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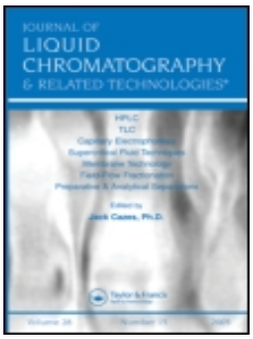

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High Performance Liquid Chromatographic Method for Iso-α-Acids

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Abstract: Using pure trans/cis-isocohumulone, trans/cis-isohumulone, and trans/cis-isoadhumulone an isocratic HPLC system has been developed for quantitation of these compounds in hop extracts and beers. The mobile phase contains acetonitrile-water-H_3PO_4 (50 : 50 : 0.01, v/v/v) and is used with a Phenomenex Hypersil 5 μ C_{18} column 250 × 4.6 mm, flow rate 1.5 mL/min. Baseline separation of all 6 isomers was achieved with a total run time of 25 min. The UV spectrum of these pure compounds using this system are quite different. The effect of different mobile phase compositions on separation was investigated as well as the chromatographic parameters, detection limit, and linearity.

Keywords: LC-UV, Iso-α-acids, Bitter acids, Hop, *Humulus lupulus*, Beer

INTRODUCTION

Hop (*Humulus lupulus*) is a climbing herbaceous plant belonging to the family of the Cannabinaceae. Hops are added to beer, providing taste and flavour and contributing to the stability of foam.[1–3] The analysis of the composition of hops constitutes, therefore, a major issue in brewing industry.

The main constituents of hop related to these properties are generically known as humulones, consisting in three α-acids: humulone, cohumulone, and adhumulone. During the brewing process, these acids are isomerized, resulting in the formation of three pairs of trans/cis iso-α-acids (**1**–**6**),

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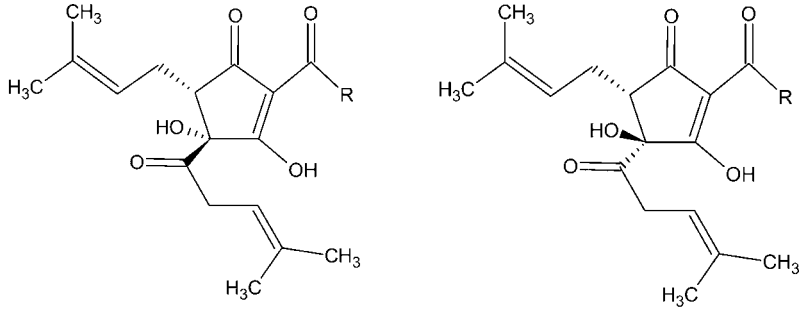
which contribute to the characteristics of beer with their bitter taste and foam-lacing properties.[1–3] In modern brewing practice, whole hops are often substituted by various hop products, such as isomerized hop extracts which contain iso-α-acids (Figure 1).[4]

Iso-α-acids need to be determined in beer, in wort, in hop extracts, and in pre-isomerized hop extract. Undoubtedly the quantitative determination of iso-α-acids in beer is the most important. Analysis of iso-α-acids in wort is also worthwhile to establish the degree of alpha acids conversion into iso-α-acids. Pre-isomerized hop extracts have to be analyzed for the iso-α-acids content claimed by the manufacturer, as such extracts can show a dramatic decrease in iso-α-acids content in only a few months.[5]

The traditional method for measuring bitterness in beer, i.e., spectrometric analysis, is incapable of distinguishing between the source of bitterness and, is therefore, wholly unsuitable when any mixture is involved.[6] HPLC with UV detection is routinely used to analyze bitter acids and several HPLC systems have been developed for this purpose.[7–11] They used dicyclohexylamine (DCHA) salt of the trans-iso-α-acids complex as the standard instead of pure trans isomers since they are not stable. In order to get the pure iso-α-acids, we have developed a simple method for the isolation of individual iso-α-acids and the method will soon appear in a patent publication. This method allowed us to use both trans- and cis-isomers in free form instead of in DCHA salts complex for HPLC determination.



cis (**1-3**) trans (**4-6**)

R = $CH_2CH(CH_3)_2$: **1, 4** isohumulone

R = $CH(CH_3)CH_2CH_3$: **2,5** isoadhumulone

R = $CH(CH_3)_2$: **3,6** isocohumulone

Figure 1. Structures of iso-α-acids.

In this study, we developed an isocratic HPLC system for the separation of the six iso-α-acids, using pure iso-α-acids as the external standard together with their UV spectrum. The influence of different eluents and columns and the linearity for quantitative analysis are also discussed.

EXPERIMENTAL

Chromatography

The HPLC system consisted of a pump type 626 (Waters, USA) with a pump controller type 600 S (Waters, USA), an autosampler, type 717 plus (Waters, USA), a photo-diode array detector type 2996 (Waters, USA). Column used was a Hypersil 5 μ C_{18}, 250 × 4.6 mm (Phenomenex, USA). Mobile phases were filtered over a 0.2 μm hydrophilic polypropylene membrane filter 47 mm type GH Polypro (Pall Corporation, Michigan, USA).

Chemicals

HPLC grade organic solvents; acetonitrile (Biosolve, The Netherlands), methanol (Biosolve, The Netherlands), and tetrahydrofuran (J.T. Baker, USA) and ortho-phosphoric acid 85% (Merck, Germany) were used in the mobile phase. Isomerized supercritical fluid extract of hop extract was obtained from Heineken Brewery, The Netherlands.

Beer Sample Preparation for Injection into an HPLC System

The iso-α-acids were extracted from beer by liquid-liquid extraction. Degassed beer (100 mL) was acidified with HCl (6 M; 5 mL) and subsequently partitioned with chloroform (100 mL) three times. After phase separation, the chloroform layer was collected and the solvent was removed under reduced pressure. The residue was redissolved in MeOH (0.5 mL; HPLC grade, Merck) prior to injection into the HPLC.

Isolation of Iso-α-Acids

The pure trans/cis-isocohumulone, trans/cis-isohumulone, and trans/cis-isoadhumulone were isolated from hop extracts in our laboratory, and the purity and quantity were checked by ^{1}H NMR using anthracene as an internal standard.[12]

Prior to HPLC analysis, all the samples were filtered through an Acrodisc LC 13 mm syringe filter with PVDF membrane (Pall Corporation, Michigan, USA).

RESULTS AND DISCUSSION

In an effort to develop an isocratic HPLC system, in which the six iso-α-acids could be separated, we used, as a starting point, a solvent system that consists of a gradient of 40% water and 60% acetonitrile with addition of 0.7% H_3PO_4 and a Macherey-Nagel C_{18} 250 × 4 mm column.[11] This system separates 5 out of 6 iso-α-acids, as the cis-isohumulone and trans-isoadhumulone are not separated.

A simple attempt to separate the cis-isohumulone from the trans-isoadhumulone peak by modification of the flow rate and acetonitrile concentration did not give any satisfactory results. In all these systems, cis-isohumulone and trans-isoadhumulone peaks overlapped. Therefore, other strategies were followed; testing different solvents and the acid concentration.

Influence of Organic Solvents and Phosphoric Acid

The initial gradient run using 0–100% methanol/water, acetonitrile/water, and tetrahydrofuran/water gave a poor separation and the peaks were broadened. The addition of acid seems necessary to suppress the ionization. Phosphoric acid was chosen, as this acid can also stabilize the sample from oxidation by inhibition of trace metal activity in the column packing material.[13]

Table 1 shows the effect of H_3PO_4 concentration on the resolution (R_s), which is defined as the degree of separation of one component from another measured as the difference in retention time of the two solutes divided by

Table 1. The effect of phosphoric acid concentration on the resolution (R_s) using acetonitrile/water mobile phase[a]

Phosphoric acid (%)	R_s[b]					Running time (min)
	TICH-CICH	CICH-TIH	TIH-CIH	CIH-TIAH	TIAH-CIAH[c]	
0.0001	1.1	0.4	0.6	0.0	1.0	11.5
0.001	1.4	0.7	0.9	0.4	1.8	28
0.01	1.6	2.2	1.3	0.6	2.1	29
0.1	2.1	3.9	1.7	0.0	1.9	30

[a]Gradient run using 60–95% acetonitrile 90% in water/acetonitrile 10% in water with addition of H_3PO_4 and Phenomenex Hypersil C_{18} 5 μ (250 mm × 4.6 mm) column during 60 min.

[b]Calculated as $t_{R2} - t_{R1}/0.5(w_1 + w_2)$ in which t_{R2} and t_{R1} are the retention time of the compound 1 and 2 and w_1 and w_2 are peak width of the compound 1 and compound 2.

[c]TICH = trans-isocohumulone, CICH = cis-isocohumulone, TIH = trans-isohumulone, CIH = cis-isohumulone, TIAH=trans-isoadhumulone, CIAH = cis-isoadhumulone.

Table 2. The effect of phosphoric acid concentration on the resolution (R_s) using methanol/water mobile phase[a]

Phosphoric acid (%)	R_s					Running time (min)
	CICH-TICH	TICH-CIH	CIH-TIH	TIH-CIAH	CIAH-TIAH	
0.0001	0.2	1.1	0.4	1.1	0.0	14.0
0.001	0.5	1.5	0.7	2.2	0.0	16.0
0.01	0.6	1.2	1.4	2.4	0.0	23.5
0.1	0.8	1.8	1.0	1.6	0.0	38.5

[a]Gradient run using 60–95% methanol 90% in water/methanol 10% in water with addition of H_3PO_4 and Phenomenex Hypersil C_{18} 5 μ (250 mm × 4.6 mm) column during 60 min.

their average peak width.[14] Phosphoric acid concentration at 0.01% gave the optimum separation of all iso-α-acids. However, separation between cis-isohumulone and trans-isoadhumulone is still poor ($R_s < 1.5$).

Another experiment using methanol instead of acetonitrile is presented in Table 2. These solvent systems gave poorer separation compared to the acetonitrile system, especially, there is no separation between cis-isoadhumulone and trans-isoadhumulone, and poor separation between trans-isocohumulone and cis-isocohumulone. A notable difference in elution pattern was determined between methanol and acetonitrile. Using acetonitrile the sequence is trans- and cis-iso-α-acids, however with methanol that sequence is reversed.

Table 3 shows the resolution of peaks by using tetrahydrofuran instead of acetonitrile or methanol. The result is worse than the use of either acetonitrile or methanol. There is no separation between trans- and cis-isocohumulone, isohumulone, and isoadhumulone.

Table 3. The effect of phosphoric acid concentration on the resolution (R_s) using tetrahydrofuran/water mobile phase[a]

Phosphoric acid (%)	R_s					Running time (min)
	TICH-CICH	CICH-TIH	TIH-CIH	CIH-TIAH	TIAH-CIAH	
0.0001	0.0	0.9	0.0	1.8	0.0	14.0
0.001	0.0	1.2	0.0	1.7	0.0	16.0
0.01	0.0	1.0	0.0	1.8	0.0	17.5
0.1	0.0	2.0	0.0	1.5	0.0	25.0

[a]Gradient run using 50–80% tetrahydrofuran 90% in water/tetrahydrofuran 10% in water with addition of H_3PO_4 and Phenomenex Hypersil C_{18} 5 μ (250 mm × 4.6 mm) column during 60 min.

Table 4. The effect of acetonitrile concentration on the resolution (R_s) under gradient elution[a]

Conc. MeCN (%)[b]	R_s					Running time (min)
	TICH-CICH	CICH-TIH	TIH-CIH	CIH-TIAH	TIAH-CIAH	
40–80	2.4	2.7	1.4	1.3	1.7	37.5
50–80	1.6	1.9	1.0	0.9	1.2	32
60–95	1.6	2.2	1.3	0.6	1.9	29

[a]Gradient run using acetonitrile 90% in water/acetonitrile 10% in water with addition of H_3PO_4 (0.01%) and Phenomenex Hypersil C_{18} 5 μ (250 mm × 4.6 mm) column during 60 min.

[b]Concentration of acetonitrile 90% in water/acetonitrile 10% in water from 0 to 60 min elution.

It can be concluded, that acetonitrile is the best choice among the tested solvents and the optimum concentration of phosphoric acid is 0.01% (v/v) of the total solvent. However, the resolution is not good enough since the resolution between cis- and trans-isohumulone and between cis-isohumulone and trans-isoadhumulone is not a base line separation.

Resolution might be increased by selecting the optimum solvents ratio and using an isocratic mode. This will increase analysis time, but may lead to improved resolution. Table 4 shows the resolution of peaks for various acetonitrile concentrations under gradient elution. A baseline separation between cis-isohumulone and trans-isoadhumulone and between trans- and cis-isohumulone could not be achieved. But, obviously the resolution is better at the lower concentration of acetonitrile. Finally, the problem could be resolved by isocratic elution as shown in Table 5.

Table 5. The effect of acetonitrile concentration and flow rate on the resolution (R_s) under isocratic elution[a]

MeCN (%); flow rate (mL/min)	R_s					Running time (min)
	TICH-CICH	CICH-TIH	TIH-CIH	CIH-TIAH	TIAH-CIAH	
45; 1.0	2.0	2.6	1.4	2.1	2.3	51
50; 1.0	1.9	2.2	1.5	1.7	2.2	33
55; 1.0	1.7	2.1	1.3	1.0	1.7	29
50; 1.5	2.0	2.5	1.5	1.5	2.0	23

[a]Isocratic run using acetonitrile in water with addition of H_3PO_4 (0.01%) and Phenomenex Hypersil C_{18} 5 μ (250 mm × 4.6 mm) column.

Various acetonitrile concentrations were tested and 50% acetonitrile in water was found to be the best. Baseline separation can be achieved for all 6 compounds with a resolution of 1.5 or higher. However, the total running time is longer then with a gradient; it can be reduced by increasing the flow rate to 1.5 mL/min. Higher flow rates (>1.5 mL/min) could not be applied with the column used in these experiments.

UV Spectra of Individual Iso-α-Acids

Complete chromatograms and the UV photodiode array spectra of iso-α-acids extracts, beers, and standard mixture using the best solvent system, are shown in Figure 2. The UV spectra are quite different for the various isomers. Co-, n-, and ad-isomers have a different λ_{max}. The different spectra of the trans- and

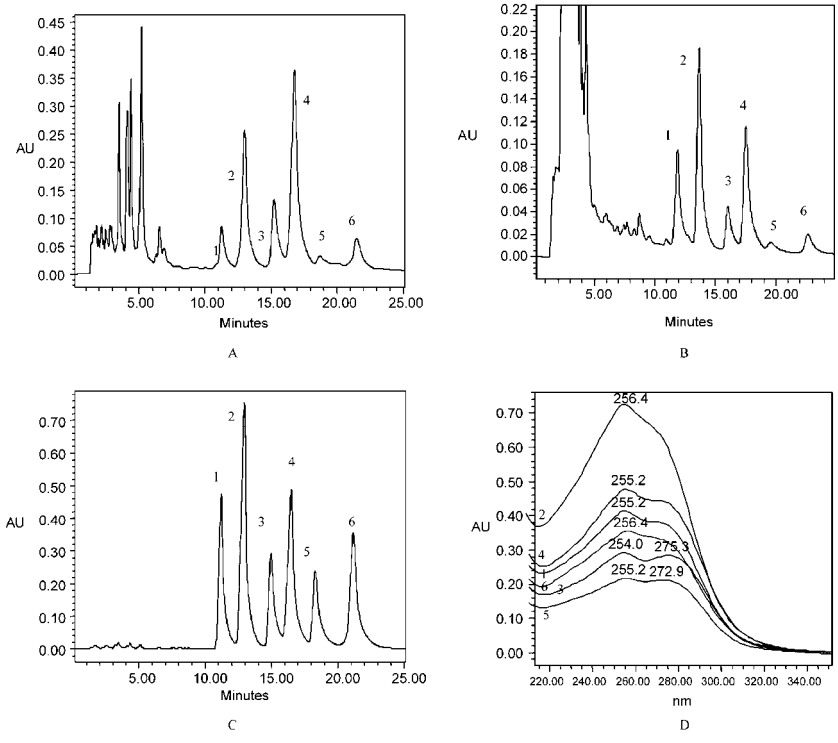


Figure 2. Chromatogram of isomerized hop extract (A) beer (B) and iso-α-acids standard mixture (C). Column: Phenomenex Hypersil 5 μ C_{18}, 250 × 4.6 mm; eluent: acetonitrile–water-H_3PO_4 (50 : 50 : 0.01, v/v/v); flow rate: 1.5 mL/min; peak number: (1) trans-isocohumulone, (2) cis-isocohumulone, (3) trans-isohumulone, (4) cis-isohumulone, (5) trans-isoadhumulone, (6) cis-isoadhumulone. (D) UV photodiode array spectra (wavelength in nm) of peaks 1–6 of (C).

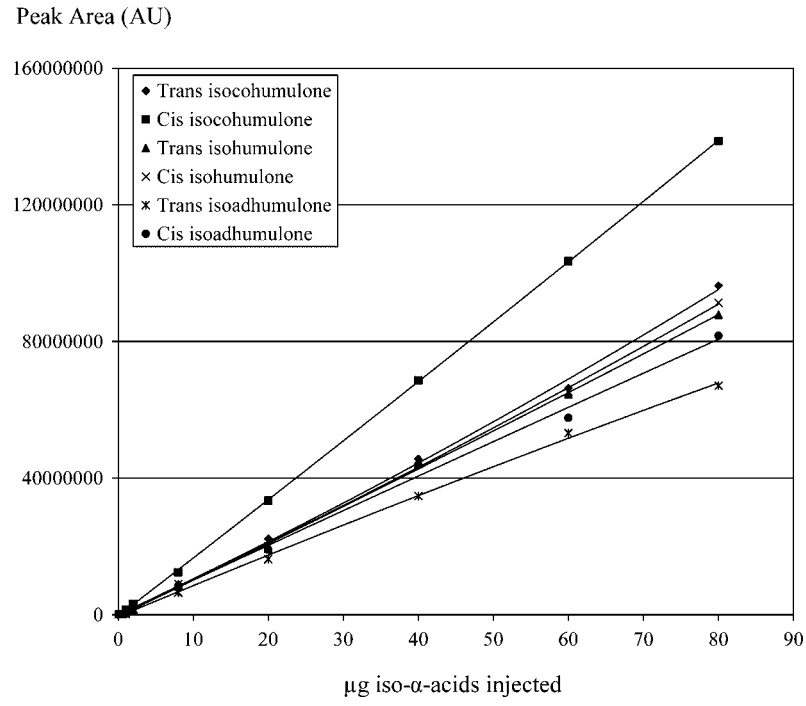


Figure 3. Peak areas as a function of injected amount of iso-α-acids. Column and eluent as in Fig. 2.

cis-isomers is also quite striking, all cis-isomers have a lower shoulder than the trans counterpart. This behavior may have implications for the iso-α-acids determinations by means of LC-UV, which allow us now to recognize different individual iso-α-acids (Figure 3).

Column Chromatographic Parameters, Detection Limits, and Linearity

Chromatographic parameters were measured from this chromatogram and the data are presented in Table 6. The k′ values are within an acceptable range to be able to perform the analysis of all six compounds in an isocratic system. Detection limits were determined after injection of a known amount of iso-α-acid standard. The detection limit was defined as the amount giving a peak height of two times the noise level, measured at the absorbance maximum. Detection limits measured were in the range of 50–100 ng.

The relationship between peak area and amount injected was investigated by injecting 10 µL pure iso-α-acids solution. The amounts injected were in

Table 6. Chromatographic parameters for the solvent system, described in text

Compounds	Capacity factor (k')[a]	Resolution (R_s)	Detection limits (ng)
trans-isocohumulone	4.7	2.0	70
cis-isocohumulone	5.6	2.5	53
trans-isohumulone	6.8	1.5	94
cis-isohumulone	7.6	1.5	75
trans-isoadhumulone	8.6	2.0	95
cis-isoadhumulone	10.0		74

[a]Calculated as $t_R - t_o/t_o$ in which t_R is the retention time of the compound and t_o the retention time of first peak appearing in chromatogram.

the range of 0.1–80 μg of iso-α-acids. The linearity of trans-isocohumulone, cis-isocohumulone, trans-isohumulone, cis-isohumulone, trans-isoadhumulone, and cis-isoadhumulone calibration curves were found to be 0.9967, 0.9999, 0.9997, 0.9988, 0.9964, and 0.9959, respectively.

CONCLUSION

Quantitative analysis of trans-isocohumulone, cis-isocohumulone, trans-isohumulone, cis-isohumulone, trans-isoadhumulone, and cis-isoadhumulone in isomerized hop extract and beer is possible with the isocratic HPLC system: acetonitrile-water-H_3PO_4 (50 : 50 : 0.01, v/v/v), combined with a Hypersil 5 μ C_{18} column 250 × 4.6 mm (Phenomenex, USA), and a flow rate of 1.5 mL/min with 25 min run time. UV spectra of individual iso-α-acids are different and it allows recognition of the purity of the peak.

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