ACTA CHEMICA SCANDINAVICA 16 (1962) 1586–1592

An Apparatus for Partition Chromatography and its Application to the Separation of the Bitter Substances of Hops

LARS-OLOV SPETSIG

Forskningslaboratoriet LKB, Bromma, Sweden

Reversed-phase partition chromatography has proved useful for the separation of hop bitter substances which are then estimated by ultraviolet spectrometry. But hand collection of fractions and determination of ultraviolet absorption are time-consuming and costly. An apparatus has therefore been constructed which measures and records the optical density of the eluant by means of a spectrophotometer connected to a recorder. The apparatus further consists of a chromatographic column with devices of gradient elution and for treating the influent with oxygen-free nitrogen. The elution is carried out with buffer solutions of continuously increasing pH, the value of which is also recorded. The eluted substances may be characterised by their pH of elution, which also enables a rough estimation of the solubility of the substances. Their relative bitterness may be determined after isolation from the eluate. For further characterisation and identification UV and IR spectrometry as well as mass spectrometry have been tried. To facilitate the collection of the samples the tube changes are noted by the recorder.

In 1955, we tried to apply the reversed-phase partition chromatography method of Silk and Hahn [1] to the analysis of humulones, a group of hop bitter substances. The column was packed with kieselguhr treated with dichlorodimethylsilane to make it non-wettable by polar solvents. To begin with, carbon tetrachloride was used as stationary solvent and aqueous methanol as eluant [2]. It soon became evident, however, that chloroform and buffer solutions containing 25 % methanol were superior as solvents [3]. By means of a mixing device gradient elution could then be performed, the pH value of the eluant being raised from pH 3 to pH 11. The separated substances could most easily be estimated by measuring the optical density of the eluate fractions.

But in applying this technique, the collecting of large numbers of fractions and the determination of their ultraviolet absorption were found to be very time-consuming. Furthermore a slow autoxidation of the substances in the fraction collector tubes leads to somewhat erroneous results. An apparatus for automatic absorption measurement was therefore needed. A paper had been published on the use of such a device for measuring and recording the optical

density of eluates continuously and simultaneously at four wavelengths [4]. This apparatus was interesting in principle but seemed too elaborate for our purpose and a simpler construction was worked out, which has now been in use for five years.

This paper will give a detailed description of this apparatus, which automatically measures the pH value of the eluate and its optical density at one wavelength by means of a pH-meter and a spectrophotometer, respectively, connected to a recorder. The application of the apparatus to reversed-phase partition chromatography of hop bitter acids with a pH gradient elution technique is discussed with illustrative examples.

APPARATUS

Most of the apparatus is mounted in a table-type cabinet. On top of the table are placed the column assembly inside a metal shield, the spectrophotometer and the pH-meter. The recorder is placed inside the cabinet as is also the fraction collector, which is thus protected from light. In the cabinet there is also a thermostat, from which water is pumped through the column jacket. A control panel is placed on the rear side of the table.

Column assembly. For analytical purposes a 800 × 8 mm column is used which may be loaded with 20—40 mg of bitter substances. Fig. 1 shows schematically the various Pyrex components of the column assembly. The adapter *A* with a medium fritted-glass disc serves as a support to the packing of the column *B*. On top of the column is placed

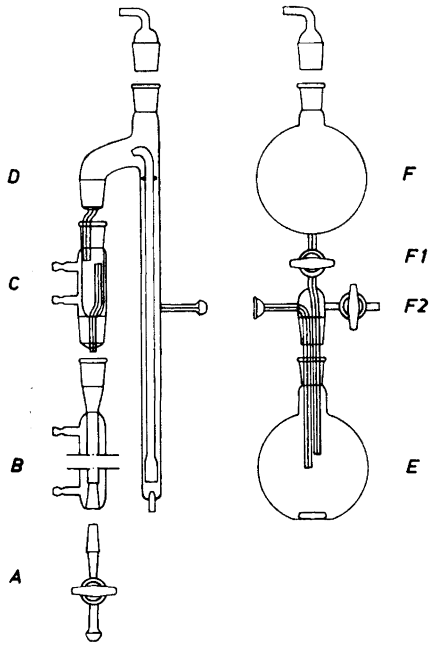

Fig. 1. Column assembly and gradient elution device.

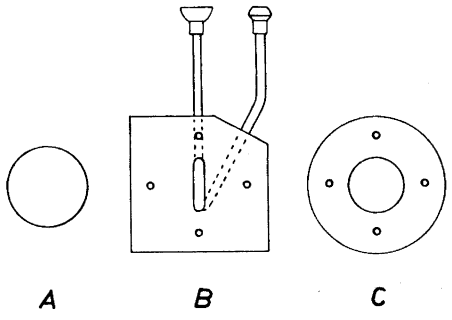

Fig. 2. Absorption cell.

another adapter *C*, in which the eluant is passed through a layer of chloroform for final saturation. Both column *B* and adapter *C* are provided with water jackets. The top piece of the adapter *C* is connected to a gas lift *D*, where the influent is blown with oxygen-free nitrogen to exclude dissolved oxygen, and then transported to the adapter *C*. The gas leaving the apparatus may be led through a series of small mercury-filled gas washing bottles serving as manostats.

For preparative purposes a column of 800 × 25 mm is used which can be loaded with more than 300 mg of bitter substances.

Gradient elution system. This system is composed of a mixing chamber *E* and a feeder *F* (Fig. 1). The mixing chamber is equipped with a magnetic stirrer and contains a buffer solution of low pH and a layer of chloroform. It may be hermetically sealed by means of the stopcock F2. The solution leaving the mixing chamber is replaced by a buffer solution of high pH value stored in the feeder. The pH of the eluant thus rises continuously during the elution, giving a gradient which is a function of the amount and composition of the two solutions. [5] Before filling, both mixing chamber and feeder are flushed with oxygen-free nitrogen.

Absorption cell. The demountable type absorption cell is composed of two optically polished fused silica windows *A* and a 5 mm stainless steel spacer *B* (Fig. 2). The windows are clamped tightly in place by means of stainless steel plates *C* and screws. Two stainless steel tubes with spherical joints form the inlet and outlet tubes of the cell, which is mounted on an extra cover of the spectrophotometer cell compartment.

Spectrophotometer and recorder. A line-operated Hilger Uvispek spectrophotometer connected to a Honeywell Electronik multichannel recorder is used. As the recorder deflections were found to be nonlinear, they have been calibrated by means of an alkaline solution of potassium chromate as described by Haupt. [6]

pH-Meter. The pH value of the eluant leaving the cell is measured by a small combined glass and calomel electrode connected to a Metrohm pH-meter and the recorder.

Fraction collector. The eluate may be fractionated by a fraction collector operated by either a timer or a siphon device. The impulse causing the fraction collector to advance one step also actuates a shutter in the exit opening of the spectrophotometer lamphouse. The shutter is kept closed for one minute by means of a time delay relay. During this period the line of 0 % transmission is recorded. This enables corrections to be made for the slight temperature drift of the spectrophotometer. In addition, the tube changes are noted in the record, which greatly simplifies the picking out of tubes for further investigation.

Nitrogen purification train. The eluant is kept free of dissolved oxygen with purified tank nitrogen. A lift-pump scrubber containing copper ribbon and ammoniacal ammonium chloride solution, as described by Altieri [7], was originally used (*cf.* Vandenheuvel and Richardson [8]). Now amalgamated zinc and vanadium(II) sulphate [9] in dilute sulphuric acid are employed as purification agents. In both cases nitrogen of high purity is obtained.

ANALYTICAL PROCEDURE

Reagents. Mixing chamber solution for hop analysis. 5.4 g of citric acid and 3.4 g of monopotassium orthophosphate in 1 liter of 25 % methanol (v/v), saturated with chloroform.

Feeder solution for hop analysis. 3.1 g of ethylolamine, 5.3 g of diethylolamine. 7.5 g of triethylolamine and 2.0 g of sodium hydroxide in 1 liter of 25 % methanol.

Mixing chamber solution for beer analysis. 5.4 g of citric acid in 1 liter of 25 % methanol, saturated with chloroform.

Feeder solution for beer analysis. 8.9 g of disodium orthophosphate in one liter of 25 % methanol, saturated with chloroform.

Preparation of kieselguhr. Hyflo Super Cel is dried at 110°C, treated with dichlorodimethylsilane, washed with methanol and dried as described earlier[3].

Packing of column. 10—20 ml of chloroform is added slowly to 11 g of hydrophobic Hyflo Super Cel in 300 ml of 35—45 % (v/v) methanol in a top drive macerator. The proper amount of chloroform will depend on the duration of the silane treatment. The slurry is stirred gently for a quarter of an hour to expel entrapped air bubbles and is then transferred to a separating funnel equipped with a stirrer and connected to an 8 mm

column by a ground joint at its neck. The kieselguhr is permitted to settle slowly by gravity into the column, which is filled with air-free 35—45 % methanol saturated with chloroform.

Especially with 25 mm columns good results are also obtained when the packing of the column is carried out in a mixing chamber solution saturated with chloroform.

Preparation of samples. Hop extracts are prepared by macerating 10 g of hops with 150—300 ml of chloroform in a macerator and filtering off the pulp on a glass filter. Beer extracts are obtained by repeated extraction of decarbonised beer in a separatory funnel with chloroform (150 ml/1000 ml beer) after addition of hydrochloric acid to give pH<1 (*cf.* Spetsig *et al.* [3]). The extracts are then evaporated at a temperature not exceeding 30°C to a volume containing 20—40 mg of hop bitter substances or 10—20 mg of beer bitter substances in 0.05 ml.

Loading. The column is loaded by means of a special device consisting of a glass tube with a ground-in glass plunge into which a suitable amount of Hyflo Super Cel is weighed. A small amount of mixing chamber solution is added and the appropriate amount of the sample dissolved in 0.05 ml chloroform. After the mixture has been stirred thoroughly with a thin glass rod, the slurry is added quantitatively by pressing in the plunge to expel the mull into the column, where some of the packing has been sucked off to give a plain surface. The kieselguhr is then gently packed by means of a glass rod with a diskshaped foot. After addition of a small amount of the sucked-off packing over the sample, the column is ready for elution.

Elution. The elution is usually carried out with 1000 ml of eluant. For hop analysis 650 ml of mixing chamber solution is added, giving a gradient from pH 3 to pH 10.5. For beer analysis 1000 ml of mixing chamber solution gives a rise from pH 3 to pH 7, which is sufficient, since beer contains no bitter substances emerging at higher pH values.

Routine analytical procedure. The column mixture is prepared and the column packed as described above. After settling overnight the column is mounted in the apparatus and water of 30°C circulated through the jacket. Then the packing is slowly washed with the appropriate mixing chamber solution in a nitrogen atmosphere. After loading, the column is protected from light with a plastic sleeve and is filled with mixing chamber solution before the adapter is put in place. The adapter, too, is filled completely to exclude air. The gradient elution system, which has previously been flushed with nitrogen, is loaded with the appropriate amount of mixing chamber solution and chloroform and after turning the stopcocks to a closed position also with feeder solution. Nitrogen is then led through the gas lift and the space over the feeder solution. The absorption cell is rinsed with 25 % (v/v) methanol used as a standard through a side tube on the glass tubing connecting column and cell. The spectrophotometer and recorder are standardised at the wavelength chosen, usually 280 or 325 mμ. The stopcock connecting the feeder and the mixing chamber and the stopcock of the column can then be opened, the timer started and the elution begun. If necessary the gas pressure is increased by means of the manostat device to give an elution velocity of 200—400 ml a day. The extinction values are read from the records with a special measuring rule constructed with the aid of the deflection values obtained by calibration and are then plotted against the eluate volume. From the diagram the tubes containing the separated substances are sorted out and the ultraviolet spectra are determined. The compounds can then be extracted with chloroform, dissolved in dilute sodium bicarbonate and their taste tested.

The column may often be used repeatedly. The sample zone is then sucked off and the column is washed with feeder solution in a nitrogen atmosphere.

RESULTS AND DISCUSSION

The bitter substances in hops and beer may be extracted with a number of solvents, *e.g.* chloroform, benzene, methanol, carbon tetrachloride and iso-octane. The most efficient of these solvents is chloroform, which is thus suitable as the stationary solvent in reversed-phase partition chromatography of the bitter substances. Chloroform was therefore used in combination with buffer solutions, which, as eluants, allow an estimation of the solubilities of the acidic

bitter substances. Addition of 25 % methanol was found suitable for producing symmetrical peaks.

Preliminary experiments showed that the front peak and the residue left on the column after the elution contained no bittertasting substances. All the bitter substances must therefore be acids and included in the chromatogram.

With the procedure adopted, sufficient amounts (1—2 mg) of each substance could be isolated from the eluate by extraction with chloroform to make possible an evaluation of the relative bitterness, the most important property of a bitter substance from the practical point of view. Only a few hop substances found in the chromatogram were without a bitter taste. Chloroform extracts of beer gave a number of very small peaks belonging to non-bitter substances, but carbon tetrachloride and isooctane extracts contained mainly bitter compounds.

The pH value of elution proved to be a convenient property for characterising an eluted substance. This pH value is closely related to the solubility of the substance and enables a rough determination of its solubility product by comparison with the values earlier determined for humulinic acid, humulone and lupulone. Hops were found to contain substances over a very wide solubility range with solubility products varying from high values down to 10^{-6}. As beer contains no bitter substances eluted at higher pH values than pH 7, substances with lower solubility products than 10^{-3} are not soluble in wort in unchanged form. The most soluble substances which are first eluted are therefore in general the most interesting.

Another suitable means of characterisation was provided by the ultraviolet absorption curves. A number of peaks gave characteristic spectra with maxima from 325 to 400 mμ. But many substances had rather similar spectra with a maximum in the range 255—275 mμ. A suitable allround wavelength for the recording of the chromatogram was 280 mμ, while 325 mμ was used for special studies, *e.g.* on hulupones, humulones and lupulones. A prefractionation could sometimes be obtained when using a selective solvent such as isooctane for the extraction, which further simplified the study of individual substances.

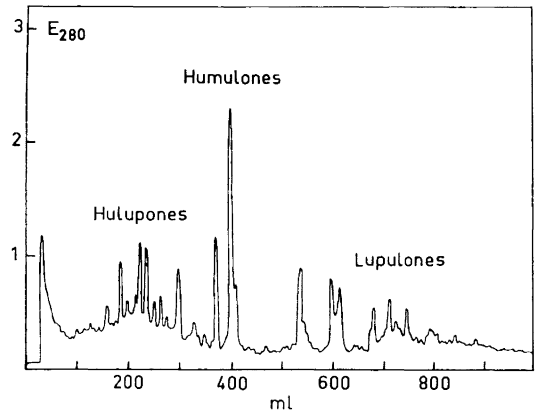

Fig. 3. Chromatogram of bitter substances from Hallertau hops.

The apparatus and procedure has been used to make a survey of all the bitter substances appearing in the brewing process and to study some of the most important of these substances in detail.

When extracts of hops were analysed an extensive resolution of the complex bitter substance mixture was obtained, as is seen from a typical chromatogram in Fig. 3. The chromatogram clearly shows two well-known groups of three substances, the humulones and lupulones. Among the forty or so hitherto unknown substances a further group of three substances named hulupones are included. Many of the other substances are probably oxidation products formed from humulones and lupulones by autoxidation during the storage of the hops. The relative heights of the peaks are altered accordingly as the hops grow older.

A chromatogram of beer bitter substances has a rather different appearance (Fig. 4). The most interesting feature is the group of five high peaks. Hitherto, beer has been supposed to contain as main bitter substances only three iso-humulones formed by rearrangement of the humulones during wort boiling. But the five peaks seem to be caused by six isocompounds, two from each humulone. In addition to these compounds beer is found to contain a number of minor substances, probably oxidation and rearrangement products of hop bitter substances.

Similar analyses have been made of bitter substances from spent hops, from technical extracts of spent hops and from trub. These products contained a mixture of the hop and the beer type bitter substances. A detailed report of all these substances will be given in a separate paper.

By the use of wide columns it became possible to separate individual bitter substances in 100 mg amounts or more for further study. The investigations were concentrated on the hulupones. By means of molecular spectrometry, a method which may very suitably be combined with chromatography, a determination of their structure has been possible [11]. Some of their derivatives have likewise been studied.

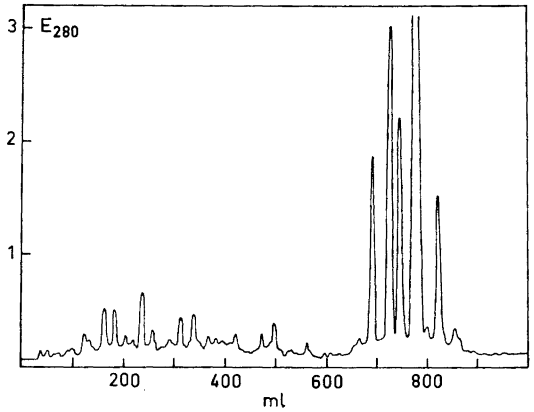

Fig. 4. Chromatogram of bitter substances from Pilsener beer.

The six rearrangement products of the humulones appearing in beer have been isolated for structure determination in combination with a kinetic study of the rearrangement. This investigation will be reported in a separate paper.

Though the method gave good qualitative results, only a semiquantitative determination of bitter substances was possible. The more slowly the elution was carried out, the smaller the areas of the peaks in the last part of the hop chromatograms were found to be. No doubt these substances were slowly oxidised in spite of the nitrogen treatment of the eluate. With a short column and stepwise elution, however, it was possible to obtain in a few hours a number of fractions whose content of substances with characteristic ultraviolet spectra could easily be determined. By a proper choice of the pH values of the eluants in the light of the results of the qualitative analyses, the main components of the bitter substance mixture could be determined with a high degree of accuracy [12].

Acknowledgement. It is gratefully acknowledged that this work has been supported by the Stockholm Brewing Company, Stockholm, as part of its research programme on hops carried out over a period of some years. Thanks are also due to Mr. J. Engblom and the workshop personnel of Forskningslaboratoriet LKB for building the apparatus, and for service and maintenance work.

REFERENCES

1. Silk, M. L. and Hahn, H. H. *Biochem. J.* **56** (1954) 406.
2. Spetsig, L.-O. and Steninger, M. *J. Inst. Brewing* **62** (1956) 333.
3. Spetsig, L.-O., Steninger, M. and Brohult, S. *Proc. European Brewery Conv.* Copenhagen 1957, p. 22.
4. Deutsch, A., Zuckerman, R. and Dunn, M. S. *Anal. Chem.* **24** (1952) 1763.
5. Drake, B. *Arkiv Kemi* **8** (1955) 1.
6. Haupt, G. W. *J. Research Natl. Bur. Standards* **48** (1952) 414.
7. Altieri, V. J. *Gas analysis*, Am. Gas Assoc. New York 1945, p. 261.
8. Vandenheuvel, F. A. and Richardson, G. H. *J. Am. Oil Chem. Soc.* **30** (1953) 104.
9. Meites, L. and Meites, T. *Anal. Chem.* **20** (1948) 984.
10. Rigby, F. L. and Bethune, J. L. *Proc. Am. Soc. Brew. Chem.* **1952** 98.
11. Brohult, S., Ryhage, R., Spetsig, L.-O. and Stenhagen, E. *Proc. European Brewery Conv.* Rome 1959, p. 121.
12. Spetsig, L.-O., *Proc. European Brewery Conv.* Vienna 1961, p. 134.

Received February 9, 1962.