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Analytical Manual for Pomegranate

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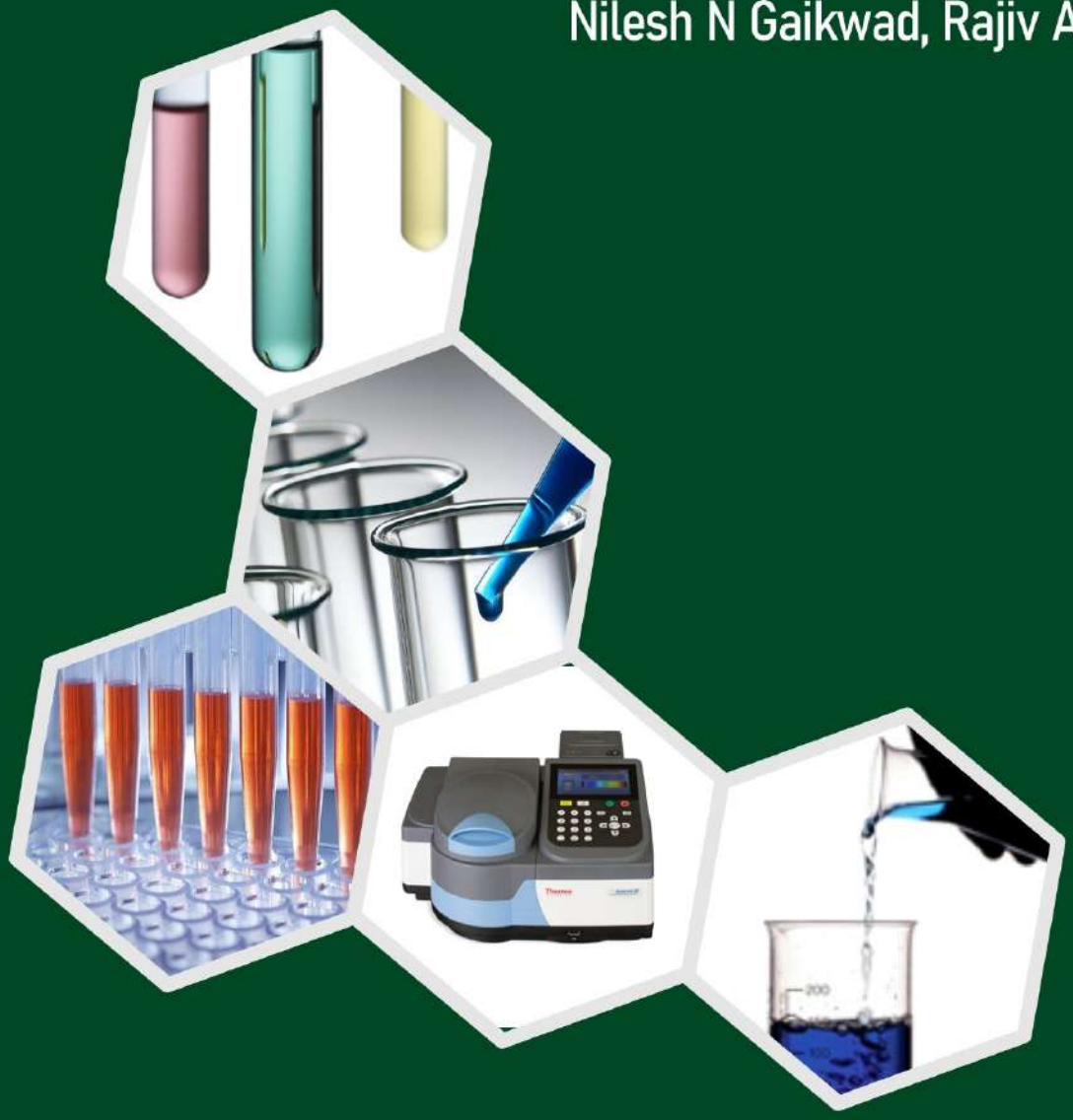

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Preface

India is the largest pomegranate producer of the world. The pomegranate crop has got renewed attention from the researcher's world over due to its amazing health benefits, nutraceutical value and therapeutic utility. The crop is at the centre of attraction for the researchers, academicians and students in the field of the horticulture, soil and plant nutrition, pharmaceutical, food processing, medicine, cosmetics etc.

Despite being at centre stage of research there is lack of compiled information on the biochemical analytical methods in pomegranate. It is often essential to estimate the stress indicators, fruit quality parameters in the quality breeding programmes for breeders and biotechnologist. The food, pharmaceutical and cosmetic researchers and industry need to evaluate processed products for quality parameters. The soil scientist and plant nutrition researchers working in pomegranate are keen to know the plant health status and to see effect of the nutrition on plant parts such as leaves, fruits etc.

This technical bulletin entitled "Analytical Manual for Pomegranate" is timely and need based attempt which provides comprehensive information about analytical methods in pomegranate. It will be of great help and serve as ready reckoner for the researchers, industry, academicians and students for the research and quality control. I congratulate the authors for their efforts in bringing out this valuable publication.

25th September 2023

Dr. R.A. Marathe
Director, ICAR-NRCP
Solapur

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Abbreviations used in the manual

min : minutes
sec : second
h : hour
M : molar
mM : millimolar
N : normal
mg : milli gram
g : gram
Kg : kilo gram
ml : milli litre
µl : micro litre
L : Litre
mm : milli meter
DW : distilled water
nm : nanometer
wt : weight
vol : volume
MW : molecular weight
OD : Optical density (absorbance)

Methods for Aril/ Rind/ Leaf Analysis

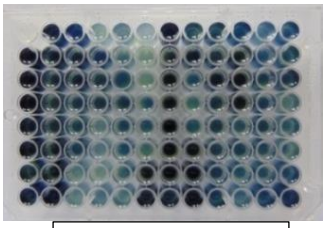
Total Phenolics

Reagents

1.	Folin-Ciocalteu reagent	
2.	7.5% sodium carbonate	Dissolve 7.5gm of sodium carbonate in 100ml distilled water
3.	Standard stock solution	Dissolve 500mg Gallic acid in 10ml ethanol and make up volume to 100ml with distilled water

Procedure

1. Take 15µl of pomegranate juice sample and dilute to 1ml with distilled water.
2. Add 0.5ml of Folin-Ciocalteu reagent, mix and incubate for 3min.
3. Add 4ml of 7.5% sodium carbonate solution.
4. Vortex and incubate for 15min at 45°C.
5. Measure the absorbance of samples at 620nm against reagent blank and calculate phenol by comparing with standard curve.



Color developed

Reference: Derakhshan D, Ferrante M, Tadi M, Ansari F, Heydari A, Hosseini MS, Conti GO, Sadrabad EK (2018) Antioxidant activity and total phenolic content of ethanolic extract of pomegranate peels, juice and seeds. Food and Chemical Toxicology. 114: 108-111

Total Monomeric Anthocyanin Content

Reagents

1.	0.025M Potassium chloride (pH 1.0)	Dissolve 1.86g of potassium chloride in 980ml DW and make up volume to 1L with DW. Adjust pH to 1.0 with HCl
2.	0.4M sodium acetate (pH 4.5)	Dissolve 54.43g sodium acetate in 960ml DW and make up the volume to 1L with DW. Adjust pH to 4.5 with HCl

Procedure

1. Take 1ml juice sample in two sets of test tubes.
2. Add 4.5 ml volume of the Potassium chloride buffer into one set of the tubes, and 4.5 ml of Sodium acetate buffer to the other set of tubes.
3. Mix and incubate the tubes in dark for 15min at room temperature.
4. Measure absorbance of samples from both set of tubes at 510nm and 700nm against distilled water blank.
5. The total anthocyanin content was reported as mg of anthocyanin equivalents per 100ml of the sample (mg cyanidin-3-glucoside equivalent /100ml) using the following equation:

Calculation

$$\text{Total Anthocyanin (mg/100ml)} = \frac{A \times MW \times DF \times 100}{\varepsilon \times L}$$

Where, A= (A_{510nm}-A_{700nm}) pH1.0 - (A_{510nm}-A_{700nm}) pH 4.5
MW (Cy-3-glu molecular weight) = 449.2 g/mol
DF (dilution factor) = 10

L (path length in cm) = 1
ε (molar extinction coefficient) = 26,900 L.mol−1.cm−1.

Reference: Chrysavgi Gardeli, Kalliopi Varela, Eleni Krokida, Athanasios Mallouchos (2019) Investigation of Anthocyanins Stability from Pomegranate Juice (*Punica Granatum* L. Cv Ermioni) under a Simulated Digestion Process. Medicines (Basel). 6(3): 90.

Ascorbic Acid

Reagents

1.	Oxalic Acid (4%)	Dissolve 4g oxalic acid in 100ml of DW
2.	Dye Solution	Weigh 42mg sodium bicarbonate into a small volume of distilled water. Dissolve 52 mg 2,6-dichlorophenol indophenol in it and make up to 200ml with distilled water.
3.	Stock Standard Solution	Dissolve 100mg ascorbic acid in 100ml of 4% oxalic acid solution in a standard flask (1mg/ml) Working Standard: Dilute 10ml of stock solution to 100ml with 4% oxalic acid. The concentration of working standard is 100ug/ml

Procedure

1. Pipette out 5ml of the working standard solution into 100ml conical flask.
2. Add 10ml of 4% oxalic acid and titrate against the dye (V1 ml). End of reaction is the appearance of pink colour, which persists for a few minutes. The amount of the dye consumed is equivalent to the amount of ascorbic acid.
3. Dilute 0.5ml sample in 4% oxalic acid and make up to a known volume (100ml) with DW and centrifuge.
4. Pipette out 0.5 ml of this supernatant, add 10ml of 4% oxalic acid and titrate against the dye (V2 ml).

Calculation

$$\text{Ascorbic acid (mg/100ml)} = \frac{0.5\text{ ml} \times V2\text{ (ml)} \times 100\text{ ml}}{V1\text{ (ml)} \times 5\text{ (ml)} \times \text{Sample weight}} \times 100$$

Where,
V1 = Titre value of standard solution; V2 = Titre value of sample

Reference: Gaikwad, N. N., Pal, R. K., Suryavanshi, S., Babu, K. D., Maity, A., & Sarkar, S. K. (2017). Effect of extraction method and thermal processing on retention of bioactive compounds of pomegrante (*Punica granatum*) (cv. Bhagwa) juice. Indian Journal of Agricultural Sciences, 87(11):1445-52.

Total Flavonoids

Reagents

1.	10% Aluminium chloride	Dissolve 10 g of Aluminium chloride in distilled water and make final volume to 100 ml.
2.	1M potassium acetate	Dissolve 9.814 g of potassium acetate in distilled water and make final volume to 100 ml.
3.	Standard Querecitin	Dissolve 10mg of querecitin in 10ml of methanol (Stock solution). Take 1 ml of stock solution and make up volume to 10ml with methanol.

Procedure

1. Take 0.1ml of juice sample and add 0.4ml of methanol, 1ml of aluminium chloride and 1ml of potassium acetate.
2. Vortex and incubate for 45min in dark condition.
3. Measure the absorbance of samples at 415nm against reagent blank and calculate flavonoid content by comparing with standard curve. Express results as mg Querecitin per 100 ml of the sample.

Reference: Giri, N. A., Sakhale, B. K., & Krishnakumar, T. (2022). Nutrient composition, bioactive components, functional, thermal and pasting properties of sweet potato flour-incorporated protein-enriched and low glycemic composite flour. Journal of Food Processing and Preservation, 46(2), e16244.

Total antioxidant capacity by FRAP method

Reagents

1.	0.3M Acetate buffer	Dissolve 0.31 g Sodium acetate trihydrate in 1.6 ml of glycial acetic acid and make up volume to 100 ml with distilled water.
2.	40mM HCl	Add 0.36 ml of HCl (33%) and make up volume to 100 ml with distilled water.
3.	10mM TPTZ (2, 4, 6-tripyridyl-s-triazine)	Dissolve 0.0312 g of TPTZ in 10 ml of 40mM HCl.
4.	20mM $FeCl_3.6H_2O$	Dissolve 0.054 g FeCl3 in 10 ml of distilled water.
5.	FRAP reagent	FRAP reagent should be pre-warmed at 37 °C and should always be freshly prepared by mixing 2.5 ml of a 10 mM TPTZ solution in 40 mM HCl with 2.5 mL of 20 mM FeCl3,.6H2O and 25 mL of 0.3 M acetate buffer pH 3.6.

Procedure

1. Take 150µl juice sample and mix it with 4.5ml FRAP reagent.
2. Vortex and incubate for 30min at 37°C.
3. Prepare standard curve using varying concentrations of ascorbic acid (20-100µg/ml).
4. Take absorbance at 595nm.
5. Express results as ascorbic acid equivalent antioxidant capacity (AEAC) and mg of Ascorbic acid per 100 ml of the sample.

Reference: Gaikwad, N. N., Pal, R. K., Suryavanshi, S., Babu, K. D., Maity, A., & Sarkar, S. K. (2017). Effect of extraction method and thermal processing on retention of bioactive compounds of pomegrante (*Punica granatum*) (cv. Bhagwa) juice. Indian Journal of Agricultural Sciences, 87(11):1445-52.

Total antioxidant activity by DPPH method

Reagents

1.	Methanol
2.	0.1 mM DPPH (2, 2-Diphenyl-1-picrylhydrazyl)

Procedure

1. Take 0.5ml juice sample in test tube.
2. Add 0.5ml methanol and 1ml DPPH reagent.

3. Incubate for 30min at room temperature.
4. Measure absorbance at 517nm.
5. Use methanol as blank, and methanol and DPPH reagent in 1:1 ratio as control.
6. Calculate using following formula:

$$\text{Antioxidant activity (\%)} = \frac{OD\ of\ control - OD\ of\ sample}{OD\ of\ control} \times 100$$

Reference: Bersuder, P., Hole, M. and Smith, G. (1998) Antioxidants from a Heated Histidine-Glucose Model System. I: Investigation of the Antioxidant Role of Histidine and Isolation of Antioxidants by High-Performance Liquid Chromatography. Journal of the American Oil Chemists' Society, 75, 181-187.

Reducing Sugars

Reagents

1.	Dinitrosalicylic acid	Dissolves simultaneously 1gm of DNS, 200mg crystalline phenol and 50mg sodium sulphite in 100ml of 1% NaOH solution by continuous stirring. Store at 4°C. Add sodium sulphate at the time of use.
2.	40% sodium potassium tartrate	Dissolve 40gm sodium potassium tartrate in 100ml distilled water
3.	80% ethanol	Dilute as per the available concentration
4.	Glucose	For standard preparation

Procedure

1. Pomegranate juice was diluted with water to 0.5:10 ratio.
2. Diluted juice (25µl) was further mixed with 1.3ml of distilled water and 1.5ml of DNS solution.
3. Vortex the reaction mixture and heat for 10min in boiling water bath.
4. After colour development, add 500µl of 40% sodium potassium tartrate to warm tubes.
5. Vortex and cool tube under running tap water.
6. Measure the absorbance at 510nm using reagent blank.
7. Calculate amount of reducing sugars in samples using standard graph prepared from standard glucose solution in same manner.

Reference: Thimmaiah SR (2009). In: *Standard methods of biochemical analysis,* Kalyani Publishers, New Delhi. Pp. 64-65.

Total Starch

Reagents

1.	80% Ethanol	Dilute as per the available concentration
2.	52% perchloric acid	Dilute as per the available concentration
3.	Anthrone reagent	Dissolve 200mg anthrone in 100ml of concentrated sulphuric acid. Prepare fresh and chill before use.

Extraction:

1. Take 100mg of aril/ dry peel sample and add 3ml of 80% ethanol. Grind the sample in ethanol properly.
2. Vortex and centrifuge at 10,000 rpm for 10 min.
3. Collect the supernatant.

4. Re-extract the residue with 3ml of 80% ethanol and centrifuge again at 10,000 rpm for 10 min.
5. Pool the supernatants received at step no. 3 and 4 in same tube.
6. Extract the residue with 5ml of distilled water and 6.5ml of 52% perchloric acid.
7. Incubate the samples in refrigerator for 10 min.
8. Centrifuge at 10,000 rpm for 10 min and collect the supernatant in 25ml volumetric flask.
9. To residue, add 6.5ml of 52% perchloric acid, vortex and centrifuge at 10,000 rpm for 10 min.
10. Pool the supernatant of step 8 and 9 and make up volume to 25ml with distilled water.

Analysis:

1. Take 0.1ml of extract from step 10 and add 0.9ml of distilled water.
2. Add 4ml of freshly prepared anthrone reagent.
3. Heat the tubes in boiling water bath for 10min.
4. Cool to room temperature and measure absorbance at 630nm against reagent blank.

Reference: Mccready RM, Guggolz J, Silviera V and Owens HS (1958) Determination of starch and amylose in vegetables. *Anal Chem* 22: 1156

Cellulose

Reagents

1.	Acetic /Nitric reagent	Mix 150ml of 80% acetic acid with 15ml of concentrated nitric acid.
2.	Anthrone reagent	Dissolve 200mg anthrone in 100ml of concentrated sulphuric acid. Prepare fresh and chill before use
3.	67% sulphuric acid	Dilute as per the available concentration.
4.	Standard	Stock solution: 100mg cellulose in 10ml of 67% sulphuric acid Working solution: Dilute 1ml of stock solution to 100ml with distilled water.

Extraction:

1. Grind 500mg aril/dry peel sample in 3ml of acetic/nitric acid reagent properly and vortex.
2. Heat the samples in water bath at 85°C for 45min.
3. Bring the samples to room temperature and centrifuge for 20min at 8000rpm.
4. Discard the supernatant and wash the residue with distilled water.
5. Add 10ml of 67% sulphuric acid to the tubes and incubate it for 1hr.

Analysis:

1. Take 1ml of sample from step 5 and dilute the sample to 100ml with distilled water.
2. To 0.5ml diluted solution, add 5ml of anthrone reagent and vortex.
3. Heat the tubes in boiling water bath for 10min.
4. Cool to room temperature and measure absorbance at 630nm against reagent blank.
5. Calculate the cellulose content from standard graph prepared using same steps as mentioned in the analysis section.

Reference: Thimmaiah SR (2009). In: *Standard methods of biochemical analysis*, Kalyani Publishers, New Delhi. Pp. 64-65.

Crude Fiber

Reagents

1. 0.255N Sulphuric acid
2. 0.313N Sodium hydroxide

Procedure

1. Take 2-4g of fat free sample in Fiber crucibles and fit them to socket of Fiber Plus system.
2. Pour 150ml of warm 0.255N sulphuric acid into crucible from the top and boil at 400°C for 30min.
3. Drain the acid and wash the sample thrice with hot DW.
4. Pour 150ml of 0.313N sodium hydroxide into the extractor from the top and boil at 400°C for 30min.
5. Drain the alkali and wash the sample thrice with hot DW.
6. Dry the crucibles in hot air oven till constant weight is obtained.
7. Then place dried crucible in muffle furnace for ashing at 550°C for 2-3hr.
8. Calculate fiber % as per the formula given below.

Instrument for fiber estimation

Calculation

$$\text{Crude Fiber \%} = \frac{(\text{Weight of residue after drying} - \text{weight of ash})\ X\ 100}{\text{weight of sample}}$$

Reference: AOAC (2011) Official methods of analysis of the association of official analytical chemists, 18th Edition, Washington DC

Pectin

Reagents

1. 20mM Nitric acid
2. Ethanol

Extraction & Analysis

1. Take 1g peel powder in 50ml of 20mM nitric acid.
2. Stir at 400rpm and 86°C for 80min.
3. Allow to cool at room temperature and filter through cheese cloth.
4. Add 2 volumes of 96% ethanol to filtrate for pectin precipitation.
5. Incubate at 4°C for 1hr.
6. Centrifuge at 8000rpm for 20min at 10°C.
7. Wash the precipitate with 50% ethanol followed by 75% ethanol and 2 times with 100% ethanol.
8. Centrifuge at 5000rpm for 10 min at 10°C.
9. Dry the precipitate at 45°C to constant weight.
10. Estimate pectin as ratio between weight of powdered pectin to weight of flour raw material (% W/W), both on dry basis.

Reference: Abid M, Cheikhrouhou S., Renard CMGC, Bureau S, Cuvelier G, Attia H, Ayadi MA (2017) Food Chemistry Characterization of pectins extracted from pomegranate peel and their gelling properties 215, 318-325

Epicuticular Wax Content (EWC)

Reagents

1.	Chloroform	
2.	Acidic potassium dichromate	Dissolve 20gm potassium dichromate in 40ml distilled water and slowly add 1L of conc. sulphuric acid.

Procedure

1. Collect mature leaf and fruit from field.
2. Clean sample using cotton and make disc of leaves/rind with cork borer (note the weight and area of sample taken).
3. Immerse the sample discs in 10ml chloroform.
4. Shake the sample tubes for 30sec. on electronic shaker.
5. Transfer chloroform to another test tube and allow to evaporate completely.
6. Add 5ml of acidic potassium dichromate to dried tubes (step 5).
7. Heat in boiling water bath for 30min.
8. Cool to room temperature and adjust the volume to 12ml with distilled water.
9. Measure the absorbance at 590nm against blank prepared using 5ml of acidic potassium dichromate raised to 12 ml with DW.

Calculation

$$\text{EWC (mg/cm}^2) = \frac{OD590 \times Weight\ of\ sample}{(Area\ of\ sample)2}$$

Reference: Ebercon A, Blum A, Jordan WR (1977) A rapid colorimetric method for epicuticular wax content of Sorghum leaves. Crop Science, 17 (1): 179-180.

Total Soluble Protein

Reagents

1.	0.1N sodium hydroxide	Take 4gm sodium hydroxide and make volume to 1L with DW
2.	15% trichloroacetic acid	Take 15gm trichloroacetic acid and make volume to 100ml with DW
3.	Solution A	2% sodium carbonate in 0.1N NaOH
4.	Solution B	0.5% copper sulphate in 1% sodium potassium tartarate
5.	Solution C	Mix 50ml solution A and 1ml solution B. Prepare fresh.
6.	Solution D	Mix Folin-Ciocalteu phenol reagent and distilled water in equal volume
7.	Standard	Stock solution: 20mg Bovine serum albumin in 100ml DW

Extraction

1. Take 1gm fresh or 100mg dry peel/ aril/leaf sample in 5ml of 0.1N NaOH.
2. Vortex and centrifuge at 10,000 rpm for 10min.
3. Collect the supernatant and make volume to 5ml with 0.1N NaOH.
4. Add 1ml of 15% trichloroacetic acid.
5. Vortex and incubate sample at 4°C for 24hr.
6. Centrifuge the sample at 5000rpm for 20min.
7. Discard supernatant.
8. Dissolve the residue (precipitates) in 5ml of 0.1N NaOH.

Analysis:

1. Take 0.5ml extract and add 5ml of solution C to it.
2. Shake the sample and keep it for 5min.
3. Add 0.5ml of solution D and incubate at room temperature for 60min.
4. Measure the absorbance at 570nm.
5. Prepare the standard curve form Bovine Serum albumin in the same way.

Reference: Lowry OH, Rosenbrough RJ, Fern AL, Randall RJ (1951). Protein measurements with Folin-phenol reagent. *Journal of Biological Chemistry.* **193**: 263-75.

Total soluble sugars (TSS)

Measure the total soluble sugars of juice sample using digital pocket refractometer values corrected to 20°C expressed as °Brix.

Reference: Gaikwad, N. N., Pal, R. K., Suryavanshi, S., Babu, K. D., Maity, A., & Sarkar, S. K. (2017). Effect of extraction method and thermal processing on retention of bioactive compounds of pomegrante (*Punica granatum*) (cv. Bhagwa) juice.

Determination of Crude Fat

Reagents

1.	Petroleum ether or Hexane

Procedure

1. Take 1-3g dry sample into thimble and plug properly with cotton plug.
2. The thimble is then place in pre-weighed empty soxlet beakers.
3. Pour 80ml of anhydrous ether in beakers.
4. Place these beakers to the SOCS-PLUS system.
5. Run the system at 70°C and cool in desiccator.
6. Weight beakers along with extract.

Instrument used for fat estimation

Calculation

$$\text{Crude Fat \%} = \frac{Weight\ of\ fat\ in\ gm}{Weight\ of\ sample\ in\ gm} \times 100$$

Reference: AOAC (2011) Official methods of analysis of the association of official analytical chemists, 18th Edition, Washington DC

Acid Value (AV) and Free Fatty acid (FFA)

Acid Value (AV)

Reagents

1.	95% Ethanol	Take 95 ml ethanol and make final volume to 100 ml with distilled water.
2.	Phenolphthalein indicator	
3.	0.1N potassium hydroxide	Dissolve 5.6 g of KOH in distilled water and make up final volume to 1000 ml with distilled water.

Procedure

1. Take 10 g of oil sample and add 25 ml of 95% ethanol.
2. The mixture was boiled for about 5 min and titrated while hot against 0.1 N KOH solution shaking vigorously during the titration.
3. A drop of phenolphthalein was used as an indicator. The endpoint of titration was the appearance of a persistent pink color.

Calculation

$$AV = \frac{56.1 \times V \times N}{W}$$

where V = volume of standard potassium hydroxide (ml), N = normality of the potassium hydroxide solution, and W = weight of the sample (g).

Free Fatty Acid (FFA) = Acid value x 0.503%

Reference: Gaikwad, N. N., Kalal, A. Y., Suryavanshi, S. K., Patil, P. G., Sharma, D., & Sharma, J. (2021). Process optimization by response surface methodology for microencapsulation of pomegranate seed oil. *Journal of Food Processing and Preservation*, *45*(6), e15561.

Iodine Value (IV)

Reagents

1.	0.1 N $Na_2S_2O_3$	Dissolve 2.48 g $Na_2S_2O_3$ in distilled water and make final volume to 1000 ml with distilled water.

Procedure

1. Oil sample of 0.15 g is weighed accurately and dissolved in 25 ml carbon tetrachloride followed by the addition of 25 ml Wij's solution.
2. The mixture is shaken gently and placed in the dark for 30 min. At the end of incubation, 15 ml of potassium iodide solution is added followed by addition of 100ml distilled water.
3. The liberated iodine was titrated against standardized 0.1 N sodium thiosulphate solution using starch as an indicator until the blue color formed disappears after thorough shaking with stopper still on. Blank is titrated separately under similar conditions.

Calculation

$$IV = \frac{12.69 \times (B - S) \times N}{W}$$

where B = volume of 0.1 N $Na_2S_2O_3$ required to titrate the blank (ml), S = volume of 0.1 N $Na_2S_2O_3$ required to titrate the sample (ml), 12.69 = atomic weight of iodine, N = normality of $Na_2S_2O_3$, and W = weight of the oil sample (g).

Reference: Gaikwad, N. N., Kalal, A. Y., Suryavanshi, S. K., Patil, P. G., Sharma, D., & Sharma, J. (2021). Process optimization by response surface methodology for microencapsulation of pomegranate seed oil. *Journal of Food Processing and Preservation*, *45*(6), e15561.

Peroxide Value

Reagents

1.	Acetic acid: Chloroform 30:70 mixture	Mix 30 ml acetic acid in 70 ml choloform
2.	Saturated potassium iodide	
3.	Starch indicator	
4.	0.01M Sodium thiosulfate	Dissolve 2.5 g Sodium thiosulfate in distilled water and make final volume to 100 ml with distilled water.

Procedure

1. Take 1g of oil in 50ml conical flask.
2. Add 5ml of acetic acid: chloroform mixture and dissolve it.
3. Add 0.1ml of saturated potassium iodide.
4. Incubate for 30min in dark condition.
5. Add 6ml of DW and 1ml of starch indicator.
6. Titrate against 0.01M sodium thiosulfate solution.
7. Note the end point pink to reddish colour changes.

Calculation

$$\text{mEq./Kg} = \frac{Titer\ value \times Strenth\ of\ sodium\ thiosulfate}{(Weight\ of\ oil\ sample \times 1000)}$$

Peroxide value = meq. Oxygen*1000

Reference: Gaikwad, N. N., Kalal, A. Y., Suryavanshi, S. K., Patil, P. G., Sharma, D., & Sharma, J. (2021). Process optimization by response surface methodology for microencapsulation of pomegranate seed oil. *Journal of Food Processing and Preservation*, *45*(6), e15561.

Acidity

Reagents

1.	0.1N sodium hydroxide
2.	Phenolphthalein indicator

Procedure

1. Take 5ml juice and dilute to 50ml with DW.
2. Take 10ml diluted juice sample in conical flask.
3. Titrate against 0.1N sodium hydroxide using phenolphthalein as indicator.
4. Note the end point pink to yellowish-green.

Calculation

$$\text{Acidity (\%)} = \frac{Titer\ value \times Normality\ of\ NaOH \times 0.64 \times 100}{Volume\ of\ aliquat\ taken}$$

Reference: Gaikwad, N. N., Pal, R. K., Suryavanshi, S., Babu, K. D., Maity, A., & Sarkar, S. K. (2017). Effect of extraction method and thermal processing on retention of bioactive compounds of pomegrante (*Punica granatum*) (cv. Bhagwa) juice.

Moisture

Procedure

1. Take weight of petri plate in which sample will be kept for drying (W1).
2. Weigh the sample along with perti plate weight (W2).
3. Dry the sample in hot air oven at 105°C till constant weight is obtained.
4. Cool sample in desiccator.
5. Weigh the dried sample along with perti plate (W3)

$$\text{Moisture \%} = \frac{(W3-W1)}{(W2-W1)} \times 100$$

Color Measurement

Procedure:

1. Switch on the LabScan XE system. Open the Easy Match QC software.
2. Select Add Sensor to install a new sensor. The Setup Sensor screen allows selection of the instrument model and the communications port. Select Next when ready.
3. Place the desired port plate at the reflectance port and snap it into place.
4. **Standardization:** The LabScan XE must be standardized on a regular basis to keep it operating properly. The instrument can be standardized at any time by selecting Sensor > Standardize or by clicking the Standardize button on the default toolbar.

a. Place black glass at the port to begin and press Next to continue.
b. Place the calibrated white tile at the port and press Next to continue.

5. After standardization, place the sample on the port plate and click on the 'read sample' ('read standard' in case of standard sample) option on the toolbar.
6. The results are generated in the form of *L*, *a*, *b* values.

Instrument used for color measurement

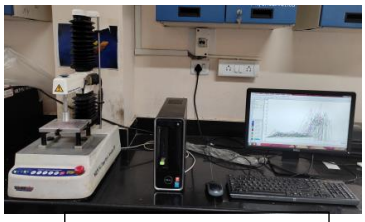
Reference: Gaikwad, N. N., Pal, R. K., Suryavanshi, S., Babu, K. D., Maity, A., & Sarkar, S. K. (2017). Effect of extraction method and thermal processing on retention of bioactive compounds of pomegrante (*Punica granatum*) (cv. Bhagwa) juice.

Texture

Procedure

1. Switch on the Stable Microsystems Texture analyser, open the Exponent lite software.
2. Attach the required probe.

3. Click on the file option > New > Graph > Ok
4. Select T.A. > T.A. Settings > Calibrate > Calibrate height
5. Click on T.A. > T.A. settings > Library > Return to start > Ok. Set the test parameters (pre- test speed, test speed, post- test speed, target mode,…..)
6. Select T.A. > Run a test. Fill the test details like file Id, file number, select path to save test data in the 'Archieve information' dialogue box. In probe selection dialogue box, select the probe to be used in the analysis depending on the sample. Set data acquisition as per the requirement. Once the parameters are set, give 'Apply' command. Place the sample on the platform and click on 'start test'.
7. A graph will be generated.



Texture analyzer

Viscosity

1. Plug in the power and turn on the instrument. Now, adjust the level of instrument by fine tunning the right and left screw on the bottom of the device until the circle on the display turns yellow and as soon as stabilize to green. Now tap apply, make sure that no spindle is attached.
2. Attach the spindle guard followed by spindle. Now position the sample beaker under the instrument and adjust the height with knob on the side. After attaching your spindle, the tool master feature automatically detects which spindle in use. Fix the spindle guard with the screw on the back side of the flange.
3. Make sure that the instrument is properly adjusted to the immersion mark of the attached spindle.
4. Go to Menu > true guard > disable > measurement settings > use unit.
5. Again menu > service > trusine (any mechanical error related to auto zero then enter trusine option and wait)
6. In display always check for green √
7. Note: Make sure to measure in the valid torque range of 10 to 100%. If you are outside of the range, choose another speed from the speed list.
8. Use the true mode feature should be used if the spindle speed combination is unknown for any sample, enter the desired time for measurement, the instrument then automatically adjusts the speed to a torque value of approximately 80%.

Viscometer

Total Tannin

1. Poly vinyl polypyrolidone (PVPP)
2. 7.5% sodium carbonate
3. Folin-Ciocalteu reagent

Procedure

1. Take 0.1gm PVPP in 1ml Distilled water.
2. Add 1ml of sample and vortex for 30sec.
3. Centrifuge at 4000rpm for 10min.

4. Incubate supernatant at 4°C for 15min.

Reaction

1. Take 0.2ml of supernatant in test tube.
2. Add 5ml of Folin-Ciocalteu reagent and 4ml of 7.5% sodium carbonate solution.
3. Incubate for 30min.
4. Measure absorbance at 765nm against reagent blank.

Total tannin content = Total phenol content (in sample extract without PVPP) – Total phenol content (in sample extract treated with PVPP)

Reference: Makkar, H.P.S., Bluemmel, M., Borowy, N.K., Becker, K. (1993). Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. J. Sci. Food Agric. 61, 161-165.

Peroxidase

Reagents

1.	0.1M potassium phosphate buffer (pH 7.0)	
2.	Extraction buffer	0.1M potassium phosphate buffer containing A) 0.5mM EDTA B) 0.1mM phenylmethylsulfonyl fluoride C) 2% polyvinylpyrrolidone(PVP) D) 5mM Ascorbate
3.	0.05M Guaiacol in 0.1M phosphate buffer	Take 5.59ml guaiacol (MW 124.14) and make volume to 1L with 0.1M phosphate buffer.
4.	0.8M Hydrogen peroxide	Take 9.07ml of hydrogen peroxide and make up volume to 100ml with DW

Extraction

1. Grind 250mg fresh tissue in pre-chilled mortar-pestle with 1.5ml of 0.1M phosphate buffer.
2. Centrifuge the homogenate at 12,000rpm for 30min in refrigerated centrifuge at 4°C.
3. Collect the supernatant and use as enzyme extract for assay of peroxidase activity.

Analysis

1. Take 3ml of 0.1M phosphate buffer containing 0.05M guaiacol in spectrophotometric cuvette.
2. Add 0.1ml enzyme extract.
3. Initiate the reaction by addition of 0.1ml of 0.8M hydrogen peroxide.
4. The rate of change in absorbance at 470nm after every 15sec for 3min on UV-spectrophotometer.
5. Reaction mixture without hydrogen peroxide was used as blank.
6. Express the enzyme activity as µmoles/min/g FW.

Note: Maintain sample at low temperature throughout the analysis.

Reference: Shannon LM, Kay E, Lew JY (1966). Peroxidase isozymes from Horseradish roots I. Isolation and physical properties. *Journal of Biological Chemistry*. **241**: 2166-72

Catalase

Reagents

1. Potassium phosphate buffer (50 mM, pH 7.0)
2. H_2O_2 solution (10 mM)

Extraction:

1. Approximately 200 mg of leaf/aril/rind tissue was weighed and extracted with chilled 50mM potassium phosphate buffer, pH 7.0 in precooled mortar and pestle.
2. The samples were centrifuged at 15,000*g* for 20 min at 4◦C.
3. Remove the supernatant and re-extract the pellet with 0.8 ml of the same buffer, and the suspension centrifuged for another 15 min at 15,000×*g*.
4. The combined supernatants were stored on ice and used to determine catalase activities.

Procedure:

1. The decomposition of H_2O_2 was followed as a decrease in absorbance at 240 nm in a UV/Vis spectrophotometer.
2. The 3ml assay mixture contained 2 ml leaf extract (diluted 200 times in 50mM potassium phosphate buffer, pH 7.0) and 10mMH_2O_2.
3. The extinction coefficient of H_2O_2 (40 mM^{-1} cm^{-1} at 240 nm) was used to calculate the enzyme activity that was expressed in terms of millimoles of H_2O_2 per minute per gram fresh weight).

Reference: Aebi, H. and Lester, P. (1984) Catalase in vitro. *Meth Enzymol*, 121–126.

Superoxide Dismutase

Reagents

1. 0.1M Potassium Phosphate Buffer (pH 7.0)
2. Extraction buffer — 0.1M Potassium Phosphate Buffer containing
   a) 0.5mM EDTA
   b) 0.1mM Phenylmethylsulfonyl Fluoride
   c) 2% Polyvinylpyrrolidone (PVP)
   d) 5mM Ascorbate
3. 200mM Methionine
4. 2.25mM Nitrobluetetrazolium Chloride (NBT)
5. 3mM EDTA
6. 100mM Phosphate Buffer (pH 7.8)
7. 1.5M Sodium Carbonate
8. 2µM Riboflavin

Extraction

1. Grind 250mg fresh tissue in pre-chilled mortar-pestle with 1.5ml of 0.1M phosphate buffer.
2. Centrifuge the homogenate at 12,000rpm for 30min in refrigerated centrifuge at 4°C.
3. Collect the supernatant and use as enzyme extract for assay of superoxide dismutase activity.

Analysis

1. Three ml of reaction mixture contained:
   a) 0.2ml of 200mM Methionine
   b) 0.1ml of 2.25mM Nitrobluetetrazolium Chloride
   c) 0.1ml of 3mM EDTA
   d) 1.5ml of 100mM Phosphate Buffer pH 7.8
   e) 0.1ml of 1.5M Sodium Carbonate
   f) 0.1ml enzyme extract
2. Finally make up volume to 3ml with DW.
3. Reaction started by adding 0.1ml of 2µM riboflavin and placing the tube under two 15W fluorescent lamps for 15min.
4. Complete reaction without enzyme to be used as control and only buffer to be used as blank.
5. Rate of reaction measured at 560nm in UV-Visible spectrophotometer.

Calculation

$$1unit\ (of\ enzyme) = \frac{Blank - sample}{Blank/2}$$

Reference: Dhindsa R.S., Plumb-Dhindsa S.P. & Thorpe T.A. (1981) Leaf senescence correlated with increased level of membrane permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase. J. Exp. Bot. 126: 93–101.

Polyphenol Oxidase

Reagents

1.	0.1M potassium phosphate buffer (pH 6.5)	
2.	0.1M potassium phosphate buffer (pH 7.2) containing 1% TritonX-100	Dissolve 5.05g of Catechol in 1L of 0.1M Potassium Potassium Phosphate buffer pH 6.5.
3.	0.5M catechol	

Extraction & Analysis

1. Take 0.5g of fresh arils and 2.5ml ice cold potassium phosphate buffer pH 7.2 containing 1% Triton-X100.
2. Homogenise for 2min in chilled condition.
3. Centrifuge at 10,000g and 4°C for 30min.
4. In test tube, add 2.5ml of 0.1M potassium phosphate buffer pH 6.5, 0.3ml of 0.5M catechol and 0.2ml of enzyme extract.
5. Record the change in absorbance every 30sec up to 3min in UV-Visible spectrophotometer.

Reference: Jaiswal A, DerMarderosian A, Porter JR (2010) Anthocyanins and polyphenol oxidase from dried arils of pomegranate (*Punica granatum* L.). Food Chemistry 118, 11–16

α-Amylase Activity

Reagents

1.	10mM Calcium Chloride	
2.	0.1M Sodium acetate buffer (pH 4.7)	
3.	1% Starch solution	Dissolve 1g starch in 100ml of sodium acetate buffer (prepare fresh, slightly warm if required)
4.	Dinitrosalicylic acid solution (DNS)	Dissolve 1g of dinitrosalicylic acid, 200mg crystalline phenol and 50mg sodium sulphite in 100ml of 1% Sodium hydroxide solution. Store at 4^0C and sodium sulphite add at the time of use
5.	40% Rochelle salt	40g potassium sodium tartrate and make up the volume to 100ml with DW
6.	Maltose solution	Dissolve 50mg maltose in 50ml DW

Extraction

1. Grind 500mg of fresh arils in 4ml of ice cold 10mM Calcium Chloride.
2. Incubate for 3hr at room temperature or overnight at 4°C.
3. Centrifuge at 5400g at 4°C for 20min.
4. Collect supernatant and use for enzyme activity estimation.

Analysis

1. Mix 1ml enzyme extract with 1ml starch solution.
2. Incubate at 27°C for 15min.
3. Stop the reaction by addition of 2ml DNS solution.
4. Heat in boiling water bath for 5min.
5. Add 1ml Rochelle solution to warm tubes and cool under tap water.
6. Make up volume to 10ml by adding 5ml DW.
7. Measure absorbance at 560nm.
8. Terminate reaction at zero time in control tubes.
9. Prepare standard curve with maltose in same way.
10. Express α amylase activity as mg of maltose produced during 5min incubation with 1% starch.

Reference: Bergmeyer HU (1983) Methods of Enzymatic Analysis, 3rd edn., vol.2, pp 151-152, Verlag Chemie, Weinheim

Polygalacturonase Activity

Reagents

1.	17mM Tris-HCL buffer containing 5mM 2-mercaptoethanol (pH 10)	
2.	Extraction buffer	0.1M sodium citrate buffer containing 1.7M sodium chloride and 15mM EDTA. Add 9.93g of sodium citrate, 2.58g of sodium chloride and 0.44g of EDTA in 80ml DW. Adjust pH to 5.5 and make up volume to 100ml with DW
3.	2M Ammonium chloride	Dissolve 10.7g of ammonium chloride in 100ml DW
4.	1% Polygalacturonic acid	Dissolve 1g polygalacturonic acid in 100ml DW
5.	5% Trichloroacetic acid	Dissolve 5g trichloroacetic acid in 100ml DW

Extraction

1. Homogenize 5g tissue and 6.5ml of 17mM Tris-HCl (pH 10) in chilled condition.
2. Centrifuge at 15000g and 4°C for 15min.
3. Incubate pellets in 2.5ml extraction buffer for 60min.
4. Centrifuge for 15000g and 4°C for 30min and use supernatant for assay.

Analysis

1. Take 0.1ml Ammonium Chloride and 1ml Polygalacturonic acid in test tube.
2. Incubate at room temperature for 5min.
3. Initiate reaction by addition of 0.1ml enzyme extract and incubate for 30min.
4. Terminate reaction by adding 0.3ml of 5% trichloroacetic acid.
5. Centrifuge at 2000g for 30min.
6. Collect the supernatant and use for sugar estimation.
7. By using 1ml aliquot and 0.5ml DW estimate sugar by DNS method (develop colour as mentioned under reducing sugar method).
8. Prepare standard curve with α-D-galacturonate or D-glucose.

Reference: Thimmaiah SR (2009). In: *Standard methods of biochemical analysis*, Kalyani Publishers, New Delhi. Pp. 64-65.

β-galactosidase

Reagents

1. Extraction buffer- 10mM Tris-HCl containing 0.1% Triton-X100	Dissolve 157.6mg Tris HCl in 100ml DW
2. 0.1M Sodium acetate buffer (pH 5)	(A) 0.1M Sodium acetate: Dissolve sodium acetate 0.8203g in 100ml DW (B) 0.1M Acetic acid: 0.6ml acetic acid in 100ml DW Mix 82.5ml solution A and 17.5ml solution B. Adjust pH to 5
3. 10mM p-nitrophenol-β-D-galactosidase (PNPG)	Dissolve 30.125mg of p-nitrophenol-β-D-galactosidase in 10ml 0.1M sodium acetate buffer, pH 5.0
4. 0.1M NaOH	Dissolve 0.4g sodium chloride in 100ml DW
5. p-nitrophenol standard	Stock solution: Dissolve 1mg in 1ml DW

Extraction

1. Homogenize 1g tissue and 10ml ice-cold extraction buffer.
2. Centrifuge at 10,000g and 4°C for 20min.
3. Collect supernatant and use for enzyme assay.

Analysis

1. Take 0.25ml of 0.1M sodium acetate buffer (pH 5) and 0.01ml of 10mM PNPG in test tube.
2. Incubate at 55°C for 10min.
3. Initiate reaction by adding 15µl of enzyme extract and incubate for 10min.
4. Stop reaction by adding 4ml of 0.1M sodium hydroxide.
5. Prepare standard of p-nitrophenol of different concentration.
6. Record OD at 410nm against reagent blank.

Reference: Thimmaiah SR (2009). In: *Standard methods of biochemical analysis*, Kalyani Publishers, New Delhi. Pp. 64-65.

Malondialdehyde

Reagents

1.	0.1% Trichloroacetic acid	Dissolve 0.1gm trichloroacetic acid in 100ml DW
2.	Thiobarbituric acid reagent	Dissolve 0.5g thiobarbituric acid in 100ml of 20% trichloroacetic acid

Extraction & Analysis

1. Take 150mg fresh leaf/ aril/ peel sample and grind in 2ml of 0.1% trichloroacetic acid.
2. Centrifuge at 12,000g for 30min.
3. Take 1ml supernatant and add 2ml of thiobarbituric acid.
4. Incubate at 90°C for 45min.
5. Stop the reaction by cooling tube in ice bath for 10min.
6. Centrifuge at 10,000g for 15min.
7. Measure the absorbance at 532nm and 600nm.

Note: The amount of malondialdehyde present is calculated from extinction coefficient of $155mM^{-1}cm^{-1}$.

Reference: Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125: 189–198

Proline

Reagents

1.	3% Sulphosalicylic acid	
2.	Glacial acetic acid	
3.	Acidic ninhydrin	Dissolve 1.25g ninhydrin, 30ml glacial acetic acid and 20ml of 6 molar orthophosphoric acid. Dissolve by vortex and gentle warming
4.	Standard Proline	10mg/100ml

Extraction

1. Homogenize 200mg of tissue in 1.5 ml of 3% Sulphosalicylic acid.
2. Centrifuge at 10000rpm for 10 min and collect supernatant.

Analysis:

1. Mix 300µl 3% Sulphosalicylic acid, 600µl of glacial acetic acid, 600µl of acidic ninhydrin and 300µl of supernatant.
2. Incubate the tubes at 96°C for 1 hr.
3. Terminate the reaction by placing the tubes on ice
4. Add 2ml toluene to each tube.
5. Vortex and incubate for 5 min at room temperature
6. Pick the clean colored layer in fresh tube.
7. Read the absorbance at 520nm against blank (toluene).
8. Prepare the standard of varying concentrations using same procedure.

Reference: Thimmaiah SR (2009). In: *Standard methods of biochemical analysis*, Kalyani Publishers, New Delhi. Pp. 64-65.

Hydrogen peroxide generation

Reagents

1.	0.1% Trichloroacetic acid	Dissolve 0.1gm trichloroacetic acid in 100ml DW
2.	1M potassium phosphate buffer (pH 7.0)	
3.	1M potassium iodide	Take 16.6g of potassium iodide and make volume to 100ml with DW
4.	Standard	Stock solution: Take 2.27ml of 30% H_2O_2 and make volume to 1L with DW

Extraction & Analysis

1. Take 150mg fresh leaf sample and grind in 2ml of 0.1% trichloroacetic acid.
2. Centrifuge at 12,000g for 30min.
3. Take 0.3ml supernatant in test tube.
4. Add 1.7ml of 1M potassium phosphate buffer and 1ml of 1M potassium iodide.
5. Incubate at room temperature for 15min.
6. Prepare standard curve of H_2O_2 using same method as mentioned above.
7. Measure the absorbance at 390nm.

Reference: Kumar N, Ebel RC (2016) Oxidative metabolism in 'Valencia' sweet orange (Citrus sinensis Osbeck) abscission zone tissue treated with the abscission agent 5-chloro-3-methyl-4-nitro-1H-pyrazole. Hortscience, 51(4): 377-382.

Membrane Thermal Stability

1. Cut 10 disc per sample (leaves) with cork borer.
2. Place disc in 20ml DW and heat at 49°C for 30min in water bath.
3. Incubate tube overnight at room temperature.
4. After incubation, measure conductivity (EC_1) using conductivity meter.
5. Autoclave tubes containing samples at 121°C and 15psi for 10min.
6. Bring to room temperature and measure conductivity (EC_2).
7. Calculate MTS using following formula:

$$\text{MTS (\%)} = [1 - \frac{(E1)}{(E2)}] \times 100$$

Note: Calibrate conductivity meter with standard KCl solution.

Reference: Shanahan JF, Edwards IB, Quick JS, Fenwick JR (1990) Membrane Thermostability and Heat Tolerance of Spring Wheat**.** Crop Ecology, Production & Management. 30(2): 247-251

Total Chlorophyll Content

Reagents

1. DMSO (Dimethyl sulphoxide)

Procedure

1. Take frozen leaf sample or chopped tissue (100mg).
2. Place in a tube containing 7ml DMSO and incubate at 65°C for 30 min.
3. Transfer the sample to a 25ml graduated tube.
4. Raise the volume to 10ml with DMSO.

5. Transfer 1ml of aliquot to a cuvette.
6. Calibrate spectrophotometer against DMSO at 645 and 663nm.
7. Record absorbance at 645 and 663nm.

***Calculation*:**

Chlorophyll 'a' (mg/g) = $[12.7(A663) - 2.69(A645)] \times \frac{Volume\ made}{1000 \times Weight}$

Chlorophyll 'b' (mg/g) = $[22.9(A645) - 4.68(A663)] \times \frac{Volume\ made}{1000 \times Weight}$

Total Chlorophyll (mg/g) = Chlorophyll 'a' + Chlorophyll 'b'

Where A is absorbance

Reference: Hiscox JD, Israelstam GF (1979). A method for the extraction of chlorophyll from leaf tissue without maceration. *Canadian Journal of Botany.* **57**:1332-1334.

Methods for Plant analysis

Plant sample washing & processing

Washing

1. Wash plant sample with tap water several times.
2. Then wash with 0.2% detergent, followed by washing with0.1N HCl and later with plenty of water.
3. Finally rinse the sample with DW.

Drying

1. Dry the sample at 60°C for 48 hr.
2. Or dry at clean surface for 2-3 days

Grinding

1. Grind the sample in electric grinder to fine powder.
2. Sieve with 0.5mm sieve.
3. Place sample in oven and dry again for few hours more to get constant weight.

Di-acid digestion procedure

1. Weigh 1gm of dried and processed plant sample in 100ml conical flask.
2. Add 2ml of concentrated HNO_3, place funnel on the flask and keep it for about 6-8 hr or overnight at covered chamber for pre-digestion.
3. After pre-digestion, when sample is no more visible, then add 10ml concentrated HNO_3 and 2-3ml perchloric acid ($HClO_4$). Or prepare mixture of concentrated HNO_3: $HClO_4$ (10:2) and add 10ml of this mixture.
4. Keep on a hot plate in acid-proof digestion chamber (or at open place) having fume exhaust system and heat about 100°C for 1st 1hr and then raise the temperature to about 200°C.
5. Continue digestion until the content become colorless and only white dense fumes appear.
6. Reduce the acid content to about 2-3ml by continuing heating at the same temperature. Do not allow to dry up completely.
7. Remove from hot plate, cool it and add 10ml of 2N HCl.
8. Warm slightly and filter through Whatman No. 42 filter paper into a 100ml volumetric flask.
9. Wash 3-4 times with double DW and make the volume to 100ml.

Plant Nitrogen

Reagents:

1.	0.1N H_2SO_4	Add 2.8ml conc. H_2SO_4 in 900ml DW and make up the volume to 1L.
2.	4% Boric acid mixed with indicator (pH 4.5)	
3.	Digestion accelerator mixture	3gm in each tube Potassium sulphate: Copper sulphate (30:1)
4.	Conc. H_2SO_4	
5.	40% NaOH	

A) Digestion

1. Weigh 1gm finely ground, dried, 0.5mm sieved plant sample in Kjeldahl flask.
2. Add 3gm of digestion accelerator mixture.
3. Add 12ml of conc. H_2SO_4.
4. Set digestion system to attain temperature of about 385°C and then place digestion tube in heating unit as per the instructions given in the operation manual.
5. Run the tap water with desired flow rate for safe disposal of fumes.
6. Allow the digestion to continue till completion.
7. Switch off the system.
8. Remove the rack of sample tubes along with the exhaust system or scrubber from heating unit. Do not stop water flow as the fumes continue to come for some more time.

B) Distillation:

1. Set the distillation unit to perform the various steps viz. dilution, addition of alkali, steam generation, titration, etc.
2. Keep boric acid 20ml in conical flask and run the distillation unit up to 100ml.
3. Titrate boric acid with 0.1N H_2SO_4.
4. Run blank similarly.

Observations to be recorded

1.	Weight of plant sample	W (g)
2.	Normality of H_2SO_4	N
3.	Volume of standard acid required to absorb liberated ammonia	X (ml)
4.	Volume of standard acid required to neutralize liberated ammonia	Y (ml)

Calculation:

$$\text{Nitrogen \%} = (X - Y) \times 0.014 \times \frac{100}{W}$$

Reference: Kalra YP (1998) Handbook of Methods for Plant Analysis. CRC Press Taylor & Francis Group.

Plant Phosphorous

Reagents

1.	Ammonium molybdate-vandate in HNO_3	A) Dissolve 25g ammonium molybdate in 400ml of DW B) Dissolve 1.25gm of ammonium metavandatein 300ml of boiling DW. C) Mix solution A and B and cool to room temperature. Add 250ml of conc. HNO_3 and dilute to 1L.
2.	Standard stock phosphorous solution (50ppm)	Dissolve 0.2195gm analytical grade potassium dihydrogen phosphate and dilute to 1L.
3.	Working standard (5ppm)	Dilute 10ml of standard stock solution (50ppm) to 100ml with DW.

A) Preparation of standard curve:

1. Transfer 0, 5, 10, 15, 20 and 25ml of working standard to 50ml volumetric flask to get 0.5, 1.0, 1.5, 2.0 and 2.5 ppm concentration, respectively.
2. Add vandate-molybdate reagent to each flask and incubate for 10 min. Make up the volume with DW.
3. Read absorbance at 470nm.

B) Determination of phosphorous

1. Take 10ml plant acid digested extract in 50ml volumetric flask.
2. Add 10ml vandate-molybdate reagent and incubate for 10 min.
3. Make the volume with DW.
4. Read absorbance at 470nm.

Observations to be recorded:

1. Flame photometer reading	R
2. Factor from standard graph	F
3. Phosphorous from standard curve (R×F)	P (ppm)
4. Volume of colored solution	VS (ml)
5. Volume of acid extract	VE (ml)
6. Aliquot taken	VA (ml)
7. Weight of sample	W (gm)

Calculation:

$$\text{Phosphorus \%} = P \times VS \times \frac{VE}{VA} \times \frac{100}{W}$$

Reference: Munk, H. (1960) The vanadate-molybdate method for phosphorus determination in Petermann's ammonium citrate extract.
Landw. Forsch. Sonderh. 14: 90-91.

Plant Potassium

Reagents:

1.	Standard stock potassium solution (1000ppm)	Dissolve 1.9069gm of oven dried AR grade crystals of KCl in 1L DW
2.	Working Standard	Prepare 5, 10, 15, 20, 30, 40 and 50ppm working standards from stock solution

Procedure

1. Switch on Flame photometer, adjust proper flame using air pressure and gas knobs.
2. Set potassium filter.
3. With DW adjust/set the reading to zero.
4. Take reading of the standards.
5. Draw standard curve in flame photometer.
6. Take reading of unknown sample (prepared by di-acid digestion procedure).
   Note: Dilute the sample, if required.

Observations to be recorded:

1. Flame photometer reading	R
2. Factor from standard graph	F
3. Volume of extract	V (ml)
4. Weight of sample	W (gm)
5. Dilution factor	DF

Calculation:

$$\text{Potassium \%} = R \times F \times V \times \frac{100}{W} \times DF$$

Reference: Mason JL (1963) Flame Photometric Determination of Potassium in Unashed Plant Leaves. Analytical Chemistry 1963 35 (7), 874-875

Plant Boron

Reagents

1.	Azomethine-H	Dissolve 0.45gm of azomethine-H in 100ml of 1% L-ascorbic acid solution in propylene reagent bottle. Prepare fresh and store in refrigerator, use for one week
2.	Buffer solution	Dissolve 250gm of ammonium acetate and 15gm EDTA (disodium) in 400ml DW. Add slowly 125ml of glacial acetic acid and mix.
3.	Boron Stock Standard solution (20mg/ml)	Dissolve 0.174g of boric acid in DW and adjust volume to 1L
4.	Working standard	Dilute 0.5, 1, 2, 3, 4, 5, 10, 20 ml of 20mg/ml Boron stock standard to 100ml DW. This will make 0.1, 0.2, 0.4, 0.6, 0.8,…..mg/L of boron respectively

Sample preparation:

1. Clean crucibles by heating on a hot plate with 10% HNO_3.
2. Place crucibles in drying oven at 80°C for 30 min.
3. Keep 1gm of sample for dry ashing.
4. Moisten ash in crucible with 8-10 drops of water followed by 3ml of 5M HCl. Care must be taken to ensure that there is no loss of sample due to effervesces.
5. Place crucible on hot plate at 80°C and add 0.25ml of conc. HNO_3.
6. Evaporate to dryness in order to solubilize phosphate and precipitate Silica.
7. Moisten dried salt from step 6 with 3ml of 5M HCl and warm on hot plate. Add 5ml water and maintain heat.
8. Filter the solution through Whatman filter paper no. 42 and make up the volume to 100ml with DW.
9. Wash crucible and filter paper with DW.
10. Make up the volume to 100ml with DW.
    Note: This solution cannot be used for As, S, Se, F or Cl analysis because of volatilization loss of compound.

Analysis:

1. Mix 1ml sample with 2ml buffer solution and 2ml of Azomethine-H reagent.
2. Mix and incubate for 30 min at room temperature.
3. Read absorbance at 420nm.

Reference: T. Powell Gaines & G. Allen Mitchell (1979) Boron determination in plant tissue by the azomethine H method, Communications in Soil Science and Plant Analysis, 10:8, 1099-1108.

Plant Calcium and Magnesium by Versanate method

Reagents

1.	Buffer solution	Dissolve 67.5gm of pure ammonium chloride in 570ml of concentrated ammonium hydroxide and raise volume to 1L (pH 10) with DW
2.	Eriochrome Black T (EBT) indicator	Dissolve 0.5gm EBT dye and 4.5gm hydroxylamine hydrochloride in 100ml ethanol

3.	EDTA solution (0.01N)	Dissolve 2gm of versenate/EDTA (disodium dihydrogen EDTA) in DW to make 1L. Standardize it by titrating against 0.01N $CaCl_2$
4.	Standard Calcium solution	Dissolve 0.5gm of pure dried $CaCO_3$ in minimum of 10ml concentrated HCl. Boil gently to expel the CO_2 and then dilute to 1L with DW.
5.	4N NaOH	160g NaOH raised to 1L
6.	Calcon indicator	Dissolve 0.5gm calcon reagent in 100ml ethanol

Procedure

Calcium + Magnesium determination

1. Pipette 5ml sample prepared by di-acid digestion in 100ml porcelain dish.
2. Add 20ml DW and 1-2 ml of ammonium hydroxide-ammonium chloride solution.
3. Add 2-3 drops of EBT indicator.
4. Titrate against standard EDTA solution till the color changes from wine red to blue.
5. Note the volume of EDTA

Observations to be recorded

1.	Volume of standard EDTA (sample- Blank)	E
2.	Volume of acid extract	V
3.	Volume of aliquot taken for analysis	V1
4.	Weight of sample	W
5.	Normality of EDTA	N (0.01)
6.	1ml of 1N EDTA	0.02g Ca

Calculation

$$\text{Ca+Mg (meq./Litre)} = \frac{E \times N \times 1000}{V} = A$$

$$\text{meg. Ca/100g} = \frac{100}{W} \times \frac{V}{1000} \times A$$

meg. Mg/100g = (meq. Ca+Mg/100g sample) – (meq. Ca/100g sample)

$$\text{Mg \%} = \frac{\frac{meq.\ Mg}{100g\ sample}}{1000} \times eq.Wt\ of\ Mg$$

Calcium Determination

1. Pipette 5ml sample prepared by di-acid digestion in 100ml porcelain dish.
2. Add 20ml DW and 5ml of 4N NaOH.
3. Add 2-3 drops of Calcon indicator.
4. Titrate against standard EDTA solution till color changes from orange red to purple.
5. Note the volume of EDTA.

Observations to be recorded

1.	Volume of standard EDTA (sample- Blank)	E
2.	Volume of acid extract	V
3.	Volume of aliquot taken for analysis	V1
4.	Weight of sample	W
5.	Normality of EDTA	N (0.01)
6.	1ml of 1N EDTA	0.02g Ca

Calculation

Calcium+Magnesium

$$\text{Calcium \%} = E \times N \times 0.02 \times \frac{V}{V1} \times \frac{100}{W}$$

$$\text{Total Ca (meg./L)} = \frac{E \times N \times 1000}{V1}$$

Reference: Kalra YP (1998) Handbook of Methods for Plant Analysis. CRC Press Taylor & Francis Group.

Plant Sulphur

1.	Hydrogen peroxide	
2.	Glacial acetic acid	
3.	Gelatin barium chloride solution	Dissolve 5g gelatin in 250ml DW in 1L capacity beaker. Heat up to dissolve. Then add 60g barium chloride and 120ml of 1N HCl. Filter through filter paper and dilute to 1L with DW
4.	Standard sulphur stock solution: 1000mg S/l	Dissolve 5.434g of oven dried K_2SO_4 in DW and dilute to 1L with DW
5.	Working standard 25mg S/l	Prepare working solution by diluting 2.5ml stock solution of 1000ppm to 100ml with DW

Procedure

A) Standard curve preparation

1. Prepare a series of standard by taking 0, 1, 2, 4, 6, 8 and 10 ml of 25ppm standard Sulphur solution in 25ml volumetric flask separately.
2. Add 2ml of 2% H_2O_2 in each flask, mix and wait for 10min.
3. Add 2ml of glacial acetic acid in each flask, mix and wait for 10min.
4. Add 2ml of gelatin barium chloride solution in each flask, mix and wait for 10min.
5. Raise the volume to 25ml with DW and measure absorbance at 420nm.
6. Make graph by plotting conc. v/s absorbance.

B) Estimation

1. Pipette out 10 ml of acid digested plant extract into 25ml volumetric flask.
2. Add 2ml of 2% H_2O_2 in each flask, mix and wait for 10min.
3. Add 2ml of glacial acetic acid in each flask, mix and wait for 10min.
4. Add 2ml of gelatin barium chloride solution in each flask, mix and wait for 10min.
5. Make the volume to 25ml with DW and measure absorbance at 420nm.

Calculation

$$\text{Sulphur (\%)} = \text{Sulphur from standard curve (ppm)} \times 0.025$$

Plant Micronutrients

1.	Standard stock solution of Zn 1000 mg/L	Dissolve exactly 1.0 g of pure Zn metal in about 10ml of dilute HCl (1:1) and make the volume to 1L.
2.	Standard stock solution of Mn 1000 mg/L	Dissolve exactly 1.583 g of pure MnO_2 in 50ml dilute HNO_3 (1:1) and make the volume to 1L.

3.	Standard stock solution of Fe 1000 mg/L	Dissolve exactly 1.0 g of pure Fe metal MnO_2 in 50ml dilute HNO_3 (1:1) and make the volume to 1L.
4.	Standard stock solution of Cu 1000 mg/L	Dissolve exactly 1.0 g of pure Cu metal MnO_2 in 50ml dilute HNO_3 (1:1) and make the volume to 1L
5.	Standard working solution	Prepare 0.5, 1.0, 2.0, 4.0 and 6.0 ppm working solution of each metal from standard stock solution

Procedure

1. Switch on AAS, adjust proper flame using air pressure and gas knobs.
2. Set desirable lamp.
3. Record readings of the standard micronutrients and prepare standard graph.
4. Extract sample using di-acid digestion method (already given under Di-acid digestion section).
5. Record sample reading in AAS.

Calculation

Micronutrient (mg/kg) = Absorbance × 100

Methods for Soil Analysis

Available Soil Nitrogen

Reagents

1.	0.32% Potassium permanganate	
2.	2.5% Sodium hydroxide Solution	
3.	0.02N Sulphuric acid	
4.	Mixed indicator	Dissolve Methyl red 0.066g and Bromocresol green 0.099g in 100ml ethanol
5.	2% boric acid mixed indicator	Add 20ml mixed indicator in 2% boric acid solution and adjust the pH 4.5 and make up the volume to 1L
6.	Paraffin oil	

Procedure

1. Process soil sample by drying and passing through 2mm sieve.
2. Transfer 20g of soil sample in Kjeldahl distillation tube and moist soil with DW.
3. Add 100ml of 0.32% potassium permanganate and 100ml of 2.5% sodium hydroxide solution.
4. Add 2 to 3 glass beads and few drop of paraffin oil to prevent bumping and frothing.
5. Immediately connect distillation tube to Kjeldahl assembly.
6. Distill content in Kjeldahl assembly at a steady rate and collect the liberated ammonia in the form of distillate in a 250ml flask containing 20ml of boric acid mixed indicator solution.
7. Collect approximately 100ml distillate and titrate with standard Sulphuric acid solution till the colour changes from green to original shade.
8. Carry out blank without soil.

Observations to be recorded

1.	Weight of soil sample taken	W (gm)
2.	Volume of standard acid required for soil	S (ml)
3.	Volume of standard acid required for blank	B (ml)
4.	Normality of sulphuric acid	N

Calculation

$$\text{Available N (kg/ha)} = \frac{(S-B)\times N\times 0.014\times 2.24\times 106}{\text{wt. of soil g}}$$

$$\text{Available N (kg/ha)} = (S - B) \times 31.36$$

$$\text{Available N (g/plant)} = \frac{\text{Available N (kg/ha)}}{740} \times 1000$$

Note: Primary standard 0.1N potassium hydrogen phthalate

i. For standardization of sodium hydroxide, potassium hydrogen phthalate is used as standard.
ii. For standardization of sulphuric acid, sodium hydroxide is used as standard.

Reference: Bremner, J.M., Mulvaney, C.S., et al., 1983. Nitrogen—Total. In: Page, A.L., et al. (Eds.), Methods of Soil Analysis–Part 2 Chemical And Microbiological Properties. Soil Science Society of America Inc, Madison, Wisc, pp. 595–624.

Available Soil Phosphorous

Reagents

1.	Olsen's reagent (0.5M $NaHCO_3$)	Dissolve 42g of P-free sodium bicarbonate in 500ml hot DW and adjust the pH to 8.5 with dil. NaOH or dil. HCL and make up the volume to 1L with DW.
2.	Activated charcoal	
3.	Ammonium molybdate	Solution A: Dissolve 12g ammonium molybdate in 250ml DW. Solution B: Dissolve antimony potassium tartrate in 100ml DW. In 1L of 5N H_2SO_4 add solution A and solution B. Mix well and make up the volume to 2L.
4.	Ascorbic acid	Dissolve 1.056g of ascorbic acid in 200ml ammonium molybdate solution and mix well
5.	p-nitrophenol indicator	Dissolve 0.5g p-nitrophenol in DW and dilute to 100ml
6.	5N sulphuric acid	
7.	Standard stock solution of phosphorous: 100mg/l	Dissolve 0.439g of AR grade dried KH_2PO_4 in 500ml DW. To this add 25ml of 7N H_2SO_4 and make up the volume to 1L with DW.
8.	5ppm working standard	5ml of 100ppm std stock solution diluted to 100ml with DW

Procedure

A) Standard curve preparation

1. Prepare a series of standards by taking 0.0, 2.5, 5.0, 7.5, 10.0 and 12.5 ml of 5ppm standard Phosphorous solution in 25ml volumetric flask separately.
2. Add 5ml of Olsen's reagent to each flask.
3. Add 4ml ascorbic acid solution, shake well and incubate for 10min at room temperature.
4. Raise the volume to 25ml with DW and measure the absorbance at 730nm on spectrophotometer.

B) Extraction

1. Take 2.5g of processed air dried soil in 100ml conical flask.
2. Add pinch of activated charcoal and 50ml of Olsen's reagent.
3. Shake for 30min on mechanical shaker and filter through Whatman no. 40 or 42 filter paper.

C) Estimation

1. Pipette out 5ml of Olsen's reagent into 25ml volumetric flask.
2. Add 2-3 drops of p-nitrophenol indicator to develop yellow colour.
3. Add 5N H_2SO_4 drop by drop to acidify Olsen's reagent to pH 5 till the yellow colour disappear (Note the volume of H_2SO_4 used).
4. Take 5ml filtrate sample in 25ml volumetric flask.
5. Add required quantity of 5N H_2SO_4.
6. Add 4ml ascorbic acid solution, shake well and incubate for 10min.

7. Make the volume to 25ml with DW and measure the absorbance at 730nm on spectrophotometer.

Observations to be recorded

1.	Concentration of Phosphorous from standard curve	Q (ppm)
2.	Volume for extraction used	V (ml)
3.	Aliquot of extract used for analysis	A (ml)
4.	Weight of soil sample taken	W (gm)

Calculation

$$\text{Available P (kg/ha)} = \frac{Q \times V \times 2.24}{A \times W}$$

$$\text{Available P (g/plant)} = \frac{\text{Available P (kg/ha)}}{740} \times 1000$$

Reference: Richards, J.E., Bates, T.E., 1989. Studies on the potassium-supplying capacities of southern Ontario soils. III. Measurement of available K. Can. J. Soil Sci. 69, 597–610.

Available Soil Potassium

Reagents

1.	1N Ammonium acetate solution (pH 7.0)	
2.	Standard stock solution of potassium: 1000mg/l	Dissolve 1.908g of AR grade dried potassium chloride in DW and dilute to 1L.
3.	Working standard	Prepare 5, 10, 15, 20, 30, 40 and 50 mg/l working standard from stock solution.

Procedure

1. Take 5g of processed air dried soil in 100ml conical flask.
2. Add 25ml of ammonium acetate solution and shake for 5min on mechanical shaker.
3. Filter through Whatman no. 40 or 42 filter paper.
4. Dilute 5ml extract to 25ml with DW.
5. Measure concentration of potassium on flame photometer.

Flame photometer

Observations to be recorded

1.	Concentration of K in ppm from standard curve	Q (ppm)
2.	Volume for extraction used	V (ml)
3.	Dilution factor	DF (ml)
4.	Weight of soil sample taken	W (gm)

Calculation

$$\text{Available K (kg/ha)} = \frac{Q \times DF \times 2.24 \times 106}{W \times 106}$$

$$\text{Available K (g/plant)} = \frac{\text{Available K (kg/ha)}}{740} \times 1000$$

Reference: Richards, J.E., Bates, T.E., 1989. Studies on the potassium-supplying capacities of southern Ontario soils. III. Measurement of available K. Can. J. Soil Sci. 69, 597–610.

Soil Organic Carbon

Reagents

1.	1N Potassium dichromate	Dissolve 49.04g of $K_2Cr_2O_7$ in DW and dilute to 1L
2.	0.5N ferrous ammonium sulphate	Dissolve 196g in 800ml DW, add 20ml conc. H_2SO_4 and dilute to 1L
3.	Diphenylamine indicator	Dissolve 0.5g of diphenylamine indicator in mixture of 100ml DW and 20ml conc. 20ml conc. H_2SO_4
4.	Conc. H_2SO_4: 96% pure	
5.	Orthophosphoric acid: 85% pure	

Procedure

1. Process soil by passing dried soil through 0.5mm sieve.
2. Take 0.5g soil in 500ml conical flask.
3. Add 10ml of 1N potassium dichromate and 20ml conc. H_2SO_4.
4. Shake the content in the flask and incubate for 30min.
5. Add 200ml DW, 10ml Orthophosphoric acid and 1ml Diphenylamine indicator.
6. Titrate the content with standard ferrous ammonium sulphate till colour changes from blue violet to brilliant green.
7. Similarly run blank without soil and note down the reading.

Observations to be recorded

1.	Weight of soil sample taken	W (g)
2.	Vol. of 0.5N FAS required for blank	B (ml)
3.	Vol. of 0.5N FAS required for soil	S (ml)

Calculation

$$\text{Organic Carbon (\%)} = \frac{10(B-S)}{B} \times 0.003 \times \frac{100}{W}$$

Reference: Walkley, A.J. and Black, I.A. (1934) Estimation of soil organic carbon by the chromic acid titration method. Soil Sci. 37, 29-38.

Soil Sulphur Content

Reagents

1.	Mono-calcium phosphate (500mg/l) or 0.15% Calcium chloride	Dissolve 2.035g of $Ca(H_2PO_4).H_2O$ in DW and dilute to 1L.
2.	Hydrogen peroxide	
3.	Glacial acetic acid	
4.	Gelatin barium chloride solution	Dissolve 5g gelatin in 250ml DW in 1L capacity beaker. Heat up to dissolve. Then add 60g barium chloride and 120ml of 1N HCl. Filter through filter paper and dilute to 1L with DW
5.	Standard sulphur stock solution: 1000mg S/l	Dissolve 5.434g of oven dried K_2SO_4 in DW and dilute to 1L with DW
6.	Working standard 25mg S/l	Prepare working solution by diluting 2.5ml stock solution of 1000ppm to 100ml with DW

Procedure

A) Standard curve preparation

7. Prepare a series of standard by taking 0, 1, 2, 4, 6, 8 and 10 ml of 25ppm standard Sulphur solution in 25ml volumetric flask separately.
8. Add 2ml of 2% H_2O_2 in each flask, mix and wait for 10min.
9. Add 2ml of glacial acetic acid in each flask, mix and wait for 10min.
10. Add 2ml of gelatin barium chloride solution in each flask, mix and wait for 10min.
11. Raise the volume to 25ml with DW and measure absorbance at 420nm.
12. Make graph by plotting conc. v/s absorbance.

B) Extraction

1. Take 5g of processed air dried soil in 100ml conical flask.
2. Add 25ml of 0.15% calcium chloride.
3. Shake for 30min on mechanical shaker and filter through Whatman no. 40or 42filter paper.

C) Estimation

6. Pipette out 10 ml of extract into 25ml volumetric flask.
7. Add 2ml of 2% H2O2 in each flask, mix and wait for 10min.
8. Add 2ml of glacial acetic acid in each flask, mix and wait for 10min.
9. Add 2ml of gelatin barium chloride solution in each flask, mix and wait for 10min.
10. Make the volume to 25ml with DW and measure absorbance at 420nm.

Observations to be recorded

1.	Concentration of S in ppm from Standard curve	A (ppm)
2.	Final Volume	B (ml)
3.	Volume for extraction used	C (ml)
4.	Aliquot of extract used for analysis	D (ml)
5.	Weight of soil sample taken	W (g)

Calculation

$$\text{Soil Sulphur (mg/kg)} = \frac{A \times B \times C}{D \times W}$$

Reference: Arora, C.L., Tandon H.L.S., 2009. Analysis of soil, plant and fertilizer for plant nutrients. In Fundamental of Soil Science, 2nd ed.ISSS, New Delhi, 491–510.

Available Soil Micronutrients

Reagents

1.	DTPA-extractant	Dissolve 1.967g of diethyline-triamine-penta acetic acid and 1.47 g of calcium chloride in about 100 ml double DW by adding 13.3ml of triethanolamine, adjust pH to 7.3 with dilute HCl and make final volume to 1L.
2.	Standard stock solution of Zn 1000 mg/L	Dissolve exactly 1.0 g of pure Zn metal in about 10ml of dilute HCl (1:1) and make the volume to 1L.
3.	Standard stock solution of Mn 1000 mg/L	Dissolve exactly 1.583 g of pure MnO_2 in 50ml dilute HNO_3 (1:1) and make the volume to 1L.
4.	Standard stock solution of Fe 1000 mg/L	Dissolve exactly 1.0 g of pure Fe metal MnO_2 in 50ml dilute HNO_3 (1:1) and make the volume to 1L.

5.	Standard stock solution of Cu 1000 mg/L	Dissolve exactly 1.0 g of pure Cu metal MnO_2 in 50ml dilute HNO_3 (1:1) and make the volume to 1L
6.	Standard working solution	Prepare 0.5, 1.0, 2.0, 4.0 and 6.0 ppm working solution of each metal from standard stock solution

Procedure

1. Weigh 20gm soil in conical flask.
2. Add 40 ml DTPA and shake for 2 hr.
3. Filter through Whatman no. 1 filter paper
4. Take observations on AAS.

Note: If required then dilute extract with double DW in 1:5 ratio.

Observations to be recorded

1.	Concentration	A (ppm)
2.	Extractant used for extraction	40 ml
3.	Weight of soil	20gm
4.	Final dilution	25
5.	Volume of aliquot used for analysis	5

Calculation

DTPA extractable micronutrients of soil (mg/kg) = $\frac{A \times 40 \times 25}{20 \times 5}$

Atomic Absorption Spectroscope

Reference: Lindsay, W. L. and Norvell, W. A. 1978. Development of a DTPA soil test for zinc, iron, manganese, and copper. Soil Sci. Soc. Amer. J. 42:421-428.

Available Soil Boron

Reagents

1.	Azomethine-H reagent	Dissolve 0.45gm of azomethine-H in 100ml of 1% L-ascorbic acid solution in polypropylene reagent bottle. Prepare fresh weekly and store in refrigerator.
2.	Buffer solution	Dissolve 250gm of ammonium acetate and 15 gm of disoudium salt EDTA in 400ml of DW. Slowly add 125ml of glacial acetic acid and mix.
3.	0.01M Calcium chloride	Dissolve 1.47gm $CaCl_2.2H_2O$ in 1L DW
4.	Boron standard solution	Stock solution 20mg/L Dissolve 0.114g of boric acid in 1L DW Working solution: Prepare 0,0.1,0.2,0.4…..1.0mg of Boron per Litre

Procedure

1. Weight 20gm soil passed through 200 mesh sieved in 250ml B-free conical flask.
2. Add 40ml 0.01M $CaCl_2$ solution.
3. Attach a water cooled regular condenser.
4. Heat for 5min from 1^{st} sign of boiling.
5. Allow to cool and filter
6. Take 1ml of aliquot, 2ml buffer, 2ml azomethine-H reagent in polypropylene or quartz tube. Incubate for 30 min.
7. Read absorbance at 420nm.
8. Prepare standard and blank similarly.

Reference: Sarkar D, Sheikh AA, Batabyal K, Mandal B (2014) Boron Estimation in Soil, Plant, and Water Samples using Spectrophotometric Methods. Communications in Soil Science and Plant Analysis, 45:1538–1550, 1538-1550

APPENDIX

Preparation of Buffers

1. Acetate buffer (0.1M)

Stock solutions

A. 0.2M solution of acetic acid (11.55ml in 1000ml)
B. 0.2M solution of sodium acetate (16.4g C_2H_2Na in 1000ml) Mix 'x' ml of A and 'y' ml of B and raise to 100 ml with DW

Acetic acid (x)	Sodium acetate (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	39.5	5.2
8.8	41.2	5.4
4.8	45.2	5.6

2. Boric acid-borax buffer (0.125M)

Stock solution

A. 0.2M solution of boric acid (12.4g in 1000ml)

B. 0.05M solution of borax (19.05g in 1000ml; 0.2M in case of sodiumborate)

Mix 50ml of A, with 'x' ml of B, dilute to a total volume of 200ml

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

3. Carbonate –bicarbonate buffer (0.1M)

Stock solutions

A: 0.2M solution of anhydrous sodium carbonate (21.2g in 1000ml)
B: 0.2M solution of sodium bicarbonate (16.8g in 1000ml)

Mix 'x'ml of A, 'y'ml of B and dilute to a total volume of 200ml

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

4. Citrate buffer (0.05M)

Stock solutions

A: 0.1M of citric acid (21.01g in 1000ml)

B: 0.1M solution of sodium citrate (29.41g $C_6H_5O_7Na_3.2H_2O$ in1000ml)

Mix 'x' ml of A and 'y' ml of B and dilute to a total volume of 100ml

Acetic acid (x)	Sodium citrate (y)	pH
46.5	3.5	3.0
40.0	10.0	3.4
37.0	13.0	3.6
35.0	15.0	3.8
33.0	17.0	4.0
31.5	18.5	4.2
28.0	22.0	4.4
25.5	24.5	4.6
20.5	29.5	5.0
18.0	32.0	5.2
16.0	34.0	5.4
13.7	36.3	5.6
9.5	41.5	6.0
7.2	42.8	6.2

5. Citrate phosphate buffer (0.1M)

Stock solutions

A: 0.1M citric acid

B: 0.2M dibasic sodium phosphate

Mix 'x' ml of A, 'y' ml of B and dilute to a total volume of 100ml

Citric acid (x)	Sodium phosphate (y)	pH
44.6	5.4	2.6
42.2	7.8	2.8
37.7	12.3	3.2
35.9	14.1	3.4
33.9	16.1	3.6
30.7	19.3	4.0

29.4	20.6	4.2
27.8	22.2	4.4
26.7	23.3	4.6
25.2	24.8	4.8
24.3	25.7	5.0
22.2	27.8	5.4
21.0	29.0	5.6
19.7	30.3	5.8
16.9	33.1	6.2
15.4	34.6	6.4
9.1	40.9	6.8
6.5	43.6	7.0

6. Glycine- HCl buffer (0.1M)

Stock solutions

A: 0.2M glycine (15.01g in 1000ml)

B: 0.2N HCl

Mix 25ml of A, 'x' ml of B and dilute to a total volume of 100ml.

HCl (x)	pH
22.0	2.2
16.2	2.4
12.1	2.6
8.4	2.8
5.7	3.0
4.1	3.2
3.2	3.4
2.5	3.6

7. Phosphate buffer (0.1M)

Stock solution

A: 0.2M solution of monobasic sodium phosphate (24.00g of NaH_2PO_4 in 1000 ml)

B: 0.2M solution of dibasic sodium phosphate (53.65g of $Na_2HPO_4.7H_2O$ in 1000 ml)

Mix 'x' ml of A, 'y' ml of B, dilute to total volume of 200 ml

A (x)	B (y)	pH
92.0	8.0	5.8
87.7	12.3	6.0
81.5	18.5	6.2
68.5	31.5	6.5
62.5	37.5	6.6
56.5	43.5	6.7
51.0	49.0	6.8
45.0	55.0	6.9
39.0	61.0	7.0
33.0	67.0	7.1
28.0	72.0	7.2
23.0	77.0	7.3
19.0	81.0	7.4

16.0	84.0	7.5
8.5	91.5	7.8
5.3	94.7	8.0

8. Tris (hydroxymethyl) aminomethane (Tris HCl) buffer (0.1M)

Stock solutions

A: 0.2M solution of Tris (hydroxymethyl) aminomethane (24.2g in1000ml)

B: 0.2N HCl

Mix 50ml of A, 'x'ml of B and dilute to a total of 200ml

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

Key References:

Thimmaiah SK (1999). *Standard methods of Biochemical analysis.* Kalyani Publishers, New Delhi.

Sadasivam S, Manickam A (1996). *Biochemical methods, second edition.* New age international (P) Limited, publishers, New Delhi.

Patil MN. Methods of soil, water and plant analysis. A Practcal manual. Dr. Panjabrao Deshmukh Krishi Vidyapeeth.

Seenappa C., Kalyana Murthy K.N., Anand M.R., Madam Vikramarjun (2020) *Handbook of Soil Water & Plant Analysis.*

Kalra YP (1998) Handbook of Methods for Plant Analysis. CRC Press Taylor & Francis Group.

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