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Investigation of Size Exclusion Chromatography (SEC) Column Performance for Detection of Microplastics in Aquatic Environment

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Abstract

Microplastics have become a problem of the modern era with the increasing use of plastics. However, the effects of microplastics on living beings are not known exactly. As stated in the booklet "Microplastics in Drinking Water" published by the World Health Organization (WHO) in 2019, there is no standard method for the detection of microplastics. Although there are various analysis methods for the determination of microplastics in the literature, some deficiencies are observed. In this study, it is aimed to analyze the microplastics in aquatic environment without using organic solvents, especially in accordance with green chemistry. In this context, Size Exclusion Chromatography was used and the performance of four different Ultra-hydrogel columns suitable for aqueous mobile phases was tested. Polyethylene glycol (PEG) standards with different molecular masses were used during this performance test. As a result of the study, the theoretical plate numbers of Ultra-hydrogel 250 Å, Ultra-hydrogel 500 Å, and Ultra-hydrogel 1000 Å columns were calculated as 256, 713, and 342, respectively, and the regression coefficients (R^2) were calculated as >0.97. The theoretical plate number of Ultra-hydrogel 2000 Å column was not calculated because of lower regression coefficient.

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Highlights

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Chromatography
Column Performance

Mikroplastiklerin Sucul Ortamda Tespitinde Boyut Eleme Kromatografisi (SEC) Kolon Performansının İncelenmesi

Özet

Mikroplastikler, artan plastik kullanımı ile modern çağın sorunu haline gelmiştir. Mikroplastiklerin canlı varlıklar üzerindeki etkileri ise tam olarak bilinememektedir. Dünya Sağlık Örgütü (WHO) tarafından 2019 yılında yayınlanan ''İçme Sularında Mikroplastikler'' isimli kitapçıkta da belirtildiği üzere, mikroplastiklerin tespitine yönelik bir standart metot bulunmamaktadır. Literatürde mikroplastiklerin tespiti için çeşitli analiz yöntemleri olmasına rağmen bazı eksiklikler gözlemlenmektedir. Bu çalışmada, özellikle yeşil kimyaya uygun olarak, mikroplastiklerin organik çözücü kullanmadan sucul ortamda analiz edilmesi amaçlanmıştır. Bu kapsamda, Boyut Dışlama Kromatografisi kullanılmış olup, sulu hareketli fazlara uygun olan dört farklı Ultrahydrogel kolonunun performansı test edilmiştir. Bu performans testi sırasında farklı molekül kütlelerine sahip polietilen glikol (PEG) standartları kullanılmıştır. Yapılan testlerin sonucunda, Ultrahydrogel 250 Å, Ultrahydrogel 500 Å ve Ultrahydrogel 1000 Å kolonlarının teorik plaka sayıları sırasıyla 256, 713 ve 342 ve çizilen kalibrasyon grafiklerinde regresyon katsayıları (R^2)>0.97 olarak hesaplanmıştır. Ultrahydrogel 2000 Å kolonu için grafik çizildiğinde regresyon katsayısı düşük olduğundan teorik plaka sayısı hesaplanmamıştır.

Anahtar Kelimeler

Mikroplastikler, Boyut Eleme Kromatografisi, Ultrahydrogel Kolon

Öne Çıkanlar

Yeşil Kimya
Kromatografi
Kolon Perfrmansı

1. Introduction

Studies on microplastics first entered the scientific literature in 1972 with the research of Carpenter et al. on small pieces of plastic floating in the oceans (Carpenter et al., 1972). However, the term "microplastic" was first used by Thompson et al. in 2004 to describe microscopic pieces of plastic smaller than 5 mm in studies on seawater (Frias & Nash, 2019; Thompson et al., 2004).

Microplastics are classified as primary and secondary microplastics, depending on their source. Microplastics that are produced in microscopic size for special purposes are called primary microplastics. These microplastics are often used in cosmetics and personal care (Zitko & Hanlon, 1991) and as air-blasting media (Gregory, 1996). Their use as carriers for drugs has also been increasingly reported (Patel et al., 2009). Also unprocessed plastic pellets used in industrial manufacturing can be included in this class (Cole et al., 2011; da Costa et al., 2017). Secondary microplastics are defined as small pieces of plastic formed by the breakdown and degradation of large plastics found both in the sea and on land (Thompson et al., 2004). An ultraviolet (UV) radiation in sunlight causes oxidation of the polymeric matrix, leading to bond cleavage. Therefore, prolonged exposure of the

plastic to sunlight can cause its photo-degradation (Barnes et al., 2009; Browne et al., 2007; Moore, 2008). That degradations may cause the leaching of additives from the plastic, which are added to give the polymer strength and increase corrosion resistance. This degradation and decomposition process varies according to environmental factors such as temperature and sunlight, as well as structural features of plastic such as size, fragility, and density (Browne et al., 2007).

It is possible to examine the effects of microplastics on the health of living beings in two groups physical and chemical effects. Considering its physical effects, it can be swallowed by aquatic creatures such as plankton, fish, and marine mammals due to its small size. Besides sea creatures, birds can also swallow microplastics on the sea surface (da Costa et al., 2017; Koelmans et al., 2015; Wang et al., 2016). The swallows of microplastics cause living beings to consume less food and therefore have less energy to carry out their life functions. It may also cause neurological and reproductive toxicity. When chemical effects are examined, it is possible that monomers, solvents, and additives that have not undergone polymerization reactions during polymer production may leak from the plastic material. The fact that most of the additives used in polymer production are lipophilic makes it possible to penetrate cell membranes and facilitate their participation in biochemical reactions (da Costa et al., 2017).

As stated in the booklet "Microplastics in Drinking Water" published by the World Health Organization (WHO) in 2019, there is no standard method for the detection of microplastics (WHO, 2019). Looking at the literature, it is seen that Fourier Transform Infrared Spectroscopy (Harrison et al., 2012), single particle-inductively coupled plasma mass spectrometry (SP– ICP/MS) (Laborda et al., 2021), Pyrolysis-Gas Chromatography-Mass Spectrometry (Py–GC/MS) (Kirstein et al., 2021) and Size Exclusion Chromatography (SEC) (Biver et al., 2018) techniques are used for the detection of microplastics.

In this study, four Ultra-hydrogel columns (250 Å – 500 Å – 1000 Å – 2000 Å) of approximately twenty years belonging to the Size Exclusion Chromatography (SEC) device, which was maintained and repaired by Bursa Uludag University within the scope of TÜBİTAK Project No. 215S620, were subjected to performance trials. Organic solvents have been used in most of the studies dealing with microplastics in the literature, such as 1,2,4-Trichlorobenzene (Hintersteiner et al., 2015). However, this study is to method development suitable for green chemistry for the qualitative and semi-quantitative analysis of microplastics in the aqueous phase by minimizing the use of organic solvents.

2. Material and Method

2.1. Material

2.1.1. Materials and reagents

In this study, ten different polyethylene glycol (PEG) standards (Waters Corporation, Germany) with molecular mass ranging from 106 to 24400 Dalton were used. A 0.2 g L^{-1} NaN_3 solution was used in the preparation of stock and intermediate stock polymer

solutions. Ultrapure water was used in the preparation of all standard polymer solutions and NaN_3 solution.

2.1.2. Instruments

For Size Exclusion Chromatography (SEC) analysis, an Empower Build 1154 software, a Waters 1515 system equipped with an isocratic HPLC pump, and 2414 Refractive Index detector was used. For the separation, four Ultra-hydrogel columns (300 x 7.8 mm, 30°C) from Waters were subjected to performance testing for the use as a stationary phase, using 0.2 g L^{-1} NaN_3 at 1.0 mL min^{-1} flow rate as the eluent. The properties of the existing Ultra hydrogel columns are given in Table.1.

Table.1 Properties of the Ultra-hydrogel columns

Pore size	Particle size
250 Å	6 µm
500 Å	10 µm
1000 Å	12 µm
2000 Å	12 µm

2.2. Method

2.2.1. Preparation of stock and intermediate stock polymer solutions

For all PEG standards, stock solutions of 100 mg mL^{-1} were prepared. For the liquid PEG standards, 300 µL was taken with a micropipette and prepared by adding 2700 µL of NaN_3 solution. For the solid PEG standards, approximately 300 mg (0.3000 ± 0.0002) weighing was taken and dissolved with some NaN_3 solution. Then it was prepared by filling to 3 mL with NaN_3 solution. A 10 mg mL^{-1} standard for all PEG standards were prepared by dilution from stock solution. All standards were filtered with 0.45 µm before being injected into SEC columns. For all PEG standards, injections were made in three repetitions.

2.2.2. Determining of column performance

Ultra-hydrogel columns were tested according to ten PEG standards, whose molecular mass ranged from 106 to 24400 Daltons (Da). During the analysis, the flow rate of the mobile phase was fixed at 1 mL min^{-1}. Calibration graphs were plotted according to retention time versus logarithm of molar mass (Log (M_W)). The slope of the line and the regression coefficient (R^2) was calculated by the Least Squares method.

Theoretical plate number and plate height were calculated with the formulas given in F.1 and F.2, respectively.

$$N = 16 \times \left(\frac{t_R}{W}\right)^2 \quad \text{(F.1)}$$

$$H = \frac{L}{N} \qquad (F.2)$$

Where N is the theoretical plate number, W is the observed peak width, t_R is the retention time, H is the plate height, and L is the length of the columns (300 mm).

Theoretical plate numbers (N) generally range from 100 to 10^6 for all chromatographic techniques (Keller & Giddings, 2022). If the theoretical plate number is greater than 100 (N>100), the plate height is less than 1 (H<1), and the regression coefficient is greater than 0.97 (R^2>0.97), the data are enough to accept that the column is working.

3. Results and Discussion

3.1. Ultra-hydrogel 250 Å

For the Ultra-hydrogel 250 Å, the prepared intermediate stock PEG standards were injected into the SEC column starting from the smallest molecular mass. With the obtained data, a graph was plotted showing the change in retention time versus increasing molecular mass. The calibration graph was plotted as retention time versus Log (M_W) and R^2 of calibration graph was calculated as 0.9977.

Chromatograms and calibration curve of Ultra-hydrogel 250 Å column are given in Figure.1 and Figure.2.

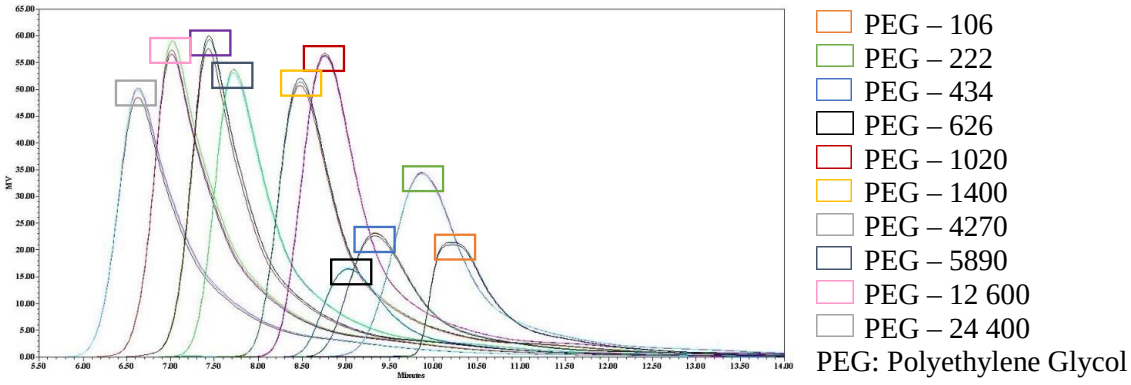

Figure.1 Chromatograms of Ultra-hydrogel 250 Å

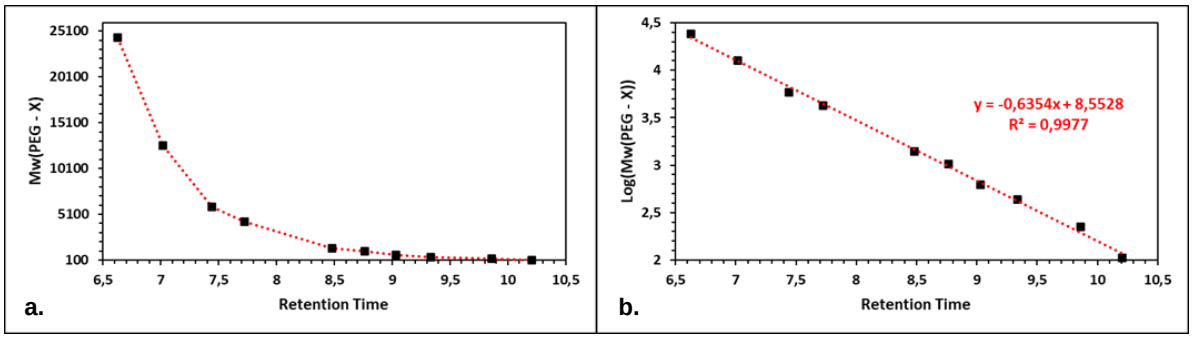

Figure.2 For the Ultra-hydrogel 250 Å column; **a.** According to molar mass distribution, **b.** Linear calibration line

As a result of the performance test for the Ultra-hydrogel 250 Å column;

- It was observed that the peak heights and peak areas were consistent between injections of three repetitions for each PEG standard.
- It was monitored that the retention times (t_R) of the standards were consistent between injections and, as expected, decreased as the molecular mass increased.
- Since the Ultra-hydrogel 250 Å column has the smallest pore size, it was observed very high peak widths depending on the holding time of the PEG standards in the column. The theoretical plate numbers and the plate heights of the Ultra- hydrogel 250 Å column were calculated using the formula F.3 and F.2 and results of them was given in Table 2.

$$N = 5{,}54 \times \left(\frac{t_R}{W/_2}\right)^2 \qquad (F.3)$$

3.2. Ultra-hydrogel 500 Å

For the Ultra-hydrogel 500 Å, the prepared intermediate stock PEG standards were injected into the SEC column starting from the smallest molecular mass. With the obtained data, a graph was plotted showing the change in retention time versus increasing molecular mass. The calibration graph was plotted as retention time versus Log (M_W) and R^2 of calibration graph was calculated as 0.9890.

Chromatograms and calibration curve of Ultra-hydrogel 500 Å column are given in Figure.3 and Figure.4.

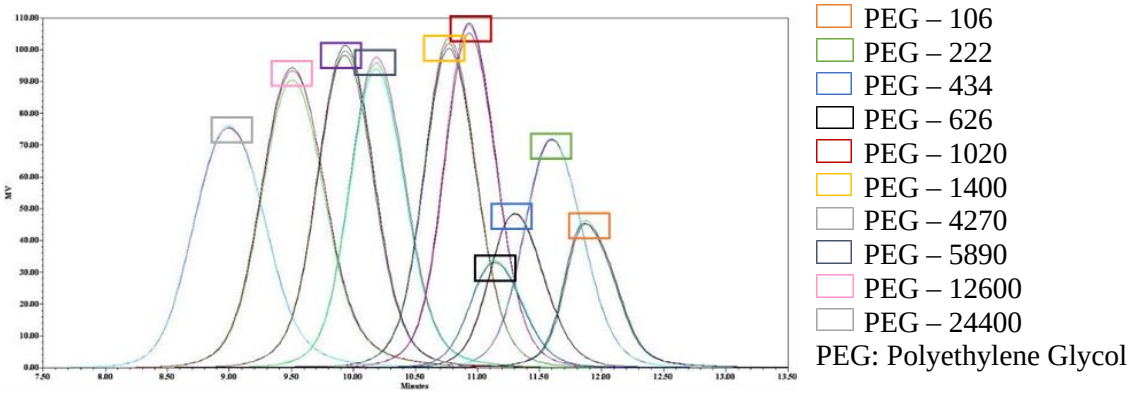

Figure.3 Chromatograms of Ultra-hydrogel 500 Å

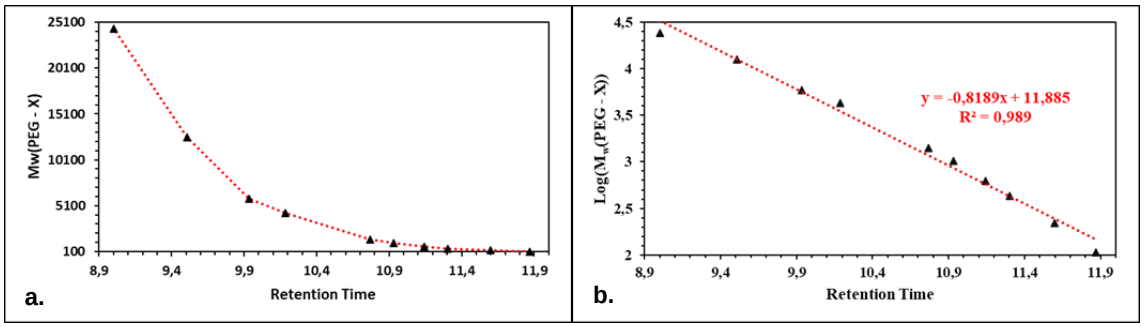

Figure.4 For the Ultra-hydrogel 500 Å column; **a.** According to molar mass distribution, **b.** Linear calibration line

As a result of the performance test for the Ultra-hydrogel 500 Å column;

- It was monitored that the peak heights and peak areas were consistent between injections of three repetitions for each PEG standard.
- It was observed that the retention times (t_R) of the standards were consistent between injections and, as expected, decreased as the molecular mass increased.
- The theoretical plate numbers and plate heights of the Ultra-hydrogel 500 Å column were calculated using the formula F.1 and F.2 and results of them was given in Table.2.

3.3. Ultra-hydrogel 1000 Å

For the Ultra-hydrogel 1000 Å, the prepared intermediate stock PEG standards were injected into the SEC column starting from the smallest molecular mass. With the obtained data, a graph was plotted showing the change in retention time versus increasing molecular mass. The calibration graph was plotted as retention time versus Log (M_W) and R^2 of calibration graph was calculated as 0.9799.

Chromatograms and calibration curve of Ultra-hydrogel 1000 Å column are given in Figure.5 and Figure.6.

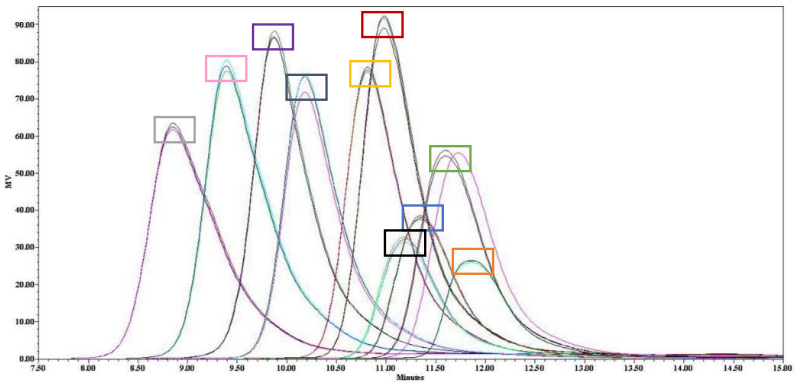

Figure.5 Chromatograms of Ultra-hydrogel 1000 Å

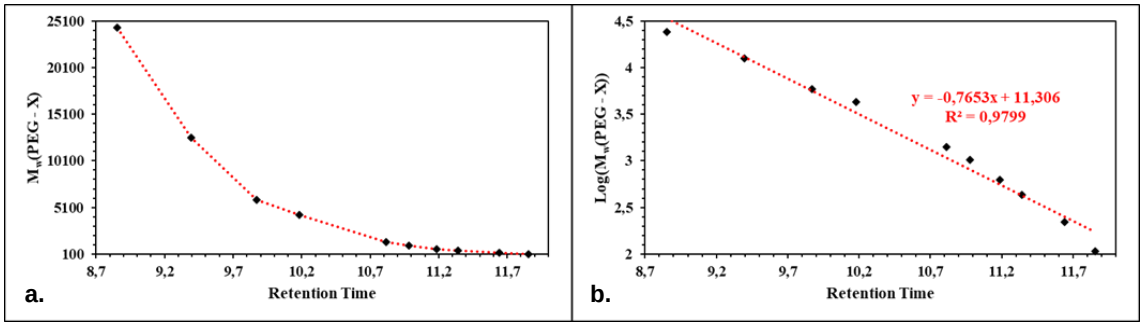

Figure.6 For the Ultra-hydrogel 1000 Å column; **a.** According to molar mass distribution, **b.** Linear calibration line

As a result of the performance test for the Ultra-hydrogel 1000 Å column;

- It was observed that the peak heights and peak areas were consistent between injections of three repetitions for each PEG standard.

- It was monitoring that the retention times (t_R) of the standards were consistent between injections and, as expected, decreased as the molecular mass increased.
- The theoretical plate numbers and the plate heights of the Ultra-hydrogel 1000 Å column were calculated using the formula F.1 and F.2 and results of them was given in Table.2.

3.4. Ultra-hydrogel 2000 Å

For the Ultra-hydrogel 2000 Å, the prepared intermediate stock PEG standards were injected into the SEC column starting from the smallest molecular mass.

When six PEG standards in the molecular mass range of 106 to 1400 Daltons were tested, the results were found to be inconsistent. Considering that there may be a blockage in the column pores, the Ultra-hydrogel 2000 Å column was connected opposite direction and washed with the 0.2 g L^{-1} NaN_3 solution for about three hours. Then the column direction was changed to its original position and a retry was made.

With the obtained data, a graph was plotted showing the change in retention time versus increasing molecular mass. The calibration graph was plotted as retention time versus Log (M_W) and R^2 was calculated as 0.1306.

Chromatograms and calibration curve of Ultra-hydrogel 2000 Å column are given in Figure.7 and Figure.8.

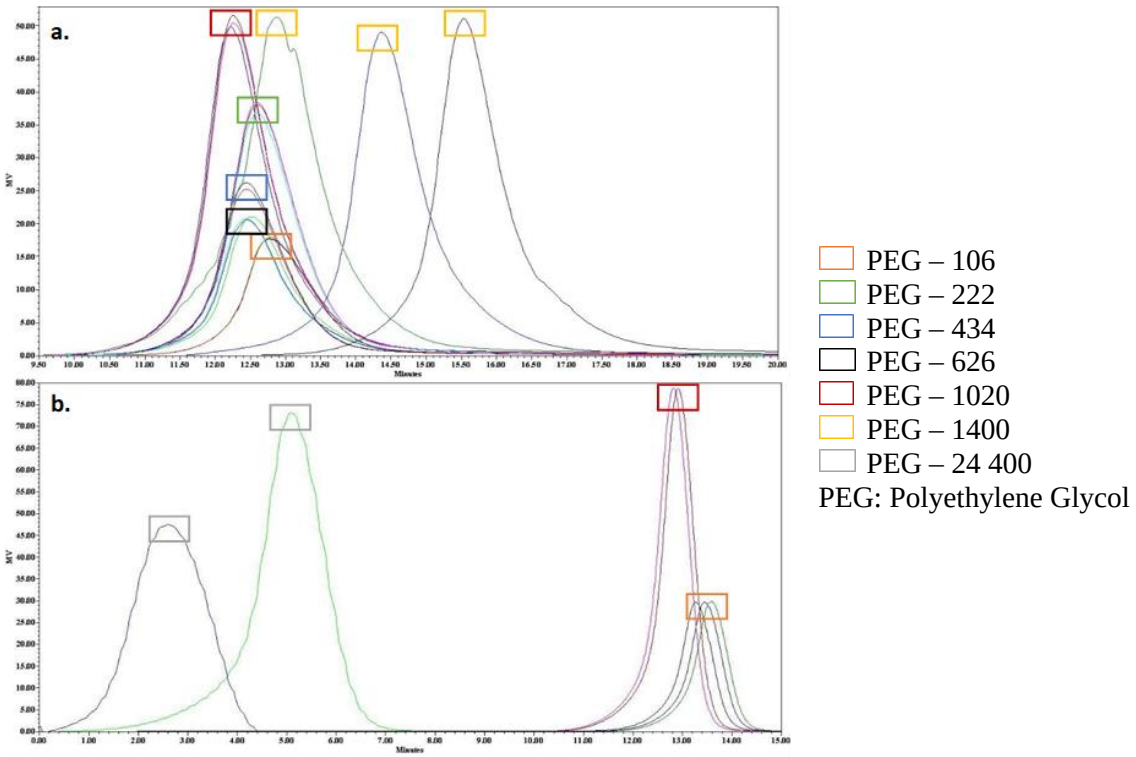

Figure.7 Chromatograms of Ultra-hydrogel 2000 Å; **a.** First injections, **b.** Second injections after column washing

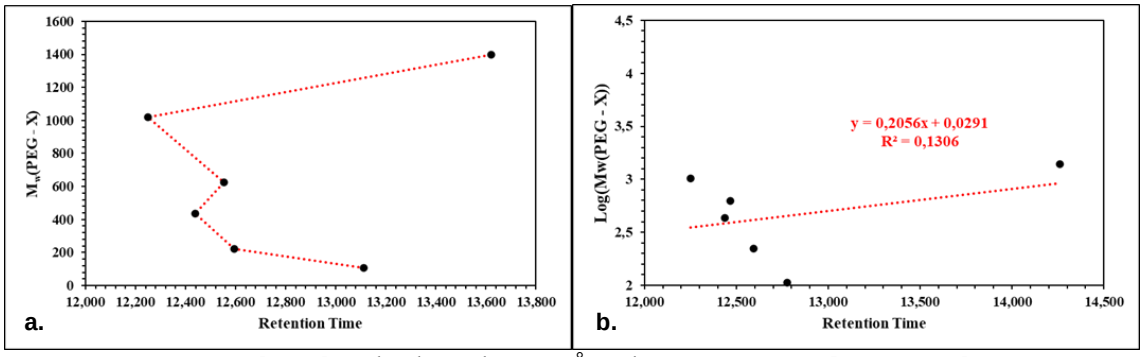

Figure.8 For the Ultra-hydrogel 2000 Å column; **a.** According to molar mass distribution, **b.** Linear calibration line

As a result of the performance test for the Ultra-hydrogel 2000 Å column;

- It was monitored that the peak heights and peak areas were inconsistent between injections of three repetitions for each PEG standard.
- As column performance was in doubt, trial injections were performed with PEG standards with molecular masses of 106 Da, 626 Da, and 1400 Da. For the PEG 106 standard, it was determined that the result of the second trial was different from the first trial.

As a result of the new trial after washing the Ultra-hydrogel 2000 Å column mentioned in the upper paragraph about three hours;

- For the PEG – 106 Da standard, the retention times were like the data obtained in the second experiment, but an approximately two-fold increase in peak heights was observed.
- For the PEG – 1020 Da standard, different results were obtained from the previous experiments in the first two injections, but the new data were found to be compatible with the PEG – 106 Da standard given in the same set. However, in the third injection, the runtime was not enough for the standard to leave the column.
- For the PEG – 24400 Da standard;
  - By looking at the peak heights of the data obtained in the first injection, it was thought that the molecular mass remaining in the column in the previous injection belonged to the PEG – 1020 Da.
  - In the second injection, by looking at the peak height, it was determined that it belonged to the first injection of the PEG – 24400 Da standard.

As the result, it was observed that the Ultra-hydrogel 2000 Å column was inoperative.

The calculated theoretical plate numbers (N) and plate heights (H) of all columns that are operational given in Table.2.

Table.2 Theoretical plate numbers (N) and plate heights (H) of columns

Columns	Theoretical Plate Numbers (N)	Plate Heights (H)
Ultra-hydrogel 250 Å	256	0.85 ± 0.40
Ultra-hydrogel 500 Å	713	0.44 ± 0.11
Ultra-hydrogel 1000 Å	342	0.96 ± 0.27

3.5. Sequential of all Ultra-hydrogel columns

Columns considered operative (Ultra-hydrogel – Seq.) were coupled to the SEC with large-to-small pore size. The prepared intermediate stock PEG standards were injected into the SEC column starting from the smallest molecular mass. With the obtained data, a graph was plotted showing the change in retention time versus increasing molecular mass. The calibration graph was plotted as retention time versus Log (M_W) and R^2 of calibration graph was calculated as 0.9948.

Chromatograms and calibration curve of Ultra-hydrogel – Seq. columns are given in Figure.9 and Figure.10.

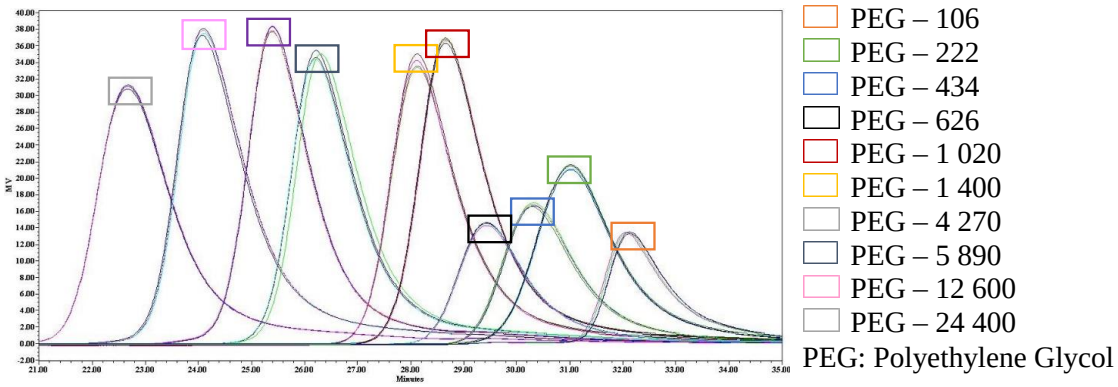

Figure.9 Chromatograms of Ultra-hydrogel – Seq.

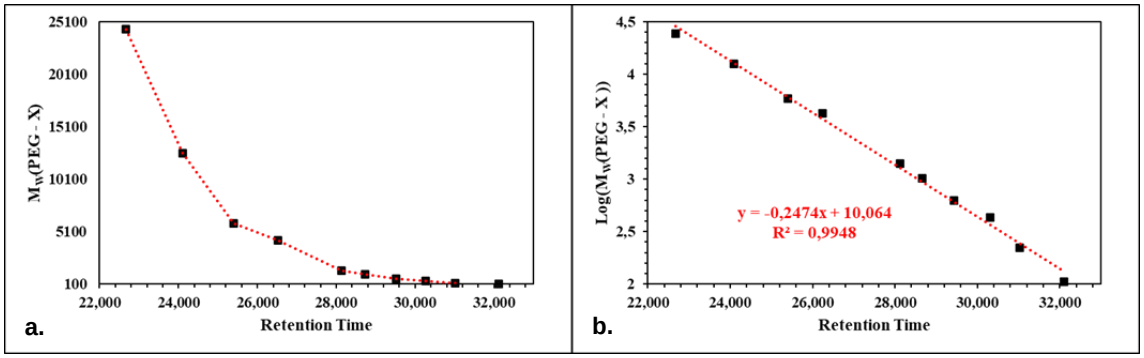

Figure.10 For the Ultra-hydrogel – Seq. column; **a.** According to molar mass distribution, **b.** Linear calibration line

3.6. Validation parameter for SEC columns

Repeatability tests were performed by comparing the retention times of injections at different time intervals for a selected PEG standard. As a result of the injections made on three different days, the t_R value of the PEG–24400 standard was found 22.66 ± 0.02. The summary of the repeatability test is given in Table.3. This test was performed with same instrument and standard by the same analyst.

Table.3 Summary of the repeatability tests

	26.01.2022	**27.01.2022**	**02.02.2022**
t_R (min)	22.67 ± 0.01	22.63 ± 0.01	22.66 ± 0.01
Width (sec)	338 ± 2.58	330.67 ± 1.53	323.67 ± 1.53
N	259	269	282

Selectivity test was carried out by injecting the solution prepared by mixing three different molecular masses of PEG at the same concentrations (10 mg mL^{-1} each) at three repetitions.

The capacity factor and the selectivity factor were calculated with the formulas given in F.4 and F.5, respectively.

$$k = \frac{(t_R - t_0)}{t_0} \quad \text{(F.4)}$$

$$\alpha = \frac{k_2}{k_1} \quad \text{(F.5)}$$

Where k is the capacity factor, α is the selectivity factor, t_R is the retention time, t_0 is the void volume of the mobile phase. The results of the selectivity test are given in Table.4.

Table.4 Results of the selectivity tests (Run time: 45 min, Flow rate: 1.0 mL min^{-1})

[i]k_1	3,02
[ii]k_2	3,99
[iii]k_3	4,63
[iv]α_1	1,32
[v]α_2	1,16

[i]: capacity factor of PEG 24400; [ii]: capacity factor of PEG 1400; [iii]: capacity factor of PEG 106; [iv]: selectivity factor of k_2 and k_1; [v]: selectivity factor of k_3 and k_2

4. Conclusion

In the user manual of the commercial Ultra-hydrogel columns, which were subjected to performance testing within the scope of this study, it is stated that if they are not used for more than 72 hours, they should be filled with (w/w) 0.05% NaN_3 solution and the end caps should be closed unless they are attached to the SEC. However, it was determined that before the maintenance and repair of the device, the columns were left exposed without being filled with any solution and without the end caps being attached.

To condition the columns, a long period of washing with milli-Q pure water was made and the pores in the column were swelled.

After optimizing the columns, each column was tested with the polyethylene glycol standards provided under the project. As a result of the tests, it was determined that the

Ultra-hydrogel 250 Å, Ultra-hydrogel 500 Å and Ultra-hydrogel 1000 Å columns were working (Table.2).

In the Ultra-hydrogel 2000 Å column, on the other hand, the fact that the peak shapes (peak height, peak area, peak width, etc.) (*Figure.7.a,b*) and retention times were not as expected (*Figure.8.a,b*), and the appropriate regression coefficient (0.1306) was not obtained when the calibration graph plotted with the obtained data proved that the column was inoperative.

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Conflict of Interest

There is no conflict of interest.

Author Contribution

Each participant has an equal share.

5. References

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