Journal of Chromatography, 244 (1982) 321–326
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,891

ANALYSIS OF BEER ISO-α-ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITHOUT SAMPLE PRE-TREATMENT

M. VERZELE*, C. DEWAELE and M. VAN KERREBROECK
Laboratory for Organic Chemistry, State University of Ghent, Krijgslaan 281 (S.4) B-9000 Ghent (Belgium)
(Received March 11th, 1982)

SUMMARY

An isocratic high-performance liquid chromatographic analysis for beer iso-α-acids is described. No sample pre-treatment is necessary; beer is injected as such into the chromatograph. One analysis run takes 20 min. The relative standard deviation is 2.0%.

INTRODUCTION

Quantitation of the iso-α-acids in beer is currently achieved by direct photometry of a beer isooctane extract[1]. In our opinion complete separation between iso-α-acids and other isooctane-extractable compounds is needed for progress in this field. It is obvious today to turn to high-performance liquid chromatography (HPLC) for this purpose. Such an HPLC procedure was recently developed in our laboratory[2], and consists in isocratic elution of an evaporated isooctane extract on an octadecyl-silica gel column.

For routine analysis in the brewing industries, it would be useful to avoid time-consuming sample pre-treatment such as the extraction mentioned above. Methanol–water gradient elution [from methanol–water (1:9) to 100% methanol; all solvents containing 0.5% orthophosphoric acid] of beer samples as such on an octadecyl stationary phase gives a complex pattern. Most of the peaks detected by UV (270–280 nm) have not previously been identified. They can be correlated with polyphenolic compounds. Using an octadecyl-silica gel phase free of traces of metals, a group of three peaks appears at the end of the chromatogram (Fig. 1). As a result of earlier work on beer bitter acids, we expected these to be the iso-α-acids. Co-chromatography with these compounds confirmed this.

The analysis of iso-α-acids using beer directly as the sample must be possible on a high-quality reversed-phase column. Such a method for the iso-α-acids in beer has been developed in this work.

EXPERIMENTAL

A Varian LC-5020 liquid chromatograph was used with a Varian UV-50 vari-

0021-9673/82/0000–0000/$02.75

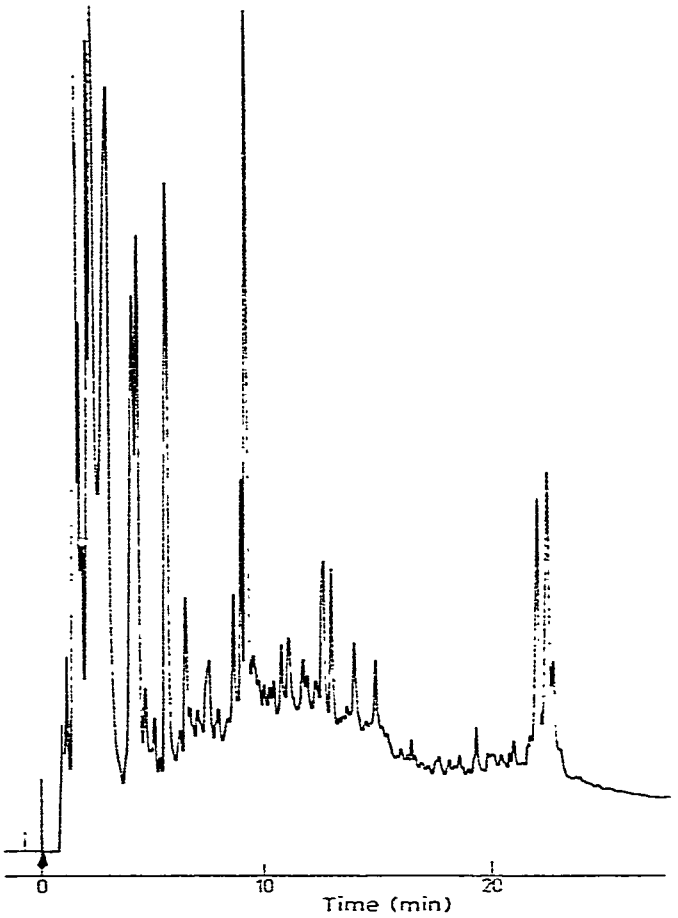

Fig. 1. Chromatogram obtained with a Varian LC-5020 chromatograph, a Varichrom detector (280 nm; attenuation 0.2), a Valco 7000 injector with a 50-μl sample loop and a 25 × 0.46 cm I.D. column made of Lichroma tubing, packed with 10-μm ROSiL-C_{18}-D. Elution at 1 ml/min with a methanol–water gradient from 1:9 to 100% methanol, both solvents containing 0.5% of orthophosphoric acid. Sample: 50 μl of beer.

able-wavelength detector. The detector wavelength setting has to be calibrated as it is our experience that variable-wavelength detectors can easily be a few nanometres off the correct value. For quantitation at a chosen wavelength, calibration is therefore essential. An easy way to do this is to turn the wavelength selector with a suitable solution of naphthalene in isooctane in the detector cell. The very sharp, intense extinction peak at 311 nm is easily detected and can be compared with the wavelength selector reading[3].

Calibration is effected with a 2-nm slit width on the detector and the actual analyses with an 8-nm slit width. The chromatograms were both plotted and integrated by a Varian Vista Data system. The Vista 401 includes a printer/plotter which plots chromatograms during a chromatographic run and then prints out a report at the end of the run. A floppy disk memory provides data storage on removable disks. Analytical methods, measured peak data, calculated results and chro-

matograms can be saved. The chromatograms can be re-plotted and re-integrated and results computed. Baselines can be included on the re-plots.

Although this sophisticated equipment greatly improves the ease of operation, we should emphasize that the analysis can be run on any HPLC instrument with a variable-wavelength detector and an electronic integrator.

The column was a Lichroma 25 × 0.46 cm I.D. tube with Valco fittings. The packing material is most important: it must be a high-efficiency material and especially free from traces of metals. A test for such trace metal activity was developed recently[4]. Silica gel can be made essentially metal-free by repeated boiling in concentrated hydrochloric acid followed by washing. Derivatization of such silica gel with octadecyl chains and deactivation with trimethylchlorosilane (TMCS) does not lead, however, to a material that passes the trace metal test[4]. On such a column the iso-α-acids are not visible under the conditions used in Fig. 1. The bonded phase itself has to be boiled twice with a mixture of concentrated hydrochloric acid and methanol to make it suitable for the analysis of iso-α-acids. We explain these facts by assuming that the octadecylsilane is itself contaminated by traces of metals. During the acid boiling procedure the phase loses some octadecyl chains, as shown by foaming of the mixture. Subsequently the phase has to be deactivated again with TMCS. This step does not seem to introduce traces of metals. We then use 5-μm ROSiL-C_{18}-D or spherical octadecylated and deactivated RSL 5-μm silica gel. This is then boiled twice with hydrochloric acid–methanol and deactivated again as explained above. This material, when packed correctly, will produce 20,000–25,000 theoretical plates for polycyclic aromatic compounds in a 25 × 0.46 cm I.D. column even with a classical detector cell; the time constant of the detector must be 0.5 sec or lower, however[5]. (Ready-made columns for this analysis are available from Altech Europe-RSL). Further chromatographic conditions are given in the legends to the figures.

The solvent system can be varied so as to give further separation of the six iso-α-acids but this leads to integration difficulties. To avoid these, it is advisable to suppress partial separation of the *cis–trans* iso-α-acid pairs. This is achieved with the chosen solvent composition. First we tried to find a suitable beer-soluble internal standard, but as we failed we resorted to the external standard method. The standard is injected first, followed immediately by the beer sample. With such a procedure it is not necessary to provide each individual beer sample with an internal standard. We used one Valco 7000 p.s.i. injector provided with a 25-μl sample loop, but for automated operation two Valco injectors can be used in series or possibly the new ten-port sampling valves can be adopted for this purpose. An external standard that satisfies the requirement of elution close to the iso-α-acids is 2,6-di-*tert.*-butylphenol. The commercial product was purified by recrystallization from isooctane. The concentration in methanol–water (75:25) was about 6 mg per 100 ml. The stability of the external standard solution was checked over several weeks and at several concentrations by chromatography against a suitable naphthalene solution; no deviations were observed.

The iso-α-acids of beer consist of three pairs of *cis–trans* isomers. Under the conditions of the analysis *cis-* and *trans*-isocohumulone elute together in the first peak, *cis-* and *trans*-isohumulone elute in the second peak and the third peak is then *cis-* and *trans* isoadhumulone. The fourth peak in the example in Fig. 2 is then the external standard, 2,6-di-*tert.*-butylphenol. The molar absorptivities of the six iso-α-

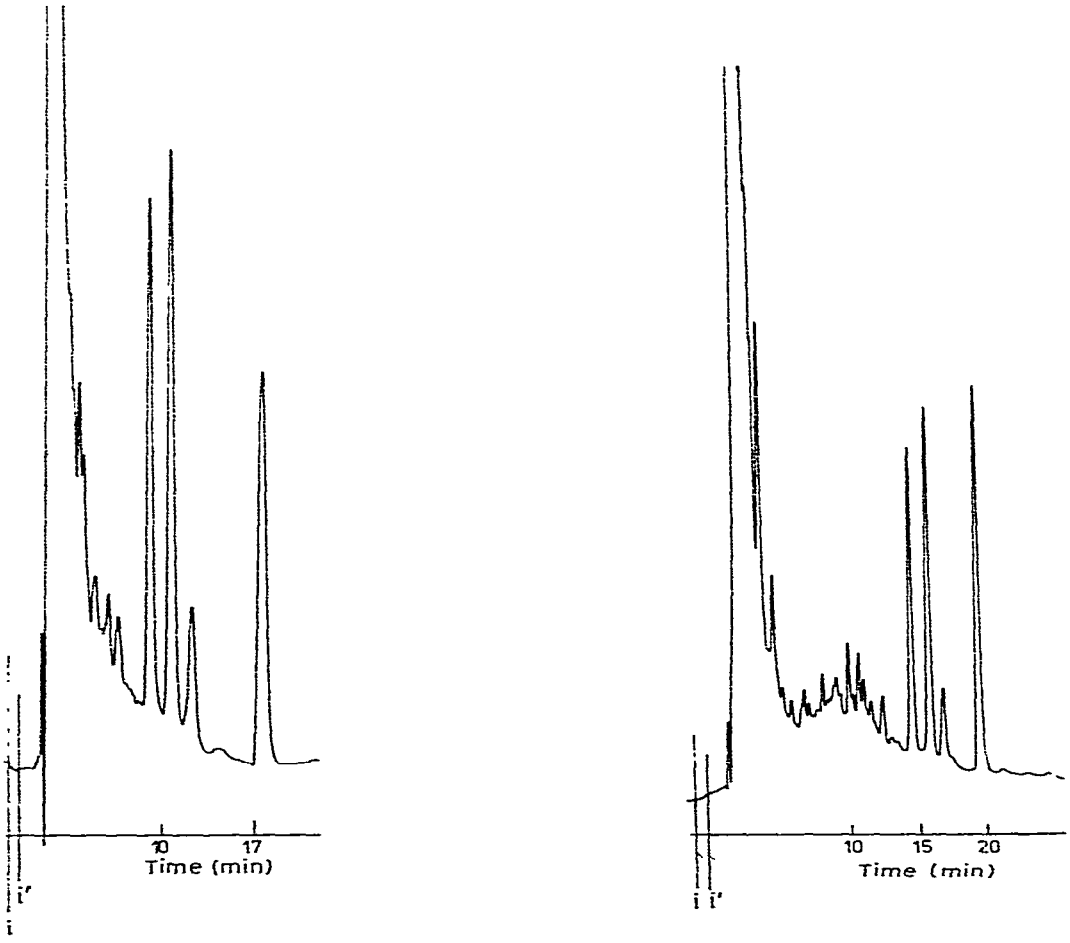

Fig. 2. Chromatogram obtained under the same conditions as in Fig. 1, except detection at 270 nm (attenuation 0.05), a 25-μl sample loop, 5-μm ROSiL-C_{18}-D, and isocratic elution with methanol–water (80:20) containing 13 g/l of 40% tetrabutylammonium hydroxide and 2% of 85% orthophosphoric acid. At point i, injection of external standard, 25 μl of 2,6-di-*tert.*-butylphenol (6.69 mg in 100 ml of methanol–water (75:25). At point i', injection of 25 μl of beer. The last peak is the external standard; the three peaks before it are the three *cis-trans* pairs of iso-α-acids.

Fig. 3. Chromatogram obtained under the same conditions as Fig. 2, except att. 0.1 and elution at 1 ml/min with a methanol–water gradient from 60% to 83% methanol (containing 1% of orthophosphoric acid) in 5 min. At point i, injection of external standard, 25 μl of 2,6-di-*tert.*-butylphenol (6.69 mg in 100 ml of methanol–water, 75:25). At point i', injection of 25 μl beer. The gradient starts at point i'. The last peak is the external standard; the three peaks before it are the three *cis–trans* pairs of iso-α-acids.

acids differ but, as explained previously[6], these differences are minimized by measuring at 270 nm. Errors resulting from this effect and from variations in the iso-α-acid composition are unavoidable. The small isoadhumulones peak introduces the greatest integration error. If it could be forced under the isohumulones peak this weakness would be avoided. We have made considerable efforts to find conditions under which the isohumulones and isoadhumulones could be co-eluted, but were

unsuccessful. A tendency in the right direction is obtained by increasing the orthophosphoric acid concentration. The time element is also important, of course. We tried to use shorter analysis times but without success so far, as the reproducibility of the resolution and integration is adversely affected. We realise, of course, that the brewing industry would be strongly in favour of shorter, analytical runs, *e.g.* less than 10 min for a potentially routine on-stream analytical technique.

We also investigated whether gradient elution would improve the method. The optimized result with a gradient is shown in Fig. 3. Although the separation looks better, the relative standard deviation, not surprisingly, is even greater than with the isocratic procedure (*ca.* 2.5–3.0% compared with 2%). Gradient elution requires a longer time for re-equilibrating the column after each run. Moreover, the solvent quality requirements are very stringent. Further details on this gradient elution technique are given in the legend to Fig. 3.

The main reason for the high standard deviation in both procedures is the double injection or the use of the external standard technique. It is therefore important to continue to search for a method for the analysis of iso-α-acids that is faster and uses the internal standard technique. More efficient HPLC is therefore the answer and we are working towards this goal.

CALCULATIONS

A calibration equation was established by plotting the ratios of weight and peak area of the external standard to those of *trans*-isohumulone. The latter compound was obtained by photoisomerization of humulone[7]. Great care was taken to ensure the purity of both humulone and *trans*-isohumulone. Unfortunately, these compounds are so unstable to air oxidation that even after a few days, even in the freezer, the molar absorptivities change.

The equation established for *trans*-isohumulone is $y = 0.195x + 0.004$, where y is the ratio of the concentrations of the two compounds and x is the ratio of the peak areas. For lack of anything better, the same equation is used for all iso-α-acids. We have shown earlier, however, that this is acceptable[6].

Each peak area is converted into concentration in parts per million as follows:

$$\text{Concentration in peak (ppm)} = \left(0.195 \cdot \frac{\text{area iso peak}}{\text{area standard}} + 0.004\right) \cdot 66.9$$

In this example the concentration of the external standard was 66.9 mg/l. The total content of iso-α-acids in beer is then found by adding the results for the three peaks.

The relative standard deviation for a typical series of results is given in Table I.

The concentration of iso-α-acids in some commercial beers as determined with the present method was compared with the results obtained by direct photometry on an isooctane extract. For the latter determination, acidified beer was extracted with 2.5 times its volume of isooctane. The absorbance at 276 nm was converted into iso-α-acid concentration by means of the equation

$$\text{Iso-α-acids (mg/l)} = (28.6 \cdot 2.5 \cdot A) - 5.8$$

TABLE I

CONCENTRATIONS OF ISO-α-ACIDS IN A COMMERCIAL BEER

	Results of replicate determinations (mg/ml)	
	28.52	27.91
	27.56	28.44
	29.27	27.93
	29.28	28.93
	28.34	28.38
Average:	28.46	
Standard deviation:	0.574	
Relative standard deviation:	2.01%	

where A is absorbance. The results are given in Table II.

The differences between the two methods (confirmed by many other measurements) range from only 3% to nearly 25%. This is most disturbing and demands further research. In any case, according to HPLC beers contain larger amounts of iso-α-acids than was hitherto suspected. Most intriguing is also the observation that bitterness as evaluated by tasting does not seem to correlate well with the HPLC results. Result A, for example, is for a French beer generally considered to be much milder in taste than the others.

TABLE II

COMPARISON OF RESULTS OBTAINED BY HPLC AND DIRECT PHOTOMETRY

Beer sample	*Iso-α-acids (mg/ml)*	
	HPLC	*Direct photometry*
A	26.43	20.95
B	25.64	22.36
C	27.49	26.56
D	28.57	25.79
E	32.91	26.23

ACKNOWLEDGEMENTS

C. D. and M. V. K. thank the Instituut voor Wetenschappelijk Onderzoek in Nijverheid en Landbouw (IWONL) for a Dr.Sc. preparation grant. The authors thank the Ministerie voor Wetenschapsbeleid, the Nationaal Fonds voor Wetenschappelijk Onderzoek (NFWO) and the IWONL for financial aid.

REFERENCES

1 *Methods of Analysis*, American Society of Brewing Chemists, St. Paul, MN, 7th ed., 1976, Beer —23. A and B.
2 M. Verzele and C. Dewaele, *J. Amer. Soc. Brew. Chem.*, 59 (1981) 67.
3 M. Verzele, J. van Dyck and H. Claus, *J. Inst. Brew.*, 86 (1980) 9.
4 M. Verzele and C. Dewaele, *J. Chromatogr.*, 217 (1981) 399.
5 M. Verzele, J. van Dyck, P. Mussche and C. Dewaele, *J. Liquid Chromatogr.*, in press.
6 M. Verzele, H. Claus and J. van Dyck, *J. Inst. Brew.*, 84 (1978) 218.
7 B. Clarke and R. Hildebrand, *J. Inst. Brew.*, 71 (1965) 26.