Characterization of Hop Acids by Liquid Chromatography with Negative Electrospray Ionization Mass Spectrometry

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ABSTRACT


The analysis of hop acids, humulones and lupulones, with negative electrospray ionization mass spectrometry (MS) is described for two different chromatographic systems. One system focuses on the complete resolution of the ad- and normal homologues implying that no optimal mass spectrometric parameters can be used, whereas the other system makes no compromise to the MS and yields a high sensitivity. The latter system is especially suitable for the fast screening of bitter acids. With this system, the detection limit with selected ion monitoring (SIM)-MS could be estimated to be 10 ng/ml for the humulones and 5 ng/ml for the lupulones. The presence of hop acids in beer was investigated and was found to be positive for humulones, whereas lupulones could not be detected.

RESUMEN

Es descrito el análisis de ácidos de lupulo, humulonas y lupulonas, por espectrometría de masas de ionización electrospray negativo, por dos sistemas cromatográficos diferentes. Un sistema se enfoca en la completa resolución de los homólogos normal y ad implicando que parámetros espectrométricos de masa no optima pueden ser usados, mientras que el otro sistema no se compromete al MS y proporciona una alta sensibilidad. El último sistema es especialmente conveniente para el rápido monitoreo de ácidos amargos. Con este sistema el límite de la determinación con espectrometría de masas por medio de monitoreo de ion selectivo (SIM) puede ser estimado en 10 ng/ml para las humulonas y 5 ng/ml para las lupulonas. La presencia de ácidos de lupulo en cerveza fue investigado y se encontró era positivo para humulonas, mientras que lupulonas no pudieron ser detectadas.

Hop (Humulus lupulus) is used for its preservative and flavoring characteristics in beer. Hop or hop extracts are added during the boil of the wort. It is during this time that the virtually insoluble α-acids (humulones) are isomerized into the more soluble iso-acids, the main bittering substances in beer. Although isomerized α-acids are the largest contributors, hops also contain β-acids (lupulones), which are claimed to add bitterness to beer. Most common hop acids are normal, co-, and ad-homologues (Fig 1).

The analysis of the hop acids in hop is important for quality control. The difficulty of this analysis with LC is caused by the isomeric form of the ad- and normal homologues of the hop acid. For a proper chromatographic system, fairly high efficiencies are needed, as it is desirable to have baseline separation for all the compounds (9).

The type of stationary phase in the chromatographic system appeared to be important. Certain types of reversed phase columns do not separate the ad- and normal homologues of the bitter acids (6). The analysis of the six major hop bitter acids using UV detection has been reported. For complete separation with micro LC, retention times of up to ≈50 min have been reported (8). Shorter retention times of 20 min are reported for a chromatographic system with coelution of the ad- and normal homologues (10). The use of other techniques like capillary electrophoresis (CE) (12), micellar electrokinetic chromatography (MEKC) (11), and centrifugal partition chromatography (CPC) (4) with UV detection did not, up to now, result in reproducible separation of all six major hop bitter acids, mainly due to the narrow pH interval in which the separation seems to be successful.

So far, the identification of the hop acids using MS is an interesting option, although the analysis of hop bitter acids has not yet been successful by GC-MS, due to their thermal instability (6). Due to this instability, a soft ionization technique has to be used for the analysis of bitter acids. Thermospray is less favorable due to thermal degradation in the vaporizer as well as the lack of sensitivity. Nevertheless, the analysis of phenolics together with the hop acids using a quadrupole mass spectrometer with thermospray interface in positive mode has been described, although incomplete separation of ad- and normal homologues is observed (1) (R. A. M. van der Hoeven, unpublished results).

With electrospray ionization (ESI) as an ionization technique, molecular mass information is obtained (7), whereas structure information is obtained either with nozzle- or skimmer-induced dissociation or collision-induced dissociation (CID).

In this article, two chromatographic systems are described. One system is based on the use of a gradient of acetonitrile and water with ammonium acetate at pH 6.5, which results in almost baseline separation of all six hop acids, although less sensitivity has to be accepted. For optimal sensitivity, an isocratic system is described with higher modifier concentration and pH 3.

EXPERIMENTAL

All experiments were performed on a Finnigan Mat TSQ-70 (San Jose, CA) triple quadrupole mass spectrometer equipped with a custom-made electrospray interface (5). The ESI needle assembly is kept at –3 kV. The stainless steel sampling capillary and ion-source were optimized with respect to sensitivity and kept at 250 and 200°C, respectively.

The analysis was performed in negative ionization mode, selected ion monitoring (SIM) of the [M-H] of the bitter acids. Optimization of the repeller voltage, which was found to be ion-source pressure-dependent, was done at 500 Pa and found to be optimal at –85 V.

Optimization of the mass spectrometer for MS and MS/MS experiments was performed in the so-called constant infusion mode. The reference compounds were dissolved in a mixture of acetonitrile and water (80:20, v/v) containing 1% acetic acid and infused with a flow rate of 2 µl/min by means of a Harvard 2400 syringe pump. For electrical contact, a sheath flow of the same composition at a flow rate of 2 µl/min was used.

The solvent delivery system consisted of two high-pressure pumps (model 2150, LKB, Bromma, Sweden), controlled by a 2152-HPLC controller (LKB). Injection was performed with an injection valve (Rheodyne 7120, Cotati, CA) with a 20-µl loop. Two systems
were used. System 1: In the gradient system, the mobile phase consisted of 100% A (mixture of acetonitrile and water, 20:80, v/v, containing 0.05M ammonium acetate) to 100% B (mixture of acetonitrile and water, 90:10, v/v, containing 0.05M ammonium acetate) at a flow rate of 0.8 ml/min. A PRP-1 column, 150 × 4 mm, was used. System 2: In the isocratic system, the mobile phase consisted of a mixture of methanol and water (90:10, v/v) containing 1% acetic acid, resulting in a pH of 3. A Zorbax C₈ column, 150 × 4.6 mm, was used.

For best performance, 25 µl/min of the delivered flow rate is directed to the MS by means of a splitting device. For electrical contact, 1 µl/min of a mixture of methanol and water (80:20, v/v) with 1% acetic acid is used as a sheath flow. Nitrogen gas (6 L/min) is used as a sheath gas to stabilize the electrospray. The experiments were performed using SIM, alternatively scanning the deprotonated molecules of m/z 347 (cohumulone), 361 (humulone and adhumulone), 399 (colupulone), and 413 (lupulone and adlupulone).

The MS/MS experiments were performed with argon as collision gas at a pressure of 0.5 Pa and a collision offset of 10 eV.

Chemicals

Hop bitter acids were isolated by A. C. Hoek (2). Acetonitrile and acetic acid (Baker, Deventer, The Netherlands) were of HPLC grade. Water was purified with a Milli-Q apparatus (Millipore, Bedford, MA). Ammonium acetate was of analytical grade from Merck (Darmstadt, Germany) and beer came from Grolsch (Grolsche bierbrouwerijen, Groenlo-Enschede, The Netherlands). Acetonitrile and water, 90:10, v/v, containing 0.05M ammonium acetate is used as a sheath flow. Nitrogen gas (6 L/min) is used as a sheath gas to stabilize the electrospray. The experiments were performed using SIM, alternatively scanning the deprotonated molecules of m/z 347 (cohumulone), 361 (humulone and adhumulone), 399 (colupulone), and 413 (lupulone and adlupulone).

The MS/MS experiments were performed with argon as collision gas at a pressure of 0.5 Pa and a collision offset of 10 eV.

RESULTS AND DISCUSSION

Hop acids can be analyzed with electrospray in the negative as well as in the positive ionization mode, but with an acidic eluent the sensitivity is superior in the negative ionization mode. The mass spectra are dominated by a deprotonated molecule.

Structure elucidation of the hop acids is done with MS/MS. The fragmentation pattern of the hop acids with collision induced dissociation of 10 eV gives specific structure information. The three α-acids fragment with a loss of the 69 (C₅H₉) side chain. Lupulones fragment with a loss of the same side chain 69 (C₅H₉) as the humulones, and a loss of 112, which is a consecutive loss of 69 (C₅H₉) and 43 (C₃H₇). The loss of 124 is again the 69 (C₅H₉) plus an subsequent loss of 55 (C₅H₇). The loss of 137 is due to the loss of two fragments of 69 (with rearrangement; 2 × C₅H₇). The loss of 180 is 137 (2 × C₅H₇) plus a subsequent loss of 43 (C₃H₇; Table I). MS/MS of the bitter acids in combination with LC does not give more information, due to the same fragmentation pattern of the homologues of humulone and lupulone.

Chromatography

The separation of the hop acids is strongly dependent on the choice of the stationary phase, the column, and pH level. For optimal separation of the hop acids, a chromatographic system is described (3) using a PRP-1 column and pH > 8.

For the humulones, pH 8.4 proved to be optimal for high resolution and low retention times. The optimal level for lupulones was pH = 10. At pH 10, all six hop acids are almost baseline separated. Due to the pKₐ of the hop acids, which for humulones are between 4.0 and 5.5 and for lupulones between 5.5 and 7.8, all hop acids are totally deprotonated and hard to detect with electrospray in negative ionization mode.

System 1

For total separation of the main six bitter acids and a retention time within 25 min, a PRP-1 column with gradient is used. Sensitivity is not very good due to the deprotonated state of the bitter acids (Fig. 2). The difference in detection limits between lupulones and humulones can be explained by the difference in pKₐ. Also, the low modifier concentration has a bad influence on the droplet evaporation (and thereby the charge transfer) of the electrospray. The use of a make-up flow of acetonitrile and water (95:5, v/v) with 5% acetic acid after the analytical column and before the splitter has no influence.

System 2

With a mobile phase with higher modifier concentration at pH 3 (methanol and water 90:10, v/v, with 1% acetic acid) and a ZORBAX SB C₈ column, better sensitivity is obtained.

The detection limit with this system improves to 10 ng/ml for the humulones and 5 ng/ml for lupulones. Retention times of the six hop acids are within 9 min. Unfortunately, with this system the ad-, and normal homologous of the lupulones and humulones are not separated (Fig. 3).

![Fig. 1. Structure and molecular weights of hop acids.](image)

<p>| TABLE I: Structure Information from MS/MS of the Bitter Acids with 10eV |
|--------------------------|----------------|-------------------------|-------------------|</p>
<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Mass</th>
<th>[M-H]* (intensity)</th>
<th>Fragments (intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohumulone</td>
<td>348</td>
<td>347 (100)</td>
<td>278 (47)</td>
</tr>
<tr>
<td>Adhumulone</td>
<td>362</td>
<td>361 (100)</td>
<td>292 (60)</td>
</tr>
<tr>
<td>Humulone</td>
<td>362</td>
<td>361 (90)</td>
<td>292 (100)</td>
</tr>
<tr>
<td>Colupulone</td>
<td>400</td>
<td>399 (100)</td>
<td>330 (15), 287 (45), 275 (10), 262 (12), 219 (10)</td>
</tr>
<tr>
<td>Adlupulone</td>
<td>414</td>
<td>413 (100)</td>
<td>344 (15), 301 (42), 289 (15), 276 (10), 233 (10)</td>
</tr>
<tr>
<td>Lupulone</td>
<td>414</td>
<td>413 (100)</td>
<td>344 (18), 301 (55), 289 (10), 276 (10), 233 (10)</td>
</tr>
</tbody>
</table>

*[M-H]* = deprotonated ion.
With this iscoratic system, a beer sample is analyzed. The α-acids (humulones) in beer are isomerized and keep the same molecular mass as before boiling of the wort. In contrast to the α-acids, β-acids (lupulones) are oxidized and have a different molecular mass. Degassed beer (20 ml) was injected into the high sensitivity system. No interference could be observed, and humulones were present at a concentration level of ≈150–200 ng/ml (Fig. 4). Lupulones were not detected.

To prove that lupulones can be measured when no interference is seen, beer was spiked to 125 ng/ml with the six main hop bitter acids. After spiking, both (lupulones and humulones) could be detected (Fig. 5).

Fig. 2. Chromatogram of 1 µg/ml of hop acids injected in the selective system, following the deprotonated ion [M-H].

Fig. 3. Chromatogram of 10 ng/ml of hop acids injected in the sensitive system, following the deprotonated ion [M-H].
CONCLUSION

The six main hop acids, ad-, co-, and normal homologues of humulone and lupulone, can be detected with negative electrospray ionization MS. MS/MS used to identify these six acids in an LC system cannot discriminate between the ad- and normal hop acids due to the similar fragmentation pattern of these compounds.

Complete separation can be achieved with gradient HPLC using a polymer PRP-1 column. However, sensitivity of the compounds is rather poor, due to the high pH level necessary for separation.

Fig. 4. Chromatogram of raw beer sample injected in the sensitive sample, following the deprotonated ion [M-H].

Fig. 5. Chromatogram of spiked beer sample injected in the sensitive system, following the deprotonated ion [M-H].
A higher sensitivity is obtained by using an alternative system with a mobile phase containing more modifier, a lower pH, and a ZORBAX SB C8 column. The detection limit is 5 ng/ml for these β-acids. For humulone homologous, the detection limit is 10 ng/ml. A beer sample was analyzed with the sensitive system and humulones were found at 150 ng/ml level. Lupulones could not be detected in beer.

LITERATURE CITED

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