Repellent Determination of Calcium Ion and Regeneration of a Chromogenic Reagent Using Chlorophosphonazo III and an Ion Exchanger in a Circulatory Flow Injection System

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The spectrophotometric determination of Ca\(^{2+}\) with chlorophosphonazo III (CPN) has been carried out by a circulatory flow injection (FI) method. A cation-exchange mini-column for the on-line regeneration of the main reagent was incorporated in this FI system, allowing a repetitive determination of Ca\(^{2+}\). A solution of \(4.0 \times 10^{-5}\) M CPN in a 0.05 M acetate buffer (pH 5.0) in a single reservoir (50 ml) was continuously circulated at a constant flow rate of 1.5 ml min\(^{-1}\). Into the stream, an aliquot (20 µl) of a sample containing Ca\(^{2+}\) was quickly injected by means of a 6-way valve. The complex formed was monitored spectrophotometrically at 670 nm in the flow system. Then, the stream passed through a cation-exchange column, which was introduced after the flow-through cell. A successful ligand-exchange reaction of Ca\(^{2+}\) between the CPN reagent and a cation exchanger, as well as a simultaneous regeneration of the free reagent took place. The stream then returned to the reservoir. The regeneration and recycling of the CPN reagent allowed as many as 300 repetitive determinations of 2.5 mg l\(^{-1}\) Ca\(^{2+}\) solutions with the same 50 ml circulating solution.

(Received June 10, 2002; Accepted August 22, 2002)

Introduction

Spectrophotometry has become the most widely used detection technique for flow injection (FI) methods. Very expensive and special reagents (synthesized or not commercially available) must be used frequently. The minimum consumption of the reagents, as well as the sample, is one of the principal advantages of FI methods compared with batch methods. Nevertheless, the sample/reagent mixing step of the flow system essentially requires disposable analytical reagents, such as chromogenic reagents, masking agents and buffers. The use of a circulatory (closed-loop) FI system, which affords re-circulation of the same reagent solution, leads to an appreciable answer to these situations. To reuse the organic reagents effectively, colored species must be physically or chemically removed from the system. A rapid regeneration system is thus desired under circulating flow conditions. The possibility of repeatable usage and automatic re-circulation of the reagent solution has not yet reached its potential. There appears to be little in the literature concerning repeated use of the reagent solution.

In industrial quality control and environmental monitoring, a large number of samples of similar nature must be analyzed for determining of a single species. A method which is rapid, repetitive, accurate, easily handled and with low cost is required. In such situations, the application of a circulatory FI system, which can lead to appreciable economies and disposal problems, is very effective. The consumption of the reagents and the waste must be kept to a minimum by recycling the reagent solution. The aim of the work described here was an attempt to regenerate and reuse the reagents in a circulatory FI system in order to develop a method for rapid and repetitive measurements. The spectrophotometric determination of Ca\(^{2+}\) with chlorophosphonazo III (CPN)\(^{6-12}\) was chosen to illustrate the application. After the spectrophotometric determination of Ca\(^{2+}\) in a flow through-cell, the reagent stream was passed through a mini-column packed with a cation exchange resin for regeneration of the free CPN. Successful removal of Ca\(^{2+}\) and the other metal ions was carried out; consequently, it will be able to circulate the reagent solution repeatedly. The ability to introduce an ion exchanger has never been reported, and should represent an important development for circulatory FI methods.

Experimental

Chemicals and reagents

All chemicals were of analytical reagent grade. Deionized water purified with a Millipore Milli-Q system was used throughout. Chlorophosphonazo III (CPN) [2,7-bis(4-chloro-2-phosphonophenylazo)-1,8-dihydroxy-3,6-naphthalenedisulfonic acid] was obtained from Dojindo Laboratories (Kumamoto, Japan) and used without further purification. A calcium standard solution (1000 mg l\(^{-1}\)) was purchased from Wako Pure Chemicals (Osaka, Japan). Cation-exchange resins, Amberlite 200 and IR-122 (strongly acidic type, 16 - 50 mesh, 1.7 and 2.1 meq ml\(^{-1}\), respectively) were used in the Na\(^+\) form. Amberlite IRC-76 (weakly acidic type, 16 - 50 mesh, 4 meq ml\(^{-1}\)) was also used in the H\(^+\) form.

Instrumentation

A schematic diagram of the flow system used in this work is
shown in Fig. 1. The FI apparatus, consisted of a double-plunger pump (DMX-2300, Sanuki, Japan), a 6-way rotary valve (9725, Rheodyne, 20 µl loop), a visible spectrophotometer (S-3250, Soma, Japan) equipped with a flow-through cell (1 cm light path, 8 µl inner volume) and a multi-range recorder (U-228, Nippon Denki Kagaku, Japan). A column was prepared by packing an appropriate amount (ca. 0.6 g) of a cation-exchange resin into a 10 cm × 4 mm i.d. glass tube for regeneration of the main reagent, and was placed after the flow-through cell. Flow lines were made of PTFE tubings (0.5 mm i.d.) and connectors.

Procedure
A typical circulating reagent solution was made up of 50 ml containing 4.0 × 10⁻⁵ M CPN and 0.05 M acetate buffer (pH 5.0). This solution in a reservoir was constantly stirred with the help of a magnetic stirrer. In the determinations, 20 µl of Ca²⁺ solutions was injected into the reagent stream at a flow rate of 1.5 ml min⁻¹. The calcium solution injected into the stream was allowed to proceed through a 60 cm long reaction coil to form a colored CPN-Ca²⁺ complex. In the flow-through cell, the complex was monitored spectrophotometrically at 670 nm and the response was fed to a strip-chart recorder. The stream then passed through a cation-exchange mini-column in order to remove any Ca²⁺ and to obtain the main free reagent, CPN. After leaving the column, the thus-formed free CPN was carried back to the reservoir ready for re-circulation. Using a cycle time of 1 min, 60 injections h⁻¹ were made. The heights of the recorded peaks were proportional to the Ca²⁺ concentration in the sample. To evaluate the proposed method, standard Ca²⁺ solutions (0 – 5 mg l⁻¹) were injected in triplicate for making the calibration graph, a 2.5 mg l⁻¹ Ca²⁺ standard solution was injected 100 times in sequence for regeneration and repetitive determination tests, and calibration followed again.

Results and Discussion
Chlorophosphonazo III reacts with several metal ions to form blue complexes. Calcium was selected as a model metal ion because it is one of the major natural water components and is suitable for monitoring environmental pollution. The proposed regeneration step of the ligand-exchange reaction between the CPN-Ca²⁺ complex and a cation exchanger is presented as follows:

\[
\text{CPN-Ca}^{2+} + \text{Ex} \rightarrow \text{CPN} + \text{Ex-Ca}^{2+},
\]

where Ex represents a cation exchanger. Due to the strong interaction between Ca²⁺ and the cation exchanger, Ca²⁺ is retained in the column and the free CPN is regenerated. Generally, quantitative exchange reactions are achieved more easily by a column technique, because an ion-exchange column can be regarded as a series of equilibrium systems. On the other hand, particular attention should be given to the adsorption of the metal complexes and the organic reagent, itself, on an ion exchanger. Yoshimura and co-workers¹⁴,¹⁵ have reported that some transition-metal complexes were adsorbed on an ion-exchange resin, and that the cell packed resin was measured directly by spectrophotometry. Preliminary batchwise experiments revealed that CPN and its metal complexes were not adsorbed on the cation exchangers,¹² whereas neutral or cationic complexes, such as 1,10-phenanthroline-Fe²⁺ derivatives, were adsorbed strongly. As they suggested,¹⁴,¹⁵ the species that were adsorbed on the ion exchanger were positively charged and converted into a complex of a higher ligand number. Because CPN has two sulfonic acid groups in a molecule and forms water-soluble anionic complexes with several metal ions, having a metal-ligand ratio of 1:1,¹¹ the adsorption of CPN, itself, and its complexes on a cation exchanger may be neglected.

Optimization of chemical and FI variables
The FI variables were optimized for the determination of Ca²⁺ using a flow system, as shown in Fig. 1. Although CPN reacts with Ca²⁺ to form a blue complex over a wide pH range of 2 - 12,⁹ it is preferable to undergo the reaction under a weakly acidic solution because of growing background absorbance.⁹-¹² The influence of the pH on the complexation reaction was studied in the range of 3.5 – 6.0 using appropriate mixing of 0.05 M acetic acid and a sodium acetate solution. The maximum complex formation was achieved for pH values ranging from 4.5 to 5.5. Thus, a pH of 5.0 was selected as being optimal.

The effect of the concentration of CPN on the determination of Ca²⁺ was investigated. The increase of the CPN concentration in the circulating reagent solution increases both the sensitivity and the background absorbance. Taking into account the sensitivity and determination range, the concentration of the final CPN solution was decided to be 4.0 × 10⁻⁵ M.

The flow rate of the circulating reagent solution varied from 0.8 to 2.5 ml min⁻¹. Increasing the flow rate showed constant peak heights, but caused the baseline noise to rise to over 2.0 ml min⁻¹. Taking into consideration the baseline stability and sampling time, the flow rate of the circulating reagent solution was adjusted to 1.5 ml min⁻¹.

In the circulatory FI system, an injection loop volume as small as possible was favorable, because the concentration of the reagent solution was diluted as increasing numbers of samples were injected. On the other hand, the sensitivity increased with increasing the volume of the injection loop. A sample loop of 20 µl was selected as a compromise between the sensitivity, determination range (0 – 5 mg l⁻¹) and sample throughput.

Other FI variables were optimized by maximizing the obtained peak height. Table 1 summarizes the optimum experimental conditions in which the assayed ranges for all variables studied are also listed.

Regeneration column and repetitive determination
The effectiveness of attaching of the column depends on the quantity of resin and on the ion-exchange capacity of the resin used. In this study, two types of cation exchange resins, having

Fig. 1 Schematic diagram of a closed-loop flow system for the determination of calcium. R, reservoir; P, pump; S, sample injector; RC, reaction coil; D, detector; Rec, recorder; Ex, ion-exchange column; St, stirrer.
sulfonic acid (Amberlite 200 and IR-122) and iminodiacetic acid (Amberlite IRC-76) groups, were examined. These are commercially available and are most often used in ion-exchange separations of elements. The column length was fixed at 10 cm (4 mm i.d.) throughout the experiments.

Experiments were conducted to see whether this reagent regeneration cycle could be used for the continuous determination of Ca²⁺. To estimate the regeneration and reuse of the main reagents, the standard Ca²⁺ solutions (0 – 5 mg l⁻¹) were injected in triplicate for calibration, a 2.5 mg l⁻¹ Ca²⁺ standard solution was injected 100 times in sequence, and calibration followed again. All injections were operated manually at a 1-min interval. The result obtained without a regeneration column in the circulatory flow system is shown in Fig. 2(A). Naturally, the detected signals produced a continuous baseline shift that limited the spectrophotometric scale useful for the determination. Figure 2(B) shows the signals obtained with a regeneration column packed with Amberlite 200. Repetitive injection of 2.5 mg l⁻¹ Ca²⁺ into the circulating reagent solution produced reproducible signals, a stable baseline and excellent repetition. The same results were obtained when another strongly acidic cation exchange resin, Amberlite IR-122, was used. Figure 2(C) shows the signals obtained using a weakly acidic resin, Amberlite IRC-76, as a regenerating resin. The results, shown in Figs. 2(B) and (C), demonstrate that the regeneration of CPN was essentially complete. Using these resins, five hours of continuous running (300 repetitive determinations for 2.5 mg l⁻¹ Ca²⁺) were carried out. Experiments showed the same stable baseline and reproducible signals. By considering the ion-exchange capacity and charging, more than 1000 determinations of 2.5 mg ml⁻¹ Ca²⁺ can be carried out. It undoubtedly greatly decreases the consumption of CPN, which is an expensive organic reagent.

It is common to incorporate a column packed with a cation exchanger before/after the injection valve in order to avoid interference of metallic ions. However, to our knowledge, no such method has been described for regenerating a chromogenic reagent. Another advantage of this method is that the column acts as a back-pressure coil at the same time.

**Reservoir volume and calibration graph**

The selection of the reservoir volume is dictated mainly by the number of Ca²⁺ samples to be determined and the approximate content. Because many practical processes call for stringent requirements, the reservoir volume was chosen to be 50 ml as small as possible in the present investigation. A linear calibration graph was obtained ranging from 0 to 5 mg l⁻¹ Ca²⁺ with a regression coefficient of r = 0.9997. After one hundred sequential injections of a 2.5 mg l⁻¹ Ca²⁺ solution, the slope of the calibration graph decreased by about 7%, as shown in Fig. 2. For this reason, the reservoir volume was found to be too small (50 ml) to maintain the concentration of the CPN reagent constant. The decrease in the CPN concentration caused a decrease in the sensitivity (peak height), as mentioned above. The effects arising from the decrease in the reagent concentration during continuous analysis could always be made negligible by employing a high initial volume.

**Robustness of the proposed method**

The robustness of the proposed method was evaluated by both long-time operation and the removal of interfering ions. The system was run continuously for 9 h, and at hourly intervals Ca²⁺ standard solutions (0 – 5 mg l⁻¹) were injected and calibrations of Ca²⁺ were carried out. The results are shown in Fig. 3. Precise and reproducible calibrations were obtained. It is clear that this method is applicable whenever an analysis is needed, and is suitable for such an urgent determination or monitoring. Another important feature of the proposed method is that after continuous use of the FI system for more than 9 h, no disturbances of the baseline has been observed. This fact shows that there is no potential adsorption of the CPN reagent or CPN-Ca²⁺ complex on the cation exchangers and wall of the PTFE tubing.

The CPN is a sensitive reagent for several metal ions, but is not selective. Most metal ions, such as alkaline earth, transition and rare earth metals, were found to interfere in the determination of Ca²⁺. To investigate the effectiveness of the column for removing interfering ions, Cu²⁺ was selected as a representative, because it caused a serious change in the

### Table 1 Optimized FI variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Optimal value</th>
<th>Range studied</th>
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<tbody>
<tr>
<td>Chlorophosphonazo III (× 10⁻¹ M)</td>
<td>4.0</td>
<td>2.0 – 6.0</td>
</tr>
<tr>
<td>pH</td>
<td>5.0</td>
<td>3.5 – 6.0</td>
</tr>
<tr>
<td>Buffer concentration (M)</td>
<td>0.05</td>
<td>0.02 – 0.2</td>
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<tr>
<td>Injection volume (μl)</td>
<td>20</td>
<td>10 – 200</td>
</tr>
<tr>
<td>Reaction coil length (cm)</td>
<td>60</td>
<td>0 – 120</td>
</tr>
<tr>
<td>Flow rate (ml min⁻¹)</td>
<td>1.5</td>
<td>0.8 – 2.5</td>
</tr>
<tr>
<td>Wavelength (nm)</td>
<td>670</td>
<td>600 – 700</td>
</tr>
</tbody>
</table>
absorption. Figure 4 shows the flow signals obtained when solutions containing Ca$^{2+}$ or Cu$^{2+}$ of 2.5 mg l$^{-1}$ solutions were injected 10 times alternatively. Copper caused serious negative peaks, because the CPN-Cu$^{2+}$ complex had an absorption maximum at 600 nm. After passing through the ion-exchange column, no significant damage was observed in either the stability of the baseline or subsequent determinations. The same results were obtained when solutions containing other interfering ions, such as Mg$^{2+}$, Sr$^{2+}$, Ba$^{2+}$, Ni$^{2+}$, Co$^{2+}$, Fe$^{3+}$, Al$^{3+}$ and La$^{3+}$, were injected. It is clear that the proposed method avoids serious damage of the reagent solution, even if the samples used are contaminated by large amounts of interfering ions.

**Conclusion**

The advantages of on-line regeneration of the reagent solution include the repetitive determination of a large number of samples, minimization of the reagents and waste, and high sample treatment. This method can probably be applied to the determination or monitoring of calcium ion, which is routinely determined in industrial, environmental and clinical laboratories.

**References**