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# SEPARATION OF GEOMETRIC ISOMERS OF RETINYL ESTER, RETINAL AND RETINOL, PERTAINING TO THE VISUAL CYCLE, BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

A method for the analytical and/or preparative separation of the 13-*cis*, 11-*cis*, 9-*cis* and all all-*trans* isomers of retinyl palmitate ester, retinal and retinol by high-performance liquid chromatography is described. A straight-phase adsorption system consisting of Si 60 silica gel as the stationary phase and mixtures of *n*-hexane and dioxane as mobile phases were employed, using photometric detection at either 320 or 360 nm, depending on the nature of the compounds being studied. With simple adaptation of the phase system, all geometric isomers within each group could be separated. The precision of the method was 0.5% at the 10-$\mu$g (about 40-nmole) level and 4% at the 10-ng (about 40-pmole) level ($n = 3$). The detection limit was about 0.5 ng (about 2 pmole). The suitability of the phase system is demonstrated by chromatograms of test mixtures and of eye extracts. The fractionation of 0.5 mg of an isomeric sample could be achieved on a column of length 250 mm and I.D. 10 mm.

## INTRODUCTION

The visual process is believed to be a cyclic one, linking the photolysis and regeneration of rhodopsin by metabolic reactions. The elucidation of this mechanism is still under investigation, but it is believed that three major classes of compounds play a crucial role, namely retinyl esters, retinals and retinols, and more specifically the 11-*cis* and all-*trans* isomers of these compounds[1]. Some thin-layer chromatographic(TLC)[2–6] separations have been reported that do not allow complete separation. The greater efficiency of high-performance liquid chromatography (HPLC) facilitates

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the complete separation of all common geometric isomers. Moreover, HPLC has some additional advantages over TLC such as more convenient collection of compounds after separation with less risk of isomerization, thermal degradation and oxidation, and a lower detection limit when using the native absorbance of these compounds.

Vecchi *et al.*[7] reported the separation of 13-*cis*-, 11-*cis*-, 9-*cis*- and all-*trans*-retinyl acetate in about 60 min by means of liquid–liquid chromatography, using 3,3'-oxydipropionitrile as the stationary phase and *n*-hexane as the eluent. They also separated 13-*cis*-retinyl acetate and all-*trans*-retinyl acetate by liquid–solid chromatography (LSC) on silica using mixtures of dioxane and *n*-hexane. In addition to the 13-*cis* and all-*trans* isomers they included 9-*cis*-retinyl acetate in their separation on alumina using *n*-hexane and 1% of diethyl ether as the mobile phase. Rotmans and Kropf[8] were the first to separate the three common mono-*cis* isomers (*i.e.*, 13-*cis*, 11-*cis* and 9-*cis*) and the all-*trans* isomer of retinal. They used silica and 2% of diethyl ether in light petroleum as the eluent. The analysis took 8 min, with a detection limit of about 20 ng (*ca.* 80 pmole). Pilkiewicz *et al.*[9] used a CN-modified silica and 1% of diethyl ether in *n*-hexane to separate all-*trans*-retinal and the three mono-*cis* isomers of retinal in about 20 min. Recently, Tsukida *et al.*[10] reported the separation of the *cis–trans* isomers of retinal in about 20 min on silica with 12% of diethyl ether in *n*-hexane as the eluent. Tsukida *et al.*[11] were also able to separate the common geometric isomers of retinol by LSC, using a mixture of 7.5% of ethyl acetate and 9.5% of dichloromethane in *n*-hexane as the eluent. Bridges[1] included only the 11-*cis* and all-*trans* isomers in his study and separated those isomers of retinyl ester (2% of diethyl ether in *n*-hexane), retinal (12.5% of diethyl ether in *n*-hexane) and retinol (20% of diethyl ether in *n*-hexane) by LSC on silica.

In general it can be stated that these methods can be improved with respect to either separation time, resolution, peak shape or detection limit. Moreover, no suitable procedure is available at the moment for the case in which all common geometric isomers (13-*cis*, 11-*cis*, 9-*cis* and all-*trans*) of retinal, retinol and retinyl ester are present. Therefore, there is a need for a more efficient HPLC method as both an analytical and a preparative tool.

## EXPERIMENTAL

### *Apparatus*

The liquid chromatographic apparatus consisted of a Du Pont chromatograph (Model 830), equipped with a sampling device (Valco valve, CV-6-UHPA) and a variable-wavelength spectrophotometer (Zeiss, PM 2D), with a linear potentiometric recorder (Goertz, Servogor RE 512). In quantitative experiments all peaks were integrated by means of an electronic integrator (Spectra Physics, Autolab System I). The columns were made of stainless steel 316. The dimensions of the analytical column were length 250 mm and I.D. 3.0 mm and those of the preparative column were length 250 mm and I.D. 10 mm. All tubing and unions (Swagelok) were made of stainless steel 316 and the crucial fittings (valve–column and column–detector) were of the low dead volume type.

### *Materials and methods*

Silica gel (Si 60; Merck, Darmstadt, G.F.R.), sieved to a specific particle size,

was used as an adsorbent. The columns were packed by means of a balanced-density technique[12], followed by activation as described elsewhere[13]. The capacity ratio was calculated from the retention time of component $i$ ($t_{Ri}$) and the retention time of the unretained component ($t_{R0}$) as:

$$\kappa_i = \frac{t_{Ri}}{t_{R0}} - 1 \qquad (1)$$

Benzene was used as the unretained component in most experiments. In phase systems in which benzene was retained, $t_{R0}$ was determined from the known mobile phase column volume (1104 $\mu$l) and the measured flow-rate. In all experiments *n*-hexane was used with dioxane in varying concentrations as a modifier. All solvents were of analytical-reagent grade. All-*trans*-, 9-*cis*- and 13-*cis*-retinaldehyde (retinal) were obtained from Sigma (St. Louis, Mo., U.S.A.). The 11-*cis*-retinaldehyde isomer was isolated by photoisomerization of all-*trans*-retinal according to the procedure of Brown and Wald[14] as modified by Rotmans[15], using toluene instead of benzene.

The retinols were prepared from the corresponding retinaldehydes by reduction with sodium tetrahydroborate(III) in ethanol and subsequent extraction with *n*-hexane. The retinyl esters were synthetised from the corresponding retinols by incubation with palmitoyl chloride and pyridine in toluene. The esters were isolated from the incubation mixture by thick-layer chromatography (PLC plates, silica 60; Merck).

All procedures involving rod outer segments (ROS), retinal pigment epithelium (RPE) and vitamin A derivatives were carried out in dim red light or darkness.

Cattle ROS were isolated using a modification of the procedure of De Grip *et al.*[16], involving mild homogenization of the retinas in 600 m*M* sucrose, 160 m*M* Tris–hydrochloric acid (pH 7.1), followed by sucrose density gradient centrifugation. The ROS band was collected and washed with 60 m*M* phosphate (pH 7.1) and either used immediately or stored at −70°.

RPE cells were isolated according to Berman and Feeney[17]. After excision of the retina from fresh bovine eyes, the RPE layer was carefully brushed off. The contaminants (*e.g.*, red blood cells and parts of the ROS) were removed by sieving through PTFE gauze and centrifuging (150 *g*) in 0.32 *M* sucrose. The latter procedure was repeated five times.

ROS suspension and suspended RPE cells were extracted repeatedly with *n*-hexane (in order to extract non-protein-bound vitamin A compounds) and subsequently with dichloromethane[18] or methanol–dichloromethane (1:1). All manipulations were carried out at about 0°.

## RESULTS AND DISCUSSION

### *Analytical aspects*

The resolution, separation time and detection limit of a chromatographic method[19,20] are determined by several parameters, such as column efficiency, selectivity and retardation. A high-speed, high-resolution separation with a low detection limit is favoured by high selectivity factors, intermediate capacity ratios and small theoretical plate heights at high eluent flow-rate. The last requirement is satisfied by

employing small particles and advanced packing techniques. In our experiments, silica gel (Si 60) was used as an adsorbent because high plate numbers can be achieved with this material[12]. The retardation and selectivity can be adjusted by the choice of the phase system. In order to cover a wide range of polarity with respect to the solutes, mixtures of *n*-hexane and dioxane in different concentrations were used as mobile phases. Retention data, obtained under isocratic conditions, are collected in Table I.

TABLE I

CAPACITY RATIOS OF THE 13-*cis*, 11-*cis*, 9-*cis* AND all-*trans* ISOMERS OF RETINYL PALMITATE ESTER, RETINAL AND RETINOL AS A FUNCTION OF THE PERCENTAGE OF DIOXANE IN *n*-HEXANE

Conditions: support, Si 60 (5 μm); detection of retinyl palmitate and retinol at 320 nm; detection of retinal at 360 nm.

| *Compound* | *Dioxane concentration (%)* | *Isomer* | | | |
|---|---|---|---|---|---|
| | | *13-cis* | *11-cis* | *9-cis* | *All-trans* |
| Retinyl palmitate | 0 | 8.81 | 8.81 | 11.15 | 13.24 |
| | 0.05 | 2.70 | 2.84 | 3.33 | 4.08 |
| | 0.1 | 2.28 | 2.41 | 2.79 | 3.36 |
| Retinal | 1.25 | 2.95 | 3.17 | 3.96 | 5.11 |
| | 2.5 | 2.17 | 2.40 | 2.84 | 3.59 |
| | 5.0 | 1.35 | 1.50 | 1.69 | 2.11 |
| Retinol | 2.5 | 21.01 | 20.14 | 23.95 | 28.07 |
| | 5.0 | 9.96 | 9.52 | 11.38 | 13.29 |
| | 10.0 | 4.37 | 4.17 | 4.85 | 5.62 |

Some characteristic high-speed separations are shown in Figs. 1–4. The small peak between 9-*cis*- and all-*trans*-retinal probably represents 7-*cis*-retinal[21]. It should be borne in mind that there is a reversal of elution order for 13-*cis*- and 11-*cis*-retinol, compared with the corresponding palmitates and aldehydes, as has been noticed before[11].

The chromatograms obtained with extracts of RPE and ROS are shown in Figs. 5 and 6. These profiles show the presence of small amounts of the 9-*cis* and 13-*cis* isomers, although these are not assumed to occur *in vivo*. Their presence is probably due to aspecific isomerization during extraction. No isomerization occurred on the column, as was checked by injection of some pure compounds (and mixtures), subsequent fractionation and re-injection. The chromatograms demonstrate that application to biological systems is feasible, as they do not show the presence of other components that interfere in the determination of the vitamin A isomers. A major improvement in the extraction of the chromophore is possible when, by analogy with the work of Pilkiewicz *et al.*[9], a detergent (Ammonyx LO) is added to the rhodopsin suspension to a final concentration of 2%, prior to extraction with dichloromethane. The overall recovery is still poor (20–25%), but almost no isomerization occurs, as 92% 11-*cis*- and 8% all-*trans*-retinaldehyde is obtained. Studies aimed at improving the extraction procedure with respect to isomerization and recovery are in progress and will be published separately[22]. A gradual decrease in column efficiency was noticed after injection of ten samples, due to the presence of

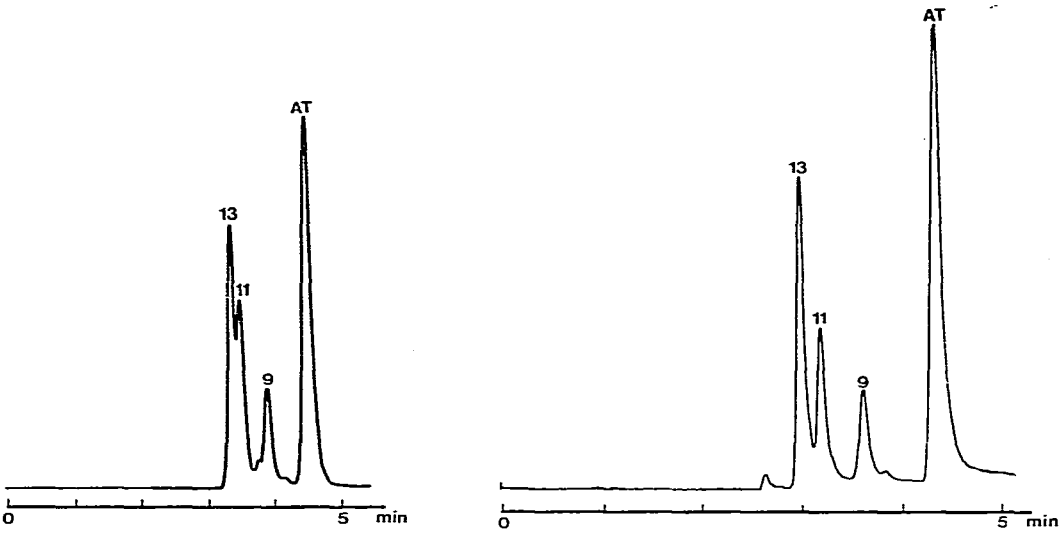



Fig. 1. Chromatogram of a test mixture of four *cis–trans* isomers of retinyl ester. Column, length 25 cm, I.D. 3.0 mm. Stationary phase, Si 60 (5 $\mu$m); mobile phase, *n*-hexane–dioxane (0.1 %); pressure 3000 p.s.i.; flow-rate, 17.5 $\mu$l/sec; wavelength, 320 nm. 11 = 11-*cis*-Palmitate; 13 = 13-*cis*-palmitate; 9 = 9-*cis*-palmitate; AT = all-*trans*-palmitate.

Fig. 2. Separation of a test mixture of four *cis–trans* isomers of retinal. Mobile phase, *n*-hexane–dioxane (2.5%); pressure, 3000 p.s.i.; flow-rate, 17.1 $\mu$l/sec; wavelength, 360 nm. 11 = 11-*cis*-Retinal; 13 = 13-*cis*-retinal; 9 = 9-*cis*-retinal; AT = all-*trans*-retinal. Column dimensions and stationary phase as in Fig. 1.

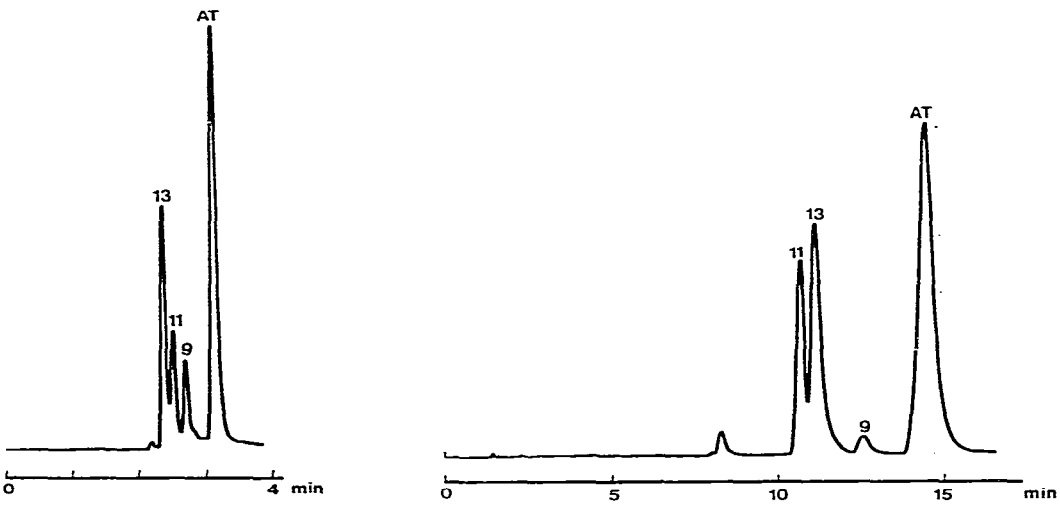
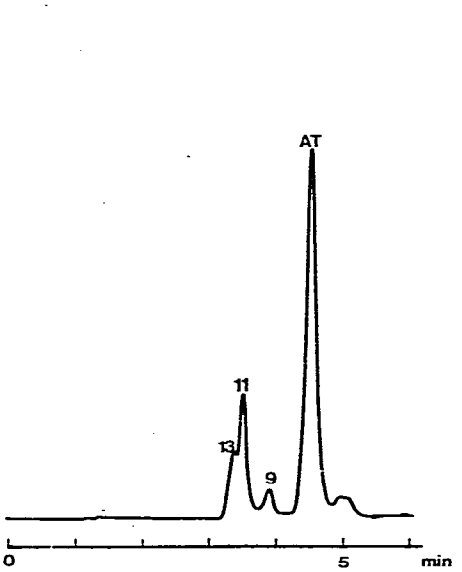



Fig. 3. Separation of a test mixture of four *cis–trans* isomers of retinal. Mobile phase, *n*-hexane–dioxane (5.0%); pressure, 3500 p.s.i.; flow-rate, 18.1 $\mu$l/sec; wavelength, 360 nm. Peaks as in Fig. 2. Column dimensions and stationary phase as in Fig. 1.

Fig. 4. Chromatogram of a test mixture of four *cis–trans* isomers of retinol. Mobile phase, *n*-hexane–dioxane (5.0%); pressure, 3500 p.s.i.; flow-rate, 18.1 $\mu$l/sec; wavelength, 320 nm. 11 = 11-*cis*-Retinol; 13 = 13-*cis*-retinol; 9 = 9-*cis*-retinol; AT = all-*trans*-retinol. Column and stationary phase as in Fig. 1.



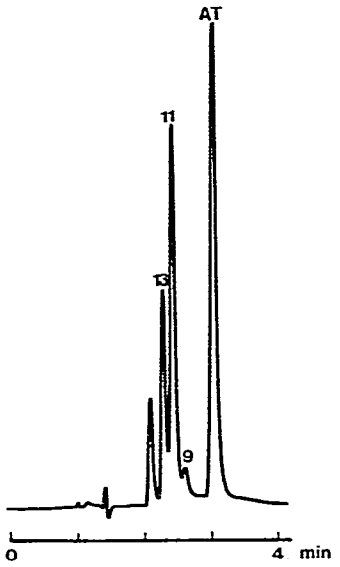




Fig. 5. Chromatogram of a retinal pigment epithelium extract. Conditions as in Fig. 1.
Fig. 6. Chromatogram of a rod outer segments extract. Conditions as in Fig. 3.

(phospho-)lipids. The efficiency can be restored by elution with acetone and subsequent activation with *n*-hexane. Alternatively, phospholipids can be removed from the sample by dissolving the extract in *n*-hexane containing 5% of dioxane and passing it over a small (2 × 0.5 cm) silica column.

*Quantitative aspects*

The sensitivity of detection for a component at its wavelength of maximum

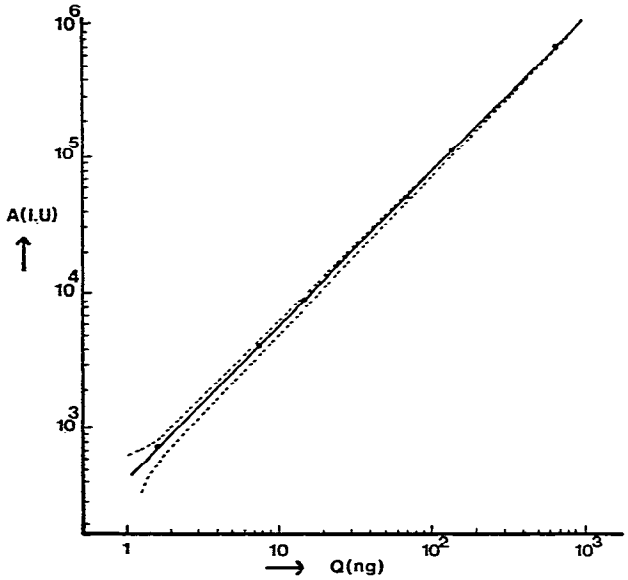



Fig. 7. Double logarithmic plot of peak area (*A*) *versus* amount injected (*Q*) for all-*trans*-retinal. Coefficient of correlation ($r$) = 0.9997. The broken lines show ±3 times the standard deviation.

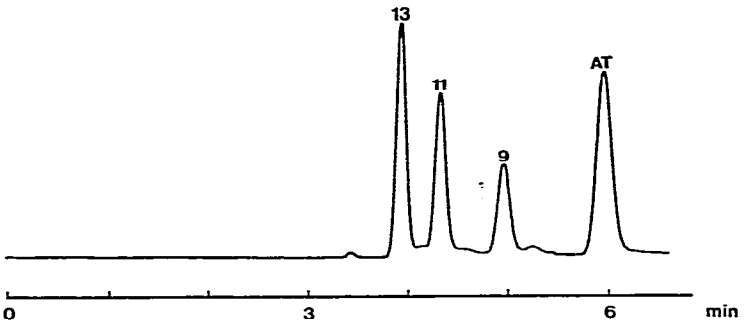



Fig. 8. Chromatogram of a test mixture of four *cis–trans* isomers of retinal. Column, length 25 cm, I.D. 10 mm. Stationary phase, Si 60 (5–8 $\mu$m); mobile phase, *n*-hexane–dioxane (2.5%); pressure, 3000 p.s.i.; flow-rate, 202 $\mu$l/sec; wavelength, 360 nm. Amount of sample injected, *ca.* 500 ng. Peaks as in Fig. 2.

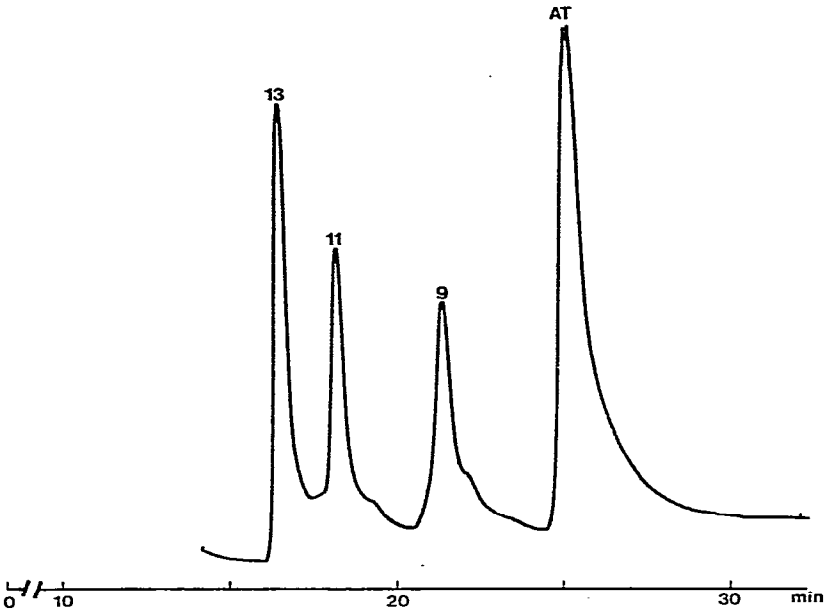



Fig. 9. Preparative separation of four *cis–trans* isomers of retinal. Pressure, 750 p.s.i.; flow-rate, 48.4 $\mu$l/sec; wavelength, 350 nm. Amount of sample injected, *ca.* 0.5 mg. Other conditions as in Fig. 8. Peaks as in Fig. 2.

absorption was determined by injection of a known volume (12 $\mu$l) of a dilution series of a stock solution and subsequent integration of the emerging peak. The detection limit is arbitrarily defined as the ratio of three times the standard deviation of the baseline noise and the sensitivity. A typical example is shown in Fig. 7 for all-*trans*-retinal, where the sensitivity was 840 I.U./ng and the standard deviation of the baseline noise 131 I.U., the detection limit thus being about 0.5 ng (about 2 pmole). The precision was about 0.5% at 10 $\mu$g (about 40 nmole) and 4% at 10 ng (about 40 pmole) ($n = 3$).

*Preparative aspects*

In view of its high resolving power, this method can also be applied in the preparative mode for the isolation of the pure isomers from an isomeric mixture.

Preliminary experiments on a preparative scale were carried out with a column of length 250 mm and I.D. 10 mm[23]. Analytical amounts (about 500 ng) were injected and the results are shown in Fig. 8. When this chromatogram is compared with that in Fig. 2, it can be seen that a better resolution is achieved, presumably owing to the smaller specific sample load and less external contribution to peak broadening on the preparative column. Fig. 9 shows the results of the separation of 0.5 mg of an isomeric mixture of retinals.

## CONCLUSION

### *Conclusion*

The separation of the geometric isomers of each of the different classes of vitamin A derivatives can be achieved by HPLC with silica gel as adsorbent and *n*-hexane–dioxane as eluent.

These different classes are often present simultaneously in biological samples. It should be possible to analyse them in one run by means of continuous or stepwise gradient elution. The need for a relatively fast re-equilibration of the column (one of the drawbacks in gradient elution) seems to be satisfied, as we could obtain this within 15 min by elution of the column with the original eluent.

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