Semipreparative Chromatographic Procedure for the Isolation of Dimeric and Trimeric Proanthocyanidins from Barley

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A semipreparative chromatographic method for the isolation of small amounts (10−20 μg) of dimeric and trimeric proanthocyanidins from barley is described. Concentrated extracts of barley were injected onto a high-performance gel filtration column (Superdex 75 HR), and were eluted with methanol. This procedure resolved the dimeric proanthocyanidins (prodelphinidin B3 and procyanidin B3), as well as the trimeric procyanidin C2 and three other trimeric prodelphinidins. The separated flavanoid peaks were collected and their contents were estimated by UV spectrophotometry, reaction with *p*-dimethylaminocinnamadehyde, and reversed phase HPLC with electrochemical detection. This method produced proanthocyanidins in sufficient amounts to calibrate a system for direct injection chromatographic analysis of beers and barley extracts. The method described may be optimized for the isolation of dimeric proanthocyanidins only, in which case the preparation can take as little as 3 h; alternatively, by extending the chromatographic separation to 9 h, the four major trimeric proanthocyanidins of barley can be recovered also in a chromatographically pure state.

Keywords: *Analysis; barley; beer; calibration standard; high-performance liquid chromatography; proanthocyanidin*

INTRODUCTION

The proanthocyanidins and catechins of barley have long been known to be implicated in the formation of haze in beer, through their participation in reactions with beer proteins (McMurrough *et al.*, 1992a; Gardner and McGuinness, 1977; Siebert *et al.*, 1996). The role of these compounds in beer flavour stability is less certain and is the subject of much current debate (Irwin *et al.*, 1991; McMurrough *et al.*, 1996). Plant flavanols are also very important in the wine-, cider-, and tea-making industries, due to their effects on both flavor and haze formation in these products. The flavanoid oligomers of barley are composed of linked units of (a) (+)-catechin [2-(3,4-dihydroxyphenyl)-3,4 dihydro-2*H*-1-benzopyran-3,5,7-triol] and/or (b) (+)-gallocatechin [2-(3,4,5-trihydroxyphenyl)-3,4 dihydro-2*H*-1-benzopyran-3,5,7-triol] (Delcour and Tuytens, 1984).

One procedure used frequently by brewers to stabilize beers against haze formation is the use of polyvinylpolypyrrolidone (PVPP) during beer filtration to remove phenolic material. To assess the efficiency of this stabilization process, it is necessary to measure the changes in the concentrations of haze-forming flavanols effected by PVPP treatments. A number of methods exist for the measurement of flavanols in various matrices (American Society of Brewing Chemists, 1992; European Brewery Convention, 1987; Delcour *et al.*, 1985; Jerumanis, 1979, 1985; McMurrough et al., 1992b; Madigan *et al.*, 1994), and these range from simple nonselective colorimetric procedures (American Society of Brewing Chemists, 1992; European Brewery Convention, 1987; Delcour and Janssens de Varebeke, 1985) to more sophisticated HPLC methods (Jerumanis, 1979, 1985; McMurrough *et al.*, 1992b; Madigan *et al.*, 1994) which may use UV absorbance detection (Jerumanis, 1979, 1985) or, for greater sensitivity and selectivity, electrochemical detection (McMurrough *et al.*, 1992b, 1993; Madigan *et al.*, 1994). In our laboratory we routinely use HPLC with dual-electrode electrochemical detection (HPLC-ED) for the determination of monomeric and dimeric flavanols in beer and barley (Madigan *et al.*, 1994), and we have clearly demonstrated the effectiveness of this analysis as a valid indicator of the effectiveness of PVPP stabilization protocols (McMurrough *et al.*, 1992a, 1993). One difficulty with this method, however, has been the prerequisite isolation of easily oxidized flavanoid dimers in sufficient amounts, and in sufficient purity, for use as reference materials in the analysis. Until now, this has involved lengthy separations of beer or barley extracts on Sephadex LH-20, followed by further purification either on LH-20 or by semipreparative reversed phase HPLC (Jerumanis, 1985; McMurrough and Baert, 1994; Delcour and Tuytens, 1984; McMurrough *et al.*, 1983). In this paper we present a more convenient method using a high-performance gel filtration column which can be used to prepare microgram amounts of both dimeric and trimeric proanthocyanidins for use as chromatographic standards. The high sensitivity of HPLC-ED means that even such small amounts of standard are sufficient for many analyses.

EXPERIMENTAL PROCEDURES

Instrumentation. The liquid chromatograph used for the semipreparative procedure consisted of a Waters model 510 HPLC pump, a Waters WISP Model 710B autosampler, and a Waters Lambda-Max model 481 LC spectrophotometer (Wa-

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ters U.K. Ltd., Hertfordshire, England). The column used was a 10 × 300 mm Superdex 75 HR 10/30 high-performance gel filtration column (Pharmacia LKB Biotechnology, Uppsala, Sweden).

For the analytical determinations, the liquid chromatograph consisted of a Perkin-Elmer Integral 4000 (Perkin-Elmer, Beaconsfield, Buckinghamshire, U.K.). The diode array detector of this chromatograph was replaced with an ESA Analytical Coulochem II electrochemical detector, equipped with a Model 5011 analytical cell (ESA Analytical, Huntingdon, Cambridgeshire, U.K.). This cell contained two working electrodes in series: the upstream electrode was a porous graphite high-efficiency (coulometric) electrode, and the downstream electrode was of the glassy carbon thin-layer amperometric type.

Electronic data acquisition and peak integration for both systems was performed using a Waters Maxima 820 chromatography workstation.

UV spectra were recorded with a Hitachi Model U-2000 double-beam spectrophotometer (Hitachi, Tokyo, Japan).

Reagents. All solvents and acids used were of analytical reagent grade, with the exception of methanol, which was of HPLC grade. Deionized water was prepared using an Elga Prima/Maxima purification system (Elga, High Wycombe, Buckinghamshire, U.K.).

p-Dimethylaminocinnamaldehyde (DAC) was supplied by BDH Chemical Co. (Poole, Dorset, U.K.). (+)-Catechin and (−)-epicatechin were supplied by Sigma Chemical Co. (Poole, Dorset, U.K.). Polyclar Super R (recoverable PVPP) was supplied by International Specialty Products (Guildford, U.K.).

Procedures. *Extraction of Barley.* Two extraction procedures were developed and were optimized either for extraction of dimeric proanthocyanidins only or for both dimers and trimers. The methods were as follows.

Method 1 (Dimers Only). A 50 g sample of barley grown in Ireland (Blenheim variety, 1994 crop) was ground for 2 min in an IKA Analysis Mill A10 (Janke and Kunkel KG, Staufen, Germany). The ground barley was extracted with 150 mL of methanol under a CO_2 atmosphere for 1 h, after which time the extract was filtered through sintered glass and reduced to 10 mL by evaporation *in vacuo* at 35 °C. The solution was filtered through an alumina membrane filter (Anotop 10 plus 0.22 μm, Whatman U.K. Ltd., Maidstone, England) and retained for semipreparative separation.

Method 2 (Dimers and Trimers). Barley was ground as described above, but the extraction was performed in 150 mL of acetone−water (3:1). The extract was filtered through sintered glass and was salted out by vigorous shaking with excess NaCl (ca. 5 g) for 10 min, followed by standing for 1−2 h. The upper acetone phase, which formed on standing, was evaporated *in vacuo* until only approximately 7 mL of an aqueous solution remained. This solution was filtered through a 0.22 μm membrane and retained for semipreparative separation.

Semipreparative Separation. Aliquots (200 μL) of the barley extracts were injected onto the semipreparative column, which was eluted with methanol at 1.0 mL/min. The column effluent was monitored at 280 nm, and fractions corresponding to the peaks of interest were collected manually. Analysis of these fractions by HPLC-ED (Madigan *et al.*, 1994) was used to provide initial identification of these peaks on the basis of retention times and collection efficiencies. For extracts prepared using method 1, the column was eluted for 3 h, and fractions were collected corresponding to the retention times of procyanidin B3 and prodelphinidin B3. Extracts prepared according to method 2 were eluted for an extended run time of 9 h to permit collection of four trimer fractions in addition to the dimeric flavanols. Fractions were collected only near the peak apices, to avoid any possible contamination due to overlapping of neighboring peaks. Collected fractions were evaporated *in vacuo* to near dryness and were reconstituted in methanol (5 mL for trimer fractions; 15 mL for dimers). This provided concentrated solutions of standards which could be diluted to the normal calibration range of the analytical HPLC system as required. Standards were stored at −40 °C under N_2 when not in use.

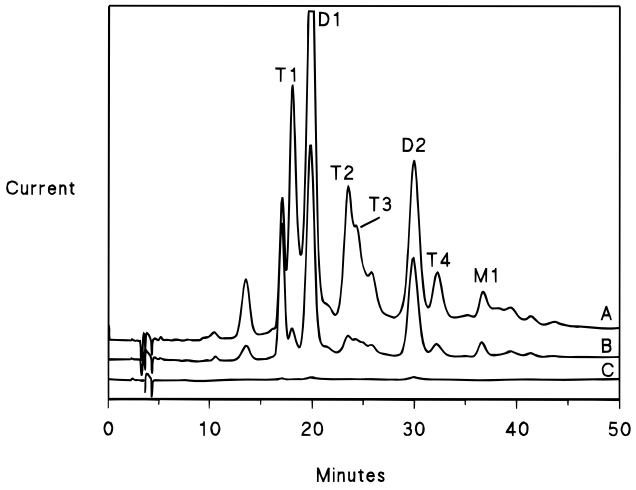

Figure 1. Gradient reversed phase HPLC-ED of barley extracts: A, acetone−water (3:1) extract; B, methanol extract; C, acetone extract. Identified peaks: T1, trimer 1; D1, dimer 1 (prodelphinidin B3); T2, trimer 2; T3, trimer 3; D2, dimer 2 (procyanidin B3); T4, trimer 4 (procyanidin C2); M1, monomer 1 [(+)-catechin].

Analytical Separation. The concentrated fractions were tested for purity by the reversed phase HPLC-ED method described previously for beers and barley extracts (Madigan *et al.*, 1994). The experimental parameters were as follows: flow rate, 1.0 mL/min; injection volume, 10 μL; mobile phase A, 2.5% v/v CH_3COOH; mobile phase B, 10% v/v CH_3COOH. The gradient program was as follows:

time (min)	A (%)	B (%)
0	100	0
60	0	100
70	0	100
80	100	0
100	100	0

The electrochemical detector settings were as follows: (channel 1, high-efficiency electrode) potential +350 mV, output range 5 μA, offset +5%, polarity positive, and filter 2 s; (channel 2, amperometric electrode) potential −650 mV, output range 2 μA, offset +5%, polarity negative, and filter 2 s.

Measurement of Concentration of Standards. Spectra of the different flavanoids in the concentrated fractions from the semipreparative separation were acquired in the range 190−400 nm, and the concentrations of the flavanols were estimated from their optical densities measured at 280 nm, using solutions of (+)-catechin at 1−30 mg/L as standards. The solutions were then analyzed by reaction with DAC using (+)-catechin (1−30 mg/L) as a calibration standard (Delcour and Janssens de Varebeke, 1985). Concentrations of the flavanol standards were expressed as (+)-catechin equivalents.

RESULTS AND DISCUSSION

Development of Extraction Conditions. *Method 1.* This procedure was developed as a convenient method for the extraction of dimeric proanthocyanidins from barley, with the aim of minimizing chromatographic pretreatment protocols. Different solvents were tested first for their effectiveness in extracting barley flavanoids. Fifty gram portions of barley were extracted with 150 mL of either methanol, acetone, or acetone−water (3:1) with stirring for 1 h under a CO_2 atmosphere. The solutions were filtered through sintered glass, and the barley samples were washed with a further 50 mL of extraction solvent. Figure 1 shows a comparison of reversed phase HPLC-ED separations of samples from these three barley extracts. Measurements for total flavanols in the extracts using DAC reagent (European Brewery Convention, 1987; Delcour

and Janssens de Varebeke, 1985) indicated that the relative efficiencies of extraction by methanol, acetone, and acetone−water (3:1) were, respectively, 6.5:1:15. Clearly, 100% acetone was a very ineffective extractant of flavanols. Methanol, however, extracted large amounts of the dimeric proanthocyanidins but showed poor extractability for the trimers and was therefore the extraction solvent of choice for method 1. We have previously used acetone−water (3:1) as an extraction solvent for the quantitative analysis of barley flavanoids (Madigan *et al.*, 1994; McMurrough *et al.*, 1983), but for preparative work this solvent is less convenient than methanol, which can be easily reduced to dryness by evaporation under vacuum.

Method 2. It was evident that acetone−water (3:1) extracted large amounts of both the dimers and the trimers, so this solvent was therefore chosen for extraction method 2. To isolate the proanthocyanidins from this extract, it was found to be convenient to salt out the extract solution, thereby removing water from the solvent mixture. HPLC-ED analysis showed that, following salting out with excess NaCl, most of the proanthocyanidins remained in the upper acetone phase. Following evaporation of the acetone phase, the bulk of the detectable proanthocyanidins were recovered in the remaining water. The small amount of residue precipitated during evaporation contained only traces of detectable flavanols and was therefore discarded.

Semipreparative Separations. *Separation Conditions.* The high affinity of Sephadex LH-20, a hydroxypropylated dextran gel, for flavanoid compounds has made it the current method of choice for the isolation of large amounts of proanthocyanidins (McMurrough and Baert, 1994; Delcour and Tuytens, 1984; McMurrough *et al.*, 1983). However, LH-20 does not have the correct physical characteristics to facilitate high-performance resolution, so further purifications by either gel filtration or HPLC are often necessary to provide isolates of sufficient quality for use as chromatographic standards. Superdex 75 HR, on the other hand, is a dextran polymer that has a mean bead diameter of 13 μm and can withstand backpressures of up to 1.8 MPa (260 psi). Although commercially available columns of this material are designed and marketed specifically for the separation of proteins, we examined its separation characteristics with a view to improving the separations of flavanoids obtainable with Sephadex LH-20. When small volumes (200 μL) of extracts from barley were injected onto the Sephadex column and eluted with methanol, the separation of dimers and trimers was superior to that previously reported for LH-20 (McMurrough and Baert, 1994; Delcour and Tuytens, 1984; McMurrough *et al.*, 1983). A separation of dimers and trimers from a barley extract is shown in Figure 2. The areas of the peaks in Figure 2 were in the ratio 3:4:1:1.1:1.3:1.5 for D2:D1:T4:T3:T2:T1, which indicates the relative proportions of the compounds present in the extract. This system was adopted as a routine method for the isolation of proanthocyanidins for use as chromatographic standards in the analysis of beers and barley extracts by HPLC-ED.

Identity of Collected Fractions. Concentrated fractions from the semipreparative separation were analyzed by HPLC-ED as described previously (Madigan *et al.*, 1994). Figure 3 shows a chromatogram of (A) the barley extract used for the semipreparative isolation and (B) an overlay of the chromatograms of the individual isolated fractions. For consistency the dimer and

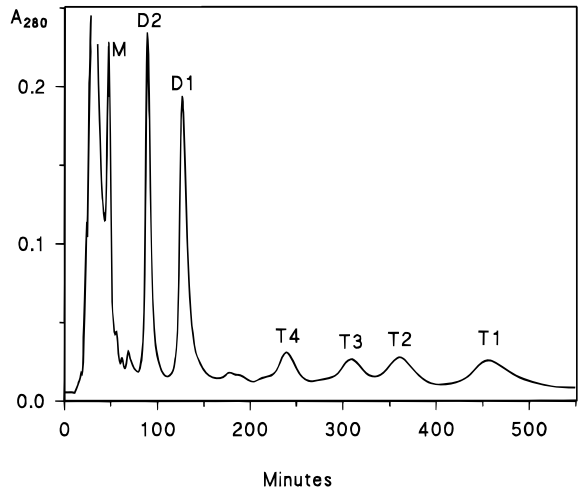

Figure 2. Separation of flavanoids in acetone−water (3:1) extract of barley by elution from a column of Superdex 75 HR 10/30 with methanol. Identified flavanoid peaks: M, monomers; D1, dimer 1; D2, dimer 2; T1−T4, trimers 1−4.

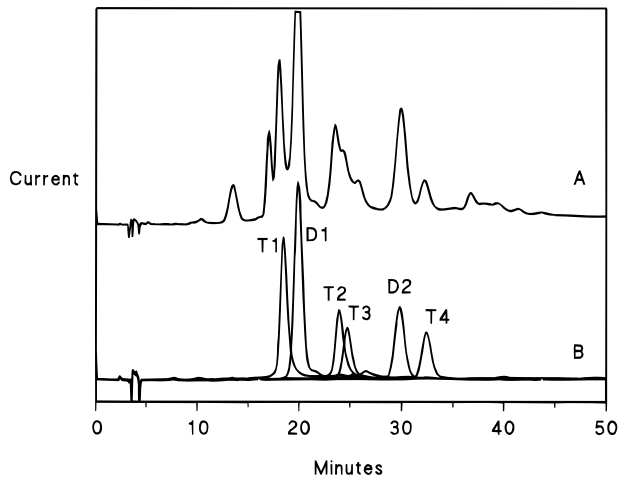

Figure 3. Reversed phase HPLC comparison of (A) acetone−water (3:1) extract of barley and (B) composite overlays of the purified fractions isolated by semipreparative chromatography on Superdex 75 HR 10/30. Identified peaks: T1, trimer 1; D1, dimer 1 (prodelphinidin B3); T2, trimer 2; T3, trimer 3; D2, dimer 2 (procyanidin B3); T4, trimer 4 (procyanidin C2).

trimer fractions were numbered D1−D2 and T1−T4, respectively, in the order of their elution from the reversed phase HPLC column, in compliance with the nomenclature described previously by Outtrup (1981) and used by McMurrough *et al.* (1983). It was evident that all of the compounds were isolated in a high state of purity, and their identities were deduced as follows.

(*1*) UV spectra in the range 190−400 nm exhibited maxima at 280 nm, identical with (+)-catechin and authentic dimeric proanthocyanidins and typical of polyhydric phenols in which no carbonyl conjugation is present.

(*2*) All of the isolated peaks gave strong positive reactions with DAC, indicating that they were flavanoid in nature.

(*3*) The relative retention times on a reversed phase C_{18} column of the compounds isolated were in agreement with those observed by Jerumanis (1985), Outtrup (1981), Mulkay *et al.* (1981a,b), and by other workers in this laboratory (McMurrough and Baert, 1994; McMurrough *et al.*, 1983) and were therefore designated as follows: D1 = prodelphinidin B3; D2 = procyanidin B3; T1 = prodelphinidin trimer with structure (gallo-

catechin−gallocatechin−catechin); T2 = prodelphinidin trimer with structure (gallocatechin−catechin−catechin); T3 = prodelphinidin trimer with structure (catechin−gallocatechin−catechin); T4 = procyanidin C2 (catechin−catechin−catechin).

(*4*) Collection efficiency (Roston and Kissinger, 1982; Madigan *et al*., 1994) is defined as the ratio of the current at the downstream electrode to that at the upstream electrode in a dual-electrode electrochemical detection system, when the downstream electrode is set at a potential sufficient to reverse the oxidative effect of the upstream electrode. For example, a reversible reaction, such as the oxidation of hydroquinone, will give a higher collection efficiency than an irreversible reaction, such as the oxidation of gallic acid (Roston and Kissinger, 1982). The collection efficiencies observed for prodelphinidin B3 and procyanidin B3 were similar to those observed previously with isolated compounds (Madigan *et al*., 1994). The collection efficiencies of the trimers increased in order of increasing catechin to gallocatechin ratios, as observed for the dimeric proanthocyanidins and as would be expected from the predicted higher stability of catechin structural units in the oxidized form.

(*5*) Hydrolysis of concentrated methanolic solutions (1 mL) of the isolated prodelphinidin B3 and procyanidin B3 fractions in 5:1 butanol−HCl (2 mL) for 1 h under reflux yielded compounds with absorbance maxima at either 556 or 546 nm, in accordance with the expected production of, respectively, delphinidin and cyanidin (Haslam, 1966; Porter, 1986).

It is significant that the orders of elution of the flavanoid dimers and trimers on normal phase Superdex 75 (Figure 2) were not the exact reverse of that displayed on the reverse phase column (Figures 1 and 3). Retention of solutes on the dextran gel through hydrogen bonding increased primarily with increasing molecular size, but with a secondary influence of the number of gallocatechin versus catechin units per molecule (cf. D2 vs D1, D2 vs T4, D1 vs T1). Thereupon, different possible sequences of one gallocatechin residue and two catechin residues provided yet another selective influence on elution order (cf. T3 vs T2). In contrast, the retention to C_{18} silica of phenolic substances is thought to increase generally with decreasing polarity and, consequently, with decreasing aromatic hydroxylation. From Figures 1, 3, and 4 it is seen that the effects of molecular size, degree of hydroxylation, and intermolecular sequence on elution order were not clearly evident. For instance, the retention of (+)-catechin oligomers increased in the order dimer (D2), trimer (T4), monomer (M1) (Figures 1 and 4).

Quantification of the Collected Fractions. To quantify the concentrations of each fraction in catechin equivalents, UV spectra of the collected fractions were acquired in the range 190−400 nm, and the absorbances of the solutions at 280 nm were related to the absorbances of standard solutions of (+)-catechin in methanol (Table 1). One milliliter aliquots of the fractions were also analyzed with DAC reagent and compared also with standard solutions of (+)-catechin between 0 and 30 mg/L. Results obtained with DAC solution (Table 1) differed significantly from those based on UV absorbance, in accordance with expectations (McMurrough and McDowell, 1978; Delcour, 1988). It has been shown previously that the molar color yields obtained with DAC reagent decrease almost in proportion with the degree of polymerization of flavanoid oligomers. Accordingly, the average ratios for the concentrations measured in catechin equivalents by UV absorbance and DAC reactivity were 2.0 for the dimers and 2.6 for the trimers (Table 1). It was decided, however, to rely on the results of the direct UV absorbance analysis to quantify the fractions (Table 1). It should be noted that the relative proportions of the flavanols that were isolated do not necessarily reflect the proportions present in the original barley extract, because fractions were collected only near the apices of the chromatographic peaks (Figure 2).

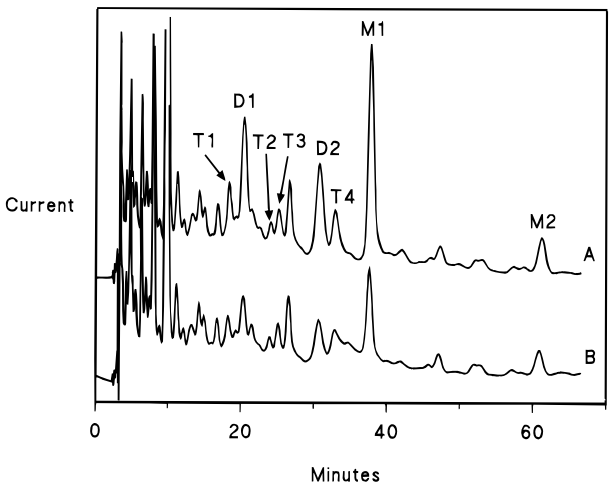

Figure 4. Reversed phase HPLC of a lager beer prepared from barley malt (A) before and (B) after stabilization with 50 g/hL PVPP for 1 h at 0 °C. Identified peaks: T1, trimer 1; D1, dimer 1 (prodelphinidin B3); T2, trimer 2; T3, trimer 3; D2, dimer 2 (procyanidin B3); T4, trimer 4 (procyanidin C2); M1, monomer 1 [(+)-catechin]; M2, monomer 2 [(−)-epicatechin].

Table 1. Quantification of Concentrated Fractions from Semipreparative Separation by UV Absorbance and Colorimetry with DAC

collected peak concentrate	concn in (+)-catechin equiv (mg/L)		
	UV (280 nm)	DAC	UV/DAC
dimer 1	14.9	8.3	1.8
dimer 2	10.3	4.7	2.2
trimer 1	18.1	9.2	2.0
trimer 2	13.1	4.4	3.0
trimer 3	11.8	4.7	2.5
trimer 4	12.9	4.4	2.9

Use of the Quantified Fractions To Calibrate Reversed Phase HPLC. The quantified dimer and trimer fractions were used to calibrate their determination in unknown samples, by area integration of those peaks separated by reversed phase gradient chromatography and detected by amperometry. As a test of this method, a sample of lager beer, brewed with an all-barley malt, was stabilized on a bench scale (1 L) by treatment with 50 g/hL of PVPP at 0 °C for 1 h. Samples of the lager before and after stabilization were centrifuged and then analyzed by HPLC-ED as described previously (Madigan *et al*., 1994) and shown in Figure 4; the results of this analysis are given in Table 2. This ability to measure trimers in beer provides an important addition to the capability of the analytical method described previously. In the beer used as an example herein, the trimeric proanthocyanidins accounted for 14% of the total flavanols measurable by HPLC-ED, but it has been claimed that their contribution to haze formation may be more significant than is their quantitative presence (Delcour, 1984). Furthermore, PVPP stabilization caused a 38% decrease in the concentration of these compounds,

Table 2. Effect of Stabilization of Beer with 50 g/hL PVPP on Contents of Proanthocyanidins and Catechins

	flavanol concn (mg/L)		
flavanol	unstabilized lager	stabilized lager	% decrease
(+)-catechin	4.3	1.7	60
(−)-epicatechin	1.3	0.9	31
total monomers	5.6	2.6	54
D1 (prodelphinidin B3)	2.5	1.1	56
D2 (procyanidin B3)	1.7	0.7	59
total dimers	4.2	1.8	57
T1 (prodelphinidin trimer)	0.7	0.4	43
T2 (prodelphinidin trimer)	0.1	0.1	0
T3 (prodelphinidin trimer)	0.3	0.2	33
T4 (procyanidin trimer)	0.5	0.3	40
total trimers	1.6	1.0	38
total flavanols measurable by HPLC	11.5	5.5	52

indicating that the measurement of trimers may be another useful marker for the assessment of PVPP stabilization protocols. It is evident from Figures 1 and 3 that the barley extracts made with acetone−water (3:1) contained electroactive compounds other than the flavanols that were separated as distinct peaks on Superdex 75. These unidentified compounds may also be oligomeric proanthocyanidins, as judged by their HPLC-ED behaviors. Their importance in the assessment of beer stabilization is in doubt, however, since there is no evidence that they were removed from beer by a PVPP treatment that is sufficient to achieve colloidal stability (Figure 4).

Conclusions. The method described is a simple and fast procedure for the preparation of dimeric and trimeric proanthocyanidins for use as chromatographic standards using a commercially available high-performance column that has been previously unexplored in this context. The resolution of proanthocyanidins observed using Superdex 75 is far superior to that achievable using Sephadex LH-20. The ability to calibrate the measurement of trimers in beer enables significant improvement of the HPLC-ED analysis of beer flavanols and is of particular interest for monitoring the removal of flavanol dimers and trimers from beer by stabilization with PVPP.

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