Studies of iso-alpha-acids: analysis, purification, and stability.

Khatib, Alfi

CHAPTER 2

High Performance Liquid Chromatography Method of Iso-α-Acids

Alfi Khatib, Hye Kyong Kim, Erica G. Wilson, and Robert Verpoorte

Division of Pharmacognosy, Section of Metabolomics, Institute of Biology, Leiden University, Einsteinweg 55, PO BOX 9502, 2300 RA Leiden, The Netherlands

ABSTRACT

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

Published in *J. Liq. Chrom. & Rel. Technol.*, 2006, 29: 293-302

2.1. INTRODUCTION

Hop (*Humulus lupulus* L.) is a climbing herbaceous plant belonging to the family of the Cannabaceae. Hops are added to beer, providing taste and flavour and contributing to the stability of foam (Bamforth, 1985; Hughes, 2000; Verzele and De Keukeleire, 1991). 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 α-acids which consist of 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, which contribute to the characterisitics of beer with their bitter taste and foam- lacing properties (Bamforth, 1985; Hughes, 2000; Verzele and De Keukeleire, 1991). In modern brewing practice, whole hops are often substituted by various hop products such as isomerized hop extracts which contain iso-α-acids (IAAs) (Hughes and Simpson, 1993).

IAAs need to be determined in beer, in wort, in hop extracts, and in pre-isomerized hop extract. Undoubtedly the quantitative determination of IAAs in beer is the most important. Analysis of IAAs in wort is also worthwhile to establish the degree of α-acids conversion into IAAs. Pre-isomerized hop extracts have to be analyzed for the IAAs content claimed by the manufacturer as such extracts can show a dramatic decrease in IAAs content in only a few months (De Cooman *et al*., 2001).

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 (Verhagen, 1988). High performance liquid chromatography (HPLC) with ultra violet (UV) detection is routinely used to analyze bitter acids and several HPLC systems have been developed for this purpose (Forster *et al*., 1997; Harms and Nitzche, 2001; Raumschuh *et al.*, 1999; Vanhoenacker *et al*., 2004). They used dicyclohexylamine (DCHA) salt of the *trans*-IAAs complex as the standard instead of pure *trans*-isomers since they are not stable. In order to get the pure IAAs, we have developed a simple method for the isolation of individual IAAs as discussed in **Chapter 4**. This method allowed us to use both *trans*- and *cis*-isomers in free form instead of in DCHA salts complex for HPLC determination.

In this study we developed an isocratic HPLC system for the separation of the six IAAs using pure IAAs 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.

2.2. MATERIALS AND METHODS

2.2.1. Chemicals

HPLC grade organic solvents: acetonitrile, methanol (Biosolve, Valkenswaard, The Netherlands) and tetrahydrofuran (J.T. Baker, Deventer, The Netherlands) and *ortho*-phosphoric acid 85% (w/v) (Merck, Darmstadt, Germany) were used in the mobile phase. Isomerized supercritical CO_2 hop extract was obtained from Botanix (Paddock Wood, Kent, UK).

2.2.2. Chromatography

The HPLC system used consisted of a Waters system equipped with a 626 pump and 600 S pump controller, a 717 plus auto sampler, and a 2996 photodiode array detector type 2996. A Hypersil 5 µ C18, 250 x 4.6 mm (Phenomenex, Torrance, CA, USA) column was used. The mobile phase was filtered using a 0.2 µm hydrophilic polypropylene membrane filter GH Polypro (Pall Corporation, Ann Arbor, MI, USA) and helium sparged.

2.2.3. Beer Sample Preparation for Injection to HPLC

The IAAs were extracted from beer by liquid-liquid extraction. Beer was degassed using sonicator under negative pressure during 15 minutes. 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 re-dissolved in methanol (0.5 ml; HPLC grade, Merck, Darmstadt, Germany) prior to injection to HPLC.

2.2.4. Isolation of Iso-α-acids

The pure IAAs were isolated following the procedure as described in **Chapter 4**, and the purity and quantity were checked by HPLC and ^{1}H NMR using anthracene as an internal standard.

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

2.3. RESULTS AND DISCUSSION

In the effort to develop an isocratic HPLC system, in which the six IAAs 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 x 4 mm column (Forster *et al.*, 1997). This system separates 5 out of 6 IAAs 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.

2.3.1. 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 stabilise the sample from oxidation by inhibition of trace metal activity in the column packing material (De Keukeleire *et al.*, 1992).

Table 2.1. 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 their average peak width (Lindsay, 1992). Phosphoric acid concentration at 0.01% gave the optimum separation

of all IAAs. However, separation between *cis*-isohumulone and *trans*-isoadhumulone is still poor (R_s<1.5).

Table 2.1. The effect of phosphoric acid concentration on the resolution (R_s) using acetonitrile/water mobile phase[a].

H_3PO_4 (%)	R_s[b]					Running time (minutes)
	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 x 4.6 mm) column during 60 minutes.

[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.

Another experiment using methanol instead of acetonitrile is presented in Table 2.2. 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*- IAAs, however with methanol that sequence is reversed.

Table 2.2. The effect of phosphoric acid concentration on the resolution (R_s) using methanol/water mobile phase[a].

H_3PO_4 (%)	R_s					Running time (minutes)
	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 x 4.6 mm) column during 60 minutes.

Table 2.3. 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.

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.

Table 2.3. The effect of phosphoric acid concentration on the resolution (R_s) using tetrahydrofuran/water mobile phase[a].

H_3PO_4 (%)	R_s					Running time (minutes)
	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 x 4.6 mm) column during 60 minutes.

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 2.4. 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 2.5.

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/minute. Higher flow rates ($\geq$1.5 ml/minute) could not be applied with the column used in these experiments.

2.3.2. UV Spectra of Individual Iso-α-Acids

Complete chromatograms and the UV photodiode array spectra of IAAs extracts, beers and standard mixture using the best solvent system are shown in Fig. 2.1. 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 *cis*-isomers is also quite striking, all *cis*-isomers have a lower shoulder than the *trans*- counterpart. This behavior may have implications for the IAAs determinations by means of LC-UV which allow us to recognize different individual IAAs.

Table 2.4. The effect of acetonitrile concentration on the resolution (R_s) under gradient elution[a].

Acetonitrile (%)[b]	R_s					Running time (minutes)
	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 x 4.6 mm) column during 60 minutes.
[b] Concentration of acetonitrile 90% in water/ acetonitrile 10% in water from 0 to 60 minutes elution.

Table 2.5. The effect of acetonitrile concentration and flow rate on the resolution (R_s) under isocratic elution[a].

Acetonitrile (%); flow rate (ml/minute)	R_s					Running time (minutes)
	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 x 4.6 mm) column.

2.3.3. Column Chromatographic Parameters, Detection Limits, and Linearity

Chromatographic parameters were measured from this chromatogram and the data are presented in Table 2.6. 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 IAAs 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.

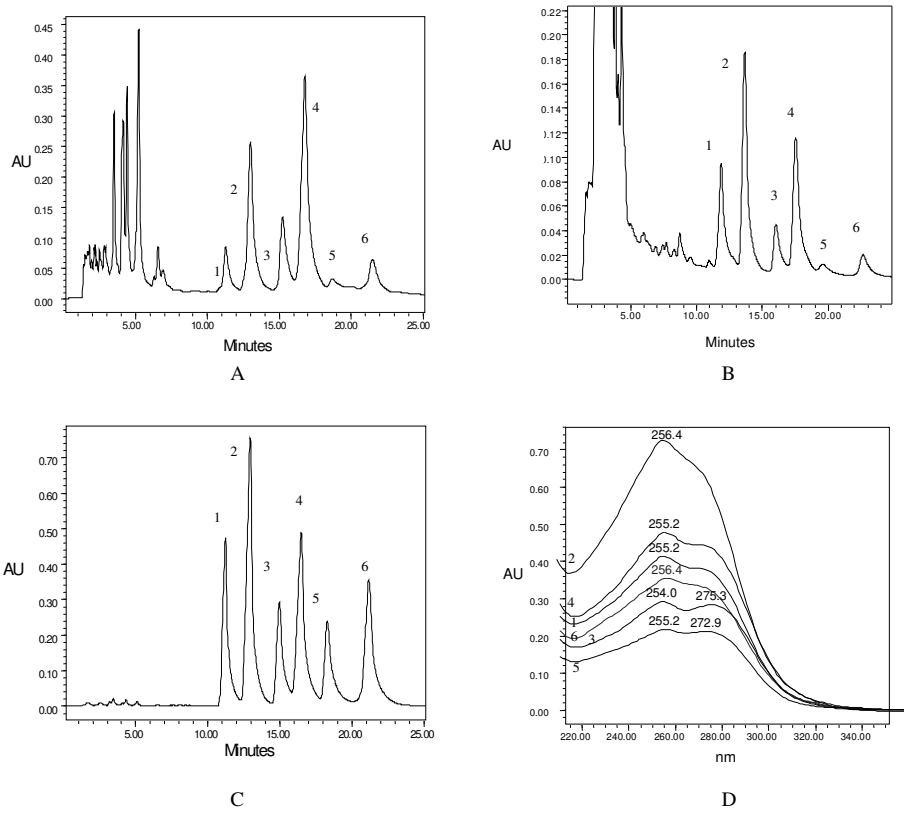

Fig. 2.1. Chromatogram of isomerized hop extract (A), beer (B), and IAAs standard mixture (C). Column: Phenomenex Hypersil 5 µ C_{18}, 250 x 4.6 mm; isocratic system; eluent: acetonitrile – water-H_3PO_4 (50:50:0.01, v/v/v) ; flow rate: 1.5 ml/minute; peak numbers: (1) *trans*-isocohumulone, (2) *cis*-isocohumulone, (3) *trans*-isohumulone, (4) *cis*-isohumulone, (5) *trans*-isoadhumulone, (6) *cis*-isoadhumulone. (D) UV spectra (wavelength in nm) of peaks 1-6 of (C).

The relationship between peak area and amount injected was investigated by injecting 10 µl pure IAAs solution. The amounts injected were in the range of 0.1-80

μg of IAAs. The regression data and linearity of each iso-α-acids are presented in Table 2.7.

Table 2.6. 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.

Table 2.7. Regression equation and linearity of each the iso-α-acids.

Standard compounds	Regression equation	r^2
trans-Isocohumulone	y = 1205634x - 1180365	0.9967
cis-Isocohumulone	y = 1721660x - 377070	0.9999
trans-Isohumulone	y = 1099664x - 687599	0.9997
cis-Isohumulone	y = 1102738x - 202000	0.9988
trans-Isoadhumulone	y = 897707x - 851042	0.9964
cis-Isoadhumulone	y =1 053246x - 681053	0.9959

2.4. 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 x 4.6 mm (Phenomenex, USA) and a flow rate of 1.5 ml/minute with 25 minutes run time. UV spectra of individual IAAs are different and it allows to recognize the purity of the peak.