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Development and Validation of LC-APCI-MS Method for the Estimation of Ellagic acid in Fresh and Processed Fruit Products

Krishna Veni N.[1]*, Meyyanathan S N.[1], Gowramma B.[1], Babu B[1] and Elango K[2]
[1]Department of Pharmaceutical Analysis, JSS College of Pharmacy, (Constituent College of JSS University, Mysore), Rocklands, Udhagamandalam – 643 001. Tamilnadu, India. [2] JSS College of Pharmacy, (Constituent College of JSS University, Mysore), Rocklands, Udhagamandalam , Tamilnadu, India.

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ABSTRACT

A rapid and simple LC/MS method has been developed for the quantification of ellagic acid in fresh and processed fruits. The interface used was Atmospheric Pressure Chemical Ionization technique. Analysis was performed using a Princeton SPHER C_{18} column (100 X 4.6 mm) by isocratic elution with 10 mM ammonium acetate: Acetonitrile (20:80) at a flow rate of 1.0 ml/min. The calibration plot was linear over the range studied (Ellagic acid: 80 – 1300 ng/ml) with a correlation of 0.999. The (M-H) peak of ellagic acid was identified at m/z of 302 in selective ion monitoring mode. Two fresh fruits and two processed fruit products were subjected to analysis by the developed method. The samples were found to contain ellagic acid in the range of 18.20 – 97.80 µg/10 gm of the products consumed. The method was also validated for the precision and recovery. Thus the method is suitable for routine analysis of ellagic acid in fresh and processed fruits.

INTRODUCTION

It is well established that a diet high in fruits and vegetables is associated with a reduced risk of oxidative stress mediated diseases such as cancer, cardiovascular and neurodegenerative diseases. The health beneficial effects of fruits and vegetables are attributed to their high levels of a wide variety of phytochemicals, of which phenolics constitute the greatest proportion. Among them, Ellagic acid, a dimeric compound of gallic acid is found in numerous fruits and vegetables including raspberries, strawberries, cranberries, walnuts, pecans, pomegranates and other plant foods in the form of hydrolysable tannins called ellagitannins. Ellagitannins are esters of glucose with hexahydroxydiphenic acid; when hydrolyzed, they yield Ellagic acid, the dilactone of hexahydroxydiphenic acid. Ellagic acid is found to have antiviral, antimutagenic and antioxidant properties. The anti proliferative and antioxidant properties of ellagic acid have spurred preliminary research into the potential health benefits of ellagic acid consumption.

Detailed review of literature for various analytical methods for the detection of ellagic acid revealed several methods based on different techniques Viz LC-ESI-MS (Navindra Seeram *et al*., 2006 & Lee J H *et al*., 2005) for the estimation of phenolics compounds in strawberry fruits and identification of ellagic acid conjugates and other polyphenolics in muscadine grapes. An LCMS (Mullen *et al*., 2003) method was also reported for the analysis of ellagitannins and quercetin in raspberry fruits. HPLC (Yoshiake *et al*., 2000; Okada *et al*., 2000) methods were also carried out to estimate the total content of phenolics and ellagic acid in fresh and processed fruits. A survey (Koponen *et al*., 2007) was also reported for the content of ellagic acid in selected foods consumed in Finland. However there was no LC MS method reported for the determination of ellagic acid in fresh and processed fruits. The purpose of the present study was to evaluate ellagic acid in fresh and processed fruits available in Indian market by an accurate and sensitive LC/MS method. The developed method was validated as per ICH guidelines (International Conference on Harmonisation, 1996).

* *Corresponding Author*
Assistant Professor, Department of Pharmaceutical Analysis, JSS College of Pharmacy, (Constituent College of JSS University, Mysore), Rocklands, Udhagamandalam – 643 001. Tamilnadu, India
Mobile: +91 9442083447
Phone: 0423 - 2443393

MATERIALS AND METHODS

Chemicals

All the solvents were of analytical grade purchased from SRL laboratories (India). Water purified by the milli Q water purification system was used.

The standard Ellagic acid was purchased from Sigma Aldrich (India).

Stock solutions

The stock solution containing 100 µg/ml of ellagic acid was prepared in methanol. This stock solution was stored in light resistant container.

Aliquots of ellagic acid (80–1300 ng/ml) were prepared in the mobile phase. Food products for analysis were obtained randomly at various supermarkets in India.

Sample preparation

Ellagic acid from four different food products (pomegranate fruit, strawberry fruit, strawberry jam, raspberry jam) were extracted by hydrolysis (Yoshiake *et al.*, 2000) of 10 g of the samples with 30 ml of methanol, refluxed for 1 hour and filtered using a Whatmann filter paper No 42. 10 ml of distilled water was added to the filtrate and evaporated to a volume of 10 ml. 0.25 ml of 0.1N hydrochloric acid was added and the volume was made up to 25 ml with distilled water.

Ellagic acid from this solution was separated using solid phase extraction technique. Phenomenex Strata C_{18} columns with 1 ml capacity were used for SPE analysis. The catridges were conditioned using 1ml of methanol and water, 1 ml of the refluxed sample was loaded and the samples were extracted with 2 ml of methanol, evaporated to dryness and reconstituted with 1 ml of mobile phase.

Apparatus and Instrument conditions

The LC/MS was performed using a Shimadzu LC 2010A system (Shimadzu Technologies, Japan). A Princeton SPHER C_{18} column (100 X 4.6 mm, 5µ ID.,), was used (Merck, India). Samples of 10µl volume were injected. LC separation was carried out using mobile phase of 10mM Ammonium Acetate: Acetonitrile (20:80 % V/V).

The flow rate was 1.0 mL/min. The working conditions for atmospheric pressure chemical ionization MS were as follows: The probe temperature was set at 400 º C, CDL temperature was set at 250 º C, block temperature was set at 200 º C and the drying gas (nitrogen) was introduced in to the capillary region at a flow of 10 L/min.

The detector voltage was held at a potential of 1.3Kv and the polarity was maintained at negative ion mode. When working in a selective ion monitoring mode (SIM), ion at m/z 302 was assigned to (M-H) of Ellagic acid. These ions were then monitored and quantified. The standard and the sample solutions were analysed by the optimised chromato-graphic conditions and the chromatograms were recorded.

VALIDATION

Calibration curve

A standard solution of 80 - 1300 ng/ml of ellagic acid was analyzed to check the linearity of response. (Table 1).

Table . 1: Linearity and range for Ellagic acid by LC-MS.

S. No	Concentration of Ellagic acid (ng/ml)	Peak area
1	80	41516
2	160	76470
3	320	168449
4	650	338333
5	1300	680049

Specificity

The specificity of the method was ascertained by analyzing the standards and the samples. The peak of ellagic acid in sample was confirmed by comparing the retention time and mass spectra of the sample with that of the standards.

Precision

Six injections at three different concentration of ellagic acid (80, 320 and 1300 ng/ml) were made and analyzed to examine the precision of the method. The mean peak area, standard deviation and % RSD were calculated (Table 2).

Table. 2: Precision studies for Ellagic acid by LC-MS.

S. No	Ellagic acid		
	80 ng/ml	320 ng/ml	1300 ng/ml
1	41516	168449	680049
2	41769	167925	680175
3	41443	168357	680957
4	41890	168470	681652
5	41866	168934	679776
6	41705	168655	679952
Mean	41698.17	168465	680426.8
SD	183.48	334.55	726.27
% RSD	0.4400	0.1985	0.1067

Accuracy

Accuracy of the method was determined by recovery experiments. The recovery of the method was determined at single level by adding a known quantity of ellagic acid to the food products of pre analyzed samples and the mixtures were reanalyzed according to the proposed method.

The average recoveries obtained from each sample were shown in Table 3.

Table. 3: Results of analysis of food products and recovery studies for Ellagic acid by LC-MS.

Sample	Amount present (µg/10 g) ± % RSD*	Amount added (µg)	% Recovery ±% RSD*
I	48.35 ± 0.2809	0.4	98.14 ± 0.2749
II	18.20 ± 0.5472	0.4	100.47 ± 0.8515
III	97.80 ± 0.2579	0.4	101.97 ± 0.6852
IV	89.73 ± 0.1793	0.4	99.57 ± 0.4680

* n=3
Sample I: Strawberry fruit
Sample II: Pomegranate fruit
Sample III: Strawberry jam
Sample IV: Raspberry jam

Limit of detection and Limit of quantification

The limit of detection and the limit of quantitation of the developed method were determined by injecting progressively low concentration of the solid solutions using the developed LC/MS method.

The limit of detection is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3). The limit of quantitation is the smallest concentration of the analyte, which gives a response that can be accurately quantified (signal to noise ratio of 10). (Table 4)

Table. 4: System suitability studies for estimation of Ellagic acid by LC-MS.

S. No.	Parameters	Ellagic acid
1	Linearity range	80 - 1300 ng/ml
2	Regression equation Y = mx + c	Y=525.3X-2744
3	Correlation coefficient	0.999
4	Asymmetric factor	0.98
5	LOD (ng/ml)	2
6	LOQ (ng/ml)	10

RESULTS AND DISCUSSIONS

In the spectral investigation by LC/MS in the SCAN mode, standard solution of ellagic acid showed major peak at m/z of 302.00, which were assigned to the (M-H) ions of ellagic acid (Fig 1). Optimisation of the method was carried out using various concentrations of acetonitrile while keeping the aqueous phase concentration constant. A solvent combination of 10 mM Ammonium acetate: acetonitrile (20:80 v/v) gave a satisfactory separation of the compound of interest. This optimized mobile phase separated ellagic acid at 1.3 min. The typical chromatograms of the standard and the sample solutions are shown in Fig 2-3.

The calibration curve of ellagic acid was linear in the range of 80–1300 ng/ml (Table 1). Linear regression equation and correlation coefficient values are shown in Table 4. Detection limit of ellagic acid was found to be 2.0 ng/ml whereas quantification limit was 10 ng/ml.

The precision of the method was demonstrated by reproducibility studies. The mean, standard deviation and % RSD were calculated and are presented in Table 2. The % RSD values less than 1**%** revealed that the methods were precise.

The selected food products were found to contain ellagic acid in the range of 18.20 µg – 97.80 µg/ 10 g. The average percent recoveries obtained from each sample were between 98.14 % and 101.97% (Table 3).

No marked changes in the chromatogram occurred on changing the operator, columns and chromatographic conditions indicating that the developed method was rugged and robust.

The peak asymmetry was calculated for the standard solutions and is presented in Table 4. The values obtained demonstrated the suitability of the system for the analysis of ellagic acid in food products.

A simple, sensitive and rapid LC/MS method has been developed and validated for estimation of ellagic acid in fresh and processed fruits.

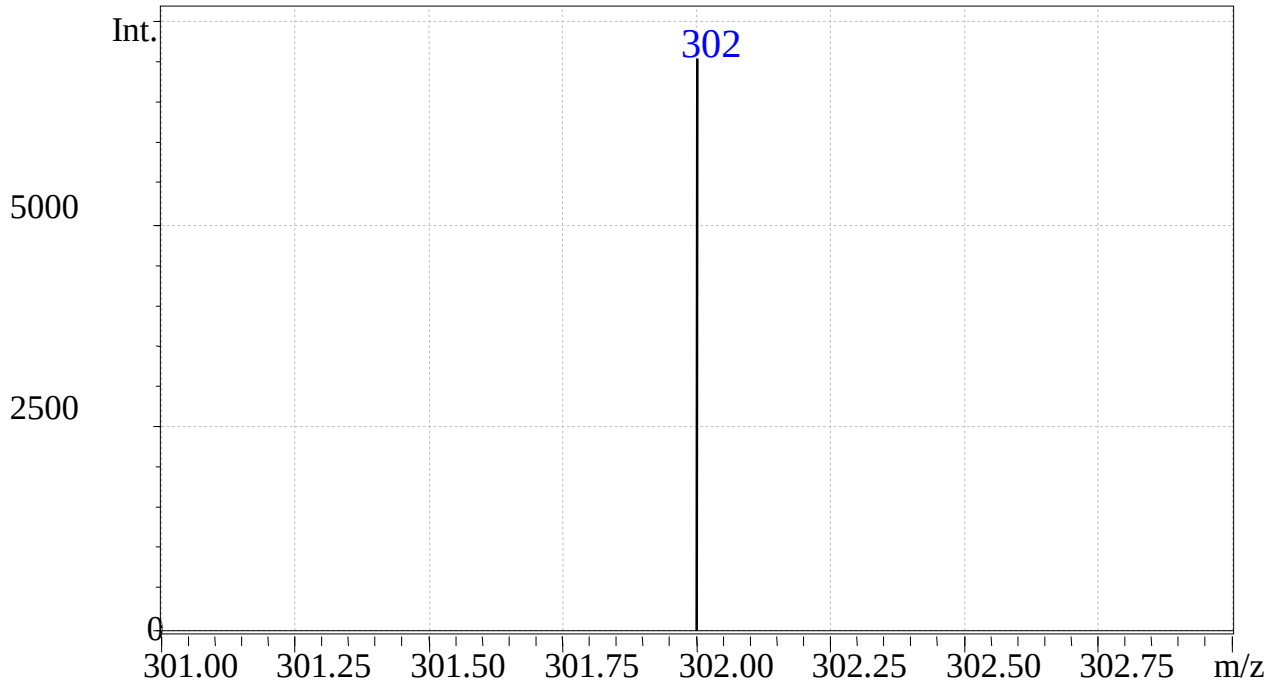

Fig. 1: Typical MS chromatogram of Ellagic acid in SIM mode.

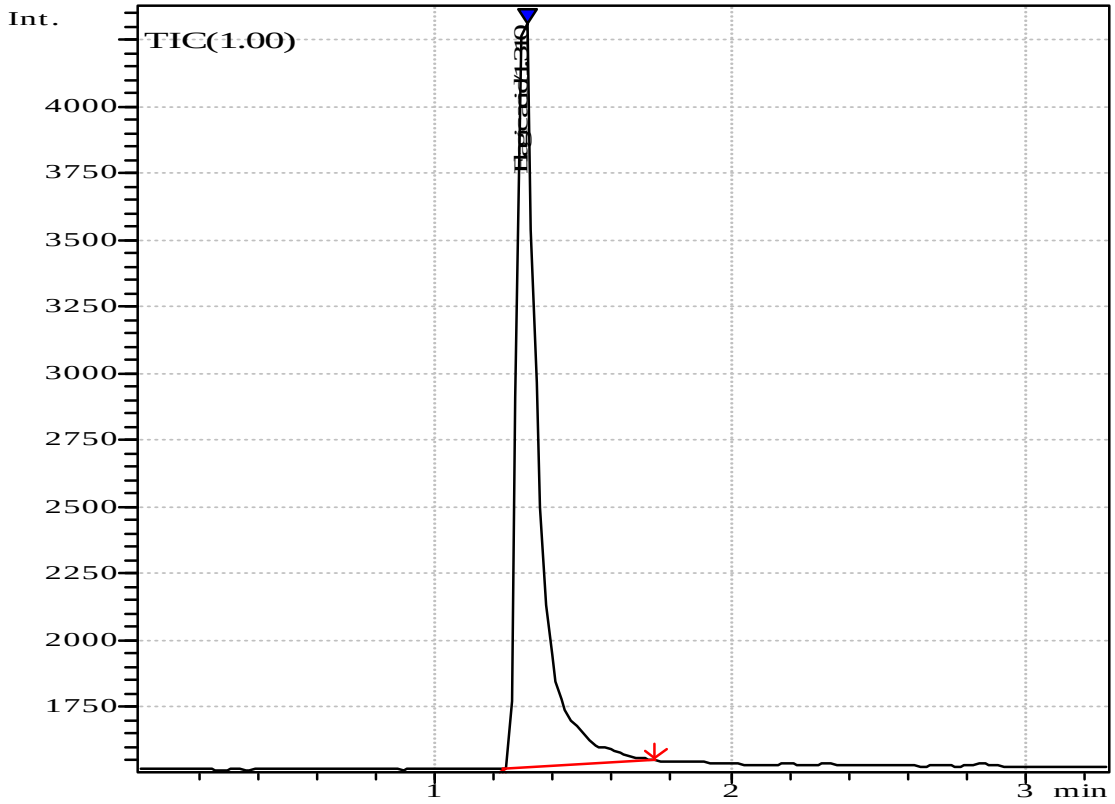

Fig. 2: Typical LC- MS chromatogram of Ellagic acid Standard solution.

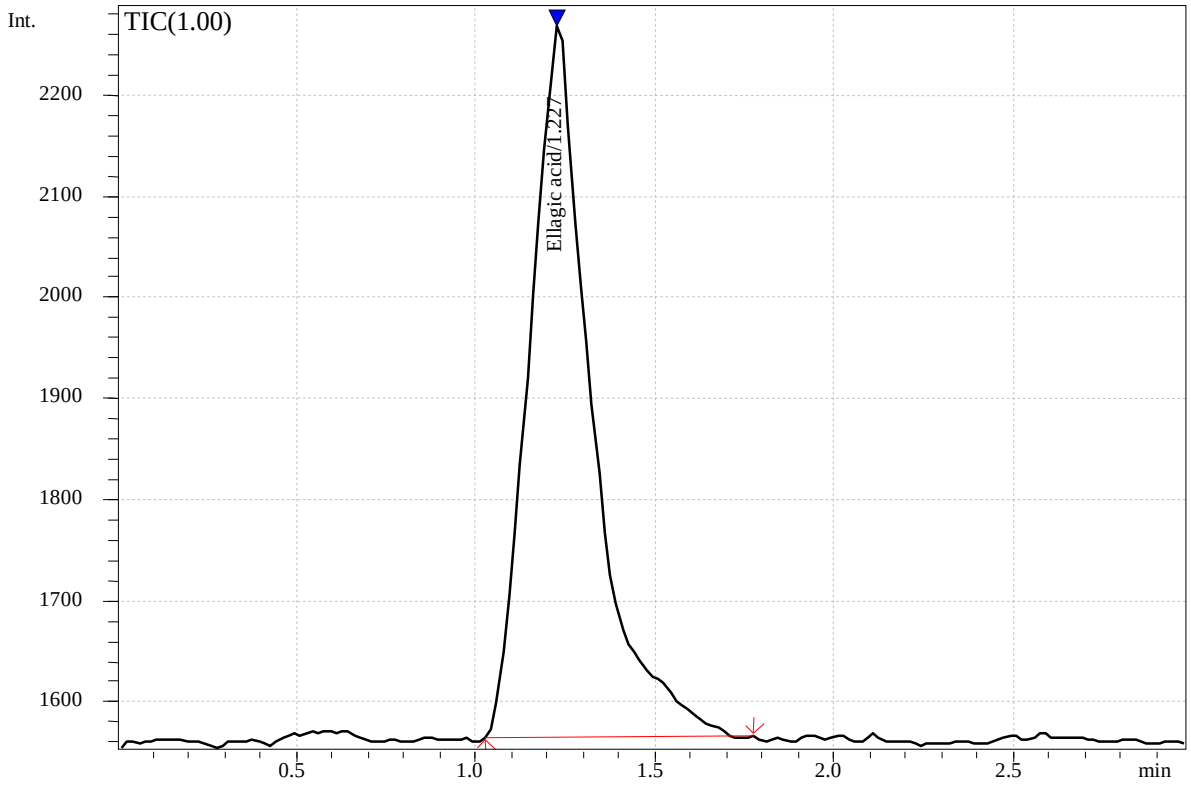

Fig. 3: Typical LC- MS chromatogram of Sample I containing Ellagic acid.

CONCLUSIONS

The proposed LCMS method for quantification of ellagic acid in fresh and processed fruits is simple, rapid, accurate, precise, linear, rugged and robust. Hence the present LCMS method is suitable for the quantification of ellagic acid in food products.

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