A Sensitive and High Throughput LC-MS/MS Method for the Determination of Resveratrol in Peanut Matrix

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Abstract

A precise and very efficient tandem mass spectrometry method (LC-MS/MS) was developed to quantify the resveratrol in the peanut matrix. Two mobile phases made up of water and acetonitrile in a ratio of 98:2 (%v/v) with 0.1% formic acid and acetonitrile and water in a percentage of 98:2 %(v/v) with 0.1% formic acid were used in the LC-MS/MS analysis of resveratrol on a BEH C-18, 130A° (50mm 2.1mm, 1.7µm) analytical column with 0.3 mL/min of flow rate.

A triple quadrupole mass spectrometer and a positive electrospray ionization system were used to quantify resveratrol in multiple reaction monitoring (MRM) modes. The technique was evaluated and was found capable of quantifying resveratrol in peanut samples starting at 10 ng/g in compliance with SANTE guidelines.

Keywords: Resveratrol, Peanut, LC-MS/MS, Validation.

Introduction

The legume plant *Arachis hypogaea* L., known as the peanut, is self-pollinated and cultivated on more than 28.5 million hectares of land worldwide, producing 45.95 million tonnes of goods[9,10]. Across Asia, Africa and the Americas, there are over 100 countries that grow it predominantly in semi-arid tropical climates. Oil (48–50%), protein (25–28%) and carbs (18%) are all abundant sources of energy in peanut seeds. They also include a variety of other vital nutrients including fatty acids, iron, zinc, calcium, magnesium, phosphorus, folate and vitamin E. The major element of the plant is the peanut kernel which is used to make peanut butter, peanut cake, chikki and a variety of other items for eating[4]. The peanut is known as the "poor man's almond", since it has excellent nutritional value and significance in the fight against malnutrition.

Peanuts contain bioactive compounds such as polyphenols, isoflavones, flavonoids and stilbenes. These bioactive compounds are highly beneficial to the body and function as an antioxidant[11]. P-coumaric acid, resveratrol and vitamin E are just a few of the numerous antioxidants in peanuts. Takaoka initially isolated resveratrol (3, 5, 4′-trihydroxy-trans-stilbene), a phytoalexin stillbenoid from the roots of white hellebore in 1940[13]. Red wines, grapes, peanut butter and peanuts are the primary sources of it[5]. The molecular weight of the trans-resveratrol is 228, which has two phenolic rings and a styrene double bond (Figure 1). The more stable form is the trans-form[15]. Hence, the trans-form is prevalent and has a lot of bioactive properties.

Resveratrol is biosynthesized from the p-coumaric acid with the help of resveratrol synthase[13]. It effectively inhibits reactive oxygen species and hence has effective antioxidant properties associated with a cardiovascular protective effect, delaying aging, neurodegenerative reduction of cancer, Parkinson's and Alzheimer's disease phytoestrogen activity and reducing pain and hyperglycemia. As per the literature, the researchers focused on determining resveratrol in red wine and peanut content through LC-MS/MS, GC-MS/MS and HPLC analysis with longer run times and higher LOQs[1,6,8].

We previously reported the LC-MS/MS method for the resveratrol content and its losses upon processing in selected peanut accessions but with a limited focus on the method development and validation[12]. Thus, the current study aimed to develop and evaluate a high throughput, sensitive and reliable method to measure the resveratrol content of peanuts. A hyphenated process known as LC-MS/MS, which combines chromatography with mass spectrometry, was developed and verified in compliance with SANTE/12682/2019[3] and SANTE/11312/2021[2] for the detection of resveratrol in peanuts. In this manuscript, the word resveratrol refers to trans-resveratrol only.

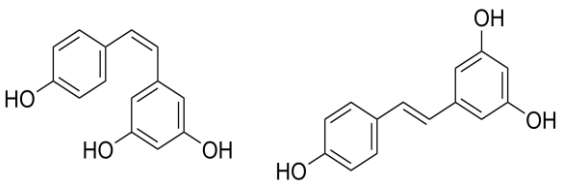

Figure 1: Chemical structures of *cis*- ((*Z*)-resveratrol, left) and *trans*-resveratrol ((*E*)-resveratrol, right)

Material and Methods

Reagents and standards: We purchased MS-grade methanol and acetonitrile from Merck (Mumbai, India). Purified water was collected through Pacific TII and Micropore UV water purification system (Thermo Scientific, California, USA). Certified reference material of resveratrol (catalog number: R150000) utilized to prepare calibration standards and quality control checks was purchased from Toronto Research Chemicals, Canada.

Instrumentation: A liquid chromatograph (Acquity UPLC H-Class) hyphenated to triple quadrupole mass spectrometer

(TQS Micro) from Waters Corporation, USA was used for method development, validation and analysis of samples. Masslynx 4.1 software was used for data acquisition and processing.

Operating conditions of LC/MS/MS: The analytical column used in the LC/MS/MS process was a BEH C18, 130 A°, 1.7 m, 2.1 mm x 50 mm column. It was purchased from the USA-based Waters Company. According to the gradient program in table 1, two mobile phases were used: A) 98% water + 2% acetonitrile with 0.1% formic acid; and B) 98% acetonitrile + 2% water with 0.1% formic acid. The addition of formic acid was reported to improve the ionization efficiency in positive ESI mode[14]. The injection had a 5µL volume and the run time was 6 min.

The mass spectrophotometer was run using electrospray ionization (positive) mode (ESI) to measure resveratrol. Multiple reaction monitoring (MRM) modes were used for each analysis with a dwell period of 50 ms and three transitions. The mass spectrometric parameters are given in table 2. The sample cooler temperature was 5°C while the column oven temperature was 45°C. The injection volume was 5µL.

Sample and standard preparation: The method reported by Lee et al[6] was used with minor modifications. Peanut samples were finely ground using a blender. 5 g of the homogenized material was accurately weighed into a 50 mL polypropylene centrifuge tube, then 25 mL of the extraction solvent (acetonitrile and water in an 8:2 ratio, v/v) was added. With a digital ultra turrax homogenizer (Make: Ika, Model: T25), the sample is homogenized at 10000 RPM for three minutes and incubated for 30 minutes at 70°C in a water bath. The extract was centrifuged for five minutes at 4000 RPM for 5 minutes after being cooled to room temperature. From the supernatant, 1 ml was collected for cleanup with C18 50 mg by using a dispersive solid phase extraction to remove the interferences.

The extract was centrifuged at 15000 RPM for five minutes while being vortexed and filtered with a 0.22 µm nylon syringe filter (Catalogue number CH2225-NN purchased from Thermo Scientific). 0.5 mL was taken from the filtrate and diluted to 1 mL using extraction solvent. The obtained extract is injected to the LC-MS/MS for resveratrol determination. Matrix-matched 6-point calibration including calibration blank, was performed in the 1 – 100 ng/mL range using resveratrol-certified reference material. Method validation and calibration acceptance criteria were followed in SANTE guidelines. The deviation back-calculated concentration should be within ±20% of the accurate concentration for the calibration to be acceptable. The calibration plot is shown in figure 2. Acquired data were quantified using the software MassLynx 4.1 version.

Results and Discussion

Method development: The primary goal of this study was to develop and validate a robust, sensitive and high throughput LC-MS/MS technique to measure resveratrol in peanut matrix. After a couple of gradient programs, the gradient program mentioned in table 1 in combination with BEH C18, 2.1 mm x 50 mm, 1.7 µm and 130 A°, was adopted due to proper separation of matrix interferences and symmetrical resveratrol peak shape.

Table 1
Mobile phase gradient profile for resveratrol determination

Time (min)	Flow Rate (mL/min)	% A	% B
0.00	0.30	90	10
2.00	0.30	20	80
4.00	0.30	10	90
4.10	0.30	90	10
6.00	0.30	90	10

Table 2
Mass spectrometric parameters for estimation of resveratrol content

Source Parameter			Value		
Polarity			+ve		
Capillary voltage			3.0 KV		
Desolvation gas flow			1000 L/hr		
Source temperature			150°C		
Cone gas flow			50 L/hr		
Desolvation temperature			500°C		
Parent Ion (Q1)	Product Ion (Q3)	Dwell time (ms)	Quantifier / Qualifier	Cone voltage (V)	Collision energy (V)
229	107	50	Quantifier	25	22
229	135	50	Qualifier	18	12
229	91	50	Qualifier*	34	21

* Second qualifier, where required

Optimization of MS-MS parameters: Since the ionization efficiency of the sample determines the method's sensitivity, optimization of capillary voltage was performed and it was found that 3.0 KV was suitable for optimum ionization with 1000 L/hr of desolation gas flow and a 50 L/hr cone gas flow rate. The dissolving temperature was 500 °C. Cone voltage and collision energies were also optimized to get proper mass fragmentation.

Method validation

Specificity: Resveratrol standard solution was prepared at 10 ng/L in diluent and injected with regent blank and control matrix samples. Figure 2a showed that no peak was found at the retention times of resveratrol.

Matrix Effect: The matrix effect was evaluated by comparing the response difference of resveratrol standard in matrix extract and solvent. The matrix effect observed was within the acceptable criteria i.e. ≤ 20%. Same was presented in figure 3a.

Determination of LOD and LOQ: The limit of detection (LOD) 2.3 ng/g and limit of quantification (LOQ) 7.1 ng/g were calculated theoretically from the linear regression model (Table 3) and established LOD and LOQ are 3.0 ng/g and 10 ng/g respectively (Tables 3). Data generated from six replicated injections at LOD and LOQ concentrations was found within acceptance criteria.

Linearity: A six-point calibration graph between 1.0 and 100.0 ng/mL verified the matrix-matched linearity of resveratrol. Plotting the area of resveratrol injections versus the concentration expressed in ng/mL resulted in the calibration curve. The data and the values in figure 3b display the slope, intercept and correlation coefficient from the linear least-square regression analysis. It demonstrates a strong correlation between peak areas and concentration of resveratrol with a correlation coefficient of more than 0.998. Back calculated concentrations were found within ± 20%.

Recovery studies: Recovery studies were performed at the LOQ level and between 2-10 times to the LOQ level in peanut samples to assess the correctness using the conventional addition procedure. The percentage recoveries were then calculated and presented in table 4. Excellent recovery values of resveratrol (80.6 -110.9%) were obtained with less than 12 % RSD.

Precision: In order to determine the repeatability, the relative standard deviation (RSD) of six measurements was computed by injecting six freshly prepared samples at 3 concentration levels in 3 different days. Repeatability (RSDr) at three levels was 6.3 to 11.4% and results are presented in table 5. Within lab, reproducibility (RSDR) at three levels was in the range of 8.6 to 10.1, % and results are presented in table 6. The low % RSD readings show that the developed method has good precision.

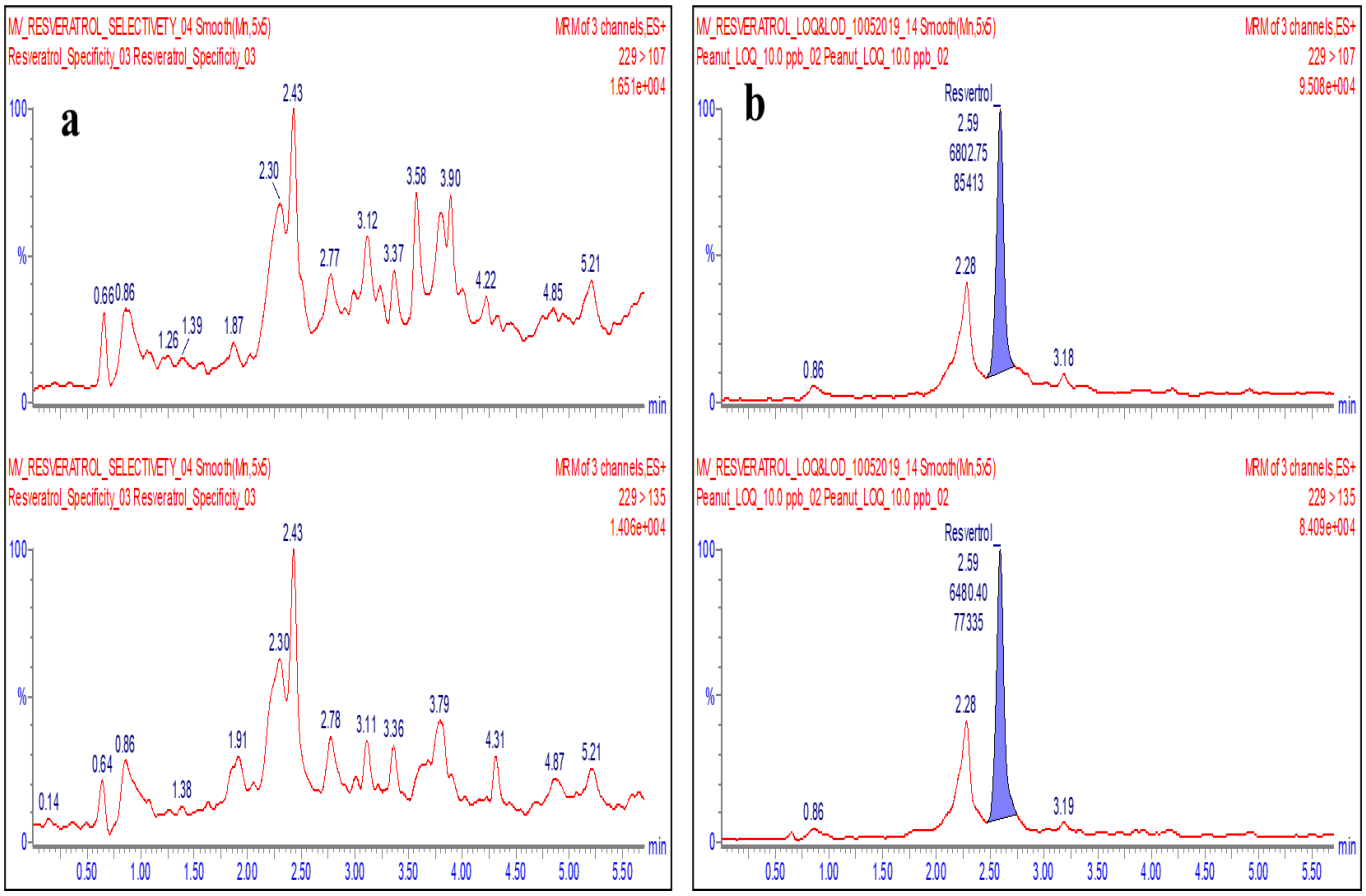

Figure 2: a) Specificity Chromatogram of resveratrol in control peanut and b) LOQ level in Matrix

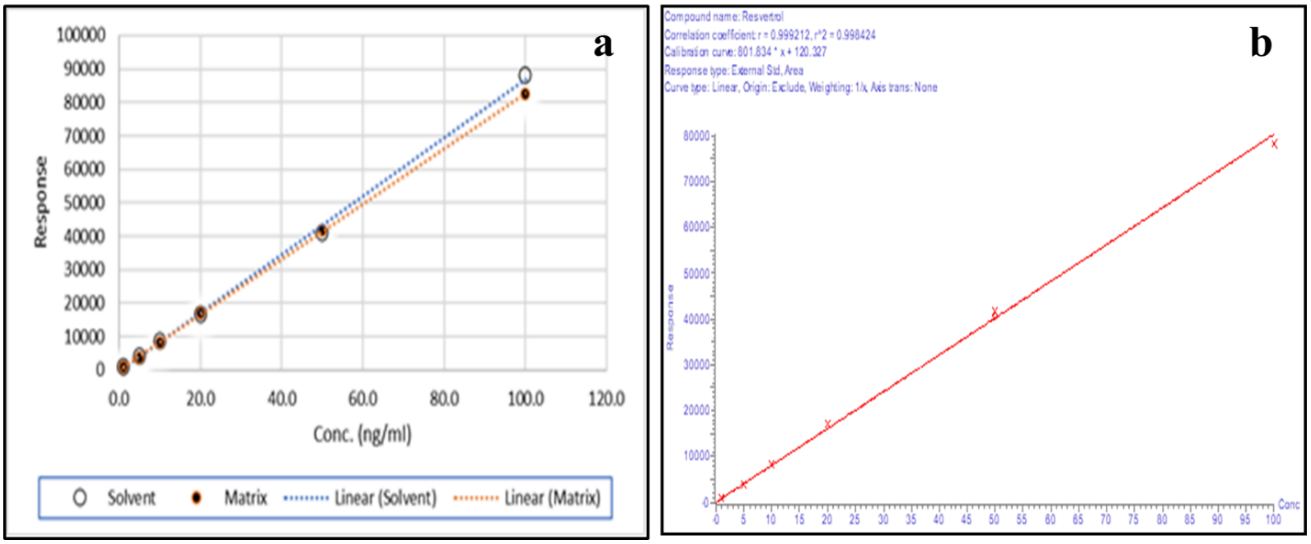

Figure 3: a) Matrix effect study b) Linearity curve of analyte in matrix

Table 3
Theoretical calculation of LOD and LOD with the linear regression model

Concentration (ng/mL)	Response	Slope Details	
1.0	1457	Slope	535
5.0	3858	Intercept	1365
10.0	6854	Standard error Y Intercept	377
20.0	12587	LoD	2.3
50.0	28211	LoQ	7.1
100	54661		

Table 4
Results of the recovery studies

Spiking Level	Spiked concentration (ng/g)	Day-01 Calculated conc. (ng/g)	Recovery (%)	Day-02 Calculated conc. (ng/g)	Recovery (%)	Day-03 Calculated conc. (ng/g)	Recovery (%)
Spike at 3 times of LOQ	30.0	27.2	90.6	25.4	84.5	28.7	95.6
	30.0	31.8	106.1	30.5	101.6	32.8	109.5
	30.0	33.0	110.1	32.2	107.2	33.3	110.9
	30.0	27.1	90.5	26.2	87.3	29.3	97.7
	30.0	31.1	103.8	29.1	97.1	32.3	107.8
	30.0	32.5	108.4	32.8	109.5	32.7	108.9
Spike at 6 times of LOQ	60.0	52.8	88.0	51.4	85.6	55.6	92.6
	60.0	63.2	105.4	57.1	95.1	63.3	105.5
	60.0	64.1	106.8	62.9	104.9	65.3	108.8
	60.0	54.0	90.0	52.4	87.3	54.6	91.0
	60.0	62.7	104.6	58.6	97.7	63.2	105.3
	60.0	65.8	109.7	64.8	107.9	65.2	108.6
Spike at 9 times of LOQ	90.0	73.5	81.7	73.9	82.1	73.8	82.0
	90.0	83.7	93.0	81.6	90.6	84.2	93.5
	90.0	96.6	107.3	91.1	101.2	88.1	97.9
	90.0	76.7	85.3	73.5	81.6	76.5	85.0
	90.0	81.8	90.9	81.4	90.4	86.3	95.9
	90.0	96.1	106.8	93.6	104.0	98.3	109.3

Table 5
Precision (RSDr) results of resveratrol

Spiking Level	Spiked concentration (ng/g)	Day-01 Calculated Conc. (ng/g)	Repeatability (%)	Day-02 Calculated Conc. (ng/g)	Repeatability (%)	Day-01 Calculated Conc. (ng/g)	Repeatability (%)
Spike at 3 times of LOQ	30.0	27.2	8.7	25.4	10.5	28.7	6.3
	30.0	31.8		30.5		32.8	
	30.0	33.0		32.2		33.3	
	30.0	27.1		26.2		29.3	
	30.0	31.1		29.1		32.3	
	30.0	32.5		32.8		32.7	
Spike at 6 times of LOQ	60.0	52.8	9.2	51.4	9.4	55.6	7.9
	60.0	63.2		57.1		63.3	
	60.0	64.1		62.9		65.3	
	60.0	54.0		52.4		54.6	
	60.0	62.7		58.6		63.2	
	60.0	65.8		64.8		65.2	
Spike at 9 times of LOQ	90.0	73.5	11.4	73.9	10.2	73.8	10.4
	90.0	83.7		81.6		84.2	
	90.0	96.6		91.1		88.1	
	90.0	76.7		73.5		76.5	
	90.0	81.8		81.4		86.3	
	90.0	96.1		93.6		98.3	

Table 6
Precision (RSDR) results of resveratrol

Spike Concentration (ng/g)	No. of Replicates	Mean Conc. (ng/g)	Mean Recovery (%)	SD	Within lab reproducibility (RSDR)
30	18	30.45	101.5	2.61	8.57
60	18	59.82	99.7	5.17	8.64
90	18	83.93	93.3	8.50	10.13

Table 7
Comparison of LoQ and Runtime of the present method with other methods

Method	Sample Matrix	LoQ	Run Time (min)	Ref.
HPLC	Peanut	0.09 µg/g	20	6
HPLC	Peanut	0.01 µg/g	22	14
HPLC	Peanut	0.03 µg/g	14	8
SPE HPLC–ESI-MS/M	Peanut	0.40 µg/g	16	16
LC-MS/MS	Peanut	7.1ng/g	6	Present work

Robustness: With deliberate changes, the method's resilience was investigated in incubation temperature, centrifugation RPM, column temperature and desolvation temperature in the ESI source of MS/MS.

The Incubation temperature was altered by 5°C i.e.70 to 65 and 75 °C. At 42°C and 48°C, column temperature's effects on the resolution of interferences were investigated (temperature altered by three units). The source temperature was also changed by 50°C from 450°C to 550°C and the findings showed that these modifications did not affect the chromatographic or extraction processes' effectiveness process.

Comparison of current method with the existing methods: The current method has a clear advantage over the existing methods reported in the literature (Table 7). The present process has the shortest run time of 6 min with a retention time of 2.5 min and a lower limit of quantification values than the methods reported in the literature.

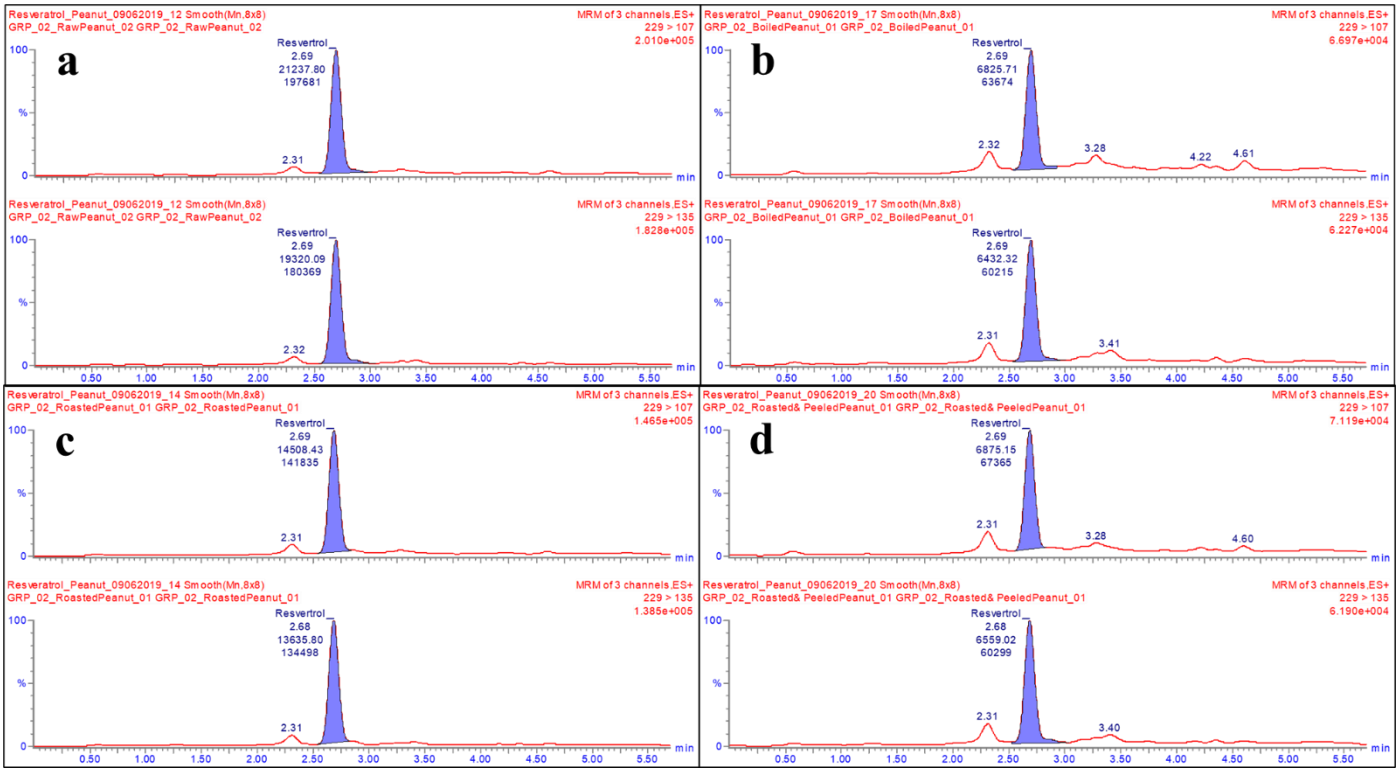

Figure 4: Chromatograms of Resveratrol in a) Raw b) Boiled c) Roasted and d) Roasted and Peeling Peanuts

Table 8
Resveratrol content in Peanut at different condition

S.N.	Sample Name	Resveratrol (Mean ± SD)	% Loss
1	Raw	475.4 ± 27.0	-
2	Boiled	151.8 ± 11.4	68.06
3	Roasted	286.5 ± 16.6	39.73
4	Roasted and Peeling	134.7 ± 8.8	71.66

Application of Method: The corresponding chromatograms are shown in figure 4. The amount of resveratrol overall and its losses during processing such as roasting, boiling and peeling subsequent to roasting, are also examined compared with the raw peanut in this study. The amount of resveratrol is seen to decrease during various processes including boiling, roasting and peeling following roasting and the results are presented in table 8. According to the study, roasting loses relatively less resveratrol than other processing methods.

Conclusion

A selective, robust and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for the determination of resveratrol in peanut matrix. The current method is specific, linear, exact, accurate and reliable. The calibration curve linearity is good and has a correlation value (r) of greater than 0.9992 between 1.0 ng/mL and 100 ng/mL.

It has been demonstrated that it is highly sensitive with a low limit of detection (LOD) of 3.0 ng/g and limit of quantification (LOQ) values of 10.0 ng/g with respect to resveratrol sample concentration. An excellent recovery value of resveratrol was obtained. The information presented in this study could be beneficial for monitoring resveratrol in peanut matrix.

Acknowledgement

We are grateful to management of National Commodities Management Services Limited's management for their support and help in this work.

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(Received 23rd April 2023, accepted 24th May 2023)