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Plant Secondary Metabolites

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Preface

Plant secondary metabolites are a diverse group of molecules that are involved in the adaptation of plants to their environment but are not part of the primary biochemical pathways of cell growth and reproduction. In general, the terms *plant secondary compounds, phytochemicals, antinutritional factors*, and *plant xenobiotics* have been used in the literature to refer to this group of compounds. There are well over 24,000 structures, including many compounds that have antinutritional and toxic effects on mammals. This number does not include the oligomeric polyphenolic compounds (proanthocyanidins and hydrolyzable tannins) that are just now being more accurately described and will increase the number by several thousand. Some major plant secondary metabolites or phytochemicals that occur in plants include protease inhibitors, lectins, alkaloids, nonprotein amino acids, cyanogenic glycosides, saponins, and tannins. These compounds are involved in defense against herbivores and pathogens, regulation of symbiosis, control of seed germination, and chemical inhibition of competing plant species (allelopathy), and therefore are an integral part of the interactions of species in plant and animal communities and the adaptation of plants to their environment.

Much of the research on plant secondary metabolites has concentrated on their toxic and antinutritional effects on livestock. Toxic plant secondary metabolites are present in plants at low concentrations (generally less than 2% of the dry matter) and have negative physiological effects when absorbed, such as neurological problems, reproductive failure, goiter, gangrene, and death. Examples are alkaloids, cyanogenic glycosides, toxic amino acids, saponins, and many others. Nontoxic phytochemicals lower digestibility of nutrients and affect palatability. Higher concentrations (>2% of dry matter) of these compounds are required for eliciting negative effects, and the primary site of activity is in the digestive tract or the sensory organs associated with feeding behavior. These plant secondary metabolites include tannins, protease, and amylase inhibitors. Compounds that have a structural role in the plant (e.g., lignin, biogenic silica, and cutin) lower the extent of microbial digestion of cell wall polysaccharides.

This division between groups of plant secondary metabolites is not exclusive. For instance, hydrolyzable tannins are potentially toxic to ruminants. The major lesions are hemorrhagic gastroenteritis, necrosis of the liver, and kidney damage with proximal tubular necrosis. Excessive and fast consumption of oaks and

other tree species that contain more than 5% hydrolyzable tannins results in high mortality and morbidity in cattle and sheep.

In addition, plant secondary metabolites are also associated with improved nutritive value and may have beneficial effects on animal health. Proanthocyanidins, more commonly called condensed tannins in the animal nutrition literature [present in forage legumes such as sainfoin (*Onobrychis viciaefolia*), bird's-foot trefoil (*Lotus corniculatus*), and *Lotus pedunculatus*], are associated with improved protein digestion and metabolism in ruminants and in protecting ruminants against legume bloat. Tannins may also protect ruminants against helminthiasis. Growing interest in the potential health-promoting effects of plant secondary metabolites in human foods has prompted research on their potential to prevent or treat cancer, circulatory disease, and viral infection. The mechanisms by which these substances have beneficial effects on health may also be related to their toxic effects, and the difference between toxicity and beneficial effects may be dose- and structure-dependent. However, mechanisms of toxicity and health-promoting effects of most of the plant secondary metabolites in human and animal diets are not well established.

Interest in plant secondary metabolites has risen dramatically in recent years among plant molecular biologists and plant breeders because of their diverse effects, which, in addition to those mentioned above, include antioxidant, antiviral, antibacterial, and anticancer effects. To name few recent developments, molecular biologists have made genetic modifications in proanthocyanidin biosynthesis in forage plants with the aim of eliminating bloat, improving the efficiency of conversion of plant protein into animal protein (increase rumen undegradable protein and thus increase protein availability postruminally), reduce greenhouse gases, and reduce gastrointestinal parasites; and plant breeders have developed and commercialized rapeseeds (canola) with low levels of glucosinolates and erucic acid, and cottonseed with low gossypol. Genetically modified rice, which expressed insecticidal cowpea trypsin inhibitor, has also been produced. The emerging molecular genetic approaches have tremendous potential to unravel the regulatory genes that control plant secondary metabolite biosynthesis. This information, together with increased knowledge of the enzymes specific for the pathway, could facilitate the genetic engineering of plants.

Most of the plant resources, especially in the tropical regions, are rich in plant secondary metabolites, and the lack of information on the appropriate methods for their determination has been the main bottleneck in better understanding the enzymes and biochemical pathways in their synthesis, the genes responsible for controlling major biochemical processes, and the physiological significance of plant secondary metabolites, and in exploiting the beneficial effects of these phytochemicals.

A number of methods are available in the literature for quantification of plant secondary metabolites. This manual does not present all the available methods; rather, based on our over-a-decade experience on quantification of plant secondary metabolites, it contains the methods for analysis of some important plant secondary metabolites that have worked well in our hands and that can be conducted in laboratories equipped with basic facilities. The methods have been written in a recipe-like format designed for direct practical use in the laboratory. The chemical nature of plant secondary metabolites, their known physiological effects, and their mechanism of action are also briefly presented. This work has originated as a result of numerous requests, especially from scientists from developing countries, for appropriate methods to quantify plant secondary metabolites.

It is hoped that the reliable assays presented in this manual will contribute to the safe and efficient use of locally available feed resources, and better equip the researchers to meet the unprecedented challenge presented by the huge demand for feed, which is driven by the increasing demand for animal protein in developing countries. This manual will also enable better understanding of plant–animal interactions, which is of importance not only for animal agriculture but also for games. The methodologies given in the manual could also be used for determination of plant secondary metabolites in human food and in studying the implications of their consumption on human health and welfare. The manual will also further the interests of molecular geneticists in genetically engineering the plants for introduction of value-added nutraceuticals and food and forage quality traits.

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Contents

1

Trypsin Inhibitor

Key Words: Trypsin inhibitor; pancreas; pancreatic hyperplasia; benzyl-DL-arginine-para-nitroanilide; trypsin inhibitor determination; expression of activity; feedback mechanism; inactivation; fermentation; germination; heat treatment.

1. Introduction

1.1. Nature, Mechanism of Action, and Biological Effects

Among the many factors that have been implicated as having an adverse effect on the nutritional value of proteins is a class of proteins, known as protease inhibitors, that has the ability to inhibit the proteolytic activity of proteases of diverse origin. The protease inhibitors that have been isolated from soybeans and other legumes fall biochemically into two main categories: (1) those that have a molecular weight of 20,000 to 25,000 Da with relatively few disulfide bonds and a specificity directed primarily toward trypsin (Kunitz inhibitor), and (2) those that have a molecular weight of only 6000 to 10,000 Da with a high proportion of cystine residues and are capable of inhibiting chymotrypsin as well as trypsin at independent binding sites (Bowman-Birk inhibitor).

Trypsin inhibitors induce pancreatic hypertrophy/hyperplasia. Concomitant with this increase in the size of the pancreas is an increase in the secretion of digestive enzymes, including trypsin, chymotrypsin, and elastase. This led to the hypothesis that the growth depression caused by the trypsin inhibitors is the consequence of an endogenous loss of amino acids in the form of enzymes being secreted by hyperactive pancreases. Pancreatic secretion is controlled by a negative feedback mechanism whereby enzyme secretion is inversely related to the level of trypsin present in the small intestine. Thus, when the level of trypsin in the gut is depressed, as would be the case when it combines with the

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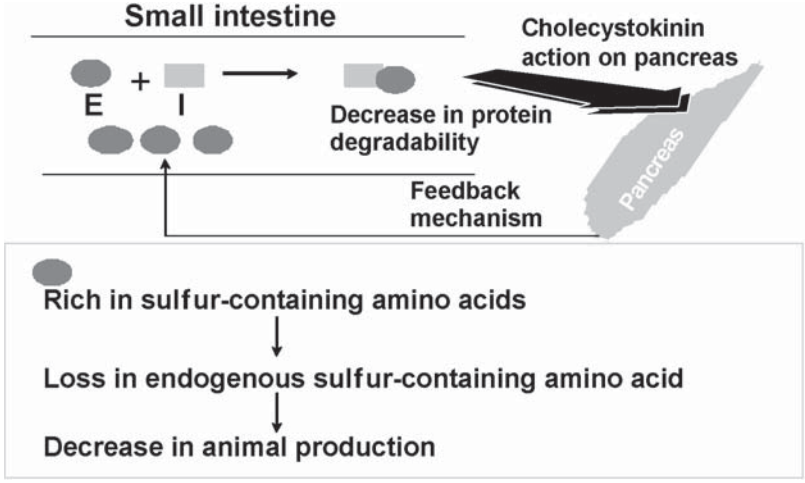

Fig. 1. Mechanism of action of protease inhibitors. Dark gray oval, E, trypsin inhibitor; light gray rectangle, I, inhibitor.

inhibitor, the pancreas as a result of higher secretion of cholecystokinin (a pancreas stimulating hormone), responds in a compensatory fashion by producing more enzymes **(Fig. 1)**. Apart from this, the inhibitor interferes in the digestion of protein in the intestinal tract, decreasing the availability of amino acids for production purposes.

Protease inhibitors can be inactivated by heat treatment. Moist heating has been found to be more effective than dry heating. Fermentation and germination are also known to decrease the inhibitors' capability to produce adverse effects. Monitoring the level of these inhibitors present in both unprocessed and processed food and feed sources is necessary to prevent their adverse effects.

1.2. Present in

Psophocarpus tetragonolobus, *Erythrina caffra, Glycine max, Phaseolus lunatus, Arachis hypogaea, Macrotyloma axillare, Vigna unguiculata, Solanum tuberosum, Triticum aestivum, Hordeum vulgare, Cucumus sativus, Cucurbita pepo, Cucurbita maxima*, rice bran, corn, black gram, chick pea, mung bean, pigeon pea, *Prosopis juliflora, Entada polystachia, E. scandens, Leucaena leucocephala, Gliricidia sepium, Bauhinia purpurea, Canavalia ensiformis, Lens culinaris, Pentaclethra macrophylla, Lupinus albus, Lupinus angustifolius, Lupinus mutablis, Phaseolus vulgaris, Vicia faba, Vigna aconitifolia, Vigna subterranea, Vigna umbellata, Ricinus communis, Mucuna pruriens*. This inhibitor is also present in some unconventional seeds, for example

nontoxic *Jatropha curcas* and *Moringa oleifera,* having the potential for addition to animal diets. Some cereals and nuts also contain this inhibitor.

1.3. Principle of Assay

Two widely used methods are presented here for quantification of trypsin inhibitor activity. In both methods the inhibitor activity is measured indirectly by inhibiting the activity of trypsin. A synthetic substrate, benzyl-DL-arginine-para-nitroanilide (BAPNA) is subjected to hydrolysis by trypsin to produce yellow-colored *p*-nitroanilide. The degree of inhibition by the plant extract of the yellow-color production, a measure of trypsin inhibitor activity, is measured at 410 nm using a spectrophotometer. In the first method ***(1)***, the results are expressed in terms of trypsin units inhibited, and the trypsin unit is an arbitrary unit representing an increase of 0.01 absorbance under the conditions of the assay; and in the second method ***(2,3)*** trypsin inhibitor activity is expressed as milligrams of pure trypsin inhibited.

2. Materials

The materials are the same for both methods:

1. *Tris-buffer (0.05M, pH 8.2) containing 0.02M $CaCl_2$.* Dissolve 6.05 g Tris (hydroxymethylamino methane) and 2.94 g $CaCl_2.2H_2O$ in 900 mL of water. Adjust the pH to 8.2, and make the volume up to 1 L with distilled water.
2. *Substrate solution.* Dissolve 40 mg of BAPNA hydrochloride in 1 mL of dimethyl sulfoxide and dilute to 100 mL with the Tris-buffer prewarmed to 37°C. Prepare the BAPNA solution daily and keep at 37°C while in use. Sometimes BAPNA falls out of the solution without any apparent reasons. To avoid this, dissolve BAPNA in dimethyl sulfoxide, which can be kept in a refrigerator for a week, and this solution can be diluted with the Tris-buffer before conducting the assay.
3. *Trypsin solution.* Dissolve 20 mg of accurately weighed trypsin (2× crystallized, salt free) in 1 L of 0.001M HCl. This solution can be stored in a refrigerator for 2 to 3 weeks without appreciable loss in activity.
4. *0.01M NaOH solution.* Take 400 mg of NaOH, dissolve in 900 mL of distilled water, and dilute to 1 L with distilled water.
5. *0.001M HCl.* Dilute 0.09 mL of concentrated hydrochloric acid [37% weight per volume (w/v)] to 1 L with distilled water.

3. Methods

3.1. Preparation of Extract

The procedure for preparation of the extract is the same for both methods.

Grind the sample, preferably using a ball mill, to pass through a sieve of 40 mesh. To 1 g of the sample add 50 mL of 0.01M NaOH and keep on a magnetic stirrer at low setting for 3 h at room temperature. The extraction can be hastened

by using a homogenizer (Ultra-Turrax, [IKA Werke GmbH and Co. KG, Staufen, Germany], at 20,000 rpm) for 2 min at 0°C. The pH of the suspension is usually 9.5 to 9.8 (if pH is below 8.4, extraction should be repeated with a stronger NaOH solution so that pH of the suspension is between 8.4 and 10.0; *see* **Note 1**). This suspension should be diluted to the point where 1 mL of the diluted suspension produces trypsin inhibition of 40% to 60% (*see* **Note 2**).

If the sample contains more than 5% fat, it should be freeze-dried and extracted with 50 mL of petroleum ether [boiling point (bp) 40–60°C] at room temperature until fat free; the residue should be allowed to air-dry before addition of 50 mL of 0.01M NaOH to 1 g of the sample.

3.2. Determination of Inhibitor [Method 1; Based on (1)]

1. Pipette aliquots (0, 0.6, 1.0, 1.4, and 1.8 mL) of the diluted suspension into duplicate sets of test tubes and make the volume up to 2.0 mL with distilled water.
2. Add 2 mL of trypsin solution to each test tube, and place the tubes in a water bath at 37°C.
3. To each tube, add 5 mL of the BAPNA solution previously warmed to 37°C, and exactly 10 min later terminate the reaction by adding 1 mL of 30% acetic acid.
4. After thorough mixing, centrifuge the content of each tube and measure the absorbance of the supernatant at 410 nm against a reagent blank.
5. Prepare the reagent blank by adding 1 mL of 30% acetic acid to a test tube containing trypsin and water (2 mL each) and then add 5 mL of the BAPNA solution.
6. A sample blank may be prepared by adding 5 mL of the BAPNA solution to 2 mL of the sample extract, incubating the mixture at 37°C for 10 min and then adding 1 mL of 30% acetic acid and 2 mL of the trypsin solution.

3.2.1. Expression of Activity

One trypsin unit (TU) is arbitrarily defined as an increase of 0.01 absorbance units at 410 nm per 10 mL of the reaction mixture under the conditions used here. Trypsin inhibitor activity is expressed in terms of trypsin-inhibiting units (TIUs).

3.2.2. Calculation

1. Plot TIU/mL versus volume of extracts (mL) taken for analysis (*see* **Note 3**).
2. TIU/g sample = extrapolated value × dilution factor [the factor by which the original plant extract (1 g in 50 mL) was diluted so as to obtain an inhibition between 40% and 60% by 1 mL of the diluted extract].
3. When the plot of TIU/mL of extracts versus volume of extracts taken for the analysis does not give a linear correlation, calculate the TIU/mL by averaging values obtained for each volume of extracts. TIU/g sample = averaged value × dilution factor.

3.3. Determination of Inhibitor [Method 2; Based on (2,3)]

1. Pipette the following solutions in a series of 10 mL tubes:
   a. Reagent blank: 2 mL of deionized or distilled water
   b. Standard (40 µg trypsin): 2.0 mL trypsin solution and 2.0 mL distilled water
   c. Sample blank(s): 1.0 mL diluted sample extract and 1.0 mL distilled water
   d. Sample(s): 1.0 mL diluted sample extract, 1.0 mL distilled water, and 2.0 mL trypsin solution
2. After mixing and preheating to 37°C for 10 min, pipette 5.0 mL of BAPNA solution (prewarmed to 37°C) into each tube and mix.
3. After exactly 10 min incubation at 37°C, add 1.0 mL of acetic acid (30%) to each tube to stop the reaction. Then add the trypsin solution (2.0 mL) to the reagent blank (a) and sample blank (c) tubes.
4. After centrifugation (at 3000 *g* for 10 min at room temperature), measure the absorbance of the clear solution at 410 nm. The color is stable for several hours.

3.3.1. Calculation

The change in absorbance (A_I) due to trypsin inhibitor/mL diluted sample extract is $(A_b - A_a) - (A_d - A_c)$, where the subscripts refer to tubes (a) to (d) above. The percentage inhibition in each sample tube is given by $A_I/(A_b - A_a)$. If this value is less than 40% or greater than 60%, the assay must be repeated, making a more suitable dilution of the sample suspension. Since 1 µg pure trypsin would give an absorbance of 0.0190, the weight of pure trypsin inhibited/mL diluted sample extract is $A_I/0.019$ µg (i.e., $50A_I/19$ mg per 50 mL). From this value, the trypsin inhibitor activity (TIA) is calculated in terms of milligrams of pure trypsin/g sample as weighed (mg/g).

$$\mathrm{TIA} = (2.632\ D\ A_I)/S$$

where D is the dilution factor [the factor by which the original plant extract (1 g in 50 mL) was diluted so as to obtain an inhibition between 40% and 60% by 1 mL of the diluted extract], and S is the sample weight (g).

Separate moisture and nitrogen determinations for each sample will allow the TIA to be expressed in terms of dry matter or of protein (N × 6.25), if this is more appropriate. The units can then be clearly stated, for example as mg/g protein.

4. Notes

1. The pH of the sample suspension should be between 8.4 and 10.0.
2. The solution should be diluted to the point where 1 mL produces trypsin inhibition of 40% to 60%. This reduces the relative standard deviation.

3. TIU is the difference between the 0 mL extract reading and the reading for each volume of extract (mL) taken minus the blank reading. TIU/mL is TIU/mL of extract taken and not TIU/mL of the assay volume (10 mL).

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2

Chymotrypsin Inhibitor

Key Words: Casein; chymotrypsin inhibitor; casein; chymotrypsin determination; chymotrypsin unit; protein degradability; amino acid; peptide availability.

1. Introduction

Similar to the trypsin inhibitor, the chymotrypsin inhibitor also decreases protein degradability in the intestine, resulting in lower availability of amino acids and peptides for production purposes. This adversely affects growth and other productive responses. This inhibitor is also heat labile.

1.1. Present in

Erythrina caffra, Glycine max, Phaseolus lunatus, Arachis hypogaea, Macrotyloma axillare, Vigna unguiculata, Solanum tuberosum, black gram, chick pea, pigeon pea, *Phaseolus vulgaris, Psophocarpus tetragonolobus, Vicia faba, Vigna subterranea, Vigna umbellata*.

1.2. Principle of Assay

The method described here is based on the spectrophotometric determination of the breakdown products of casein at 280 nm produced by a given concentration of chymotrypsin, in the presence and absence of the inhibitor. It is based on the method of Kakade et al. ***(1)***.

2. Materials

1. *Borate buffer, 0.1M, pH 7.6. Stock solution A:* 0.2M solution of boric acid (12.4 g in 1 L of distilled water). *Stock solution B:* 0.05M solution of borax (19.05 g in 1 L of distilled water; 0.2M in terms of sodium borate). 50 mL of solution A +2 mL

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of solution B and dilute to a total of 200 mL with distilled water. Check pH, which should be 7.6.

2. *Casein solution*. Suspend 1 g of casein in 80 mL of 0.1M the borate buffer, pH 7.6, and completely dissolved by heating on a steam bath for 15 min. Cool this solution, adjust the pH to 7.6 and make the volume up to 100 mL by the borate buffer.
3. *Chymotrypsin stock (40 μg/mL)*. Dissolve 4 mg chymotrypsin in 100 mL of 0.001M HCl containing 0.08M $CaCl_2.2H_2O$.
4. *Trichloroacetic acid (TCA) reagent*. Take 18 g of TCA and 18.0 g of anhydrous sodium acetate, add 20 mL of glacial acetic acid, and make the volume up to 1000 mL with distilled water.
5. *HCl (0.001M) containing 0.08M $CaCl_2.2H_2O$*. Dilute 0.09 mL concentrated HCl [37% weight/volume (w/v)] to approximately 900 mL distilled water. To it add 11.76 g $CaCl_2.2H_2O$ and make the volume up to 1 L with distilled water.
6. *NaOH (1M)*. Dissolve 4 g NaOH in 100 mL distilled water.

3. Methods

3.1. Preparation of Extract

Take 1 g of defatted (by using petroleum ether) and ground sample (ground preferably using a ball mill) and suspend in 10 mL of distilled water (*see* **Note 1**). Adjust its pH to 7.6 using 1M sodium hydroxide solution. After shaking for 1 h on a magnetic stirrer, centrifuge (3000 g, 10 min) the suspension.

3.2. Preparation of Calibration Curve

1. Pipette the stock solution of chymotrypsin (0.2 to 1.0 mL) into a triplicate set of tubes (one set for each level of enzyme) and make the volume up to 1.0 mL with 0.001M HCl containing 0.08M Ca^{2+}.
2. Add 1 mL of 0.1M borate buffer (pH 7.6) to each tube, and transfer the tubes to a water bath at 37°C. To one of the triplicate tubes add 6 mL of the TCA reagent (this tube serves as a blank for the other two). Then to the other two tubes in each set add 2 mL of the casein solution prewarmed to 37°C.
3. Allow the tubes to remain at 37°C for exactly 10 min, and then stop the reaction by adding 6 mL of the TCA to the tubes.
4. After allowing it to stand at room temperature for at least 30 min, filter the suspension, and measure the absorbance of the filtrate at 275 nm against the appropriate blank.
5. One chymotrypsin unit (CU) is arbitrarily defined as an increase of 0.01 absorbance unit at 275 nm in 10 min per 10 mL of the reaction mixture under the conditions described here.

3.3. Determination of Inhibitor

1. Take the sample extract (0.25, 0.5, 0.75 mL) into a triplicate set of test tubes (one set for each level of the extract), bring the volume to 1.0 mL with the borate buffer,

and add 1 mL of the stock chymotrypsin. Transfer these tubes to a water bath adjusted at 37°C.

2. To one of the triplicate tubes add 6 mL of the TCA reagent (this tube serves as a blank for the other two). Then to each tube add 2 mL of the casein solution (prewarmed to 37°C). After exactly 10 min, stop the reaction by adding 6 mL of the TCA to the other two tubes.
3. After allowing it to stand at room temperature for at least 30 min, filter the suspension and measure the absorbance of the filtrate at 275 nm against the appropriate blank.
4. The chymotrypsin inhibitor activity is defined as the number of chymotrypsin units inhibited (CUI), and the results can be expressed as CUI per milligram of protein or per gram of the sample. For expressing CUI per milligram of protein, the protein content of the extract can be determined using the method of Lowry et al. ***(2)***. True chymotrypsin inhibitor activity may be obtained by taking different volumes of the sample extract and then extrapolating to zero volume of the inhibitor (sample) solution.

4. Note

1. The 0.1M borate buffer (pH 7.6) can also be used for the extraction of chymotrypsin inhibitor from the samples.

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3

α-Amylase Inhibitor

Key Words: α-Amylase inhibitor; *Phaseolus vulgaris*; starch; sorghum; dinitrosalicylic acid; maltose; hyperglycemia; hyperinsulinemia; α-amylase inhibitor activity.

1. Introduction

1.1. Nature, Mechanism of Action, and Biological Actions

α-Amylase inhibitors are heat-labile proteins that are active against salivary, pancreatic, bacterial, or insect α-amylases. Two thirds of the albumin fractions of wheat are composed of multiple protein components capable of inhibiting α-amylases of diverse origin. Three major groups of α-amylase have been characterized, based on molecular weight: 60,000, 24,000, and 12,500 Da. The inhibitor forms a complex with amylase. The complex formation can inactivate the amylase and in turn cause reduction in starch digestion. The fact that the wheat amylase inhibitors are highly effective toward insect amylases suggests that they are part of a defense mechanism of the seed against insect attack, and as far as animal nutrition is concerned, they decrease the availability of starch. On the other hand, in human subjects it was also demonstrated that the wheat amylase inhibitors could reduce hyperglycemia and hyperinsulinemia in diabetic patients. The purified inhibitors extracted from the beans are a glycoprotein (10% carbohydrate) with a molecular weight of 40,000 to 50,000 Da, 1 mol of which reacts with pancreatic amylase to form a 1 : 1 complex. The detection of starch in the feces of rats fed diets containing raw beans with high antiamylase activity suggests that this factor may be active in vivo. Extracts from Leoti sorghum grain have been reported to be active against salivary and pancreatic amylases, implying that they inhibit dietary starch digestion in the gastrointestinal tract. Administration of amylase inhibitors to chickens depressed growth

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and caused pancreatic hypertrophy. To offer potentially available starch in the animal diet to increase the productivity of monogastric animals, screening of amylase inhibitor (specific and nonspecific) in the potential diet constituents could be one of the parameters of feed quality evaluation.

1.2. Present in

Lens culinaris, Psophocarpus tetragonolobus, Cicer arietinum, Vigna aconitifolia, Phaseolus vulgaris, wheat, oats, sorghum, rye, barley, mango seeds, and potatoes.

1.3. Principle of Assay

α-Amylase hydrolyses α-1,4 linkages of starch molecules in a random manner. The reducing sugars (mainly maltose) produced by the action of α-amylase react with dinitrosalicylic acid and reduce it to a brown/orange-red–colored product, nitroaminosalicylic acid. The starch hydrolyzed product concentration under a specified level of α-amylase enzyme, with and without inhibitor, is used to express the α-amylase inhibitor activity ***(1)***.

2. Materials

1. *Starch solution*. Take 1 g of potato amylopectin or soluble starch and dissolve in 100 mL of 0.02M phosphate buffer (pH 7.0).
2. *Dinitrosalicylic acid reagent*. It can be prepared in two ways: (a) To 300 mL of 4.5% carbonate free NaOH, add 880 mL of 1% dinitrosalicylic acid and 255 g of Rochelle salt (potassium sodium tartarate, $KNaC_4H_4O_6.4H_2O$), mix until dissolved and keep in tightly stoppered brown bottle ***(2)***. (b) Dissolve at room temperature 1 g of 3,5-dinitrosalicylic acid in 20 mL of 2N NaOH, add 50 mL of distilled water followed by 30 g of Rochelle salt and make the volume up to 100 mL with distilled water. Protect this solution from CO_2 and store at 4°C ***(3)***.
3. *α-Amylase enzyme solution*. Dissolve 6 mg of α-amylase in 200 mL of 0.2M phosphate buffer (pH 7.0) containing 0.006M NaCl. From this stock solution take 10 mL and dilute to 100 mL with the same buffer solution. The final concentration of enzyme in the solution is 30 μg/mL.
4. *Maltose standard solution*. Dissolve 50 mg of maltose in 50 mL of distilled water and store at 4°C.
5. *NaOH (4.5%)*. Weigh 4.5 g NaOH, dissolve in approximately 80 mL distilled water, and make the final volume up to 100 mL with distilled water.
6. *NaOH (2N)*. Weigh 8 g NaOH, dissolve in approximately 80 mL distilled water, and make the final volume up to 100 mL with distilled water.
7. *Phosphate buffer (0.2M, pH 7.0)*. Take 39 mL of 0.2M monobasic sodium phosphate solution and mix with 61 mL of 0.2M dibasic sodium phosphate solution and dilute to a total volume of 200 mL.

8. *Phosphate buffer (0.02M, pH 7.0)*. Take 10 mL of the above phosphate buffer (0.2M) and dilute it to 100 mL with distilled water.

3. Method

3.1. Preparation of Maltose Calibration Curve

Pipette aliquots of 0.1 to 1.0 mL of maltose (100–1000 µg) solution into test tubes and make up the volume to 1 mL with suitable addition of distilled water. To each tube, add 2.0 mL of the dinitrosalicylic acid reagent. Cover the tubes with marbles, keep the tubes in a boiling water bath for 10 min, cool the tubes, and add 10 mL of distilled water to each tube. The orange-red color formed is measured at 540 nm against a reagent blank using a spectrophotometer and a calibration curve is developed ***(2)***.

3.2. Determination of α-Amylase Enzyme Activity

1. Preincubate all the reagents for 15 min at 37°C in a water bath.
2. Pipette 0.5 mL of 1% starch solution, add to it 0.25 mL of the phosphate buffer (0.2M, pH 7.0) and 0.25 mL of α-amylase enzyme solution.
3. Similarly, prepare a second set of test tubes (blank) by using the phosphate buffer in place of the enzyme solution. Prepare the third set of test tubes containing 0.5 mL of starch solution, 2 mL of dinitrosalicylic acid reagent, 0.25 mL of the phosphate buffer, and 0.25 mL of α-amylase enzyme solution; this set is called the zero time control.
4. Incubate all the tubes at 37°C for 3 min. At the end of the incubation, add 2 mL of dinitrosalicylic acid reagent to the first and second set of tubes to stop the reaction and transfer all the tubes to a boiling water bath for 10 min.
5. After cooling under cold water, add 10 mL of distilled water, mix thoroughly, and take absorbance at 540 nm against the blank. Liberated reducing sugars are expressed as maltose equivalent using the calibration curve ***(3)***.
6. One unit of enzyme activity is defined as that amount which liberates 1 µmol of reducing groups (calculated as maltose)/min from soluble starch at 37°C, pH 7.0, and under the specified experimental condition.

3.3. Preparation of Extract and Quantification of α-Amylase Inhibitor Activity

1. Take 1 g of ground sample (ground preferably using a ball mill) and extract with 10 mL of distilled water for 12 h at 4°C using a magnetic stirrer.
2. Centrifuge the suspension at 5000 *g* for 20 min. Collect the supernatant in a test tube (*see* **Note 1**). From this supernatant, take an aliquot of 0.25 mL and incubate with 0.25 mL of the enzyme solution for 15 min at 37°C. To this mixture, add 0.5 mL of 1% starch solution (prewarmed at 37°C; *see* **Note 2**).

3. The assay is conducted as described above. The sample blank should be prepared with the addition of 0.25 mL enzyme solution at the end of 3 min incubation after adding the dinitrosalicylic acid reagent.
4. One unit of α-amylase activity inhibited is defined as one α-amylase inhibitory unit (AIU) ***(1)***.

4. Notes

1. Samples containing a high amount of phenolic constituents, may provide nonspecific α-amylase inhibitor activity similarly to that of a specific one. The relative reduction of such components (using insoluble polyvinylpyrrolidone) in the samples before extraction may reduce nonspecific interference.
2. Before starting the assay, preincubation of all the reagents at 37°C is necessary.

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4

Phytohemagglutin/Lectin

Key Words: Lectin; phytohemagglutin; hemagglutination; agglutination; human blood erythrocytes; cattle blood erythrocytes; intestinal epithelium; hyperplasia; polyamines; cell membranes; insulin; hemagglutinating unit; Con A; protein catabolism; fat; glycogen; lectin inactivation; trypsinized erythrocyte suspension.

1. Introduction

1.1. Nature, Mechanism of Action, and Biological Effects

Lectins are proteins in nature with molecular weight ranging from 60,000 to 100,000 Da. Many lectins contain covalently bound sugar moieties and are glycoprotein in nature. These are also called phytohemagglutins because they agglutinate red blood cells.

Lectins are widely distributed in the plant kingdom and have the unique property of binding to carbohydrate-containing molecules, with a high degree of specificity toward the sugar component. One obvious manifestation of this property is their ability to agglutinate the red blood cells from various species of animals, which is because of the interaction of multiple binding sites on the lectin molecule with specific glycoconjugate receptors on the surface of the cell membrane **(Fig. 1)**. Lectins inhibit growth of the animals. About 60% of the lectin survives intestinal transit and becomes bound to the intestinal epithelium **(Fig. 1)**, where it causes disruption of the brush border and atrophy of the microvilli, and reduces the viability of the epithelial cells. As a consequence of the interaction of lectin with the epithelial surface of the proximal small intestine, there is an increase in the weight of the small intestine. This is because of the hyperplasia of the crypt cells, an effect that is believed to involve the accumulation of polyamines, mostly spermidine, a known stimulant of cellular

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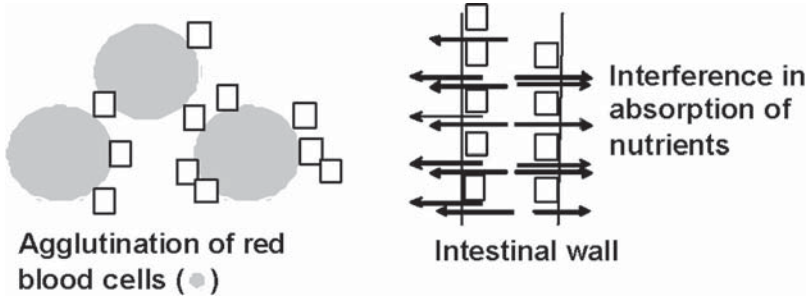

Fig. 1. Mechanism of action of lectins (□).

proliferation. Other physiological effects are lowering of the insulin levels in the blood, inhibition of the disaccharidases and proteases in the intestine, degenerative changes in the liver and kidneys, increased endogenous loss of nitrogen, increased protein catabolism and breakdown of stored fat and glycogen, disturbances in mineral metabolism, and interference with absorption of nonheme iron and lipid from the diet. These also impair immune systems of animals.

Lectin activity can also be destroyed by heat treatment. Moist heat treatment is more effective than the dry heat treatment.

1.2. Present in

Lens culinaris, L. esculenta, Pisum sativum, Phaseolus vulgaris, Glycine max, Phaseolus lunatus, Dolichos biflorus, Vicia faba, Vigna radiata, Amaranthus cruentus, Abrus precatorious, Ricinus communis, Lupinus angustifolius, Canavalia ensiformis, *Canavalia gladiata, Canavalia maritima, Vigna unguiculata, Dolichos lablab, Psophocarpus tetragonolobus, Mucuna pruriens*, *Jatropha curcas, Moringa oleifera, Parkia javanica*, and *Phaseolus aureus.*

1.3. Principle of Assays

Two methods are described in this chapter, both of which are based on agglutination of red blood cells. A series of serial twofold dilutions are made in both methods, which are added to a solution of red blood cells. The first method is based on observing the agglutination visually ***(1,2)***, and the second method is done spectrophotometrically ***(3)***. The visual method is not very precise and does not permit the detection of small differences in haemagglutinating activity. The second method, which measures the absorbance of the layer of unsedimented erythrocytes, is more quantitative and has higher sensitivity.

2. Materials

2.1. Visual Method

1. *Phosphate-buffered saline (PBS) (pH 7.2).* Dissolve 8.2 g NaCl, 0.136 g KH_2PO_4, 0.224 g KCl, and 1.14 g Na_2HPO_4 in 1 L of distilled water (composition is 140 mM NaCl, 3 mM KH_2PO_4, 8 mM KCl, and 1 mM Na_2HPO_4).
2. *Cattle blood erythrocyte suspension (1%).* Collect the blood into a flask containing ethylenediaminetetraacetic acid (EDTA)-sodium salt (a pinch), store in a refrigerator (4° to 6°C) and use within 7 days. Centrifuge the blood at 1500 *g* for 5 min, discard the supernatant, and wash the erythrocytes three to four times with the PBS in the ratio of 1 : 5 [volume per volume (v/v)]. Add washed erythrocytes (4 mL) to 95 mL of the PBS. To this, add 10 mg of crystalline trypsin dissolved in 1 mL of the PBS and keep it for 90 min at 37°C. After repeated washing (three to four times) with the PBS, prepare 1% erythrocytes suspension using the PBS for the assay (*see* **Note 1**).

2.2. Spectrophotometric Method

1. *Saline.* Dissolve 0.9 g NaCl in 100 mL distilled water.
2. *Phosphate-buffered saline (PBS).* Same as in the visual method.
3. *Alsever's solution.* Dissolve 2.05 g of glucose, 0.8 g of sodium citrate, and 0.42 g of NaCl in 100 mL of distilled water and bring to pH 6.1 by adding solid citric acid.
4. *Anticoagulant.* Dissolve 8 g of sodium citrate in 54 mL of 37% formaldehyde and 100 mL saline.
5. *Trypsin (1%).* Dissolve 10 mg of crystalline trypsin in 1 mL of the PBS.
6. *Stock blood suspension.* Take rabbit/cattle/human blood and to it add an equal volume of Alsever's solution containing 1/30 volume of the anticoagulant. This suspension can be stored for 2 weeks at 4°C.
7. *Concanavalin A (ConA) (for example, from Sigma, St. Louis, MO).* 0.01% solution of the ConA in saline is freshly prepared prior to each run.

3. Methods

3.1. Visual Method

3.1.1. Preparation of Extract

Weigh 1.0 g of the defatted sample, add 20 mL of the PBS, and stir the suspension using a magnetic stirrer for 16 h at approximately 1° to 4°C (*see* **Note 2**). Centrifuge the contents at 4000 *g* for 20 min and collect the supernatant (the contents can also be filtered through Whatman No. 540 filter paper and filtrate used for the assay).

3.1.2. Determination of Lectin

1. Prepare a twofold serial dilution of the sample with the PBS in the wells of a microtiter plate and mix with equal volume of 1% erythrocyte suspensions (final volume 0.1 mL) (*see* **Note 2**).
2. Examine the sedimentation of 1% erythrocyte suspensions after 2 h incubation at room temperature.
3. A positive pattern, indicating agglutination, is a uniform effacement of the bottom of the well by erythrocytes, and a negative pattern, indicating no agglutination, is a circular clump of erythrocytes surrounded by a concentric clear zone (*see* **Note 3**).
4. Also examine the contents of the wells microscopically at 2 and 20 h for agglutination, by resuspending the contents of each well with a Pasteur pipette and transferring a drop to a glass microscope slide and covering with a glass coverslip. The formation of erythrocyte aggregates of at least four or five cells that are not disturbed by gentle movement is considered as evidence of agglutination.
5. Hemagglutination activity is defined as the inverse of the amount of material per milliliter in the last dilution giving positive agglutination ***(4)*** (*see* **Note 4**).

3.2. Spectrophotometric Method

3.2.1. Preparation of Standard Trypsinized Erythrocyte Suspension

Trypsinized erythrocytes should be prepared on the day of the assay. Erythrocytes are collected from the stock blood suspension by centrifugation at room temperature in a clinical centrifuge (1500 *g*, 5 min). These are washed three to four times with saline (approximately 5 mL of saline for each milliliter of packed erythrocytes). The packed erythrocytes are added to the PBS (about 4 mL of cells per 100 mL of the PBS) to give a suspension with an absorbance of 2 at 620 nm. To 10 mL of this suspension is added 1 mL of 1% trypsin solution, and the mixture is incubated at 37°C for 1 h. The trypsinized erythrocytes are then washed four to five times with saline, as above, to remove the last traces of trypsin and are finally suspended in sufficient saline to give a standard erythrocyte suspension with an absorbance of 1 at 620 nm (1.2 to 1.5 mL packed cells/100 mL). About 80 mL of standard erythrocyte suspension is obtained from 5 mL of the stock blood suspension (*see* **Note 5**).

3.2.2. Preparation of Extract

Weigh 1.0 g of the defatted sample, add 10 mL of the PBS, and stir the suspension using a magnetic stirrer for 16 h at approximately 1° to 4°C. Centrifuge the contents at 4000 *g* for 20 min and collect the supernatant (the contents can also be filtered through Whatman No. 540 filter paper and filtrate used for the assay).

3.2.3. Determination of Hemagglutination Activity

1. Prepare a twofold serial dilution of the starting solution (supernatant) in a final volume of 1 mL of the PBS in disposable plastic cuvettes.
2. To each cuvette, add 1 mL of the standard erythrocytes suspension and mix the contents of each cuvette. Place the cuvettes in a rack that holds them in an exactly vertical position.
3. After 2.5 h at room temperature, the cuvettes are read in a spectrophotometer at 620 nm, due care being taken not to agitate the contents. Each experiment should include a set of two to four control cuvettes, containing 1 mL of the PBS and 1 mL of standard blood suspension.

3.2.4. Calculation of Hemagglutinating Activity

Hemagglutinating activity is expressed in arbitrary units. The number of units equals the dilution number, which causes a decrease of 50% in the absorbance of the erythrocyte suspension in 2.5 h under the condition described above. This dilution number (x) is calculated from the readings of the two cuvettes that have absorbance nearest to half of the absorbance of the control (E_{50}), one of the readings (E_A) being lower and the other (E_B) being higher than E_{50}. The following equation is then used ***(5)***:

$$\text{Log}\, x = \text{Log}\, A + \text{Log}\, 2\ (E_{50} - E_A)/(E_B - E_A)$$

where

A = dilution number of tube A (nearest tube having an absorbance less than E_{50})

E_A = absorbance of tube A

E_B = absorbance of tube B (nearest tube having an absorbance greater than E_{50}).

The specific hemagglutinating activity of the material tested, HU/mg protein, is calculated from the value of x and from the protein concentration in the starting solution (*see* **Note 6**).

Example

The soybean extract (SBE): 1 g defatted soybean sample is extracted by using 10 mL of saline. Protein content of the extract is determined by Lowry's method ***(6)*** is 0.12 mg protein/mL).

A twofold serial dilution is made and E_A, E_B, and A are 0.23, 0.28, and 16, respectively. E_{50} equals 0.25; absorbance of 1 mL each of PBS; and the erythrocyte suspension = 0.50.

$$\begin{aligned}\text{Log}\, x &= \text{Log}\, 16 + \text{Log}\, 2\ (0.250 - 0.230)/(0.280 - 0.230)\\ &= 1.2040 + (0.301 \times 0.4)\\ &= 1.2040 + 0.1204\\ &= 1.3241\end{aligned}$$

The antilog of both sides gives x = 21.

Hemagglutinating activity of SBE = 21 units/mL.

Specific hemagglutinating activity of the SBE, HU/mg protein = (21/0.12) = 175.

The specific hemagglutinating activity of any seed sample extracts may also be compared with a standard, for example ConA or purified soybean lectin, soyin.

4. Notes

1. Human blood erythrocytes (A, B, and O group) can also be used (1–3% erythrocyte suspension) for the phytohemagglutinating assay.
2. Ultra-Turrax (IKA Werke GmbH and Co. KG, Staufen, Germany), (20,000 rpm) for 5 min (2 × 2.5 min) under ice-cold water bath conditions can also be used for the extraction. Extraction of sample, preparation of blood erythrocytes, and two-fold dilution can also be done using normal saline solution (0.9% NaCl), pH 7.2 instead of the PBS.
3. The limit of experimental accuracy for this technique is ±1 dilution.
4. The results can also be expressed using a standard lectin, concanavalin A (ConA), with the assay performed under the specified experimental conditions simultaneously with the sample extract.
5. The method is highly reproducible (within ±5%) when the assay is done with the same preparation of standard erythrocyte suspension. With different preparations of erythrocytes, the variation can be higher. It is therefore advisable to include in each assay a standard ConA with known specific activity, for comparison.
6. The protein content of the test sample can be estimated from N content (N × 6.25) or by the Lowry et al. ***(6)*** method.

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5

Phytic Acid

Key Words: Phytic acid; myoinositol; Wade reagent; metal ions chelater; Amberlite AG1-X8 anion exchange resin; antioxidant; legumes; cereals; phytate precipitation.

1. Introduction

1.1. Nature, Mechanism of Action, and Biological Effects

Phytic acid, a cyclic compound (1,2,3,4,5,6-hexakis dihydrogen phosphate myoinositol) is a common storage form of phosphorus in seeds and is also considered as an antinutritional factor. Phytic acid, as a result of possessing negative charge at a wide range of pH values, has strong affinity to bind metal ions such as with calcium, zinc, and iron **(Fig. 1)**. This leads to interference in the absorption of these minerals from small intestine and adversely affects various metabolic processes. In addition, phytic acid is also known to complex with proteins and starch, resulting in reduced digestibility of these nutrients. The phosphorus in phytic acid is not nutritionally available to monogastric animals. Nonetheless, non-antinutritive concentration of phytic acid in dietary sources is recently considered to be a potential antioxidant. Reduction in iron-induced oxidative injury and reversal in initiation of colorectal tumorigenesis have also been observed. Phytic acid has recently been suggested to have a protective role in carcinogenesis.

1.2. Present in

Glycine max, Cicer arietinum, Vigna mungo, Vigna radiata, Entada scandens, Cajanus cajan, Lablab purpureus, Lens culinaris, Phaseolus lunatus, Phaseolus vulgaris, Moringa oleifera, Jatropha curcas, Entada scandens, Sesbania sesban, Sesbania bispinosa, Triticum vulgare, Mucuna pruriens, Vicia

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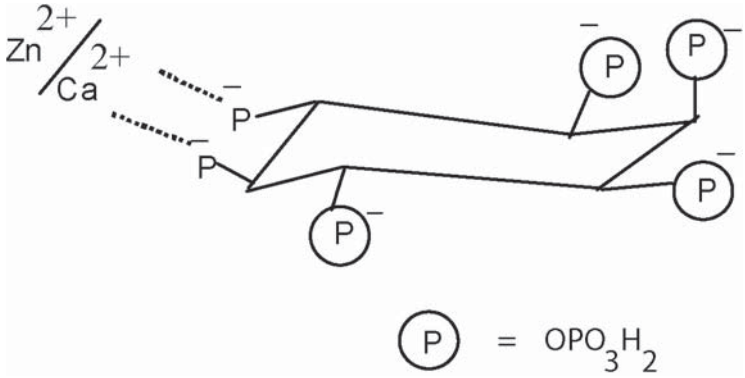

Fig. 1. Interaction of phytic acid, hexadihydrogenphosphate with metal ions.

faba, Vigna aconitifolia, Treculia africana (African breadfruit), *Artocarpus altilis* (Polynesian breadfruit), and rapeseed.

1.3. Principle of Assays

This chapter presents two methods for determination of phytic acid (phytate).

In the first method, phytate is extracted with trichloroacetic acid and precipitated as ferric salt. The iron content of the precipitate is determined spectrophotometrically and the phytate phosphorus content is calculated from this value, assuming a constant 4 Fe/6 P molecular ratio in the precipitate ***(1)***.

In the second method, phytate is extracted with 3.5% [weight/volume (w/v)] HCl and further purified through an AG1–X8 chloride anion exchange column. The pink color of the Wade reagent is due to the reaction between ferric ion and sulfosalicylic acid with an absorbance maximum at 500 nm. In the presence of phytate, the iron becomes bound to the phosphate ester and is unavailable to react with sulfosalicylic acid, resulting in a decrease in pink color intensity ***(2)***.

2. Materials

2.1. Method 1: Based on Precipitation of Phytate (1)

1. *Trichloroacetic acid (TCA), 3%*. Weigh 3 g TCA and dissolve in 100 mL distilled water.
2. *Sodium sulfate (3%) in 3% TCA*. Weigh 3 g sodium sulfate and dissolve in 100 mL of 3% TCA.
3. *NaOH (1.5M)*. Weigh 6 g sodium hydroxide and dissolve in 100 mL distilled water.
4. *HNO_3 (3.2N)*. Take 20.5 mL nitric acid and make the volume up to 100 mL with distilled water.

5. *$FeCl_3$ solution.* Dissolve 583 mg $FeCl_3$ in 100 mL of 3% TCA.
6. *Potassium thiocyanate (KSCN), 1.5M.* Dissolve 29.15 g of potassium thiocyanate in 200 mL distilled water.
7. *Stock standard $Fe(NO_3)_3$ solution.* Weigh 433 mg $Fe(NO_3)_3$ and dissolve in 100 mL of distilled water in a volumetric flask.

2.2. Method 2: Based on Reaction with Wade's Reagent (2)

1. *HCl (3.5% w/v).* Take 50 mL of concentrated HCl (37% w/v) and dilute to 529 mL with distilled water.
2. *NaCl (0.7M).* Dissolve 40.91 g NaCl in 1 L of distilled water.
3. *NaCl (0.1M).* Take 100 mL of 0.7 M NaCl solution and add to it 600 mL of distilled water.
4. *Wade reagent.* Take 30 mg of $FeCl_3.6H_2O$ and 300 mg of sulfosalicylic acid in a 100-mL volumetric flask, dissolve in approximately 70 mL distilled water, and make the volume up to 100 mL with distilled water.
5. *Amberlite AG1–X8 (200–400 mesh) anion-exchange resin.* Take 0.5 g of resin (commercially available; Bio-Rad Laboratories, Richmond, CA) and fill in a 0.75 cm × 25 cm column plugged with a small quantity of glass wool.

3. Methods

3.1. Method 1

1. Weigh a finely ground (40 mesh, ground preferably using a ball mill) sample estimated to contain 5 to 30 mg phytate-P into a 125-mL Erlenmeyer flask. Generally, the amount weighed for cereals and legumes is 500 to 700 mg.
2. Extract phytate in 50 mL of 3% TCA by shaking on a magnetic stirrer for 30 min or with occasional swirling by hand for 45 min.
3. Centrifuge the suspension (3000 *g*, 10 min) and transfer a 10-mL aliquot of the supernatant to a 40-mL conical centrifuge tube.
4. Add rapidly 4 mL of $FeCl_3$ solution to the aliquot in the centrifuge tubes. Heat the contents in a boiling water bath for 45 min. If the supernatant is not clear after 30 min, add one or two drops of 3% sodium sulfate in 3% TCA and continue heating.
5. Centrifuge (3000 *g*, 10–15 min) and carefully decant the clear supernatant. Wash the precipitate twice by dispersing it well in 20 to 25 mL 3% TCA. Heat it in boiling water for 5 to 10 min and then centrifuge (3000 *g*, 10 min). Repeat the washing of the precipitate with distilled water.
6. Disperse the precipitate in a few milliliters of water and add 3 mL of 1.5N NaOH with mixing. Bring volume to approximately 30 mL with distilled water and heat in boiling water for 30 min.
7. Filter hot (quantitatively) through a moderately retentive paper (Whatman No. 2). Wash the precipitate with 60 to 70 mL of hot distilled water and discard the filtrate.

8. Transfer and dissolve the precipitate that is on the filter paper into the 100 mL volumetric flask containing 40 mL of hot 3.2N HNO_3. Wash paper with several portions of distilled water and collect the washings in the same flask.
9. Cool flask and contents to room temperature and bring the volume to 100 mL with distilled water.
10. Transfer a 5-mL aliquot to another 100-mL volumetric flask and dilute to approximately 70 mL with distilled water.
11. Add 20 mL of 1.5M KSCN and bring the volume to 100 mL with distilled water, and read the color immediately (within 1 min) at 480 nm using a spectrophotometer.
12. Run a reagent blank with each set of samples.

3.1.1. Preparation of $Fe(NO_3)_3$ Calibration Curve

Take 2.5 mL of the stock $Fe(NO_3)_3$ solution and make the volume up to 250 mL in a volumetric flask. Pipette 2.5-, 5-, 10-, 15- and 20-mL aliquots of this working standard into a series of 100-mL volumetric flasks and dilute them to approximately 70 mL with distilled water. Then proceed from step 11 in **Section 3.1**.

3.1.2. Calculation

Determine the micrograms of iron present in the test from the calibration curve, and calculate the phytate P as per the following equation:

$$\text{Phytate P mg/100 g sample} = [\text{Fe } (\mu g) \times 15]/\text{Weight of sample in g}$$

Correct the values obtained for dry matter of the sample.

3.2. Method 2

1. Extract 5 g of plant materials (40 mesh, ground preferably using a ball mill) with 100 mL of 3.5% HCl for 1 h at room temperature using a magnetic stirrer. Centrifuge the contents at 3000 *g* for 10 min at room temperature and collect the supernatant.
2. Dilute an aliquot, between 1 mL and 5 mL of the supernatant (depending on the level of phytate) to 25 mL with distilled water (*see* **Note 1**). Pass 10 mL of the diluted sample extract through an AG1 X8 chloride anion exchange (200–400 mesh) column (0.5 g) (*see* **Note 2**).
3. Inorganic phosphorus and other interfering compounds are eluted with 15 mL of 0.1M NaCl, and subsequently the phytate is eluted with 15 mL of 0.7M NaCl ***(3)***.
4. Take 3 mL of the above-eluted sample in a separate test tube and add 1 mL of the Wade reagent. Vortex and then centrifuge the mixture at 3000 *g* for 10 min. Measure the absorbance value at 500 nm against a reagent blank.

3.2.1. Preparation of Calibration Curve

Sodium phytate (for example, from Sigma, St. Louis, MO) is used as a standard since it is soluble in water and does not require conversion to free phytic acid. Prepare a series of standard solutions containing 5 to 40 µg/mL phytic acid in distilled water (*see* **Note 3**). Pipette 3 mL of the standard solution into 15-mL conical centrifuge tubes. The blank tube contains 3 mL of distilled water. To each tube add 1 mL of the Wade reagent. Mix the solution on a vortex mixture for 5 s. The mixture is centrifuged at 3000 *g* for 10 min and the absorbance of the supernatant is read at 500 nm by using water to zero the spectrophotometer ***(2)***.

4. Notes

1. If samples contain less than 1% phytic acid, a dilution of 5 : 25 in distilled water is recommended, whereas a 1 : 25 dilution is enough for samples containing 1% or more phytic acid.
2. If the interference substances are negligible either in the parent extracts or in the diluted extracts, the purification of phytic acid through a 200- to 400-mesh AG1 X8 anion exchange column is not necessary and the direct assay of phytic acid can be conducted.
3. One hundred grams of sodium phytate equals 59.9 g of phytic acid.

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6

Oxalic Acid

Key Words: Oxalic acid; oxalate; metal chelator; kidney stones; hyperoxaluria; potassium permanganate; washing liquid; tungstophosphoric acid reagent; oxylate precipitation; titration; permanganate solution.

1. Introduction

1.1. Nature, Mechanism of Action, and Biological Effects

Oxalate is a common constituent of many plant species. Some crop plants accumulate high levels of this dicarboxylic acid anion (oxalate). Depending on the species, oxalate accumulates primarily as soluble oxalate, insoluble calcium oxalate, or a combination of these two forms. Oxalate, because of possessing negative charges, has high affinity for minerals, such as calcium, magnesium, and zinc. Through ionic interaction it binds to these metal ions **(Fig. 1)** and affects their availability and metabolism **(Fig. 1)**. The high capacity of soluble oxalate to bind to calcium in blood serum can lead to acute intoxication in humans and cattle if ingested in high doses. On the other hand, a high oxalate uptake from the diet is also thought to play a role in hyperoxaluria, which can lead to the formation of calcium oxalate in kidneys (commonly called kidney stones). High levels of oxalate in the blood have also been reported to be associated with arthritis. Furthermore, metal ions like cadmium-incorporated calcium oxalate crystals are also responsible for the heavy metal mediated health risks.

1.2. Present in

Foods that contain significant concentrations of oxalic acid, such as buckwheat, star fruit (carambola), black pepper, parsley, poppy seed, spinach, chard,

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Fig. 1. Binding of oxalate to metal ions.

beets, banana, cocoa, chocolate, leaves of the tea plant (*Camellia sinensis*), and rhubarb. In the case of rhubarb the only edible portion is the stalk because the root and leaves contain dangerously high concentrations of oxalic acid.

Seeds, nuts, and fruits of *Dolichos biflorus, Vigna aconitifolia, Lathyrus sativus, Prunus amygdalus, Anacardium occidentale, Sesamum indicum*, lentils, Drumstick, mango seed kernel, *Emblica officinalis, Grewia asiatica*, pumpkin, eggplant, and tomatoes.

Leaves, tubers, and green of *Amaranthus gangeticus, Amaranthus spinosa, Murrya konigii, Portulaca oleracea, Spinacea oleracea, Tamarindus indica, Moringa oleifera*, Cassava, *Beta vulgaris* (beet root), *Nelumbium nelumbo* (lotus stem), *Musa sapientum* (plantain flower and greens), and cabbage.

Some other feeds/foods containing oxalic acid, such as *Opuntia* spp. cladodes, Niper grass, rice straw, water hyacinth (Eichhornia crassipes), *Atriplex* spp., and *Maireana brevifolia*.

1.3. Principle of Assay

The oxalic acid from the plant material is extracted with HCl, which is precipitated as calcium oxalate. This precipitate is treated with dilute sulfuric acid to form a solution of oxalic acid. The oxalic acid is then quantified by titration with a standard $KMnO_4$ solution ***(1,2)***.

2. Materials

1. *Standardization of $KMnO_4$ stock solution (0.1 N)*. Transfer 0.3 g oven-dried (60°C, 10 min) Na-oxalate ($Na_2C_2O_4$) to a 600-mL beaker. Add 250 mL H_2SO_4 (30 mL H_2SO_4 + 270 mL of distilled water, previously boiled for 10 to 15 min and then cooled to approximately 25°C). Stir until Na-oxalate dissolves. Add 39 to 40 mL $KMnO_4$ solution (3.16 g of $KMnO_4$ dissolved in 800 mL of distilled water and made up to 1000 mL with distilled water in a volumetric flask, ca. 0.1 N) until its color disappears (ca. 45 s). If a pink color persists because $KMnO_4$ solution is too concentrated, discard and begin again by adding few milliliters less of $KMnO_4$ solution. Heat to 55° to 60°C and complete titration by adding $KMnO_4$ solution until a faint pink color persists for approximately 30 s. Add the last 0.5 to 1 mL drop by drop, letting each drop decolorize before adding the next one. Determine the excess $KMnO_4$ solution required to turn the solution pink by matching with the color obtained by adding $KMnO_4$ solution to the same volume of the boiled

and cooled dilute H_2SO_4 at 55° to 60°C. The correction is usually 0.03 to 0.05 mL. From the net volume (i.e., by subtracting this correction volume from the volume of $KMnO_4$ required for complete titration):

$$\text{Normality} = \text{gram } Na_2C_2O_4 \text{ weighed} \times 1000/\text{mL } KMnO_4 \times 66.99$$

2. *Calcium-containing acetate buffer, pH 4.5.* Solution A: dissolve 2.5 g of anhydrous $CaCl_2$ in 50 mL of 50% acetic acid. *Solution B:* dissolve 33 g sodium acetate.$3H_2O$ in 50 mL distilled water. Mix both solutions, check pH, and, if necessary, adjust the pH to 4.5.
3. *Washing liquid.* Dilute 12.5 mL of concentrated acetic acid (100%) to 250 mL with distilled water. Prepare a saturated solution of Ca-oxalate by adding the salt (5–6 g) until no salt remains in the bottom of the beaker. Cool to 4°C and store in a refrigerator. Only filter just before use and keep cold while using.
4. *Tungstophosphoric acid reagent.* Dissolve 2.5 g sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) in a mixture of 4 mL H_3PO_4 (70% w/v) and 50 mL distilled water and dilute to 100 mL with distilled water.
5. *HCl (3 N).* Dilute 62.5 mL concentrated HCl (37% w/v) to 187.5 mL with distilled water.
6. *Potassium permanganate solution (0.01 N).* Dilute 1 : 10 the 0.1 N solution of potassium permanganate solution.

3. Method

3.1. Preparation of Extract

Add 1 g of sample in 10 mL of 3 N HCl. Ultra-Turrax, (IKA Werke GmbH and Co. KG, Staufen, Germany), (20,000 rpm) for 2 min and wash turrax-probe into the sample by using 4 to 5 mL of 3 N HCl. Centrifuge the contents for 5 min at 3000*g* at 4°C. Collect the supernatant in a 25-mL volumetric flask. Repeat the extraction procedure using approximately 8 mL of 3 N HCl and collect the supernatant in the same flask and make the volume up to 25 mL with 3 N HCl.

3.2. Determination of Oxalate

1. Pipette 10 mL filtrate into 40-mL capacity plastic centrifuge tubes, and add 15 mL distilled water and 5 mL of the tungstophosphoric acid reagent. Mix thoroughly, let stand for 5 h, centrifuge at 3000*g* for 5 min, and collect the supernatant.
2. Take 20 mL of the supernatant into a 50-mL conical centrifuge tube (for blank, take 20 mL of 3 N HCl) and add NH_4OH (12.5% w/v; ≈4.2 mL) drop by drop to adjust to pH 4.0 to 4.5. Add 5 mL of the calcium containing acetate buffer solution, stir with a glass rod, and store overnight at room temperature.
3. Centrifuge at 3000*g* for 15 min to obtain a compact pellet. Decant the supernatant with one smooth continuous inversion of the centrifuge tube. Hold the tube upside down and let remaining supernatant drip completely onto clean filter paper. Do not disturb Ca-oxalate precipitate.

4. Wash precipitate by completely breaking it into fine suspension with a fine jet stream of 20 mL of the filtered cold wash liquid. Repeat centrifugation and decanting steps, taking care that precipitate is drained completely.
5. Add 5 mL of H_2SO_4 (1 mL concentrated sulfuric acid + 9 mL distilled water; prepared as above) to the precipitate.
6. Heat sample and blank (5 mL H_2SO_4; prepared as above) in a 50-mL centrifuge tube by keeping it in a boiling water bath. Titrate hot solution (55–60°C) with 0.01 N $KMnO_4$ until the first pink color persists for approximately 30 sec (*see* **Note 1**).

$$\text{Oxalic acid (mg)/100 g product} = [(\text{milliliter of } 0.01\text{ N } KMnO_4 \text{ used} \times 0.45\text{ mg} \times (25/10))/\text{Weight of the sample taken (g)}] \times 100$$

where 0.45 = mg anhydrous oxalic acid $(COOH)_2$ equivalent to 1 mL of 0.01 N $KMnO_4$, 25/10 is the dilution factor, and 100 is the conversion factor for oxalic acid content per 100 g sample.

4. Note

1. The normality of permanganate solution suggested in ***(1)***, i.e., 0.01 N, is too weak in the case of halophytes and other oxalic acid-rich species like cactus pads. Therefore, it is recommended to titrate with more concentrated permanganate solution, for example 0.1 N or 0.05 N.

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7

Nitrate and Nitrite

Key Words: Nitrate; nitrite; methemoglobin; blood pressure; asphyxia; spinach; spongy cadmium column; zinc metal; sodium nitrate; sodium nitrite; ammonia buffer solution; Jones reductor.

1. Introduction

1.1. Nature, Mechanism of Action, and Biological Effects

Occasionally forages accumulate nitrates in quantities that are toxic to some farm animals. Under certain environmental conditions, some plants may accumulate high concentrations of nitrates. The factors that can influence the accumulation of nitrates in the plants are drought, shade, use of herbicides, and application of nitrogenous fertilizers. Nitrates are not very toxic, but they are readily converted by bacteria in the alimentary tract into nitrites, which are much more toxic. In cattle and sheep this conversion takes place in the rumen and in horses in the cecum. Nitrites pass easily from the gastrointestinal tract into the blood, where they combine with hemoglobin in the red blood cells to form methemoglobin, a compound that is incapable of taking up and transporting oxygen **(Fig. 1)**. Consequently the clinical signs of nitrite poisoning are those associated with oxygen deficiency and include general weakness and a fall in blood pressure. Death may follow from asphyxia. Young animals and babies are particularly at risk because the small volume of blood that they contain requires only a small amount of nitrite to convert all the hemoglobin to methemoglobin. Pregnant animals that are affected, but do not die, may abort later.

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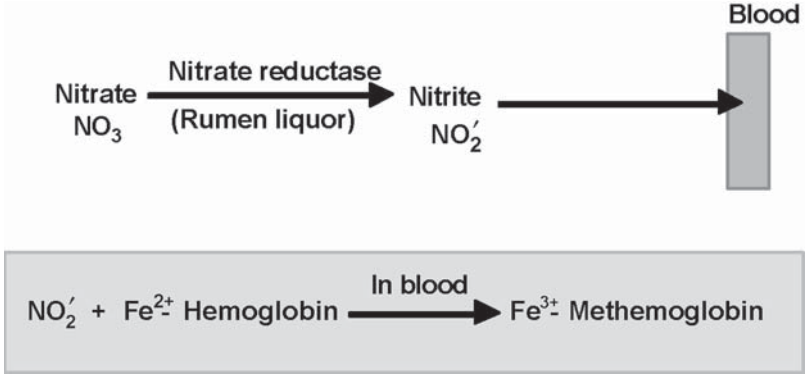

Fig. 1. Mechanism of action of nitrate.

1.2. Present in

Spinach, raps, sorghum, grasses.

1.3. Principle of Assay

The sample is extracted with distilled water and the aqueous extract is clarified with zinc hydroxide. Nitrate is reduced to nitrite on a spongy cadmium column (the nitrite originally present is unaltered); sulfanilic acid is diazotized by the nitrite and coupled with *N*-(1-naphthyl)-ethylenediamine dihydrochloride to form a pink azo dye, the absorbance of which is measured at 550 nm using a spectrophotometer. For those samples containing both nitrite and nitrate, the nitrite content is first determined from the unreduced sample filtrate, and then the total nitrite content (existing nitrite and nitrite formed from nitrate) from the column eluate. The nitrate content is then calculated by difference ***(1–4)***.

2. Materials

1. *Ammonia buffer solution, pH 9.6–9.7.* Dilute 20 mL of concentrated HCl (37%) to 500 mL with distilled water, mix, and add 50 mL concentrated NH_4OH (25%). Dilute this solution to 1 L with distilled water and mix thoroughly. Check the pH of the solution and adjust it to 9.6 to 9.7 using HCl or NH_4OH as necessary.
2. *Zinc sulfate solutions (0.42 M).* Dissolve 12 g $ZnSO_4.7H_2O$ in approximately 70 mL distilled water and then make the volume up to 100 mL with distilled water.
3. *Sodium hydroxide solution (2%).* Dissolve 2 g sodium hydroxide in 100 mL distilled water.
4. *Cadmium sulfate solutions (0.14 M).* Dissolve 3.7 g $CdSO_4.8H_2O$ in approximately 70 mL distilled water and make the volume up to 100 mL with distilled water.

5. *Zinc metal (about 10 cm sticks)*—Fisher Scientific (Scwerte, Germany), Catalogue No. Z-13, or equivalent.
6. *Sulfanilic acid solution, 1% in 30% acetic acid.* Dissolve 10 g of sulfanilic acid in 700 mL of distilled water, then add 300 mL of acetic acid (100%) and mix thoroughly (do not add acetic acid until the sulfanilic acid has dissolved completely). Store at room temperature.
7. *N-(1-naphthyl)-ethylenediamine dihydrochloride (Marshall`s reagent), 0.1% in 60% acetic acid.* Weigh 100 mg *N*-(1-naphthyl)-ethylenediamine dihydrochloride and dissolve in 100 mL of 60% acetic acid. Store in a refrigerator. It is stable for 1 week.
8. *Color reagent.* Mix equal volumes of the sulfanilic acid and Marshall's reagent solutions just before use.
9. *Sodium nitrate standard solutions. I. Stock solution, 1 mg/mL*: Dissolve 500 mg of $NaNO_3$ in approximately 300 mL distilled water in a 500-mL volumetric flask. Add to it 50 mL of the NH_4Cl buffer and make the volume up to 500 mL with distilled water. The solution is stable at 4°C for 1 to 2 weeks. *II. Working solution, 10 µg/mL*: Transfer 1 mL of stock solution to a 100-mL volumetric flask, make the volume to 100 mL with distilled water and mix thoroughly. Prepare the solution every day.
10. *Sodium nitrite standard solutions. I. Stock solution, 500 µg/mL*: Dissolve 250 mg $NaNO_2$ in approximately 300 mL distilled water in a 500-mL volumetric flask. Add 100 mL of the ammonia buffer and make the volume up to 500 mL with distilled water. Mix thoroughly and store it at 4°C (stable for 1 week). *II. Working solution, 5 µg/mL*: Transfer 1 mL stock solution to a 100-mL volumetric flask, make the volume up to 100 mL with distilled water, and mix the solution thoroughly. The solution should be prepared fresh every day.

3. Method

3.1. Preparation of Standard Curve for Sodium Nitrite

1. Add 0.0, 1.0, 2.0, 4.0, 6.0, and 10 mL of $NaNO_2$ working solution to separate 50-mL volumetric flasks. Add 9.0 mL of the ammonia buffer and 5 mL of 60% acetic acid solution to each flask and immediately proceed to step 2 (*see* **Note 1**).
2. Add 10 mL of the color reagent, make the volume up to 50 mL with distilled water, mix thoroughly, and let it stand for 25 min in the dark.
3. Set the spectrophotometer at 550 nm and measure absorbance of the above solutions against the blank (0 standard solution).
4. Prepare a calibration curve by plotting absorbance against micrograms of $NaNO_2$ (range: 5 to 50 µg).
5. The absorbance range should extend from 0 to 0.6 approximately.

3.2. Preparation of Modified Jones Reductor

1. Place three to five zinc (Zn) sticks in each of the two 800-mL beakers containing 500 mL $CdSO_4$ solution.

2. Remove Zn sticks every 2 to 3 h [or as soon as a thick layer of cadmium (Cd) forms] and scrape off the spongy metallic Cd by rubbing sticks against each other (important: Cd must be kept covered with aqueous solution at all times).
3. After 6 to 8 h, wash deposits with two 500-mL portions of distilled water.
4. Transfer Cd with distilled water to a high-speed blender and blend for 2 to 3 s.
5. Retain 8 to 40 mesh particles and repeat blending to increase yield of particles.
6. Wash particles with 0.1 N HCl, stir occasionally with a glass rod, and leave it overnight in acid.
7. Stir once to degas and decant. Then wash with two 500-mL portions of distilled water.
8. Fill the modified Jones reductor in a chromatographic column (glass tube of approximately 300 mm in length and 10 mm in internal diameter) plugged with a glass wool and fixed with a stopcock.
9. Add Cd to a depth of 8 to 10 cm and drain occasionally during filling but do not allow liquid level to fall below top of Cd bed. During filling, Cd bed should always be dipped in distilled water.
10. Eliminate bubbles in Cd bed by tapping sides of column and wash Cd column with 25 mL of the ammonia buffer and drain to top of Cd bed (*see* **Note 2**).

3.3. Testing Efficiency of Cadmium Column

1. Mix 6 mL $NaNO_3$ working solution (10 µg/mL) and 5 mL of the ammonia buffer and pour on the Cd column.
2. Adjust flow rate to 3 to 5 mL/min. After column is emptied, wash with 15 mL of distilled water.
3. Collect the eluate in a 50-mL volumetric flask and wash the column with additional approximately 10 mL of distilled water and again collect the eluate in the same 50-mL volumetric flask. Add to the flask 5.0 mL of 60% acetic acid.
4. Add 10 mL of the color reagent, make the volume up to 50 mL with distilled water, and mix well. Keep the solution in the dark for 25 min.
5. Prepare a blank in the same manner by taking 6 mL of distilled water through steps 1 and 4 (*see* **Note 3**).
6. Read the absorbance in the spectrophotometer at 550 nm. The $NaNO_2$ concentration, as determined from the standard curve, should be about 48.7 µg/mL if 100% conversion occurs.
7. If there is less than 90% conversion, recondition the column by passing 25 mL of 0.1 N HCl followed by two 25-mL portions of distilled water and 25 mL of the ammonia buffer and repeat steps 1–6. Check efficiency of each column once a week.

3.4. Preparation of Extract

1. Weigh a 10-g sample (ground if dry, and cut into small pieces if fresh) and blend with 70 mL of distilled water and 12 mL of 2% NaOH solution in a blender until smooth slurry is formed (generally takes about 5 min) (*see* **Note 4**).

2. Transfer the slurry into a 200-mL volumetric flask, and rinse blender with 30 to 50 mL of distilled water. Then mix the suspension well by swirling. Take out one or two drops of suspension from the flask and check the pH with pH paper. If the pH is between 8 and 10, heat the contents in a water bath (50–60°C) until the temperature of the suspension reaches close to 50°C. If the pH is less than 8, add additional amounts of 2% NaOH solution until the pH rises to 8 to 10, and then heat as above.
3. Occasionally swirl the contents while heating. Maintain the temperature at about 50°C for an additional 10 min, mixing occasionally.
4. Add 10 mL of $ZnSO_4$ solution and mix by swirling. If no white precipitate of $Zn(OH)_2$ becomes visible after the addition of $ZnSO_4$ solution, add 2 to 5 mL of 2% NaOH solution and keep mixing to avoid excessive addition of 2% NaOH solution.
5. Cool to room temperature in a water bath and dilute to volume 200 mL with distilled water and mix thoroughly.
6. Filter the content through filter paper (Whatman No. 1), discarding the first 20 mL filtrate, into a 250-mL glass-stoppered flask. Re-filter if the extract is not clear.

3.5. Determination of Nitrite

1. Transfer a 10-mL aliquot of the above filtrate to a 50-mL volumetric flask. Add 9.0 mL of the ammonia buffer and 5.0 mL of 60% acetic acid.
2. Add 10 mL of the color reagent and make the volume up to 50 mL with distilled water.
3. Mix thoroughly and place in the dark for 25 min.

3.6. Determination of Nitrate Plus Nitrite

1. Mix a second 10-mL aliquot of filtrate with 5 mL of the ammonium buffer and pass through the modified Jones Reductor (*see* **Note 5**).
2. Wash the column with about 15 mL of distilled water.
3. Collect the eluate in a 50-mL volumetric flask and wash the column with additional approximately 10 mL of distilled water and again collect the eluate in the same 50-mL volumetric flask. Add to the flask 5.0 mL of 60% acetic acid.
4. Add 10 mL of the color reagent and make the volume up to 50 mL with distilled water. Then mix well and leave it in the dark for 25 min.

3.7. Preparation of Reagent Blank

Carry 70 mL of distilled water through steps 2 to 7 described in Section 3.4.

3.8. Calculations

1. After color development, read the absorbance of unreduced blank and unreduced sample filtrates against the blank (0 standard solution; step 3 in **Section 3.1.**). This corresponds to the presence of nitrite in the sample.

2. Read the absorbance of reduced blank and reduced sample eluates against the same blank (0 standard solution; step 3 in **Section 3.1.**). This corresponds to the presence of nitrate plus nitrite in the sample.
3. Subtract the blank values from the corresponding sample values.
4. Determine the concentration of nitrite in each case from the standard curve.
5. Calculate the concentration of nitrite in the unreduced and reduced sample filtrates.
6. The difference between the two values is a measure of the nitrate concentration, equivalent to nitrite.
7. Finally, calculate the concentrations of nitrite and nitrate in the original sample.

Example

Absorbances:	Reagent blank, unreduced	NO_2	a
	Reagent blank, reduced	NO_2/NO_3	b
	Sample, unreduced	NO_2	c
	Sample, reduced	NO_2/NO_3	d

I. $NaNO_2$ determinations:

Absorbance (due to $NaNO_2$) = A (sample NO_2) − A (blank NO_2)
$= c - a$
$= x$

From standard curve, absorbance $x = m\,\mu g$

Since this is from a 10-mL aliquot from a total of 200 mL, nitrite in 200 mL = (200/10) $m = 20m\,\mu g$.

Since the original sample wt. was 10 g, the $NaNO_2$ concentration is $20m\,\mu g/10\,g = 2m\,\mu g/g = 2m$ ppm.

II. $NaNO_3$ determination:

Absorbance (due to $NaNO_3/NaNO_2$) = A (sample NO_3/NO_2) − A (blank NO_3/NO_2)
$= d - b$
$= y$

From standard curve, absorbance $y = n\,\mu g$

Since this is from a 10-mL aliquot, from a total of 200 mL, total $NaNO_2$ (from $NaNO_3$ plus $NaNO_2$ present in the sample) = (200/10) × $n = 20n\,\mu g$.

Since the original sample was 10 g, total $NaNO_2$ is $20n\,\mu g/10\,g = 2n\,\mu g/g$ or $2n$ ppm.

$NaNO_2$ from $NaNO_3$ in the sample = $2n - 2m = 2\,(n - m)$ ppm.

The conversion factor from $NaNO_2$ [molecular weight (MW) 69] to $NaNO_3$ (MW 85) is 1.23.

Therefore, $NaNO_3$ level in the sample is 2.46 $(n - m)$ ppm.

4. Notes

1. Since nitrite is unstable in acidic solutions the color reagent should be added immediately after adding the 60% acetic acid solution.
2. When the column is drained, the liquid level should be just at top of the Cd bed (±2 mm).
3. If high blank is obtained for NO_3 on Cd column, follow the following washing schedule to remove absorbed residual NO_3 from Cd column:

   Pass 50 mL of 1 N NaOH through Cd column. Wash column with distilled water until the pH of the effluent is close to neutral pH (pH 7–8). Then pass 25 mL of 0.1 N HCl and again wash column with distilled water. Finally, wash column with 25 mL of the ammonia buffer. This should be done each day and the columns be kept ready for later use. Omit the above steps if columns are new, i.e., being used for the first time.

4. If the results are beyond the range of the standard curve, then dilutions must be made to the sample extract. Do not dilute the final reaction mixture after color development has occurred. Appropriate dilutions must also be made to the sample blanks. Conversely, if the absorbance is low on using 10 mL of the sample extract, higher volumes can be taken for the color development, and absorbance should be recorded against appropriate blanks.
5. Recondition the column after each analysis and store the Cd under the ammonia buffer between analyses.

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8

L-Mimosine (β-(3-Hydroxy-4-Pyridone-1-yl)-L-Alanine)

Key Words: Mimosine; *Leucaena leucocephala;* 3-hydroxy-4-(*1H*)-pyridone; 2,3-dihydroxypyridine; hair loss; goiter; *Synergistes jonesii;* ferric chloride; activated charcoal; p-nitroaniline; sodium nitrite; pyridoxalphosphate.

1. Introduction

1.1. Nature, Mechanism of Action, and Biological Effects

L-mimosine is a nonprotein amino acid. Despite the consideration of *Leucaena leucocephala* as a promising alternate source of protein for fodder, the presence of mimosine to the extent of 2% to 10% dry matter in the leaf and 2% to 5% dry matter in the seed has limited its use as a livestock feed since mimosine and its degradation products 3-hydroxy-4-(*1H*)-pyridone (3,4-DHP) and 2,3-dihydroxypyridine (2,3-DHP) have been known to be toxic to many species **(Fig. 1)**. Ingestion of mimosine results in hair loss, goiter, reproductive disorders, epithelial damage, reduced feed intake, and ultimately death in both nonruminants and ruminants. Outbreaks of human alopecia have also been reported in some areas. Certain segments of the human population are known to consume portions of the leucaena in their diet, and a loss of hair has been frequently observed among those individuals who eat the leaves, pods, and seeds in the form of a soup. Resistance to mimosine toxicity in ruminants of certain geographical areas has been attributed to the capability of their rumen microorganisms to restrictively metabolize mimosine and DHP. From the rumen of goats in Hawaii resistant to mimosine toxicity, a microorganism (*Synergistes jonesii*), capable of metabolizing mimosine and DHP to innocuous products has been successfully transferred to the rumen of cattle in Australia that were

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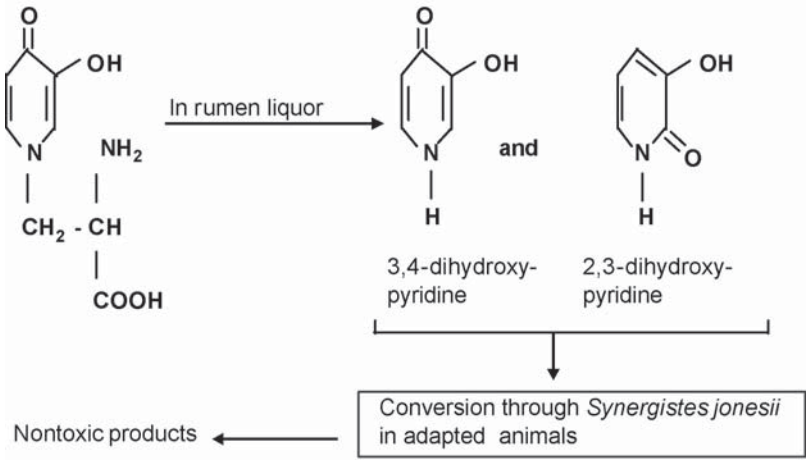

Fig. 1. Degradation of mimosine.

susceptible to mimosine toxicity **(Fig. 1).** This gave these cattle the capability to utilize effectively the mimosine-containing forage.

Mimosine is considered to bind minerals and pyridoxalphosphate, required for activities of various enzymes **(Fig. 2)**. This binding decreases activity of enzymes such as tyrosine decarboxylase, tyrosinase, and ribonucleotide

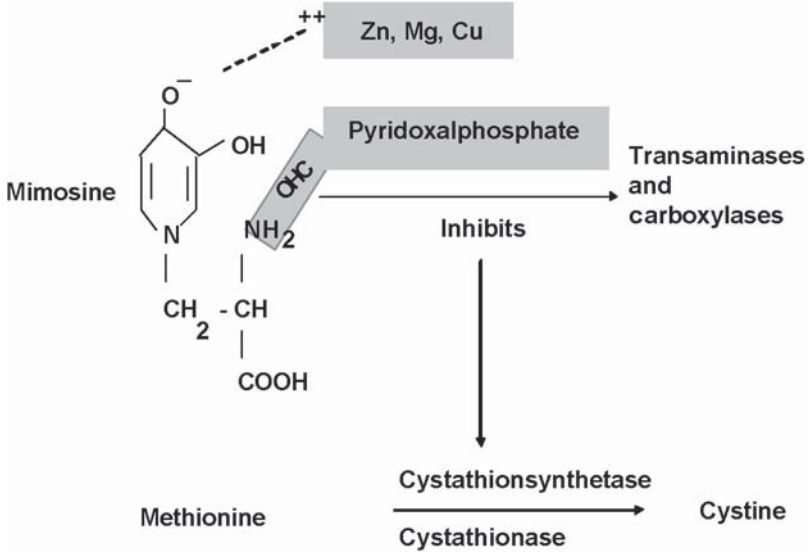

Fig. 2. Mode of action of mimosine.

reductase, leading to various metabolic disorders, including inhibition of conversion of methionine to cystine, which is an important component of wool/hair. Mimosine also has general antimitotic activity. It inhibits the proliferation of various animal cell in tissue culture and arrests cell division in cultured human cells.

1.2. Present in

Leucaena leucocephala and *Mimosa pudica*.

1.3. Principle of Assays

Two assays are presented in this chapter. In the first method, mimosine reacts quantitatively with ferric chloride, producing an intense violet-colored compound in slightly acid solution. The color produced is measured at 535 nm using a spectrophotometer ***(1)***. The second assay is based on the reaction between mimosine and p-nitroaniline to produce an intense yellow-colored azodye, which has a sharp absorption maximum at 400 nm ***(2)***. This method is more sensitive and specific for mimosine.

2. Materials

2.1. Method 1: Based on Ferric Chloride

1. *Mimosine solution (0.1%) in 0.1 N HCl.* Weigh exactly 10 mg of mimosine (for example, from Sigma, St. Louis, MO) and dissolve in 10 mL of 0.1 N HCl.
2. *Ferric chloride (0.5%) in 0.1 N HCl.* Take 500 mg of ferric chloride in a 100-mL volumetric flask and make the volume up to 100 mL with 0.1 N HCl.
3. *Activated charcoal (activated carbon).*
4. *0.1 N HCl.* Take 1.1 mL of 37% (w/v) HCl and dilute it to 100 mL distilled water.

2.2. Method 2: Based on p-Nitroaniline

1. *A stock solution of mimosine (5 mM) in 0.05 M HCl. Step I:* Take 2.08 mL concentrated HCl in a 500-mL volumetric flask and make the volume up to 500 mL with distilled water to obtain 0.05 M HCl. *Step II:* Weigh 99.1 mg of pure mimosine and transfer it into a 100-mL volumetric flask and make the volume up to 100 mL with 0.05 M HCl.
2. *Working mimosine solution.* The stock solution (5 mM) is suitably diluted with distilled water to get a working standard of 50 µM solution of mimosine. (Take 1 mL of stock solution and make the volume up to 100 mL with distilled water.) Both stock and working solutions are stored in frozen conditions.
3. *p-Nitroaniline solution (0.05%).* Dissolve 50 mg *p*-nitroaniline in 5 mL of methanol and dilute it to 100 mL with 0.033 M H_3PO_4.
4. *Sodium nitrite solution (0.1%).* Weigh 100 mg sodium nitrite and dissolve in 100 mL distilled water.

5. *Diazotized p-nitroaniline reagent*. Prepare it 1 to 2 min prior to the assay by mixing equal volumes of the *p*-nitroaniline solution (0.05%) and sodium nitrite solution (0.1%).
6. *Sodium phosphate buffer (0.25 M, pH 7)*. Prepare 0.25 M monobasic sodium phosphate (NaH_2PO_4), solution 1 (dissolve 34.75 g in 1000 mL distilled water) and dibasic sodium phosphate ($Na_2HPO_4.7H_2O$), and solution 2 (dissolve 67.06 g in 1000 mL distilled water). Take 39 mL of solution 1 and 61 mL of solution 2, and dilute it to 200 mL with distilled water.
7. *Na_2CO_3 solution (5%)*. Dissolve 5 g sodium carbonate in 100 mL distilled water.
8. *HCl (0.2 M)*. Take 8.3 mL of 37% (w/v) HCl and make the volume up to 500 mL with distilled water.
9. *HCl (0.05 M)*. Take 2.08 mL of 37% (w/v) HCl and make the volume up to 500 mL with distilled water.
10. *H_3PO_4 (0.033 M)*. Take 0.92 mL of concentrated phosphoric acid (18.1 M) and make the volume up to 500 mL with distilled water.

3. Methods

3.1. Method 1: Based on Ferric Chloride

3.1.1. Preparation of Extract

Weigh 1.25 g of dried leucaena leaf meal sample in a 250-mL beaker and digest with 100 mL of 0.1 N HCl at 80° to 90°C for approximately 1 h with frequent stirring (*see* **Note 1**). Allow the digest to cool and transfer the entire material including the solids to a 250-mL volumetric flask. After thorough shaking, allow the flask to stand until most of the solids get settled on the bottom.

3.1.2. Clarification of Extract

Transfer 10 mL of the above supernatant liquid into a 150-mL beaker containing 30 mg activated carbon. Add distilled water to bring the volume to about 25 mL. Cover the beaker with a watch glass and boil the liquid for 15 min on a hot plate. Allow the material to cool and filter the solution with suction through a fritted-glass crucible capable of retaining the carbon. Collect the filtrate in a test tube placed inside the suction flask. Transfer the filtrate into a 50-mL capacity volumetric flask. Wash the beaker and the material on the crucible with 15 to 20 mL of 0.1 N HCl, split in three to four portions, and collect the filtrate in the test tube. Combine all the filtrates in a 50-mL volumetric flask and make up the final volume to 50 mL by using 0.1 N HCl.

3.1.3. Determination of Mimosine

Take 2 to 5 mL of the filtrate (depending on the amount of mimosine present) into a 100-mL volumetric flask (*see* **Note 2**). To it add 10 mL of 0.1 N HCl and 4 mL of 0.5% ferric chloride in 0.1 N HCl. Make the volume of the solution up to 100 mL with distilled water. The pH of the solution should be between

1.5 and 2.5. Read the color intensity of the reaction mixture at 535 nm using a spectrophotometer. The quantity of mimosine is estimated from a curve prepared by the following procedure.

3.1.4. Preparation of Calibration Curve

Dissolve exactly 0.100 g of pure mimosine (for example, from Sigma, St. Louis, MO) in 100 mL of 0.1 N HCl in a volumetric flask. Take 0- (blank), 1-, 2-, 3-, 4-, and 5-mL aliquots of this solution in 100-mL volumetric flasks. To this, add 10 mL of 0.1 N HCl and 4 mL of 0.5% ferric chloride solution and then make the volume up to 100 mL with distilled water. Measure the intensity of the violet color at 535 nm using a spectrophotometer. Calculate the level of mimosine in the sample using the linear curve (from 10 to 50 µg mimosine in the reaction mixture), and express the results as g/100 g dry matter.

3.2. Method 2: Based on p-Nitroaniline

3.2.1. Preparation of Calibration Curve

Suitable volumes of mimosine working standard are placed in test tubes to get a range of mimosine concentrations from 1.25 to 50 nmol, in a volume of 3.5 mL of distilled water. To this, add 1 mL of sodium phosphate buffer (0.25 M, pH 7.0) to maintain neutral pH. Then add 0.5 mL of the diazotized *p*-nitroaniline reagent to each tube, mix well, and incubate at room temperature for 15 min for development of color. Measure absorbance at 400 nm using a spectrophotometer.

3.2.2. Preparation of Extract

1. Take fresh leaves of *L. leucocephala* (2 g), immerse in 20 mL of boiling water, and continue boiling for 3 to 5 min (*see* **Note 3**).
2. After cooling, add an equal volume of 0.2 M HCl and extract mimosine by homogenization (Ultra-Turrax (IKA Werke GmbH and Co. KG, Staufen, Germany), 20,000 rpm for 5 min on ice), followed by centrifugation at 4000 *g* for 25 min. Adjust the supernatant volume to 40 mL.
3. To perform the decolorization process, boil 10 mL of this extract with 10 to 20 mg of activated charcoal for 15 min.
4. After cooling, filter the solution through Whatman No. 42 filter paper and make the volume up to 10 mL with distilled water. Instead of filtration, centrifugation at 4000 *g* for 20 min may also be used.

3.2.3. Separation by Paper Chromatography and Estimation of Mimosine

Aliquots, 10 to 50 µL of suitably diluted mimosine standards containing 2 to 50 nmol of mimosine, are applied onto Whatman No. 1 filter paper (*see* **Note 4**). Similarly, suitable aliquots of the plant extracts are also spotted on the paper. The paper is developed overnight with a solvent mixture of *n*-butanol/

acetic acid/water (4 : 1 : 2, v/v) by the ascending method. Before starting to develop the spotted chromatographic paper, the chromatographic chamber must be saturated with the same solvent for about 3 to 5 h. After the solvent front reaches 1 cm below the top edge of the paper, it is taken out carefully and dried at 80°C. The dried paper is sprayed with the diazotized *p*-nitroaniline reagent and dried at room temperature. Subsequently, Na_2CO_3 solution (5%) is sprayed to enhance the color of spots. The yellow spots are marked and the paper containing these spots is cut into squares of 20 × 20 mm (*see* **Note 5**). These are placed in test tubes containing 3 mL of water and the test tubes are vortexed for 3 to 5 min. The pH of the solution is carefully brought to neutral pH by the addition of 0.2 M HCl (tested with pH paper), made up to 3.5 mL, and color is developed by the addition of 1 mL of the sodium phosphate buffer followed by 0.5 mL of the diazotized *p*-nitroaniline reagent. The contents are mixed well, and the absorbance at 400 nm is measured against blanks prepared in the same manner with paper of the same size as for the test samples, cut from blank portions. A calibration curve is drawn for concentration vs. absorbance at 400 nm for mimosine. Concentration of mimosine in the plant samples is determined from these plots. The results are expressed on percent dry matter basis.

4. Notes

1. Leaf and seed [both fresh (1–3 g) and dried (0.2–0.5 g)] sample can be placed in the boiling water for 1 to 5 min, and after cooling, an equal volume of 0.2 N HCl should be added. The mimosine can be extracted by homogenization (Ultra-Turrax, IKA Werke GmbH and Co. KG, Staufen, Germany) at 20,000 rpm for 5 min at approximately 0°C), followed by centrifugation at 3000 *g* for 20 min. This could prevent the formation of 3-hydroxy-4-(1H)-pyridone (3,4-dihydroxypyridine).
2. Proper dilution of sample extracts and decolorization with activated carbon could avoid or minimize the interference in the assay by pigments, and by 3,4-dihydroxypyridine since 3,4-dihydroxypyridine also complexes with ferric chloride.
3. Too long boiling of sample with water should be avoided.
4. The pigments, phenolics, and mimosine precursors may interfere in the method. Therefore, it is advised to develop the color after separation of mimosine by paper chromatography.
5. The location of mimosine spot from the sample on the developed chromatographic paper must be carefully authenticated with standard mimosine.

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9

Canavanine

Key Words: Canavanine; 2-amino-4-(guanidinooxy) butanoic acid; antimetabolite; arginine antagonist; autoimmune disease; lupus erythematosus; pentacyanoaminoferate; potassium persulfate; *Canavalia* spp.; *Sesbania* spp.

1. Introduction

1.1. Nature, Mechanism of Action, and Biological Effects

Canavanine [2-amino-4-(guanidinooxy) butanoic acid] occurs in over 350 species of the papilionoideae, a subfamily of the Leguminosae. It is an analogue of arginine **(Fig. 1)**. The highest concentration of canavanine (13% dry weight) has been reported in *Dioclea megacarpa* and it occurs up to 5% in seeds of *Canavalia ensiformis*. L-canavanine exhibits potent antimetabolic properties in organisms ranging from viruses and prokaryotes to whole animals. Canavanine acts primarily as an arginine antagonist and gets incorporated into the cell nucleus and other proteins and interferes with deoxyribonucleic acid and ribonucleic acid formation. In monkeys it causes hematological and serological abnormalities. These abnormalities are similar to those seen in human systemic lupus erythematosus, an autoimmune disease that adversely affects the kidney and skin. Canavanine affects the charged surface of membrane of autoimmune β cells, and it has been suggested that such alterations may be associated with abnormal autoimmune response.

1.2. Present in

Canavalia spp., *Medicago sativa, Sesbania* spp., *and Dioclea megacarpa*.

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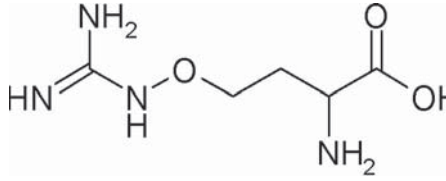

Fig. 1. Structure of canavanine.

1.3. Principle of Assay

The guanidoxy group, $-O \cdot NH \cdot C(:NH) \cdot NH_2$, in canavanine reacts with trisodium pentacyanoammonioferrate in aqueous solution at pH 7.0 and forms a magenta-colored chromophore. The color formed is measured at 520 to 530 nm ***(1)***.

2. Materials

1. *Potassium phosphate buffer (0.2 M, pH 7.0)*. Take 50 mL of 0.2 M KH_2PO_4 (27.20 g/L) and 29.1 mL of 0.2 M NaOH and dilute to 100 mL. Check pH and adjust to pH 7, if needed.
2. *Potassium persulfate (1%)*. Weigh 1 g of potassium persulfate and dissolve in 100 mL distilled water.
3. *Preparation of pentacyanoaminoferate (PCAF)*. Dissolve 10 g of sodium nitroprusside in 55 mL of concentrated ammonia solution (25% or 32% w/v). Keep this solution in the dark at 0°C for 24 h and filter a yellow-green precipitate containing a mixture of sodium pentacyanoaminoferate (II) and (III). Add ethanol to the filtrate until no precipitates are obtained. Filter it and combine this precipitate with the first precipitate. Wash the pooled precipitate with absolute ethanol until all ammonia is eliminated. Dry the precipitate over H_2SO_4 (a desiccator containing the acid) and then store in the dark over $CaCl_2$ (a desiccator containing dry $CaCl_2$).
4. *Photoactivated pentacyanoaminoferate solution (1%)*. Take 1 g of PCAF in a volumetric flask, add 50 mL of distilled water to dissolve it, and make up the volume to 100 mL with distilled water. Transfer the solution to a separate 200-mL capacity beaker and illuminate the whole solution under a light source (60 W table lamp) for 1 h. Finally, transfer the photoactivated PCAF solution into an amber-colored screw-capped bottle. Store this solution in the dark condition. The PCAF solution should be prepared every day.
5. *HCl (0.1 M)*. Take an aliquot of 1.1 mL HCl (w/v) and make the volume up to 100 mL with distilled water.
6. *NaOH (0.2 M)*. Weigh 8 g sodium hydroxide, dissolve in approximately 500 mL distilled water, and then make the volume up to 1 L with distilled water.
7. *Canavanine solution*. Dissolve 1 mg of canavanine in 1 mL of 0.2 M potassium phosphate buffer, pH 7.

3. Method

3.1. Preparation of Extract

The preparation of extract is based on Cacho et al. ***(2)***. Take 5 g of finely ground plant sample, de-fat in a Soxhlet extractor with hexane for 5 h, and after drying in air add 25 mL of 0.1 M HCl. Stir the mixture using a magnetic stirrer for 6 h at room temperature and centrifuge at 4000 *g* for 20 min. Save the supernatant and once again repeat the extraction using 20 mL of 0.1 M HCl. Combine both the supernatants, adjust the pH to 7.0 with 0.2 M NaOH solution and dilute to a final volume of 100 mL using 0.2 M phosphate buffer, pH 7.0.

3.2. Preparation of Calibration Curve

1. Take 1 mL of canavanine solution (5–80 µg of canavanine in 0.2 M potassium phosphate buffer of pH 7.0 in a 10-mL volumetric flask).
2. To this solution, add 6.5 mL of the pH 7.0 phosphate buffer, 1 mL of 1% potassium persulfate and 0.5 mL of 1% aqueous PCAF. Make the volume up to 10 mL with distilled water. Mix the solution thoroughly and after 15 min read the absorbance at 520 to 530 nm against reagent blank (the phosphate buffer in place of canavanine solution).

3.3. Determination of Canavanine

Take 1 mL of the plant extract in a 10-mL volumetric flask instead of 1 mL of canavanine solution and follow the procedure given in **Section 3.2**. Determine the concentration of canavanine in the sample using the calibration curve and express the results as grams of canavanine/100 g dry matter.

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10

L-DOPA (L-3,4-Dihydroxyphenylalanine)

Key Words: L-DOPA; 3,4-dihydroxyphenylalanine; favisum; hemolytic anemia; glutathione content; protein digestibility; semiquinone; Parkinson's disease; *Mucuna* spp.; *Vicia faba*; HPLC; C_{18} nucleosil 120 column.

1. Introduction

1.1. Nature, Mechanism of Action, and Biological Effects

The nonprotein amino acid, 3,4-dihydroxyphenylalanine (L-DOPA) **(Fig. 1)** is present in high concentrations (about 3–6%) in *Mucuna* spp. The in vitro addition of L-DOPA to the red blood cells from individuals deficient in glucose 6-phosphate dehydrogenase decrease the glutathione content in red blood cells and this ultimately induces the favisum (hemolytic anemia; breakup of red blood cells). The inclusion of L-DOPA in fish feed has been reported to significantly reduce the growth performance. L-DOPA is susceptible to oxidation, giving a semiquinone that complexes with proteins. This decreases protein digestibility. On the other hand, it has been used for the treatment of Parkinson's disease because of its being a precursor of dopamine, a neurotransmitter.

1.2. Present in

Mucuna spp. and *Vicia faba*.

1.3. Principle of Assay

The DOPA is extracted from the sample in acidic condition and separated on a reverse-phase C_{18} nucleosil 120 column using a high-performance liquid chromatography (HPLC). The absorbance is measured at 282 nm and the peak area is converted into concentration using a standard DOPA solution ***(1)***.

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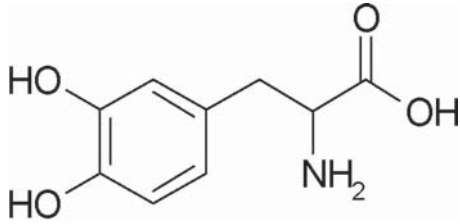

Fig. 1. Structure of 3,4-dihydroxyphenylalanine.

2. Materials and Methods

2.1. Preparation of Extract

Take 200 mg finely ground (ground preferably using a ball mill) and defatted seed flour in a centrifuge tube (*see* **Note 1**), add 20 mL of 0.1 N HCl and keep the mixture on a magnetic stirrer for 10 min at room temperature (*see* **Note 2**). Subject the mixture to Ultra-Turrax (IKA Werke GmbH and Co. KG, Staufen, Germany), (approximately 20,000 rpm) for 30 s in an ice bath condition and subsequently keep it on a magnetic stirrer for 1 h at room temperature. Collect the supernatant by centrifugation at 4000*g* for 15 min. Repeat the extraction procedure twice, pool all three supernatants, and make the volume to 100 mL with 0.1 N HCl. Filter the supernatant through a 0.2-µm glass filter and maintain it at 2° to 5°C until analysis. Sample should be analyzed within 8 h of preparation ***(2)***.

2.2. HPLC Instrument

The chromatography system used by us consisted of a Merk-Hitach model L-7100 HPLC pump, a 7450 UV detection and photo diode array detector, an L-7200 autosampler with injector valve containing a 100-µL sample loop, a D-7000 interphase module, and an LC organizer (Hitachi Instruments Inc., San Jose, CA). The analytical column was reverse-phase C_{18} (nucleosil 120, mean particle diameter 5 µm, 250 × 4.6 mm ID, Macherey-Nagel GmbH and Co., Düren, Germany). A guard pre-column was packed with the material as in the main column.

2.3. Preparation of Standard L-DOPA

Dissolve 0.1 g of L-DOPA (for example, from Sigma, St. Louis, MO) in 100 mL of 0.1 N HCl. Prior to analysis, dilute the stock solution to appropriate concentrations (2–100 µg/mL) with 0.1 N HCl and keep it at a temperature between 2° and 5°C.

2.4. Chromatographic Conditions

2.4.1. Solvents, Gradient System, and Separation of L-DOPA

Solvent A: Water, methanol, and *o*-phosphoric acid in the ratio of 975.5 : 19.5 : 1 (v/v/v).

Solvent B: 70% methanol.

These solvents are stable at 22°C for 1 week. All the aqueous solutions are passed through a 0.2-µm glass filter. Eluents are degassed in an ultrasonic bath.

The following gradient system is used for the elution of L-DOPA: start with 100% of solvent A and 0% of solvent B for 12 min, in the next 5 min increase solvent B from 0% to 100% and decrease Solvent A from 100% to 0%, in the next 5 min increase solvent A to 100% and decrease solvent B to 0%, and then for the next 15 min wash the column with solvent A to bring the column to the starting condition (solvent A 100% and solvent B 0%).

Separation is performed at room temperature with a flow rate of 1.2 mL/min. Absorbance is monitored at 282 nm and peak areas are determined. The peak area is converted to the DOPA amount using a calibration curve prepared by injecting 100 µL (the same amount as the sample extract) of the DOPA solution (2–100 µg/mL in 0.1 N HCl). Results are expressed as g/100 g on a dry matter basis ***(2)***.

3. Notes

1. Raw samples should be stored under a dry, dark, and low temperature condition.
2. During the acid extraction, ascorbic acid (50–100 mg/L) can be used to inhibit the polyphenoloxidase mediated oxidation of L-DOPA.

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11

Glucosinolates

Key Words: Glucosinolates; pyridine acetate buffer; goiter; lead and barium acetate; myrosinase; ferricyanide reduction; anticarcinogenic; antioxidants; rapeseed; isothiocyanates; thiocyanates; nitriles; glucose; DEAE-Sephadex A-25.

1. Introduction

1.1. Nature, Mechanism of Action, and Biological Effects

Glucosinolates, a group of plant thioglucosides, occur naturally in many plant species. These consist of a β-thioglucose moiety, a sulfonated oxime moiety, and a variable side chain derived from an amino acid. More than 120 different side chains have been described, although glucosinolates are often divided into three groups according to the nature of their side chain: aliphatic, aromatic, and indolyl. The difference between the glucosinolates with regard to chemical properties and biological activity and the hydrolyzed products formed are largely determined by the side-chain structure.

Glucosinolates impair the nutritional quality of rapeseed and rapeseed meal and restrict its use as high-quality protein animal feed. Glucosinolates are always accompanied in plant tissues by the thioglucosidase enzyme, myrosinase (thioglucoside glucohydrolase), which catalyzes the cleavage of the thioglucoside bond of glucosinolates. In the intact plant, enzyme and substrate occur in separate compartments, presumably as an adaptive measure to avoid autotoxicity. But following cell disruption, enzyme and substrate come into contact. The resulting hydrolysis involves cleavage of the thioglucoside bond and yields free glucose and an aglucone intermediate, which undergoes spontaneous degradation to one of a number of toxic metabolites (isothiocyanates, thiocyanates, and nitriles). These released compounds are responsible for the characteristic biting

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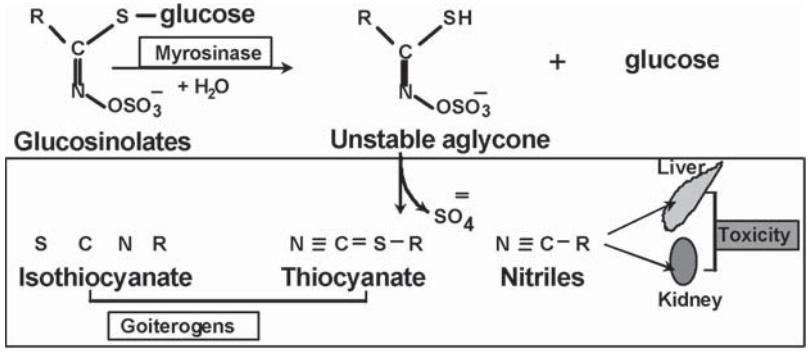

Fig. 1. Mechanism of action of glucosinolates.

taste of important condiments, such as radish and mustard. High levels of the hydrolysis products produce an unpleasant bitter taste **(Fig. 1)**.

When humans consume glucosinolates at low levels, as part of the normal diet, some of the hydrolysis products exhibit anticarcinogenic properties. Recently, these compounds have also been reported to be potential antioxidants. However, excessive consumption of glucosinolate-containing vegetables leads to increased risk of goiter. When consumed in large amounts by animals as part of their feed, the hydrolysis products reduce palatability and are toxic. Feeding of rapeseed to chickens caused enlarged thyroids, depressed growth, and lowered egg production. Moreover, liver hemorrhage and reticulolysis in poultry fed rapeseed meal leads to increased mortality.

1.2. Present in

Armoracia lapathifolia, Brassica campestris, B. napus, B. oleracea, B. carinata, B. juncea, B. hirta, Carica papaya, Rhaphanus sativus, Capparis spinosa, Cranbe abyssinica, Lepidium sativum, Camelina sativa, and *Moringa oleifera.*

1.3. Principle of Assays

Two methods are described in this chapter. The first method is based on the reduction of ferricyanide by the breakdown product, 1-thioglucose produced by the alkaline treatment of glucosinolates, and measurement of the absorbance at 420 nm ***(1)***. The second method is based on glucose determination after endogenous myrosinase hydrolysis of the intact glucosinolates adsorbed on the DEAE-Sephadex A-25 minicolumns ***(2,3)***.

2. Materials

2.1. Method 1: Based on Reduction of Ferricyanide

1. *Acetate buffer (0.2 M, pH 4.2). Solution A:* 0.2 M acetic acid (11.55 mL acetic acid in 1 L distilled water). *Solution B:* 0.2 M sodium acetate (16.4 g of sodium acetate

in 1 L distilled water). Take 36.8 mL of solution A and 13.2 mL of solution B, and dilute to a total volume of 100 mL with distilled water. Check pH and adjust if necessary.
2. *Lead and barium acetate (0.5 M).* Dissolve 189.5 g lead acetate $3H_2O$ in 600 mL distilled water and to this solution add 127.7 g barium acetate, dissolve, and make the total volume up to 1 L.
3. *Na_2SO_4 saturated solution (>1.4 M).* Dissolve 10.65 g anhydrous Na_2SO_4 in 50 mL distilled water.
4. *2 M NaOH.* Dissolve 80 g in 800 mL H_2O and make the volume up to 1 L with distilled water.
5. *Concentrated HCl (37%, w/v).*
6. *Sodium phosphate buffer 0.2 M, pH 7.0. Solution A:* 0.2 M monobasic sodium phosphate (27.8 g monobasic sodium phosphate in 1 L distilled water). *Solution B:* 0.2 M dibasic sodium phosphate (53.65 g of $Na_2HPO_4 \cdot 7H_2O$ or 71.7 g of $Na_2HPO_4 \cdot 12H_2O$ in 1 L distilled water). Take 39 mL of solution A, 61.0 mL of solution B, and dilute to a total volume of 200 mL with distilled water.
7. *Potassium ferricyanide ($K_3Fe(CN)_6$), 2 mM.* Dissolve 658 mg potassium ferricyanide in 1 L phosphate buffer (0.2 M, pH 7).

2.2. Method 2: Based on Hydrolysis to Glucose

1. *Pyridine acetate buffer (0.5 M, pH 5.0).* Take 40 mL pyridine and to it add 30 mL glacial acetic acid, and make the volume up to 1 L with distilled water.
2. *Pyridine acetate buffer (0.02 M, pH 5.0).* Take 40 mL of the above buffer and dilute to 1 L with distilled water.
3. *Myrosinase (thioglucosidase, EC 3.2.3.1., for example T-4528 from Sigma, St. Louis, MO).* Dissolve 2.5 mg of the enzyme in 1 mL of the pyridine acetate buffer (0.02 M, pH 5). Use 250 µL of this solution for each determination.
4. *Glucose kit No. 510-A (for example from Sigma, St. Louis, MO).*
5. *Methanol (70% v/v).* Take 70 mL of methanol in a 100 mL volumetric flask and make the volume up to 100 mL with distilled water.
6. *DEAE-Sephadex A-25* (*for example from Pharmacia, Hounslow, Middlesex, England*) *suspension.* Prepare a suspension of DEAE-Sephadex A-25 by first stirring the dry gel with an excess of 0.5 M the pyridine acetate buffer. Filter the suspension, wash it with 0.02 M pyridine acetate buffer, and finally suspend it in the 0.02 M pyridine acetate buffer, the volume of which was twice that of the settled gel.

3. Methods

3.1. Method 1: Based on Reduction of Ferricyanide

3.1.1. Preparation of Extract

Take 500 mg ground sample in a glass test tube and pipette 7.5 mL of near-boiling acetate buffer (0.2 M, pH 4.2). Keep the mixture in a boiling water bath for 15 min. After cooling (5 min), mix it with 1.5 mL of the barium and lead

acetate solution and vortex thoroughly. Add to it about 0.4 g of insoluble polyvinylpolypyrrolidone, stir, and incubate the mixture at room temperature for 15 min (*see* **Note 1**). Then add 1.5 mL of sodium sulfate solution while stirring. Vortex it and centrifuge the content for 10 min at 4000 *g* and collect supernatant. For a blank run the above procedure, without the sample.

3.1.2. Determination of Glucosinolates

1. Mix 0.9 mL of the clear supernatants (test and blank separately) with 0.9 mL of 2 M of NaOH, incubate it for 30 min at room temperature, and then add 0.138 mL of concentrated HCl (37%) to neutralize the solution.
2. Centrifuge the resulting mixture (4000 *g* for 10 min). Take 0.5 mL of the supernatant and mix with an equal volume (0.5 mL) of potassium ferricyanide (2 mM) prepared in phosphate buffer (pH 7, 0.2 M).
3. Vortex the mixture, centrifuge at 4000 *g* for 3 min, and measure the absorbance of the supernatant at 420 nm within 15 s against the blank. Convert the absorbance to the concentration using the following calibration curve.

3.1.3. Preparation of Calibration Curve

Sinigrin (for example, from Fluka Chemical Co., Taufkirchen, Germany) must be hydrolyzed with 2 M NaOH and continued in the same manner as the sample. Dissolve 5 mg sinigrin in 1 mL distilled water. Take 500 µL of the sinigrin-stock solution and to it add 500 µL of 2 M NaOH. After mixing, incubate the solution for 30 min at room temperature. Then add 77 µL of concentrated HCl (37%). The final concentration of sinigrin monohydrate is 2.3364 mg/mL. From this, take 0 to 500 µL and add the phosphate buffer to reach the final volume of 500 µL. Add 500 µL of potassium ferricyanide in each tube, mix thoroughly, and centrifuge at 4000 *g* for 3 min. Collect the supernatant and measure the absorbance at 420 nm against the phosphate buffer pH 7.0.

3.2. Method 2: Based on Hydrolysis to Glucose

3.2.1. Preparation of Extract

Take 200 mg of ground seed sample (ground preferably using a ball mill; *see* **Note 2**) into a clean and dry test tube. After preheating the tube (2 min) in a water bath or heating block, set at 75° to 76°C, add approximately 3 mL of 70% v/v methanol (preheated to boiling point). Stir the suspension continuously for 10 min. Centrifuge it at 4000 *g* for 10 min and carefully decant the supernatant. Repeat the extraction and centrifugation steps twice, heating for 5 min each time. Then combine the three supernatants and remove the methanol by rotary evaporation at approximately 40°C to give a volume of about 1 mL. Adjust the volume of this solution to exactly 10 mL with distilled water, mix well, and freeze the solution (−18°C) until analysis. This solution is designated as solution A.

3.2.2. Preparation of DEAE-Sephadex A-25 Minicolumns

Warm the end of a Pasteur pipette (short-form) in a flame and insert a 2-µL microcap pipette such that it protrudes approximately 1 cm. Hold the Pasteur pipette horizontal and introduce a drop of molten paraffin wax into the gap between the two pipettes, allowing the wax to fill the gap. Firmly plug the bottom of the Pasteur pipette with fine glass wool and wash with distilled water. Add a suspension of DEAE-Sephadex A-25 (1 mL) and allow the column to drain. After washing with distilled water the column is ready for use. With practice it is simple to produce columns with reproducible flow rates and performance and at very low cost.

3.2.3. Enzymatic Hydrolysis of Glucosinolates

For each determination, prepare a Sephadex minicolumn as described above. The flow through the column ceases when the water or solution reaches the surface of the gel. Add solution A (4 mL) to the Sephadex minicolumn, taking care not to disturb the gel bed. Allow the column to drain and wash it with distilled water (2×0.5 mL), followed by the addition of 0.02 M pyridine acetate buffer (2×0.5 mL), allowing each addition to drain completely. Discard all effluents. Wipe the column tip clean, place a clean, dry vial beneath the column, add 0.25 mL of the myrosinase (thioglucosidase) solution to the surface of the gel, and collect the effluent. After 2 to 15 h at room temperature (25°C), elute the liberated glucose by adding 0.5 mL distilled water and allowing the column to drain fully before adding a further aliquot of water (0.5 mL). Collect the effluent (final volume 1.25 mL) (designated as solution B). If necessary, solution B may be kept frozen (−18°C) until required for glucose assay ***(3)***.

3.2.4. Determination of Glucose

The glucose kit No. 510 A is used in accordance with the manufacturer's instructions. In the event of low absorbance resulting from an aliquot of solution B, a higher volume of the test solution should be used. A linear response is obtained up to approximately 1.2 mg/mL of glucose, and above this concentration there is a marked deviation from linearity. If glucose value higher than 1.0 mg/mL is obtained, dilute solution B and repeat the assay.

3.2.5. Calculation

Glucosinolates in seed (µ mol/g)

$$= [\text{glucose in solution B (mg/mL)} \times 1.25 \times 10 \times 1000]/(M \times V \times 180)$$

$$= [(\text{glucose in solution B (mg/mL)} \times 69.4]/M \times V$$

where M is the weight of the seed sample (g); V is the volume of solution A applied to the Sephadex-minicolumn (mL) and 10 mL is the total volume of solution A; 1.25 mL is the total volume of solution B; and 180 is the relative molecular mass of glucose.

4. Notes

1. During the extraction, the phenolics that interfere in the assay could be removed by adsorption with polyvinylpolypyrrolidone.
2. The ground seed sample product should be thoroughly mixed and it should be fine enough to pass through a standard 40-mesh sieve.

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12

Cyanogenic Glucosides/Cyanogens

Key Words: Cyanogenic glucoside; cyanogens; cyanide; β-glucosidase; linamarin; ATP production; nitrosamine; cassava leaves; drying; soaking; fermentation; *Phaseolus lunatus* seeds; sodium picrate; potassium cyanide; toxicity; goiter.

1. Introduction

1.1. Nature, Mechanism of Action, and Biological Effects

Cyanide (hydrogen cyanide, HCN) in trace amounts is widespread in the plant kingdom and occurs mainly in the form of cyanogenic glucosides (also called as cyanogens). Cyanogens are glycosides of sugar and cyanide-containing aglycon. These generally taste bitter. Relatively high concentrations are found in certain grasses, pulses, root crops, and fruit kernels. Some cassava varieties are capable of producing over 1 g HCN/kg of fresh tissue. The poisoning generally occurs due to consumption of new and young plants that contain higher amounts compared to the mature ones. Toxicity problems are highly variable and depend on the rate and amount of cyanogenic glucoside consumed, the presence of the β-glucosidase, and detoxification of cyanide. Poisoning and death may occur when an uninitiated animal rapidly consumes a plant with moderate to high levels of cyanogenic glucoside. Chronic problems such as goiter may be caused by long-term consumption of lower levels.

Intact cyanogenic glucosides are not toxic. The liberated cyanide upon enzymatic or acid hydrolysis is toxic. The lethal dose of HCN taken by mouth in humans has been estimated to be between 0.5 and 3.5 mg/kg body weight. A relatively large dose of HCN will cause death within a few minutes. The toxicity symptoms of ingested HCN are peripheral numbness and light-headedness followed by mental confusion and stupor, cyanosis, twitching, and convulsion with

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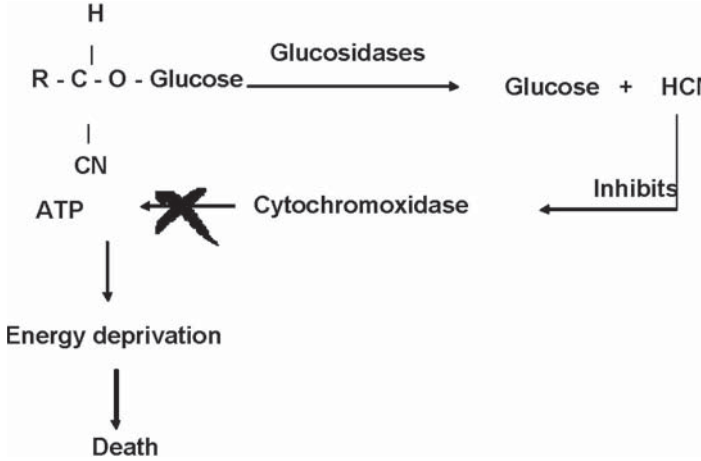

Fig. 1. Mechanism of action of cyanogens.

terminal coma. A high dose of cyanide inhibits cytochrome oxidase, a vital enzyme in the tricarboxylic acid cycle responsible for adenosine triphosphate (ATP) production. This could stop natural respiration and extend the cardiac arrest **(Fig. 1)**. With respect to frequently ingested cyanoglucosides, linamarin, in addition to HCN and acetone, is produced through decarboxylation of acetoacetate, and amygdalin liberates benzaldehyde, which has mild toxicity **(Fig. 2)**. Cyanide has been shown to inhibit pancreatic α-amylase activity, but the significance of this for digestion is not yet clear. Ruminants are more susceptible to poisoning of plant cyanogens than nonruminants. Cyanide is known to get converted to thiocyanate, which may enhance nitrosamine formation. Nitrosamine is linked to tumor incidence in human populations.

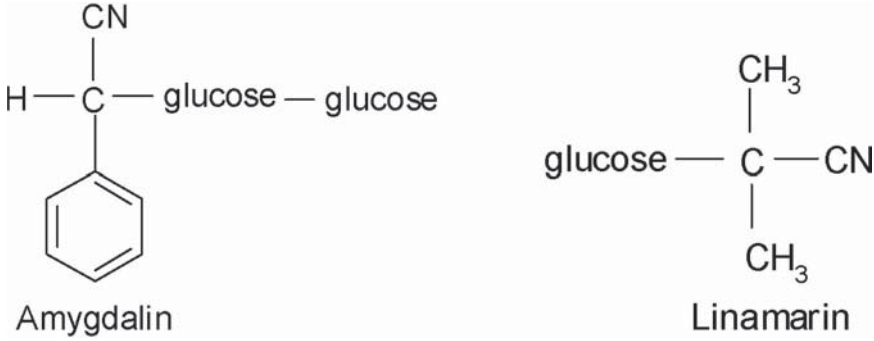

Fig. 2. Structures of amygdalin and linamarin.

Detoxification of small amounts of cyanide occurs mainly in the liver and to some extent in the kidney and thyroid in monogastrics. The enzyme rhodanase, present in animal tissues, detoxifies cyanide by conjugation with sulfur to form thiocyanate. This thiocyanate may cause goiter.

Cyanogens can be removed by drying; for example, hay-making removes cyanogens by volatizing the cyanide released. Soaking and fermentation of cassava and discarding the water containing cyanide makes the cassava safe for human or livestock consumption.

1.2. Present in

Linseed, bitter cassava, sweet potato, yam, maize, sorghum vulgare, bamboo (tip of immature shoots), sugarcane, lima bean, *Phaseolus lunatus*, kernel of almond, lemon, lime, apple, pear, cherry, apricot, prune, plum, white clover, rubber cake, kosum cake, and bamboo leaves.

1.3. Principle of Assays

Two methods are presented in this chapter. In the first method, hydrocyanic acid (HCN), evolved from the sample, reduces sodium picrate to a red-colored compound, in proportion to its amount evolved. The color intensity is measured at 510 nm ***(1–3)***. The second method is based on reaction of the evolved hydrocyanic acid with potassium hydroxide to form potassium cyanide, which then reacts with sodium picrate, forming a red-colored compound. The color intensity is measured at 520 nm ***(4)***.

2. Materials

2.1. Method 1: Based on Reduction of Picrate

1. *Sodium phosphate buffer (0.2 M, pH 5 and pH 8). Stock solution A*: 0.2 M $NaHPO_4$ (27.8 g in 1 L distilled water). *Stock solution B*: 0.2 M $Na_2HPO_4{\cdot}2H_2O$ (35.6 g in 1 L distilled water).

   Take 93 mL of solution A, mix with 6 mL of solution B, adjust the pH to 5, and make the volume up to 200 mL with distilled water. Take 5.3 mL of solution A, mix with 94.7 mL of solution B, adjust the pH to 8, and make the volume up to 200 mL distilled water.
2. *Sodium carbonate (2.5%).* Dissolve 2.5 g Na_2CO_3 in approximately 80 mL distilled water and make the volume up to 100 mL with distilled water.
3. *Picric acid (1.4%) in 2.5% Na_2CO_3.* Dissolve 1.4 g moist picric acid in 100 mL of 2.5% Na_2CO_3. After mixing the solution, prepare 1 : 500 dilutions with distilled water and check the absorbance value at 385 nm (absorbance should be at least 1.0). Note: 1% dry picric acid in 2.5% Na_2CO_3 after dilution (1 : 500) gives an absorbance of 0.999.

4. *Picrate paper.* Dip a sheet of Whatman filter paper in the picric acid solution. Allow the paper to air dry and cut it into 3 cm × 1 cm strips. Paper can be stored for at least 4 weeks at 4°C in a screw-capped bottle.

2.2. Method 2: Based on Formation of Potassium Cyanide and Reaction with Picrate

1. *Potassium hydroxide (2%).* Dissolve 2 g KOH in 100 mL distilled water.
2. *Alkaline picrate solution.* Dissolve 50 g of sodium carbonate and 5 g of picric acid in 1 L of distilled water brought nearer to boiling point to obtain dissolution.

3. Methods

3.1. Method 1: Based on Reduction of Picrate

3.1.1. Preparation of Extract

Weigh 20 to 100 mg of the ground powder or leaf sample into a flat-bottomed screw-capped glass bottle/vial and transfer 1 mL of phosphate buffer (0.2 M, pH 8.0). Insert a picrate paper attached with circular rubber cap and place in a proper position (on the neck of the bottle/vial) so that it just hangs on the empty space above the liquid phase. Close bottle/vial immediately with a screw cap. After incubation for 16 to 24 h at 30°C, take out the picrate paper carefully and immerse in a glass tube containing 5.0 mL distilled water and stir gently for 30 min at room temperature. Take out the colorless filter paper, and place the tube in a boiling water bath for 5 min. Centrifuge the solution at 4000 *g* for 5 min and measure absorbance at 510 nm against the reagent blank.

3.1.2. Preparation of Calibration Curve

Dissolve 241 mg of KCN in 1 L distilled water. This solution is equivalent to 100 µg of HCN/mL. From this solution, take aliquots of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 mL in a screw-capped glass bottle/vial separately and add 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, and 0.0 mL of distilled water, respectively. Then add 4 mL distilled water in each tube, immerse the picrate paper strip, and stir gently for 30 min. Take out the colorless filter paper and place the tube in a boiling water bath for 5 min. Centrifuge the solution at 4000 *g* for 5 min and measure absorbance at 510 nm against the reagent blank. Use this calibration curve for determination of HCN concentration of the sample. The results can be expressed as milligrams of HCN/100 g of sample.

3.2. Method 2: Based on Formation of Potassium Cyanide and Reaction with Picrate

3.2.1. Extraction and Quantification of Total Cyanide

1. Take 4 g of air-dried seed flour/leaf sample in an 800-mL long-necked Kjeldhal flask and add 125 mL of distilled water and 2.5 mL of chloroform.

2. Pass the steam through this suspension to distill the HCN, which is trapped into a 50-mL test tube containing 5 mL of 2% KOH. Keep the delivery end of the condenser below the surface of the KOH solution. Collect approximately 20 mL of distillate and stop the distillation. Make up the volume to 50 mL with distilled water.
3. Pipette a 5-mL aliquot of the well-mixed distillate into a test tube. To this, add 5 mL of the alkaline picrate solution. Mix the contents of the tubes and heat in a boiling water bath for 5 min for color development.
4. Measure the color intensity at 520 nm against the reagent blank.
5. Prepare the blank with 5 mL of distilled water and 5 mL of the alkaline picrate solution.
6. Prepare a standard curve by using potassium cyanide (241 mg of KCN in 1 L distilled water, which gives 100 µg of HCN/mL) in the range of 10 to 100 µg equivalent of HCN.
7. Calculate the total cyanide content of the sample from the calibration curve and express the results as milligrams per 100 g seed flour/leaf sample on a dry matter basis.

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13

Tannins

Key Words: Hydrolyzable tannins; condensed tannins; proanthocyanidins; polyvinylpolypyrrolidone; Folin-Ciocalteu reagent; butanol-HCl reagent; gallic acid; tannic acid; polyethylene glycol; ^{14}C-labeled polyethylene glycol; ferric reagent; protein precipitation assay; biological activity of tannins; in vitro rumen fermentation buffer.

1. Introduction

1.1. Nature, Mechanism of Action, and Biological Effects

Tannins are polyphenolic compounds that are broadly categorized into two major groups: (1) hydrolyzable tannins, consisting of a central core of carbohydrate to which phenolic carboxylic acids are bound by ester linkage **(Fig. 1)**; and (2) condensed tannins, or proanthocyanidins, consisting of oligomers of two or more flavan-3-ols, such as catechin, epicatechin, or the corresponding gallocatechin **(Fig. 2)**. Tannins have a very high affinity for proteins and form protein-tannin complexes. The ingestion of a plant containing condensed tannins decreases nutrient utilization, protein being affected to a great extent, and decreases feed intake. On the other hand, hydrolyzable tannins are potentially toxic to animals. Consumption of feeds containing high levels of hydrolyzable tannins cause liver and kidney toxicity and lead to death of animals. Oak and yellow wood poisonings are attributed to hydrolyzable tannins ***(1)***.

The tannin assays are generally categorized into two groups: chemical methods and protein precipitation methods. Other methods that do not fall under these categories are the gravimetric assays, a tannin bioassay, and ^{14}C-labeled polyethylene glycol binding assay ***(2)***.

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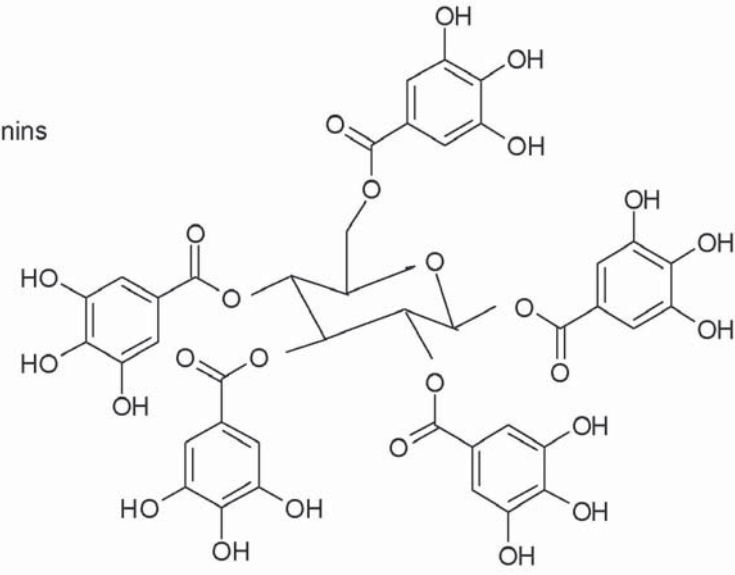
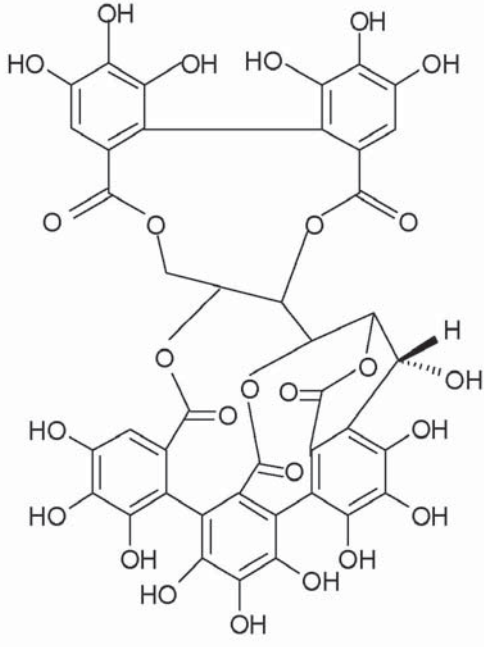
1. Gallotannins



Pentagalloyl glucose (β-1,2,3,4,6-pentagallyol-O-D-glucopranose)

2. Ellagitannins



Castalagin

Fig. 1. Structure of hydrolyzable tannins.

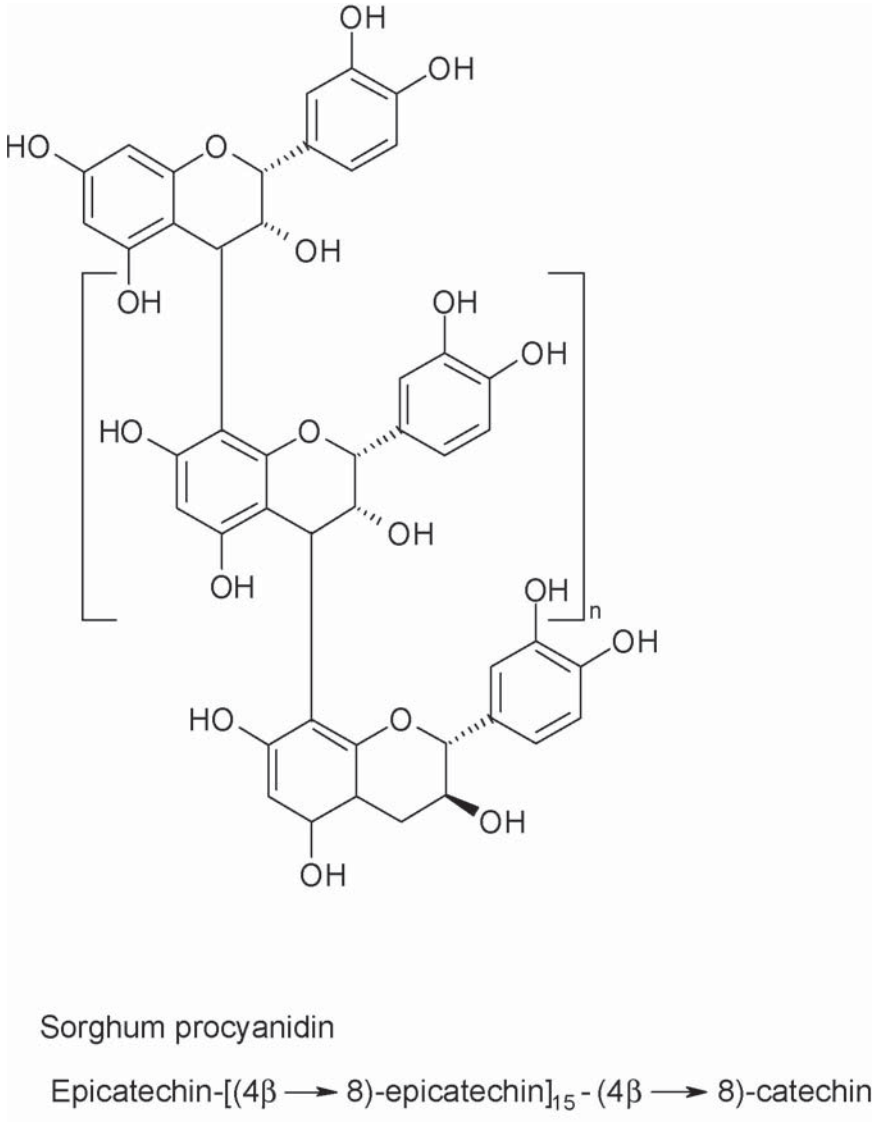


Fig. 2. Structure of hydrolyzable tannins.

1.2. Chemical Assays

The most commonly used procedures in the category of chemical assays are described in the following subsections.

1.2.1. Total Phenol and Total Tannin Assays

These assays are based on the oxidation-reduction principle and use the Folin-Ciocalteu or Folin-Denis reagents. Another method using the ferric chloride reagent is based on the metal-complexing property of phenolics ***(3)***. The Folin-Ciocalteu method is used widely for total phenols because of its high sensitivity and reproducibility. However, the presence of reducing agents interferes in the assay. Nontannin phenolics cannot be distinguished from tannins with both the oxidation-reduction and metal-complexing methods. Tannins are distinguished from nontannins by using a solid matrix, polyvinylpolypyrrolidone (PVPP). A portion of the plant extract is treated with PVPP. This method assumes that the phenolics that bind to proteins are the same as those that bind to PVPP. Total phenols are measured in a plant extract using the Folin-Ciocalteu method before and after treatment with PVPP. Polyvinylpolypyrrolidone has a high affinity for tannins and its removal using centrifugation, following the PVPP treatment, removes tannins from the extract. The difference between total phenol values before and after the PVPP treatment is a measure of tannins ***(4)***.

1.2.2. Vanillin Assay and Butanol-HCl Methods for Condensed Tannins

Vanillin assay is based on the metal-complexing properties of tannins ***(5,6)***, and the butanol-HCl assay with and without addition of iron on the oxidative depolymerization of tannins ***(7)***. The vanillin assay has been widely used for measuring condensed tannins in sorghum and other beans. This assay is not very specific since it measures flavan-3-ols and dihydrochalcones, which are nontannin, in addition to condensed tannins. In addition, in this method monomeric flavans give higher color yield as compared to condensed tannins, and proanthocyanidins based on 5-deoxyflavanols, such as profistinidin in quebracho tannin, do not react; the presence of acetone interferes in this assay, and reproducibility of this method is not good ***(8)***. The acidic butanol method is simple and more specific compared to the vanillin assay. This method yields pink-colored anthocyanidins on oxidative cleavage of the interflavan bonds of condensed tannins in the presence of mineral acids in alcoholic solutions at about 95°C. This method was later modified by inclusion of iron in the butanol-HCl reagent. The addition of iron was considered to enhance the sensitivity and reproducibility of the assay ***(7)***.

1.2.3. Hydrolyzable Tannin Assays

The approach used for the determination of hydrolyzable tannins is based on their conversion to a common unit and the determination of the common units by spectrophotometric or high-performance liquid chromatography (HPLC) methods. These methods are useful for simple hydrolyzable tannins but may provide limited information for complex oligomeric hydrolyzable tannins. The rhodanine method ***(9)*** determines gallotannins as gallic acid equivalent. In this method, gallotannins are hydrolyzed under anaerobic and acidic conditions to gallic acid, which is reacted with rhodanine to give pink chromophore (measured at 520 nm). The specificity and sensitivity of the assays could be increased by measuring gallic acid by the HPLC method ***(2)***. Recently, Hagerman's group ***(10)*** modified the potassium iodate method to include a first step in which all of the hydrolyzable tannins are converted to a single chemical species, methyl gallate. The advantage of this method is that the effect due to the difference in reactivity of the parent compounds is thus eliminated, and results from various laboratories can be compared directly since they are measured in terms of methyl gallate, which is commercially available.

1.3. Protein Precipitation Assays

The methods for quantification of tannins based on their property of complexing with proteins (protein precipitation assays) are considered to provide better information on the biological value of feeds and fodders containing tannins. In these methods, protein-tannin complexes are prepared. The protein in these complexes is measured using the ninhydrin assay of amino acids released by alkaline hydrolysis of the complex ***(11,12)***, which represents protein precipitation capacity; and when phenolics in the complex are determined by the ferric chloride method ***(3)*** the protein precipitable phenolics are measured. The method based on binding of ^{125}I-labeled bovine serum albumin ***(13)*** for determination of the protein precipitation capacity of tannins is accurate and sensitive. Recently, this method has been simplified ***(14)***. However, it requires special equipment and some degree of expertise.

In another method ***(15)***, the tannin-protein complexes are formed on a chromatography paper and reacted with the protein bovine serum albumin (BSA). Unbound BSA is washed off, and the protein in the tannin-protein complex is stained with Ponceau S, a dye specific for proteins. Protein-bound dye is eluted and the absorbance of the eluate is measured at 525 nm. The absorbance is converted to protein by using a calibration curve. This method is about 20 times more sensitive than the other methods ***(11,12)***. The protein bound to tannins can also be measured using amido black dye, which can be eluted and measured or can be measured using an image analyser ***(16)***. Another

advantage of these methods ***(14–16)*** is that acetone containing plant extracts can be used, whereas the presence of acetone interferes in other protein precipitation assays ***(3,11–13)***. The preparation of plant extract in aqueous acetone (generally 70%) is desirable because the solubility of tannins and phenolics is higher in aqueous acetone solution, and acetone prevents oxidation of phenols.

Another protein precipitation method that is insensitive to acetone is the radial diffusion assay ***(17)***. The tannin-protein complex is formed in the gel containing BSA, which appears as a ring. The diameter of the ring is a measure of protein precipitation/binding capacity of tannins.

1.4. Gravimetric Methods

The spectrophotometric methods determine tannins relative to one or another standard, namely tannic acid, gallic acid, catechin, quebracho tannins, leukoanthocyanins, etc. The gravimetric method ***(18)*** based on the precipitation of phenolics by ytterbium acetate measures total phenolics and not tannins. This method is not specific for phenolics. It precipitates other moieties in addition to phenolics. In addition, the precipitation is not complete at low phenolic concentration, and some phenolics, for example, rutin, is not precipitated ***(19)***, leading to underestimation of phenolics. Another gravimetric method ***(4)*** is based on weighing the tannin extract before and after removal of the tannin by treatment with PVPP to bind tannin and removal of the PVPP-tannin complexes by centrifugation. Gravimetric methods have generally lower sensitivities than colorimetric methods and are time-consuming. The gravimetric method ***(4)*** also suffers from these disadvantages. So it should be used when the dry matter digestibility of tannin-rich feeds needs to be corrected for the presence of tannins. For other routine applications the principle of the gravimetric method is used in conjunction with a spectrophotometric method. Total phenols are determined spectrophotometrically using the Folin-Ciocalteu reagent in the extract before and after the PVPP treatment. The difference in these phenolic values is a measure of tannins. This difference (tannins) when expressed as tannic acid equivalent is quite close to the tannin levels determined gravimetrically in leaves from various trees and browses; each gram of tannins (by mass) had a reducing power equivalent to 0.76 to 1.25 g tannic acid ***(4)***.

1.5. Tannin Bioassay

All available protein precipitation assays measure tannins under conditions different from those of the rumen, and therefore the results obtained have limited applicability for predicting the nutritive value of tannin-containing feedstuffs. Polyethylene glycol and polyvinylpyrrolidone bind to tannins and make them inert ***(20)***. This property has been exploited together with the in

vitro gas method to quantify the effects of tannins on rumen fermentation. Incubation of polyethylene glycol 6000 along with a tannin-containing feedstuff in the in vitro system increases gas production. The percentage increase in gas production, for example at 24 h of incubation, represents the effects of tannins. The higher the percentage increase in gas production, the greater is the effect ***(20)***.

1.6. ^{14}C-Labeled Polyethylene Glycol Binding Assay

Polyethylene glycols (PEGs) of molecular weights 4000 or 6000 have a very high affinity for both hydrolyzable and condensed tannins over a wide range of pH ***(20)***. In this method the feed samples are kept in contact with PEG spiked with ^{14}C-labeled PEG, and the radioactivity bound to feed sample is a measure of tannins; the higher the activity, the higher the tannins. The method is reportedly useful since there is no need to extract tannins, and it is considered to be a measure of both bound and extractable tannins ***(21,22)***. The formation of soluble tannin-PEG complexes, which is not recovered in the feed sample, can underestimate the tannin values. There is a need to study the extent of formation of soluble versus precipitable PEG-tannin complexes under the conditions of the assay (and to study the nutritional significance of these soluble complexes); to standardize the assay for parameters such as optimum particle size of the sample, temperature, and treatment time; and to investigate the extent of nonspecific binding of ^{14}C-PEG. Additionally, correlations should be established between the PEG-binding assay and protein precipitation assays or the tannin bioassay using the in vitro gas method or the increase in the nitrogen degradability of feeds on addition of PEG. Inclusion of in vivo parameters such as intake, nitrogen balance, and degradability, production parameters (growth, wool production, etc.) for these feeds in the correlation studies, would reveal the usefulness of the in vitro methods in predicting nutritional and physiological effects of feeding tannin-containing diets to ruminants.

An increase in nitrogen degradability of a feed when incubated in an in vitro rumen fermentation system in the presence of PEG is also a measure of tannin activity, and this increase in nitrogen degradability has also been found to predict the effects of tannins in ruminants ***(23,24)***.

1.7. Near-Infrared–Based Method

Tannins in legume forages were quantified using near-infrared reflectance spectroscopy. The wavelength, 2.150 μm, was identified for prediction of condensed tannins ***(25)***. This method may be applicable to the determination of tannins in large sample sets of homogeneous feeds such as forage legumes.

Among the above methods, only three methods are presented here:

1. Total phenolic and tannin assays
2. Condensed tannin assay
3. Biological activity of tannins

For other methods, see *A Laboratory Manual on Tannin Assays* (Makkar, 2003).

2. Materials

2.1. Measurement of Total Phenolics and Tannins Using the Folin-Ciocalteu Method, Based on Makkar et al. (4)

1. *Folin-Ciocalteu reagent (1 N).* Dilute commercially available Folin-Ciocalteu reagent (2 N) with an equal volume of distilled water. Transfer it into a brown bottle and store in a refrigerator (4°C). It must be golden-yellow. Do not use it if it turns olive green or green.
2. *Sodium carbonate (20%).* Weigh 40 g sodium carbonate.10 H_2O, dissolve it in about 150 mL distilled water, and make the volume up to 200 mL with distilled water.
3. *Insoluble polyvinylpyrrolidone (polyvinylpolypyrrolidone, PVPP).* This is commercially available from Sigma, St. Louis, MO (P 6755).
4. *Standard tannic acid solution (0.1 mg/mL).* Dissolve 25 mg tannic acid obtained from Merck in 25 mL distilled water and then dilute 1 : 10 in distilled water (always use a freshly prepared solution).

2.2. Measurement of Condensed Tannins (Proanthocyanidins, Based on Porter et al. (7)

1. *Butanol-HCl reagent (butanol-HCl 95 : 5 v/v).* Mix 950 mL *n*-butanol with 50 mL concentrated HCl (37% w/v).
2. *Ferric reagent (2% ferric ammonium sulfate in 2 N HCl).* To prepare 2 N HCl, take 16.6 mL of concentrated HCl and make the volume up to 100 mL with distilled water. Dissolve 2.0 g ferric ammonium sulfate in 100 mL of 2 N HCl. This reagent should be stored in a dark-brown bottle.

2.3. Determination of Biological Activity of Tannins, Based on Makkar et al. (20)

1. *Bicarbonate buffer solution:* Dissolve 35 g sodium bicarbonate ($NaHCO_3$) and 4 g ammonium carbonate (NH_4HCO_3) in approximately 500 mL distilled water and then make the volume up to 1 L with distilled water.
2. *Macromineral solution:* Dissolve 6.2 g potassium dihydrogen phosphate (KH_2PO_4), 5.7 g disodium hydrogen phosphate (Na_2HPO_4), and 0.6 g magnesium sulfate ($MgSO_4 \cdot 7H_2O$) in approximately 500 mL distilled water and then make the volume up to 1 L with distilled water.
3. *Micromineral solution:* Dissolve 10 g manganese chloride ($MnCl_2 \cdot 4H_2O$), 13.2 g calcium chloride ($CaCl_2 \cdot 2H_2O$), 1 g cobalt chloride ($CoCl_2 \cdot 6H_2O$), 8 g ferric

chloride ($FeCl_3 \cdot 6H_2O$) in approximately 50 mL distilled water and then make the volume up to 100 mL with distilled water.
4. *Resazurin:* Dissolve 0.1 g resazurin in 100 mL distilled water.
5. *Reducing solution:* Dissolve 996 mg sodium sulfide ($Na_2S \cdot 9H_2O$) in 94 mL distilled water and then add 6 mL of 1 N sodium hydroxide solution (dissolve 4 g sodium hydroxide in 100 mL distilled water for 1 N sodium hydroxide).

3. Methods

3.1. Measurement of Total Phenolics and Tannins Using Folin-Ciocalteu Method, Based on Makkar et al. (4)

3.1.1. Extraction of Phenolics (Simple Phenolics and Tannins)

Dried and finely ground (ground preferably using a ball mill) plant sample (leaves, seeds, root, stem, etc.) is placed in a glass beaker of approximately 25-mL capacity (*see* **Note 1**); 10 mL of aqueous acetone (70%) is added and the beaker is suspended in an ultrasonic water bath and subjected to ultrasonic treatment at 300 W for 20 min at room temperature (*see* **Notes 2** and **3**). The content of the beaker is then transferred to centrifuge tubes and centrifuged for 10 min at approximately 3000 *g* at 4°C (if refrigerated centrifuge is not available, cool the contents by keeping the centrifuge tube on ice and then centrifuge at 3000 *g* using an ordinary clinical centrifuge). Collect the supernatant and keep it on ice (*see* **Notes 4** and **5**).

3.1.2. Preparation of Calibration Curve

Take 0.0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µL of standard tannic acid solution in 5-mL glass test tubes separately. Then add an adequate quantity of distilled water for respective test tubes to bring the volume to 500 µL. To this solution, add 250 µL of the Folin-Ciocalteu reagent (1 N) and 1.25 mL of the sodium carbonate solution (final volume will be 2.0 mL; tannic acid concentration will be 0.0 to 10 µg). Vortex the contents and incubate at room temperature under dark condition. After 40 min read absorbance at 725 nm against the reagent blank.

3.1.3. Analysis of Total Phenols

Take suitable aliquots of the above sample extract in test tubes and make the volume to 500 µL with distilled water. Add to it 250 µL of the Folin-Ciocalteu reagent (1 N) and then 1.25 mL of the sodium carbonate solution. Vortex the tubes and record absorbance at 725 nm after incubation for 40 min under dark conditions. Calculate the amount of total phenols as tannic acid equivalent from the above calibration curve. Express the results as total phenolics as grams per 100 g on a dry matter basis.

3.1.4. Determination of Total Tannins

Weigh 100 mg PVPP in a 100 × 12 mm test tube. Add 1.0 mL distilled water and then 1.0 mL of the sample extract (100 mg PVPP is sufficient to bind 2 mg of total phenols; if total phenolic content of feed is more than 10% on a dry matter basis, dilute the extract). Vortex and keep the tube at 4°C for 15 min, vortex it again, and centrifuge at 3000 *g* for 10 min. Collect the supernatant. This supernatant has only simple phenolics other than tannins (the tannins get bound to PVPP). Measure the phenolic content of the supernatant as mentioned above and express the content of nontannin phenolics on a dry matter basis. From the above results, the tannin content of the sample can be calculated as follows:

$$\text{Total phenolics (\%)} - \text{Nontannin phenolics (\%)} = \text{Tannin (\%)}$$

The results can be expressed as tannic acid equivalent on a dry matter basis.

3.2. Measurement of Condensed Tannins (Proanthocyanidins), Based on Porter et al. (7)

3.2.1. Determination of Condensed Tannins

In a 100 mm × 12 mm glass test tube, take a suitable aliquot (0.1 to 0.5 mL) of the sample extract (for preparation of the extract see **Section 3.1.1**; also *see* **Note 6**) and dilute it with 70% aqueous acetone to 0.5 mL (the aliquot volume depends on the amount of condensed tannins in the extract). To the tubes add 3.0 mL of the butanol-HCl reagent and 0.1 mL of the ferric reagent. Vortex the tubes and cover the mouth of each tube with a glass marble and transfer the tubes in a heating block adjusted at 97° to 100°C or in a boiling water bath for 60 min. After cooling the tubes, record absorbance at 550 nm. Subtract absorbance of a suitable blank, which is usually the unheated mixture. If the extract has flavan-3,4-diols or flavan 4-ol, a pink color develops without heating. If this happens, use one heated blank for each sample, composed of 0.5 mL extract, 3 mL *n*-butanol (no HCl), and 0.1 mL ferric reagent. Condensed tannins (% in dry matter) as leukocyanidin equivalent is calculated by the following formula:

$$(\text{Absorbance at 550 nm} \times 78.26 \times \text{Dilution factor})/(\text{\% Dry matter})$$

This formula takes the effective $E^{1\%,1cm,550nm}$ of leukocyanidin to be 460. The dilution factor is equal to 1 if 0.5 mL of the sample extract is taken before addition of 3 mL of the butanol-HCl reagent and the extract was made from a 200-mg sample in 10 mL 70% aqueous acetone (the dilution factor would be 5 if, for example, 0.1 mL of the sample extract is taken and to it is added 0.4 mL of 70% aqueous acetone before the addition of 3 mL of the butanol-HCl

reagent; the amount of sample extract depends on the amount of condensed tannins in the extract; if by taking 0.5 mL of the sample extract the absorbance is greater than 0.6, take the lower amount of the sample extract and dilute to 0.5 mL with 70% aqueous acetone). *See* **Note 7**.

3.3. Determination of Biological Activity of Tannins, Based on Makkar et al. (20)

3.3.1. Sample Preparation

Dried leaves should be passed through a 1-mm sieve.

3.3.2. Weighing of Samples and Preparation of Syringes

Tare a specially made scoop (approximately 4 cm in length and 1 cm in depth/radius; a standard sodium hydroxide–containing plastic container can be cut horizontally in half to form the scoop) on an analytical balance. Weigh a 500-mg feed sample (passed through a 1-mm sieve) in the scoop and then insert a 5-mL-capacity pipette or a glass rod into the narrow end of the scoop and transfer the sample from the scoop into 100-mL calibrated glass syringes. Weigh 1 g tannin-complexing agent, polyethylene glycol, PEG (molecular weight 4000 or 6000) on the scoop and transfer it also into syringes similar to those for the feed samples. The feedstuffs with and without the tannin-complexing agent are incubated at least in triplicate.

3.3.3. Preparation of In Vitro Rumen Fermentation Buffer Solution

Collect the rumen fluid and particulate matter before the morning feed from two cattle fed on a roughage diet, homogenize, strain, and filter through four layers of cheese cloth. Keep all glassware at approximately 39°C and flush these with carbon dioxide before use. Carbon dioxide is heavier than air and hence it remains in the glassware for a reasonable period, provided the container is not inverted upside down. The strained rumen fluid is kept at 39°C under carbon dioxide and should be prepared just before the start of the incubation. As the amount of feed taken is 500 mg, the composition of the medium is based on Tilley and Terry ***(26)***. Menke et al. ***(27)*** reduced the rumen buffer volume per syringe by half as they used 200 mg of the substrate because of the limited volume of the syringes and the inconvenience of emptying the syringes. Here, besides recording the gas volume, the fermented material is taken for various analyses and hence the amount of substrate taken is 500 mg. There is an inherent error associated with gravimetric determination of the fermented residue, which is large if 200 mg feed is taken in place of 500 mg.

Medium composition and incubation procedure (following volume in milliliters)	
Rumen buffer solution	630.00
Macromineral solution	315.00
Micromineral solution	0.16
Resazurin	1.60
Distilled water	975.00
Freshly prepared reducing solution	60.00
The rumen fluid (see above for collection and preparation)	660.00

The above volume is sufficient for 60 syringes (40 mL/syringe) plus 10% extra.

Except for the rumen fluid and the reducing solution, mix all the above-mentioned solutions in the order listed above in a 3- or 5-L capacity glass container.

3.3.4. Incubation and Determination of Gas Released

The container holding the above solutions is kept in a water bath adjusted at 39°C. This water bath is a plastic rectangular container (400 cm × 300 cm × 200 cm) filled with water, the temperature of which is adjusted at 39°C using a portable thermostat suspended from the top of the plastic container in water. This plastic water bath is kept on a magnetic stirrer. The contents are flushed with carbon dioxide and kept stirred using a magnetic stirrer. After about 5 min, add the reducing solution and keep the mixture stirring and flushing with carbon dioxide at 39°C. When the mixture has been reduced (blue color of the dye changes to pink and then to colorless; it takes about 15 to 20 min for the reduction process to complete, and during this time we generally homogenized and strained the rumen liquor and the particulate material collected from cattle), add 660 mL of the rumen fluid. Keep this mixture stirring and flushing with carbon dioxide for another 10 min. Transfer a portion (40 mL) of the rumen-fluid medium into each syringe using a dispenser, and incubate in a water bath at 39°C. Filling 60 syringes, after some practice, should take 30 to 35 min. After completion of the filling-up process, shake the syringes well and then transfer them to the water bath. Shake all the syringes every hour for the first 4 hours and then every 2 hours. Generally, the incubation is started at about 7.30 a.m. and after 12 h of the incubation, the syringes are not shaken until the termination of the incubation (16 h and/or 24 h). The gas volume is recorded after 2, 4, 6, 8, 10, 12, and 16 or 24 h. The net gas production is calculated by subtracting values for the blank. The blanks (at least three in number) contain only the

rumen-fluid medium and no feed material. The addition of PEG to blanks does not affect the gas production from blanks, suggesting that it is inert.

3.3.5. Determination of Biological Activity of Tannins

The difference between the net volume of gas produced from syringes with and without PEG, generally at 16 h and 24 h, is measured, which is a measure of tannin effect. The PEG binds tannins and inactivates them. The higher the percent increase in gas (without PEG being 100%) on addition of PEG, the higher the biological activity of tannins with regard to rumen microbes ***(20)***.

4. Notes

1. Pigments and fat can be removed from the dried leaf sample by extracting with diethyl ether containing 1% acetic acid before extracting phenolics.
2. A first extraction with 50% aqueous methanol followed by a second extraction with 70% aqueous acetone can also be used for extraction of phenolics.
3. A very long period of phenolic extraction and extraction at high temperature may lead to inactivation of phenolics.
4. Freshly prepared extract should be used for phenolic and tannin analysis.
5. Tubes/containers containing the extract should be kept under cold conditions until the analysis is complete.
6. The presence of pigments may interfere with this method. The pigments can be removed by extracting the dried leaves with petroleum ether containing 1% acetic acid. Ascorbic acid (generally added to prevent oxidation of phenolics) does not interfere in the condensed tannin assay and can be added while preparation of the plant extract (ascorbic acid interferes in the phenolic assay by Folin-Ciocalteu method).
7. Vanillin-HCl ***(5,6)*** is also used for determination of condensed tannins but this method is not specific ***(8,28)***. It measures condensed tannins as well as simple flavonoids. This method also has several other disadvantages.

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14

Gossypol

Key Words: Gossypol; free gossypol; cottonseed; antioxidant; constipation; depressed appetite; aniline; *Gossypium* spp.; thiourea.

1. Introduction

1.1. Nature and Biological Effects

Gossypol is a polyphenolic aldehyde **(Fig. 1)** that is an antioxidant and polymerization inhibitor. It is toxic to monogastric animals; pigs and rabbits are the most sensitive, whereas poultry are relatively more tolerant. The general symptoms of gossypol toxicity are constipation, depressed appetite, loss of weight, and death, which usually results from circulatory failure. Although acute toxicity is low, ingestion of small amounts over a prolonged period can be lethal. It is important to distinguish between free (soluble in 70–30 v/v aqueous acetone) and bound gossypol since only the former is considered to be physiologically active.

1.2. Present in

Gossypium spp.

1.3. Principle of Assays

This chapter describes methods for determination of total and free gossypol. Total gossypol includes gossypol and gossypol derivatives, both free and bound. These react with 3-amino-1-propanol in dimethylformamide solution to form a diaminopropanol complex, which then reacts with aniline, giving dianilinogossypol. Gossypol analogues and gossypol derivatives having an available aldehyde moiety are measured by the method ***(1)***. This method is applicable to

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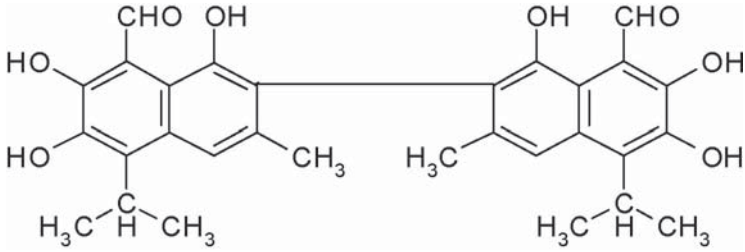

Fig. 1. Structure of gossypol.

decorticated, glanded and glandless cottonseed, cottonseed flour, cooked cottonseed meals, cottonseed press cake, cottonseed meal, and crude cottonseed oil.

The term *free gossypol* defines gossypol and gossypol derivatives in cottonseed products that are soluble in 70% aqueous acetone under the conditions of the method. The gossypol and gossypol derivatives react with thiourea and aniline in acidic conditions to give yellowish orange complex, which is measured at 440 nm ***(2)***. The method is applicable for cottonseed, cottonseed meals, cottonseed slab, and sized cake (smaller cake). The applicability of the method for chemically treated meals should be verified before use.

2. Materials

2.1. Determination of Total Gossypol

1. *Aniline.* Distil reagent grade aniline over a small amount of zinc dust (place 1 to 2 g of zinc dust in a round bottom flask or distillation flask containing aniline) using a water-cooled condenser. Discard the first and last 10% of the distillate. Store the distillate in a glass-stoppered brown bottle in a refrigerator.
2. *Isopropyl alcohol-hexane mixture.* Isopropyl alcohol and *n*-hexane (60 : 40 v/v).
3. *Complexing reagent.* Pipette 2 mL of 3-amino-1-propanol and 10 mL glacial acetic acid into a 100-mL volumetric flask. Cool the mixture to room temperature, and make the volume up to 100 mL with dimethylformamide. Prepare reagent weekly and store in refrigerator when not in use.
4. *Gossypol or gossypol acetic acid (for example, from Sigma, St. Louis, MO).* Gossypol and gossypol acetic acid standards are commercially available. Gossypol acetic acid contains 89.62% gossypol by weight, or 0.8962 mg gossypol per milligram of gossypol acetic acid.
5. *Standard gossypol solution.* Weigh 25 mg of gossypol or 27.9 mg of gossypol acetic acid into a 50-mL volumetric flask. Dissolve in, and make the volume up to 50 mL with, the complexing reagent. The solution is stable up to 1 week if stored in a refrigerator. If exact amounts of either gossypol or gossypol acetic acid were weighed as indicated, either solution contains 0.50 mg gossypol per

milliliter. Multiply the milligrams of gossypol acetic acid by 0.8962 to obtain milligrams of gossypol.

2.2. Determination of Free Gossypol

1. *Aqueous acetone (70%)*. Mix 700 mL acetone and 300 mL of distilled water.
2. *Aqueous isopropyl alcohol (80%)*. Mix 800 mL isopropyl alcohol and 200 mL of distilled water.
3. *Thiourea solution*. Dissolve 10 g of reagent grade thiourea in approximately 60 mL distilled water and dilute to 100 mL with distilled water.
4. *Hydrochloric acid (1.2 N)*. Dilute 106 mL of concentrated hydrochloric acid (37% w/v) to 1 L with distilled water.
5. *Aniline*. Distill reagent grated aniline as described above.
6. *Standard gossypol solution*. Weigh accurately 25 mg of gossypol or 27.9 mg of primary standard gossypol acetic acid into a 250-mL volumetric flask. Transfer approximately 100 mL of 70% acetone to the flask. Add 1.0 mL glacial acetic acid and 75 mL distilled water and dilute to 250 mL with 70% acetone. Mix these solutions well. Pipette 50 mL of the above solution into a 200-mL volumetric flask, add 100 mL 70% acetone and 60 mL distilled water, dilute to 200 mL with 70% acetone, and mix well. This standard gossypol solution contains 0.025 mg of gossypol per milliliter. It is stable for 24 h when protected from the light.

3. Methods

3.1. Determination of Total Gossypol

3.1.1. Preparation of Sample

De-hull about 50 g sample (seeds) and grind in a Wiley mill using a 2-mm sieve. Avoid overheating of the sample.

3.1.2. Determination of Total Gossypol

1. Weigh 1 or 2 g of de-hulled sample (or meal) and transfer to a 50-mL volumetric flask (*see* **Note 1**). Add 10 mL of the complexing reagent along the walls of the flask and take down the sample adhering to neck of flask. A rapid delivery pipette may be used.
2. Prepare reagent blank containing 10 mL of the complexing reagent in a 50-mL volumetric flask. Heat sample and blank in a water bath (95–100°C) for 30 min. Cool them to room temperature and make the volume up to 50 mL with the isopropyl alcohol-hexane mixture and mix well (*see* **Note 2**).
3. Filter sample extract through a medium retention paper. Discard first 5 mL of the filtrate and collect the rest.
4. Pipette duplicate aliquots of the filtrate (2 mL each) into 25-mL volumetric flasks. Also pipette duplicate aliquots (2 mL) of the reagent blank into 25-mL volumetric flasks.

5. Dilute one set of the sample and blank aliquots to 25 mL with the isopropyl alcohol-hexane mixture, and reserve as reference solutions for absorbance measurement.
6. Add 2 mL aniline to the other set of the sample and reagent blank aliquots and heat in a water bath (95–100°C) for 30 min. Cool to room temperature, make the volume up to 25 mL with the isopropyl alcohol-hexane mixture, and mix well. Allow to stand for 1 h at room temperature after dilution (*see* **Note 3**).
7. Using a spectrophotometer measure absorbance at 440 nm of the reagent blank reacted with aniline against the blank aliquot without aniline (absorbance A).
8. Determine absorbance of the sample aliquot reacted with aniline against the sample aliquot without aniline (absorbance B). Subtract the absorbance of the reagent blank (A) from that of sample aliquot (B) to obtain the corrected absorbance. From the corrected absorbance of the sample, determine gossypol content using the calibration curve.

3.1.3. Preparation of Calibration Curve

1. Pipette in duplicate 1-, 2-, 3-, 4-, 6-, 8-, and 10-mL aliquots of the standard gossypol solution in 50-mL volumetric flasks. Add the complexing reagent to make the volume up to 10 mL. Use 10 mL of the complexing reagent as a blank.
2. Heat flasks in a water bath (95–100°C) for 30 min, cool to room temperature, make the volume up to 50 mL with the isopropyl alcohol-hexane solution, and mix well.
3. Pipette duplicate 2-mL aliquots of each standard and the reagent blank into 25-mL volumetric flasks.
4. Dilute one set of the standard aliquots and reagent blank to 25 mL with the isopropyl alcohol-hexane solution and reserve as reference solutions for absorbance measurements.
5. Add 2 mL aniline to the other set of the standard aliquots and the blank, heat in water bath (95–100°C) for 30 min, cool to room temperature, dilute to make volume 25 mL with the isopropyl alcohol-hexane solution, and mix well. Allow to stand for 1 h at room temperature before determining absorbance (*see* **Note 3**).
6. Using a spectrophotometer measure absorbance at 440 nm of the reagent blank reacted with aniline against the blank aliquot without aniline (absorbance Ac).
7. Determine absorbance of the sample aliquot reacted with aniline against the sample aliquot without aniline (absorbance Bc). Subtract the absorbance of the reagent blank (Ac) from that of sample aliquot (Bc) to obtain corrected absorbance.
8. Draw a calibration curve using different concentrations of gossypol taken and their absorbances recorded.

3.2. Determination of Free Gossypol

3.2.1. Preparation of Extract and Determination of Free Gossypol

1. De-hull 50 g of the sample and remove the seed kernel from the hulls and lint. Grind the kernel in a Wiley mill to pass through a 2-mm screen. Do not preheat cottonseed and avoid heating during grinding.

2. Weigh (1–2 g) of the ground sample into a 250-mL Erlenmeyer flask and place five or six glass beads on the bottom of the flask.
3. Add 50 mL of 70% aqueous acetone, stopper the flask with a leak proof glass stopper or polyethylene stopper, and shake vigorously on a mechanical shaker for 1 h.
4. Filter through a medium retentive filter paper (for example, Whatman No. 2). Discard the first 5 mL of filtrate, and collect the rest of the filtrate in a small flask. Place a watch glass over the funnel to reduce evaporation during filtration.
5. Pipette appropriate duplicate aliquots of the filtrate (2–10 mL) into 25-mL volumetric flasks.
6. To one sample aliquot, designated as solution A, add two drops of 10% aqueous thiourea, one drop of 1.2 N HCl, and make the volume up to 25 mL with 80% aqueous isopropyl alcohol.
7. To the other sample aliquot, designated as solution B, add two drops of 10% aqueous thiourea, one drop of 1.2 N HCl, and 2 mL of redistilled aniline. A rapid delivery pipette may be used for dispensing aniline.
8. Prepare a reagent blank containing a volume of 70% aqueous acetone solution, equal to that of the sample aliquot, and add two drops of 10% aqueous thiourea (do not add any 1.2 N HCl), and 2 mL of aniline.
9. Heat the sample aliquot (at serial number 7) and the reagent blank (at serial number 8) in a boiling water bath (100°C) for 30 min.
10. Remove the solutions from the bath, add 10 mL of 80% aqueous isopropyl alcohol, and cool to room temperature in a water bath. Make the volume up to 20 mL with 80% aqueous isopropyl alcohol.
11. Determine the absorbance of sample aliquot A (at serial number 6) at 440 nm using the aqueous isopropyl alcohol to set the instrument at 0 absorbance (*see* **Note 3**).
12. With the instrument set at 0 absorbance with the aqueous isopropyl alcohol, determine the absorbance of the reagent blank (at serial numbers 8 and 9). If the reagent blank exceeds 0.022 absorbance, the analysis must be repeated using freshly distilled aniline.
13. Determine the absorbance of sample aliquot B (at serial numbers 7 and 9) at 440 nm using the reagent blank (at serial numbers 8 and 9) to set the instrument at 0 absorbance.
14. Calculate the corrected absorbance of the sample aliquot as follows: Corrected absorbance = [Absorbance solution B − Absorbance solution A].
15. From the corrected absorbance of the sample aliquot (at serial number 14), determine the gossypol content in the sample aliquot by reference to a calibration graph prepared using the following procedure.

3.2.2. Preparation of Calibration Curve

1. Pipette in duplicate 1-, 2-, 3-, 4-, 5-, 7-, 8-, and 10-mL aliquots of the standard gossypol solution (0.025 mg/mL) into 25-mL volumetric flasks.

2. To one set of aliquots, designated as A, add two drops of 10% aqueous thiourea, one drop of 1.2 N HCl, and make the volume up to 25 mL with the aqueous isopropyl alcohol.
3. Determine the absorbance as outlined in **Section 3.2.1**.
4. To the other set of standard gossypol aliquots, designated as B, add two drops of 10% aqueous thiourea, two drops of 1.2 N HCl, and 2 mL of redistilled aniline. Prepare a reagent blank containing 10 mL of aqueous acetone, two drops of 10% aqueous thiourea, and 2 mL of aniline (do not add any 1.2 N HCl to the reagent blank).
5. Heat the standards and the reagent blank in a boiling water bath (100°C) for 30 min, cool to room temperature, and make the volume up to 25 mL with the aqueous isopropyl alcohol.
6. Determine the absorbance as directed in **Section 3.2.1**. at serial numbers 12 and 13.
7. Calculate the corrected absorbance for each standard gossypol aliquot as follows: Corrected absorbance = [Absorbance solution B − Absorbance solution A].
8. Plot this corrected absorbance of each gossypol standard against the corresponding milligrams of gossypol in the 25 mL volume, to obtain the calibration graph.

4. Notes

1. This method may not be applicable to feeds containing whole, unprocessed cottonseed. Components such as minerals, protein, oil, and polysaccharides in the feed interfere with this method and may give false-positive results.
2. Extracts of sample treated with the complexing reagent are exceptionally stable, and if necessary, can be stored under refrigeration for several days before conducting the aniline spectrophotometer reaction.
3. After the aniline reaction, a slight increase in absorbance is observed up to 1 h, and the color is stable for 1 to 5 h. The absorption maxima of the gossypol-aniline reaction product should be at 440 nm. However, depending on the wavelength accuracy of the spectrophotometer and the band isolated, the maxima may be in the range of 440 to 450 nm. All absorbance measurements should be taken at the actual maxima for the spectrophotometer used.

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15

Chlorogenic Acid

Key Words: Chlorogenic acid; antioxidant; carcinogenic inhibitor; sunflower; titanium reagent.

1. Introduction

1.1. Nature and Biological Effects

Chlorogenic acid is a phenolic compound **(Fig. 1)**. It is an antioxidant and a carcinogenic inhibitor; however, its presence in oilseeds and grains poses nutritional problems. Chlorogenic acid found in the range of 2 to 4 g/100 g of defatted sunflower meal is a matter of concern. Inclusion of high levels of sunflower and rapeseed meal in animal diets decreases the hydrolytic enzymes and interferes with the utilization of various nutrients including protein, amino acids, and minerals. Breeding for a low-chlorogenic-acid–containing sunflower and developing the technology to remove chlorogenic acid from the defatted meal are major areas of sunflower research today.

1.2. Present in

Coffee beans, sunflower seeds, and rapeseed.

1.3. Principle of Assay

Chlorogenic acid is extracted with alcohol. Alcohol is removed using vacuum drying, and the dried material is dissolved in acetone. It is reacted with titanium ion to form a colored complex, which is measured at 450 nm ***(1)*** (*see* **Note 1**).

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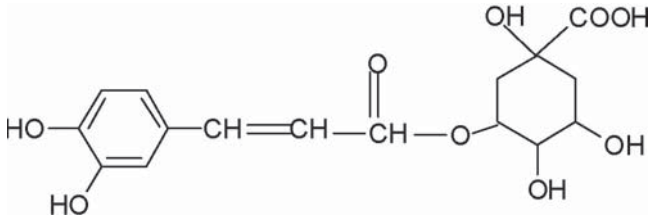

Fig. 1. Structure of chlorogenic acid.

2. Materials

1. *Titanium reagent*. Weigh 20 g titanium chloride ($TiCl_4$) and dissolve in approximately 85 mL of concentrated HCl (37% w/v), and make the volume up to 100 mL with the concentrated HCl.
2. *HCl (2.5 N)*. Take 100 mL concentrated HCl (37% w/v) and make the volume up to 360 mL with distilled water.

3. Methods

3.1. Determination of Chlorogenic Acid

Reflux twice a known quantity of defatted sunflower meal in 80% aqueous ethanol (adjusted to pH 4.0 with 2.5 N HCl) for 30 min (125 mL to 1 g meal). Filter and collect 250 mL of the extract. Then remove 0.5 mL of the extract and dry in a vacuum oven at 50°C (can take 2 h). It can be dried by flushing with nitrogen gas, which takes 10 to 15 min. Dissolve the dried extract in 9.5 mL of acetone and to this add 0.5 mL of the titanium reagent. After incubating for 5 min at room temperature, mix the solution thoroughly and read the color intensity at 450 nm against the reagent blank (acetone plus titanium reagent; 9.5 and 0.5 mL, respectively). Prepare a calibration curve and determine chlorogenic acid content in the sample using the calibration curve. Express chlorogenic acid content as grams per 100 g sample.

3.2. Calibration Curve

Prepare a series of standard solutions of chlorogenic acid (0–200 µg/mL) in 10 mL of acetone in 15-mL screw-top glass tubes. Add to each tube 0.5 mL of the titanium reagent and thoroughly mix the solution using a vortex mixture. A colored complex between the chlorogenic acid and titanium is formed immediately. Measure the absorbance at 450 nm in a spectrophotometer against an equivalent blank.

4. Note

1. The titanium chloride method can be followed for other phenolic compounds too. But the wavelength of maximum absorbance differs. Total phenols can be measured at 410 nm, and catechol and catechin at 430 nm.

Reference

1. Eskin, M. M. A., Hochn, E., and Frenkel, C. (1978) A simple and rapid quantitative method for total phenols. *J. Agric. Food Chem.* **26**, 973–975.

16

Saponins

Key Words: Steroidal saponins; triterpenoid saponins; sapogenins; hemolytic activity; foaming; hypocholesterolemic effects; medicagenic acid; sapogenin; diosgenin; vanillin-perchloric acid reagent; TLC plate; fenugreek; Quillaja; Yucca.

1. Introduction

1.1. Nature, Mechanism of Action, and Biological Effects

Saponins comprise a large family of structurally related compounds containing a steroid or triterpenoid aglycone (sapogenin) linked to one or more oligosaccharide moieties by glycosidic linkage **(Fig. 1)**. The carbohydrate moiety consists of pentoses, hexoses, or uronic acids. The presence of both polar (sugar) and nonpolar (steroid or triterpene) groups provides saponins with strong surface-active properties that then are responsible for many of its adverse and beneficial effects. The primary biological effect of saponins is the interactions with cellular and membrane components. For example, saponins hemolyze red blood cells by nonspecific interactions with membrane proteins, phospholipids, and cholesterol of erythrocytes.

Saponins are characterized by their hemolytic activity and foaming properties and are responsible for imparting a bitter taste and astringency to plant materials containing high concentrations of saponins. Nonetheless, saponins are reported to affect the permeability of the small intestinal mucosal cells and thus have effect on active nutrient transport. Saponins have also been shown to inhibit various digestive enzymes, including trypsin and chymotrypsin, and are also known to inhibit protein degradation by forming saponin-protein complexes. On the other hand, positive nutritional effects of specific saponins such as hypocholesterolemic effects and improvement of growth in various

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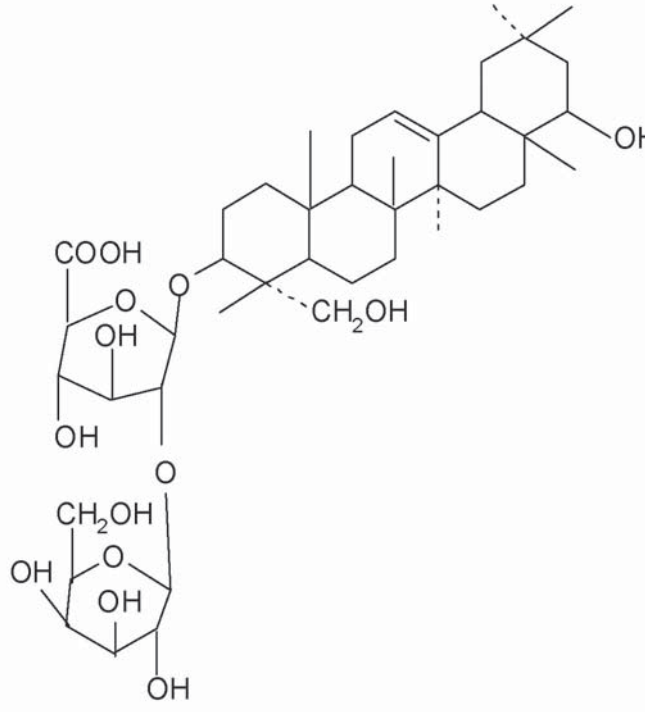
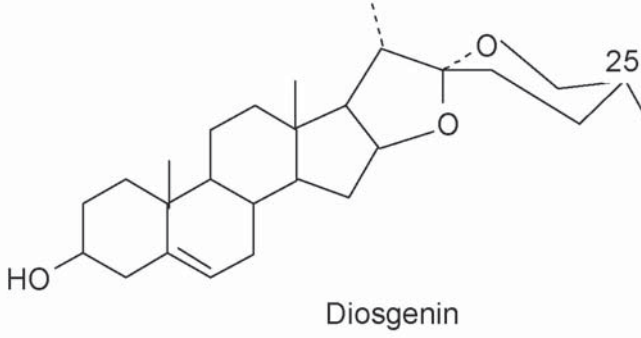
I. Triterpene saponins



Soyasaponin III

2. Steroid saponins



Diosgenin

Fig. 1. Structure of triterpenoid and steroid saponins.

animal species have also been reported. *Medicago sativa* (Alfalfa, Lucerne) contains many saponins. Medicagenic acid is unique to alfalfa. Alfalfa saponins may lower growth rate in chicks and egg production of hens when included in poultry diets above 5%.

The cardiac glycosides are also saponins. Digitalin is the cardiac glycoside in *Digitalis* species and other *Serophulariaceae*. The closely related cardiac glycoside, strophanthin, is found in the Apocynaceae. Livestock poisoning caused by strophanthin has occurred in Kenya when sheep and cattle consumed leaves of *Acokanthera longiflora* and *Acokanthera schimperi*. Death is caused by heart failure. Extracts of these plants are used as arrow poisons.

1.2. Present in

Glycine max, Medicago sativa, M. lupulina, M. media, Chenopodium quinoa, Glycyrrhiza glabra, Phaseolus vulgaris, Vigna angularis, Asparagus officinalis, dried pea, mung bean, runner bean, butter bean, kidney bean, haricot bean, field bean, broad pea, lentil, yellow split pea, chickpea, sunflower, sugar beat, spinach, oats, yam, fenugreek, gilla bean, moth bean, *Amaranthus caudatus, Thea sinensis, Quillaja saponaria, Yucca mohavensis, Y. schidigera, Aloe barbadensis, Lathyrus hirsutus, Smilax aristolochiifolia, Saponaria officinalis, S. sapindus, Aesculus hippcastum* (horse chestnut), *Chenapodium ambrosioides, C. quinoa, Sesbania sesban, S. bispinosa, Trigonella monspeliaca, Zizyphus jujuba, Treculia africana* (African breadfruit), *Artocarpus altilis* (Polynesian breadfruit), *Lotus corniculatus.*

1.3. Principle of Assays

This chapter presents four methods: determination of total steroidal saponins, based on Baccou et al. ***(1)***; determination of total saponins, based on Hiai et al. ***(2)***; determination of saponins based on hemolytic activity, based on Francis et al. ***(3)***; and qualitative evaluation of saponins, based on Burbano et al. ***(4)***.

The method for quantification of total steroidal saponins is based on the reaction of steroidal saponins with anisaldehyde and ethyl acetate in acidic medium to give a colored complex, the absorbance of which is measured at 430 nm ***(1)***.

In the method for determination of total saponins, steroidal sapogenins with or without double bond at C-5, triterpenoid sapogenins, and sterol and bile acids that have an OH group at their C-3 position react with vanillin in acidic medium to give chromogens with the absorbance maxima at 455 to 460 nm or 460 to 480 nm or at 544 nm, depending on the nature of the saponins ***(2)***. The chromogen formed is not dependent on the nature of sugar moieties.

In the third method based on hemolytic properties, saponins in a hemolytic unit are determined visually by twofold serial dilution of the plant extract ***(3–5)***. The qualitative evaluation of saponins is based on separation of saponins on the thin-layer chromatography (TLC) plate and location of saponin spots by their violet blue color produced by spraying the vanillin–perchloric acid or sulfuric acid reagent. The principle that saponins have hemolytic activity is also used for detecting hemolytic saponins. The developed TLC plates are sprayed with 6% cattle erythrocyte solution. The clear zones against the red blood erythrocyte area indicate the presence of saponins.

2. Materials

2.1. Determination of Total Steroidal Saponins

1. *Reagent A:* Add 0.5 mL of anisaldehyde to 99.5 mL of ethyl acetate and mix thoroughly.
2. *Reagent B:* 50 mL of concentrated sulfuric acid (95–98% w/w) plus 50 mL of ethyl acetate.
3. *Standard saponin solution:* Weigh 10 mg of diosgenin and dissolve it in 100 mL of ethyl acetate (0.1 mg/mL).

2.2. Determination of Total Saponins

1. *Vanillin reagent (8%):* Dissolve 800 mg of vanillin in 10 mL of 99.5% ethanol (analytical grade).
2. *72% (v/v) sulfuric acid:* To 28 mL of distilled water, add 72 mL of sulfuric acid (analytical grade, 95%, w/w).
3. *Standard saponin solution:* Weigh 10 mg of diosgenin, dissolve in 16 mL of methanol, and add 4 mL of distilled water. The final concentration of diosgenin in the solution is 0.5 mg/mL of 80% aqueous methanol. Mix thoroughly and start pipetting immediately.

2.3. Determination of Saponins Based on Hemolytic Property

1. *Phosphate buffer saline, PBS (pH 7.2):* Dissolve 8.2 g NaCl, 0.136 g KH_2PO_4, 0.224 g KCl, and 1.14 g Na_2HPO_4 in 1 L of distilled water (composition is 140 mM NaCl, 3 mM KH_2PO_4, 8 mM KCl, and 1 mM Na_2HPO_4).
2. *Preparation of 3% suspension of red blood cells in phosphate-buffered saline (PBS):* Take blood from cattle into heparinized tubes containing beads. The beads can be removed soon after taking the blood. Centrifuge the blood at 1500 *g* for 5 min and wash the packed erythrocyte cells three times with the PBS (pH 7.0) by centrifugation and subsequent removal of supernatants. The remaining layer of packed erythrocyte cells is diluted to 3% with the PBS.

2.4. Qualitative Evaluation of Saponins

1. *Vanillin-perchloric acid reagent:* Prepare 1% vanillin in ethanol (w/v) and 2% perchloric acid in ethanol (v/v) in a separate bottle. Combine equal volumes of vanilline and perchloric acid reagents.
2. *Sulfuric acid reagent:* Prepare freshly a mixture of ethyl acetate/ethanol/concentrated H_2SO_4 (10 : 10 : 18; v/v/v). Acid should be added drop by drop to the mixture kept on an ice-bath.
3. *Phosphate buffer saline, PBS (pH 7.2):* Dissolve 8.2 g NaCl, 0.136 g KH_2PO_4, 0.224 g KCl, and 1.14 g Na_2HPO_4 in 1 L of distilled water (composition is 140 mM NaCl, 3 mM KH_2PO_4, 8 mM KCl, and 1 mM Na_2HPO_4).
4. *Preparation of 6% suspension of red blood cells in PBS:* Take blood from cattle into heparinized tubes containing beads. The beads can be removed soon after taking the blood. Centrifuge the blood at 1500 *g* for 5 min and wash the packed erythrocyte cells three times with the PBS (pH 7.0) by centrifugation and subsequent removal of supernatants. The remaining layer of packed erythrocyte cells is diluted to 6% with the PBS. This erythrocyte solution for spraying on the plates should be prepared freshly.

3. Methods

3.1. Determination of Total Steroidal Saponins

3.1.1. Preparation of Extract

Take 10 g of defatted sample (finely ground, preferably using a ball mill) in a 250-mL flask and add 100 mL of 50% aqueous methanol. Keep it on a magnetic stirrer overnight at room temperature. Centrifuge the contents at 3000 *g* for 10 min and collect the supernatant. Repeat extraction with the same solvent by stirring on a magnetic stirrer for overnight. After centrifugation combine the first supernatant with the second one. If any particles are floating on the surface of the solvent, the filtration through Whatman filter paper is necessary. Evaporate methanol from the solution under vacuum at approximately 42°C by using a rotary-evaporator. Then centrifuge the aqueous phase at 3000 *g* for 10 min to remove the water insoluble materials. Transfer the aqueous phase into a separating funnel and extract with equal volume of chloroform (three times) to remove pigments. Finally extract concentrated saponins in the aqueous solution with equal volume of n-butanol (two times). Evaporate the solvent n-butanol under vacuum at a temperature not higher than 45°C or by nitrogen flushing. Dissolve the dried fraction containing saponins in 5 to 10 mL of distilled water and transfer the solution into a separate preweighed container. Freeze-dry the fraction and calculate the percent recovery of saponins (*see* **Notes 1** to **3**).

3.1.2. Preparation of Calibration Curve

1. Place 0, 20, 40, 60, 80, and 100 µl of the diosgenin standard solution (0, 2, 4, 6, 8, and 10 µg) in test tubes and make up the volume to 2 mL with ethyl acetate.
2. Add 1 mL of reagent A and 1 mL of reagent B.
3. After stirring, place the test tubes at room temperature for 30 min.
4. Measure absorbance at 430 nm against the reagent blank (0 µl of the diosgenin standard solution).

3.1.3. Determination of Saponins

1. Dissolve a known amount of extracted (freeze-dried) crude saponins in 80% aqueous methanol.
2. Take its suitable aliquots (corresponding to a sapogenin content of between 0 and 40 µg) in test tubes. Place the tubes in a boiling water bath or in a hot air bath at 100°C in order to remove alcohol (flushing with nitrogen gas could also be done).
3. After cooling, add 2 mL of ethyl acetate and carry out the determination as for diosgenin described in **Section 3.1.2**.

3.2. Determination of Total Saponins

3.2.1. Preparation of Calibration Curve

1. Place 0, 50, 100, 150, 200, and 250 µl of the diosgenin standard solution (0, 25, 50, 75, 100, and 125 µg) in test tubes and make the volume up to 0.25 mL with 80% aqueous methanol.
2. Add 0.25 mL of the vanillin reagent, and then 2.5 mL of 72% (v/v) sulfuric acid slowly on the inner side of the wall.
3. Mix the solution well and transfer the tubes to a water bath adjusted at 60°C.
4. After 10 min, cool the tubes in ice-cold water for 3 to 4 min, and measure absorbance at 544 nm against the reagent blank (0 µl of the diosgenin standard solution).

3.2.2. Quantitation of Total Saponins

Dissolve a known amount of extracted freeze-dried saponin residue (*see* **Section 3.1.1.**) in 80% aqueous methanol. From this, take an aliquot of 0.25 mL and carry out the determination as for the standard saponin.

3.3. Determination of Saponins Based on Hemolytic Property

1. Place 50 µL of the red blood cells suspension (3%) in separate wells of a microtiter plate.
2. Dissolve 10 to 20 mg of the freeze-dried saponin-enriched fraction (*see* **Section 3.1.1.**) in 1 mL of the PBS. Prepare a series of twofold diluted solution with the PBS.

3. Add a 50-µL aliquot of these twofold diluted solutions of saponins with the PBS to each well and incubate the mixture at room temperature for 2 h.
4. At the end of the incubation, visually determine which hemolyzed erythrocyte well is just before the well completely containing the nonhemolytic erythrocytes. A clear concentric circle around the red blood cells is indicative of a nonhemolytic well, and the spread of red color in the well and absence of a clear zone around red blood cells shows hemolysis.
5. The hemolytic activity is expressed as the inverse of the minimum amount of saponin extract/mL assay medium in the highest dilution that started producing the hemolysis. The results can also be expressed as compared to the hemolytic activity of a commercial standard triterpenoid saponin mixture from Quillaja bark, run simultaneously with the test sample. Any other commercially available hemolytic saponin can also be used as a reference.

3.4. Qualitative Evaluation of Saponins

1. Prepare developing solvent mixture of chloroform/methanol/water (65 : 35 : 10, v/v/v). Pour about 120 mL of this solvent mixture into a chromatographic tank and saturate the tank for overnight.
2. Dissolve 5 mg of the freeze-dried crude saponin residue (*see* **Section 3.1.1.**) in 1 mL of 50% aqueous methanol. From this stock, take 5 µl and load on each lane, 2 to 2.5 cm above from the bottom of plate, on a TLC plate (20 cm × 20 cm, Silica gel 60; Merck catalogue No. 1.05721).
3. After completely drying the spots, insert the TLC plate in the chromatographic tank containing the developing solvent mixture and place in the proper position.
4. When the developing solvent reaches approximately 1 cm below the top of the TLC plate (could take 3 to 4 h), remove the plate carefully and allow it to air-dry in room temperature.
5. Then spray the spraying reagent (the vanillin-perchloric acid reagent or sulfuric acid reagent) and heat at 100°C for 5 min. Saponins as violet or blue spots are located visually on the plates.
6. For evaluating the hemolytic nature of saponins, develop another set of the TLC plates in the same manner. Soon after air-drying the developed plates, spray uniformly the 6% blood erythrocytes on the surface of the plate. After 2 to 3 min, the location of white spot on a red background indicates a hemolytic activity of the separated saponins.
7. The results can be interpreted in terms of how many and which saponin spots out of the total spots located visually using the vanillin-perchloric acid reagent or sulfuric acid reagent produce hemolytic activities.

4. Notes

1. For leaf samples, after extraction of saponins in 80% aqueous methanol, the pigment can be removal by using chloroform. Pigment removal is not necessary for the grain samples.

2. Without the purification step, 80% aqueous methanol extract of sample may be directly used for the estimation of total saponins. However, many moieties present in the extract could interfere in the assay, giving an overestimate of the saponin values.
3. To get more purified and specific targeted saponins, the acetone precipitation of saponins from the *n*-butanol fraction may be carried out.

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17

Phorbol Esters

Key Words: Phorbol esters; *Jatropha curcas* seed kernels; Euphorbiaceae; carcinogenic; mutagenic; giddiness; phorbol-12-myristate 13-acetate; HPLC; *O*-phosphoric acid; acetonitrile; tetrahydrofuran.

1. Introduction

1.1. Nature and Biological Effects

The term *phorbol* is loosely used to describe a family of naturally occurring compounds referred to as tiglian diterpenes. These are widely distributed in the plant species of the families Euphorbiaceae and Thymelaeaceae. This chapter describes a method for determination of phorbol esters in physic nut (*Jatropha curcas*), which belongs to Euphorbiaceae family.

Poisoning in humans by accidental ingestion of jatropha seeds has been reported. Symptoms are giddiness, vomiting, and diarrhea. Toxicity to sheep, goats, calves, chicks, and fish by consumption of jatropha seed or seed meal has been observed ***(1,2)***. Phorbol esters are carcinogenic, mutagenic, and tumor promotors ***(3)***. They are also highly irritant ***(4)***. The ester groups of these compounds are recognized as being essential for their undesirable activities.

1.2. Principle of Assay

Phorbol esters are extracted from defatted seed kernels in methanol or dichloromethane. An aliquot of this extract is loaded on a high-performance liquid chromatography (HPLC) reverse-phase C_{18} LiChrospher 100 (Merck, Darmstadt, Germany), end-capped 5 µm column. The separation is done using a gradient elution with solvents comprising diluted *o*-phosphoric acid, acetonitrile, and tetrahydrofuran. The absorbance is recorded at 280 nm. The method

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presented here is based on Adolf et al. ***(4)*** and Makkar et al. ***(5)***, except that the gradient has been changed to reduce the analysis time. The results are expressed as equivalent to a standard, phorbol-12-myristate 13–acetate ***(5–7)***.

2. Materials

2.1. HPLC Instrument

The Merck Hitachi L-7100 HPLC pump, L-7450 photodiode array detector, L-7200 autosampler, D-7000 interphase module, and an LC organizer are used. The analytical column is reverse-phase C_{18} (LiChrospher 100, end-capped 5 μm) 250 × 4 mm I.D. (Lichrocart; Merck catalogue No. 1.50838), protected with a guard column containing the material as in the main column.

2.2. Solvent Systems

1. *Solvent A:* Add 1.75 mL of *o*-phosphoric acid (85%) in 1 L of distilled water.
2. *Solvent B:* Acetonitrile
3. *Solvent C:* Tetrahydrofuran

Before use, filter Solvent A. Solvents B and C could be used without filtration if these are of HPLC gradient grade. Degas all the solvents by ultrasonication and application of vacuum.

3. Methods

3.1. Preparation of Extract

Any of the following two methods could be used.

3.1.1. Method I

1. Take 2 g of ground seed kernels/defatted seed kernel meal in a 50-mL capacity plastic centrifuge tube and add 15 mL of methanol (100%). Sonicate the content at 240 W for 3 min under ice-cold conditions.
2. Centrifuge the content at 3000 *g* for 8 min at room temperature.
3. Collect the supernatant in a 150-mL capacity round-bottomed flask. Repeat the methanol extraction of the residue twice and collect the supernatants in the same round-bottomed flask.
4. Remove methanol from the supernatant using a rotary evaporator at a temperature not exceeding 40°C. Soon after removal of the solvent, pipette approximately 2 mL methanol into the flask containing the dry residue. Transfer this flask in a sonication bath for 3 to 4 min. Pipette out the dissolved material in a 10-mL graduated tube. Again pipette approximately 2 mL methanol into the flask, sonicate, and transfer the dissolved material in the same 10-mL graduated tube. Repeat

this step once more. Remove the methanol by flushing with nitrogen atmosphere and adjust the volume to 2 mL with methanol and vortex thoroughly.

5. Centrifuge at 17,000 *g* for 10 min at room temperature (some pellets can be seen in the centrifuge tube), pass the supernatant through a 0.2-µm Teflon or nylon filter, and transfer the filtrate into a HPLC vial. Inject 10 to 40 µL (depending on the concentration of phorbol esters) to the HPLC column.

3.1.2. Method II

1. Take 3 to 5 g of seed kernel/defatted seed kernel in a pestle and mortar and grind with a small amount of acid washed sand (about 200 mg).
2. Add 20 mL of dichloromethane and grind the mixture again for 5 min with the mortar. Allow the material to settle and filter the liquid phase using a filter paper.
3. Transfer about 20 mL of dichloromethane to the pestle and grind the mixture again for about 5 min using the mortar. Allow the material to settle and filter the liquid phase. Repeat this extraction procedure three more times. Pool the filtrate from all five extractions.
4. Transfer the residue containing sand plus kernels in the pestle and the filter paper in a beaker. Add 50 mL of dichloromethane and subject it to ultrasonic waves (105 W for 3 min). Filter the suspension and pool the filtrate with the pooled filtrates from the previous five extractions.
5. Remove the solvent of the pooled filtrate under vacuum at a temperature not more exceeding 40°C. Dissolve the dried residue in 5 mL of tetrahydrofuran, pass it through a 0.2 µm Teflon or nylon filter, and inject a volume of 10 to 20 µL (depending on the concentration of phorbol esters) into the HPLC.

3.2. Quantitative Determination of Phorbol Esters by HPLC

3.2.1. The Gradient System and the Procedure

Start with 40% of solvent A and 60% of solvent B, then decrease solvent A to 25% and increase solvent B to 75% in the next 20 min, then decrease solvent A to 0% and increase solvent B to 100% in the next 8 min, and maintain solvent B at 100% for the next 2 min. Then wash the column with solvent C by increasing it to 100% in the next 3 min and maintaining it for the next 1 min. Adjust the column to the starting conditions (40% solvent A and 60% solvent B) in the next 2 min, and maintain these conditions for the next 2 min before injecting another sample.

Separation is performed at room temperature (22°C) at a flow rate of 1.3 mL/min. Phorbol esters peaks (four) appear between 26.5 and 29.5 min **(Fig. 1)**. The four peaks are integrated at 280 nm, and the results are expressed as equivalent to phorbol-12-myristate 13-acetate (from Sigma, St. Louis, MO), which appears at 31.5 min.

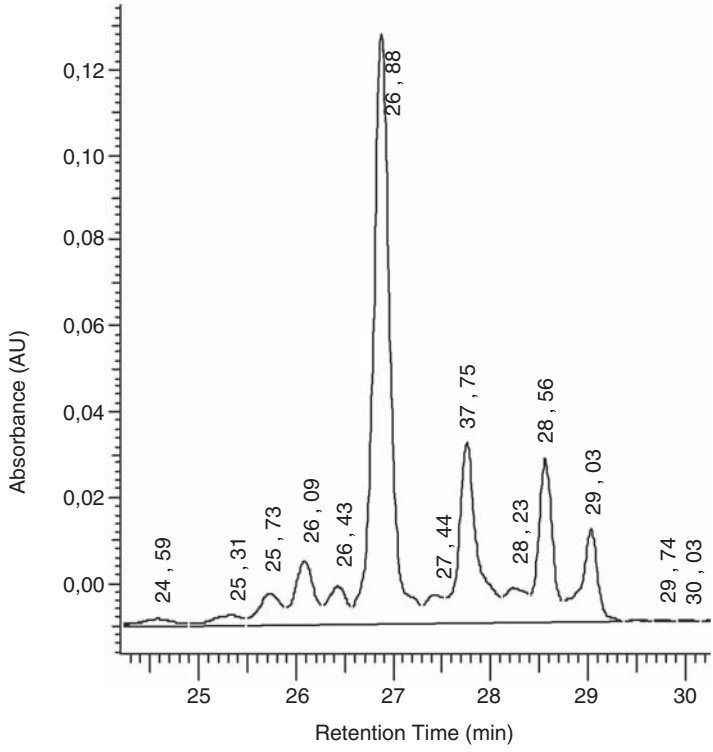

Fig. 1. A typical chromatogram showing four phorbol ester peaks at 26.88, 27.75, 28.56, and 29.03 min.

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18

Alkaloids

Key Words: Alkaloids; strychnine; solanine; tryptamine alkaloids; potato; *Lupinus* spp.; Dragendorff reagent; Marquis reagent; p-dimethylamino benzaldehyde reagent; potassium permanganate reagent; bitter taste; Silica gel-G60; TLC plate; ammonium hydroxide; chloroform.

1. Introduction

1.1. Nature and Biological Effects

Alkaloids are a diverse group of organic bases containing secondary, tertiary, or cyclic amines. Some 5500 alkaloids are known, comprising the largest single class of secondary plant substances. There is no one definition of the term *alkaloid,* which is completely satisfactory, but alkaloids generally include "those basic substances which contain one or more nitrogen atoms, usually in combination as part of a cyclic system." ***(1)*** Chemically, alkaloids are a very heterogeneous group, ranging from simple compounds like coniine, the major alkaloid of hemlock, *Conium maculatum*, to the pentacyclic structure of strychnine, the toxin of the *Strychnos* bark. Many alkaloids are terpenoid in nature, and some (e.g., solanine, the steroidal alkaloid of the potato, *Solanum tuberosum*) steroidal. Others are mainly aromatic compounds (e.g., colchicine).

Alkaloids are often toxic to humans, and many have dramatic physiological and neurological activities. Some plants rich in alkaloids are *Phalaris* species, *Datura stramonium*, or rye grass (*Lolium perenne* L.), and infected by endophytic fungus (*Acremonium lolii*). *Phalaris* species contain tryptamine alkaloids and cause a staggering gate and death. *D. stramonium* contains tropane alkaloids such as hyoscyamine, the consumption of which causes paralysis and rapid heart beat, leading to death. A simple but by no means infallible test for

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alkaloids in fresh leaf or fruit material is the bitter taste they often impart. The alkaloid quinine, for example, is one of the bitterest substances known and is significantly bitter at a molar concentration of 1×10^{-5}.

In general, the angiosperm families are particularly rich in these bases, but it should be borne in mind that alkaloid distribution is very uneven and many families lack them altogether. Alkaloids are generally absent or infrequent in the gymnosperms, ferns, mosses, and lower plants.

1.2. Present in

Ricinus communis, Sphenostylis stenocarpa, Pentaclethra macrophylla, Lupinus albus, L. angustifolius, L. luteus, L. mutabilis, L. polyphyllus, Coffea arabica, Camellia sinensis, Theobroma cacao, and *Lycopersicon esculentum*.

1.3. Preliminary Detection

Alkaloids cannot be identified or quantified by a single method because these are highly heterogeneous chemically and there are so many of them. In general, it is difficult to identify an alkaloid from a new plant source without knowing approximately what type of alkaloid is likely to be found there. Also, because of the wide range of solubility and other properties of alkaloids, any general screening procedure for alkaloids in plants may fail to detect particular compounds.

2. Materials

2.1. Alkaloid Spray Reagents

2.1.1. The Dragendorff Reagent

Two stock solutions are prepared: ***(2)*** 0.6 g bismuth subnitrate in 2 mL concentrated HCl (37% w/v) and 10 mL distilled water, and ***(1)*** 6 g potassium iodide in 10 mL distilled water. These stock solutions are mixed together with 7 mL HCl (37% w/v) and 15 mL distilled water and diluted to 400 mL with distilled water.

2.1.2. Iodoplatinate Reagent

For spraying papers, the iodoplatinate reagent is prepared by mixing 10 mL of 5% platinum chloride and 240 mL of 2% potassium iodide, and diluting to 500 mL with distilled water. For spraying plates, this reagent is prepared by mixing 10 mL of 5% platinum chloride, 5 mL concentrated HCl, and 240 mL 2% potassium iodide.

2.1.3. Marquis Reagent

Marquis reagent can only be applied to the thin-layer chromatography (TLC) plates and it consists of 1 mL of formaldehyde (40%) in 10 mL concentrated sulfuric acid.

2.1.4. p-Dimethylaminobenzaldehyde Reagent

One gram of *p*-dimethylaminobenzaldehyde is dissolved in 100 mL of ethanol and to it 10 mL of concentrated HCl is added.

2.1.5. Potassium Permanganate Reagent

Dissolve 1 g potassium permanganate in approximately 70 mL distilled water and make up the volume to 100 mL with distilled water. It is also used for detection of some alkaloids.

3. Methods

3.1. General Procedure for Alkaloid Detection

Isolation and analysis of alkaloids generally rely on the differences in solubility of the free-base form and the acid form. The free base is usually soluble in hydrophobic solvents.

Take 4 g of the sample and to it add 40 mL of 10% acetic acid in ethanol or methanol. Keep the mixture for 4 h under dark conditions on a shaking bath at room temperature, and filter or centrifuge the contents. Concentrate the extract to one quarter of the original volume and precipitate alkaloid by drop-by-drop addition of concentrate ammonium hydroxide. Collect the precipitate by centrifugation (3000 *g*, 10 min), wash with 1% NH_4OH, followed by centrifugation, and dissolve it in a few drops of ethanol or chloroform. If no precipitate occurs on addition of ammonium hydroxide, extract alkaloids using chloroform by following the procedure described below.

Extract three times with chloroform, 40 mL each time. Reduce the volume using a vacuum rotary evaporator at 35°C to about 2 to 4 mL. Use this for application on the TLC plates (silica gel G). Even if precipitates form on addition of the ammonium hydroxide, the remaining supernatant should also be extracted with chloroform to see the presence of alkaloids that do not get precipitated with ammonium hydroxide.

Apply an aliquot (5 to 10 µl) on the silica gel plates and develop these in methanol/concentrated ammonium hydroxide (200 : 3, v/v). Tanks should be equilibrated for at least 1 h with the methanol-ammonium hydroxide solution before inserting the TLC plates. Also place a sheet of filter paper on the sides of the wall to facilitate evaporation and equilibration. After two runs, the solvent

Table 1
R_f values and color properties of some well-known alkaloids

Alkaloid	R_f on paper	R_f on TLC plate	Behavior in UV light	Recommended reagent for detection	Spectral max (nm) in 0.1 M H_2SO_4
Cytisine	03	32	Blue	Dragendorff	303
Nicotine	07	57	Absorbs	Iodoplatinate	260
Tomatine	08	62	Invisible	Iodoplatinate	—
Morphine	14	34	Absorbs	Iodoplatinate	284
Solanine	15	52	Invisible	Marquis	—
Codeine	16	35	Absorbs	Iodoplatinate	284
Berberine	25	07	Fluorescent yellow	Iodoplatinate	228
Strychnine	30	22	Absorbs	Iodoplatinate	254
Thebaine	32	41	Absorbs	Iodoplatinate	284
Atropine	37	18	Absorbs	Iodoplatinate	258
Quinine	46	52	Bright blue	Iodoplatinate	250
Coniine	56	26	Invisible	Iodoplatinate	268

Source: Data from Clarke ***(2)***.

should be changed and reequilibrated. Detect the presence of alkaloids on the developed plates, after drying, first by observing fluorescence in ultraviolet (UV) light and then by applying three spray reagents: Dragendorff, iodoplatinate, and Marquis. The R_f and colors of twelve common alkaloids are shown in **Table 1**. Confirm the presence of a particular alkaloid by using commercially available standards and by measuring the UV spectrum in 0.1 M sulphuric acid. Typical maxima values range from 250 to 303 nm. Alkaloids with aromatic rings in their structures may also absorb at longer wavelengths; for example, the lambda max of colchicines are at 243 nm and 351 nm and that of berberine 265 nm and 343 nm. This test cannot be applied if more than one major alkaloid is present in the extract under examination.

Instead of the TLC plates, sodium citrate–buffered paper can also be used to separate alkaloids using *n*-butanol-aqueous citric acid. As for the TLC plates, alkaloids are detected initially by their fluorescence in UV light and then by application of the three spray reagents: Dragendorff, iodoplatinate, and Marquis.

Generally,

- Dragendorff reagent gives orange-brown spots on a yellow background.
- Marquis reagent gives yellow to purple spots. Codeine and morphine give bluish purple, most of the phenothiazine derivatives give red-purple, and amphetamine gives orange color.

- Iodoplatinate reagent produces a violet color (or dark blue-black spots), except for cinchonidine and dihydrocinchonidine, which give a grey-blue color.
- *p*-Dimethylaminobenzaldehyde reagent gives a blue color with the ergot alkaloids, and a bright yellow with compounds containing a primary aromatic amino group.

Solvent on paper is previously buffered with 5% sodium dihydrogen citrate/*n*-BuOH in aqueous citric acid (870 mL/4.8 g citric acid in 130 mL H_2O); solvent on silica gel is MeOH : NH_4OH (200 : 3, v/v).

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Appendix

I. Boiling Points of Certain Common Solvents

The following boiling points are correct to the nearest degree centigrade (°C)

Acetone	56	Chloroform	62
Acetic acid	118	Ether	34
Amyl alcohol	130	Ethyl alcohol	78
Benzene	80	Hexane	68
Butyl alcohol	118	Methyl alcohol	65
Caprylic alcohol	180	Toluene	111
Carbon disulphide	46	Water	100
Xylene	138–144		

II. Constants of Acids and Bases: Commercial Concentrated Reagent

Substance	Formula	Molecular weight	Specific gravity	Per cent by weight	Molarity	Normality
Acetic acid	CH_3COOH	60.1	1.05	99.5	17.4	17.6
Ammonium hydroxide	NH_4OH	35.0	0.89	28.0	14.8	15.1
Hydrochloric acid	HCl	36.5	1.18	36.0	11.6	11.7
Nitric acid	HNO_3	63.0	1.42	71.0	16.0	15.6
Perchloric acid	$HClO_4$	100.5	1.67	70.0	11.6	9.2
Phosphoric acid	H_3PO_4	80.0	1.70	85.0	18.1	45.0
Sulfuric acid	H_2SO_4	98.1	1.84	96.0	18.0	35.9

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III. Equivalent Weights of Some Common Chemicals

Name	Formula	Molecular weight (MW)	Fraction of MW	Equivalent weight
Hydrochloric acid	HCl	36.46	1.0	36.46
Sulfuric acid	H_2SO_4	98.00	0.5	49.00
Oxalic acid	$(COOH)_2\,2H_2O$	126.05	0.5	63.03
Sodium hydroxide	NaOH	40.00	1.0	40.00
Potassium hydroxide	KOH	56.10	1.0	56.10
Sodium carbonate	Na_2CO_3	106.00	0.5	53.00
Borax	$Na_2B_4O_7$	381.44	0.5	190.72
Potassium permanganate	$KMnO_4$	158.00	0.2	31.60
Potassium dichromate	$K_2Cr_2O_7$	294.20	0.17	49.03
Ferrous ammonium sulfate	$FeSO_4\,(NH_4)_2\,SO_4.6H_2O$	392.10	1.0	392.10
Ferrous sulfate	$FeSO_4.7H_2O$	278.00	1.0	278.00
Sodium oxalate	$(COONa)_2$	134.00	0.5	67.00
Ammonium oxalate	$(COONH_4)_2.H_2O$	144.08	0.5	72.04
Iodine	I_2	253.80	0.5	126.90
Hypo (sodium thiosulphate)	$Na_2S_2O_3.5H_2O$	248.20	1.0	248.20

IV. Preparation of Common Buffers

The accuracy of the following tables is within ±0.05 pH at 23°C. The molarity of the buffer described is between 0.05 M and 0.1 M.

(*Source:* (1955) *Methods in Enzymology*, vol. I, Colowick, S.P. and Kaplan, N.O., eds., Academic Press, New York, pp. 138–146.)

1. Acetate buffer pH 3.6–5.6
   Stock solutions

   A: 0.2 M solution of acetic acid (11.55 mL in 1 L distilled water)
   B: 0.2 M solution of sodium acetate (16.4 g of C_2H_2Na or 27.2 g of $C_2H_3O_2Na.3H_2O$ in 1 L distilled water)

   x mL of A *plus* *y* mL of B, dilute to a total of 100 mL with distilled water.

x	*y*	pH
46.3	3.7	3.6
44.0	6.0	3.8
41.0	9.0	4.0
36.8	13.2	4.2
30.5	19.5	4.4
25.5	24.5	4.6
20.0	30.0	4.8
14.8	35.2	5.0
10.5	39.2	5.2
8.8	41.2	5.4
4.8	45.2	5.6

2. Glycine–HCl buffer pH 2.2–3.6
   Stock solutions

   A: 0.2 M glycine (15.01 g in 1 L distilled water)
   B: 0.2 M HCl

   25 mL of A *plus* *x* mL of B, dilute to a total of 100 mL with distilled water.

x	pH
22.0	2.2
16.2	2.4
12.1	2.6
8.4	2.8
5.7	3.0
1.1	3.2
1.2	3.4
2.5	3.6

3. Sodium phosphate buffer pH 5.7–8.0
   Stock solutions

   A: 0.2 M solution of monobasic sodium phosphate, NaH_2PO_4 (27.8 g in 1 L distilled water)
   B: 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7H_2O$ or 71.7 g of $Na_2HPO_4.12H_2O$ in 1 L distilled water)

   x mL of A *plus* *y* mL of B, dilute to a total of 200 mL with distilled water.

x	*y*	pH	*x*	*y*	pH
93.5	6.5	5.7	45.0	55.0	6.9
92.0	8.0	5.8	39.0	61.0	7.0
90.0	10.0	5.9	33.0	67.0	7.1
87.7	12.3	6.0	28.0	72.0	7.2
85.0	15.0	6.1	23.0	77.0	7.3
81.5	18.5	6.2	19.0	81.0	7.4
77.5	22.5	6.3	16.0	84.0	7.5
73.5	26.5	6.4	13.0	87.0	7.6
68.5	31.5	6.5	10.5	89.5	7.7
62.5	37.5	6.6	8.5	91.5	7.8
56.5	43.5	6.7	7.0	93.0	7.9
51.0	49.0	6.8	5.3	94.7	8.0

4. Potassium phosphate buffer 5.8–8.0
   Stock solutions
   50 mL of 0.1 M KH_2PO_4 (13.60 g/L distilled water) *plus* *y* mL of 0.1 M NaOH (4 g/L distilled water), dilute to a total of 100 mL with distilled water.

y	pH	*y*	pH	*y*	pH
3.6	5.8	16.4	6.6	39.1	7.4
4.6	5.9	19.3	6.7	40.9	7.5
5.6	6.0	22.4	6.8	42.4	7.6
6.8	6.1	25.9	6.9	43.5	7.7
8.1	6.2	29.1	7.0	44.5	7.8
9.7	6.3	32.1	7.1	45.3	7.9
11.6	6.4	34.7	7.2	46.1	8.0
13.9	6.5	37.0	7.3		

5. Citrate buffer pH 3.0–6.2
   Stock solutions

   A: 0.1 M solution of citric acid (21.01 g in 1 L distilled water)
   B: 0.1 M solution of sodium citrate (29.41 g $C_6H_5O_7Na_2.2H_2O$ in 1 L distilled water)

   X mL of A *plus* *y* mL of B, dilute to a total of 100 mL with distilled water.

x	*y*	pH	*x*	*y*	pH	*x*	*y*	pH
46.5	3.5	3.0	33.0	17.0	4.0	18.0	32.0	5.2
43.7	6.3	3.2	31.5	18.5	4.2	16.0	34.0	5.4
40.0	10.0	3.4	28.0	22.0	4.4	13.7	36.3	5.6
37.0	13.0	3.6	25.5	24.5	4.6	11.8	38.2	5.8
35.0	15.0	3.8	23.0	27.0	4.8	9.5	40.5	6.0
			20.5	29.5	5.0	7.2	42.8	6.2

6. Barbital buffer pH 6.8–8.4
   Stock solutions

   A: 0.2 M solution of sodium barbital (veronal) (41.2 g in 1 L distilled water)
   B: 0.2 M HCl

   50 mL of A *plus* y mL of B, dilute to a total of 200 mL with distilled water.

y	pH	*y*	pH	*y*	pH
1.5	9.2	12.7	8.2	32.5	7.4
2.5	9.0	17.5	8.0	39.0	7.2
4.0	8.8	22.5	7.8	43.0	7.0
6.0	8.6	27.5	7.6	45.0	6.8
9.0	8.4				

7. Tris-HCl buffer pH 7.2–9.0
   Stock solutions

   A: 0.2 M solution of Tris (hydroxymethyl) aminomethane (24.2 g in 1 L distilled water)
   B: 0.2 M HCl

   50 mL of A *plus* *x* mL of B, dilute to total volume of 200 mL.

x	pH	*x*	pH
5.0	9.0	26.8	8.0
8.1	8.8	32.5	7.8
12.2	8.6	38.4	7.6
16.5	8.4	41.4	7.4
21.9	8.2	44.2	7.2

8. Boric acid-borax buffer pH 7.6–9.2
Stock solutions

A: 0.2 M solution of boric acid (12.4 g in 1 L)
B: 0.05 M solution of borax (19.05 g in 1 L; 0.2 M in terms of sodium borate)

50 mL of A *plus* *x* mL of B, diluted to a total of 200 mL.

x	pH	*x*	pH
2.0	7.6	22.5	8.7
3.1	7.8	30.0	8.8
4.9	8.0	42.5	8.9
7.3	8.2	59.0	9.0
11.5	8.4	83.0	9.1
17.5	8.6	115.0	9.2

9. Sorensen's phosphate buffer pH 4.9–8.0
Stock solutions

A: Sodium phosphate dibasic, $Na_2HPO_4.2H_2O$ (11.86 g in 1 L distilled water)
B: Potassium phosphate monobasic, KH_2PO_4 (9.08 g in 1 L distilled water)

The desired pH can be obtained by adding enough solution B to solution A given below to make a total volume of 100 mL.

A	pH	A	pH
0.6	4.9	72.6	7.2
2.3	5.3	77.7	7.3
4.9	5.6	81.8	7.4
12.1	6.0	85.2	7.5
26.4	6.4	88.5	7.6
49.2	6.8	93.6	7.8
61.2	7.0	96.9	8.0
67.0	7.1		

10. Carbonate-bicarbonate buffer pH 9.2–10.7
Stock solutions

A: 0.2 M solution of anhydrous sodium carbonate (21.2 g in 1 L distilled water)
B: 0.2 M solution of sodium bicarbonate (16.8 g in 1 L distilled water)

x mL of A *plus* *y* mL of B, dilute to a total volume of 200 mL with distilled water.

x	*y*	pH	*x*	*y*	pH
4.0	46.0	9.2	27.5	22.5	10.0
7.5	42.5	9.3	30.0	20.0	10.1
9.5	40.5	9.4	33.0	17.0	10.2
13.0	37.0	9.5	35.5	14.5	10.3
16.0	34.0	9.6	38.5	11.5	10.4
19.5	30.5	9.7	40.5	9.5	10.5
22.0	28.0	9.8	42.5	7.5	10.6
25.0	25.0	9.9	45.0	5.0	10.7

11. Glycine-NaOH buffer 8.6–10.6
Stock solutions

A: 0.2 M solution of glycine (15.01 g in 1 L distilled water)
B: 0.2 M NaOH (8 g in 1 L distilled water)

50 mL of A *plus* *x* mL of B, dilute to total of 200 mL with distilled water.

x	pH	*x*	pH
4.0	8.6	22.4	9.60
6.0	8.8	27.2	9.80
8.8	9.0	32.0	10.0
12.0	9.2	38.6	10.4
16.8	9.4	45.5	10.6

V. Method of Expressing Concentration of a Solution

Following are some of the common ways of expressing the concentration of solutions:

1. Molarity (M)
The molarity of a solution is the number of moles of the solute dissolved per liter of the solution. A solution that contains one mole (1 g molecular weight) of the solute in one litre of the solution is called a molar solution. Molarity of a solution can be calculated as follows:

$$\text{Molarity} = \frac{\text{Weight of a solute in g/L of solution}}{\text{Molecular weight of solute}}$$

It may be noted that in case of molar solutions, the combined total volume of the solute and solvent is 1 L. Thus for preparing 0.1 M NaOH, one has to proceed as follows:

Mol. wt. of NaOH = 40

Required molarity of solution = 0.1 M

Therefore, amount (in grams) of NaOH per liter of solution
= Molecular weight of NaOH × molarity
= 40 × 0.1 = 4 g
Thus, weigh 4 g of NaOH, dissolve it in a small volume of solvent (water), and make the final volume up to 1 L with the solvent.

Sometimes it is desirable to know the number of moles of a substance in a reaction mixture. This can be calculated using a simple relationship:

1 M solution = 1 mole of the substance/L of solution
= 1 mmol/mL of solution
= 1 µmol/µL of solution

1 mM solution = 1 mmol/L of solution
= 1 µmol/mL of solution

2. Molality (M)
A solution that contains 1 mole of the solute dissolved in 1 kg of the solvent is called a molal solution. Hence,

$$\text{Molality} = \frac{\text{Weight of a solute in g/kg of solvent}}{\text{Molecular weight of solute}}$$

It is important to remember that in a molal solution, the amount of solvent is 1000 g. Thus in the case of aqueous solution, 1 molal solution is obtained by dissolving 1 mole of the solute in 1 L of water (since specific gravity = 1). For example, for preparing 1 M Na_2CO_3 solution, dissolve 106 g of Na_2CO_3 (molecular weight of Na_2CO_3 = 106) in 1 kg of water (or 1 L of water).

3. Normality (N)
The normality of a solution is the number of gram equivalents of the solute per liter of the solution. A solution having 1 g equivalent of the solute per liter of solution is called a 1 N solution. Therefore,

$$\text{Normality} = \frac{\text{Amount of a substance in g/L of solution}}{\text{Equivalent weight of substance}}$$

For preparing 0.1 N Na_2CO_3 solution (equivalent weight of Na_2CO_3 = 53), dissolve 5.3 g Na_2CO_3 in a final volume of 1 L of solution.

4. Parts per million (ppm)
This is generally employed for those solutions in which a substance is present in a very small quantity. It represents grams of a solute per million grams of solution or grams of a solute per million mL of the solution.

$$\text{ppm} = \frac{\text{Mass of the component}}{\text{Total mass of the solution}} \times 10^6$$

or

$$\text{ppm} = \frac{\text{g or mL of solute or substance}}{\text{g or mL of solution}} \times 10^6$$

Thus, 1 ppm of solution of NaCl in water represents

$$1\,\text{ppm} = 1\,\text{mg NaCl/L of solution (or)}$$
$$= 1\,\mu\text{g NaCl/mL of solution}$$

5. Equivalent weight
   Equivalent weight of a substance is the number of grams of the substance required to react with, replace, or furnish one mole of H_2O^+ or OH^-. The equivalent weight of an acid is the weight that contains one atomic weight of acidic hydrogen, that is, the hydrogen that reacts during neutralization of acid with base.

   For example, the equivalent weight of H_2SO_4 is 49. Since H_2SO_4 contains two replaceable hydrogens, equivalent weight is molecular weight /2, 98/2 = 49.
6. Mass concentration
   Substance concentration is expressed in terms of weight per unit volume rather than moles per unit volume. The unit of volume is L, so all concentrations should be expressed with L (g/L, mg/, µg/L, etc.). The term *percent* (%) is also quite commonly used. However, to avoid any ambiguity it is necessary to properly define the basis of % solution as illustrated by the following example. A 5% solution of acetic acid could mean:

   5 g of acetic acid per 100 g of solution (w/w)
   5 g of acetic acid per 100 mL of solution w/v)
   5 mL of acetic acid per 100 mL of solution (v/v)

   Thus, 1% (w/v) solution of casein would imply 1 g of casein dissolved in solvent to give a final volume of 100 mL of the solution.
7. pH
   pH is a value taken to represent the acidity or alkalinity of an aqueous solution. It is defined as logarithm of the reciprocal of the hydrogen ion concentration of the solution.

$$\text{pH} = \log \frac{1}{[H^+]}$$

VI. International Systems of Units Conversion

A. Temperature

$$\text{Temperature in °F} = (\text{Temperature in °C} \times 1.8) + 32$$

$$\text{Temperature in °C} = (\text{Temperature in °F} - 32) \times 5/9$$

$$\text{Temperature in K} = (\text{Temperature in °F} + 459.67) \times 5/9$$

B. Milliequivalents

To convert mg/100 mL into milliequivalents and vice versa:

$$\text{Milliequivalents} = \frac{\text{mg per 100 mL} \times \text{Valency} \times 10}{\text{Molecular weight}}$$

$$\text{mg per 100 mL} = \frac{\text{Milliequivalents} \times \text{Molecular weight}}{\text{Valency} \times 10}$$

VII. Atomic and Molecular Weight

A. Atomic Weight

Atomic weight of an element is the relative weight of the atom on the basis of oxygen as 16. For example, atomic weight of sodium is 23.

B. Molecular Weight

The sum of the atomic weights of all the atoms in a molecule is its molecular weight. For example, molecular weight of H_2SO_4 is 98, since

Hydrogen	$2 \times 1 = 2$
Sulfur	$1 \times 32 = 32$
Oxygen	$4 \times 16 = 64$
	98

Index