# Preparative Thin-Layer Chromatography of Semi-Xylenol Orange and Xylenol Orange by Development with Solvents Giving Discrete pH Change on the Plate*

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A new procedure is proposed to obtain pure Semi-Xylenol Orange (SXO) and Xylenol Orange (XO) from *ca.* 100 mg of synthetic mixture using preparative thin-layer chromatography on cellulose layer *ca.* 2 mm thick. Development with a composite solvent, 55—60 ml of 1-butanol saturated with 25% aqueous acetic acid-0.6—0.8 ml of 28% aqueous ammonia, forms two discrete zones of different pH values on the layer. The zones favor a sharp separation of polybasic Brønsted acids with structures similar to those of SXO and XO.[1] SXO and XO are in the protonated forms corresponding to the pH values of the given zones. XO is located near the starting line in the lower zone of higher pH and SXO migrates through the layer in the upper part of the same zone. Both SXO and XO constitute the compact and concentrated bands. Each run takes only 3 hr. 18 mg of SXO and 36 mg of XO are obtained as sodium salts from 100 mg of the synthetic mixture.

Semi-Xylenol Orange, 3-[*N*,*N*-bis(carboxymethyl)-aminomethyl]-*o*-cresolsulfonphthalein, is an appropriate chelating agent for spectrophotometric determination of zirconium(IV)[2] and for interpreting the kinetic behavior for the metal complex formation in a strongly acid medium.[3]

Xylenol Orange, 3,3′-bis[*N*,*N*-bis(carboxymethyl)-aminomethyl]-*o*-cresolsulfonphthalein, is a well-known metallochromic indicator.[4] However, there has been considerable disagreement in the results obtained by different authors on the solution chemistry of the metal complex formation.[5] The disagreement is caused by the use of "*impure*" XO containing iminodiacetic acid (IDA) and SXO with complexing properties and the lack of attention to the existence of metal complex species other than mononuclear 1:1 complex.[6]

Two methods have been reported for the purification of XO and/or SXO: (1) Selective precipitation of XO with ethanol from a saturated aqueous sodium acetate solution containing the mixture,[7] and (2) column chromatography on cellulose powder with the eluent, 1-butanol–acetic acid–water.[2,8] By the former, XO is partly decomposed[2,8] and cannot sufficiently be separated from SXO and IDA.[8] The latter causes serious tailing and cross-contamination of XO and SXO.

In the present paper we propose a new procedure to obtain "*pure*"[9] SXO and XO from a synthetic mixture. The procedure is preparative thin-layer chromatography with a developing solvent, 55—60 ml of 1-butanol saturated with 25% aqueous acetic acid-0.6—0.8 ml of 28% aqueous ammonia. The solvent yields on the cellulose layer two distinct zones I and II with stepwise different pH values. Zones I and II correspond to the regions of lower and higher pH values, respectively. Development with the composite solvent satisfactorily separates SXO, XO and *o*-Cresol Red (*o*-CR). SXO is thus concentrated in the upper part of zone II, while XO near the starting line in the same zone. *o*-CR moves much faster than both SXO and XO and is located in zone I at the top of the plate. The band of XO partly overlaps that of IDA and further separation is required. Development with the solvent, acetone–water–1 M hydrochloric acid (160/59/1, v/v/v), is found to be very effective for this purpose. "*Pure*" specimens of SXO and XO in the forms of free acid are prepared by cation exchange.

## Experimental

*Materials.* *Reagents*: All the chemicals used were of analytical grade, unless otherwise specified. Water was deionized and distilled. *n*-BuOH was distilled.

*SXO and XO*: These compounds were synthesized by Mannich condensation.[4] 1.91 g ($0.5\times10^{-2}$ mol) of *o*-CR, 1.33 g ($1.0\times10^{-2}$ mol) of IDA and 1 g of NaOH were dissolved in 50 ml of glacial acetic acid followed by dropping 1 ml of 37% HCHO and kept at 65 °C for 10 h with stirring. The solvent was distilled off under reduced pressure to give a dry sample.

*Cellulose Powder*: Toyo-Roshi cellulose powder (300 or 200—300 mesh) was suspended in 0.5 M HCl and left to stand overnight. The suspension was sucked off, washed with water until washings became free from chloride, washed with acetone and dried in an oven at 80 °C for 2 h.

*Ion Exchanger*: A macroreticular strongly acid cation-exchange resin of low cross-linkage, Diaion PK-204 (Mitsubishi Kasei, Ltd.), was treated with HCl to remove trace of metal impurities and used in the hydrogen-form.

*Preparative Thin-Layer Chromatography.* *Adsorbent Layer*: A slurry was prepared by adding 90 ml of water to 30 g of the pretreated cellulose powder. A layer was obtained by the pouring procedure:[10] The slurry was rapidly poured on the middle of a glass plate (20×20 cm) and uniformly distributed over the plate by gentle tilting and shaking. The layer was sufficiently dried in the air at room temperature to avoid cracking during the course of heating. The plates were heated in an oven at 110 °C for 80 to 100 min, and stored in a desiccator over silica gel. Fairly uniform layers *ca.* 2 mm thick were usually prepared.

*Chromatographic Chamber*: A sandwich chamber (20×20×0.3 or 0.6 cm) was used. The terminal edge of the chamber was kept open during the development in order to allow free evaporation of the solvent at the top of the plate.[10]

*Application of the Sample*: About 100 mg sample powder was dissolved in 0.36 ml of water and successively applied with a 0.06 ml band-pipet (2.6 cm wide) in a line *ca.* 15 cm wide on

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the layer 1.5 cm apart from the solvent surface in a sandwich chamber trough. After the application of the sample the plate was stored in a desiccator over silica gel until the water of the sample solution evaporated.

*Developing Solvents*: *Fifty-five* to *sixty* milliliters — a volume sufficient for one run — of *n*-BuOH saturated with 10, 20, or 25% aqueous HOAc was poured into a sandwich chamber trough (19×207×23 mm), and 0.6—0.8 ml of 28% aqueous $NH_3$ was added drop by drop so as to form an emulsion as uniformly as possible. Developing was started immediately after the preparation of the emulsion.

*Identification of Each Band on the Chromatogram*: The bands of *o*-CR, SXO and XO were readily identified by their intense coloration on the plate. It was necessary to confirm a position of the colorless band of IDA under similar experimental conditions beforehand, otherwise the band was inevitably covered with the intense coloration of XO. The band of IDA was detected by spraying a copper(II) acetate solution on the layer.

*Transfer of SXO and XO from the Adsorbent to Solutions*: The bands of SXO and XO on the layer were carefully scraped off with a glass rod, extracted with a small amount of water and dried *in vacuo*.

*Ion Exchange.* The extracts of SXO and XO were subjected to cation exchange to yield their free acids.

*$H_4SXO$*: About 50 mg of the sodium salt of purified SXO was dissolved in 25 ml of water, passed through a column containing 4 ml of pretreated cation-exchange resin, and eluted with 25 ml of water. A small part of SXO remaining on the resin phase was thoroughly washed out with a small amount of *n*-BuOH saturated with water.

*$H_6XO$*: About 50 mg of the sodium salt of purified XO was dissolved in 25 ml of water, passed through a column containing 9.5 ml of resin, and eluted with 75 ml of water. A small part of XO retained on the resin was washed out with a small amount of *n*-BuOH saturated with water.

Each effluent was evaporated to dryness at room temperature under reduced pressure and further kept *in vacuo* for 3 days to yield a dried sample. For the XO portion a further separation from IDA was required.

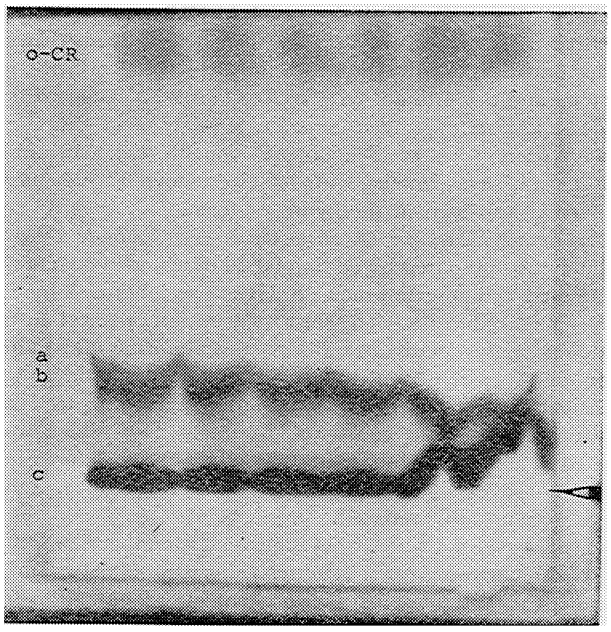



Fig. 1. Chromatogram developed for 3 hr with 60 ml of *n*-BuOH satd. with 10% HOAc–0.6 ml of 28% $NH_3$. a) $NH_4$-front, b) SXO, and c) XO.

## Results

*Separation of SXO and XO.* Figure 1 shows a chromatogram with the developing solvent, 60 ml of *n*-BuOH saturated with 10% HOAc–0.6 ml of 28% $NH_3$.

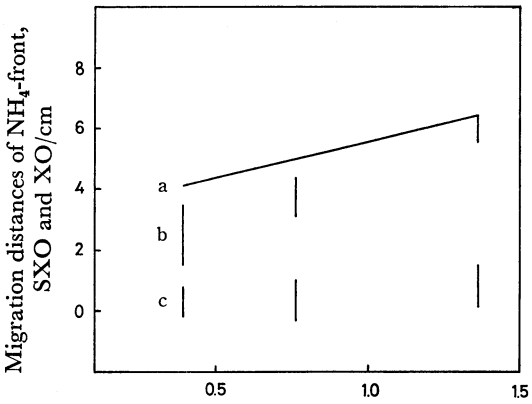



Fig. 2. Dependence of the migration distances of SXO, XO, and $NH_4$-front on the concentrations of HOAc contained in the *n*-BuOH satd. with 10, 20, and 25% HOAc.
The developing solvents used are 60 ml of *n*-BuOH saturated with 10, 20, and 25% HOAc–0.6—0.8 ml of 28% $NH_3$. a) $NH_4$-front, b) SXO, and c) XO.

Figure 2 shows the dependence of the migration distances of SXO, XO and $NH_4$-front on the concentrations of HOAc contained in *n*-BuOH saturated with 10, 20 and 25% HOAc. The developing solvents are 60 ml of *n*-BuOH saturated with 10, 20 and 25% HOAc–0.6—0.8 ml of 28% $NH_3$. It is found that as far as the experimental conditions are concerned the greater the concentration of HOAc, the better the

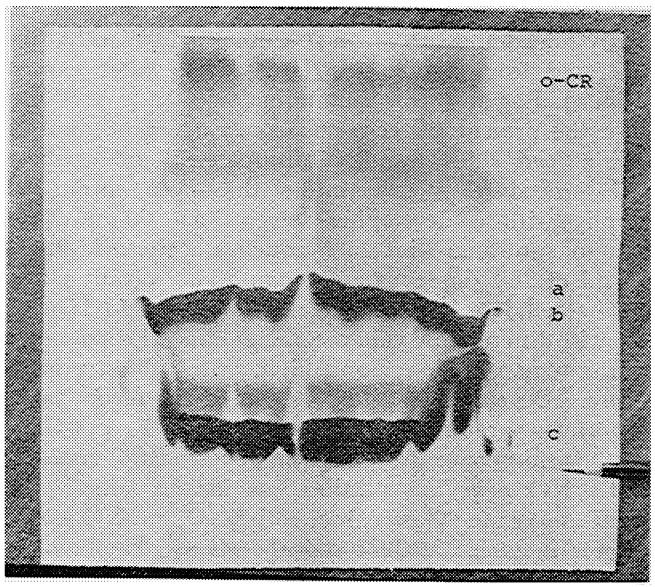



Fig. 3. Chromatogram developed for 3 hr with 60 ml of *n*-BuOH satd. with 25% HOAc–0.8 ml of 28% $NH_3$. a) $NH_4$-front, b) SXO, and c) XO.

separation of SXO and XO. Figure 3 gives the chromatogram obtained in the case of 25% HOAc and 0.8 ml of 28% $NH_3$ added.

SXO is finely separated from XO under the following conditions: The sample is applied on the layer 1.5 cm apart from the solvent surface in the trough, and developed with an emulsion freshly prepared in the trough by adding 0.6—0.8 ml of 28% $NH_3$ drop by drop to 55—60 ml of *n*-BuOH saturated with 25% HOAc.

The solvent, 55—60 ml of *n*-BuOH saturated with 10, 20 and 25% HOAc–0.6—0.8 ml of 28% $NH_3$ forms zones I and II with discrete different pH values, the zones being divided by a distinct boundary. After 3 hr development using 60 ml of *n*-BuOH saturated with 25% HOAc–0.8 ml of 28% $NH_3$ without the sample applied, three acid-base indicators, Bromophenol Blue, Bromocresol Green and Bromothymol Blue, are sprayed on the layer. They exhibit uniform visible colorations on the layer corresponding to the pH value of both zones I and II as shown in Table 1.

Table 1. The coloration of the layer sprayed with acid-base indicators

| Zone | Acid-base indicators | | |
|---|---|---|---|
| | BPB[a)] | BCG[b)] | BTB[c)] |
| I | Yellow | Yellow | Yellow |
| II | Blue | Blue | Bluish green |

a) Bromophenol Blue. b) Bromocresol Green.
c) Bromothymol Blue.

Uniformity of the sensitive coloration indicates that the given solvent forms two distinct zones of discrete pH values as defined by the acid-base indicators. The pH of zone I is estimated to be lower than 3.0 and that of zone II between 5.4 and 6.0.

*Separation of XO and IDA.* In this stage of separation the band of XO is inevitably contaminated with IDA. Acetone–water (8/2, v/v) is useful for the separation of the sodium salts of aminopolycarboxylic acids by paper chromatography.[11] *Seventy* milliliters of a solvent, acetone–water–1 M HCl (160/59/1, v/v/v) was found to be effective to separate 5 mg mixture a band-pipet. The mixture contains *ca.* 90% XO and *ca.* 10% IDA in free acid form. It is necessary to confirm a position of the band of IDA under similar experimental conditions beforehand. In Fig. 4 the band of IDA is schematically reproduced by d, where 10 mg of pure IDA in the acid form is applied. *Thirty* milligrams of the mixture in the free acid form was applied on the layer (prepared from cellulose powder of 200—300 mesh) 1.0 cm apart from the solvent surface in a trough and developed with 70 ml of the solvent, acetone–water–1 M HCl (160/59/1, v/v/v) (Fig. 4), each run taking 1 hr. A sandwich trough (34×205×29 mm) was used.

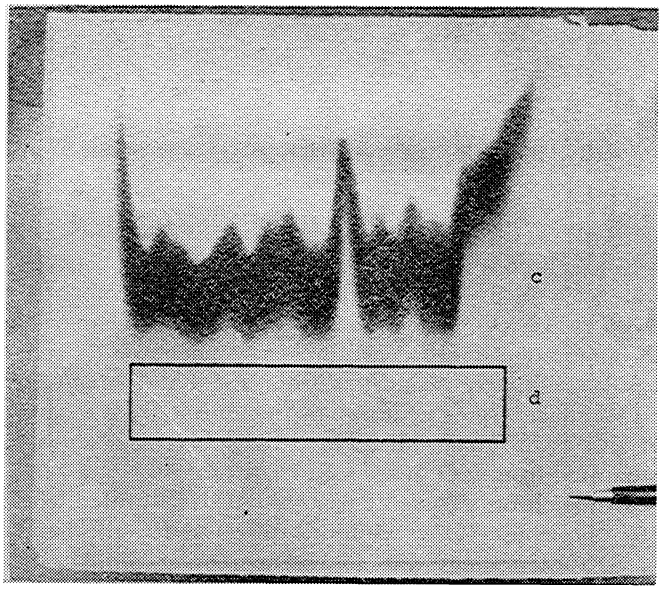



Fig. 4. Chromatogram developed for 1 hr with 70 ml of acetone–water–1M HCl (160/59/1, v/v/v).
c) XO and d) IDA.

*Elemental Analysis.* Table 2 gives the results of elemental analyses together with the decomposition points for the purified specimens of SXO and XO in free acid form, which have no melting points. The decomposition points were determined by a Rigaku Denki Differential Scanning Calorimeter Model 8001 SL/C. The temperature was calibrated with anthracene (216 °C) and anthraquinone (286 °C).

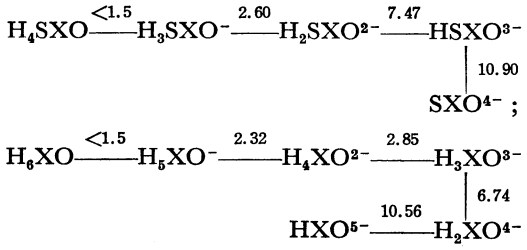
## Discussion

The solvent *n*-BuOH–HOAc–$H_2O$–$NH_3$ forms on the layer two zones I and II with stepwise different pH values. Zone I consists of *n*-BuOH–HOAc–$H_2O$ system which primarily acts as an organic phase for the solute species. Zone II consists of $NH_4OAc$–HOAc–*n*-BuOH–$H_2O$ system which primarily acts as an aqueous phase for solutes.

The polybasic Brønsted acids SXO and XO dissociate to the species corresponding to the pH of zone II. They dissociate in aqueous solution with acid dissociation constants, $pK_a$,[8] as follows.

Table 2. Analytical data of the purified specimens of SXO and XO

| Compound | Formula | Dp (°C) | | Elemental analyses | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | C, % | H, % | N, % | S, % | C/N |
| SXO | $C_{26}H_{25}O_9NS \cdot H_2O$ | 201 | Calcd | 57.24 | 4.99 | 2.57 | 5.87 | 22.3 |
| | | | Found | 56.05 | 4.97 | 2.53 | 5.68 | 22.2 |
| XO | $C_{31}H_{32}O_{13}N_2S \cdot 2H_2O$ | 281 | Calcd | 52.54 | 5.12 | 3.95 | 4.52 | 13.3 |
| | | | Found | 52.40 | 4.93 | 3.95 | 4.20 | 13.3 |




Since there is a discrete change in pH value between zones I and II, SXO exists only as a diprotonated species $H_2SXO^{2-}$ in zone II, and cannot migrate through zone I; it constitutes a concentrated "*one*" band completely separated from the band of *o*-CR. XO is concentrated near the starting line as $H_3XO^{3-}$ and $H_2XO^{4-}$ in zone II. Thus SXO is satisfactorily separated from XO and *o*-CR.

In the chromatography using *n*-BuOH saturated with 10% aqueous HOAc without aqueous $NH_3$, the band of SXO lengthens due to the increasing number of species in protolytic equilibria and causes serious cross-contamination with XO and *o*-CR.[1)]

The best conditions for the separation of SXO and XO in preparative thin-layer chromatography (p-tlc) differ from those in thin-layer chromatography (tlc).[1)] In tlc, XO migrates through zone II beneath the $NH_4$-front and SXO migrates through zone I. In p-tlc, both XO and SXO migrate through zone II. The difference is due to the fact that the amount of $NH_4OAc$ formed in zone II is in large excess relative to that of the solute species applied. Thus $NH_4OAc$ forms a saturated adsorption band in zone II and XO migrates through zone II beneath the $NH_4$-front. In p-tlc, however, the $NH_4OAc$ cannot form a saturated adsorption band in zone II under the conditions mentioned above owing to the increased amount of the solutes applied. Therefore, both SXO and XO remain in zone II. With increase in the amount of $NH_3$ added the chromatographic behavior of p-tlc approaches that of tlc.

However, in the deeper portion of the layer the band of SXO becomes considerably overlapped with that of XO.

With a thick layer some substances show different rates of migration in the surface and the deeper portion of the layer.[12)] We observed that the band of SXO moves faster in the surface of the layer than in the deeper layer, but this is not the case for XO. Under the given conditions, the band of SXO is not contaminated with XO and IDA either in the surface or in the deeper layer.

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