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Analyses of synthetic antioxidants by capillary electrochromatography using poly(styrene–divinylbenzene–lauryl methacrylate) monolith

Hsi-Ya Huang*, Yi-Jie Cheng, Cheng-Lan Lin

Department of Chemistry and Center for Nanotechnology at CYCU, Chung Yuan Christian University, Chung-Li, 320, Taiwan

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ABSTRACT

In this study, several organic polymer-based monoliths prepared by single step *in situ* copolymerization of styrene- and methacrylate ester-based monomers (styrene (S), divinylbenzene (DVB) and lauryl methacrylate (LMA)) were developed as stationary phases of capillary electrochromatography (CEC) for the analyses of synthetic antioxidants. These monoliths were characterized by examining the SEM image, IR spectrum, and measuring the pore size, surface area, conversion yield, and thermal decomposition temperature. The polymerization procedure was optimized by varying the reaction temperature, the reaction time, and the LMA–styrene ratio. The LMA–styrene ratio had the most significant influence on the peak symmetry of butylated hydroxyanisole (BHA) and 2, 6-di-*tert*-butyl-4-methyl phenol (BHT), the latter being greatly affected by excessive peak tailing in the poly(S–DVB) monolith. It showed that the interaction between the poly(S–DVB) monolith and the antioxidant (BHT or BHA) was significantly altered by the insertion of LMA. Compared with the best HPLC and CE methods previously reported, this proposed CEC method provides a comparable separation ability for the five antioxidants analyzed. This study demonstrates that the potentiality of poly(S–DVB–LMA) monolith as stationary phase, especially for CEC system, because of high thermal stability and good column reproducibility.

1. Introduction

Organic polymer-based monolithic columns have attracted a great deal of attention in HPLC and capillary electrochromatography (CEC) mainly because they are simple to make and no retaining frits are required [1–10]. Presently, four organic polymer monoliths including acrylate-, acrylamide-, methacrylate ester-, and styrene- based polymer have been developed as separation columns in HPLC system [11–17]. Among them, only methacrylate ester-based polymers are widely used as stationary phases of CEC system. The methacrylate ester-based monolithic columns, which were first developed by Fréchet and co-workers [18,19], provided high separation ability with reliable results over a wide range of compounds either in micro HPLC or CEC system [20–23]. In contrast to methacrylate ester-based monolithic column, the styrene-based polymeric column has higher chemical stability under a wide pH range and better column reproducibility (over 3 months) [24–27], but was less reported in CEC because a serious peak tailing often occurred for aromatic compound separation [28–30]. In order to combine the advantages of these two types of polymers (high separation ability of methacrylate ester-based polymer and good chemical stability of styrene-based polymer) in a single column, the development of a mixed styrene- and methacrylate ester-based monolith as chromatographic stationary phase is worth exploring. Buszewski et al. reported an organic polymer-based monolithic column prepared by both styrene- and methacrylate ester-based monomers for the first time. In this case, a macroporous poly(styrene–divinylbenzene) (poly(S–DVB)) monolith was first prepared by *in situ* copolymerization of styrene with divinylbenzene (DVB), and then it was modified further with octadecyl chains either by Friedel–Crafts reaction with 1-chlorooctadecane or grafting with octadecyl methacrylate [27,31]. Subsequently, the same authors demonstrated that these types of alkylated poly(S–DVB) monoliths were able to generate electroosmotic flow (EOF) without the charged groups. This octadecyl methacrylate grafted poly(S–DVB) column gave a better separation efficiency for several polar phenolic compounds than the unmodified poly(S–DVB) column. The grafting protocol is a fast and versatile surface functionalization way of monoliths, which avoids the need of reoptimizing the composition of polymerization mixture by direct-polymerization approach [32].

So far, analytical methods including GC, HPLC and CE [33–42] have been developed for the analyses of food-grade antioxidants with reliable results. Antioxidants are chemical compounds, which are used to inhibit the decomposition of foods and organic materials caused by reactions with oxygen. Natural antioxidants are usually

* Corresponding author. Tel.: +886 3 2653319; fax: +886 3 2653399.
E-mail address: hyhuang@cycu.edu.tw (H.-Y. Huang).

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unstable, as a result, synthetic antioxidants containing a benzene structure with varying degrees of hydroxylation and side-chain substitutions are commonly employed as food grade antioxidants. Excess synthetic antioxidants added to food might produce toxicities or mutagenicities, thus the allowable amount of synthetic antioxidants added to food products is strictly limited. Since these synthetic antioxidants have large diversity of hydrophobic property, either a HPLC with gradient elution or a CE with micellar pseudostationary phase was employed to rapidly separate synthetic antioxidants. Several reports showed that the separation of synthetic antioxidants by HPLC is usually completed in less time than by CE because of its ability to perform gradient elution [39,41].

In this study, a CEC method, which used a mixed styrene- and methacrylate ester-based polymeric monolith as separation column, was developed for the separation of five common synthetic antioxidants (propyl gallate (PG), octyl gallate (OG), butylated hydroxyanisole (BHA), 2,6-di-*tert*-butyl-4-methyl phenol (BHT), and *tert*-butylhydroquinone(TBHQ)). In order to simplify the fabrication procedure, these polymeric monoliths were prepared by single-step *in situ* polymerization of styrene- and methacrylate ester-based monomers (styrene (S), divinylbenzene (DVB) and lauryl methacrylate (LMA)) in various ratio. Furthermore, a two-step gradient elution coupled to the CEC system was employed to achieve a speedy separation. Furthermore, the analytical performances of five antioxidants on the poly(S–DVB) and poly(styrene–divinylbenzene–lauryl methacrylate) (poly(S–DVB–LMA)) monolithic columns were also compared.

2. Experimental

2.1. Chemicals and reagents

OG (pK_a ~7.8) and TBHQ (pK_a ~10.8) were obtained from Aldrich (St. Louis, MO, USA). BHA (pK_a ~11.8), BHT (pK_a ~12.8) and PG (pK_a ~7.8) were purchased from Sigma (Steinheim, Germany). The pK_a values of the five standards studied in this paper were obtained from SciFinder® [43]. The above five antioxidant standards used as test analytes in the study were individually dissolved in methanol at a stock concentration of 2 mg mL^{-1}. Styrene was obtained from Showa (Tokyo, Japan). DVB (80.1%, a mixture of *m*-DVB (55.5%) and *p*-DVB (24.6%)) was obtained from Fluka (Buchs, Switzerland). LMA (96%) and vinylbenzene sulfonic acid (VBSA) were purchased from Aldrich (Steinheim, Germany). Styrene was purified by distillation under vacuum prior to use. DVB, which is a cross linker, was washed with 10% (w/v) aqueous sodium hydroxide to remove the inhibitors (*tert*-butylcatecohol, 30 μg mL^{-1}) before use. This *tert*-butylcatecohol was able to convert to ion form in a basic solution, and thus it was separated from DVB monomer by washing aqueous sodium hydroxide. All other materials were reagent-grade and were used as received. Polyimide coated fused-silica capillaries with 100-μm I.D. and 375-μm O.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA). Mobile phases were prepared by mixing acetonitrile and phosphate buffer (5 mM) in different volume ratios. 1.0 M HCl or NaOH was then added to mobile phase solution until the desired pH was achieved.

2.2. Edible oil products and pretreatment

Commercially available edible oil products, which were obtained from supermarkets in Taiwan, were used as test samples. In order to be analyzed by CEC, 5 mL of edible oil sample was mixed with 20 mL of methanol and acetonitrile (in the ratio of 1:1). This mixture was sonicated for 15 min, and the supernatant was collected and centrifuged for 10 min at 6000 rpm. The upper organic solvent was kept in a deep-freezer for 1 h. The resulting clear liquid was concentrated to dry residue by a rotary evaporator. The residue was dissolved by a mixture solution of methanol and acetonitrile (in the ratio of 1:7, 2.5 mL), and then was ready for CEC analysis. The above extraction procedure was followed the previous method [37]. Because the recovery of extraction protocol was over 98.5% for these antioxidants, thus no degradation problem was observed during the dryness treatment of sample.

2.3. Apparatus

The CEC experiments were performed with a Beckman Coulter MDQ capillary electrophoresis system equipped with a photodiode array detector (Fullerton, CA, USA). Beckman Coulter MDQ 32 Karat software was used for instrumental control and data analysis. A Waters instrument model 515 HPLC pump (Milford, MA, USA) was used for washing and equilibrating the polymeric monolithic column. A scanning electron microscope model Hitachi S-4100 (Ibraraki, Japan) was used for the polymeric morphology observation. A surface area analysis equipment model Micromeretics Tri-star 3000 (Norcross, GA, USA) was employed for surface area measurement. A thermo Nexus 6700 FT-IR spectrometer (Waltham, MA, USA) coupled to a thermo Nicolet Continuum microscope equipped with a Ge tip slide-on ATR crystal was used to acquire IR spectra. A thermogravimetry model TG/DTA 6200 from SII Nano Technology (Chiba, Japan) was used for thermal decomposition temperature (T_d) measurement. A mercury intrusion porosimeter model Micromeretics Autopore IV 9500 (Norcross, GA, USA) was used for pore size measurement. The measurement of porous properties used in this study was performed with materials in the dry state, while the column actually operated in the swollen state. As a result, the porous data obtained in the dry state may not exactly reflect the pore size during the chromatography. Previous reports have demonstrated there was a strong correlation between the porous property of "dry" monolith and its chromatographic performance [24], and the available means in determining the porous structure in the swollen state are still limited, thus the porous data obtained from mercury intrusion porosimetry was still used in the study.

2.4. Preparation of polymeric monolithic column

Prior to the preparation of a polymeric monolithic column, the inner wall of a 100-μm I.D. capillary column was treated according to the following procedure. The capillary was conditioned by first washing with 0.1 M sodium hydroxide (5 min), followed by deionized water (20 min), and finally with methanol (5 min). After the capillary was dried by N_2 gas, it was filled with 3-trimethoxysilyl propyl methacrylate and methanol in a volume ratio of 1:1. Both ends of the capillary were then sealed and submerged in a 35 °C water bath (17 h). Finally, the capillary was washed with methanol (13 min), then with water (13 min), and dried by N_2 gas.

After conditioning, a solution composed of monomer, porogenic solvent, charged monomer and initiator was used to prepare the monolithic columns. The monomer amount in solution was kept at 18% and 24% (v/v) for poly(S–DVB) and poly(S–DVB–LMA), respectively. For poly(S–DVB), the monomers were composed of styrene (714 μL, 40%, v/v) and DVB (1071 μL, 60%, v/v), while it included styrene (0–366 μL), LMA (0–927 μL) and DVB (1391 μL) for poly(S–DVB–LMA). The total amounts of styrene and LMA were maintained at 3.18×10^{-3} mol for poly(S–DVB–LMA). For example, 50% LMA (mole ratio) was prepared with styrene of 183 μL (1.59×10^{-3} mol) and LMA of 462 μL (1.59×10^{-3} mol). Porogenic solvent was consisted of cyclohexanol (3750 μL), *N*,*N*-dimethylacetamide (DMAc, 3750 μL) and water (375 μL) for poly(S–DVB), while it included cyclohexanol (3484 μL), DMAc (3483 μL), and water (375 μL) for poly(S–DVB–LMA). After dissolv-

ing charged monomer (vinylbenzene sulfonic acid, VBSA, 0.0448 g) and initiator (azobisisobutyronitrile, 0.0155 g) in monomer and porogenic solvent, the mixture solution was sonicated for 15 min until it became homogeneous, then it was used to fill the preconditioned capillary (33 cm) to a total length of 20 cm by syringe injection. The remainder of the homogeneous mixture was sealed in a glass vial. After both ends of the capillary were sealed with adhesive resin, the capillary and the glass vial were submerged in a 70 °C water bath for 15 h. An LC pump was used to wash the monolithic column first with methanol then with the mobile phase. A detection window was fabricated by using a microtorch to remove the polyimide coating at the 20 cm position on the column where a polymer bed was absent. The monolithic polymer formed in the vial was Soxhlet extracted with methanol for 17 h, and vacuum dried overnight. The polymer was directly used for the analyses of surface area, pore size and conversion yield, while it was pressed into a thin wafer for the FT-IR measurement.

2.5. Operation condition for CEC

The monolithic column was placed in the CE instrument and was equilibrated with the mobile phase under 10 kV applied voltage and 50 psi pressure at both ends of the column until a stable baseline was obtained. Samples and standards were electrokinetically injected into the capillary for 3 s at a voltage of 10 kV. An internal standard, propyl paraben (100 μg mL^{-1}), was added into samples or standards in order to improve the reproducibility of sample injection. Separation was achieved either with an isocratic elution or a two-step gradient method. Several solutions of pH 3.0 composed of acetonitrile and phosphate solution in different volume ratio (55:45–85:15) were used as mobile phases for isocratic elution. The two-step gradient method was initiated with a mobile phase of pH 3 consisting 60% acetonitrile and 40% phosphate solution for poly(S–DVB) or 55% acetonitrile and 45% phosphate solution for poly(S–DVB–LMA). And then was followed by a mobile phase of pH 3 consisting 85% acetonitrile and 15% phosphate solution at a given time point (4 or 5 min). Separations were carried out using an electrical voltage of 20 kV, and the temperature of the capillary was maintained at 25 °C, while 200 nm or 214 nm was selected as the detection wavelength. Thiourea was added to standards or samples as the EOF marker for the determination of the EOF mobility and the retention factor of antioxidants.

3. Results and discussion

3.1. Antioxidant separations in poly(S–DVB) column

3.1.1. Effect of polymerization condition

A poly(S–DVB) monolithic column produced by single step *in situ* copolymerization of styrene, divinylbenzene and vinylbenzene sulfonic acid, was able to achieve reproducible separation with good separation efficiency in a previous report [44], was first employed to analyze the five synthetic antioxidants in the study, to evaluate the effects of polymerization conditions on antioxidants. Several variables such as the amount of charge-bearing monomer, porogenic solvent ratio, and the level of total monomers used in the poly(S–DVB) preparation were examined to optimize antioxidant separation. The results indicated that in these poly(S–DVB) columns prepared with different polymerization conditions, thiourea (EOF marker) and all the antioxidants, except for BHT, were separated well within 10 min. However, BHT, which has the lowest water solubility among the five antioxidants, was not detected until 35 min.

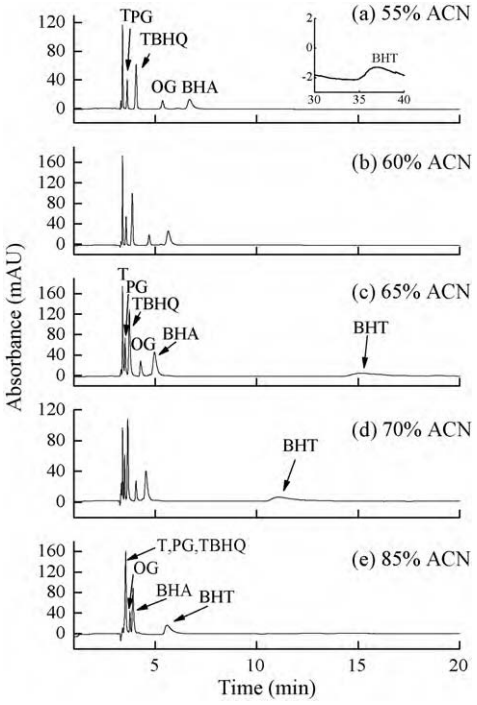

Fig. 1. Antioxidant separations on poly(S–DVB) columns with different acetonitrile level in mobile phase. Mobile phases of pH 3.0 were composed of acetonitrile and 5 mM phosphate buffer in the volume ratio of 55:45 to 85:15. Monolithic capillaries were prepared by three monomers of styrene (714 μL), divinylbenzene (1070 μL) and VBSA (0.045 g). T (thiourea, EOF marker), PG (propyl gallate), TBHQ (*tert*-butylhydroquinone), OG (octyl gallate), BHA (butylated hydroxyanisole), and BHT (2, 6-di-*tert*-butyl-4-methyl phenol).

3.1.2. Effect of mobile phase composition

Next, the effect of mobile phase composition on the antioxidant separations was examined. The results indicated that the retention behavior of the five antioxidants was highly altered by the volume fraction of acetonitrile in the mobile phase (Fig. 1). The resolution (R) of PG, TBHQ, OG and BHA were larger than 2 within 7 min when acetonitrile concentration is lower than 65%. However, BHT had a relatively strong retention in the poly(S–DVB) stationary phase in these mobile phase conditions (e.g. 35 min for 55% acentonitrile). For this reason, the acetonitrile level in mobile phase had to be raised to 70% or 85% in order to speed up BHT migration (i.e. its retention time was shortened to 6 min at 85% acetonitrile), but it caused inadequate resolutions for the other antioxidants.

3.1.3. Step-gradient elution

As mentioned earlier, baseline separations of all the antioxidants were not obtained under isocratic elution mode. Hence, a two-step gradient method was employed. This was performed by stopping the EOF and changing the mobile phase elution strength, which was achieved by varying the concentrations of acetonitrile (60% and 85%) in the phosphate solution. In order to separate all antioxidants rapidly, the optimal time interval for each mobile phase was examined. The results indicated that the time interval of 4 or 5 min provided acceptable resolution and retention time for the five analytes ($R > 1.5$, $t_R < 10$ min), but BHT still had relatively broaden signal in the two-step gradient elution (29 000 plates/m for BHT) (Fig. S1, supplementary data). Similar to previous reports on polystyrene-based stationary phase, in which serious peak tailing often happened in aromatic compound analyses [28–30], the same drawback (peak tailing) also occurred for BHT separation in this study.

3.2. Antioxidant separation in poly(S–DVB–LMA) column

3.2.1. Optimal polymerization condition of poly(S–DVB–LMA) column

Several polymeric monoliths which were prepared by both styrene- and methacrylate ester-based monomers (styrene, DVB and/or LMA), were employed as stationary phases. The polymerization procedure was optimized by varying the three parameters (the reaction temperature, the reaction time, and the LMA–styrene ratio) likely to have the most significant effect on the tailing factor of BHA and BHT, as well as the resolution of PG and thiourea (EOF marker). According to the experimental design [31,45], the polymerization was performed at three levels of each parameter (the reaction temperature of 60, 65 and 70 °C, the reaction time of 5, 10 and 15 h, and the LMA–styrene ratio of 50%, 75% and 100%). The polymerization conditions are shown in Table S1 (supplementary data), and the effect of each parameter on the tailing factor or resolution is shown in Fig. S2 (supplementary data). The change in the reaction temperature did not cause obvious difference in the tailing factor of BHT and BHA, but the resolution of thiourea and PG was improved at a higher reaction temperature (70 °C) (Fig. S2(a)). Furthermore, a longer reaction time (15 h) improved the peak symmetry of BHT, and maintained a good resolution for PG and thiourea ($R \sim 2$)(Fig. S2(b)). Among these three parameters, the LMA amount which was instead of styrene monomer had the most influence on the peak symmetry of BHT and BHA (Fig. S2(c)), in which the best peak shape was acquired at 50% LMA for BHA and 100% LMA for BHT. Consequently, the optimal polymerization conditions (i.e. the reaction temperature of 70 °C and the reaction time of 15 h), were used for the poly(S–DVB–LMA) column preparation in this study, while the optimal LMA amount still needed to further examination.

3.2.2. Antioxidant separation in poly(S–DVB–LMA) column by step-gradient elution

As mentioned in the previous section, the chromatographic behaviors of BHA and BHT were predominantly affected by the LMA–styrene ratio, so its effect on the chromatographic behavior of all the antioxidants was further studied. The electrochromatograms of the antioxidants derived from several poly(S–DVB–LMA) columns using a two-step gradient elution were shown in Fig. 2. A mobile phase composed of 55% ACN was used in the initial separation of 5 min followed with a mobile phase composition of 85% ACN until the separation ended (Table 1). In these poly(S–DVB–LMA) columns, the DVB amount was maintained at 1391 μL, but the styrene amount was replaced with LMA partially to totally (i.e. 0%, 50%, 75% and 100% LMA mole ratio), while the total amount of styrene and LMA was kept at 3.18×10^{-3} mol. As a result, benzene moieties reduced with the addition of LMA, as well as included C_{12} carbon chains in the polymers. The results indicated that the poly(S–DVB–LMA) columns provided baseline separation within 11 min for the tested analytes, furthermore the peak symmetry for BHT compound was improved with the inclusion of LMA in the polymeric columns. For instance, the number of theoretical plates raised from 32 000 plates/m (0% LMA) to 98 000 plates/m (75% LMA), while the tailing factor was improved from 4.2 (0% LMA) to 1.3 (100% LMA) (BHT, Table 2). On the other hand, although the same two-step gradient elution was used in these columns, a higher LMA amount could speed up the migration of all tested analytes, but in turn caused inadequate resolution for T, PG, and TBHQ ($R < 1.0$ at 100% LMA, Fig. 2). The above results demonstrated that the insertion of LMA monomer to poly(S–DVB) column indeed changed the retention of aromatic compound in the polystyrene-based stationary phase, even if their elution order was still the same in both poly(S–DVB) and poly(S–DVB–LMA) columns. By comparison, the poly(S–DVB–LMA) column with 50% LMA and the two-step gradient elution offered better peak symmetry with highest efficiency for most analytes (Table 2, Fig. 2), and thus it was chosen as the optimal condition for the antioxidant analyses.

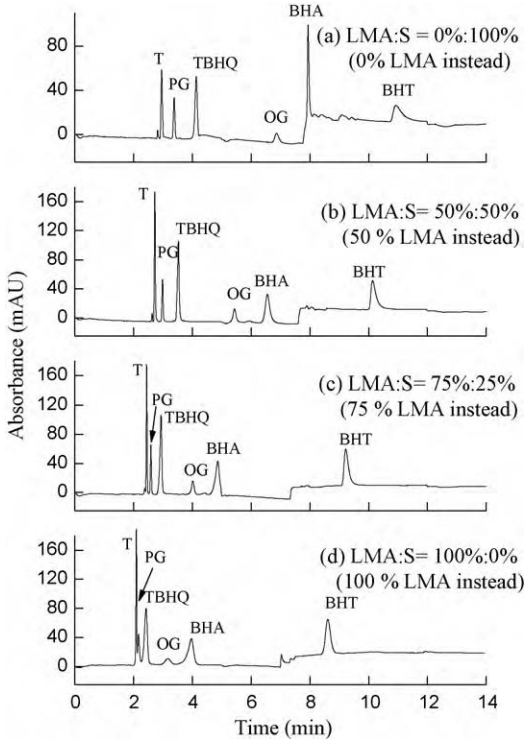

Fig. 2. Antioxidant separation on poly(S–DVB–LMA) columns prepared with different LMA–styrene ratios. Poly(S–DVB–LMA) monoliths were prepared by LMA and styrene in the mole ratio of (a) 0:100, (b) 50:50, (c) 75:25, and (d) 100:0, in which DVB amount was maintained at 1391 μL. 250 μg mL^{-1} of each analyte was electrically injected by 10 kV for 3 s, 200 nm was selected as the detection wavelength. The condition of step-gradient elution was listed in Table 1.

The plots of plate height (H) versus linear flow rate (i.e. EOF velocity) in the optimal poly(S–DVB–LMA) column also indicated that the plate heights of all the analytes were only slightly changed with the linear flow rate (e.g. 6.2, 6.6, 15.6, 14.5, 28.6 and 34.5 μm for thiourea, PG, TBHQ, OG, BHA and BHT in the flow rate of 0.54–1.1 mm s^{-1}). Thus, the proposed poly(S–DVB–LMA) column had comparable efficiency with previous CEC report on polystyrene-based monoliths [31], in which the plate height of thiourea was 5 μm over a broad range of linear flow rate.

Table 1
Two-step gradient elution used in the proposed poly(S–DVB–LMA) column.

Step-gradient elution	Mobile phase[a]	Applied voltage (ramping time)[b]	Elution time
Step 1	A mobile phase of pH 3 composed of 55% ACN and 45% phosphate solution	20 kV (0.5 min)	0–5 min
Step 2	A mobile phase of pH 3 composed of 85% ACN and 15% phosphate solution	20 kV (0.5 min)	5–12 min
Run-to-run condition	A mobile phase of pH 3 composed of 55% ACN and 45% phosphate solution	10 kV (2.0 min)	12–20 min

[a] The mobile phase of both inlet vial and outlet vial were changed at the same time. ACN: acetonitrile.
[b] The ramping time is the time interval that rises from 0 kV to 20 kV.

Table 2
Effect of LMA–styrene ratio on separation efficiency and peak symmetry of antioxidants[a].

	LMA versus styene mole ratio (%)							
	Tailing factor (F_t)[b]				Theoretical plate number (plates m^{-1})			
	0:100	50:50	75:25	100:0	0:100	50:50	75:25	100:0
PG	0.89	1.11	0.94	–[c]	105 000	153 000	115 000	52 000
TBHQ	0.77	0.78	0.61	0.71	74 000	81 000	56 000	16 000
OG	1.5	0.96	0.85	0.98	57 000	67 000	49 000	5000
BHA	1.6	0.96	0.55	0.51	–[c]	46 000	31 000	10 000
BHT	4.2	2.68	2.3	1.3	32 000	90 000	98 000	56 000

[a] Poly(S–DVB–LMA) monoliths were prepared by LMA and styrene in the volume ratio of 0:100, 50:50, 75:25, and 100:0, respectively, in which DVB amount was maintained at 1391 μL. All other conditions were the same as in Fig. 1.
[b] The data for tailing factor (F_t) were obtained by the equation, $F_t = b/a$, where b and a were the distances between the centre and the tailing and leading edge of the analyte peak, respectively, measured at 10% of peak height.
[c] The PG or BHA peak was obviously overlapped with the thiourea signal or the background signals resulted from the change of the mobile phase composition (Fig. 1a), thus its tailing factor or theoretical plate number was not included.

3.2.3. Morphology and surface property of poly(S–DVB–LMA) columns

The morphology and surface property of poly(S–DVB–LMA) monolith was also evaluated in order to characterize the stationary phases. Several experiments including scanning electron microscopy (SEM) image, analyses of pore size and surface area, conversion yield of polymerization, FT-IR spectrum and thermal decomposition pattern were carried out. The SEM images indicated that the morphology of poly(S–DVB–LMA) monolith was slightly altered with the LMA level, and the polymers prepared by 50% LMA ratio had smaller linked nodules (Fig. 3). The surface analysis data shown in Table 3 indicated that the surface areas of these polymers were greatly reduced with the LMA amount (549 $m^2\,g^{-1}$ for 0% LMA, 215 $m^2\,g^{-1}$ for 50% LMA, 29.3 $m^2\,g^{-1}$ for 75% LMA, and 33.9 $m^2\,g^{-1}$ for 100% LMA). Although the pore size formed in these polymers had no certain correlation with the LMA amount

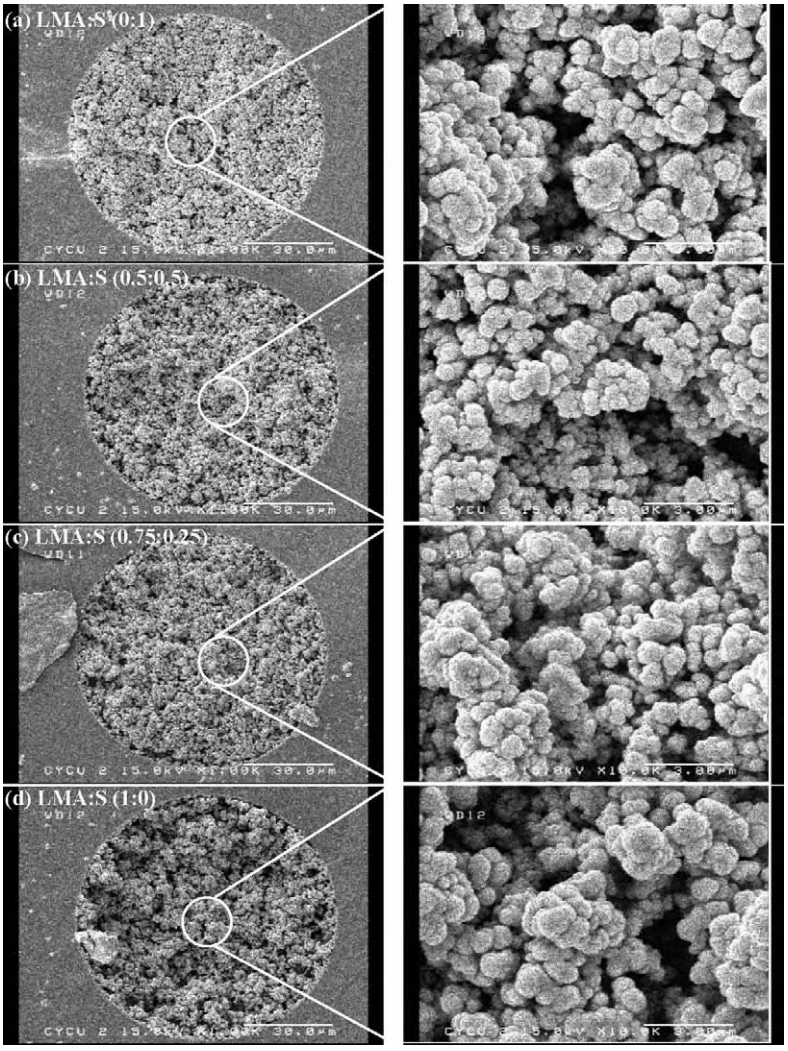

Fig. 3. SEM micrographs of poly(S–DVB–LMA) monolithic columns prepared with different LMA–styrene ratios.

Table 3
Properties of various polymer monoliths.

LMA versus styene mole ratio (%)	Conversion yield[a] (w/w, %)	Surface area ($m^2\ g^{-1}$)	Pore diameter (nm)	T_d (°C)[b]		
				5%	10%	50%
0:100	95.0	549	282	300	325	405
50:50	97.4	215	95.0	294	317	379
75:25	88.3	29.3	678	292	314	373
100:0	96.8	33.9	434	285	308	369
Poly(LMA–EDMA) monolith column[c]	–	–	–	214	215	218

[a] Conversion yield = (weight of formed polymer)/(weight of used monomers) × 100%.
[b] T_d, the decomposed temperature at 5%, 10% and 50% weight loss, was measured by a thermogravimetry (TGA).
[c] The poly(LMA–EDMA) monolith column was prepared by two methacrylate-based monomers (2.56 g LMA and 0.96 g ethylene dimethacrylate (EDMA)), in which a solution consisted of water (0.36 g), 1, 4-butanediol (1.0 g) and 1-propanol was used as ternary porogenic solvent. The composition was referred to previous CEC report on methacrylate ester-based polymer [47].

(282 nm for 0% LMA, 95.0 nm for 50% LMA, 678 nm for 75% LMA and 434 nm for 100% LMA) (Table 3), a higher LMA level caused a narrower distribution of pore size of the polymeric monolith. As mentioned earlier, the poly(S–DVB–LMA) column prepared with 50% LMA provided the best separation efficiency for the antioxidants (Fig. 2b). This was likely due to the smallest pores produced by this poly(S–DVB–LMA) column that resulted to a significant sieving effect, therefore caused a better separation ability of the column.

3.2.4. Polymerization reactivity of poly(S–DVB–LMA) columns

Because of the differences in the chemical structures of styrene, DVB and LMA, a different reactivity among these monomers is possible. Thus, the monomer reactivity in the poly(S–DVB–LMA) preparation needs to be examined. First, the conversion yield of the polymeric monolith, which was obtained by comparing the weights of the originally used monomers and the formed polymer (i.e. the weight of formed polymer divided by the weight of original monomers used in polymerization) was used to evaluate the reactivity. The results indicated that the conversion yield of poly(S–DVB–LMA) was over 96% (w/w) either in 50% or 100% LMA amount, which was similar with poly(S–DVB) (Table 3). It seemed to indicate that the reactivity between the methacrylate ester- and styrene-based monomers was relatively good under the polymerization condition. Therefore, the "clusters" formation of polymer, which consisted of one monomeric unit (LMA or styrene) in the poly(S–DVB–LMA) monolith should not be significant.

To further clarify the hypothesis, several FT-IR spectra were used to assess the difference in the organic functional groups of these polymers. For the poly(S–DVB) monolith, only the characteristic absorptions of benzene group were measured (1600 and 1450 cm^{-1} for the C=C group stretching vibrations, Fig. 4a). Moreover, in addition to the benzene signals resulted from styrene or DVB monomer (1600 and 1450 cm^{-1}), the characteristic absorptions of LMA (i.e. ester group) were also found in the poly(S–DVB–LMA) monoliths (1730 cm^{-1} and 1200/1120 cm^{-1} for the stretching vibrations of C=O and C—O groups; Fig. 4b and c). Note that the absorptions of 1600 and 1450 cm^{-1} appeared in Fig. 4c (LMA: S = 100%:0) should be attributed to the benzene moiety of DVB monomer. The above results demonstrated that the proposed polymerization condition produced a homogenous styrene- and methacrylate ester-based copolymer, and thus the reactivity of LMA and styrene-based monomer (styrene or DVB) was acceptable.

3.2.5. Thermal properties of poly(S–DVB–LMA) columns

In contrast to HPLC, a significant Joule heating usually happens in the CEC system because a high electric voltage needs to be applied. Significant temperature increases inside the capillary due to Joule heating certainly lead to the change of solute partitioning between mobile and stationary phase, and even composition change of polymeric stationary phase. As a result, the thermal stability of stationary phase is also an important issue in CEC system. To evaluate the thermal stability of these polymeric stationary phases (poly(S–DVB), poly(S–DVB–LMA) and poly(lauryl methacrylate–ethylene dimethacrylate) (poly(LMA–EDMA)) monoliths), a thermogravimetric analysis (TGA) method was subsequently used (Fig. S3, supplementary data), and their thermal decomposition temperatures (T_d) were also summarized in Table 3. Compared to the other polymers shown in Table 3, the poly(LMA–EDMA) monolith, which was only prepared by methacrylate ester-based monomers had the poorest thermal stability (its T_d was about 214 °C and 218 °C at 5% and 50% weight loss, respectively). In contrast, the poly(S–DVB) monolith, which had the highest T_d either at 5% or 50% weight loss (300 °C and 405 °C, respectively), provided the strongest resistance to thermal degradation. With the combination of LMA and styrene-based monomers, the poly(S–DVB–LMA) monoliths

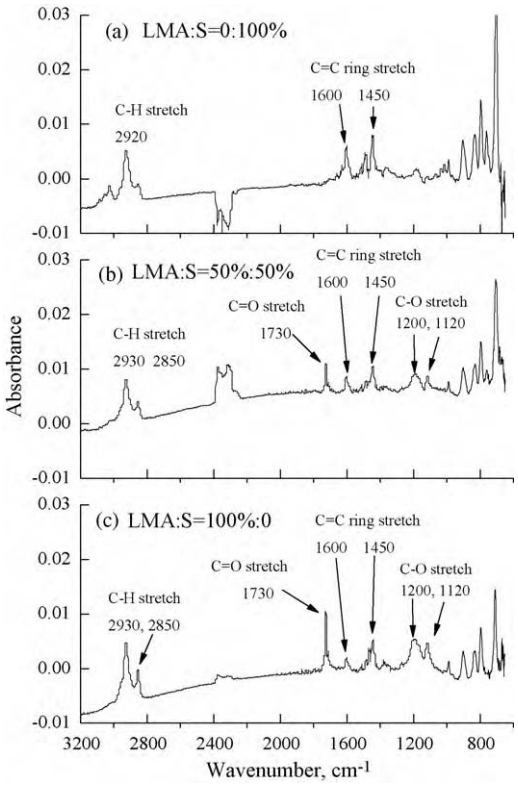

Fig. 4. IR spectra of poly(S–DVB–LMA) monoliths with different LMA-styrene ratios. All other conditions were the same as in Fig. 2.

have markedly improved thermal stabilities. For example: the T_d of the poly(S–DVB–LMA) with 50% LMA was about 294 °C and 379 °C at 5% and 50% weight loss, respectively; that was very close to the poly(S–DVB) column. Therefore, both the poly(S–DVB) and the poly(S–DVB–LMA) columns had comparable thermal stabilities. Upon examination of the T_d data shown in Table 3, the higher thermal stabilities of poly(S–DVB–LMA) monoliths over poly(LMA–EDMA) may be attributed to the greater rigidity of aromatic structures in the poly(S–DVB–LMA) material, which prevents the movement of the fragments from bond scission, and even leads to the collision of aromatic rings (carbonization) at higher temperatures [46]. As a result, it commenced degradation at higher temperatures than poly(LMA–EDMA).

On the other hand, the relative standard deviation (RSD) of the retention time of the five antioxidants, which was injected triplicate every 7 days for a period of 35 days, was in the range of 0.58–0.97% for the same poly(S–DVB–LMA) column with 50% LMA. It indicated that there was a highly reproducible and stable chromatographic behavior in the poly(S–DVB–LMA) column. Consequently, the poly(S–DVB–LMA) column was demonstrated as a highly potential stationary phase for CEC system because of its high thermal stability and good column reproducibility (at least 3 months).

3.3. Qualitative and quantitative performances under optimal CEC condition

The qualitative and quantitative performances of the antioxidant compounds under the optimal poly(S–DVB–LMA) column (i.e. 50% LMA) and step-gradient elution program were evaluated, and the results were summarized in Table 4. The RSD of retention time and peak area of the five analytes with concentration of 250 μg mL^{-1} for each one was in the range of 0.0–0.70% and 1.37–4.53%, respectively, for three intra-day replicated injections in the same column, and was in the range of 0.05–1.96% and 1.13–8.48%, respectively, for nine replicated injections in three different columns prepared from different batches (Table 4). It was noted that there was a higher RSD for peak area in the test of three different columns, which was possibly due to the two-step gradient elution used in the CEC separation. In addition, the correlation coefficients (r) of the calibration curves were greater than 0.999 for each of the analytes after internal standard calibration. The detection limits for the analytes were in the range of 0.09–1.37 μg mL^{-1} based on S/N ratio of 3. These results indicated that the poly(S–DVB–LMA) column indeed provided relatively good quantitative performance for the antioxidant analyses.

A previous HPLC method reported for these synthetic antioxidants with the fastest separation was completed within 8 min by gradient elution, and with good resolution and baseline [37]. On the other hand, the best CE method reported for the synthetic antioxidants so far indicated that a high resolution and baseline separation was acquired within 12 min, with good reproducibility (RSD in the range of 1.7–3.0%, and 3.0–10% for intra-day retention time and peak area, respectively; n = 10) [41]. In contrast to the best HPLC and CE methods [37,41], in which only three to four synthetic antioxidants were studied, the proposed CEC method coupled with two-step gradient elution provided a comparable separation ability for the five antioxidants analyzed (Table 4).

3.4. Analyses of edible oil products

Since the five tested analytes are commonly used as antioxidants in commercially available food products, edible oil products were chosen as real samples in this study. Fig. 5a and b showed the electrochromatograms of the antioxidants found in edible oil prod-

Table 4
Separation performance of antioxidant standards in CEC method with poly(S–DVB–LMA) stationary phase[a].

Antioxidants	Column repeatability[b]		Run-to-run (n = 3)		Column reproducibility[b]	Column-to-column (n = 3)	Theoretical plate numbers (plates m^{-1})	Detection limit (μg mL^{-1}) (S/N = 3)	Calibration curves[c]
	Retention time (min)	RSD of peak area	Retention time (min)	RSD of peak area					
PG	2.98 (0.00%)	1.92%	2.99 (0.55%)	2.70%			153 000	0.09	$Y = 0.0142X - 0.0142$
TBHQ	3.51 (0.24%)	3.34%	3.54 (0.93%)	2.20%			81 000	0.34	$Y = 0.0057X - 0.0910$
OG	5.39 (0.70%)	2.67%	5.50 (1.86%)	8.48%			67 000	0.31	$Y = 0.0155X - 0.4493$
BHA	6.49 (0.64%)	4.53%	6.63 (1.96%)	1.64%			46 000	1.37	$Y = 0.0051X - 0.0184$
BHT	10.0 (0.09%)	1.37%	10.0 (0.05%)	1.13%			90 000	0.83	$Y = 0.0081X - 0.1227$

[a] Separation conditions: the volumes of acetonitrile and 5 mM phosphate buffer in the mobile phase was in a ratio of 55:45 in the initial 5 min, then was changed to the ratio of 85:15 after 5 min. The detection wavelength was kept at 214 nm. Propyl paraben (100 μg mL^{-1}) was used as internal standard (IS) in the method. The data of detection limit and calibration curves did not include sample pretreatment.

[b] Values of column repeatability were means of three intra-day replicates on the same column, and data of column reproducibility were means of nine replicates on three columns prepared by different batches. The value in parenthesis indicates the RSD of migration time in percentage.

[c] The calibration curves were constructed from three replicate measurements at each concentration in the range of 20 μg mL^{-1} to 2000 μg mL^{-1} (20, 100, 200, 250 and 2000 μg mL^{-1}).

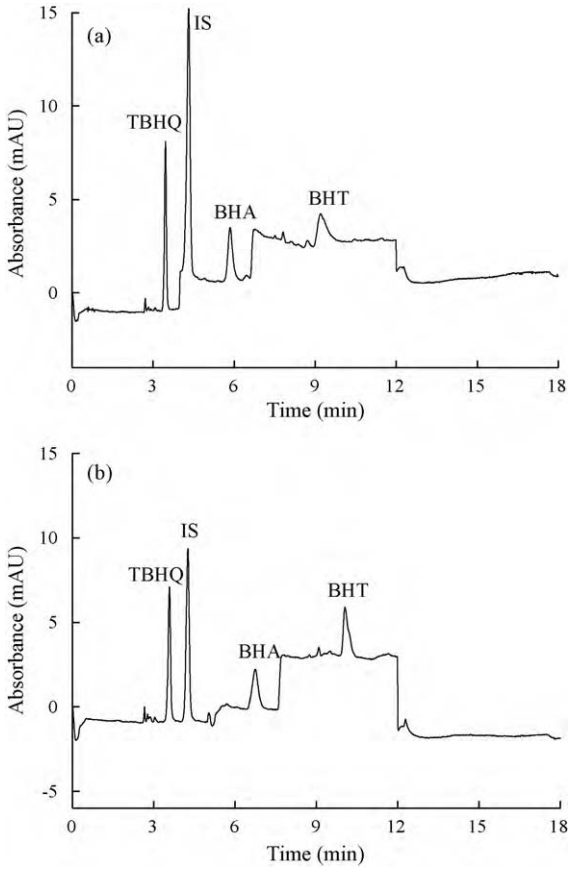

Fig. 5. The electrochromatograms of commercially edible oil product determined by step-gradient elution CEC method. (a) poly(S–DVB), and (b) poly(S–DVB–LMA) monolithic columns were used as separation columns. The mobile phase composition (acetonitrile: 5 mM phosphate buffer, volume percentage ratio) was (a) 60:40 for 0–4 min, and 85:15 for 4–11 min, and (b) 55:45 for 0–5 min, and 85:15 for 5–12 min. The detection wavelength was 214 nm. IS (Propyl paraben).

ucts separated by the poly(S–DVB) and poly(S–DVB–LMA) columns, respectively; the latter one provided a better peak symmetry for BHT. TBHQ, BHA and BHT were determined in the tested sample and the antioxidant contents were in the range of 59.2–77.9 $\mu g\,mL^{-1}$, which was lower than the maximum allowable amounts of Food and Drug Administration in Taiwan (200 $\mu g\,mL^{-1}$). The RSD of the antioxidant contents in these samples was in the range of 2.23–4.92% with triplicate measurements, so indicating that the poly(S–DVB–LMA) CEC method did provide a good quantitative reproducibility.

4. Conclusion

In this paper, several polymeric stationary phases, which were prepared by mixed styrene- and methacrylate ester-based monomers (S, DVB and LMA), were developed for the antioxidant analyses. A two-step gradient elution with different strengths of mobile phases was also used to shorten the separation time of these antioxidants. The increase in LMA amount in the poly(S–DVB–LMA) column markedly improved the retention behavior of the antioxidants (higher peak symmetry and faster separation time). The study demonstrated that the poly(S–DVB–LMA) monoliths, which provided higher separation performance than conventional styrene-based polymer columns, as well as better thermal stability than methacrylate ester-based polymer columns, are highly potential CEC stationary phases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.07.014.

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