Aroma Profile of Hops, *Humulus lupulus*, as a Function of Boil Time by GC-TOFMS and GCxGC-TOFMS

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1. Introduction

Hops, *Humulus lupulus*, are one of the primary ingredients in beer and serve as both a natural preservative and as a flavoring agent. These leafy green flowers, shown in Figure 1, are responsible for the characteristic bitterness in beer, but can also impart other flavors such as floral, tangy, piney, or citrusy notes. One factor that impacts the eventual flavor profile of beer is the selection of hop variety, as different strains lead to different flavors and aromas. Another important aspect is the timing of the hop addition during beer brewing. Brewing is a multi-step process that begins by mixing grains with hot water to convert starches in the grain to a sugary solution called wort. The wort is filtered and then boiled together with hops and other specialty ingredients to further develop flavors. At the completion of the boil, yeast is added to initiate fermentation. Hops can be added at any point during or after the boil to bring out desired flavors. Generally, hops are added earlier to draw out bitterness and later to highlight aroma and flavor.

Figure 1. Photograph of hop flowers grown in Seattle, WA, USA.

Bitterness in beer is primarily derived from alpha acids that are present in hop flowers. These alpha acids require extended exposure to elevated temperatures in order to isomerize to iso-alpha acids which are more stable and soluble in the finished product. The thermal energy from the extended boil, however, simultaneously leads to a loss of the essential oils responsible for flavor and aroma that are extracted from the hops into the wort. For this reason, hops are usually added more than once during the beer brewing process, and the timing of the additions is generally closely controlled. Hops added earlier in the boil supply bitterness as their alpha acids have ample time and heat to isomerize, while aroma and flavor come from hops added later in the boil since they are exposed to less heat which maintains the extracted essential oils.

In this application note, a method is developed to characterize aroma and flavor compounds associated with hops throughout the boil process. Headspace solid phase micro-extraction (HS-SPME) was used to sample the volatile and semi-volatile aroma and flavor compounds in the headspace of a boiled hop flower extract. Both one- and two-dimensional gas chromatography (GC and GCxGC) with Time-of-Flight Mass Spectrometry (TOFMS) were subsequently used to separate, quantify, and identify these compounds. Target analytes were monitored throughout the boil and quantified in order to determine aroma and flavor changes as a function of boil time.

2. Experimental Conditions

Samples

Cascade Leaf Hops were purchased from Label Peelers (Kent, OH, USA) and stored in a freezer for preservation. A hop extract was prepared by adding 3 g of hop flowers to 0.5 L of boiling water to mimic the boil process. This ratio of hops to water is roughly equivalent to 4 oz. of hops in a 5 gallon batch. Sample aliquots of 20 mL were removed from the boil at 5, 10, 20, 40, and 60 min of boil time and cooled. The overall volume of the boil was maintained at approximately 0.5 L with the addition of boiling water as needed. For SPME analysis, 4.0 mL of each hop extract (5, 10, 20, 40, and 60 min) were transferred by pipet into 20 mL glass headspace vials then sealed with septum caps.

SPME Conditions

SPME was automated using a Gerstel MPS2 Auto Sampler through LECO's ChromaTOF® software. Samples were incubated at 50°C for 10 min immediately prior to extraction. Extraction was accomplished by exposing a 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber (Supelco, Bellefonte, PA, USA) to the sample headspace for 30 min at 50°C. Analytes were desorbed from the fiber and injected for analysis by exposing the fiber in a 250°C GC-inlet for 2 min.

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GC-TOFMS Conditions

GC-TOFMS analyses were performed with LECO's Pegasus® HT. The Pegasus HT consists of an Agilent 6890 GC paired with LECO's Pegusus TOFMS. Analytes were separated with a 30 m x 0.25 mm i.d. x 0.25 μm film thickness Rxi-5Sil MS column from Restek (Bellefonte, PA, USA). Helium was used as the carrier gas at a flow of 1.0 mL/min. A temperature program was employed that held at 35°C for 4 min, ramped 10°C/min to 250°C, and held for 4 min. The transfer line was maintained at 260°C throughout the separation and the ion source temperature was set to 250°C. Full mass range spectra were collected at a 20 spectra/s acquisition rate and the 30-400 m/z mass range was saved.

GCxGC-TOFMS Conditions

GCxGC-TOFMS analyses were performed with LECO's Pegasus 4D. The Pegasus 4D consists of the Pegasus HT, and an Agilent GC modified with a secondary oven and dual stage quad jet thermal modulator. The carrier gas flow was maintained as in the GC-TOFMS method. A secondary column, 1.5 m x 0.25 mm i.d. x 0.25 μm film thickness Stabilwax column from Restek (Bellefonte, PA, USA), was joined in series to the Rxi-5Sil MS column. The temperature program was adjusted to hold at 35°C for 4 min, ramp by 5°C/min to 250°C, and hold for 4 min. The secondary oven followed the same temperature program, but was maintained 10°C higher throughout to a maximum temperature of 250°C. The modulator temperature was maintained 20°C higher than the primary oven and a 6 s modulation period was set. The transfer line and the ion source temperatures were maintained at 250°C. Full mass range spectra were collected at a 100 spectra/s acquisition rate and the mass range of 30-400 m/z was saved.

3. Results

These methods provided good characterization of the complex hop aroma samples and allowed for monitoring time dependencies of specific flavor and aroma compounds. HS-SPME collects the volatile and semi-volatile headspace analytes onto the fiber for concentration prior to injection. Clear differences between the TIC chromatograms of the GC-TOFMS analysis for the 5 and 60 min boil times can be observed in Figure 2.
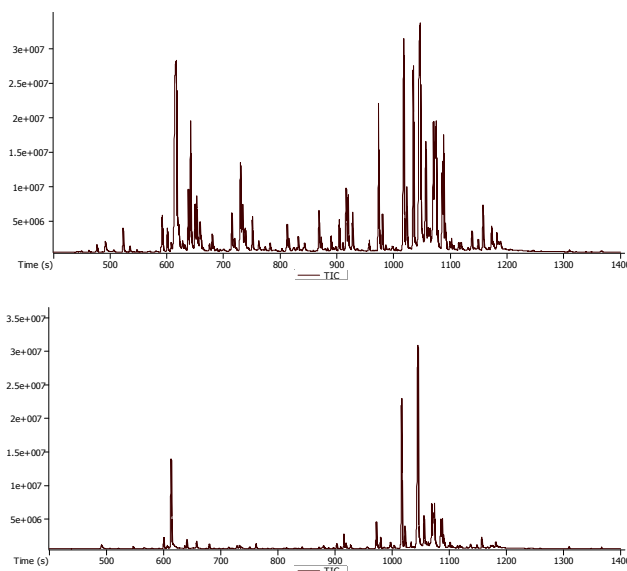

Figure 2. GC-TOFMS TIC chromatograms for the 5 min boil time (top) and 60 min boil time (bottom).

Samples exposed to longer time periods of boiling, have fewer volatile and semi-volatile headspace analyte peaks in their chromatograms. A total of 607 peaks with S/N >200 were detected in the hops sample boiled for 5 min and 373 peaks were detected in the sample boiled for 60 min. The overall signal intensity in each chromatogram also decreased with increased boil time. The total area for each sample was acquired with the ChromaTOF software and plot as a function of boil time, shown in Figure 3.
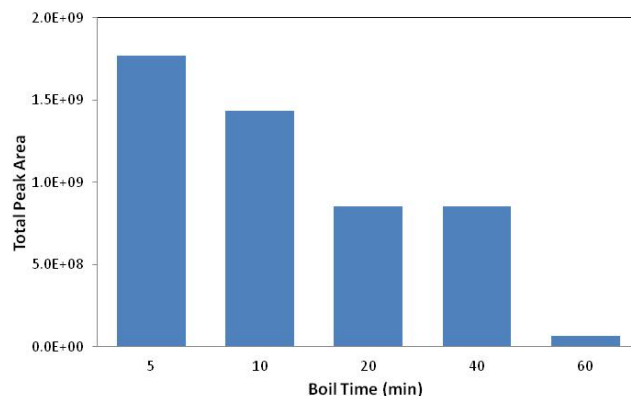

Figure 3. Total chromatographic intensity per boil time.

This gives useful characterization of the hops' overall aroma profile which indicates clear time dependencies. This method can also offer more specific insight due to the ability to isolate individual analytes within the complex matrix. These data contain some unresolved regions, but chromatographically overlapped analytes can often be separated based on differences in their mass spectral patterns. This is accomplished through deconvolution of the mass spectral information via ChromaTOF's True Signal Deconvolution® and Automated Peak Find algorithms. An example is shown in Figure 4. It appears that only one analyte is present at this retention time from

the TIC view. However, the True Signal Deconvolution (TSD®) algorithm isolates two unique analytes in this retention time window. When m/z unique to the overlapped analytes (103 and 100) are plotted instead of the TIC, both analytes can clearly be observed.
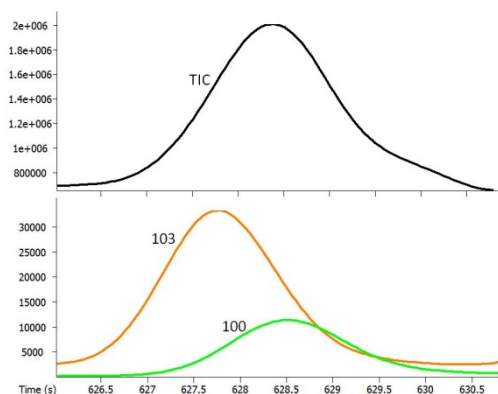

Figure 4. ChromaTOF's TSD algorithm resolves overlapped analytes based on their mass spectral patterns.

Deconvolution of the mass spectral data allows for quantification of each analyte using unique m/z and for identification through library matching of pure spectra. Prior to deconvolution, the mass spectral data across the width of what appeared to be a single peak, shown in the top box of Figure 5, did not yield a good match to any library spectra. Library searching of the deconvoluted mass spectral data, however, produces identification information for each analyte. The middle box of Figure 5 shows the peak true spectrum of the first peak and its library match, 2-methyl 2-methylpropyl ester butanoic acid, and the bottom box shows the second peak true spectrum and its library match, octanal. With identification information, flavor and aroma properties can also be found. Fruity and citrusy flavors are associated with 2-methyl 2-methylpropyl ester butanoic acid while citrus and orange flavors are associated with octanal.
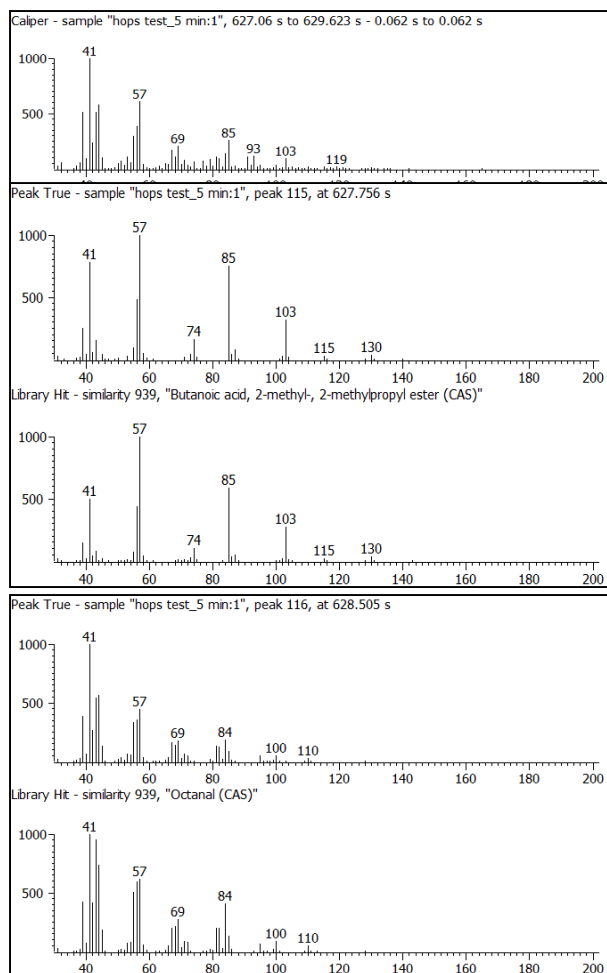

Figure 5. MS data for the analytes highlighted in Figure 4. Mass spectral deconvolution can isolate coeluting GC peaks.

Another way to separate analytes which were not chromatographically resolved in a 1D separation, or when mass spectral deconvolution is unable to separate coeluting analytes, is to add another separation dimension, as with GCxGC. In GCxGC, two complementary columns are connected in series and effluent from the first column is collected and injected to the second column at the set modulation period. Each sample is simultaneously subjected to two separation mechanisms, such as boiling point and polarity. Analytes with similar properties in one regard can often be separated by differences in the other. TIC contour plot chromatograms for the GCxGC analyses are shown in Figure 6.
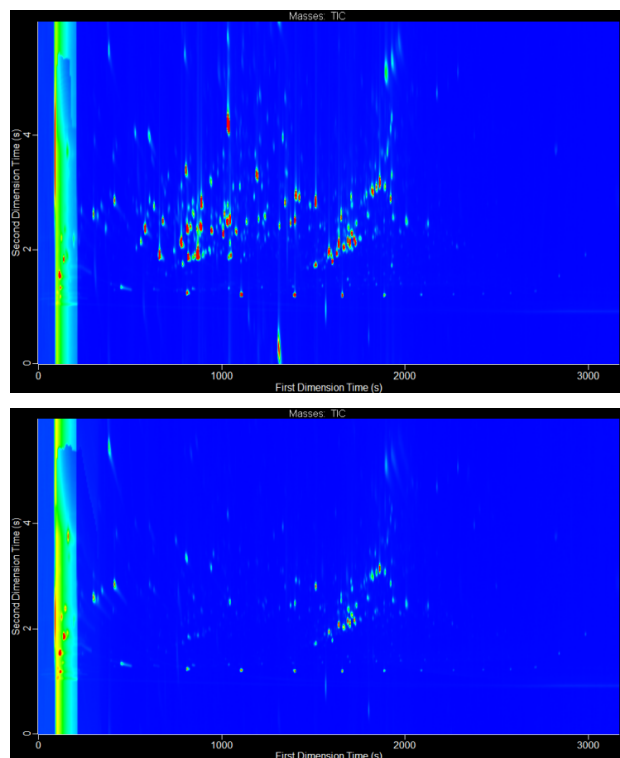
Figure 6. GCxGC-TOFMS TIC chromatograms for the 5 min boil time (top) and 60 min boil time (bottom).

Figure 7. A) GC-TOFMS and B) GCxGC-TOFMS chromatograms showing 2-decanone through decanal, both indicated with asterisks.

In the contour plot, the first dimension separation is displayed along the x-axis and the second dimension separation is displayed along the y-axis. Analytes appear as color spots with intensity proportional to color scale. The complexity of the aroma profile can be observed by the large number of peaks present in the chromatogram and clear difference between the boil times can be observed in this data, as well. A total of 1057 peaks with S/N >200 were detected in the hops sample boiled for 5 min and 500 peaks were detected in the sample boiled for 60 min.

GCxGC provides two main benefits for the analysis of complex samples. First, an improved peak capacity is achieved by having two complementary separations. Second, the cryogenic focusing effects of thermal modulation provide low level detection capabilities. Both of these can lead to an increased number of detected analytes. In the 5 minute boil time sample, 450 more peaks (1057 vs. 607) were detected by GCxGC as compared to GC.

Both of these benefits can be observed in the region of the chromatogram between 2-decanone and decanal (815 to 829 and 1200 to 1224 in the 1D and 2D data, respectively), shown in Figure 7.
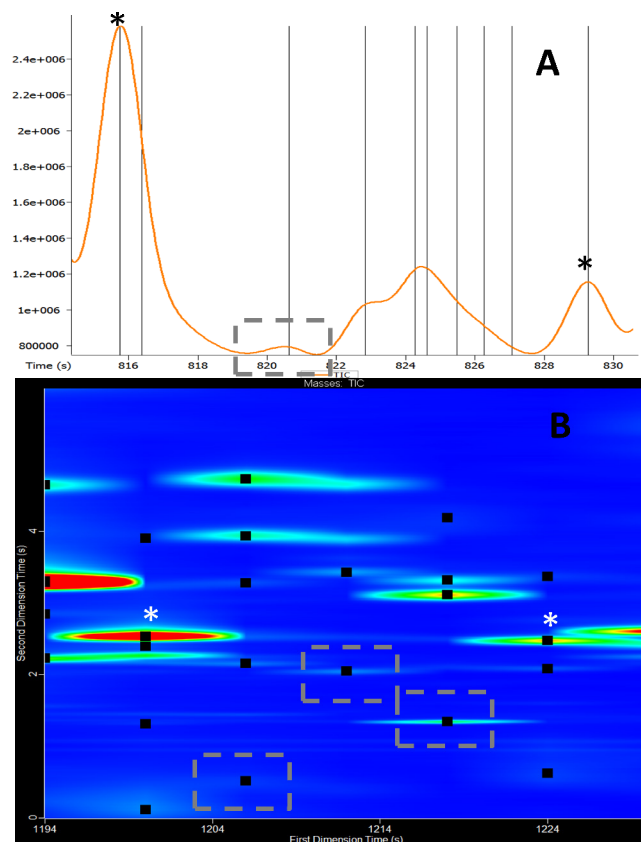
In the 1D separation, peaks are indicated by vertical lines and 10 are found between and including these two analytes. In the 2D separation 20 peaks are found, indicated by black dots. Some of the additional peaks are a result of lower level detection while others are due to the ability to separate previously coeluting analytes.

For example, in the GC-TOFMS data shown in Figure 7A, the small peak at 820.6 s, enclosed in the dashed gray box, is identified as 2-hydroxy-methyl ester benzoic acid with a match value of 638. The library and true peak spectra are shown in the top box of Figure 8. Multiple interfering m/z (55, 71 81, and 96) all lead to the fairly low match value. In the GCxGC separation, this peak is chromatographically separated into 3 separate peaks, shown enclosed in gray dashed boxes in Figure 7B. The spectra for these three analytes are shown in the bottom three boxes of Figure 8 and roughly combine to the single peak spectra from the GC-TOFMS data. This additional peak capacity leads to both a higher match value for 2-hydroxy-methyl ester benzoic acid (916 instead of 638) and the ability to measure these additional analytes.
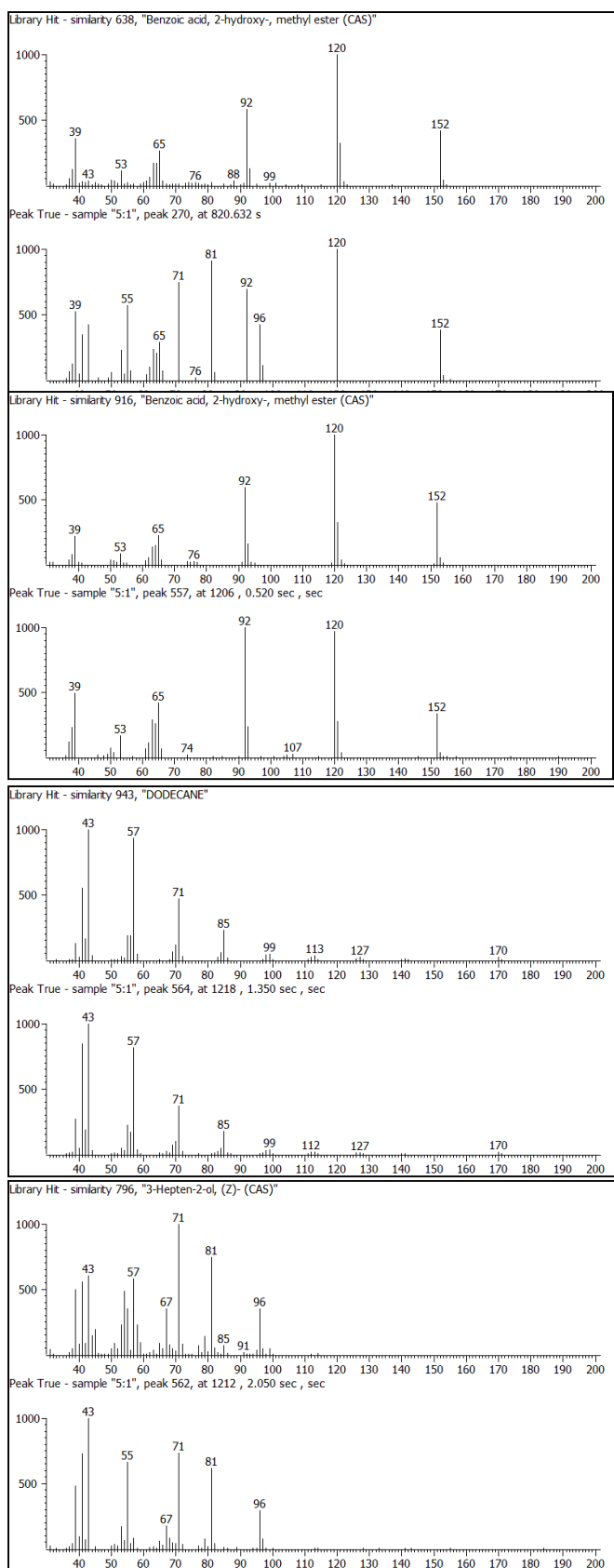
Library Hit - similarity 638, "Benzoic acid, 2-hydroxy-, methyl ester (CAS)"

Peak True - sample "5:1", peak 270, at 820.632 s

Library Hit - similarity 916, "Benzoic acid, 2-hydroxy-, methyl ester (CAS)"

Peak True - sample "5:1", peak 557, at 1206 , 0.520 sec , sec

Library Hit - similarity 943, "DODECANE"

Peak True - sample "5:1", peak 564, at 1218 , 1.350 sec , sec

Library Hit - similarity 796, "3-Hepten-2-ol, (Z)- (CAS)"

Peak True - sample "5:1", peak 562, at 1212 , 2.050 sec , sec

Figure 8. MS data for the analytes highlighted in Figure 7. GCxGC chromatographically resolves coeluting GC peaks.

In either case, coupling GC or GCxGC to TOFMS provides information for both identification and quantification. The ChromaTOF software identifies analyte peaks after deconvolution by matching the acquired mass spectral data to libraries of known spectra and quantifies by peak area and/or height. The information for all peaks within the data is compiled into Peak Tables that can be reviewed for both target and non-target analytes. Reverse Library Searches can also facilitate locating specific target analytes by matching library spectra to the data to locate the analyte peak with the best match. These tools were utilized to locate and tentatively identify 18 hop aroma compounds in the GC-TOFMS data, listed in Table 1. These identifications could be further confirmed with the addition of retention time matching to standards, if desired.

Additional data analysis features of the ChromaTOF software were utilized to further evaluate the hop aroma compounds. The Reference Feature allows the user to set a specific sample (and/or specific analytes in a specific sample) as a Reference to which the other samples are compared with user-input match criteria. The Reference Feature was used to rapidly quantify the target analytes in all of the samples for comparison purposes. The Reference Feature could also be implemented in a non-targeted way to locate differences between samples. These comparisons are done automatically through data processing methods, and relative quantification information is assembled in Peak Tables. In this application of the Reference Feature, the 5 min boil time was set as the Reference and the 18 target analytes were quantified relative to the 5 min sample in all other boil time samples. The quantification information is compiled graphically in Figure 9.

Table 1. **Target Aroma and Flavor Compounds from GC-TOFMS**

HOP AROMA COMPOUNDS	tR	m/z	MV
Humulene and Caryophyllene Oxidation Products			
Humulene	1149.7	80	827
Caryophyllene	1047.82	80	906
Caryophyllene oxide	1138.72	109	854
Caryophyllene epoxide	1158.19	109	822
Floral/Estery Compounds			
Geraniol	868.855	93	897
Geranyl acetate	974.086	121	900
Geranyl isobutyrate	957.612	45	901
Linalool	730.327	93	819
Citrus/Piney Compounds			
D-Cadinene	1176.81	161	868
Citral (Z)	858.821	119	861
Citral (E)	883.931	84	885
Nerol	847.139	136	890
Limonene	659.091	68	933
a-Muurolene	1102.73	105	892
Selinene	1190.19	189	859
Other			
Myrcene	617.108	53	805
Farnesene (b)	1035.84	69	905
Farnesene (a)	1061.7	107	828

Consistently across all 18 target aroma compounds, a loss of intensity is observed after the 10 min boil time. On average, the levels at 20 min are less than 40% of those observed after 5 min of boiling. Many analytes are not detected or are detected at only low levels by the 60 min boil time. These data are consistent with the understanding that hops should be added late in the boil to optimize their aroma and flavor contributions as additional boiling will lead to a loss of flavor and aroma. The detection of the iso-alpha acids, responsible for bitterness, is not compatible with these sampling conditions, so their presumed increase is not apparent in these data.

4. Conclusions

The experiments described in this application note demonstrate a food, flavor, and fragrance analysis for the characterization of hops' aroma profile. HS-SPME was used to pre-concentrate volatile and semi-volatile compounds in a hops extract. LECO's Pegasus HT GC-TOFMS efficiently separated, quantified, and identified analytes within the complex sample matrix. Full mass range acquisition allowed for identification of both target and non-target compounds through mass spectral matching to library spectra. The target aroma compounds were quantified across all boil time samples to determine temporal dependencies, providing information on the relative contribution of aromatic and flavor notes based on the timing of the hop addition. LECO's Pegasus 4D GCxGC-TOFMS also efficiently separated individual analytes for identification and quantification. GCxGC offers additional peak capacity and lower level detection in order to increase the number of measured analytes.
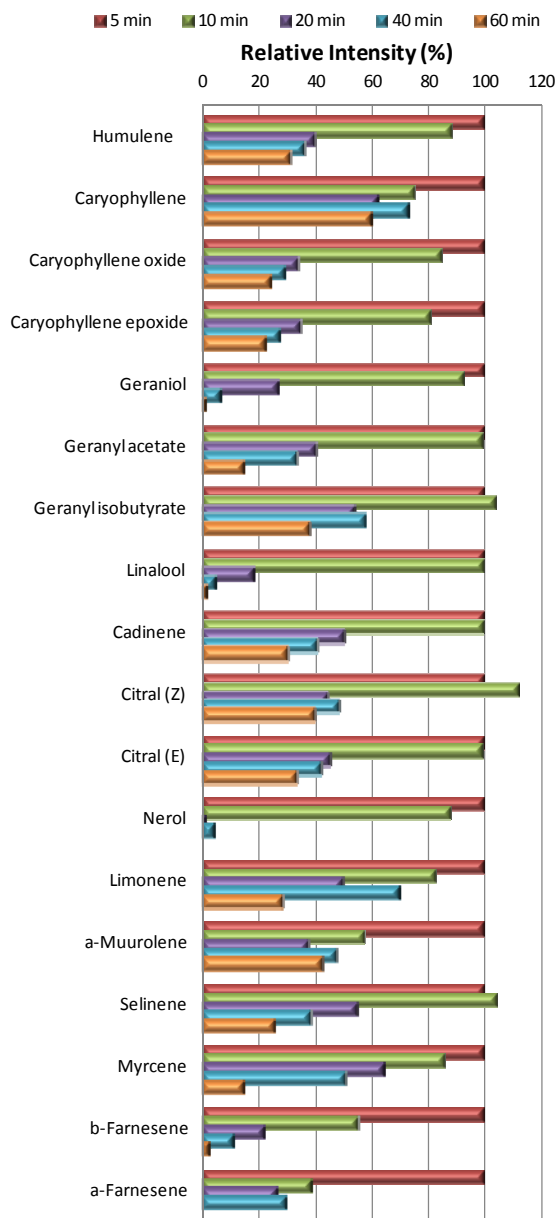

Figure 9. Target analytes (tentatively identified by mass spectral matching) are shown with relative intensity from GC-TOFMS data as a function of boil time. The 5 min boil time is set to 100%.