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Short communication

Preparative regime for the purification of bitter acids derived from hops (*Humulus lupulus* L.)

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Abstract

The preparation of pure iso-α-acids from hop-derived extracts is described. The method relies on the chemical separation of *cis*- and *trans*-isomers of the iso-α-acids, by the precipitation of the *trans*-isomers with dicyclohexylamine (DCHA), followed by C_{18} reversed-phase preparative HPLC using a mobile phase of ethanol–water–glacial acetic acid (60:40:0.2, v/v). The selectivity of ethanol as an organic modifier allows the purification of more than 500 mg of mixture per day. The materials are of high purity and suitable for research in the areas of sensory analysis, foam behaviour and antibacterial activity.

Keywords: *Humulus lupulus*; Preparative chromatography; Beer; Acids; Dicyclohexylamine; Isocohumulone; Isohumulone; Isoadhumulone

1. Introduction

Iso-α-acids are derived from the α-acids of the cultivated hop (*Humulus lupulus* L.). They occur in beer as a mixture of six closely related compounds (*trans*- and *cis*-isocohumulone, *trans*- and *cis*-isohumulone, *trans*- and *cis*-isoadhumulone; see Fig. 1). They influence beer flavour, beer foam, and have antibacterial properties [1].

The iso-α-acid preparations used in many experiments have been mixtures of various compounds, as their chemical similarity and instability to light have limited their routine separation. Pfenninger et al. reported the synthesis of iso-α-acids in high yields, as mixtures of the *cis*- and *trans*-isomers [2]. Synthetic routes to iso-α-acids are best avoided because racemic mixtures are generally produced giving isomers that are not native to beer and synthetic routes are generally more costly than alternative

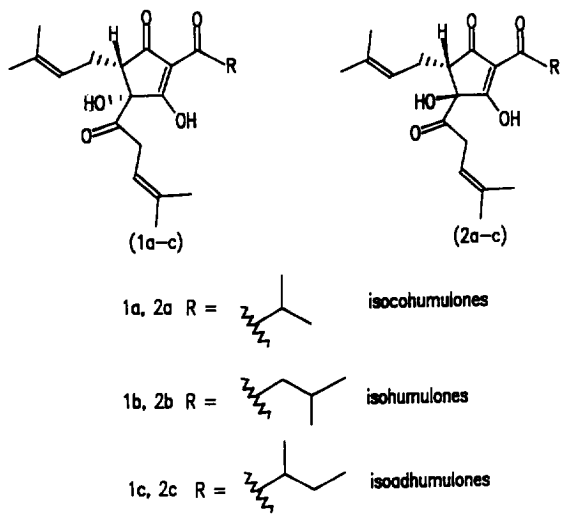

Fig. 1. Structures of six iso-α-acids.

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methods. Schwarzenbach [3] showed that chromatography on buffered silica gel systems allowed the separation of a wide range of hop-derived substances. However, Verzele et al. [4] have noted that there are problems recovering compounds from such systems. They do show that it is possible to separate small quantities of single iso-α-acids by reversed-phase preparative chromatography. Preparation of small quantities presents a problem in itself as the components may be unstable and degrade while sufficient material is being prepared.

Thus, for research purposes, a preparative method for iso-α-acids has to meet several criteria. It should allow the preparation of sufficient quantities in a working day and produce materials which are free of contaminants, especially toxic compounds (this is particularly important if the compounds are to be used for organoleptic trials) and substances of low volatility such as ion-pair reagents, salts and phosphoric acid. The method should be based on readily available equipment and should not be labour-intensive. A new method for separating and isolating pure iso-α-acids is described in this paper.

Dicyclohexylamine (DCHA) specifically complexes with *trans*-iso-α-acids [5,6] allowing their separation from *cis*-iso-α-acids. The *trans*-iso-α-acids complex forms a precipitate in ethyl acetate, which leaves free *cis*-iso-α-acids in solution. Furthermore, the complex formed between *trans*-iso-α-acids and DCHA has similar chromatographic properties to those of the free iso-α-acids, presumably because it is fully dissociated in the mobile phase. DCHA precipitation simplifies the chromatographic separation, as the mixture of six compounds is reduced to two mixtures, each of three components. Reversed-phase preparative HPLC of each of these three-component mixtures allows the rapid, reliable preparation of iso-α-acids on a 500+ mg scale. A preliminary report of this methodology has been published [7].

2. Experimental

2.1. Chemicals

Trifluoroacetic acid (99.9%; HPLC grade), formic acid (98%) and magnesium acetate (99.5%) were obtained from BDH. Dicyclohexylamine (99%) and propan-1-ol (99.5%; HPLC grade) were obtained from Aldrich. Glacial acetic acid (99.7%), ethyl acetate (99.8%), acetonitrile (99.98%) and 2,2,4-trimethylpentane (99.5%) were obtained from Fisons. Ethanol (96%) and methanol (99.85%) were obtained from Hayman. Mixtures of iso-α-acids (92% iso-α-acids) were a gift from Hop Developments (Eardiston, UK). All chemicals were used without further purification. Water was glass-distilled before use. Mobile phases were filtered through a 0.45-μm Durapore Phobic HV membrane (Millipore) before use and continually sparged with helium gas (50 ml min^{-1}).

2.2. Sample preparation

A mixture of iso-α-acids (10 g) was dissolved in ethyl acetate (30 ml) and DCHA (5.1 g; ca. 1.02 mole equivalents) added. The mixture was left at 20°C in the dark for 2–4 days, after which a precipitate was evident. The solid was recovered by filtration under reduced pressure, washed with ice-cold ethyl acetate (2×3 ml) and recrystallized from ethyl acetate. The white, fine crystalline solid complex of *trans*-iso-α-acids and DCHA was filtered off under vacuum and dried by suction. The *cis*-isomers which were contained in the filtrate were washed with 2 *M* hydrochloric acid (50 ml) and the solid hydrochloride salt of DCHA removed by filtration. Excess ethyl acetate was removed from the filtrate by rotary evaporation (ca. 2 kPa; 35°C) to yield a crude *cis*-iso-α-acids fraction which was not further purified.

2.3. Liquid chromatography

Preparative-scale separations were carried out on a Waters Delta Prep 4000 chromatograph, using a Waters Prep NovaPak HR 6 μm C_{18} cartridge (100×25 mm I.D.) under radial compression (ca. 7 MPa). For analytical purposes a Waters NovaPak 4 μm C_{18} cartridge (100×8 mm I.D.) under radial compression (ca. 18 MPa) was used. Analytical samples were introduced via a Rheodyne 7010 injector fitted with a 20-μl loop. The compounds were detected using a Waters 486 UV–Vis variable-wavelength detector. In the preparative mode the

detector was set to 290 nm, 2.0 absorbance units full scale (AUFS). The UV spectra of iso-α-acids are dependent on the pH of the solvent and water content [8]. For preparative separations, the detector was deliberately 'de-tuned' to 290 nm, to prevent the detector response being saturated by high concentrations of iso-α-acids. However, for analytical runs, settings were altered to 280 nm, 0.2 AUFS. Data were collected using a Perkin-Elmer LCI-100 laboratory computing integrator. The mobile phase (A) for both analytical and preparative separations was ethanol–water–glacial acetic acid (60:40:0.2, v/v) delivered at 0.9–1.1 ml min^{-1} for analytical purposes and 20–25 ml min^{-1} for preparative purposes. A method which separates *cis*- and *trans*-isomers of iso-α-acids was used to evaluate the purity of the products of preparative HPLC. For this purpose, a mobile phase (B) of acetonitrile–methanol–water–0.2 *M* magnesium acetate–formic acid–trifluoroacetic acid (840:490:480:24.0:3.60:1.20, v/v) delivered at 2.0–2.2 ml min^{-1} was employed.

2.4. Recovery of fractionated material

The solutions collected were applied to the preparative column with water (1:1, v/v). This was continued until all of the collected fraction had been applied. The retained material was eluted from the column with ethanol (ca. 100 ml, first 20 ml discarded). The ethanol was removed from the eluate by rotary evaporation (ca. 2 kPa; 35°C). The residual aqueous suspension was extracted with 2,2,4-trimethylpentane (5×5 ml), then dried over sodium sulphate and the sample concentrated under a stream of oxygen-free nitrogen gas. Solid *trans*-isomers were recovered by filtration, while the *cis*-isomer oils were collected by removal of the supernatant solvent. The residue was then concentrated under a stream of oxygen-free nitrogen gas.

3. Results and discussion

Considerable care has to be taken in the design of the mobile phase for reversed-phase preparative chromatography. Water must be effectively removed from the prepared fractions and ion-pair reagents or non-volatile acids need to be removed if the prepared compounds produced are to be free of contaminants.

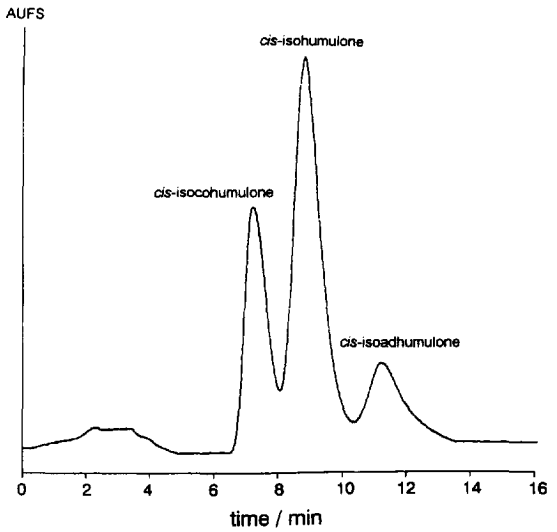

Fig. 2. Preparative separation of *cis*-iso-α-acids. Conditions: mobile phase A; flow-rate, 22 ml min^{-1}; λ=290 nm; 2.0 AUFS. Sample loading: 60 mg of *cis*-iso-α-acids in 500 μl ethanol. Although under these conditions, *cis*- and *trans*-isomers are not resolved, subsequent analytical HPLC of the prepared fractions demonstrated the absence of *trans*-isomers from these fractions.

Fig. 2 illustrates the separation of a mixture of *trans*-iso-α-acids by preparative HPLC. Sample loading was as high as possible without sacrificing resolution (typically 50 mg per run). Each separation took 15–20 min which allowed 500 mg of material to be separated into fractions in 3–4 h. It is interesting that ethanol exhibited unique selectivity as an organic modifier for the separation of isomeric isohumulones and isoadhumulones. Neither methanol or propan-1-ol, as organic modifiers, were able to separate isohumulones from isoadhumulones (results not shown). Sample purity was assessed by ^{1}H NMR (270 MHz) and UV–Vis spectrophotometry and was quantified by HPLC. Samples were >95% pure by HPLC, based on peak area of analyte relative to the total integrated area of the chromatogram (see for example, Fig. 3).

This method meets the criteria described in Section 1 and has been successfully employed, on a routine basis, for the preparation of single iso-α-acids for sensory, antimicrobial and beer foaming studies.

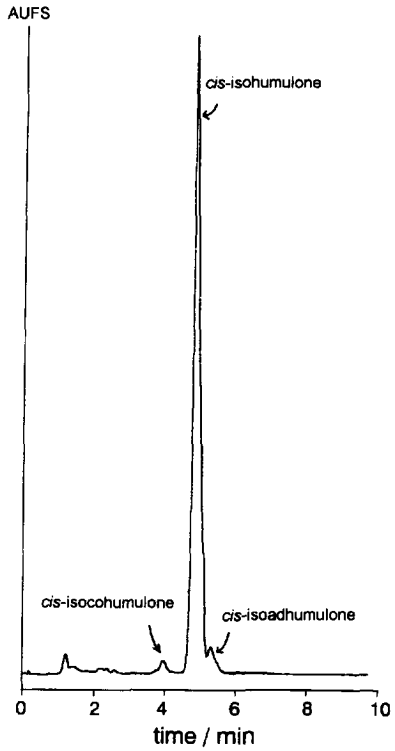

Fig. 3. Purity check for *cis*-isohumulone. Conditions: mobile phase B; flow-rate, 2.1 ml min^{-1}; λ=280 nm; 0.2 AUFS. Sample loading: 20 μl of 1 m*M* solution of *cis*-isohumulone fraction in mobile phase B. Purity=96%, by peak area relative to the total integrated area of the chromatogram. Note the two small peaks either side of the major peak: these correspond to *cis*-isocohumulone (1%) and *cis*-isoadhumulone (2%).

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References

[1] J.S. Hough, D.E. Briggs, R. Stevens and T.W. Young, Malting and Brewing Science, Vol. II, Chapman and Hall, London, 1985.
[2] H.B. Pfenninger, F. Schur, B.P. Vaterlaus, T. Sigg and J. Wild, Proceedings of the European Brewery Convention, Nice, 1975, pp. 159–169.
[3] R. Schwarzenbach, J. Am. Soc. Brew. Chem., 37 (1979) 180.
[4] M. Verzele, G. Steenbeke, L.C. Verhagen and J. Strating, J. Chromatogr., 484 (1989) 361.
[5] H.A. Thornton, J. Kulandai, D.B. Hawthorne and T.E. Kavanagh, J. Inst. Brew., 96 (1990) 367.
[6] H.A. Thornton, J. Kulandai, M. Bond, M.P. Jontef, D.B. Hawthorne and T.E. Kavanagh, J. Inst. Brew., 99 (1993) 473.
[7] P.S. Hughes and W.J. Simpson, Proceedings of the EBC Symposium on 'Hops', Zouterwoude, May/June 1994, Verlag Hans Carl Getränke-Fachverlag, Nürnberg, 1994, p. 128.
[8] W.J. Simpson, J. Inst. Brew., 99 (1993) 317.