Table V. Quantity of Saponin Found in Sugar and Efficiency of Recovery of Saponin from Saponin-Free Filtrates

Sample No.	Mg. Saponin Originally Present/100 G. Sugar		Saponin Added to Filtrate, Mg.	Saponin Recovered from Filtrate, Mg.		Saponin Recovered from Filtrate, %	
1	0.56	0.56	0.25	0.26	0.23	104	92
2	0.39	0.46	0.25	0.23	0.26	92	104
3	0.72	0.74	0.25	0.26	0.26	104	104

Table VI. Suitability of Sugars Graded by Pepsi-Cola Co. Visual Estimation as Compared to Quantity of Saponin Found by Colorimetric Method

Sample No.	Mg. Saponin/ 100 G. Sugar	Visual Floc Evaluation
1	0.02	Negative floc
2	0.03	Border line pin point
3	0.14	Border line pin point
4	0.50	Heavy positive floc
5	0.32	Heavy positive floc

except in acid solution where it produces an unsightly flocculent precipitate. Fats are also considered to be a component of this floc. However, the contribution of fats to the formation of floc was not pursued here. The problem is to provide a quantitative relationship of saponin content in sugar to the visually observed floc. Table VI shows the saponin found to be present in various sugar samples which had been evaluated visually by analysts of the Pepsi-Cola Co.

As the visual evaluation is entirely subjective, the correlation might be considered very good.

The method described also provides a suitable and fairly rapid method which can be used for the study of saponin removed in the beet sugar refinery.

Acknowledgment

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MOISTURE MEASUREMENT

Determination of Moisture in Hops

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Moisture determination in hops by titration with Karl Fischer reagent was compared with oven drying and solvent distillation. Lower moisture values were obtained by Karl Fischer titration and by solvent distillation with iso-octane than by oven drying or solvent distillation with toluene or benzene. Higher results obtained by the latter three methods were due in large part to the liberation of water from hop components owing to chemical decomposition and concurrent decrease in the number of hydroxyl groups present. The Karl Fischer method is convenient and valid for the determination of moisture in hops.

OFFICIAL METHODS suggested for the determination of moisture in hops by the American Society of Brewing Chemists (7) and the Association of Official Agricultural Chemists (2) include oven drying and solvent distillation. Both methods suffer from serious disadvantages.

While these methods are good for the determination of moisture in many materials, special difficulties arise in hops, owing to the very heat-sensitive nature of several hop constituents and to the presence of up to 1% volatile oils.

During an evaluation of the Karl Fischer reagent for moisture determination on raw materials used in the brewery, this reagent was also studied for hops.

Comparative data on the various methods for the determination of hop moisture, with special emphasis on the use of the Karl Fischer reagent, are presented.

Methods for Moisture Determination

The amount of moisture in a material is often determined by heating in an oven and measuring the weight loss; however, this is unsuitable for materials which are heat sensitive.

Solvent distillation, employing a water-immiscible solvent, is another commonly used method. This test is based on the boiling point of water-solvent mixtures which is lower than that of either the water or solvent alone. For this distillation, the method of Dean and Stark (8, 16) was employed.

Drying in vacuo over a desiccant is one of the most accurate methods, but it takes a very long time for many materials to reach constant weight.

A rapid method, specific for water,

Table I. Hop Moisture vs. Extraction Time in the Karl Fischer Titration

Method of Shaking	Time, Min.	Moisture, %
By hand 100 times		7.49
Mechanical	5	7.57
	10	7.48
	15	7.41
	30	7.33
	45	7.94
	60	7.52
	90	7.50

Table II. Per Cent Moisture Found in Hop Samples by Various Analytical Procedures

Sample	Oven Drying 1 Hr. at 104 C.	Karl Fischer Titration	Benzene Distillation	Iso-octane Distillation	Toluene Distillation	Vacuum Drying 1 Wk. at Room Temp.
1	8.9	6.8	8.8	6.8	7.8	6.8
2	5.8	3.3	5.7	3.4	5.1	3.9
3	7.4	7.5	8.8	7.6	8.3	7.8
4	7.4	5.6	6.2	5.5	5.8	5.5
5	9.1	5.4	8.0	5.4	7.4	...
6	6.2	4.3	6.1	4.5	5.5	...
7	8.1	7.8	8.5	7.8	8.0	8.0
8	6.7	4.1	5.5	4.1	5.2	4.0

uses the Karl Fischer reagent, which contains iodine, sulfur dioxide, methanol, and pyridine, and which changes from the familiar reddish-violet color of iodine to colorless in the presence of water. The end point may be determined potentiometrically or visually. The visual end point was employed in this study.

The Karl Fischer reagent has been applied to many different problems of moisture determination (10, 13). It was mentioned as a possible method for the determination of moisture in raw materials of the brewery by De Clerck (9), Klang and Sandegren (11), and others (3, 4, 7). While this work has been in progress, the use of the Karl Fischer reagent for moisture determination in hops has been mentioned by the Analysis Committee of the European Brewery Convention (5). Their report shows lower results for the Karl Fischer than for other methods. Because of similar findings in this study, the reasons were sought why the Karl Fischer method gives lower results than the oven-drying and solvent-distillation methods.

The approach of this study differed in one point from the European workers—the use of ground vs. unground hops. Ground hops were used for closest possible conformity with approved American methods (1, 2), which normally employ ground hops, and because of accelerated contact with the solvent by increase in surface area. As work was carried out in an air-conditioned laboratory, the error introduced by the

Table III. Per Cent Moisture Found in Hop Samples by the Karl Fischer Titration and by Acetyl Chloride Reaction

Sample	Moisture, % Acetyl chloride	Karl Fischer
1	3.97	3.68
	4.17	3.69
	3.81	3.68
	Av. 3.98	3.68
2	3.87	3.80
	3.84	3.79
	Av. 3.86	3.80

short exposure during grinding was negligible. Some comparative experiments with ground and unground hops showed no difference in moisture results.

Reagents

Karl Fischer reagent, Fisher Scientific Co. Unified reagent, usual water equivalent is 1 ml. per 5 mg. of water.

Water in methanol, 1 mg. of water per ml. of anhydrous methanol.

Anhydrous methanol, Fisher reagent grade was satisfactory. With other grades, there was at times a high blank value; however, such methanol can be dried satisfactorily by distillation from magnesium methoxide (18).

Procedure

The moisture determination on hops with Karl Fischer reagent is essentially very simple. For other brewing materials, such as malt and adjuncts, refluxing was found necessary for the efficient extraction of water, while simple shaking by hand was sufficient for hops.

Hops, 0.5 gram (weighed on an analytical balance to the nearest 0.001 gram), previously ground in a Waring Blendor, is placed into a 100-ml. glass-stoppered flask. [A Waring Blendor was used for grinding because of its speed, efficacy, and the fact that little heat is generated in the sample. This generation of heat could alter the properties of the sample. The use of similar equipment for hop grinding has also been described by Verzele and Govaert (17).] Anhydrous methanol, 50 ml., is added and the mixture shaken rapidly by hand or by machine for 5 minutes. It is then titrated directly with Karl Fischer reagent. The blank value for the methanol is determined and subtracted. In order to obtain good reproducibility with the Karl Fischer method, all glassware must be dry, as any moisture in the flask would show up as a positive error. The titration should be carried out rapidly so that no moisture is taken up from the

atmosphere. Under very humid atmospheric conditions, extra precautions may be necessary.

The Karl Fischer reagent is standardized by titration against a standard water-in-methanol solution. (Titer equals milligrams of water per ml. of reagent.)

Moisture may then be calculated from the formula:

$$\% \text{ moisture} = \frac{(\text{ml. Karl Fischer for sample} - \text{ml. Karl Fischer for blank}) \times (\text{titer}) \times 100}{(\text{mass of sample}) \times 1000}$$

In order to check this procedure for optimal extraction of moisture from hops, experiments were carried out, and the results are tabulated in Table I. The data demonstrate that shaking by hand is satisfactory. These figures also show the reproducibility of the Karl Fischer determination.

Results and Discussion

The Karl Fischer method was compared with oven drying for 1 hour at 104° C. [in an Elconap air convection oven according to the American Society of Brewing Chemists' (1) procedure], with drying in vacuo, and with distillation from various solvents. The procedure of the European Brewery Convention (5) was used for solvent distillation. The effect of the solubility of water in the solvents was obviated by saturating the solvents with water before use. The apparatus used was that of Dean and Stark (8). Results are shown in Table II. For solvent distillation, three solvents were chosen: benzene (boiling point 80.1° C.) and toluene (boiling point 110.8° C.) both of which form minimum boiling azeotropes with water, and iso-octane (2,2,4-trimethylpentane), (boiling point 99.3° C.), which does not do so.

Oven drying and distillation with benzene and toluene tend to give considerably higher results than drying in vacuo, distillation with iso-octane, or Karl Fischer titration. Values obtained by these latter three methods tend to agree. The difference between the two sets of methods was considerably larger

Table IV. Loss in Acetylatable Groups by Hops during Oven Drying and Solvent Distillation

Sample	Moisture Method	Temp., ° C.	Heating Time, Hr.	Moisture Found, %	Increase in Moisture Found from Zero Hour[a], %	Acetylatable Groups Found, %	Decrease in Acetylatable Groups from Zero Hour, Expressed as Water, %
	Karl Fischer	Room	0	5.40	...	13.52	...
	Oven	104	1	9.13	3.73	10.25	3.47
	Oven	104	3	9.71	4.31	8.49	5.33
A	Benzene	80.1	2	8.0	2.60	11.11	2.56
	Iso-octane	99.3	2	5.4	0	13.59	0
	Toluene	110.8	2	7.4	2.00	11.80	1.90
B	Karl Fischer	Room	0	5.00	...	11.66	...
	Benzene	80.1	2	6.18	1.18	10.59	1.13
	Iso-octane	99.3	2	4.99	0	11.61	0.05
	Toluene	110.8	2	5.73	0.73	10.92	0.79
C	Karl Fischer	Room	0	2.80	...	11.20	...
	Oven	104	1	4.92	2.92	8.66	2.60
	Oven	104	3	5.58	2.78	8.08	3.30

[a] Karl Fischer values were taken as the zero-hour moisture.

than could be accounted for by the presence of volatile oils.

In order to check the amount of moisture present by a different method working on an independent principle, the reaction of water with acetyl chloride was used. The acid formed was determined by titration with standard sodium hydroxide (13, 15).

This method was carried out as follows: The hop sample was shaken with a mixture of acetyl chloride and pyridine in toluene, and the excess reagent destroyed by the addition of anhydrous ethyl alcohol. The liberated acid was titrated with $1.0N$ sodium hydroxide with phenolphthalein as indicator. A reagent blank was run without hops. The water present was calculated from the equation:

$$\% \text{ moisture} = \frac{(\text{ml. std. alkali used in titration} - \text{blank}) \times (\text{normality of std. alkali}) \times 0.018 \times 100}{\text{mass sample in grams}}$$

Table III shows that there is fairly good agreement between this method and the Karl Fischer procedure.

It was of interest to determine the reason for the discrepancy between these two sets of methods:

Karl Fischer determination
Acetyl chloride procedure
Vacuum drying at room temperature   vs.
Distillation with iso-octane

Oven Drying at 104° C. for 1 hour
Distillation with benzene
Distillation with toluene

The question arose whether the high results obtained, by the methods employing heat, could at least in part be accounted for by liberation of water from hydroxyl groups as a result of decomposition of hop components. This possibility seemed worth investigating as hops contain considerable amounts of highly hydroxylated substances, such as pectins and other carbohydrates, tannins, and the notoriously heat-sensitive hop resins.

The number of hydroxyl groups was

determined by an acetylation procedure (12). The hops are heated with a known excess of acetic anhydride in pyridine. The excess acetic anhydride was decomposed with water, and the acetic acid formed was titrated with standard sodium hydroxide. The acetic anhydride consumed is a measure of the groups which may be acetylated, particularly the amino and hydroxyl groups.

Table IV shows results of determinations of acetylatable groups at various stages of moisture removal. For purposes of this discussion, the acetylatable groups will be called hydroxyl groups, as formol titration indicated no change in amino groups. There was a considerable loss of these groups, and this loss could account for all of the additional water found by oven drying over and above that determined by the Karl Fischer reagent. This will not be the only change on heating hops; there will be a loss in volatile oils, as mentioned earlier, and also oxidative changes, which may contribute to weight increase

and consequent too-low moisture results.

In any case, oven drying is not a valid method for the determination of moisture in hops, as has been pointed out by numerous investigators (6, 14, 19).

At this time, it cannot be fully explained why benzene and toluene distillations bring about a loss of hydroxyl groups, while iso-octane distillation does not do so. In the benzene and toluene distillations, there is a loss of the green color of the hops, while with iso-octane the green color persists, showing that with the

other two solvents there is more decomposition.

The Karl Fischer method appears to be considerably more valid for the determination of moisture in hops than oven drying or solvent distillation with benzene or toluene. Solvent distillation with iso-octane may also be a useful method, but more experiments on different hops should be carried out before arriving at a definite conclusion.

As mentioned earlier, the European report (5) also shows somewhat lower results (by an average of 1.6%) by the Karl Fischer method than by other methods. These workers also found that toluene distillation may bring about hop decomposition, but explained this merely on the basis of high boiling point. The fact that benzene (boiling point 80.1° C.) gives as high a result as toluene, while iso-octane (boiling point 99.3° C.) gives results comparable to the Karl Fischer method, shows that there must be other factors involved—i.e., formation of low-boiling azeotropes. The European workers recommend oven drying at 98°C. This particular temperature was not used in our study, but a method entirely avoiding heat appears preferable; hence, the Karl Fischer determination is recommended.

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MINT OILS

Chromatographic Measurement of Variations in Essential Oils within a Single Plant

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Techniques have been developed for the study of essential oils from just one or two leaves. Leaves from different areas on the peppermint and spearmint plants have been steam distilled and the oils chromatographed. Measurement of the area of the chromatographic spots shows that the newer leaves contain considerably more oil per unit leaf area. The relative amounts of the constituents vary greatly from one area of the plant to another and it is possible to correlate these with increasing amounts of the more reduced forms of constituents in the older leaves.

THE FORMATION OF ESSENTIAL OILS in plants has been a subject of many proposed schemes of biogenesis. Some of these schemes have been based simply upon logical chemical reactions. Others have been the result of comparison of compounds in the oils isolated at different times of the year. A more direct method of comparison of oil of different ages would be desirable. At any one time on an actively growing plant, there are leaves formed several weeks earlier as well as leaves in the very young stages of growth. Presumably these leaves could contain oil of different ages also. Any differences between the oil from the newer leaves and the oil from the older leaves would give new insight into the processes of oil formation.

The chromatographic characterization of essential oils has been developed so that small amounts of oil can be used (*2, 3*). Peppermint and spearmint oils have been studied in detail both chemically and chromatographically and these have been used for the initial testing of the leaf comparison method. Steam distillation is a convenient method of separating the essential oils from other plant constituents. New distillation and

isolation techniques were developed so that less than 1 λ of oil can be isolated efficiently from leaf areas of as small as 1.5 square inches.

Experimental

Leaves were taken from various locations on the plant and their areas measured with a planimeter. As some leaves contained considerably less oil than others, it was convenient to use 3 to 6 square inches of leaf area to provide enough oil for chromatography from most plants, irrespective of the particular growth conditions or location of the leaves on the plant.

Three areas were arbitrarily defined to serve as a uniform basis of description of plant areas, as illustrated in Figure 1. The leaves on the central stem at the bottom of the plant (location *A*) are unquestionably the oldest leaves. The leaves on the same control stem toward the top of the plant (location *B*) are next in order of appearance. The newest leaves are those at the top of the plant and on side branches at the top of the plant (location *C*).

The leaves were ground in a mortar with a little sand, 2 ml. of hexane, and enough water to cover them. The ground mixture was added to a 50-ml.

flask and about 15 ml. of water was used to rinse the contents into the flask. After addition of a foam inhibitor the mixture was then steam distilled, using a very short simple condenser, into a 30-ml. separatory funnel containing 1 ml. of hexane. About 10 ml. of condensate
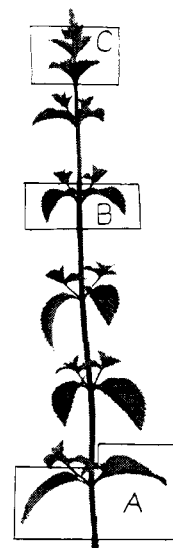

Figure 1. Mint plant illustrating location from which leaves were harvested