IDENTIFICATION OF FLAVONOIDS IN HOPS (*HUMULUS LUPULUS* LINNE) BY THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

A number of publications have appeared on the bitter principles and other constituents of hops, but very little has been published regarding the flavonoid constituents. The earlier workers reported the presence of quercitrin[1,2], isoquercitrin and rutin[3–5], but LEBRETON[6] approached this problem in a different way by hydrolysing the flavonoid mixture as such instead of isolating the individual compounds. Quercetin and kaempferol were identified in the hydrolysate as the aglucones and glucose and rhamnose as the sugars. The form in which the aglucones and sugars are attached to each other was not suggested.

In the present investigation use has been made of thin-layer chromatography with polyamide Woelm for isolating and identifying the various flavonoids in hops.

EXPERIMENTAL AND RESULTS

Thin-layer chromatoplates were prepared by pouring a suspension of the adsorbent in ethanol or ethanol–water mixture according to a method developed earlier[7]. A suspension of 1 g polyamide in 13.5 ml ethanol, or 3 g magnesium silicate in 13.5 ml ethanol + 1.5 ml water, or 2 g MN-Cellulosepulver 300 in 13.5 ml ethanol + 1.5 ml water, was sufficient for a plate measuring 20 × 20 cm. Except for the MN-Cellulosepulver 300, which was obtained from Macherey, Nagel and Co., the thin-layer adsorbents were made by Woelm and did not contain any binder.

1 kg of powdered hops was boiled 3 times with distilled water for 15 min each time. The filtered extract (*ca.* 30 l) was concentrated in vacuum to about 800 ml and poured into 4 l of methanol. The resulting precipitate (which was free of flavonoids) was separated by filtration. The filtrate was freed of methanol and the volume reduced to about 300 ml. This aqueous solution was shaken 50 times with 200 ml of ethyl acetate. Each extract was filtered separately and vacuum concentrated and then chromatographed on a thin-layer plate using polyamide as adsorbent and solvent mixture I for development. The first 30 ethyl acetate fractions showed four clear spots and a fifth spot of very weak intensity. The substances corresponding to the above spots have been provisionally named as substances A (R_F 0.33), B (R_F 0.24), 'C (R_F 0.16), D (R_F 0.07) and E (R_F 0.43). As all the thirty extracts showed identical spots they were pooled and vacuum evaporated to dryness. The residue was dissolved

in a small quantity of water, filtered and the filtrate shaken many times with ethyl acetate. This ethyl acetate fraction was concentrated and termed as Fraction I.

The remaining ethyl acetate (30–50) extracts were worked up in the same way as the first 30. Thin-layer chromatograms showed almost the same picture as for Fraction I except that the spots with R_F values 0.33 and 0.43 were almost absent. These twenty extracts were pooled, concentrated and termed Fraction II. The aqueous solution left after shaking with ethyl acetate was concentrated under vacuum, passed through a polyamide Woelm column and eluted with dilute methanol and finally with methanol. The various eluates were chromatographed as before. Thin-layer chromatograms showed three flavonoid spots with R_F values 0.08, 0.12 and 0.18. The main spot had the R_F value 0.12 (substance F); the other two being of very weak intensity. Since all the eluates gave the same chromatographic picture, they were pooled and concentrated (Fraction III).

A series of plates with polyamide were prepared and Fraction I was put on the entire length of the starting line. It was then developed with solvent mixture I. All the zones were marked under U.V. light. After scraping off the individual zones they were extracted with methanol. These methanolic extracts were concentrated separately and purified through active charcoal and allowed to crystallise. In this way the substances A, B, C, D and E from Fractions I and II and substance F from Fraction III were isolated. All the substances were crystallised a number of times from methanol.

Substance A

The melting point of substance A agreed with that of an authentic sample of astragalin[8] (176–178°) and on admixture the melting point was not depressed. The U.V. spectrum shows three maxima, *viz.* 267, 298 and 350 mμ like astragalin, for which GEISSMAN[8] gives maxima at 267, 298, 350 mμ and ÖISETH AND NORDAL[9] mention the wavelengths 269, 350 mμ and 265, 347 mμ. Colour reaction with Benedict's reagent[10] points to the fact that it is a kaempferol glucoside. Reaction with zirconium oxychloride–citric acid[11] and tests on paper with zinc–hydrochloric acid[12] indicate that it is substituted in the 3-position.

Hydrolysis. A small quantity of the substance was dissolved in methanol and boiled with 1 % H_2SO_4 for 3 h. After removal of methanol the solution was cooled and shaken with ether. After concentration the ether extract was put on a thin-layer chromatogram of cellulose acetate; a water-saturated mixture of chloroform and acetic acid (2:3) was used for development and 1 % methanolic aluminium chloride as spray reagent. A solution of authentic kaempferol was chromatographed with the ether extract and a mixture[13] of both for comparison. The thin-layer chromatogram showed that all the test substances had the same R_F value as authentic kaempferol. The aqueous extract left after ether extraction was freed of sulphuric acid by shaking with an anion exchanger (Amberlite I.R. 45) and filtered. After concentration, it was subjected to thin-layer chromatography using magnesium silicate[14] as an adsorbent and propanol–ethyl methyl ketone–water (2:1:1) as solvent. The chromatogram was dried and sprayed with 1 % $KMnO_4$ solution. The thin-layer chromatogram showed that glucose (R_F 0.49) was present in the hydrolysate of substance A.

Substance A was chromatographed in different solvent mixtures to test its purity and compared with authentic astragalin and also a mixture[13] of the two. In all the three cases the substances had the same R_F values (Table I). The above facts seem to

TABLE I

R_F VALUES OF SUBSTANCES A, B, AND F IN DIFFERENT SOLVENT MIXTURES

S	Compound	Solvent mixture				
		I	*II*	*III*	*IV*	*V*
I	Substance A	0.33	0.26	0.476	0.29	0.44
II	Astragalin (authentic)	0.33	0.26	0.476	0.29	0.44
III	Mixture of I + II	0.33	0.26	0.476	0.29	0.44
IV	Substance B	0.24	0.193	0.393	0.26	—
V	Isoquercitrin (authentic)	0.24	0.193	0.393	0.26	—
VI	Mixture of IV + V	0.24	0.193	0.393	0.26	—
VII	Substance F	0.12	0.09	0.50	0.46	—
VIII	Rutin (authentic)	0.12	0.09	0.50	0.46	—
IX	Mixture of VII + VIII	0.12	0.09	0.50	0.46	—

Solvent mixtures: I = Ethyl methyl ketone–toluene–glacial acetic acid–methanol–water (80:10:2:5:6); II = Ethyl methyl ketone–ethyl acetate–formic acid–water (3:5:1:1); III = Methanol–glacial acetic acid–water (90:5:5). IV = Water–ethanol–ethyl methyl ketone–acetylacetone (15:3:3:1); V = Ethanol–water (3:2).

indicate that substance A is identical with astragalin. The yield of substance A is very low and perhaps this is the reason for it not being reported by earlier workers.

Substance B

This substance melts at 224–226° and in admixture with an authentic sample of isoquercitrin did not depress the melting point. Hydrolytic studies show that the molecule is composed of quercetin and glucose. The zirconium oxychloride–citric acid test shows that the molecule is substituted in 3-position. A mixed and comparative thin-layer chromatogram with authentic isoquercitrin showed that substance B is identical with it. In all cases it gave the R_F value of 0.24. This was further confirmed by comparison of R_F values obtained by paper chromatography using a solvent mixture of isoamyl alcohol–glacial acetic acid–water (5:3:2) as suggested by PARIS[15]. The R_F value obtained here, *viz.* 0.54, compares well with the value of 0.56 quoted by him.

Substance C

A very high state of purity could not be obtained as the yield is very small. Hydrolytic studies show that it contains principally kaempferol and glucose together with very small traces of quercetin. The Benedict and the zirconium oxychloride–citric acid tests indicate that it is probably a 3-substituted kaempferol glucoside.

Substance D

Hydrolysis of this substance shows that the molecule is constituted of quercetin and glucose. The zirconium oxychloride–citric acid test indicates that the C-3 position of the flavonoid molecule is substituted. The quantity of the substance available was too small for further studies. This substance has a lower R_F value (0.07) than that of rutin (0.12) in solvent mixture I.

Substance E

Only very small amounts of this substance could be isolated. Benedict's colour test suggests that it is a quercetin glycoside.

Substance F

This has a m.p. of 188–189°. On admixture with authentic rutin there was no depression of the melting point. Hydrolytic studies revealed quercetin, glucose and rhamnose in the molecule. The colour tests and thin-layer chromatograms were compared with those of authentic rutin and found to be identical.

The amounts of the substances corresponding to the spots with R_F values 0.08 and 0.18 (from the aqueous extract) were too small to be isolated in a pure enough state for further investigations.

Beer

It was interesting to investigate whether the same flavonoids could be identified in a beer sample or whether they underwent any change. A concentrated solution of beer (Andreas Kloster-Bräuerei, Eschwege) when worked up as above showed identical spots to those in hops on a thin-layer chromatogram.

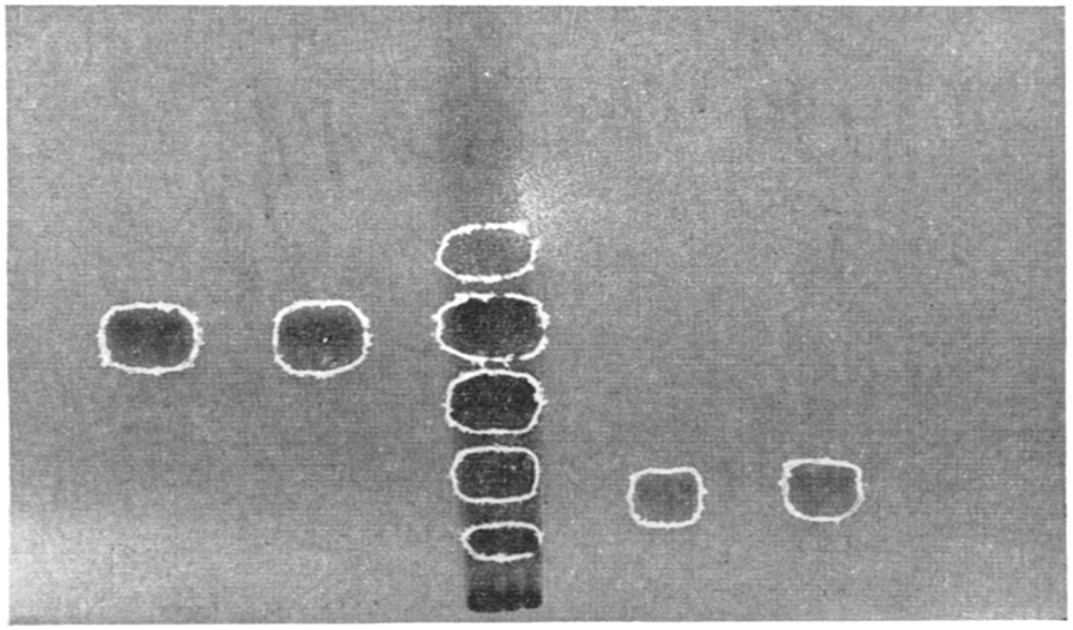

Fig. 1. Thin-layer chromatograph of hops on a polyamide plate with solvent mixture I. From left to right: (1) astragalin; (2) substance A; (3) ethyl acetate-soluble Fraction I; (4) rutin; (5) substance F.

DISCUSSION

The use of preparative thin-layer chromatography with polyamide was found to be very helpful in the isolation of various flavonoids from hops. As already indicated at least six flavonoid substances were isolated out of which three could be identified. Astragalin was found for the first time in hops. The other two flavonoids which have been identified are isoquercitrin and rutin.

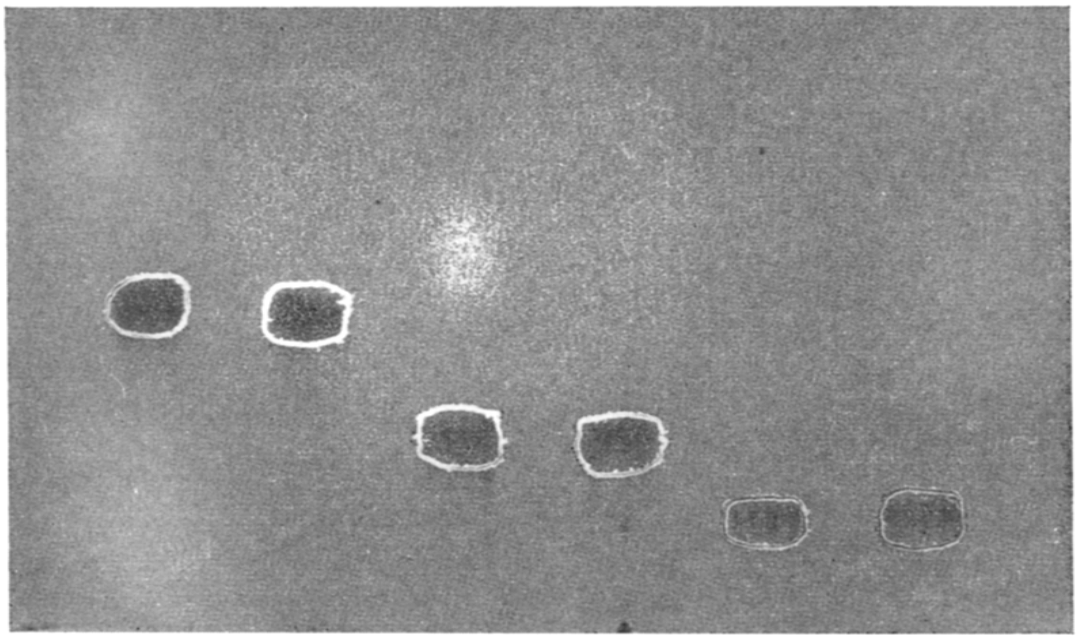

Fig. 2. Thin-layer chromatograph of substances A, B and F from hops on a polyamide plate with solvent mixture I. From left to right: (1) astragalin; (2) substance A; (3) isoquercitrin; (4) substance B; (5) rutin; (6) substance F.

The presence of quercitrin, found by earlier workers[1,2] during their investigations by paper chromatography and spectrophotometry, could not be confirmed. A paper chromatogram (Schleicher & Schüll 2043a Mgl) using Partridge mixture as solvent gave the R_F value of 0.68 for both astragalin as well as authentic quercitrin. A thin-layer chromatogram with polyamide with solvent mixture I shows however that astragalin has a slightly lower R_F value (0.33) than quercitrin with R_F 0.36. The difference is small but clear enough. The possibility of our mistaking quercitrin for astragalin is not possible because not only the colour reactions but also the hydrolytic products of the two glycosides are entirely different. It is difficult to say whether the amount of quercitrin in the particular charge of hops which we worked up was so low that it could not be isolated or whether the earlier workers[1,2] mistook astragalin for quercitrin. Some of the earlier workers[3,4,6] also failed to confirm the presence of quercitrin in hops. It should not be forgotten that the yield of flavonoids varies considerably according to the source[3].

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SUMMARY

Using polyamide Woelm for thin-layer chromatography it has been possible to

identify astragalin for the first time in hops. The thin-layer chromatogram shows three more unidentified flavonoid spots besides those of rutin and isoquercitrin.

Benedict's and zirconium oxychloride–citric acid tests indicate that of these three, two seem to be quercitin glycosides and the third a kaempferol glycoside.

Beer was also investigated for its flavonoids. The thin-layer chromatogram showed spots identical to those from hops.

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