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EVIDENCE FOR CARRIER AMPHOLYTE–PEPTIDE INTERACTIONS DURING SOMATOMEDIN PURIFICATION

J. A. BAKKER*, J. L. VAN DEN BRANDE and C. M. HOOGERBRUGGE
Wilhelmina Children's Hospital, Nieuwe Gracht 137, 3512 LK Utrecht (The Netherlands)
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SUMMARY

The application of gel filtration on Sephadex G-50, hydrophobic interaction on octyl-Sepharose and mixed ion-exchange on AG 50 1-X8 were examined for the removal of carrier ampholytes from peptide mixtures containing somatomedins. With respect to gel filtration, the results suggest peptide–ampholyte interactions, particularly at pH 2.5, which prevent complete separation. Hydrophobic interaction chromatography and mixed ion-exchange chromatography appear potentially suitable, although the use of the latter is limited to peptides having molecular weights >4000 daltons.

INTRODUCTION

Somatomedins are growth hormone-dependent peptide growth factors[1], the fundamental biological characteristic of which is to stimulate the incorporation of sulphate into chondroitin sulphate in cartilage[2]. *In vitro* and *in vivo* studies have yielded evidence that somatomedins are the growth stimulators which transmit to the tissues the signal issued by growth hormone and perhaps other hormones[3,4].

Previously extracted somatomedins have a molecular weight of approximately 6000 daltons[1], and during their purification we were confronted with the problem of their separation from ampholytes[5]. As dialysis was not effective, in this study we investigated the use of gel filtration on Sephadex G-50, hydrophobic interaction on octyl-Sepharose and mixed ion exchange on AG 50 1-X8 as possible alternatives, paying special attention to the conservation of the biological activity of the peptides.

EXPERIMENTAL

Somatomedin-containing preparations

Semi-purified extracts were prepared from porcine plasma through a sequence of heating to 80°C, concentration and acid diafiltration on a hollow-fibre device, with cut-offs at 5000 and 50,000 daltons (Amicon Europe, Oosterhout, The Netherlands) and gel filtration on Sephadex G-50.

The preparations used were derived from different batches, which had been treated in an identical fashion. However, their K_{av} values on Sephadex G-50 differed.

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Somatomedin preparations (SM prep) A and B were obtained by pooling the zone with K_{av} 0.4–0.6; SM prep C was from the zone between K_{av} 0.2 and 0.4, and SM prep D and E between K_{av} 0.4 and 0.8. Further details of the procedures used for large-scale extraction from porcine plasma have been published previously[5].

Bioassay and receptor assay

Somatomedin activity (SM-act) was determined by the porcine cartilage bioassay[6], according to incubation schedule A. [^{35}S]Sulphate and [^{3}H]methylthymidine (Radiochemical Centre, Amersham, Great Britain) were used to measure the stimulation of their incorporation into chondroitin sulphate and DNA by somatomedins.

Insulin-like activity (ILA) was estimated with a placental radioreceptor assay for insulin as described by Van Buul-Offers and Van den Brande[7]. Porcine crystalline insulin (Organon, Oss, The Netherlands) was iodinated by the method of Thorell and Johansson[8].

Gel filtration on Sephadex G-50

For practical reasons gel filtration was performed on a column (40 × 1 cm) of Sephadex G-50 (fine) connected serially to a column (30 × 1 cm) filled with G-50 (superfine), both from LKB (Bromma, Sweden). The elution rate was 3–3.5 ml/h and elution was effected either at pH 2.5 with 1 % formic acid or at pH 5 by adjustment with ammonia.

Samples of 1.0 ml, containing 0.5–2.0 mg of peptide with or without 100 µl (approximately 40 mg) of carrier ampholytes, were applied. Ampholines (LKB) and Pharmalytes (Pharmacia, Uppsala, Sweden) were used.

Hydrophobic interaction chromatography on octyl-Sepharose

Octyl-Sepharose CL-4B (Pharmacia). after a recommended washing procedure, in a 30 × 1.6 cm column was loaded at 20 ml/h with 30 mg of a peptide in 15 ml of 0.02 *M* ammonium acetate solution of pH 6.1 (starting buffer). Elution (60 ml/h) was carried out with stepwise increments of ethanol concentration up to 96 % (v/v) in the starting buffer. If necessary, ethanol was removed by rotating vacuum distillation at 25–30°C. All fractions were lyophilized and tested for biological activity.

Mixed ion-exchange chromatography on AG 50 1-X8

The mixed ion-exchange resin AG 50 1-X8 (Bio-Rad Labs., Richmond, CA, U.S.A.) was used in columns of 1 cm diameter and lengths between 4 and 30 cm at elution rates from 4 to 30 ml/h. Washing and elution were performed with distilled water. Before testing all samples were lyophilized.

Isoelectric focusing (IEF)

Analytical focusing was performed on flat-bed Multiphor equipment (LKB) using PAG-plates pH 3.5–9.5 (LKB) at constant power, according to LKB instructions. Cooling water was circulated at 4°C. Staining and fixation were performed simultaneously for 1 h in the destaining solution [8 % (v/v) acetic acid and 25 % (v/v) ethanol in water], which was supplemented with 15 % (w/v) trichloroacetic acid, 0.1 % (w/v) Coomassie Brilliant Blue R-250 (Merck, Darmstadt, G.F.R.) and 0.1 % (w/v) Coomassie Brilliant Blue G-250 (Eastman-Kodak, Rochester, NY, U.S.A.). The gel

slab was prepared for conservation by submersion for 1 h in a 9:1 (v/v) mixture of destaining solution and glycerol.

Preparative focusing was carried out on the same LKB equipment in a bed of Sephadex G-75 (superfine) in 2% (w/v) Pharmalyte 3-10 (Pharmacia) according to Radola[9].

Materials

Gramidicin (1200 daltons), Lima bean trypsin inhibitor (9000 daltons) and RIA-grade bovine serum albumin (69,000 daltons) were obtained from Sigma (St. Louis, MO, U.S.A.), bovine insulin-B (3495 daltons) and glucagon (3483 daltons) from Serva (Heidelberg, G.F.R.), $ACTH^{1-24}$ (2935 daltons) from Ciba (Basle, Switzerland) and porcine insulin (5600 daltons) from Nordisk (Gentofte, Denmark).

Peristaltic pumps from Pharmacia and LKB were used. Uvicord-III equipment (LKB) was used to follow elution profiles at 254 and 280 nm.

All chemicals were of analytical-reagent grade from Merck unless specified otherwise.

If not stated otherwise, all procedures were performed at 4°C.

RESULTS AND DISCUSSION

Gel filtration on Sephadex G-50

It is usual in the isolation of somatomedins to perform gel filtration of the peptide preparations in dilute organic acids[10,11]. Formic acid, acetic acid and propionic acid are recommended for suppressing adsorption to gels[12]. Further, these solvents are easily removed by lyophilization.

The behaviour of standard peptides and of both Ampholine pH 3.5–10 and Pharmalyte pH 3–10 at pH 2.5 and 5.0 was examined. The resulting K_{av} values are represented in Figs. 1 and 2. At both pH values the standard compounds between 1200 and 9000 daltons behave identically, as they yield almost the same slopes and intercepts for the relationship between K_{av} and log M (molecular weight). Under the conditions used, the tested carrier ampholytes have their peak concentration at apparently higher molecular weights than the assumed 600 daltons. This might indicate self-aggregation, especially at pH 5.0. The difference between pH 2.5 and 5.0 (K_{av} 0.88 and 0.78, respectively) may be a consequence of adsorption to the gel at pH 2.5.

In view of its anticipated application for studying hitherto poorly defined somatomedins with isoelectric points in this region[5], the behaviour of Ampholine pH 5–7 and Pharmalyte pH 5–8 on Sephadex G-50 in 1% (v/v) formic acid was studied in more detail. Based on the absorption at 254 nm, Ampholine pH 5–7 emerged at K_{av} 0.45 and had a peak at 0.88, and Pharmalyte pH 5–8 emerged at K_{av} 0 and also peaked at 0.88 (Fig. 3). The possibility that self-aggregation was the cause of the recovery of ampholytes between K_{av} 0 and 0.8 was investigated by re-chromatography of the Pharmalyte pH 5–8 recovered in this zone using a lower concentration (15 mg instead of 40 mg in the sample). As hardly any material was recovered at K_{av} 0.88 (Fig. 3), the results suggest over-polymerization of the product rather than self-aggregation. These findings eliminate the possibility of a complete separation of these ampholytes from our peptide preparations. A similar phenomenon was also observed on Bio-Gel P-4 by Gelsema *et al.*[13].

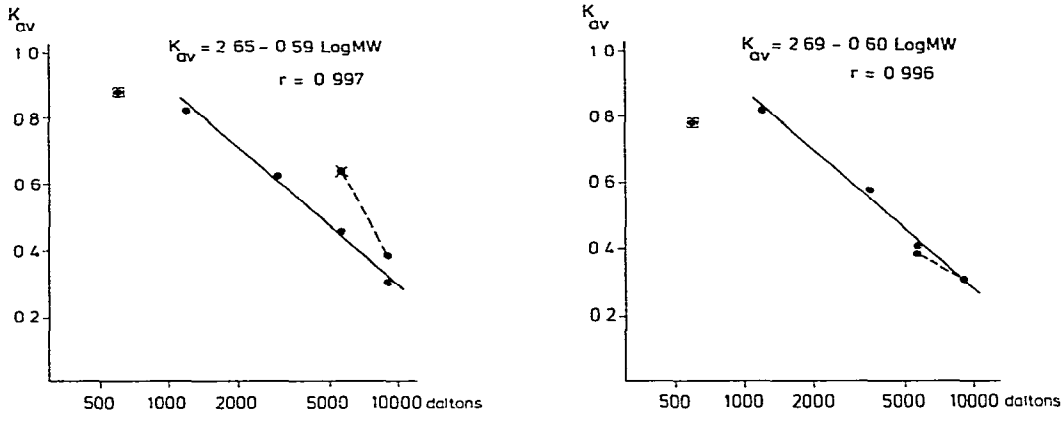

Fig. 1. Gel filtration on Sephadex G-50 at pH 2.5 (1 % formic acid). ●—●, Gramicidin, ACTH$^{1-24}$, insulin and trypsin inhibitor without carrier ampholytes; ●---●, insulin and trypsin inhibitor in the presence of Ampholine pH 3.5–10 or Pharmalyte pH 3–10 (40 mg/ml); ●, insulin in the presence of NaCl (300 mg/ml); ■, carrier ampholytes, assumed 600 daltons.

Fig. 2. Gel filtration on Sephadex G-50 at pH 5.0 (1 % formic acid + ammonia). ●—●, Gramicidin, insulin-B, insulin and trypsin inhibitor without carrier ampholytes; ●---●, insulin and trypsin inhibitor in the presence of Ampholine pH 3.5–10 or Pharmalyte pH 3–10 (40 mg/ml); ■, carrier ampholytes, assumed 600 daltons.

The problem is further complicated by our finding that the distribution of SM-act and ILA by gel filtration on Sephadex G-50 in 1 % formic acid of pools obtained after preparative IEF of SM prep A for most pH intervals conflicted with the K_{av} value of 0.40–0.60 of the starting peptide mixture. The K_{av} values were so high that good separations of ampholytes and peptides were impossible, as shown in Table I. These findings prompted a further study of the gel filtration behaviour of some model peptides in the presence of carrier ampholytes (Figs. 1 and 2).

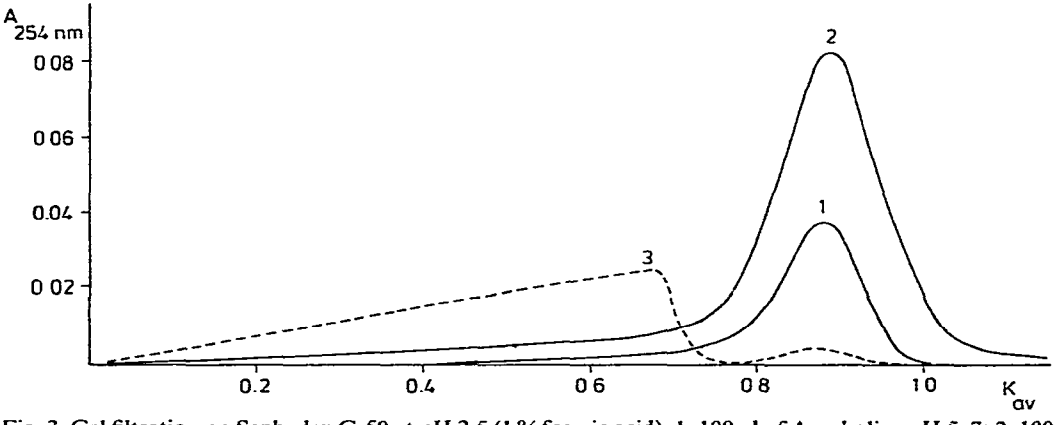

Fig. 3. Gel filtration on Sephadex G-50 at pH 2.5 (1 % formic acid). 1, 100 µl of Ampholine pH 5–7; 2, 100 µl of Pharmalyte pH 5–8; 3, re-chromatography of 15 mg of Pharmalyte (K_{av} = 0–0.8) derived from 400 µl of Pharmalyte pH 5–8.

TABLE I

DISTRIBUTION ON SEPHADEX G-50 (IN 1% FORMIC ACID) OF SM-ACT AND ILA IN POOLED FRACTIONS OF PREPARATIVE ISOELECTRIC FOCUSING

The starting material for IEF was fraction 3 of the Sephadex G-50 eluate (SM prep A). Results are expressed as a percentage of total recovered activity.

Material	*Fraction No.*	K_{av} *(Sephadex G-50)*	*Sulphation (%)*	*Thymidine incorporation (%)*	*ILA (%)*
Starting material	2*	0.2–0.4	0	0	0
	3	0.4–0.6	86	86	72
	4*	0.6–0.8	14	14	28
Pools after preparative IEF of fraction 3					
Pool I		0–0.29	0	0	0
(pH 6.9–7.6)		0.29–0.42	0	0	2
		0.42–0.55	0	16	0
		0.55–0.68	26	27	3
		0.68–0.87	74	57	95
Pool II		0–0.29	0	0	0
(pH 8.1–8.7)		0.29–0.42	Inhibition	Inhibition	0
		0.42–0.55	0	0	0
		0.55–0.68	50	0	22
		0.68–0.81	50	0	78
Pool III		0–0.29	0	0	0
(pH 8.7–9.1)		0.29–0.42	0	0	0
		0.42–0.55	19	0	0
		0.55–0.68	26	0	26
		0.68–0.81	55	0	74

* Fractions not applied to IEF.

At pH 2.5 a large increase in K_{av} from 0.45 to 0.63 was found for insulin when 100 µl/ml of carrier ampholytes were added to the samples. Under the same conditions, trypsin inhibitor also showed a shift of K_{av} from 0.30 to 0.38. A concentration of 300 mg/ml of sodium chloride had the same effect as ampholytes on the position of insulin, whereas 40 mg/ml had no effect. At pH 5.0 the addition of carrier ampholytes (100 µl/ml) to insulin or trypsin inhibitor did not significantly influence their K_{av} values (Fig. 2).

As hydrophobic interactions are enhanced by low pH values and high salt concentrations[14,15], our observations during gel filtration at pH 2.5 can be explained by ionic interactions between carrier ampholytes and peptides, resulting in an enhancement of the hydrophobicity of peptides. On a molar basis, carrier ampholytes are more effective than sodium chloride in this respect, although at higher concentrations salts exert the same effect. Indeed, when somatomedin-containing peptide mixtures derived from fraction Cohn IV of human plasma[5] in a highly concentrated salt solution [*ca.* 8% (w/v)] were chromatographed at the usual protein concentration of 2–3% (w/v) on Sephadex G-50 in 1% (v/v) formic acid, the biological activity was recovered at K_{av} values up to 0.9, but on re-chromatography the activity was found at

K_{av} 0.4–0.6. When a desalting step with hollow fibres (H_5P_2, Amicon) was introduced prior to gel filtration, these shifts in K_{av} did not occur.

To investigate further the possible interaction between carrier ampholytes and peptides we placed SM prep E at opposite sides near the electrode paper strips on a polyacrylamide slab in which the pH gradient was already established. After 3 h stabilization was attained at 3 mA and 900 V. Fig. 4 shows that not all bands have the same intensity and, depending on the starting position, some broad and vague bands can be seen. The broadness of these bands may indicate that the equilibrium position has not yet been reached, possibly as a consequence of interactions with some carrier ampholytes (Ampholine). Fig. 4 also shows some minor differences when the same experiment was carried out with a p*I*-marker mixture (Serva).

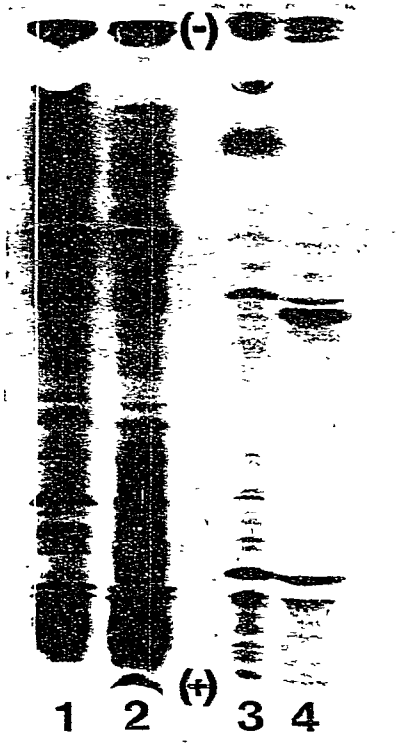

Fig. 4. Analytical isoelectric focusing on slabs with established pH gradient. 1 and 2 = p*I* markers, starting position at cathode and anode, respectively; 3 and 4 = peptide mixture, starting position at cathode and anode, respectively.

These observations and the influence of carrier ampholytes on the gel filtration behaviour of peptides support the existence of interactions between peptides and carrier ampholytes.

There are many other indications of such interactions. Gel filtration resulting in the separation of proteins from ^{14}C-labelled Ampholine on Sephadex G-75 in 0.01 *M* buffers by Dean and Messer[16] and on Sephadex G-50 in 0.1 *M* buffers by Vesterberg[17] illustrate the reversible nature of these interactions. This was confirmed by

Righetti and Chrambach[18], who showed that amino acids focus as single peaks at or close to their isoelectric points, provided that 0.1 *M* potassium chloride is present during the focusing.

All of these observations indicate that peptide–carrier ampholyte interactions may be suppressed by inorganic ions. However, the presence of salts may induce enhanced hydrophobicity of peptides.

Under the present conditions it has not been possible to use gel filtration as an effective method for the removal of ampholytes from the peptides studied. More dilute samples in combination with larger columns during gel filtration will diminish the interactions between carrier ampholytes and peptides, but may also result in larger losses owing to adsorption to a larger surface of the gel.

Hydrophobic interaction chromatography on octyl-Sepharose CL-4B

In a preliminary study[14], evidence was presented that peptides with sufficient hydrophobicity can be separated from carrier ampholytes by hydrophobic interaction chromatography, even if molecular size alone would not allow such separations. It was shown that on octyl-Sepharose most of a peptide mixture derived from human plasma[5] did not elute together with carrier ampholytes, which were not retarded to the column. Using a gradient of ethanol up to 40 % (v/v), only 32 % was recovered on a weight basis.

The present results, as shown in Table II, indicate recoveries of 65 % when 96 % (v/v) ethanol was used for the elution of SM prep B.

TABLE II

RECOVERY OF WEIGHT AND BIOLOGICAL ACTIVITY OF SM PREP B AFTER CHROMATOGRAPHY ON OCTYL-SEPHAROSE CL-4B

Ethanol in starting buffer (%, v/v)	*SM activity (U)*		*Weight (mg)*
	Sulphation	*Thymidine incorporation*	
0	–	–	15.4
9.6	–	–	–
19.2	1.9	5.0	1.0
38.4	0.8	1.9	1.0
76.8	1.0	1.9	1.0
96.0	0.2	–	1.0
Observed yield	3.9	8.8	19.4
Expected yield*	6.0	8.0	*ca.* 30
Starting material	18.0	24.0	30.0

* Calculated on the basis of usual losses of biological activity without material loss as exemplified in Table III, sample D.

Recovery of biological activity is good when an expected loss of 50 % by lyophilization is taken in account (Table III, sample D). As shown in Table II, about half of the applied material (derived from porcine plasma) was not bound to the column and did not possess SM activity. In this instance, therefore, the somatomedins had a

different behaviour from the carrier ampholytes, which are not bound to the octyl-Sepharose.

Mixed ion-exchange chromatography on AG 50 1-X8

This method, which is based on the difference in the number of ionic groups per mole[17], is mixed ion-exchange on AG 50 1-X8, which was shown to be effective in separating carrier ampholytes from proteins by Brown and Green[19] and by Baumann and Chrambach[20].

Table III shows the recovery of SM-act and ILA for two peptide mixtures (SM prep C and D) after mixed ion-exchange chromatography.

TABLE III

RECOVERY OF ILA AND SM ACTIVITY AFTER MIXED ION-EXCHANGE CHROMATOGRAPHY ON AG 50 1-X8

Sample	*Treatment*	*Conditions*		*ILA (mU/mg)*	*SM activity (U/mg)*	
		Column dimensions (cm)	*Elution rate (ml/h)*		*Sulphation*	*Thymidine incorporation*
C	Ion exchange + lyophilization	4 × 1	4	–	0.27	0.5
C	Lyophilization only	–	–	–	0.25	0.4
D	No treatment	–	–	3.6	4.1	4.1
D	Ion exchange + lyophilization	14 × 1	14	2.3	1.5	1.1
D	Lyophilization only	–	–	1.7	1.2	1.8

Both samples ranged from 1000 to 8000 daltons, as measured by gel filtration on Sephadex G-50 in 1% (v/v) formic acid. By analytical isoelectric focusing the sample composition was found not to have changed after ion-exchange, although small differences cannot be excluded in view of the reduced sensitivity of the staining technique for peptides below about 3000 daltons. On a weight basis 70–80% of these samples was recovered.

Using a 50 × 1 cm column at an elution rate of 40 ml/h the recovery of [^{125}I]insulin, mixed with 1 mg of bovine serum albumin, was about 90%; on a 30 × 1 cm column at an elution rate of 25 ml/h, 70% of [^{125}I]insulin (without albumin addition) was recovered, compared with only 20% and 11% (w/w), respectively, for gramicidin and glucagon.

With regard to SM-act and ILA (Table III, sample D), the conservation of activities of tested mixtures is satisfactory compared with those after a single lyophilization.

Small columns (4 × 1 cm) at low elution rates (4 ml/h) have the drawback of a 10-fold dilution of samples, although they are as effective in binding the full-range carrier ampholytes tested as larger columns at higher elution rates. Based on absorb-

ance at 254 nm, binding of Ampholine pH 3.5–10 and 5–7 and Pharmalyte pH 3–10 was almost 100%, but it was only 90% for Pharmalyte pH 5–8. This last figure may be explained by the apparently larger molecular weights of up to 20,000 daltons constituting about 9% (w/w) of Pharmalyte pH 5–8 during gel filtration (Fig. 3).

CONCLUSION

Gel filtration on Sephadex G-50

Removal of carrier ampholytes from peptides by gel filtration on Sephadex G-50 in strongly acidic environments (pH 2.5) is not advisable. A higher pH (*e.g.*, pH 5) will reduce adsorption. High ionic strengths will reduce interactions between peptides and carrier ampholytes[17,18]. However, high ionic strengths will promote hydrophobic interactions. Complete removal of carrier ampholytes from peptides below about 5600 daltons ($K_{av} = 0.45$) is not possible in one step under the conditions examined (Fig. 3).

Hydrophobic interaction chromatography on octyl-Sepharose CL-4B

This technique is of interest only for the removal of carrier ampholytes from peptides possessing enough hydrophobic amino acid residues[14]. The recovery of SM-act from the peptides studied was lower than expected. However, this technique may be of interest as it can add a further separation principle to a particular purification scheme.

Mixed ion-exchange chromatography on AG 50 1-X8

In general, this technique is not advisable for the removal of carrier ampholytes from peptides below about 4000 daltons (see glucagon and gramicidin).

With regard to SM and ILA recoveries from the peptide mixture studied, this technique appears in principle to be appropriate for separations from ampholytes. Further work will be needed to establish this with certainty.

A general conclusion may be that none of the techniques examined can guarantee the removal of carrier ampholytes from peptides smaller than about 4000–6000 daltons in a single step. If these smaller peptides are sufficiently hydrophobic, hydrophobic interaction chromatography may offer a solution.

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