Polyphenol and colour stability through beer ageing: comparison of polyphenol quantification assays

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Beer quality is known to deteriorate greatly with time. In the last decade, many papers have underlined the key role played by different off-flavours (*trans*-2-nonenal, etc.). As far as polyphenols are concerned, scarce information is available. NP-HPLC-ESI(-)-MS/MS data show that the level of small "natural" flavanoids (monomers, dimers, and trimers) decreases through ageing, leading to the formation of yellow/brown adducts. RP-HPLC-ESI(-)-MS/MS analyses of aged model media and beers enabled us to identify the coloured structures as dehydrodi(tri)catechin A di(tri)mers. Thioacidolysis with toluene-α-thiol was unable to depolymerise such structures, leading to underestimated polymerisation degree values in aged samples. Different usual global assays were applied in order to identify which ones can help to predict the extent of beer polyphenol degradation. Whatever the beer degradation state, the Bishop EBC total polyphenol test yielded the same absorbance. On the other hand, total flavanoids (absorbance in the presence of *p*-dimethylaminocinnamaldehyde) were slightly lower after a few days. Antioxidant activity (AAPH assay) unsurprisingly decreased through storage, especially in the case of natural ageing.

INTRODUCTION

Proanthocyanidins are known to be responsible for colloidal instability through beer storage (*1*). As recently described (*2*), they could also be involved in the colour of aged beer. NP-HPLC-ESI(-)-MS/MS analyses have shown that one-year storage at 20°C leads to degradation of 75% of small flavanoids (final concentration of small procyanidins under 0.5 mg/L). As depicted in **Figure 1**, storage of model media containing catechin and procyanidin dimers (P2) and trimers (P3) revealed that only the monomeric fraction is responsible for a significant colour change. Light and storage temperature emerged as key factors.

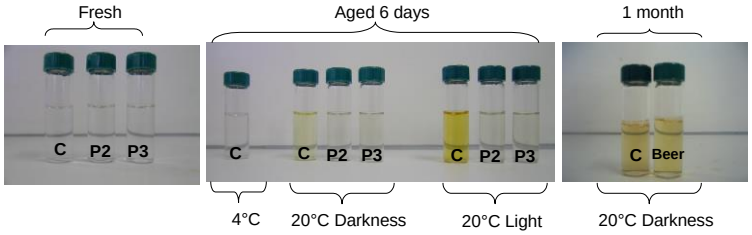

Figure 1. Pictures of aqueous model media containing (+)-catechin (C), dimers (P2) and trimers (P3). Fresh and stored samples (6 days and 1 month), compared to a beer aged 1 month at 20°C in a brown bottle.

The appearance of colour revealed to correlate with (+)-catechin degradation. As shown in **Figure 2**, colourless dehydrodicatechin B dimers (n°4+7, M_w 578) and dehydrotricatechin B trimers (n° 15, M_w 866) are gradually formed. They will be further transformed into yellow-brown dehydrodicatechin A dimers (n°11+13, M_w 576) and dehydrotricatechin A trimers (n°17+19, M_w 864) (*2*).

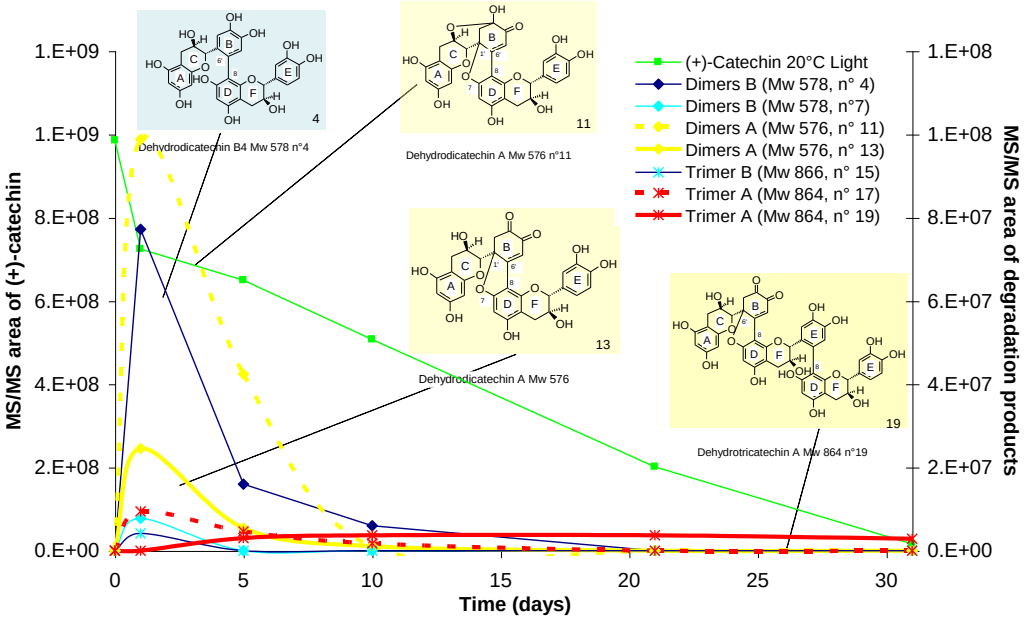

Figure 2. RP-HPLC-ESI(-)-MS/MS areas (arbitrary units-counts) of (+)-catechin and its degradation products through storage (20°C with light, model medium).

The aim of the present paper was to find which, among the usual global assays, might help us to predict the extent of beer polyphenol degradation. Assays for total polyphenols (complexation reaction with ferric ions in alkaline solution) and total flavanoids (nucleophilic addition on *p*-dimethylaminocinnamaldehyde) were applied to model media and aged beers. The antioxidant efficiency and polymerisation degree (DP) were also assessed (AAPH and thioacidolysis methods, respectively).

MATERIALS AND METHODS

Chemicals. (-)-Epicatechin (98%), (+)-catechin (98%), (-)-gallocatechin (98%), (-)-epigallocatechin (98%), and B2 ((-)-epicatechin-4β-8-(-)-epicatechin, 90%) were obtained from Sigma-Aldrich (Bornem, Belgium). Methanol (99.9%) was purchased from Romil (Cambridge, UK). Toluene-α-thiol (99%) was obtained from Fluka (Buchs, Switzerland). Hydrochloric acid (37%) was from Fischer Scientific (Leicestershire, UK). 3,4-β-Epicatechin benzylthioether, 3,4-α- or β-catechin benzylthioether, and 3,4-α- or -β-gallocatechin benzylthioether were obtained as previously described (*3*). Pure fractions of procyanidins dimers-P2 and trimers-P3 were obtained as described in (*4*).

Beers and model media. Four pilot plant lager beers were obtained in brown bottles from a Belgian brewery (1, 1' and 2 stabilized with PVPP, 3 filtrated on silica gel). They were aged at 20°C in a dark room. Beer samples were directly analysed in duplicate. For thioacidolysis, a medium without water was required. Powder extracts were obtained by freeze-drying of degassed samples concentrated under vacuum at low temperature.
(+)-Catechin and B2 were prepared in non degassed mQ water (each 50 ppm) and stored at 20°C under white light. Samples were collected after 2, 6, 15, and 60 days.

Total polyphenols and total flavanoids contents. Total polyphenols concentration was determined according to Bishop (*5-7*). Flavanoids were quantified by means of a colorimetric assay based on the reaction with *p*-dimethylaminocinnamaldehyde (*8*).

Antioxidant assay - AAPH method. The reduction power was measured by a method developed in our laboratory (*9*). The oxidation of linoleic acid (aqueous dispersion) is induced by 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) in the absence or presence of antioxidant. The rate of oxidation at 37 °C is monitored by recording the increase in absorption at 234 nm caused by conjugated diene hydroperoxides. A Shimadzu UV-visible 240 spectrophotometer (Antwerp, Belgium) equipped with an automatic sample positioner allowed analysis of six samples per minute. In all cases, the measurements were run in duplicate against the buffer and compared with a separate AAPH-free control to check for any spontaneous oxidation.

Determination of the mean polymerization degree by thiolysis (*3*). In a polypropylene vial, 40 µL sample (or 5 mg freeze-dried powder), 40 µL methanol with 3.3% HCL (v/v), and 80 µL toluene-α-thiol (5% v/v in methanol) were mixed together. The vials were placed at 40°C for 30 min. To ensure complete degradation, the reaction medium was further kept at room temperature for 10 h. Separations were carried out on a 2-µm, 150 x 2.1 mm i.d. reverse phase C18 Prevail column (Alltech, Deerfield, IL, USA). A flow rate of 0.2 mL/min was applied with a linear gradient from water with 1% acetonitrile and 0.1% formic acid (A) to acetonitrile (B). Gradient elution was 97-91% A, 0-5 min; 91-84% A, 5-15 min; 84-50% A, 15-45 min; 50-10% A, 45-48 min; 48-51 min isocratic and then return to the initial conditions for 15 min. 5 µL of sample was injected into the column kept at 25°C. For the ESI source, the following inlet conditions were applied : source voltage, 4.9 kV; capillary voltage, -4 V; capillary temperature, 200°C; and sheath gas 40 psi. Collision-induced dissociation spectra were recorded at 30%. Quantification of terminal and extension units was obtained after calibration with each standard (MS/MS on m/z 289 for (+)-catechin and (-)-epicatechin, MS/MS on m/z 305 for (+)-gallocatechin and (-)-epigallocatechin; the same ion was selected for free and nucleophile-bounded flavan-3-ols). The mean polymerization degree was obtained with the following equation: mDP = (terminal units+ extension units)/terminal units. The undegraded medium was used to quantify monomeric native structures.

RESULTS AND DISCUSSION

Evolution of total polyphenols and total flavanoids through storage. Total polyphenols and total flavanoids were first quantified in aqueous model media containing (+)-catechin or dimer. As depicted in **Table 1**, (+)-catechin gave a response similar to P2 in the Bishop assay. As previously shown by McMurrough and McDowell (*10*), monomers gave a higher total flavanoid value than natural P2 procyanidin.

Table 1. Total polyphenols and flavanoids in aqueous model media through storage*.

		(+)-Catechin	P2 (B2)
Total polyphenols (mg/L eq. polyphenols isolated from malt and hop) – Abs. at 600 nm (CV, %)			
Fresh		6.9 (1.2)	7.0 (0.4)
Ageing at 20°C, light	2 days	7.0 (0.5)	-
	6 days	7.0 (0.3)	-
	15 days	7.0 (1.0)	-
	2 months	6.5 (1.0)	-
Total flavanoids (mg/L eq catechin) – Abs. at 640 nm (CV, %)			
Fresh		5.0 (0.0)	1.9 (0.3)
Ageing at 20°C, light	2 days	5.9 (0.0)	-
	6 days	8.7 (1.4)	-
	15 days	7.1 (0.3)	-
	2 months	5.1 (0.3)	

- Not determined; *data given for 5 ppm, r^2= 0.9999

To assess the impact of storage, these global assays were further applied to the aged model media. Surprisingly, the Bishop EBC total polyphenol test yielded similar absorbance, whatever the degradation state. On the other hand, total flavanoids were higher in intermediate samples. These results suggest that *p*-dimethylaminocinnamaldehyde could bind to dehydrodi(tri)catechin B di(tri)mers to create adducts with high molar extinction coefficients. Over a long period, more brown dehydrodi(tri)catechin A di(tri)mers appeared to be synthesised and the absorbance slightly decreased.

The same methodology was then applied to three lager beers (**Table 2**). Massive degradation of small oligomers was confirmed for all three beer samples by ESI(-)-MS/MS (*2*). Total polyphenol values were relatively stable for from one month to one year whilst total flavanoids decreased. Expectedly, values were two times higher in silicagel-filtered beer 3 than in beers 1 and 2, stabilised by PVPP treatment.

Table 2. Total polyphenols and flavanoids in three beers through storage.

		Beer 1	Beer 2	Beer 3
Total polyphenols (mg/L eq. polyphenols isolated from malt and hop) – Abs. at 600 nm (CV, %)				
Fresh		87.0 (1.0)	88.0 (1.8)	162.0 (0.0)
Natural ageing at 20°C	1 month	86.9 (0.0)	87.7 (0.0)	162.3 (0.0)
	6 months	95.5 (0.4)	84.0 (0.5)	162.7 (0.3)
	10 months	92.2 (0.4)	93.8 (1.3)	161.9 (0.3)
	12 months	88.9 (0.5)	92.2 (1.3)	160.3 (0.3)
Accelerated ageing	2 days at 60°C	82.4 (1.4)	-	-
	5 days at 40°C	82.4 (5.4)	-	-
	5 days a 50°C	84.0 (4.5)	-	-
	5 days at 60°C	84.4 (4.8)	-	-
Total flavanoids (mg/L eq catechin) – Abs. at 640 nm (CV, %)				
Fresh		13.5 (1.2)	12.2 (1.4)	29.1 (1.7)
Natural ageing at 20°C	1 month	13.0 (0.0)	11.0 (0.0)	25.3 (0.6)
	6 months	13.4 (0.0)	11.5 (1.4)	26.2 (0.6)
	10 months	11.8 (1.4)	9.7 (0.0)	20.9 (0.8)
	12 months	7.2 (2.3)	7.8 (2.1)	17.9 (0.9)
Accelerated ageing	2 days at 60°C	16.9 (1.0)	-	-
	5 days at 40°C	16.6 (1.0)	-	-
	5 days at 50°C	16.7 (0.0)	-	-
	5 days at 60°C	16.9 (3.0)	-	-

- Not determined

Evolution through storage of antioxidant activity and mDP. The antioxidant efficiency was measured in the model media by the AAPH assay. The mDP was assessed by thioacidolysis. As shown in **Table 3**, the higher the polymerisation degree of fresh "natural" procyanidins the higher the antioxidant efficiency. Thioacidolysis gave the correct mDP when natural P1, P2, and P3 reacted with toluene-α-thiol. The Tinh was found to increase through storage despite the loss of (+)-catechin. This means that the new structures (compounds n°4, 11, 17 and 19 in **Figure 2**) formed by oxidation of (+)-catechin exhibit high antioxidant activity. On the other hand, they are unable to react with toluene-α-thiol, leading to an unmodified mDP=1.

Table 3. Antioxidant efficiency and mDP in aqueous model media through storage (analyses for 50 ppm).

		(+)-Catechin	P2	P3
Antioxidant efficiency, inhibition time = Tinh (min/ppm) (CV, %)				
Fresh		128.7 (0.0)	171 [a]	286 [a]
Ageing at 20°C, light	2 days	147.6 (3.6)	-	-
	6 days	166.0 (5.9)	-	-
	15 days	165.4 (1.8)	-	-
	2 months	191.0 (3.4)		
mDP, measured by thiolysis				
Fresh [b]		1	2	3
Ageing at 20°C, light	2 days	1	-	-
	6 days	1	-	-
	15 days	1		
	2 months	1	-	-

[a] Results of (*11*) and [b] Results of (*3*); -: Not determined

In the case of lager beers (**Table 4**), the inhibition time decrease was obvious, suggesting that storage over the longer period investigated here leads to degradation of the best antioxidants (coloured structures probably). Thioacidolysis directly applied to the global freeze-dried beer extract yielded a constant mDP whatever the storage time.

Table 4. Antioxidant efficiency and mDP of three beers through storage.

Antioxidant efficiency, inhibition time = Tinh (min for beer diluted 400x)				
		Beer 1 (CV %)	**Beer 2 (CV %)**	**Beer 3 (CV %)**
Fresh		70.4 (0.6)	56.5 (0.7)	80.2 (0.3)
Natural ageing at 20°C	4 months	54.4 (0.7)	47.9 (2.3)	61.2 (1.3)
	6 months	33.6 (1.5)	32.0 (1.0)	46.0 (1.6)
	10 months	34.5 (0.4)	28.4 (1.4)	34.0 (0.4)
	12 months	29.8 (4.7)	31.1 (1.4)	38.6 (1.9)
Accelerated ageing	2 days at 60°C	50.5 (3.2)	-	-
	5 days at 40°C	63.6 (1.3)	-	-
	5 days at 50°C	65.8 (1.0)	-	-
	5 days at 60°C	53.2 (1.0)	-	-
mDP measured by thiolysis *				
		Beer 1	**Beer 1'**	
Fresh		3.3	3.3	-
Natural ageing at 20°C	1 month	2.6	2.9	-
	2 months	-	3.5	-
	3 months	3.0	-	-

- Not determined

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