

Thin-Layer Chromatography of Metal Ions on Immobilized Chondroitin Sulfate Plate Prepared by Plasma Polymerization Technique

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ABSTRACT: Chondroitin sulfate (CS) as a sodium salt was impregnated within micropores of silica gel on a thin-layer chromatographic plate and coated by a thin film of plasma-polymerized propargyl alcohol (PPPA) for immobilization of CS macromolecules. Chromatographic behavior of cations such as Co^{2+} , Fe^{2+} , and Cu^{2+} was observed on the prepared thin-layer plate to explore ion-exchange functions from the acidic groups of CS. Affinity of the cations to the immobilized CS plate was enhanced with increasing CS impregnated in the silica gel, regardless of the kind of cation. Moreover, affinity was significantly altered by the mode of preliminary washing of the thin-layer plate, use of either aqueous or organic solvent, and adjusted pH of the solvent. The PPPA film itself indicated certain affinity to the cations, likely due to the oxygen-containing groups, e.g., $-\text{OH}$, partly dissociable in the polymer matrix. The proposed technique suggests other new thin-layer chromatographic plates in which various ion-attractive or ion-exchange materials are immobilized.

KEY WORDS Plasma-Polymerized Propargyl Alcohol / Immobilization of Chondroitin Sulfate / Thin-Layer Chromatography of Cations /

A number of studies have been reported for materials which separate or distinguish various ions, because such function is applicable to broad fields of chemical processes or analytical chemistry. A recent paper reports an attempt to prepare ion-exchange membrane by plasma polymerization.¹ The plasma polymerization technique has been also applied to thin-layer chromatography (TLC) and Yoshimura *et al.* reported successful results by coating a silica gel TLC plate with a plasma-polymer thin film prepared by use of propargyl alcohol (2-propyn-1-ol, PA) as a monomer.^{2,3} Although the TLC plate coated with plasma-polymerized PA (PPPA) altered the migration rates of organic substances in comparison with non-coated TLC plate, the plasma technique was further applied to a TLC plate preliminarily impregnated with sodium salt of chondroitin sulfate (CS).⁴ The immobilization of CS was carried out by coating a CS-impregnated plate with PPPA in a similar manner to the preparation of an immobilized enzyme membrane by the PPPA-coating.⁵ Immobilized CS exhibited affinity to some biochemical substances, which is likely due to the resemblance of the molecular structure of CS or the acidic groups of the CS molecule.

This paper reports ion-exchange functions for metal cations using the immobilized CS plates. Different ways of preliminary washing of the plates were involved in this experiment for enhancement of the ion-exchange ability. Metal cations tested were Co^{2+} , Fe^{2+} , and Cu^{2+} , since they are important biochemically and individual spots can be detected visibly.

The CS molecule shown in Figure 1 is constructed with alternately repeated segments of D-glucuronic acid and N-acetylchondrosamine (N-acetyl-2-amino-D-galactose), the latter of which is esterified as 4-sulfate or 6-sulfate.^{6,7} Sulfonic and carboxyl groups are therefore considered ion-exchange sites within the CS molecule.

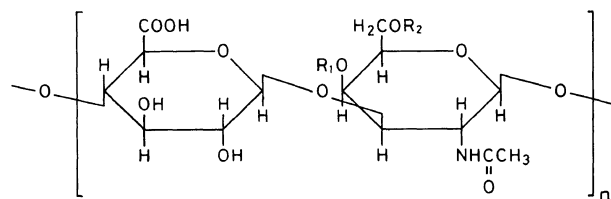
EXPERIMENTAL

Materials

Sodium CS (CS-Na) and acetone were supplied from Kanto Chemicals Inc. (Tokyo) and all the other reagents were from Nacalai Tesque Inc. (Kyoto). Quality of the reagents was guaranteed grade except that PA was an extrapure reagent. The TLC plate was purchased from Whatman Ltd. (United Kingdom) as a Silica Gel-Precoated Plate (thickness of silica gel layer, 250 μm ; back plate, aluminum).

Preparation of Thin-Layer Plate

The PPPA-coating of the TLC plate was carried out as described previously.^{4,8} The TLC plate ($5 \times 10 \text{ cm}^2$) was placed between a pair of parallel electrodes installed within the Plasma Deposition System Model BP-1 (Samco International Lab. Inc., Kyoto), and the plasma chamber was evacuated with High Vacuum Pump E2M-12 (3001 min^{-1} , Edwards Inc., United Kingdom). The rate of evacuation was controlled by a throttle valve, monitoring by a Thermocouple Vacuum Gauge (Thermocouple 1 and Gauge Head TC1, Edwards Inc.), so as to hold the pressure at the desired level. After



$\text{R}_1 = \text{SO}_3\text{H}$, $\text{R}_2 = \text{H}$
chondroitin sulfate A (chondroitin 4-sulfate).
 $\text{R}_1 = \text{SO}_3\text{H}$, $\text{R}_2 = \text{H}$, C-5' epimer
chondroitin sulfate B (β -heparin).
 $\text{R}_1 = \text{H}$, $\text{R}_2 = \text{SO}_3\text{H}$
chondroitin sulfate C (chondroitin 6-sulfate).

Figure 1. Chemical structure of chondroitin sulfate (CS).

preliminary evacuation, vapor of PA was slowly transported from a glass reservoir into the plasma chamber through a thin metal tube.

At approximately 0.1 Torr, plasma discharge was initiated and sustained by a radio-frequency electric power (13.56 MHz) of 5 W, while a thin film of PPPA was uniformly deposited on the TLC plate. Film thickness on the TLC plate could be controlled by the time of deposition. The CS-immobilized TLC plate was prepared by soaking a new TLC plate in aqueous CS-Na solution, air-drying overnight, and plasma processing as above.

Thin-Layer Chromatography

Chromatography was carried out using the intact plate, PPPA-coated plate, and immobilized CS plate. Preliminary washing was applied to the plate with distilled water or 3% HCl solution. Time for washing was regulated within 1 min for either case, but 1 min more with distilled water was added to the latter case for neutralization. After drying at room temperature, sample solutions of 2 μ l were spotted on the TLC plate. Sample solutions of Co^{2+} , Fe^{2+} , and Cu^{2+} were prepared by dissolving $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, FeCl_2 , and $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ in water at 5 g l^{-1} . Development of the sample ions was continued until the solvent front reached 8 cm from the origin.

Various developing solvents were considered, referring to solvent mixtures for ion-exchange chromatography and volatile buffer solutions for zone electrophoresis. The solvent mixtures were prepared as follows: (a) 0.1 M ethylenediamine/0.2 M tartaric acid, (pH 4); (b) 0.5 M nitric acid/10% pyridine in acetone/water (4:1), (pH 6); (c) pyridine/acetic acid/water, (pH 3.1, pH 4.7, pH 6.5); (d) 0.5 M nitric acid in acetone/water (4:1), (pH 1). The spots of Co^{2+} , Fe^{2+} , and Cu^{2+} were detected by coloration after spraying saturated acetone solutions of ammonium thiocyanate, *o*-phenanthroline (1,10-phenanthroline), and cuproine (2,2'-biquinoline), respectively. R_f of the individual spot was measured and relative ratios of R_f calculated as "Processed plate/Intact plate" were evaluated.

RESULTS AND DISCUSSION

Time for Coating

Since CS still holds ion-exchange functions after irradiation of PA plasma and PPPA film exhibits certain interactions with H^+ ,⁹ the CS-impregnated and/or the PPPA-coated plates were naturally assumed to interact with cations by the same mechanism as ion-exchange chromatography. In the previous study,⁴ the plasma processed plates were simply washed with developing solvent to remove weakly bound PPPA and CS on the TLC plate. However, in this work, a variety of washing processes was applied to improve ion-exchange ability.

An appropriate time for coating of the PPPA film on the TLC plate was selected because the film thickness is an important factor in interactions between sample cations and the surface of the TLC plate. A comparative study was made with a number of TLC plates processed by various conditions. The results are tabulated in Table I.

Table I. Effects of PPPA-coating and impregnation of CS on R_f of cations

(A) R_f -Value					
Cation	R_f value (SD)				
	I^a	P_{10}^b	P_{30}^c	C^d	$C+P^e$
Co^{2+}	0.85 (0.04)	0.85 (0.06)	0.88 (0.05)	0.51 (0.06)	0.44 (0.09)
Fe^{2+}	0.86 (0.02)	0.81 (0.03)	0.84 (0.02)	0.60 (0.07)	0.54 (0.03)
Cu^{2+}	0.80 (0.06)	0.88 (0.04)	0.87 (0.01)	0.40 (0.02)	0.56 (0.09)

(B) Ratios of R_f				
Cation	Ratio of R_f value			
	P_{10}^b/I^a	P_{30}^c/I	C^d/I	$C+P^e/I$
Co^{2+}	1.00	1.04	0.59	0.51
Fe^{2+}	0.94	0.98	0.70	0.63
Cu^{2+}	1.10	1.09	0.50	0.70

Silica gel TLC plate: ^a I , Intact. ^b P_{10} , PPPA-coated (10 min). ^c P_{30} , PPPA-coated (30 min). ^d C , Impregnated with CS-Na (3%). ^e $C+P$, Impregnated with CS-Na (3%) and PPPA-coated (10 or 20 min).

Developing solvent, 0.1 M ethylenediamine/0.2 M tartaric acid. Washing, water (1 min). Number of measurement, 3—6.

Mean R_f of the each cation is indicated in column (A) with standard deviation ($n=3-6$) and the ratio of R_f is in column (B). The data was obtained from five types of test plates, namely, the intact TLC plate, TLC plate coated with PPPA for 10 min, the same plate coated for 30 min, CS-impregnated plate using 3% CS-Na solution, and finally the CS-impregnated and PPPA-coated plate. The test plates were washed with water for 1 min and air-dried before chromatography with a mixed solvent of 0.1 M ethylenediamine/0.2 M tartaric acid.

In terms of R_f , the migration rates of the cations on the simple PPPA-coated plates were almost independent of time for coating in spite of large differences of film thickness. Since the deposition rate of PPPA was determined as roughly 450 \AA min^{-1} , the thickness of the polymer film would grow up to 4500 \AA or 13500 \AA during a deposition time of 10 min or 30 min, respectively. Elliptical extension of sample spots along the direction of solvent flow was reduced on the PPPA-coated plates in comparison with intact TLC plate. Since the elliptical extension of a sample spot appears when solvent flow is comparably fast with respect to adsorption force,¹⁰ the PPPA-coating should bring about a proper balance between the rate of development and adsorption force, hence reducing the elliptical extension of the spots. Relatively higher R_f was observed with Co^{2+} and Cu^{2+} , while lower values were obtained with Fe^{2+} . This implies certain differences of interactions between the metal cations and PPPA film. The PPPA network contains abundant oxygen atoms as hydroxyl, carbonyl and etheric groups.⁸ Partial dissociation of the first group acts as a cation exchange site and the other groups capture cations due to weak negativity. It is therefore understandable that the different cations showed individual interactions with the three oxygen groups within

the PPPA network.

In the case of the CS-impregnated TLC plate, significant decrease of R_f of three cations was observed, although CS retained in the TLC plate was simply adsorbed on microstructure of silica gel. The physico-chemical state of the CS in the TLC plate has not been studied in detail, and CS-Na molecules seem to be trapped fairly well in the micropores of silica gel possibly bound by intermolecular forces between the CS-Na and the silanol groups of silica gel. Noticeable cation-capture ability of CS was thus evident. The sample spots, however, showed some distortion and tailing. Such spots unsuitable for TLC suggest that the CS adsorbing the cations is gradually carried by the developing solvent.

When the CS-impregnated TLC plate was further coated with PPPA, the distortion and tailing of the spots were much reduced and R_f were not affected by the time of PPPA-coating for either 10 min or 20 min. Therefore, the CS-Na molecules adsorbed on the silica gel were immobilized by enclosure with the PPPA film and time needed for the immobilization should be 10 min or less.

The ratio of R_f tabulated in (B) indicates more clearly the effects from PPPA-coating and impregnation of CS on the migration rates of the cations. The plate processed with CS-impregnation and PPPA-coating lowered the ratio of R_f to 50–70% compared with the intact TLC plate. Although this would be due to the presence of CS, Cu^{2+} showed somewhat higher ratios of R_f probably because of weaker interactions with the PPPA film than other cations. It is concluded that the overall effect of CS-immobilization on cations was derived from both cation-CS and cation-PPPA interactions.

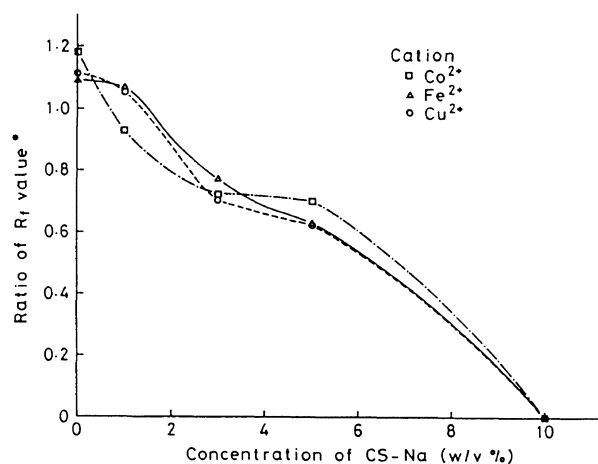
Development with Aqueous Solvent

Investigation was continued on cation-capture ability of the immobilized CS plate, whether it was dependent on the amount of CS impregnated and way of washing before the development. The developing solvent was an aqueous solution of 0.1 M ethylenediamine/0.2 M tartaric acid. Figure 2 shows the results of different pretreatments, (A) without washing, (B) washing with water, and (C) washing with 3% HCl. The abscissa indicates the concentration of aqueous CS-Na solution used for impregnation within the TLC plate. The ordinate is employed for the ratio of R_f obtained by comparison with the intact TLC plate.

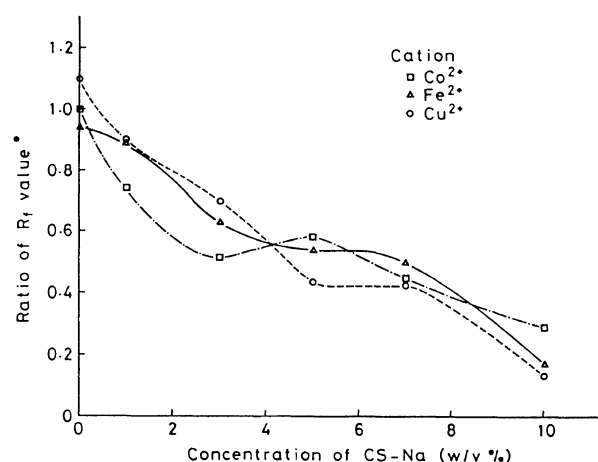
Regardless of the mode of preliminary washing, the ratio of R_f of any cation mostly decreased with increasing concentration of CS-Na solution (0–10%) for impregnation. However, fairly characteristic curves drawn changed in dependent on the way of washing. Inconsistency of the curves of three cations, which was hardly recognized in the case of (A), became prominent in the case of (B) and more in the case of (C). It was clear that the preliminary washing with water removed low molecular species of PPPA and some CS-Na molecules due to incomplete immobilization. The washing with water seemed to promote partial desalination of CS-Na and conversion to CS form. Washing with 3% HCl considerably converted CS-Na to free CS, so that the evaluation of the cations-CS interactions should become more substantial.

Comparison of cases (B) and (C) showed large dif-

(A) Without washing



(B) Washing with water



(C) Washing with 3% HCl

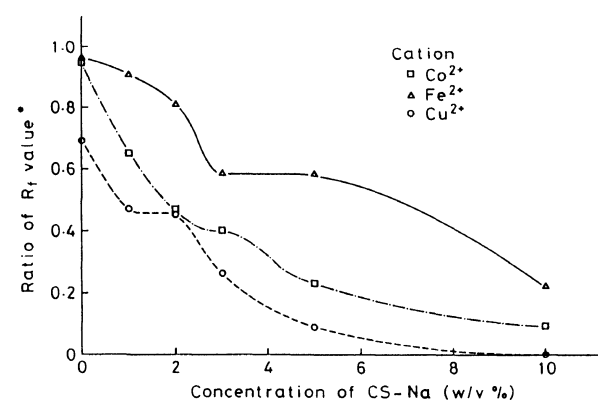


Figure 2. Effects of CS-immobilization on ratios of R_f of cations using aqueous developing solvent: substrate, silica gel TLC plate; developing solvent, 0.1 M ethylenediamine/0.2 M tartaric acid. *CS-Immobilized/Intact. (A) without washing; (B) washing with water; (C) washing with 3% HCl.

ferences of the ratios of R_f for Co^{2+} and Cu^{2+} , while small changes with Fe^{2+} . It is supposed that the ion-exchange rate between H^+ and Fe^{2+} is not far from that between Na^+ and Fe^{2+} or that other complex surface interactions caused the above results.

Exceptionally different behavior of Cu^{2+} was observed when the plate was washed with 3% HCl. The TLC plate

Table II. Time for development of cations using aqueous and organic solvents^a

Concentration of CS-Na w/v %	Time for development/h	
	0.1 M ethylenediamine /0.2 M tartaric acid	0.5 M HNO ₃ /10% pyridine /acetone: water (4:1)
0	0.5	0.5
1	1.0	0.5
3	2.0	0.5
5	5.0	0.5
10	≥ 7.0	0.5

^a Distance of development, 8 cm.

coated only with PPPA showed the ratio of R_f of 0.69 for Cu^{2+} when the plate was preliminarily washed with 3% HCl. Therefore, the characteristic behavior of the Cu^{2+} in the case of (C) seems to result from two types of cation-capture ability from free CS and PPPA film after the acid treatment. This implies that PPPA itself may be utilized as cation-capture material after proper pretreatment.

Developing Solvent and Time for Development

Although the ion-capture ability of the immobilized CS plate is enhanced with increasing concentration of CS-Na solution utilized for impregnation of the TLC plate, the time necessary for development of the cations is markedly longer. Table II shows the time needed for development of 8 cm using aqueous and organic solvents.

Using 0.1 M ethylenediamine/0.2 M tartaric acid aqueous solvent, the time for development varied from 30 min to 7 hrs or more, owing to the concentration of CS-Na for impregnation from 0 to 10%. Such time for development was thought caused by gelation of CS where a large amount of water in the developing solvent was retained for the swelling of CS.

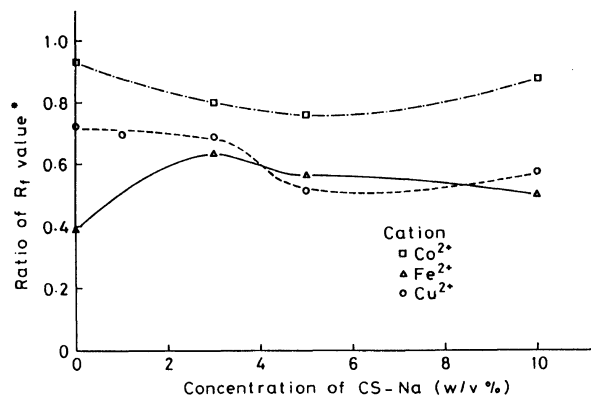
The organic solvent was therefore used. Acetone-water (4:1) mixed solvent was employed to make up 0.5 M nitric acid/10% pyridine solution. In contrast to the aqueous developing solvent, the organic solvent migrated on the immobilized CS plate at a constant speed (30 min/8 cm) regardless of the amount of CS on the plate.

Development with Organic Solvent

The relationship between the concentration of CS-Na solution used for impregnation and ratio of R_f (CS-Immobilized/Intact) was studied with the organic developing solvent in the same way as in the case of the aqueous solvent. The results are illustrated in Figure 3, where (A) indicates the data obtained after the preliminary washing with water and (B) is with 3% HCl.

In the case of (A), all curves of three cations exhibited no decrease in the ratio of R_f with increasing CS-Na impregnated. Such behavior was noticeably different from the case using the aqueous developing solvent. However, the ratios of R_f for Co^{2+} and Cu^{2+} with the simply PPPA-coated (without CS) plate indicated similar values to that with the aqueous solvent, while Fe^{2+} showed an exceptionally low ratio of 0.4 to the intact TLC plate.

(A) Washing with water



(B) Washing with 3% HCl

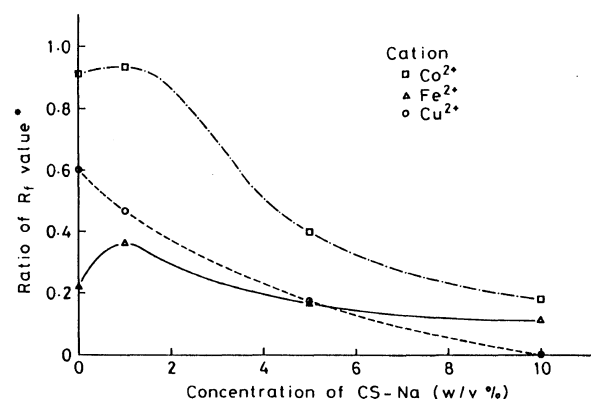


Figure 3. Effects of CS-immobilization on ratios of R_f of cations using organic developing solvent: substrate, silica gel TLC plate; developing solvent, 0.5 M HNO₃/10% pyridine/acetone-water (4:1). *CS-Immobilized/Intact. (A) washing with water; (B) washing with 3% HCl.

The preliminary washing with 3% HCl led to reduction in the ratio of R_f with increasing CS-Na as shown in (B). These results suggest that development with organic solvent such as the case of (A) suppressed gelation and partial desalination of CS-Na impregnated. On the other hand, preliminary washing with 3% HCl in such case as (B) produced much free CS within the PPPA matrix, so that cation-capture ability was enhanced with increasing CS-Na impregnated even if the development was carried out with organic solvent.

By detailed comparison of the affinity to the stationary phase, Cu^{2+} was more efficiently captured than Co^{2+} whenever developed with the organic solvent or on the processed plate preliminarily washed with 3% HCl. Great increase in affinity of Fe^{2+} was brought about by the organic solvent, and Fe^{2+} showed the strongest affinity after preliminary washing with 3% HCl in all cases of the TLC plate simply coated with PPPA. This affinity of PPPA for Fe^{2+} should also contribute to the affinity between Fe^{2+} and the immobilized CS plate prepared by PPPA-coating, while the affinity of the plate for Cu^{2+} became comparable to and exceeded that for Fe^{2+} by increasing the amount of CS-Na impregnated. Overall, these results imply that the affinity of metal cations to the immobilized CS plate is derived not only from the acidic functional groups of CS but also from

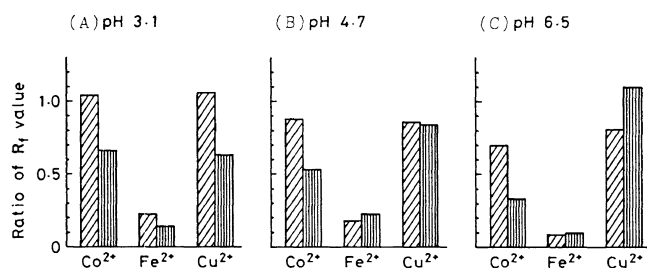


Figure 4. Effects of pH of developing solvent on R_f of cations: TLC plate, silica gel on aluminum foil; washing, water (1 min); developing solvent, pyridine/acetic acid/water. Ratio of R_f -value: , [PPPA-coated (10 min)]/[intact]; , [impregnated with CS-Na (3%) and PPPA-coated (10 min)]/[intact].

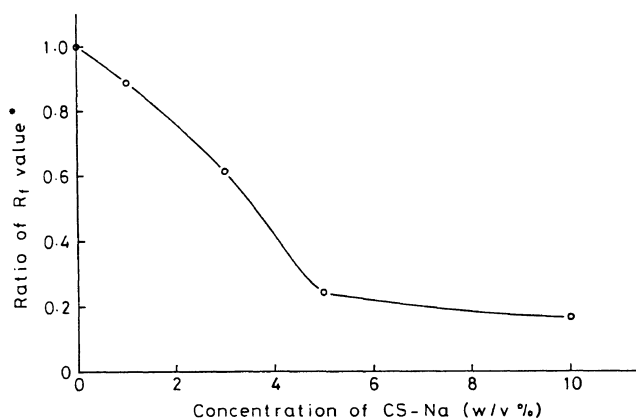


Figure 5. Effects of CS-immobilization on ratios of R_f of Fe²⁺: substrate, silica gel TLC plate; developing solvent, 0.5 M HNO₃/acetone-water (4:1); washing, 3% HCl. *CS-Immobilized/Intact.

the properties of PPPA.

pH of Developing Solvent

Generally, the chromatographic behavior of cations is dependent on pH of the eluent during ion-exchange chromatography. Considering the existence of ion-exchange sites such as sulfonic and carboxylic groups in CS, the pH of developing solvent should affect R_f of cations on the immobilized CS plate. This point was investigated with the immobilized CS plate and the simply PPPA-coated plate by changing pH of the developing solvent composed of pyridine/acetic acid/water. The mixed solvent is "volatile buffer solution" excluding metal ions and the pH is adjusted by changing the compositions of pyridine and acetic acid over a wide range. The effect of pH on R_f was examined by comparison of the ratios of R_f (Processed/Intact). The results are shown in Figure 4 as a bargraph with data for pH 3.1, 4.7, and 6.5.

It was observed that the bargram for the PPPA-coated plate generally reduced ratio of R_f at higher pH, suggesting ion-exchange ability of PPPA with cations. The immobilized CS plate prepared by impregnation of 3%

CS-Na showed different results for the cations, probably due to competitive interactions between PPPA and CS which both were reactive to pH.

As a comparative example, Fe²⁺ was developed on the immobilized CS plate with strongly acidic solvent and the data are shown in Figure 5. Solvent composition was 0.5 M nitric acid/acetone-water (4:1) with an apparent pH of 1. A wide range of ratio of R_f was obtained by changing the amount of CS on the TLC plate, and time for development was within 30 min. Therefore, this TLC condition was judged to be favorable for development of Fe²⁺ in terms of the time needed for the development and the range of controllable R_f by reference to the results shown in Figures 2, 3, and Table II. The instance suggests that ratios of R_f for cations were largely altered by pH of the developing solvent on the processed TLC plate.

CONCLUSIONS

It may be concluded that the CS-immobilized TLC plate displays cation-capture ability largely affected by the presence of PPPA film, amount of CS immobilized, way of preliminary washing of the processed plate, and developing solvent adjusted at different pH. PPPA film contributed not only to immobilization of CS but also to capture of metal cations. Cation-capture ability of the immobilized CS plate was derived from synthetic properties of PPPA film and CS immobilized. The plasma polymerization technique should be extensively applicable to the preparation of other chromatographic media or biochemical sensors.

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