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RESEARCH ARTICLE

Development and validation of method for molecular weight determination of cellulose using GPC column in HPLC.

Satish N. Patkar and Prasad D. Panzade*

Analytical Science & Technology, Aditya Birla Science and Technology Company Private Ltd, Plot no. 1 & 1-A1, MIDC, Taloja, District Raigad, Maharashtra, 410206, India.

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Corresponding Author

Prasad D. Panzade.

Abstract

The longer analysis time required to estimate the molecular weight on traditional GPC. The purpose of this study is to establish method for molecular weight analysis of cellulose using lesser number of GPC columns, shorter analysis time and lesser cost of analysis using HPLC in GPC mode. The method is validated to cover system precision, linearity, accuracy, method precision, robustness and solution stability to support the study. Pullulan polysaccharide is used as standard and 0.5% LiCl in N,N-dimethylacetamide as eluent. The comparative data of four columns versus two columns and HPLC versus traditional GPC is represented in the results and discussion section.

Introduction:-

Size Exclusion Chromatography (SEC) or Gel Permeation Chromatography (GPC) is the preferred method of determining molecular weight (MW) and molecular weight distribution (MWD). Differences in effective molecular size in solution separate molecule in sample. The fundamental of SEC / GPC is the separation of solvated molecules on column packed with material having a broad distribution of pore sizes. The column packing does not retain molecules too large to penetrate any of the pores so they elute first. The column retains smaller molecules that penetrate some pores causing them to elute later [1]. (Refer Figure 1).

Previous studies have characterized values of MW of pulp celluloses by methods such as fractionation or GPC or SEC of cellulose derivatives. Fractionation methods are tedious and impractical for routine analysis. GPC is more practical, but it requires converting cellulose into a derivative that dissolves in organic solvents. This derivatization exposes the cellulose chains to degradation [2].

In the production of pulp & cellulose fibre, there is occurrence of vigorous polymerization that can precede either uniformly or non-uniformly. Accurate information about changes in the MW and MWD of the wood pulp is necessary [3]. A discovered solvent system for cellulose is a mixture of N-N,-dimethylacetamide (DMAc) and lithium chloride (LiCl) [4-5]. Evidence suggests that this solvent system effects dissolution by complex formation [4].

In the dissolution mechanism (Refer Figure 2) by McCormick, suggests that the hydroxyl protons of the hydroglucose units and the chloride anions from the dissociated salt form hydrogen bonding [6]. The chloride anion also associates with a Li^{+}(DMAc) macrocation. Each hydroxyl group in a cellulose molecules complexes with only one LiCl molecule [7]. Dausey postulated that the interaction of the chloride anion with the hydroxyl protons would result in competitive hydrogen bond structure. The accumulated associations of Cl along the cellulose chain would

produce an anionically charged polymer with the macrocation $(Li\text{-}DMAc)^+$ as the counter ion. The net effect on molecule would be a charge-to-charge repulsion. Continuous influx of the solvent would further disrupt the cellulose binding forces until there was complete solvation of the polymer [8].

GPC technique is used to determine the molecular weights of four polymeric arms of asymmetric star polymer [9]. The influence of cellulose properties and pre-treatment on the mode of enzymatic hydrolysis is studied by GPC analysis [10]. MW of Cellulose of micro and nanofibers can be possible by GPC / SEC technique [11]. The removal of lignin by holocellulose pulping and hemicellulose removal by acid hydrolysis was shown to have little effect on the crystalline ultrastructure components of cellulose based on NMR and GPC results of microcrystalline cellulose [12]. A method for the molar mass distribution of softwood kraft pulps dissolved in 0.5% LiCl /DMAc by SEC is mentioned [13]. Hardwood kraft pulps can be completely dissolved in LiCl /DMAc. The cellulose and hemicellulose components can be separated by SEC. The MWD that corresponds to cellulose is extended up to the high molecular weight region and the weight average molecular weight (MW) relative to pullulan of this distribution is high [14]. MWD determination of the unaged and artificially aged papers were studied using 0.5% LiCl/DMAc [15].

The present method was developed to measure the molecular weight distribution of the cellulose on HPLC in GPC mode. On traditional GPC, four GPC columns were used, which takes 50 minutes to complete the analysis. This leads to more consumption of solvent, time and other resources. In view of reducing the factors related to cost and time for analysis, the method was developed. This new method deals with two GPC columns instead of four columns. The results obtained by comparative study of four columns versus two columns as well as HPLC versus traditional GPC were mentioned in results and discussion section. The developed method was validated to support the study.

Experimental:-

Reagents: N,N-Dimethylacetamide: GC Grade, Assay 99.0%,
Lithium Chloride: Reagent Plus, Assay 99.0%
Pullulan Polysaccharide standards (Peak Molecular Weight (Mp): 180, 667, 6100, 9600, 21100, 47100, 107000, 194000, 344000 and 708000).
Instrument: Waters HPLC equipped with Refractive Index detector, Auto sampler, Temperature Control mode and Empower2 GPC Software.
Columns: Agilent column PLgel 20µ Mixed-A LS 300 x 7.5mm and a guard column.
Column temperature: 70°C
Mobile phase flow rate: 1.0 ml/min

Procedure:-

Mobile phase preparation (0.5% LiCl in N,N-Dimethylacetamide): Take 5 gm of LiCl in 1000ml of N,N-Dimethylacetamide. Stir at 500 rpm for one hour and filter using Ultipor N Nylon 6,6 membrane or equivalent with pore size of filter0.2 µm and diameter of 47 mm.

Standard solution preparation (Standard solution concentration 0.1%): Weigh 10mg of Pullulan Polysaccharide standard in culture vial. Add 10 ml of mobile phase. Stir vigorously for 24 hours on magnetic stirrer without heating. Filter the solution using Syringe filters Nylon or equivalent with pore size of filter 0.2 µm and diameter of 25 mm.

Sample preparation: Weigh 100 mg of sample. Add 100 ml of deionised water and boil at 100°C for 2 hours. Stir occasionally with glass rod. Filter the solution using Ultipor N Nylon 6,6 membrane or equivalent with pore size of filter 0.2 µm and diameter of 47 mm. Give 25ml hot deionised water wash three times and collect the residue. Take residue and add 40ml of methanol. Stir for 30 min on magnetic stirrer without heating. Filter the solution using Ultipor N Nylon 6,6 membrane or equivalent with pore size of filter 0.2 µm and diameter of 47 mm. Give 15 ml methanol wash three times and collect the residue. Soak the residue with N,N-Dimethylacetamide for 30 min with stirring and filter using Ultipor N Nylon 6,6 membrane or equivalent with pore size of filter 0.2 µm and diameter of 47 mm. Give 15 ml N,N-Dimethylacetamide wash three times and collect the residue. Take freshly prepared 10ml of 8% LiCl in N,N-Dimethylacetamide (0.8gm LiCl in 10ml N,N-Dimethylacetamide) in culture vial. Add 20 mg of cellulose with respect to initial amount (one-fifth of the initial 100mg sample) in culture vial. Stir continuously with heating till 40°C for two hours. The dissolved cellulose sample is diluted with N,N-Dimethylacetamide to make the concentration of 0.5% of LiCl in the sample solution. Filter the sample using Syringe filters Nylon or equivalent

with pore size of filter 0.2 µm and diameter of 25mm. Fill the filtered sample in sample vial and run the sample for analysis using HPLC equipped with GPC software and RI detector.

Results and discussion:-

Comparative study - Four columns versus two columns:-

The comparative study between four columns versus two columns was carried out to support the method development. The results obtained by using four columns versus two columns indicates very less significant variation in data for final assessment of the cellulose using pullulan polysaccharide as standard. It is inferred that the method is equally accurate and reproducible as well as economic using two columns instead of four columns. The method also has enhanced the productivity of analysis by two folds. *(Refer table no.1&2)*

System precision study:-

System precision of the system was performed by six replicates injection of standard solution of (Mp) 180, 107000 and 708000 on HPLC converted GPC. Since on traditional GPC Mp from 667 to 344000 can be measured. Thus six replicates injection of standard solution of Mp 667, 47100 and 344000 were carried out on traditional GPC. The criteria set for system precision are RSD of six replicates injection is Not More Than (NMT) 15.0%, Number of Theoretical Plates (N) is Not Less Than (NLT) 1000 and Tailing Factor (T) is NMT 1.08. Results obtained are average of six replicates of each molecular weight for system precision. The set criteria are achieved thus concludes that the HPLC converted GPC system is precise. Also infers that range for analysis is broad compare to traditional GPC. (Refer table no. 3 and 4, figure 3, 4 and 5).

Linearity study:-

Linearity parameter was demonstrated by preparing ten solutions ranged from Mp = 180 to Mp = 708000 and performed triplicate injections for each solution. Similarly for traditional GPC, range from Mp 667 to 344000 were carried out. Linear regression analysis was performed. The criteria set for linearity was $r^2 \geq 0.98$. Results obtained for linearity is summarized and shown in table no. 5; linearity graph of log MW vs. retention time are shown in figure 6 and 7.The study derives $r^2 = 0.9996$ and 0.9995 for HPLC converted GPC and traditional GPC respectively, which meets the set criteria and concludes that the system is linear.

Accuracy study:-

Accuracy of the method was performed by triplicate of a standard solution of Mp = 180, 107000 and 708000. Similarly, triplicate injections of sample solution of different molecular weight were injected for accuracy study. The criteria set for accuracy was % Recovery = 90% to 110%. Results obtained from accuracy study are shown in table no. 6 and 7 for standard and sample respectively. The result obtained from the study for standard and sample concludes that the method is accurate.

Method precision study:-

Method precision is carried out for repeatability and intermediate precision. One chemist performed six replicates of standard solutions of Mp = 107000 for molecular weight distribution determinations. Similarly, second chemist performed six replicate standard solutions of Mp = 107000 for molecular weight distribution determinations. The criteria's set for method precision were %RSD NMT 15.0% and absolute difference NMT 10%. Results obtained are average of six replicates from method precision are shown in table no.8 for first and second chemist. Similarly, absolute differences between two chemists are shown in table no.9. The study concludes that the %RSD is less than 15.0% and absolute differences are also less than 10%. This shows that method is precise and reproducible.

Robustness study:-

Robustness is the parameter in which deliberate changes are made to the system or equipment parameters like change in the column temperature by ± 5°C, change in the flow rate by ± 0.2 ml/min and change in the concentration of LiCl in DMAC by ± 20% from the value specified in the method. Injected standard solution of Mp = 180, 107000 and 708000. The criteria's set for robustness were %RSD of six replicates injection is NMT 15.0%, Number of Theoretical Plates (N) is NLT 1000 and Tailing Factor (T) is NMT 1.08. Results obtained are summarized by taking average of six replicates for the robustness study and are shown in table no. 10. From the detail study of robustness, it can be concluded that the method is robust to various changes in equipment parameters.

Solution stability study:-

Solution stability parameter is the study of stability of working standard and sample solution. It was carried out by performing injection of pullulan polysaccharide of molecular weight 107000 after every six hours of interval for 60 hours and data was collected. Similarly, sample was injected after every six hours of interval for 60 hours and data is collected. The criteria's set for solution stability were %RSD NMT 15.0% and % Stability 90 to 110%. % stability is calculated based on the molecular weight distribution at the time zero (T_0) and the molecular weight distribution at the time x (T_x) as per equation below.

% Stability = (T_x x100) /T_0

Results obtained by solution stability study are shown in table no. 11 and 12 for standard and sample respectively. The study concludes that the solution of standards and samples are stable for 60 hours. (Refer figure no. 8, 9, 10 and 11)

Figures:-

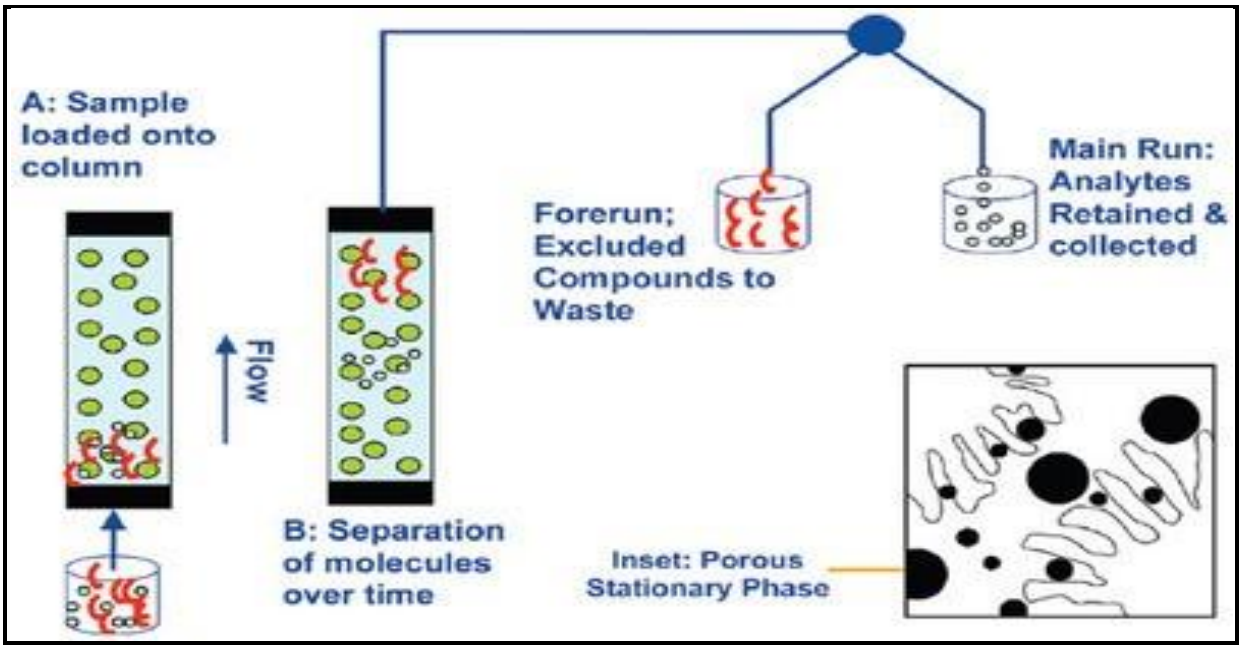

Figure 1: Schematic diagram of GPC. [16]

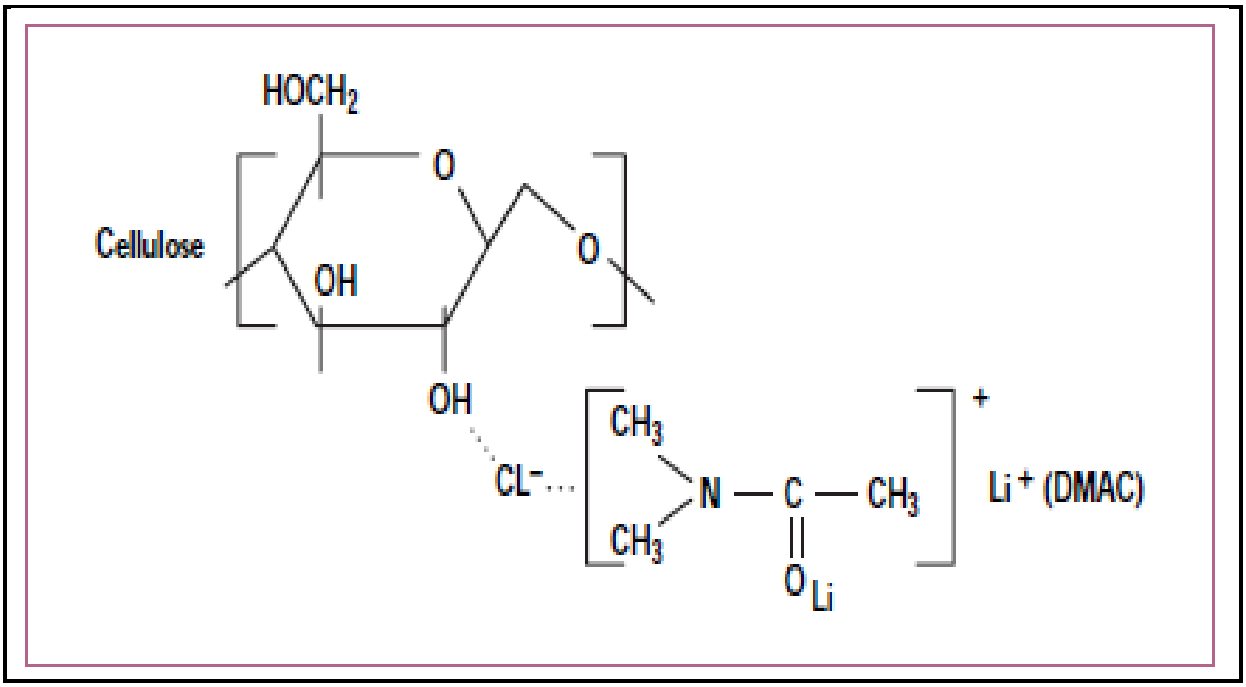

Figure 2: Mechanism of cellulose dissolution in the LiCl / DMAc solvent system. [6]

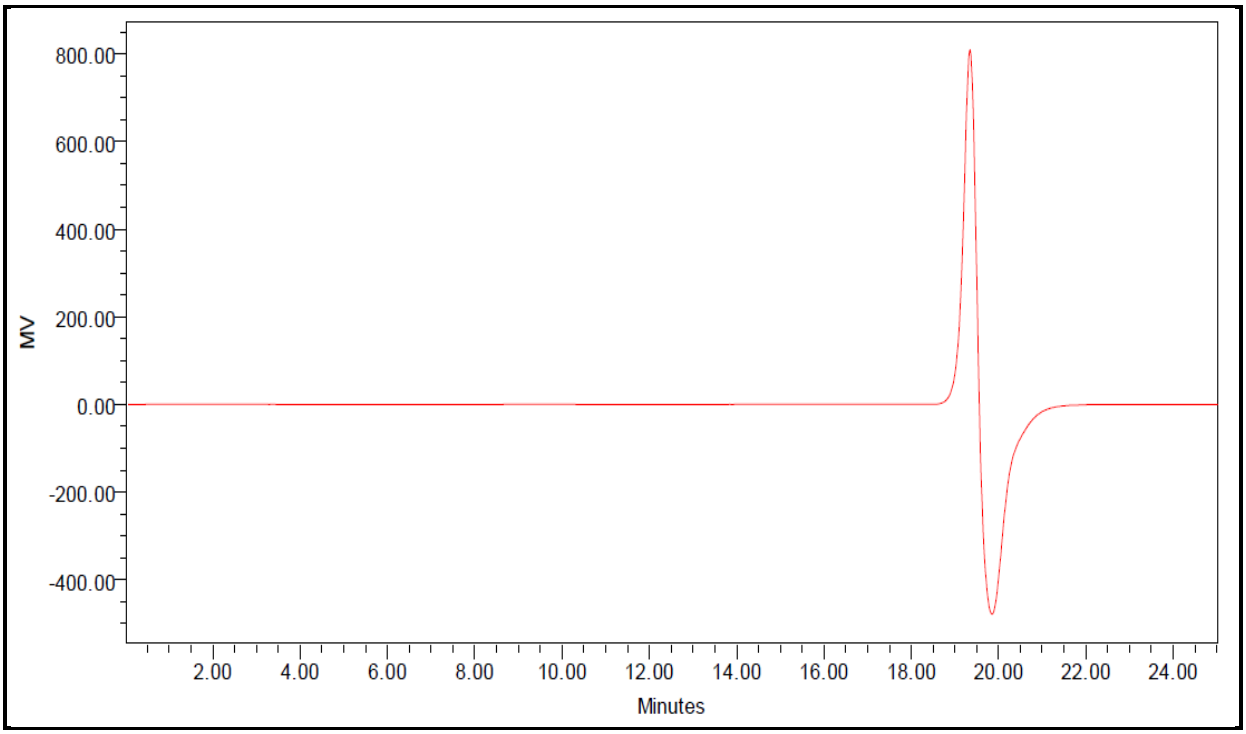

Figure 3: Typical chromatograph of Blank

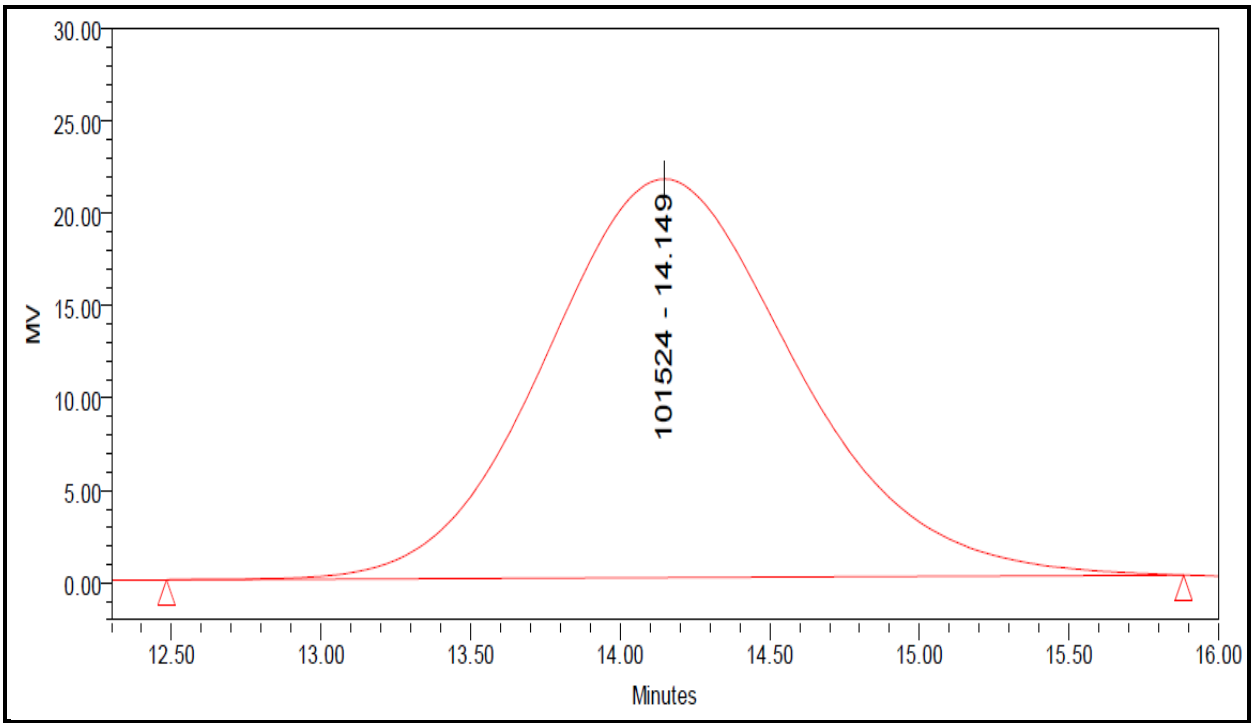

Figure 4: Typical chromatograph of pullulan polysaccharide standard

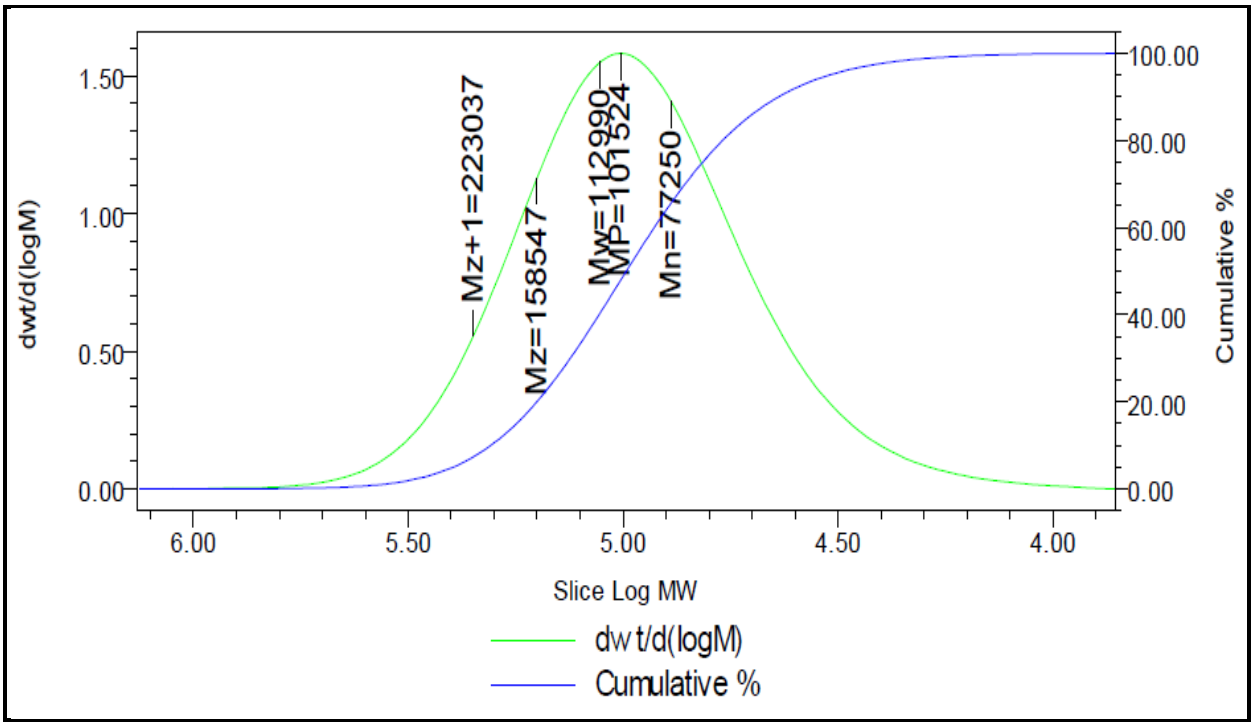

Figure 5: Typical chromatograph of pullulan polysaccharide standard with MWD plots

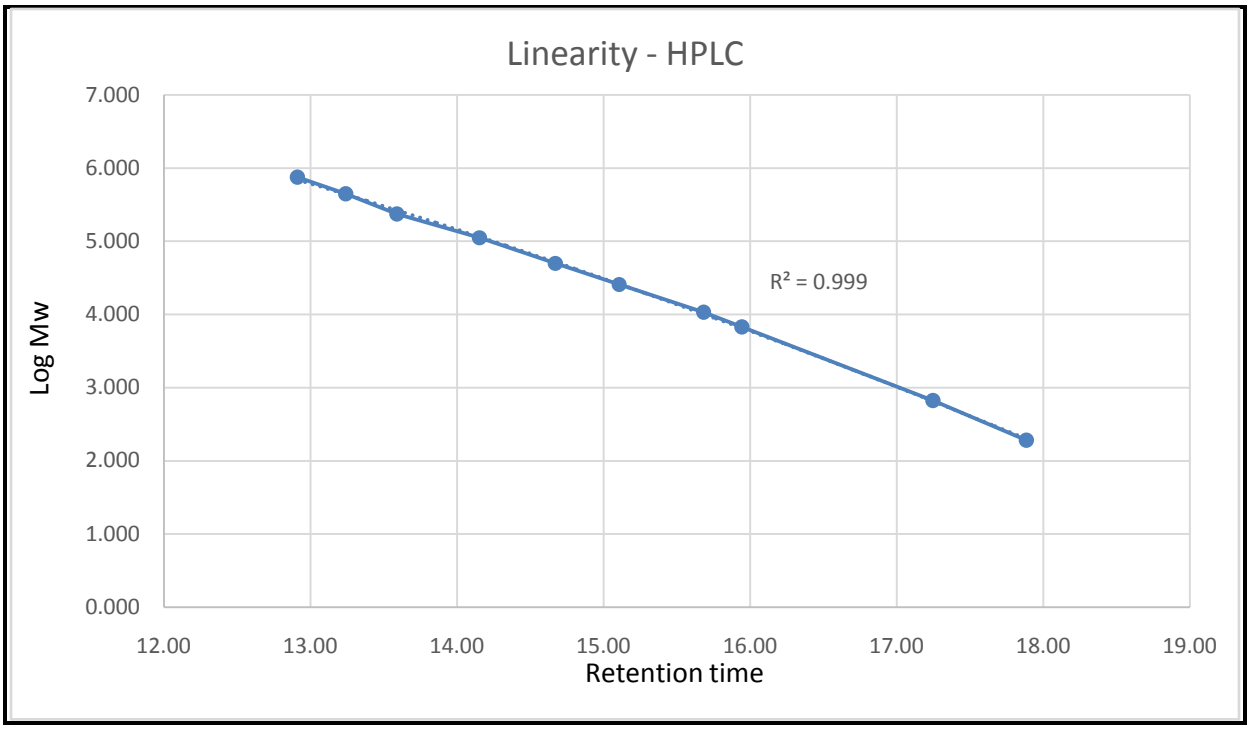

Figure 6: Linearity study on HPLC

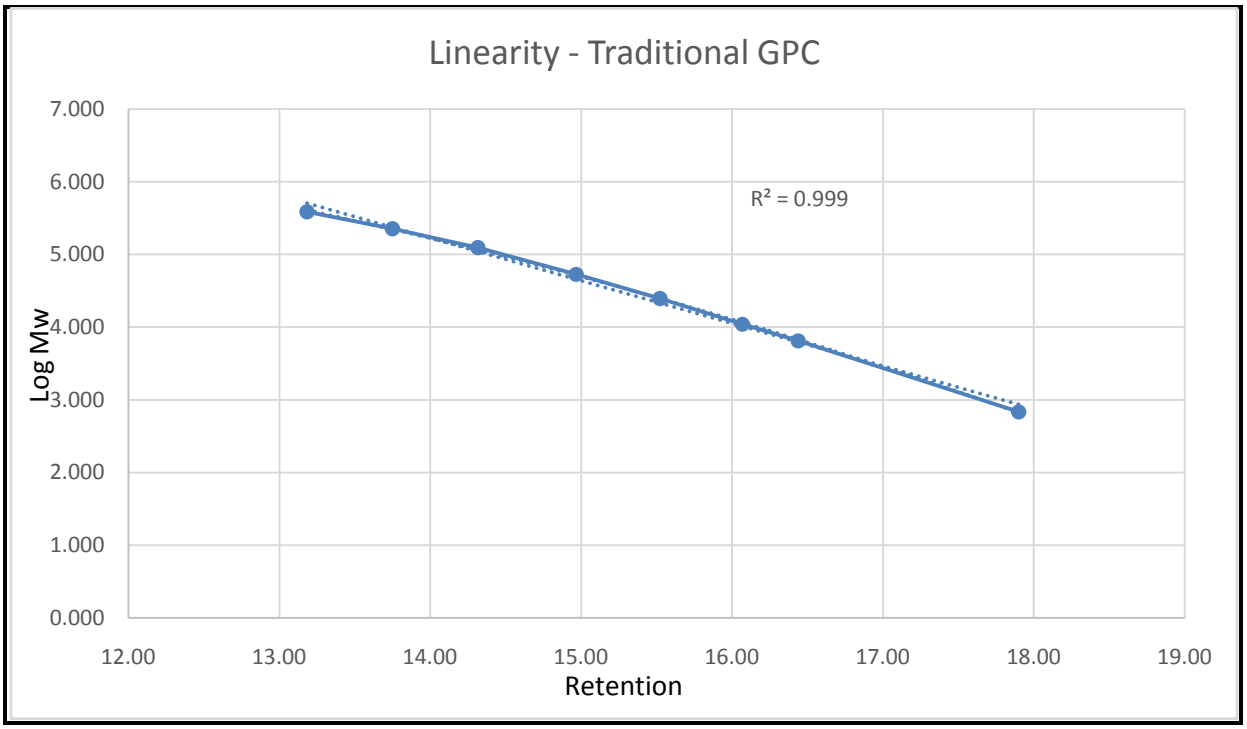

Figure 7: Linearity study on traditional GPC

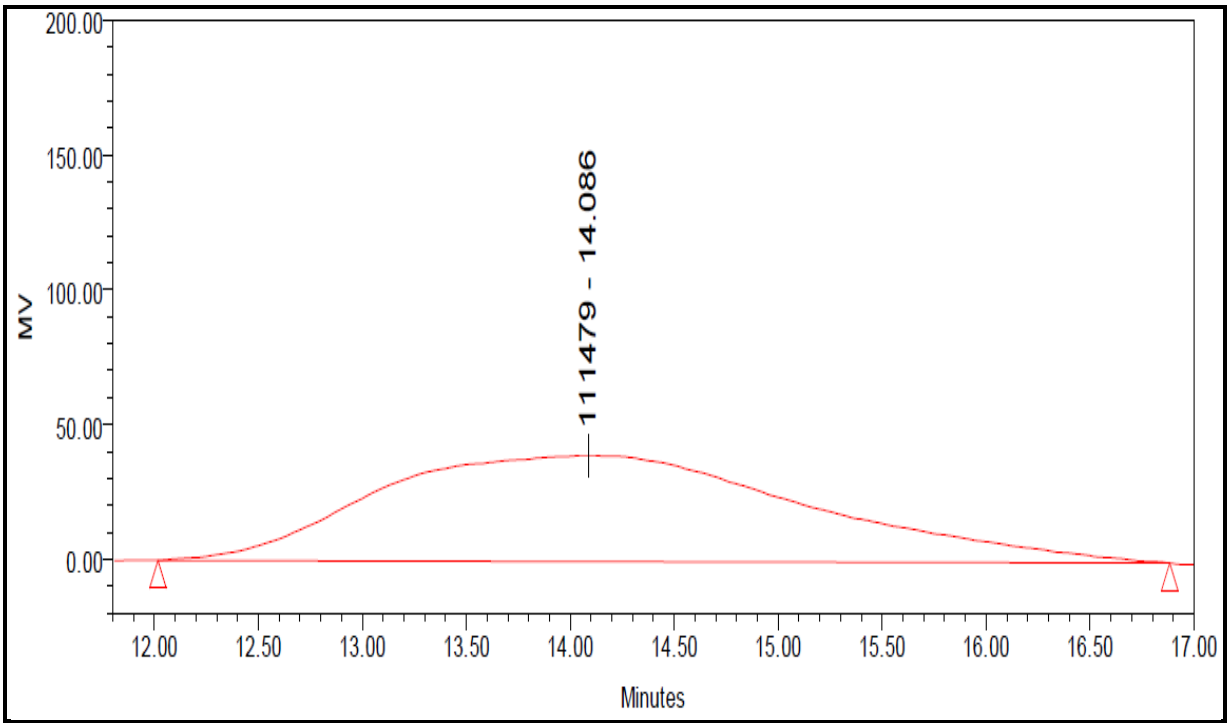

Figure 8: Typical chromatograph of sample

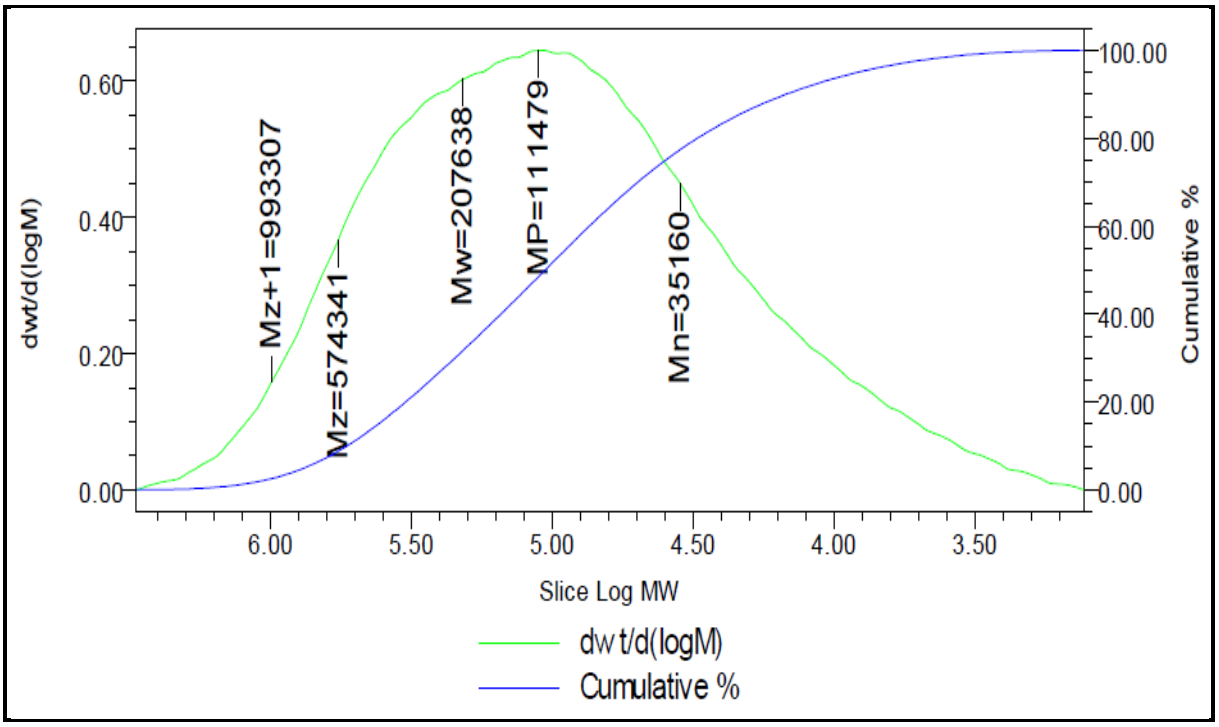

Figure 9: Typical chromatograph of sample with MWD plots

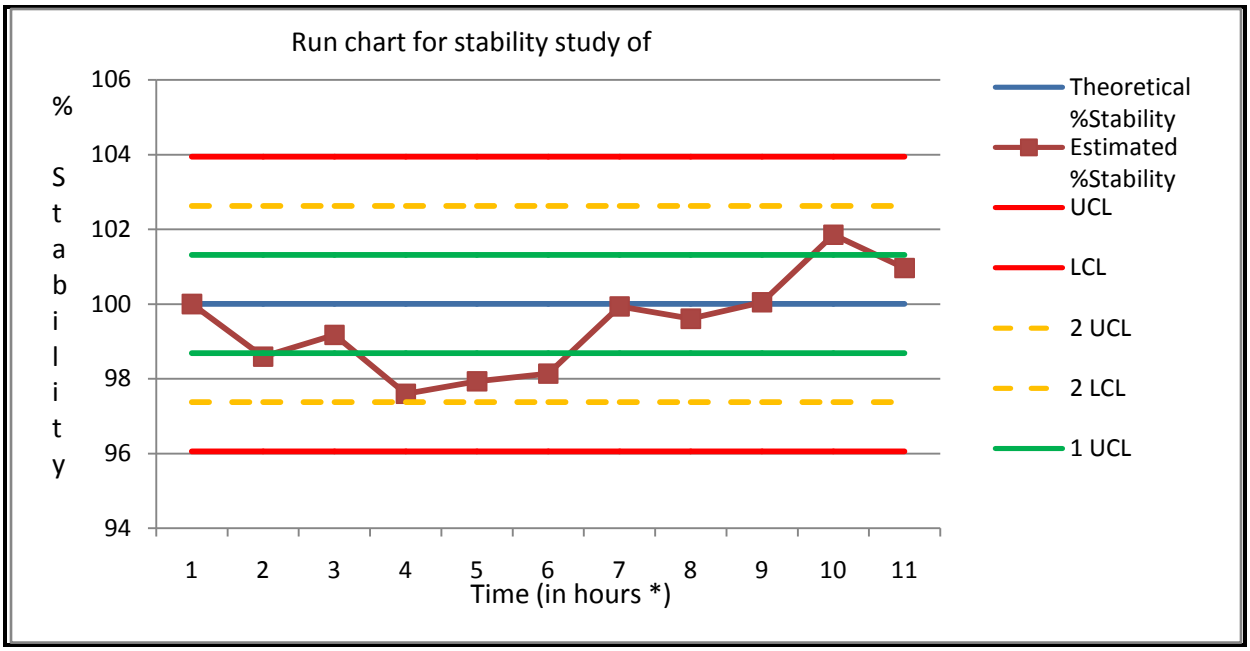

Figure 10: Run chart for stability study of standard.
*Standard injection done every 6 hours interval

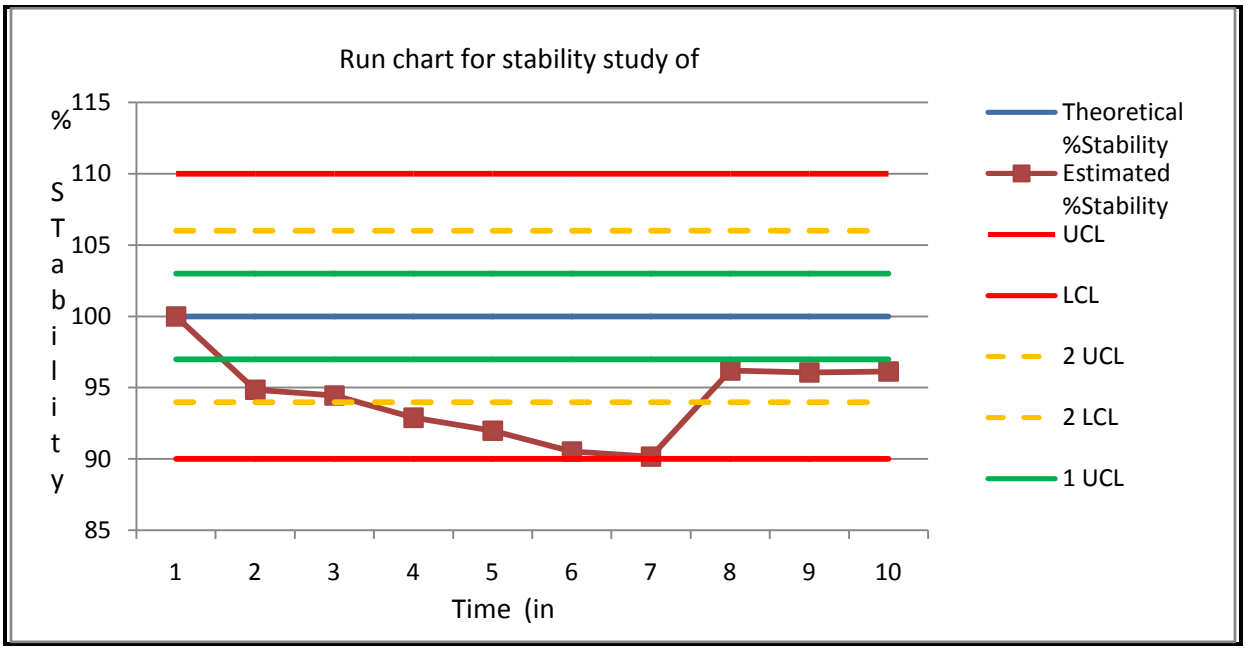

Figure 11: Run chart for stability study of sample.
*Sample injection done every 6 hours interval

Tables:-

Table 1. Comparative study – Four columns

Mp	Theoretical MW	Observed Mw	R^2	Retention Time
180	180	-	0.9988	-
667	667	659		32.37
6100	6200	6300		29.85
9600	10000	9933		29.38
21100	21700	20229		28.36
47100	48800	46913		27.22
107000	113000	98191		26.20
194000	200000	200287		25.20
344000	366000	401314		24.25
708000	805000	635295		23.63

Table 2. Comparative study – Two columns

Mp	Theoretical MW	Observed Mw	R^2	Retention Time
180	180	181	0.9995	17.82
667	667	650		17.20
6100	6200	6415		15.90
9600	10000	9991		15.62
21100	21700	20425		15.16
47100	48800	43927		14.65
107000	113000	101128		14.09
194000	200000	209281		13.60
344000	366000	377800		13.21
708000	805000	652902		12.86

Table 3. System Precision for HPLC

Mp	Theoretical MW	Calculated MW	Standard deviation	% RSD	Theoretical plate (N)	Tailing factor (Tf)	Retention time
180	180	192	1	0.6	7033	1.06	17.88
107000	113000	114364	2242	2.0	1388	1.03	14.14
708000	805000	747803	2327	0.3	1512	0.64	12.91

Table 4. System Precision for traditional GPC

Mp	Theoretical Mw	Calculated MW (Average of 6 replicates)	Standard deviation	%RSD
667	667	680	3	0.4
47100	48800	52840	219	0.4
344000	366000	382257	778	0.2

Table 5. Summary table for linearity of HPLC and traditional GPC

System	R^2	Intercept	Slope
HPLC	0.9996	15.083	-0.710
Traditional GPC	0.9995	13.435	-0.587

Table 6. Summary table of accuracy for standard

Mp	Theoretical MW	Observed MW	% Recovery
180	180	191	107
107000	113000	112469	100
708000	805000	749356	93

Table 7. Accuracy study for samples

Sample Id	Observed MW	Average of Observed MW	% Recovery *
	236472		101
	222523		95
I1C	245198	234731	104
	227488		104
	212110		97
S101-Ctrl	217374	218991	93
	244109		99
	252692		102
X102-Ctrl	245815	247539	99

***As theoretical molecular weight of sample is unknown, thus average of observed molecular weight is compared with the observed molecular weight of each sample for % recovery calculation.**

Table 8. Method precision study

Chemist	Observed MW	Observed Mn	Average of MWD = MW / Mn	Standard Deviation	% RSD	Theoretical Plate (N)	Tailing factor (T)	Retention time
One	114364	78435	1.46	0	0.3	1388	1.03	14.14
Two	112257	73885	1.52	0	4.6	1366	0.86	14.15

Table 9. Absolute differences between two chemists

Chemist	% RSD	Average % RSD	Average of MWD	Mean of Average MWD	Difference in Average MWD	Absolute differences (%)
One	0.3	2.5	1.46	1.49	0.06	4
Two	4.6		1.52			4

Table 10. Robustness study – Summary table

Mp	Theoretical MW	Average MW of six replicate each	Standard Deviation	% RSD	Average Theoretical plate of six replicate each	Average Tailing factor of six replicate each
Parameter: Change in column temperature by +5°C (i.e., 75°C)						
180	180	188	1	0.52	6967	1.01
107000	113000	112809	509	0.45	1397	0.94
708000	805000	729015	11338	1.56	1421	0.97
Parameter: Change in column temperature by -5°C (i.e., 65°C)						
180	180	188	1	0.52	6685	1.01
107000	113000	111966	534	0.48	1350	0.96
708000	805000	743954	6801	0.91	1365	0.93
Parameter: Change in flow rate by +0.2ml (i.e., 1.2ml)						
180	180	187	3	1.56	6617	1.02
107000	113000	113046	411	0.36	1277	0.98
708000	805000	658281	5026	0.76	1159	0.92
Parameter: Change in flow rate by -0.2ml (i.e., 0.8ml)						
180	180	188	1	0.43	7228	1.04
107000	113000	115411	1206	1.05	1447	0.98
708000	805000	707924	9820	1.39	1151	1.01
Parameter: Change in concentration of LiCl in DMAC by +20% (i.e., 0.6%)						
180	180	186	1	0.53	6891	1.01
107000	113000	112648	1227	1.09	1370	0.98
708000	805000	693441	3355	0.48	1440	0.98
Parameter: Change in concentration of LiCl in DMAC by -20% (i.e., 0.4%)						
180	180	190	1	0.43	6582	1.02
107000	113000	112360	705	0.63	1395	0.98
708000	805000	733671	5509	0.75	1492	1.02

Table 11. Solution stability study for standard with Mp = 107000

Time	Mw	Mn	MWD = Mw/Mn	Average	Standard Deviation	% RSD	Retention Time	% Stability
T_0	116557	76367	1.53	1.55	0	1.51	14.13	100.00
T_1	114915	74549	1.54				14.13	98.59
T_2	115595	75476	1.53				14.13	99.17
T_3	113761	71472	1.59				14.14	97.60
T_4	114145	74627	1.53				14.14	97.93
T_5	114388	73649	1.55				14.14	98.14
T_6	116482	76124	1.53				14.12	99.94
T_7	116099	76749	1.51				14.13	99.61
T_8	116611	74959	1.56				14.12	100.05
T_9	118718	76614	1.55				14.11	101.85
T_{10}	117681	74643	1.58				14.12	100.96

Table 12. Solution stability study for sample

Time	Mw	Mn	MWD = Mw/Mn	Average	Standard Deviation	% RSD	Retention Time	% Stability
T_0	258974	82683	3.13	3.25	0	11.15	13.71	100.00
T_1	245641	69782	3.52				13.98	94.85
T_2	244627	63437	3.86				14.07	94.46
T_4	240584	79141	3.04				14.09	92.90
T_5	238210	77276	3.08				14.07	91.98
T_6	234463	67463	3.48				14.12	90.54
T_7	233533	86752	2.69				14.00	90.18
T_8	249155	68150	3.66				14.00	96.21
T_9	248780	83817	2.97				14.08	96.06
T_{10}	248968	82011	3.04				14.07	96.14

Injection at time T3 is outlier which was investigated and proved.

Notes:-

Names of the companies or commercial products are given solely for the purpose of providing specific information; their mention does not imply recommendation or endorsement by the Aditya Birla Science and Technology Company Private Limited or Aditya Birla Group over others not mentioned.

Conclusion:-

The method validated for the determination of molecular weight distribution of cellulose using pullulan polysaccharide as a standard from the range 180 to 708000 using HPLC exhibits precise, linear and accurate. Also the method was shown to be robust with the change in the temperature, flow rate of mobile phase and concentration of LiCl in DMAc as critical criteria. The stability study for standard as well as sample is carried out for 60 hours which concludes stable. Thus, the developed method is economic with less turnaround time.

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