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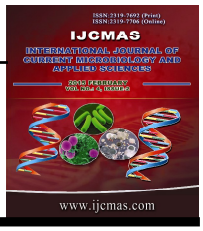

Original Research Article

Sequential Extraction of Plant Metabolites

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A B S T R A C T

Photosynthetic pigments, carotenoids and metabolites are being routinely extracted and estimated by scientists from leaf samples to elucidate effects of various nutrients and stress conditions on plant growth and productivity. Such extraction procedures and estimation techniques involve use of leaf samples and various solvents and reagents. Many a times leaf samples obtained from single plant are insufficient for extraction of all the metabolites and thus leaf samples taken from randomly selected plants are used for extractions and estimations. Hence, a sequential extraction method was suggested to extract primary and secondary metabolites from single leaf and small samples. Results clearly indicate that the sequential extraction protocol used in present investigation showed similar values with slight variations (statistically insignificant) as compared to routine standard methods.

Introduction

Sunlight provides energy for photosynthesis and help in synthesis of carbohydrates required for the regulation of plant development. Plant scientists are studying the role of light in plant development, in the biogenesis of chloroplast and in signalling between chloroplast and nucleus. The simple carbohydrates produced during photosynthesis are used as energy by plant for biosynthesis of primary metabolites like amino acids, proteins, complex carbohydrates and lipids. Primary metabolites are the key metabolites, which mainly involved in plant growth, development and reproduction.

The chlorophyll biosynthesis and photosynthetic capacity of plant leaf depend on genetic makeup of plant and environmental factors. Chlorophyll pigments synthesize carbohydrates in leaf tissues and then these are partitioned to different parts of the plant for various metabolic activities required by the plant for its growth, reproduction and productivity. Plant physiologists have been involved in research on regulation of synthesis and transport of primary carbohydrate metabolites as well as on complex carbohydrate biosynthesis and partitioning. Various environmental factors like abiotic and biotic stresses influence photosynthetic pigments, carbohydrate and protein synthesis as well as utilization in plants.

Amino acids are building blocks of proteins;

plants utilize these primary metabolites as precursors for synthesis of a large number of structurally diverse secondary metabolites. Plant biochemists have been studying the regulation and transport of amino acids for protein synthesis and as precursors to secondary metabolism. Some of the plant scientists are also involved in research on regulation of metabolic processes influenced by environmental factors such as cold temperatures, drought stress, insect attacks or attack by fungal and bacterial pathogens.

Primary metabolites and photosynthetic pigments are being routinely extracted and estimated by scientists from leaf samples to elucidate effects of various nutrients and stress conditions on plant growth and productivity. Such extraction procedures and estimation techniques involve use of leaf samples and various chemical reagents. Extraction procedures in particular are labour intensive, time consuming and require large volume of solvents and reagents. Many a times leaf samples obtained from single plant are insufficient for extraction of all the metabolites and thus leaf samples taken from randomly selected plants are used for extractions and estimations. Data obtained from such samples are ambiguous and lead to a wrong interpretation. Hence, a sequential extraction method was planned to extract primary and secondary metabolites from single leaf and small samples. The results of sequential extraction method and routine extraction methods are compared and discussed.

Materials and Methods

Peanut var. TAG-24 and okra var. *Phule Utkarsha* were grown in pots. The fully matured and physiologically active third leaf from top at the time of flowering was used for extraction of pigments and metabolites from peanut and okra plants.

Step-1: Extraction of photosynthetic pigments

Fresh leaves were chopped into small pieces and exactly 0.1 g material was weighed and macerated in mortar and pastel with 2 ml of 80% acetone. The content was centrifuged at 10000 g for 10 minutes. The residue was re-extracted in 1 ml of 80% acetone for two times and centrifuged at 10000 g for 10 minutes. The supernatants were pooled and made to 10 ml with 80% acetone. Appropriately diluted extracts were read on spectrophotometer for estimation of pigments. After estimation samples were saved and replaced in original sample.

Step-2: Extraction of soluble sugars, phenols and amino acids.

The residue obtained in step-1 was extracted in 2 ml of 80% methyl alcohol in boiling water bath for 30 minutes. The content was cooled and centrifuged at 10000 g for 10 minutes and the residue obtained was re-extracted with fresh 2 ml of 80% alcohol. The supernatant obtained in step-1(chlorophyll extract) and step-2 were pooled and condensed in water bath to 1-2 ml and diluted to 10 ml with distilled water and centrifuged at 10000 g for 10 minutes. Supernatant obtained was used for estimation of reducing sugars, total sugars, total phenols, total falvonoids and free amino acids.

Step-3: Extraction of starch

The residue obtained after step-2 was re-suspended in digestion mixture (0.65 ml of 52% perchloric acid + 0.5ml distilled water) and subjected to digestion in cold condition at 0 ^{0}C in refrigerator for 30 minutes. After cold incubation the content was centrifuged at 10000 g for 10 minutes and supernatant was collected as source of starch. The pellet

was further extracted with same volume of (52 % PCA and water) and supernatants were pooled. The extracted starch solution was neutralized with sodium carbonate. Final volume was made to 2.5 ml with distilled water.

Step-4: Extraction of proteins

The residue obtained in step-3 was used for extraction of proteins. The residue was treated with 2.0 N solution of NaOH (1 ml) for 30 minutes and centrifuged at 10000 g for 10 minutes and supernatant was collected as source proteins. The extraction was repeated with fresh NaOH solution (1 ml) and centrifuged. The supernatants were pooled and final volume was made to 2.0 ml with distilled water. The pooled supernatant was saved as source of proteins.

Extraction of metabolites by routine methods

The leaf materials 0.1 g from peanut and okra respectively were also extracted with 80 % acetone for estimation of pigments. Reducing sugars, soluble sugars, total phenols, total flavonoids and free amino acids were extracted with 80 % methyl alcohol from 0.1 g leaf material of both the plants. Starch was extracted from 0.1 g material with 52 % perchloric acid at 0 ^{o}C after extracting and washing out soluble sugars with methanol. Proteins were extracted in 0.2 N NaOH from 0.1 g leaf materials after treating materials with 80% acetone, 80 % methanol and 52% perchloric acid in a sequence for washing out pigments, phenols, sugars, free amino acids, and starch.

Estimation of pigments

Chlorophyll pigments and carotenoids were estimated by Lichtenthaler and Welburn (1983) method. After estimation samples were saved and replaced in original sample.

Chlorophyll **a** (µg/ml) = 12.21 (A663) - 2.81 (A646)
Chlorophyll **b** (µg/ml) = 20.13 (A646) - 5.03 (A663)
Carotenoids (µg/ml) = $(1000A_{470}$ - 3.27[chl a] - 104[chl b])/227

1. **Estimation of soluble carbohydrates:** Soluble carbohydrates were estimated by Antrone reagent as per the method given by Hansen and Moller (1975). D-Glucose at the concentration of 20 to 100µg ml^{-1} was used to prepare the standard curve.
2. **Estimation of reducing sugars:** Reducing sugars were estimated with DNSA reagent according to Miller (1959) method. Maltose at the concentration of 20 to 100µg ml^{-1} was used to prepare the standard curve.
3. **Estimation of starch:** Starch was estimated with Antrone reagent as per the method given by Hansen and Moller (1975). D-Glucose at the concentration of 20 to 100µg ml^{-1} was used to prepare the standard curve.
4. **Estimation of free amino acids:** Total free amino acid content was estimated according to the Ninhydrin method given by Moore and Stein, (1948). L-lysine was used as standard amino acid to prepare standard curve.
5. **Estimation of phenols:** Total phenols were estimated as per the method given by Farkas and Kiraly (1962). Catachol at the concentration of 20 to 100µg ml^{-1} was used to prepare the standard curve.
6. **Estimation of flavonoids:** Aluminium chloride method was used for flavonoid determination (Chang *et al.*, 2002). Calibration curve was prepared with quercetin at concentrations from 12.5 to 100µg ml^{-1} in methanol.

7. **Estimation of proteins:** Protein content was estimated by Lowry *et al.* (1951) method. Bovine serum albumin-fraction V (BSA) was used at the concentration of 0.2 to 1.0 mg ml^{-1} as a standard protein to prepare the standard curve.

Experiments were carried out in four replications. Data recorded from four replications were subjected to single way analysis of variance (ANOVA) and critical differences were calculated at p=0.05 level.

Results and Discussion

The results related to methods of extraction of photosynthetic pigments, carbohydrates, proteins, amino acids, phenols and falvonoids are given in table 1-3. Results clearly indicate that the sequential extraction protocol used in present investigation showed similar values with slight variations (statistically insignificant) as compared to routine standard methods. Results on carbohydrate content i.e. soluble sugar, reducing sugar, non reducing sugars and starch profiles of both the extracts are fairly close, in spite of the extraction methods.

Generally the selection of an extraction method depends on working requirements and availability of laboratory facilities. Any metabolite extraction includes extraction instruments, labour cost, operational cost, and cost of consumables as well as the extraction time.

Table.1 Methods of extraction and values of photosynthetic pigments and carotenoids

Phyto-constituents	Peanut var. TAG-24			Okra var. *Phule Utkarsha*		
	Routine extraction methods	Sequential extraction method	CD 5%	Routine extraction methods	Sequential extraction method	CD 5%
Chlorophyll a (mg g^{-1})	1.34	1.33	0.22	1.54	1.56	0.18
Chlorophyll b (mg g^{-1})	0.44	0.45	0.06	0.48	0.52	0.08
Chlorophyll a+b (mg g^{-1})	1.78	1.78	0.16	2.02	2.08	0.14
Carotenoids (mg g^{-1})	1.62	1.72	0.58	1.42	1.48	0.66

CD= critical difference

Table.2 Methods of extraction and values of carbohydrates, proteins and free amino acids

Phyto-constituents	Peanut var. TAG-24			Okra var. *Phule Utkarsha*		
	Routine extraction methods	Sequential extraction method	CD 5%	Routine extraction methods	Sequential extraction method	CD 5%
Reducing sugars (mg g^{-1})	19.18	18.96	1.04	24.09	25.16	1.08
Soluble sugars (mg g^{-1})	41.52	42.08	1.56	54.68	54.12	1.66
Starch (mg g^{-1})	81.02	80.96	1.84	70.26	69.58	1.98
Proteins (mg g^{-1})	84.12	82.62	1.26	32.88	31.12	1.14
Free amino acids (mg g^{-1})	70.24	68.64	1.84	62.82	60.14	1.82

CD= critical difference

Table.3 Methods of extraction and values of total phenols and flavonoids

Phyto-constituents	Peanut var. TAG-24			Okra var. *Phule Utkarsha*		
	Routine extraction methods	Sequential extraction method	CD 5%	Routine extraction methods	Sequential extraction method	CD 5%
Total phenols (mg g^{-1})	4.52	4.48	0.82	2.38	2.32	0.12
Total flavonoids (mg g^{-1})	0.82	0.84	0.09	0.62	0.66	0.06

CD= critical difference

Generally reducing sugars and soluble sugars are extracted with an 80% methyl alcohol. Monosaccharides and oligosaccharides are soluble in alcoholic solutions, whereas proteins, polysaccharides and dietary fiber are insoluble. Similarly phenolic and flavonoid compounds are extracted in alcohol and insoluble components like starch and proteins are discarded with residue. In the aforesaid methods soluble components are separated from the insoluble components by centrifugation techniques, where the supernatants collected for soluble components. The supernatant contains sugars, free amino acids and phenolic compounds. During these extractions residue is generally discarded, which otherwise contain valuable insoluble carbohydrates and proteins.

On the other hand during protein extraction plant material is first treated with alcohol for removal of chlorophyll pigments and free sugars and then with perchloric acid for digestion of starch. In this process, supernatants containing sugars, amino acids, phenols, flavonoids, alkaloids, and starch obtained after each step are discarded and proteins are extracted with NaOH solution. Thus, during such extractions large quantities of organic solvents are utilized and discarded. However, the present sequential extraction method if followed for extraction one can save organic solvents and utilize the same sample for extraction.

The metabolic profile carried out in present investigation by extracting the sample with conventional methods and by sequential extraction method is at par. This indicates that one can save solvents and material, which can be used for multiple extractions and same quality of results. This indicate that the sequential extraction method can be suitable for extraction of chlorophyll pigments, caroteinoids, primary metabolites and well as some secondary metabolites like phenols, flavonoids and alkaloids to compare metabolite profile of healthy and infected plants, normal and stressed crop plants as well as to study phenological variations in plants.

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