Electrophoresis

See **III / ION ANALYSIS / Capillary Electrophoresis**

High-Speed Countercurrent Chromatography

E. Kitazume, Iwate University, Morioka, Iwate, Japan

High-speed countercurrent chromatography (HSCCC), developed by Ito, is a useful method of separating many organic materials, such as biologically active substances, natural and synthetic peptides and various plant hormones. Like other countercurrent chromatography (CCC) methods, it is also free from problems based on solid supports, such as adsorption or irreversible binding and contamination of the sample.

HSCCC has been applied to preconcentration and separation of inorganic elements since the late 1980s and since then, inorganic elements, including rare earth elements, have been separated by HSCCC. Also the preconcentration and separation of inorganics from geological samples have been studied. The liquid systems for inorganics are more complicated than those for separation of organics, because they usually contain significant amounts of an extracting agent, which influences kinetic properties and viscosities of the whole two-phase system.

In order to achieve high sensitivity for analysing trace inorganic elements in a solution using atomic absorption spectrometry (AAS) or inductively coupled plasma atomic emission spectrometry (ICP-AES), conventional preconcentration methods such as evaporation, ion exchange and solvent extraction have been used. However, there are several problems in these methods for the determination of ultra trace elements, for example, peak broadening for ion exchange and a small enrichment factor for solvent extraction. It is difficult to achieve under 0.5 mL concentrated sample solutions by conventional methods. If there were effective methods to concentrate traces into 0.1 mL volume or less, absolute detection limits for trace analysis such as AAS, ICP-AES and ICP–mass spectrometry (ICP-MS) would be greatly decreased, and matrix effects would be eliminated.

Recently, pH-zone refining CCC, which is a unique technique based on the neutralization reaction between the mobile and stationary phase, has been developed for the separation and enrichment of organic acids, basic derivatives of amino acids and acidic peptide derivatives. It can initiate chemical reactions in quite a limited thin area, the interface between the organic and aqueous phase. Therefore, if there is a reversible pH area between the mobile and stationary phase, the pH in the column can be continuously controlled. This means that another flow rate, concerned with pH and different from real flow rate, can be realized in the column. Impurities in sample solutions can be concentrated in the pH boundary in the column. As it can be successfully applied to the enrichment of inorganic trace elements in solution, it has great potential for on-line enrichment and subsequent analysis, when HSCCC is combined with instruments such as AAS, ICP-AES and ICP-MS.

Mechanism of Two-phase Separation in HSCCC

In HSCCC, a stationary sun gear is mounted around the central stationary axis of the centrifuge to prevent the flow tubes twisting. This gear arrangement gives a planetary motion of the column holder – one rotation about its own axis for one revolution around the central stationary axis of the centrifuge in the same direction.

Figure 1 shows a schematic diagram of two-phase separation in an HSCCC column. The heavier mobile phase (black) is introduced into the column from the right side (column head). The upper stationary phase (grey) is retained in the column by a rotational force field and Archimedean screw effect (ASE), in spite of being pushed to the column end by the mobile phase. For separation and enrichment of inorganic elements, the stationary phase commonly contains one or more extracting reagents, such as di(2-ethylhexyl)phosphoric acid (DEHPA), dissolved in the appropriate stationary organic phase. The mobile phase is commonly composed of inorganic acids and their salts. Water-soluble complexing reagents forming

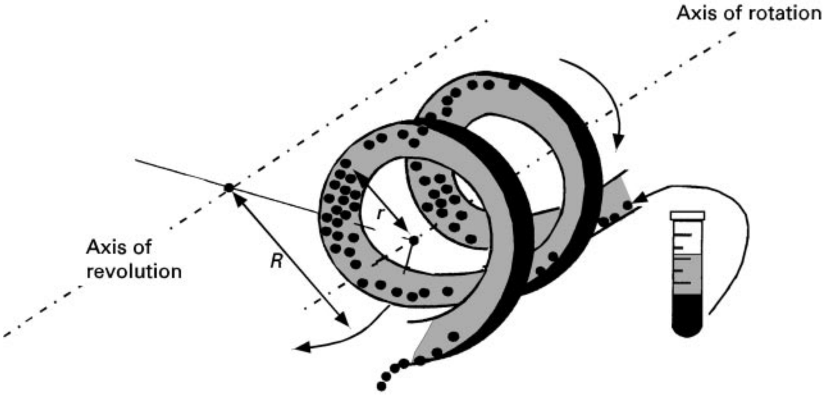

Figure 1 Schematic diagram of two-phase separation in HSCCC column. r, Rotation radius; R, revolution radius.

stable compounds with specified elements in the sample can also be used as an aqueous mobile phase. The column is revolved in the radius R and rotated with the radius r at the same time. When the coil column is facing the axis of revolution (top left in Figure 1), the two phases are vigorously mixed because of the weak force field. In contrast, when facing the outer side of the column, the mobile phase is clearly separated by a stronger force field than that at the inner side. The inner quarter turn of the coil is the most vigorous mixing area. As one phase usually occupies the column head by an Archimedean screw effect, another phase can be introduced as an eluent. After being introduced from the column head, the heavier mobile phase emerges from the end of the column (bottom left in Figure 1). When the column makes one revolution around the axis, it rotates once on the orbital of revolution. Therefore, as a specific point in the column, one turn in the coil proceeds towards the column head per revolution. At the same time, one cycle of each mixing and separation process at the specific point occurs in the column. As the HSCCC column rotates at over several hundred rpm, this is a rapid process. For example, there are 13 processes per second taking place at 800 rpm, giving efficient separation and mixing in the column.

Extracting Reagent for Separation of Inorganic Elements

As mentioned above, the existence of extracting reagent in the mobile phase is an essential factor in the separation of inorganic elements. It complicates the determination of several important factors, such as distribution coefficients, peak resolution and separation efficiency. Research reveals that the kinetic properties of a specific system used in HSCCC affect the separation efficiency. The mass transfer rate into the organic stationary phase is responsible for using either stepwise or isocratic elution. In addition, the value of the distribution coefficients, determined by batch extraction measurements on systems, are sometimes considerably different from the dynamic distribution coefficients calculated from the elution curve. Further theoretical and basic investigations are necessarily concerned with extraction kinetics, as well as the hydrodynamic behaviour of the two phases in the HSCCC column.

In **Table 1**, typical extracting reagents used for separation and enrichment of inorganic elements are summarized. Organophosphorus extractants are often used because of their solubility properties.

Table 1 Typical extracting reagents for separation and enrichment of inorganic elements using high-speed countercurrent chromatography

Extracting reagent	*Two-phase system*	*Inorganic element*
Di(2-ethylhexyl)phosphoric acid (DEHPA)	HCl, organic acid–heptane	Rare earth, heavy metals
2-Ethylhexylphosphonic acid mono-2-ethylhexyl ester (EHPA)	Carboxylic acid–toluene	Rare earth
Dinonyltin dichloride	HCl, HNO_3^- Methylisobutylketone (MIBK)	Ortho- and pyrophosphate
Cobalt dicarbolide	HNO_3-nitrobenzene	Cs and Sr
Tetraoctylethylenediamine (TOEDA)	HCl, HNO_3, organic acid–chloroform	Alkali, alkaline earth, rare earth, heavy metals, Hf, Zr, Nb, Ta

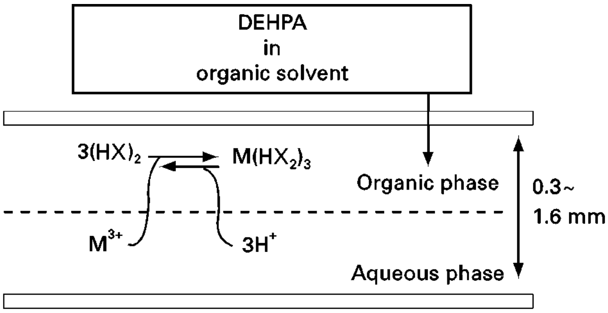

Figure 2 Extraction equilibrium in HSCCC column using di (2-ethylhexyl) phosphoric acid (DEHPA) as an extracing reagent for trivalent metal ion (M^{3+}). $(HX)_2$, dimer of DEHPA.

Figure 2 shows the equilibrium for extraction of trivalent metal ions, such as lanthanide, using the extracting reagent (DEHPA). DEHPA is commonly applied in industrial separation due to its high extractability and high separation factors between many inorganic elements, especially for rare earth elements. Trivalent metal ions (M^{3+}) are extracted as shown in Figure 2 in PTFE tubing of 0.3–1.6 mm in diameter. The long thin rectangles in Figure 2 show the wall of the PTFE tubing. $(HX)_2$ is a dimer of DEHPA. Metal ions in the aqueous phase are extracted into organic phase as $M(HX_2)_3$.

Mutual Separation of Inorganic Elements

Figure 3 shows a typical flow diagram of the instrumentation assembly for the separation of rare earth elements and other inorganic elements by HSCCC. In this case, the spectrophotometer is used as a detector for each element. If an alternative measurement system such as AAS, ICP-AES or ICP-MS is used, all the equipment depicted after the splitter is not required. Each separation is usually initiated by filling the entire column with the stationary nonaqueous phase, followed by injection of an appropriate volume of the sample solution through the sampling port. Then, the mobile phase is eluted through the column at a flow rate of 0.1–5 mL min^{-1} while the apparatus is rotated at several hundred rpm. In Figure 3, continuous detection of the inorganic elements is effected by means of a post-column reaction with an appropriate compound such as arsenazo III, and the elution curve is obtained by monitoring the effluent with a spectrophotometer. The effluent is divided into two streams with a tee adapter and a low-dead-volume pump (pump II). Pump II delivers a portion of the effluent at the required flow rate to the spectrophotometer and pump III adds reagent for post-column reaction to the effluent stream. The resulting stream first passed through a narrow mixing coil and then leads through an analytical flow cell in a spectrophotometer. The other effluent stream through the tee adapter is either collected or discarded.

Figure 4 shows a one-step separation of all 14 lanthanides (except for promethium) performed by applying an exponential gradient of hydrochloric acid in the mobile phase. The main problem in gradient elution is that the optimum range of the ligand concentration in the stationary phase is substantially different between the lighter and heavier groups of the rare earth elements. Because the separation of the heavy lanthanide elements, including thulium, ytterbium and lutetium, is more difficult, a ligand concentration of 0.003 mol L^{-1} was selected for best resolution. With an isocratic separation mode, using a constant eluent concentration, even heavy lanthanide elements such as Tm, Yb and La are well separated.

Figure 5 shows a typical chromatogram of Ni(II), Co(II), Mg(II) and Cu(II) obtained by eluting with

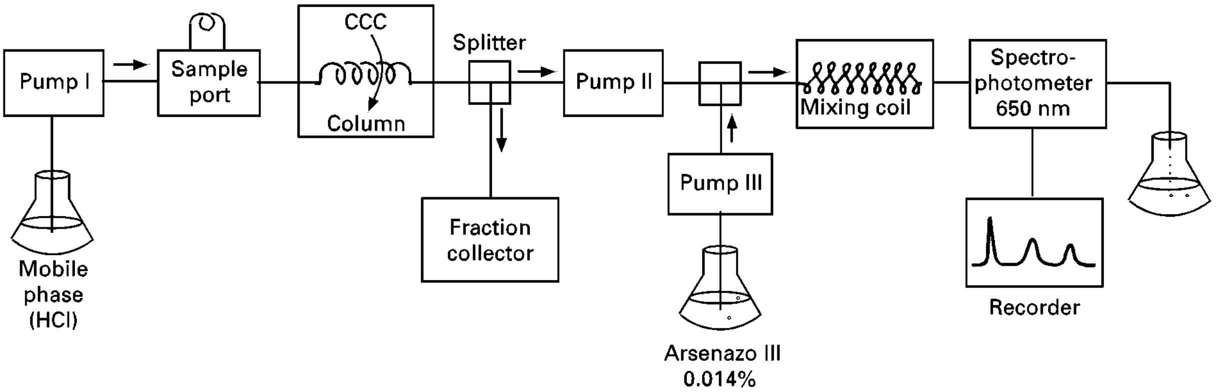

Figure 3 Flow diagram of instrumentation assembly for separation of rare earth elements by HSCCC. CCC, countercurrent chromatography.

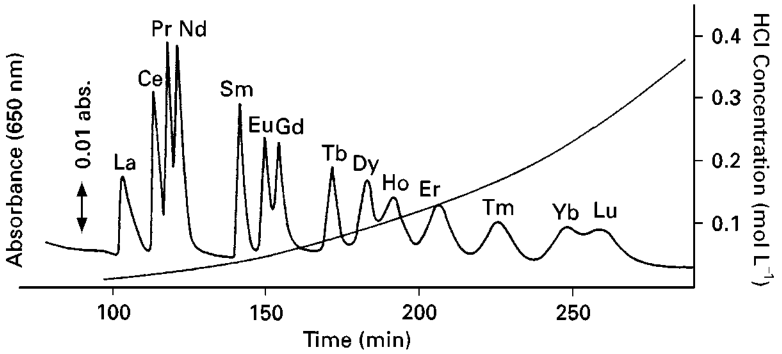

Figure 4 Gradient separation of 14 lanthanides obtained by HSCCC. Apparatus: HSCCC centrifuge with 7.6 cm revolution radius; column: three multilayer coils connected in series, 300 m × 1.07 mm i.d., 270 mL capacity; stationary phase: 0.003 mol L^{-1} DEHPA in *n*-heptane; mobile phase: exponential gradient of hydrochloric acid concentration from 0 to 0.3 mol L^{-1}, as indicated in the chromatogram; sample: 14 lanthanide chlorides, each 0.001 mol L^{-1} in 100 μL water; speed: 900 rpm; flow rate: 5 mL min^{-1}; pressure: 300 p.s.i.

7 mmol L^{-1} citric acid at a flow rate of 5 mL min^{-1}. The efficiencies range from 1600 theoretical plates (Ni) to 200 (Cu).

Group Separation of Inorganic Elements

There are numerous methods, such as X-ray fluorescence (XRF), spectroscopy, AAS, AES and ICP-MS, for the determination of trace inorganics including rare earth elements. Of these methods, ICP-AES is one of the most popular for the determination of metals such as Ta, Zr, Hf, etc., including rare earth elements. However, there may be problems with spectral interference because of the many spectral lines. To ensure sufficient precision and accuracy, especially for the determination of trace elements, separation of matrix elements, which interfere with the determination, must be undertaken. HSCCC has great potential as a separation technique which preconcentrates trace elements before determination.

Figure 6 shows an example of the quantitative group separation of the rare earth elements from the constituents of rocks, which interfere with the determination of trace elements by ICP-AES. A 0.5 mol L^{-1} solution of DEHPA in decane was used as the stationary phase. Alkaline, alkaline earth elements and iron(II) were separated from the total amount of rare earth elements at the first stage of the eluent concentration with 0.1 mol L^{-1} hydrochloric acid. Then the total amount of rare earth elements, including yttrium, was eluted from the stationary

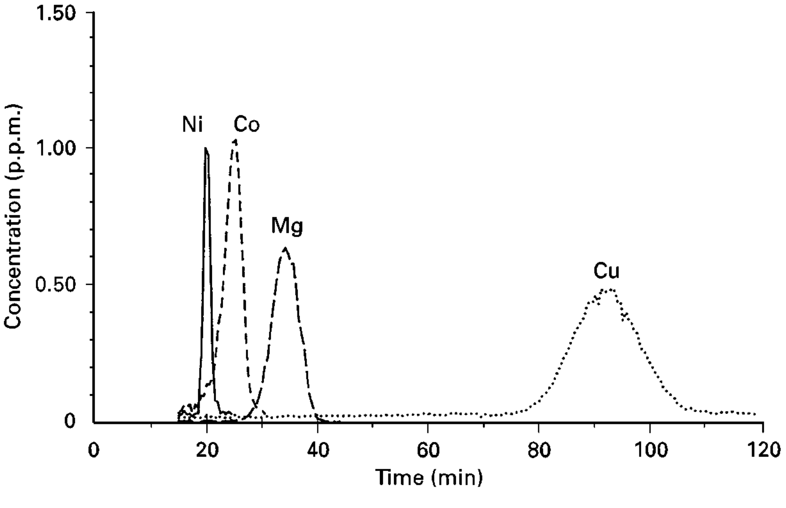

Figure 5 Isocratic separation of nickel, cobalt, magnesium and copper by HSCCC. Apparatus: HSCCC centrifuge with 10.0 cm revolution radius; column: one multilayer coil, 150 m × 1.6 mm i.d., 300 mL capacity; stationary phase: 0.2 mol L^{-1} DEHPA in heptane; mobile phase: 7 mmol L^{-1} citric acid; sample 10 μg Ni, each 20 μg Co and Mg, 40 μg Cu; speed: 800 rpm; flow rate: 5.0 mL min^{-1}; pressure: 140 p.s.i.; observed wavelength: 341.4 nm (Ni), 345.3 nm (Co), 280.2 nm (Mg), 324.7 nm (Cu).

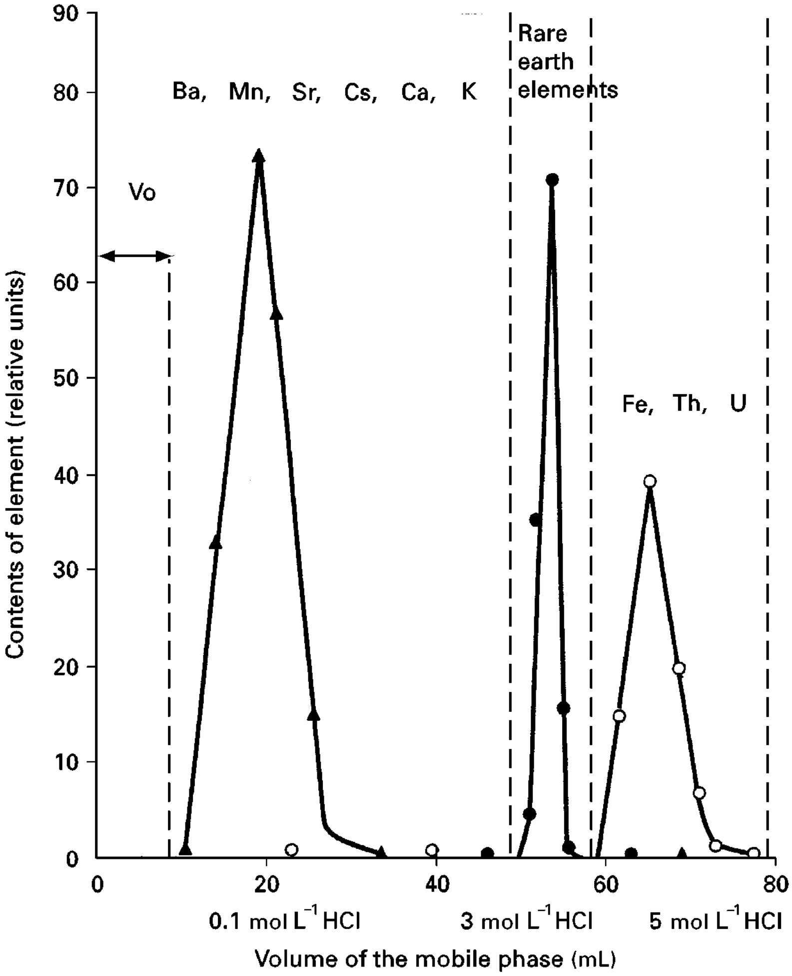

Figure 6 Example of quantitative group separation of the rare earth elements from the constituents of rocks. Apparatus: HSCCC centrifuge with 14.0 cm revolution radius; column: one monolayer coil, 1.13 m × 1.5 mm i.d., 20 mL capacity; stationary phase: 0.5 mol L^{-1} DEHPA in decane Kr: 30%; mobile phase: 1–0.1 mol L^{-1} HCl, 2–3 mol L^{-1} HCl, 3–5 mol L^{-1} HCl; sample: Mn 0.3, Fe 6.4, Ca 8.2, Na 4.2, K 4.1 (%) and Ba 440, La 1350, Ce 2290, Nd 760, Pr 239, Sm 97, Eu 18.3, Gd 120, Tb 15, Dy 139, Ho 29, Er 88, Tm 12.5, Yb 71, Lu 8.6, Y 716, Cs 2.6, Sr 307, U 682, Th 940 (p.p.m.); speed: 350 rpm; flow rate: 2.0 mL min^{-1}. Kr, the retained stationary phase volume relative to the total column volume.

phase with 3 mol L^{-1} hydrochloric acid. To elute other elements, including iron(III), from the stationary phase, 5 mol L^{-1} hydrochloric acid was introduced into the column. Separation of the mixture was performed within 40 min at a pumping rate of the mobile phase of 2 mL min^{-1}.

Enrichment of Inorganic Elements

Large-scale Enrichment of Inorganics

HSCCC has great potential as an enrichment technique for trace elements before determination. Enrichment prior to determination can overcome problems such as interference, toxic or radioactive samples.

The p.p.b. level of metal ions in a 500 mL of the mobile phase was continuously concentrated into a small volume of the stationary phase retained in the column. Concentrated metal ions were simultaneously eluted with nitric acid and determined by a plasma atomic emission spectrometer. The recoveries of Ca, Cd, Mg, Mn, Pb and Zn were over 88% at the 10 p.p.b. level in 500 mL of the sample solution. The versatility of this method has been further demonstrated by the determination of trace metals in tap water and deionized water.

Rare earth elements have been enriched in a stationary phase of toluene: 2-ethylhexylphosphonic acid mono-2-ethylhexyl ester (EHPA) from a litre of aqueous solution, and eluted with a stepwise pH gradient.

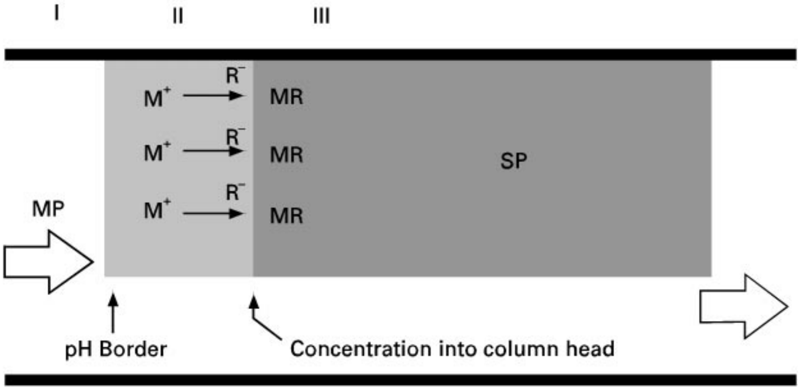

Figure 7 Concentration procedure just after revolving the HSCCC column. I, Mobile phase (MP) with HCl; II, sample with NH_3; III, stationary phase (SP) with NH_3.

Large-scale enrichment is very useful to determine extremely low levels of metals in solution, when a large amount of sample is available.

Enrichment Using the pH-zone Refining Technique

Even if a large volume of sample is not available, enrichment techniques that concentrate trace metals in microlitre samples are sometimes quite useful because modern instrumental methods do not need a large sample size. Moreover, if trace metals separated from their major matrices can be concentrated in an extremely small area of the polytetrafluoroethylene (PTFE) tube in HSCCC, this would be ideal for flow-injection analysis. From this point of view, the recently developed pH-zone refining technique has great potential for enrichment of trace inorganic elements.

In pH-zone refining, a basic organic solution containing a complex-forming reagent such as DEHPA as a stationary phase is used. After sample introduction into the column, metal ions stay close to the sharp pH border region in the small-bore PTFE tube. Then the trace inorganic ions in the sample are moved by the acid effluent (dilute HCl or HNO_3) to the tail of the column while concentrating in the sharp-moving pH interface, and finally eluted as small fractions containing concentrated inorganic ions.

This concentration procedure is shown in **Figures 7** and **8**. In Figure 7, sample ion is concentrated into the column head soon after the concentration procedure is started. After the stationary phase and the sample are introduced into the system, HSCCC is started at an appropriate revolution rate, followed by the mobile-phase pump. Metal or inorganic ions (M^+) are concentrated into the column head as MR. Zone III shows the stationary phase, which includes DEHPA and ammonia in an organic solvent such as ether or heptane. Zone II shows a sample phase with the pH adjusted by ammonia. When the column revolution is started, as the organic stationary phase is lighter than

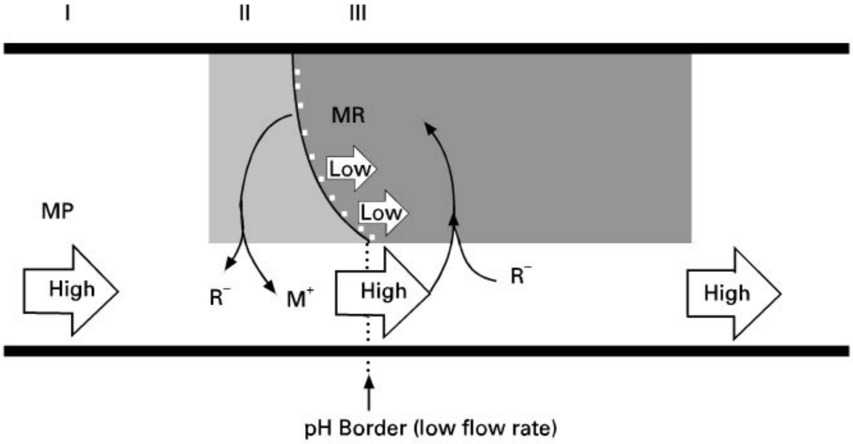

Figure 8 Concentration procedure at the pH interface in the HSCCC column. I, Mobile phase (MP) with HCl; II, stationary phase after being neutralized with HCl; III, stationary phase with NH_3.

the mobile phase (diluted acid solution), it moves to the head of the column by ASE. The driving force based on ASE increases with the revolution speed of the column. Therefore, the stationary phase can be retained in the column by selecting an appropriate speed of revolution and pump rate, even if the mobile phase is introduced from the head into the column. The retention ratio of the stationary phase to the whole column can be varied from 20 to 70%, but is stable when all conditions including pump or revolution rate are constant. So, the position of the mobile phase is stable in the column when in operation. Inorganic ions (M^+) form complexes with ligand ions (R^-) and are mainly concentrated on the column head, shown as MR in Figure 7. Hydrogen and counter ions of the ligand are not shown in the figure.

Figure 8 shows a concentration procedure in the HSCCC column after all the ions in the sample are extracted on top of the zone III in Figure 7. After Zone II in Figure 7 (sample) has completely passed through the top of the stationary phase, ammonia in the stationary phase begins to be neutralized with hydrochloric acid in the mobile phase. The neutralization area, where reaction between the acid and the base has just finished, is shown as the pH border in Figure 8. The pH border proceeds from left to right. Also the concentrated ions proceed circulating (extracting and back-extracting process in Figure 8) around the pH interface. The rectangular zone (II + III) shows the total stationary phase.

The mobile phase that includes acid solution (hydrochloric acid in Figures 7 and 8) moves at a constant flow rate from the head of the column (left) to the tail of the column (right). The three arrows labelled High show a constant stream of the mobile phase.

As the mobile phase is an acidic solution, it reacts with ammonia in the stationary phase as it proceeds to the tail of the column in Figure 8. The left area (II) in the rectangular zone shows the stationary phase that has been saturated with hydrochloric acid. In the right area (III) in the rectangular zone, ammonia is not yet neutralized with hydrochloric acid. The dark gradation zone between areas II and III shows a pH interface, where the neutralization between acid and base has just finished. As the stationary phase and the mobile phase are mixed well in the HSCCC column, there must be a pH interface in the mobile phase.

The pH interface moves to the right (the same direction as the mobile phase); however, its flow rate is lower than that of the mobile phase because of the delay based on the neutralization. So, concentrated ions shown as MR on the top left of the zone III in Figure 7 move slowly as shown by the flow rate arrow Low in Figure 8, and circulate around the pH interface by repeating extraction and back-extraction into the stationary phase and the mobile phase, respectively. The flow rate of the pH interface in the mobile phase is shown as the Low arrow in the stationary phase in Figure 8. The flow rate of the pH interface in the mobile phase may be made the same as that in the stationary phase by vigorous mixing in HSCCC column. The pH interface in the mobile phase is shown by the dotted line at the pH border in Figure 8. When the inorganic ions combined with ligand (MR) pass through the pH interface from zone III to zone II in the stationary phase, they are back-extracted into the mobile phase. Then, as the flow rate of the mobile phase is faster than that of the pH interface, the ions (M^+) pass through the pH border (shown by the black dotted line in Figure 8) from left to right in the mobile phase. Just after passing the pH border, the pH is rapidly rising, so, the ions react with the ligand again and are extracted back into the stationary phase. By repeating this cycle, the ions are moved to the tail (right) of the column without diffusion.

In the concentration mechanisms described above, there is no diffusion process observed as in elution procedures with ion exchange or other chromatographic separation methods, such as conventional HSCCC and HPLC. If there is no basic compound such as ammonia in the stationary phase, ions move to the tail with a different flow rate as a function of the distribution ratio between the stationary phase and the mobile phase. Many ions can be separated from each other in this way but because there is no sharp pH interface in the column, concentration is not effected, but only separation with diffusion.

Figure 9 shows the typical concentration results for a 10 p.p.m. solution of cadmium, magnesium and zinc. The injected sample solution contained 50 μg of each in 5 mL of 0.1 mol L^{-1} tartaric acid solution adjusted to pH 8.8. The mobile phase was pumped at a flow rate of 0.05 mL min^{-1}. Revolutional speed was 950 rpm. The eluent was collected every 2 min (0.1 mL fractions). The fractions were diluted 1 : 10 with water and the emission intensity for each element was measured by direct current plasma atomic emission spectrometer. The emission intensities for each element were increased 20-fold compared to the original sample solution. The results of this study demonstrate the high performance capabilities of the pH-zone refining technique. Trace elements in the sample solution can be successfully concentrated into a small volume with enormous enrichment.

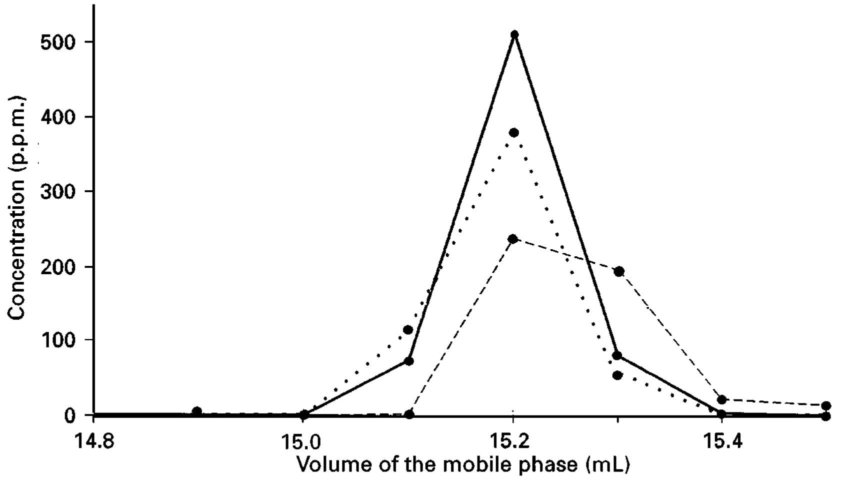

Figure 9 Typical concentration results for 10 p.p.m. solution of cadmium (continuous line), zinc (dashed line), magnesium (dotted line). Apparatus: HSCCC centrifuge with 10.0 cm revolution radius; column: one multilayer coil; sample: 5 mL of each 10 p.p.m. solution (pH 9.25) in 0.1 mol L^{-1} tartaric acid; mobile phase: 0.1 mol L^{-1} HCl saturated with ether; stationary phase: 6 mL of 0.2 mol L^{-1} DEHPA and 0.18 mol L^{-1} ammonia in ether; column: 0.5 mm i.d. × 32 m; flow rate: 0.05 mL min^{-1}; speed: 950 rpm.

Conclusions

In constrast to HPLC, the unique feature of HSCCC is that there is no solid support in the column. As the distribution abilities including the capacity of the stationary phase are easy to control, HSCCC can be applied to the separation, enrichment and purification of inorganics over a wide range of concentration. In particular, enrichment of trace elements using pH-zone refining technique will be an ideal preconcentration method for subsequent determination by modern instrumental methods. HSCCC can be combined directly with the flow injection technique, and shows great potential for preconcentration of selected desired trace element prior to detection. On-line enrichment and subsequent analysis may thus take the place of conventional sample preparation using a beaker and separating funnel, in future investigations in this field.

See also: **III/Ion Analysis:** Capillary Electrophoresis; Liquid Chromatography; Thin-Layer (Planar) Chromatography.

Further Reading

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