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VALIDATED RP-HPLC METHOD FOR THE ESTIMATION OF CAFFEIC ACID IN SWEET POTATO *(IPOMOEA BATATAS)*

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ABSTRACT

Sweet potato is an edible tuber reported to be rich in antioxidants and other nutrients. Results of the previous studies have presumed that it is a rich source of caffeic acid which is an important antioxidant of medicinal values. Hence, a simple, precise and rapid HPLC method was developed for the estimation of caffeic acid and applied to the analysis of sweet potato extract. For the separation a Phenomenex C_{18} column was used as a stationary phase, 0.2% ortho-phosphoric acid in water and acetonitrile in a ratio of (81:19) was used as a mobile phase. The flow rate has been maintained at 1.2 ml/min and detection was carried at a wavelength of 325 nm. Good separation of caffeic acid was obtained with a retention time of 6.5 mins. The calibration curve showed good linearity (r^2= 0.997) in the concentration range of 1.6 µg/ml to 25.6µg/ml. The relative standard deviation of intra-day and inter-day precision for caffeic acid was found to be less than 3% for caffeic acid. The limit of detection was found to be 28.00 ng/ml and the limit of quantitation was found to be 77.00 ng/ml. The content of caffeic acid, was quantified in methanolic extracts of different parts of sweet potato using the newly developed HPLC method. Among the samples sweet potato peel extract has been shown to contain 0.1415±0.015 mg of caffeic acid and sweet potato leaf was shown to contain 0.00108±0.0022 mg/100mg of the extract respectively, while the rhizome extract has shown no appreciable response to the detector system. Thus, the present research suggests that sweet potato peel which is generally removed off before its consumption has rich caffeic acid content than the other parts of the tuber and may be consumed along with the peel which has no detrimental effects on its taste and culinary values.

KEYWORDS: Caffeic acid, Sweet potato, RP-HPLC, antioxidant.

1. INTRODUCTION

Antioxidants are the compounds capable of preventing or reducing harmful effects of free radicals, which may cause a broad range of diseases like inflammation, stroke, heart disease, diabetes mellitus, cancer, Parkinson's disease and Alzheimer's disease. Undeniably, antioxidant constituents of plants act as radical scavengers and help in converting the free radicals into less reactive species. Most naturally occurring antioxidants of plants viz., flavonoids, vitamins, phenols, carotenoids and dietary glutathione have been reported to quench singlet oxygen, scavenge free radicals, inhibit enzymes and decompose peroxides.[1-14] Phenolic compounds constitute a large group of secondary plant metabolites viz., polyphenols (flavonoids and tannins) and simple phenols (phenolic acids and coumarins). Phenolic acids are hydroxylated derivatives of benzoic and cinnamic acids and the most common among them are caffeic acid, gallic acid, p-coumaric acid, ferulic acid, p-hydroxybenzoic acid, vanillic acid and procatechuic acid, which frequently occur in fruits and vegetables as esters or glycosides.[15,35]

Among these, caffeic acid is an important phenolic compound that exhibits appreciable degree of antioxidant activity. Sweet potato is an edible tuber belongs to the family *Convulaceae*. Pharmacological studies have been conducted on anti-diabetic, anti-hypertensive, anti-inflammatory, anti-microbial and anti-oxidant activities of sweet potato. These physiological properties are possible because of the numerous phytochemicals present in sweet potato that includes polyphenols, such as anthocyanins, acylated anthocyanins and phenolic acids viz., caffeic acid, chlorogenic acid, di and tricaffeoylquinic acids, sesquiterpenoids, alpha-tocopherol and storage proteins[36-39]

A prerequisite for investigating the biochemical effects of plant species is to know its qualitative and quantitative composition of constituents precisely. The content of

phenolic compounds in plant samples can be determined by various analytical instrumental methods, such as gas chromatography, thin-layer chromatography and capillary electrophoresis[40-55] However, high performance liquid chromatography (HPLC) has been proved to be the most appropriate method challenging the structural similarity and diversity of phenolic compounds, allowing the analysis with sufficient precision, selectivity and reasonable run time. Therefore, in the present research a HPLC method was developed and validated to quantify caffeic acid in methanolic extract of different parts of sweet potato. This would help diet and nutraceutical developers to select the plant part to be picked for extraction of the constituents.[56]

Objective

In the present study, the goal was to extract caffeic acid in sweet potato has been proved by high performance liquid chromatography (HPLC) and the objective behind the study was to investigate the biochemical effects of plant species is to know its qualitative and quantitative composition of constituents.

2. RESEARCH METHODOLOGY

2.1. Plant material

Fresh rhizomes and leaves used for the study were collected from Palakkad, Kerala (India). The samples were cleaned and the peel was removed off from the rhizomes and dried along with the rhizome and leaves at room temperature under shade for three days. The dry samples were powdered well prior to the methanolic extraction.[57]

2.2. Reagents

HPLC grade acetonitrile and analytical grade ortho-phosphoric acid were used for the preparation of mobile phase. For the extraction of plant materials HPLC grade methanol was used. Water for chromatography was prepared using Millipore MilliQ water purification system. Standard marker compound of caffeic acid was obtained from Sigma-Aldrich (Mumbai, India).

2.3. HPLC instrumentation

Chromatography was performed on a Shimadzu Prominence LC-20 AT HPLC system connected with SPD-M20, diode array detector along with a Rheodyne mannual injector. The analysis has been carried out on an Phenomenex C18 column(150mm × 4.6mm, 5µm) fitted to a guard column (10mm ×4mm). The mobile phase used composed of acetonitrile and water in the ratio of 81:19; pumped at a flow rate of 1.2 ml/ min and the detection wavelength was set at 325nm. The sample injection being 20µL and the temperature was kept at ambient throughout the analysis.

2.4. Preparation of extracts.

Dried and powdered plant materials viz., leaf, peel and rhizome of sweet potato in quantities of 2 gms were weighed and soaked in 50 ml of HPLC grade methanol.[58] This was then sonicated for 20 minutes and the contents were set aside for a day, then filtered through Whatmann filter paper No.1. The filtrate was evaporated to dryness at room temperature and the residue was collected, dissolved in 2ml of methanol and stored at 4º C before use. It is then passed through the syringe filter (0.25µm) before introducing in to the HPLC column**.**

2.5 HPLC method validation

Validation of the HPLC-UV/DAD method was performed in agreement with the guidelines provided by ICH for analytical tachniques meant for the quality control of pharmaceuticals.[59]

2.5.1. Determination of linearity

The stock standard solution was prepared by dissolving 1 mg of caffeic acid in 10 ml of methanol in a standard flask. From this stock solution concentrations between 0.25- 125 µg/ml was analysed to determine the linearity of the HPLCmethod.

2.5.2. Preparation of calibration curve

The standard solution was diluted with mobile phase to obtain a series of standard solution with concentarions of 1.6, 3.2, 6.4, 12.8 and 25.6 µg/ml. From this 20µL aliquots of each standard solution were used for the HPLC analysis. The injections were performed in triplicate for each concentration level. The calibration curve was constructed by plotting the peak area versus the concentration. The content of caffeic acid was determined by regression equation using the calibration curve and it is given below.[60]

$$Y = mx+c \quad \text{--------} \quad (1)$$

$$Con = y/mx-c \quad \text{---} \quad (2)$$

Where, y= sample response, mx= regression co-effecient, c= constant

2.5.3. Determination of LOD and LOQ

The limit of detection (LOD) is the lowest quantity or concentration of the analyte that can be detected by the given analytical method (LOD=3.3×D/S) and the limit of quantification is the smallest concentration of the analyte, which shows response that can be accurately quantified (LOQ = 10 × D/S) where, D is the standard deviation of y – intercepts of regression line and S is the slope of the calibration curve. For reference compounds, the LOD and LOQ values were experimentally determined using regression analysis.

2.5.4. Determination of precision

The precision of the chromatographic system was tested by performing intra-day and inter-day multiple injections of a methanolic extract and then checking the %RSD of retention times and peak areas. Six injections were performed on each day for three consecutive days.

2.5.5. Determination of accuracy

The accuracy of the analytical procedure was evaluated using the recovery study. This involved the addition of a known quantity of standard compound to half the amount

of a methanolic extract of the sample to reach 100% of the test concentration. The spiked samples were then analyzed by the proposed method.

2.6. Extraction of phenolic compounds

After boiling, the cubes were separated from the boiling water and it was cooled to room temperature and homogenized in 15 ml of 80% methanol. The homogenate was centrifuged (1500 × g, 10mins) and the precipitate was washed with methanol for two more times. The supernatants were collected and 80% methanol was added to make up 50ml and the solution was filtered through cartridge filter and used for HPLC analysis.

3. RESULTS AND DISCUSSION

3.1. Optimization of chromatographic conditions

To obtain a HPLC method with better separation of the phyto-constituents with an appreciable analysis time, the chromatographic conditions were optimised. The resolution of the compounds were tested and compared with different ratios of mobile phases. Because of low wavelength threshold of absorption and better resolution compared with methanol, acetonitrile, was fixed as the organic phase. Considering the nature of the compound, acidic condition was maintained for the mobile phase; hence, 0.2% ortho-phosphoric acid in water was used as the aqueous phase. The column was set at room temperature (28°C) and the wavelength of detection was set at 325nm for caffeic acid.

3.2. Results obtained for the HPLC method

3.2.1. Specificity

The specificity was assessed by comparing the chromatogram obtained from the analysis of a blank sample, standard solution and sample solution. The integration of peak in the chromatogram of the sample solution was corresponding to the peak obtained for standard solution. Further, no other peak in the retention time of the compound of interest appeared in the chromatogram due to the solvent or any other interfering substances. This indicates that the newly developed HPLC method is specific for the qualitative and quantitative analysis of caffeic acid.

3.2.2. Linearity

Series of concentrations ranging from 0.25-125µg/ml were analysed by the newly developed HPLC method. The results obtained revealed that caffeic acid was linear between concentration range of 0.25 to 70 µg/ml.

3.2.3. LOD and LOQ

Sample extracts were analysed by the newly developed HPLC procedure and the content of phenolic acid was calculated using regression analysis. The LOD of the method was found to be 28.00ng/ml and LOQ was found to be 77.00 ng/ml.

3.2.4. Precision

The intra-day and inter-day variability of precision was analysed by replicate samples at three different concentrations on each consecutive days. The RSD of intra-day and inter-day variability of precision were in the range of standard prescribed values. The results obtained for the accuracy study is given in the Table.1.

3.2.5. Accuracy

To assess the accuracy of the proposed method, recovery studies were performed by standard addition method at three different levels (50%, 100% and 150%). A known amount of standard drug was added to the pre-analyzed herbal extract and the sample was then analyzed by the proposed method. The results of the recovery studies were found to be satisfactory and are given in the Table.2.

3.2.6. Analysis of the sample extract

The sample extract of leaf, rhizome and peel were introduced in to the HPLC system in 6 replicate injections (n= 6) and the average of 6 injections has been calculated to get the final concentration. The results obtained through the analytical method are shown in Table 3.

Table 1: Results obtained for precision analysis (inter-day).

SI. No.	Conc.of Drug (ng/ml)	Mean peak area*		Accuracy (%)		RSD (%)	
		DAY I	DAY II	DAY I	DAY II	DAY I	DAY II
1	3200	66181	66043	98.54	98.13	0.35	0.78
2	6400	133702	130640	99.25	99.86	1.86	1.63
3	12800	226926	218093	99.13	99.32	1.48	1.47

*Average of 5 injections

Table 2: Results obtained for the accuracy studies.

Levels	Amount added (µg/ml)*	Amount recovered*					Percentage recovery
		Sample-1	Sample-2	Sample-3	Sample-4	Sample-5	
I – 50%	0.8	0.79	0.81	0.84	0.77	0.78	99.75
II – 100%	1.6	1.57	1.52	1.66	1.49	1.68	99.00
III – 150%	2.4	2.43	2.45	2.38	2.43	2.49	101.50

Table 3: Quantification of caffeic acid in different parts of sweet potato.

Parts of plant	Amount of caffeic acid (mg/100mg of sample)
Sweet potato peel	0.1415±0.0150
Sweet potato leaf	0.00108±0.0002
Sweet potato rhizome	Not detected

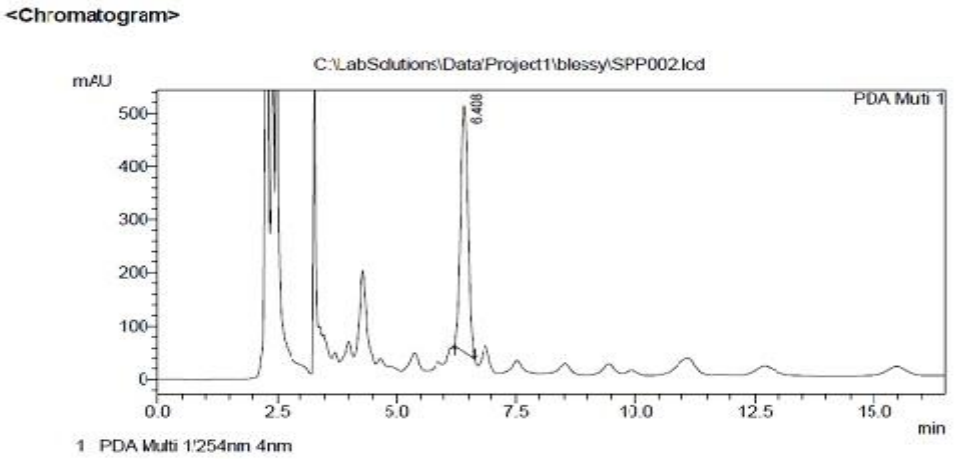

Fig. 4: Chromatogram of sweet potato peel extract.

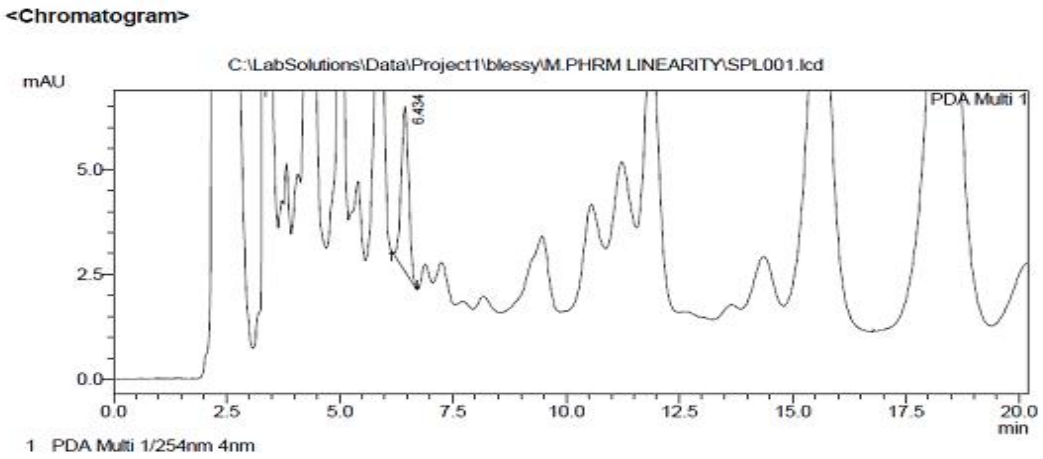

Fig. 5: Chromatogram of sweet potato leaf extract.

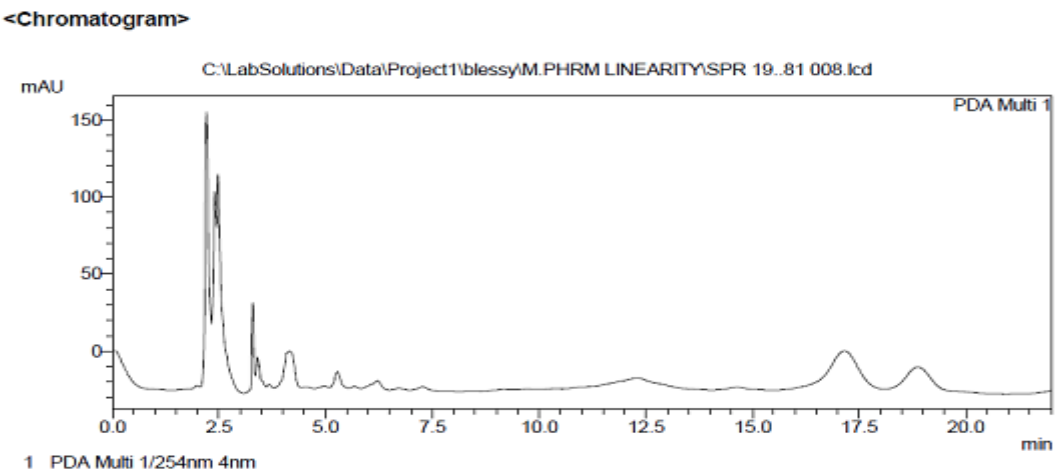

Fig. 6: Chromatogram of sweet potato rhizome extract.

CONCLUSION

A validated HPLC method with high precision, repeatability and stability was developed for the estimation of caffeic acid in sweet potato. The newly developed HPLC method was shown to be simple, precise, accurate and rapid. It can be successfully applied to the analysis of extracts obtained from sweet potato to quantify the amount of caffeic acid present in it. The peak for the caffeic acid present in the plant extract was identified by comparison with the UV spectra and peak purity profile of the standard marker compound. Amount of caffeic acid was found to be abundant in sweet potato peel compared to the leaf; the rhizome was shown to contain an 'undetectable' proportion of caffeic acid. The overall result of the analysis suggests that sweet potato may be consumed wholly without removal of the peel in order to gain the phyto anti-oxidant caffeic acid to the optimum extent.

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