Journal of Chromatography, 196 (1980) 51–58
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,843

STUDY OF THE FEASIBILITY OF SEPARATION OF CARRIER AMPHOLYTES FROM PEPTIDES BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY ON OCTYL-SEPHAROSE

W. J. GELSEMA*, C. L. DE LIGNY, W. M. BLANKEN, R. J. HAMER and A. M. P. ROOZEN
Analytical Chemistry Laboratory, State University of Utrecht, Croesestraat 77a, 3522 AD Utrecht (The Netherlands)
and
J. A. BAKKER
Wilhelmina Children's Hospital, Nieuwe Gracht 137, 3512 LK Utrecht (The Netherlands)
(Received March 18th, 1980)

SUMMARY

The merits of hydrophobic interaction chromatography on octyl-Sepharose as a method for the separation of carrier ampholytes from peptides have been investigated. K values for the association of octyl groups with small model peptides and with carrier ampholytes in aqueous sodium chloride-containing phosphate buffers were determined as a function of ionic strength and pH. A separation of a somatomedin-containing peptide mixture into seven fractions by hydrophobic interaction chromatography on octyl-Sepharose is shown.

INTRODUCTION

Isoelectric focusing is a widely used technique with high resolution for the separation of proteins or peptides. It has, however, a serious drawback, *viz.*, the sample is necessarily "contaminated" with substances (the carrier ampholytes) that resemble in many respects the compounds to be separated. As a result, it is sometimes necessary to remove, subsequent to isoelectric focusing, the carrier ampholytes from the focused proteins or peptides. In the preparative mode of the technique, the most obvious method for this separation is gel filtration. However, from the great number of alternative separation methods proposed* and from some literature reports of failure of separation by gel chromatography[2] or dialysis[3,4], it seems that it is not satisfactory in all instances. In principle, this can be due to the following causes: (i) carrier ampholytes contain relatively high-molecular-weight species; (ii) peptides or proteins are retarded on the molecular sieving gel; and (iii) carrier ampholytes associate with proteins or peptides.

On the basis of our recent work[5,6], we do not consider, contrary to the view of

* For a survey of the alternatives that have been, or could be, tried, see Vesterberg[1].

0021-9673/80/0000–0000/$02.25

Baumann and Chrambach[7], the first cause to be responsible in most instances: the molecular weights of the constituents of carrier ampholytes are so low that they should have free access to the pores of such a dense gel as Sephadex G-50, which means that peptides with molecular weights above, say, 3000 should be completely separable from carrier ampholytes on this gel.

The second cause is, in the case of peptides, a more probable source of failure of the separation by gel chromatography: it is well known that peptides containing aromatic amino acids are retarded on Sephadex gels.

With regard to the third cause, we think it is often operative. Here we refer to the recent survey by Righetti[8] of the conflicting reports and opinions about protein–carrier ampholyte association. Since the appearence of this survey, evidence for association of carrier ampholytes with proteins[9], amino acids[10] and with themselves[6,11] has been growing. Inasmuch as these associates are formed by electrostatic interactions, the addition of some electrolyte would in principle overcome problems in separation by gel chromatography. In fact, Vesterberg[1] recommends the use of a high ionic strength. This procedure, however, adds another drawback to preparative isoelectric focusing: in order to remove efficiently the unavoidable "contaminants" (the carrier ampholytes), another "contaminant" (a salt) has to be introduced.

One can, however, take advantage of this situation. It is well known that a high ionic strength favours the hydrophobic bonding of peptides and proteins with alkyl-agarose matrices. As the carrier ampholyte constituents generally have very small hydrophobic regions in comparison with peptides and proteins, a separation by hydrophobic interaction chromatography in the presence of salt seems feasible. This would have the advantage over gel chromatography in the presence of salt that an additional separation criterion is added to the isolation procedure. For, if the mixture of peptides or proteins to be separated by isoelectric focusing is obtained from a previous gel filtration step (as is often the case), no further separation of the peptides or proteins from each other can be expected by gel filtration of the focused fractions in the presence of salt, whereas this may be obtained by hydrophobic interaction chromatography in the presence of salt. In fact, Vesterberg[1] mentions the potential usefulness of this approach, but to our knowledge it has never been studied experimentally.

In this paper, we present the results of an investigation undertaken with the aim of exploring the merits of hydrophobic interaction chromatography on octyl-Sepharose for the separation problem in question. Therefore, we studied the retention of some model peptides and of carrier ampholytes on octyl-Sepharose as a function of salt content and pH of the eluent. As a practical example, we also performed on this gel hydrophobic interaction chromatography of a peptide mixture containing somatomedins[12,13] (growth hormone-regulated peptide growth factors[14]). A previous study[15] had indicated that dialysis or gel filtration on Sephadex G-50 was not efficient enough for the separation of these peptides from carrier ampholytes.

EXPERIMENTAL

Hydrophobic interaction chromatography of carrier ampholytes and small model peptides

Octyl-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) was washed several times with the 25 m*M* phosphate buffer–sodium chloride solution to be used as an

eluent. It was subsequently packed in a column (K16/70, Pharmacia) to a bed height of about 65 cm (bed volume about 130 ml) at an eluent flow-rate of about 15 $ml \cdot h^{-1}$. The column was equipped with a sample valve (LV4, Pharmacia), a flow adaptor (A16, Pharmacia), a peristaltic pump (Minipuls 2, Gilson, Villiers-le-Bel, France) and a UV monitor (Uvicord S; LKB, Stockholm, Sweden) with a 70-μl flow cell.

As a routine, 15-μl aliquots of commercially supplied Ampholines (LKB), Servalytes (Serva, Heidelberg, G.F.R.) and Pharmalytes (Pharmacia), diluted with eluent to 1 ml, were applied to the column and eluted at a flow-rate of about 15 $ml \cdot h^{-1}$. Alternatively, 1–2 mg of a peptide (all composed of L-amino acids; Sigma, St. Louis, MO, U.S.A.) were dissolved in 1 ml of eluent and applied to the column. Flow-rates were determined gravimetrically. The UV absorbance was measured at 206 nm. Analytical-reagent grade compounds (Baker, Deventer, The Netherlands) were used to make up the eluents. All eluents contained 0.005% of sodium azide.

In some control experiments unsubstituted Sepharose CL-4B was used. In one experiment, sucrose (Baker) instead of sodium chloride was added to the phosphate buffer.

For the determination of the total volume of the column a 2-ml sample of a 0.8 m*M* copper(II) sulphate solution in the eluent was chromatographed. In that case the eluent was collected in 2.2-ml fractions, 0.5 ml of concentrated ammonia solution was added to the fractions and the absorbance at 754 nm was measured with a Vitatron Type MPS spectrophotometer.

Hydrophobic interaction chromatography of a peptide mixture

This peptide mixture was obtained in the following way. A diluted suspension of the Cohn-IV fraction of human plasma in 0.025 *M* sodium acetate (pH 5.5) was heated to 80°C and, after cooling, centrifuged. The supernatant was subsequently submitted to ultrafiltration through hollow fibres (H10 P50, Amicon Europe, Oosterhout, The Netherlands) and ion exchange at pH 5.6 on CM-cellulose (CM52, preswollen; Whatman, W. & R. Balston, Maidstone, Great Britain)[12,13]. The non-bound material was loaded at pH 4 and a specific conductivity of $2 \cdot 10^{-3}\ \Omega^{-1} \cdot cm^{-1}$ on a SP-Sephadex C-25 (Pharmacia) column and eluted with a combined salt–pH gradient composed of ammonium formate buffers. The fraction eluting between pH 5.2 and 10.3 (specific conductivity $3.9 \cdot 10^{-3}$–$4.2 \cdot 10^{-3}\ \Omega^{-1} \cdot cm^{-1}$) was finally chromatographed in 1% (v/v) formic acid on Sephadex G-50 (Pharmacia) and the fraction having K_{av} values from 0.4 to 0.5 was lyophilized. Except for the heating, all procedures were performed at 4°C.

An aliquot of 11 mg of the lyophilized peptide mixture was dissolved in 1 ml of 25 m*M* phosphate buffer (pH 6.5) and applied to an octyl-Sepharose CL-4B column (1.6 × 61 cm), equilibrated at 20°C in the same buffer. Elution (eluent rate 17.3 $ml \cdot h^{-1}$) was performed with this buffer for 16 h, then with a gradient of this buffer in water–ethanol up to 40% (v/v) ethanol. The UV absorbance at 206 nm was measured as described above and the eluate was collected in fractions of 3.7 ml.

Pooled fractions were lyophilized and desalted by gel filtration in 1% (v/v) formic acid on Sephadex G-50, using a Uvicord III detector (LKB) at 254 and 280 nm. The peptide-containing fractions were again lyophilized and aliquots of these fractions were analysed by isoelectric focusing on a polyacrylamide gel (PAG plate, pH 3.5–9.5, LKB).

RESULTS

Hydrophobic interaction chromatography of carrier ampholytes and small model peptides

The elution peaks of the carrier ampholytes and peptides were symmetrical; the theoretical plate height values ranged from 0.3 to 0.5 mm for the peptides and from 0.4 to 0.7 mm for the carrier ampholytes.

The association constant, K, was calculated from the elution volume, V_e, using the equation

$$K = \frac{V_e - V_t}{V_t c^{\circ}_{octyl}}$$

where V_t is the elution volume of copper(II) sulphate and c°_{octyl} is the concentration (40 mM) of octyl groups in the gel bed[16]. In Table I the influence of the sodium chloride concentration and of the presence of 20% (w/v) of sucrose, at constant pH, on the K values of several small peptides and of carrier ampholytes is shown.

TABLE I

ASSOCIATION CONSTANTS, K (M^{-1}), OF SOME TRI- AND TETRAPEPTIDES AND OF CARRIER AMPHOLYTES WITH OCTYL GROUPS OF OCTYL-SEPHAROSE CL-4B IN 25 mM PHOSPHATE BUFFER (pH 3.65) AT 25°C

Peptide or carrier ampholyte	*Sodium chloride concentration (M)*			*20% (w/v) sucrose*
	0	*1*	*2*	
Gly–Gly–Gly	0	0.2	0	0
Gly–Gly–Val		0.3	0	
Gly–Gly–Leu		0.4	0.4	
Gly–Gly–Phe	1.0	1.6	2.3	
Gly–Phe–Phe	3.6	5.2	7.8	2.3
Phe–Phe–Phe	8.7	24.5	38.1	8.9
Leu–Leu–Leu	0.8	2.6	4.0	0.8
Phe–Gly–Gly–Phe		2.2	4.3	
Phe–Gly–Phe–Gly		3.2	4.6	
Ampholine, pH 4–6	0	0	0	
Ampholine, pH 3.5–10*			0	
Servalyte, pH 2–11*			0	
Pharmalyte, pH 5–8*			0	
Pharmalyte, pH 8–10*			0	

* At pH 4.

Some experiments with trileucine in 25 mM phosphate buffer (pH 3.65) + 2 M sodium chloride were performed at lower eluent rates (about 5 and 10 ml·h^{-1}), giving identical K values. Control experiments with triglycine, glycylphenylalanylphenylalanine and Ampholine (pH 4–6) in 25 mM phosphate buffer (pH 4) + 2 M sodium chloride on unsubstituted Sepharose CL-4B all resulted in identical elution volumes equal to V_t.

In Fig. 1 the effect of pH at constant sodium chloride concentration is demonstrated for one tripeptide. The pH had no effect on the elution volume of carrier ampholytes.

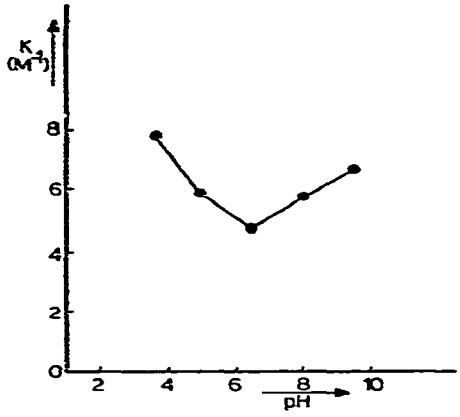

Fig. 1. Association constant, K_a of glycylphenylalanylphenylalanine with octyl groups of octyl-Sepharose as a function of pH in 25 m*M* phosphate buffer in 2 *M* sodium chloride at 25°C.

As a check on the quantitative elution of carrier ampholyte in the "unretarded peak", the following experiment was performed. Octyl-Sepharose CL-4B was regenerated by extensive washing with 80% ethanol and *n*-butanol, equilibrated in 25 m*M* phosphate buffer (pH 3.65) + 2 *M* sodium chloride and packed in a column. Ampholine (pH 3.5–10) was chromatographed in the usual way. Then the column was eluted with 25 m*M* phosphate buffer: no UV-absorbing material was eluted. Upon subsequent elution with 25 m*M* phosphate buffer (pH 3.65) in 80% (v/v) ethanol, some UV-absorbing material eluted in the total volume of the column, but a UV spectrum of this material showed that it was not Ampholine (as it appeared to absorb noticeably at 280 nm, it was attributed to aromatic contaminants of the deionized water used). Upon final elution with *n*-butanol no more UV-absorbing material was eluted.

Hydrophobic interaction chromatography of a peptide mixture

The elution of the peptide mixture is shown in Fig. 2; fractions were pooled as indicated. Based on the weights of the lyophilized pooled fractions, the recovery was about 32%.

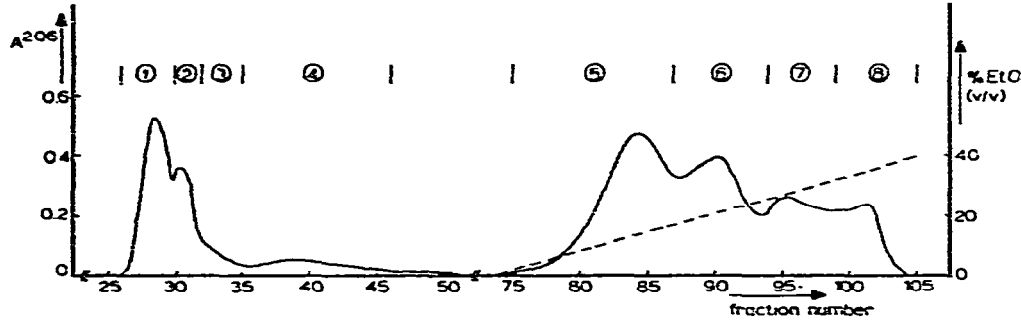

Fig. 2. Elution pattern of the peptide mixture on octyl-Sepharose. ——, Absorbance at 206 nm; - - - - - -, ethanol concentration.

DISCUSSION

The K values for the peptides at constant pH (Table I) show the expected dependence on the hydrophobicity of the constituent amino acids: they increase in the order Gly < Val < Leu < Phe and with increasing content of the more hydrophobic amino acid. This is in accordance with literature reports[17,18] on hydrophobic interactions of dipeptides with silica-bonded alkyl groups (studied by reversed-phase high-performance liquid chromatography (HPLC) on octyl silica[17] or octadecyl silica[18] in aqueous buffer systems).

The expected K-enhancing effect of increasing salt concentration is also borne out by the results (Table I).

The K values in 20% sucrose are of interest from a theoretical point of view. The effect of salts on the interaction of biopolymers (*e.g.* proteins) with hydrophobic surfaces (*e.g.*, alkyl chains) in water has been related[19] to the increase in the surface tension of water on the addition of salt. With this simple concept it is also possible to explain the elution of a protein (and even the elution order of different proteins) bound to octyl-Sepharose by increasing concentrations of ethylene glycol, which has a lower surface tension than water. On the basis of this view one expects sucrose to have an analogous K-enhancing effect [for comparison: 1 M sodium chloride and 20% (w/v) sucrose increase the surface tension of water by 1.6 and 1.0 dyne·cm^{-1}, respectively[20]]. Clearly, this simple theory is not able to explain our K values for the tripeptides in 20% sucrose (Table I).

Fig. 1 shows that the interaction of Gly–Phe–Phe with octyl groups is minimal at a pH value near to the isoelectric point of the peptide (p$I \approx 5.7$). Similar effects have been reported by Kroeff and Pietrzyk[17] for the interaction of several dipeptides with octyl groups on octylsilica and their explanation also holds here.

Of special interest to our problem is the fact that all of the investigated carrier ampholytes show no retention on octyl-Sepharose (Table I), even in the presence of 2 M sodium chloride and at pH values remote from their mean isoelectric point. This means that an efficient separation is feasible of carrier ampholytes from peptides that contain some hydrophobic residues. Based on the observed HETP values (about 0.6 mm), a minimal K value for the peptide of about 3 M^{-1} is necessary for a satisfactory (*i.e.*, a 4 σ or 2% overlap) separation from a carrier ampholyte.

It is possible to estimate the minimal size of a peptide of average composition for which such a good separation can be expected. From the data in Table I it can be inferred that a peptide containing two Phe residues will be sufficiently retarded in 2 M sodium chloride. The data of Lundanes and Greibrokk[18] indicate that the hydrophobic interaction with alkyl chains increases for amino acid residues in the order Leu < Phe < Trp, of which the last two are by far the most hydrophobic. As the sum of the frequencies of occurrence[21] of Phe and Trp residues in natural peptides or protein fragments is 4.6%, the chance of finding none or one of these residues in a peptide is less than 10% if it contains more than 85 amino acids[22]. This would mean that a sufficient separation from carrier ampholytes is guaranteed only for more than 90% of the peptides in a mixture if the molecular weights of these peptides exceed about 10,000.

As only the contributions to the hydrophobicity of Phe and Trp are taken into account, this estimation of the lower limit of the applicability of the method might seem rather pessimistic: a peptide with 85 amino acids contains, on average, in addi-

tion to four Phe + Trp residues, six Leu, four Ile and three Tyr residues, all of which might contribute to the overall hydrophobicity. However, a peptide of this size can be expected to have a native conformation in which some of the hydrophobic residues are less available for interaction with octyl groups: an HPLC study[23] of a large number of peptides and proteins on octadecylsilica has shown that for small peptides (< 15 residues) the retention can be described reasonably well with additive individual residue contributions to the hydrophobicity, but that for larger peptides (> 16 residues) the retention is generally smaller than expected.

We conclude, therefore, that the separation of peptides from carrier ampholytes by hydrophobic interaction chromatography on octyl-Sepharose in the presence of salt is in general not feasible for peptides with molecular weights in the most interesting range (say 2000–8000). For this separation, more hydrophobic adsorbents are required.

It should be realized that the method has yet another drawback: part of the peptides might be so tightly bound that elution with relatively mild eluents cannot be achieved from an adsorbent as hydrophobic as octyl-Sepharose.

The mixture of somatomedin-containing peptides that we used as an example gives an illustration of such a situation. This mixture has K_{av} values of 0.4–0.5 on Sephadex G-50, so its mean molecular weight is 5600, corresponding with 44 amino acid residues. The chance that a peptide of this size contains no or one Phe or Trp residues is 40%. As the mixture was applied to the octyl-Sepharose column in aqueous buffer without sodium chloride, peptides containing one and two Phe or Trp residues should have K values of about 1 and 2 M^{-1} respectively. Hence, we expect 40% of the material to be eluted within an eluent volume corresponding to $K = 1.5\ M^{-1}$. In reality, 5% is found (see Fig. 2). On the other hand, 68% of the material cannot be eluted with a gradient of up to 40% ethanol. So, to separate this peptide mixture completely from carrier ampholytes, one should have at ones disposal a range of adsorbents of varying hydrophobicity: first, the more hydrophobic peptides are separated from the less hydrophobic ones and the carrier ampholytes on an adsorbent of low hydrophobicity, then the less hydrophobic peptides are separated from the carrier ampholytes on a strongly hydrophobic adsorbent. An additional reward of this procedure is that the peptides are separated according to their hydrophobicity: in the elution pattern in Fig. 2 seven clear-cut fractions can be observed.

The somatomedin-containing peptides thus appear to be rather hydrophobic. It is not unrealistic to expect that this applies to all hitherto characterized somatomedins since they appear to be firmly bound to carrier proteins in plasma. However, a few further observations on the pooled fractions of the octyl-Sepharose chromatography should be mentioned.

The ratio of the sum of the absorbances at 254 and 280 nm to the weight of peptide seems to increase gradually from fractions 1 to 4, but to decrease from fractions 5 to 8. If this ratio as taken is indicative of the Phe + Trp + Tyr content of the peptides, this decrease is not in accord with hydrophobic bonding through these residues.

The isoelectric focusing patterns of the pooled fractions in comparison with that of the original mixture reveal that, whereas fractions 1–4 contain hardly any basic peptides, fractions 6–8 contain an increasing number of peptides in the basic range, while the most basic peptides in the original mixture are apparently bound to the octyl-Sepharose. This result also cannot be readily explained by hydrophobic bonding alone.

Possibly a type of binding different from hydrophobic bonding is also involved in this instance; the binding of the peptides eluted by the ethanol gradient might be of the same non-ionic non-hydrophobic nature as that described by Hofstee and Otillio[24] for γ-globulin components.

CONCLUSIONS

Hydrophobic interaction chromatography on alkyl-agarose seems to be feasible for the separation of carrier ampholytes from peptides if a range of adsorbents of varying hydrophobicity is available. Peptides containing two or more phenylalanine or tryptophan residues can be separated from carrier ampholytes on octyl-Sepharose. An additional reward of this separation procedure is that the peptides are separated according to their hydrophobicity.

ACKNOWLEDGEMENTS

This work was supported in part by the Dutch Foundation for Medical Research (Fungo, grant no. 13-24-12). Thanks are due to Dr. H. G. J. Brummelhuis of the Central Laboratory of the Dutch Bloodtransfusion Service for supplying Cohn fraction IV, and to Mr. C. M. Hoogerbrugge (Wilhelmina Children's Hospital) for technical assistance with somatomedin purification.

REFERENCES

1 O. Vesterberg, in N. Catsimpoolas (Editor), *Isoelectric Focusing*, Academic Press, New York, 1976, p. 53.
2 M. B. Hayes and D. Wellner, *J. Biol. Chem.*, 244 (1969) 6636.
3 A. Carlström and O. Vesterberg, *Acta Chem. Scand.*, 21 (1967) 271.
4 N. Catsimpoolas, C. Ekenstam and E. W. Meyer, *Cereal Chem.*, 46 (1969) 357.
5 W. J. Gelsema, C. L. de Ligny and N. G. van der Veen, *J. Chromatogr.*, 173 (1979) 33.
6 W. J. Gelsema, C. L. de Ligny and W. M. Blanken, *J. Chromatogr.*, submitted for publication.
7 G. Baumann and A. Chrambach, *Anal. Biochem.*, 69 (1975) 649.
8 P. G. Righetti, in P. G. Righetti, C. J. van Oss and J. W. Vanderhoff (Editors) *Electrokinetic Separation Methods*, Elsevier, Amsterdam, 1979, p. 389.
9 F. P. Guengerich, *Biochim. Biophys. Acta*, 577 (1979) 132.
10 U. Ernström and K. Nordlind, *J. Chromatogr.*, 175 (1979) 202.
11 E. Gianazza, C. Astorri and P. G. Righetti, *J. Chromatogr.*, 171 (1979) 161.
12 J. L. Van den Brande and C. M. Hoogerbrugge, in A. Pecile and E. E. Müller (Editors), *Growth Hormone and Other Biologically Active Peptides*, Exerpta Medica, Amsterdam, 1980, in press.
13 S. van Buul-Offers and J. L. Van den Brande, *Acta Endocrinol. (Kbh)*, 92 (1979) 242.
14 W. H. Daughaday, K. Hall, M. S. Raben, W. D. Salmon, J. L. Van den Brande and J. J. van Wyk, *Nature (London)*, 235 (1972) 107.
15 J. A. Bakker and J. L. Van den Brande, in preparation.
16 *Affinity Chromatography, Principles and Methods*, Pharmacia, Uppsala, Sweden, 1979.
17 E. P. Kroeff and D. J. Pietrzyk, *Anal. Chem.*, 50 (1978) 1353.
18 E. Lundanes and T. Greibrokk, *J. Chromatogr.*, 149 (1978) 241.
19 C. J. van Oss, D. R. Absolom and A. W. Neumann, *Separ. Sci. Technol.*, 14 (1979) 305.
20 E. W. Washburn (Editor), *International Critical Tables*, Vol. 4, McGraw-Hill, New York, 1st ed., 1928.
21 M. M. Klapper, *Biochem. Biophys. Res. Commun.*, 78 (1977) 1018.
22 W. J. Dixon and F. J. Massey, *Introduction to Statistical Analysis*, McGraw-Hill, New York, 2nd ed., 1957, p. 435.
23 M. J. O'Hare and E. C. Nice, *J. Chromatogr.*, 171 (1979) 209.
24 B. H. J. Hofstee and N. F. Otillio, *J. Chromatogr.*, 159 (1978) 57.