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Methods of analysis of herbs and spices

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Abstract: The quality of spices depends largely on the chemical signatures of the essential oils and oleoresins contained within them. Every importing country has developed quality requirements for spices and in this context standard analytical techniques for quality assessment of spices have been established. This chapter describes some of these methodologies, discussing general analytical methods, distillation and extraction techniques, and methods of identifying the constituents of essential oils and of estimating the oleoresins in spices. Methods for quantifying antioxidant capacity and estimation of crude fibre are also described.

Key words: volatile oil, oleoresin, piperine, curcumin, capsaicin, anthocyanins, phenols, carotenoids, antioxidant, crude fibre.

5.1 Introduction

Spices are storehouses of many chemical constituents that impart flavour, fragrance and piquancy. Most spices owe their flavouring properties to volatile oils and in some cases to a combination of oils and resinoids, which are known as oleoresins. No single compound is responsible for the characteristic flavour of a spice; but a combination of different chemical constituents such as alcohols, phenols, aldehydes, esters, terpenes and alkaloids in various proportions impart flavour. The quality of spices is assessed by intrinsic and extrinsic characteristics of spices. The former consists of chemical quality while the latter emphasizes the physical quality. This includes the appearance, shape, texture, colour, presence or absence of extraneous things, etc. In addition to this, certain quality standards of spices, namely, pesticide residue, aflatoxin, heavy metals, solvent residues, sulphur dioxide and microbiological quality are stipulated. The physicochemical qualities remain the ultimate attribute while considering the export requirement of spices. The physicochemical characteristics vary depending on the variety, agroclimatic conditions existing in the area of cultivation, harvest and post-harvest operations.

Every importing country has developed quality requirements for spices. Cleanliness specifications for spices of ASTA (American Spice Trade Association) are a universally adopted manual for the assessment of physical quality of spices. The US Food and Drug Administration (USFDA) has also provided quality specifications

for spices. Besides this, Indian agencies namely, AGMARK and Bureau of Indian Standards (BIS), have also developed quality regulations. Similarly, European Spice Association (ESA), British Standard Institution (BSI), International Standards Organization (ISO), Essential Oil Association (EOA) are other reputed agencies providing quality specifications for spices. This underlines the importance of adopting standard analytical techniques for quality assessment of spices.

5.2   General analytical methods

5.2.1   Sieve analysis
Purpose: To determine the particle size distribution of prepared samples.

Apparatus required:
Shaking device, vibratory sieve – Model VS1 OR VE1, or equivalent. Sieves, US Standard or equivalent, 20 cm in diameter, with cover and bottom pan (clean and free of oil and other contamination). Faucet washers, rubber, srewcap.

Procedure:
Obtain the tare weight of each sieve containing three faucet washers and nest the sieves in order of decreasing fineness; attach the cover and tared bottom pan. Weigh 50.0 g sample into tared 240 ml screw cap jar. Weigh 1.0 g of Syloid 244, or equivalent, and add it to the contents of the jar. Cap the jar tightly and mix by inversion until a uniform or homogeneous mixture results. Transfer the mixture quantitatively to the top sieve. Mount the nested sieves in the shaking device and shake 2 min. plus additional 2 min. for each sieve (do not include the pan). Reweigh each sieve with washers and pan and then subtract the tare weight to obtain the fraction retained (ASTA, 1997).

Calculation:

$$\frac{\text{grams retained/screen} \times 100}{\sum \text{g retained on screens} + \text{pan}} = \% \text{ retained on each sieve}$$

5.2.2   Bulk index/bulk density (manual method)
Purpose: To determine bulk index and bulk density.

Apparatus required:
Nalgene graduated cylinders, 250 ml, 500 ml and 1000 ml capacity. Ring stand and ring clamp.

Procedure:
Weigh 100 g of sample and place in graduated cylinder of appropriate size. Weigh 50 g of parsley flakes or other bulky materials exceeding 1000 ml volume. Place cylinder on ring stand and adjust ring clamp so that, when base of cylinder is raised to touch the ring, the bottom surface of the cylinder is exactly 1 in. from the base

of ring stand. Drop cylinder (1 in) 50 times and record to nearest 2 ml for 250, 5 ml for 500, or 10 ml for 1000 ml graduated cylinder (ASTA, 1997).

Calculation:

$$\text{bulk index} = \frac{\text{volume of product (ml)}}{\text{sample weight (g)}} \times 100$$

$$\text{bulk density (g/ml)} = \frac{\text{sample weight (g)}}{\text{volume of product (ml)}}$$

5.2.3   Bulk index/bulk density (machine method)
Purpose: To determine bulk index and bulk density.

Apparatus required:
Nalgene graduated cylinders, 250 ml, 500 ml and 1000 ml capacity. Stop watch. Bulk index machine.

Procedure:
Weigh 100 g of sample and place in graduated cylinder of appropriate size. Weigh 50 g of parsley flakes or other bulky materials exceeding 1000 ml volume. Clamp cylinder in bulk index machine and allow tapping to proceed for 60 seconds. Record volume to nearest 2 ml for 250, 5 ml for 500 or 10 ml for 1000 ml graduated cylinder (ASTA, 1997).

Calculation:

$$\text{bulk index} = \frac{\text{volume of product (ml)}}{\text{sample weight (g)}} \times 100$$

$$\text{bulk density (g/ml)} = \frac{\text{sample weight (g)}}{\text{volume of product (ml)}}$$

5.2.4   Moisture (distillation method)
Purpose: To determine the moisture content by distillation with toluene.

Apparatus required:
Round-bottomed flask, receiver trap, condenser.

Reagent:
Toluene, analytical grade.

Procedure:
Weigh 20–40 g of spice or sample sufficient to yield 2–5 ml. of water. Place the weighed sample in a distilling flask and add sufficient toluene to cover the sample completely (never less than 75 ml). Insert the attiring bar when magnetic stirring is to be used. Assemble the apparatus and fill the receiver trap with toluene by pouring

it through the condenser until the toluene begins to overflow into the distillation flask. Insert a loose cotton plug in the top of the condenser to prevent condensation of atmospheric moisture in the tube. Bring to boil and slowly distill at c. two drops per second until most of the water is collected in the trap, then increase the distillation rate to c. four drops per second. Continue distillation until two consecutive readings taken at 15 min. intervals show no change of water volume in the trap, dislodge any water droplets in the condenser tube with the brush or wire loop and rinse with c. 5 ml of toluene. Continue the distillation for 3–5 min. and cool the receiver trap to room temperature either by allowing it to stand in air or by immersing it in water. The toluene and water layers should now be clear; if not, allow to stand until clearing occurs, then read the volume of water, estimating the nearest 0.01 ml.

Calculation:

$$\text{moisture (\%)} = \frac{\text{volume of water (ml)} \times 100}{\text{weight of sample (g)}}$$

5.2.5   Total ash
Purpose: To determine, as ash, the residue remaining after ignition.

Apparatus required:
Muffle furnace, flat-bottomed crucible.

Procedure:
Ignite the flat-bottomed dish or crucible to a dull redness, cool to room temperature in desiccator and weigh. Weigh accurately 2 g of well-mixed sample in the tared flat-bottomed dish or crucible. Place the dish or crucible in the entrance of the open muffle furnace until the sample is well carbonized. The sample must not catch fire. Place the carbonized sample in the furnace at 600°C ± 2° for 2 hours. If carbon remains, leach ash with hot water, filter through quantitative filter paper, wash paper thoroughly, and transfer paper and contents to the original dish. Dry and ignite in the muffle furnace at 600°C until the ash is white. Cool dish, add filtrate, evaporate to dryness on steam bath and heat in muffle for 30 mins. When carbon-free ash is obtained, place the dish in desiccator, cool and weigh (ASTA, 1997).

Calculation:

$$\text{ash (\%)} = \frac{\text{weight of ash (g)} \times 100}{\text{weight of sample (g)}}$$

5.3   Extraction techniques: determining essential oil content of plant material

A laboratory distillation of essential oil from plant material is often necessary in order to evaluate raw material to be used for large-scale commercial distillation.

The determination of essential oil may be conveniently carried out in a special apparatus devised by Clevenger. The apparatus consists of specially designed oil traps and a small condenser of the cold-finger type. Two types of traps are supplied; one, for oils lighter than water and the other for oils heavier than water.

Volatile oils give the aroma and the oleoresins impart the taste. Aroma compounds play a significant role in the production of flavourants, which are used in the food industry to flavour, improve and increase the appeal of their products. They are classified as per the functional groups, *viz*; alcohols, aldehydes, amines, esters, ethers, ketones, terpenes, thiols and other miscellaneous compounds. In spices, the volatile oils constitute these components (Parthasarathy *et al.*, 2008).

Essential oil is a complex mixture of the following primary compounds:

*   terpene hydrocarbons: monoterpene hydrocarbons, sesquiterpenes;
*   oxygenated compounds, phenols, alcohols;
*   monoterpene alcohols;
*   sesquiterpene alcohols;
*   aldehydes, ketones, esters, lactones, coumarins, ethers, oxides.

Therefore gas chromatography (GC) coupled with flame ionization detection (FID) or mass spectroscopy (MS) is used to characterize the components in an essential oil.

Depending up on the nature of the constituents, polar and non-polar columns are used.

5.3.1   The modified Clevenger method
Purpose: To determine the amount of steam volatile oil (most adopted laboratory method).

Apparatus required:
Boiling flask, short neck, 1 or 2 l, with T.S. 24/40 ground joint. Suitable electric heating mantle or oil bath. Volatile oil traps, Clevenger with T.S. 24/40 ground joints, either

*   designed for lighter-than-water oils, or
*   designed for heavier-than-water oils.

West condenser, 400 mm length with drip tip and T.S. 24/40 ground joints.

Procedure:
Weigh accurately sufficient size sample to yield 2–5 ml of oil and transfer quantitatively to flask – using water if necessary. Add about 500 ml of water. If magnetic stirring is to be used, insert stirring bar. Assemble apparatus, select the trap depending upon the density of the oil to be trapped. Heat the flask to boiling and maintain a reflux rate of one to two drops per second. Reflux until two consecutive readings taken at 1 hour intervals show no change of oil volume in the trap. Cool to 20°C either by allowing to stand in air or immersing trap in a suitable water bath. If the calculated volume of oil is below 2 ml or above 5 ml, the test should be repeated with appropriate adjustment to the amount of sample used (AOAC, 2007).

Calculation:

$$\text{volatile oil \% (v/w)} = \frac{\text{volume of oil (ml) 20°C}}{\text{weight of sample (g)}} \times 100$$

For oils heavier than water when xylene is used, calculate the volatile oil by:

$$\frac{\text{volume of oil (ml) – volume of xylene in blank}}{\text{weight of sample (g)}} \times 100$$

Note: Antifoam agent (containing no volatile oil) and sodium alkylbenzene sulphonate can also be used if severe frothing is observed.

The Clevenger method is the most popular method for extraction of oil. Other related methods are also:

- Supercritical fluid extraction (SFE), mainly by supercritical carbon dioxide (SC-CO_2), can be used to extract volatile oils from natural products and does not produce substantial thermal degradation or organic solvent contamination (Chempakam and Parthasarathy, 2008).
- Solvent-free microwave extraction (SFME) (Lucchesi *et al.*, 2004) a combination of microwave heating and dry distillation, performed at atmospheric pressure without the use of any solvent or water, is a new development in the field. SFME was compared with hydrodistillation, for the extraction of essential oil from three aromatic herbs, namely basil (*Ocimum basilicum* L.), garden mint (*Mentha crispa* L.) and thyme (*Thymus vulgaris* L.), and it was found that the essential oils extracted by SFME for 30 min. were similar to those obtained by conventional hydrodistillation for 4.5 hours, in terms of quantity (yield) and quality (aromatic profile) (Lucchesi *et al.*, 2004). The SFME method yielded an essential oil with higher amounts of oxygenated compounds, and substantial savings of costs, in terms of time, energy and plant material. SFME is a green technology and appears as a good alternative for the extraction of essential oils from aromatic plants.

5.3.2   The Lee and Ogg method

Purpose: To determine the amount of water-insoluble steam volatile oil.

Apparatus required:

One litre, T.S. 24/40 short neck, round-bottom flask. Volatile oil traps, either

- lighter-than-water designed for oils with densities less than that of water, or
- heavier-than-water designed for oils with densities greater than water.

Wet condenser, 400 mm in length, with T.S. 24/40 drip tip.

Procedure:

Weigh a sample that will yield 0.5–1.5 ml of oil and transfer quantitatively to the 1 l flask. Add about 500 ml water and place the flask in the heating mantle. Assemble the apparatus using the proper trap. Heat the flask with occasional shaking and maintain a distillation rate of 1 to 1½ drops per second. If foaming occurs, reduce

the voltage, and increase the temperature gradually until the proper distillation is obtained. Distill until two consecutive readings taken at half hour intervals show no change of oil volume in the trap. Cool to the room temperature, allow to stand until the oil layer is clear and read the volume of the oil collected, estimated to the nearest 0.05 ml. If the calculated volume of oil is below 0.5 ml or above 1.5 ml, the test should be repeated with appropriate adjustment to the amount of sample used (AOAC, 2007).

Calculation:

$$\text{volatile oil \% (v/w)} = \frac{\text{volume of oil (ml)}}{\text{weight of sample (g)}} \times 100$$

When xylene is used, calculate the volatile oil by:

$$\frac{\text{volume of oil (ml) – volume of xylene in blank}}{\text{weight of sample (g)}} \times 100$$

5.3.3   Estimation of steam volatile oil in cassia

Purpose: To determine the amount of water insoluble steam volatile oil in cassia.

Apparatus required:

One litre, T.S. 24/40 short neck, round-bottom flask. ASTM D322 crankcase dilution trap, 5 ml capacity. West condenser with T.S. 24/40 drip tip.

Procedure:

Cleaned trap is rinsed with acetone and then rinsed well with water. Weigh 35 g sample to nearest 0.01 g and quantitatively transfer to the 1 l flask. Add 500 ml of 10 % sodium chloride solution. Add small amount of water to the trap followed by 2.00 ml xylene using the volumetric pipette. Assemble the apparatus, using a small amount of non-volatile stopcock grease on the ground joints. Heat the flask and maintain a distillation rate of 30 drops/minute for 5 hours after boiling begins. Cool the trap to 20 °C by placing it in suitable water bath and hold until the oil layer is clear. Read the trap to the nearest 0.01 ml. The volume of the oil is obtained by deducting the blank for xylene (ASTA, 1997).

Calculation:

$$\text{volatile oil (\%) (v/w)} = \frac{\text{vol. of oil (ml) – vol. of xylene in blank}}{\text{weight of sample (g)}} \times 100$$

Analysis of essential oil constituents

Essential oil of any spice consists of terpenes and hydrocarbons with a boiling range of 70–210 °C. It can be separated using GC–FID. Zachariah *et al.* (2010) conducted detailed analysis of leaf and berry constituents of black pepper oil by GC and GC–MS. The instrument techniques are same for all spice essential oils.

5.4   Identifying the physical properties of essential oils

5.4.1   Specific gravity

Specific gravity is an important criterion of the quality and purity of an essential oil. Specific gravities of essential oils vary between 0.696 and 1.188 at 15 °C; in general, the value is less than 1.000.

The specific gravity of an essential oil at 15 °C/15 °C may be defined as the ratio of the weight of a given volume of oil at 15 °C to the weight of an equal volume of water at 15 °C. Pycnometers offer the most convenient and rapid method for determining specific gravity.

Apparatus required:

Pycnometer: A conical-shaped pycnometer having a volume of about 10 ml with a ground-in thermometer and a capillary side tube with a ground glass cap proves very satisfactory.

Procedure:

Clean the pycnometer by filling it with a saturated solution of chromium trioxide in sulphuric acid and allow it to stand for at least three hours. Empty the pycnometer and rinse thoroughly with distilled water. Fill the pycnometer with recently boiled distilled water which has been cooled to a temperature of about 12 °C and place it in a water bath, previously cooled to 12 °C. Permit the temperature to rise slowly to 15 °C. Adjust the level of water to the top of the capillary side arm, removing any excess with a blotter or cloth, and put the ground glass cap in place. Remove the pycnometer from the water bath, dry carefully with a clean cloth, permit it to stand for 30 min. and weigh accurately. Empty the pycnometer, rinse several times with alcohol and finally with ether.

Remove the ether fumes with the aid of an air blast and permit the pycnometer to dry thoroughly. Weigh accurately after standing 30 min. The 'water equivalent' of the pycnometer may be found by subtracting the weight of the empty pycnometer from its weight when full.

Fill the clean, dried pycnometer with the oil previously cooled to a temperature of 12 °C. Following the same procedure as above, place the pycnometer in a water bath and permit it to warm slowly to 15 °C. Adjust the oil to the proper level, put the cap in place and wipe the pycnometer dry. Accurately weigh after 30 min.

The weight of the oil contained in the pycnometer divided by the water equivalent gives the specific gravity of the oil at 15 °C/15 °C. For a given pycnometer the water equivalent need be determined only once; therefore, it is important that the determination be performed with great care and accuracy.

5.4.2   Optical rotation

Most essential oils when placed in a beam of polarized light possess the property of rotating the plane of polarization to the right. The extent of the optical activity of oil is determined by a polarimeter and is measured in degrees of rotation. The angle of rotation is dependent upon the nature of the liquid, the length of the column through which the light passes, the wavelength of the light used and the temperature.

Both the degree of rotation and its direction are important as criteria of purity. In recording rotations, it is customary to indicate direction by the use of a plus sign (+) to indicate dextrorotation (rotation to the right), or a minus sign (−) to indicate laevorotation (rotation to the left).

Since the scale reading for an optically active liquid is directly proportional to the length of the transmitting column of liquid, it is necessary to use standard tube, 100 mm long. It has become customary in polarimetric work to use sodium light.

Liquids:

The oil or liquid should be free from suspended material. Often oils are hazy owing to the presence of small amounts of water; such oil should be dried with anhydrous sodium sulphate and filtered before a determination is attempted.

Procedure:

Place the 100 mm polarimeter tube containing the oil or liquid under examination in the trough of the instrument between the polarizer and the analyser. Slowly turn the analyser until both halves of the field, viewed through the telescope, show equal intensities of illumination. At the proper setting, a small rotation to the right or to the left will immediately cause a pronounced inequality in the intensities of illumination of the two halves of the field.

Determine the direction of rotation. If the analyser was turned counterclockwise from the zero position to obtain the final reading, the rotation is laevo(−); if clockwise, dextro(+).

After the direction of rotation has been established, carefully readjust the analyser until equal illumination of the two halves of the field is obtained. Adjust the eyepiece of the telescope to give a clear, sharp line between the two halves of the field. Determine the rotation by means of the protractor; read the degree directly, and the minutes with the aid of either of the two fixed verniers; the movable magnifying glasses will aid in obtaining greater accuracy. A second reading should be taken; it should not differ by more than ±5° from the previous reading.

5.4.3   Refractive index

When a ray of light passes from a less dense to a more dense medium, it is bent or 'refracted' towards the normal. If e represents the angle of refraction and i the angle of incidence, according to the law of refraction:

$$\frac{\sin i}{\sin e} = \frac{N}{n}$$

where n is the index of refraction of the less dense, and N the index of refraction of the more dense medium. Refractometers offer a rapid and convenient method for the determination of this physical constant.

5.4.4   Solubility

Solubility in alcohol

Since most essential oils are only slightly soluble in water and are miscible with absolute alcohol, it is possible to determine the number of volumes of dilute alcohol

required for the complete solubility of one volume of oil. The determination of such a solubility is a convenient and rapid aid in the evaluation of quality of an oil. Oils rich in oxygenated constituents are more readily soluble in dilute alcohol than oils rich in terpenes.

Adulteration with relatively insoluble material will often greatly affect the solubility. The solubility of an oil may change with age. Polymerization is usually accompanied with a decrease in solubility. Alcohols of the following strengths are customarily used in determining solubilities of essential oils:

50 %– 60 %–70 %–80 %–90 %–95 % and occasionally 65 % and 75 %

In preparing dilute alcohols, it is convenient to weigh the alcohol and the distilled water to give the proper volume percentage. The strength of the alcohol should be checked by determining specific gravity at 15.56 °C/15.56 °C.

Solubility in non-alcoholic media
Several solubility tests have been introduced for the rapid evaluation of oils.

- **Carbon disulphide solubility for the presence of water**: Oils rich in oxygenated constituents frequently contain dissolved water. Such oils fail to give a clear solution when diluted with an equal volume of carbon disulphide or chloroform. This is the basis of a rapid test to ascertain whether or not an oil has been sufficiently dried.
- **Potassium hydroxide solubility for phenol-containing oils**: Phenolic isolates and synthetics as well as oils consisting almost exclusively of phenolic bodies may be evaluated rapidly by dissolving 2 ml of the oil in 20 or 25 ml of a 1 N aqueous solution of potassium hydroxide in 25 ml glass-stoppered graduated cylinder. It is well to examine critically the odour of the solution or any insoluble portion, whereby additions of foreign, odour-bearing substances may be detected.
- **Sodium bisulphite solubility for aldehyde-containing oils**: Oils and synthetics and isolates may reveal impurities by their incomplete solution in dilute bisulphite solution. This test is usually carried out in a 25 ml glass-stoppered, graduated cylinder: shake 1 ml of the oil with 9 ml of a freshly prepared saturated solution of sodium bisulphite and then add 10 cc of water with further shaking. The odour of the resulting solution should be carefully examined.

Congealing point
The congealing point offers distinct advantages over the melting point and the titre, in the case of mixtures such as essential oils. In determining the congealing point, the oil is super-cooled so that, upon congelation, immediate crystallization with liberation of heat occurs. This results in a rapid rise of temperature, which soon approaches a constant value and remains at this temperature for a period of time. This point is the congealing point.

5.4.5   Evaporation residue
An important criterion of purity is the evaporation residue; i.e., the percentage of the oil which is not volatile at 100 °C. A determination of the evaporation residue

is of special value in the case of the citrus oils; a low value for an expressed oil suggests the possibility of the addition of terpenes, or other volatile compounds; a high value may indicate the addition of foreign material.

It is important to study the odour of an oil as it volatilizes during the heating. The consistency of the residue, both when hot and cool, and the colour sometimes indicates the presence of particular adulterants.

The fact that essential oils are complex mixtures makes an exact determination of the non-volatile residue very difficult.

5.5   Estimation of oleoresin in spices

The total extracts or oleoresins reflect the flavour profile more closely than the volatile oil. The oleoresins also extract non-aromatic fats, waxes, resinoids, colour and other components soluble in the chosen extracting solvent. Oleoresins freed from fat components and prepared as concretes are useful in perfume industries.

5.5.1   Determining the non-volatile ether content of spices and contents
Purpose: To extract with diethyl ether, removal of the volatile fractions, removal of the insoluble substances, drying of the non-volatile residue and weighing.

Reagent:
Diethyl ether, anhydrous.

Apparatus required:
Apparatus for continuous extraction (Soxhlet apparatus).

Procedure:
- **Test portion:** Weigh, to the nearest 1 mg, approximately 2 g of the test spice sample.
- **Determination:** Extract the test portion with the diethyl ether in the continuous extraction apparatus for 18 hours. Eliminate the diethyl ether by distillation, using the extractor flask connected to a rotary evaporator. Dry the flask at 110 °C, until the difference in mass between two successive weighings is not more than 2 mg. By shaking gently, mix the residue in the flask with 2–3 ml of the diethyl ether at laboratory temperature, allow to settle and decant the supernatant solution. Repeat the extraction procedure and eliminate the diethyl ether as before, until no more of the residue dissolves. Dry the flask again as before until the difference in mass between two successive weighings is not more than 2 mg.

Calculation:
The non-volatile ether extract, expressed as a percentage by mass on the dry basis, is given by

$$\frac{m_1 - m_2}{m_0} \times 100 \times \frac{100}{100 - II}$$

where:

m_0 is the mass, in grams, of the test portion;
m_1 is the mass, in grams, of the flask plus residue obtained after drying;
m_2 is the mass, in grams, of the flask and the insoluble residue obtained;
II is the moisture content expressed as a percentage by mass, of the sample as received.

5.5.2   Analysis of constituents: determining the piperine content of pepper

Piperine is the major constituent of pepper oleoresin. Investigations have demonstrated, that piperine is the major pungent principle and chavicine is a mixture of piperine and several minor alkaloids. The presence of chavicine and isopiperine has not been confirmed in pepper extracts while isochavicine is shown to occur as an artifact of photolytic transformation of piperine. Five minor alkaloids possessing a degree of pungency have been identified in pepper extracts. They are piperettine, piperylin, piperolein A and B and piperanine (Zachariah and Parthasarathy, 2008). Piperine is a yellow crystalline substance having a melting point of 128–130 °C. Piperine, $C_{17}H_{19}O_3N$, was shown to be a weak base which, on hydrolysis with aqueous alkali or nitric acid, yielded a volatile base $C_5H_{11}N$, later identified as piperidine. Wood *et al.* (1988) developed the reversed-phase high-performance liquid chromatographic (HPLC) method for piperine determination in black pepper and its oleoresins. It employs C18-bonded stationary phase (ODS-2) and acetonitrile aqueous acetic acid mobile phase with UV detection. As the spectrophotometric method which invariably yields higher results because of the contributions from other alkaloids such as piperyline and piperettine, the HPLC method relates more to piperine.

Principle:
Extraction with ethanol under reflux, then determination of piperine by HPLC.

Reagents:
Use only reagents of recognized analytical grade and distilled or demineralized water or water of equivalent purity.

Reference substance:
Piperine of at least 98 % purity, ethanol, 96 % (v/v), acetonitrile, acetic acid, 1 % (v/v) aqueous solution.

Elution solvent:
Mix 52 volumes of acetic acid solution and 48 volumes of acetonitrile.

Calibration method:

1. **Reference solution:**
    Prepare for immediate use a 1 g/L stock solution of the piperine in ethanol.

2. **Calibration curve:**
    (a) From the reference solution prepare at least three standard solutions of piperine with concentrations ranging from 0.05 g/L to 0.2 g/L. Inject each solution into the chromatograph. Repeat the determination at least once.
    (b) Plot the calibration curve, i.e. the mass of piperine injected versus the peak area.
    (c) Define the mean slope of the curve.

Calculation of the response factor, K:
Calculate the response factor K using the following formulae:

$$K = m'/A$$

$$m' = m \times P_1$$

where:

m is the mass of piperine, in milligrams;
A is the area of the piperine peak, in integrator units;
P_1 is the purity of the reference piperine;
m' is the corrected mass of piperine, in milligrams.

Calculation – peppers, whole or ground:
Calculate the piperine content, as a percentage by mass, using the following formula:

$$A \times K \times \frac{25}{10} \times \frac{100}{m_x} \times 100$$

where:
A is the area of the piperine peak, in integrator units;
m_x is the mass of the sample, in milligrams;
K is the response factor determined for the reference substance.

Calculation – oleoresins of pepper
Calculate the piperine content, as a percentage mass, using the following formula

$$A \times K \times \frac{50}{10} \times \frac{100}{m_x} \times 100$$

where A, m_x, and K have the same meaning as in the above equation.

5.5.3   Analysis of constituents: determining the curcumin content of turmeric

Turmeric oleoresin is the organic extract of turmeric and is added to food items as a spice and colouring agent. Oleoresin yield ranges from 7.0–14.0 %. Curcumin, the principal colouring matter, forms one-third of a good-quality oleoresin. The rhizomes contain curcuminoids (2.5–6 %) and are responsible for the yellow colour. Curcuminoids comprises Curcumin I (diferuloylmethane), Curcumin II (demethoxycurcumin) and Curcumin III (bisdemethoxycurcumin) which are found to be natural antioxidants (Chempakam and Parthasarathy, 2008).

Purpose: To determine the percentage of curcumin present in turmeric preparations by spectrophotometric procedure.

Apparatus required:
Spectrophotometer, visible (tungsten) light source, capable of accurately measuring absorbance at 415–425 nm cuvettes, 1 cm square, silica. Erlenmeyer flask, 125 ml, TS 24/40, amber. Condenser, West type, 400 mm, TS 24/40. Volumetric flasks, 100 and 200 ml, TS, amber.

Reagents:
acetone, reagent grade.

Preparation of sample:
- Raw turmeric rhizome powder with a composite mixture of mother, primary and secondary rhizome (20:60:20) of 40 mesh size.
- Oleoresins – stir well, use as is.

Procedure:
- **Raw turmeric rhizome powder:**
  a. Weigh to nearest 0.01 g, 1 g of sample into 125 ml Erlenmeyer flask; add *c.* 75 ml of acetone and stir bar; gently reflux on stirring hot plate with West condenser for 1 hour.
  b. Cool to room temperature and filter quantitatively into 250 ml volumetric flask. Transfer extracted residue to filter with acetone. Wash thoroughly and dilute with acetone.
  c. Pipette 1 ml of (b) solution in to a 100 ml volumetric flask; dilute to volume with acetone and mix.
  d. Compare the absorbance with a pure standard curcumin preparation (0.0025 g/L).
- **Turmeric oleoresins:**
  a. Weigh to the nearest 0.001 g appropriate weight of well-mixed sample and transfer into 100 ml volumetric flask. Dissolve in acetone and mix.
  b. Pipette 1 ml in to a 100 ml volumetric flask; dilute to volume with acetone and mix.
  c. Compare the absorbance with a pure standard curcumin preparation.

Calculation:

$$\% \text{ curcumin} = \frac{D_s}{100} \times \frac{A_s}{W_s \times 1650} \times 100\%$$

where:
D_s is dilution volume of sample (ml) which, if using the dilution schedule as presented in this method, is 20000 for raw spice and 10000 for oleoresins;
W_s is weight of sample (g);
A_s is absorbance of sample;
1650 = E_{1m} for curcumin.

5.5.4   Analysis of constituents: determining the gingerol and shogaol content of ginger

Ginger oleoresin should contain predominantly the aroma and pungency contributed mainly by the volatile oils, gingerols and related compounds. The pungent group includes gingerols, shogaols, paradols and zingerone that produce a 'hot' sensation in the mouth. The gingerols, a series of chemical homologues differentiated by the length of their unbranched alkyl chains, were identified as the major active components in the fresh rhizome. The major constituents were found to be condensation products of zingerone with saturated straight-chain aldehydes of chain length 6, 8 and 10. These compounds were named [6]-, [8]- and [10]-gingerols and their dehydration products [6]-, [8]- and [10]-shogaols, according to the length of the aldehyde unit, and the relative abundance of these compounds in the sample was estimated as 53:17:30, respectively (Zachariah, 2008).

Principle:
From ground and dried ginger, extraction of the pungent components by methanol at atmospheric temperature and concentration at reduced pressure of part of the extract. The oleoresins are dissolved in methanol. The resulting solutions are analysed directly by reversed phase HPLC on octadecyl-silyl silica column, with a mixture of acetonitrile and aqueous acetic acid as the mobile phase and by UV detection at 280 nm.

The quantification is done by external standardization with nonanoic acid vanillyl amide (NVA) which has a retention time comparable to that of [6]-gingerol.

Procedure:
Refer to ISO/ ASTA manual.

Note the area of the peak of NVA for each chromatogram and calculate the mean value for each concentration of NVA (about 0.2 mg/ml and 0.4 mg/ml).

Calculation:
Calculate the NVA as follows:

$$K_{NVA} = \frac{C_{NVA}}{A_{NVA}} \times 100 \qquad \text{mg/100 ml/unit area}^2$$

where:
C_{NVA} is the concentration of NVA (mg/ml);
A_{NVA} is the mean area of the peak of NVA at that concentration.

For a linear response, the values of K_{NVA} calculated for the two concentrations must not differ by more than 2 %.

$$K_{[6]\text{-}G} = K_{NVA} \times \frac{\text{molecular weight of [6]-G (294.38)}}{\text{molecular weight of NVA (293.41)}}$$

$$= K_{NVA} \times 1.003 \text{ mg/100 ml/unit area}$$

In the same way,
$$K_{[8]\text{-}G} = K_{NVA} \times 1.099 \text{ mg/100 ml/unit area}$$
$$K_{[10]\text{-}G} = K_{NVA} \times 1.194 \text{ mg/100 ml/unit area}$$

$K_{[6]\text{-}S} = K_{NVA} \times 0.942$ mg/100 ml/unit area
$K_{[8]\text{-}S} = K_{NVA} \times 1.037$ mg/100 ml/unit area
$K_{[10]\text{-}S} = K_{NVA} \times 1.133$ mg/100 ml/unit area

Expression of results:
Calculate the concentration of each gingerol or shogaol in the sample of dried ginger or oleoresin as illustrated below for [6]-gingerol:

$$\text{content of [6]-G in the sample} = \frac{A_{[6]\text{-}G} \times K_{[6]\text{-}G}}{C} \% \text{ (m/m)}$$

where:
   $A_{[6]\text{-}G}$ is the area of the peak of [6]-G in the chromatogram of the sample;
   $K_{[6]\text{-}G}$ is the response factor for [6]-G (mg/100 ml/unit area);
   C is the concentration of dried ginger or of oleoresin in the sample solution (mg/ml).

5.5.5   Analysis of constituents: determining the carotenoid content of capsicum
The colour of chilli spice powder is due to the presence of red-pigmented carotenoids. The main pigments are capsanthin, capsorubin, zeaxanthin and cryptoxanthin. Carotenoids are very stable in intact plant tissue. However, when chillies are processed by drying and grinding into spice powder, the carotenoids easily autooxidize due to effects of heat, light and oxygen. This leads to a more orange and less intense colouration that devalues the spice powder. Carotenoids control pod colour with approximately 20 carotenoids contributing to the colour of the powder. Carotenoid compounds are yellow to red pigments of aliphatic or alicyclic structures composed of isoprene units, which are normally fat-soluble colours. The keto-carotenoids, capsanthin, capsorubin and cryptocapsin are unique *Capsicum* carotenoids. The major red colour in chilli comes from the carotenoids capsanthin and capsorubin, while the yellow-orange colour is from β-carotene and violaxanthin. Capsanthin, the major carotenoid in ripe fruits, contributes up to 60 % of the total carotenoids. The total carotenoid content of the ripe fruits was about 3.2 g/100 g DW of which capsanthin constituted 42 %, zeaxanthin 8 %, cucurbitaxanthin A 6.6 %, capsorubin 3.2 % and β-carotene 7 % (Zachariah and Gobinath, 2008).
   The colour of chilli powder can be measured either as extractable red colour or surface colour. Extractable colour is estimated by ASTA (ASTA, 1997) in international trade. Generally, in trade the lower limit allowable for chilli powder is 120 ASTA units and for non-pungent paprika 160–180 ASTA units.

Extractable colour in capsicums and their oleoresins
Purpose: To determine the extractable colour in capsicums and their oleoresins by measuring the absorbance of an acetone extract at 460 nm.

Apparatus required:
Spectrophotometer capable of accurately measuring absorbance (A) at 460 nm. Glass referenced standard: NIST SRM 2030 OR 930, glass filter with absorbance specified by NIST in range 0.4–0.6 AT 465 nm, or equivalent. Volumetric flasks 100 ml with ground glass stoppers.

Reagents:
Acetone.

Preparation of sample:
• Capsicums–ground samples.
• Oleoresins – mix the sample well by shaking.

Procedure:
• **Capsicums:** Accurately weigh 70–100 mg of ground capsicum sample and transfer quantitatively to a 100 ml volumetric flask. Dilute to volume with acetone, and stopper tightly. Shake the flask and let it stand for 16 hours at room temperature in a dark area. Shake the flask and allow sufficient time for the particles to settle. Fill the spectrophotometer cell with acetone. Set the wavelength on the spectrophotometer to 460 nm. Place the acetone-filled cell in the instrument. Zero the spectrophotometer. Measure the absorbance at 460 nm. Avoid transferring particles to absorption cell. Determine the absorbance of the glass filter at 465 nm.
• **Oleoresins:** Accurately weigh 70–100 mg of ground capsicum sample and transfer quantitatively to a 100 ml volumetric flask. Dilute to volume with acetone, shake and let stand for 2 min. Pipette 10.00 ml of this extract into another 100 ml volumetric flask. Dilute to volume with acetone and shake. Transfer a portion to the spectrophotometer cell and measure the absorbance at 460 nm. Determine the absorbance of standard glass filter at 465 nm (ASTA, 1997).

Calculation:
• Capsicums extractable colour:

$$\text{ASTA colour} = \frac{\text{absorbance at 460 nm} \times 16.4}{\text{sample weight (g)}}$$

• Oleoresins extractable colour:

$$\text{ASTA colour} = \frac{\text{absorbance at 460 nm} \times 164}{\text{sample weight (g)}}$$

• Instrument correction factor (I_f):

$$I_f = \frac{\text{NIST declared absorbance for glass filter at 465 nm}}{\text{Lab observed absorbance of glass filter at 465 nm}}$$

• Corrected ASTA colour:

$$\text{ASTA colour (corrected)} = \text{ASTA colour} \times I_f$$

5.5.6   Analysis of constituents: determining the capsaicinoid content of capsicum
Pungency of chilli is produced by the capsaicinoids, a group of alkaloid compounds that are found only in the plant genus, *Capsicum*. The nature of the pungency has been established as a mixture of seven homologous branched-chain alkyl vanillylamides. They often are called capsaicin after the most prevalent one.

Dihydrocapsaicin is usually the second most prevalent capsaicinoid, while the other five compounds, norcapsaicin, nordihydrocapsaicin, nornordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin, are considered minor capsaicinoids because of their relative low abundance in most natural products. Capsaicin is a powerful and stable alkaloid that can be detected by human taste buds in solutions of ten parts per million. Capsaicin's composition ($C_{18}H_{27}NO_3$) is similar to piperine ($C_{17}H_{19}NO_3$) that gives black pepper its bite (Zachariah and Gobinath, 2008). The most common instrumental method of analysis is HPLC. It provides accurate and efficient analysis of content and type of capsaicinoids present in a chilli sample. HPLC analysis has become the standard method for routine analysis by the processing industry. A spectrophotometric method is also available.

Determining the total capsaicinoid content
Purpose: To determine total capsaicinoid content from methanolic extracts of chillies or chilli oleoresins at wavelengths of 248 nm and 296 nm.

- **Chillies in powder form:** Extraction with tetrahydrofuran, then determination of the capsaicinoids by spectrometry.
- **Whole chillies:** Preparation by grinding the sample, then extraction with tetrahydrofuran, followed by determination of the capsaicinoids by spectrometry.
- **Oleoresins of chillies:** Dilution of the oleoresin in methanol, then determination of the capsaicinoids by spectrometry.
- **Reagents:** Carbon black, for analysis. Methanol, spectrometric grade. Methanol solution, obtained by mixing 70 parts by volume of methanol with 30 parts by volume of water. Hydrochloric acid solution, freshly prepared, 1 mol/L. Tetrahydrofuran, freshly distilled or spectrometric grade.

Procedure – Preparation of sample:
- **Chillies in powder form:** Check that all of the powder passes through the 500 µm sieve. Weigh, to the nearest 0.01 g, about 10 g of homogenized powder, and transfer it quantitatively to the continuous extraction apparatus. Extract for 8 hours using 100 ml of tetrahydrofuran. Evaporate the solvent to the maximum extent possible in the rotary vacuum evaporator under reduced pressure in a 250 ml round-bottomed flask on the water bath.
- **Whole chillies:** Grind the chillies until the powder obtained passes entirely through the 500 µm sieve. Homogenize the powder after sieving. Weigh, to the nearest 0.01 g, about 10 g of homogenized powder, and transfer it quantitatively to the continuous extraction apparatus. Extract for 8 hours using 100 ml of tetrahydrofuran. Evaporate the solvent to the maximum extent possible in the rotary vacuum evaporator under reduced pressure in a 250 ml round-bottomed flask on the water bath.
- **Oleoresins of chillies:** Thoroughly homogenize the oleoresin. Weigh, to the nearest 0.0001 g, 0.5–1 g of the homogenized oleoresin in a 250 ml volumetric flask with a ground glass stopper.

Procedure – Preparing test solutions:
- **Chillies, whole or in powder form:** To the extract add 0.05–0.1 g of carbon black so as to maintain a ratio of the order of 10 between the extract and carbon black.

Add about 90 ml of methanol solution. Agitate on the magnetic stirrer for 30 min. Allow the solution to stand for 5 min. Filter through the membrane filter into a 100 ml volumetric flask. Dilute to the mark with methanol solution. The filtrate shall be clear.
- **Oleoresins of chillies:** To the test portion, add 0.05–0.1 g of carbon black so as to maintain a ratio of the order of 10 between the oleoresin and carbon black. Add about 90 ml of methanol solution. Agitate on the magnetic stirrer for 30 min. Allow the solution to stand for 5 min. Filter through the membrane filter into a 100 ml volumetric flask. Dilute to the mark with methanol solution. The filtrate shall be clear (ASTA, 1997; Sadasivam and Manickam, 2008).

Preparing dilutions for spectrometric measurement:
1. Transfer the following to a 25 ml volumetric flask: 3 ml of water, 2 ml of hydrochloric acid. Dilute to the mark with methanol. This solution is the 'blank acid solution' (A).
2. Transfer the following to a 25 ml volumetric flask: 3 ml of water, 2 ml of sodium hydroxide solution. Dilute the mark with methanol. This solution is the 'blank alkali solution' (B).
3. Take three 25 ml volumetric flasks and mark them **a1**, **a2** and **a3**, respectively. Transfer to each flask: 1 ml of the filtrate, 2.7 ml of water, 2 ml of hydrochloric acid. Dilute each flask to the mark with methanol.
4. Take three 25 ml volumetric flasks and mark them **b1**, **b2** and **b3**, respectively. Transfer to each flask: 1 ml of the filtrate, 2.7 ml of water, 2 ml of sodium hydroxide solution. Dilute each flask to the mark with methanol.

Taking spectrometric measurements using a *double-beam spectrometer*:
1. Adjust the zero and the 100 % absorption with methanol solution.
2. Measure the blank absorbances at wavelengths of 248 nm and 296 nm by placing first the blank alkali solution (B) in the measuring cell and the blank acid solution (A) in the reference cell.
3. Measure the absorbances of each sample solution at wavelengths of 248 nm and 296 nm by placing the solution from flask **b1** in the measuring cell and the solution from flask **a1** in the reference cell. Then measure the absorbances with solutions from flasks **b2** and **a2**, and flasks **b3** and **a3**, respectively.

Calculation:
Calculate the total capsaicinoid content, w_{248}, as a percentage by mass, at a wavelength of 248 nm, using the following formula:

$$w_{248} = \frac{(A_S - A_b) \times d}{314 \times m}$$

where
A_s is the absorbance of the sample solution;
A_b is the absorbance of the blank solution;
d is the dilution factor;
m is the mass (g) of the test portion.

Carry out an additional dilution when the absorbance is greater than 0.8. Calculate the total capsaicinoid content, w_{296}, as a percentage by mass, at a wavelength of 296 nm:

$$w_{296} = \frac{(A'_s - A'_b) \times d}{127 \times m}$$

where

A'_s is the absorbance of the sample solution;
A'_b is the absorbance of the blank solution;
d is the dilution factor;
m is the mass (g) of the test portion.

5.5.7 Determining the pungency of capsicums and their oleoresins: high-performance liquid chromatographly (HPLC) method

Purpose: To determine pungency levels in crushed red pepper, chilli pepper, jalapeno pepper and red pepper oleoresins.

Apparatus required:

Liquid chromatograph equipped with: (i) integrator; (ii) 20 µL sample loop injection valve; (iii) fluorescence detector and/or ultraviolet detector. Chromatographic column stainless steel, 150 × 4.6 mm packed with 5 µm LC-18. Glass beads.

Reagents:

Ethyl alcohol (EtOH), 95 % or denatured. Acetone, pure, mobile phase. Use LC grade solvents, or equivalent: (i) deionized H_2O, add 1 % HOAc (v/v); (ii) acetonitrile; (iii) mix 400 ml of (ii) with 600 ml of (i). (iv) De-gas with helium or by other suitable means. Standard: N-vanillyl-*n*-nonanamide, 99 + %. C18 Sep-Pak cartridge, 6 ml capacity.

Preparation of sample:
- **Ground or crushed peppers:** Weigh accurately about 25 g pepper into a 500 ml boiling flask. Pipette 200 ml EtOH into the flask and add several glass beads to aid boiling. Reflux gently for 5 hours. Allow to cool. Filter 3–4 ml through a 0.45 µm syringe filter into glass vial.
- **Oleoresins:** Accurately weigh 1–2 g oleoresin (increase sample size if total capsaicinoid concentration is below 1 %) into 50 ml volumetric flask, being sure not to allow any oleoresin to coat the sides of the flask. Add 5 ml of acetone to flask and swirl acetone until sample is completely dispersed. Filter through a 0.45 µm syringe filter into a glass vial.

Procedure:
1. Prepare all standard solutions with EtOH. Keep solutions out of direct sunlight.
   - Standard solution A – 0.15 mg/ml. Accurately weigh and transfer 75 mg of standard into a 500 ml volumetric flask, dissolve, dilute to volume and mix.

   - Standard solution B – 0.015 mg/ml. Pipette 10 ml standard solution A into a 100 ml volumetric flask, dilute to volume and use with chilli peppers.
   - Dilute standard solution C – 0.00075 mg/ml. Pipette 5 ml of working standard solution B into a 100 ml volumetric flask, dilute to volume and mix. (Use with samples, which contain capsicum heat levels below 5000.)
2. Chromatographic conditions: Mobile phase: 40 % acetonitrile and 60 % deionized H_2O with 1 % acetic acid (v/v). Flow rate: 1.5 ml/minute, isocratic. Column: LC-18 150 × 4.6 mm i.d., 5 µm particle size. Injection volume: 20 µL. Detection: excitation 280 nm; emission 325 nm for fluorescence or 280 nm for ultraviolet.

Using a sample loop injection valve, inject in duplicate 20 µL of the prepared sample solution onto the column. Inject the appropriate standard solution before first sample injection and after no more than six sample injections. Purge the column with 100 % acetonitrile for 30 min. at 1.5 ml/min after no more than 30 sample injections. Return to previous mobile phase for further determinations (Hoffman *et al.*, 1983).

Calculation:

Scoville heat units (SHU) are the sum of SHU of the three major capsaicinoids. Calculate SHU as follows:

- Nordihydrocapsaicin, $SHU_N = (N/A) \times (C_S/C_X) \times (H_N/R_N)$
- Capsaicin, $SHU_C = (C/A) \times (C_S/C_X) \times (H_C/R_C)$
- Dihydrocapsaicin, $SHU_D = (D/A) \times (C_S/C_X) \times (H_D/R_D)$
- Total $SHU_T = SHU_N + SHU_C + SHU_D$

where:

A is average peak area of standard;
N, C and D are average peak areas for respective capsaicinoids (nordihydrocapsaicin, capsaicin and dihydrocapsaicin) from duplicate injections;
C_S is concentration of standard (mg/ml);
C_X is concentration of sample in extract (mg of sample/ml);
H_N, H_C and H_D are heat factors for respective capsaicinoids;
R_N, R_C and R_D are response factors of respective capsaicinoids relative to standard.

Accepted heat factors and response factors and relative retention times are given in Table 5.1.

## 5.6 Antioxidant potential of plant extracts

For an aerobic organism, oxygen is a double-edged sword since although essential for aerobic life processes, 5 % or more of the inhaled oxygen is converted to reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and the extremely reactive hydroxyl radical. Cellular antioxidant enzymes catalase, superoxide dismutase and various peroxidases and free-radical scavengers like glutathione, vitamin C and vitamin E normally protect the cells from the damaging effects of ROS but, when the dynamic equilibrium is upset, pathological conditions result from the oxidative damage to the cellular macromolecules such as lipids, proteins and nucleic

**Table 5.1** Heat factors, response factors and retention times of capsaicinoids

	Heat factor	Response factor		Retention time
		UV	FLU	
Nordihydrocapsaicin (N)	$H_N = 9.3 \times 10^6$	$R_N = 0.98$	0.92	0.90
Capsaicin (C)	$H_C = 16.0 \times 10^6$	$R_C = 0.89$	0.88	1.00
Dihydrocapsaicin (D)	$H_D = 16.0 \times 10^6$	$R_D = 0.93$	0.93	1.00
N-vanillyl-n-nonanamide		$R = 1.00$	1.00	1.58

acids. These include cardiovascular dysfunction, neurodegenerative diseases, gastroduodenal pathogenesis, metabolic dysfunction of almost all vital organs, cancer, premature ageing, gallstones and such. It is in this context that antioxidant therapy becomes relevant. Polyphenols are widely distributed in the plant kingdom, and one of their key nutraceutical attributes is to lend protection against oxidative damage by scavenging free radicals. Protection against peroxidation of low-density lipoprotein (LDL) is likely to be beneficial to heart disease, whereas protection against oxidative DNA damage is likely to protect against cancer and genomic instability. Also, certain polyphenols may induce phase II enzymes such as glutathione transferase (GST) that will enhance the excretion of oxidizing species, or induce antioxidant enzymes such as metallothionein (a metal-binding protein with antioxidant property).

Polyphenols may also inhibit cytochrome P450s (CYPs) or enzymes such as cyclo-oxygenase or lipoxygenase that have oxidant activities. The ability of certain polyphenols to bind minerals may be beneficial in some cases, since copper and iron can be initiators of hydroxyl radical production by the Fenton and Haber-Weiss reactions. For example, tannic acid has been shown to inhibit hydroxyl radical formation from the Fenton reaction by complexing ferrous ions. Most antioxidant polyphenols function as terminators of free radicals and may also chelate metal ions that are capable of catalyzing lipid peroxidation. They interfere with the oxidation of lipids and other molecules by rapid donation of a hydrogen atom to radicals as follows:

$$RO^{\cdot} + PPH \rightarrow ROH + PP^{\cdot}$$
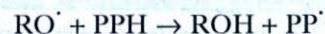
The phenoxy radical intermediates are relatively stable, and also act as terminators of the propagation route by reacting with other free radicals:

$$RO^{\cdot} + PP^{\cdot} \rightarrow ROPP$$
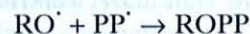
Protection against oxidative stress in intact cells or tissues is often measured by the presence of malondialdehyde, one of the end-points of the peroxidation of polyunsaturated fatty acids. Two principle mechanisms of action have been proposed for antioxidants. The first is a chain-breaking mechanism, by which the primary antioxidant donates an electron to the free radical present in the system (e.g., lipid radical). The second mechanism involves removal of ROS/RNS initiators (secondary antioxidants) by quenching chain-initiating catalysts.

The ability of antioxidants to chelate transition metal ions can be followed spectroscopically. High molecular weight proteins bind directly or indirectly to redox active metals and thus inhibit the production of metal-catalyzed free radicals. Some low molecular weight compounds, such as polyphenols, in addition to their ability to donate hydrogen atom and thus to act as chain-breaking antioxidants, can also chelate transition metal ions and hence inhibit free radical formation.

Another simple, but very informative technique for quantifying antioxidant activity is the reaction of an antioxidant with 1,1-diphenyl-2-picryl-hydrazyl (DPPH) or with galvinoxyl. The principle of this method is that, in the presence of a molecule consisting of a stable free radical (DPPH), an antioxidant with the ability to donate a hydrogen atom will quench the stable free radical, a process which is associated with changes in absorption which can be followed spectroscopically. This simple method can be applied either when the antioxidant is in its pure form, or in a mixture (e.g., a natural extract). Using this method, it is possible to follow the kinetics of the reaction, the number of electrons an antioxidant molecule can donate, and also to estimate the structure of the oxidized antioxidant after it has donated hydrogen atom(s).

5.6.1 Sample preparation

Fresh plant samples (0.5–1.0 g) are homogenized in appropriate solvents (water, methanol, ethanol, petroleum ether, chloroform, etc.) to obtain extracts. The extracts are centrifuged at 10 000 rpm for 20 min. The volume of the supernatant is adjusted to yield a 0.01 g/ml solution, which is used for the following assays.

DPPH radical scavenging assay
Principle: 1,1-Diphenyl-2-picryl hydrazyl is a nitrogen centred free radical, the colour of which changes from violet to yellow on reduction by H^+ or e^- donation. Substances able to perform this reaction are antioxidants and therefore radical scavengers like ascorbic acid, BHA, BHT and gallic acid are references.

Reagents:
0.004 % DPPH in ethanol.

Procedure:
Ethanol is added to appropriate volumes of test solution to bring the total volume to 4.0 ml; 1 ml of 0.004 % DPPH in ethanol is added. A control of 4 ml ethanol and 1 ml 0.004 % DPPH is maintained; absolute blank consisted of only ethanol. The tubes are incubated in the dark for 30 min., and absorbance read at 517 nm in a spectrophotometer (Braca *et al.*, 2001).

Calculation:

$$\text{Percentage radical scavenging capacity} = \frac{\text{ABS}_{\text{control}} - \text{ABS}_{\text{sample}}}{\text{ABS}_{\text{control}}}$$

Total antioxidant capacity by phosphomolybdenum method
Principle: Antioxidants present in the extract react with the molybdenum in the reagent, and reduction of Mo(VI) to Mo(V) results in green colour.

Reagents:
- **Reagent solution:** 0.6 M H_2SO_4, 25 mM Na_2HPO_4, 4 mM ammonium molybdate.
- **Stock solution:** 10 mM ascorbic acid; working standard: 1 mM.

Procedure:
Ethanol is added to appropriate volumes of test solution to bring the total volume to 3.0 ml. The working curve is obtained by pipetting aliquots of the working standard in the concentration range of 200–600 µM and the final volume is made up to 3 ml with ethanol. A blank of 3 ml ethanol is maintained. 1 ml of reagent solution is added to all the tubes, capped well, boiled for 90 min. at 95 °C and cooled. The absorbance is read at 695 nm in a spectrophotometer (Prieto *et al.*, 1999).

Calculation:
Antioxidant capacity is expressed as ascorbic acid equivalents (µmol/g of the sample).

Ferric reducing power method
Principle: This method monitors the reductive ability of the antioxidants manifested by the transformation of Fe(III) to Fe(II). The presence of reductants (antioxidants) causes the reduction of Fe^{3+}/ferricyanide complex to Fe^{2+} forms. This Fe^{2+} can be monitored by measuring the complex at 700 nm in the spectrophotometer. Increase in absorbance indicates increased reducing power of the sample.

Reagents:
0.2 M phosphate buffer, pH 6.6, 1 % potassium ferricyanide [$K_3Fe(CN)_6$], 10 % tri-chloroacetic acid (TCA), 0.1 % ferric chloride ($FeCl_3$). Stock solution: 10 mM ascorbic acid; working standard: 1 mm.

Procedure:
An appropriate volume of the extract is made up to 1.0 ml with distilled water; 2.5 ml of 0.2 M phosphate buffer pH 6.6 and 2.5 ml of 1 % potassium ferricyanide [$K_3Fe(CN)_6$] are added. The tubes are incubated for 30 min. at 50 °C, after which 2.5 ml of 10 % TCA is added to the mixture. 2.5 ml aliquot is taken from the above solution and diluted with 2.5 ml distilled water. 0.1 % $FeCl_3$ (0.5 ml) is added and absorbance measured at 700 nm in a spectrophotometer. Ascorbic acid is used as the working standard (0.25–1 mM) and phosphate buffer as the blank. The Fe^{3+} to Fe^{2+} reducing power is expressed in terms of ascorbic acid equivalents (Oyaizu, 1986).

Calculation:
Fe (III) reducing activity is expressed as ascorbic acid equivalents (µmol ascorbic acid/g extract).

Thiobarbituric acid reactive species (TBARS) assay
Principle: TBARS assay quantifies oxidative stress by measuring the peroxidative damage to lipids that occurs with free radical generation. Free radical damage to lipids

results in the production of malondialdehyde (MDA), which reacts with TBA under conditions of high temperature and acidity generating a chromogen that can be measured either spectrophotometrically or spectrofluorometrically.

Reagents:
Egg yolk homogenate, as lipid-rich media, 0.07 mol/l 2,2′-azobis (2-amidinopropane) dihydrochloride, 20 % acetic acid, 0.8 % TBA in 1 % (w/v) SDS solution.

Procedure:
A modified TBARS assay is used to measure the potential antioxidant capacity using egg yolk homogenates as lipid-rich media. Briefly, 0.5 ml of 10 % (w/v) egg yolk homogenate and appropriate volumes of extracts are added to a test tube and made up to 1.0 ml with distilled water. 0.05 ml of 2,2′-azobis (2-amidinopropane) dihydrochloride solution (0.07 M) in water is added to induce lipid peroxidation. 1.5 ml of 20 % acetic acid (pH 3.5) and 1.5 ml 0.8 % (w/v) TBA in 1.1 % (w/v) SDS solution is added and the resulting mixture vortexed, and then heated at 95 °C for 60 min. After cooling, 5.0 ml of butan-1-ol is added to each tube, then extensively vortexed and centrifuged at 1200 g for 10 min. The absorbance of the organic upper layer is measured using a spectrophotometer at 532 nm. All the values are based on the percentage antioxidant index (AI%):

$$AI\% = (1 - A_T/A_C) \times 100$$

where A_C is the ABS of the fully oxidized control and A_T is the ABS of the test sample (Ruberto and Baratta, 2000).

Non-site-specific hydroxyl radical mediated 2-deoxy-D-ribose degradation
Principle: Site- and non-site-specific hydroxyl radical mediated deoxyribose degradation occurs by hydroxyl radicals generated by Fenton reaction. Activities of extracts are compared to mannitol, which is an effective hydroxyl radical scavenger.

Reagents:
5.6 mM 2-deoxy-D-ribose in KH_2PO_4-sodium hydroxide buffer (50 mM, pH 7.4), premixed 100 µM ferric chloride and 104 mM EDTA (1:1 v/v solution), 1 mM H_2O_2, 1 mM ascorbic acid, 2.8 % TCA, 1 % TBA, standard mannitol: 1 mM.

Procedure:
To appropriate volumes of the sample made up to 1 ml with distilled water is added 0.5 ml of 2-deoxy-D-ribose in KH_2PO_4-sodium hydroxide buffer, 0.2 ml of premixed 100 µM ferric chloride and 104 mM EDTA (1:1 v/v solution), 0.1 ml of 1 mM H_2O_2 and 0.1 ml of 1 mM ascorbic acid. The tubes were incubated at 50 °C for 30 min., and 1 ml each of 2.8 % TCA and 1 % TBA were added. The tubes were vortexed, heated in a water bath at 50 °C for 30 min. and the absorbance read at 532 nm in a spectrophotometer. Antioxidant activity of the extracts is expressed as mannitol equivalents per gram of sample (Halliwell *et al.*, 1987).

5.6.2   Estimation of phenols

Phenols and polyphenols are associated with antioxidant activity of plant extracts, so a quantification of the total phenols and profiling of the type of phenols present in an extract is relevant in any study involving measurement of antioxidant potential (Oyaizu, 1986).

Principle: Phenols react with the oxidizing agent phosphomolybdate in Folin–Ciocalteau reagent under alkaline conditions, resulting in the formation of a blue-coloured complex, the molybdenum blue, which is measured at 760 nm colorimetrically.

Reagents:

80 % ethanol, Folin–Ciocalteau reagent, Na_2CO_3, 20 %, standard (100 mg catechol in 100 ml water), dilute 10 times for a working standard.

Procedure:

An appropriate volume of extract is made up to 4 ml with water, and 0.250 ml of Folin–Ciocalteu reagent added. After standing the tubes for 1 min., 0.750 ml of 20 % Na_2CO_3 is added, mixed thoroughly, placed in a boiling water bath for exactly 1 min. and cooled, or incubated at room temperature, for 2 hours. The absorbance is read at 760 nm in a spectrophotometer. The working curve is obtained by running a working standard in the concentration range of 20–100 µM. A blank was maintained with 3 ml of water (Singleton *et al.*, 1999).

Calculation:

The amount of total phenols was expressed as g%.

Estimation of anthocyanin
Reagents:
Ethanolic HCl: 95 % ethanol in 1.5 N HCl (85:15 ratio).

Procedure:

Fresh sample extracted in ethanolic HCl and read the absorbance at 535 nm.

Calculation:

Total absorbance per 100 g of plant material is given by:

$$ABS_T = \frac{V_{EXT} \times V_T \times 100}{\text{extract used (ml)} \times W}$$

where:

ABS_T is total absorbance per 100 g of the plant material;
V_{EXT} is volume made up of the extracts (ml);
V_T is total volume used for colour measured (ml);
W is weight of sample taken (g).

The ε value for 1 % solution (i.e 10 mg/1 ml) at 535 nm is equal to 982.
   Therefore the absorbance of a solution containing 1 mg /ml is equal to 98.2.

total anthocyanin content in mg/100 g of material

$$= \frac{ABS_T/100 \text{ g of plant material}}{98.2}$$

Estimation of carotenoid
Reagents:
Acetone.

Procedure:

Acetone extract to be read at 480 mn and 510 nm.

Calculation:

Total carotenoid content in mg/1 g tissue is given by:

$$7.6\,(480 \text{ nm}) - 1.49\,(510 \text{ nm}) \times \frac{F_V}{\text{weight}} \times 1000$$

where F_V is the fluorescence parameter (maximum fluorescence minus minimum fluorescence – $F_M - F_0$).

5.7   Estimation of fibre

5.7.1   Fibre (crude) in animal feed and pet food (Ceramic fibre filter method)

Principle: Crude fibre is loss on ignition of dried residue remaining after digestion of sample with 1.25 % (w/v) H_2SO_4 and 1.25 % (w/v) NaOH solutions under specific conditions. Method is applicable to materials from which the fat can be and is extracted to obtain a workable residue, including grains, meals, flours, feeds, fibrous materials and pet foods.

Reagents:

Sulphuric acid solution – 0.128 ± 0.003M. 1.25 g H_2SO_4/ 100 ml.

$$ABS_T = \frac{V_{EXT} \times V_T \times 100}{\text{extract used (ml)} \times W}$$

Apparatus required:

Digestion apparatus with built-in heaters and filter system are to be adopted.

Procedure:

Pre-weighed crucible suitable for the extraction units is loaded with coarsely ground ginger sample. Extract with hot acid for 30 min., followed by washing with hot water to neutral pH. Extract the residue with hot alkali for 30 min. followed by washing with water to neutral pH. Evaporate the residue to dryness. Ash the residue in a muffle furnace, cool and weigh (AOAC, 2007).

Calculation:

Crude fibre in ground test portion, % (C) is given by

$$\frac{\text{Weight of crucible after digestion} - \text{Weight of crucible after ashing}}{\text{weight of test portion}} \times 100$$

$$= \frac{W_1 - W_2}{W} \times 100$$

where:

W_1 is weight of crucible after digestion;

W_2 is weight of crucible after ashing;

W is weight of test portion in gm;

5.8   References

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