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Spectroscopic & Chromatographic Methods for Quantitative Analysis of Phospholipid Complexes of Flavonoids – A Comparative Study

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Abstract

Introduction- Polyphenols belong to a chemical class of flavonoids which are widely distributed in vegetables and plants. They possess a number of biological activities and thus received much attention in phytotherapy research. The phospholipid complexes were originally coined as "Phytosomes". No literature has been reported on the reactivity of polyphenols towards phospholipids depending upon refluxing time, cooling temperature, nature of phospholipids and the polyphenols in mixture form or in an isolated form. We aim to compare different analytical techniques for quantification of biomarkers and their phospholipid complexes. In this study biomarkers were taken at standard concentrations and its phospholipid complex were prepared. LC-MS/MS, HPLC, FTIR and NMR analytical techniques were used to quantify, characterize and monitor the complexes. Methods used for complexation of biomarkers gave good yields. 1H-NMR and FT-IR were reported for confirmation of phospholipids complex with natural polyphenols but the present study highlighted the need of spectrometry and chromatographic system in order to get better clarity regarding the formation of phospholipids complexes. NMR spectra can only give us the information if the complex has formed or not but doesn't give the required information which flavonoid has complexed. Mass spectrometry revealed good precision and gave superior results over NMR of the respective complexes formed. More over the purity of these complexes which were formed during the reaction can be understood by the chromatographic system. This work found novelty in terms of revealing the molecules involved and their efficiency in the process of complexation.

Keywords: Flavonoids; HPLC; NMR; LC-MS/MS; FTIR

Introduction

Polyphenols belong to the chemical class of flavonoids which are widely distributed in vegetables and plants. They have been demonstrated to possess a number of biological activities and thus received much attention in phytotherapy research. All these polyphenols possess catechol group which make them a potential antioxidant. But large structures of flavonoid polyphenols restrict their diffusion across the biological membrane which limits their absorption. Poor absorption is one of drawback in polyphenol efficacy *in vivo* upon per-oral administration. To overcome these problem a lipid compatible complexes of these flavonoids has been reported. These complexes were called as phospholipid complex or "Phytosomes". Considerable reported literature has demonstrated the phospholipid complex formation between polyphenols like flavonoids and terpenoids with phospholipids. As per reported literature phospholipids form a supramolecular complex by H-bonding with the flavonoids or terpenoids in 1:1 or 1:2 ratios [1,2]. In this study we aim to highlight the comparison and quantification of polyphenols and its phospholipid complex by chromatographic and spectroscopic analytical methods as no literature has reported it before.

Experimental

Materials, chemicals and reagents

All the biomarkers and phospholipids (Phosphatidylcholine – 60%) were purchased from Sigma (USA). All technical grade solvents were procured from SD fine chemicals (India).

Preparation of biomarker mixture for HPLC

Three standard polyphenols catechin, quercetin and myricetin prepared in methanol to give a concentration of 10 µg/ml. All the solutions were stored in the dark at 4°C. HPLC analysis indicated these solutions were stable within 2 months. Standard quercetin, catechin and myricetin solution was mixed in equiproportion (1 ml each of 10 µg/ml solution) to give 10 µg/ml solution of standard mixture of polyphenols. The final solution of standard polyphenol mixture was made in the range of 20-15000 ng/ml. These three polyphenols were chosen because these three polyphenols possess different solubility in various solvents as only myricetin is soluble in water, catechin is soluble in methanol and ethanol and not in water and quercetin is soluble in acetone. Methanolic extract was prepared for the polyphenols to successfully quantify using suitable analytical tools.

High Performance Liquid Chromatographic (HPLC) conditions

The chromatographic system (Jasco) consisting a PDA detector was used. A reverse phase Qualisil BDS-C_{18} column (4.6 mm φ x 25. mm) packed with 5 µm diameter particles was used. In order to estimate the three polyphenols simultaneously a gradient phase chromatographic methods was developed. In the course of experiments, several ratio of acetonitrile:water in isocratic system were studied and the effect of o-phosphoric acid to regulate the pH value was examined.

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LC instruments and analytical condition

Polyphenols analysis by LC-ESI-MS/MS were 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, the reference solution was used were 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°C. Quantification was achieved using selected ion monitoring system (SIM) mode of ion. The flow rate was 0.5 ml/min.

Preparation of standard solution and working reference solution for LC-MS/MS

Standard solution was prepared by dissolving biomarkers like quercetin, catechin and myricetin in methanol. The concentration prepared was in the range of 5 ppm-200 ppm 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.

Preparation of phospholipid complex of biomarker mixture

Biomarkers were taken into a solution of phospholipid in dichloromethane (DCM) in 2:1 proportion (1gm 25 mg of phospholipids and 450 mg of biomarker mixture). Two types of reactions conditions were maintained during preparation of phospholipid complex. After refluxing for certain time the mixture was filtered and kept for evaporation under vacuum. The residue was re-dissolved in the DCM and added slowly to a non-solvent n-hexane. The resultant mixture was kept on cooling at room temperature (R-1). In second method all the steps were repeated except the last step where the resultant mixture was kept on cooling in refrigerator for overnight (R-2) (Table-1).

Characterization of phospholipid complexes

Phospholipids complexes were evaluated by FT-IR, ^{1}H-NMR, and Mass spectrometry.

Recording of FT-IR spectra

Individual biomarkers and phospholipid complexes were taken for KBr pellet method to record IR graph in diffuse reflectance FT-IR.

Recording of 1H-NMR spectra

^{1}H-NMR spectra of individual biomarkers and phospholipids complexes were recorded and furnished the data.

Recording of mass spectra

In mass spectra an ESI total ion chromatogram (TIC) scans was performed and analysed. Mass spectrometry was performed with an Agilent 6460 LC/MS/MS triple quadruple (Agilent Technologies, Santa Clara, CA) and an electrospray ionization source (ESI). To reduce the surface contact with the interior of the system, samples were directly infused via the Agilent 1290 automated injection system, with the column adaptation bypassed; 20 lL of each sample were infused at 100 lL/min. Between each run, the syringe and insert tubing were cleaned using buffer and chloroform to avoid sample to sample contamination. Phospholipids were identified by detection of specific leaving groups from precursor ions after collision-induced dissociation (CID) for example, the leaving group of fragmented phosphatidylcholine (PC) [3] (Figure 1).

Monitoring of phospholipid complexes reaction condition by HPLC

A gradient HPLC method for the polyphenols catechin, quercetin and myricetin was taken into consideration in monitoring the reaction condition during preparation of phospholipids. Phospholipids complex was not soluble in acetonitrile unlike polyphenols. Several solvent has been tested to dissolve the phospholipid complex and finally dioxane has been selected because its miscibility with water. Immiscibility of solvent and insolubility of complex in the solvent can leads to precipitation of complex in the mobile phase leading to clogging on the column thereby with the dioxane this problem was conquered.

Results and Discussion

Preparation of phospholipid complex of biomarker mixture

Two types of conditions were maintained during preparation of phospholipid complex (Table 2). Formation of bond is an exothermic reaction and it liberates energy after forming bond. Formation of phospholipids complex also requires liberating energy and hence can be facilitated by keeping them into cold condition (in order to liberate energy rapidly). This can be a possible justification for enhancing the yield of final complex.

Characterization of phospholipid complexes of polyphenols, phospholipids and polyphenols in isolated form

Phospholipids complexes showed typical behaviour observed by FT-IR, ^{1}H-NMR, and Mass spectrometry than its free form.

Recording of FT-IR spectra

Individual biomarkers and phospholipid complexes were taken for KBr pellet method to record IR graph in a diffuse reflectance FT-IR. A non-super imposable IR between the biomarkers and their phospholipid complexes reveals that the complexes are not a mere mixture but a separate chemical entity. Figure 2-4 deals with FTIR

Reaction condition	Refluxing time	Cooling condition
R-1	30 min	Room temperature
R-2	60 min	Refrigeration

Table 1: Reaction condition of phospholipids complex

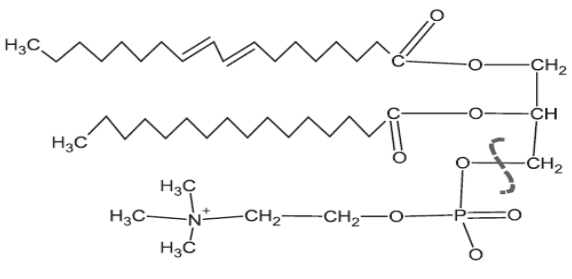


Figure 1: Fragmentation of Phospholipids

Reaction condition	Refluxing time	Cooling condition	Percent Yield
R-1	30 min	Room temperature	11.4% (175 mg /from 1.5 gm)
R-2	60 min	Refrigeration	13% (195 mg/from 1.5 gm)

Table 2: Yield of complexes

spectra of flavonoids and Figure 5-7 deals with phospholipid complex of flavonoids [4].

Recording of 1H-NMR spectra

^{1}H- NMR revealed the fact that complex formation leads to disappearance of peaks between 7-9 ppm with a broadened peak around 0.8-1 ppm. These evidences suggest the existence of H-bond formation between flavonoids and phospholipids. Figure 8 and 9 depicts quercetin and catechin NMR spectra, Figure 10 and 11 depicts NMR of phospholipid complex from the methods used and Figure 12 depicts NMR of pure phospholipids [4].

Recording of mass spectra

Mass spectra were recorded for polyphenols, phospholipids and complexes (Figure 13). The characteristic peak was observed in negative ion mode for all three polyphenols, phospholipids and complexes (Table 3). Figure 14 depicts fragments of flavonoids by LC-MS/MS. Mass spectra of phospholipids differ from variety to variety. Phospholipid used over here is L-α-Phosphatidylcholine. Synonyms of L-α-Phosphatidylcholine are L-α-Lecithin, 1, 2-Diacyl-sn-glycero-3-phosphocholine, 3-sn-Phosphatidylcholine, and Azolectin. Typical lots of egg yolk phosphatidylcholine have fatty acid contents of approximately 33% 16:0 (palmitic), 13% 18:0 (stearic), 31% 18:1(oleic), and 15% 18:2 (linoleic) (other fatty acids being minor contributors). An already reported MS/MS spectrum of L-α-Phosphatidylcholine was recorded (Figure 15). Major fragments that arise from L-α-Phosphatidylcholine were recorded which was quite matching with earlier reported literature. Molecular weight of L-α-Phosphatidylcholine = 760.09 molecular formula = C_{42} H_{82} NO_8P (Table 4) (Figure 16-20). The loss of choline molecule is most pre dominant product in ion spectra (Loss of 184 m/z fragment) .Phospholipids complexes of catechin, quercetin and myricetin were evaluated by same manner and characteristic peaks were observed Table 5. Fragments corresponding to quercetin and catechin phospholipids were observed. But no myricetin phospholipid complex peak corresponding to 1077 molecular weight was detected in both the complexes obtained from the methods R-1 and R-2 during study. Catechin phospholipid complex was detected in phospholipids complex obtained after R-2 method. This suggests that polarity can play a very important role in formation of phospholipids complexes Figure 21 and 22. High polarity compound like myricetin and catechin always may have less chance to form phospholipids complex under moderate reaction conditions. A reported literature of phospholipid complex of curcumin demonstrated the involvement of non-polarity in phospholipid complex formation. Changing of solvent during reaction may enhance the yield further. A Total Ion chromatogram (TIC) of the catechin, myricetin and quercetin shown in Figure 23. Complete MS/MS spectrum of each flavonoid (Figure 24) and (Table 8) detailing about the fragments of flavonoids and its TIC details.

Monitoring of phospholipid complexes reaction condition by gradient RP-HPLC

A gradient HPLC method developed for the polyphenols

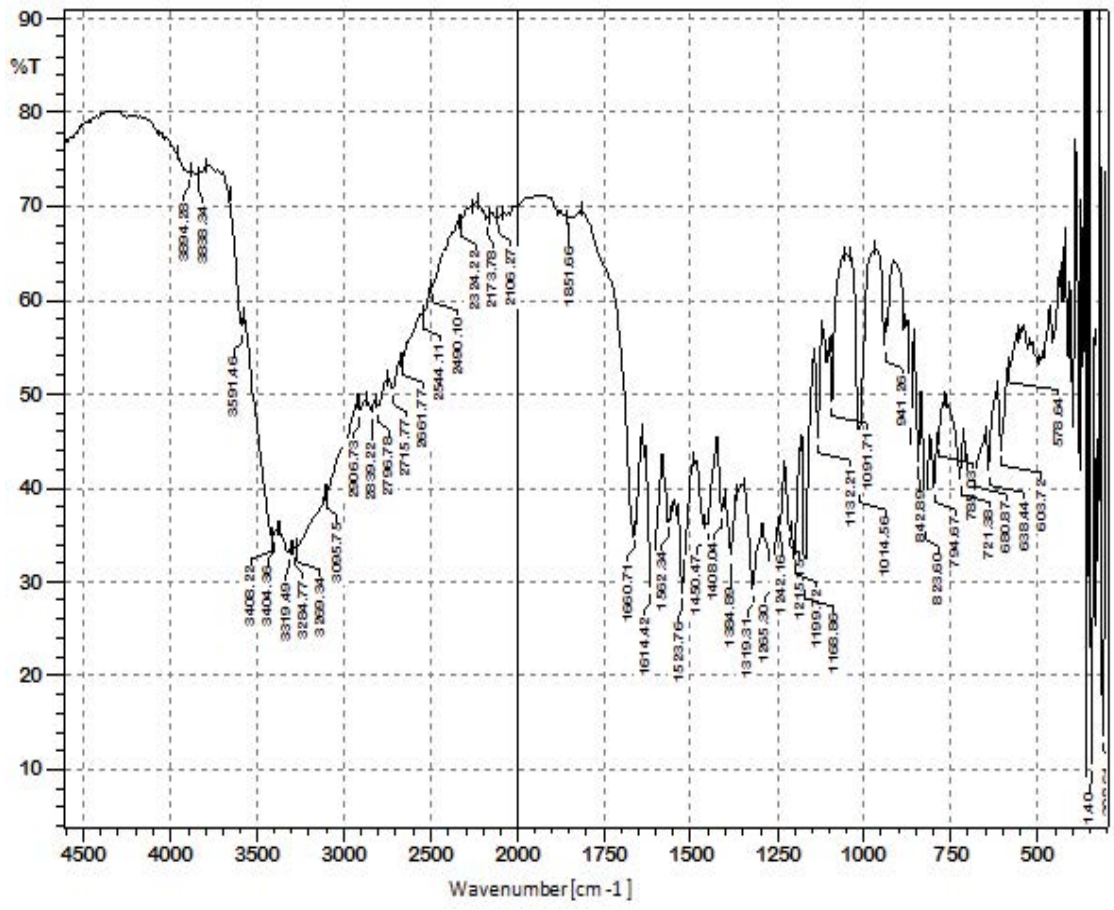


Figure 2: Myricetin FT-IR spectrum

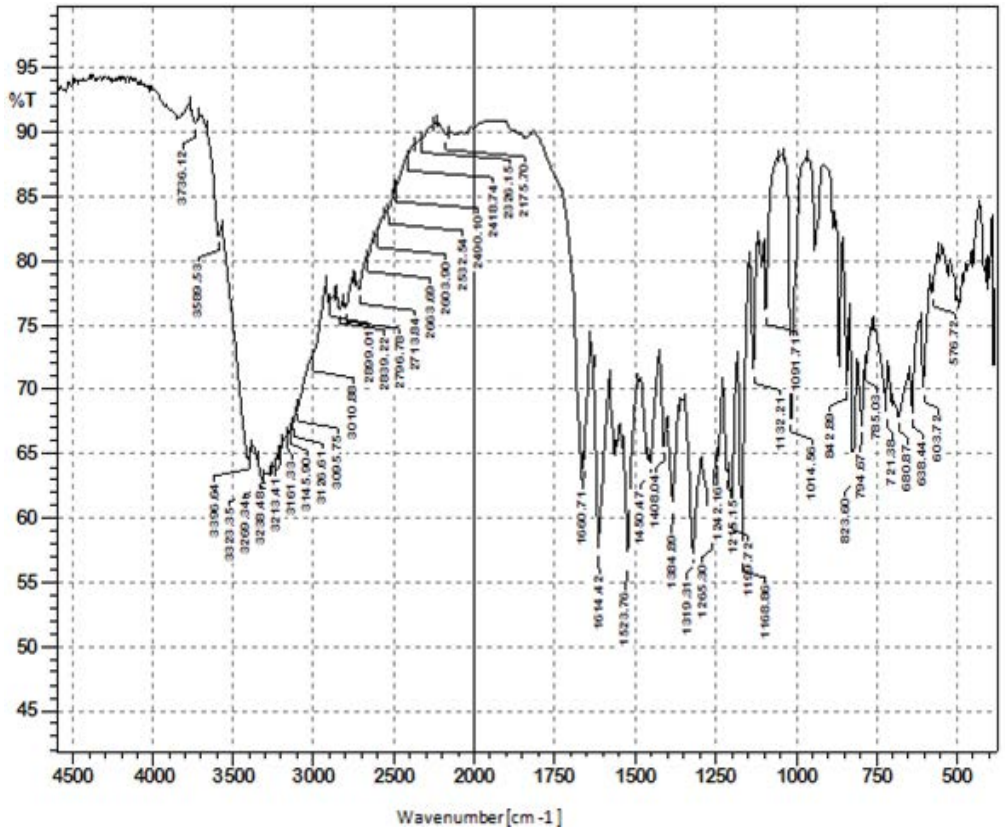


Figure 3: FT-IR- Spectrum of Quercetin

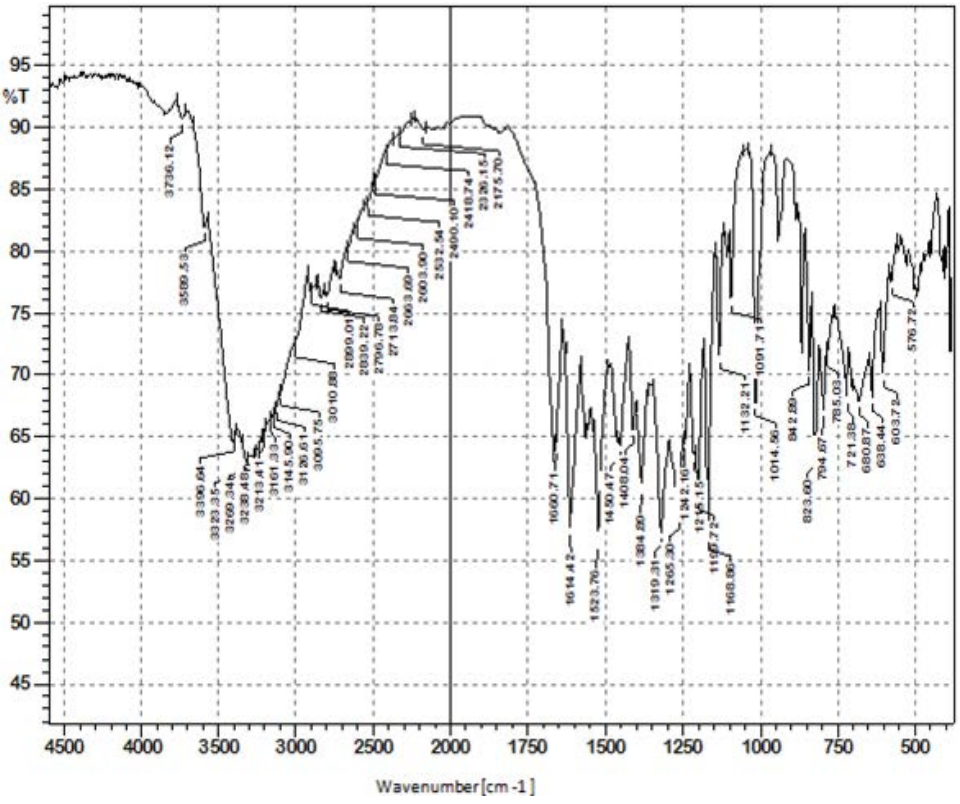


Figure 4: Catechin FT-IR spectrum

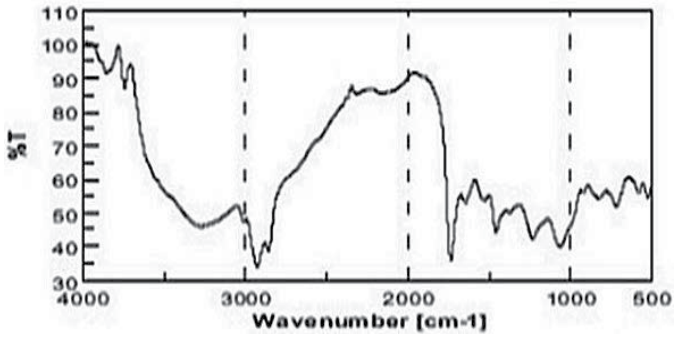


Figure 5: Phospholipids FT-IR-spectrum (soya lecithin)

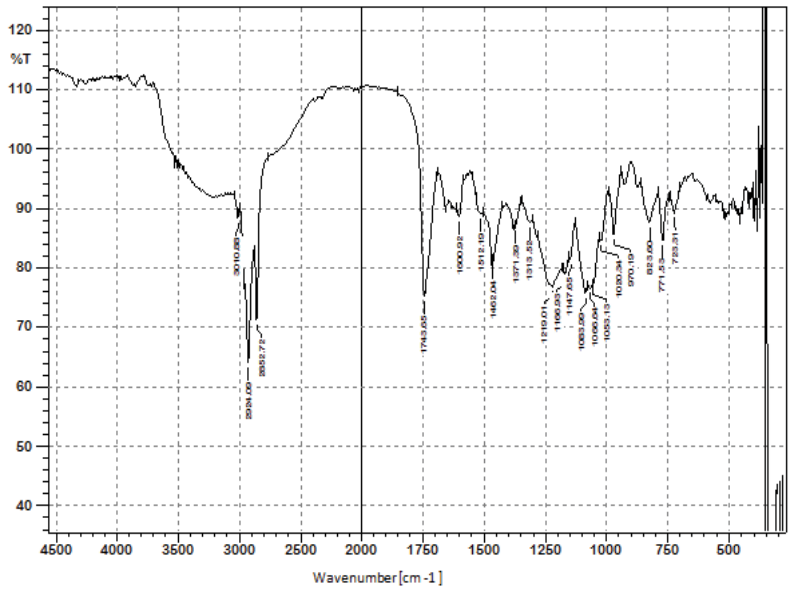


Figure 6: FT-IR spectrum of Biomarker complex for R-1

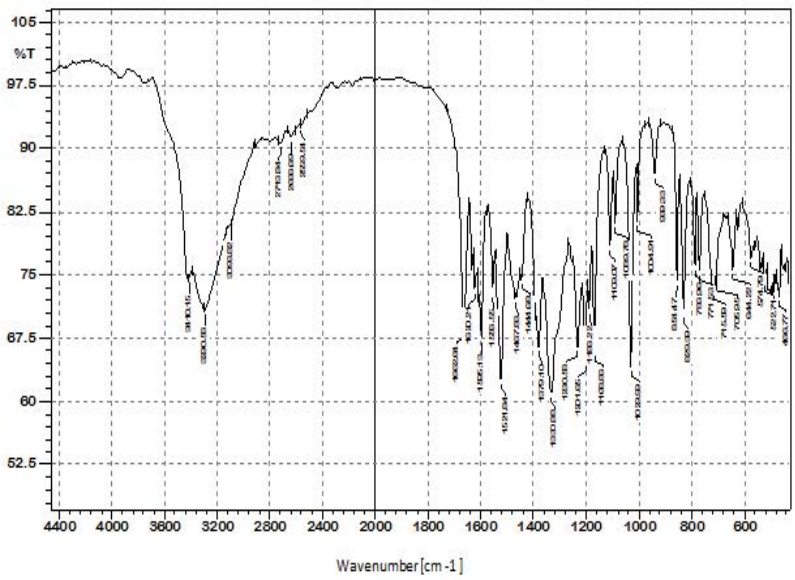


Figure 7: FT-IR spectrum of Biomarker complex for R-2

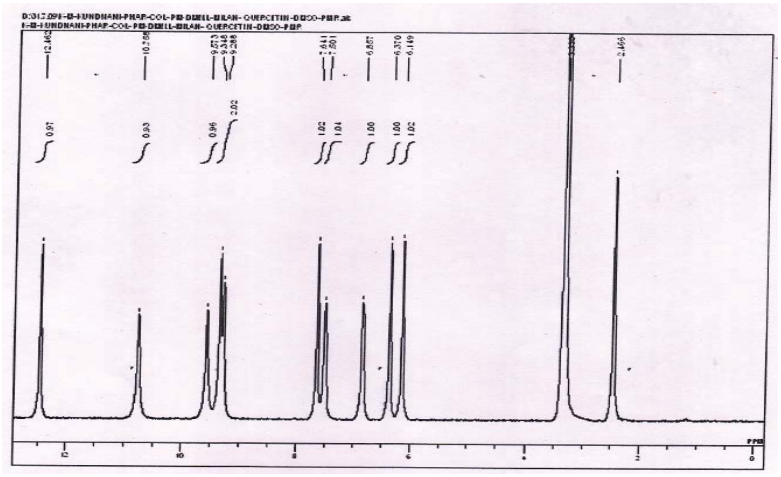


Figure 8: Quercetin NMR

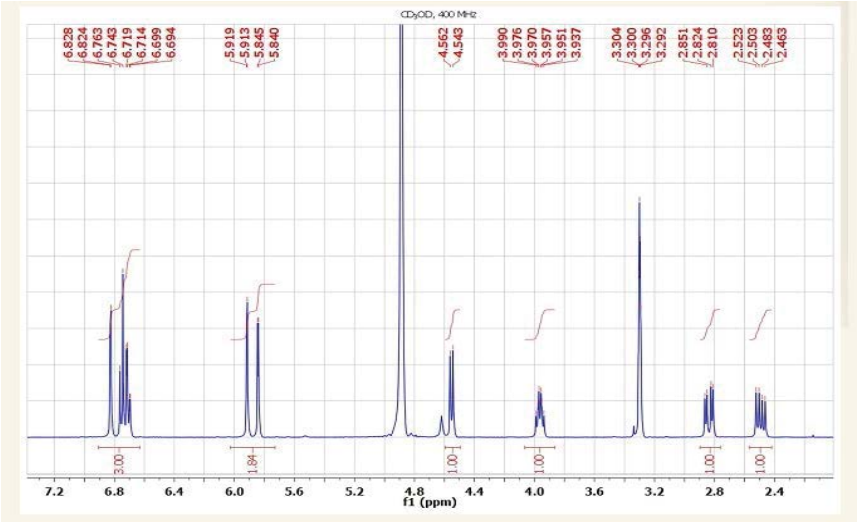


Figure 9: Catechin NMR

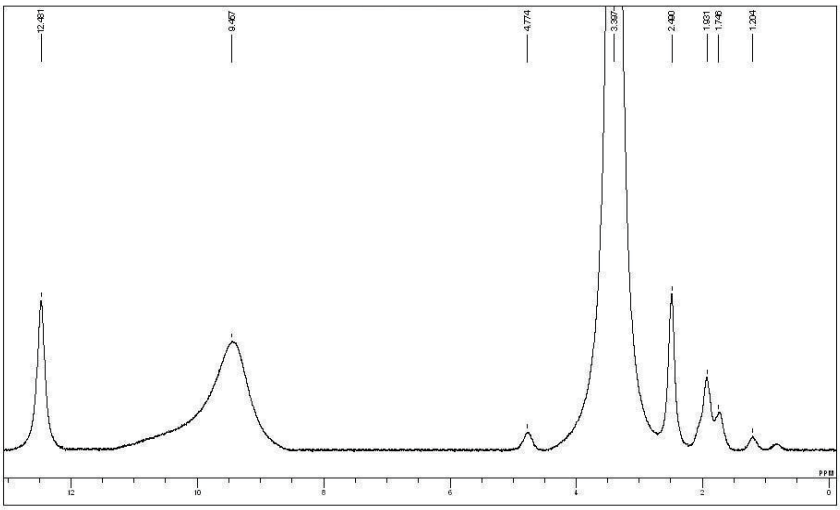


Figure 10: NMR of phospholipid complex from method R-1

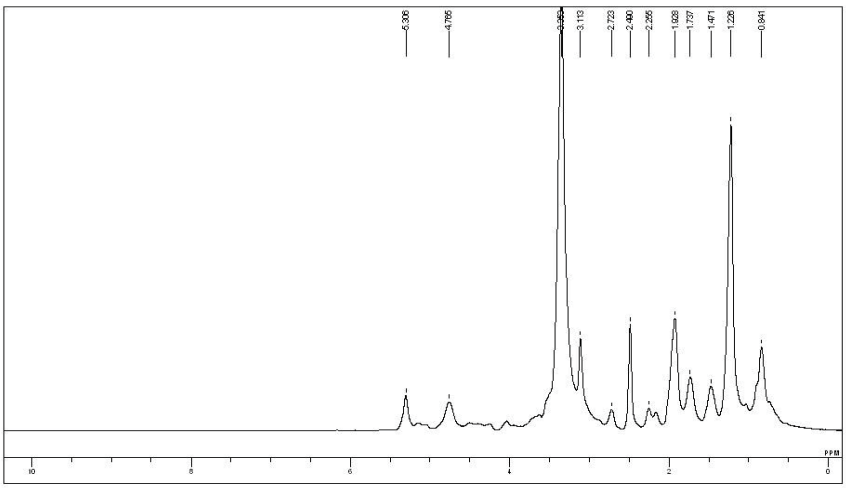


Figure 11: NMR of phospholipid complex from method R-2

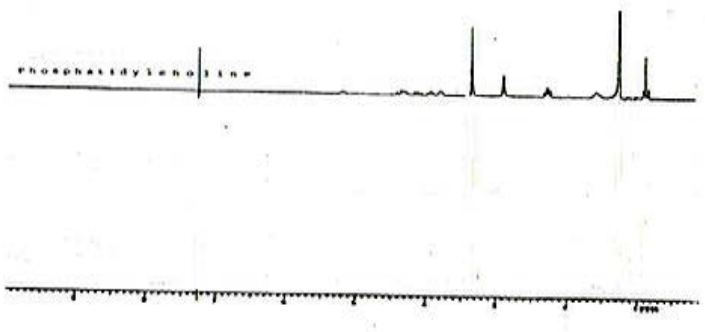


Figure 12: NMR of Phospholipids

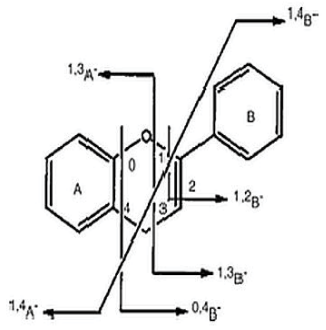


Figure 13: Fragmentation pattern for flavonoids

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	

Table 3: MS/MS fragments of polyphenols in biomarker mixture

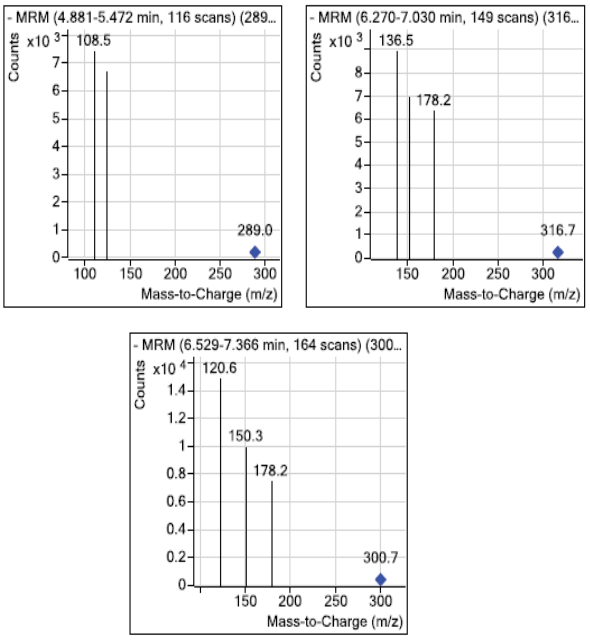


Figure 14: Fragments of catechin, myricetin and quercetin recorded by LC-MS/MS

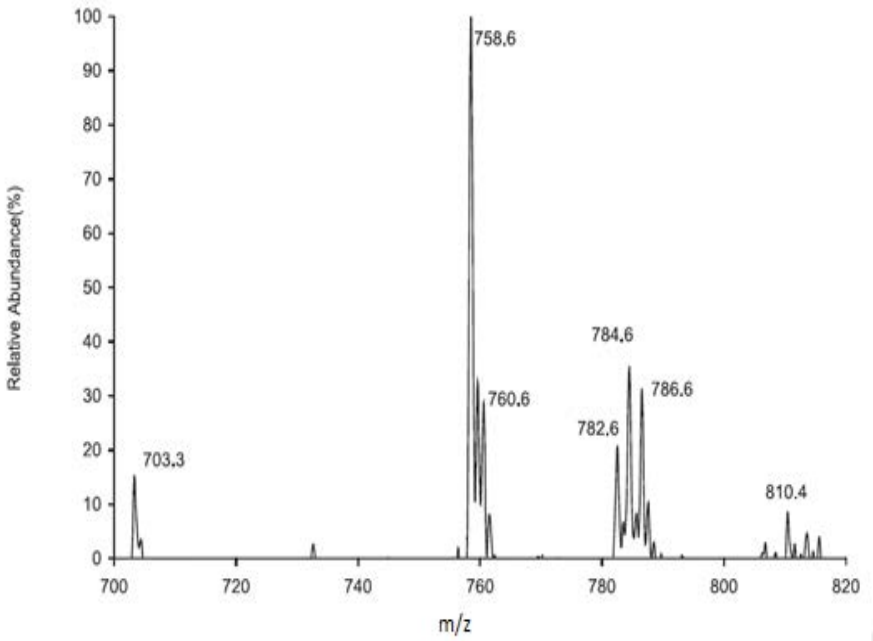


Figure 15: MS Fragments of Phospholipids as per earlier reported literature

MS/MS fragments	Phosphatidylcholine m/z
From $[M-H]^-$ loss of 50	760
From $[M-H]^-$ loss of 28	782
$[M-H]^-$	810

Table 4: MS/MS fragments of phospholipids

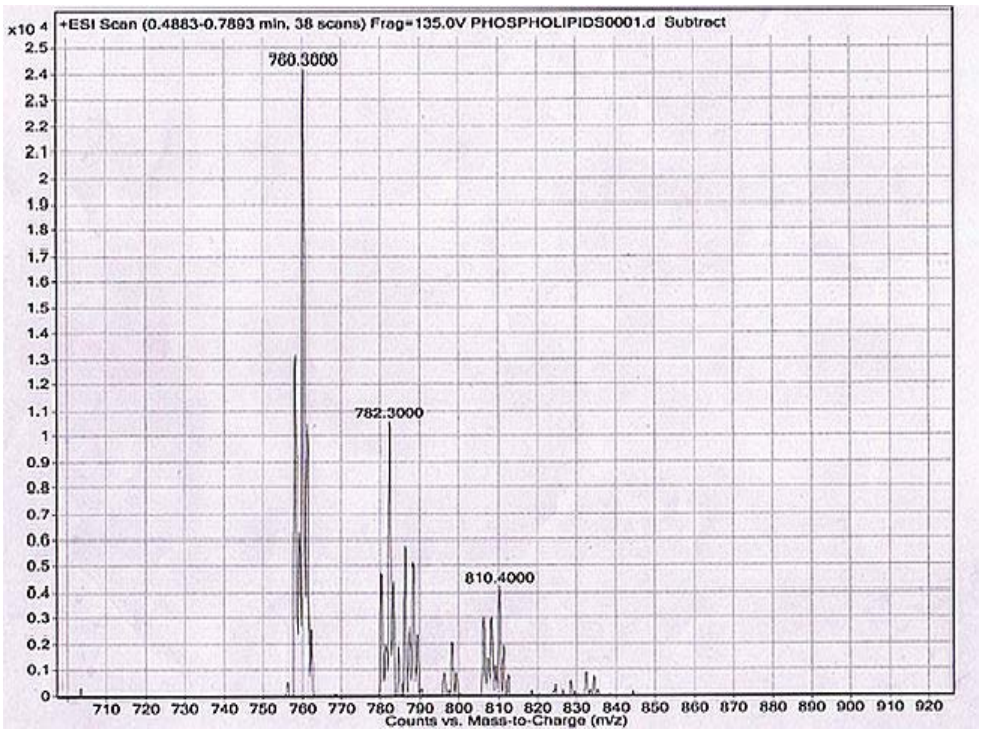
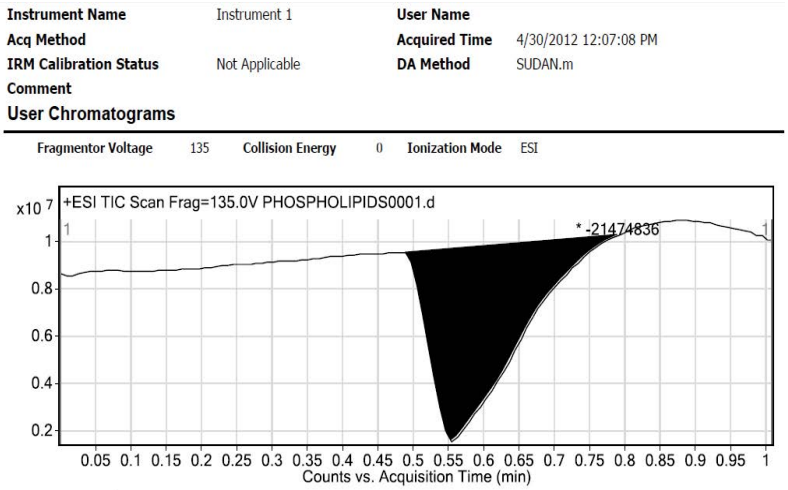
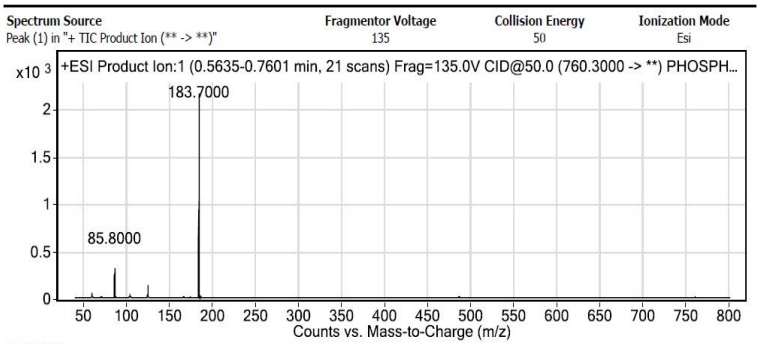


Figure 16: MS/MS fragmentation peaks of L-α-Phosphatidylcholine (60%)



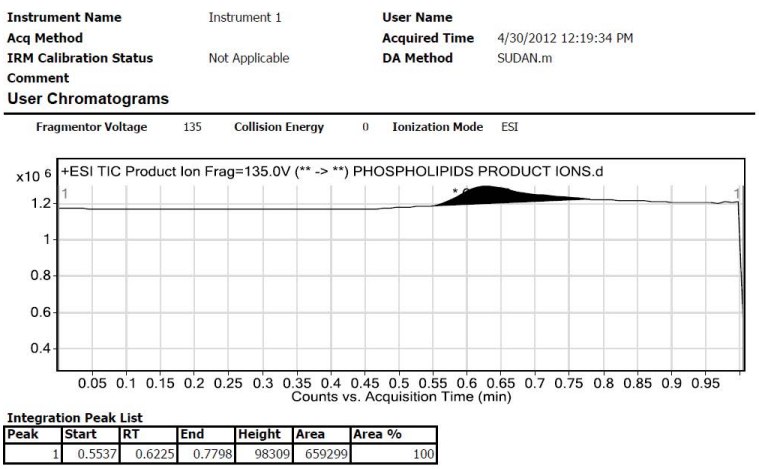
Peak	Start	RT	End	Height	Area	Area %
1	0.4883	0.7893	0.7893	0	-21474836	0

Figure 17: LC-ESI-MS/MS chromatogram of phospholipids



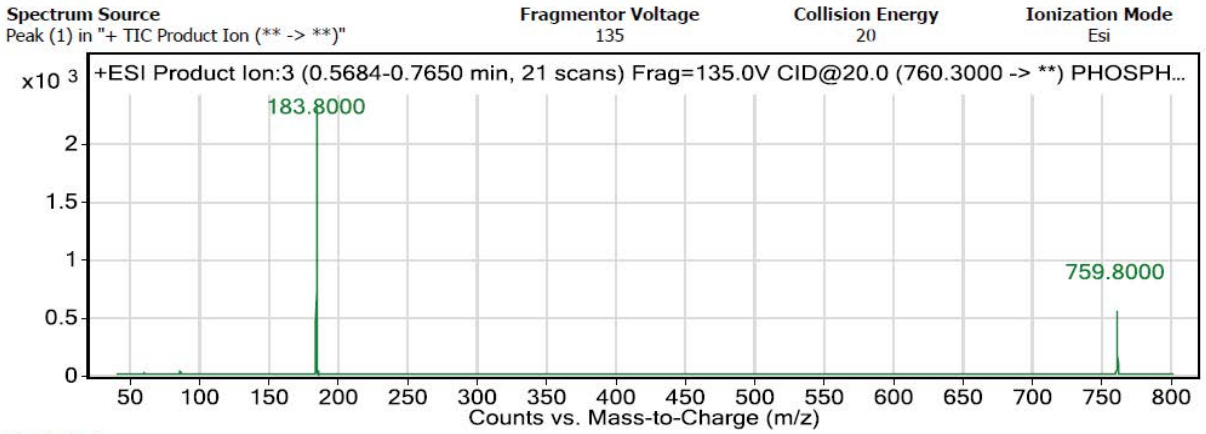
m/z	Abund
85.8	343
124.5	168
183.7	2178

Figure 18: Product ion fragments of L-α-Phosphatidylcholine



Peak	Start	RT	End	Height	Area	Area %
1	0.5537	0.6225	0.7798	98309	659299	100

Figure 19: LC-ESI chromatogram of L-α-Phosphatidylcholine



Peak List

m/z	Abund
183.8	2303
759.8	568

Figure 20: Product ion of L-α-Phosphatidylcholine

MS/MS fragments	Phospholipid complex (m/z) of polyphenols
$[M-H]^-$	760, 782, 810 (phospholipids) Molecular weight = 760.09 molecular formula = C_{42} H_{82} NO_8P
$[M-H]^-$	1061 (Querecetin phospholipids complex)
$[M-H]^-$	1049 (Catechin Phospholipids complex)
$[M-H]^-$	289 (catechin)
$[M-H]^-$	317 (Myricetin)
$[M-H]^-$	301 (Quercetin)

Table 5: MS/MS spectra for phospholipids complex of polyphenols

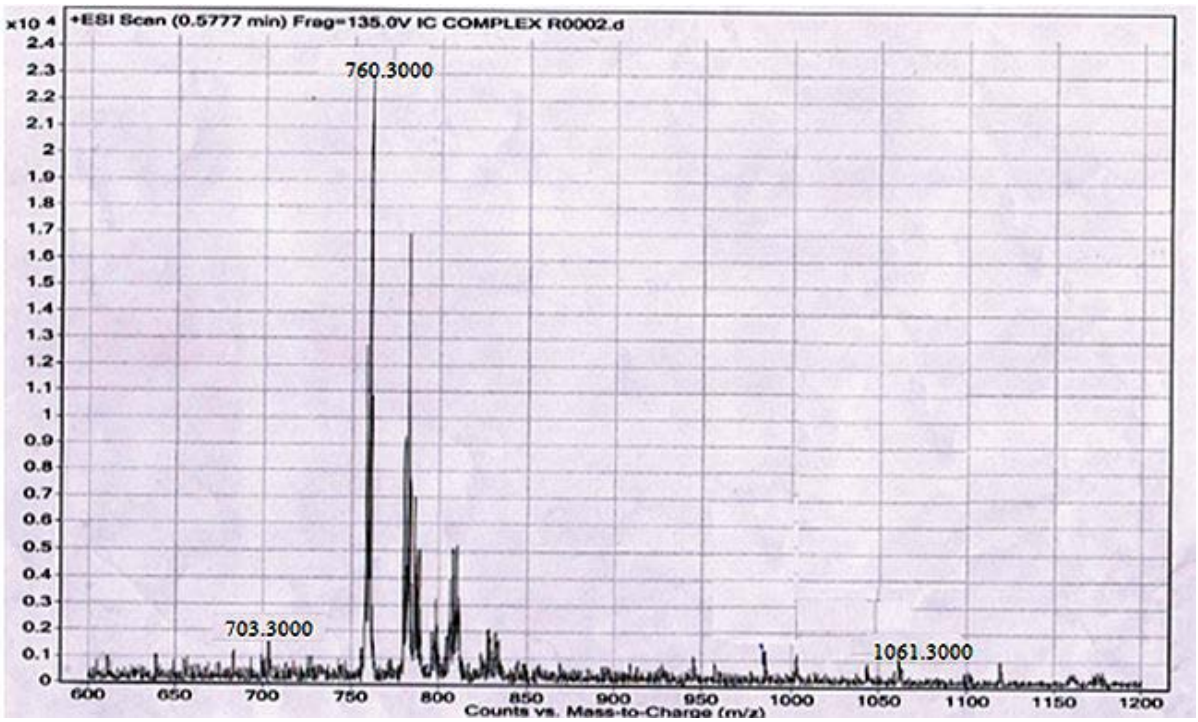


Figure 21: MS/MS fragments peaks of Phospholipids complex of biomarker mixture for R-1 method.

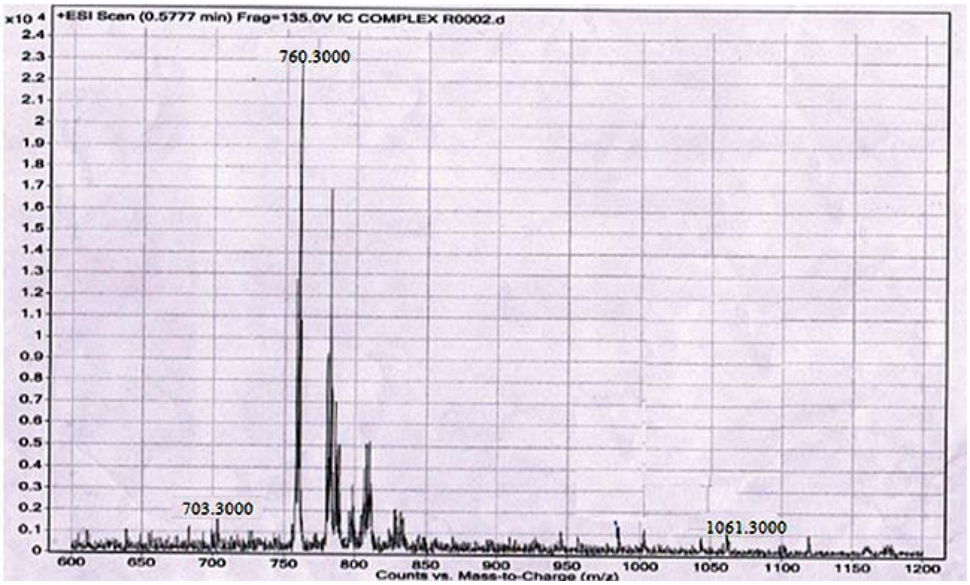


Figure 22: MS/MS fragments peaks of Phospholipids complex of biomarker mixture for R-2 method.

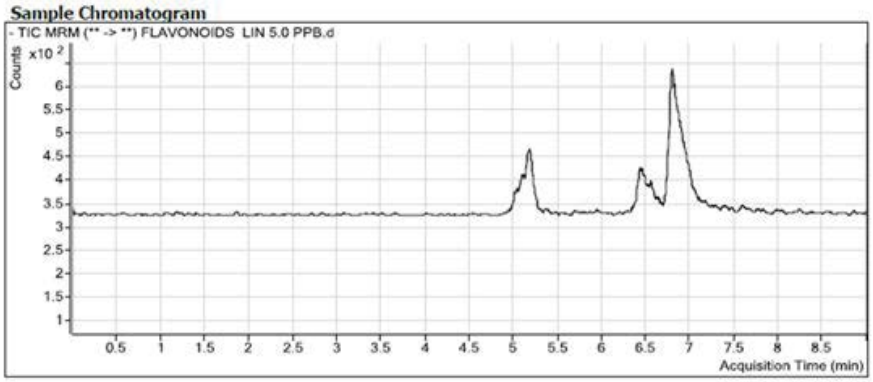


Figure 23: Total Ion chromatogram (TIC) showing the profiles of catechin, myricetin and quercetin.

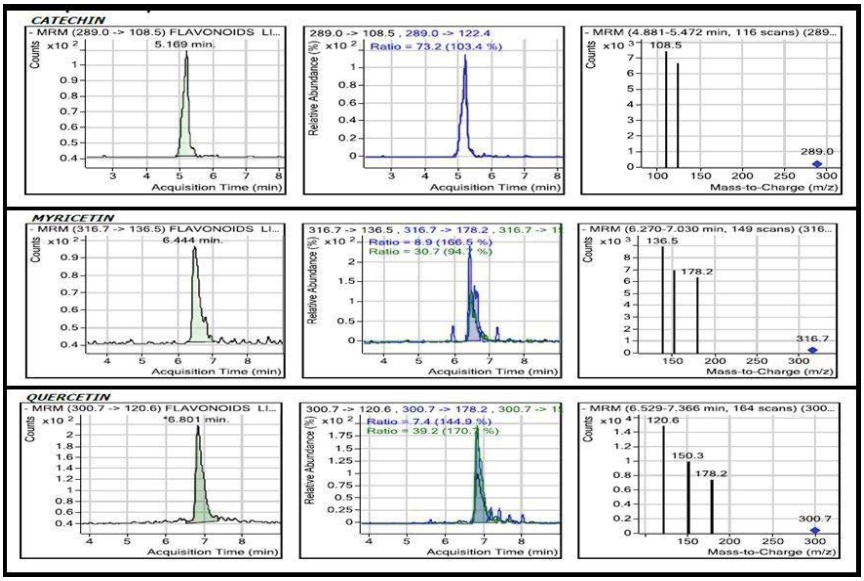


Figure 24: MS/MS spectra of Catechin, Myricetin and Quercetin

Biomarkers	Catechin	Quercetin	Myricetin
Peak area	72.6	178.5	11.9
Concentration	6.096 µg	4.49 µg	.2096 µg

Table 6: Estimated unreacted biomarkers after the complexation for R-1 method

Biomarkers	Catechin	Quercetin	Myricetin
Peak area	Absent	3.43	Absent
Concentration	0	.0214 µg	Absent

Table 7: Estimated unreacted biomarkers after the complexation from the method R-2

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

Table 8: Fragments and TIC details of the biomarkers

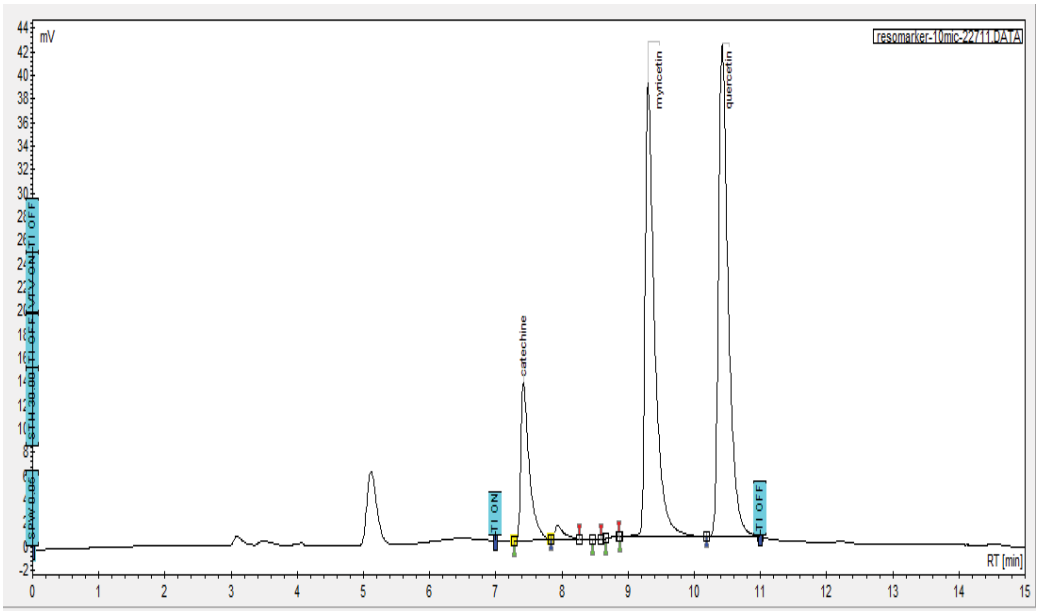


Figure 25: HPLC chromatogram of catechin, myricetin and quercetin in gradient system

like catechin, quercetin and myricetin (Figure 25) was taken into consideration for monitoring the reaction condition of preparation of phospholipids. Chromatogram (A) is all about phospholipids and (B) and (C) about phospholipid complexes which were prepared by the help of R-2 method. HPLC helps to determine the purity of complex as shown in chromatogram (B). It revealed that the complex needed to be purified. Purification steps were achieved by re-dissolving the complex in DCM and filtered them. This process may take in continuous manner to achieve the desired purity. As results chromatogram (C) revealed the absence of unreacted polyphenols with traces of quercetin. These may also suggest the higher affinity of quercetin with the phospholipids (Figure 26-28). 100 µg/ml of phospholipid complex was injected into the column and area of un-reacted biomarkers were measured and mentioned in the Table 6 and 7. A total 10.795 µg biomarkers out of 100 µg were unreacted in a mixture. The purity of complex was found to be 89.205 % by method R-1. So purity of the complex was found to be 99.97% in the second method after purification process. Plant phospholipid complex also exhibited the same phenomena and revealed the complexation of all other plant components selectively during complexation process. As a result no other interference was observed in the chromatogram of plant phospholipids complex. A significant difference in retention time was observed between standard polyphenols and phospholipids complexes of polyphenols on a reverse phase column which ensured different physicochemical properties between the above two candidates in a gradient system described in earlier section. The developed and validated system efficiently separated the complexes and un-reacted polyphenols after a reaction between them.

So far only ^{1}H-NMR and FT-IR were reported for confirmation of phospholipids complex with natural polyphenols. But the present study highlighted the need of mass spectrometry and chromatographic system in order to get better clarity regarding the formation of phospholipids complexes. Phospholipids complexes can be confirmed by ^{1}H-NMR but mass spectrometry and chromatography plays a vital role in concluding which complexes are formed between which flavonoid with purity. NMR spectra can only give us the information if the complexed has formed or not but doesn't give the required information which flavonoid has complexed. If complexes are formed with the biomarkers

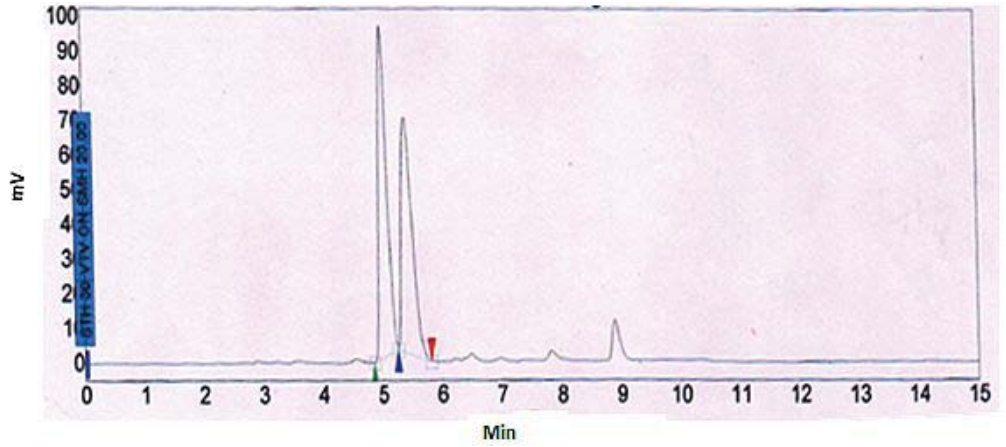


Figure 26: HPLC chromatogram of phospholipids (A)

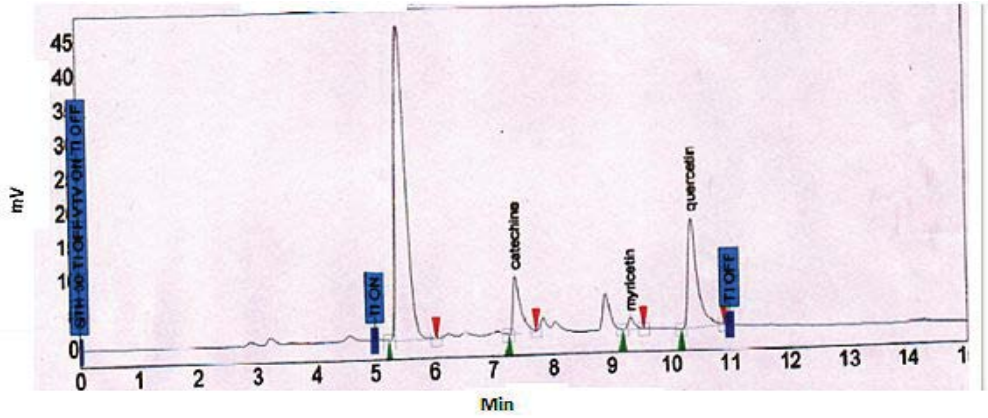


Figure 27: HPLC chromatogram of phospholipid complexes of biomarkers after method R-1 (B)

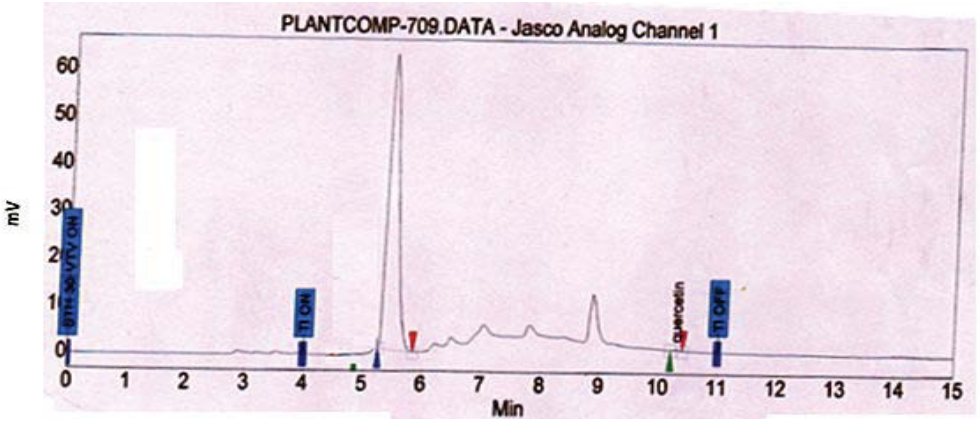


Figure 28: HPLC chromatogram of phospholipid complexes of biomarkers after method R-2 (C)

then the molecules that are involved in the complexation process that can be revealed with precision only with the help of mass spectrometric process from its fragmentation patterns. More over the purity of these complexes or whether the complexes are formed properly during the reaction can be understood by the chromatographic system. So this work found novelty in terms of revealing the molecules involved and their efficiency in the process of complexation with suitable analytical methods.

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