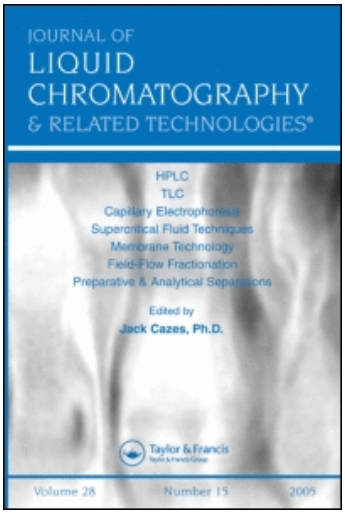

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AN APPLICATION OF THE LIGAND EXCHANGE CHROMATOGRAPHY TO THE ANALYSIS OF SOME PROTEIN COMPONENTS

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ABSTRACT

The resolution of a mixture containing some α-aminoacids, their β-isomers and dipeptides is realized on column by the ligand-exchange (LE) chromatography. The support used is a chelating exchanger bounded with a copper(II)- or a nichel(II)-ammonia complex.

INTRODUCTION

The resolution of a mixture of α-, β-aminoacids and dipeptides represents an argument of considerable significance for its applications both in clinical and in food chemistry. In the latter field the interest for this separation is particularly remarkable because of the recent topic on "single cell protein"(namely bioproteins). Methods for the separation of aminoacids from peptides are known (1-4), but no one of them per-

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mits a complete separation of the three classes of compounds.

The ability of the considered compounds to form chelate species of different stability and electrical charge with some metal ions suggests the possibility to obtain this separation by the ligand-exchange (LE) chromatography, which is a technique often applied for the resolution of mixtures of species having different coordination power (5) and for the purification and concentration of solutions (6). In order to prove the validity of the above hypothesis, in the present paper we report, as an example, the resolution of a mixture containing three compounds of each class: α-alanine, α-aminobutyrric acid, α-aminoisobutyrric acid, their β-isomers and three dipeptides: glycylglycine, alanylalanine and glycylalanine.

The form of the chelating exchanger is chosen as $Me(NH_3)_x^{2+}$, where Me^{2+} is Ni^{2+} or Cu^{2+}. Among the ions generally employed in the ligand-exchange technique (7) they are those which form complex species with the considered ligands, the stability constants of which are sufficiently strong and appreciably different (8) to suppose a separation by the LE method.

Preliminary experiments were done on thin layer and the resolution of the mixture was carried out by column chromatography.

MATERIALS

All chemicals were analytical grade products: L-alanine, β-alanine and glycylglycine were "Merck for biochemi-

stry", while the other aminoacids and peptides were "Fluka". An exchanger of the polystyrene-iminodiacetic type (Chelex 100, BioRad, 100-200 mesh) and a cellulose powder (MN 300 for TLC, Macherey Nagel) were used. Supporting plates (5x20 cm) and glass columns (h =25 cm, i.d. = 1cm) were employed.

The pH measurements were made on a Radiometer pH M-22r instrument equipped with external glass and saturated calomel electrodes.

Preparation of the Exchanger in the $Ni(NH_3)_6^{2+}$ Form

An amount of 10g of the Chelex resin (Na^+ form) was exhaustively washed with several portions of a 1M HCl solution until free from Fe^{3+} (thyocianate essay), then with deionized water up to neutrality (exchanger in the H^+ form). A 1:1 ammonia solution of $[Ni(NH_3)_6]Cl_2$ prepared according to the litterature (9), was added in a beaker to the exchanger and the suspension was stirred for about twenty hours, until no colour variation could be detected.

For thin layer chromatography equal amounts of the resin so prepared and of cellulose powder were suspended in a 10^{-3} M ammonia solution and stirred for about four hours, then a uniform film was spread on the plates in such a way as to obtain layers 0.25 mm thick. After the plates were air dried over night.

For column chromatography the exchanger in the $Ni(NH_3)_6^{2+}$ form was placed in the column which was loaded until the packed resin was 10 or 20 cm high. The

system was stabilized by washing it with 1:1 NH_3 solution (200 ml) and 10^{-3} M NH_3 solution (300 ml), until the effluent was free from Ni^{2+} ions (dimethylglyoxime essay).

Preparation of the Exchanger in the $Cu(NH_3)_x^{2+}$ Form

The resin in the H^+ form was stirred in a beaker with a solution 0.5 M of $CuCl_2.2H_2O$ until no colour variation could be detected and washed with deionized water. Successively the exchanger was stirred with about 100 ml of 3 M NH_3.

METHODS

For TL chromatography the resin in the Cu^{2+} form was treated as described before, while for column chromatography the resin was packed in the column and stabilized with a 10^{-3} M ammonia solution until the pH of the effluent was between 8 and 9.

In TLC an amount of 20 µl of an aqueous solution of each substance under examination (1 mg/ml) was placed on the starting point and then the plates were air dried. The considered compounds were detected with ninhydrine spray for TLC.

The elution mixtures employed during the chromatographic runs are reported in the tables 1 and 2.

In the column chromatography preliminary tests were carried out by loading some columns with 0.5 ml of a 10^{-1} M solution of each compound separately and checking concentrations of the NH_3 solutions between 10^{-5}M

TABLE 1

Rf Values of some α- and β-Aminoacids and Dipeptides on Copper(II)-Chelex Plates in Propanol-Ammonia solution (40:60) with various Concentrations of the latter Constituent.

Compounds	NH_3= 0.3 M	NH_3= 0.6 M	NH_3= 0.8 M
α-alanine	0.48	0.45	0.68
α-aminobut. ac.	0.47	0.46	0.66
α-aminoisobut. ac.	0.42	0.44	0.61
β-alanine	0.82	0.77	0.85
β-aminobut. ac.	0.86	0.88	0.86
β-aminoisobut. ac.	0.86	0.88	0.86
glycylglycine	0.89	0.80	0.89
alanylalanine	0.92	0.87	0.87
glycylalanine	0.90	0.92	0.90

and 1 M. The flow rate of the elution was 0.5 ml/min. Then 1.5 ml of a mixture containing all the compounds, in equal molar quantity, were allowed to pass through the column and fractions of 2 ml were collected and examined. The detection of each compound was performed by colour development with ninhydrine method on TLC (10), obtaining violet-red spots for the β-aminoacids, violet-blue spots for α-aminoacids and brown or yellow spots for dipeptides. The Rf value of each component was compared with that of a reference.

TABLE 2

Rf Values of some α- and β-Aminoacids and Dipeptides on Nichel(II)-Chelex Plates in Propanol-Ammonia solution (40:60) with various Concentrations of the latter Constituent.

Compounds	NH_3= 0.3 M	NH_3= 0.6 M	NH_3= 0.8 M
α-alanine	0.38	0.73	0.72
α-aminobut. ac.	0.43	0.72	0.71
α-aminoisobut. ac.	0.56	0.71	0.72
β-alanine	0.64	0.80	0.83
β-aminobut. ac.	0.65	0.80	0.81
β-aminoisobut. ac.	0.65	0.83	0.82
glycylglycine	0.64	0.82	0.83
alanylalanine	0.70	0.87	0.89
glycylalanine	0.65	0.84	0.85

RESULTS AND DISCUSSION

Exchanger in the $Cu(NH_3)_x^{2+}$ Form

On the basis of the preliminary tests on thin layer we observe that the ligand-exchange effect is operating only for α-aminoacids: in fact their Rf values (tab.1) are smaller than those of the other classes of compounds which are quite equal to one. In addition an increase of the ammonia concentration in the eluent mix-

ture does not appreciably affect the Rf values of dipeptides and of β-aminoacids, showing that there is no LE effect for these compounds (11), on the contrary the α-aminoacids Rf values raise as the ammonia concentration increases, as it could have been foreseen. These results were confirmed by the column chromatography: only the α-aminoacids are retained on the resin and are eluted with a 0.5 M ammonia solution, whereas the other two classes of substances are eluted by water and their separation is realized only by chromatographic absorption.

$Cu(NH_3)_x^{2+}$ ions were detected in the effluent only during the elution of the peptidic component of mixture.

Exchanger in the $Ni(NH_3)_6^{2+}$ Form

The TLC tests (tab.2) show that, in this case, the LE effect is operating for all compounds.

The relative values of Rf are in general smaller than those observed on $Cu(NH_3)_x^{2+}$-Chelex layers. By increasing the NH_3 concentration of the eluent, all the Rf values raise, so much so that with a 0.3 M NH_3 concentration no more separation can occur.

On column chromatography we observe the retention of all compounds under examination by LE and we realize the resolution of the mixture with different eluent solutions. In particular β-aminoacids are displaced by eluting the column with NH_3 aqueous sol.-EtOH (80:20; actual concentration of NH_3 is 4 10^{-3} M), then dipeptides are eluted with a 10^{-3} M NH_3 solution and α-aminoacids with 10^{-1} M NH_3.

Note that, before the hydroalcoholic eluent, few ml of a 10^{-3} M NH_3 solution were allowed to pass through the column to avoid bubble formation inside the packed resin. During all chromatographic runs Ni^{2+} ions were never eluted together with the ammonia solutions.

CONCLUSIONS

The results of the present paper show that a successful resolution of a mixture containing α-aminoacids, their corresponding β-isomers and dipeptides can be realized by the ligand-exchange chromatography. In particular, if the separation of the first class of substances from a mixture of proteinic compounds is required, it can be usefully employed a chelating exchanger in the $Cu(NH_3)_x^{2+}$ form, on which only α-aminoacids are retained. Otherwise the Chelex-resin in the $Ni(NH_3)_6^{2+}$ form can be better employed in the separation of compounds of the three classes because, in this case, the selective retention of all substances is achieved.

The detailed analysis of the results and the comparison with the formation constants values of the related complexes (8) prove, once again (7,11), that the stability of coordination compounds in aqueous solution is not always an index of the behaviour of the same complexes on the chelating exchangers. While it could have been foreseen the LE effect with the Chelex-resin both in the nichel(II) and in the copper(II) form, our results show that this is true only with regard to the former metal ion. In fact, even if the log K values of the

copper(II)-complexes are greater and appreciably different among the three classes compared with those of the nichel(II)-complexes, our purpose was achieved using the exchanger chelated with the latter ion.
Neither with copper(II) nor with nichel(II), as central metal ion bound to the resin, the experimental order of retention (α-aminoacids > dipeptides > β-aminoacids) corresponds to the formation constants trend.
From all the data, we can also confirm that the preliminary results obtained by thin layer chromatography on metal-Chelex plates are substantially consistent with those realized by column.

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