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Research Article

Simultaneous Estimation of Flavonoidic Compounds of *Punica granatum* Extract by LC-ESI-MS/MS Technique

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ABSTRACT

Pomegranate pericarp was reported to contain ingredients like, phenolic punicalagins, gallic acid, flavan-3-ol like catechin, EGCG (Epicatechin Gallocatechin),3, 4-oxo-flavonoids like quercetin, myricetin, rutin.So far no simultaneous determination of quercetin, catechin and myricetin flavonoids in pomegranate peel has been reported. In this study LC-ESI-MS/MS was used for the simultaneous detection of three flavonoids quercetin, catechin and myricetin (bio-markers) from pomegranate peel extract.Good separation was obtained using standard solution of bio-markers within 7.5 min and analysed by electro spray ionization (ESI) techniques with the gradient mobile phase system. Selective ion monitoring (SIM) afforded by tandem mass spectrometry has greater advantage of reducing interference and enhancing sensitivity over selected ion monitoring, the peaks at the retention time of 5, 6.4 and 7 min were identified as catechin, myricetin and quercetin respectively. The technique was optimized having good specificity, linearity and precision of RSD value was less than 5%. The developed method was validated and has been applied for quantitative determination of catechin, quercetin and myricetin in the extract of agro waste of *Punica granatum* with negative SIM (Selective ion monitoring) mode with good precision and accuracy.

Keywords: *Punica granatum,* LC-ESI-MS/MS, Polyphenols, Selective ion monitoring

1. INTRODUCTION

Flavonoids are large group of compounds that ubiquitously exist in natural products and have been considered as active ingredients of many medicinal plants. Flavonoids are polyphenols with diphenylpropanes (C_6-C_3-C_6) skeletons. Phenolic compounds may exert beneficial effects through their free radical scavenging and anti-oxidant potential. Pomegranate pericarp was reported to contain ingredients like, phenolic punicalagins, gallic acid, flavan-3-ol like catechin, EGCG (Epicatechin Gallocatechin),3, 4-oxo-flavonoids like quercetin, myricetin, rutin, anthocyanidineetc[1]. The biomarkers of the peel of this plant were targeted for the following work as catechin, quercetin and myricetin. The LC-MS characterization previously reported mainly concentrated on tannins of pomegranate peels. The LC-MS study also revealed the presence of anthocyanin's and other non-phenolic entities in the peel extracts. Presence of quercetin was also detected from peel extract. Up till now different kind of ionization methods have been developed among which electrospray ionization (ESI-MS) has been used for the analysis of natural products in either positive or negative ion mode. So far no simultaneous determination of quercetin, catechin and myricetin flavonoids in pomegranate peel is reported. In this study LC-ESI-MS/MS was used for the simultaneous detection of three flavonoids quercetin, catechin and myricetin from pomegranate peel extract[2,3].

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2. MATERIALS AND METHODS

2.1 Materials, Chemicals and Reagents

All the biomarkers were purchased from Sigma (USA). All technical grade solvents were procured from SD Fine Chemicals (India). Peels of *P. granatum* were obtained from local markets, Mumbai. Water was purified by Milli-Q water Purification system (Millipore, MA, and USA).

2.2 LC instruments and analytical condition

Polyphenol analysis by LC-ESI-MS/MS was carried out using an Agilent 1100 series LC and LC/MSD Trap VL mass spectrometer (Agilent Technologies, USA) equipped with electrospray ionization (ESI) interface. In order to obtain optimum ionizing conditions, using the reference solution, both atmospheric pressure chemical ionization (APCI) and electrospray ionization interface were tested in positive and negative ion modes by scanning between m/z 200-550 per second. The column temperature was maintained at 25^0C. Quantification was achieved using selected ion monitoring system (SIM) mode. The flow rate was 0.5ml/min.

2.3 Preparation of standard solution and working reference solution

Standard solution was prepared by dissolving biomarkers like quercetin, catechin and myricetin in methanol. The concentration prepared was in the range of 5ppb-200ppb both for the biomarkers and the extract. Biomarkers like quercetin, catechin and myricetin were taken in combination to prepare biomarker mixture as working reference solution for simultaneous determination of these three polyphenols.

2.4 Preparation of calibration curve and quality control sample

Different concentrations of the standard solution ranging from 5ng/ml-200ng/ml were prepared for calibration samples. Quality control samples were generated to yield three final different concentration levels (10ng/ml, 50ng/ml and 100ng/ml) to determine accuracy. All the samples were stored at 4^0C and brought to room temperature before use.

2.4 Preparation of High Yield Polyphenolic Extract (HYPE) of *Punica granatum*

High yield Polyphenolic extracts were prepared as per reported in the literature[4]. Around 10 gm peel was stirred with acetate buffer (pH 4.6) and 5000 ppm of pectinolytic and cellulolytic enzyme preparation at 40^0C for 1hr. The solution was evaporated under vacuum. The dried residue of powder and enzyme was taken in soxhalation process of extraction for 2h and filtered and concentrated under vacuum at 40^0C.

2.5 Method validation

Calibration curves of the standard compounds (catechin, myricetin and quercetin) were prepared using standard solutions in various concentrations. The linearity of the calibration curves was validated using measurements in triplicate. The limit of detection (LOD) was determined as the concentration giving a signal-to-noise (S/N) ratio of 3:1. The lower limit of quantification (LLOQ) was defined as the lowest concentration in the standard curve at which the percentage coefficient of variation (%CV) was below 15%. Specificity was established by the lack of interference peaks at the retention times for the standards. Linearity was evaluated at six concentration levels, encompassing a range between 5ppb-200ppb. The linear regression equations and correlation coefficients were calculated using the least-squares method.

3. RESULTS AND DISCUSSION

3.1 Optimization of chromatographic conditions

Under the optimized reverse phase LC-MS/MS conditions mentioned in experimental section, good separation of biomarkers was obtained within 7.5 min and analysed by electro spray ionization (ESI) techniques with the gradient mobile phase consisting of A = Methanol: Water with 5mM ammonium formate buffer (9:1) B = Water: Methanol,5mM ammonium formate buffer (8:2). Gradient elution was employed and was used as follows: starting with 10%B and increasing linearity to 90%B within 3 min, and then increasing linearity to 100%B within 7 min and at last re-equilibrating linearity to 10%B within 10 min. In the course of experiments, several ratio of methanol-water in isocratic system were studied and the effect of pH value was examined. Effect of pH was studied with the help of volatile buffer used in LC-MS.

3.2 Validation of assay

A developed LC-MS/MS method is already described above. Mass spectrometric conditions were optimized so as to achieve the maximum stable response of the parent ions and major

production of the analyte fragments. Selective ion monitoring (SIM) afforded by tandem mass spectrometry has greater advantage of reducing interference and enhancing sensitivity over selected ion monitoring. The representative total ion chromatogram (TIC) obtained in negative and positive ESI mode is shown in (**Figure- 1**) for catechin, myricetin and quercetin. The peaks at the retention time of 5, 6.4 and 7 min were identified as catechin, myricetin and quercetin respectively and (**Table- 1**) showing MS/MS fragments information of polyphenol standards.

Table 1: MS/MS fragments details of polyphenol standards

MS/MS fragments	Catechin	Quercetin	Myricetin
$[M-H]^-$	289	301	317
Loss of B ring (Catechol)	108.5		
$^{1,2}B^-$		178.2	178.2
$^{1,2}B^-$ - CO		150.3	
Loss of trihydroxy benzene methane from $[M-H]^-$			136.5
Loss of Catechol methyl from $[M-H]^-$		120.6	

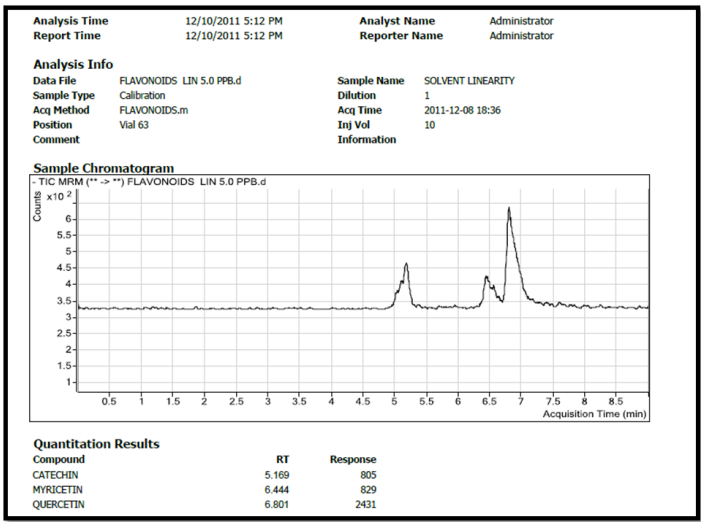

Figure 1: Total Ion chromatogram (TIC) of catechin, myricetin and quercetin respectively

3.3 Optimization of MS/MS detection condition

For each injection the negative MS/MS spectra was obtained by collision-assisted dissociation (CAD) to form $[M-H]^-$ ions. To optimize electrospray ionization (ESI) conditions for catechin, myricetin and quercetin a full scan was carried out in negative ion detection mode as per literature. **(Table- 2)**

Table 2: Fragments and TIC details of the biomarkers

Peak No	Biomarkers	Retention time (min)	$[M-H]^-$ (m/z)	Fragment ion (m/z)
1	Catechin	5.2	289	108.5
2	Myricetin	6.5	317	178.2, 136.5
3	Quercetin	7	301	178.2, 150.3, 120.6

3.3.1 Specificity

Specificity of the flavonoids catechin, myricetin and quercetin was evaluated by MRM spectra. (**Figure- 2, 3, 4**)

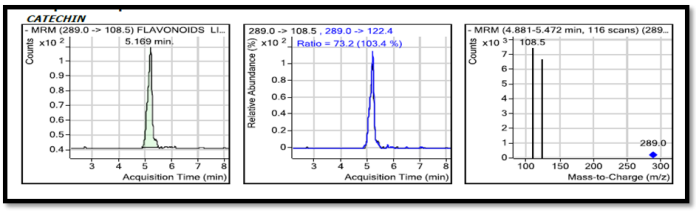

Figure 2: MS/MS spectra of catechin

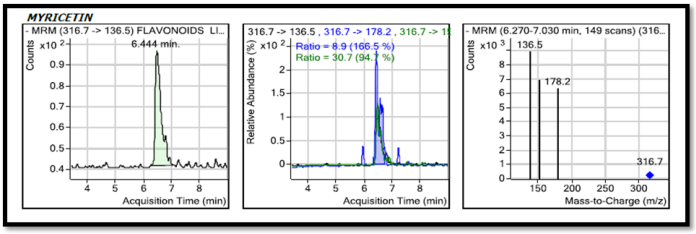

Figure 3: MS/MS spectra of myricetin

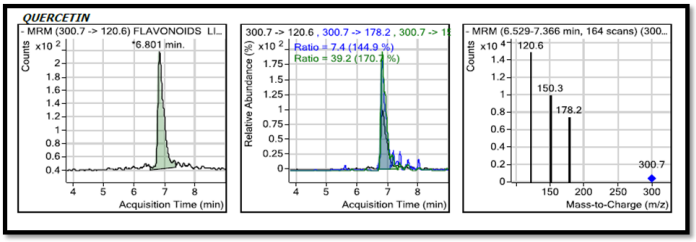

Figure 4: MS/MS spectra of quercetin

3.3.2 Linearity

The calibration curves were linear in the range of 9-200 ng/ml for catechin, myricetin and quercetin. The calibration equations, Linear range, LOD and LOQ of catechin, myricetin and quercetin respectively illustrated in (**Table- 3**). A solvent blank was analysed for determining limit of detection (LOD) and no peaks at m/z 289 (catechin), m/z 316 (myricetin) and m/z 300 (quercetin) was found.

Table 3: Standard curves, linear ranges, LOD and LOQ of the three compounds

Compounds	Linear ranges	Linear equations	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
Catechin	9-200[a]	Y = 104.872661X – 261.726272 (.9993),	1.55379	5.1793
Quercetin	9-200[a]	Y = 279.340377X – 996.312061 (.99784272)	1.54071	5.1357
Myricetin	9-200[a]	Y = 113.071783X + 258.300790 (.9973),	1.5282	5.094

[a]ng mL^{-1}

3.3.3 Precision / repeatability

In this study, the precision was measured by means of repeatability by six replicate analyses of quality control samples of catechin, quercetin and myricetin. In all cases the RSD value was less than 5%, which was considered to be acceptable. **(Table- 4, 4.1, 4.2)**

RSD = SD/Mean X100

3.3.4 Accuracy/Recovery

The recovery of the method was determined by analysing relative error (RE) at three quality control level. This experiment was carried out by analysing replicates (n=3) at three QC level. The accuracy was in the order of -.0832-4.68 (1.66-9.37%) for all three flavonoids. The recovery was found to be 86.1512%-101.664%. As showed in the (**Table 5)** the recovery and precision of the developed method were very satisfactory.

Table 4: Precision table for Catechin

Precision- Catechin	At 5ng	At 20ng	At 50ng
Mean	4.58142	18.5845	46.78963
SD	0.14181	0.178904	0.31421
RSD	3.09	0.9626	0.67153

Table 4.1: Precision table for Quercetin

Precision- Quercetin	At 5ng	At 20ng	At 50ng
Mean	5.083283	17.5058	46.65763
SD	0.08878	0.202224	0.339845
RSD	1.7465	1.15	0.7283

Table 4.2: Precision table for Myricetin

Precision- Myricetin	At 5ng	At 20ng	At 50ng
Mean	4.30756	18.57478	45.31468
SD	0.091741	0.156095	0.439506
RSD	2.1297	0.8403	0.9698

Table 5: Results for recovery analysis

Compounds	Added	found	Relative error (%)	Percent recovery
Catechin	5	4.58142	0.41858 (8.3)	91.6284
	20	18.5845	1.4155 (7.0775)	92.9225
	50	46.7896	3.2104 (6.4208)	93.5792
Quercetin	5	5.0832	-0.0832 (1.664)	101.664
	20	17.5058	2.4942 (12.47)	87.52
	50	46.6576	3.3424 (6.6848)	93.3152
Myricetin	5	4.30756	0.69244 (13.84)	86.1512
	20	18.5747	1.4253 (7.1265)	92.8735
	50	45.3146	4.6854 (9.3708)	90.6292

3.3.5 Application of LC-ESI-MS-MS methods for qualitative and quantitative study of biomarkers in High Yield Polyphenolic Extract (HYPE) of an agro waste of *punica granatum*

The developed method has been applied for quantitative determination of catechin, quercetin and myricetin in the extract of an agrowaste of *Punica granatum.* The typical LC: ESI: MS: MS chromatogram of extract of an agro waste of *Punica granatum*is displayed in (**Fig-5)**.The retention time of the unidentified peaks did not overlay the peaks of the standard polyphenols. **(Table- 6)**

Table 6: Results of polyphenols in extract (HYPE) of *P.granatum*

Polyphenols	Amount present in 1000 µg of extract	Retention time (mins)
Catechin	125 µg	4.927
Quercetin	18.3 µg	6.033
Myricetin	0.6 µg	6.452

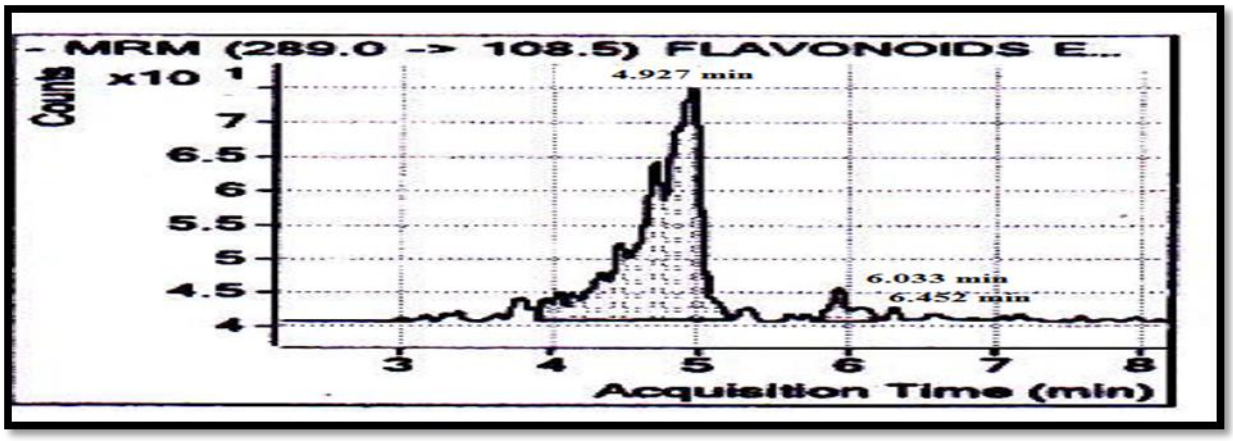

Fig 5: LC-ESI-MS-MS chromatogram for *Punica granatum* peel extract

4. CONCLUSION

A LC-ESI-MS/MS method for the qualitative and quantitative estimation of flavonoids in *P.granatum* extract was developed. Three flavonoids like catechin, quercetin and myricetin were selectively targeted for the estimation and were quantified with good precision and accuracy. With negative SIM (Selective ion monitoring) mode, a LC-ESI-MS/MS method was validated for quantitative determination.

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