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Research Article

PROFILING AND QUANTIFICATION OF IMPURITIES IN HESPERIDIN BY USING HPLC-PDA AND LCMS TECHNIQUES

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Abstract

The understanding, identification, quantification and control of impurities in drug substances are essential as new molecular entities are evaluated in clinical development. Monitoring of drug substance impurities is routinely accomplished by using HPLC. It is widely used for separating and quantifying impurities, and this technique is most frequently used in coupled with spectroscopic methods in the identifying and elucidating the structure of impurities. Advances in computer application techniques and instrumentation for HPLC coupled to photodiode array detection (PDA) and to mass spectrometry (MS) provide more information and make qualitative analysis accurate and faster.

A high performance liquid chromatography-photodiode array detection system was used to analyze impurities in Hesperidin. The chromatographic analysis was performed on a C_{18} column with an isocratic elution of methanol and water containing formic acid to adjust pH of mobile phase to 4.5. The flow rate was adjusted to 0.7 ml/min and detection was carried out using a photodiode array detector at 284 nm.

LC-MS study was performed using Agilent 1200 Series having 1260 BinPump model G1312B system equipped with autosampler 1260 ALS model G1329B, Thermostated Column Compartment of SL model G1316B was used for the separation. Gradient elution was performed with methanol-water (90:10) containing 5 mM Ammonium Formate at a constant flow rate of 0.4 ml/min. Detection was carried out using UV at 284 nm. Electrospray ionization (ESI) technique was used as ion source. Total seven impurities were identified, out of which 5 are flavonoids, they might have come along with solvent during extraction process.

Keywords: Hesperidin, HPLC-PDA, Impurity, LC-MS.

Introduction

Hesperidin is chemically Hesperetin 7-rhamnoglucoside which is extracted from the citrus fruits like C. aurantum, C. limonis etc. It has been widely used for its antioxidant, anti-inflammatory, hypolipidemic, vasoprotective , cholesterol lowering actions, antiulcer activity, antiallergic effects, antioxidant effect, analgesic, antipyretic activity, Ultraviolet protecting activity, anticarcinogenic activity and for antifertility activity (1).

In the pharmaceutical world, an impurity is considered as any other organic material besides the drug substance or ingredients arise out of synthesis or unwanted chemicals that remain with API (Active Pharmaceutical Ingredient). The impurity may be developed either during formulation or upon aging of both API and formulated API in medicines. The presence of these unwanted chemicals even in small amount may influence the efficacy and safety of the pharmaceutical products. The impurities are not necessarily always inferior, from the standpoint of its usage; the drug substance is compromised in terms of purity even if it contains another material with superior pharmacological or toxicological properties. Impurity can also be defined as any substance coexisting with the original drug such as starting material or intermediates or the substance that is formed due to any side reactions (2).

There is no precise definition for impurity profile. Impurity profile of a substance under investigation gives maximum possible types of impurities present in it. It also estimates the actual amount of different kinds of impurities present. Impurity profile is a description of the identified and unidentified impurities present in a typical batch of API produced by a specific controlled production process. It includes the identity or some qualitative analytical designation (e.g. retention time), the range of each impurity observed and type of each identified impurity. For each API, there should be an impurity profile describing the identified and unidentified impurities present in a typical batch. The impurity profile is normally dependent upon the process or origin of the API. Quantitative determination of these impurities could be used as a method for the quality control and validation of drug substances. Regulatory authorities such as I.C.H. (International Conference of Harmonization), USFDA (United States Food and Drug Administration), CGMP (Current Good Manufacturing Practice), TGA (Thermo Gravimetric Analysis), and MCA (Ministry of Corporate Affairs) insist on the impurity profiling of drugs (3).

Impurity can be of three types:

1. Impurities closely related to the product and coming from the chemical or from the biosynthetic route itself.
2. Impurities formed due to spontaneous decomposition of the drug during the storage or on exposure to extreme conditions.
3. The precursors that may be present in the final product as impurities.

Impurities present in excess of 0.1% should be identified and quantified by selective methods. The suggested structures of the impurities can be synthesized and will provide the final evidence for their structures, previously determined by spectroscopic methods. Therefore, it is essential to know the structure of these impurities in the bulk drug in order to alter the reaction condition and to reduce the quantity of impurity to an acceptable level. Isolation, identification and quantification of impurities help us in various ways, to obtain a pure substance with less toxicity and safety in drug therapy (4).

So, it is necessary to find out chemical composition or impurity composition of hesperidin sample. Generally, the impurities in Hesperidin are believed to be mainly the flavonoids imported during the extraction process of crude herb. The purpose of this study is to develop a method using HPLC coupled to mass spectrometry for profiling the impurities in procured sample of hesperidin.

Materials and Methods

Chemicals and reagents

Hesperidin sample was kindly supplied by Aaisland chemical products, Aurangabad. The hesperidin sample was labeled as citrus flavonoids 80%. The methanol, water, formic acid and ammonium formate for analysis was of HPLC grade.

HPLC-PDA analysis

The various solvents were studied for solubility of Hesperidin. It was soluble in DMF, DMSO, pyridine, formic acid, sparingly soluble in water, methanol and acetonitrile. After suitable literature survey, practical experience and taking above factors into considerations the suitable solvents selected were methanol and water.

The HPLC system consisted Intelligent LC pump with sampler programmed at 20 µl capacity per injection was used. The detector consisted of PDA operated at a wavelength of 284 nm. The column used was C_{18} and the mobile phase consisted of methanol: water (pH=4.5 adjusted with formic acid) in a ratio of 45:55. The flow rate was set as 0.7 ml/min throughout the experiments.

HPLC/mass analysis

LC-MS study was performed using Agilent 1200 Series having 1260 BinPump model G1312B system equipped with autosampler 1260 ALS model G1329B, Thermostated Column Compartment of SL model G1316B was used for the separation. Gradient elution was performed with methanol-water (90:10) containing 5 mM Ammonium Formate at constant flow rate of 0.4 ml/ min. Detection was carried out using UV at 284 nm. Electrospray ionization (ESI) technique was used as ion source.

The HPLC system described in above Section was online coupled to an Agilent jet stream mass spectrometer equipped with ESI interface. Instrument control and data acquisition were performed using 1200 series. The MS spectra were acquired in negative ion mode. High purity nitrogen (N_2) was used as both drying gas with a flow rate of 8 l/min and as nebulizing gas with a pressure of 40 psi. Ultrahigh pure helium (He) was used as the collision gas. The dry temperature was set at 350 °C and the capillary voltage was set at 3500V. The mass spectra were recorded in the scale from 50 to 700 *m*/*z*. MS*n* data were acquired in the Auto MS*n* mode.

Results and Discussion

HPLC-PDA studies

The objective of doing this analysis was to check chemical nature of impurities present in hesperidin powder. The PDA UV spectra of hesperidin and its impurities were recorded in range of 210-400 nm. Figure 1 represent HPLC chromatogram of Hesperidin using PDA detector at 284 nm. According to table 1 total 5 impurities are present in hesperidin sample. Hesperidin was eluted at retention time 11.06 and all impurities were eluted within retention time of 3-10 minutes that means all impurities are highly polar in nature than Hesperidin. The percent of Hesperidin in Hesperidin sample is 93.45 and impurities present in sample are 6.55%. The fig. 2 indicates that hesperidin and its impurities show high absorption at about 280-285 nm with very similar spectra.

The impurity eluted at retention time of 9.149 min had similar spectra with that of naringin. The literature survey reveals that naringin gives λ_{max} at 283.6 nm and impurity eluted at 9.149 min also has λ_{max} value of 283 nm.

Table 1 HPLC-PDA result of Hesperidin sample.

Name	Ret. Time	Area	Area %	Theoretical plates	Tailing Factor	Resolution
RT 3.716	3.714	11307	1.072	915.847	0.828	0.000
RT 5.707	5.739	955	0.094	7813.648	0.721	5.393
RT 6.018	6.013	8697	0.825	3280.560	1.371	0.806
RT 7 .125	7.113	13947	1.322	2550.609	1.594	2.238
RT 9.149	9.148	34136	3.236	3227.207	1.262	3.372
RT 11.060	11.061	985659	93.450	2873.871	1.081	2.603
		1054742	100.000			

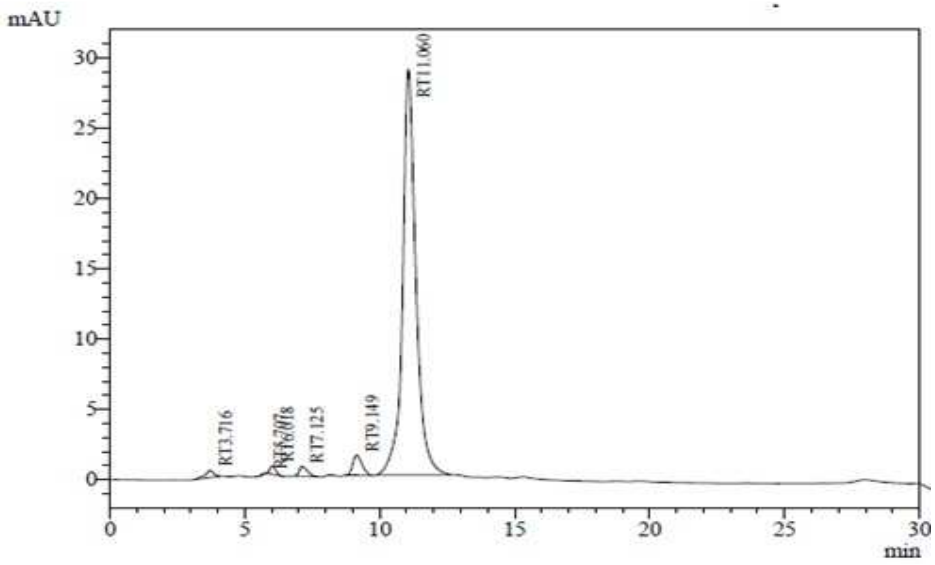


Figure 1 HPLC chromatogram of Hesperidin using PDA detector at 284 nm.

HPLC/MS analysis

The chromatographic conditions of gradient program and mobile phase for the identification of the impurities in hesperidin samples were optimized in order to acquire overall impurities peaks with good resolution within a short

analysis time. Subsequently, the MS conditions were optimized in order to acquire the extensive information of the impurities. The representative HPLC/MS chromatogram for hesperidin powder is shown in fig. 3 and details of LCMS result of Hesperidin shown in table 2. The fig. 4 represent spectrum of counts versus acquisition time for Hesperidin blank. According to fig. 3 and fig. 4 the components of Hesperidin sample were eluted upto 20 minutes only. So, peaks obtained after 20 min were not considered. The fig. 8 shows mass spectrum of Hesperidin and table 6 gives details of Hesperidin. The fig. 5, fig. 6, fig. 7, fig. 9, fig. 10, fig. 11 and fig. 12 describes the mass spectra of impurities eluted at retention times of 3.1639-3.6511 min., 9.3403-10.1522 min., 12.1121-12.4200 min., 15.7667-16.2259 min., 16.7448-17.0752 min., 17.1872-17.9655 min., and 19.5073-19.8768 min respectively. The details of these impurities are mentioned in table 3, table 4, table 5, table 7, table 8, table 9 and table 10 according to order as per they had eluted. The identity of the known impurities was obtained by their m/z ratio and their UV spectra by comparing with the reported data available in literatures. These impurities were identified as seen in table 11.

Table- 2: LC-MS result of hesperidin powder.

Peak	Start	RT	END	Height	Area	Area %
2	9.1	9.3912	9.5562	1.4	18.09	0.52
3	9.6242	9.7892	10.2745	1.47	29.98	0.86
4	11.8222	12.1139	12.4537	4.35	59.25	1.71
5	12.5216	12.7837	13.6088	184.43	3471.48	100
6	15.6132	15.8753	16.2635	3.71	55.03	1.59
7	16.9818	17.2245	17.7681	4.05	63.9	1.84
12	22.7767	22.9708	23.3397	1.98	26.7	0.77
13	24.0386	24.354	24.8005	1.04	19.93	0.57
15	26.3876	26.6497	27.1301	2.89	52.99	1.53
16	29.3918	29.6005	29.8286	6.05	77.24	2.22

Table-3: Details of impurity eluted at retention time 3.1639-3.6511 min.

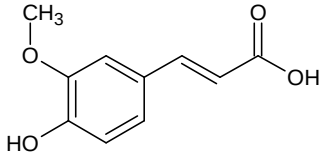
m/z	190
Name of impurity	Ferulic acid
Structure	

Table-4: Details of impurity eluted at retention time 9.3403-10.1522 min.

m/z	474
Name of impurity	unknown

Table- 5: Details of impurity eluted at retention time 12.1121-12.4200 min.

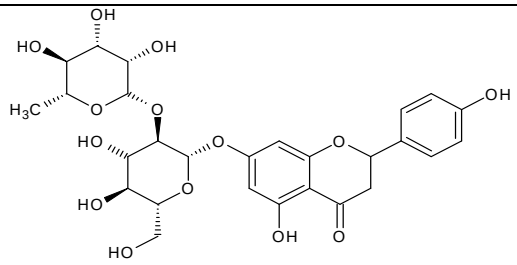
m/z	578
Name of impurity	Naringin
Structure	

Table- 6: Details of hesperidin.

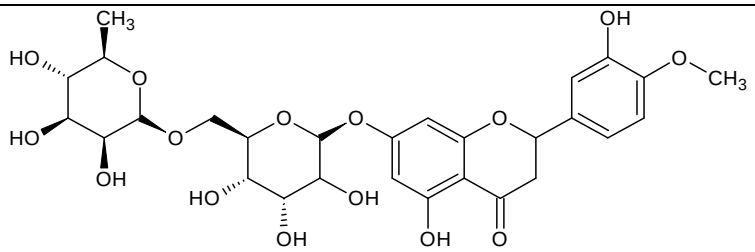
m/z	608
Name	Hesperidin
Structure	

Table- 7: Details of impurity eluted at retention time 15.7667-16.2259 min.

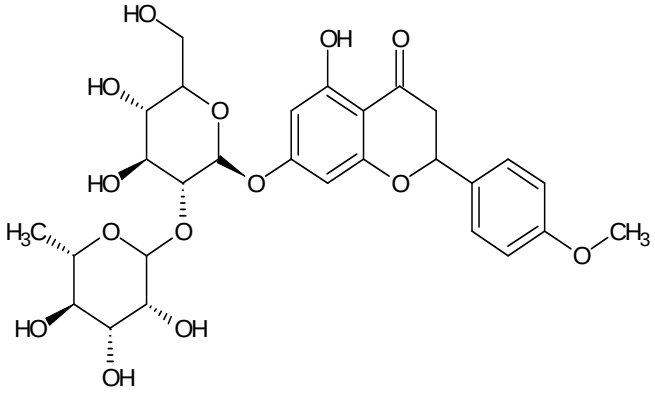
m/z	592
Name of impurity	Poncirin
Structure	

Table- 8: Details of impurity eluted at retention time 16.7448-17.0752 min.

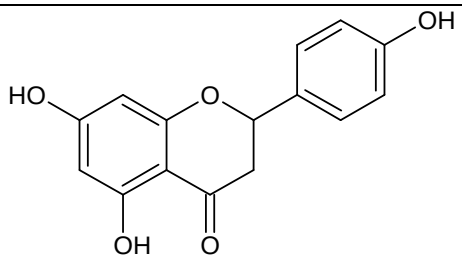
m/z	270
Name of impurity	Apigenin
Structure	

Table- 9: Details of of impurity eluted at retention time 17.1872-17.9655 min.

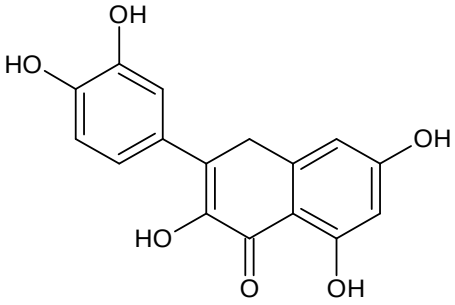
m/z	300
Name of impurity	Quercetin
Structure	

Table-10: Details of impurity eluted at retention time 19.5073-19.8768 min.

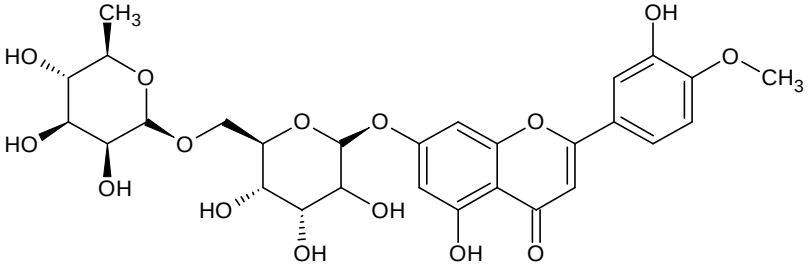
m/z	608
Name of impurity	Diosmin
Structure	

Table-11 Details of impurities present in hesperidin powder.

Sr. No.	Name of impurity	Molecular weight	m/z	Percentage (%)
1	Ferulic acid	194	190	0.46
2	Unknown	-	474	0.61
3	Naringin	580	578	1.59
4	Poncirin	594	592	1.44
5	Apigenin	272	270	1.52
6	Quercetin	302	300	0.12
7	Diosmin	608	608	0.12

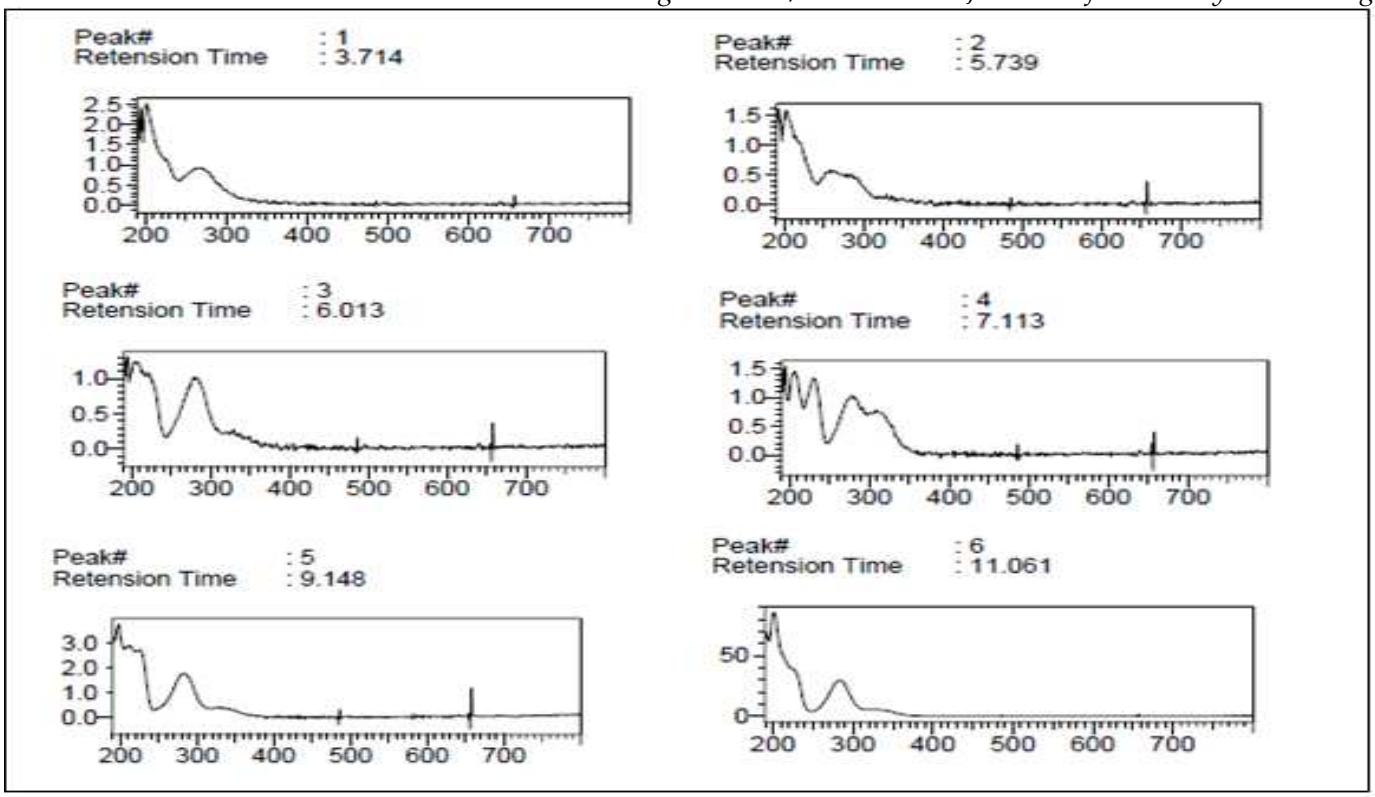


Figure-2: PDA-UV spectra Hesperidin.

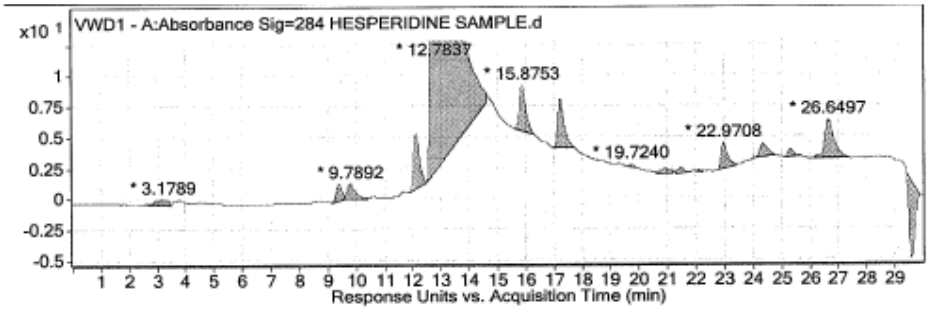


Figure-3: HPLC-MS chromatogram of hesperidin powder.

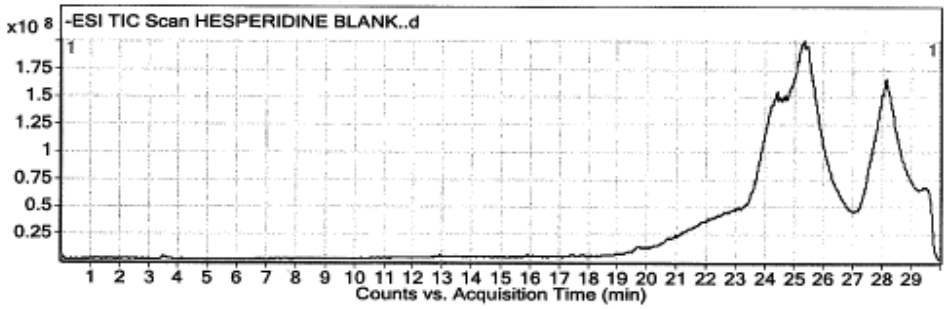


Figure-4: Counts Vs Acquisition time (min) of blank.

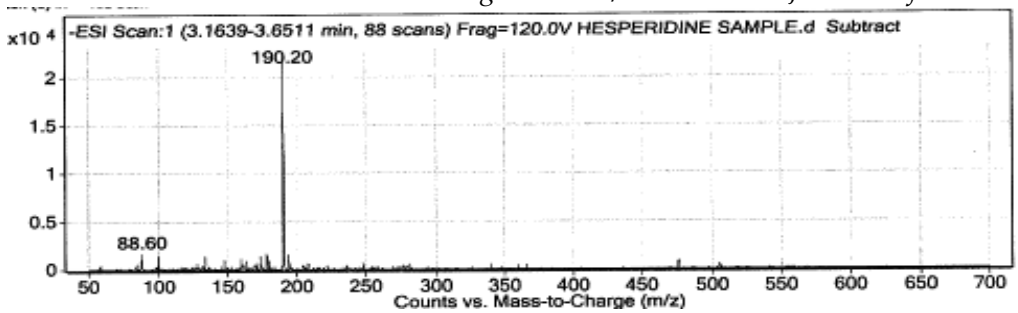


Figure-5: Mass spectrum of impurity eluted at retention time 3.1639-3.6511min.

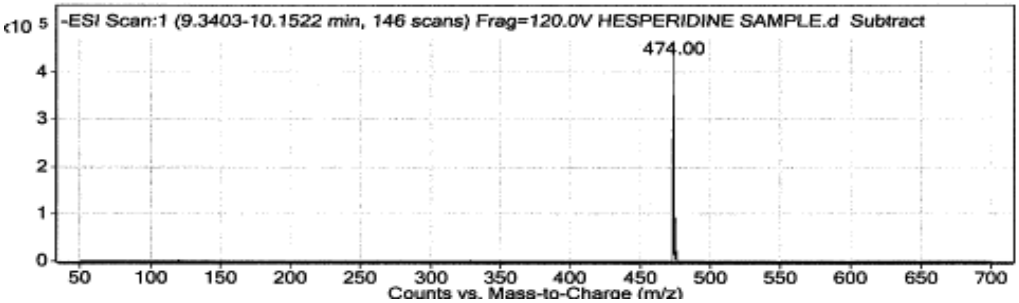


Figure-6: Mass spectrum of impurity eluted at retention time 9.3403-10.1522 min.

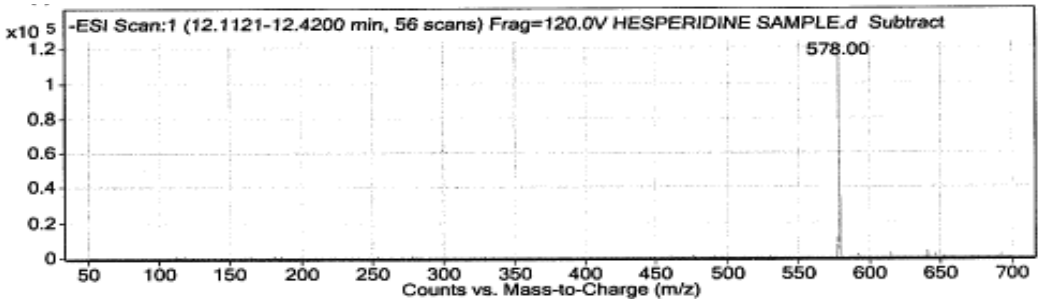


Figure-7: Mass spectrum of impurity eluted at retention time 12.1121-12.4200 min.

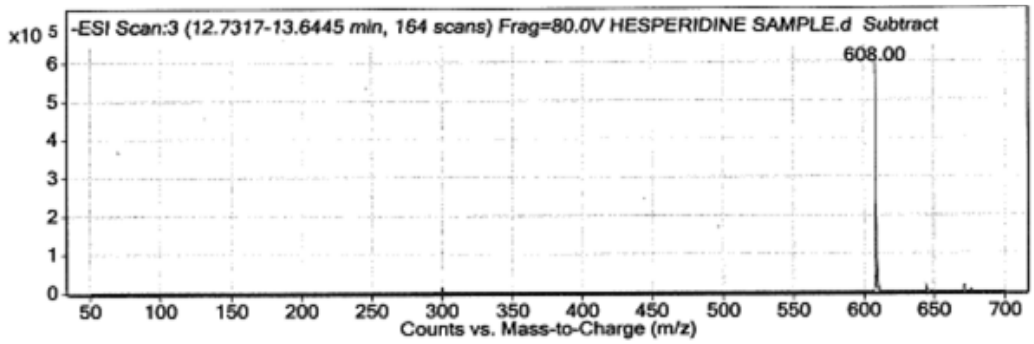


Figure-:8 Mass spectrum of hesperidin.

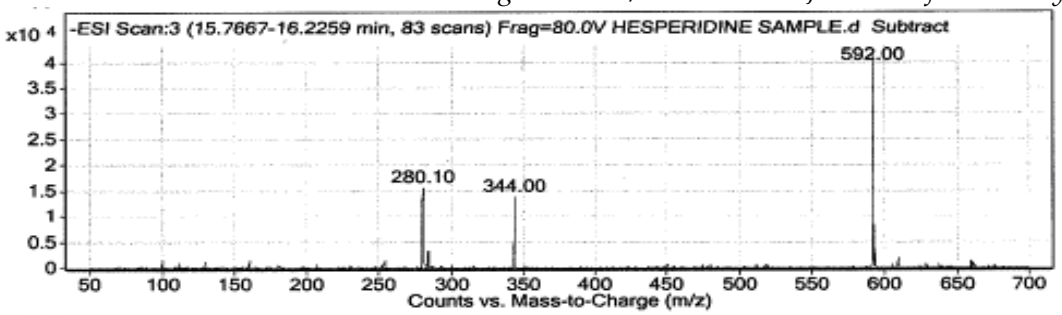


Figure-9: Mass spectrum of impurity eluted at retention time 15.7667-16.2259 min.

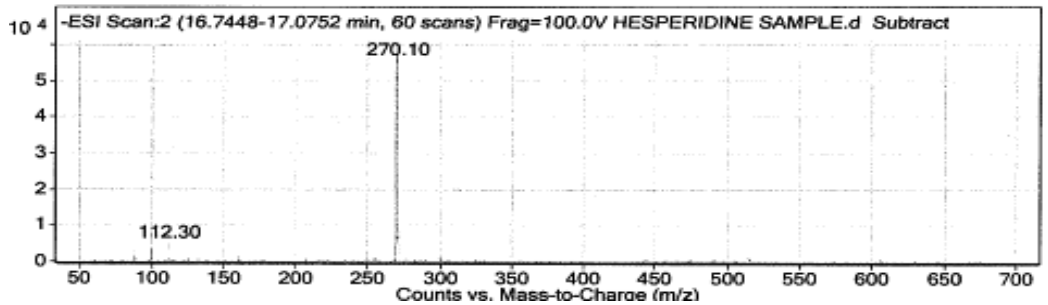


Figure-10: Mass spectrum of impurity eluted at retention time 16.7448-17.0752 min.

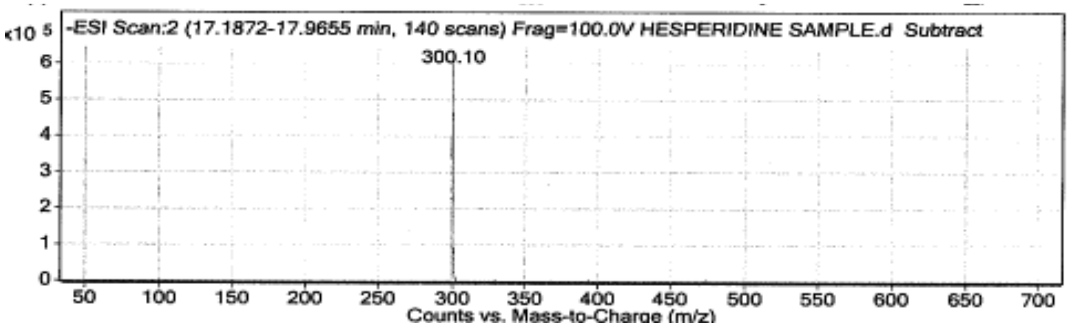


Figure-11: Mass spectrum of impurity eluted at retention time 17.1872-17.9655 min.

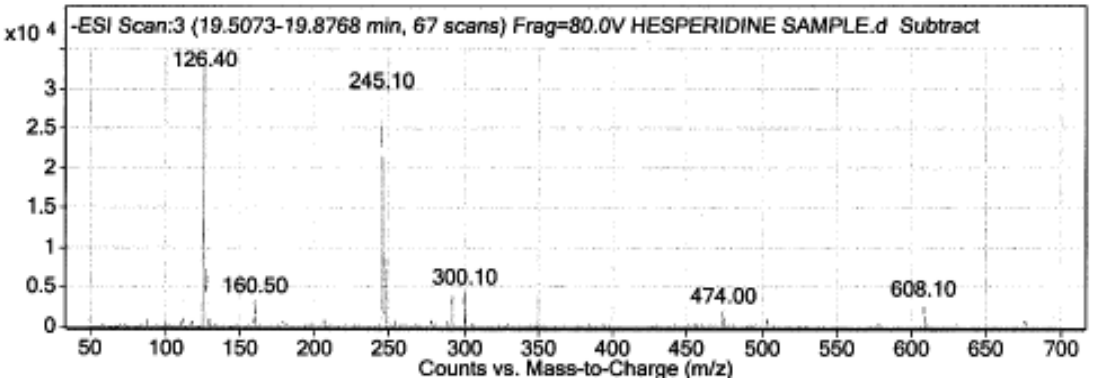


Figure-12: Mass spectrum of impurity eluted at retention time 19.5073-19.8768 min.

Conclusion

The HPLC method was developed to separate impurities from hesperidin powder. The developed HPLC method was applied in PDA to obtain UV spectra of impurities. The UV spectra of hesperidin and its impurities were compared in range of 210-400 nm. The hesperidin and its impurities showed high absorption at about 280-285 nm with very similar spectra.

Finally, LCMS study was performed to obtain molecular weight of impurities. The identity of impurities was done by their m/z ratio and their UV spectra by comparing with the reported data in literatures. Total seven impurities were identified out of which 5 are flavonoids, they might have come along with solvent during extraction process.

So, from the above discussion we can conclude that a fully automated comprehensive impurity profiling method employing HPLC separations, hyphenated LC-UV and LC-MS detections has been developed. It is also concluded as all the impurities belong to same pharmacological class, they are chemical impurities but they are not pharmacological impurities.

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