

## **Better knowledge of flavanoids in fresh lager beers: comparison of extraction methods as regards low- and higher-molecular-weight flavanoid recovery, use of thiolysis hyphenated to RP-HPLC-ESI(-)-MS/MS to determine flavanoid subunits**

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*Descriptors:*

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### **SUMMARY**

Elucidation of polyphenols involved in beer organoleptic properties is of prime importance for brewers. Unfortunately, the assays used to quantify them are too often general ones. This approach fails to take into account that each molecule can impart different properties to beer. To investigate beer flavanoids, five extraction methods were tested. As compared to acetone/water (70/30, v/v) elution of an LH-20 resin, small oligomers (P1, P2, and P3) were poorly recovered by liquid-liquid extraction with ethyl acetate or butan-2-one or by dimethylformamide elution of a polyamide cartridge. Higher oligomers were extracted by polyamide SPE or dialysis. To obtain a more detailed composition of higher oligomers, the dialysate was thiolized. Results were compared to previous data obtained on the LH-20 extract. It was concluded that "natural" beer oligomers exhibit a relatively low degree of polymerization whilst "chemically" synthesized oligomers most probably account for most of the "heavy" flavanoid content.

### **INTRODUCTION**

Beer proanthocyanidins have been considered mostly for their involvement in haze (1), but they can also be responsible for other important organoleptic alterations. Given their low concentration and high complexity, specific extraction and detection methods are required for their study. Polarity, acidity, and molecular size are three physico-chemical properties enabling investigators to distinguish phenols from other co-constituents in beer.

Recently a Sephadex LH-20 solid phase extraction (SPE) with acetone/water (70/30, v/v) elution was developed to analyse beer flavanoids up to trimers of flavan-3-ols (2). Quantification was achieved by NP-HPLC-ESI(-)-MS/MS. Detailed structural

information of these small oligomers was obtained by thioacidolysis in the presence of toluene- $\alpha$ -thiol (2). It was concluded that catechin, procyanidin dimers (two catechin units), and prodelphinidin trimers (catechin in terminal unit and gallo catechin or catechin in extension units) amount to a polyphenol concentration of 9-16 ppm in beer (see figure 1 for structures).

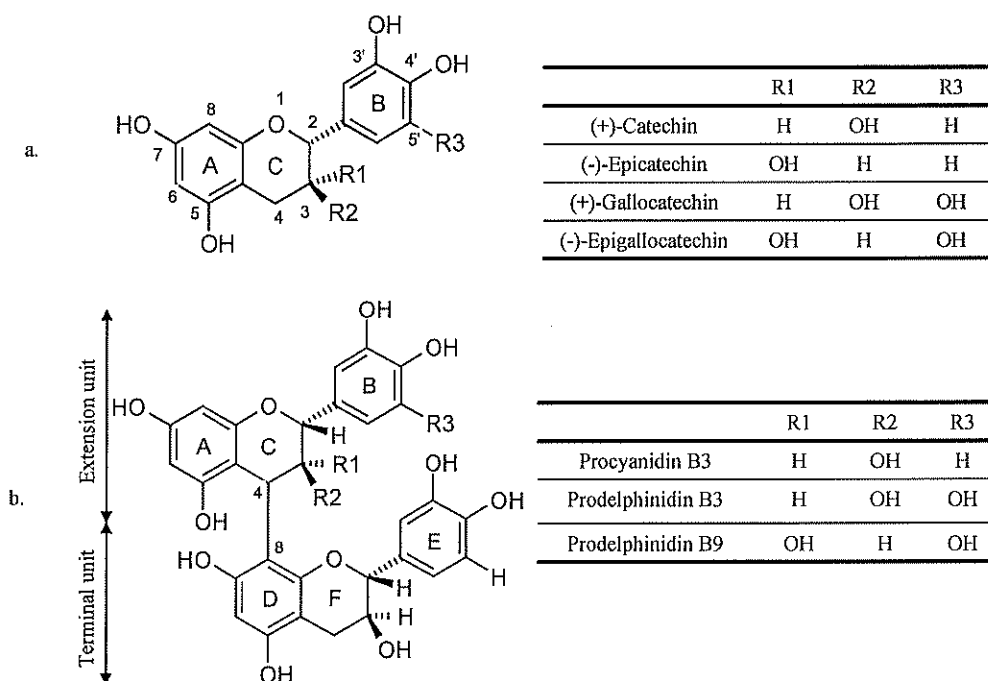


Figure 1: a. Structure of the flavanol-3-ol units and b. B-type C4-C8 proanthocyanidin dimers.

The aim of this paper was first to compare the Sephadex LH-20 SPE with other usual flavanoid extraction methods. In each extract, the monomer-to-trimer content was analysed by NP-HPLC-ESI(-)-MS/MS. Total polyphenols, total flavanoids, and antioxidant activity were also measured in global assays. The detailed composition of higher-molecular-weight flavanoids was further obtained by thioacidolysis.

## MATERIALS AND METHODS

### Chemicals

Acetone (99.9 %), *N,N*-dimethylformamide (99.9 %), (-)-epicatechin (98 %), (+)-catechin (98 %), (-)-gallocatechin (98 %), and (-)-epigallocatechin (98 %) were obtained from Sigma-Aldrich (Bornem, Belgium). Methanol (99.9 %) and dichloromethane (99.9 %) were purchased from Romil (Cambridge, UK). Acetic acid (99.8 %) was obtained from Acros (Geel, Belgium). Toluene- $\alpha$ -thiol (99 %) and ammonium acetate (99 %) were obtained from Fluka (Buchs, Switzerland). Acetonitrile (99.99 %) and hydrochloric acid (37 %) were from Fischer Scientific (Leicestershire, UK). Formic acid (99 %) was obtained from Janssen Chemica (Geel, Belgium). 3,4- $\beta$ -Epicatechin benzylthioether, 3,4- $\alpha$ - or  $\beta$ -catechin benzylthioether and 3,4- $\alpha$ - or  $\beta$ -gallocatechin benzylthioether were obtained as previously described (2).

### **Extraction of beer flavanoids**

Beer was first degassed by sonication for 10 min.

*Solid phase extraction on LH20 (2).* Three grams of Sephadex LH-20 (Sigma-Aldrich, St Louis, USA) packed in a 12-ml filtration tube with polyethylene frits (Supelco, Bellfonte, USA) was preconditioned for 4 h with methanol/water (30/70, v/v). After loading 50 ml degassed beer, the column was washed with 40 ml methanol/water (30/70, v/v). Proanthocyanidins were recovered with 70 ml acetone/water (70/30, v/v). The eluates were concentrated to dryness by rotary evaporation and dissolved in 2 ml methanol. By the standard addition method, a 100 % recovery factor was calculated for (+)-catechin (spiked with increasing amounts of (+)-catechin before extraction).

*Liquid-liquid extraction with (a) ethyl acetate (3,4) or with (b) butan-2-one (3,5).* 200 ml degassed beer were mixed for 10 min at 1000 rpm with: (a) 900 ml HCl/H<sub>2</sub>O/isooctane (1/3/5, v/v) or (b) 80 ml then 25 ml isooctane. Aqueous phases were recovered and concentrated. In a successive 10 min steps at 1000 rpm, the aqueous phases were extracted successively with: (a) 300, 300, and 300 ml ethyl acetate or (b) 150, 100 and 75 ml butan-2-one. The organic phases were pooled, concentrated to dryness and dissolved in 4 ml methanol.

*Solid phase extraction on polyamide (6,7).* The polyamide cartridge 1000 mg (Macherey-Nagel, Easton, USA) was preconditioned with water for 10 min. Fifty milliliters degassed beer was loaded. The cartridge was then washed with 10 ml water, dried with N<sub>2</sub> and eluted with 2.5 ml dimethylformamide.

*Dialysis.* 40 ml degassed beer were poured into a dialysis 3500 Da tube (Spectrum, USA) which was further immersed in 2-l water (replaced every day). After three days, beer dialysate was freeze-dried. The powder was diluted in methanol/water (50/50, v/v) (40000 ppm) before HPLC and AAPH analyses.

### **NP-HPLC-ESI(-)-MS/MS of proanthocyanidins (8)**

A SpectraSystem (Finnigan Mat, San Jose, USA) equipped with an SCM degasser, an AS3000 autosampler, and a P4000 quaternary pump was used. A 5- $\mu$ m, 250  $\times$  2.1 mm i.d. Silica Alltima HP column (Alltech, Deerfield, IL, USA) 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. 5  $\mu$ l 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]<sup>+</sup> ions of monomers, dimers, and trimers. Quantification was done using the calibration curves of (+)-catechin (commercial standard).

### **Thiolysis coupled to RP-HPLC-ESI(-)-MS/MS (2)**

In a polypropylene vial, 40  $\mu$ l sample (or 5 mg dialysate freeze-dried powder), 40  $\mu$ l methanol with 3.3 % HCL (v/v), and 80  $\mu$ l toluene- $\alpha$ -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- $\mu$ m, 150  $\times$  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  $\mu$ l 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.

### **Total polyphenols and total flavanoids content**

Total polyphenols were determined according to Bishop (9). Total flavanoids were quantified by means of a colorimetric assay based on the reaction with *p*-dimethylaminocinnamaldehyde (DAC) (10).

### **Antioxidant assay - AAPH method**

The reduction power was measured by a method developed in our laboratory (11). 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.

## **RESULTS AND DISCUSSION**

### **Comparison of different beer flavanoid extraction methods**

Liquid-liquid extraction with ethyl acetate or butan-2-one, polyamide cartridge SPE with dimethylformamide elution, or dialysis (3500 Da) was applied to a lager beer stabilized by PVPP treatment. All these methods were compared with recent data obtained on LH-20 SPE extracts (2). Total polyphenols, total flavanoids, and antioxidant capacity were also quantified.

As shown in table 1 and figures 2a-d, NP-HPLC-ESI(-)-MS/MS analyses revealed that ethyl acetate, butan-2-one, polyamide SPE, and dialysate extracts were very poor in catechin (P1,  $R_t$  = 11.8 min), dimers (P2,  $R_t$  = 20.1 min), and trimers (P3,  $R_t$  = 25.5

min). Only elution of the LH-20 resin with acetone/water (70/30, v/v) allowed detection of monomers to trimers (figure 2e, recovery factor of 100 % for catechin).

Table 1: Comparison of methods for the extraction of beer flavan-3-ols and proanthocyanidins.

Solvent for flavanoid extraction	Total polyphenols (CV, %)	Total flavanoids (CV, %)	T <sub>inh</sub> (CV, %)	NP-HPLC-MS/MS	NP-HPLC-UV
Ethyl acetate	5.5 (3.0)	1.0 (5.0)	5.2 (3.9)	P1, P2	0
Butan-2-one	6.2 (8.0)	2.1 (1.0)	5.4 (2.5)	P1, P2	0
SPE Polyamide dimethylformamide	/	/	13.1 (0.3)	P1, P2	Polymer
Dialysis 3500 da	/	/	8.2 (0.0)	0	Polymer
SPE LH-20 acetone/water 70/30 (v/v)	5.7 (0.0)	1.7 (5.0)	3.5 (3.0)	P1, P2, P3	0
Complete beer	78.7 (1.0)	13.5 (1.2)	70.4 (0.6)	0	0

Total polyphenols (mg/l of beer in eq. polyphenols isolated from malt and hop) and Total flavanoids (mg/l of beer in eq. catechin) obtained with the EBC methods; T<sub>inh</sub> = Inhibition time measured with the AAPH assay (in min for a 400x diluted beer); /: not determined.

As depicted in table 1, the antioxidant capacities of the polyamide SPE (T<sub>inh</sub> = 13.1 min) and dialysate (T<sub>inh</sub> = 8.2 min) extracts were high compared to those of the other extracts (T<sub>inh</sub> = 3.5 to 5.4 min). Both of these extracts were also characterized by a large peak of polymers (12) at the end of the NP-HPLC-UV chromatogram (figure 2c and d, R<sub>t</sub> = 59 min).

We conclude that small oligomers (P1, P2, and P3) are poorly recovered by ethyl acetate extraction, butan-2-one extraction, and polyamide SPE. Higher oligomers, characterized by a high antioxidant capacity, can be recovered by both polyamide SPE and dialysis. The dialysate was selected for examining, by thioacidolysis, the proanthocyanidin composition of the higher-molecular-weight fraction.

#### Detailed composition of beer higher oligomers

The oligomeric fraction of the dialysate was investigated by thioacidolysis. In this assay, all hydrolyzed oligomers can be quantified by a few HPLC peaks of monomers and monomer adducts (figure 3). As depicted in figure 3 (left), all catechins in extension units react with toluene- $\alpha$ -thiol to form both 3,4- $\alpha$ - and - $\beta$ -catechin benzylthioether. Epicatechin yields only 3,4- $\beta$ -epicatechin benzylthioether. Similar reactions take place with galocatechin and epigallocatechin extension subunits. All terminal units are released as free flavan-3-ol.

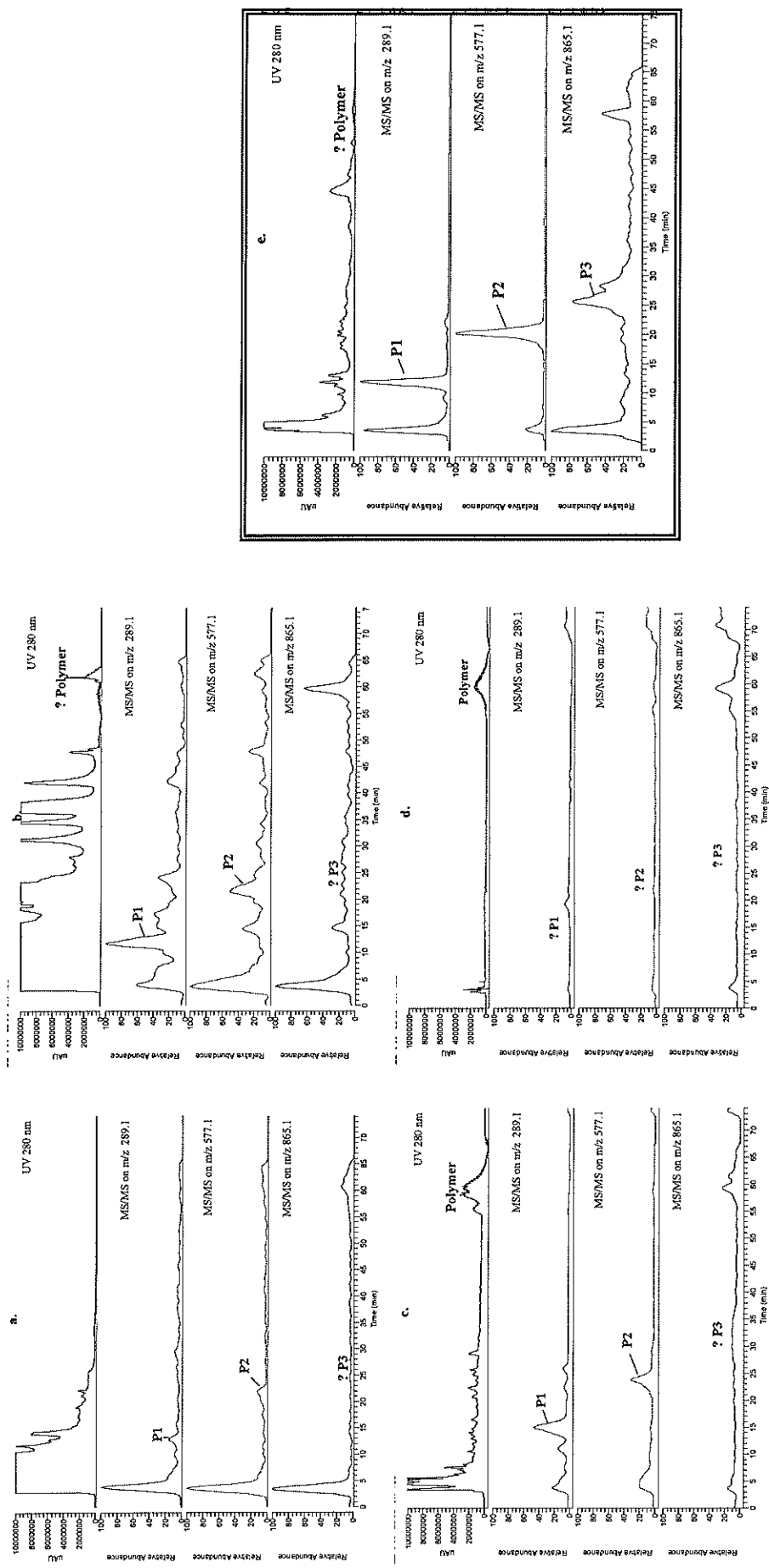


Figure 2: NP-HPLC-UV and -ESI(-)-MS/MS chromatograms of lager beer extracts obtained by means of: a. ethyl acetate or, b. butan-2-one liquid-liquid extractions, c. dimethylformamide elution of the polyamide cartridge, e. dialysis cut-off = 3500 Da, and e. acetone/water (70/30, v/v) elution of the LH20 resin SPE.

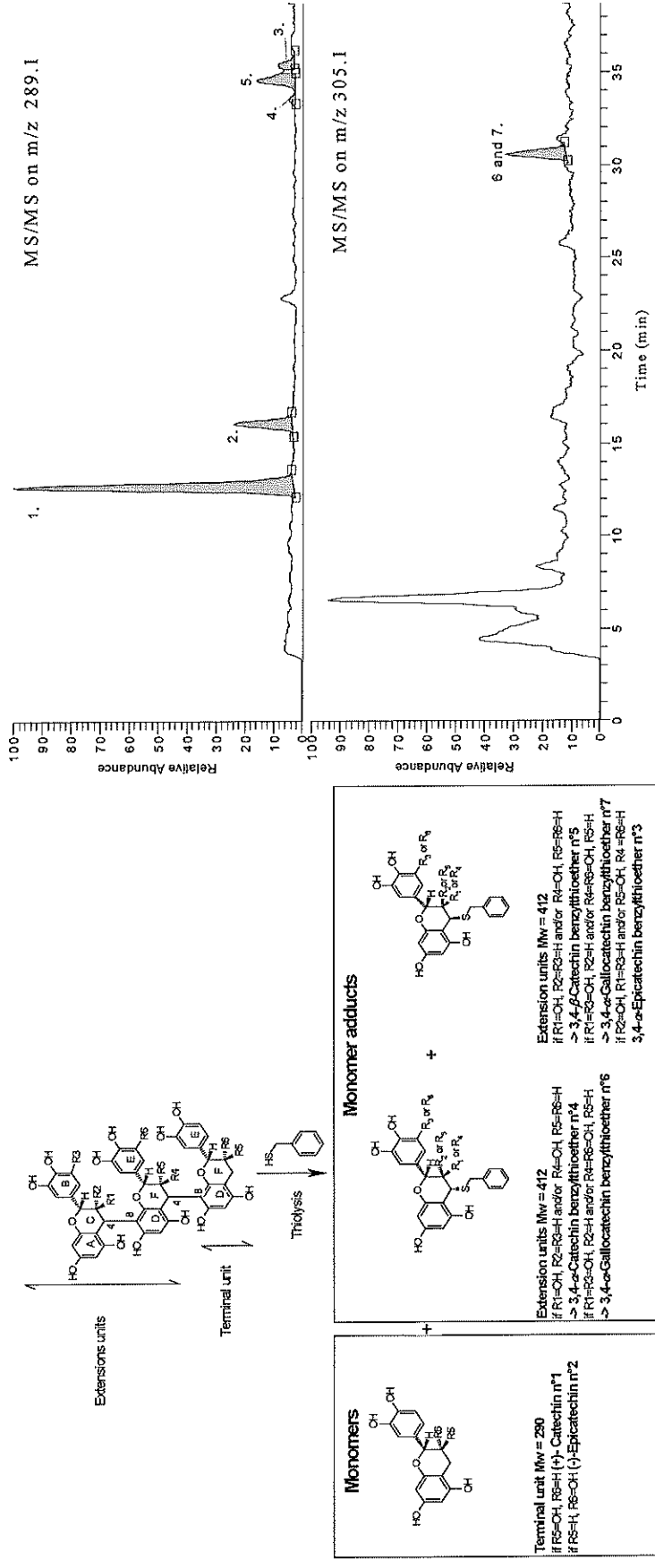


Figure 3: Scheme of the thioacidolysis and RP-HPLC-ESI(-)-MS/MS chromatograms of a dialysate extract after thiolysis; 1. Catechin, 2. Epicatechin, 3. 3,4-β-Epicatechin benzylthioether, 4. 3,4-α-Catechin benzylthioether, 5. 3,4-β-Catechin benzylthioether, 6. 3,4-α-Gallocatechin benzylthioether, and 7. 3,4-β-Gallocatechin benzylthioether.

As previously observed on the LH-20 extract (figure 4 left), catechin emerged as the main terminal unit of higher oligomers (84 % vs. 16 % epicatechin; no galocatechin) (figure 4 right). Catechin proved to be the main constituent of the extension units (76 % vs. 18 % galocatechin and 6 % epicatechin). This differs significantly from the results obtained for the small oligomer fraction (LH-20), where galocatechin accounted for up to 46 % of the extension units. The observed mDP was only 5, considerably lower than the value of more than 12 expected because of the cut-off at 3500 Da. This result indicates the presence in beer of more complex polyphenolic structures not completely degraded by thiolysis. We conclude that “natural” oligomers exhibit a relatively low DP in beer, whilst “chemically” synthesized oligomers most probably account for most of the “heavy” flavanoid content.

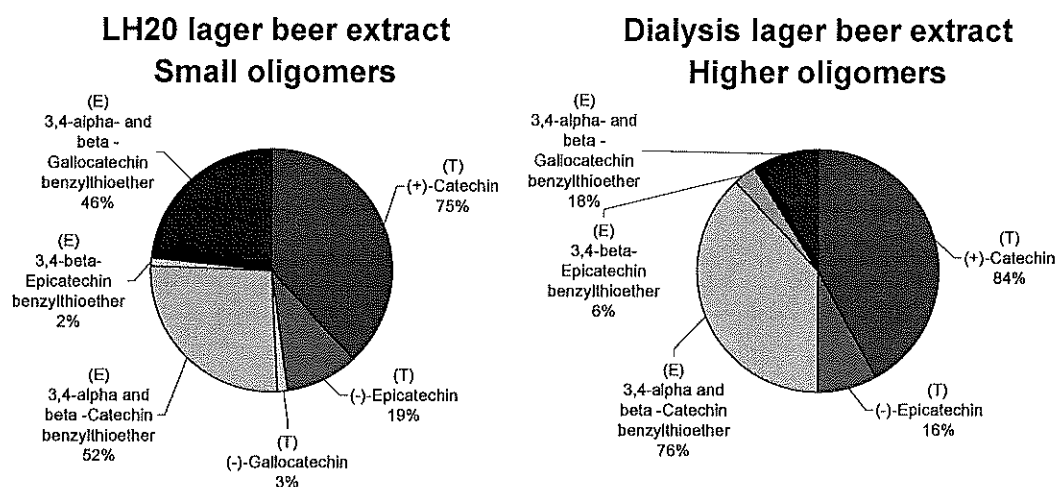


Figure 4: Proportion (%) of terminal (T) and extension (E) units obtained after thioacidolysis on beer Sephadex LH-20 and dialysate extracts.

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