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Uniaxially aligned electrospun cellulose acetate nanofibers for thin layer chromatographic screening of hydroquinone and retinoic acid adulterated in cosmetics

Siripran Tidjarat, Weerapath Winotapun, Praneet Opanasopit, Tanasait Ngawhirunpat, Theerasak Rojanarata *

Pharmaceutical Development of Green Innovations Group (PDGIG), Faculty of Pharmacy, Silpakorn University, Nakhon Pathom 73000, Thailand

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ABSTRACT

Uniaxially aligned cellulose acetate (CA) nanofibers were successfully fabricated by electrospinning and applied to use as stationary phase for thin layer chromatography. The control of alignment was achieved by using a drum collector rotating at a high speed of 6000 rpm. Spin time of 6 h was used to produce the fiber thickness of about 10 μm which was adequate for good separation. Without any chemical modification after the electrospinning process, CA nanofibers could be readily devised for screening hydroquinone (HQ) and retinoic acid (RA) adulterated in cosmetics using the mobile phase consisting of 65:35:2.5 methanol/water/acetic acid. It was found that the separation run on the aligned nanofibers over a distance of 5 cm took less than 15 min which was two to three times faster than that on the non-aligned ones. On the aligned nanofibers, the masses of HQ and RA which could be visualized were 10 and 25 ng, respectively, which were two times lower than those on the non-aligned CA fibers and five times lower than those on conventional silica plates due to the appearance of darker and sharper of spots on the aligned nanofibers. Furthermore, the proposed method efficiently resolved HQ from RA and ingredients commonly found in cosmetic creams. Due to the satisfactory analytical performance, facile and inexpensive production process, uniaxially aligned electrospun CA nanofibers are promising alternative media for planar chromatography.

1. Introduction

Electrospinning is a facile, robust and versatile technique for the fabrication of nanofibers for laboratory and industrial use. In this process, polymer liquid, i.e. solution or melt, loaded in a syringe is charged by a high voltage. Once the electrostatic force overcomes the surface tension on the polymer droplet, the liquid is ejected from the nozzle tip towards the grounded collector while solvent evaporates or melt solidifies. This phenomenon results in the formation of extremely small fibers with high surface area, high porosity and controllable compositions and size. Taking advantages of these features, electrospun nanofibers are attractive for a wide array of applications ranging from filtration, medical wound dressings, tissue engineering, drug delivery and so on [1]. So far, nanofibers electrospun by conventional methods are collected as nonwoven layers with randomly arranged structures. Later, aligned nanofibers can be produced through different means e.g. by using high speed rotating mandrel, electrostatic metallic staple and a pair of permanent magnets [2]. It is known that, for some applications, an ordered structure such as uniaxially or radially aligned nanofiber is more desirable. For instance, uniaxially aligned nanofibers with anisotropic properties has been shown to satisfy electrical, optical and mechanical purposes [3–5]. In addition, the aligned nanofiber scaffolds used in cell culture and tissue engineering not only mimic the parallel structure of fibrous tissues e.g. collagen in tendon or nerve, but also promote the migration and extension of cells [6,7].

In analytical chemistry, electrospun nanofibers have been currently applied to electrochemical and optical based detection systems, solid phase extraction and membrane separation [8]. Besides, electrospinning has recently become a new route to produce stationary phase for thin layer chromatography (TLC). There have been the reports about the fabrication and use of electrospun nanofibers as TLC stationary phase for the separation of amino

* Corresponding author at: Department of Pharmaceutical Chemistry and Pharmaceutical Development of Green Innovations Group (PDGIG), Faculty of Pharmacy, Silpakorn University, Nakhon Pathom 73000, Thailand. Tel.: +66 34 255800; fax: +66 34 255801.
E-mail addresses: rtheerasak@yahoo.com, teerasak@su.ac.th (T. Rojanarata).

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acids, laser dyes, steroidal compounds and food preservatives. However, the polymers used for the fabrication were limited to glassy carbon [9], polyacrylonitrile (PAN) [10–12], polyvinyl alcohol (PVA) [13] and cellulose acetate (CA) [14] In other aspects, electrospun nanofiber layers were improved for the detection of ultraviolet-active compounds by incorporation of photoluminescence indicator and were hyphenated with electrospray-ionization mass spectrometry [12]. Concerning the aligned electrospun nanofibers, Olesik et al. reported the use of aligned PAN nanofibers to separate a mixture of β-blockers and steroidal compounds. Compared to those with random orientation, the aligned nanofibers improved reproducibility and separation efficiency as well as shortened run time [11]. The alignment of PAN nanofibers was also observed in the work done by Kampalanonwat et al. However, in that study the delicate investigation aimed to control the aligment such as the optimization of rotating speed of drum collector has not yet been conducted [12]. These reported performance enhancements motivate further pursuit of new electrospun TLC materials.

CA is an environmentally degradable material made from the most abundant biopolymer on earth i.e. cellulose [15]. It is much cheaper than some synthetic polymers such as PAN. In addition, nanofibers made from CA can be readily used after electrospinning process for TLC without additional steps such as pyrolysis or chemical crosslinking which are usually required for the fabrication of glassy carbon and PVA nanofibers, respectively. Recently, TLC plates made from non-aligned CA nanofibers have been prepared and used with eco-friendlier hydro-alcoholic mobile phases for the analysis of prohibited steroids in traditional medicines [14]. However, no reports related to the fabrication of aligned CA nanofibers used for TLC have been available.

The present study extends this work by optimizing drum collector rotation speed and spin time to produce new reversed-phase TLC plates with uniaxially aligned CA nanofibers. TLC performance of the aligned nanofibers was compared to that of non-aligned ones as well as commercially available silica based plates. Finally, their application was demonstrated by using them for screening hydroquinone (HQ) and retinoic acid (RA) adulterated in cosmetic creams since both of them are prohibited substances for use in cosmetics in many countries due to the side effects including scaling of skin, stinging, flushing and ochronosis [16,17]. From the study, the aligned CA nanofibers were proven to be the efficient TLC media for resolving the analytes within a shorter run time. Furthermore, this work reports for the first time about the advantage of aligned nanofibers over the non-aligned ones in the term of the lower mass of the analyte which could be visualized on the plates.

2. Materials and methods

2.1. Materials

Cellulose acetate (CA; M_w = 30 kDa; degree of acetylation ≈ 2.4), hydroquinone (HQ; purity ≥ 99.0%), retinoic acid (RA; purity ≥ 98.0%), vitamin C (VC; purity ≥ 99.0%), vitamin E (purity ≥ 96.0%), resorcinol (RS; purity ≥ 99.0%), α-arbutin (AR; purity ≥ 98.0%), sodium metabisulfite (purity ≥ 98.0%) and phosphomolybdic acid were purchased from Sigma–Aldrich, St. Louis, MO. Solvents used in this study i.e. *N*,*N*-dimethylacetamide (Labscan, Thailand; purity ≥ 99.5%), acetone (Carlo, Italy; purity ≥ 99.5%), glacial acetic acid (Merck, Germany; purity ≥ 99.8%), hexane (Merck, Germany; purity ≥ 99.0%), methanol and ethanol (Merck, Germany; purity ≥ 99.9%) were of analytical grade. Distilled water was used throughout the experiments. Commercial TLC plates i.e. silica gel 60 and silica gel 60 RP-18 plates (particle size of 10–12 μm, layer thickness of 200 μm on aluminum backing) were purchased from Merck. Cosmetic cream samples were collected from local markets in Nakhon Pathom province, Thailand, during January–March 2014.

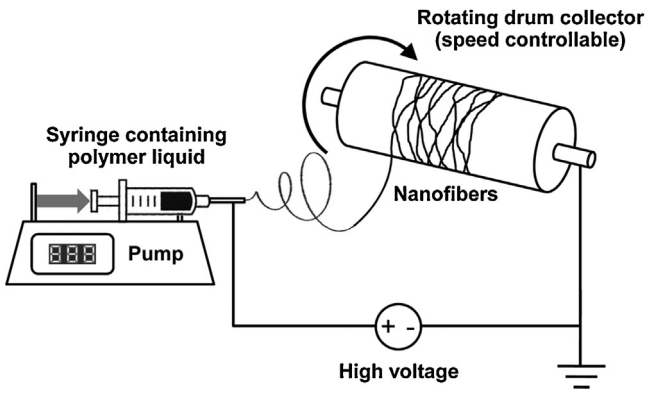


Fig. 1. Setup of electrospinning instrumentation for fabrication of nanofibers.

2.2. Fabrication and characterization of electrospun CA nanofibers

2.2.1. Electrospinning of CA nanofibers

Initially, 17% (w/v) CA solution was prepared by dissolving 1.02 g of CA powder in 6 mL of 2:1 (v/v) acetone/*N*,*N*-dimethylacetamide at room temperature (30 °C). Then, 5 mL of the solution was loaded into a glass syringe equipped with a blunted stainless steel needle (diameter ≈ 0.9 mm) attached to a pump (Fig. 1). Electrospinning was performed by ejecting CA solution from the needle tip at the feeding rate of 0.4 mL h^{-1} towards the rotating drum which functioned as a grounded collector under the electric field using electrical potential of 17.5 kV from a high voltage supply. The distance between the needle tip and the aluminum foil (standard household quality with the thickness of about 15 μm) that was wrapped around the drum (circumference ≈ 19 cm) was 15 cm. To fabricate vertically aligned nanofibers, the drum was set to rotate at different high speeds (4500, 6000 and 7500 rpm) and the nanofibers were collected at different spin times (1, 2, 4, 6, and 8 h). Non-aligned nanofibers were prepared in a similar way except that the drum rotation speed was 350 rpm. After electrospinning, the aluminum foil containing CA nanofiber layer was removed from the collector and cut into rectangular shape (7.5 × 2.5 cm^2) by selecting the area with the uniform thickness. The cut foil was then fixed onto the glass backing using double sided adhesive tape to produce CA nanofiber plate which was ready to use for TLC separation. It should be noted that this fixation is limited with regard to solvent resistance; thereby the use of thicker foils can be an option to avoid the tape.

2.2.2. Characterization of CA nanofibers

To observe the effect of drum rotation speed and spin time on the morphology and alignment, electrospun CA nanofibers fabricated under different conditions were subjected to scanning electron microscopy (SEM) (Camscan, MX-2000). The specimens for SEM were prepared by coating nanofibers with thin layer of gold (thickness ≈ 150 Å) by using sputtering device. The diameters of fibers and the thickness of layers were measured directly from SEM images (n = 50), using the image analysis software (JMicroVision V.1.2.7, University of Geneva, Geneva, Switzerland). The alignment of nanofibers was assessed as the number of fibers oriented in a determined direction with respect to a vertical reference line (0° angles). The angles from which individual fibers were deviated from this line were determined (n = 50) and then plotted as a function of frequency in a histogram. The narrow distribution of nanofiber angles indicated more alignment.

2.3. TLC screening method for HQ and RA

2.3.1. Preparation of standard and sample solutions

The standard solutions containing 0.125 mg mL^{-1} HQ and 0.67 mg mL^{-1} RA were freshly prepared in absolute ethanol. Cosmetic creams were extracted prior to TLC analysis following ASEAN guidelines [18,19]. Briefly, 1 mL of ethanol was added to 0.5 g of sample in a microcentrifuge tube. The mixture was then mixed and sonicated for 10 min to improve the dissolution and the release of HQ and RA from sample matrix. Subsequently, it was put on ice until the separation of wax occurred, centrifuged and the clear supernatant was finally collected for TLC. Since the adulteration level might vary among the collected samples, some extracts were further evaporated under nitrogen gas at room temperature to increase the concentration of target analytes for 2–10 folds.

2.3.2. TLC procedures

For general protocol, the separation of HQ and RA on electrospun CA nanofiber plate was carried out by manually spotting 0.5 μL of standard solutions of HQ and RA and sample solutions side by side, 1 cm from the bottom edge with the aid of calibrated micropipette and tips. The plate was then developed vertically in a closed chamber containing mobile phase (methanol/water/acetic acid) which was previously saturated at 30 °C for 30 min. The mobile phase was allowed to migrate for a distance of 5 cm from the start. Subsequently, the plate was removed from the chamber and air-dried. The spots of HQ and RA were detected by spraying the plate with 5% (w/v) phosphomolybdic acid solution in ethanol following the ASEAN guidelines [18,19] and blowing with hot air (95 °C) from hair dryer at 5 cm distance from the plate for 2.5 min. Alternatively, the plate might be incubated in hot air oven set at 95 °C for 2.5 min. HQ and RA were directly visualized as grey spots under visible light. For comparison purposes, TLC was also run on two types of commercial plates i.e. silica gel 60 and silica gel 60 RP-18 plates using the same procedures, except that the volume of solutions spotted on the plate was increased to 2 μL.

To investigate the optimal mobile phase, the solvents consisting of methanol and water at various ratios in the presence of 2.5% acetic acid were tested. The separation quality was evaluated from R_f values, the resolution between HQ and RA spots and the spot shape. In addition, the chromatographic performance of aligned and non-aligned CA nanofibers was compared in the aspects of run time, migration constant as calculated from (migration distance)^2/time and size of spot appeared on the plates. For this purpose, the images of the TLC plates ($n = 4$) were captured by a scanner (HP Deskjet 1050, Hewlett Packard, China) at a resolution of 300 dpi and saved as TIF files. The image was then opened with Photoshop CS2 software (Adobe System, Mountain View, CA, USA). The mode of color was changed to grayscale and the threshold level was adjusted to 220. The area of spot was measured as pixels by using Magic Wand Tool and Histogram.

2.3.3. Method validation

The masses of HQ and RA which could be visualized on aligned and non-aligned CA electrospun nanofibers as well as commercial silica-based plates were determined by spotting series of standard solutions of HQ and RA on the plates to obtain 5, 10, 20, 40, 80 ng of HQ and 25, 50, 100, 200, 400 ng of RA. The specificity was validated by examining the interference effects from the ingredients commonly used in cosmetic creams i.e. VC, RS, AR, vitamin E and sodium metabisulfite. Finally, the analytical results obtained from TLC method run on the aligned CA nanofiber plates were verified by the confirmatory HPLC method according to ASEAN guidelines [18,19].

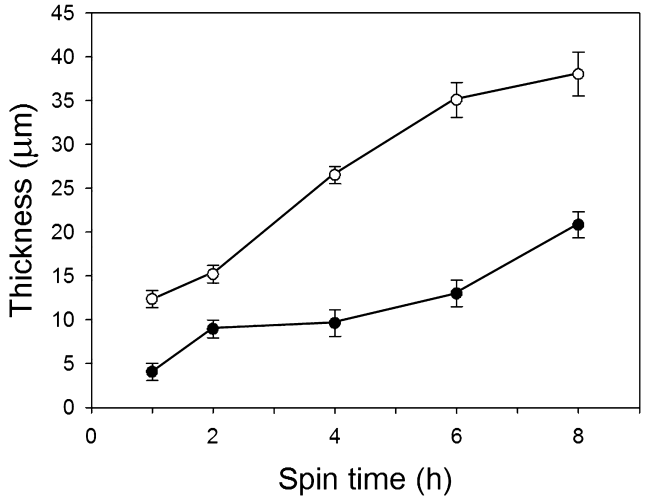


Fig. 2. Thickness of aligned (●) and non-aligned (○) nanofiber layers as a function of spin time.

3. Results and discussion

3.1. Morphological characteristics of CA nanofibers

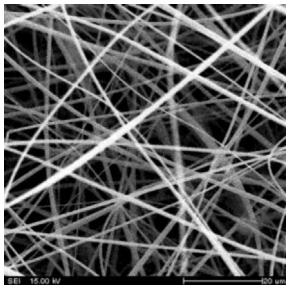
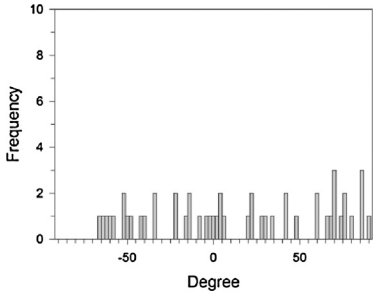
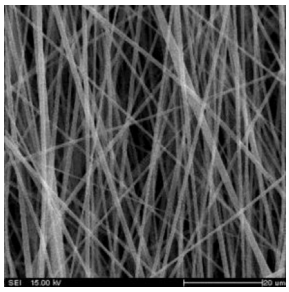
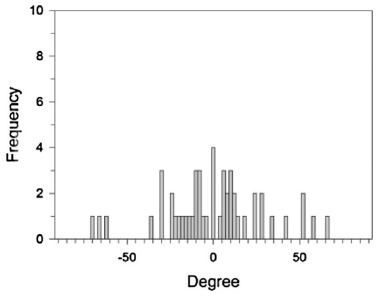
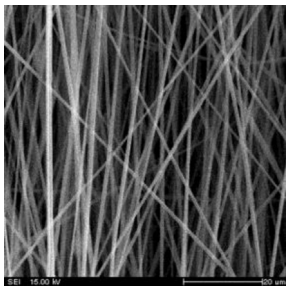
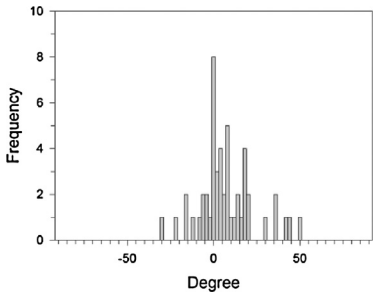
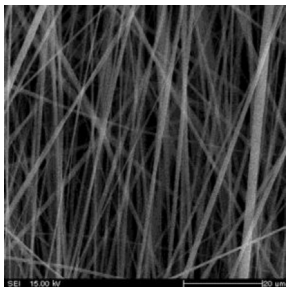
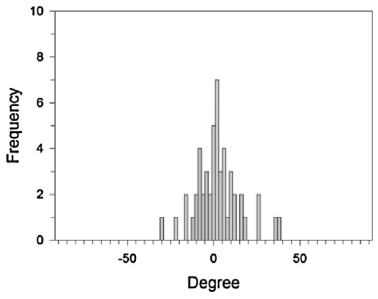
Of several means to control the alignment of electrospun nanofibers, a mechanical method through the use of a drum collector rotating at a high speed was employed in this study. As shown in Table 1, the drum rotation at 350 rpm was apparently too slow to orient the deposited fibers in the uniaxial direction. By raising the collector speeds, nanofibers were found to be more aligned as indicated by the narrower distribution of nanofiber angles. Along with the improved alignment, the average diameter of nanofibers decreased with the increasing rotation speeds due to the higher stretch of the polymer jets wounded around the drum. Nonetheless, no evidence of fiber breakage was observed at all tested rotation speeds as seen under SEM. Since the nanofibers collected at 6000 rpm showed satisfactory alignment and insignificant morphological difference from those collected at the higher rpm, this rotation speed was chosen for the subsequent study.

To investigate the effect of spin time, the aligned nanofibers were electrospun at times of 1, 2, 4, 6, 8 and 10 h, maintaining the optimized collector rotation speed at 6000 rpm. Spin time is an important process parameter because the longer time generates more fibers on the collector and the electrical charge accumulated on the deposited fibers may interfere with the incoming ones resulting in the fibers with less alignment [20]. In another way, too short spin time produces insufficient thickness of fiber layers and may give rise to the poor chromatographic performance in terms of loading capacity of sample solution and separation quality. As shown in Fig. 2, the thickness of aligned nanofiber layers increased with the increasing spin time. Besides, the aligned nanofiber mats were thinner than the non-aligned ones which were electrospun at 350 rpm using the equal spin time. This effect was probably caused by the more compact deposit of aligned nanofibers with smaller diameter size on the aluminum backing. In this study, the alignment of nanofibers was not adversely affected by the increasing spin time, up to 10 h.

3.2. Silica gel TLC plates incompatible with green mobile phase

The separation of two target analytes namely HQ and RA and a possible interference VC was used for the evaluation of the chromatographic performance of different types of plates. HQ and RA are prohibited substances for use in cosmetics in many countries

Table 1
Effect of drum rotation speed on fiber morphology and alignment.

Speed of drum collector (rpm)	Characteristics of nanofibers		
	SEM	Diameter (nm)	Alignment
350		730 ± 60	
4500		650 ± 70	
6000		500 ± 40	
7500		500 ± 70	

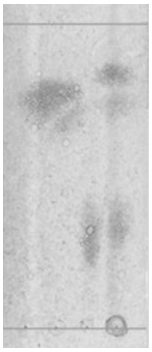
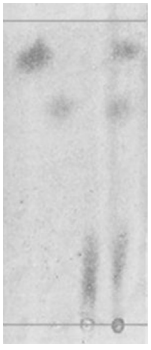
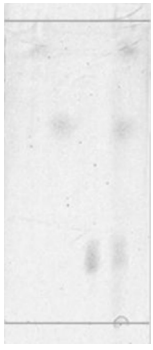
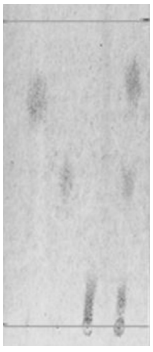
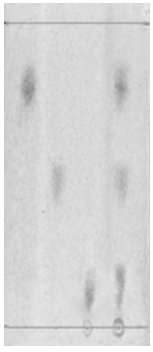
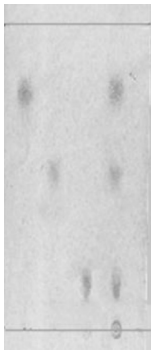
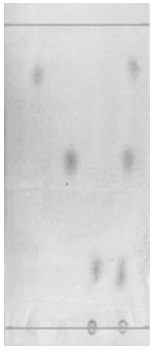
due to their side effects and safety profiles while VC is an ingredient commonly found in skin whitening and anti-wrinkle formulations. Currently, the screening methods for HQ and RA on commercial TLC plates employ organic solvents e.g. toluene, ethyl acetate and hexane as mobile phase [18,19,21,22]. However, since hydro-alcoholic mixture is considered as safer and greener solvent and is easy to prepare and handle in the field outside laboratories, it was selected as the mobile phase of choice in this study.

By using the mobile phase composed of methanol and water in the presence of 2.5% acetic acid with silica gel 60 plates, the compounds could not be resolved at any percentages of methanol (data not shown). After changing to use the more expensive silica gel 60 RP-18 plates, low polar RA appeared to stick tightly to the start spots and did not move up along the solvents containing 0–70% methanol. The increase of elution strength by raising the content of methanol in the mobile phase to higher than 80% resulted in the poor resolution between HQ and VC. Because of the failed attempts at using methanol/water/acetic acid with silica gel TLC, organic solvents i.e. n-hexane/acetone were employed with this type of plate in the standard method [18,19] in this study and the opportunity of using electrospun CA nanofibers as TLC media compatible with safer and greener solvents was investigated.

3.3. Chromatographic performance of CA nanofibers with green mobile phase

To select the electrospun CA nanofibers which were suitable for TLC, aligned and non-aligned nanofibers produced at various spin times were evaluated using 65:35:2.5 methanol/water/acetic acid as the mobile phase. It was noticed that the initial spots of samples on the aligned nanofibers were more elliptical than those on non-aligned ones due to the preferential diffusion along the

Table 2
Effect of spin time on TLC separation of HQ, RA and VC.*

Type of nanofibers	Spin time (h)				
	1	2	4	6	8
Non-aligned					
Run time (min)	47 ± 3	40 ± 4	38 ± 3	37 ± 3	35 ± 2
Migration constant (cm^2/min)**	0.53	0.63	0.66	0.68	0.71
Aligned					
Run time (min)	34 ± 3	15 ± 2	13 ± 2	13 ± 2	16 ± 2
Migration constant (cm^2/min)**	0.74	1.67	1.92	1.92	1.56

* The order of spots from bottom to top was RA, HQ and VC.
** Migration constant was calculated from (migration distance)^2/time.

Table 3
Area of analyte spots on aligned and non-aligned CA nanofibers ($n = 4$).

CA nanofibers	Area of spot (pixel)	
	HQ	RA
Aligned	1260 ± 130	1020 ± 140
Non-aligned	2520 ± 300	1770 ± 330

vertical direction of nanofibers. Compared with the silica based plates, both aligned and non-aligned CA nanofibers were able to separate HQ, RA and VC with superior performance, as illustrated in Table 2. Good resolution without the solutes remaining at the initial spots was obtained from aligned and non-aligned nanofibers electrospun for at least 6 h and 4 h, respectively. The nanofibers collected at the shorter time than these periods were too thin for the efficient separation due to the inadequate chromatographic beds. Additionally, they took longer run time, probably related to the low capillary action to draw the solvent up the plate.

In spite of the comparable separation ability, the aligned and non-aligned nanofibers were found to be different in some aspects. By visual analysis, the spots on the aligned nanofibers were generally smaller and appeared darker compared to those on the non-aligned ones which were more diffused in broad areas (Table 2). The measurement of spot dimension by digital imaging software revealed that the spots on the non-aligned nanofiber plates were almost twice as large as those on the aligned ones in pixels (Table 3). This difference arose due to the fact that while the solutes were moving up in the flow of mobile phase on the non-aligned nanofibers, they were likely to disperse in all directions due to the random arrangement of nanofiber mesh. On the other hand, the solute migration on the aligned nanofibers occurred preferentially along the vertical channels, giving rise to the lower extent of transverse (across-channel) diffusion. In addition, the reduced spot diffusion might associate with the lower thickness and higher density of the aligned media compared to the non-aligned one. For practical application, this feature offered the advantage of aligned nanofibers over the non-aligned ones in the terms of the ease of spot visualization and the lower mass of the analyte which could be detected.

Also interesting is the markedly faster separation ability of the aligned nanofibers. From the results, about two to three times reduction of developing time could be achieved by using the aligned nanofibers instead of the random ones (Table 2). In addition, the solvent migration on the aligned nanofibers used in this study (migration constant $\approx 1.9\,cm^2$/min) was more rapid than that on the non-aligned ones reported in the previous work (migration constant $\approx 1.0\,cm^2$/min), using the similar mobile phase [14]. It was hypothesized that the vertical channeling generated from the aligned nanofibers facilitated the solvent to move straight up the plate and minimized the lengthy distracted traveling along the random paths. In another point of view, the faster run time also lowered the chance of solute diffusion happening in the flow of mobile phase, thereby promoting the compact size of spots. In overall, since easy visual detection of spots and fast analysis are

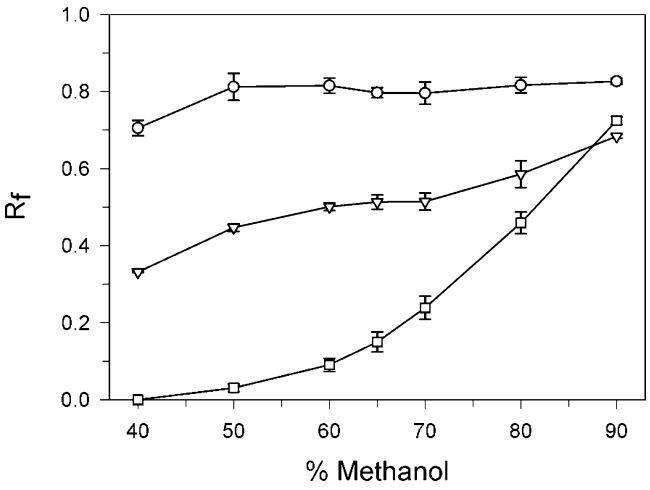


Fig. 3. TLC separation of RA (□), HQ (▽) and VC (○) on aligned CA nanofiber plates (electrospun by using collector speed of 6000 rpm and spin time of 6 h) using mobile phases containing different percentages of methanol to water in the presence of 2.5% acetic acid.

Table 4
Masses of HQ and RA which could be visualized on different types of TLC plates.

Stationary phase	Mobile phase	Mass (ng)	
		HQ	RA
Aligned CA	65:35:2.5 methanol:water:acetic acid	10	25
Non-aligned CA	65:35:2.5 methanol:water:acetic acid	20	50
Silica gel 60	60:40 n-hexane:acetone	50	125

In all cases, 5% (w/v) phosphomolybdic acid in ethanol was used as a visualization reagent.

preferred for screening tasks, uniaxially aligned CA nanofibers with the adequate thickness were appropriate and practical for use.

3.4. Optimal conditions for TLC and method validation results

After the aligned CA nanofibers collected at the drum speed of 6000 rpm over 6 h of spin time were selected as the suitable stationary phase, the optimal mobile phase was investigated by varying the ratio of methanol to water. For reversed-phase separation, 2.5% acetic acid was added in the mobile phase to render the analytes non-ionized. The results showed that all solutes were eluted from the original application spots ($R_f > 0$) and well separated by using the mobile phase containing 60–70% methanol (Fig. 3). Therefore, 65:35:2.5 methanol/water/acetic acid was chosen as the optimized mobile phase.

To ascertain the analytical performance, TLC method was studied in the aspect of the masses of the analytes which could be visualized on the plates and the specificity. Interestingly, the masses of HQ and RA which could be visually detected on the aligned CA nanofiber plates were twice lower than those on the non-aligned nanofibers (Table 4) due to the darker spots on this type of fibers as previously discussed. Moreover, the masses of both compounds which could be detected on the aligned CA nanofibers were five times lower than those on typical silica plates which were used in the ASEAN method using the same visualization reagent. It was probable that the thinner chromatographic bed of electrospun nanofibers (8.5 μm) compared to that of commercial silica gel plates (200–250 μm) contributed to the better ability to visualize the analyte at the lower mass. To the best literature review, this is the first report about the advantage of nanofibrous stationary phases, especially in the aligned orientation, over conventional TLC plates in the aspect of the lower mass of the analyte which

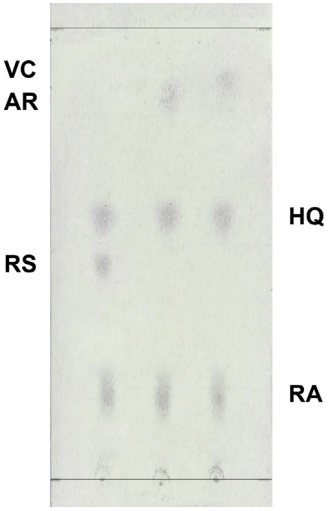


Fig. 4. Results of interference study for the proposed TLC screening method. The masses of HQ, RA, VC, AR and RS are 62, 335, 500, 500 and 50 ng per spot, respectively.

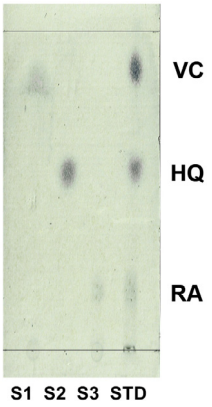


Fig. 5. TLC results for the analysis of three different samples on aligned CA nanofiber plates. S1 contained neither HQ nor RA whereas S2 and S3 were adulterated with HQ and RA, respectively. Standard (STD) RA, VC and HQ are in the right lane.

could be detected. For the specificity, the proposed method was free from the interference by the ingredients commonly found in cosmetic creams. As depicted in Fig. 4, HQ and RA were efficiently separated from the substances which could form colored spots with phosphomolybdic acid i.e. VC, RS and AR. For vitamin E and sodium metabisulfite, they did not react with phosphomolybdic acid and thus did not interfere with the test. Finally, the applicability of the proposed method was evaluated by the analysis of cosmetic creams collected from the local markets and it was found that the screening results were in agreement with the confirmatory HPLC method according to ASEAN guidelines [18,19]. Fig. 5 presents the TLC results of analysis of three different samples run on the aligned CA nanofiber plates. It was found that the sample S1 contained neither HQ nor RA whereas S2 and S3 were adulterated with HQ and RA, respectively.

4. Conclusion

Uniaxially aligned CA electrospun nanofibers were successfully fabricated by using high speed rotating drum collector and applied to use as TLC media. Besides the efficient separation ability, the aligned nanofibers with the appropriate layer thickness provided two to three times shorter run time than the randomly oriented ones. Furthermore, the masses of HQ and RA which could be detected on this material were two times lower than those on the

non-aligned nanofibers and five times lower than those on typical silica gel plates. In this work, the applicability of the aligned CA nanofibers was demonstrated in the screening of HQ and RA adulterated in cosmetics. Due to the satisfactory analytical performance as well as facile and inexpensive production process, aligned CA electrospun nanofiber is promising stationary phase for use in planar chromatography.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2014.09.043.

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