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A New Reversed Phase HPLC Method for Separation of PEG 600 on C8 Column Coupled with Evaporative Light Scattering Detector

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Abstract : The baseline separation of poly(ethylene glycol) (PEG) compounds are essential among researchers, involve in calculation of exact molecular distribution. A binary gradient elution consisting of water and methanol was performed. Baseline separation for higher molecular weight PEG compounds, up to PEG 600 was successfully obtained. The suitability of the packing materials of C8 column and mobile phase plays a major role, to achieve baseline separation. The baseline resolution (Rs) values were observed in the following sequence: PEG 200 > PEG 300 > PEG 400 > PEG 600 > PEG 1000 > PEG 1500 > PEG 2000. The above order was a result of the physical and chemical properties of PEG compounds that changed as Mr increased. Under the same separation conditions, methanol shows a better baseline separation than acetonitrile in Rs of PEC compounds. This is due to an optimum physico-chemical interaction between solvents and adsorptive. The less solubility of PEG compounds in methanol required a longer time for them to interact with the stationary phase. It is concluded that exact information of molecular heterogeneity will facilitate molecular distribution estimation, based on the baseline separation.

Keywords: Poly (ethylene glycol), Adsorption, C8 column, liquid chromatography

Abstrak : Pemisahan sebatian poli(etil glikol) (PEG) pada garis tapak adalah diperlukan oleh para penyelidik yang terlibat dalam pengiraan nombor taburan molekul. Satu sistem yang terdiri daripada pemisahan secara perubahan kecerunan dengan dua cecair yang melibatkan air dan metanol atau asetonitril. Satu corak pemisahan pada aras tapak dan resolusi (Rs) yang lebih baik telah diperoleh sehingga PEG 600 dengan menggunakan air dan metanol disebabkan kesesuaian bahan pemadat dalam turus C8. Satu siri pada garis asal mengikuti urutan PEG 200 > PEG 300 > PEG 400 > PEG 600 > PEG 1000 > PEG 1500 > PEG 2000. Siri ini adalah disebabkan perubahan seragam keadaan fizikal dan kimia sebatian PEG apabila Mr semakin meningkat. Pemisahan Rs pada aras tapak yang lebih baik dengan metanol berbanding asetonitril. Fenomena ini disebabkan wujud ikatan optimum fizikal-kimia antara pelarut dan bahan penjerap. Keputusan ini memberi maklumat kehomogenan molekul untuk memudahkan pengiraan penyerakan molekul berpandukan pemisahan pada garis tapak.

Kata kunci: Poli (etil glikol), Penjerapan, turus C8, kromatografi cecair

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Introduction

Poly(ethylene glycol) (PEG) compounds are widely used in food, including baby products, lipsticks, protective creams and pharmaceuticals [1]. Moreover, PEG compounds are also used as intermediates to produce emulsifiers, surfactants, detergents and cosmetics [2,3]. Thus, it is desirable to develop a highly efficient baseline separation of the individual oligomers for PEG compounds. This is because, many applications required exact information of its molecular heterogeneity. For example, baseline separation could provide information on the identity of the free hydroxyl groups that reacted with an appropriate reagent during synthesis of intermediates [4]. Baseline separation could also be use to identify these compounds, based on their chromatographic pattern or chromatographic fingerprint [5]. In addition, sufficient baseline separation of the oligomer facilitates the calculation of average molecular weight (Mr), average molecular mass (Mw), and polydispersity index (Mw/ Mr) [4,5].

High performance liquid chromatographic (HPLC) technique has been widely used to separate PEG compounds. However, detection and separation of PEG compounds are still very challenging. The separations of PEG compounds are difficult, either by reversed-phase (RP) or normal-phase (NP) HPLC. For example, the higher Mr PEG compounds often cause excessively strong retention on NP, such as silica gel, diol, CN and aminoproply [5]. PEG compounds have also been investigated using different chromatographic methods, such as gas chromatography (GC), gel

permeation chromatography/size exclusion chromatography (GPC/SEC), thin layer chromatography (TLC) and supercritical fluid chromatography (SFC) [5]. GC gave satisfactory peak Rs but unfortunately, it was only restricted to low Mr samples, e.g. PEG 200. SEC [6,7] covers the whole Mr range but associated with poor resolution. TLC [8,9] exhibits poor Rs compared to HPLC. SFC [10,11] gave promising Rs but is restricted to certain suitable mobile phase. Although, several methods are available for analysis of PEG compounds, they still lack baseline or near baseline separation for PEG compounds with higher Mr. Recently, Rissler utilized NP-HPLC to obtain baseline separation of PEG up to 1000 molecular weight [5]. However, due to environmental concerns, NP-HPLC is not encouraged. This is because organic solvent was utilized as mobile phase. More recently, some researchers [12] have reported an alternative method using NP-HPLC with aqueous-organic solvent, as mobile phase to obtain baseline separation of higher molecular weight PEG compounds. This technique was known as "pseudo-reversed-phase process". However, the disadvantage of this technique is the column lifetime is much shorter than that generally used in RP matrices. This is because NP stationary phase (e.g. bare silica gel) is easily hydrolyzed in the presence of water [13]. Although, RP-HPLC also uses organic solvent, it employs aqueous-organic solvent, as mobile phase. Consequently, RP-HPLC is more environmentally friendly and column lifetime is longer. Recently, Andersen et. al reported a baseline separation of higher molecular weight PEG compounds using packed capillary RP-HPLC [4]. They used inverse temperature programming, ranging from 80 to 25ºC, which required column compartment modification to control the heating rate. On the other hand, RP-HPLC operated at room temperature was only able to obtain a baseline separation of native PEG compounds not more than 400 molecular weight using water and acetonitrile, as mobile phase [12,14].

Moreover, another challenge of native PEG analysis was detection of these compounds. The native PEG compounds, which lacks chromophore and fluorophore can only be detected at wavelength below 200 nm [10,15-18]. Therefore, tedious derivatives of PEG compounds are needed to obtain chromophore and fluorophore signal or response [13,15,19-21]. Capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) have been developed for separation of derivatized PEG compounds [18]. PEG compounds often required gradient elution to give satisfactory signal Rs or complete elution. This is because they have large difference in Mr, broad Mr distribution, and large difference in the retention time. Furthermore, isocratic elution was only applicable for low Mr PEG because at higher Mr,, separation of oligomers was either broad or poor [5]. Hence, detection using refractive index detector (RID) [20-25] with gradient elution is problematic [15,22]. In the present study, we have utilized evaporative light scattering detector (ELSD) [1,4,7,11,15,26-29], as an alternative and useful tool for the detection of native PEG compounds.

In this paper, we attempted to obtain a linear baseline separation of native PEG compounds, for molecular weight higher than 400 using RP-HPLC. A binary gradient elution, consist of aqueous-organic solvent have been applied.

Experimental

1. Analytical Equipment

The HPLC system consisting of a 1100 Series quaternary pump, 1100 Series auto sampler, 1100 Series thermostat column compartment with Chemstation software, were obtained from Agilent Technologies (Germany). The ELSD used was from Alltech Model 2000 ELSD system (Alltech Associates Inc, USA)

2. Chromatography

Chromatographic separation was achieved using a reversed phase Zorbax Eclipse XDB-C8 column (150 X 4.6 mm I.D., 5 µm particle size, 80 Å pore size), purchased from Agilent Technologies. Solvent A (water) and Solvent B was either methanol or acetonitrile. A 25 µL injection volume was used for all samples. The column was eluted with the following gradient: 0 min, 10% B; 35 min 80% B; 38 min 10% B and 45 min 10% B at a flow rate of 0.5 mL/min. The column temperature was set at 40°C. For detection of PEG, the drift tube temperature and nitrogen flow rate of ELSD were set at 104.0°C and 2.60 L/min, respectively. The optimum drift tube temperature and gas flow were calculated according to eq.1 and eq. 2 respectively. However, when the mobile phase flow rate is below 1 mL/min, the drift temperature and gas flow rate calculated may be lower.

The drift tube temperature

$$\begin{aligned} &= (\%\ \text{solvent} \times \text{absolute boiling point of solvent}) \times (\%\ \text{aqueous} \times \text{absolute boiling point of water}) \\ &= (\%\ \text{methanol} \times \text{absolute boiling point of methanol}) \times (\%\ \text{water} \times \text{absolute boiling point of water}) \\ &= (0.10)(60) \times (0.9)(115) \\ &= 109.5\ ^\circ\text{C} \end{aligned} \qquad \text{(eq. 1)}$$

The gas flow rate

$$\begin{aligned}&= (\%\ \text{solvent} \times \text{optimum gas flow rate of methanol}) \times (\%\ \text{water} \times \text{optimum gas flow rate of water})\\ &= (\%\ \text{methanol} \times \text{optimum gas flow rate of methanol}) \times (\%\ \text{water} \times \text{optimum gas flow rate of water})\\ &= (0.10)(1.6\ ) \times (0.9)(3.2)\\ &= 3.04\ \text{L/min}\end{aligned} \quad \text{(eq. 2)}$$

3. Chemicals

HPLC-grade methanol and acetonitrile were obtained from Merck. Demineralized water was used, after double distillation. Eight commercial grades of PEG compounds (PEG 200, PEG 300, PEG 400, PEG 600, PEG 1000, PEG 1500 and PEG 2000) were purchased from Merck. All PEG compounds were dissolved in methanol to 0.0125 mol dm-3, and 20 µL samples was injected.

4. Solubility test

PEG compounds (solute) were weighted to ca. 1.0 g. 20 mL of solvent was added and swirled. After that, the solution was filtered and dried. The filter paper weights before and after filtration were taken. As a control, a blank of each solvent was performed. The purpose of the blank was to correct uncertainty from the solvent, such as moisture contamination. Calculation of PEG compounds solubility is shown in eq. 3. Besides that, an insoluble solvent (e.g. hexane) was used, to rectify PEG compounds contamination, especially environment moisture. In general, hydrophobic solvent such as hexane does not absorb moisture from the environment. However, PEG compounds easily absorb moisture from the environment and cause error in the calculation. Therefore, solubility index (eq. 4) was calculated based on the solubility of insoluble solvent, to provide an accurate and actual solubility of PEG compounds. This would mean, the higher the solubility index, the better solubility of PEG compounds in the solvent.

The solubility

$$= \frac{\text{weight of solute} - \text{weight difference of filter paper (before and after filtration)}}{20\ \text{mL solvent}} \quad \text{(eq. 3)}$$

The solubility index

$$= \frac{\text{solubility in solvent} - \text{solubility in hexane}}{\text{solubility } in \text{ hexane}} \quad \text{(eq. 4)}$$

Results and discussion

1. Separation of native PEG compounds

The separations of PEG compounds, for Mr ranging from 200 to 2000 are shown in Figure 1. A baseline separation of PEG compounds with Mr less than 600 is successfully obtained (Figure 1(a-d)). This is achieved using a binary solvent gradient consisting of methanol and water. Recently, Rissler et al. [12,15,14] have successfully obtained a baseline separation a native PEG compounds, up to PEG 400 on RP-HPLC. In this study, a baseline separation improvement up to PEG 600 was achieved due to different gradient elution system and better column packing material used. When tuning the gradient elution system, a shallower gradient elution with less organic solvent should be employed, to resolve PEG compounds into individual oligomer. It is interesting to note, that even we have employed a more rapid gradient elution than reported in the literature [14], the separation was still better. Therefore, it is noteworthy the separation enhancement is mainly due to the better column selectivity, based on the packing material. The column material was treated with double endcapping and extra dense bonding to give better peak shape and longer column lifetime.

The separations of higher Mr PEG compounds, ranging from PEG 1000 to PEG 2000 are shown in Fig. 1(e-g). The PEG compounds were still sufficiently separated into own characteristics peaks. As shown in Fig. 1(a-g), Rs of PEG decreases in the following order: PEG 200 > PEG 300 > PEG 400 > PEG 600 > PEG 1000 > PEG 1500 > PEG 2000. This clearly demonstrated that the Rs and capacity factor (k) value were dependent on Mr of PEG compounds. The k and Rs value up to PEG 1000 were in the range of 1.88 to 5.59 and 10.32 to 0.74, respectively. These values indicated that a sufficient time for separation of each individual oligomer up to PEG 1000 was accomplished. The poor Rs when Mr of PEG compounds are more than 1000 could be due to the following reasons. First, a weaker interaction between the alkyl chains of C8 matrix (octyl) and the more hydrophilic nature of the higher Mr PEG compounds. As the polarity of PEG compounds increased with Mr, the interaction with the non polar stationary phase will be reduced. Secondly, column particle pores size also influenced the retention time of the bigger solute molecules which tend to remain longer through maximum exposure

to the surface area of the particles, thus sufficiently separated. Average pore sizes of 80 Å for C8 column did not provide sufficient Rs because high Mr of PEG molecules, up to 2000 were difficult to enter large surface area inside the pores for adsorption and desorption processes, due to small pores obstruct. This limitation is well-known as diffusion factor, which can be improved by using larger pore sizes. Some researchers [14], have reported baseline separation of other higher Mr compounds using larger pores size (100 Å). Thirdly, individual oligomers of higher Mr PEG compounds have lower relative mass difference between individual oligomers. As a consequence, they exhibit lower differences in solute-matrix interaction [12], which resulted in poor Rs of higher Mr PEG compounds. The fourth reason is that higher Mr PEG compounds tend to form coiled structure in solution. Therefore, the active sites are hindered and this decreased the interaction between PEG molecules and stationary phase. It is suggested that these factors collectively contributed to the poor Rs, as the Mr increases.

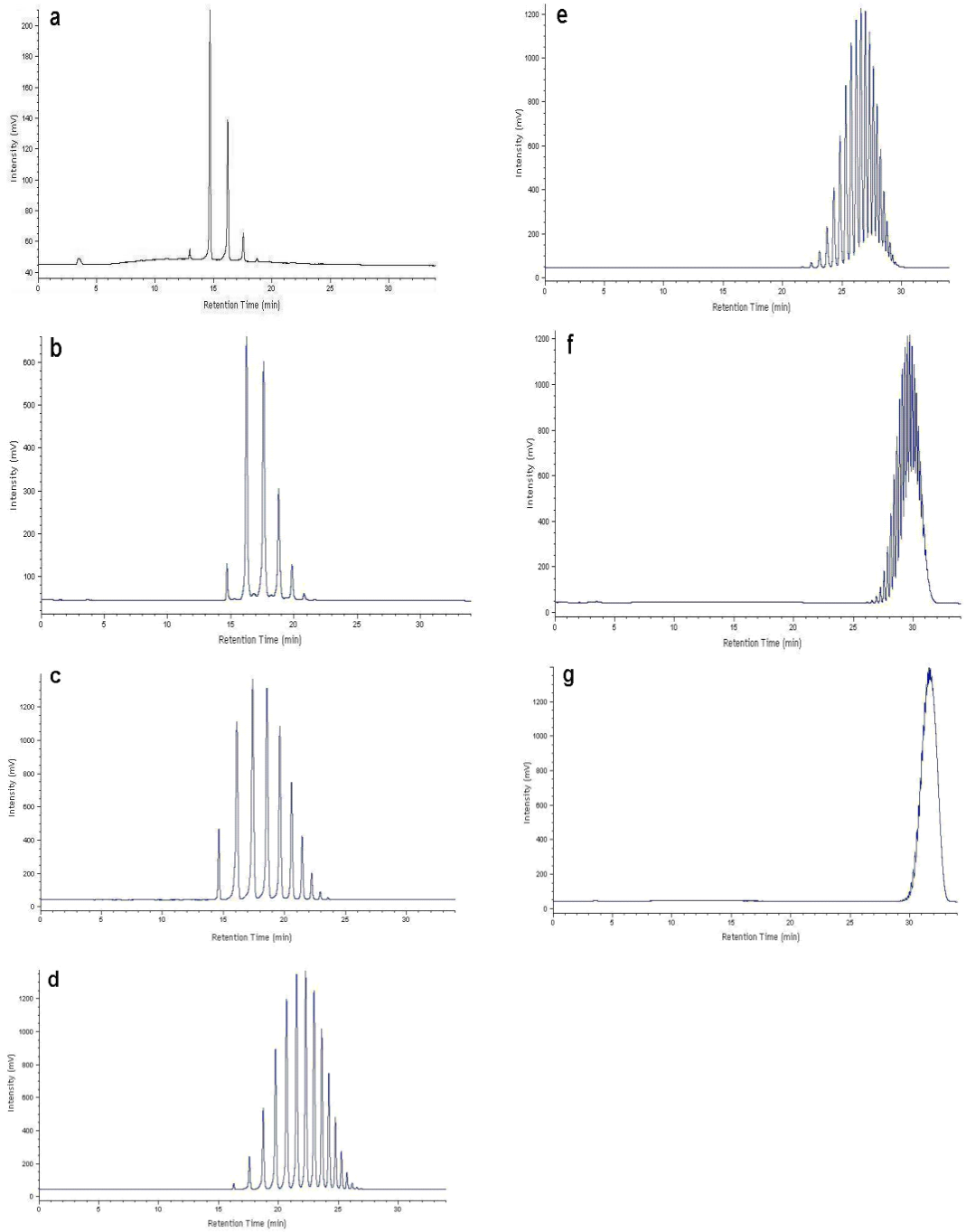

Figure 1 : Chromatograms of (a) PEG 200, (b) PEG 300, (c) PEG 400, (d) PEG 600, (e) PEG 1000, (f) PEG 1500 and (g) PEG 2000 using methanol as organic solvent.
Conditions as described in the text.

2. Influence of solvent

Influence of solvent was also investigated by comparing methanol and acetonitrile, as the organic mobile phase. The separations of PEG 400 using acetonitrile are shown in Fig. 2. It is noted that methanol provides better separation than acetonitrile. The finding is related to the retention mechanisms. The used of acetonitrile gave less retention time (tR) or k value, compared to methanol. This agreed with results reported in the literature [12], which shows acetonitrile and acetone exhibit smaller tR value than methanol. The remarkable result could be due to polarity effect of acetonitrile. Based on solvent polarity parameter (P') and strength parameter (S'), if the value of P' and S' are higher, the k value should be smaller. This is because PEG molecules and mobile phase will compete with each other for the adsorptive sites. If the polarity strength of the mobile phase is less, PEG molecules would easily adsorbed on the non polar stationary phase and give smaller k value. According to NP-HPLC, the P' for acetonitrile and methanol is 5.8 and 5.1, respectively [30]. In addition, the S' for RP-LC is 3.1 and 3.0 corresponds to acetonitrile and methanol, respectively [30]. Both P' and S' shows the values of acetonitrile were slightly higher than methanol. Therefore, in terms of polarity effect, acetonitrile is only slightly more polar compared to methanol, and should give a slightly smaller k value. It is noteworthy that k value of acetonitrile is two folds smaller than methanol as shown in Figure 1(c) and Figure 2.

The possible explanation is the solubility effect of PEG compounds in the solvent. The k value is inversed to masses or volume of solute in the mobile phase. This means that when PEG compounds are more soluble in the mobile phase, they will be retained longer on the non polar stationary phase and tends to give a smaller k value. PEG compounds are more soluble in acetonitrile than methanol because acetonitrile is able to form stronger hydrogen bonds with PEG molecule, compared to methanol. It is also known that nitrile group is more electronegative than hydroxyl and this favors stronger it bonding between nitrile and polyether oxygen groups of PEG. Furthermore, the solubility of PEG 400 in methanol and acetonitrile was found to be 0.0474 ± 0.0003 g/mL and 0.0492 ± 0.0002 g/mL, respectively. Based on the solubility of hexane 0.0164 ± 0.0004 g/mL, the solubility index for methanol and acetonitrile is 1.89 and 2.00, respectively. The solubility effects show that PEG 400 is slightly more soluble in acetonitrile. Both methanol and acetonitrile are able to form hydrogen bond with hydroxyl terminal of PEG molecule, as shown in Figure 3(a) & (b). However, acetonitrile is more soluble because of the higher electronegativity of the nitrile group. It is concluded that retention and resolution of PEG compounds are significantly affected by solubility of PEG compounds in the solvent. These also help to explain the enhancement on the Rs value of PEG compound by using methanol compared to acetonitrile. The delay in the retention of PEG solutes is an added advantage because the solute requires sufficient time to interact with the stationary phase.

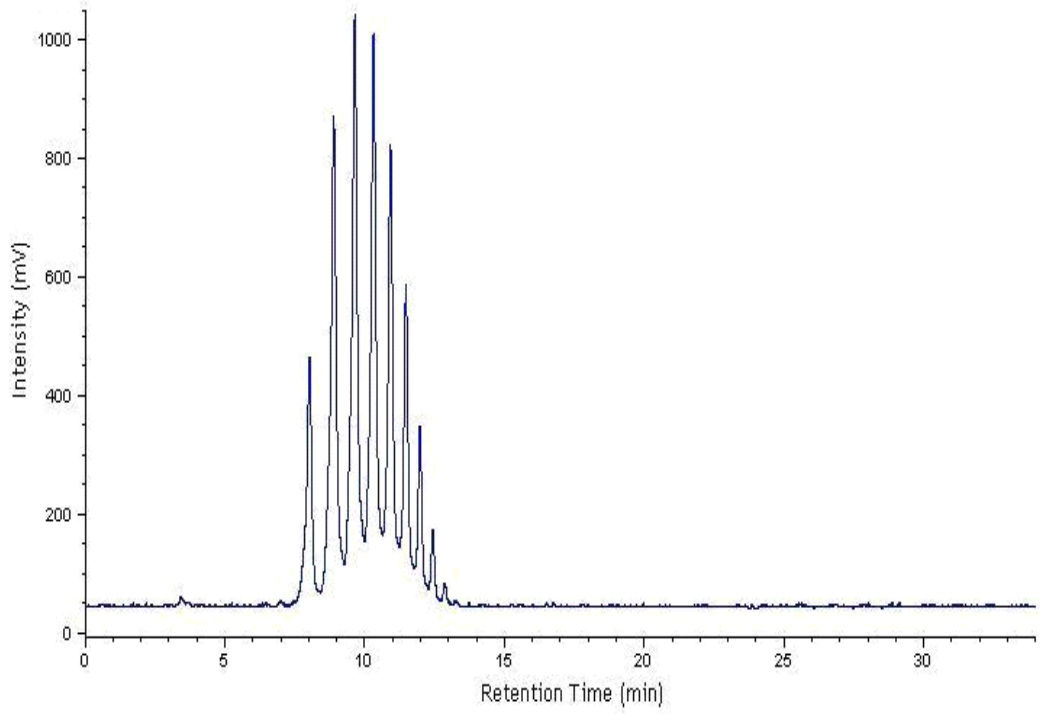

Figure 2 : Chromatograms of PEG 400 using acetonitrile as organic solvent, instead of methanol. Conditions are described in the text.

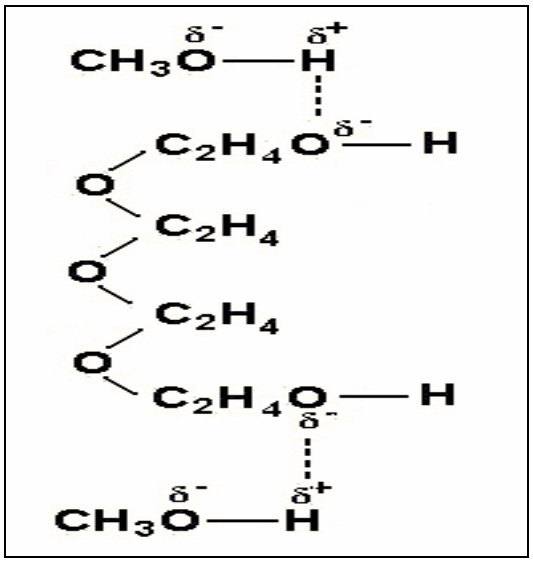

Figure 3(a).

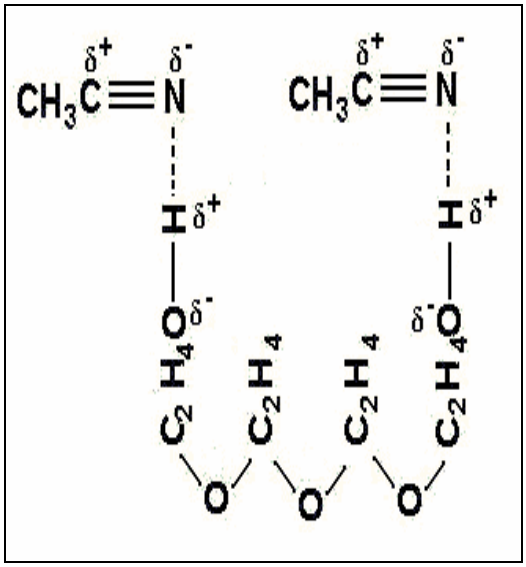

Figure 3(b).

Figure 3(a) : Interaction between methanol and PEG molecules via hydrogen bonding.
Figure 3(b) : Interaction between acetonitrile and PEG molecules via hydrogen bonding.

3. ELSD response

For the same concentration, Fig. 1(a) & (b) show the intensity of PEG 200 and PEG 300 are much lower than the rest of the higher molecular weight PEG compounds. During operation of ELSD, the drift tube temperature and gas flow rate were set based on the volatility of mobile phase and flow rate. Hence, when volatile sample, such as PEG 200 was used, much of the sample particles were evaporated in the drift tube and therefore not detected. PEG compounds having higher molecular weight than PEG 400 and show high intensity and maximum response compared to that of PEG 300 and PEG 200 because they exhibit low volatility. The lack of "Gaussian distribution" in PEG 200 and PEG 300 is also due to high volatility of lower molecular weight PEG compounds.

Conclusions

Separation of PEG compounds with RP-HPLC on C8 column using aqueous-organic mobile phase coupled with ELSD detector gave good baseline separation. The native PEG compounds having Mr up to 600 was sufficiently separated into individual oligomers with linear baseline separation enabling recognizable pattern of PEG compounds of different Mr based on their chromatographic pattern. It is understood that separation of PEG compounds is closely related to the solvent chosen and characteristics of the packing materials. Binary gradient elution of aqueous-organic solvent with methanol provides better separation than acetonitrile. The role of solubility of the PEG compounds in the solvent was more important compared to polarity. The lower solubility of PEG compounds in methanol allowed sufficient interaction time between PEG molecules with the adsorptive. High volatility of the PEG samples gave poor signal intensity because the samples evaporated before it could be detected. Hence, a suitable drift temperature must be selected when analyzing volatile samples.

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