Customer application note | 004238

Food and beverage

Efficient sustainable online SPE-UHPLC workflow for the determination of bitter acids in hops

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Keywords

Humulones, lupulones, online SPE, Hypersil GOLD column, Vanquish Online SPE UHPLC system, SureStart vials

Application benefits

- The Thermo Scientific™ Vanquish™ Online SPE UHPLC System eliminates the need for an additional solid phase extraction (SPE) kit, simplifying the workflow, reducing costs, and enhancing automation.
- The use of a Thermo Scientific™ Hypersil GOLD™ C18 Selectivity Column provides separation of α- and β-acids and their derivatives, ensuring more accurate and distinct quantification of these compounds in hops.
- The use of online SPE with a Thermo Scientific™ Hypersil GOLD™ C8 Column and less toxic extraction solvents minimizes solvent consumption and reduces plastic disposal from single-use cartridges, enhancing environmental sustainability.

Goal

Determination of α- and β- bitter acids in hops using the Vanquish Online SPE UHPLC system.

Introduction

Hops (*Humulus lupulus L.*) are primarily recognized as a flavor ingredient for beer, with α- and β- bitter acids being the key substances that add flavor and bitterness. The α - and β - acids, also known as humulones and lupulones, include several isomers with different side chains (Figure 1 and Table 1). The three major α-acids in hops are humulone, adhumulone, and cohumulone, while the three major β -acids in hops are lupulone, adlupulone, and colupulone. The levels of bitter acids are the primary information provided to brewers, enabling them to prepare recipes that meet the desired International Bitterness Units (IBU) and aroma. Since hops can vary in their bitter acid content due to conditions such as climate and soil composition, it is crucial to identify the bitter acids profile to achieve the desired flavor.[1]

Hops present a complex matrix that necessitates meticulous sample pretreatment and preparation to effectively isolate bitter acids and eliminate interfering matrix components. Extraction with ethanol has replaced the use of more toxic solvents such as toluene and diethyl ether, making it a greener approach. To remove prenylated flavonoids that interfere with the separation of α- and β-acids, as well as nonpolar compounds, sample pretreatment steps like offline SPE are often employed. However, manual SPE is very time-consuming, with the complete analysis of a single sample taking at least an hour from sampling to result, and often longer.[2,3] Moreover, these multi-step methods generate considerable volumes of chemical waste. The Vanquish Online SPE UHPLC system streamlines the sample preparation process by removing additional sample preparation steps (Figure 2). This approach provides time savings and reduces solvent consumption, thereby offering a more efficient and environmentally sustainable solution.

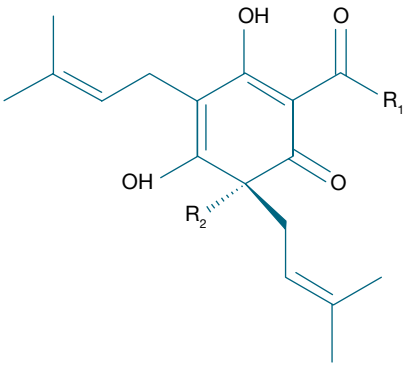

Figure 1. General chemical structure of hop bitter acids.

Table 1. R_1 and R_2 substituents of hop bitter acids.

	R_1	R_2
Adhumulone	$CH(CH_3)CH_2CH_3$	OH
Cohumulone	$CH(CH_3)_2$	OH
Humulone	$CH_2CH(CH_3)_2$	OH
Adlupulone	$CH(CH_3)CH_2CH_3$	$CH_2CH(CH_3)_2$
Colupulone	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$
Lupulone	$CH_2CH(CH_3)_2$	$CH_2CH(CH_3)_2$

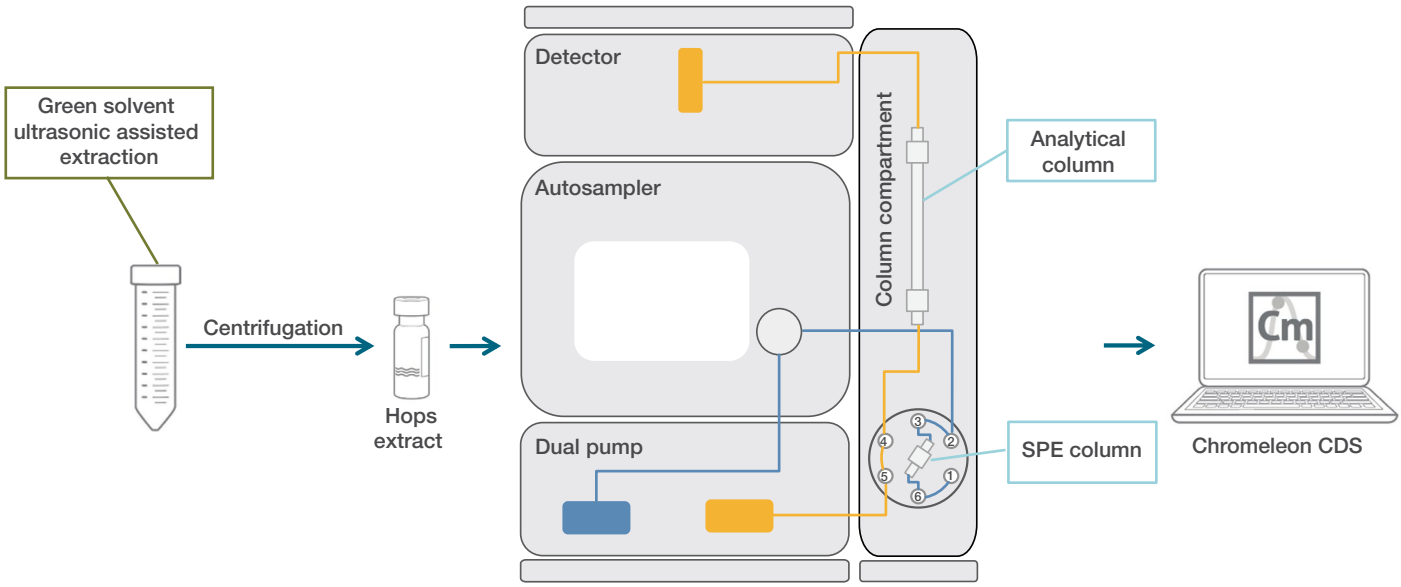

Figure 2. Schematic workflow for the determination of bitter acids in hops. The process involves extraction with a green solvent, followed by instrumental analysis using the Vanquish Online SPE UHPLC system. Data acquisition and processing are performed using the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS).

Experimental

Chemicals

- Fisher Scientific™ Water with 0.1% Formic Acid (v/v), Optima™ LC/MS grade (Cat. No. LS118)
- Fisher Scientific™ Acetonitrile with 0.1% Formic Acid (v/v), Optima™ LC/MS grade (Cat. No. LS120)
- Fisher Chemical™ Acetonitrile, UHPLC grade, for gradient analysis (Cat. No. A/0650/PB15)
- Fisher Chemical™ Methanol, for HPLC (Cat. No. M/4056/17)
- Deionized water, 18.2 MΩ·cm, Thermo Scientific™, Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System (Cat. No. 50136149)

Sample handling

- Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipette: 10–100 µL (Cat. No. 4641070N)
- Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipette: 1–10 µL (Cat. No. 4641030N)
- Fisherbrand™ Mini Vortexer (Cat. No. 4-955-152)
- Thermo Scientific™ SureSTART™ 2 mL Amber Glass Short Thread Screw Top Vials, 100/pack, Level 2 (Cat. No. 6ASV9-2P)
- Thermo Scientific™ SureSTART™ Glass Inserts for 2 mL Vials, Level 3 High Performance Applications (Cat. No. 6PME03C1SPG)
- Thermo Scientific™ SureSTART™ Blue Polypropylene 9 mm AVCS™ Screw Caps with Soft Blue Silicone/Clear PTFE Septa, 100/pack, Level 3 (Cat. No. 6PSC9ST101)

Instrumentation

Thermo Scientific™ Vanquish™ Flex Online SPE UHPLC System consisting of:

- Thermo Scientific™ System Base Vanquish™ Duo (Cat. No. VF-S02-A-02)
- Thermo Scientific™ Vanquish™ Dual Pump F (Cat. No. VF-P32-A-01)
- Thermo Scientific™ Vanquish™ Split Sampler FT (Cat. No. VF-A10-A-03)
- Thermo Scientific™ Vanquish™ Column Compartment H (Cat. No. VH-C10-A-03)
- Thermo Scientific™ Vanquish™ Variable Wavelength Detector F (Cat. No. VF-D40-A)
- Semi-micro flow cell, 2.5 µL, 7 mm, SST (Cat. No. 6077.0360)

Sample preparation

Hop extracts were prepared by grinding 100 mg of hops with 5 mL of 80% ethanol, followed by ultrasonic treatment for 45 minutes at room temperature. The samples were then centrifuged for 5 minutes at 4,700 *g*. The supernatant was then transferred to a vial and centrifuged for 5 minutes at 11,180 *g* to avoid the filtration step. Samples were stored at -20°C until analysis.

Standard preparation

International Calibration Extract 4 (ICE-4, Labor Veritas AG) was used for the preparation of the calibration curve. The composition of ICE-4 has been determined and validated by the International Hop Standards Committee (IHSC), containing 10.98% cohumulone, 13.02% colupulone, 31.60% n+adhumulone, and 13.52% n+adlupulone, with a total of 42.58% α-acids and 26.54% β-acids. For the calibration curve, a stock solution was prepared by dissolving 0.1139 g of ICE-4 in 100 mL of acetonitrile, resulting in a concentration of 1.139 mg/mL. This stock solution was then serially diluted to obtain concentrations of 75%, 50%, and 25% of the original concentration, creating a four-point calibration curve.

Chromatographic conditions

Table 2A. Instrument conditions for online SPE.

Online SPE conditions			
Column	Thermo Scientific™ Hypersil GOLD™ C8, 5 µm, 20 × 2.1 mm (Cat. No. 25205-022130)		
Solvent A	0.1% formic acid in water		
Solvent B	0.1% formic acid in acetonitrile		
Gradient	Time (min)	Flow rate (mL/min)	%B
	0	2	25
	2.5	2	25
	2.5	2	100
	5	2	100
	6	0.2	25
	13	0.2	25
	14	2	25
	15	2	25

Table 2B. Instrument conditions for analytical separation.

Analytical separation conditions	
Column	Thermo Scientific™ Hypersil GOLD™ C18 Selectivity, 1.9 µm, 100 × 2.1 mm (Cat. No. 25002-102130)
Solvent A	0.1% formic acid in water
Solvent B	0.1% formic acid in acetonitrile
Gradient	Time (min) %B 0 50 4 50 6 60 7 60 8 70 11 80 15 60
Flow rate	0.65 mL/min
Column temperature	35°C (still air)
Autosampler temperature	5°C
Needle wash solution	10% MeOH in water (v/v)
Needle wash mode	After draw
Injection volume	5 µL standards 1 µL hops samples
Detector settings	270 nm, data collection rate: 20 Hz, response time: 0.2 s

Chromatography Data System

The Thermo Scientific™ Chromeleon™ 7.3.2 Chromatography Data System (CDS) was used for data acquisition and analysis.

Results and discussion

Online SPE in bitter acids analysis

The online SPE approach offers several significant benefits over the traditional offline SPE approach. Notably, the total solvent consumption is less than 8 mL of solvent A and B, making the process more efficient, cost-effective, and environmentally friendly. The SPE process is completed in just 2 minutes, compared to the usual 30 minutes to 1 hour required for offline SPE,[3] significantly speeding up the workflow. The automated nature of the online SPE method minimizes manual intervention, reducing the risk of human error and enhancing reproducibility. Additionally, the use of a Vanquish dual pump system with separate flow paths for SPE and the analytical column allows simultaneous washing and re-equilibration of the SPE column while separation occurs on the analytical column, optimizing the use of time and resources. The use of Thermo Scientific™ Viper™ TQ UHPLC fittings further simplifies the setup, ensuring leak-free connections and virtually zero-dead volume. The setup streamlines the workflow, enhances automation, and improves the overall efficiency of the analytical process.

Bitter acids analysis in hops

The ICE-4 standard contains a precise composition of bitter acids, including cohumulone, colupulone, n+adhumulone, and n+adlupulone. While ICE-4 is essential for accurate quantification of these compounds in hops analysis, the chromatogram provided in the standard report does not fully resolve some critical pairs of compounds (humulone and adhumulone, lupulone and adlupulone) during the runtime of 30 minutes, necessitating quantification based on the sum of these co-eluting compounds. The optimized method using the Hypersil GOLD C18 selectivity column allowed achieving a runtime of 15 minutes, with successful separation of the critical pairs of peaks—humulone and adhumulone (resolution 2.29), lupulone and adlupulone (resolution 1.06) (Figure 3).

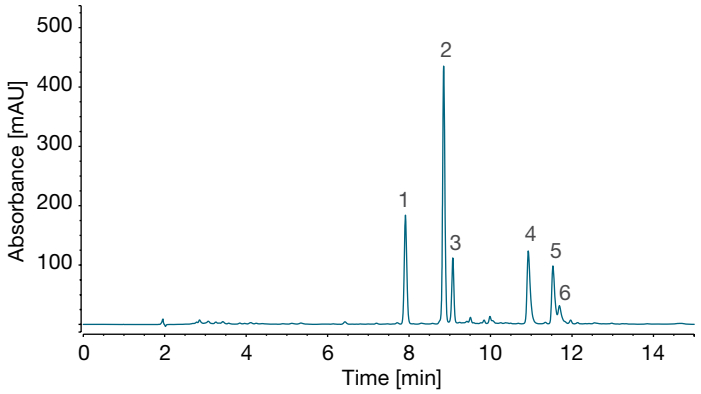

Figure 3. Chromatogram of hop bitter acids in ICE-4 standard (1-cohumulone, 2-humulone, 3-adhumulone, 4-colupulone, 5-lupulone, 6-adlupulone).

Calibration curves were obtained from serial dilutions of the ICE-4 standard, as described in the standard preparation section. Even though Hypersil GOLD C18 columns can separate humulone and adhumulone, as well as lupulone and adlupulone, quantification is performed on the combined sum of these compounds, in accordance with the reported values for the ICE-4. Table 3 presents the relative standard deviations (RSD) for peak area of three replicate injections and the coefficient of determination (R^2) values for the linear calibration curves, all above 0.995, thereby demonstrating high precision and linearity.

Four hop species—Sultana, Barbe Rouge, Azacca, and Sabro—were analyzed for their bitter acids profiles. The α-acids were predominant for all species, with concentrations ranging from 5.78% in Barbe Rouge to 12.5% in Sultana hops. β-acids were detected at lower levels ranging from 3.44% in Barbe Rouge to 4.75% in Sabro hops. Each hop species exhibited distinct profiles in terms of α-acid and β-acid content and their respective ratios (Figure 4).

Table 3. RSD of peak area and R^2 values (n=3) of ICE-4 calibration standard.

	Cohumulone		Humulone + adhumulone		Colupulone		Lupulone + adlupulone	
	RSD	R^2	RSD	R^2	RSD	R^2	RSD	R^2
25% ICE-4	0.60%	0.995	0.13%	0.995	0.13%	0.996	0.31%	0.996
50% ICE-4	0.51%		0.06%		0.06%		0.13%	
75% ICE-4	0.44%		0.04%		0.04%		0.14%	
100% ICE-4	0.07%		0.21%		0.08%		0.27%	

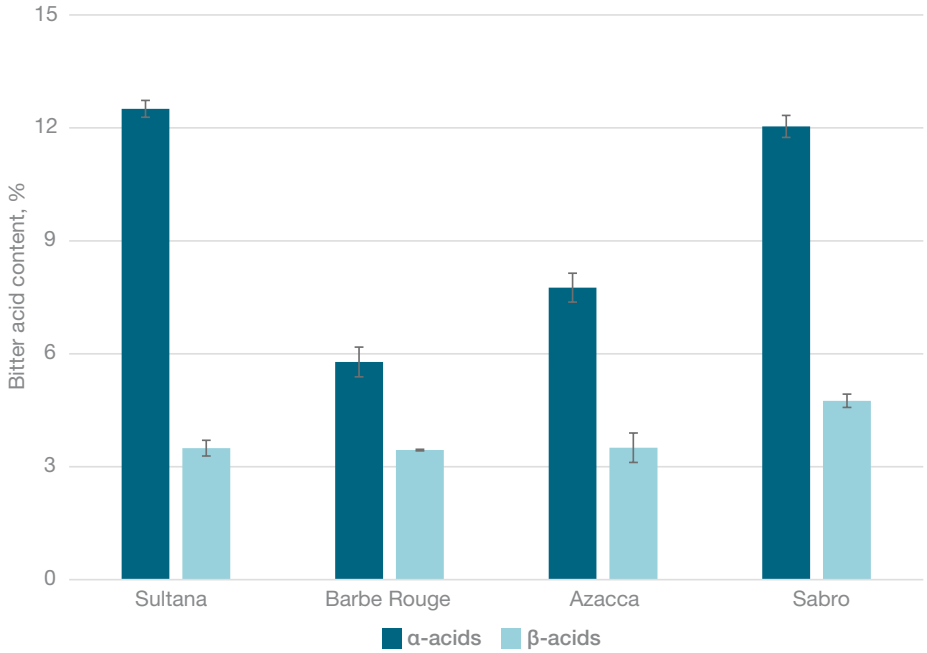

Figure 4. Bitter acid profiles expressed as percent by mass in four different hop species, errors bars display SD (n=6).

Each extract was measured six times, and the standard deviation (SD) was below 0.50% in hop species. This high level of reproducibility and precision in the measurements ensures reliable characterization of the hop samples, confirming the consistency and accuracy of the analytical method used.

Although chromatographic resolution enables quantification of all six individual bitter acids, the American Society of Brewing Chemists (ASBC) Methods of Analysis (Hops-6) recommends reporting humulone + adhumulone and lupulone + adlupulone as summed pairs, an industry-standard practice considered sufficient for comprehensive characterization of hop bitter compounds.

The profiles of bitter acids varied across different hops species (Figure 5). Humulone + adhumulone were the main components across all species, reaching 9.15% ± 0.11% in Sultana, 9.30% ± 0.15% in Sabro, 4.39% ± 0.20% in Barbe Rouge, and 4.67% ± 0.19% in Azacca. Cohumulone levels varied by species, with the highest in Sultana at 3.35% ± 0.11%, followed by Azacca at 3.08% ± 0.19%, and Sabro at 2.73% ± 0.14%, and the lowest in Barbe Rouge at 1.39% ± 0.20%. Lupulone + adlupulone levels were comparable among Sultana (1.79% ± 0.11%), Barbe Rouge (1.81% ± 0.10%), and Sabro (2.39% ± 0.09%), but notably lower in Azacca (1.29% ± 0.20%). No significant differences in colupulone content were observed across hops varieties, with values ranging from 2.21% ± 0.19% (Azacca) and 2.36% ± 0.09% (Sabro) to 1.71% ± 0.10% (Sultana) and 1.63% ± 0.10% (Barbe Rouge). Precision was high across all measurements (RSD < 0.50%), supporting the robustness of the profiling and confirming distinct bitter-acid fingerprints for each hop variety.

IBU measure the bitterness of beer, with higher values indicating greater bitterness. Different hop varieties contribute to this bitterness in varying degrees, making them suitable for specific types of beer. Based on their bitter-acid profiles, Sultana and Sabro hops are well-suited for beers with higher IBU due to their strong α-acid compositions, which support efficient bittering. Conversely, Barbe Rouge hops are better suited for beers that seek smoother, gentler bitterness, while Azacca hops are less suitable as primary bittering hops and are more appropriate for beers requiring softer bitterness.

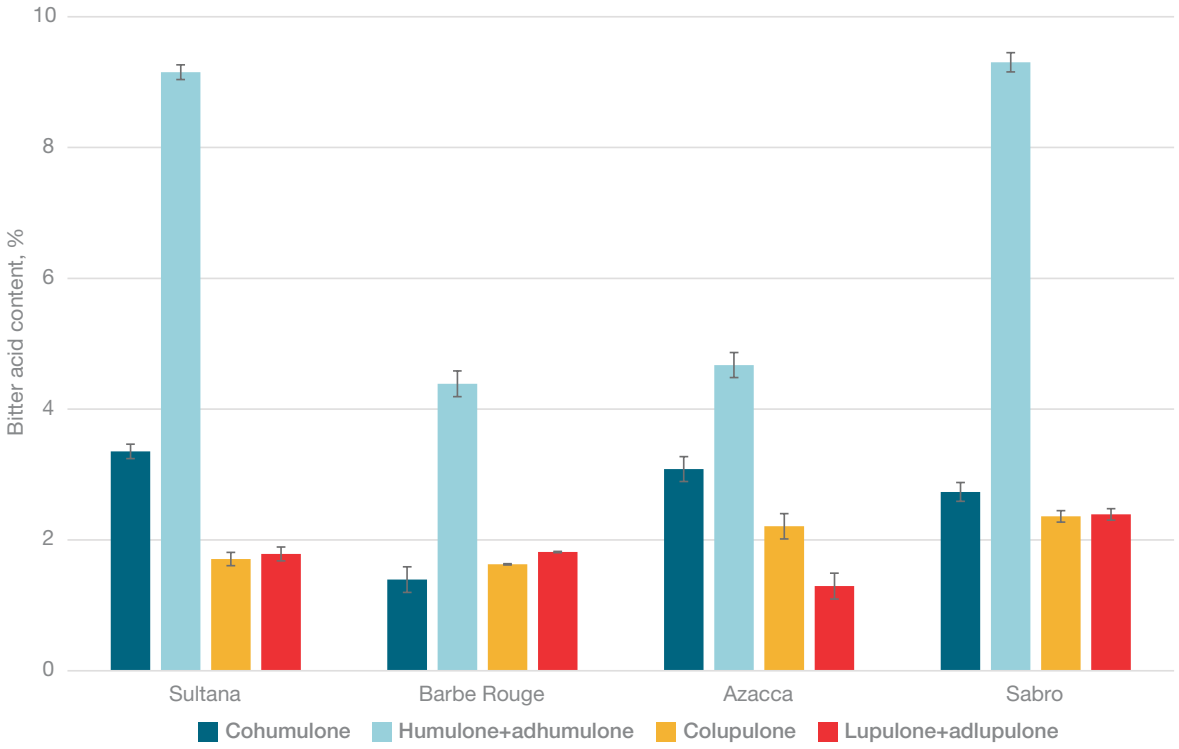

Figure 5. Bitter acid content expressed as percent by mass in four different hop species, error bars represent SD (n=6).

Conclusion

The analysis of four hop species using the Vanquish Online SPE UHPLC system demonstrated efficient and environmentally sustainable isolation of bitter acids. The method provided accurate and reproducible quantification of α- and β-acids, essential for the brewing process.

- The Vanquish Online SPE UHPLC system and Hypersil GOLD C8 online SPE column significantly reduce sample preparation time and solvent consumption, streamlining the workflow, enhancing automation, and improving the overall efficiency of the analytical process.
- The use of online SPE and green extraction solvents supports a green chemistry approach and promotes environmental sustainability by minimizing solvent consumption and reducing plastic waste from single-use cartridges.
- Calibration curves showed good linearity ($R^2 > 0.995$) and high reproducibility across all concentration levels.
- In all hop species, α-acids were consistently higher than β-acids. While β-acid levels did not show significant differences across species, variation in α-acid content across hop varieties largely determines their suitability for different bitterness intensities in brewing.

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