CHAPTER 13

Analysis of Volatile Oils

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13.1 INTRODUCTION

Essential oils have been used in pharmaceutical preparations throughout the ages. Their systematic analysis, however, is of comparatively recent origin. A satisfactory evaluation of these products, generally complex mixtures of compounds of widely different chemical characteristics, requires careful interpretation of analytical results.

Preliminary examination of an oil involves measurement of its physical characteristics, such as specific gravity, refractive index, optical rotation, solubility, congealing point, and determination of chemical properties associated with the amount of acids, esters, alcohols, carbonyl compounds, and phenols present.

Many essential oils are valued for some particular constituent or constituents, which require special methods of estimation. Certain components, though not contributing to the value of the oil, serve as indices of purity or quality. Determination of such valuable or characteristic constituents will be described later.

13.2 MEASUREMENT OF PHYSICAL PROPERTIES

A. SPECIFIC GRAVITY

Specific gravity of an essential oil is one of the important criteria for determining its authenticity. It may be defined as the ratio of the weight of a given volume of oil at a certain temperature to that of the same volume of water at the same temperature and is usually denoted as d_t^t. The temperatures at which specific gravity of essential oils is most frequently determined are 20° and 25°C. The measurements are most conveniently carried out by using a 10-ml conical pycnometer fitted with a standard-joint thermometer and a capillary side tube equipped with a ground glass cap [Fig. 13.1(*a*)].

Procedure

Fill the clean pycnometer with distilled water which has been recently boiled and cooled to about 20°C and place it in a constant temperature bath at 25°C for 30 min. As the temperature of the pycnometer and its contents

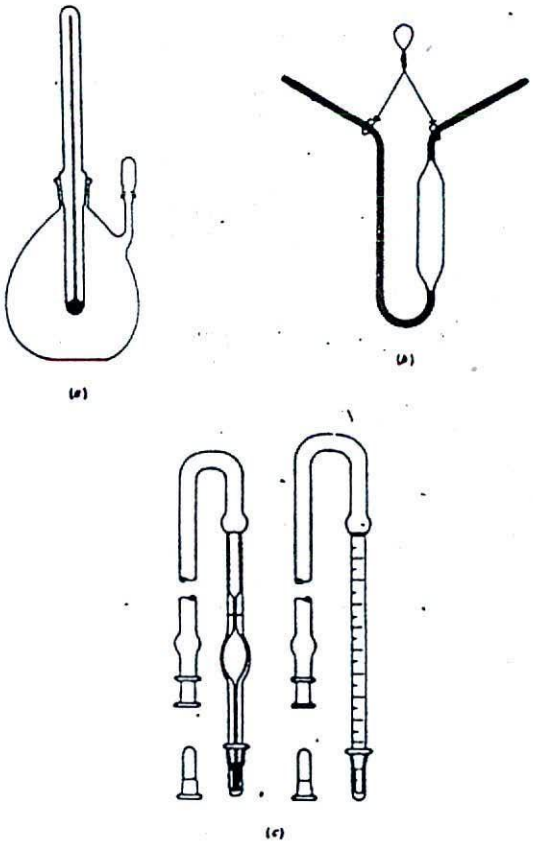

FIGURE 13.1: Apparatus for determination of specific gravity.

rises, the level of water in the capillary side arm first goes down and then rises. Keep the level of water adjusted to the required mark on the side arm by soaking excess water with a blotter. Cover the tip of the side arm with the ground-glass cap, remove the pycnometer from the water bath, and wipe it dry with a soft cloth or tissue paper. Allow it to stand for 30 min and then weigh accurately.

Empty the pycnometer and dry it by washing first with alcohol and then with ether, finally removing the ether vapors in a current of air. Let it stand for 30 min and weigh accurately.

Next fill the pycnometer with oil, previously cooled to about 20°, and keep it in a constant temperature bath for 30 min. Adjust the level of oil to the required mark, cover the capillary side arm with the cap, wipe the pycnometer dry, and accurately weigh after 30 min.

Calculate the specific gravity (d_{15}^{25}) of the oil as follows:

$$d_{15}^{25} = \frac{w_2 - w}{w_1 - w}$$

where w is the weight of the empty pycnometer and w_1 and w_2 are weights of the pycnometer when filled with water and oil, respectively.

For essential oil samples, available only in small quantities, a small sprengel tube [Fig. 13.1(*b*)] of 0.5- to 2-cc capacity is more suitable. The measurements are carried out in essentially the same manner as with a pycnometer. Semimicrodeterminations of specific gravity employ micropipettes [Fig. 13.1(*c*)] which can hold about 0.1ml and allow an accuracy of 0.05% when a semimicrobalance is used.

For routine analyses, density d_4^t is sometimes measured. It may be expressed as grams of mass per milliliter and measured by comparison of the weights of equal volumes of the oil at t°C and water at 4°C. Values of density, d_4^t and specific gravity d_t^t are related to each other as follows:

$$d_4^t = d_t^t \times d'^t_4$$

where d'^t_4 is density of water.

If the measurements were not carried out at a standard temperature the values could be reduced to that temperature by applying a correction of 0.00064 per degree centigrade. Quite often, in routine analyses, the specific gravity of an oil is determined as d_{15}^t and reduced to d_{15}^{15} by applying an average correction factor of 0.00084 per degree centigrade. However, the correction factors for different essential oils vary over a wide range and, therefore, for more accurate results values for individual oils and compounds (Tables 13.1 and 13.2) should be used. The correction will be added when the value is to be reduced to a lower temperature and subtracted, if the value were to be corrected to a higher temperature.

B. REFRACTIVE INDEX

Refractive index is an important physical characteristic of an essential oil. It is defined as the ratio of the velocity of light in air to that in the experimental sample, and can also be expressed as the ratio of the sine of the angle of incidence to the sine of the angle of refratcion for a ray of light passing from air into the experimental material.

The value of refractive indices varies with the wavelength of light and the temperature at which measurements are made. The wavelength of light most commonly used is 589.3 mμ corresponding with the D line of sodium light on Frauenhofer scale, and the temperature preferred for measuring refractive indices of essential oils is 20°C, unless the substance is solid at this temperature. These factors are mentioned while recording refractive index data as subscripts and superscripts, respectively, of the letter n, for example, refractive index of an oil for D line of sodium at 20°C will be expressed as a figure following the notation n_D^{20}.

TABLE 13.1: Variations in Specific Gravity per Degree Centigrade for Essential Oils*

Almond, bitter	0.00089	Linaloe	0.00083
Anise	0.00082	Mace	0.00082
Bay	0.00085	Mirbane	0.00098
Bergamot	0.00081	Orange, sweet	0.00078
Bois de Rose, Brazilian	0.00081	Origanum	0.00076
Cade	0.00074	Palmarosa	0.00073
Camphor	0.00081	Patchouly	0.00073
Cananga	0.00074	Pennyroyal	0.00078
Caraway	0.00078	Peppermint	0.00076
Cassia	0.00081	Petitgrain	0.00081
Cedarwood	0.00071	Pine	0.00079
Citronella, Ceylon	0.00081	Rosemary	0.00081
Citronella, Java	0.00093	Sandalwood, East Indian	0.00070
Clove	0.00085	Sassafras, artificial	0.00087
Eucalyptus (*Eucalyptus globulus*) 70 to 80%	0.00084	Spearmint	0.00079
Geranium, African	0.00076	Spike	0.00082
Geranium, Bourbon	0.00076	Tansy	0.00080
Ho	0.00083	Thyme	0.00079
Lavender	0.00082	Vetiver	0.00071
Lemon	0.00077	Wintergreen (*Gaultheria procumbens*)	0.00099
Lemongrass	0.00079	Ylang Ylang	0.00073

* Reprinted with the courtesy of *Industrial and Engineering Chemistry*.[1]

Refractive index measurements are conveniently carried out by using a refractometer. Of the various types, the Pulfrich or the Abbe refractometer is most popular. Either can give reproducible readings, correct up to fourth decimal place and requires only a drop to two of the oil. The temperature at which measurements are made should be controlled.

Procedure

Circulate a stream of water through the refractometer to maintain the prisms at the required temperature. Clean the prism surfaces first with ethyl alcohol and then with ethyl ether. Place the sample on the unpolished surface of the lower prism. Screw up the two prisms and wait for a couple of minutes to allow the sample to attain the required temperature. Illuminate the refractometer with diffused daylight or an electric lamp. Achromatize the critical boundary by rotating the compensator and bring it exactly at the intersection of the cross wires. Read the refractive index from apparatus scale.

If the refractive index is not measured at the specific temperature, it can be calculated by applying a correction of 0.0004 per degree centigrade; thus,

$$n_D^t = n_D^{t'} + 0.0004(t' - t)$$

where $n_D^{t'}$ is the value of the refractive index noted at t'°C and t is the specified temperature.

TABLE 13.2: Variations in Specific Gravity per Degree Centigrade for Isolates and Synthetics*

Acetaldehyde	0.00129	Heptaldehyde	0.00086
Acetophenone	0.00086	Heptyl alcohol	0.00073
α-Amyl cinnamic aldehyde	0.00076	Hydroxycitronellal	0.00077
Amyl salicylate	0.00085	Ionone	0.00076
Anisic aldehyde	0.00085	Isoeugenol	0.00087
Benzaldehyde	0.00089	Isopulegol	0.00083
Benzyl acetate	0.00092	Isosafrole	0.00088
Benzyl alcohol	0.00076	Lauryl alcohol	0.00067
Benzyl benzoate	0.00081	*d*-Limonene	0.00077
Bornyl acetate	0.00086	Linalool	0.00082
Butyraldehyde	0.00105	Linalyl acetate	0.00084
n-Caproic acid	0.00087	Methyl acetophenone	0.00081
n-Caprylic acid	0.00082	Methyl anthranilate	0.00088
Carvacrol	0.00076	Methyl benzoate	0.00095
Carvone	0.00080	Methyl heptenone	0.00084
Cinnamic aldehyde	0.00080	Methyl nonyl ketone	0.00076
Cinnamyl alcohol	0.00074	Methyl phenylacetate	0.00093
Citral	0.00080	Methyl salicylate	0.00098
Citronellal	0.00082	Octyl alcohol	0.00068
Citronellol	0.00070	Phellandrene	0.00078
p-Cresyl acetate	0.00093	Phenyl acetate	0.00098
p-Cymene	0.00079	Phenylethyl acetate	0.00090
Decyl alcohol	0.00068	Phenylethyl alcohol	0.00074
Dipentene	0.00080	Phenylpropyl alcohol	0.00073
Diphenyl oxide	0.00085	Pinene	0.00082
Ethyl acetate	0.00120	Rhodinol	0.00071
Ethyl benzoate	0.00092	Safrole	0.00089
Eugenol	0.00087	Salicyl aldehyde	0.00097
Geraniol	0.00071	Terpineol	0.00078
Geranyl acetate	0.00385	Terpinyl acetate	0.00082
Heliotropin	0.00093	Valeric acid	0.00091

* Reprinted with the courtesy of *Perfumery and Essential Oil Record*.[3]

Variation in refractive index per degree centigrade for a large number of essential oils, isolates, and synthetics was investigated by Bosart.[3] A summary of his findings is given in Tables 13.3 and 13.4.

C. MOLECULAR REFRACTION

A knowledge of the refractive index and density of a pure liquid substance enables us to derive another property called *specific refraction*, a constant which is independent of temperature. The product of specific refraction and molecular weight of the substance, is its *molecular refraction*. The values of both these constants can be obtained by using the Lorentz and Lorenz expression:

$$R = Mr = \frac{n^2 - 1}{n^2 + 2} \cdot \frac{M}{d}$$

TABLE 13.3: Change in Refractive Index per Degree Centigrade for Essential Oils*

Almond, bitter	0.00049	Lemongrass	0.00044
Anise	0.00049	Linaloe	0.00044
Bay leaves	0.00047	Mace	0.00046
Bergamot	0.00044	Mawah	0.00041
Bois de Rose	0.00044	Mustard	0.00054
Cajuput	0.00045	Orange, sweet	0.00045
Camphor, brown, sp. gr., 0.95–0.97	0.00043	Origanum	0.00042
Camphor, sp. gr., 1.020	0.00044	Palmarosa	0.00040
Camphor, white	0.00045	Patchouly	0.00042
Cananga	0.00041	Pennyroyal	0.00042
Caraway	0.00044	Peppermint	0.00040
Cassia	0.00048	Petitgrain	0.00044
Cedarwood	0.00040	Pimenta	0.00047
Cinnamon, Ceylon	0.00048	Pine	0.00042
Citronella, Ceylon	0.00046	Rosemary	0.00044
Citronella, Java	0.00047	Sandalwood, E. I.	0.00039
Clove	0.00045	Sassafras, artificial	0.00045
Copaiba	0.00040	Savin	0.00044
Coriander	0.00047	Spearmint	0.00043
Erigeron	0.00046	Spike	0.00045
Eucalyptus (*Eucalyptus globulus*)	0.00044	Sweet birch (*Betula lenta*)	0.00045
Fennel	0.00047	Tansy	0.00042
Geranium, African	0.00040	Thyme, red, 40–45%	0.00044
Geranium, Bourbon	0.00040	Turpentine	0.00046
Ho	0.00043	Vetiver	0.00039
Lavender	0.00043	Wintergreen (*Gaultheria procumbens*)	0.00045
Lemon	0.00046	Ylang Ylang	0.00042

* Reprinted with the courtesy of *Perfumery and Essential Oil Record.*

where R is molecular refraction, r is specific refraction, M is molecular weight, n is refractive index, and d is density.

Molecular refraction of a pure substance is an additive and constitutive property and can be calculated by adding up atomic refractions and the values for various functional groups of the molecule. A comparison of the calculated and observed values of molecular refraction of a compound can be used for confirmation or rejection of the structure assigned to the substance.

D. OPTICAL ROTATION

Many essential oil constitutents, in liquid state or in solution have the power to rotate the plane of polarization of plane polarized light. This property, called optical activity, is consequently exhibited by most essential oils and when expressed numerically, as optical rotation (for liquids) or specific rotation (for substances in solution), forms an important physical characteristic. Substances are called dextro- or levorotatory, depending on

TABLE 13.4: Change in Refractive Index per Degree Centigrade for Isolates and Synthetics[a]

Acetophenone	0.00047	Hydroxycitronellal	0.00040
α-Amyl cinnamic aldehyde	0.00050	Ionone	0.00044
Amyl salicylate	0.00042	Isoeugenol	0.00050
Anisic aldehyde (Aubepine)	0.00046	Isopulegol	0.00045
Benzaldehyde	0.00047	Limonene	0.00045
Benzyl acetate	0.00045	Linalool	0.00046
Bornyl acetate	0.00043	Linalyl acetate	0.00043
Carvacrol, technical	0.00043	Methyl anthranilate	0.00048
Cineole	0.00046	Methyl benzoate	0.00048
Cinnamic alcohol	0.00044	Methyl heptenone	0.00046
Cinnamic aldehyde	0.00052	Methyl phenylacetate	0.00046
Citral	0.00045	Methyl salicylate	0.00047
Citronellal	0.00044	Orange terpenes	0.00046
Citronellol	0.00040	Phenylethyl acetate	0.00046
p-Cresyl acetate	0.00046	Phenylethyl alcohol	0.00041
p-Cymene	0.00049	Phenyl methyl carbinyl acetate	0.00046
Diphenyl oxide	0.00049	Phenylpropyl alcohol	0.00038
Eugenol	0.00046	Rhodinol	0.00040
Geraniol	0.00041	Safrole	0.00045
Geranyl acetate	0.00045	Terpineol	0.00044
Geranyl butyrate	0.00043	Terpinyl acetate	0.00041
Geranyl formate	0.00043		

[a] Reprinted with the courtesy of *Perfumery and Essential Oil Record*.

TABLE 13.5: Atomic and Molecular Refractions[a]

Structural unit or molecule	Refraction
C	2.418
H	1.100
O′	2.211
O<	1.643
O′	1.525
Double bond (C=C)	1.733
Triple bond (C≡C)	2.398
Monoterpenes ($C_{10}H_{16}$)	
Acyclic	46.98
Monocyclic	45.25
Bicyclic	43.51
Tricyclic	41.78
Sesquiterpenes ($C_{15}H_{24}$)	
Acyclic	69.60
Monocyclic	67.87
Bicyclic	66.14
Tricyclic	64.40
Tetracyclic	62.67

[a] After Eisenlohr.[4]

whether the plane of polarization is rotated right or left, and the direction of rotation is indicated by prefixing a (+) or (−) sign, respectively, to the numerical value of rotation.

Optical rotation is the angle through which the plane of polarization of light is rotated when the polarized light passes through a 100-mm layer of the liquid at a specified temperature. It is expressed as α_x^t where t is the specified temperature in degrees centigrade and x is the wavelength of light employed, which is, usually, the sodium-D line (589.3 mμ), but sometimes the mercury green line (546.1 mμ).

The specific rotation $[\alpha]_x^t$ can be calculated from the value of optical rotation. If α is the optical rotation for the sodium-D line at the temperature t°C the specific rotation for a liquid is given by:

$$[\alpha]_D^t = \frac{\alpha}{ld}$$

where l is the length of the polarimeter tube in decimeters and d is the density of the liquid at t°C. For a solid substance in solution, the expression used is:

$$[\alpha]_D^t = \frac{100\alpha}{lc}$$

where c is the concentration of the substance in solution expressed as grams per 100 ml.

When reporting the specific rotation of a substance in solution, the concentration and the solvent must be stated.

Procedure

Carefully fill the polarimeter tube with the sample, avoiding the formation of any air bubbles. If the value of the optical rotation is to be reported at a specific temperature, use a jacketed tube and circulate water adjusted to that temperature. Check the temperature of the sample by placing a thermometer in the pocket provided for that purpose.

Place the tube in the trough of the instrument and rotate the analyzer, at the same time viewing through the telescope, until the two segments of the field of view (three in some instruments) are equally illuminated. Note the direction of rotation and read the angle of rotation on the scale with the help of verniers.

When the observed rotation is too small, a longer polarimeter tube should be used. On the other hand, shorter tubes are more convenient with dark-colored oils. In either instance the value of optical rotation should be calculated for a 100-mm tube.

Sometimes it is advisable to ascertain the direction of rotation caused by a sample. Thus, when measuring rotation of an essential oil with an instrument calibrated up to 180, an optical rotation of +80 may be mistaken for −100. The ambiguity can be clarified by repeating measurements with a 50-mm tube.

If true rotation is +80, a reading of +40 will be obtained, otherwise the reading will be −50, corresponding to optical rotation of −100.

E. SOLUBILITY IN ALCOHOL

Solubility of a substance in ethyl alcohol of a specified strength is constant at a given temperature. Adulterated or improperly stored samples of essential oils quite often show a marked change in their solubility, and, as such, the determination of this property offers a convenient and rapid means for checking the authenticity of the sample.

Alcohols of various strengths used for solubility determinations can be prepared by suitably diluting commercial 95% alcohol with distilled water as shown in Table 13.6. Correctness of dilution can be checked by determining

TABLE 13.6: Preparation of Dilute Alcohols*

Alcohol, % by volume	Specific gravity 15.56°/15.56°, 60°F/60°F	Proportions for mixing: 95% alcohol by volume, g	Proportions for mixing: Distilled water, g
50	0.9342	460	540
60	0.9133	564	436
65	0.9019	619	381
70	0.8899	676	324
75	0.8771	734	266
80	0.8636	796	204
90	0.8336	927	73
95	0.8158	1000	0

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the specific gravity of alcohols so obtained. Determination of solubility is carried out at a specified temperature usually 20° or 25°C.

Procedure

Introduce exactly 1 ml of the essential oil in a 10-ml glass-stoppered cylinder, graduated in 0.1-ml divisions. Slowly add small volumes of alcohol of the specified strength and shake thoroughly after each addition. Record the volume of alcohol when a clear solution is first obtained. Continue addition of alcohol until 10 ml has been added; at the same time note any change in appearance of the mixture, for example, opalescence, cloudiness, turbidity, or fluorescence and also record the volume of alcohol added up to the time the change takes place. If a clear solution is not obtained at any stage of the addition of alcohol, repeat the experiment with an alcohol of higher strength. If the determinations are to be carried out at a specified temperature, immerse the cylinder frequently in a water bath maintained at that temperature.

F. CONGEALING POINT

The congealing or solidifying temperature of an essential oil is a physical characteristic, which can often be employed for determination of purity. When congelation commences in a super cooled oil, crystallization is accompanied with liberation of heat. The highest temperature remaining constant for about 1 min during congelation is defined as the congealing point of the oil. The method for determination of congealing point is given below.

Procedure

Into a dry test tube of 18- to 20-mm internal diameter place about 10 ml of the dried sample. Cool in a suitable freezing mixture, the temperature of which should be about 5° below the expected congealing point of the oil. Vigorously rub the walls of the test tube with a thermometer graduated into tenths of a degree. Read the thermometer frequently. At first the rise of temperature is rapid but it soon approaches a value that remains constant for about 1 min. Take this temperature as the congealing point of the oil. Repeat several times to get concordant values.

Determination of the congealing point is important in the evaluation of anise, eucalyptus, fennel, and sassafras oils. The higher the congealing point of these oils, the more they are valued. An unusually low congealing temperature indicates the partial removal of the characteristic constituent for which the oil is valued or the addition of some extraneous matter.

13.3 DETERMINATION OF FUNCTIONAL GROUPS

A. ACIDS

Most essential oils contain small quantities of free acids, which in many instances originate, during the process of steam distillation, from the esters present in the plant material. Acid content of an oil may increase on aging, more particularly when it has been improperly stored, and it possesses aldehydic constituents. Several essential oils contain large quantities of some characteristic acids, for example, oil of orris root contains about 85% myristic acid, oil of ambrette seed contains chiefly palmitic acid, and oil of valerian roots contains about 10% of lower fatty acids. The acid content of an essential oil is, therefore, a valuable criterion of its quality.

The amount of free acids present in an essential oil is usually expressed as a number termed *acid value*. It is defined as the number of milligrams of potassium hydroxide required to neutralize the free acids present in 1 g of the oil.

The acid value is determined by titrating a solution of the oil in alcohol with an alcoholic solution of potassium hydroxide. The titration is carried out in a saponification flask so that the neutralized sample of the oil can further be used for determining its saponification value, described in the next section. To avoid the hydrolysis of esters during neutralization of the free acids, a weak solution (0.1 N) of alkali is usually employed. Phenolphthalein is generally used as the indicator, except for essential oils containing large quantities of phenolic constituents, when phenol red may be employed.

Procedure

Weigh accurately about 2 g of the oil into a 100-ml saponification flask and dissolve in 5 ml of ethanol. Add 5 drops of a 0.2% phenolphthalein solution and titrate with a standard alcoholic caustic potash solution until the appearance of a red color, which is stable for at least 10 sec.

Express the result to one decimal, using the following formula:

$$\text{acid value} = \frac{56.1vN}{w}$$

where v is the volume in milliliters of the potassium hydroxide solution, N is the normality of the potassium hydroxide solution, and w is the weight in grams of the sample.

For an essential oil containing a large amount of free acids, the result may be expressed as a percentage in terms of a particular acid and may be calculated as follows:

$$\text{free acids} = \frac{vNW}{10w}$$

where W is the equivalent weight of the acid.

B. ESTERS

A number of essential oils are evaluated on the basis of their ester content. Thus oil of wintergreen is valued for its chief constituent, methyl salicylate; determination of linalylacetate serves to establish the quality of the oils of bergamot and lavender, and official standards have set minimum requirements for menthyl acetate in peppermint oils.

The esters present in an essential oil may be determined by saponification with standard alkali solution, the amount of alkali consumed being ascertained by acidimetric determination of the unreacted alkali. For an ester of a monobasic acid, the saponification reaction may be expressed as follows:

$$RCOOR' + KOH \rightarrow RCOOK + R'OH$$

Since an essential oil may contain a number of esters, quite often possessing different molecular weights, it is more convenient to express the ester content of the oil in terms of *ester value*, which may be defined as the number of

milligrams of potassium hydroxide required to saponify the esters present in 1 g of the oil. If appreciable quantities of free acids are present in the oil, these will also be neutralized during saponification. The amount of alkali consumed during the process will, therefore, correspond to both the acid content as well as the ester content of the oil, and may be used for the calculation of another chemical constant, the *saponification value*, which may be defined as the number of milligrams of potassium hydroxide required to neutralize the free acids and saponify the esters present in 1 g of the oil. The ester value of the oil may be calculated from the saponification value as follows:

$$\text{ester value} = (\text{saponification value}) - (\text{acid value})$$

For determining ester values, it is more convenient to neutralize the free acids beforehand or to continue with the sample used for determination of acid value.

Procedure

Weigh accurately about 2 g of the oil and neutralize the free acids as described under procedure for determining acid value. Add 20 ml of approximately 0.5 *N* ethanolic potassium hydroxide solution and boil under a reflux condenser, on a water bath, for 1 hr. Carry out a blank determination by taking 5 ml of neutralized ethanol, 5 drops of phenolphthalein solution, and 20 ml of the same 0.5 *N* ethanolic potassium hydroxide solution in a similar saponification flask and refluxing it for the same length of time. Allow the contents of both the saponification flasks to cool at room temperature. Titrate with standard 0.5 *N* aqueous hydrochloric acid, adding more indicator dropwise, if needed, near the end point.

Calculate the ester value of the oil by means of the formula

$$\text{ester value} = \frac{56.1(v_1 - v_2)N}{w}$$

where v_1 is the volume in milliliters of standard hydrochloric acid used for the blank, v_2 the volume in milliliters of standard hydrochloric acid used for the determination, N the strength in normality of the standard hydrochloric acid, and w the weight of the sample in grams.

Percentage in terms of a particular ester, may be readily calculated as follows:

$$\text{percentage of ester} = \frac{ME}{561.04b} = \frac{M(v_1 - v_2)N}{10bw}$$

where E is the ester value of the essential oil, M is the molecular weight of the ester, and b is the number of ester groups in the molecule.

For oils containing large amounts of free acids, the step involving initial neutralization of acids is omitted. The expression for ester value now gives the saponification value, from which the ester value may be calculated, as discussed before, by subtracting the acid value.

Some esters, for example, geranyl formate, undergo instantaneous saponification even at room temperature. Such a reaction is carried out at room temperature and the unreacted alkali is titrated immediately. Many esters, on the other hand, are not completely saponified in 1 hr and require longer refluxing. Comparatively rapid saponification of the difficultly saponifiable esters may also be achieved by preparing the solution of potassium hydroxide in a high boiling solvent, for example, ethylene glycol or its monoethyl ether.

Saponification of some essential oils yields reaction mixtures which are too intensely colored to give a sharp end point with phenolphthalein, and hence the use of thymolphthalein is recommended.

C. ALCOHOLS

The determination of alcohols present in an oil is an important step toward the appraisal of its quality. Several essential oils are valued mainly for the alcohols they contain. Thus the quality of sandalwood oil, peppermint oil, and rosewood oil depends to a great extent on the percentage of santalols, menthol, and linalool, respectively. Many other essential oils, though not predominantly composed of alcohols, contain significant amounts of these compounds which contribute to the quality of the oils.

The general methods for determination of alcohols in essential oils involve esterification followed by estimation of esters so formed. The procedure most commonly employed consists of acetylation of the oil with acetic anhydride using fused sodium acetate as catalyst, and determination of the ester values of the acetylated oil as well as of the original oil.

Procedure

Place 10 ml of the sample in a 100-ml acetylation flask and add 10 ml of acetic anhydride (analytical grade) and 2 g of fused sodium acetate. Attach an air condenser to the flask and reflux the contents gently for exactly 2 hr, on a sand bath. Cool the flask for 15 min at room temperature. Add 50 ml of distilled water to the reaction mixture through the air condenser, and heat the flask on a steam bath for a further 15 min with frequent shaking. Pour the contents of the flask into a separatory funnel. Rinse the flask with two 10-ml portions of distilled water and add the rinsings to the separatory funnel. Shake thoroughly and allow the mixture to separate into two layers. Reject the aqueous layer and wash the oil with 100-ml portions of saturated sodium chloride solution until the washings are neutral to litmus paper. Dry the oil over anhydrous sodium sulfate.

Determine the ester value of the acetylated oil as described before, and calculate the percentage of free alcohols in the oil by means of the following expression:

$$\text{percentage of free alcohols} = \frac{M(v_1 - v_2)N}{10w - 0.42(v_1 - v_2)}$$

where v_1 is the volume in milliliters of standard hydrochloric acid used for the blank, v_2 the volume in milliliters of standard hydrochloric acid used for the determination, N the strength in normality of the standard hydrochloric acid, w the weight of the sample in g., and M the molecular weight of the alcohol. For essential oils containing a significant quantity of esters, the following formula may be applied:

$$\text{percentage of free alcohols} = \frac{Md}{561.04 - 0.42d}$$

where d is difference between the ester value of acetylated oil and ester value of the original oil.

This method is not suitable for most tertiary alcohols, for example, linalool and terpineol, which are incompletely acetylated and are also partially dehydrated. Certain aldehydes and ketones, as well as phenols can also undergo acetylation under the experimental conditions described, thereby interfering with the determination of alcohols. Special methods devised for some particular alcohols are described later.

1. Determination of Tertiary Alcohols

Dehydration of tertiary alcohols during acetylation may be considerably reduced by diluting the essential oil with a suitable substance. Boulez[5] used turpentine oil as the diluent, whereas Schimmel & Co., recommended the use of xylene.[6] The diluted essential oil has to be acetylated for longer periods—5 hr for terpineol and 7 hr for linalool.

A more convenient method for the estimation of tertiary alcohols is based on their property to undergo formylation on treatment with a mixture of acetic anhydride and formic acid. The method was developed by Glichitch[7] and has been used satisfactorily for a number of years.

Procedure. Cool 2 volumes of acetic anhydride to about 0°C and add to it slowly 1 volume of 100% formic acid. Warm the mixture cautiously to 50°C and immediately cool in an ice bath. Place 15 ml of this mixture into a glass-stoppered Erlenmeyer flask, kept immersed in an ice bath, and slowly add 10 ml of the essential oil. Leave the flask in the bath to gradually attain room temperature. After 72 hr add 50 ml of ice-cold water, shake well, and transfer to a separatory funnel. Discard the aqueous layer and wash the oil with 50 ml of water, then 50-ml portions of 2% sodium carbonate solutions until the washings are alkaline; finally wash again with 50 ml of distilled water. Dry the oil over anhydrous sodium sulfate and saponify with 0.5 N

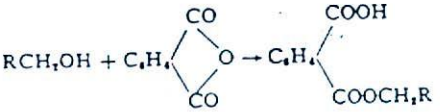
alcoholic caustic potash, as described in Section 13.3,B. Calculate the percentage of alcohols from the following formula:

$$\text{percentage of alcohols} = \frac{M(v_1 - v_2)N}{10w - 0.14(v_1 - v_2)}$$

where v_1 is the volume in milliliters of standard hydrochloric acid used for the blank, v_2 is the volume in milliliters of standard hydrochloric acid used for the determination, N is the strength in normality of the standard hydrochloric acid, w is the weight of the sample in grams, and M is the molecular weight of the alcohol.

2. Determination of Primary Alcohols

Primary alcohols may be determined in the presence of secondary and tertiary alcohols by esterification with phthalic anhydride[8]; they form acid phthalates readily at about 100°C (steam bath). Secondary alcohols react with the anhydride on prolonged heating or at a higher temperature, whereas the tertiary alcohols remain practically unreacted. Percentage of primary alcohols is calculated from the quantity of phthalic anhydride consumed in esterification.



Procedure. Place 2 g of the oil into a 100-ml acetylation flask and add 2 g of powdered, pure phthalic anhydride and 2 ml of benzene. Heat on a steam bath with frequent shaking for 2 hr. Cool the flask for 30 min and add 60 ml of 0.5 N aqueous potassium hydroxide solution. Stopper the flask and shake thoroughly for 10 min. Titrate the unreacted alkali with 0.5 N hydrochloric acid using 3 drops of 1% phenolphthalein solution. Perform a blank determination. Calculate the percentage of primary alcohols as follows:

$$\text{percentage of primary alcohols} = \frac{M(b - a)}{20w}$$

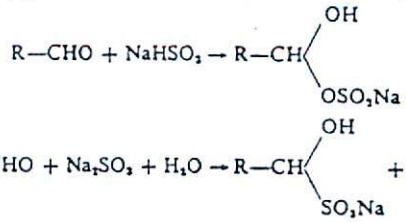
where M is the molecular weight of the primary alcohol, b is the milliliters of 0.5 N potassium hydroxide required for the amount of phthalic anhydride used in the determination as calculated from the blank estimations, a is the milliliters of 0.5 N, potassium hydroxide consumed in the determination, and w is the weight of the sample in grams.

D. ALDEHYDES AND KETONES

The conventional methods for the determination of aldehydes and ketones fall into two classes: absorption methods and titration procedures.

1. Absorption Methods

The absorption methods are based on the property of aldehydes and ketones to react with sodium bisulfite or neutral sodium sulfite to yield water soluble addition compounds. The reactions may be expressed by the following equations:



The methods generally consist of allowing a known volume of the essential oil to react with a saturated solution of either reagent and measuring the percentage of the oil absorbed. The bisulfite method has been employed for the determination of cinnamic aldehyde in cassia oil, benzaldehyde in bitter almond oil, citronellal in *Eucalyptus citriodora* oil, and citral in lemongrass oil. The neutral sulfite method has proved satisfactory for the determination of cinnamic aldehyde in cassia oil and citral in lemongrass oil, as well as of carvone in spearmint, dill and caraway oils, of pulegone in pennyroyal oil, and of piperitone in Eucalyptus oil.

Procedure[9]

a. *Bisulfite method.* To a 10-ml sample of the essential oil taken in a 150-ml cassia flask, add 75 ml of a saturated solution of sodium bisulfite. Heat the flask in a water bath with frequent shaking to dissolve the solid addition compound and to ensure complete reaction of the aldehyde with the reagent. Add an additional 25 ml of sodium bisulfite solution and continue heating. Allow the unreacted oil to form a separate layer. Drive the oily layer into the neck of the cassia flask by slowly adding sodium bisulfite solution. Measure the volume of the unreacted oil.

b. *Neutral Sulfite Method.* Neutralize a freshly prepared saturated solution of sodium sulfite with a 50% acetic acid solution, employing phenolphthalein as indicator. Proceed as in the *bisulfite method.* Neutralize the reaction mixture from time to time with 50% acetic acid solution until no further pink color is observed when further phenolphthalein solution is added dropwise. Force the unreacted oil into the neck of the cassia flask by adding an additional quantity of the neutralized sulfite solution, and note the volume of the unabsorbed oil.

Amount of aldehyde or ketone (by either method) is equal to 10(10 — volume of unreacted oil in milliliters) per cent by volume.

Both these methods are applicable only to essential oils containing large

percentages of aldehydes and ketones, and suffer from the disadvantage that some noncarbonyl unsaturated substances capable of yielding water-soluble sulfonates as well as other water-soluble constituents are also estimated as carbonyl compounds. In some respects, the neutral sulfite method is superior to the bisulfite method. The former permits exact determination of the end point of the reaction. Many aldehydes and ketones react with neutral sodium sulfite completely and in a shorter time than with sodium bisulfite.

2. Titration Procedures

The earliest titration method, employing phenylhydrazine,[10] is rarely used today. It is more suited to the determination of aldehydes and is based on the following reaction:

$$R{-}CHO + H_2N{\cdot}HN{\cdot}C_6H_5 \rightarrow R{-}CH{=}N{\cdot}NHC_6H_5 + H_2O$$

The method consists of allowing the essential oil to react with a known quantity of phenylhydrazine and titrating the excess phenylhydrazine with hydrochloric acid. A blank determination is carried out at the same time and the aldehyde is estimated from the difference in volume of hydrochloric acid consumed in the two titrations.

The two most widely applied methods for the determination of aldehydes and ketones are based on the reaction of hydroxylamine with the carbonyl compounds:

$$R{\cdot}CH{=}O + H_2N{\cdot}OH \rightarrow R{\cdot}CH{=}NOH + H_2O$$

$$\begin{matrix} R \\ \\ R_1 \end{matrix}\!\!>\!C{=}O + H_2N{\cdot}OH \rightarrow \begin{matrix} R \\ \\ R_1 \end{matrix}\!\!>\!C{=}NOH + H_2O$$

One of the techniques known as the *standard method*, involves reaction of the oil with hydroxylamine hydrochloride solution, followed by titration of the liberated hydrochloric acid.[11] The other procedure called the *Stillman–Reed method* employs for oximation a solution of free hydroxylamine prepared by neutralization of the free and combined hydrochloric acid present in hydroxylamine hydrochloride solution.[12] The details of these two methods follow.

Standard Method. Prepare the hydroxylamine hydrochloride solution by dissolving 34.7 g of the reagent in 40 ml of distilled water and adding enough 95% alcohol to make up to a liter. Add 15 ml of a 0.1% solution of bromophenol blue indicator in 50% alcohol. Neutralize free hydrochloric acid by gradual addition of 0.5 *N* alcoholic sodium hydroxide until the yellow color of the solution changes to a greenish-yellow.

Accurately weigh the required quantity of the sample in a 100-ml saponification flask and add 35 ml of the reagent solution prepared as above. Allow

the reaction to proceed at room temperature or on a steam bath as specified for the particular essential oil. Titrate with 0.5 *N* alcoholic sodium hydroxide to the original greenish-yellow color. Carry out a blank determination under identical conditions.

Stillman–Reed Method. Dissolve 20 g of hydroxylamine hydrochloride in 40 ml of water and dilute to 400 ml with 95% alcohol. To this solution add with stirring 300 ml of 0.5 *N* alcoholic potassium hydroxide and 2.5 ml of the indicator solution prepared by dissolving 0.1 g of bromophenol blue in 3 ml of 0.05 *N* sodium hydroxide, adding enough distilled water to make up to 25 ml. Filter the resulting solution.

Accurately weigh the requisite amount of the oil into a 200-ml Erlenmeyer flask equipped with a reflux condenser. Add 75 ml of the hydroxylamine solution as prepared above. Allow the reaction to proceed at room temperature or on a steam bath. Titrate with standard 0.5 *N* hydrochloric acid to a greenish-yellow end point. Carry out a blank determination under the same conditions.

Calculate the carbonyl content of the essential oil, for either method, using the following formula:

$$\text{percentage of the carbonyl compound} = \frac{0{\cdot}1MNv}{w}$$

where M is the molecular weight of the carbonyl compound, N is the normality of the sodium hydroxide solution in the standard method or of the hydrochloric acid solution in the Stillman–Reed method, v is the difference in milliliters between the titer values for the blank and the sample solution, and w is the weight of the sample in grams.

The reaction between hydroxylamine and aldehydes is rapid and is practically complete in 15 min for most of the substances. With ketones, the reaction is slow but can be expedited by refluxing the reactants on a steam bath. Tables 13.7 and 13.8 illustrate the proper size of sample and appropriate conditions of reaction for some carbonyl compounds and essential oils.

For dark-colored oils, the end point of the titration cannot be measured accurately. This difficulty can be overcome by carrying out the titration potentiometrically. Both the blank and the sample solutions should be titrated to pH 3.5.[13–15]

E. PHENOLS

Phenols are important constituents of several essential oils. To some oils, they impart antiseptic and germicidal properties, whereas in many others, they are responsible for the characteristic flavor. Thus oils of ajowan, origanum, and thyme owe their medicinal importance to thymol and carvacrol, and oils of bay, cinnamon, and clove are valued for their eugenol content.

TABLE 13.7: Conditions for Oximation of Carbonyl Compounds

Carbonyl compound	Size of sample, g	Reaction time[a]
Acetophenone	1.0	15 min
Anisic aldehyde	1.0	15 min
Benzaldehyde	1.0	Immediate
Camphor	0.5	7 hr (reflux on steam bath)[b]
Carvone	0.5	2 hr (reflux on steam bath)[b]
Cinnamic aldehyde	1.0	15 min
Citral	1.0	15 min
Citronellal	1.0	15 min (at −10°C)
Cuminic aldehyde	1.0	15 min
Decyl aldehyde	1.0	30 min
Furfural	1.0	15 min
Menthone	0.5	2 hr (reflux on steam bath)[b]
Octyl aldehyde	1.0	15 min
Salicyl aldehyde	1.0	15 min
Thujone	0.5	24 hr
Vanillin	1.0	15 min

[a] Room temperature, unless mentioned.
[b] In Tables 13.7 and 13.8 values marked [b] are based on author's work; others are reprinted from Guenther, *The Essential Oils*, Vol. I, copyright 1948, D. Van Nostrand Company Inc., Princeton, N.J.

TABLE 13.8: Conditions for Oximation of Essential Oils

Oil	Main carbonyl compound present	Size of sample, g	Reaction time[a]
Almond, bitter	Benzaldehyde	1.0	immediate
Caraway	Carvone	1.0	2 hr (reflux on steam bath)[b]
Cassia	Cinnamic aldehyde	1.0	15 min
Cedar leaf	Thujone	1.0	24 hr
Cinnamon	Cinnamic aldehyde	1.0	15 min
Citronella	Citronellal	1.0	15 min
Cumin	Cuminic aldehyde	1.0	15 min
Dill	Carvone	1.0	2 hr (reflux on steam bath)[b]
Grapefruit	Decyl aldehyde	5.0	30 min
Lemon	Citral	5.0	15 min
Lemon (terpeneless & sesquiterpeneless)	Citral	1.0	15 min
Lemongrass	Citral	1.0	15 min
Mint	Menthone	0.5	2 hr (reflux on steam bath)[b]
Orange	Decyl aldehyde	5.0	30 min
Orange (terpeneless & sesquiterpeneless)	Decyl aldehyde	1.0	30 min
Peppermint	Menthone	0.5	2 hr (reflux on steam bath)[b]
Spearmint	Carvone	1.0	2 hr (reflux on steam bath)[b]
Tansy	Thujone	0.5	24 hr
Wormwood	Thujone	0.25	24 hr

[a] Room temperature, unless mentioned.

The quantity of phenols in an essential oil may be estimated by allowing the oil to react with an alkali hydroxide, forming phenolates which go into the aqueous phase. The volume of the unabsorbed nonphenolic portion of the oil is measured.

The method suffers from the drawback that nonphenolic water-soluble and alkali-soluble substances may also be included in the phenol determination.

Procedure

Accurately measure 10 ml of the oil into a 100-ml cassia flask. Add 75 ml of a 1 *N* potassium hydroxide solution and shake thoroughly for 5 min, then allow the flask to stand undisturbed for 1 hr or more. Carefully add enough alkali solution to force the unreacted oil into the graduated neck of the flask, and measure the volume.

TABLE 13.9: Experimental Conditions for Assay of Phenols

Essential oil	Phenol to be assayed	Concentration of KOH solution	Condition of reaction
Bay (terpeneless)	Eugenol	0.5 *N*	Shake with 125 ml of alkali
Cinnamon (leaf & bark)	Eugenol	1 *N*	Shake 5 ml of the sample with 50 ml of alkali for 3 min
Clove and pimenta	Eugenol and acetyleugenol: reported as eugenol	1 *N*	Shake for 5 min in cold and heat for 10 min on a steam bath with frequent shakings
Origanum and thyme	Thymol and carvacrol (total as well as individual)	1 *N*	Follow standard procedure for total phenols; determine the melting point of mixed phenols isolated from alkaline solution and read the % of individual phenol from a standard curve (16)

If the oil forms an emulsion with the alkali solution, repeat the experiment after addition of 2 ml of xylene. Subtract 2 ml from the measured volume of the unabsorbed oil:

$$\text{percentage of phenols} = 10(10 - V)$$

where V is the volume in milliliters of the unabsorbed portion of the oil.

Differences in the reactivity of phenols and phenolic esters towards alkali, solubility of nonphenolic substances in aqueous alkali and alkali phenolates, and formation of emulsions often make it necessary to estimate the phenol content of an oil by carrying out the experiments under specified conditions. The conditions of reaction for some essential oils are described in Table 13.9.

13.4 DETERMINATION OF SOME SPECIFIC CONSTITUENTS

A. ALLYL ISOTHIOCYANATE

Allyl isothiocyanate is the main constituent of mustard essential oils. Several methods have been proposed for its assay, the most satisfactory being the procedure adopted by the USP.[17] The method is based on the reaction of allyl isothiocyanate with silver nitrate in the presence of ammonium hydroxide. The excess silver nitrate is determined by titration with standard ammonium thiocyanate solution, employing ferric ions as indicator.

$$CH_2{=}CH{\cdot}CH_2NCS + NH_4OH + AgNO_3 \rightarrow AgNCS + CH_2{=}CH{\cdot}CH_2OH + NH_4NO_3$$

Procedure

Into a 100-ml volumetric flask weigh accurately about 4 ml of the oil and make up to the mark with alcohol. Pipette 5 ml of this solution into a 100-ml volumetric flask equipped with a reflux condenser. Add 50 ml of 0.1 *N* silver nitrate solution and 5 ml of 10% ammonia solution. Reflux on a steam bath for 1 hr. Cool the contents of the flask and make up to the mark with distilled water. Mix well and filter through a dry filter paper. Reject the first 10 ml of the filtrate. To 50 ml of the subsequent filtrate, accurately measured, add about 5 ml of concentrated nitric acid and 2 ml of an 8% solution of ferric ammonium sulfate. Titrate the excess of silver nitrate with 0.1 *N* ammonium thiocyanate. Carry out a blank determination simultaneously, using 5 ml of alcohol in place of the solution of mustard oil. Calculate the percentage of allyl isothiocyanate as follows:

$$\text{percentage of allyl isothiocyanate} = \frac{19.832(v_1 - v_2)}{w}$$

where v_1 is volume in milliliters of ammonium thiocyanate solution required for the blank, v_2 is volume in milliliters of ammonium thiocyanate solution required for the determination and w is weight of the sample in grams.

B. ASCARIDOLE

Ascaridole is an important constituent of chenopodium oils. However, no satisfactory procedure is yet available for its determination. The method of Nelson,[18] which involves absorption of ascaridole in dilute acetic acid and measurement of the volume of undissolved oil, suffers from the disadvantages inherent in an absorption process and gives unreliable results.

Another method, developed by Paget,[19] is based on the reduction of ascaridole with titanium chloride. The procedure is tedious and inconvenient,

because the solution has to be stored under hydrogen and the titrations have to be carried out in an atmosphere of carbon dioxide. If all the precautions are taken, the method gives satisfactory results.

An equally accurate yet simple method is that developed by Cocking and Hymas.[20] It is based upon the oxidizing action of ascaridole on potassium iodide and has been widely adopted.[21,22]

Procedure

Dissolve about 2.5 g of accurately weighed sample in sufficient 90% acetic acid to yield 50 ml of solution and pour the solution into a burette graduated in 0.05-ml divisions. Into a glass-stoppered Erlenmeyer flask, place about 3 ml of 83% (w/v) aqueous potassium iodide solution, 5 ml of concentrated hydrochloric acid, and 10 ml of glacial acetic acid. Cool the mixture to −3°C and add about 5 ml of the solution from the burette, mixing as quickly as possible. Take the burette reading after allowing 2-min draining time. Set the stoppered flask aside for 10 min and, without diluting, titrate the liberated iodine with 0.1 *N* sodium thiosulfate solution. Carry out a blank determination, diluting the mixture with 20 ml of distilled water before titration. Calculate the quantity of ascaridole present in the solution withdrawn from the burette by the difference between the two titrations. Each milliliter of 0.1 *N* sodium thiosulfate solution is equivalent to 0.00665 g of ascaridole.

The factor 0.00665 is empirical and is based on the results of a specimen of ascaridole; however, validity of the same factor for chenopodium oils has been questioned.[23] In spite of this drawback, the method continues to be the most satisfactory among those available.

C. CAMPHOR

The estimation of camphor in pharmaceutical preparations and essential oils has been the subject of several investigations. The general techniques for the assay of carbonyl compounds described before are not applicable to this terpenoid, since the reactions are slow and incomplete.

Aschan[24] proposed a gravimetric method in which a solution of the oil in glacial acetic acid is warmed with semicarbazide hydrochloride and fused anhydrous potassium acetate. The resulting semicarbazone is precipitated by dilution with water. The percentage of camphor is calculated from the weight of the semicarbazone obtained.

Hampshire and Page[25] observed that camphor can be quantitatively precipitated from alcoholic solutions by treatment with 2,4-dinitrophenylhydrazine and proposed a method of assay for camphor which has been adopted by the BP.[26]

Procedure

Dissolve about 0.2 g of accurately weighed sample in 25-ml of aldehyde free alcohol in a 300-ml Erlenmeyer flask. Add, with constant shaking, 75 ml of 2,4-dinitrophenylhydrazine solution prepared by dissolving 1.5 g of the reagent in 20 ml of 50% (v/v) sulfuric acid, diluting to 100 ml with water and filtering. Reflux on a steam bath for 4 hr, cool, and dilute to 200 ml with 2% (v/v) sulfuric acid. Allow to stand for 24 hr, and filter through a tared sintered-glass or Gooch crucible containing a paper mat. Wash the precipitate with 10-ml portions of cold water until the washings are neutral to litmus and dry to constant weight at 80°C. Calculate the percentage of camphor as follows:

1 g precipitate = 0.4580 g camphor

Anderson[27] has pointed out that a correction of 0.2% should be applied to the calculated results to compensate for the solubility of camphor dinitrophenylhydrazone.

The above methods for the determination of camphor are not suitable when other aldehydes and ketones are also present in the sample. In such instances, the application of gas-liquid chromatography is likely to give the most satisfactory results.[28]

D. CINEOLE

Cineole is an important constituent of many essential oils, for example, eucalyptus, cajuput, camphor, rosemary, and lavender. Several procedures have been proposed for the assay of this terpenoid.

A method by Scammell,[29] later modified by Baker and Smith,[30] involves treatment of a known volume of the essential oil with phosphoric acid. The resulting cineole-phosphoric acid complex is isolated and decomposed with water in a cassia flask. Cineole is liberated, and from the volume read, its percentage is calculated in the essential oil. A similar method based on formation of an addition compound between cineole and arsenic acid has also been proposed.[31]

Kleber and von Rechenberg observed that the congealing point of the oil itself is a measure of the cineole present.[32] The correlation between the congealing points and the cineole content of essential oils is given in Table 13.10. The accuracy of the method is ±1%.

The most widely accepted method for the determination of cineole is that of Cocking which is based on the reaction between cineole and *o*-cresol. The details of the procedure[33] are as follows.

Procedure

Into a stout-walled test tube, about 15 mm in diameter and 80 mm in length, weigh accurately 3 g of dry oil and 2.1 g of melted, pure, dry *o*-cresol. Insert a thermometer graduated in fifths of a degree, and stir the contents of the tube with a wire loop until crystallization commences. Note the highest

TABLE 13.10: Determination of Cineole Content by Congealing Point*

Congealing point °C	Cineole content, % w/w	Congealing point °C	Cineole content, % w/w
1.2	100.0	−9.0	80.3
1.0	99.4	−10.0	78.5
0.0	97.3	−11.0	76.5
−1.0	95.3	−12.0	75.3
−2.0	93.4	−13.0	73.7
−3.0	91.5	−14.0	72.2
−4.0	89.6	−15.0	70.6
−5.0	87.5	−16.0	69.2
−6.0	85.7	−17.0	67.5
−7.0	83.7	−18.0	66.2
−8.0	82.0	−19.0	64.8

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reading of the thermometer. Warm the tube until the contents are melted and suspend it in a wide-mouthed bottle through a cork. Allow the mixture to cool slowly until crystallization commences or the temperature falls to the point previously noted. Stir the mixture vigorously, rubbing the loop on the sides of the tube with an up and down motion. Continue stirring as long as the temperature rises and take the highest temperature attained as the "freezing point." Remelt the mixture and repeat the procedure until two concordant results are obtained. Refer to Table 13.11 to obtain the percentage of cineole which corresponds to the observed freezing point.

The method gives accurate results for essential oils containing a relatively high percentage of cineole. If the cineole content is less than 50%, a mixture of equal parts by weight of the oil and pure cineole is prepared and used for determination. The cineole content of the original oil may be calculated as follows:

$$\text{per cent cineole in original oil} = 2(\%\ \text{cineole in mixture} - 50)$$

A spectrophotometric method for the determination of small quantities of cineole was proposed by Martin and Harrison.[34] It consists in treating a methanolic solution of the oil with a solution of *p*-dimethylamino benzaldehyde in aqueous sulfuric acid and reading the absorption at 555 mμ after 6 min.

E. CITRAL

The bisulfite and the neutral sulfite methods are not suited to the assay of citral in essential oils containing a low percentage of this aldehyde. The titrimetric methods employing phenylhydrazine or hydroxylamine are of wider applicability but suffer from the disadvantages that other aldehydes and ketones present in the oil also react with these reagents, thereby giving a higher citral content.

TABLE 13.11: Determination of Cineole Content by the o-Cresol Method*

Freezing point, °C	Cineole content, % w/w	Freezing point, °C	Cineole content, % w/w
24	45.6	41	68.6
25	46.9	42	70.5
26	48.2	43	72.3
27	49.5	44	74.2
28	50.8	45	76.1
29	52.1	46	78.0
30	53.4	47	80.0
31	54.7	48	82.1
32	56.0	49	84.2
33	57.3	50	86.3
34	58.6	51	88.8
35	59.9	52	91.3
36	61.2	53	93.8
37	62.5	54	96.3
38	63.8	55	99.3
39	65.2	55.2	100.0
40	66.8		

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Among the more specific methods based on the reactions of citral, that of Hiltner, involving formation of a colored addition product with *m*-phenylenediamine yields satisfactory results.[35] The assay has been given official status by the Association of Official Agricultural Chemists.[36]

Procedure

Dissolve 1 g of dry crystals of *m*-phenylenediamine hydrochloride (free of colored impurities by digesting with alcohol) in 45 ml of 85% alcohol. Prepare another solution of 1 g of oxalic acid crystals in 45 ml of 85% alcohol. Pour the two solutions into a 100-ml volumetric flask, add 2 to 3 g of Fuller's Earth, make up to the mark with 85% alcohol, shake well, and filter.

Into a 50-ml volumetric flask, weigh accurately about 25 g of the sample. Make up to the mark with 95% alcohol for extracts prepared from the oils or with 50 to 95% alcohol for extracts containing terpeneless oils. Pipette a suitable volume of these solutions into colorimeter tubes, add 10 ml of the phenylenediamine solution prepared as above, and dilute to a suitable volume. Compare the resulting color with sets of standards containing known quantities of citral.

Although *m*-phenylenediamine was originally considered a specific reagent for α,β-unsaturated aldehydes, it has since been shown that α,β-unsaturated ketones, as well as other reactive aldehydes and ketones, interfere.[37,38]

Two selective methods have been recently developed for the analysis of citral in essential oils, extracts, and other preparations. The method of Levi and Laughton[39] involves condensation of citral with barbituric acid and measurement of the absorbance at 336 mμ. Another method proposed by Stanley et al.[40] is based on the observation that citral, on treatment with a reagent mixture of vanillin and piperidine in absolute alcohol, forms an alcohol soluble green complex exhibiting absorption maximum at 605 mμ.

F. CITRONELLAL

The hydroxylamine methods, as described previously, are not suitable for the determination of citronellal. The aldehyde is unstable in the presence of free hydrochloric acid, whereas its reaction with hydroxylamine proceeds rapidly only in slightly acidic medium. This difficulty can be overcome to a great extent by neutralizing, as quickly as possible, most of the free acid liberated in the reaction, without allowing the solution to become alkaline. The following modified method[41] was found to yield satisfactory results.

Procedure

Dissolve 6.95 g of pure hydroxylamine hydrochloride solution in 95 ml of 90% (v/v) alcohol, and add 0.4 ml of 0.2% dimethyl yellow solution. Allow the solution to attain the full-yellow color of the indicator by gradual addition of 0.5 *N* alcoholic potassium hydroxide. Make up to 100 ml with 90% alcohol and cool the reagent solution to 0°C. Weigh accurately a suitable quantity of the oil to contain approximately 0.8 g of citronellal and cool to at least 0°C. Add 10 ml of the cooled hydroxylamine hydrochloride reagent solution prepared as above. Titrate immediately with 0.5 *N* alcoholic potassium hydroxide taking care to avoid going beyond the orange color of the indicator. Continue the titration cautiously, as long as the red color develops, then allow the mixture to stand at room temperature for 1 hr and complete the titration to the full-yellow color of the indicator. Calculate the aldehyde content, in terms of citronellal by the following formula:

$$\text{percentage of aldehydes (as citronellal)} = \frac{7.7vN}{0.5w} \times 1.008$$

where v is the volume in milliliters of 0.5 *N* alcoholic potassium hydroxide consumed in titration, N the normality of the alcoholic potassium hydroxide used for titration, and w the weight of the sample in grams.

Since the end point of the titration occurs at a pH different from that of 1 *N* hydroxylamine hydrochloride, a correction factor (1.008) has been incorporated in the above formula.

A method proposed by de Miranda and Lemmens[13] involves the addition of small quantities of hydroxylamine hydrochloride solution and immediate

titration of the liberated acid with standard alcoholic potassium hydroxide to a constant pH, employing a potentiometer which has been equipped with a glass-calomel electrode combination. The method should yield good results, since there is no accumulation of free acid at any time, and the glass electrode is more sensitive than a visual indicator for maintaining the media at a constant pH.

G. CITRONELLOL IN PRESENCE OF GERANIOL

The general method for the determination of alcohols by acetylation cannot be applied for citronellol when it occurs in association with geranoil, for example, in oils of rose or geranium. Since a knowledge of the percentage of citronellol in these oils is useful for the evaluation of their quality, several methods permitting estimation of citronellol alone have been proposed from time to time. These involve either the separation of geranoil in the form of its calcium chloride adduct[42] or its phthalate,[43] or the destruction of geraniol by treatment with such reagents as pyruvic acid,[44] alcoholic potassium hydroxide,[45] or formic acid.[46] Detailed experimental conditions for a method of employing hot formylation were described by Glichitch and Naves.[47] The procedure, outlined below, permits an accuracy of 1 to 2%.

Procedure

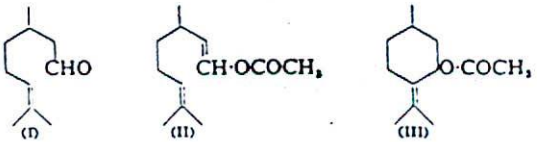
Place 10 ml of the oil and 20 ml of 90% formic acid in an acetylation flask. Attach an air condenser and heat on a steam bath for 1 hr with frequent shaking. Cool the reaction mixture to room temperature, transfer to a separatory funnel, and wash successively with 50 ml of 10% sodium chloride solution, 50 ml of 10% sodium carbonate solution, and two 50-ml portions of a solution containing 10% sodium chloride and 2% sodium carbonate. Finally, wash with 25 ml of distilled water and dry over anhydrous sodium sulfate. Saponify the formylated oil as described in Section 13.3,B and calculate the percentage of citronellol using the formula for calculation of alcohols by the method of Glichitch (Section 13.3,C).

The method gives satisfactory results in the presence of linalool also, but is useless when the essential oil contains alcohols which are not decomposed by formic acid. Actually geraniol is not completely dehydrated during formylation, but this and other discrepancies balance one another yielding satisfactory results.[48]

H. GERANIOL IN CITRONELLA OILS

Citronella oil contains, in addition to geraniol, small quantities of other alcohols, for example, borneol or citronellol, all of which are determined along with and calculated as geraniol. When the oil is acetylated for determination of alcohols, not only are the alcohols converted to esters but

citronellal(I) also undergoes acetylation forming an enol acetate (II) which suffers cyclization yielding isopulegol acetate (III).[49,50]



(I) (II) (III)

The method, therefore, estimates all constituents prone to acetylation in terms of geraniol. The amount of citronellal present in the oil may be determined by the method described in Section 13.4,F. The percentage of geraniol in the oil is obtained by subtracting the percentage of citronellal from the "total percentage of acetylated constituents."

I. HYDROCYANIC ACID

Hydrocyanic acid is present in the essential oil of many seed kernels, for example, bitter almond, cherry laurel, and apricot. It often occurs in combination with benzaldehyde in the form of cyanohydrin. The acid can be estimated satisfactorily by titration with silver nitrate. The oil is first treated with freshly precipitated magnesium hydroxide, which serves to liberate the combined hydrocyanic acid. The acid is then precipitated by addition of silver nitrate. The end point of the reaction is determined by employing potassium chromate as indicator. The details of the method[51] are as follows.

Procedure

Dissolve 0.75 g of magnesium sulfate in 45 ml of distilled water, add 5 ml of 0.5 *N* sodium hydroxide and 2 drops of 10% solution of potassium dichromate. Titrate the solution with 0.1 *N* silver nitrate until the red color of silver chromate persists. Pour the resulting mixture into a 100-ml Erlenmeyer flask containing 1 g of the sample, accurately weighed. Mix well and titrate rapidly with silver nitrate solution again, until a permanent red color is obtained. Calculate the hydrocyanic acid content as follows:

$$\text{percentage of hydrocyanic acid} = \frac{0.2703v}{w}$$

where v is the number of milliliters of 0.1 *N* silver nitrate consumed in the titration and w is the weight of the sample in grams.

J. LINALOOL

It was discussed previously that the general method for the determination of alcohols by acetylation is not suitable for the estimation of linalool. The method of Glichitch, employing cold formylation, has been successfully used

for the determination of linalool and other tertiary alcohols. However, recent investigations by Holness[52] have shown that satisfactory results obtained by this method are due to balancing of discrepancies rather than to any inherent superiority of the method. It appears that by far the most reliable method is that of Fiore, published by the Essential Oil Association of U.S.A.[53] The method is described below.

Procedure

Introduce 10 ml of the sample, dried with sodium sulfate, into a 125-ml glass-stoppered Erlenmeyer flask cooled with ice and water. To the cooled oil add 20 ml of dimethyl aniline (monomethyl free), mix thoroughly, and then add 8 ml of acetyl chloride (reagent grade) and 5 ml of acetic anhydride. Cool the mixture for a few minutes, allow to stand at room temperature for 30 min, then immerse in a water bath at 40° (±1) for 3 hr. Wash the acetylated oil three times with 75 ml of ice water, then with 25-ml portions of 5% sulfuric acid until the separated acid layer fails to liberate any dimethyl aniline in the presence of sodium hydroxide. Thereafter, wash the oil with 10 ml of 10% sodium carbonate solution and finally with water until neutral. Dry the acetylated oil over anhydrous sodium sulfate and determine its ester value as described previously. Calculate the linalool content of the oil from the following equation:

$$\text{percentage of linalool} = \frac{A \times 7.707}{w - (A \times 0.021)}$$

where A is the volume in milliliters of 0.5 N alkali required for saponification and w is the weight in grams of the sample used for saponification.

For essential oils containing a significant amount of esters, calculate the percentage of total linalool from the following equation:

$$\text{percentage of total linalool} = \frac{A \times 7.707}{w - (A \times 0.021)} \cdot (1 - 0.0021E)$$

where E is the per cent of esters, calculated as linalyl acetate, in the original oil.

K. MENTHOFURAN

Determination of menthofuran is important in the evaluation of mint oils. This terpenoid has long been considered to be present only in oils of *Mentha piperita* and its reaction with glacial acetic acid-nitric acid solution was adopted as the official method for distinguishing between oils derived from *M. piperita* and *M. Arvensis*.[54,55] The test may be carried out as follows.

Procedure

In a dry test tube, mix 3 drops of the sample and 5 ml of a mixture of 1 volume of nitric acid and 300 volumes of glacial acetic acid. Warm the tube in a beaker of boiling water. Within 5 min the liquid in the test tube develops

a blue color, which on continued heating deepens and shows a copper fluorescence, then fades, leaving a golden-yellow solution. The oil from *M. Arvensis* does not show these characteristic color changes.

Recent investigations have, however, shown that menthofuran is a natural constituent of oils derived from *M. Arvensis* and several other mint species.[56]

A procedure for the assay of methofuran has been described by Krupski and Fischer.[57] It involves reaction of menthofuran with trichloroacetic acid and measurement of the absorption of the pink solution so obtained at 505 mμ. Ohloff[58] proposed a method in which menthofuran is allowed to react with *p*-benzoquinone and the excess of the reagent is determined iodometrically. An infrared-spectrophotometric method for the determination of menthofuran in peppermint oils was reported by Naves.[59] Based on measurement of absorption intensities at 735 cm^{-1}, it permitted detection of 1% of the terpenoid in genuine preparations.

A semiquantitative method, employing the technique of coupled gas-liquid-thin layer chromatography, and suited for the assay of trace quantities of menthofuran, has been described by Nigam and Levi.[56]

L. SAFROLE IN SASSAFRAS OILS

The safrole content of sassafras oil may be estimated by determination of its congealing point with reference to a calibration graph,[60] Table 13.12. Some samples of sassafras oil congeal with great difficulty only. Seeding with a crystal of safrole accelerates this process.

TABLE 13.12: Safrole Content*

Congealing point, °C	Safrole content, % w/w	Congealing point, °C	Safrole content, % w/w
2.4	69.1	8.3	90
3.7	73.3	8.6	91
4.4	76	8.8	92
5.2	79	9.2	93
6.1	82	9.4	94
6.8	85	9.7	95
7.2	86	10.0	96
7.5	87	10.3	97
7.8	88	10.6	98
8.0	89	11.0	99.5

* Reprinted with the courtesy of *Analytical Chemistry*.

M. STEAROPTENE IN ROSE OILS

Stearoptene is the name given to solid paraffinic hydrocarbons occurring in rose oils. Since these constituents are very unreactive, their assay is based

on physical isolation. The most widely accepted method,[61] which takes advantage of the insolubility of these compounds in dilute alcohol at low temperatures, is described below.

Procedure

Dissolve about 5 g of the sample, accurately weighed, in 50 ml of 75% alcohol with gentle warming, if necessary. Cool the solution in an ice bath for 2 hr and filter off the stearoptene through a tared well-cooled sintered-glass crucible, under suction. Wash the stearoptene with 50 ml of 75% alcohol cooled to 5° and dry in a vacuum desiccator to a constant weight. Calculate the stearoptene content of the oil from the weight obtained.

The method is not convenient in tropical countries, and sufficient care has to be taken to keep the solution cool during filtration and subsequent estimation of the stearoptene. Another disadvantage is that it requires a large quantity of the oil. These shortcomings have been overcome in a method standardized by Nigam et al.[62] The procedure consists of chromatographing 1 g of the oil over 50 g Grade I (Brockman) neutral alumina, collecting the first 70 ml of the effluent in a tared flask, and evaporating the solvent (eluant: hexane).

The USP describes a limiting test for minimum stearoptene content of rose oils.[63] One milliliter of the oil is dissolved in 1 ml of chloroform in a graduated cylinder and the solution is diluted with 19 ml of 90% alcohol (v/v). The mixture is set aside at 25°C. Crystals of stearoptene should appear within 24 hr.

13.5 DETERMINATION OF ESSENTIAL OILS IN NATURAL PRODUCTS AND ALCOHOLIC PREPARATIONS

Evaluation of essential-oil-bearing plants and quality control of their finished products requires determination of their essential-oil content.

The assay of essential oils in crude drugs, spices, and oleoresins involves water distillation of the comminuted material and measurement of the volume of the separated oil. Several methods have been devised for carrying out these determinations. The official procedures[64,65] employ all glass distillation units designed to minimize the loss of volatile oils, to allow cohobation of the distillation waters, and to permit accurate reading of the volume of the separated oil. Figure 13.2 shows an apparatus devised by Clevenger[66] and adopted by the USP.[64] The still head contains a graduated trap where the essential oil collects and the volume of the oil is read. Two different designs of still heads are used, one for oils lighter than water and the other for oils heavier than water. Another apparatus designed by Cocking and Middleton,[67] has been adopted by the BP.[65]

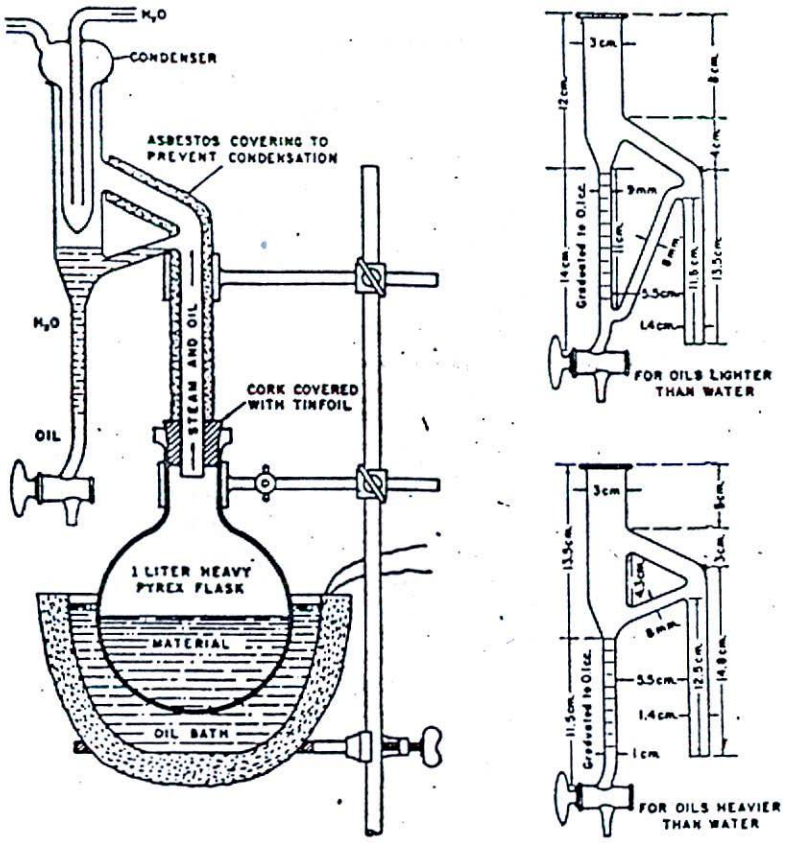


FIGURE 13.2: Apparatus for determination of essential oil in plant material.

Procedure

Place into a 1-liter short-necked round-bottom flask a sufficient quantity of properly comminuted material to yield 2 to 4 ml of essential oil. Add 3 to 6 times as much water as drug and mix well. Attach the proper still head and fill the trap with water. Boil the contents of the flask slowly on an oil bath, keeping the rate of distillation to about 1 drop of condensate water per sec. Continue distillation until no further increase in the volume of the essential oil is observed.

If the essential oil does not separate well or crystallizes on cooling, distill 1 ml of xylene with the quantity of water that would be required for the plant material. Cool and measure the volume of xylene in the trap. Introduce the plant material in the flask and resume distillation until the xylene solution of the essential oil collecting in the trap attains a constant volume. The

difference in volume of the oily layer in the trap before and after introduction of the plant material is the volume of the oil.

In the case of an oleoresin, place a few clay chips and the requisite quantity of water into the flask and heat to boiling. Rapidly add the sample of the oleoresin and some additional clay chips. Carry out the distillation as before but at a faster rate:

$$\text{percentage of essential oil in the sample} = \frac{v}{w}\,100\ (\text{v/w}) \quad \text{or} \quad \frac{vD}{w}\,100\ (\text{w/w})$$

where v is the volume of separated oil, w is the weight of plant material used for determination, and D is the density of the separated oil (determined after isolating and drying the sample).

Adherence of oil drops to the inner surface of the trap and other parts of the still head may cause significant error in the results. Hence, it is necessary that the apparatus be thoroughly cleaned before each determination.

The estimation of the essential-oil content of spirits and extracts is based upon its separation by means of an immiscible solvent and measurement of the change in the volume of the solvent. The extraction is carried out after adding hydrochloric acid and a salt solution, for example, calcium chloride,[68] sodium chloride,[69] or magnesium sulfate.[70] The salt serves to facilitate separation of the layer containing the oil and the solvent, from the alcoholic mixture. The acid ensures the absence of alkali which would tend to dissolve organic acids and some carbonyl compounds.

13.6 COMPOSITION OF ESSENTIAL OILS

Investigation of the composition of an essential oil involves isolation and identification of its constituents. Since essential oils differ widely in the nature and relative proportions of their constituents, no comprehensive procedure can be recommended which may apply generally. As a first step, the physicochemical properties of the oil are determined in accordance with the procedures described previously. This is to be followed by isolation of specific constituents. Methods must be mild because many of the terpenoids readily undergo decomposition, rearrangement, and polymerization. Some of these reactions have been observed during column and gas chromatographic analysis.

A. ISOLATION OF CONSTITUENTS

Some techniques which may be used for isolating the constituents of an essential oil are the following.

1. Chemical Treatment

If the oil has a high percentage of free acids or phenols, it is advisable to remove these components by treatment with alkali prior to analysis. Aldehydes and ketones when present in large amounts, may be isolated by reaction with sulfite, bisulfite, or Girard's reagent. However, if phenols and carbonyl compounds are present in small amounts only, it is preferable to apply chemical separations for these constituents from enriched fractions obtained by distillation or chromatography of the oil.

2. Separation of Constituents by Freezing

Sometimes essential oils or some of their fractions may deposit crystals on cooling (for example, camphor, menthol, thymol, safrole, or anethole). It is advisable to isolate these compounds by freezing either the oil or enriched fractions, followed by filtration and centrifugation.

3. Fractional Distillation

Fractional distillation permits the isolation of specific fractions of the essential oil for further analysis. To minimize decomposition, rearrangement, and polymerization of thermally labile components, it is advisable to carry out the fractionation at reduced pressures. For effective separation of the constituents, it is necessary that distillation be carried out very slowly. Usually a reflux-take-off ratio of 10:1 is satisfactory, though higher ratios may be necessary for the resolution of mixtures composed of closely boiling substances. Figure 13.3 shows one of the several types of units which may be employed for fractionation of essential oils under reduced pressures.

For accurate work, a large number of fractions of equal volumes are collected, and their physical properties are measured. Compilation of the data in the form of graphs in which boiling point, refractive index, specific gravity, and optical rotations are plotted along the ordinate and the number of fractions along the abscissa yields useful information regarding the purity of the various fractions. A plot of refractive index against density can also be successfully employed for the identification and determination of monoterpenes.[71–73]

4. Column Chromatography

Adsorption chromatography on alumina, or partition chromatography on silica gel offers a mild method of separating essential-oil constituents. The oil or the fraction is adsorbed on a column of the active solid and then eluted with one or a series of suitable solvents. In general, the hydrocarbons are eluted first, followed by other oxygenated compounds in order of increasing polarity. The technique may be successfully used for samples from less than 100 milligrams to a few kilograms.

Chromatography of essential oils and their fractions on active alumina

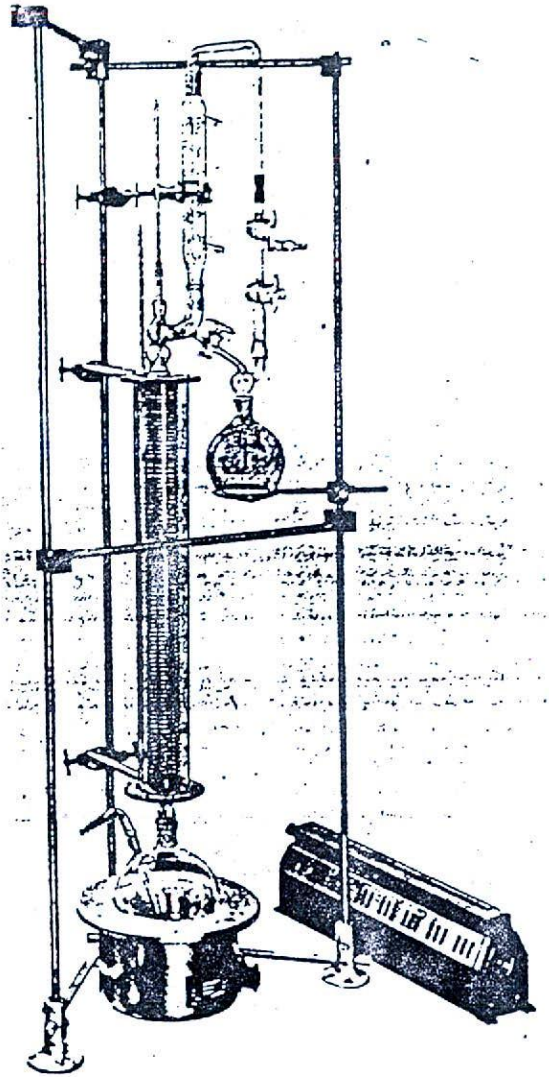


FIGURE 13.3: Fractional distillation unit. Reprinted with the courtesy of *Gallenkamp*.

was standardized and extensively applied by Sorm and his co-workers.[74,75] Since then the application of this technique to the analysis of essential oils has been described in a large number of research papers.

5. Thin-Layer Chromatography

The technique of thin-layer chromatography was first applied to the study of terpene compounds by Kirchner and Miller,[76] and by Reitsema.[77] Experimental details of the method, as a potential tool in organic microanalysis, were described by Stahl.[78] The technique is rapid and inexpensive, and permits analysis of essential oils on microgram scale.[79] It can also be adapted to preparative work allowing isolation of constituents for further characterization by spectroscopy or gas chromatography.

6. Gas-Liquid Partition Chromatography

Within a short span of time, gas-liquid partition chromatography has become a technique of choice for the investigation of essential oils and related substances. The scope and the applications of the method have been discussed in many reviews.[80,81] It involves volatilization of the oil and passage of the gaseous mixture with a stream of inert gas through a column packed with a solid which has been coated with a substrate of low volatility. The components of the oil undergo partition between the stationary liquid phase and the mobile gas phase, thereby traveling through the column at different speeds. The emergence of each constituent at the exit of the column is recorded by means of a "detector." Under given experimental conditions, each constituent is retained in the column for a definite length of time, which is referred to as the *retention time*. The technique, therefore, not only serves to separate but also to identify constituents by comparison with retention data of standard substances. The use of preparative columns permits collection of effluent fractions in quantities sufficient for further physicochemical analysis by infrared and ultraviolet spectroscopy as well as the formation of characteristic derivatives.

The scope of the technique may be further increased by combining it with thin-layer chromatography.[82]

B. IDENTIFICATION OF CONSTITUENTS

Several conventional as well as modern methods are available for the identification of the isolates.

1. Measurement of Physical Properties

Depending on whether the isolate is a solid or a liquid, its melting or boiling point is determined and its refractive index and density are measured.

Optical rotation is also determined. These properties, though helpful in the identification of the substance, can rarely be solely depended upon.

2. Preparation of Derivatives

The classical method for identification of a compound by preparation and study of its derivatives is still most popular and reliable. A description of the methods for the preparation of derivatives of different classes of compounds is beyond the scope of the present chapter. Sterrett[83] and Bedoukian[84] have discussed the suitability of various procedures as applied to essential oil constituents.

3. Spectroscopic Methods

Ultraviolet and infrared spectroscopy provide nondestructive and reliable methods of identification. NMR spectroscopy and mass spectroscopy have also proved useful in special instances. These techniques are of particular advantage when the isolates do not readily form derivatives (for example, hydrocarbons and ethers) or are available only in small quantities.

4. R_f and Retention-Time Data

R_f data and retention times, obtained by thin-layer and gas-liquid partition-chromatographic analysis, respectively, provide useful parameters for establishing the identity of essential oil constituents. The compounds are chromatographed on thin-layer plates and developed in different solvent systems or they are examined by gas chromatography on two or more columns. Results are compared with data obtained from the analysis of pure reference standards examined under similar experimental conditions.

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