Measurement of Terpenes in Plant Extracts via LC-MS/MS

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S C I E N T I F I C

OVERVIEW

Purpose: To demonstrate the measurement of ~20 relevant terpenes in hops by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Methods: Standard mixtures containing 22 terpenes were separated with a 14 minute gradient liquid chromatography (LC) method by using a 150 mm length solid-core LC column on the Thermo Scientific™ Vanquish™ Flex Binary UHPLC system. Tandem mass spectrometry (MS/MS) detection of terpenes was accomplished via selected reaction monitoring (SRM) on the Thermo Scientific™ TSQ Quantis™ Triple Quadrupole Mass Spectrometer.

Results: Optimization of the LC gradient conditions allowed for adequate separation of most of the terpenes examined. Measurement of 21 of 22 terpenes exhibited limits of detection (LODs) between 2-25 ppb. Methanol extracts of 4 hops samples showed the expected terpenes (e.g., alpha-Humulene, Linalool) in the moderate parts per million (ppm) concentration range. Beta-Myrcene was observed at lower than expected values, possibly due to losses from the age and storage conditions of the hops samples prior to extraction.

INTRODUCTION

Analytical characterization of the terpene content in plants is important for understanding the fragrance and flavoring of that plant. Owing to their volatility, terpenes can have an effect on taste, smell and even emotions when plants are consumed. Typically, gas chromatography mass spectrometry (GC-MS) is used to characterize monoterpenes, but in cases where both volatile and non-volatile compounds need to be analyzed, for example sesquiterpenoids, LC-MS/MS may be a viable option. Here, we show the performance of LC-MS/MS for measuring approximately 20 terpenes from plant extracts using hops as a model.

MATERIALS AND METHODS

Sample Preparation

Neat terpene standards were purchased from Millipore Sigma (St. Louis, MO). Stock solutions were prepared at 1 mg/mL in methanol and diluted for use in LC-MS/MS method development. Medical Cannabis Terpenes Standards #1 & #2 were purchased from Restek (Bellefonte, PA) and used as received for making calibration solutions by serial dilution in methanol. Four types of hops in pellet form were manually crushed with mortar and pestle to a fine powder. 1 g (+/- 0.02 g) of each hops sample was added into 50 mL centrifuge tubes along with 10 mL methanol. Samples were extracted by shaking for 40 minutes. Following centrifugation and supernatant filtration, samples were analyzed by LC-MS/MS.

LC-MS/MS

The Vanquish Flex Binary UHPLC system was used for LC separation of terpenes. A 2 uL aliquot was injected onto a 2.1 x 150 mm, 2.6 um Thermo Scientific™ Accucore™ Polar Premium column, which was thermostated at 50 ºC. Gradient separation of terpenes was used at a flow rate of 0.3 mL/min. over 14 minutes. Mobile phases were (A) H₂O and (B) Methanol (no modifiers).

Detection of LC separated terpenes was accomplished on the TSQ Quantis triple quadrupole mass spectrometer. Atmospheric Pressure Chemical Ionization (APCI) in positive ion mode was used for ionization of terpenes. Timed-SRM was employed to detect all target terpenes using a cycle time of 0.45 s, allowing at least 10 acquisition points under a peak.

Data Analysis

All data were collected and processed with Thermo Scientific™ Chromeleon™ 7.2 Chromatography Data System (CDS) software.

RESULTS

LC Method Development – Column Selection

To obtain the necessary chromatographic separation of numerous terpene isomers, which yield the same fragments by MS/MS, a 150 mm length column was employed to most easily increase chromatographic resolution and still maintain reasonable LC run times. Columns based on solid-core packing materials (e.g., Thermo Scientific™ Accucore™ columns) were employed, as they provide excellent chromatographic speed and resolution while reducing backpressure versus sub-2 um, fully porous packed columns.

Figure 1. LC separation of monoterpene isomers Camphene [black trace], beta-Pinene [blue trace] and alpha-Terpinene [green trace] using (A) Thermo Scientific™ Accucore C30 LC (B) Accucore Polar Premium LC and (C) Thermo Scientific™ Accucore™ C18 LC columns.
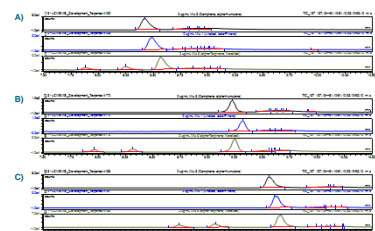

Figure 1 shows the evaluation of three Accucore LC columns for separation of three monoterpenes: Camphene, beta-Pinene and alpha-Terpinene. For two reasons, the Accucore Polar Premium, a polar embedded C18 column, was chosen for the final LC-MS/MS method. One, better observed separation between certain isomeric terpenes (e.g., beta-Pinene and Camphene/alpha-Terpinene). Two, clear separation between the sesquiterpenoids and monoterpenes. As seen in the green traces, the two earliest eluting LC peaks are from sesquiterpenoids cis- and trans-Nerolidol. Although they have higher molecular weights and different SRM transitions, Nerolidol fragments in the APCI source to produce the m/z 137 ion. These source-induced fragment ions then yield the same product ions after Q1 isolation and collision-induced dissociation as the monoterpenes. For the Accucore C30 and C18 columns, trans-Nerolidol interfered with the detection of Ocimene and beta-Myrcene, respectively (data not shown).

LC Method Development – Optimization of Terpene Separation

Different mobile phase modifiers (e.g., ammonium acetate) and organic solvents (e.g., acetonitrile) were employed to improve separation efficiency between the monoterpenes. However, no mobile phase modifications significantly improved chromatographic resolution versus unbuffered water and methanol.

Figure 2. LC separation of monoterpene isomers Limonene, delta-3-Carene and alpha-Pinene as a function of column temperature [15 C = black trace; 30 C = blue trace; 50 C = green trace].
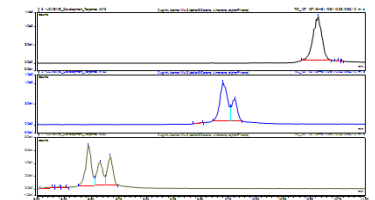
LC Method Development – Optimization of Terpene Separation (cont.)

LC column temperature was evaluated to improve separation between terpene isomers, as described previously with cis/trans isomers of Vitamin K1.[1] Unexpectedly, monoterpene chromatographic resolution improved with *increasing* column temperature contrary to that reported for separation of Vitamin K1 isomers (see Figure 2). Monoterpenoids isomers did follow the general trend shown in reference 1. Linalool and Geraniol were baseline resolved at a column temperature of 15 C yet coelute at 50 C (data not shown). Interestingly, isomers Eucalyptol and Isopulegol coelute at 30 C, but are baseline separated at 15 C and at 50 C, transposing their retention time order between these column temperatures.

Despite extensive LC method development efforts, certain monoterpenes could not be separated under any conditions attempted (e.g., gamma-Terpinene and Terpinolene).

Table 1. Terpenes measured by LC-MS/MS. Legend (Class): AH = aromatic hydrocarbon; MO = monoterpenoid; SO = sesquiterpenoid; M = monoterpene; S = sesquiterpene

Legend (LOD): est. = estimated; nd = not determined

| Compound	Class	Ret. Time (min)	SRM Transitions	LOD (ppb) LC-MS/MS	Measured Conc. Extracts (ppm)
p-Cymene	AH	2.60	135 > 43	250 (est)	ND, ND, ND, ND
Isopulegol	MO	3.27	137 > 81, 95	5	ND, ND, ND, 0.37
Eucalyptol	MO	3.47	137 > 81, 95	2.5 (est)	2.5, 0.41, 0.58, 1.3
Geraniol	MO	3.51	137 > 81, 95	nd	
Linalool	MO	3.52	137 > 81, 95	2 (est)	21, 3.3, 3.7, 9.0
Caryophyllene Oxide	SO	6.10	203 > 147, 105	2.5 (est)	474, 92.6, 73.4, 67.3
cis-Nerolidol	SO	6.68	205 > 149, 93	10	ND, ND, ND, ND
Guaiol	SO	6.87	205 > 149, 93	2 (est)	22.7, 1.9, 2.8, ND
trans-Nerolidol	SO	6.96	205 > 149, 93	5	ND, ND, ND, ND
Alpha-Bisabolol	SO	7.05	205 > 149, 93	5	111, 16.7, 14.2, 72.1
Beta-Myrcene	M	7.47	137 > 81, 95	25	5.7, 5.3, 6.6, 6.7
Ocimene	M	7.60	137 > 95, 81	25	ND, ND, ND, ND
Alpha-Terpinene	M	7.81	137 > 81, 95	25	ND, ND, ND, ND
Camphene	M	7.83	137 > 81, 95	nd	ND, ND, ND, ND
Beta-Pinene	M	7.90	137 > 81, 95	25	0.67, 0.67, 0.76, 2.4
Gamma-Terpinene	M	8.10	137 > 81, 95	10	
Terpinolene	M	8.12	137 > 81, 95	nd	1.3, ND, ND, ND
Limonene	M	8.16	137 > 81, 95	25	ND, ND, ND, 7.6
Delta-3-Carene	M	8.28	137 > 81, 95	25	60.4, 0.88, 0.67, 2.0
Alpha-Pinene	M	8.37	137 > 81, 95	25	ND, 1.3, 1.5, 1.7
Alpha-Humulene	S	11.81	205 > 149, 93	5	572, 16.9, 17.9, 148
Beta-Caryophyllene	S	12.07	205 > 149, 93	10	148, 4.0, 4.6, 23.6

Quantitation of Terpenes – Standards

Table 1 presents figures of merit on measured terpenes by LC-MS/MS. Compounds highlighted in color represent terpenes that were not adequately resolved chromatographically using the final LC method. Except for p-Cymene, two SRM transitions were used with the first as the quantifier and the second as the qualifier.

Figures 3A and 3B (top) show example chromatograms near LODs for beta-Myrcene and alpha-Humulene, respectively. LODs by LC-MS/MS (Table 1) were best for oxygenated terpenes (e.g., monoterpenoids, sesquiterpenoids), ranging from 2-10 ppb. LODs for monoterpenes were generally 25 ppb, reflecting the poorer ionization efficiency of hydrocarbons without a functional group.

Calibration curves for beta-Myrcene and alpha-Humulene (Figures 3A and B, bottom) are linear with LLOQs to 10,000 ppb. All terpenes which had adequate chromatographic resolution yielded linear regression curves using with 1/x weighting and had R² values >0.990.

Figure 3A. Top – Extracted chromatograms for beta-Myrcene near LOD (25 ppb). Bottom – Calibration curve for beta-Myrcene from 25-10,000 ppb.

Figure 3B. Top – Extracted chromatograms for alpha-Humulene near LOD (5 ppb). Bottom – Calibration curve for alpha-Humulene from 10-10,000 ppb.
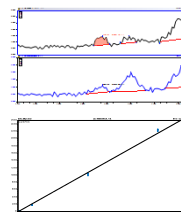
Quantitation of Terpenes – Hops Extracts

Measured concentrations of terpenes from extracted hops (Table 1, last column) are reported in ppm, and are corrected for the extraction volume (i.e., LC-MS/MS concentration [ppb] divided by 1000 [ppm] times 10 mL [extraction volume]). Each number represents the concentrations of the measured terpenes from the four hops samples. ND indicates the terpene was not detected. An asterisk indicates that the terpene could not be accurately quantified since it was not chromatographically resolved from its adjacent terpene (e.g., Geraniol and Linalool). Concentrations of Caryophyllene Oxide could be in part from Humulene Epoxide. Alpha-Humulene standard solutions exhibited an LC-MS/MS peak at same retention time as Caryophyllene Oxide (data not shown), which is hypothesized to be Humulene Epoxide.

Extracted concentrations of beta-Myrcene from hops samples were lower than expected (Figure 4; Table 1).[2] This is likely due in part to the age of the samples (> 6 months) and that they were stored at room temperature. Observation of high concentrations of oxygenated sesquiterpinoids, both identified (e.g., Caryophyllene Oxide, alpha-Bisabolol) and unidentified (e.g., see Figure 5, between RT 5.5-6.0 min), supports the hypothesis of sample aging.

Figure 5 shows high concentrations of alpha-Humulene (11.81 min) and beta-Caryophyllene (12.07 min) in all hops extracts, as reported previously.[3,4,5] However, other putative sesquiterpenes are observed in the same retention time range (11.0-13.5 min). Based on literature reports,[4,5] these may be Farnesene and/or Cadinene isomers. Analysis by GC-MS could help identify these and other unidentified terpenes by LC-MS/MS.

Figure 4. LC-MS/MS chromatograms for monoterpenes. Black trace is 1000 ppb standard; other traces are hops sample extracts. Arrow is retention time for beta-Myrcene.
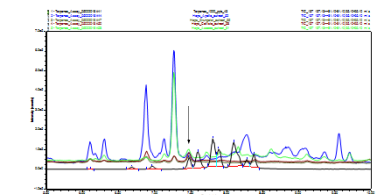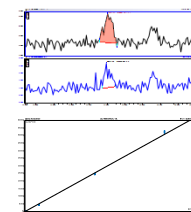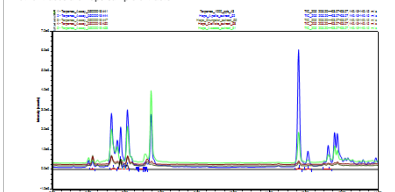

Figure 5. LC-MS/MS chromatograms for sesquiterpenoids. Black trace is 1000 ppb standard; other traces are hops sample extracts.

CONCLUSIONS

- LC-MS/MS method was successfully developed and optimized for measuring ~20 terpenes in plant extracts using the Vanquish Flex Binary UHPLC system and TSQ Quantis triple quadrupole mass spectrometer.

- LODs ranged from 2-10 ppb for oxygenated terpenes and sesquiterpenes, while monoterpenes had measured LODs of 25 ppb.

- Simple methanol extraction of hops samples showed low to moderate ppm concentrations of expected terpenes. Beta-Myrcene concentrations in extracted hops samples were lower than expected, possibly due to age and storage of hops.

- Many unidentified terpenes were observed in the hops extracts. Further analyses using GC-MS should help confirm the identity of these unknown terpenes.

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TRADEMARKS/LICENSING

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