



ELSEVIER

Journal of Chromatography A, 789 (1997) 21–27

JOURNAL OF  
CHROMATOGRAPHY A

## Review

# Chromatographic ion-exchange and change-partner reactions New mechanisms concerning ion–ion interactions of inorganic compounds in Sephadex G-15 columns

T. Okada<sup>1</sup>

*Department of Biochemistry, Kanazawa Medical University, Daigaku 1-1, Uchinada, Ishikawa 920-02, Japan*

### Abstract

This review describes ion-exchange and change-partner reactions, the new mechanisms concerning ion–ion interactions of inorganic compounds, during elution in Sephadex G-15 column. These reactions were revealed by observing the elution profiles of all of the ions employed when sodium or potassium chloride was eluted with sodium or potassium phosphate buffer in various sample–eluent combinations. The general conditions under which the reactions occur are discussed.  
© 1997 Elsevier Science B.V.

*Keywords:* Reviews; Ion–ion interactions; Potassium salts; Phosphate salts; Sodium salts

### Contents

1. Introduction .....	22
2. Experimental .....	22
2.1. Chemicals .....	22
2.2. Columns .....	22
2.3. Samples .....	23
2.4. Eluents .....	23
2.5. Sample–eluent systems .....	23
2.6. Quantitation of ions .....	23
3. Results and discussion .....	23
3.1. Ion-exchange reaction .....	23
3.1.1. Ion pairs from sample and eluent exchanged the partner ion during elution in heterocationic systems .....	23
3.1.2. Na <sup>+</sup> P <sup>-</sup> ion pair was eluted more rapidly than K <sup>+</sup> P <sup>-</sup> ion pair .....	24
3.1.3. Cation and anion from the sample were separated more efficiently with higher concentration of the eluent .....	24
3.1.4. Ion-exchange reaction occurred with extremely low concentration of sample salt .....	24
3.1.5. Ion-exchange reaction occurred also in homocationic systems .....	24
3.1.6. Ion-exchange reaction did not occur appreciably in Sephadex G-25 column .....	25
3.1.7. When eluted with distilled water, cation and anion from the sample were eluted together .....	25
3.1.8. Mechanism of ion-exchange reaction .....	25

<sup>1</sup>Present address: Midoridai 1-45, Uchinada, Ishikawa 920-02, Japan.

3.2. Change-partner reaction.....	25
3.2.1. Na <sup>+</sup> and P <sup>-</sup> of the Na <sup>+</sup> P <sup>-</sup> ion pair changed the partner ion, during elution .....	25
3.2.2. Mechanism of the change-partner reaction .....	25
3.3. Ion-exchange and change-partner reactions were assumed to be coupled .....	26
4. Conclusions .....	26
Acknowledgements .....	26
References .....	26

## 1. Introduction

The chromatographic behaviour of inorganic compounds in the Sephadex G-15 column has been extensively studied by many workers from early 1960 to late 1970 [1–3]. They reported that, when inorganic compounds are eluted from Sephadex, various side effects such as solute–gel matrix interactions (ion-exchange [4–6], adsorption [5,7–13]) as well as solute–solute interactions (complexation [4,7] and ion exclusion [4,5,11]) alter the elution volume predicted from the sizes of the hydrated ions [1].

In some of these experiments, the sample and eluent salts differed in either cation [7] or anion [4] and in others pure water was used as an eluent [4,5,11]. In few experiments in which sample and eluent salts differed from each other in both the cation and anion, the elution profiles were observed only with refractometer, spectrophotometer, or atomic absorption spectrometer or by determining the amount of either the cation [6,7] or anion [10,13]. Therefore, side effects were not completely elucidated.

In 1979, we eluted potassium chloride solution from a Sephadex G-15 column with 0.025 M sodium phosphate buffer (pH 7.0) to double-check the data described in a booklet published from Pharmacia Fine Chemicals [14], which showed that potassium chloride ion pair was eluted in a single peak under the above-mentioned conditions. However, the refractory index curve of the eluate we obtained was unexpectedly complex. Therefore, we determined the concentration distribution of all of the ions in the eluate and found that the potassium and chloride ions from the sample were eluted separately apparently against the electrical neutrality. This result prompted us to perform further experiments to investigate the phenomenon and propose new mechanisms con-

cerning ion–ion interaction, ion-exchange [15] and change-partner reactions [16].

This review describes the selected and rearranged results we obtained [15,16] and discusses the general conditions required for the ion-exchange and change-partner reactions to occur.

The changing-partner reaction coined in our previous paper [16] is called change-partner reaction in this review.

## 2. Experimental

### 2.1. Chemicals

All reagents were of analytical grade from Wako Pure Chemical Industries (Osaka, Japan). Sodium-22 chloride (<sup>22</sup>NaCl; 61.60 mCi/mg; 99% pure) was obtained from New England Nuclear (Boston, MA, USA) and phosphorus-32 labelled phosphoric acid in 0.08 M hydrochloric acid solution (H<sub>3</sub><sup>32</sup>PO<sub>4</sub>; carrier free, 99% pure) produced by the Japan Atomic Energy Research Institute, was obtained from Japan Radioisotope Association (Tokyo, Japan).

### 2.2. Columns

Sephadex G-15 (Pharmacia Fine Chemicals, Uppsala, Sweden; dry particle diameter, 40–120 μm) was packed according to a standard procedure in two kinds of glass tube, to make a short column (300 × 13 mm; bed height, 25 cm) and a long column (Excel Type SE-1000, 1000 × 19 mm; bed height, 90 cm; porous polystyrene support). In an experiment in our previous paper [17], it was found that the phosphate ion (P<sup>-</sup>) was bound tightly on the gel and did not appreciably exchange with the P<sup>-</sup> in the eluent under the described conditions. Therefore, to prevent adsorption of the P<sup>-</sup>, the gel was pre-

equilibrated with phosphate buffer in some experiments where  $\text{NaH}_2\text{PO}_4$  solution was eluted with distilled water. Sephadex G-25 (Pharmacia; dry particle diameter, 50–150  $\mu\text{m}$ ) packed in a long glass tube was also used.

When a sample solution was eluted from a short column, no peristaltic pump was used for the elution, and a volume of 0.1 ml of sample solution was applied to the top of the column. When the long column was employed, a peristaltic pump (LKB Instrument, Rockville, USA; gear box 3:250) was inserted between the elution reservoir and the top of the column to maintain a constant flow-rate (12 ml/h) and a volume of 0.6 ml of the sample solution was applied. In both cases, the eluate was continuously monitored with a Refractometer Model SF-1107 (Mitsumi Scientific, Tokyo, Japan) and collected in 10-min fractions using a LKB7000 Ultrac fraction collector (LKB Instrument). All columns were operated in a cold room at 4°C to avoid changing the elution behaviour of ions by temperature shift.

### 2.3. Samples

Various concentrations of sodium chloride (NaCl) and/or potassium chloride (KCl) dissolved in the eluent were used as sample solutions. In some experiments,  $^{22}\text{NaCl}$ , sometimes diluted in 0.72 M NaCl, and/or the  $\text{H}_3^{32}\text{PO}_4$ , which were dissolved in the eluent were employed. The radioactivities of both  $^{22}\text{NaCl}$  and  $\text{H}_3^{32}\text{PO}_4$  were 0.03  $\mu\text{Ci}$  in 0.6 ml of the sample solution applied. Sodium dihydrogenphosphate ( $\text{NaH}_2\text{PO}_4$ ; 1 M) was also eluted with distilled water in an experiment.

### 2.4. Eluents

The eluents were 0.025 M potassium or sodium phosphate buffer (pH 7.0) ( $\text{NaP}^-$  or  $\text{KP}^-$  buffer) unless otherwise stated.

### 2.5. Sample–eluent systems

The sample–eluent systems employed were two homocationic, heteroanionic systems (referred to as homocationic systems in this paper), i.e., potassium chloride and potassium phosphate (KCl–KP) buffer

and sodium chloride–sodium phosphate (NaCl–NaP) buffer, and two heterocationic, heteroanionic systems (referred to as heterocationic systems in this paper), i.e., sodium chloride–potassium phosphate (NaCl–KP) buffer and potassium chloride–sodium phosphate buffer (KCl–NaP).

### 2.6. Quantitation of ions

The amounts of potassium ion ( $\text{K}^+$ ) and sodium ion ( $\text{Na}^+$ ) were determined in a Na–K flame photometer Model 450 (Corning Eel, Sudbury, UK). Chloride ion ( $\text{Cl}^-$ ) was measured with a Chloridometer Model 920 (Corning Eel). The amounts of  $\text{P}^-$ , irrespective of whether the ion was  $\text{H}_2\text{PO}_4^{1-}$ ,  $\text{HPO}_4^{2-}$  or  $\text{PO}_4^{3-}$ , were determined by the method of Fiske and Subbarow [19]. Sodium-22 ion ( $^{22}\text{Na}^+$ ) was counted in an Auto-well gamma system Model JDC-751 (Aloka, Tokyo, Japan), and phosphorus-32-labelled phosphate ion ( $^{32}\text{P}^-$ ) in a liquid scintillation counter Model LSC-900 (Aloka). The Auto-well gamma system counted  $^{32}\text{P}^-$  about 1.2% as efficiently as the liquid scintillation counter, and the liquid scintillation counter counted  $^{22}\text{Na}^+$  about 5% as efficiently as the Auto-well gamma system.

## 3. Results and discussion

### 3.1. Ion-exchange reaction

#### 3.1.1. Ion pairs from sample and eluent exchanged the partner ion during elution in heterocationic systems

When NaCl (0.68 M) or KCl (0.20 M) solutions were eluted from a short column with KP or NaP buffer, respectively (NaCl–KP or KCl–NaP system), the refractory index curve of the eluate showed that, in NaCl–KP system, two positive peaks with a negative peak in between, while, in KCl–NaP system, one small negative peak followed by two positive peaks. These complex elution curves were unexpected one. Therefore, all of the ions employed were determined of the eluate. Then, it was revealed that, in these heterocationic systems, the cation and anion from the sample were not eluted together but separately, apparently against the electrical neutrality. However, the cation from the sample was

accompanied by anion from the eluent ( $P^-$ ) and eluted in the early fractions corresponding to the first positive peak of the refractory index curve, and the anion from the sample ( $Cl^-$ ) was accompanied by the cation from the eluent and eluted in the late fractions, corresponding to the second positive peak of the refractory index curve. This phenomenon clearly indicated that the ion pairs from the sample and eluent exchanged the partner ion during elution in the column. We called this phenomenon chromatographic ion-exchange reaction (referred to as ion-exchange reaction) [15] which has never been reported previously.

Ogata et al. [7] eluted various concentrations of magnesium chloride ( $MgCl_2$ ) from a Sephadex G-15 column with sodium sulfate ( $Na_2SO_4$ ) solution (heterocationic, heteroanionic system), but observed the elution profile of  $Mg^{2+}$  alone. Therefore, they did not ascribe the phenomenon to an ion-exchange reaction.

### 3.1.2. $Na^+P^-$ ion pair was eluted more rapidly than $K^+P^-$ ion pair

The next question was why a negative peak appeared between two positive peaks in the  $NaCl$ – $KP$  system, and before the two positive peaks in the  $KCl$ – $NaP$  system.

These phenomena are explained as follows; in the  $NaCl$ – $KP$  system, the  $Na^+P^-$  ion pair produced by ion-exchange reaction is eluted more rapidly than the  $K^+P^-$  ion pair of the eluent, and the  $K^+Cl^-$  ion pair produced by ion-exchange reaction elutes more slowly than the  $K^+P^-$  ion pair, resulting in producing a low concentration area of electrolytes (negative peak) between the two positive peaks. On the other hand, in the  $KCl$ – $NaP$  system, not only the  $Na^+Cl^-$  ion pair but also the  $K^+P^-$  ion pair, which are produced by ion-exchange reaction, are eluted more slowly than the  $Na^+P^-$  ion pair of the eluent, resulting in a negative peak followed by the two positive peaks of  $K^+P^-$  and  $Na^+Cl^-$  ion pairs.

### 3.1.3. Cation and anion from the sample were separated more efficiently with higher concentration of the eluent

The assumption described above was supported by the observation that when 0.56 M  $KCl$  solution was eluted with a 5-fold higher concentration (0.125 M)

of  $NaP$  buffer, the  $K^+$  peak formed a symmetrical single peak and the elution volume of  $Cl^-$  increased. Therefore, the peaks of  $K^+$  and  $Cl^-$  were not overlapped but separated from each other. This phenomenon indicated that the higher concentration of the eluent caused rapid and efficient ion-exchange between the two ion pairs from the sample and eluent.

### 3.1.4. Ion-exchange reaction occurred with extremely low concentration of sample salt

The ion-exchange reaction also occurred with an extremely low concentration of sample salt. When a solution of  $^{22}NaCl$  at  $5.5 \cdot 10^{-9}$  M was eluted with  $KP$  buffer from a long column, a sharp peak of radioactivity of  $^{22}Na^+$  was formed in approximately the same early fractions as those of  $Na^+P^-$  peak produced by ion-exchange reaction in the  $NaCl$ – $KP$  system. This elution profile indicated that ion-exchange reaction occurred. The reaction was also assumed to occur because the  $^{22}Na^+Cl^-$  ion pair met a higher concentration of ion pair from the eluent continuously flowing in the column.

### 3.1.5. Ion-exchange reaction occurred also in homocationic systems

In contrast to the heterocationic systems, in the  $NaCl$ – $NaP$  system (a homocationic system), single positive peak corresponding to the  $Na^+Cl^-$  ion pair and no negative peak were observed. In addition to this, since the cation from the sample and the eluent were the same, it was not clear from the elution profiles whether ion-exchange reaction occurred or not. However, the reaction was expected to occur in the systems as well, theoretically.

To examine this possibility, the  $Na^+$  of the sample was labelled with  $^{22}Na^+$  to differentiate it from the  $Na^+$  of the eluent. Then,  $^{22}Na^+$  and  $^{22}Na^+$  in 0.72 M  $NaCl$  solutions and 0.72 M  $NaCl$  solution containing no  $^{22}Na^+$  were eluted independently from a long column with  $NaP$  buffer. The elution profile showed that the radioactivity was within the sharp peak in early fractions and a positive peak of  $Na^+Cl^-$  ion pair, in late fractions. This result indicated that ion-exchange reaction occurred in the homocationic system.

Although we have never performed the experiment in the other homocationic system ( $KCl$ – $KP$  system),

the ion-exchange reaction would be expected to occur in the system as well.

### 3.1.6. Ion-exchange reaction did not occur appreciably in Sephadex G-25 column

To examine the effect of Sephadex gel on the elution behaviour of ions, 1.13 M KCl was eluted with NaP buffer from a Sephadex G-25 column. The elution profiles showed that the cation and anion from the sample were eluted in almost identical fractions. Therefore, the pore size of Sephadex G-15 is particularly suitable for occurrence of the ion-exchange reaction.

### 3.1.7. When eluted with distilled water, cation and anion from the sample were eluted together

When the sample solutions of 0.68 M NaCl and/or 0.54 M KCl were eluted with distilled water, the ion-exchange reaction scarcely occurred and the bulk of the cation and anion from the sample were eluted together.

### 3.1.8. Mechanism of ion-exchange reaction

Since  $\text{Na}^+$  was eluted earlier than  $\text{K}^+$  in NaCl–KP system, and  $\text{K}^+$  was eluted earlier than  $\text{Na}^+$  in KCl–NaP system, the elution volumes of  $\text{Na}^+$  and  $\text{K}^+$  ions were independent of both the sizes of the cations and the preferential adsorption to, or exclusion from the gel matrix. Since  $\text{Na}^+$  from the sample was associated with  $\text{P}^-$  in one heterocationic system, and  $\text{K}^+$  from the sample was associated with  $\text{P}^-$  in the other, there was no preferential association of a cation with  $\text{P}^-$ .

On the other hand,  $\text{P}^-$  is eluted more rapidly than  $\text{Cl}^-$  in all the systems employed. This might be due to the difference of the penetrabilities into gel particles between the  $\text{Cl}^-$  (from the sample) and  $\text{P}^-$  (from the eluent), which causes the separation of the two anions topographically, weakening the cation  $\text{P}^-$  bond and causing the difference of the elution velocities of the two anions.

Since the sizes of  $\text{Na}^+$  and  $\text{K}^+$  are similar, cation  $\text{P}^-$  ion pair from the eluent is eluted more rapidly than cation  $\text{Cl}^-$  ion pair from the sample. Although some of the ion pairs from the sample and eluent may exchange their partner ions immediately after the sample is applied to a column as Saunders and

Pecsok [20] observed, the bulk of the two ion pairs exchange their ions during elution in the column.

This reaction repeats until all cation of the cation– $\text{Cl}^-$  ion pair is replaced by the cation from the eluent. Thus, cation (from the sample)  $\text{P}^-$  and cation (from the eluent)  $\text{Cl}^-$  ion pairs are produced; the former is eluted more rapidly than the latter.

Therefore, it was concluded that the ion-exchange reaction might not be ascribed to solute–gel matrix interaction, but to ion–ion interactions between the ion pairs from sample and eluent. That is, definite amounts of cation  $\text{Cl}^-$  ion pair met many cation  $\text{P}^-$  ion pairs flowing through later during elution.

## 3.2. Change-partner reaction

### 3.2.1. $\text{Na}^+$ and $\text{P}^-$ of the $\text{Na}^+\text{P}^-$ ion pair changed the partner ion, during elution

The next question was whether the cation and  $\text{P}^-$  ion of the cation (from the sample)  $\text{P}^-$  ion pair are eluted together or separated during the elution. To resolve this question, a mixture of  $^{22}\text{NaCl}$  and  $\text{H}_3^{32}\text{PO}_4$  solution in NaP buffer was eluted from a long column with NaP buffer ( $^{22}\text{NaCl}\cdot\text{H}_3^{32}\text{PO}_4$ –NaP system) [17]. Then,  $^{22}\text{Na}^+\text{P}^-$  and  $\text{Na}^+^{32}\text{P}^-$  ion pairs would be formed promptly. As the elution velocities of  $^{22}\text{Na}^+$  and  $^{32}\text{P}^-$  ions were supposed to be the same as that of  $\text{Na}^+$  and  $\text{P}^-$  ions, respectively, it was assumed that the elution behaviours of  $^{22}\text{Na}^+\text{P}^-$  and  $\text{Na}^+^{32}\text{P}^-$  reflected those of  $\text{Na}^+\text{P}^-$  ion pair.

The elution profiles of radioactivity showed that the  $^{32}\text{P}^-$  peak and  $^{22}\text{Na}^+$  peak were separated; the former was in Fraction No. 83 and the latter, in Fraction No. 90 which was about the same early fraction as that of the cation  $\text{P}^-$  peak produced in heterocationic system. The late fraction in which the  $\text{Na}^+\text{Cl}^-$  peak produced by ion-exchange reaction appeared in homocationic system was No. 105 approximately. In other words,  $^{32}\text{P}^-$  was eluted in an earlier fraction than that of the  $^{22}\text{Na}^+\text{P}^-$  peak, and the  $^{22}\text{Na}^+\text{P}^-$  peak, than  $\text{Na}^+\text{Cl}^-$  peak produced by ion-exchange reaction.

### 3.2.2. Mechanism of the change-partner reaction

This phenomenon might be explained as follows; as the penetrability of the  $\text{Na}^+$  and  $\text{P}^-$  ions into the gel particles is different,  $\text{Na}^+$  tend to be eluted more slowly than  $\text{P}^-$ . As a result,  $\text{Na}^+$  is pulled down by

the partner  $P^-$ , released gradually from the  $P^-$  and pairs with other  $P^-$  flowing through later. On the other hand,  $P^-$  is pulled up by  $Na^+$ , but released from the  $Na^+$  and pairs with other  $Na^+$  flowing through earlier. This reaction repeats during passage through the column, and the  $Na^+$  and  $P^-$  ions of a  $Na^+P^-$  ion pair are separated during elution. The ion-exchange reaction also supports the idea that the association of  $Na^+$  with  $P^-$  is not so tight.

Another possibility is that the  $^{32}P^-$  in the sample is excluded from the gel (ion exclusion), whereas the  $^{32}Na^+$  is adsorbed (adsorption) on the gel and retarded. However, this possibility is unlikely because the amounts of  $Na^+$  and  $P^-$  adsorbed on the gel were  $3.2 \cdot 10^{-3}$  and  $6.3 \cdot 10^{-12}$  mmole/g of the gel, respectively [17], which are negligible against the high concentration of  $Na^+$  and  $P^-$  in the eluent. In addition to this, when 1 M  $NaH_2PO_4$  was eluted from fresh and phosphate-treated column with distilled water,  $Na^+$  and  $P^-$  ions were eluted together in approximately the same fractions in both instances. Furthermore, as described in Section 3.1.8, the possibility of adsorption and exclusion of cations are unlikely. Therefore, ion exclusion and adsorption, if they occurred, does not seem to affect the elution behaviour of the bulk of the  $Na^+$  and  $P^-$  in the sample.

### 3.3. Ion-exchange and change-partner reactions were assumed to be coupled

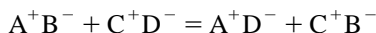
We then performed the experiments, in which 1 M sodium or potassium phosphate solution was eluted with 0.05 M NaCl or KCl solution from a long column; i.e., sample and eluent salts were reversed. Then, the elution profile showed that a negative peak of  $Cl^-$  was coeluted with a positive peak of cation  $P^-$  ion pair in all sample–eluent systems employed. We assumed that the phenomenon is attributable to the coupling of ion-exchange reaction to change-partner reaction [18].

## 4. Conclusions

When KCl or NaCl is eluted with KP or NaP buffer in various sample–eluent combination sys-

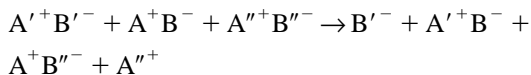
tems, ion-exchange and change-partner reactions occur during elution.

The ion-exchange reaction is formulated as follows,



In our case,  $Na^+$  and  $K^+$  correspond to  $A^+$  and  $C^+$ , and  $Cl^-$  and  $P^-$  correspond to  $B^-$  and  $D^-$ . The  $P^-$  is eluted more rapidly than  $Cl^-$ , and  $K^+$  is eluted slightly more slowly than  $Na^+$ . Therefore, when the elution velocities of  $A^+B^-$  ion pair from the sample and  $C^+D^-$  ion pair from the eluent are different, ion-exchange reaction will occur.

The change-partner reaction is formulated as follows;



with a proviso that  $A'^+$ ,  $A^+$  and  $A''^+$  are the same cation and  $B'^-$ ,  $B^-$  and  $B''^-$  are the same anion.

In our case, the  $Na^+P^-$  or  $K^+P^-$  ion pair corresponds to the  $A^+B^-$  ion pair. Therefore, when the elution velocities of  $A^+$  and  $B^-$  of the  $A^+B^-$  ion pair are different, the change-partner reaction would occur.

## Acknowledgements

The author is indebted to Honorary Professor T. Ohashi at Kanazawa Medical University for his advice and discussion on our work. His thanks go to the members who collaborated on this work with him.

## References

- [1] N. Yoza, J. Chromatogr. 86 (1973) 325.
- [2] E.R. Nightingale Jr., J. Phys. Chem. 63 (1959) 1381.
- [3] T.C. Laurent, J. Killander, J. Chromatogr. 14 (1964) 317.
- [4] P.A. Neddermeyer, L.B. Rogers, Anal. Chem. 41 (1969) 94.
- [5] B.Z. Egan, J. Chromatogr. 34 (1968) 382.
- [6] Y. Ueno, N. Yoza, S. Ohashi, J. Chromatogr. 52 (1970) 321.
- [7] T. Ogata, N. Yoza, S. Ohashi, J. Chromatogr. 58 (1971) 267.
- [8] M. Sinibaldi, M. Lederer, J. Chromatogr. 107 (1975) 210.
- [9] N. Yoza, T. Ogata, S. Ohashi, J. Chromatogr. 52 (1970) 210.
- [10] T. Deguchi, J. Chromatogr. 108 (1975) 409.

- [11] P.A. Neddermeyer, L.B. Rogers, *Anal. Chem.* 40 (1968) 755.
- [12] N. Yoza, S. Ohashi, *J. Chromatogr.* 41 (1969) 429.
- [13] J. Porath, *Metab. Clin. Exp.* 13 (1964) 1004.
- [14] Sephadex—Gel Filtration in Theory and Practice, Pharmacia Fine Chemicals, Uppsala, Sweden, Sept. 1978, p. 14.
- [15] T. Okada, K. Sugata, Y. Nakabayashi, K. Teraoka, M. Miyakoshi, M. Inoue, *J. Chromatogr.* 481 (1989) 299.
- [16] T. Okada, M. Miyakoshi, M. Inoue, M. Miyanabe, Y. Ueno, Y. Nakabayashi, *J. Chromatogr.* 604 (1992) 219.
- [17] T. Okada, M. Miyakoshi, M. Inoue, *J. Chromatogr.* 475 (1989) 412.
- [18] T. Okada, Y. Nakabayashi, F. Jisaki, (1997) unpublished data.
- [19] C.H. Fiske, Y. Subbarow, *J. Biol. Chem.* 66 (1925) 375.
- [20] D. Saunders, R.L. Pecsok, *Anal. Chem.* 40 (1968) 44.