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Analytical Methods

Use of thiolysis hyphenated to RP-HPLC-ESI(-)-MS/MS for the analysis of flavanoids in fresh lager beers

Delphine Callemien [a], Sylvain Guyot [b], Sonia Collin [a,*]

[a] Unité de Brasserie et des Industries Alimentaires, Université catholique de Louvain, Croix du Sud, 2/bte 7, B-1348 Louvain-la-Neuve, Belgium
[b] INRA, Unité de Recherches Cidricoles, Biotransformation des Fruits et Légumes (URC-BFL), Domaine de la Motte, BP 35327, 35 653 Le Rheu, Cedex, France

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ABSTRACT

Proanthocyanidins are well known for their involvement in haze and colour development during beer ageing. New methodologies are needed, however, to understand what happens to them in the bottled beer. For the first time in the brewing field, thiolysis was hyphenated to RP-HPLC-ESI(-)-MS/MS to investigate these flavanoids. Thirty minutes at 40 °C followed by 10 h at room temperature emerged as the best conditions for complete depolymerisation. NP-HPLC-ESI(-)-MS/MS was used to quantify and isolate fractions from monomers to trimers in a Sephadex LH20 acetone/water (70/30, v/v) beer extract. Unsurprisingly, a lower dimer/monomer ratio was evidenced in PVPP-treated beers than in silica gel-filtered beers. Most beer dimers are procyanidins B3 (two catechin units) whilst most trimers are prodelphinidins (catechin in terminal units and gallocatechins or catechins in extension units). Gallocatechin appeared to come mainly from malt. Despite the absence of chromatographic peaks corresponding to oligomers above trimers, an apparent degree of polymerisation close to six was calculated in the total LH20 extract. Still higher mean degrees of polymerisation (mDPs) were calculated for malt and hop, indicating selective extraction or depolymerisation from raw materials to beer. The main part of beer polyphenols is composed of complex undefined structures not degraded by toluene-α-thiol.

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1. Introduction

Up to now, beer flavanoids have been considered most for their involvement, with proteins, in haze development (Siebert, 1999). Among them, two monomeric flavan-3-ols ((+)-catechin and (-)-epicatechin), a few B-type (with a single C4–C8 bond between successive units) procyanidin and prodelphinidin dimers (B3 and B9), and two A-type (with a single C4–C8 or C4–C6 bond and an additional ether bond between C2 and O–C7 or O–C5) prodelphinidin dimers have been found (Fig. 1 (Delcour, & Tuytens, 1984; Gerhauser, 2005; Madigan, McMurrough, & Smyth, 1994; McMurrough, & Baert, 1994; McMurrough, Madigan, Kelly, & Smyth, 1996)). The presence of oligomers up to hexamers has also been mentioned (Gu, Kelm, Hammerstone, Beecher, Holden, Haytowitz, & Prior, 2003). Despite the fact that 100 times more malt is used, up to 30% of beer proanthocyanidins are known to derive from hops (Jerumanis, 1985; Li, & Deinzer, 2006; McMurrough, 1981; Mulkay, Touillaux, & Jerumanis, 1981; Stevens, Miranda, Wolthers, Schimerlik, Deinzer, & Buhler, 2002).

Strong contradictions exist in the literature between reported concentrations of individual flavanoids (often quantified by HPLC) and total polyphenol levels assessed by global assays, such as Folin-Ciocalteau (an oxido-reduction reaction with phosphotungstomolybdic acids (George, Brat, Alter, & Amiot, 2005)), the Bishop EBC test (a complexation reaction with ferric ions in alkaline solution leading to a red colour quantified at 600 nm (Bishop, 1972)) or total flavanoids (nucleophilic addition on *p*-dimethylaminocinnamaldehyde, leading to absorption at 640 nm (Delcour, & Janssens de Varebeke, 1985)).

Monomers to trimers can be directly quantified by NP or RP-HPLC combined with ESI-MS/MS (Callemien, & Collin, 2007). Given the low concentration and the high complexity of oligomers, development of specific methods for beer proanthocyanidins is required to elucidate the molecules involved in colloidal and colour instability of beer. The acid-catalysed cleavage of the interflavanyl linkage can be obtained in the presence of a nucleophilic reagent such as toluene-α-thiol (Gu, Kelm, Hammerstone, Beecher, Cunningham, Vannozzi, & Prior, 2002; Gu et al., 2003; Guyot, Marnet, Sanoner, & Drilleau, 2001; Kennedy, & Jones, 2001; Matthews, Mila, Scalbert, Pollet, Lapierre, Herve du Penhoat, Rolando, & Donnelly, 1997). The extension units combined with the nucleophile are released. Only the terminal unit is detected as a free flavan-3-ol. In this way, terminal and extension units can be quantified, and a mean degree of

Abbreviation: mDP, mean degree of polymerization; P1–P10, procyanidins from monomers to decamers; EBC, European brewery convention.

* Corresponding author. Tel: +32 (0) 10 47 29 13; fax: +32 (0) 10 47 21 78.
E-mail address: collin@inbr.ucl.ac.be (S. Collin).

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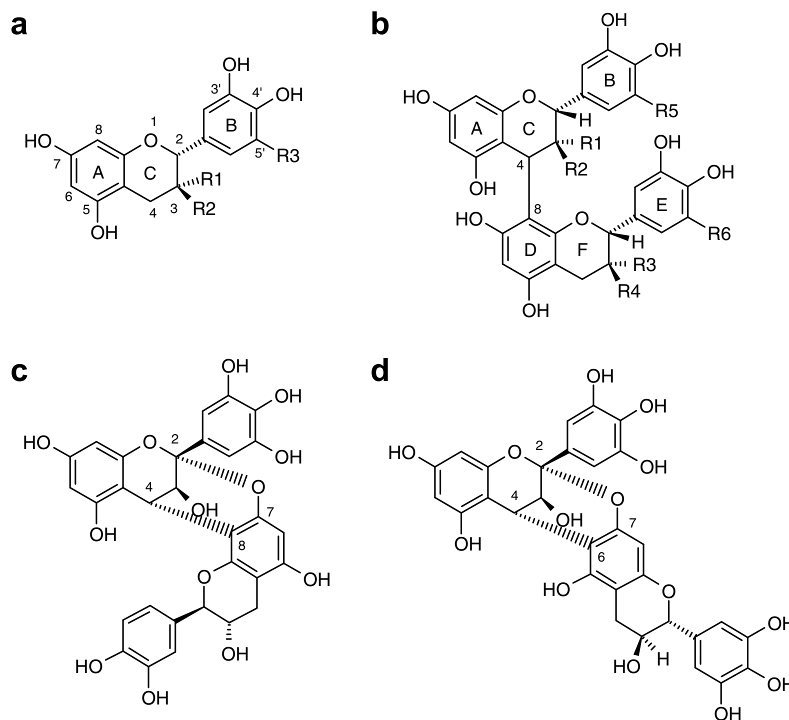

Fig. 1. (a) Structure of the flavanol-3-ol units: (+)-catechin (R1 = H, R2 = OH, R3 = H), (-)-epicatechin (R1 = OH, R2 = R3 = H), (-)-gallocatechin (R1 = H, R2 = R3 = OH), and (-)-epigallocatechin (R1 = OH, R2 = H, R3 = OH); (b) B-type dimer proanthocyanidins C4–C8. *Procyanidin:* B3 (R1 = H, R2 = OH, R3 = H, R4 = OH, R5 = R6 = H); *Prodelphinidins*: B3 (R1 = H, R2 = OH, R3 = H, R4 = R5 = OH, R6 = H), B9 (R1 = OH, R2 = H, R3 = H, R4 = R5 = OH, R6 = H); (c) A-type dimer prodelphinidins: *ent*-epigallocatechin-(4α-8, 2α-O-7)-catechin and (d) A-type dimer prodelphinidins: *ent*-epigallocatechin-(4α-6, 2α-O-7)-catechin.

polymerisation calculated. This method, initially proposed to investigate grape seeds (Prieur, Rigaud, Cheynier, & Moutounet, 1994) and cider apples (Guyot, Doco, Souquet, Moutounet, & Drilleau, 1997), has been optimised here on beer-flavanoid-like components from cocoa.

Beer proanthocyanidin composition was also investigated. Dimers and trimers were quantified and isolated as pure fractions by NP-HPLC-ESI(-)-MS/MS applied to a Sephadex LH20 acetone/water (70/30, v/v) beer extract. Thiolysis was then investigated both on these isolated fractions and on the total Sephadex LH20 extract. Similar experiments were finally conducted on malt and hops.

2. Materials and methods

2.1. Chemicals

Acetone (99.9%), *n*-hexane (99.8%), (-)-epicatechin (98%), (+)-catechin (98%), (-)-gallocatechin (98%), and (-)-epigallocatechin (98%) were from Sigma-Aldrich (Bornem, Belgium). Methanol (99.9%) and dichloromethane (99.9%) were from Romil (Cambridge, UK). Acetic acid (99.8%) was from Acros (Geel, Belgium). Toluene-α-thiol (99%) and ammonium acetate (99%) were obtained from Fluka (Buchs, Switzerland). Acetonitrile (99.99%) and hydrochloric acid (37%) were from Fisher Scientific (Loughborough, UK). Formic acid (99%) was from Janssen Chemica (Geel, Belgium). 3,4-β-Epicatechin benzylthioether was provided by the "Unité de Recherches Cidricoles, Biotransformation des Fruits et Légumes" (INRA, France). Isolated fractions of cocoa liquor procyanidins (P1–P7) were obtained as described in previous works (Counet, & Collin, 2003). Barley Angora was provided by INRA (Clermont-Ferrand, France). Two pilot (A and D) and one commercial (B) lager beers treated by polyvinylpoly pyrrolidone (PVPP) as well as one pilot lager beer filtered with silica gel (C), were offered by a Belgian brewery. Both malts used for these productions and T90 hop pellets (a bitter hop: Tomahawk, and an aromatic variety: Willamette) were kind gifts of the Belgian brewery and Yakima Chief (Strombeeck-Bever, Belgium), respectively.

2.2. Beer Flavanoids solid-phase extractions (Sephadex LH20)

These were conducted according to Callemien and Collin (2007). By the standard addition method, 100% recovery in catechin was calculated (spike with increasing amounts of (+)-catechin before extraction). The same recovery factor was used for P2 and P3.

2.3. NP-HPLC-ESI(-)-MS/MS of proanthocyanidins

A SpectraSystem (Finnigan Mat, San Jose, CA) equipped with an SCM degasser, an AS3000 autosampler, and a P4000 quaternary pump was used. A 5-μm, 250 × 2.1 mm i.d. Silica Alltima HP column (Alltech, Deerfield, IL) was used at a flow rate of 0.2 ml/min. Chromatographic separation was obtained with a multilinear dichloromethane (A) methanol (B) gradient containing a constant 4% level of acetic acid/water (1/1 v/v). Gradient elution was 82–72% A, 0–20 min; 72–61% A, 20–50 min; 61–10% A, 50–55 min; 55–60 min isocratic and return to the initial conditions for 15 min. A post-column addition of ammonium acetate (10 mM in methanol) at 0.05 ml/min was applied. Five microlitres of sample was injected into the column kept at 25 °C. Mass spectra were acquired with an LCQ ion trap mass spectrometer equipped with an ESI source. The system was controlled with the Xcalibur software version 1.2. The ESI inlet conditions were as follows: source voltage, 4.5 kV; capillary voltage, –6 V; capillary temperature, 200 °C; and sheath gas, 20 psi. Collision-induced dissociation spectra were recorded at relative collision energies of 30%, 35%, and 40%, respectively, for singly charged $[M–H]^-$ ions of monomers, dimers, and

trimers. Quantification was done using the calibration curves of (+)-catechin (commercial standards).

2.4. Collection of beer proanthocyanidin fractions by NP-HPLC-UV

Separation was carried out on a 5-μm, 250 × 4.6 mm i.d. Luna silica column (Phenomenex, Torrance, CA) at a flow rate of 1 ml/min with the same gradient elution as described above for the MS/MS analysis. Twenty microlitres of a lager beer LH20 extract were injected twenty times onto the column, which was kept at 25 °C. Procyanidins were monitored at 280 nm (9-nm bandwidth) with a UV6000LP diode array detector. Each minute of eluate was collected by the automatic collector (Pharmacia, Uppsala, Sweden). The contents of suitable vials (P1: from 11 to 12 min; P2: from 18 to 19 min; P3: from 23 to 24 min) were pooled, concentrated to dryness, and dissolved in 2 ml methanol.

2.5. Thiolysis coupled to RP-HPLC-ESI(-)-MS/MS

This method is adapted from that of Guyot et al. (2001). In a polypropylene vial, 40 μl sample, 40 μl methanol containing 3.3% HCL (v/v), and 80 μl toluene-α-thiol (5% v/v in methanol) were mixed together. For pale malt and hop, 5 mg was mixed with 400 μl methanol containing 3.3% HCL (v/v) and 800 μl toluene-α-thiol (5% v/v in methanol). The vials were incubated at 40 °C for 30 min, and 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 × 2.1 mm i.d. reversed phase C18 Prevail column (Alltech). 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. Five microlitres of sample were injected onto 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%. Identification was done by three methods: comparison of mass spectra obtained by ESI(-)-MS/MS, the pseudomolecular ions by full scan ESI(-)-MS, and the retention times with those of commercial and isolated products. Quantification of terminal units and of the extension unit was done with the calibration curves obtained for each standard (commercial and isolated), using tandem mass spectrometry MS/MS on *m/z* 289 for the analysis of (+)-catechin or (-)-epicatechin and *m/z* 305 for (+)-gallocatechin or (-)-epigallocatechin (the same ion has been selected for free and nucleophile-bounded flavan-3-ols). The mDP was obtained with the following equation:

$$\mathrm{mDP} = (\text{terminal units} + \text{extension units})/\text{terminal units}.$$

The medium without degradation was used to quantify the monomeric native structures.

2.6. Preparation of 3,4-β- or -α-catechin benzylthioether and 3,4-β- or -α-gallocatechin benzylthioether standards

Barley grain (100 g) was reduced to powder. By successive 10-min steps, lipids were removed at room temperature under gentle stirring with 200 ml *n*-hexane (5 times). At the end of each step, the sample was centrifuged for 10 min at 3000g. Barley powder was finally dried under vacuum to get rid of residual solvent.

Defatted barley powder was extracted 5 times with 200 ml acetone/water/acetic acid (70/28/2, v/v/v), each time for 10 min under gentle stirring. After each extraction, the sample was centrifuged for 10 min at 5000*g* and the supernatant collected. After filtration to remove residual particles, the combined supernatants were concentrated by rotary evaporation to dryness and dissolved in 100 ml methanol.

In a TSK Toyopearl cartridge (preconditioned with methanol/water (30/70, v/v), the methanolic extract was loaded and washed with 160 ml methanol/water (30/70, v/v). Proanthocyanidins were then eluted with 280 ml acetone/water (70/30, v/v). The eluates were concentrated by rotary evaporation and freeze-dried.

Sixty milligrams of a freeze-dried barley acetone extract were mixed with 250 μl methanol containing 3.3% HCL (v/v), and 500 μl toluene-α-thiol (5% v/v in methanol), incubated (same thiolysis conditions as described in Section 2.5), and injected onto a preparative reversed-phase HPLC system. The HPLC gradient system (Rainin Instrument Company Inc., Woburn, MA) was equipped with two high-pressure pumps (100 WTI pump heads, Dynamax SD 300, Rainin), a manual injection valve (5-ml injection loop) and a UV detector (Dynamax UV1, Rainin). Data were acquired and processed with EZ-Chrom software (Scientific Software, Inc., Pleasanton, CA). The semi-preparative column consisted of a C18 Lichrosphere (Merck, Germany) PrepLC column (200 × 50 mm, 12-μm). A flow rate of 30 ml/min was applied, with a linear gradient from water containing 2.5% of acetic acid (A) to acetonitrile (B). The gradient elution was the same as described above for thiolysis analysis and peaks were monitored at 280 nm. Two fractions were recovered and freeze dried. Pure standards were then obtained by means of a second purification on a reversed-phase C18 Novapack semi-prep column (100 × 10 mm, 6-μm, Merck) with a guard column of the same material (25 × 10 mm, 6 μm) and by applying the gradient and conditions described above. Identifications were based on direct ESI-MS/MS, HPLC retention times, and UV–Vis absorption spectra.

5. Results and discussion

5.1. Direct analysis of beer monomers, dimers, and trimers by NP-HPLC-ESI-MS/MS

As depicted in Fig. 2, NP-HPLC-ESI(-)-MS/MS allowed quantification of monomers to trimers in a Sephadex LH20 acetone/water (70/30, v/v) beer extract. As shown in Fig. 3, major differences were observed, unsurprisingly, between PVPP-treated (A and B) and silica gel-filtered (C) beers, PVPP being able to bind flavanoid oligomers (Siebert, & Lynn, 1997). This leads to very low levels of P1-P3 (3.0 vs. 10.3 mg/l in the silica gel-filtered beer) and a very low P2/P1 ratio (0.8 *vs.* 2.6 in the silica gel-filtered beer). In the three beers, oligomers above P3 were not concentrated enough to be individually quantified in the extract. In order to check if it was possible to quantify those proanthocyanidins, thioacidolysis was tested on beer A and D; since but this assay allowed the analysis of only a few HPLC peaks of monomers and monomer adducts.

5.2. Optimisation of thiolysis conditions

RP-HPLC-UV has often been used in published studies to quantify and characterise thiolysis reaction media (Gu et al., 2002; Gu et al., 2003; Guyot et al., 1997; Guyot et al., 2001; Kennedy, & Jones, 2001; Matthews et al., 1997). In this work, ESI-MS/MS was preferred to elucidate unknown structures and to lower the detection limit as much as possible (LQ = 0.05 mg/l and LD = 0.01 mg/l). Ionisation in negative mode proved very efficient, making it possible to select just two ions: *m/z* 289 and 305, the former being issued from all catechin and benzylthioether catechin units and the latter from gallocatechins and benzylthioether gallocatechins. In a few cases, a small peak just after 3,4-β-epicatechin benzylthioether was detected as an artifact (the same mass spectrum

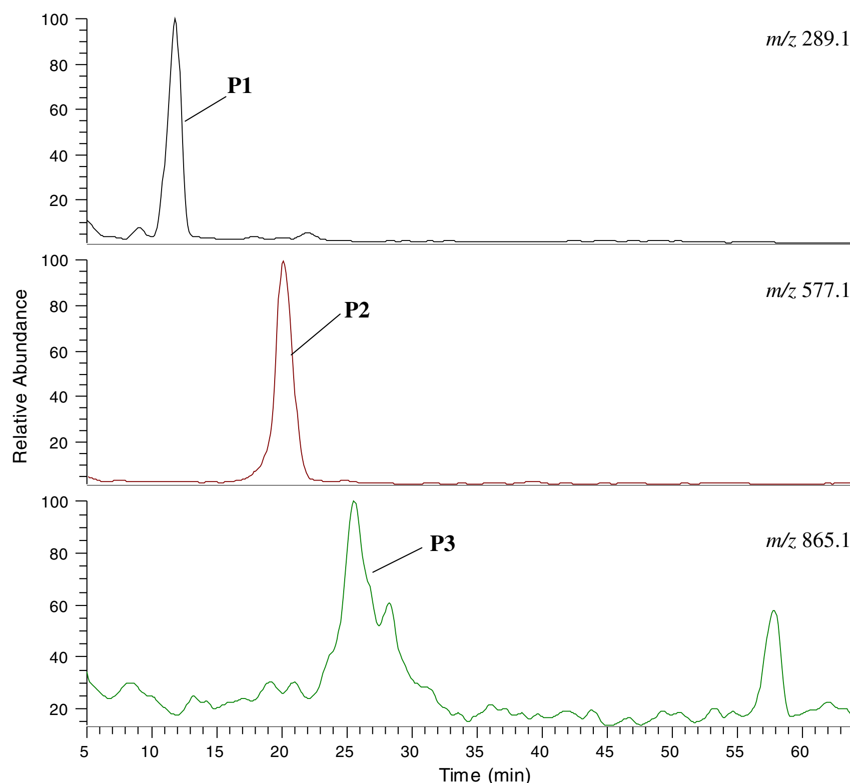

Fig. 2. NP-HPLC-ESI(-)-MS/MS analysis of a Sephadex LH20 lager beer A extract. P1: 289.1 *m/z*; P2: 577.1 *m/z*; P3: 865.1 *m/z*.

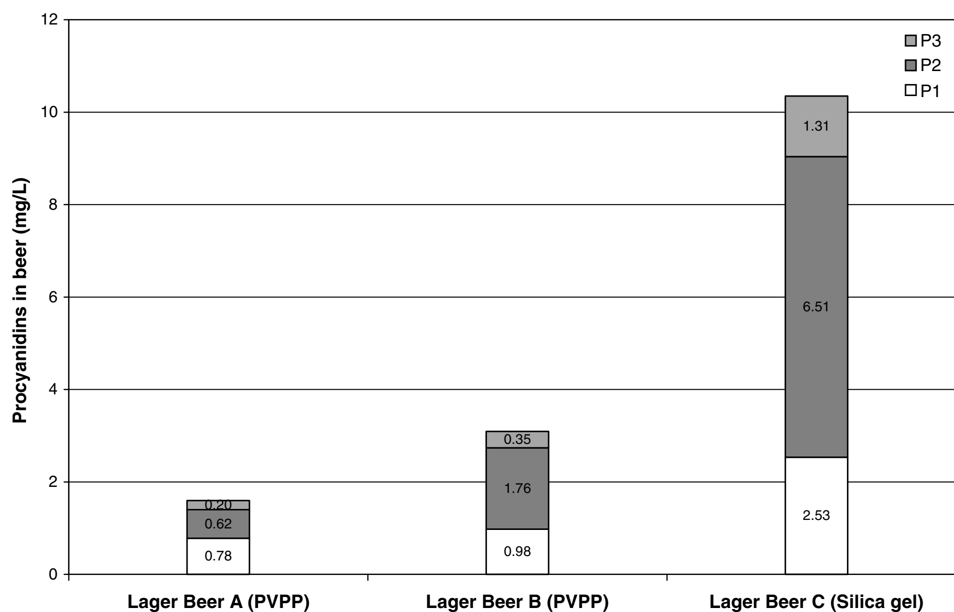

Fig. 3. Procyanidins concentrations (mg/l in (+)-catechin equivalents) detected in two lager beers stabilised by PVPP (A and B) and one beer filtered on silica gel (C).

as the monomers and UV_{max} at 280 nm). In fact, toluene-α-thiol can attack position 2 of flavan-3-ols instead of position 4 (Betts, Brown, & Shaw, 1969; Gu et al., 2002), leading by heterocyclic cleavage to a compound with a Mw of 414 (*m/z* 413 in negative mode, giving a fragment at *m/z* 289.1). This reaction can also induce slight epimerization of (+)-catechin and (-)-epicatechin, as previously mentioned (Betts et al., 1969; Gu et al., 2003; Matthews et al., 1997).

Optimal thiolysis conditions (incubation temperature and time) were determined on pure procyanidin fractions issued from cocoa (see experimental methods). Although 30 min at 40 °C was strong enough, as previously suggested by Guyot et al. (2001), to depolymerise flavan-3-ol oligomers up to tetramers, 10 additional hours at room temperature proved necessary to reach the true DP for bigger structures (Table 1).

5.3. *Thiolysis of beer monomers, dimers and trimers issued from the Sephadex LH20 beer A extract*

Purified beer fractions isolated by normal-phase HPLC were thiolysed. As depicted in Fig. 4 and 5, all catechins in the extension

Table 1
Degree of polymerisation obtained on isolated fractions of cocoa procyanidins after thiolysis during different times at 20 °C and at 40 °C (CV < 2%)

Fractions	Thiolysis conditions applied				
	10 h 20 °C	24 h 20 °C	30 min. 40 °C	30 min. 40 °C 10 h 20 °C (Selected conditions)	30 min. 40 °C 24 h 20 °C
P5	5.0	5.2	4.4	5.2	5.9
P6	5.4	5.2	5.2	6.1	6.3
P7	7.2	6.6	5.6	7	7.3

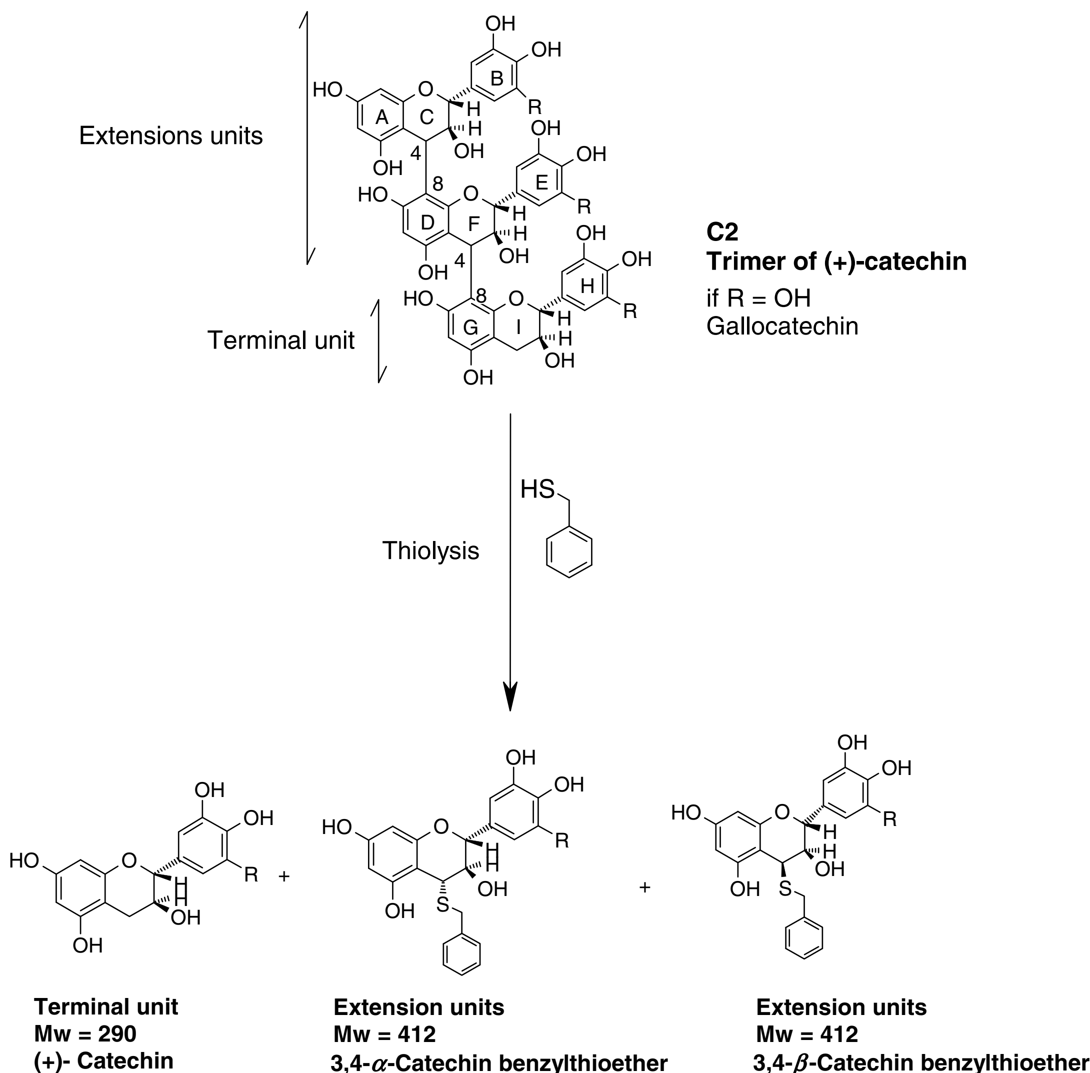

Fig. 4. Scheme of the thioacidolysis applied on a C2 – trimer of (+)-catechin.

units were able to react with toluene-α-thiol to form both the 3,4-β-catechin benzylthioether (the major product, due to hydrogen bonding between the 3-hydroxyl-group and the sulfur atom) and 3,4-α-catechin benzylthioether. On the other hand, epicatechin yielded only 3,4-β-epicatechin benzylthioether (Matthews et al., 1997; Thompson, Jacques, Haslam, & Tanner, 1972). Similar reactions took place with gallocatechin and epigallocatechin extension subunits. All terminal units were released as free flavan-3-ol. As expected, most monomers (97%) were catechins (Table 2). In the dimer, both terminal and extension units were mainly constituted of catechin (92 and 89%, respectively). This means that procyanidin B3 is the major dimer in beer. On the other hand, the occurrence of gallocatechins was obvious in trimers (73% in the extension units), indicating the presence of prodelphinidin C (Fig. 5a).

5.4. Thiolysis on the total Sephadex LH20 extract of two beers (A and D) stabilized by PVPP

After thiolysis, the RP-HPLC-ESI-MS/MS chromatogram of the Sephadex LH20 acetone/water (70/30, v/v) lager beer extract led

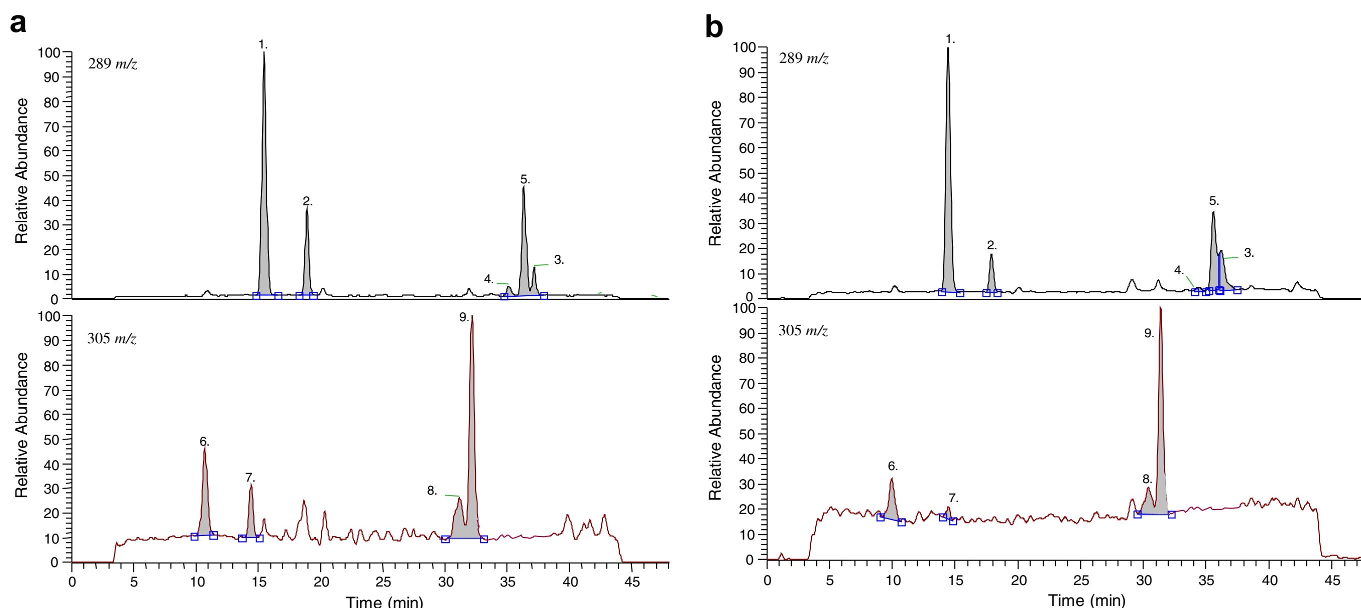

Fig. 5. RP-HPLC-ESI(-)-MS/MS (*m/z* 289 and 305) analysis after thiolysis of: (a) pure fraction of beer A trimers and (b) the Sephadex LH20 beer A extract; 1. catechin, 2. epicatechin, 3. 3,4-*β*-epicatechin benzylthioether, 4. 3,4-*α*-catechin benzylthioether, 5. 3,4-*β*-catechin benzylthioether, 6. gallocatechin, 7. epigallocatechin, 8. 3,4-*α*-gallocatechin benzylthioether, and 9. 3,4-*β*-gallocatechin benzylthioether.

Table 2
Thiolysis results obtained on the SPE Sephadex LH20 extracts of two lager beers (A and D) stabilized with a PVPP treatment and on proanthocyanidin pure fractions issued from beer A: concentration of native monomers (mg/L) and proportion (%) of terminal or extension units after thiolysis, mDP, and total concentrations in proanthocyanidins (mg/L of beer)

	Pure fractions			Lager beer A		Lager beer D	
	Monomers (%)	Dimers (%)	Trimers (%)	Native (mg/l)	Oligomers (%)	Native (mg/l)	Oligomers (%)
(+)-Catechin	97	92	85	0.9	76	1.3	74
(-)-Epicatechin	3	8	8	0.2	18	0.3	20
(-)-Gallocatechin	–	–	6	0.1	3	0.1	2
(-)-Epigallocatechin	–	–	1	0.1	3	0.1	4
3,4-*α*- and *β*-Catechin benzylthioether	–	89	25	–	51	–	54
3,4-*β*-Epicatechin benzylthioether	–	5	2	–	2	–	1
3,4-*α*- and *β*-gallocatechin benzylthioether	–	6	73	–	47	–	45
mDP	1	2	3	5.0		6.8	
Total concentration in proanthocyanidins (mg/l of beer)				8.0 (9.3*)		13.9 (15.7*)	

CV < 2%.
– Not detected; *including native flavan-3-ol monomers.

to the same peaks as the pure dimeric and trimeric fractions (Table 2 and Fig. 5b). Native monomers were quantified before thiolysis: 0.9–1.3 mg/l (+)-catechin, 0.2–0.3 mg/l (-)-epicatechin, 0.1 mg/l (-)-gallocatechin, and <0.1 mg/l (-)-epigallocatechin. After subtracting these native flavan-3-ols, it was concluded that the terminal units were essentially constituted of catechin (75%) and epicatechin (19%). Catechin (53%) and gallocatechin (46%), however, emerged as the major constituents of the extension units, with less than 2% epicatechin. Apparent mDPs of 5 and 6.8 were calculated for both beer extracts. Total proanthocyanidin concentrations of 9.3 and 15.7 mg/l beer were calculated by RP-HPLC-ESI-MS/MS. The contribution of native monomers was very low (14% and 11%, respectively). When native monomers are excluded, total proanthocyanidins thus amount to 8.0 and 13.9 mg/l beer. More complex undefined structures not degraded by toluene-*α*-thiol may explain why our total polyphenol level in beer, quantified by a global assay, can reach 71.8 mg/l (method used by Bishop (1972).

5.5. Thiolysis of malt and hop flavanoids (Table 3)

The spring and winter malts used for beer production were found to contain up to 79–92% catechin as terminal units and more gallocatechin (57%) in extension units. Surprisingly, traces of epicatechin were also detected. We suspect that (+)-catechin epimerisation could take place during kilning, leading to (+)-epicatechin in malt. A relatively high mDP was calculated for malt (9.4 to 10.0), suggesting selective partition, due to better extraction of the lowest DP or depolymerisation from malt to beer. Hops are the principal source of (-)-epicatechin in beer. It was found both in terminal and extension units. The aromatic variety unsurprisingly exhibited higher concentrations of proanthocyanidins (43,300 mg/kg *vs.* 27,200 mg/kg in the Tomahawk bitter hop) (Lermusieau, Liegeois, & Collin, 2001). Thiolysis also revealed less epicatechin in the investigated aromatic cultivar. Epigallocatechin was identified here for the first time in hops.

In conclusion, thiolysis followed by RP-HPLC-ESI(-)-MS/MS analysis is a very powerful means of investigating beer flavanoids. Procyanidin B3 is the major dimer of beer whilst trimers are more frequently prodelphinidins C. Much higher oligomeric fractions present in the raw materials are selectively extracted, or partially depolymerised through the brewing process. In beer, procyanidins with epicatechins in their constitutive units are mainly issued from hop whilst procyanidins with gallocatechins in their extension units derive from malt. The main part of beer polyphenols is

Table 3
Thiolysis results obtained on raw materials: concentration of native monomers (mg/kg) and proportion (%) of terminal or extension units after thiolysis, mDP, and total concentration in proanthocyanidins (mg/kg)

	Winter malt		Spring malt		Aromatic hop Willamette		Bitter hop Tomahawk	
	Native (mg/kg)	Oligomers (%)	Native (mg/kg)	Oligomers (%)	Native (mg/kg)	Oligomers (%)	Native (mg/kg)	Oligomers (%)
(+)-Catechin	312	92	187	79	1488	78	492	54
(-)-Epicatechin	40[a]	8[a]	14[a]	21[a]	239	14	299	42
(-)-Gallocatechin	–	–	–	–	66	6	-	2
(-)-Epigallocatechin	–	–	–	–	–	2	-	2
3,4-α- and β-Catechin benzylthioether	–	43	–	42	–	41	-	48
3,4-β-Epicatechin benzylthioether	–	–	–	1	–	14	-	13
3,4-α- and β-Gallocatechin benzylthioether	–	57	–	57	–	14	-	10
3,4-β-Epigallocatechin benzylthioether	–	–	–	–	–	31	-	29
mDP	9.4		10.0		7.5		7.0	
Total concentration in proanthocyanidins (mg/kg)	7660 (8010[b])		3880 (4080[b])		43300 (45100[b])		27200 (28000[b])	

CV < 2%.
– Not detected.
[a] Probably (+)-epicatechin issued from (+)-catechin epimerization.
[b] Including native flavan-3-ol monomers.

composed of complex undefined structures not degraded by toluene-α-thiol. Compared to the bitter variety, the aromatic hop contained a higher concentration of proanthocyanidins (mainly catechin units in terminal positions). Enantiomeric separation should be now investigated in order to differentiate flavan-3-ols from malt and hops.

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