as follows: 0–10 min 0% B, 10–28 min 0–25% B, 28–30 min 25% B, 30–35 min 25–50% B, 35–40 min 50–80% B and 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 minutes. 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

Table 1. Stability of catechins and procyanidins (mg/g) in dark chocolate samples (n = 5) during storage

Storage temperature (°C)	Time (days)	c_{sr} ± SD[a]	RSD (%)	Storage temperature (°C)	Time (days)	c_{sr} ± SD[a]	RSD (%)
(+)-Catechin				**(–)-Epicatechin**			
4	7	0.185 ± 0.005	2.70	4	7	0.476 ± 0.009	1.89
	15	0.178 ± 0.004	2.25		15	0.461 ± 0.009	1.95
	30	0.169 ± 0.004	2.37		30	0.448 ± 0.008	1.79
	45	0.154 ± 0.003	1.95		45	0.427 ± 0.008	1.87
22	7	0.180 ± 0.005	2.78	22	7	0.469 ± 0.009	1.92
	15	0.169 ± 0.003	1.78		15	0.445 ± 0.008	1.82
	30	0.153 ± 0.003	1.96		30	0.418 ± 0.007	1.79
	45	0.138 ± 0.002	1.45		45	0.391 ± 0.007	1.89
35	7	0.172 ± 0.005	2.91	35	7	0.457 ± 0.008	1.75
	15	0.158 ± 0.003	1.90		15	0.426 ± 0.008	1.88
	30	0.135 ± 0.003	2.22		30	0.377 ± 0.007	1.86
	45	0.115 ± 0.002	1.74		45	0.339 ± 0.007	2.06
Procyanidin B1				**Procyanidin B2**			
4	7	0.044 ± 0.001	2.27	4	7	0.197 ± 0.006	3.05
	15	0.042 ± 0.001	2.38		15	0.192 ± 0.006	3.13
	30	0.039 ± 0.001	2.56		30	0.186 ± 0.005	2.69
	45	0.038 ± 0.001	2.63		45	0.179 ± 0.005	2.79
22	7	0.042 ± 0.001	2.44	22	7	0.193 ± 0.006	3.11
	15	0.038 ± 0.001	2.63		15	0.185 ± 0.006	3.24
	30	0.034 ± 0.001	2.94		30	0.174 ± 0.005	2.87
	45	0.031 ± 0.001	3.23		45	0.161 ± 0.005	3.11
35	7	0.039 ± 0.001	2.56	35	7	0.187 ± 0.006	3.21
	15	0.035 ± 0.001	2.86		15	0.176 ± 0.006	3.41
	30	0.030 ± 0.001	3.33		30	0.161 ± 0.005	3.11
	45	0.0264 ± 0.0008	3.03		45	0.142 ± 0.004	2.82
Procyanidin B3				**Procyanidin B4**			
4	7	0.119 ± 0.003	2.52	4	7	0.042 ± 0.001	2.38
	15	0.116 ± 0.003	2.59		15	0.040 ± 0.001	2.50
	30	0.112 ± 0.003	2.68		30	0.038 ± 0.001	2.63
	45	0.109 ± 0.003	2.75		45	0.036 ± 0.001	2.78
22	7	0.116 ± 0.003	2.59	22	7	0.040 ± 0.001	2.50
	15	0.111 ± 0.003	2.70		15	0.038 ± 0.001	2.63
	30	0.105 ± 0.002	1.91		30	0.035 ± 0.001	2.86
	45	0.097 ± 0.002	2.06		45	0.032 ± 0.001	3.13
35	7	0.111 ± 0.003	2.70	35	7	0.037 ± 0.001	2.70
	15	0.104 ± 0.002	1.92		15	0.034 ± 0.001	2.94
	30	0.092 ± 0.002	2.17		30	0.029 ± 0.001	3.45
	45	0.081 ± 0.002	2.47		45	0.0255 ± 0.0008	3.14

[a]means ± standard deviations for quintuple samples; c_{sr} – mean value of five samples; RSD – relative standard deviation

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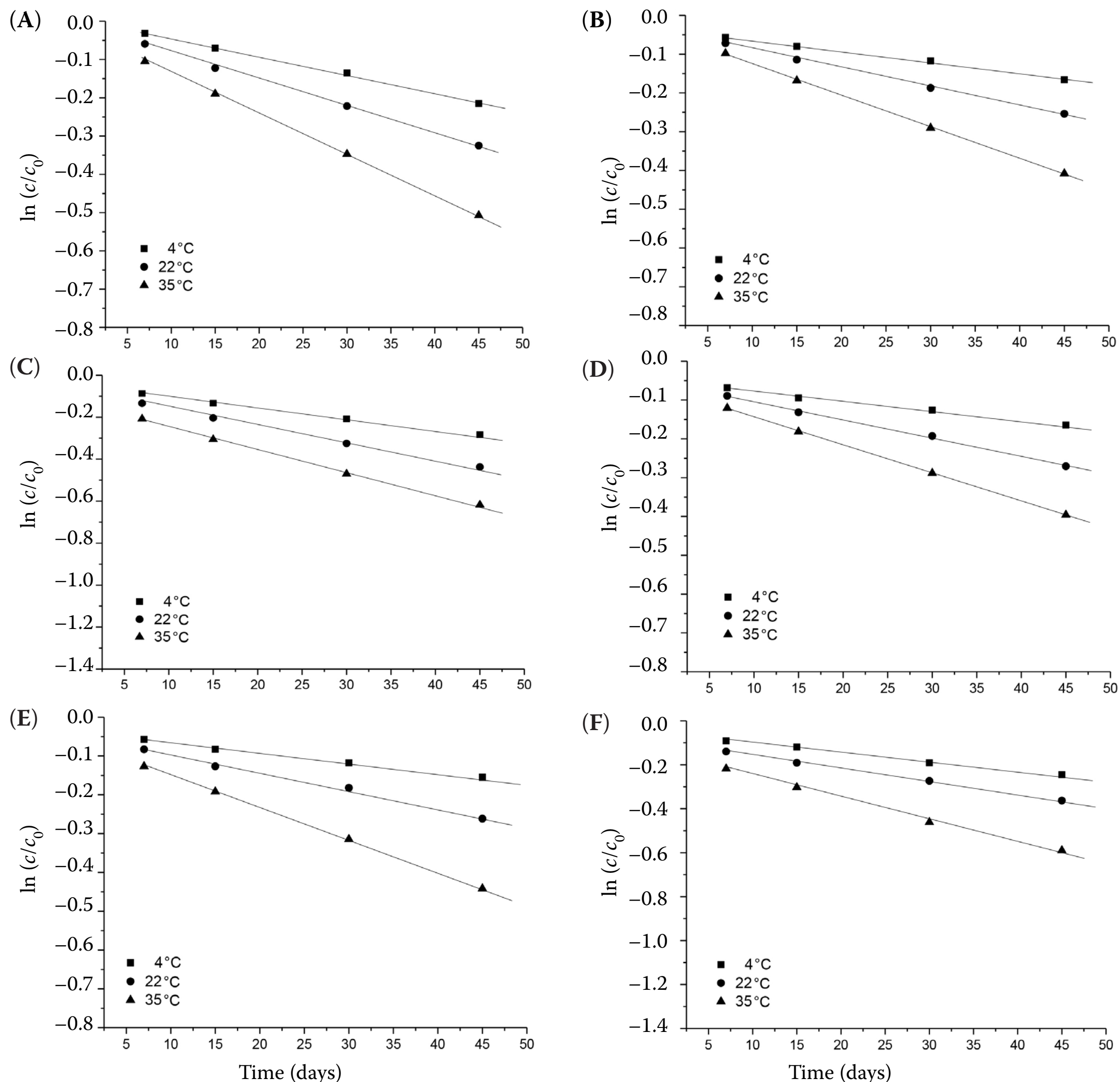

Figure 1. Degradation of catechins and procyanidins in dark chocolate samples during storage: (**A**) (+)-catechin, (**B**) (–)-epicatechin, (**C**) procyanidin B1, (**D**) procyanidin B2, (**E**) procyanidin B3, (**F**) procyanidin B4

phenolic compounds in samples was achieved using calibration lines.

RESULTS AND DISCUSSION

The stability of individual catechins and procyanidins in dark chocolate samples at 4, 22, and 35°C over the course of 45 days of storage is presented in Table 1. Among the catechin-type of flavonoids, (–)-epicatechin content was higher than (+)-catechin in all tested samples. The most abundant procyanidin was B2, followed by B3, B1, and B4. Similar findings were reported by Gu *et al.* (2006), Cooper *et al.* (2007), Langer *et al.* (2011), and Brcanović *et al.* (2013). HPLC analysis revealed that after 45 days of storage of dark chocolate in the temperature range 4–35°C the percentage decomposition of (+)-catechin ranged from 16.8% to 33.1%, (–)-epicatechin was in the range of 10.3–25.8%, procyanidin B1 ranged from 13.6% to 33.3%, procyanidin B2 from 9.1% to 24.1%, procyanidin B3 from 8.4% to 27.0%, and procyanidin B4 was ranged from 14.3% to 32.4%. Linear regression confirmed that degradation of individual flavonoids (Figure 1) in dark chocolate samples followed first-order reaction kinetics. The degradation of total flavonoid content also followed a first-order reaction model (Table 2 and Figure 2). To verify the applicability of a first-order kinetic model, ln (c/c_0) is plotted against time. The first-order reaction rate constants (k) and half-lives ($t_{1/2}$), i.e., the time needed for the degradation of 50% of total flavonoid content, were calculated using the following equations (Kirca

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Table 2. Effect of storage temperature on the degradation of total flavonoids and antioxidant activity in dark chocolate samples (n = 5)

Storage temperature (°C)	Time (days)	c_{sr} ± SD[a] (mgCE/g)	RSD (%)	Storage temperature (°C)	Time (days)	c_{sr} ± SD[a] (μmol TE/g)	RSD (%)
4	7	11.5 ± 0.3	2.61	4	7	24.9 ± 0.4	1.61
	15	11.3 ± 0.3	2.65		15	24.7 ± 0.4	1.62
	30	11.0 ± 0.2	1.82		30	24.4 ± 0.4	1.64
	45	10.7 ± 0.2	1.87		45	24.1 ± 0.4	1.66
22	7	11.2 ± 0.3	2.68	22	7	24.4 ± 0.4	1.64
	15	10.8 ± 0.2	1.85		15	24.1 ± 0.4	1.66
	30	10.2 ± 0.2	1.96		30	23.6 ± 0.4	1.69
	45	9.7 ± 0.2	2.06		45	23.0 ± 0.4	1.74
35	7	10.7 ± 0.2	1.87	35	7	23.9 ± 0.4	1.67
	15	10.1 ± 0.2	1.98		15	23.4 ± 0.4	1.71
	30	9.2 ± 0.2	2.17		30	22.5 ± 0.3	1.33
	45	8.3 ± 0.2	2.41		45	21.6 ± 0.3	1.39

[a]means ± standard deviations for quintuple samples; c_{sr} – mean value of five samples; RSD – relative standard deviation; c_0 = 11.8 ± 0.3 mg/g; c_0 = 25.1 ± 0.4 μmol TE/g

& Cemeroglu 2003; Harbourne *et al.* 2008; Atkins & de Paula 2010):

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

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

where: c_0 – initial flavonoid content (mg/g); c_t – flavonoid content after t days of storage at a given temperature

The rate of degradation of total flavonoid content increased with increasing storage temperature and time. The high R^2-values confirm this finding (Table 3). Storage at 35°C resulted in faster flavonoid degradation as compared to refrigerated storage at 4°C. For example, $t_{1/2}$ values of dark chocolate flavonoids were 319 days at 4°C and 89 days at 35°C (Table 3). After 45 days of storage at 4°C, dark chocolate samples still contained over 93% of their total flavonoid content, while at 22°C over 86% remained and at 35°C over 77% of total flavonoids remained.

The Arrhenius equation was used to describe the temperature dependence of the degradation rate constants (Atkins & de Paula 2010; Moldovan *et al.* 2012):

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

where: k_0 – frequency factor/min; E_a – activation energy (kJ/mol); R – universal gas constant (8.314 J/mol K); T – absolute temperature (K)

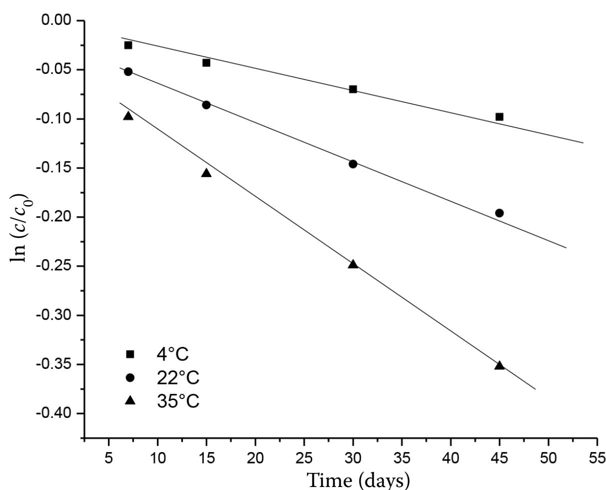

Figure 2. Degradation of total flavonoids in dark chocolate samples during storage

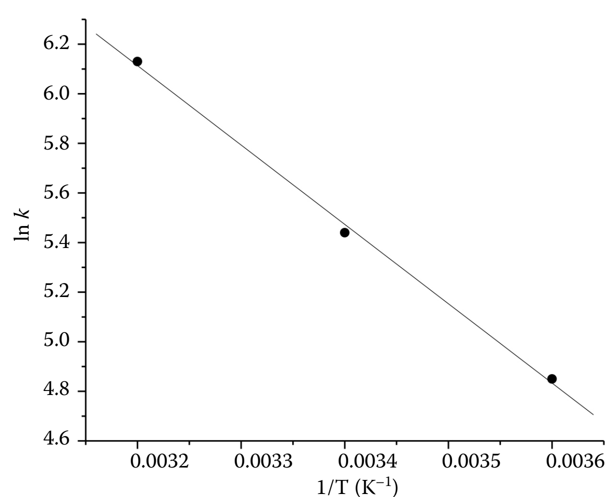

Figure 3. Arrhenius plots for degradation of total flavonoids in dark chocolate samples during storage

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Table 3. Effect of storage temperature on degradation of dark chocolate flavonoids

Temperature (°C)	$k \times 10^3$ (day^{-1})	R^2	$t_{1/2}$ (day)	E_a (kJ/mol)	Q_{10}	
					4–22°C	22–35°C
4	2.2 ± 0.1	0.9974	319			
22	5.4 ± 0.2	0.9911	159	61.2	1.7	1.3
35	7.8 ± 0.3	0.9965	89			

The Arrhenius plot is shown in Figure 3. Activation energy was found to be 61.2 kJ/mol.

Q_{10} values were also calculated from Eq. (4) to describe the sensitivity of storage temperatures to temperature changes (Atkins & de Paula 2010; Moldovan & David 2014):

$$Q_{10} = (k_2/k_1)^{10/T_2 - T_1} \quad (4)$$

Q_{10} values of 1.7 at 4–22°C and 1.3 at 22–35°C were obtained (Table 3). The higher Q_{10} value for storage temperatures of 4–22°C indicate that low storage temperatures are more sensitive to temperature elevations than high storage temperatures (22–35°C).

In their review article, Ioannou *et al.* (2012) discuss a few studies which have focused on the modelling of degradation kinetics of flavonoids during storage. According to these studies, most of which employed the Arrhenius model, increasing storage temperatures lead to a rapid degradation of flavonoids. Very few studies have evaluated the effect of storage on antioxidant activity. In our study (Table 2), antioxidant activity slightly decreased during storage. After 45 days of storage at 4°C, the antioxidant activity of samples decreased by about 3%, at 22°C by about 6% and at 35°C by about 9%. This finding is in accordance with literature data on the change in antioxidant activity during storage of strawberries (Odriozola-Serrano *et al.* 2009) and pineapple juice (Zheng & Lu 2011).

CONCLUSIONS

In this research, the degradation of total flavonoids in dark chocolate during storage was investigated. Degradation kinetics could be adequately described by a first-order model, and the reaction rate constants were more temperature-dependent at higher temperatures than at refrigeration temperatures. A good understanding of the behaviour of total and individual flavonoids during storage could be of benefit to confectionery companies, allowing them to produce high-quality chocolate with a long shelf life.

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