Available online at www.sciencedirect.com
SCIENCE DIRECT
Talanta 68 (2005) 465–469
Talanta
www.elsevier.com/locate/talanta

Speciation of mercury in salmon egg cell cytoplasm in relation with metallomics research

Takuya Hasegawa, Motoki Asano, Kohei Takatani, Hirotaka Matsuura, Tomonari Umemura, Hiroki Haraguchi *

Department of Applied Chemistry, Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan

Received 11 May 2005; received in revised form 22 September 2005; accepted 22 September 2005
Available online 20 October 2005

Abstract

Speciation of mercury in salmon egg cell cytoplasm was investigated by surfactant-mediated high-performance liquid chromatography/inductively coupled plasma mass spectrometry (HPLC/ICP-MS), where an ODS (octadecylsilica) column coated with a bile acid derivative, CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate), was used for species separation. Prior to the speciation analysis, total Hg in the cell cytoplasm was determined by ICP-MS at *m*/*z* 202 in a flow injection mode. For the precise measurement, salmon egg cell cytoplasm was diluted five-fold with 0.1 M Tris (Tris(hydroxymethyl)aminomethane)–HNO_3 buffer solution, and the standard addition method was employed. Thus, the total concentration of Hg in cell cytoplasm was estimated to be 12.4 ng g^{-1} on the wet weight basis. Next, the cell cytoplasm diluted five-fold with 0.1 M Tris–HNO_3 buffer solution was analyzed by surfactant-mediated HPLC with the dual detection system of a UV absorption detector and an ICP-MS instrument. Two peaks corresponding to some proteins and small molecules were mainly observed in those chromatograms. When salmon egg cell cytoplasm was diluted five-fold with 0.01 M Tris buffer solution or pure water, some precipitates appeared probably because of precipitation of hydrophobic proteins in cytoplasm. After the precipitates were eliminated with a membrane filter, the filtrate was subjected to the analysis by surfactant-mediated HPLC/UV/ICP-MS. As a result, the peaks for small molecular species of Hg were clearly observed at the retention time near 4.0 min (corresponding to low-molecular weight zone) in the chromatograms with UV absorption detection as well as with Hg- and S-specific ICP-MS detections. The small molecule bound with Hg was identified as cysteine through the cysteine-spiked experiment. In addition, the protein fraction on the chromatogram obtained by using the CHAPS-coated ODS column was further analyzed by SEC (size exclusion chromatography). Consequently, several protein peaks with molecular weight of 300, 50 and 12 kDa were observed in all the detections of UV absorption, Hg and Se, although two peaks among them were coincident in the case of S. These results indicate that Hg in salmon egg cell cytoplasm binds with proteins containing selenocysteine and/or cysteine residues in proteins.

Keywords: Mercury specification; Salmon egg cell cytoplasm; Surfactant-mediated HPLC; ICP-MS; Metallomics

1. Introduction

It has been pointed out that chemical speciation of trace metals is substantially important in the metallomics research [1–3], because their bio-availabilities, essentialities and toxicities depend on their chemical forms. Thus, the research on chemical speciation of trace elements has been extensively carried out in various scientific fields, such as biochemistry, geochemistry, environmental science, medicine and so forth [1,3–7]. In general, speciation analysis is performed by a hyphenated system of a chromatographic separation and a highly-sensitive spectrometric detection such as atomic absorption spectrometer (AAS), inductively coupled plasma atomic emission spectrometer (ICP-AES) or inductively coupled plasma mass spectrometer (ICP-MS).

In recent years, the present authors have been doing multi-element determination of major-to-ultratrace elements in salmon egg cell [1,8], with special aim of achieving all-elements analysis of one biological cell, and so far about 40 elements have been determined in whole salmon egg cell as well as in cell cytoplasm. In these experiments, salmon egg cell samples were digested with HNO_3–$HClO_4$ [8] and subjected to the determination by ICP-AES and ICP-MS. In the acid-digestion experiment, however, Hg could not be determined because of its volatilization

* Corresponding author. Tel.: +81 52 789 4603; fax: +81 52 789 5290.
E-mail address: haraguch@apchem.nagoya-u.ac.jp (H. Haraguchi).

0039-9140/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2005.09.038

during digestion. Thus, it is required to exploit a new analytical method for the determination of Hg in the biological samples even in the case of total-concentration of Hg. In addition, a direct sample injection method is also necessary for speciation of Hg in the biological fluids and extracts.

An ODS (octadecylsilica) column coated with CHAPS (3-[3-cholamidopropyl)dimethylammonio]-1-propane sulfonate) has been developed for electrostatic ion chromatography [9,10] as well as for surfactant-mediated HPLC [11–13]. In electrostatic ion chromatography, cations and anions in aqueous solutions were simultaneously determined with conductivity and ICP-AES detections [9,10]. In surfactant-mediated HPLC, for example, some drugs in human blood serum were determined without any sample pretreatment or deproteinization [12]. In addition, it has been found that the CHAPS-coated ODS column can be used for simultaneous separation of large molecules (proteins) and small molecules/inorganic ions [1,11,13,14]. This is because the CHAPS-coated ODS column provides multi-separation modes, such as hydrophilic exclusion function produced by the hydroxyl groups in CHAPS, electrostatic interaction with the sulfonate and ammonium groups in CHAPS, and hydrophobic interaction with the original ODS groups. These separation characteristics of the CHAPS-coated ODS column allow us to analyze the biological fluid samples with direct sample injection. In the present experiment, hence, surfactant-mediated HPLC using the CHAPS-coated ODS column was applied to speciation analysis of Hg species in salmon egg cell cytoplasm, with detection of UV absorption and ICP-MS (Hg, S and Se). Subsequently, the protein fraction in cell cytoplasm separated on the CHAPS-coated ODS column was further separated by a size exclusion chromatography (SEC) column.

2. Experimental

2.1. Instrumentation

A schematic diagram of the HPLC/ICP-MS system is shown in Fig. 1, which was used in flow injection mode as well as in HPLC mode. In the latter case, a CHPAS-coated ODS column was used as a stationary phase for simultaneous separation of large and small molecules/ions. The SEC column (Superose 12HR) used for large molecule separation was purchased from Amersham Bioscience (Piscataway, NJ, USA). This column has an exclusion limit of 300 kDa and a linear fractionation range

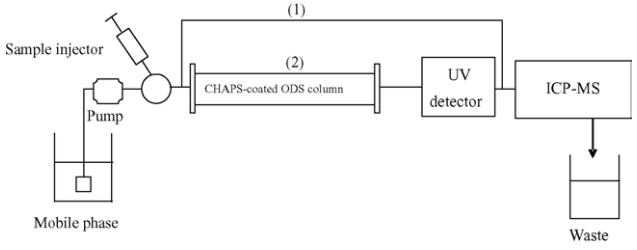

Fig. 1. A schematic diagram of HPLC/UV/ICP-MS system. (1) Flow injection mode, (2) HPLC mode. Column: CHAPS-coated ODS column; mobile phase: 0.1 M Tris–HNO_3 (pH 7.4) containing 0.2 mM CHAPS; flow rate: 0.7 ml min^{-1}; injection volume: 100 μl.

Table 1
Operating conditions for ICP-MS instrument

ICP-MS	Seiko Instruments model SPQ-8000A
Plasma conditions:	
Incident power (kW)	1.2
Coolant gas flow rate (l min^{-1})	Ar 16.0
Auxiliary gas flow rate (l min^{-1})	Ar 1.0
Carrier gas flow rate (l min^{-1})	Ar 0.85
Sampling depth	10 mm from load coil
Nebulizer:	Glass concentric-type
Sample uptake rate (ml min^{-1})	0.8
Data acquisition:	
Measurement mode	Peak hopping
Dwell time (ms/point)	100
Data point (points/peak)	1
Time interval (s)	5

of 1–300 kDa, whose calibration for molecular weights was performed using standard proteins, as usual [11,12].

An ICP-MS instrument (model SPQ-8000A from Seiko Instruments, Chiba, Japan), equipped with a quadrupole mass spectrometer, was used for Hg-, S- and Se-detections in HPLC. The operating conditions of the ICP-MS instrument are shown in Table 1. The chromatographic detection by ICP-MS was performed in a peak popping mode with the time-interval of 5 s in time-sequential program, which allowed simultaneous chromatographic measurements of at most 15 elements. An UV absorption detector (model UV-970; Jasco, Tokyo, Japan) was also used in the system of Fig. 1.

2.2. Chemicals and Samples

The 0.1 M Tris buffer solution (pH 7.4) was prepared from Tris–HNO_3, purchased from Merck Japan (Tokyo, Japan). This buffer solution was used for dilution of cell cytoplasm as well as for the mobile phase in HPLC. The zwitterionic surfactant CHAPS was purchased from Dojin Laboratories, Kumamoto, Japan, and it was used as the additives for the mobile phase and the coating reagent of the ODS column. When the Tris buffer solution was used as the mobile phase, 0.2 mM CHAPS was added into the mobile phase solution to avoid deterioration of the CHAPS-coated ODS column.

Salmon egg cells were obtained in the local supermarket. After washing with pure water repeatedly, cytoplasm was taken out by squeezing egg cells with a Teflon needle and tweezers. Then, egg cell cytoplasm was diluted five-fold with 0.1 or 0.01 M Tris buffer solution, depending on the following experiments. These diluted cytoplasm solutions were filtered with the membrane filter (pore size 0.45 μm).

2.3. Preparation of CHAPS-coated ODS column

The ODS column (L-column; 4.6 mm i.d. × 250 mm long) was purchased from the Chemicals Evaluation and Research Institute (Tokyo, Japan). The CHAPS-coated ODS column was prepared by a dynamic coating method, where 20 mM

CHAPS aqueous solution was passed through the ODS column at 0.7 ml min^{-1} for 2 h. After coating, the column was rinsed with pure water for 1 h, as described in the previous papers [9,10].

3. Results and discussion

3.1. Determination of total concentration of Hg in salmon egg cell cytoplasm by ICP-MS in flow injection mode

The total concentration of Hg in salmon egg cell cytoplasm was determined by ICP-MS at m/z 202 in a flow injection mode. In the flow injection analysis, a Teflon tubing (0.28 mm i.d. × 30 cm) was inserted between the sample injector and the nebulizer of the ICP-MS instrument as the bypass of the measurement system, as is shown in Fig. 1. For the precise measurement, the standard addition method was employed, and 5, 10, 15 and 20 ng g^{-1} of Hg were spiked in salmon egg cell cytoplasm diluted five-fold with 0.1 M Tris–HNO_3 buffer solution. The flow injection signals for spiked cytoplasm samples together with non-spiked sample are shown in Fig. 2, where each spiked sample was measured three times. In the standard addition for flow injection analysis, some blank signal was observed in the case of the not-added sample, i.e., blank solution, which was 0.1 M Tris–HNO_3 buffer solution, as is seen in Fig. 1. The blank level was 1.2 ng g^{-1}, and this blank was subtracted from the signals for the spiked salmon egg cytoplasm samples as reagent contamination. From the calibration curve for the standard addition method, the total concentration of Hg in salmon egg cell cytoplasm was estimated to be 12.4 ng g^{-1}.

3.2. Chromatograms for salmon egg cell cytoplasm diluted with 0.1 M Tris–HNO_3 buffer solution

The chromatograms for salmon egg cell cytoplasm diluted with 0.1 M Tris buffer solution were measured by HPLC using the CHAPS-coated ODS column with UV absorption detection and Hg detection by ICP-MS. The chromatograms thus obtained are shown in Fig. 3. As can be seen in the UV absorption-detected chromatogram in Fig. 3(a), a large peak was observed at ca. 3.0 min. It is known that all proteins are eluted near the void volume (t_0 = 2.8 min) in this separation system, and the mutual separation of proteins cannot be carried out with the CHAPS-coated ODS column [11]. Thus, the large peak observed near 3.0 min was certainly ascribed to some proteins in cell cytoplasm. It should also be noted here that small but clear peak with the retention time near 3.9 min was observed, although it was partly overlapped with the large peak.

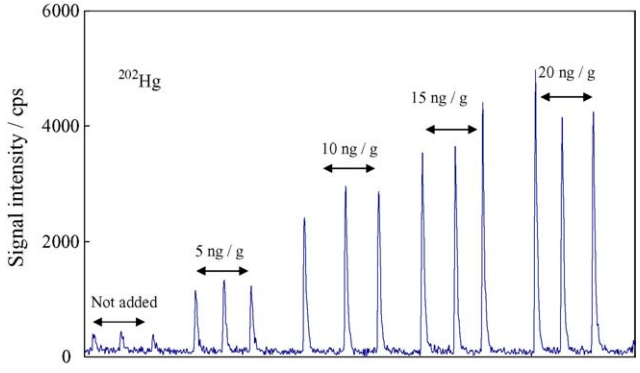

Fig. 2. Flow injection signals for ^{202}Hg in salmon egg cell cytoplasm, observed by the standard addition method. Samples: salmon egg cell cytoplasm diluted five-fold with 0.1 M Tris–HNO_3 buffer solution (pH 7.4) with and without addition of Hg. Added amount of Hg: 5, 10, 15 and 20 ng g^{-1}.

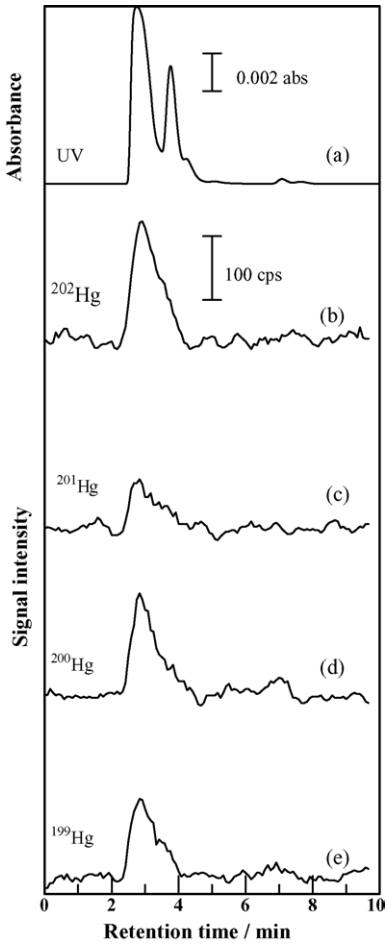

Fig. 3. Chromatograms for salmon egg cell cytoplasm diluted with 0.1 M Tris–HNO_3 buffer. (a) UV absorption detection, (b–e) Hg detection at *m/z* 199, 200, 201 and 202, respectively. Sample: salmon egg cytoplasm diluted five-fold with 0.1 M Tris–HNO_3 buffer solution (pH 7.4). Other experimental conditions are the same as Fig. 1.

In Fig. 3(b)–(e), the Hg-detected chromatograms for cytoplasm diluted with 0.1 M Tris buffer are shown, where Hg was detected at *m/z* 199, 200, 201 and 202 by ICP-MS. It is seen in Fig. 3(b)–(e) that the retention times of the main Hg peaks in the chromatograms were ca. 3.0 min, which were coincident with the larger peak in the UV-detected chromatogram shown in Fig. 3(a). These results indicate that Hg in salmon egg cell cytoplasm mainly binds with proteins in cytoplasm, although those proteins and their molecular weights could not be identified from the chromatograms using the CHAPS-coated ODS column, as mentioned above. The isotope abundances of ^{199}Hg, ^{200}Hg, ^{201}Hg and ^{202}Hg were 16.9, 23.1, 13.2 and 29.7%, respectively. The relative peak heights (or areas) of ^{199}Hg, ^{200}Hg, ^{201}Hg and ^{202}Hg

at the retention time of ca. 3.0 min were almost consistent with the ratios of isotope abundances. These results, thus, support that Hg observed in the chromatograms shown in Fig. 3(b)–(e) may be attributed to protein-binding Hg species. In addition, it should be noticed here that some shoulder peaks were observed in all the Hg-detected chromatograms, which were at almost the same position as the smaller peak (ca. 3.9 min) detected by UV absorption.

3.3. Chromatograms for salmon egg cell cytoplasm diluted with 0.01 M Tris–HNO_3 buffer solution

When salmon egg cell cytoplasm was diluted with pure water or the lower concentration (0.01 M) of Tris–buffer solution, some white precipitates appeared and were suspended in the solution. Since cell cytoplasm generally contains a large amount of hydrophobic proteins, maybe lipid proteins, it was reasonably considered that such lipid proteins were precipitated, when diluted with water. After the precipitates were excluded with a membrane filter (pore size 0.45 μm), the filtrate, in which hydrophilic or water-soluble proteins would remain together with soluble small molecules/ions, was subjected to the analysis by surfactant-mediated HPLC/ICP-MS.

The chromatograms for the filtrate of cell cytoplasm are shown in Fig. 4; (a) chromatogram detected by UV absorption at 254 nm, and (b) chromatogram detected at ^{202}Hg isotope by ICP-MS. It should be noted that the large peak of proteins at ca. 3.0 min, which was observed in the UV-detected chromatogram shown in Fig. 3(a), became very small in the chromatogram in Fig. 4(a). This may indicate that most of hydrophobic proteins were removed from the filtrate of cell cytoplasm. Instead, it is seen in Fig. 4(a) that a clear peak was observed at ca. 3.9 min, which corresponded to the smaller peak in Fig. 3(a). These results indicate that some water-soluble compounds with small molecular weight may exist in the filtrate.

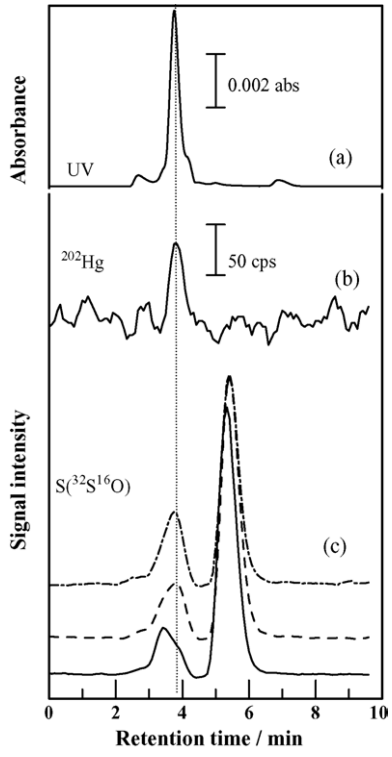

Fig. 4. Chromatograms for the filtrate of salmon egg cell cytoplasm diluted with 0.01 M Tris–HNO_3 buffer. (a) UV absorption detection, (b) Hg detection at m/z 202, (c) S detection at m/z 48 (^{32}S^{16}O). Sample: the filtrate of salmon egg cell cytoplasm diluted five-fold with 0.01 M Tris–HNO_3 buffer solution (pH 7.4). Other experimental conditions are the same as Fig. 1. In the experiment (c), cysteine was added in the filtrate as follows: (—) cysteine not added, (- - -) 100 μg g^{-1} cysteine added, (– · –) 200 μg g^{-1} cysteine added.

In the Hg-detected chromatogram at m/z 202 shown in Fig. 4(b), the peak for Hg was observed at the same retention time of ca. 3.9 min as that in the UV-detected chromatogram. These results suggest the existence of small molecule-binding Hg in cell cytoplasm.

In order to identify the peak observed at ca. 3.9 min in the chromatogram shown in Fig. 4(b), sulfur was further detected at m/z 48 (^{32}S^{16}O) by ICP-MS, with and without addition of cysteine. The results are shown in Fig. 4(c). As can be seen in Fig. 4(c), the sulfur peak at ca. 3.9 min increased with the increase in the amount of cysteine added, although without addition it was small because of the peak overlapping with sulfate ion (t = 3.5 min) in cell cytoplasm [13]. It is well known that cysteine is sulfur-containing amino acid, and it can bind strongly with Hg. Thus, the peak observed at 3.9 min was identified as cysteine-binding Hg. It is noted here that the large peak at ca. 5.5 min in Fig. 4(c) was due to spectral interference with ^{48}Ca in cytoplasm, which was confirmed by the spectral survey of Ca.

3.4. Tandem column chromatograms for Hg in salmon egg cell cytoplasm

The protein fraction between 2.5 and 3.8 min on the chromatogram for cell cytoplasm diluted with 0.1 M Tris–buffer solution, which is shown in Fig. 3, was further subjected to the analysis by SEC with the detection by UV absorption and ICP-MS (^{202}Hg). The SEC chromatograms are shown in Fig. 5, in which the chromatograms detected by S (^{32}S^{16}O) and ^{78}Se are also shown. The SEC molecular weights shown on the top of Fig. 5 was calibrated with standard proteins of known molecular weights, as usual [11,15]. It is seen in Fig. 5 that three clear peaks were observed at the retention times corresponding to the molecular weights of >300, 50 and 12 kDa in the UV-detected chromatogram. These results indicate that the protein fraction on the chromatogram of Fig. 3 contained at least three kinds of proteins.

It should be further noticed that Hg and Se provided three peaks at almost the same retention times as those in the UV-detected chromatogram of Fig. 5, although the peak overlapping of S seemed two at >300 and 50 kDa. These results indicate that Hg in salmon egg cell cytoplasm binds with seleno-proteins, some of which contain cysteine. Also, a clear fourth peak of Se was observed at 37 min out of the permeation limit, which may be ascribed to selenoamino acid.

The proteins observed in Fig. 5 could not be identified only from the present experiment. However, the identification of such proteins in salmon egg cell cytoplasm must be interesting,

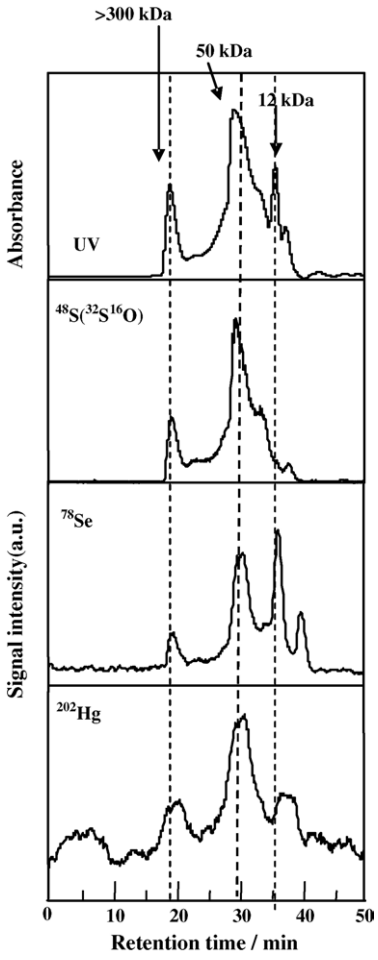

Fig. 5. SEC chromatograms for the protein fraction in Fig. 3, with detection of UV absorption, sulfur ($^{32}S^{16}O$), selenium (^{78}Se) and mercury (^{202}Hg). Sample: protein fraction collected between 2.5 and 3.8 min in Fig. 3; mobile phase: 0.1 M Tris–HNO_3 buffer solution (pH 7.4); sample injection volume: 100 μl.

and such an experiment is now in progress by using MALDI-TOFMS.

Acknowledgements

The present research has been supported partly by the Grant-in-Aid (No. 16002009) of Specially Promoted Research, by the COE Basic Research Formation of “Isotopes for the Prosperous Future” (2003–2004), and by the Grant-in-Aid (No. 15000604) for Scientific Research of Young Scientists from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

[1] H. Haraguchi, J. Anal. Atom. Spectrom. 19 (2004) 5.
[2] H. Haraguchi, T. Matsuura, in: S. Enotomo, K. Seko (Eds.), Proceedings of the International Symposium on Bio-trace Elements 2002 (BITRE 2002), October 27–November 1, 2002, Rikken, Wako, 2002, pp. 1–4.
[3] J. Szpunar, J. Anal. Bioanal. Chem. 400 (2004) 35.
[4] H. Haraguchi, Bull. Chem. Soc. Jpn. 72 (1999) 1163.
[5] J.L. Gomez-Ariza, T. Garcia-Barrera, F. Lorenzo, V. Bernal, M.J. Villeges, V. Olivaira, Anal. Chim. Acta 524 (2004) 15.
[6] Y. Ogra, K.T. Suzuki, J. Anal. Atom. Spectrom. 20 (2005) 35.
[7] R. Lobinski, I.R. Pereiro, H. Cha-saigne, A. Wasik, J. Szpunar, J. Anal. Atom. Spectrom. 13 (1998) 859.
[8] H. Matsuura, H. Haraguchi, Anal. Sci. 17 (Suppl.) (2001) i975.
[9] W. Hu, T. Takeuchi, H. Haraguchi, Anal. Chem. 65 (1993) 2204.
[10] T. Umemura, R. Kitaguchi, H. Haraguchi, Anal. Chem. 70 (1998) 937.
[11] K. Inagaki, T. Umemura, H. Matsuura, H. Haraguchi, Anal. Sci. 16 (2002) 787.
[12] T. Umemura, R. Kitaguchi, K. Inagaki, H. Haraguchi, Analyst 123 (1998) 1767.
[13] H. Matsuura, T. Hasegawa, H. Nagata, K. Takatani, M. Asano, A. Itoh, H. Haraguchi, Anal. Sci. 18 (2003) 117.
[14] H. Matsuura, H. Haraguchi, Biomed. Res. Trace Elem. 13 (2002) 230.
[15] H. Matsuura, K. Takatani, T. Hasegawa, M. Sugita, T. Umemura, H. Haraguchi, Biomed. Res. Trace Elem. 15 (2004) 345.