Use of RP-HPLC-ESI(–)-MS/MS to Differentiate Various Proanthocyanidin Isomers in Lager Beer Extracts

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ABSTRACT

Normal-phase high-performance liquid chromatography electrospray ionization(–) tandem mass spectrometry (NP-HPLC-ESI(–)-MS/MS) and thioacidolysis analyses recently have revealed the presence of (+)-catechin, (–)-epicatechin, procyanidin dimers, and prodelphinidin trimers in acetone/water (70/30, vol/vol) LH-20 extracts of lager beers. In this study, detailed structures were determined by reversed-phase (RP) HPLC-ESI(–)-MS/MS. Four dimers were identified: three procyanidins (B1, B3, and B4) and one prodelphinidin (B3). Previously detected in hop or malt, three trimers (the procyanidin C-4α-8-C-4α-8-C and two prodelphinidins, GC-4α-8-C-4α-8-C and GC-4α-8-GC-4α-8-C) were distinguished for the first time in beer. As expected based on previous thioacidolysis data, most beer proanthocyanidins carried a catechin as the terminal unit.

Keywords: Beer, Flavan-3-ols, HPLC-ESI(–)-MS/MS, Proanthocyanidins

RESUMEN

La fase normal cromatografía líquida de alto resolución de ionización electroroció y espectrometría de masa en tandém (NP-HPLC-ESI(–)-MS/MS) y tioacidólisis análisis recientemente han puesto de manifiesto la presencia de (+)-catequina, (–)-epicatequina, dímeros de procianidina, y trímeros de prodelfinidina en acetona/agua (70/30, vol/vol) LH-20 extractos de las cervezas lager. En este estudio, las estructuras detalladas se determinaron por modo de fase inversa (RP) HPLC-ESI(–)-MS/MS. Cuatro dímeros se identificaron: tres procianidinas (B1, B3, y B4) y un prodelfinidina (B3). Previamente detectadas en malta o lúpulo, tres trímeros (la procianidina C-4α-8-C-4α-8-C y dos prodelfinidinas, GC-4α-8-C-4α-8-C y GC-4α-8-GC-4α-8-C) se distinguen por primera vez en la cerveza. Como era de esperar teniendo en datos de tioacidólisis, la mayoría de procianidinas en cerveza llevaba catequina como la unidad terminal.

Palabras claves: Cerveza, Flavan-3-ols, HPLC-ESI(–)-MS/MS, Proantocianidinas

Few investigations have focused on beer or malt flavanoids compared with hop flavanoids (Table I). (+)-Catechin, (–)-epicatechin, (–)-gallocatechin, nine dimers, and five trimers have been detected in hop (7,15,18,19,21,26,29). In malt, (+)-catechin, two dimers, and four trimers have been found (10,15,21,26,28,31). The monomeric units identified in beer are (+)-catechin and (–)-epicatechin, and only three dimers have been detected so far (1,2,8,9,11–14, 16,17,20,22,25).

Flavanoids are known to be responsible for colloidal instability during storage (23). Very little haze is produced with (–)-epicatechin and (+)-catechin monomers, while procyanidin B3 and especially prodelphinidin B3 are closely related to haze formation (23,24, 27). With respect to color, little information is available in the brewing literature. Polyphenol oxidation and subsequent degradation could account for the color increase found during storage (30). Improvement of beer shelf life requires a deeper knowledge of all the chemicals involved.

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doi:10.1094/ASBCJ-2008-0215-01

In a previous work, normal-phase high-performance liquid chromatography electrospray ionization(–) tandem mass spectrometry (NP-HPLC-ESI(–)-MS/MS) analyses of an acetone/water (70/30, vol/vol) LH-20 beer extract enabled us to detect up to trimers of proanthocyanidins (5,6). To obtain more structural information, thioacidolysis also was applied to isolated dimer and trimer fractions. Our results indicated that catechin forms the main terminal unit in dimers and trimers, whereas both catechin and gallocatechin (especially in trimers) are the major constituents of the extension units.

The aim of this study was to establish detailed structures of beer proanthocyanidins. The acetone/water (70/30, vol/vol) LH-20 extract was analyzed by both NP- and reversed-phase (RP) HPLC-ESI(–)-MS/MS. Tandem mass spectrometry analysis was chosen to obtain complete fragmentation patterns. A systematic analysis of retention times and fragments obtained by retro-Diels-Alder (RDA) fission, heterocyclic ring fission (HRF), and quinone methide (QM) fission enabled identification of most procyanidins and prodelphinidins.

EXPERIMENTAL

Chemicals

Acetone (99.9%), (–)-epicatechin (98%), (+)-catechin (98%), (–)-gallocatechin (98%), and (–)-epigallocatechin (98%) were obtained from Sigma-Aldrich (Bornem, Belgium). Procyanidin B1 ((–)-epicatechin-4β-8-(–)-catechin) was provided by the Unité de Recherches Cidricoles, Biotransformation des Fruits et Légumes (INRA, Paris Cedex, France). Methanol (99.9%) and dichloromethane (99.9%) were purchased from Romil (Cambridge, UK). Acetic acid (99.8%) was obtained from Acros (Geel, Belgium). Ammonium acetate (99%) was obtained from Fluka (Buchs, Switzerland). Acetonitrile (99.99%) was obtained from Fischer Scientific (Leicestershire, UK). Formic acid (99%) was obtained from Janssen Chemica (Geel, Belgium). The commercial lager beers stabilized with polyvinylpolypyrrolidone were supplied by a Belgian brewery.

Extraction of Beer Flavanoids

Beer flavanoids were extracted as described previously (4,6). Prior to extraction, beer was degassed using sonication for 10 min. Three grams of Sephadex LH-20 (Sigma-Aldrich, St. Louis) packed in a 12-mL filtration tube with a polyethylene frits (Supelco, Bellfonte, PA) was preconditioned for 4 hr with methanol/water (30/70, vol/vol). After loading 50 mL of degassed beer, the column was washed with 40 mL of methanol/water (30/70, vol/vol). Proanthocyanidins were recovered with 70 mL of acetone/water (70/30, vol/vol). The eluates were concentrated to dryness by rotary evaporation and dissolved in 2 mL of methanol.

HPLC Separation and MS Detection

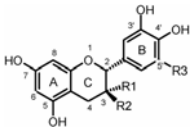
HPLC separation and MS detection were performed as described previously (3,6). A SpectraSystem (Finnigan Mat, San Jose, CA) equipped with an SCM degasser, an AS3000 autosampler, and a P4000 quaternary pump was used. Mass spectra were acquired with an LCQ ion-trap mass spectrometer equipped with an ESI source.

The system was controlled with Xcalibur software, version 1.2. Pseudomolecular ions were determined by full-MS analysis and complete fragmentation patterns were obtained by tandem mass spectrometry.

NP-HPLC-MS

Chromatographic separation was done on a 5-µm, 250 × 2.1 mm i.d. Silica Alltima HP column (Alltech, Deerfield, IL) at a flow rate of 0.2 mL/min, with a multilinear dichloromethane (A)–methanol

TABLE I
Proanthocyanidins in Malt, Hop, and Beer According to the Literature

Proanthocyanidin	Flavanoid Compound[a]		Literature Reference[b]		
			Malt	Hop	Beer
Flavan-3-ol					
	(+)-Catechin	R_1 = H, R_2 = OH, R_3 = H	10, 15, 21, 26, 31	7, 15, 18, 19, 21, 29	1, 2, 11, 14, 16, 17, 20, 22, 24, D
	(–)-Epicatechin	R_1 = OH, R_2 = R_3 = H	–	7, 18, 19, 21, 29	11, 13, 14, 16, 17, 20, 22, 24, D
	(–)-Gallocatechin	R_1 = H, R_2 = R_3 = OH	–	18, 19	17, D
	(–)-Epigallocatechin	R_1 = OH, R_2 = H, R_3 = OH	–	–	17, D
Procyanidin B					
	B1 (–)-epicatechin-(4β-8)-(+)-catechin		–	18, 19, 21, 29	–, N
	B2 (–)-epicatechin-(4β-8)-(–)-epicatechin		–	18, 19, 21, 29	–
	B3 (+)-catechin-(4α-8)-(+)-catechin		15, 21, 28, 31	15, 18, 19, 21, 29	8, 9, 17, 17, 20, 22, 24, D
	B4 (+)-catechin-(4α-8)-(–)-epicatechin		–	18, 19, 21, 26, 29	–, N
Prodelphinidin B					
	B3 (–)-gallocatechin-(4α-8)-(+)-catechin		15, 21, 28, 31	18, 19	8, 17, 17, 20, 22, 24, D
	B9 (–)-epigallocatechin-(4β-8)-(+)-catechin		–	–	8
	(+)-Catechin-(4α-8)-(–)-gallocatechin		–	18, 19	–
	(+)-Catechin-(4α-6)-(–)-gallocatechin		–	18, 19	–
	(–)-Gallocatechin-(4α-6)-(+)-catechin		–	18, 19	–
Prodelphinidin A					
	ent-(–)-Epigallocatechin-(4α-8, 2α-O-7)-(+)-catechin		–	–	12
	ent-(–)-Epigallocatechin-(4α-6, 2α-O-7)-(+)-catechin		–	–	12
Propelargonidin B					
	(+)-Afzelechin-(4α-8)-(+)-catechin		–	18, 19	–
Procyanidin C					
	C2 (+)-catechin-(4α-8)-(+)-catechin-(4α-8)-(+)-catechin		15, 28, 31	15, 18, 19	–, N
	(–)-Epicatechin-(4β-8)-(+)-catechin-(4α-8)-(+)-catechin		–	18, 19, 29	–
	(–)-Epicatechin-(4β-8)-(–)-epicatechin-(4β-8)-(+)-catechin		–	18, 19	–
Prodelphinidin C					
	(–)-Gallocatechin-(4α-8)-(–)-gallocatechin-(4α-8)-(+)-catechin		15, 31	18, 19	–, N
	(–)-Gallocatechin-(4α-8)-(+)-catechin-(4α-8)-(+)-catechin		15, 31	–	–, N
	(+)-Catechin-(4α-8)-(–)-gallocatechin-(4α-8)-(+)-catechin		15, 31	18, 19	–

[a] 4α = substituent in position 4 below the plane of the flavanoid, 4β = substituent in position 4 above the plane of the flavanoid, and *ent*- = enantiomer (nonsuperimposable mirror images).
[b] N = new compound detected in this work, D = also detected in this work, – = not determined.

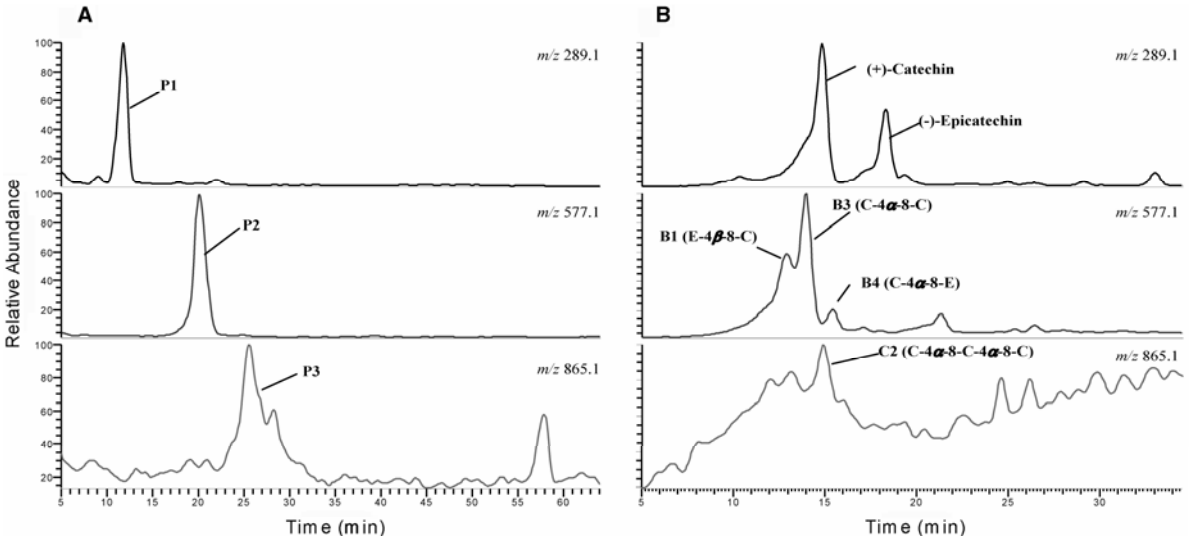


Fig. 1. Comparison of normal-phase high-performance liquid chromatography electrospray ionization(–) tandem mass spectrometry (NP-HPLC-ESI(–)-MS/MS) (**A**) and reversed-phase (RP) HPLC-ESI(–)-MS/MS (**B**) chromatograms of procyanidins (P1 = 289.1 *m/z*, P2 = 577.1 *m/z*, and P3 = 865.1 *m/z*) in an acetone/water (70/30, vol/vol) LH-20 extract of SPE lager beer. C = catechin and E = epicatechin.

TABLE II
Retention Times on Normal-Phase (RT_{NP}) and Reversed-Phase (RT_{RP}) High-Performance Liquid Chromatography Electrospray Ionization(–) Tandem Mass Spectrometry Fragmentation Patterns of Flavanoids Detected in the LH-20 Extract of a Lager Beer[a]

Compound	M_w	RT_{NP} (min)	RT_{RP} (min)	RDA/–H_2O *m/z* (%)	QM_{Upper}/RDA and –H_2O *m/z* (%)	QM_{Lower} *m/z* (%)	HRF *m/z* (%)
(+)-Catechin	290	11.8	14.8		245.1(100), 205.1(33), 246.2(14), 179.1(13), 289.1(12)		
(–)-Epicatechin	290	11.8	18.3		245.1(100), 205.1(33), 246.2(14), 179.1(13), 289.1(12)		
(–)-Gallocatechin	306	16.5	9.9		179.1(100), 219.1(72), 221.1(63), 307.0(33), 261.0(30)		
(–)-Epigallocatechin	306	16.5	13.7		179.1(100), 219.1(72), 221.1(63), 307.0(33), 261.0(30)		
Procyanidin B1							
(–)-Epicatechin-(4β-8)-(+)-catechin	578	20.1	12.9	425.1(100)/407.2(37)	289.1(13)/287.0(5)		451.1(20)
Procyanidin B3							
(+)-Catechin-(4α-8)-(+)-catechin	578	20.1	13.9	425.1(100)/407.2(37)	289.1(13)/287.0(5)		451.1(20)
Procyanidin B4							
(+)-Catechin-(4α-8)-(+)-epicatechin	578	20.1	15.4	425.1(100)/407.2(37)	289.1(13)/287.0(5)		451.1(20)
Prodelphinidin B3							
(–)-Gallocatechin-(4α-8)-(+)-catechin	594	22.5	10.6	425.1(100)/407.2(22)	289.1(11) + 303.0(5)	289.1(11) + 303.0(5)	467.0(10)
Unknown 1[b]	594	17.5	19.4		556.1(100), 431.1(10), 311.1(8), 473.1(5), 341.2(5)		
Unknown 2[b]	594	17.5	26.3		285.1(100), 286.1(13), 299.1(3), 257.2(3)		
Unknown 3[b]	594	24.4	20.7		311.2(100), 431.1(96), 473.1(48), 556.0(5), 341.2(5)		
Unknown 4[b]	594	24.4	23.7		311.2(100), 413.1(25), 503.1(23), 353.2(17), 473.1(5)		
Unknown 5[c]	610	16.9	26.6		299.2(100), 563.1(35), 301.1(38), 285.2(18), 301.1(17)		
Unknown 6[c]	610	19.8	24.4		301.2(100), 300.2(57), 302.1(13), 271.2 (9), 343.1(8)		
Unknown 7[c]	610	24.1	8.6		441.1(100), 423.1(31), 457.1(14), 483.0(12), 591.1(12)		
Unknown 8[c]	610	26.9	18.3		447.1(100), 327.2(24), 448.1(20), 357.1(24), 489.1(3)		
Unknown 9[c]	610	59.4	21.5		453.2(100), 297.1(28), 429.1(25), 454.2(21), 327.1(20)		
Procyanidin C2							
(+)-Catechin-(4α-8)-(–)-catechin-(4α-8)-(+)-catechin	866	25.5	14.8	713.1(44)/695.1(100)	287.1(10) + 577.1(58)/ 425.0(19) + 407.1(24)	289.1(5) + 575.1(40)	739.1(45)
Prodelphinidin							
(–)-Gallocatechin-(4α-8)-(–)-catechin-(4α-8)-(+)-catechin	882	27.1	11.4	713.1(44)/695.1(100)	303.1(5) + 577.1(26)/ 425.0(22) + 407.1(14)	289.1(8) + 591.1(43)	755.1(51)
(–)-Gallocatechin-(4α-8)-(–)-gallocatechin-(4α-8)-(+)-catechin	898	28.8	10.6	729.0(51)/711.1(100)	303.1(3) + 593.1(15)/ 425.0(10) + 407.1(17)	289.1(3) + 607.0(15)	771.1(22)

[a] RDA = retro-Diels-Alder fission, QM = quinone methide fission cleavage, and HRF = heterocyclic ring fission.
[b] Dimer with monomer units = one gallocatechin or epigallocatechin and one catechin or epicatechin.
[c] Dimer with monomer units = gallocatechin or epigallocatechin.

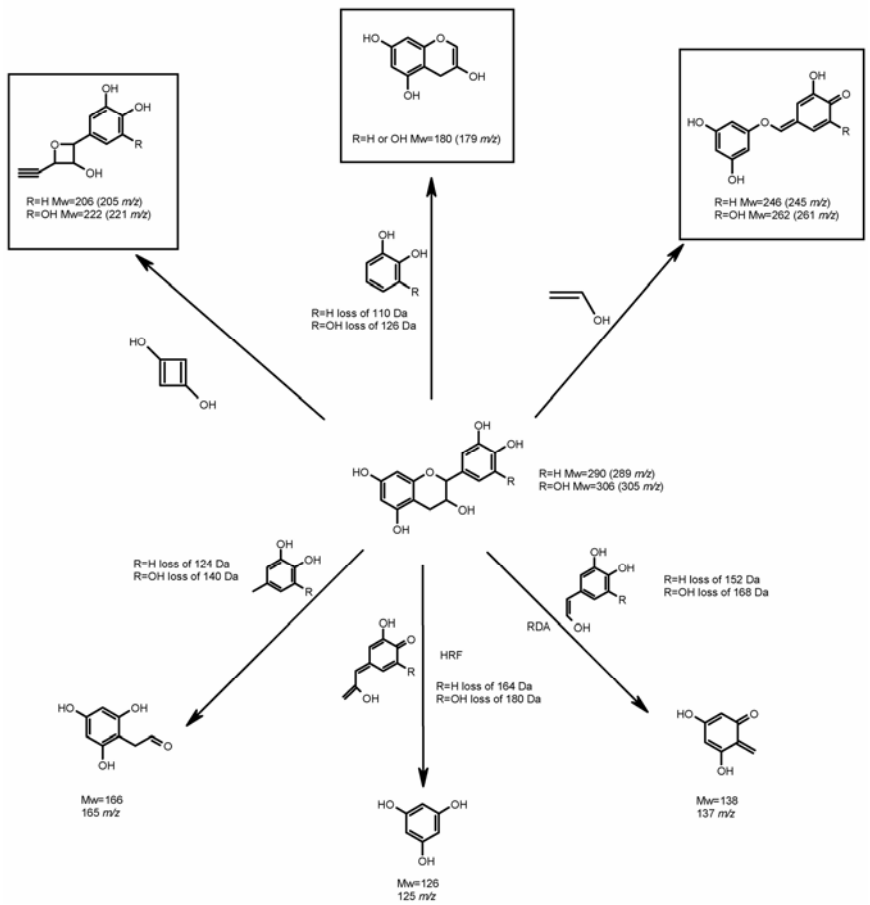


Fig. 2. Hypothetical electrospray ionization(–) tandem mass spectrometry (ESI(–)-MS/MS) fragmentation pattern for monomers. RDA = retro-Diels-Alder fission and HRF = heterocyclic ring fission. R = H = (+)-catechin and R = OH = (–)-gallocatechin.

(B) gradient containing a constant 4% level of acetic acid/water (1/1, vol/vol). 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 elution; and return to initial conditions for 15 min. A 5-µL sample was injected into the column kept at 25°C. The ESI (negative mode) 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% for singly charged $[M-H]^-$ ions of monomers, dimers, and trimers, respectively.

RP-HPLC-MS

Separations were performed on a 2-µm, 150 × 2.1 mm i.d. C18 Prevail column (Alltech) at a flow rate of 0.2 mL/min, with a multilinear gradient of water containing 1% acetonitrile and 0.1% formic acid (A) and 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 elution; and return to initial conditions for 15 min. A 5-µL sample was injected into the column kept at 25°C. For the ESI (negative mode) 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 as described for the normal-phase experiment.

RESULTS AND DISCUSSION

Identification of Beer Procyanidins

Monomers (M_w 290), dimers (M_w 578), and trimers (M_w 866) of (epi)catechin units were analyzed by selecting their $[M-H]^{-1}$ pseudomolecular ions. Identification was achieved by comparing the MS fragmentation patterns and retention times with ones acquired previously (3). As expected, procyanidins were separated by normal-phase chromatography according to their polymerization degree and by reversed-phase chromatography according to their polarity. Although only one peak emerging from the normal-phase column characterized each polymerization degree ($Rt_{NP\text{-}P1}$ = 11.8 min, $Rt_{NP\text{-}P2}$ = 20.1 min, and $Rt_{NP\text{-}P3}$ = 25.6 min) (Fig. 1A), the reversed-phase column (Fig. 1B) allowed separation of different oligomers that had the same molecular weight.

As monomers (289 *m/z*), both (+)-catechin (C, Rt_{RP} = 14.8 min) and (–)-epicatechin (E, Rt_{RP} = 18.3 min) were identified in the beer extracts (same mass spectrum) (Table II). As shown in Figure 2 and Table II, for monomers, three rearrangements involving the loss of small molecules were favored, leading to 245.2, 205.1, and 179.1 *m/z* for both catechins. For dimers (577 *m/z*), three peaks (Rt_{RP} = 12.9, 13.9, and 15.4 min) (Fig. 1B) with the same mass spectrum were assigned as B1 (E-4β-8-C), B3 (C-4α-8-C), and B4 (C-4α-8-C), respectively. Only one procyanidin trimer, C2

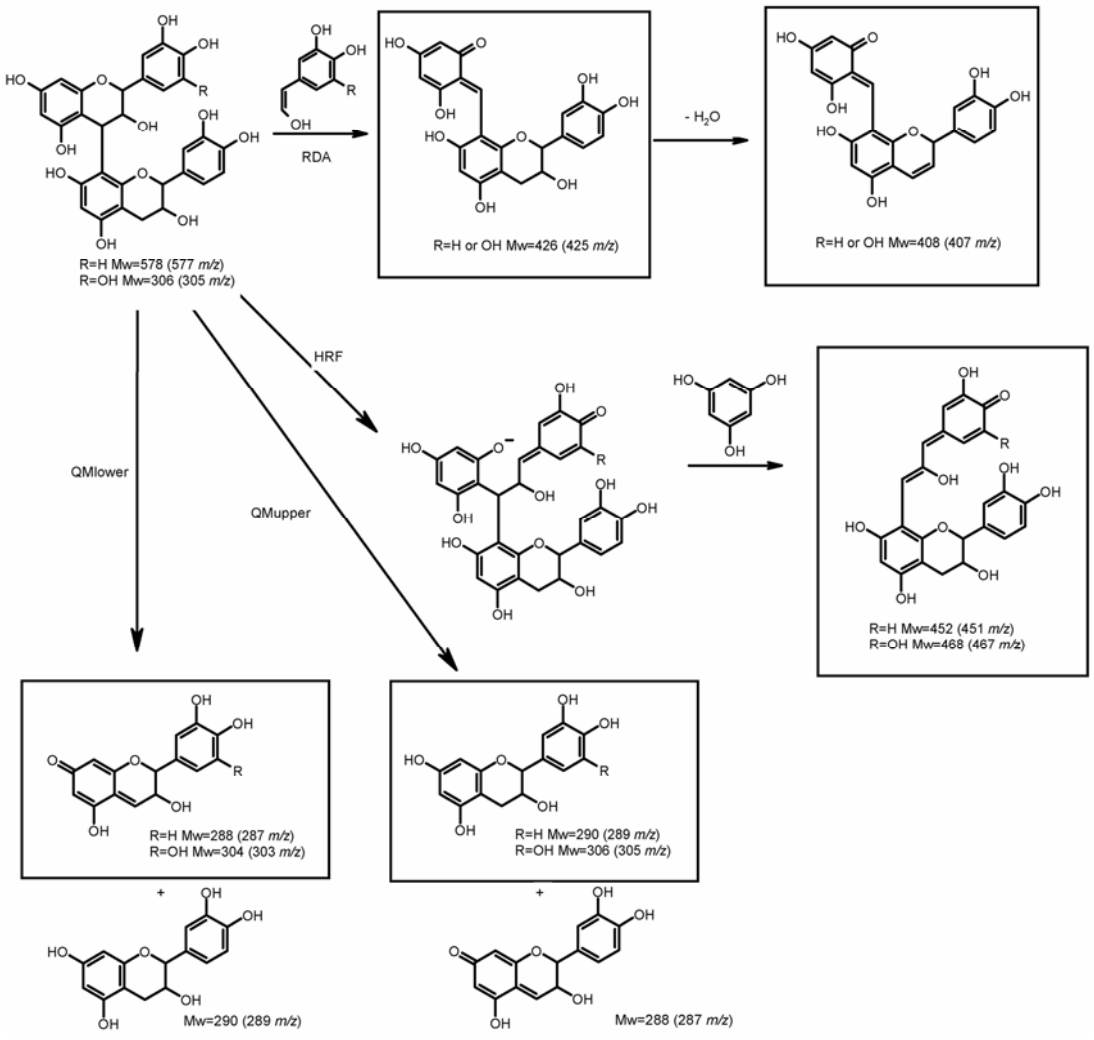


Fig. 3. Hypothetical electrospray ionization(–) tandem mass spectrometry (ESI(–)-MS/MS) fragmentation pattern for proanthocyanidin dimers. RDA = retro-Diels-Alder fission, QM = quinone methide fission cleavage, and HRF = heterocyclic ring fission. R = H = C-4α-8-C and R = OH = GC-4α-8-C. C = catechin and GC = gallocatechin.

(C-4α-8-C-4α-8-C), was significantly detected in the LH-20 extract (Rt_{RP} = 14.8 min, 865.1 *m/z*) (Fig. 1B). For dimers and trimers, HRF, RDA reaction, and QM fission cleavage were favored, leading to 451.1 or 739.1 *m/z*, 425.1 (+407.2) or 713.1 (+695.1) *m/z*, and 289.1 or 577.1 *m/z*, respectively (Figs. 3 and 4; Table II).

Identification of Beer Prodelphinidins

Monomers (M_w 306), dimers with one (M_w 594) or two (M_w 610) gallo units, and trimers with one (M_w 882), two (M_w 898), or three (M_w 914) gallo units were studied by selecting the corresponding $[M-H]^{-1}$ pseudomolecular ions. In the absence of commercial stan-

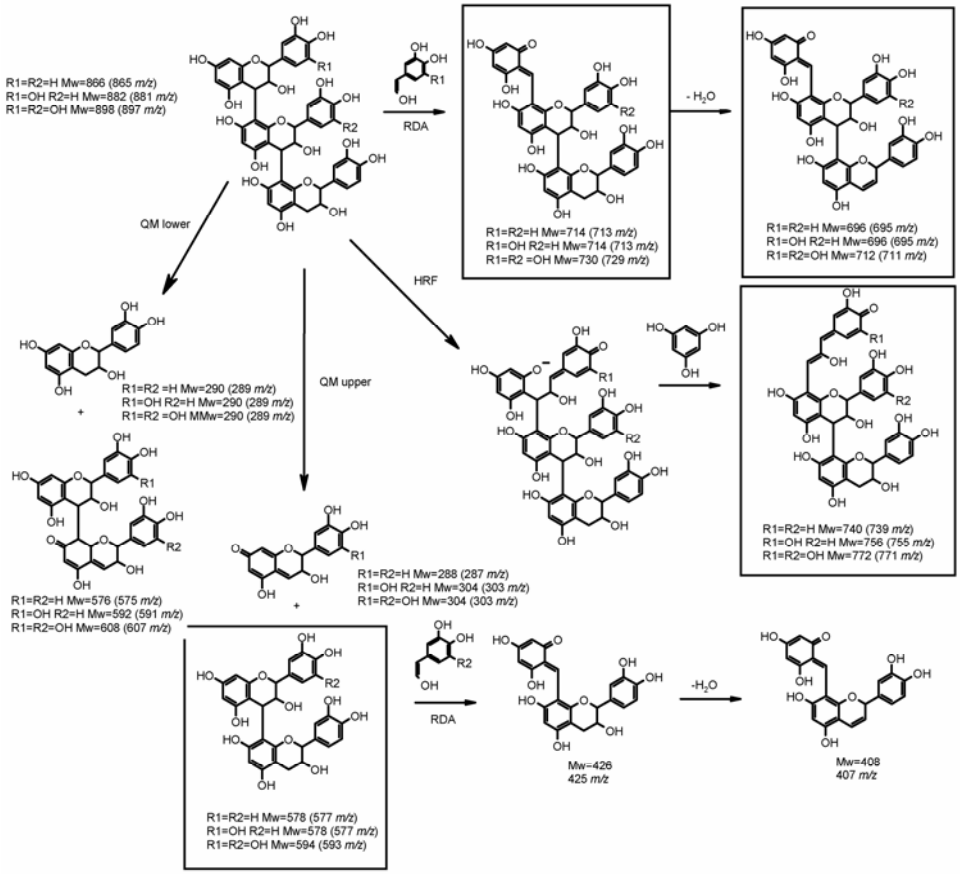


Fig. 4. Hypothetical electrospray ionization(–) tandem mass spectrometry (ESI(–)-MS/MS) fragmentation pattern for proanthocyanidin trimers. RDA = retro-Diels-Alder fission, QM = quinone methide fission cleavage, and HRF = heterocyclic ring fission. R1 = R2 = H = C-4α-8-C-4α-8-C; R1 = OH, R2 = H = GC-4α-8-C-4α-8-C; and R1 = OH, R2 = OH = GC-4α-8-GC-4α-8-C. C = catechin and GC = gallocatechin.

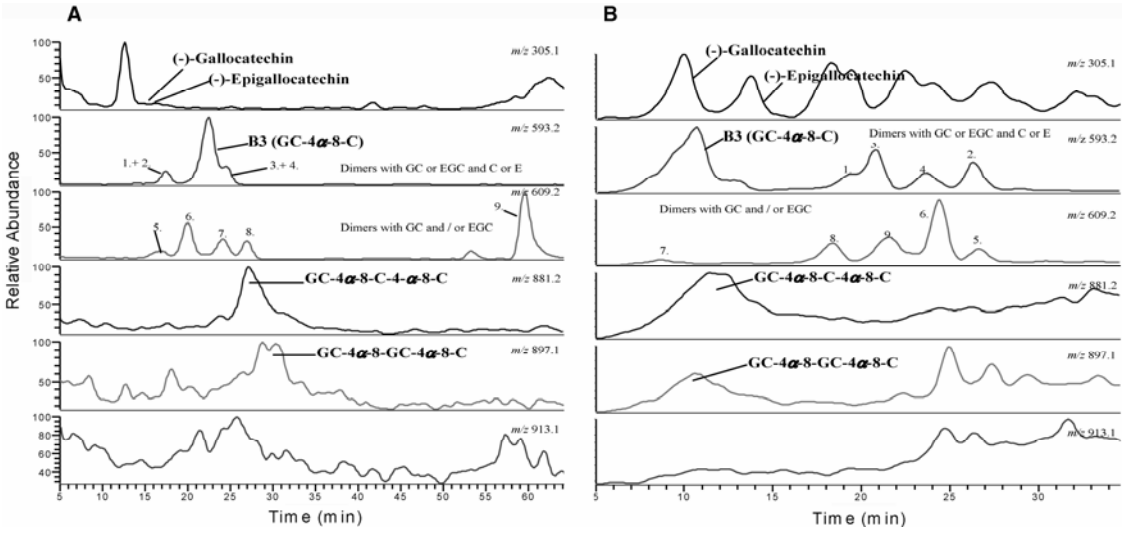


Fig. 5. Comparison of normal-phase high-performance liquid chromatography electrospray ionization(–) tandem mass spectrometry (NP-HPLC-ESI(–)-MS/MS) (**A**) and reversed-phase (RP) HPLC-ESI(–)-MS/MS (**B**) chromatograms of prodelphinidins (Pr1 = 305.1 *m/z*; Pr2 = 593.1 and 609.2 *m/z*; and Pr3 = 881.2, 897.1, and 913.1 *m/z*) in an acetone/water (70/30, vol/vol) LH-20 extract of SPE lager beer. C = catechin, E = epicatechin, GC = gallocatechin, and EGC = epigallocatechin.

dards, structures of dimers and trimers were assigned when possible according to their RDA, HRF, and QM fragmentations (Table II; Figs. 3 and 4).

As depicted in Figure 5B, traces of (–)-gallocatechin (Rt_{RP} = 9.9 min) and (–)-epigallocatechin (Rt_{RP} = 13.7 min) were found in the beer extract. Both exhibited the same ESI(–) mass spectrum (Table II; Fig. 2). Interestingly, even though the fragmentation followed similar pathways, the fragment issued from the loss of the C ring was more intense here compared with(+)-catechin or (–)-epicatechin (179.1(100) versus 245.1(100)), potentially due to better stabilization with three hydroxyl groups (Table II). With regard to dimers and trimers of prodelphinidins, the MS/MS fragmentation followed the same scheme as procyanidins (RDA, HRF, and QM). At 593 *m/z*, the main dimer was prodelphinidin B3 (GC-4β-8-C; Rt_{RP} = 10.6 min) (Fig. 5B). The gallocatechin position was assigned on the basis of the RDA 425 *m/z* and HRF 467 *m/z* fragments (Fig. 3). Four other dimer isomers (no. 1–4, 593 *m/z*) and five dimers with two gallocatechins (no. 5–9, 609 *m/z*) also were suspected. Two prodelphinidin trimers were found at 881 *m/z* (Rt_{RP} = 11.4 min) and 897.1 *m/z* (Rt_{RP} = 10.6 min) (Fig. 5B). In both cases, catechin was assigned at the terminal position according to the QM_{upper} (425 and 407 *m/z*) and QM_{lower} (289 *m/z*) fragments. The gallocatechin position in the M_w 882 oligomer was determined on the basis of its RDA (695 *m/z*), HRF (755 *m/z*), QM_{upper} (303 and 577 *m/z*), and QM_{lower} (289 and 591 *m/z*) fragments (Fig. 4), leading us to suspect the GC-4α-8-C-4α-8C structure.

CONCLUSIONS

Complementary data on beer proanthocyanidins have been obtained through RP-HPLC-ESI(–)-MS/MS analysis (all the N entries in Table I). Four dimers were identified here: three procyanidins (B1, B3, and B4) and one prodelphinidin (B3). Although previously detected in hop or malt, three trimers were distinguished for the first time in beer: one procyanidin (C-4α-8-C-4α-8-C) and two prodelphinidins (GC-4α-8-C-4α-8-C and GC-4α-8-GC-4α-8-C). As expected based on previous thioacidolysis data, most beer proanthocyanidins carry a catechin as the terminal unit. Procyanidins or prodelphinidins higher than trimers were not detected in this study.

ACKNOWLEDGMENTS

D. Callemien thanks the Inbev-Baillet Latour Foundation and InBev (Leuven, Belgium) for financial support.

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