Chalconoids and Bitter Acids in Beer by HPLC with UV and Electrochemical Detection

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Key Words
Polyphenols, bitter acids, xanthohumols, electrochemical array detection, differentiation, stability

Goal

To develop gradient HPLC methods using a spectro-electro array platform to either measure specific analytes in beer samples or in a metabolomic approach to distinguish between different beer samples, as well as study beer stability.

Introduction

Beer is the most widely consumed alcoholic beverage in the world and the third most popular drink after water and tea. It is typically brewed from four basic ingredients: water, a starch source such as 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 followed.

Hops are the female flower clusters of a hop species, *Humulus lupulus*. They are used as a flavoring and stability agent in beer, for various purposes in other beverages, and as an herbal medicine. Hops contain a number of important phytochemicals including xanthohumol (a prenylated chalconoid) and alpha- and beta-acids. As part of the beer brewing process, hops or hop extracts are added during the boiling of the wort. The alpha-acids (humulone, cohumulone, and adhumulone) are slowly isomerized into the more soluble iso-acids, the main bittering substances in beer. Unfortunately, alpha-acids can react with riboflavin and light to produce compounds that give beer an off or skunky taste and smell. Beta-acids (lupulone, colupulone, and adlupulone) do not isomerize during boiling and do not impart bitterness initially. However, during fermentation and storage, beta-acids slowly create bitterness through oxidation affecting the long-term character of aged beers. Furthermore, some secondary metabolites contribute to the degradation of beer during storage with the formation of haze (e.g., catechins and their polymers, the proanthocyanidins).

In this study, two targeted assays were developed for the measurement of either polyphenols (including catechins and proanthocyanidins) or xanthohumols and bitter acids. The bitter acid method was used to study beer stability. A metabolomic approach was also developed where patterns of both known and unknown analytes were used to study differences between beer samples–an approach that is relevant to quality control.

Experimental

Methods were developed for the measurement of either polyphenols or xanthohumols and bitter acids.

Standard Preparation

For the polyphenol method, standards were prepared in ethanol, methanol, or methanol/water solutions, depending upon solubility, at 100 or 1000 µg/mL. Working standards were prepared at 0.20, 0.50, and 1.0 µg/mL in 10% methanol containing 0.2% ascorbic acid/0.02% EDTA.

For the bitter acids method, standard mixes were obtained from the American Society of Brewing Chemists: International Calibration Standards for the HPLC Analyses of Isomerized alpha-acids including: DCHA-Iso, ICS-12; DCHA-Rho, ICS-R2; Tetra, ICS-T2; and DEHA-Hexa, ICS-H1. All acids were obtained as their dicyclohexylamine salt. Stock standard solutions were prepared by dissolving 100 mg of the standard material with 100 mL of acidified methanol (0.1% phosphoric acid in methanol containing gallic acid (10 mg/L) as preservative and antioxidant) and sonicating for 15 minutes. Calibration standards were prepared in 50% acetonitrile containing 0.1% phosphoric acid and gallic acid (10 mg/L) in the range of 0.10–2.0 µg/mL.

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Sample Preparation

Various beer samples were obtained from the local liquor store, including domestic beer and a light equivalent, numerous domestic microbrews, European examples (Bavaria and Belgium), and a domestic extremely bitter (highly-hopped) beer. Beer samples were treated as follows: 0.50 mL of beer + 0.50 mL of acidified acetonitrile (0.1% phosphoric acid and gallic acid (10 mg/L) in acetonitrile) were mixed, centrifuged, and the clear supernatant analyzed. For the stability study, beer samples were transferred to a sealed container and kept at 4 °C in the dark, and processed as needed.

Liquid Chromatography Method 1: Polyphenols

Pump:	Thermo Scientific Dionex LPG-3400BM with SR-3000 solvent rack
Autosampler:	Thermo Scientific Dionex WPS-3000TBSL
UV Detector:	Thermo Scientific Dionex DAD-3000RS diode-array detector
	Channel 1: 218 nm Channel 2: 240 nm
	Channel 3: 254 nm Channel 4: 275 nm
EC Detector:	Thermo Scientific Dionex CoulArray detector with thermal organizer
EC Parameters:	16 channel array from 0 to +900 mV in +60 mV increments
Column:	Thermo Scientific Acclaim 120, C18 (3.0 × 150 mm, 3 µm particle size)
Flow Rate:	0.65 mL/min
Injection:	10 or 20 µL
Mobile Phase A:	20 mM monobasic sodium phosphate, 3% acetonitrile, 0.2% tetrahydrofuran, pH 3.35
Mobile Phase B:	20 mM monobasic sodium phosphate, 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, Concave, 45 min: 97%B/3%C

Liquid Chromatography Method 2: Bitter Acids

Column:	Acclaim™ 120, C18 (3.0 × 150 mm, 3 µm particle size)
Flow Rate:	0.65 mL/min
Injection:	20 µL
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
EC Parameters:	Thermo Scientific Dionex model 5011A dual channel coulometric electrochemical cell
E1:	+550 mV
E2:	+850 mV

Data Analysis

Data were analyzed using Thermo Scientific Dionex Chromeleon Chromatography Data System 6.8 (SR 9) and CoulArray™ software 3.1. EC-array data were transferred to Pirouette® software for chemometric analysis (Infometrix Inc., Bothell, WA) using a CoulArray version 2.0 software utility (Pattern Recognition Setup Wizard). UV data were tabularized prior to transfer to Pirouette.

Results and Discussion

The spectro-electro array makes use of both spectrophotometric and electrochemical data (EC).[1] While UV data provides identification and quantitation of the major components in a sample, EC array detection provides additional information. First, the EC array is incredibly sensitive with low picogram limits of detection (LOD), and it is capable of measuring compounds missed by UV. Second, it voltammatrically resolves compounds that co-elute chromatographically. Third, the EC array is fully gradient compatible, thereby extending the number of analytes that can be measured in a sample. Fourth, the redox behavior of a compound reacting across the array provides qualitative information and can be used for analyte identification/authentication.

Polyphenol Method – Targeted Analysis

The analytical figures of merit for this assay were described previously.[1] Briefly, the limits of detection were typically 10–50 pg on column by ECD and 100–500 pg by UV. The limits of quantification were 200–1000 pg on column by ECD and 500–5000 pg by UV. The response range was over seven orders of magnitude by ECD and five by UV. Typical R^2 values were ~0.99 or better for all compounds. Average intra-day retention time precision for all analytes averaged 0.55% RSD over a 10 day period, with a range of 0.30%–1.22%. Examples of multi-channel EC array chromatograms for two different beer samples are presented in Figure 1A for high-hops beer and 1B for regular domestic beer. The high-hops beer contains a great abundance of analytes, as confirmed in Table 1. Analyte levels are in agreement with previously published data.

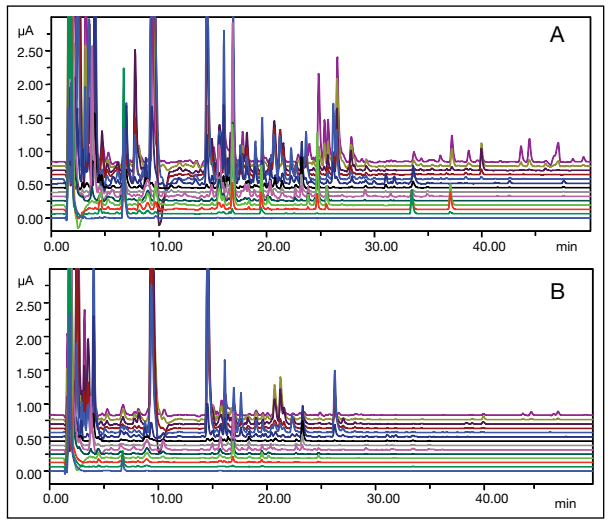

Figure 1. Polyphenol method chromatograms of (A) high-hops beer and B) regular domestic beer

Table 1. Polyphenol data presented in mg/L. Beer 1 = high-hops; Beer 3 = regular

Compound	Beer 1	Beer 3
Hydroxybenzyl alcohol	2.32	ND
2-Hydroxybenzyl alcohol	0.04	ND
3,4-Dihydroxybenzoic acid	2.10	ND
4-Hydroxybenzaldehyde	0.16	ND
4-Hydroxybenzoic acid	0.38	0.36
4-Hydroxyphenyl acetic acid	0.14	0.24
4-Hydroxycoumarin	11	0.46
Apigenin	0.2	0.02
Caffeic acid	0.14	0.1
Carnosol	0.38	ND
Catechin	4.48	1.8
Carvacrol	1.2	0.25
Chlorogenic acid	0.52	0.04
Chrysin	0.12	0.26
Epicatechin	1.76	0.40
Epicatechingallate	1.54	0.26
Epigallocatechin	0.14	0.16
Ellagic acid	1.38	0.64
Ethyl vanillin	0.2	0.02
Ferulic acid	1.42	2.54
Gentisic acid	0.24	0.06
Hesperidin	0.40	0.04
Isorhamnetin	0.94	ND
Isoxanthohumol	1.16	0.14
Kaempferol	1.04	ND
Myricetin	0.5	0.2
Naringin	2.08	5.1
p-Coumaric acid	2.02	2.8
Quercetin	1.56	ND
Salicylic acid	0.18	0.04
Sinapic acid	0.68	0.58
Syringaldehyde	1.39	ND
Syringic acid	0.08	0.1
Thymol	0.78	0.09
Umbelliferone	0.34	0.64
Vanillic acid	0.22	0.15
Vanillin	1.06	0.26
Xanthohumol	0.26	0.04

Polyphenol Method – 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 domestic normal and light beers, a variety of domestic microbrews, two European beers (from Bavaria and Belgium), an Irish stout, and a domestic Ultra (extra hopped) 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 2A) and UV data (Figure 2B). The EC data gave the best differentiation between samples and could distinguish between the light and normal beer, Irish stout, Belgian beer, and the extra hopped IPA. UV data were less selective with no ability to distinguish between light and normal beers.

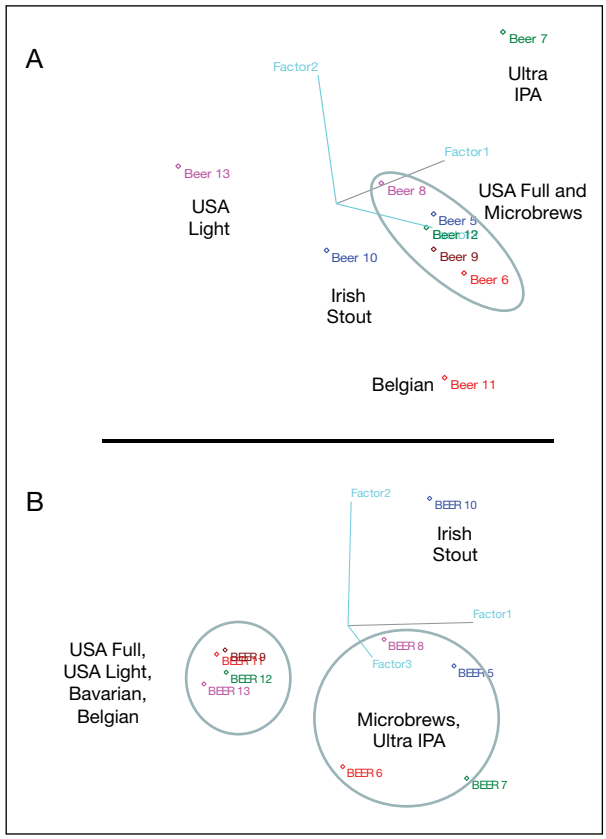

Figure 2. Principal component plots for (A) ECD and (B) UV Data

Bitter Acid Method – Targeted Analysis

The bitter acid method showed similar figures of merit to the polyphenol method above. All calibration curves showed good linear regression ($R^2 > 0.996$). Peak area RSDs over a twenty-hour run were as follows: isoxanthohumol and xanthohumol, 1.2%; alpha and beta bitter acids, 2.5%; and *trans*- and *cis*-iso-alpha acids, 2.4%. Limits of detection for most analytes were in the picogram (on-column) range. Rather than using an 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, and isoxanthohumol, and the *cis*- and *trans*-iso bitter acids on the second higher potential channel. Example chromatograms are shown in Figure 3A (standards) and Figure 3B (extra hopped IPA). As expected, the Ultra IPA beers were abundant in the bitter acids and related compounds, more so than matched normal and light beers (Table 2).

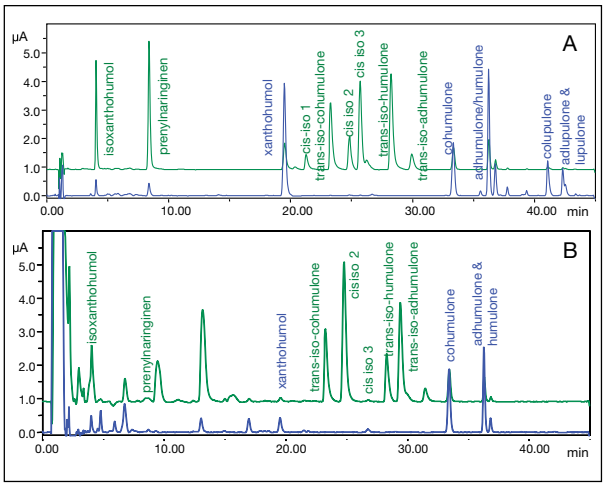

Figure 3. Chromatograms of (A) bitter acids standard mixture; (B) Ultra IPA beer sample. Blue trace at 550 mV; green trace at 850 mV. The *cis*-isomers were *cis*-rho-isocohumulone, *cis*-rho-isohumulone, and *cis*-rho-isoadhumulone. Absolute identification was not possible due to the lack of single standards.

Table 2. Hops bitter acids data presented in mg/L

Compound	Beer 1 Ultra IPA	Beer 2 Ultra IPA	Beer 3 Regular	Beer 4 Light Beer
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	3.8	1.6
trans-iso-humulone	8.4	7.6	0.20	ND
trans-iso-adhumulone	12.8	11.0	3.2	2.6
Cohumulone	6.8	6.4	0.03	0.02
Adhumulone/Humulone	9.2	9.0	ND	ND

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 the gradual decrease of beer bitterness. The current bitter acids method was used to evaluate the stability of a selection of the beers. Data for the stability of a variety of analytes in an Ultra IPA sample tested over a two week period are presented in Figure 4. Marked decreases in many analytes were seen but particularly for *cis*-iso 2 (61%), *trans*-iso-humulone (-67%), *trans*-iso-adhumulone (-56%), and *trans*-iso-cohumulone (-55%). These data are consistent with previous studies.[2]

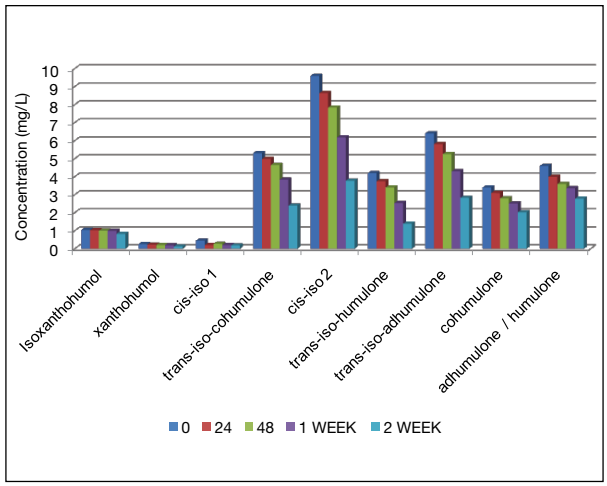

Figure 4. Stability data for Ultra IPA beer (Beer 1)

Conclusion

- The polyphenol method can be used in a targeted approach to accurately and sensitively measure numerous phenols, phenolic acids, and polyphenols in beer and other samples not possible by methods employing UV detection alone.
- Metabolomic approaches using the patterns of numerous known and unknown analytes can be used to differentiate between different samples. Such an approach can be used to study fermentation, product stability, and authenticity.
- The bitter acid method enabled 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 eliminated the need for solid phase extraction procedures for sample pre-concentration commonly used in UV detection methods. This approach was used to measure beer stability over a two week period.

References

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