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A comparison of vancomycin and sulfated beta-cyclodextrin as chiral selectors for enantiomeric separations of selenoamino acids using capillary electrophoresis with UV absorbance detection

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The enantiomeric separation of three selenoamino acids, D,L-selenomethionine, D,L-selenoethionine and D,L-selenocystine is described. Both sulfated β-cyclodextrin and vancomycin have been successfully used to separate all enantiomers of the compounds with UV detection. Reproducible separations, in terms of peak area and migration time were obtained using sulfated β-cyclodextrin with reversed polarity and UV detection. With vancomycin as a chiral selector, reversed polarity was found to be more reproducible than positive polarity in terms of peak migration times.

Introduction

Selenium is an essential trace element at concentrations of 0.8 to 1.7 μmol l^{-1}. Deficiency is associated with muscle disease in mammals and several different types of cancer.[1] It has been shown that supplemental selenium is required for cellular defense against oxidative damage and thus may participate in the prevention of cancer.[2] A more recent study concluded that selenium treatment does not protect against the development of basal or squamous cell carcinomas of the skin but results support the hypothesis that supplemental selenium may reduce the incidence of carcinomas at several sites in the human body.[3] At higher concentrations, selenium toxicity may result, which is characterized in humans by symptoms including dermatitis, fatigue, dizziness and hair loss.[4] There is a narrow range between the beneficial and toxic levels of selenium[5] although these effects are dependent on the chemical form of the selenium species.[6]

Selenium is often found in association with sulfur in both organic and inorganic compounds and may either replace the sulfur atom or be complexed to sulfur by covalent bonding.[7] Inorganic forms of selenium include the selenate and selenite ions (the analogs of sulfates and sulfites), elemental selenium and metal selenides. In addition, organisms may replace the sulfur atom in many amino acids. A natural selenium cycle links selenate and selenite with the more complex selenoamino acids; however, many of the chemical and biochemical pathways of the cycle are unknown.[8] Supplemental selenium usually takes the form of selenomethionine, which is incorporated into proteins. Excess selenium is converted to dimethyl selenide, prior to being converted to the water-soluble trimethylselenonium ion, which may be excreted. When a significant excess of selenium is ingested, the body excretes dimethyl selenide *via* expired air, which yields a characteristic 'garlic breath'.[7]

Most α-amino acids contain an asymmetric α-carbon and thus have both L- and D-enantiomers. The majority of the amino acids, which are incorporated into proteins by organisms, are of the L-form. Low levels of the D-form (relative to the L) may be found in various human body fluids. *e.g.*, urine, owing to conditions such as pregnancy.[9,10] For this reason, analytical techniques that can differentiate between the D- and L-enantiomers can be used to monitor the amount of the beneficial enantiomer delivered in a selenium supplement. In this way, the beneficial dosage of a supplement can be increased so that more of the L-enantiomer can be incorporated into proteins without the toxic effects that an excess of the seleno-containing D-enantiomer may cause.

High-performance liquid chromatography may be used to perform speciation of D,L-selenoamino acid enantiomers when a chiral column is used. Pérez Méndez *et al.*[11] used a β-cyclodextrin column for the separation and determination of D- and L-selenomethionine using fluorimetric and element specific ICP-MS detection with on-line hydride generation. Chiral columns may, however, be expensive and chiral separations are now increasingly being performed using capillary electrophoresis. A current review of the state-of-the-art of chiral separations by capillary electrophoresis has been published.[12]

The macrocyclic antibiotic, vancomycin is established as being a very versatile chiral selector for enantiomeric separations of a wide variety of compounds, including several amino acids.[13,14] Vespalec *et al.*[15] described the addition of vancomycin to the electrophoretic buffer to achieve a separation of the enantiomers of some selenoamino acids. In a subsequent paper, the group described improvements to the technique using a coated capillary.[16] UV absorbance detection was used in all instances.

Another versatile chiral selector for enantiomeric separations using capillary electrophoresis is sulfated β-cyclodextrin.[17,18] Stalcup and Gahm[19] used sulfated cyclodextrins as chiral additives for the enantioseparation of 56 compounds of pharmaceutical interest. A large number of structurally diverse analytes, both cationic and neutral were analysed and enantioresolved using this chiral additive.

In this study, the primary goal was to evaluate several chiral separation techniques for the enantioresolution, in a single electrophoretic run, of three selenoamino acids: D-, L- and *meso*-selenocystine; D- and L-selenomethionine; and D- and L-selenoethionine. Vancomycin and sulfated β-cyclodextrin as chiral selectors were examined using UV absorbance detection. Application to selenoamino acids in nutritional supplements were attempted but lower levels of detection are necessary such as by inductively coupled plasma mass spectrometry (ICP-MS)[20–26] and this is currently ongoing.

Experimental

Instrumentation

The CE experiments with UV detection were performed on a Bio-Rad Biofocus 3000 Capillary Electrophoresis system (Bio-

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Rad, Hercules, CA, USA) equipped with a high speed scanning UV detector. Polyimide-coated fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA), of dimensions 75 μm id and 75 cm length were used throughout the study. A window was created 4.6 cm from the end of the capillary and 254 nm was selected as the absorbing wavelength. The capillary was thermostatted at 20 °C. Hydrodynamic injections were made at 5 psi for 0.4 s (2 psi s^{-1}). The system was used in either positive polarity (injection made at positive electrode) or reversed polarity. Electropherograms were analysed using Bio-Rad Biofocus Integration software (version 5.20).

Reagents and chemicals

All reagents used in this study were analytical reagent grade. Vancomycin buffer solutions were prepared daily using 1 or 2 mM vancomycin hydrochloride (Spectrum Quality Products Inc., Gardena, CA, USA) and 20 mM MOPS (3-(*N*-morpholino)propanesulfonic acid) (Sigma Chemical Co., St. Louis, MO, USA), adjusted to pH 7 with Tris (Sigma). The buffer was refrigerated when not in use. Sulfated β-cyclodextrin buffers were prepared daily using 35 mM citric acid (Fisher Scientific, Fair Lawn, NJ, USA) and 1% m/v sulfated β-cyclodextrin, adjusted to pH 3 using 0.1 M sodium hydroxide (Fisher). To improve the enantiomeric resolution, 1% v/v methanol was added to the sulfated β-cyclodextrin buffer. All buffers were degassed prior to use and filtered through 0.20 μm nylon membrane filters (Fisher).

D,L-Selenomethionine, D,L-selenoethionine, D,L-selenocystine, L-selenomethionine and L-selenocystine were from Sigma. For these compounds, the L-enantiomer is the '+' isomer. Selenocystine was prepared as a 1 mM solution and adjusted to pH 9 with 0.1 M NaOH while selenomethionine and selenoethionine were prepared as 2 mM solutions and adjusted to pH 9.[15] The selenoamino acids were derivatized to enable UV detection according to a method based on that used by Pawlowska *et al*.[27] A fluorescent tagging agent, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) reacts with nucleophiles to produce highly stable and UV absorbant urea compounds.[27]

Aliquots of the amino acid solutions (200 μl) were mixed with 700 μl of the 0.2 M borate buffer provided in the AQC kit (Waters, Milford, MA, USA). The AQC was prepared by adding 1 mL of pyridine (from the AQC kit) to dry AQC reagent and the mixture was heated for 20 min at 50 °C. Aliquots of the AQC solution (200 μl) were then added to the buffered amino acid solutions and sonicated for 10 min before they were left to stand for 1 h at room temperature. The solutions were refrigerated when not in use and diluted by a factor of ten for UV analysis. All standards and electrolytes were prepared using 18 MΩ distilled, deionized water (Sybron Barnstead, Boston, MA, USA).

For the vancomycin separation with positive polarity, it was found necessary to condition the capillary between runs by drawing 0.1 M NaOH for 10 min through the capillary, followed by water for 5 min and buffer for 10 min. Using this method it was found that run to run reproducibility was maintained. For the vancomycin separation with reversed polarity and the sulfated β-cyclodextrin separation, it was not necessary to condition the capillary between runs. All capillaries were stored in water when not in use.

The electroosmotic flow was measured using 20 mM nitromethane (Fisher) as a neutral marker.

Results and discussion

Enantioresolution using sulfated β-cyclodextrin with reversed polarity

A 35 mM citric acid buffer was used with 1% sulfated β-cyclodextrin with 1% methanol added. Methanol was added to the buffer to improve resolution. Sulfated β-cyclodextrin is negatively charged at the pH used (pH 3) and migrates towards the detector (positively charged electrode). Under these conditions, the electroosmotic flow is greatly reduced (2.04×10^{-4} cm^2 V^{-1} s^{-1}) and carries the *N*-derivatized selenoamino acids away from the detector. The complexes of the selenoamino acids with sulfated β-cyclodextrin migrate towards the detector, with the strongest complexes being detected first. A separation of the selenoamino acids, using these conditions is shown in Fig. 1. The migration order was L-selenocystine reaching the detector first, followed by *meso*-selenocystine, D-selenocystine, L-selenomethionine, D-selenomethionine, L-selenoethionine and D-selenoethionine.

One of the attractive features of using a sulfated cyclodextrin (SCD) buffer is that, in this case, the capillary only requires flushing with buffer between runs to maintain reproducible separations. Table 1 shows the reproducibility of the separation and it can be seen that, in terms of peak migration time and peak area, the reproducibility of the separation was excellent, although the leading peaks are tightly bunched (Fig. 1) and the run time is well over an hour.

Enantioresolution using vancomycin with positive polarity

Separation of enantiomers of the three selenoamino acids was evaluated using vancomycin under positive polarity separation conditions, as described by Vespalec and co-workers.[15] In this buffer system (pH 7), vancomycin is positively charged and its electrophoretic mobility is in the same direction as the electroosmotic flow. The magnitude of the electroosmotic flow was 2.24×10^{-4} cm^2 V^{-1} s^{-1}. The *N*-derivatized selenoamino acids have mobilities in a direction opposite to the electroosmotic flow at this particular pH. When complexed with vancomycin, the complex migrates towards the detector.[14] However, it was found that as the concentration of vancomycin was increased from 0.4 mM to 1.0 mM, the migration time of all of the analytes increased and the resolution was improved. A possible explanation for this seemingly anomalous result is that

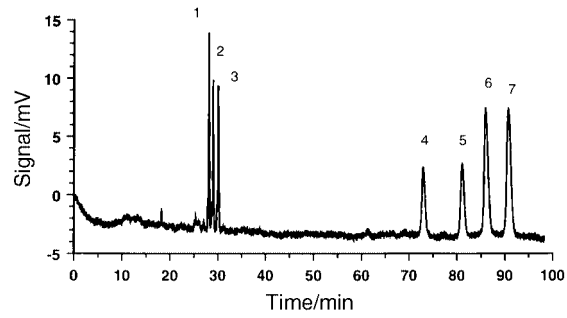

Fig. 1 Separation achieved with UV detection (254 nm) with sulfated β-cyclodextrin as the chiral selector, the addition of methanol and reversed polarity. (1) L-Selenocystine, (2) *meso*-selenocystine, (3) D-selenocystine, (4) L-selenomethionine, (5) D-selenomethionine, (6) L-selenoethionine, (7) D-selenoethionine. Buffer: 35 mM citric acid. 1% m/v sulfated β-cyclodextrin, pH 3 with NaOH, 1% v/v methanol. 2×10^{-5} M injection of each analyte.

Table 1 Precision values for enantioresolution using sulfated β-cyclodextrin with reversed polarity and UV detection (n = 3)

Selenoamino acid	Average peak migration time/min	RSD (%)	
		Migration time	Peak area
L-Selenocystine	28.3	0.74	0.21
meso-Selenocystine	29.2	0.77	1.3
D-Selenocystine	30.3	0.81	2.2
L-Selenomethionine	73.9	1.6	4.0
D-Selenomethionine	82.1	1.6	1.0
L-Selenoethionine	87.2	1.6	0.58
D-Selenoethionine	92.5	1.7	4.1

the positively charged vancomycin tends to adsorb onto the negatively charged wall of the capillary and has the net effect of decreasing the electroosmotic flow.[14] Indeed, the electroosmotic flow decreased accordingly from 3.36×10^{-4} to 2.24×10^{-4} cm^2 V^{-1} s^{-1} as the concentration of vancomycin increased. The concentration of vancomycin for optimum peak resolution was found to be 1.0 mM.

The separation obtained using the 1 mM vancomycin buffer conditions is shown in Fig. 2. It may be seen that all peaks are baseline resolved. For selenomethionine and selenoethionine two peaks may be observed which represent the D- and the L-isomers of the compounds. For selenocystine, which has two chiral centers, three peaks are observed which represent the D-, L- and *meso*-forms of the compound. In all cases the D-isomer migrated before the L. The elution order was verified by running enantiomerically pure selenoamino acids. The run was completed in just less than 1 h, which highlighted the need for buffer reservoirs to be replaced at the beginning of every run to prevent buffer depletion occurring.

Precision values for the separation, in terms of peak area and peak migration time are shown in Table 2. These values were based on three replicate injections. It may be seen that the reproducibility becomes worse as the migration time increases. This is also consistent with a gradual coating of the capillary wall with vancomycin during an electrophoretic run. It proved necessary to condition the capillary between runs using a solution of 0.1 M NaOH, water and buffer for discrete amounts of time. If the conditioning time varied in any way, it was found that the migration times and peak areas were significantly affected. Conditioning the capillary removes the gradual build-up of vancomycin on the wall of the capillary and so maintains the electroosmotic flow in the direction of the detector. If the capillary is not conditioned, the vancomycin apparently builds up on the capillary wall surface and eventually reverses the electroosmotic flow.

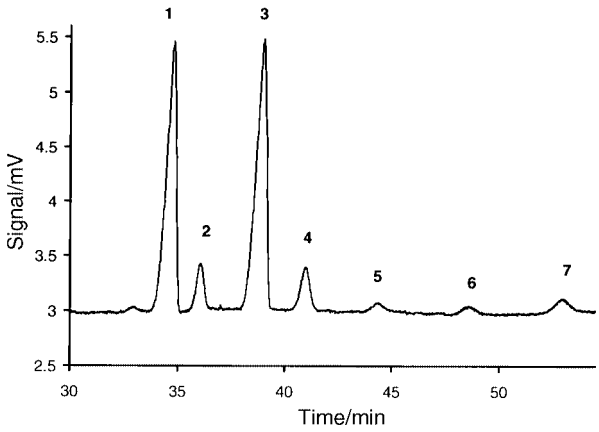

Fig. 2 Separation achieved with UV detection (254 nm) with vancomycin as the chiral selector and positive polarity. (1) D-Selenoethionine, (2) D-selenomethionine, (3) L-selenoethionine, (4) L-selenomethionine, (5) D-selenocystine, (6) *meso*-selenocystine, (7) L-selenocystine. Buffer: 1 mM vancomycin hydrochloride, 20 mM MOPS, pH 7 with Tris. 2×10^{-5} M injection of each analyte.

Table 2 Precision values for enantioresolution using vancomycin with positive polarity and UV detection (n = 3)

Selenoamino acid	Average peak migration time/min	RSD (%)	
		Migration time	Peak area
D-Selenoethionine	34.8	3.9	2.1
D-Selenomethionine	36.1	4.1	4.3
L-Selenoethionine	39.0	5.1	1.4
L-Selenomethionine	40.1	4.3	3.8
D-Selenocystine	44.5	5.7	4.5
meso-Selenocystine	48.8	6.3	7.0
L-Selenocystine	53.0	8.9	6.1

Enantioresolution using vancomycin with reversed polarity

Upon further review of the vancomycin separation with positive polarity, it was postulated that by completely coating the negatively charged surface of the capillary with positively charged vancomycin, it might be possible to reproducibly reverse the electroosmotic flow. Simply reversing the polarity might then facilitate a separation of the selenoamino acids and possibly reduce the time needed for column pre-conditioning.

The MOPS buffer, as used in the experiments with positive polarity, was therefore used with reversed polarity. The buffer containing vancomycin was run through the capillary for 30 min before the first run to ensure that the surface of the capillary was completely covered with vancomycin. Because this separation depends on the complete coating of the surface of the capillary with vancomycin, no capillary conditioning was required between runs.

The presence of the vancomycin coated on the fused silica surface had the effect of reversing the electroosmotic flow (EOF) once the voltage was applied. The EOF was therefore in the direction of the detector, while the intrinsic mobility of the vancomycin was in the opposite direction to the detector. The magnitude of the EOF (3.12×10^{-4} cm^2 V^{-1} s^{-1}), however, was greater than the intrinsic mobility of the vancomycin. Therefore, the analytes, complexed with vancomycin, were transported to the detector. The most strongly complexed analytes have the longest migration times, both because of interactions with the free vancomycin as well as interactions with the wall-coated vancomycin.

The vancomycin concentration in the buffer was optimized for peak resolution and it was found that the best separations were obtained using 2 mM vancomycin and 20 mM MOPS, adjusted to pH 7 using Tris (Fig. 3). Increasing the vancomycin concentration from 1 mM (used in the experiments with positive polarity) to 2 mM, decreased the migration time of the analytes but peak resolution was still obtained. Again, this seemingly anomalous result arises from the vancomycin-concentration-dependent EOF. Indeed, the magnitude of the electroosmotic flow increased from 3.12×10^{-4} cm^2 V^{-1} s^{-1} for 1 mM vancomycin to 4.86×10^{-4} cm^2 V^{-1} s^{-1} for a 2 mM concentration. The separation was very reproducible as shown in Table 3. Baseline resolution was achieved for all the enantiomers and identification of the peaks was accomplished by running the pure enantiomers.

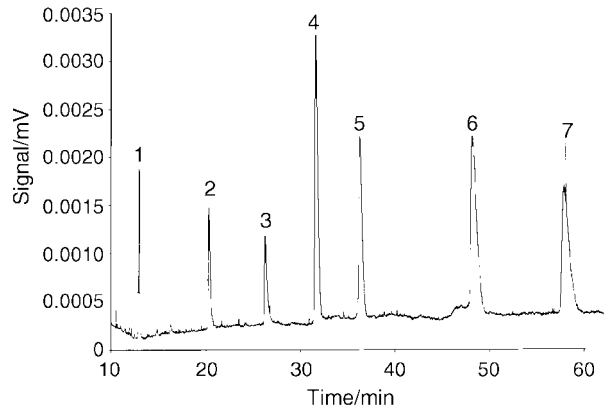

Fig. 3 Separation achieved with UV detection (254 nm) with vancomycin as the chiral selector and reversed polarity. (1) L-Selenocystine, (2) *meso*-selenocystine, (3) D-selenocystine, (4) L-selenomethionine, (5) D-selenomethionine, (6) L-selenoethionine, (7) D-selenoethionine. Buffer: 2 mM vancomycin hydrochloride, 20 mM MOPS, pH 7 with Tris. 2×10^{-5} M injection of each analyte.

Interestingly, although the enantiomeric elution order of the individual amino acids was the complete reverse of that obtained using positive polarity, the elution order of the selenoamino acids was not the complete reverse of that seen when using positive polarity. With positive polarity, the migration was in the order: D-selenoethionine, D-selenomethionine, then the corresponding L-enantiomers of selenoethionine and selenomethionine, respectively, followed by the D-, *meso*- and L-forms of selenocystine. With reversed polarity the enantiomers of the particular compounds came out together, *i.e.*, L-, *meso*- and D-selenocystine, L- and D-selenomethionine and L- and D-selenoethionine.

An attempt at application of a complex sample by using the methods described above was made by digesting commercially available selenium dietary supplements using a gastric enzymolysis digest.[28] However, UV absorbance detection is insufficiently sensitive to quantify the levels of selenoamino acids in commercial selenium dietary supplements without preconcentration. In addition, this method is not sensitive to inorganic seleno compounds which might be present. For the species investigated in this study, the limits of detection using UV absorbance are in the 0.5–1.0 mg range (as selenium). The various manufacturers of selenium dietary supplements claim to have concentrations of selenium between 50 and 200 μg in the supplement tablets. Studies are now continuing to improve the detection limits by using ICP-MS as an alternative detector to UV absorbance. ICP-MS has been shown to offer significantly improved limits of detection, of up to three orders of magnitude, compared to UV absorbance detection when low flow pneumatic nebulizers are used.[26,29] Improving the CE/ICP-MS interface is critical and complex. However, these studies are ongoing.

Conclusions

It has been demonstrated that very reproducible enantiomeric separations of three selenoamino acids (D,L-selenomethionine, D,L-selenoethionine and D,L-selenocystine) may be obtained using either sulfated β-cyclodextrin or vancomycin as chiral additives with UV detection. It was found that using sulfated β-cyclodextrin as a chiral additive, with reversed polarity enabled separation of all the enantiomers and resolution was improved using methanol in the buffer. When vancomycin is used as a chiral selector, both reversed and positive polarities may be used to separate the analytes. However, it was found that better reproducibility was obtained when the capillary was purged with the vancomycin-containing buffer for a significant period of time and reversed polarity was used. In this way the electroosmotic flow direction is reversed and better resolution is achieved.

Table 3 Precision values for enantioresolution using vancomycin with reversed polarity and UV detection (n = 3)

Selenoamino acid	Average peak migration time/min	RSD (%)	
		Migration time	Peak area
L-Selenocystine	7.8	0.52	2.9
meso-Selenocystine	12.9	0.72	0.69
D-Selenocystine	20.2	1.3	2.2
L-Selenomethionine	30.8	1.7	2.5
D-Selenomethionine	35.5	1.9	3.2
L-Selenoethionine	46.1	2.4	3.6
D-Selenoethionine	56.2	3.2	1.9

Future studies will attempt to enhance the sensitivity of the analysis using ICP-MS as a selenium specific detector with the aim of quanitying the levels of selenoamino acids in commercially available selenium supplements. The evaluation of different CE/ICP-MS interfaces is ongoing.

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References

1 Z. Wang, S. Xie and A. Peng, *J. Agric. Food Chem.*, 1996, **44**, 2754.
2 M. J. Axley, A. Bock and T. C. Stadman, *Proc. Natl. Acad. Sci. U.S.A.*, 1991, **88**, 8450.
3 L. C. Clark, G. F. Combs, B. W. Turnbull, E. H. Slate, D. C. Chalker, J. Chow, L. S. Davis, R. A. Glover, G. F. Graham, E. G. Gross, A. Krongrad, J. L. Lesher, H. K. Park, B. B. Sanders, C. L. Smith and J. R. Taylor, *J. Am. Med. Assoc.*, 1996, **276**, 1957.
4 C. Duttagupta and E. Seifter, in *Analysis of Foods and Beverages: Modern Techniques*, ed. G. Charalambous, Academic Press, London, 1974, pp. 362.
5 C. Zapsalis and R. A. Beck, *Food Chemistry and Nutritional Biochemistry*, John Wiley and Sons, New York, 1985, pp. 1015–1016.
6 J. A. Favier, *Selenium Metabolism*, ed. J. A. Favier, Walter de Gruyter, New York, 1989.
7 M. L. Scott, *Nutrition of Humans and Selected Animal Species*, John Wiley and Sons, New York, 1974.
8 G. Kolbl, K. Kalcher, K. J. Irgolic and R. J. Magee, *Appl. Organomet. Chem.*, 1993, **7**, 443.
9 D. W. Armstrong, M. P. Gasper, S. H. Lee, N. Ercal and J. Zukowski, *Amino Acids*, 1993, **5**, 299.
10 D. W. Armstrong, M. P. Gasper, S. H. Lee, J. Zukowski and N. Ercal, *Amino Acids*, 1993, **5**, 375.
11 S. Pérez Méndez, E. Blanco Gónzalez, M. L. Fernández Sánchez and A. Sanz Medel, *J. Anal. At. Spectrom.*, 1998, **13**, 893.
12 R. Vespalec and P. Bocek, *Electrophoresis*, 1997, **18**, 843.
13 R. Vespalec, H. A. H. Billiet, J. Frank and P. Bocek, *Electrophoresis*, 1996, **17**, 1214.
14 D. W. Armstrong, K. L. Rundlett and J.-R. Chen, *Chirality*, 1994, **6**, 496.
15 R. Vespalec, H. Corstijens, H. A. H. Billiet, J. Frank and K. C. A. M. Luyben, *Anal. Chem.*, 1995, **67**, 3233.
16 R. Vespalec, H. A. H. Billet, J. Frank and K. C. A. M. Luben, *J. High Resolut. Chromatogr.*, 1996, **19**, 137.
17 H. Nishi and S. Terabe, *J. Chromatogr.*, 1995, **694**, 245.
18 S. Terabe, K. Otsuka and H. Nishi, *J. Chromatogr. A*, 1994, **666**, 295.
19 A. M. Stalcup and K. H. Gahm, *Anal. Chem.*, 1996, **68**, 1360.
20 J. A. Kinzer, J. W. Olesik and S. V. Olesik, *Anal. Chem.*, 1996, **68**, 3250.
21 V. Majidi and N. Miller-Ihli, *Analyst*, 1988, **123**, 803.
22 V. Majidi and N. Miller-Ihli, *Analyst*, 1988, **123**, 809.
23 B. Michalke and P. Schramel, *J. Chromatogr. A*, 1996, **750**, 51.
24 B. Michalke, S. Listig and P. Schramel, *Electrophoresis*, 1997, **18**, 196.
25 B. Michalke and P. Schramel, *Electrophoresis*, 1998, **19**, 270.
26 K. L. Sutton, C. B'Hymer and J. A. Caruso, *J. Anal. At. Spectrom.*, 1998, **13**, 885.
27 M. Pawlowska, S. Chen and D. W. Armstrong, *J. Chromatogr. A*, 1993, **641**, 257.
28 *The United States Pharmacopeia*, United States Pharmacopeia Convention, Inc., Rockville, MD, 1989, 22nd revision.
29 K. L. Sutton and J. A. Caruso, *LC–GC*, 1999, **17**, 36.

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