Gradient HPLC Method for Analysis of Beer Polyphenols, Proanthocyanidins, and Bitter Acids Using a Novel Spectro-Electro Array Platform

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Application Note 1065

Key Words
Catechins, Xanthohumols, Iso-Alpha Acids, Electrochemical Detection, Diode Array Detection

Introduction

Beer is the most widely consumed alcoholic beverage in the world and the third most popular drink after water and tea.[1] Beer is typically brewed from four basic ingredients: water, a starch source (e.g., malted barley), brewer's yeast, and a flavoring agent such as hops. Many varieties of beer result from differences in these ingredients, the additives used, and the brewing process. Beer contains a complex mixture of phenolic compounds extracted from the starch source and hops.

A recent trend from microbreweries in the U.S. is the development of a wide range of extremely bitter beers created by the addition of extra hops during the brewing process. The hop-derived xanthohumol and the iso-alpha acids formed are primarily responsible for the perceived bitterness. Many of these secondary metabolites are not only purported to offer health benefits[2–4] but also are essential to the flavor and stability of the beer itself.[5,6] Conversely, some secondary metabolites contribute to the degradation of beer during storage with the formation of haze (e.g., catechins and their polymers, the proanthocyanidins).[7]

Goal

To develop gradient high-performance liquid chromatography (HPLC) methods using a spectro-electro array platform to measure specific analytes in beer samples and—in a metabolomic approach—to distinguish between different beer samples and study beer stability

Equipment

- Thermo Scientific™ Dionex™ UltiMate™ 3000 HPLC system, including:
  - LPG-3400BM Biocompatible Quaternary Micro Pump
  - SR-3000 Solvent Rack without Degasser
  - WPS-3000TBSL UltiMate 3000 Biocompatible Thermostatted Analytical Split-Loop Autosampler
  - DAD-3000RS UltiMate 3000 Rapid Separation Diode Array Detector
- Thermo Scientific™ Dionex™ CoulArray™ Coulometric Array Detector, Model 5600, with CoulArray Thermal Organizer Module
- Model 5011A High Sensitivity Analytical Cell, Dual Channel
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software version 6.8 (SR 9)
- CoulArray software version 3.1

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Consumables

- Centrifugal Filters, 0.22 µm, nylon
- Sample Tubes, 40 mL

Standards and Reagents

Standards		
Gallic Acid	Fisher Scientific™	P/N AC410860050
4-Hydroxybenzyl Alcohol	Fisher Scientific	P/N 50-700-3921
p-Aminobenzoic Acid	Fisher Scientific	P/N ICN1025690
3,4-Dihydroxybenzoic Acid	Fisher Scientific	P/N ICN15642110
Gentisic Acid	Fisher Scientific	P/N AC165200050
2-Hydroxybenzyl Alcohol	Fisher Scientific	P/N 50-014-36177
Chlorogenic Acid	Fisher Scientific	P/N ICN15061801
4-Hydroxyphenylacetic Acid	Fisher Scientific	P/N AC121710250
p-Hydroxybenzoic Acid	Fisher Scientific	P/N ICN10257780
Catechin	Fisher Scientific	P/N 50-749-8352
Vanillic Acid	Fisher Scientific	P/N AAA1207414
4-Hydroxybenzaldehyde	Fisher Scientific	P/N AC16277-0500
Syringic Acid	Fisher Scientific	P/N AC13289-0100
Caffeic Acid	Fisher Scientific	P/N ICN10479705
Vanillin	Fisher Scientific	P/N AC140821000
Syringaldehyde	Fisher Scientific	P/N 50-701-9419
Umbelliferone	Fisher Scientific	P/N AC12111
p-Coumaric Acid	Fisher Scientific	P/N ICN10257610
3,4-Dimethoxybenzoic Acid	Fisher Scientific	P/N AC11545-0250
Sinapic Acid	Fisher Scientific	P/N 50-121-8328
Salicylic Acid	Fisher Scientific	P/N AC14770
Ferulic Acid	Fisher Scientific	P/N AC15636
Ellagic Acid Dihydrate	Fisher Scientific	P/N AC11774
Coumarin	Fisher Scientific	P/N AC11053
Rutin	Fisher Scientific	P/N AC13239
Ethyl Vanillin Bourbonal	Fisher Scientific	P/N ICN15795980
4-Hydroxycoumarin	Fisher Scientific	P/N AC12110
Hesperidin	Fisher Scientific	P/N AC12346
Naringin	Fisher Scientific	P/N AC20691
Rosemarinic Acid	Fisher Scientific	P/N ICN15979210
Fisetin	Fisher Scientific	P/N 50-749-1075
Myricetin	Fisher Scientific	P/N 50-328-725
trans-Resveratrol	Fisher Scientific	P/N 50777-94
Luteolin	Fisher Scientific	P/N 50-148-702
cis-Resveratrol	Fisher Scientific	P/N NC9905571
Quercetin Dihydrate	Fisher Scientific	P/N ICN15200310
Kaempferol	Fisher Scientific	P/N ICN15514310
Isorhamnetin	Fisher Scientific	P/N 50-908-546
Eugenol	Fisher Scientific	P/N AC11911
Isoxanthohumol	ChromaDex®	P/N ASB-00009638
Naringenin	Fisher Scientific	P/N ICN10243001
Prenylnaringenin	Sigma Aldrich	P/N P3496
Chrysin	Fisher Scientific	P/N AC11032
Carvacrol	Fisher Scientific	P/N 50-014-24614
Thymol	Fisher Scientific	P/N AC15033
Carnosol	ChromaDex	P/N ASB-00003199
Xanthohumol	ChromaDex	P/N ASB-00024010
Carnosic Acid	ChromaDex	P/N ASB-0000319

Reagents		
Acetonitrile	Fisher Scientific	P/N A9981
Ethanol	Fisher Scientific	P/N A995-4
Methanol	Fisher Scientific	P/N A-456-1
Sodium Phosphate Monobasic	Fisher Scientific	P/N ICN19485083
Tetrahydrofuran (THF)	Fisher Scientific	P/N T425-1
Phosphoric Acid	Fisher Scientific	P/N A260-500
Ascorbic Acid	Fisher Scientific	P/N AC105021000
Ethylenediaminetetraacetic Acid (EDTA)	Fisher Scientific	P/N S311-100
Dimethylformamide (DMF)	Fisher Scientific	P/N AC116220010

Conditions

Method 1: Polyphenols	
Column:	Thermo Scientific™ Acclaim™ 120 C18, 3 µm Analytical (3.0 150 mm, P/N 063691)
Mobile Phase A:	20 mM Sodium Phosphate Monobasic, 3% Acetonitrile, 0.2% Tetrahydrofuran, pH 3.35
Mobile Phase B:	20 mM Sodium Phosphate Monobasic, 50% Acetonitrile, 10% Tetrahydrofuran, pH 3.45
Mobile Phase C:	90% Methanol
Gradient:	0–2 min, 2% B, 3% C; 30 min, 97% B, 3% C; 45 min, 97% B, 3% C; Curve 7 (concave)
Flow Rate:	0.65 mL/min
Inj. Volume:	20 µL
Temperature:	35º C
Detection:	UV; Channel 1, 218 nm; Channel 2, 240 nm; Channel 3, 254 nm; Channel 4, 275 nm
EC Detector Parameters:	16 Channel Array from 0 to +900 mV, relative to Pd, in 60 mV increments

Method 2: Bitter Acids	
Column:	Acclaim 120 C18, 3 µm Analytical (3.0 150 mm, P/N 063691)
Mobile Phase A:	25 mM Sodium Perchlorate, 50% Acetonitrile, 2.5 mM Perchloric Acid
Mobile Phase B:	25 mM Sodium Perchlorate, 90% Acetonitrile, 2.5 mM Perchloric Acid
Mobile Phase C:	90% Methanol
Gradient:	0–3 min, 0% B, 3% C; 30 min, 40% B, 3% C; 40 min, 97% B, 3% C; 45 min, 97% B, 3% C
Flow Rate:	0.65 mL/min
Inj. Volume:	20 µL
Temperature:	35° C
EC Detector Parameters:	E1 +550 mV; E2 +850 mV, relative to Pd

Standards Preparation

Method 1: Polyphenols

For Method 1, depending upon solubility, prepare individual standard stock solutions in ethanol, methanol, or methanol/water (1:1) at 1 or 0.1 mg/mL. Prepare substock solution A–G by mixing aliquots of different individual standards into 10 mL glass volumetric flasks. Add 0.5 mL solution containing 2% ascorbic acid/0.02% EDTA as a preservative. Dilute to 10 mL with a solution of 25% methanol at pH 3.2 adjusted with phosphoric acid. Prepare working standards at 0.20, 0.50, and 1.0 mg/L in water. See Table 1 for polyphenol standards preparation details.

Table 1. Details for standards preparation.

Compound Name	Stock Std Concn (mg/mL)	Solvent	Aliquot (mL) to 10 mL	Substock Concn (mg/L)
Mix A				
Gallic Acid	1	50% Methanol	0.10	10
3,4-Dihydroxybenzioc Acid	1	50% Methanol	0.10	10
Catechin	1	Methanol	0.20	20
Syringic Acid	1	50% Methanol	0.10	10
Caffeic Acid	1	50% Methanol	0.10	10
Umbelliferone	1	Methanol	0.10	10
Salicylic Acid	1	50% Methanol	0.20	20
Naringin	1	Ethanol	0.20	20
Fisetin	0.1	Ethanol	1.00	10
Luteolin	0.1	Ethanol	1.00	10
Isorhamnetin	0.1	Ethanol	1.00	10
Carvacrol	1	Methanol	0.10	10
Carnosic Acid	0.1	Methanol	1.00	10
Mix B				
4-Hydroxybenzyl Alcohol	1	50% Methanol	0.10	10
Chlorogenic Acid	1	Methanol	0.20	20
4-Hydroxyphenylacetic Acid	1	50% Methanol	0.10	10
Vanillic Acid	1	Methanol	0.10	10
Vanillin	1	Methanol	0.10	10
Sinapic Acid	1	Methanol	0.10	10
Ferulic Acid	1	Ethanol	0.10	10
4-Hydroxycoumarin	1	Methanol	0.20	20
Hesperidin	1	DMF or Formamide	0.20	20
Myricetin	0.1	Ethanol	1.00	10
Kaempferol	0.1	Ethanol	1.00	10
Thymol	1	Methanol	0.10	10
Mix C				
p-Aminobenzoic Acid	1	50% Methanol	0.10	10
Gentisic Acid	1	50% Methanol	0.10	10
2-Hydroxybenzyl Alcohol	1	50% Methanol	0.10	10
p-Hydroxybenzoic Acid	1	50% Methanol	0.10	10
4-Hydroxybenzaldehyde	1	50% Methanol	0.20	20
Syringaldehyde	1	Methanol	0.10	10
p-Coumaric Acid	1	Ethanol	0.20	20
Ethyl Vanillin Bourbanol	1	Methanol	0.10	10
Rosemarinic Acid	0.1	Ethanol	1.00	10
Quercetin Dihydrate	1	Ethanol	0.20	20
Eugenol	1	50% Methanol	0.20	20
Carnosol	0.1	50% Methanol	1.00	10

Compound Name	Stock Std Concn (mg/mL)	Solvent	Aliquot (mL) to 10 mL	Substock Concn (mg/L)
Mix D: UV Compounds				
3,4-Dimethoxybenzoic Acid	1	Methanol	0.10	10
Coumarin	1	Methanol	0.10	10
Methoxybenzaldehyde	1	Methanol	0.10	10
Cinnamic Acid	1	50% Methanol	0.10	10
Apigenin	0.1	Ethanol	1.00	10
Chrysin	1	Ethanol	0.10	10
Mix E				
Rutin	0.1	Ethanol	1.00	10
Ellagic Acid Dihydrate	0.1	Ethanol	1.00	10
trans-Resveratrol	0.1	Ethanol	1.00	10
cis-Resveratrol	0.1	Ethanol	1.00	10
Mix F				
Isoxanthohumol	0.1	Ethanol	1.00	10
Xanthohumol	0.1	Ethanol	1.00	10
Mix G				
Gallocatechin	0.1	Methanol	1.00	10
Epigallocatechin	0.1	Methanol	1.00	10
Catechin	1	Methanol	0.10	10
Epicatechin	1	Methanol	0.10	10
Epigallocatechin Gallate	1	Methanol	0.10	10
Gallocatechin Gallate	1	Methanol	0.10	10
Epicatechin Gallate	1	Methanol	0.10	10
Catechin Gallate	0.1	Methanol	1.00	10

Method 2: Bitter Acids

Bitter acid standard mixes were obtained from the American Society of Brewing Chemists, International Calibration Standards for HPLC Analysis of Isomerized -Acids, and included: DCHA-Iso, ICS-12; DCHA-Rho, ICS-R1; Tetra, ICS-T2; and DCHA-Hexa, ICS-H1. All acids were in the form of their dicyclohexylamine salt.

To prepare stock standard solutions, dissolve 100 mg of the standard material with 100 mL of acidified methanol (0.2% phosphoric acid in methanol) and sonicate for 15 min. Prepare calibration standards in 50% acetonitrile containing 0.2% phosphoric acid in the range of 0.10–2.0 µg/mL.

Sample Preparation

A random variety of beer samples were obtained from a liquor store and included regular American beer and a light equivalent, numerous American microbrews, European samples (from Bavaria and Belgium), and an extremely bitter (high-hops) American ultra India pale ale (IPA).

For all beer samples, mix 0.50 mL of beer with 0.50 mL of acidified acetonitrile (0.2% phosphoric acid in acetonitrile), centrifuge, and analyze the clear supernatant. For the stability study, transfer beer samples to a sealed container and maintain at 4 °C in the dark, then process as needed.

Data Analysis and Processing

Analyze data using Chromeleon CDS and CoulArray software. Transfer electrochemical (EC) array data to Pirouette® software for chemometric analysis using the CoulArray version 2.0 software utility, Pattern-Recognition Setup Wizard. Tabularize UV data prior to transfer to Pirouette software.

Results and Discussion

The spectro-electro array uses both spectrophotometric and EC data.[8] While UV data provides identification and quantitation of the major sample components, EC array detection provides additional information:

- The EC array is incredibly sensitive with low-pg limits of detection (LODs) that allows it to measure compounds missed by UV detection.
- The EC array voltammetrically resolves compounds that coelute chromatographically.
- The EC array is fully gradient compatible, thereby extending the number of analytes that can be measured in a sample.
- The redox behavior of a compound reacting across the array provides qualitative information and can be used for analyte identification/authentication.

Method 1 Targeted Analysis

The analytical figures of merit for this assay are not covered here but are discussed by Ullucci et al.[8]

In summary, the LODs were typically 10–50 pg on column by EC detection and 100–500 pg by UV detection. The limits of quantification were 200–1000 pg on column by EC detection and 500–5000 pg by UV detection. Response range was over 7 orders of magnitude by EC detection and 5 orders of magnitude by UV detection. Typical coefficient of determination values were ~0.99 or better for all compounds. Average intraday retention time precision for all analytes averaged 0.55% RSD over a 10-day period with a range of 0.30–1.22%.

Multichannel EC array chromatograms for two different beer samples are presented in Figure 1 with (A) American high-hops ultra IPA and (B) regular American beer. As shown, the high-hops ultra IPA contains a great abundance of analytes compared to the regular American beer, as confirmed in Table 2. Analytes are in agreement with previously published data.[9–11]

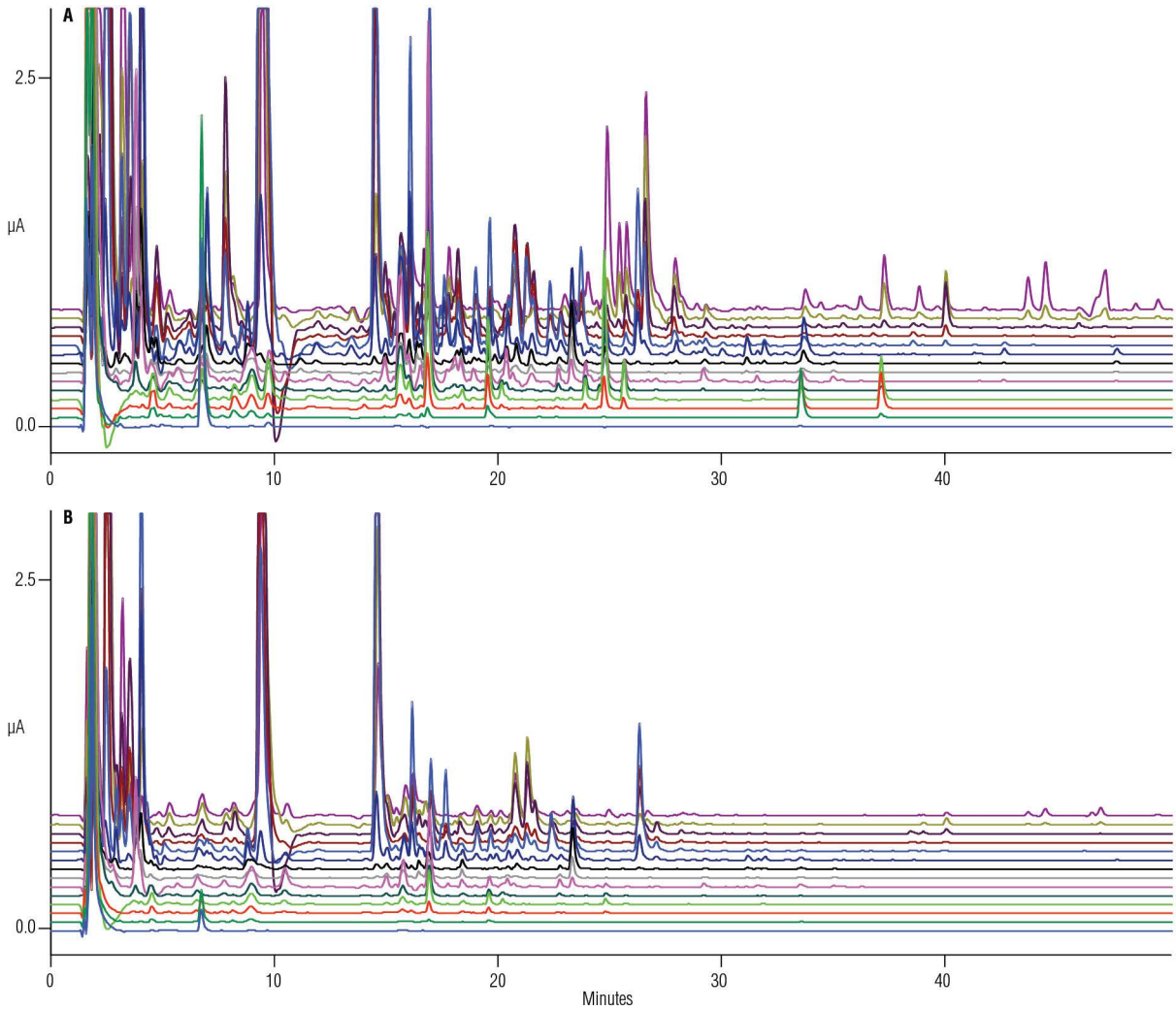

Figure 1. Polyphenol method chromatograms of (A) American high-hops ultra IPA and (B) regular American beer.

Table 2. Polyphenol data presented in mg/L for the American high-hops ultra IPA Beer 1 and the regular American Beer 3.

Compound	Beer 1	Beer 3	Compound	Beer 1	Beer 3
4-Hydroxybenzyl Alcohol	2.32	ND	Ferulic Acid	1.42	2.54
2-Hydroxybenzyl Alcohol	0.04	ND	Gentisic Acid	0.24	0.06
3,4-Dihydroxybenzoic Acid	2.10	ND	Hesperidin	0.40	0.04
4-Hydroxybenzaldehyde	0.16	ND	Isorhamnetin	0.94	ND
4-Hydroxybenzoic Acid	0.38	0.36	Isoxanthohumol	1.16	0.14
4-Hydroxyphenylacetic Acid	0.14	0.24	Kaempferol	1.04	ND
4-Hydroxycoumarin	11.0	0.46	Myricetin	0.50	0.20
Apigenin	0.20	0.02	Naringin	2.08	5.10
Caffeic Acid	0.14	0.10	p-Coumaric Acid	2.02	2.80
Carnosol	0.38	ND	Quercetin Dihydrate	1.56	ND
Catechin Hydrate	4.48	1.80	Salicylic Acid	0.18	0.04
Carvacrol	1.20	0.25	Sinapic Acid	0.68	0.58
Chlorogenic Acid	0.52	0.04	Syringaldehyde	1.39	ND
Chrysin	0.12	0.26	Syringic Acid	0.08	0.10
Epicatechin	1.76	0.40	Thymol	0.78	0.09
Epicatechin Gallate	1.54	0.26	Umbelliferone	0.34	0.64
Epigallocatechin	0.14	0.16	Vanillic Acid	0.22	0.15
Ellagic Acid	1.38	0.64	Vanillin	1.06	0.26
Ethyl Vanillin	0.20	0.02	Xanthohumol	0.26	0.04

ND = Not Determined

Metabolomic Study

A simple metabolomics experiment was conducted to evaluate whether the spectro-electro array platform could be used to differentiate between different beer types, including: matched regular and light American beers, a variety of American microbrews, a European beer from Belgium, an Irish stout, and an American high-hops ultra IPA. Metabolomic profiles containing several hundred analytes—including both known (Table 1) and unknown compounds—were measured in each sample. Principal component analysis (PCA) was then used to differentiate samples for both EC data (Figure 2, Plot A) and UV data (Figure 2, Plot B). The EC data differentiated samples the best and distinguished between the American light and regular beers, Irish stout, Belgian beer, and the American high-hops ultra IPA. UV data were less effective with no ability to distinguish between light and normal beers.

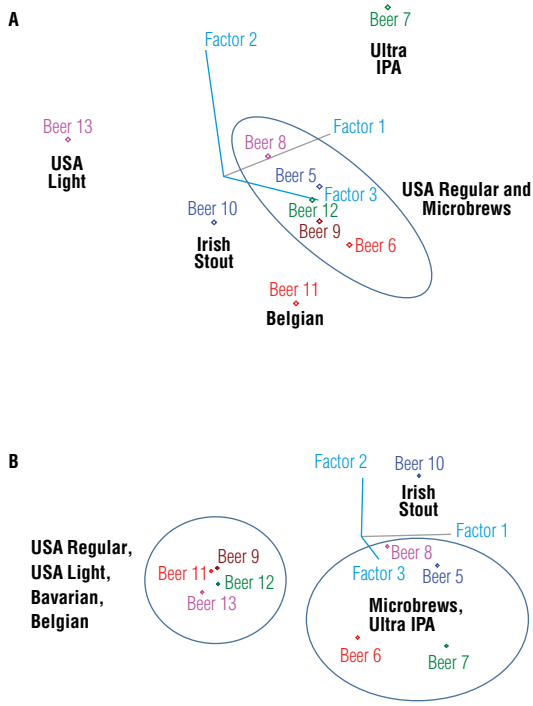

Figure 2. Principal component plots for (A) EC and (B) UV data (USA = American) blue trace at 550 mV, green trace at 850 mV, relative to Pd.

Method 2 Targeted Analysis

This method showed analytical figures of merit similar to those of Method 1. Rather than the EC array approach, a simple two-channel cell was used to detect xanthohumol and the alpha and beta bitter acids on the first lower-potential channel, as well as isoxanthohumol, prenylnaringenin, and the *cis*- and *trans*-iso bitter acids on the second higher-potential channel. Example results are shown in Figure 3, Chromatogram A (bitter acids standard mixture) and Figure 3, Chromatogram B (American high-hops ultra IPA). As expected, the American high-hops ultra IPA beers were abundant in the bitter acids and related compounds, more so than matched American regular and light beers (Table 3).

Table 3. Polyphenol data presented in mg/L for two samples of American high-hops ultra IPA (Beers 1 and 2) and the American regular (Beer 3) and light (Beer 4) beers.

Compound	Beer 1	Beer 2	Beer 3	Beer 4
Isoxanthohumol	2.10	1.3	0.38	0.28
Xanthohumol	0.52	0.48	ND	ND
cis-Iso Acid 1	0.90	0.4	ND	ND
trans-Iso-Cohumulone	10.6	7.0	ND	ND
cis-Iso Acid 2	19.1	12.2	3.80	1.60
trans-Iso-Humulone	8.40	7.6	0.20	ND
trans-Iso-Adhumulone	12.8	11.0	3.20	2.60
Cohumulone	6.80	6.4	0.03	0.02
Adhumulone/Humulone	9.20	9.0	ND	ND

ND = Not Determined

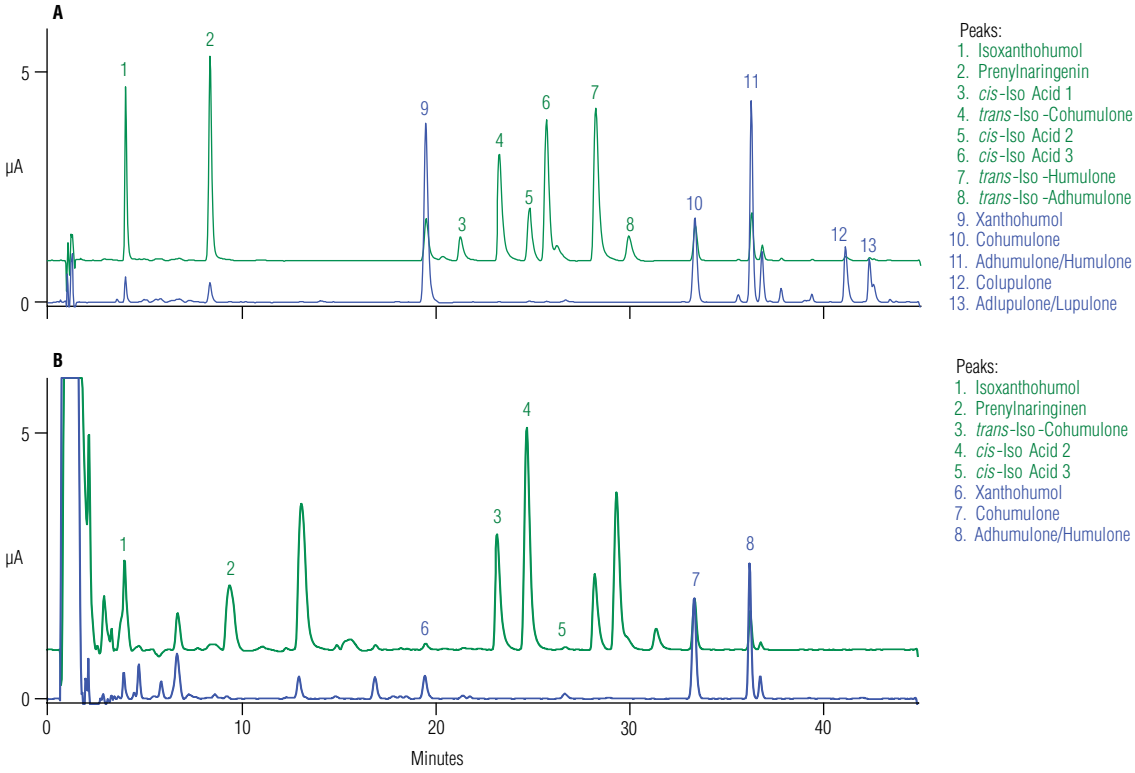

Figure 3. (A) Bitter acids standard mixture and (B) American high-hops ultra IPA sample: blue trace at 550 mV, green trace at 850 mV, relative to Pd.

Beer Stability Study

Auto-oxidation, including decomposition of iso-alpha-acids, plays an important role in the deterioration of taste and flavor qualities of beer during aging. It is believed that degradation of iso-alpha-acids is the cause of gradual decrease in beer bitterness.[12,13]

The Method 2 approach was used to evaluate the stability of a selection of the beers. Data for the stability of a variety of analytes in an American high-hops ultra IPA sample tested over a two-week period are presented in Figure 4. Marked decreases in many analytes were seen but particularly in *cis*-iso acid 2 (-61%), *trans*-iso-humulone (-67%), *trans*-iso-adhumulone (-56%), and *trans*-iso-cohumulone (-55%). These data are consistent with previous studies.[14]

Conclusion

- Method 1 can be used in a targeted approach to accurately and sensitively measure numerous phenols, phenolic acids, and polyphenols in beer and other samples. Such measurements cannot be obtained using UV detection alone.
- Metabolomic approaches using the patterns of numerous known and unknown analytes can be used to differentiate between different beers. Such approaches can be used to study fermentation, product stability, and authenticity issues that are relevant to quality control.
- Method 2 enables the sensitive targeted measurement of isoxanthohumol, xanthohumol, prenylnaringinen, the *trans*- and *cis*-iso-alpha bitter acids, and the alpha and beta bitter acids in a single run. Use of EC detection eliminates the need for solid phase extraction procedures for sample preconcentration commonly used in UV detection methods. In this study, Method 2 was used to measure beer stability over a two-week period.

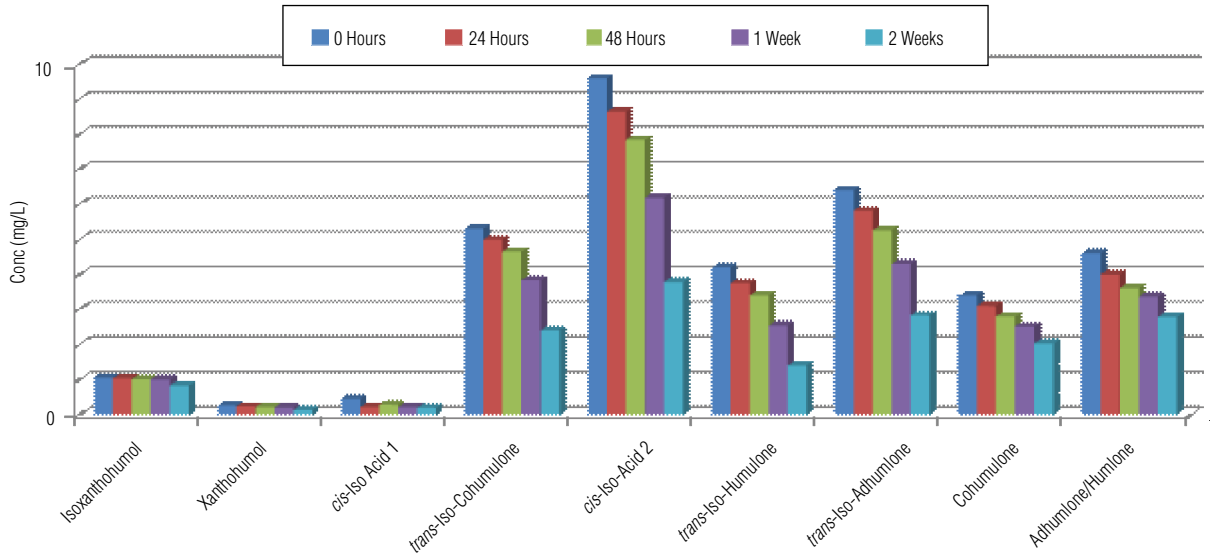

Figure 4. Stability data for American high-hops ultra IPA (Beer 1).

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