

Analysis of Hop Bittering Constituents

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CONCLUSIONS

1. Statistical interpretation of the screened data from the analysis of two pairs of hop extract samples by the ASBC spectrophotometric, Dowex, and Sephadex ion-exchange (IEC) methods indicated that there was no significant difference among results obtained by the three methods.
2. 80:20 methanol:water is an adequate solvent system for dispersion of hop extracts prior to ion-exchange chromatography.
3. Substitution of amyl acetate (AmAc) for isopropyl ether in the spectrophotometric method was considered unacceptable because it gave significantly lower results for extracts containing water solubles.
4. A literature survey indicated that reliable procedures exist for stabilizing IPE against the formation of peroxides. Including these in requirements for the spectrophotometric method would render the solvent sufficiently safe for use.
5. A survey of key members of the hop industry indicated that the Hop Storage Index was useful and should be included in the instructions for analysis and reporting of α -acids in hops, hop powders, and hop pellets.

RECOMMENDATIONS

1. Test substitution of Freon TF (1,1,2 trichloro-1,2,2-trifluoroethane) for IPE in the method for α -acids in nonisomerized hop extracts (2.6).
2. Test Hop Storage Index on hops, hop powders, and hop pellets, but not on hop extracts.
3. Reexamine the applicability of the Sephadex IEC method for iso- α -acids in isomerized hop extracts. If possible, this will be done in conjunction with the EBC Analysis Committee.
4. Lay the ground work for initiating collaborative tests using Sephadex IEC for determining hop constituents in beer.

This Subcommittee was given the principal assignment of evaluating the Dowex (7) and Sephadex (10) ion-exchange chromatography (IEC) procedures for determining α -acids in unmodified (nonisomerized) hop extracts. Since both procedures are specific in that they separate α -acids from oxidation products, it was hoped they could serve as reference procedures against which rapid methods could be compared.

The Subcommittee was also asked to evaluate substitution of AmAc for IPE in the IPE method (6) for measuring α -acids in unmodified hop extracts. The unsafe feature of IPE being a peroxidizable solvent led to this portion of the assignment.

EXPERIMENTAL

Evaluation of IEC Procedures

A preliminary study was done by four Subcommittee members to test 80:20 methanol:water as a solvent for use with the ion-exchange methods. As a result of this work, it was decided that methanol-water was an adequate solvent for IEC analyses.

The analytical program was designed according to the Youden (12) unit block test. A pair of standard hop extracts and a pair of hop extracts containing water solubles were sent to each collaborator. They were instructed to do both IEC methods and the IPE method on every sample.

Evaluation of AmAc vs. IPE as Extracting Solvent

Two Subcommittee members made a preliminary survey of solvents which could serve as potential substitutes for IPE. Hydrocarbon solvents tended to give emulsions when used in a two-phase system and gave low results. Alcohols could not be used in the two-phase extraction system of the IPE method. Lower esters gave extracts which either gave very high conductometric values (C.V.) or the end point was not identifiable. AmAc was considered best of the solvents surveyed.

Two pairs of extract samples, one containing water solubles (pair III) and one without (pair IV), were sent to seven of the collaborators who were experienced with the IPE method. They

were asked to do a single analysis using each solvent on all four samples. The instructions were as given in ASBC, *Proc. 1970*, p. 233, and no special instructions applied to AmAc.

RESULTS AND DISCUSSION

Evaluation of IEC Procedures

Nine laboratories reported on the spectrophotometric and Dowex methods and eight members reported on the Sephadex method.

Table I shows the individual data for all analyses. Outliers were identified by the Dixon test (4). Table II shows a statistical summary of α -acids analysis by the three methods. All methods performed equally well, based on the within-lab error (S_r) and the between-lab error (S_b). No problems of any consequence were reported by the members. This indicates that, if a sample is thoroughly dispersed in 80:20 methanol:water, the α -acids will be dissolved and ion-exchange analysis can be performed.

The t-test was applied to IPE vs. Dowex, IPE vs. Sephadex, and Dowex vs. Sephadex to test for differences in mean α -acids values given by the three methods. Calculated t-values ranged from 0.8 to 1.3 with 12 to 14 degrees of freedom and none was significant at $p = 0.05$ for either sample pair. Observations that the IEC methods are independent, and that both are specific for α -acids, support the assumption of accuracy. Further, since the IPE spectrophotometric

TABLE I
Per Cent α -Acids—Individual Data for Hop Extracts

Lab I. D. No.	Sample I-A			Sample I-B			Sample II-A			Sample II-B		
	Spectro	Dowex	Sephadex	Spectro	Dowex	Sephadex	Spectro	Dowex	Sephadex	Spectro	Dowex	Sephadex
1	37.2	36.6	34.2	28.8	31.7	31.1	16.0	13.6	13.3	14.0	14.1	14.1
2	39.5 ^{a,b}	34.2	34.9	32.0 ^b	32.2	29.8	15.7	14.1	14.2	15.6	14.2	13.6
3	35.8	36.6	...	29.2	29.1	...	14.9	13.6	...	12.6	14.3	...
4	37.6	34.7	37.8	29.3	31.3	28.7	14.5	13.8	13.1	14.3	15.0	13.2
5	37.6	30.8 ^b	33.2 ^b	28.8	27.6 ^b	27.2 ^b	13.6	10.2 ^{a,b}	13.8	14.4	14.4 ^b	14.6
6	37.1	35.5	35.9	30.9	29.2	29.8	13.8	13.2	13.0	14.1	14.9	14.6
7	37.2	35.1	37.1	30.2	30.4	30.3	14.6	13.5	15.1	14.9	15.1	14.9
8	36.5	31.5	34.4	30.3	31.6	30.3	14.9	13.4	15.2	15.5	15.2	15.6
9	35.2 ^b	33.4	33.9	25.6 ^{a,b}	31.0	31.0	11.4 ^{a,b}	15.4 ^{a,b}	11.6	15.0 ^b	10.4 ^{a,b}	14.6
Mean	37.0	34.7	35.5	29.6	30.8	30.1	14.8	13.6	13.7	14.4	14.7	14.4

^aOutliers at $p = 0.05$ by the Dixon test (4).

^bValue not used for means or in calculations shown in Table II.

TABLE II
Summary of α -Acids Analyses of Two Pairs of Hop Extracts by Three Procedures

Pair	Method	No. Labs.	Grand Mean $(\bar{x} + \bar{y})/2^c$	Between-Lab.		Within-Lab. ^a		Combined ^b		F ^c
				S_b	CV (S_b) ^d	S_r	CV (S_r)	$\sqrt{S_b^2 + S_r^2}$	CV Com.	
I	Spectro	7	33.3	0	0	0.79	2.4	0.79	2.4	0.763
	Dowex	8	32.8	0	0	1.74	5.3	1.74	5.3	0.390
	Sephadex	7	32.8	0	0	1.57	4.8	1.57	4.8	0.213
II	Spectro	8	14.6	0.33	2.3	0.83	5.7	0.90	6.1	1.318
	Dowex	7	14.1	0	0	0.66	4.7	0.66	4.7	0.188
	Sephadex	8	14.0	0.59	4.2	0.80	5.7	0.99	7.1	2.060

^aWithin-lab. error (s_r) is the standard deviation of a single analysis in a single laboratory.

^bCombined error, $\sqrt{S_b^2 + S_r^2}$, is equivalent to the standard deviation of a single analysis in any laboratory.

^cNo significant difference between methods for either sample pair at $p = 0.05$.

^dCV = Coefficient of variation.

^eNo value for F exceeded the critical F at $p = 0.05$.

TABLE III
Individual Data for Comparing IPE with AmAc as Extracting Solvents for Unmodified Hop Extracts

Pair	Collab.	Spectrophotometric							
		% α -Acids		% β -Acids		Storage index		Conduct. value	
		IPE	AmAc	IPE	AmAc	IPE	AmAc	IPE	AmAc
III-A	1	17.8	17.4	13.5	13.0	0.32	0.32	18.6	17.5
	2	17.0	16.1	13.8	13.3	0.29	0.30	18.2	17.6
	3	17.4	16.9	14.1	14.1	0.31	0.32	17.6 ^{a,b}	20.1 ^{a,b}
	4	16.5 ^b	...	14.0	...	0.31	...	18.6 ^b	...
	5	17.1	16.2	14.6	14.0	0.32	0.31	18.7	18.3
	6	17.8	17.9	13.4	13.3	0.31	0.29	18.6	17.9
	7	17.6	16.5	14.0	13.6	0.31	0.32	18.8	18.4
	Mean	17.45	16.83	13.91	13.55	0.310	0.310	18.58	17.94
III-B	1	17.4	16.0	13.6	12.8	0.36	0.37	19.5	18.3
	2	16.5	14.9	13.1	11.9	18.9	16.1
	3	17.9	16.3	13.5	13.9	0.33	0.35	19.1 ^{a,b}	20.7 ^b
	4
	5	16.7	16.0	13.5	13.2	0.36	0.37	19.3	18.6
	6	17.8	15.8	13.1	13.1	0.34	0.38	18.5	17.7
	7	17.3	16.6	13.6	13.3	0.34	0.34	18.1	17.6
	Mean	17.27	15.93	13.40	13.03	0.346	0.362	18.86	17.66
IV-A	1	34.7	33.7	24.5	24.0	0.34	0.35	35.4	34.0
	2	31.6	30.4	22.1	22.1	0.33	0.32	33.3	33.4
	3	28.0	29.3	21.0	20.9	0.34	0.35	25.1 ^{a,b}	31.9 ^b
	4	29.0 ^b	...	21.3	...	0.33	...	31.9 ^b	...
	5	33.6	33.3	25.8	25.5	0.34	0.34	35.8	35.8
	6	32.6	32.4	23.0	23.7	0.34	0.34	33.5	32.7
	7	34.1	33.6	24.1	24.1	0.34	0.34	35.0	34.9
	Mean	32.43	32.12	23.11	23.38	0.337	0.340	34.60	34.16
IV-B	1	40.9	37.1	31.8	29.8	0.31	0.31	40.5	37.2
	2	38.4	38.3	31.7	31.1	39.8	37.0
	3	40.1	38.7	31.7	30.9	0.28	0.29	39.7 ^b	31.2 ^{ab}
	4
	5	39.0	38.1	31.4	30.9	0.31	0.31	40.4	39.2
	6	41.5	40.3	30.2	30.2	0.29	0.29	39.3	37.7
	7	40.0	39.1	30.7	30.5	0.29	0.29	39.8	38.7
	Mean	39.98	38.60	31.25	30.56	0.296	0.298	39.96	37.96

^aOutlier, either in sample, total for the pair, or in difference between values for a pair at $p = 0.05$ (4).

^bValue not used in means or in calculations shown in Table IV.

TABLE IV
Summary of Comparison of IPE vs. AmAc as Extracting Solvents for Unmodified Hop Extracts

Pair	Method	No. Labs.	Grand Mean ($\bar{x} + \bar{y}$)/2	Between-Lab.		Within-Lab.		Combined		F
				S_b	CV (S_b) ^b	S_r	CV (s_r)	$\sqrt{S_b^2 + S_r^2}$	CV Com.	
Spectrophotometric III	IPE	6	17.4 ^b	0.39	2.24	0.27	1.55	0.47	2.70	5.24 ^c
	AmAc	6	16.4 ^b	0.29	1.77	0.58	3.54	0.65	3.96	1.49
IV	IPE	6	36.2	0.63	1.74	1.80	4.97	1.90	5.25	1.25
	AmAc	6	35.4	0	0	1.61	4.55	1.61	4.55	0.77
Conductometric III	IPE	5	18.1	0.42	2.32	0.56	3.09	0.70	3.87	2.12
	AmAc	5	17.8	0.37	2.08	0.64	3.60	0.74	4.16	1.68
IV	IPE	5	36.2	0.82	2.26	0.65	1.80	1.05	2.90	4.22
	AmAc	5	36.1	0.98	2.71	0.50	1.39	1.10	3.05	8.62 ^c

^aCV = Coefficient of variation.

^bSignificant difference between methods at $p = 0.05$.

^cSignificant difference between labs at $p = 0.05$.

method gave results indistinguishable from either of the specific methods, it can be assumed that it gives equally accurate results for α -acids, even though it is a nonspecific method.

Evaluation of AmAc vs. IPE as an Extracting Solvent

Collaborators reported α -acids, β -acids, and the Hop Storage Index (9) from the spectrophotometric analyses and also reported conductometric values, all shown in Table III. A statistical summary of the α -acids and conductometric results is presented in Table IV. Results for β -acids and Storage Index, listed in Table III, were not treated statistically since they were not directly involved in the objective of the trial.

No clear advantages for either within-laboratory error (S_w) or between-laboratory error (S_b) could be associated with either solvent. Similarly, the combined error (the standard deviation for a single analysis performed in any one of the participating laboratories) showed no advantage by use of either solvent. Significant F values, indicating differences between laboratories, were noted in two instances; one associated with IPE and one with AmAc.

AmAc gave lower values than IPE by the spectrophotometric procedure for sample pair III, but not for sample pair IV. The difference, about one part in 17, was significant at $p=0.05$. In view of the performance of IPE when compared to IEC, as noted earlier, use of AmAc may actually lead to low results.

Those members of the Subcommittee who dealt with this part of the work had mixed reactions to the choice of solvents and to the results. Most disliked the odor of AmAc. Some noted the formation of emulsions with AmAc which were difficult to break. Two felt that further work should be done with AmAc. Several expressed preference for IPE and some submitted references showing that IPE could be stabilized against formation of peroxides (3,5,8,11). They maintained that, for this particular use (where no distillation is involved), IPE protected by a recommended stabilizer, along with periodic tests for the presence of peroxide, would meet the safety standards of the Society.

One collaborator submitted results obtained when Freon TF (1,1,2 trichloro 1,2,2 trifluoroethane) was substituted for IPE. Mean results for ten different extract samples containing water solubles were 16.07% α -acids using IPE and 16.03% using Freon. Both solvents gave 41.42% α -acids for five samples of resin extract. The collaborator also noted that Freon and benzene extracted α -acids from hops equally well.

Several key members of the hop and brewing industry were asked whether they thought the Hop Storage Index should be included with α -acids analysis of hops and pellets. They were unanimously in favor of including the index in the analysis and in the report. They suggested past data from Subcommittee work be reviewed and, if not applicable, that additional studies be initiated next year.

piece of previously weighed glassine paper approximately 3 cm² and place it into a 100-ml volumetric flask without getting any sample on the neck of the flask. Add 50 ml of 80:20 methanol:water and five glass beads. Stopper the flask and shake for 30 min on a wrist-action shaker. Dilute to volume with 80:20 methanol:water, mix well, and allow the solids to settle. Pipet an aliquot of the solution containing 3–4 mg of α -acids onto the prepared resin and allow the liquid to drain to the top of the resin. Rinse the walls of the column with 2 ml of methanol and again drain the liquid to the top of the resin bed.

Procedure

Elute the column with acetic acid solutions in the order indicated in Table I.

TABLE I
Elution of Acetic Acid Solutions

Acetic Acid %	Min. Vol. ml	Approx. Vol. Needed ml	Terminating A_{330}	Components Eluted
4.25	100	150	0.05	β -acids and interferences
6.50	150	200	0.05	α -acids

Calculations

$$\text{Wt on column} = \text{wt sample (g)} \times \frac{\text{aliquot onto column (ml)}}{\text{vol extracted into (ml)}}$$

$$\% \alpha\text{-acids} = \frac{A_{330} \times \text{vol eluate (ml)}}{E_{1cm}^{1\%} \times \text{wt on column (g)}}$$

APPENDIX B DETERMINATION OF α -ACIDS IN NONISOMERIZED HOP EXTRACTS BY SEPHADEX ION-EXCHANGE CHROMATOGRAPHY

Instructions for the Sephadex method were as reported in ASBC Journal 34(3): 97 (1976)(1) except:

Sample Preparation

As for Dowex, given in APPENDIX A, above.

APPENDIX A DETERMINATION OF α -ACIDS IN NONISOMERIZED HOP EXTRACTS BY DOWEX ION-EXCHANGE CHROMATOGRAPHY

All instructions were as for analysis of iso- α -acids and reduced iso- α -acids by dowex ion-exchange chromatography in ASBC Proc. 1974, p. 25, and ASBC Proc. 1973, p. 167, except:

Reagents

Acetic acid solutions (prepared daily):

- 4.25% acetic acid in 80% methanol (v/v).
- 6.50% acetic acid in 80% methanol (v/v).

Sample Preparation

Heat the hop extract sample to 65°C for 30 min and mix well with a glass rod. Accurately weigh about one g of the extract onto a

APPENDIX C

Note

The following note is recommended to be added to the Isopropyl Ether (IPE) Method (6) for α -acids in unmodified hop extracts. IPE can form unstable peroxides upon extended storage and/or exposure to strong light. Three precautions must be taken to ensure the safety of this solvent.

- IPE must be stabilized against formation of peroxides by addition of a chemical inhibitor. Most suppliers include an inhibitor (usually 100 mg/l. hydroquinone) in their product. If the IPE is known not to have a stabilizer, 100 mg/l. of either hydroquinone or 1-naphthol (11) should be added.

IPE should be purchased in special metal containers with safety seals such as offered by J. T. Baker.

Upon receipt, the containers must be labeled with the date and tested at least every 3 months for the presence of peroxide. If absent, the label should be redated. If peroxide is present, the solvent must be disposed of or freed of peroxide and redated.

- Isopropyl ether should always be regarded as containing peroxides. Accordingly, the practice of routinely testing for peroxides should be adopted (8). Preferred qualitative tests depend on the oxidation of iodide to iodine by peroxide. The procedure recommended by the Manufacturing Chemists Association follows:

Add 1.0 ml of freshly prepared 10% solution of potassium iodide to 10 ml isopropyl ether in a 25 ml glass stoppered cylinder of colorless glass in an area protected from sunlight. Color development takes from 1 to 10 min, and presence of yellow color represents a *serious hazard*.

Color	% Peroxide as H_2O_2
Barely discernible yellow	0.001–0.005
Definite yellow color	0.01
Brown color	?

- If the test for peroxides is positive, the solvent must be disposed of or freed of peroxide. The most popular method (3,5) for

removing peroxide is by shaking the solvent with 10% potassium metabisulfite, followed by re-testing for peroxides.

Literature Cited

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