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Repeatability in column preparation of a reversed-phase C18 monolith and its application to separation of tocopherol homologues

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ABSTRACT

This work investigated the repeatability of column preparation for a reversed-phase C18 monolith, namely stearyl methacrylate-co-ethylene glycol dimethacrylate (SMA-EDMA). The columns were thermally polymerised using three commonly available heating devices (GC oven, hot air oven and water bath) and their chromatographic performance evaluated using micro-liquid chromatography for separation of five test compounds. Precision in terms of %RSD of retention times were 9.0, 6.5, and 12.5 using GC oven, hot air oven and water bath, respectively. Between-batch precision for the hot air oven ($n = 3$ days) was less than 10.4% for retention time. The SMA-EDMA monolith was applied to the separation of tocopherol homologues by capillary electrochromatography. Usually tocopherol homologues cannot be completely separated by conventional reversed-phase C8- or C18-packed bed or C18-silica based monolithic columns. Polymer monolith has been shown to give remarkable selectivity towards the tocopherols compared to the conventional microparticulate phase and silica based monolith. Successful separation of the tocopherol isomers was achieved on the SMA-EDMA monolith without any column modification.

1. Introduction

Monolithic materials have been widely used for chromatography due to their advantages in terms of high porosity, simple preparation and the possibility of modification [1–4]. The high porosity provides these materials with low flow resistance and thus can perform fast separations and be possibly coupled to low flow systems such as flow injection techniques for either separation or sample pretreatment purposes. Although silica-based monoliths are well known at providing better separation efficiency and more rigidity, most applications have been carried on organic polymer based monoliths due to their simpler preparation and modification [5–7]. Preparation of the organic polymer monolith can be performed by polymerising organic monomers in a porogenic solvent under thermal or photochemical initiation. Fréchet et al. has shown that the two initiation methods provided good repeatability in column preparation for poly (butyl methacrylate-co-ethylene dimethacrylate) monolith [8]. The photochemical polymerisation can be performed in a short period. However the method requires a UV transparent capillary, which is more expensive than that used for the thermal initiation method. Various types of organic monomers have been reported for the synthesis of organic polymer monolithic columns such as butyl methacrylate [9,10], lauryl methacrylate [11], hexyl methacrylate [12], long alkyl chain stearyl methacrylate [13,14]. Some research groups have evaluated stability and repeatability in column preparation of monolithic columns [8,15–19]. Intensive investigations of the properties and repeatability of column fabrication have been reported for commercial silica-based monolith [15,16]. For organic polymer monoliths, Claessens et al. investigated preparation and characterisation of polybutyl methacrylate-based monoliths at various ratios of monomers to porogen, for both micro-liquid chromatography (μ-LC) and capillary electrochromatography (CEC) [17]. Good reproducibility and stability of the capillary columns were observed by comparing the retention time of thiourea (EOF marker), retention factor and column efficiency of column obtained from the same batch and between batches. Other works have investigated repeatability for poly (butyl methacrylate-co-ethylene

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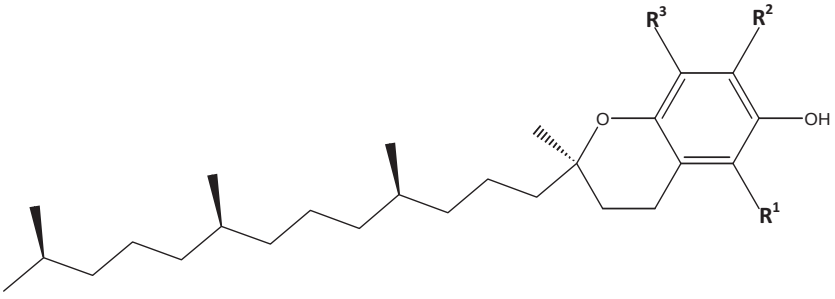

Homologues	R^1	R^2	R^3
α -tocopherol	CH_3	CH_3	CH_3
β - tocopherol	CH_3	H	CH_3
δ -tocopherol	H	CH_3	CH_3
γ -tocopherol	H	H	CH_3

Fig. 1. Chemical structure of tocopherol isomers.

dimethacrylate) monolith [8], acrylamide based monoliths [18] and poly (2-hydroxyethyl methacrylate-co-pentaerythritol triacrylate) monolith [19].

There have been only a few investigations of column preparation of polymer monolith, in term of reproducibility. We therefore aim to study repeatability in producing polymer monolithic capillary column. In this work, we investigated the repeatability in the production of a long chain monolithic material, stearyl methacrylate-co-ethylene glycol dimethacrylate (SMA-EDMA). For this type of monolith, there has been no report of repeatability study. In order to control monolithic column production, polymerisation temperature must be controlled. Three basic apparatuses available in most laboratories, namely water bath, hot air and GC ovens, were compared in their performance for temperature control of polymerisation of the monolith. The prepared monolithic columns were then tested using micro-liquid chromatography. In addition, the synthesized columns were used for a complete separation of tocopherol homologues by capillary electrochromatography.

Tocopherol (TOH) is the major form of fat-soluble vitamin E, an anti-oxidant for scavenging free radicals [20]. Tocopherols exist in four isomers (Fig. 1) which have different biological activities. Liquid chromatography (LC) was mostly used for separation of the TOHs based on interaction of the TOHs to stationary phase. The main challenge is separation of the β- and γ-isomers due to their structural similarities. In reversed-phase LC, standard C8 or C18 microparticulate stationary phases cannot provide sufficient selectivity for separation of the problematic β-and γ-isomers [21–23]. However C30 hydrophobic [24] and polar reversed-phase microparticulate phases [25,26] have been shown to give complete separation of the four tocopherol isomers. Recently, Núñez et al. [27] and Chaisuwan et al. [28] reported use of monoliths for separation of the TOHs. The separation was achieved on a silica monolith coated with poly(octadecyl methacrylate) and a mixed-mode polymer-based monolith containing polar hydroxyl and non polar C17 functional groups. This work demonstrated selectivity of the C18 monolith to TOH isomers over standard C18 microparticulate column.

2. Experimental

2.1. Chemicals and reagents

Sodium hydroxide (NaOH), and 2,2-azo-bis(isobutyronitrile) (AIBN) were purchased from BDH (Lutterworth, UK). Tris(hydroxymethyl)methylamine (Tris), methanol (MeOH), and acetonitrile (ACN) were from Fisher Chemicals (Loughborough, UK), ethylene glycol dimethacrylate (EDMA), 3-(trimethoxysilyl)propyl methacrylate) (γ-MAPS), 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS), naphthalene, phenol, and α-, β-, γ-, δ-tocopherols (TOHs) were all supplied from Sigma–Aldrich (Poole, UK). Thiourea and anisole were purchased from Merck (Darmstadt, Germany). Benzene and toluene were from J.T. Baker (Phillipsburg, Canada). All chemicals were AR and HPLC grade with purity ≥97%. The test mixture for chromatographic performance of the columns comprised 20 ppm thiourea, 40 ppm phenol, 30 ppm anisole, 200 ppm benzene, 50 ppm toluene and 10 ppm naphthalene in 50:50 (v/v) methanol:water. Stock 1000 ppm solution of standard TOHs was prepared in methanol and kept in dark vials at 4° C. Working solutions was prepared by diluting the stock solutions in 2% (v/v) water in methanol. Tris buffer was prepared by dissolving Tris salt in Milli-Q water and pH adjusted with 5 M HCl.

2.2. Apparatus

The SMA-EDMA monoliths were prepared by thermal polymerisation using a gas chromatograph oven (Model 6890 plus, Agilent Technology, USA), a hot air oven (Model 750F, Fisher Science, USA) or a water bath (Model W350, Memmert GmbH, Germany). The prepared capillary monolithic columns were tested for chromatographic properties using μ-LC. The μ-LC was constructed from a high pressure pump (Model 400, Applied Biosystems, USA) together with a UV-Visible absorbance detector (Model 785A, Applied Biosystems, USA). A tee splitter (1/16 in., Valco Instruments, USA) was connected between the pump and the micro-injector (Model 7520, Rheodyne, USA) in order to reduce the flow rate of the solution delivered from the pump. For the separation of the TOHs, the

experiments were performed on a HP^{3D} CE system from Hewlett Packard (Waldbronn, Germany), which was equipped with a photodiode array detector. Fused-silica capillaries (100 μm i.d., 375 mm o.d.) were purchased from Composite Metal Service (Worcestershire, UK).

2.3. Preparation of SMA-EDMA capillary monolithic columns

The SMA-EDMA monoliths were prepared according to the method previously reported by Jiang et al. [14]. The polymerisation solution comprised 23.0% (v/v) SMA, 12.5% (v/v) EDMA, 51.8% (v/v) isoamyl alcohol and 12.2% (v/v) 1,4-butane diol, 0.2% (v/v) AMPS and 0.3% (v/v) AIBN. The solution was homogenised by sonication and purged with nitrogen gas for 5 min and subsequently filled into nine 100 μm i.d. γ-MAPS-pre-treated capillaries [14]. After sealing with rubber septa, the capillaries were kept overnight in GC oven (55 °C), hot air oven (55 °C), or water bath (60 °C), using three capillaries for each method. After polymerisation, the capillaries were washed with methanol for 1 h. Detection windows were made by pyrolysing the monoliths at the outlet end to give the desired separation length.

2.4. μ-LC experiment

Chromatographic testing of the prepared columns was performed using μ-LC. The column (100 μm i.d., 50 cm effective length, 55 cm total length) was connected between the microinjector and UV-Visible absorbance detector, with 55:45 (v:v) acetonitrile:water as a mobile phase. The flow rate delivered by the pump was reduced to a flow in the order of μL/min by means of a tee splitter connected to a 50 μm i.d., 20 mm long bare capillary. The absorbance was continuously monitored at 214 nm.

2.5. CEC experiment

For separation of TOHs, a SMA-EDMA capillary column (100 μm i.d., 23.5 cm effective length, 32 cm total length) was first conditioned with mobile phase of 7:93 (v:v) 20 mM Tris buffer pH 9:acetonitrile at applied voltage of 5 kV for 5 min, followed by 30 kV (separation voltage) for 30 min or until stable current and signal baseline were observed. Sample introduction was by electrokinetic injection at 10 kV for 30 s. All experiments were performed at 30 °C. The absorbance was monitored at 200 nm.

3. Results and discussions

3.1. Effect of polymerisation temperature on retention times

Since temperature has been shown to have a dramatic affect on the structure and properties of the obtained monolith [29,30], the polymerisation temperature is an important factor in the synthesis of monolithic columns by thermal polymerization. In this work, we therefore first tested the effect of the polymerisation temperature for synthesizing the capillary SMA-EDMA with C18 groups. Fig. 2 shows retention times of the test compounds separated on a monolith obtained from GC oven at various polymerisation temperatures. A small change in the polymerisation temperature could significantly affect the retention times of the compounds. Longer retentions were observed when increasing the polymerisation temperature. Similar results were observed from the columns prepared by using the water bath (data not shown). As described by previous research groups, the use of a higher temperature resulted in a faster polymerisation rate and subsequently smaller pores [29,30]. At high temperature, more initiator free-radicals are produced. Large number of nuclei and globules were thus formed resulting in small pore size and high surface area. The compounds thus retained longer on the SMA-EDMA monolithic column.

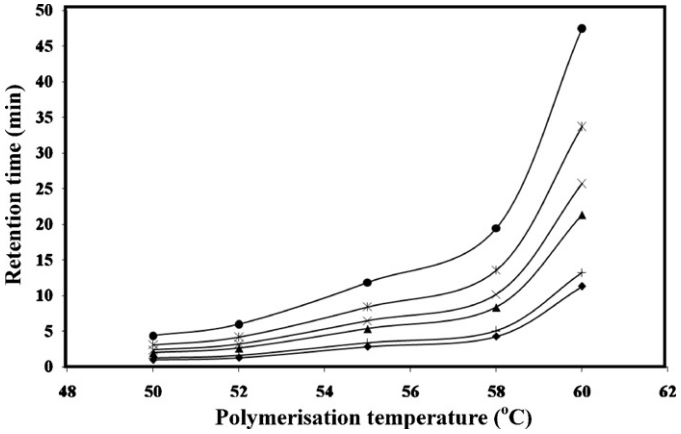

Fig. 2. Effect of polymerisation temperature on retention time of the test compounds separated on a C18 SMA-EDMA monolithic column (100 μm ID × 50 cm long) polymerised using a GC oven (♦ thiourea, ■ phenol, ▲ anisole, × benzene, * toluene, ● naphthalene). Mobile phase: 55:45 (v:v) acetonitrile:water. Detection wavelength: 214 nm.

3.2. Performance of ovens and water bath in monolithic column production

In order to obtain a uniform structure within the prepared monolith and also repeatability in column preparation, the polymerisation temperature must be strictly controlled. Preparation of the SMA-EDMA monoliths was performed with three heating devices (GC oven, hot air oven and water bath) for comparing performance in temperature control. From our preliminary results (Fig. 2), a temperature of 55 °C was selected for ovens and 60 °C for the water bath since this temperature provided a complete separation of the test compounds within a suitable short time. The temperature of 60 °C for the water bath was used so as to give the same separation and retention times as for the air ovens. The prepared capillary SMA-EDMA columns were tested for reproducibility in their chromatographic performance using the test solution.

The average retention time observed from column to column between heating methods are shown in Fig. 3. The %RSD obtained from the ovens and water bath was compared in order to evaluate repeatability in the column preparation. In this study, the %RSD for retention time obtained from just three columns prepared in a GC oven, hot air oven and water bath were 9.0, 6.5, and 12.5, respectively. Since all columns were prepared using the same polymerisation solution, the variation of the observed retention times should be due to temperature gradient in the different heating apparatuses. The use of only three columns/heating apparatuses was sufficient to show the difference in the performance of the oven/water bath.

Since the hot air oven provided the most reproducible preparation for both physical and chemical properties and such oven also can be found in most laboratories, preparation by using this oven was further studied for between batches preparation. Good precision for inter-day preparation was observed, with %RSDs for retention time and capacity factor smaller than 10.4 and 2.63, respectively. This work clearly showed that the heating system is

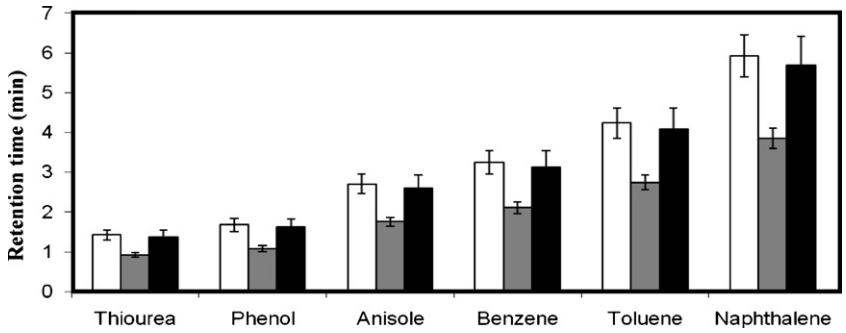

Fig. 3. Retention time obtained from μ-LC for the test compounds separated on monolithic capillary columns from □GC oven, ▒ hot air oven and ■ water bath. SMA-EDMA monolithic column (100 μm ID × 50 cm long). Mobile phase: 55:45 (v/v) acetonitrile:water as mobile phase, detection wavelength: 214 nm.

an important parameter in order to obtain good repeatability in column preparation of the monolithic materials.

3.3. Selectivity of the C18 SMA-EDMA monolith for tocopherol isomers by CEC

Tocopherol (TOH) or vitamin E homologues have been shown to be difficult to completely separate on common microparticulate C8 or C18 columns. For reversed-phase microparticulate columns with CEC technique, only extremely non polar phases [24] and polar reversed-phase [25,26] have been shown to successfully separate the tocopherol homologues. For porous monolithic material, Núñez et al. [27] and Chaisuwan et al. [28] reported the use of monoliths for the analysis of TOHs. Núñez et al. showed that in order to separate these homologues using C18 silica based monoliths, the monolith must be modified by coating with poly(octadecyl methacrylate) [27]. Chaisuwan et al. has shown that a mixed-mode polymer based monolith containing both polar hydroxyl and non polar C17 functional groups, provided good selectivity for the TOHs including the problematic isomers (β- and γ-TOHs) [28]. However in this work, we found that the methacrylate polymer based monolith with C18 as functional group, could separate the TOH homologues (Fig. 4). Resolution between β- and γ-TOHs obtained from the SMA-EDMA compared to other stationary phases is shown in Table 1. Comparing its chemical structure to that of the C18 silica-based phase, the SMA-EDMA contains non-polar C18 groups from the monomer and relatively polar ester groups on its surface (Fig. 5). The relatively polar ester groups can interact with the hydroxyl group on the structure of the TOHs resulting in better selectivity compared to the C18-microparticulate and C18-silica based monolithic phases. This shows that the ester groups on the surface of the monolith play a significant role in the separation by providing hydrophilic interaction for compounds containing polar groups and thus improve the selectivity of the separation. Acceptable precision for retention time, peak area and efficiency for the TOHs and thiourea (EOF maker) was obtained as shown by the data in Table 2. The efficiency was calculated by the Chemstation software version 10.4.

Table 1
Resolution between β- and γ-TOHs obtained from the SMA-EDMA column compare to other stationary phases.

Stationary phase	Type of column	R_s (β–γ)[a]	Ref.
1. C8, C18	Microparticulate	0.0[b]	[21–23]
2. Monomeric C30	Microparticulate	1.5	[24]
3. Pentafluorophenylsilica	Microparticulate	–[c]	[25]
4. ULTIMA C18[d]	Microparticulate	1.2	[26]
5. Silica monolith[e]	Monolith	–[c]	[27]
6. PEDAS-EDMA[f]	Monolith	1.2	[28]
7. C18 SMA-EDMA	Monolith	1.1	This work

[a] Resolution between β- and γ-TOH, $R_s = 2[(t_r)_\gamma - (t_r)_\beta]/(W_\beta + W_\gamma)$, t_r = retention time and W = peak width.
[b] Cannot separate β- and γ-TOHs.
[c] Not reported.
[d] Bifunctional groups of amide and C18.
[e] Silica monolith coated with poly(octadecyl methacrylate).
[f] Bifunctional groups of hydroxyl and C17.

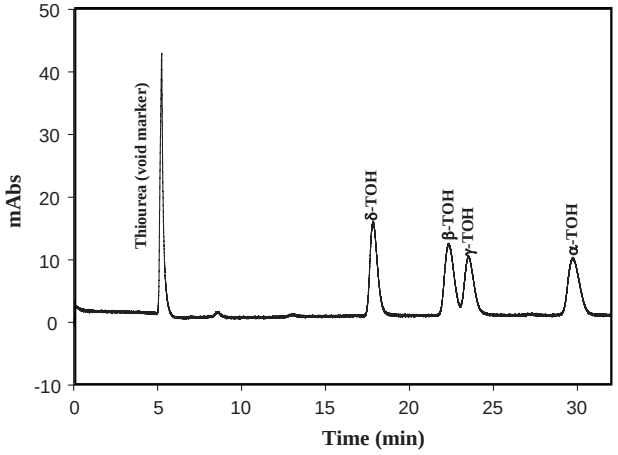

Fig. 4. Results from a CEC experiment for the separation of tocopherol homologues on the SMA-EDMA monolithic column. Capillary column, 23.5 cm effective length, 32 cm total length × 100 μm ID; applied voltage, 7:93 (v:v) 20 mM Tris buffer pH 9: acetonitrile, +30 kV; column temperature, 30 °C; electrokinetic injection at +10 kV for 30 s; detection wavelength, 200 nm.

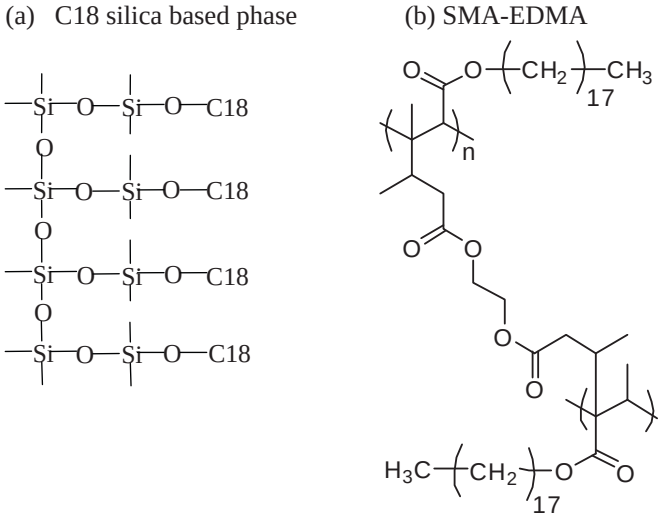

Fig. 5. Chemical structures of C18 silica based and SMA-EDMA phases.

Table 2
%RSD for retention time (t_r), peak area and efficiency of the TOHs and thiourea (EOF maker) separated on SMA-EDMA column.

Compounds	Intraday repeatability (n = 7 injections)			Interday repeatability (3 days)		
	t_r	Peak area	Efficiency	t_r	Peak area	Efficiency
EOF	0.55	–	–	1.63	–	–
δ-TOH	0.74	3.1	3.0	3.00	9.3	7.6
β-TOH	0.78	6.4	2.4	2.73	7.9	7.6
γ-TOH	0.78	4.0	5.4	2.73	6.1	12.5
α-TOH	0.76	5.5	2.8	2.56	8.2	6.0

4. Conclusions

Repeatability studies on thermal preparation of SMA-EDMA monolithic capillary columns based on polymerisation using a GC oven, a hot air oven and a water bath have been investigated. The %RSD for retention time for the five test compounds obtained from μ-LC technique, were considered for repeatability comparison. Among the studied heating devices, the most reproducible preparation was achieved by using hot air oven. Advantage of the studied monolith over conventional C18-microparticulate phase in terms of selectivity has also been shown. The SMA-EDMA column provided good selectivity to tocopherol homologues which could not be completely separated on C18-microparticulate or C18-silica based monolith. The better selectivity can be credited to the relatively polar ester groups on the surface of the monolith. The column can thus be applied for separation of the tocopherol homologues. Successful separation of the tocopherol isomers on the SMA-EDMA monolith using CEC can be achieved within 30 min with good precision for retention time, peak area and efficiency with %RSD less than 5.38 and 12.5 for intra-day and inter-day repeatability, respectively.

Acknowledgments

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