TECHNICAL NOTE

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Analysis of nickel species in cytosols of normal and malignant human colonic tissues using two dimensional liquid chromatography with ICP-sector field MS detection

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A method has been developed that allows the nickel species in healthy and neoplastic tissues from cancer subjects to be compared. It is based on the coupling of sequentially applied anion-exchange and size-exclusion chromatography using an ICP-sector field mass spectrometer as detector. The method allows the resolution of seven nickel species in tissue extracts, which can be considered as fingerprints of nickel speciation. A qualitative difference in Ni-binding biomolecules between cytosols of normal and malignant colon tissues could not be found.

Introduction

Chronic exposure to nickel and nickel compounds can have adverse effects on human health because of their toxic and carcinogenic activity.[1] While the molecular mechanisms of nickel-induced cell transformation are intensively investigated only a poor knowledge exists of the nickel binding partners in human tissues, especially in colon tissue.[2] The effect of nickel on the carcinogenesis of colon cancer is obvious because the daily uptake of nickel *via* diet is more than triple the daily need.[1,2] Because of the medical problem an analytical method should be developed to characterise nickel species in human tissues.

The determination of nickel and nickel species in biological matrices requires powerful analytical instrumentation because of the relatively low total nickel concentrations in the various human tissues.[3] Inductively coupled plasma sector field mass spectrometry (ICP-SFMS) offers high sensitivity and specific detection of the metals of interest. Additionally, direct sample introduction subsequent to separation of the metal binding species is possible. The use of hyphenated techniques, such as the coupling of ICP-MS with liquid chromatography[4–12] or capillary electrophoresis[13–18] is an attractive methodological approach to trace element speciation analysis in biological samples.[19–21] The various techniques of liquid chromatography hyphenated with ICP-MS offer a number of possibilities for comprehensive characterisation of metal-containing biomolecules.[20] Size exclusion chromatography (SEC) is the initial step in a multidimensional chromatography procedure that enables partial elimination of the sample matrix. SEC allows an estimation of the molecular weight of the species.[22–25] For further information on the metal compound, anion exchange chromatography seems to be a useful tool.[26] The successful use of multidimensional LC-ICP-MS has already been demonstrated for the analysis of selenium species in yeast,[27,28] nickel species in the latex of hyperaccumulating trees,[29] arsenic species in oysters[30] or metal binding phytochelatin in plants[31] and metallothioneins.[32–34]

The objective of this research was the development of an analytical method on basis of the two-dimensional chromatographic procedure method hyphenated with ICP-SFMS for a first characterisation of nickel species in cytosols of normal and neoplastic colons.

Experimental

Instrumentation

A HPLC system 626/606 S (Waters, Eschborn, Germany) equipped with an in-line degasser and a Model 6005 controller was used as the separation device for both anion exchange and size exclusion chromatography. An ICP sector-field mass spectrometer (Element Finnigan MAT, Bremen, Germany) fitted with a Meinhard-type nebulizer and a cooled Scott spray chamber was applied as an element-specific detector in online as well as in off line operation.

Online operation. In the case of anion exchange chromatography the LC system was directly coupled to the ICP-SFMS *via* the Meinhard nebulizer. The separation was performed by the analytical column PRP-X100 PEEK hardware (Hamilton, Reno, NV, USA).

Off line operation. In the case of size exclusion chromatography the preparative column HiLoad 16/60 Superdex 75 (Pharmacia Biotech, Uppsala, Sweden) was used. One ml of the cytosol was injected *via* a sample loop. Fractions were collected using a Foxy Jr. 8 (Isco Inc, Lincoln, NE, USA) fraction collection system. In all fractions collected, the cytosol and the tissues digested were directly analysed by ICP-SFMS.

Determination of molecular mass. To determine the approximate molecular mass of the nickel species found the SEC column was calibrated. Therefore, six protein standards with known molecular masses were detected, all at 280 nm, using the Agilent 1100 series UV detector. The retention times were related to the approximate molecular masses for calibration.

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Chemicals

Analytical reagent grade chemicals purchased from Aldrich (Germany) and ultra-pure water (18 MΩ) produced by a Milli-Q unit (Millipore, Eschborn, Germany) were used throughout unless stated otherwise. Tris buffer solution (20 mmol l^{-1}) was prepared by dissolving 2.42 g of Tris (tris(hydroxymethyl)-aminomethane) (Merck, Darmstadt, Germany) in 1 l of water. The pH of the buffer solution was adjusted to 7.4 with nitric acid. The buffer solution was purified by passing through Chelex 100 ion exchange resin (Fluka, Buchs, Switzerland). Ammonium acetate buffer solution (10 mmol l^{-1}) was prepared by dissolving 0.771 g of ammonium acetate (Merck, Darmstadt, Germany) in 1 l of water. The pH was adjusted with concentrated ammonia to 7.4. The anion exchange gradient buffer was prepared by dissolving 23.13 g of ammonium acetate (Merck, Darmstadt, Germany) in 1 l of 20 mmol l^{-1} Tris buffer solution. The pH was then adjusted to 7.4. The mobile phases were de-gassed prior to use.

The protein standards α-chymotrypsinogen A, aprotinin, myoglobin and transferrin were purchased from Sigma (Steinheim, Germany); bovine albumin and ovalbumin were bought from Acros Organica, Geel, Belgium.

Samples

Histologically normal and neoplastic tissues from six patients with colorectal cancer were supplied from the hospital in Viersen-Dülken, Germany. An aliquot of the resected colon (normal and malignant tissues for the purpose of comparison) was directly frozen at −30 °C in polyethylene bags, transported on solid carbon dioxide to the analytical laboratory and stored at −20 °C for subsequent analysis. The remaining tissues were transported to a pathological laboratory for the purpose of histopathological diagnosis. All pathological data were available in consideration of the data safety. The tumours obtained were of different size, due to different tumour stages and different malignancy.

Procedures

Digestion of tissues. Approximately 1 g of the tissue was cut into small pieces (with a quartz knife to avoid any metal contamination) and digested in a mixture of a 25% concentrated tetramethylammonium hydroxide (TMAH) and ultra-pure water (mixing ratio 2:1) for determination of the overall nickel concentration. 100 µl of the visually digested sample were then diluted with 10 ml of a 2% concentrated nitric acid for analysis *via* ICP-SFMS.

Cytosol preparation. Approximately 2 g of the tissue was cut into small pieces and homogenised using a Potter–Elvehjem homogenizer in 2 ml Tris buffer (20 mmol l^{-1}, pH 7.4) in an ice bath. This procedure was repeated four times. The extracts were combined and centrifuged at 25 000 rpm for 1 h (at 4 °C). The supernatant (8 ml crude cytosol) was decanted, frozen at −18 °C, and lyophilised overnight. The freeze-dried cytosol was dissolved in 1.5 ml of Tris buffer (20 mmol l^{-1}, pH 7.4) prior to analysis.

Chromatographic conditions. The chromatographic experimental conditions are summarised in Table 1(a). For two-dimensional LC fractions were collected from the SE chromatographic column every 2 min. Each fraction was analysed by ICP-SFMS to determine its nickel concentration. Fractions corresponding to the same chromatographic peak were pooled and freeze dried. Each lyophilizate was dissolved in 200 µl of Tris buffer (20 mmol l^{-1}, pH 7.4) and analysed using anion exchange chromatography (AEC)-ICP-SFMS. After each step, the samples were stored immediately under argon and cooled.

ICP-SFMS operating conditions. The ICP-sector field mass spectrometer was operated in the low resolution mode in order to obtain the lowest detection limits. All ICP-SFMS operating conditions are summarised in Table 1 (c). The detection limit of Ni in the low resolution mode was calculated by 3 × RSD divided by the slope of the

Table 1 Operating conditions

1 (a) Operating conditions for SEC	
Column	Superdex 75 Hiload 16/60 (60 cm × 1.6 cm id)
Mobile phase	Ammomium acetate (10 mmol l^{-1}) pH 7.4
Gradient	Isocratic
Flow rate	1 ml min^{-1}
Injection volume	1 ml
1 (b) Operating conditions for AEC	
Column	PRP-X100 Peek hardware (25 cm × 4.6 mm id)
Mobile phase	A: 20 mmol l^{-1} Tris buffer, pH 7.4
	B: 20 mmol l^{-1} Tris buffer, 300 mmol l^{-1} ammonium acetate, pH 7.4
Gradient	0–2 min 100% A
	2–25 min linear ramp to 100% B
	25–35 min 100% B
Flow rate	1 ml min^{-1}
Injection volume	100 µl
1 (c) Operating conditions for ICP-SFMS	
Cool gas	15 l min^{-1}
Auxiliary gas	0.9 l min^{-1}
Nebulizer gas	0.95 l min^{-1}
Power	1100–1150 W
Mass resolution	Low resolution mode (300)
Scan duration	500 ms
Isotopes monitored	^{60}Ni, ^{63}Cu, ^{64}Zn
Spray Chamber	Double-pass Scott-type, glass
Sampler and skimmer	Platinum
Nebulizer	Meinhard (1 ml min^{-1})
Spray chamber temperature	5 °C

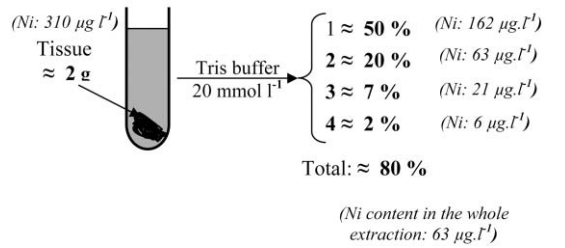

Fig. 1 Recovery rates of the nickel content in four successive extractions of a tissue sample.

calibration curve, in accordance to the IUPAC guideline, and was found to be 1.2 and 1.0 ng l^{-1}, respectively. The nickel standards were prepared in 2% concentrated nitric acid and in Tris buffer in the range between 2.5 and 100 ng l^{-1}, respectively.

Results and discussion

Nickel extraction efficiency

The overall Ni concentration in the different tissues analysed varied between 7 and 310 µg kg^{-1} (wet weight). For the reason of a limited quantity of samples ($N = 5$) no significant differences in the total Ni content between the malignant and healthy tissues were found. In Fig. 1 the yields of the successive Potter–Elvehjem extraction steps were summarised, with an example of the concentration obtained for one of the tissues analysed. After the four steps 80% of the Ni initially present was recovered in the supernatant. The 4th extract contained only 2% of Ni.

In order to concentrate the extract, freeze drying was used. The concentration of the solution after dissolving the lyophilisate was thus between 50 and 200 µg l^{-1}, depending on the concentration of nickel in the original tissue. The recovery of Ni during the preconcentration procedure was quantitative (>98%). After preconcentration, an aliquot of the 1.5 ml cytosol was analysed by anion exchange chromatography and the rest was inject on the preparative SEC.

Analysis of the cytosol using anion exchange chromatography (AEC)

A similar chromatographic gradient as described by Bayon *et al.*[6] was applied to the separation of nickel species in the analytical method presented. In order to prevent any modification of the native nickel containing biomolecules during the extraction, Tris buffer at the physiological pH of 7.4 was employed. With the operating chromatographic conditions (Table 1(b)) used here, it was possible to separate more than 5 nickel containing compounds in the cytosol of colon tissues (Fig. 2 (c), 2 (d) and Fig. 3).

Depending on the cytosol, the first peak in the anion exchange chromatogram contains 3 other different peaks, as shown in Fig. 2 (b) and Fig. 3. This first peak was detected in all the cytosol analysed. Peaks 2, 3, 4 and 5 could be found in tissues with the highest nickel concentration (310 µg l^{-1}), as shown in Fig. 2 (c), 2 (d) and Fig. 3 (b), (c)).

The shaded area in Fig. 3 corresponds to the degradation of some nickel compounds and contamination of the column which still appears in the blank (see Fig. 3 (c)). This contamination of the column does not appear in the analysis of less concentrated cytosol (see Fig. 2 (a)).

Figs. 2 and 3 show that the nickel containing compounds present in the tissues strongly depend on the total concentration of nickel, with an increase in the nickel concentration leading to the appearance of some new species.

In order to investigate the role of nickel on the carcinogenesis

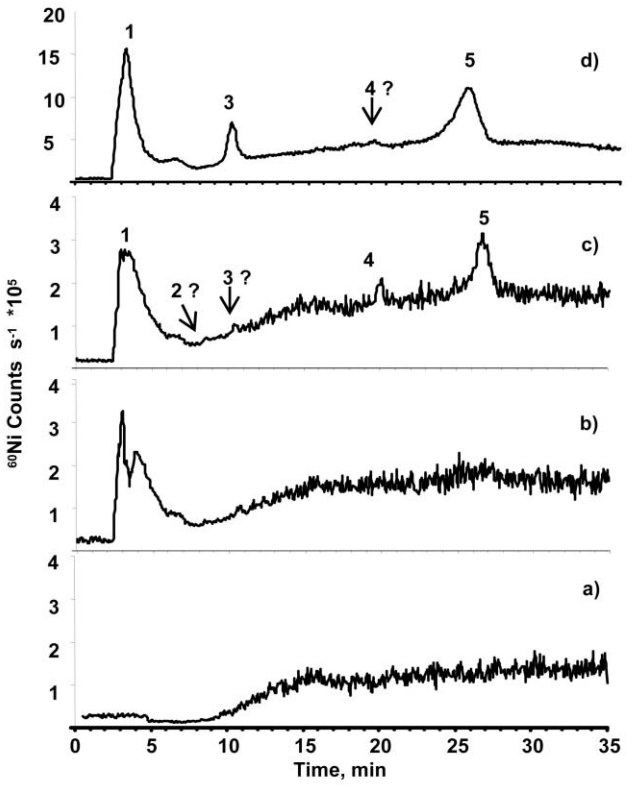

Fig. 2 Elution profile of nickel in a crude cytosol using AEC-ICP-SFMS. Chromatogram of (a) blank, crude cytosol obtained from malignant tissues with (b) 9.95 µg l^{-1} Ni, (c) 34.7 µg l^{-1} Ni and (d) 63.6 µg l^{-1} Ni.

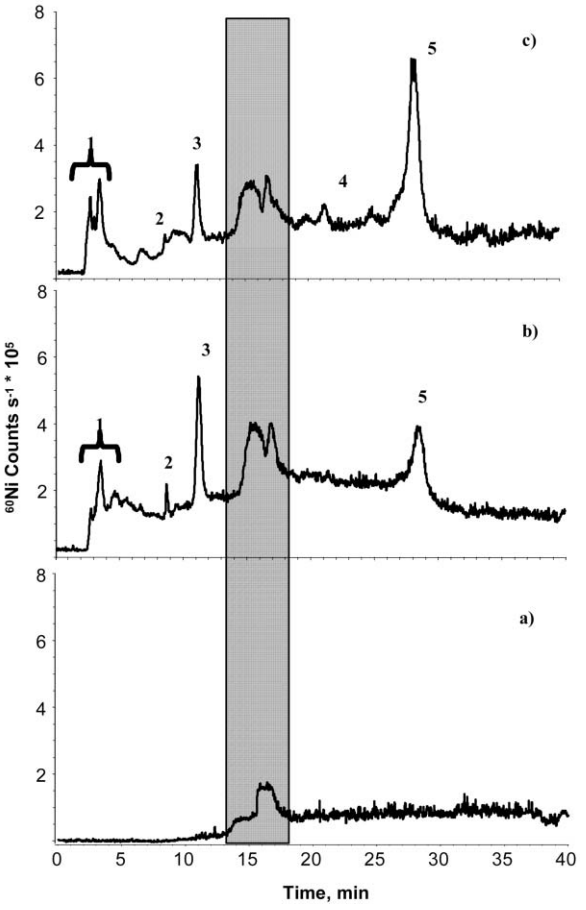

Fig. 3 Elution profile of nickel in a crude cytosol obtained from (a) normal tissue, (b) malignant tissue with a total nickel content of 310 µg l^{-1} and (c) blank by AEC-ICP-SFMS.

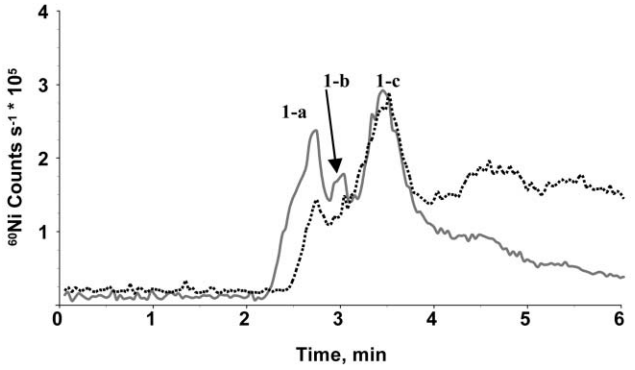

Fig. 4 Elution profile for a retention period of 6 min in a crude cytosol obtained from a malignant tissue (—) and a normal tissue (– – –) using AEC-ICP-SFMS.

of colorectal cancer, the speciation of nickel in two tissues from the same part of the colon, one malignant and one normal, was performed. Fig. 3 and Fig. 4 show the results of the analysis of these two corresponding cytosols using anion exchange chromatography. Several differences appear between these two tissues, but all these differences concern the peak intensity and the disappearance of peaks on the chromatogram obtained with the malignant tissue. It can be concluded that no other species appear in the malignant tissues compared with the normal tissues.

Fractionation of the cytosol using SEC

For an estimation of the approximate size of the nickel species observed, size-exclusion chromatography (SEC) is the method of choice. After calibration of the column with different protein standards, a correlation of each nickel peak after SEC to the approximative molecular mass is possible. The fractionation by SEC and the subsequent analysis of each fraction using anion exchange chromatography provide this information on the approximative molecular mass of the species producing the peaks, shown in Fig. 2 and Fig. 4.

The elution profiles and the elution time of the protein standards are given in Fig. 6a. The chromatogram obtained for the crude cytosol (Fig. 5b) shows 4 well resolved peaks and a small shoulder between the peaks 3 and 4. The first two peaks have correspondence in the UV chromatogram (Fig. 5a) but the two most intense ICP-SFMS peaks do not evidence correspond to the UV profile.

The calibration of the SEC column by injection of protein standards (Fig. 5) gives the size information on the four species generating the peaks. The first peak is in the void volume with a corresponding size species of more than 80 kDa, the second peak corresponds to a size species of about 65 kDa, the third one and the last one correspond to size species of about 10 kDa and 5 kDa, respectively. On the basis of the information on size it can be speculated that the two less abundant compounds could be nickel protein compounds.

The analysis of these five fractions using anion exchange chromatography (Fig. 6) gives size information on the elution profile of the crude cytosol (Figs. 2 and 3). Fig. 6 shows that compound number 1 corresponds to the fraction 5 by SEC (about 5 kDa), with some part in fractions 3 and 4. The compound number 5 corresponds to a size about 10 kDa (fraction 3) and the species 2 and 3 correspond respectively to fractions 2 and 1 (65 kDa and more than 80 kDa).

The analysis of these SEC fractions using anion exchange chromatography shows that the peak that appears at the retention time 15 min is in all fractions. This peak could result from the degradation of the nickel compound in the anion exchange column.

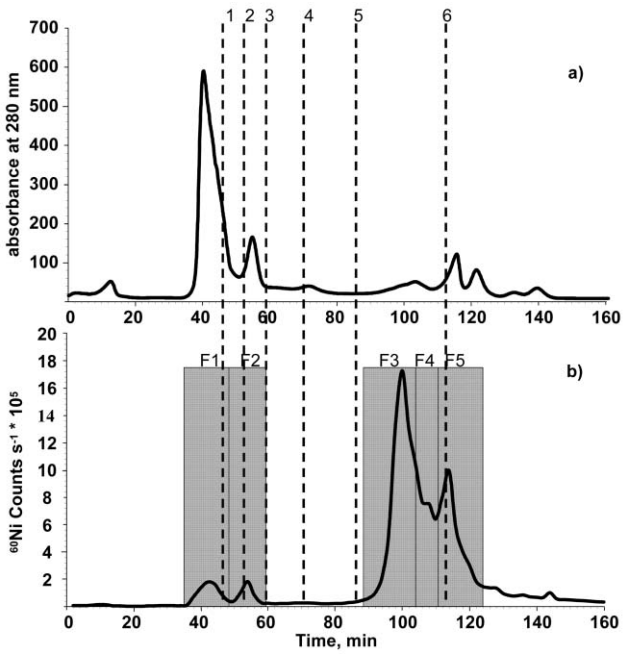

Fig. 5 SEC of a crude cytosol extract from a malignant tissue: (a) UV detection at 280 nm, (b) ICP-SFMS off line detection after fraction collection. The broken lines correspond to: 1, transferrin (80 kDa); 2, bovine albumin (66 kDa); 3, ovalbumin (44 kDa); 4, α-chymotrypsinogen (25.5 kDa); 5, myoglobin (18.5 kDa); 6, aprotinin (6.5 kDa). The shaded areas correspond to the fractions collected, mixed and freeze dried.

Conclusions

The analytical method developed in the investigations presented makes possible the separation of nickel compounds in

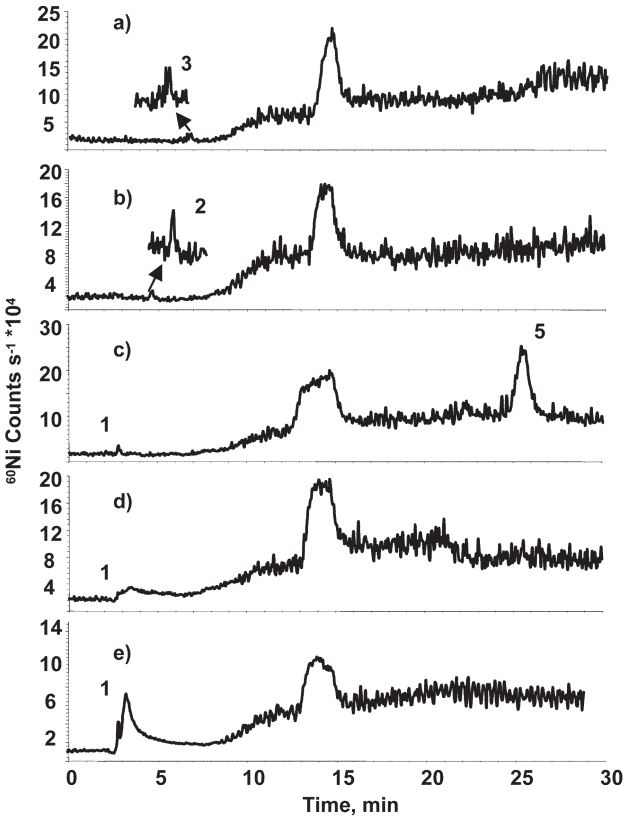

Fig. 6 Elution profile of nickel in the fraction of crude cytosol used for Fig. 4 after SEC fractionation. (a) Fraction 1; (b) fraction 2; (c) fraction 3; (d) fraction 4; (e) fraction 5. The number on the elution profiles of nickel corresponds to the peaks in Fig. 4.

normal and malignant tissues in order to get a better knowledge of the role of nickel in the carcinogenesis of colon cancer. On the basis of two-dimensional chromatography, anion exchange and size exclusion chromatography, a few unknown nickel containing compounds in the cytosol of malignant as well as normal tissues could be separated and detected by ICP-SFMS. The results show that there are no qualitative differences between the nickel species in the crude cytosol of malignant and normal tissues, but there are quantitative differences in the peak area ratios. The dependence of the nickel species occurring on the total concentration of nickel in the tissue was shown.

This study also demonstrates the power of the hyphenation between HPLC and ICP-SFMS, which is sensitive enough to detect nickel in very low amounts. This sensitivity is necessary for the analysis of tissues with total nickel concentrations of about 10 μg l^{-1}.

References

1 D. G. Barceloux, *Clin. Toxicol.*, 1999, **37**, 239–258.
2 E. Denkhaus and K. Salnikow, *Crit. Rev. Oncol. Hemat.*, 2002, **42**, 35–56.
3 E. Coni, A. Alimonti, L. Fornarelli, E. Beccaloni, E. Sabbioni, R. Pietra, G. B. Bolis, E. Cristallini, A. Stacchini and S. Caroli, *Acta. Chim. Hung.*, 1991, **128**, 563–572.
4 D. M. Templeton, F. Ariese, R. Cornelis, L. G. Danielsson, H. Muntau, H. P. Van Leeuwen and R. Lobinski, *Pure Appl. Chem.*, 2000, **72**, 1453–1470.
5 J. Szpunar, H. Chassaigne, A. Makarov and R. Lobinski, *Chem. Anal.*, 1999, **44**, 351–362.
6 M. M. Bayon, A. B. S. Cabezuelo, E. B. Gonzalez, J. I. Garcia Alonso and A. Sanz-Medel, *J. Anal. At. Spectrom.*, 1999, **14**, 947–951.
7 C. N. Ferrarello, M. M. Bayon, R. F. de la Campa and A. Sanz-Medel, *J. Anal. At. Spectrom.*, 2000, **15**, 1558–1563.
8 K. Inagaki, T. Umemura, H. Matsuura and H. Haraguchi, *Anal. Sci.*, 2000, **16**, 787–788.
9 C. A. P. de Leon, K. L. Sutton, J. A. Caruso and P. C. Uden, *J. Anal. At. Spectrom.*, 2000, **15**, 1103–1107.
10 M. A. Quijano, P. Moreno, A. M. Gutierrez, M. C. Perez-Conde and C. Camara, *J. Mass Spectrom.*, 2000, **35**, 878–884.
11 A. D. Madsen, W. Goessler, S. N. Pedersen and K. A. Francesconi, *J. Anal. At. Spectrom.*, 2000, **15**, 657–662.
12 M. Wind, H. Wesch and W. D. Lehmann, *Anal. Chem.*, 2001, **73**, 3006–3010.
13 B. Michalke, *Spectroscopy*, 2000, **15**, 30–34.
14 S. Mounicou, K. Polec, H. Chassaigne, M. Potin-Gautier and R. Lobinski, *J. Anal. At. Spectrom.*, 2000, **15**, 635–642.
15 L. Bendahl, B. Gammelgaard, O. Jons, O. Farver and S. H. Hansen, *J. Anal. At. Spectrom.*, 2001, **16**, 38–42.
16 W. H. Chen, S. Y. Lin and C. Y. Liu, *Anal. Chim. Acta*, 2000, **410**, 25–35.
17 A. Prange, D. Schaumlöffel, P. Brätter, A. N. Richarz and C. Wolf, *Fresenius' J. Anal. Chem.*, 2001, **371**, 764–774.
18 D. Schaumlöffel, A. Prange, G. Marx, K. G. Heumann and P. Brätter, *Anal. Bioanal. Chem.*, 2002, **372**, 155–163.
19 J. Szpunar, R. Lobinski and A. Prange, *Appl. Spectrosc.*, 2003, **57**, 102A–112A.
20 J. Szpunar, *Analyst*, 2000, **125**, 963–988.
21 M. B. D. C. Guntinas, G. Bordin and A. R. Rodriguez, *Anal. Bioanal. Chem.*, 2002, **374**, 369–378.
22 J. Szpunar, A. Makarov, T. Pieper, B. K. Keppler and R. Lobinski, *Anal. Chim. Acta*, 1999, **387**, 135–144.
23 J. Wang, D. Dreessen, D. R. Wiederin and R. S. Houk, *Anal. Biochem.*, 2001, **288**, 89–96.
24 C. S. Muniz, J. M. M. Gayon, J. I. Garcia Alonso and A. Sanz-Medel, *J. Anal. At. Spectrom.*, 2001, **16**, 587–592.
25 R. Koplik, M. Borkova, O. Mestek, J. Kominkova and M. Suchanek, *J. Chromatogr. B*, 2002, **775**, 179–187.
26 T. Nakazato, T. Taniguchi, H. Tao, M. Tominaga and A. Miyazaki, *J. Anal. At. Spectrom.*, 2000, **15**, 1546–1552.
27 C. Casiot, J. Szpunar, R. Lobinski and M. Potin-Gautier, *J. Anal. At. Spectrom.*, 1999, **14**, 645–650.
28 H. Chassaigne, C. C. Chery, G. Bordin and A. R. Rodriguez, *J. Chromatogr. A*, 2002, **976**, 409–422.
29 D. Schaumlöffel, L. Ouerdane, B. Bouyssiere and R. Lobinski, *J. Anal. At. Spectrom.*, 2003, **18**, 120–127.
30 S. McSheehy, P. L. Pohl, R. Lobinski and J. Szpunar, *Analyst*, 2001, **126**, 1055–1062.
31 V. Vacchina, R. Lobinski, M. Oven and M. H. Zenk, *J. Anal. At. Spectrom.*, 2000, **15**, 529–534.
32 R. Lobinski, H. Chassaigne and J. Szpunar, *Talanta*, 1998, **46**, 271–289.
33 C. N. Ferrarello, M. D. F. de la Campa, J. F. Carrasco and A. Sanz-Medel, *Spectrochim. Acta, Part B*, 2002, **57**, 439–449.
34 C. N. Ferrarello, M. D. F. de la Campa, J. F. Carrasco and A. Sanz-Medel, *Anal. Chem.*, 2000, **72**, 5874–5880.