Journal of Chromatography, 197 (1980) 189–197
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,955

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BEER BITTER ACIDS

C. DEWAELE and M. VERZELE*

Laboratory of Organic Chemistry, State University of Ghent, Krijgslaan, 271 (S. 4), B-9000 Ghent (Belgium)

(Received May 19th, 1980)

SUMMARY

The high-performance liquid chromatography of the six beer bitter iso-α-acids on different modified silica gel stationary phases is described. Octadecyl-, nitrophenyl- and cyanopropyl-substituted and buffered silica gels were studied with a variety of solvent systems. The aim was to find a system suitable for the routine isocratic analysis of the beer bitter compounds. Although the six iso-α-acids can be separated, it is concluded that a separation in three groups for each *cis* and *trans* pair corresponding to the three α-acids is preferable for this purpose. This is possible with an ion-pairing method on octadecyl-silica gel.

INTRODUCTION

The bitter-tasting compounds in beer mostly occur in concentrations around 25 ± 10 ppm. They are formed during the brewing process by isomerization of the three hop α-acids: humulone, adhumulone and cohumulone. Each α-acid yields a *cis* and a *trans* isomer and therefore there are six major iso-α-acids in beer. Their bitter tastes are almost identical and therefore it has been convenient to determine them as a group. Many contributions to this analysis can be found in the literature, but a really satisfactory method for iso-α-acids in beer is still lacking. Numerous ring analyses, inter-laboratory exercises and comparative evaluations organized by the European Brewery Convention (EBC), the American Society of Brewing Chemists (ASBC) or others have given inadequate results, considering the efforts made.

It is advantageous today to turn to high-performance liquid chromatography (HPLC) to obtain the required separation efficiency for the type of analysis involved. This investigation was a study of the HPLC of beer bitter acids. Our main aim was to establish the potential of commercially available derivatized silica gel HPLC stationary phases for the routine analysis of iso-α-acids. A similar study, mainly on the unisomerized hop bitter acids, the α- and β-acids, has recently been published[1].

0021-9673/80/0000–0000/$02.25

EXPERIMENTAL AND RESULTS

All separations were carried out on a Varian 5000 LC chromatograph, equiped with a 10-μl Valco 7000 sample loop injector and a Varichrom variable-wavelength detector. The columns were 25 × 0.46 cm I.D. Lichroma tubing unless otherwise specified. The stationary phases, all 10 μm, were obtained from RSL (Eke, Belgium) and were used without further treatment. The columns were packed either with a carbon tetrachloride or a glycerol–methanol suspension slurry. All analyses were optimized in the isocratic mode as this greatly facilitates eventual routine analysis.

The chemicals used were pure or were appropriately purified. Special attention was paid to the quality of the solvents (water and methanol).

Beer bitter substances were extracted from acidified beer with 2.5 times their volume of isooctane. Iso-α-acids in the extract form were obtained from PRB (Wetteren, Belgium). Pure individual bitter compounds were prepared from hops according to the techniques developed in this laboratory[2,3].

Reversed-phase chromatography

Reversed-phase C_{18} silica gel or octadecyl-silica gel is generally the most successful of all derivatized silica gels. An example of a separation of hop acids on RSil-C18-HL is shown in Fig. 1. This trace contains only one iso-α-acid peak, namely that of *trans*-isohumulone. The other peaks normally do not occur in beer, or in only very small concentrations, but for some applications it is interesting to know the elution sequence of Fig. 1.

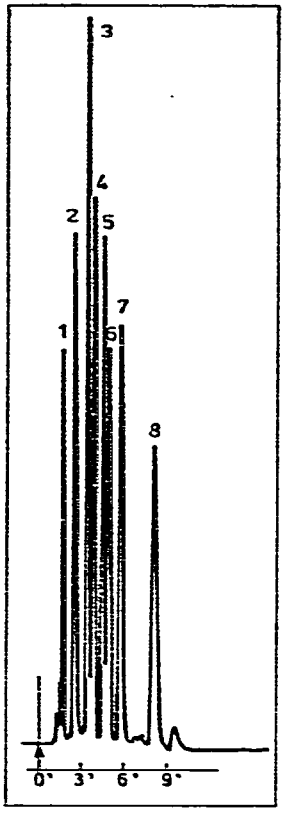
When the percentage of water is increased it becomes possible to separate nearly all of the iso-α-acids. A chromatogram obtained on isomerized hop extract is shown in Fig. 2. The analysis time is about 30 min. Complete resolution, which would permit easy quantitation, is not attained, however. With the high water and buffer concentrations, a high counter pressure is generated (*ca.* 300 kg/cm^2 at 100 ml/h) and a slight loss in column efficiency occurs[4]. With 5-μm RSiL-C18-HL the separation can be complete, but the analysis takes 1 h and is clearly more difficult. A trace of an isomerized hop extract in these conditions is given in ref. 1.

Another possibility is to use four coupled 10-μm columns. The resolution is much improved, as shown in Fig. 3. However, the analysis time is excessive.

As the complete resolution of the six iso-α-acids appears to be impractical, we can aim for partial or group separation, *e.g.* of all the *trans*- and all the *cis*-iso-α-acids together. The complete separation of the *cis–trans* pairs derived from each α-acid would even be better, as this can be related to hop origin and quality. This last aim can be partly achieved on octadecyl-silica gel by using a buffer, as shown in Fig. 4. The isoadhumulones are co-eluted with the isohumulones in Fig. 4. The analysis time is fairly long.

Although our interest was in isocratic analyses, in this instance it is worth mentioning what can be achieved by judicious gradient elution. Such an optimized separation of a large number of six- and five-membered hop and beer bitter acids is shown in Fig. 5.

It must also be emphasized that the octadecyl-silica gel must be of high quality in order to be able to obtain the separations shown here and that it must especially have a very low concentration of trace metal impurities. Trace metals can



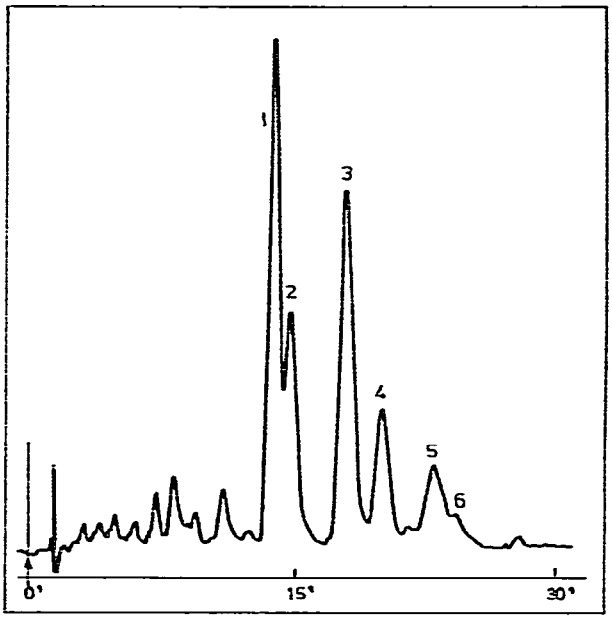


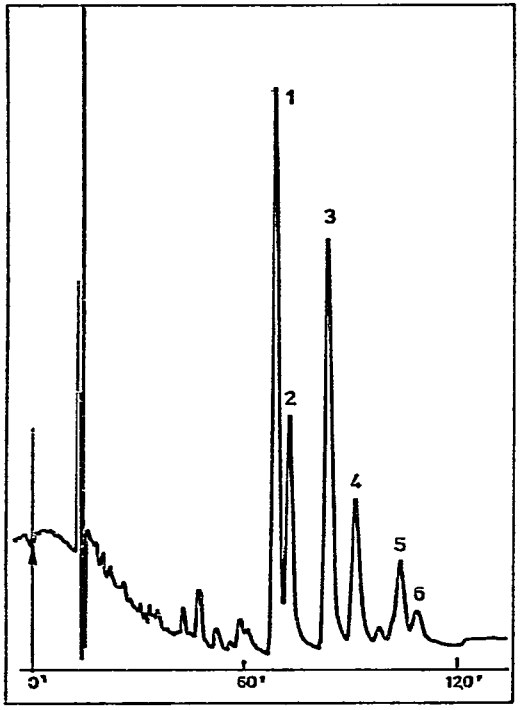
Fig. 1. Reversed-phase chromatogram of a synthetic mixture of hop bitter acids. Eluent: methanol–water–85% orthophosphoric acid (90:10:0.25) at 2 ml/min and 160 kg/cm². Detection wavelength: 280 nm. Peaks: 1 = deacylated anti-isohumulone; 2 = *trans*-humulinic acid; 3 = *trans*-isohumulone; 4 = humulone; 5 = *exo*-tricyclooxycolupulone; 6 = colupulone; 7 = lupulone; 8 = hexahydrocolupulone.

Fig. 2. Reversed-phase chromatogram of isomerized hop extract. Eluent: methanol–0.5 *M* citric acid buffer, pH 3.0 (60:40) at 100 ml/h and 250 kg/cm². Detection wavelength: 280 nm. Isomerized hop extract showing the six major iso-α-acids mentioned in the text in the sequence 1 = *cis*-isocohumulone, 2 = *trans*-isocohumulone, 3 = *cis*-isohumulone, 4 = *trans*-isohumulone, 5 = *cis*-isoadhumulone and 6 = *trans*-isoadhumulone.

be removed from the reversed-phase material by repeated boiling in 1 *N* or stronger hydrochloric acid–methanol (1:1) mixtures, followed each time by extensive washing with methanol[5]. For identification purposes the elution sequence and relative retention times in Table I are useful.

Ion-pair chromatography

Whitt and Cuzner[6] developed an ion-pair chromatographic group separation of the isohumulones and the isocohumulones. The isoadhumulones are co-eluted with the isohumulones. Whitt and Cuzner[6] used 0.005 *M* tetrabutylammonium phosphate and a methanol gradient from 50 to 60%. This separation, which takes only 8–10 min, looks promising for the routine HPLC analysis of the beer bitter acids.



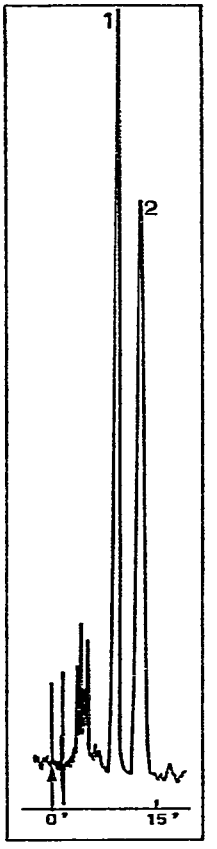


Fig. 3. Reversed-phase chromatogram of isomerized hop extract on a 1-m column in four 25 × 0.2 cm sections. Solvent as in Fig. 2 at 10 ml/h and 360 kg/cm². Peaks as in Fig. 2.

Fig. 4. Reversed-phase chromatogram of isomerized hop extract. Eluent: methanol–0.5 *M* citric acid buffer, pH 4.0 (60:40) at 100 ml/h and 250 kg/cm². Detectionwavelength: 280 nm. Peaks: 1 = *cis*- and *trans*-isocohumulone; 2 = the four other *iso*-α-acids.

We have studied systematically changes in the nature (cetyltrimethylammonium, tetrabutylammonium, tetraethylammonium, tetramethylammonium and ammonium) and concentration (0.1, 0.01, 0.005 and 0.001 *M*) of the counter ion. The retention decreased with size and concentration, but no substantial changes in resolution were noted. The selectivity, however, was sometimes dependent on very small changes in eluent composition. With the system methanol–water–85% orthophosphoric acid (72.5:27.5:1 –0.02 *M*) tetrabutylammonium salt the chromatogram shown in Fig. 6 is obtained isocratically (β-phenylchalkone was used as a possible internal standard).

The isoadhumulones were separated from the isohumulones. The slightly longer analysis time was compensated for by the separation of the isoadhumulones from the other two pairs. The difference from the Whitt and Cuzner[6] procedure is thus that the separation is run isocratically and that three peaks (for each pair of

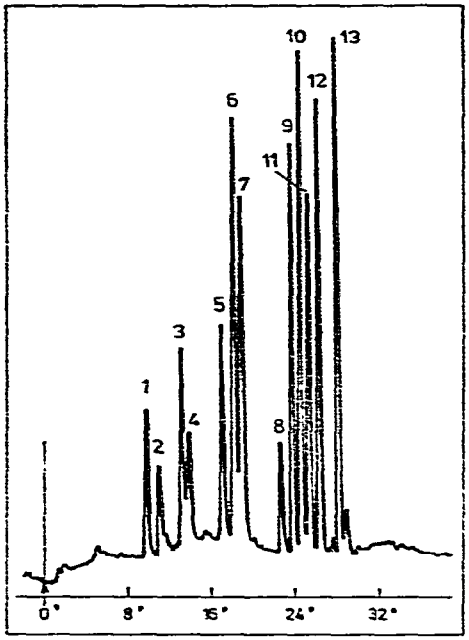


Fig. 5. Reversed-phase gradient chromatography of a synthetic mixture of hop and beer bitter acids. Eluent: from methanol–water–85% orthophosphoric acid (50:50:0.5) to methanol–85% orthophosphoric acid (100:0.5) in 30 min at 2 ml/min and with pressure from 300 to 150 kg/cm². Detection wavelength: 280 nm. This trace can be produced only with very pure components of the eluent on a suitable demineralized reversed-phase material. Peaks: see Table I.

cis–trans-iso-α-acids) are obtained instead of two. For routine beer analysis this is considered to be important.

Normal-phase chromatography

Silica gel. Gill[7] has used di-*n*-butylammonium acetate as an ion-pair former

TABLE I

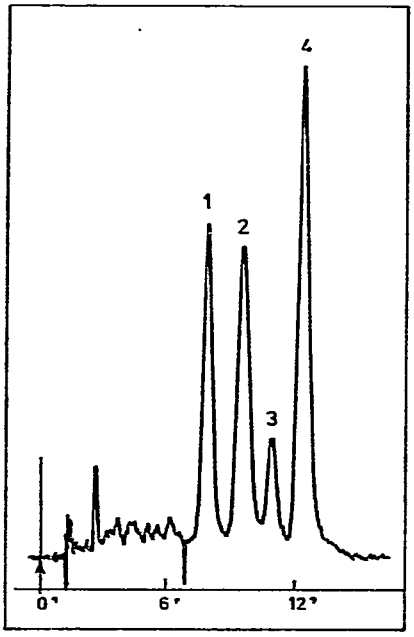
RETENTIONS RELATIVE TO HUMULONE FOR HOP AND BEER BITTER ACIDS IN THE GRADIENT CONDITIONS IN FIG. 5

Peak No.	*Compound*	*Relative retention time*
1	Deacylated *anti*-isohumulone	0.410
2	*trans*-Cohumulinic acid	0.465
3	*trans*-Humulinic acid	0.550
4	*trans*-Adhumulinic acid	0.595
5	*trans-allo*-Isohumulone	0.718
6	*trans*-Isohumulone	0.769
7	*trans*-Isoadhumulone	0.785
8	Cohumulone	0.957
9	Humulone	1.000
10	*exo*-Tricyclooxycolupulone	1.056
11	Colupulone	1.073
12	Lupulone	1.111
13	Hexhydrocolupulone	1.188

in chloroform–light petroleum as the carrier solvent and with silica gel as the stationary phase. The separation of the iso-α-acids was incomplete, however. Buffered silica gel has been studied extensively for the separation of hop bitter acids[8,9], and it is possible to separate the six iso-α-acids in this way. The three *cis*-iso-α-acids elute before the three *trans*-iso-α-acids. Chromatograms of this separation can be found in the references cited. Buffered silica gel is not easy to work with, however.

The reproducibility and lifetime of the columns are unsatisfactory. It is almost impossible to keep the water content of the columns constant. With progressive drying out of the column, the resolution of the iso-α-acids decreases. At some stage (not specifically investigated) it becomes possible to elute all *trans*- and all *cis*-iso-α-acids together in seemingly well resolved peaks (Fig. 7). This result is important in relation to the isomerization procedure leading to synthetic or natural iso-α-acids, and the *cis*/*trans* ratio is dependent on this factor. In our hands, however, normal-phase liquid chromatography with buffered silica gel did not seem suitable for the routine determination of the iso-α-acids.

Nitro-silica gel. Nitro-silica gel (in this case we also used Nucleosil 5 μm NO_2)



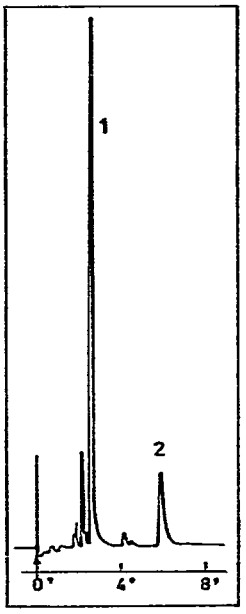


Fig. 6. Ion-pair chromatography of an evaporated isooctane extract of beer containing the six major iso-α-acids. Eluent: prepared by mixing 72.5 ml of methanol, 27.5 ml of water, 1.29 g of 40% tetrabutylammonium hydroxide (making the mixture 0.02 *M*) and 1 ml of 85% orthophosphoric acid; flow-rate, 120 ml/h. Detection wavelength: 280 nm. Peaks: 1 = *cis*- and *trans*-isocohumulone; 2 = *cis*- and *trans*-isohumulone; 3 = *cis*- and *trans*-isoadhumulone; 4 = β-phenylchalkone as possible internal standard.

Fig. 7. Normal-phase partition chromatogram of isomerized hop extract. Fairly dry 5-μm RSiL buffered with citric acid–potassium hydroxide buffer at pH 3.0. Eluent: 10% diethyl ether in isooctane at 100 ml/h. Detection wavelength: 280 nm. Peaks: 1 = the three *cis*-iso-α-acids; 2 = the three *trans*-iso-α-acids. This separation is heavily dependent on the state of hydration and is very difficult to reproduce.

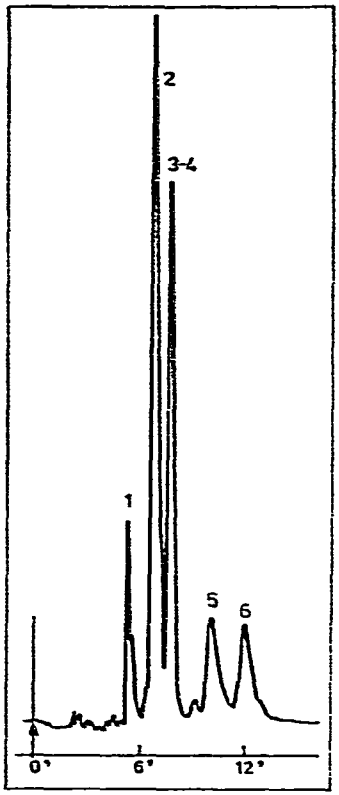


Fig. 8. Adsorption chromatography of isomerized hop extract on Nucleosil 5 μm NO_2. Eluent: isooctane–0.2% isopropanol at 120 ml/h. Detection wavelength: 280 nm. Peaks: 1 = *cis*-isoadhumulone; 2 = *cis*-isohumulone; 3 = *cis*-isocohumulone; 4 = *trans*-isoadhumulone; 5 = *trans*-isohumulone; 6 = *trans*-isocohumulone.

is obtained by derivatization of silica gel with a nitrophenyl group. This phase is very polar and is intended to be used in the normal-phase mode. This leads to the usual difficulties of a substantial influence of trace concentrations of small polar molecules (modifiers, water) and the great difficulty of keeping retention times and selectivity

TABLE II

INFLUENCE OF ISOPROPANOL CONCENTRATION IN ISOOCTANE ON THE RETENTION OF ISO-α-ACIDS

k' values calculated against solvent peak.

Isopropanol concentration, C%	k'*					
	cis-ad	*cis-h*	*cis-co*	*trans-ad*	*trans-h*	*trans-co*
1	1.35	1.40	1.55	1.65	1.75	1.95
0.5	2.25	2.35	2.60	2.95	3.15	3.50
0.25	4.00	4.35	4.80	5.75	6.30	7.00
0	Long					

* ad = isoadhumulone; h = isohumulone; co = iso-cohumulone.

constant. By first washing the column with methanol–water–85% orthophosphoric acid and then with methylene chloride, before applying isooctane–methylene chloride–isopropanol as the eluting solvent, the trace shown in Fig. 8 can be produced for a relatively short number of analyses. The retention of the iso-α-acids increases gradually and becomes excessive.

Cyanopropyl-silica gel. To compensate for the disadvantages of buffered silica gel and very polar nitro-silica gel, we examined a cyanopropyl derivatized silica gel (RSiL-CN). Isooctane, methylene chloride or their mixtures always gave tailing peaks. By washing with methanol–water–85% orthophosphoric acid (90:10:1) followed by methylene chloride, and then applying isooctane–isopropanol, a fairly good separation was obtained. The influence of the isopropanol concentration was studied (Table II).

Separations with 0.2% isopropanol in isooctane of a mixture of iso-α-acids and of an isomerized extract are shown in Fig. 9.

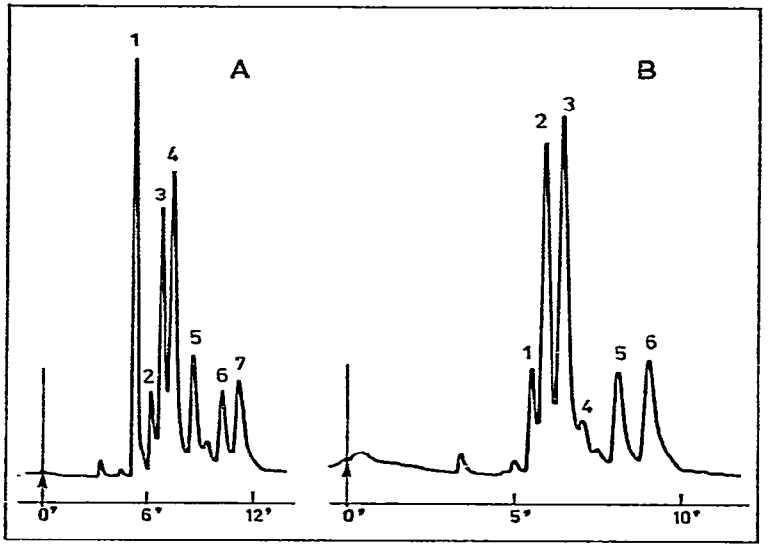


Fig. 9. (A) Adsorption chromatography on RSiL-CN of a synthetic mixture of bitter acids. Eluent: isooctane–0.2% isopropanol at 120 ml/h and 60 kg/cm². Detection wavelength: 280 nm. Sequence of elution: 1 = chalkone; 2 = *cis*-isoadhumulone; 3 = *cis*-isohumulone; 4 = *cis*-isocohumulone; 5 = *trans*-isoadhumulone; 6 = *trans*-isohumulone; 7 = *trans*-isocohumulone. (B) The same as in A but on isomerized hop extract and with isooctane–0.25% isopropanol. Peaks as in A.

The attractive feature of this method is that the counter pressure is very low compared with the usual pressure in reversed-phase liquid chromatography and therefore the analysis times can be controlled. The elution time can then easily be less than 10 min with virtually complete separation of the six iso-α-acids. The elution sequence is completely different from that on reversed-phase silica gel. Considering the low counter pressure, the smaller 5-μm packings could easily be used. We studied this in detail, but it was found that the typical difficulties of normal-phase liquid chromatography (reproducibility and influence of modifiers, as explained above) must be considered too great for cyanopropyl-silica gel be adopted in a routine method for iso-α-acids.

CONCLUSION

Although reversed-phase silica gel does not separate the six iso-α-acids completely, it is concluded that it is suitable for the routine analysis of beer bitter iso-α-acids. A modification of the Whitt and Cuzner[5] ion-pair technique, separating the isohumulones, isocohumulones and isoadhumulones, appears to be the best choice. Complete development of such a procedure is currently in progress[10].

ACKNOWLEDGEMENTS

Financial aid to the laboratory from the Ministerie voor Wetenschapsbeleid and the Nationaal Fonds voor Wetenschappelijk Onderzoek —NFWO, is gratefully acknowledged. One of us (C.D.) thanks the Instituut voor Wetenschappelijk Onderzoek in Nijverheid en Landbouw —IWONL for a doctorate study grant. We thank Heineken Breweries for their continued interest in and support of our hop and beer research.

REFERENCES

1 M. Verzele and M. De Potter, *J. Chromatogr.*, 166 (1978) 320.
2 M. Verzele, in J. Pollock (Editor), *Brewing Science*, Academic Press, New York, London, 1979, p. 279.
3 L. De Taeye, D. De Keukeleire and M. Verzele, *Tetrahedron*, 35 (1979) 489.
4 J. Kraak, H. Poppe and F. Smedes, *J. Chromatogr.* 122 (1976) 147.
5 M. Verzele, M. De Potter and J. Ghysels, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 3 (1979) 151.
6 J. Whitt and J. Cuzner, *Proc. Amer. Soc. Brew. Chem.*, 37 (1979) 13.
7 R. Gill, *J. Inst. Brew.*, 85 (1979) 15.
8 F. Slotema, L. Verhagen and M. Verzele, *Brauwissenschaft*, 5 (1977) 145.
9 R. Schwarzenbach, *Proc. Amer. Soc. Brew. Chem.*, 37 (1979) 180.
10 C. Dewaele and M. Verzele, in preparation.