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REVIEW

Identification and quantification of inorganic and organic selenium compounds with high-performance liquid chromatography

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Selenium appears in the natural selenium cycle in the form of several organic and inorganic compounds. The biologically beneficial and detrimental effects of 'selenium' must be ascribed to particular selenium compounds. The identification and quantification of selenium compounds in biological and environmental samples is required for an understanding of the role of selenium. The high-performance liquid-chromatographic (HPLC) methods for the separation, identification and quantification of selenite, selenate, hydrogen selenide, methaneselenol, bis(organothio) selenides, trimethylselenonium salts, selenoamino-acids, selenium derivatives of carbohydrates, selenoproteins, selenonucleosides and other miscellaneous selenium compounds are summarized (193 references) and pertinent detection modes discussed. Advantages and disadvantages of the methods are pointed out. The literature is covered since 1974, the year of the first publication in this field.

Keywords: Selenium, selenium compounds, high-performance liquid chromatogaphy, selenium-specific detectors

INTRODUCTION

Before 1957 the year in which the paper reporting the essentiality of selenium was published,[1] selenium compounds were thought to be only highly toxic and carcinogenic. Since that time selenium has been claimed to influence beneficially the functions of the human heart, to decrease death rates from cancer,[2] and to diminish or prevent the toxic effects caused by an excess of toxic elements such as arsenic and mercury.[3] The early investigations into selenium in biological systems used methods that provided concentrations of 'total selenium' and no information about the compounds in which selenium occurred.

During the past two decades several inorganic and organic selenium compounds (Fig. 1) were identified in biological samples (animals, plants, microorganisms). Among these compounds are selenite, selenate, dimethyl selenide, trimethylselenonium salts, selenium derivatives of carbohydrates,[4] selenoamino-acids, selenium-containing proteins (enzymes),[5] selenium-containing nucleosides,[6] and perhaps selenolipids. Additional selenium compounds will certainly be identified as research in this very active field continues.[7–10] These selenium compounds participate in a natural selenium cycle (Fig. 1) that links selenate and selenite with simple methylated and more complex organic selenium compounds. Many of the chemical and biochemical pathways in this cycle are still unknown.

To elucidate the biochemical transformations of selenium compounds, to identify still unknown, 'natural', selenium compounds, to explore the interactions of selenium compounds with biochemically important molecules leading to beneficial or detrimental effects, to seek selenium compounds best suited for dietary supplementation with the goal of preventing selenium deficiency diseases, and to increase our knowledge about suitable methods to prevent or decrease selenium toxicoses in plants, animals and men, the determination of 'total selenium' provides insufficient information and must be followed by the identification of the selenium compounds.

This paper summarizes and critically evaluates the techniques for the identification and quantification of inorganic and organic selenium compounds based on high-performance liquid chromatography.

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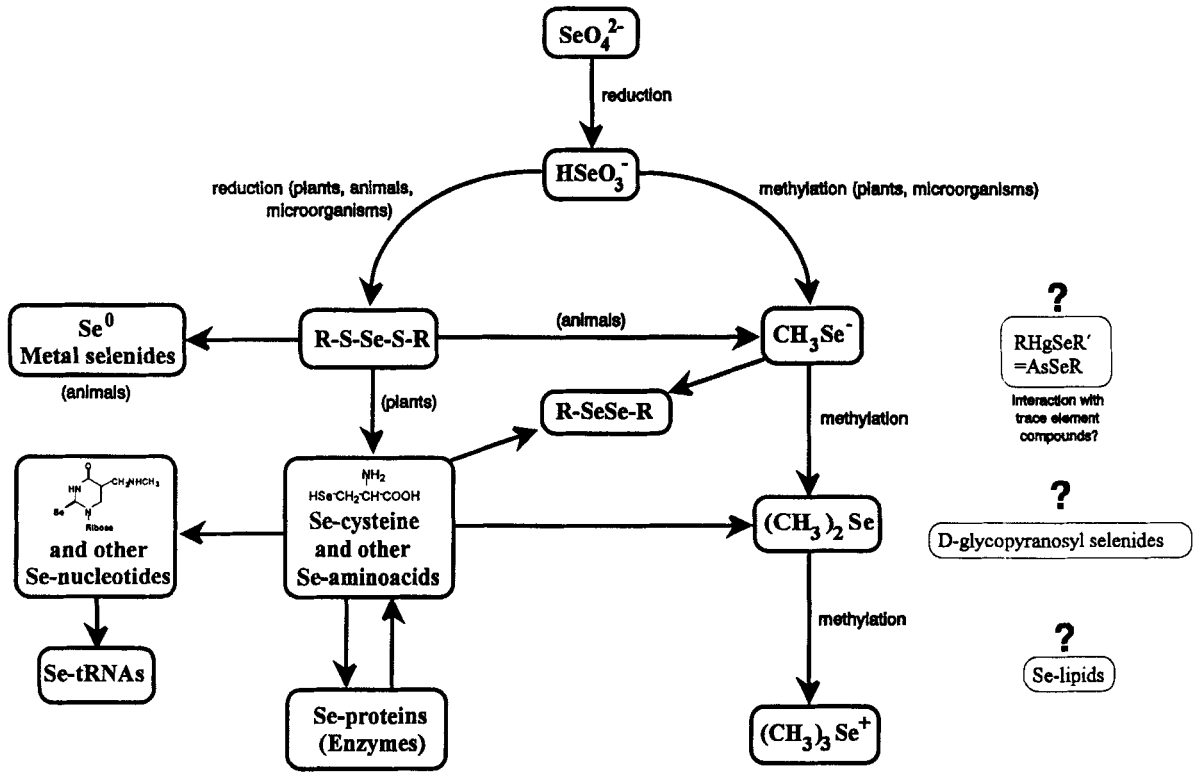


Figure 1 Selenium cycle in nature.

DETERMINATION OF TOTAL SELENIUM

The determination of total selenium—generally in a digest obtained by mineralization of a biological or environmental sample—is the first step in the sequence of analytical procedures leading to the identification and quantification of selenium compounds. Concentrations of total selenium are needed to judge whether or not the identification of selenium compounds is feasible, and to establish mass balances for selenium.

The analytical chemistry of selenium in regard to total selenium determinations has been reviewed repeatedly. In 1981, Bem summarized the methods for the determination of selenium in biological and environmental materials.[11] Raptis *et al.* published in 1983 an overview of the most common analytical techniques for the determination of selenium and pointed out their limitations.[12] In 1984, Dilli and Sutikno compared and critically evaluated the methods for the determination of selenium, based on gas chromatography.[13] A review published by Olson *et al.* includes historical information on the methods recommended for the determination of this element.[14] Ihnat, in 1992, reviewed the methods for the determination of selenium with emphasis on sampling and sample treatment.[15] Several shorter reviews—often covering particular aspects of the analytical chemistry of selenium—are available in the literature: general methods,[16] spectrophotometry,[17] atomic absorption spectrometry,[18] hydride generation–atomic absorption spectrometry for raw and potable waters,[19] electroanalytical techniques,[20] biological samples,[21,22] environmental samples,[22] geological samples,[23] foodstuff,[24] water,[25] urine,[26,27] and blood.[27]

IDENTIFICATION AND QUANTIFICATION OF SELENIUM COMPOUNDS

The determination of volatile selenium compounds (hydrogen selenide, methaneselenol, dimethyl selenide, dimethyl diselenide) by gas chromatography was reviewed.[28,29] Paper chromatography, thin-layer chromatography and

liquid chromatography were used to separate selenium compounds such as selenoamino-acids and related selenium derivatives.[28] Since Wheeler and Lott in 1974 had first suggested the use of high-performance liquid chromatography for the identification and quantification of selenium compounds,[30] many papers have appeared describing such applications. Cappon summarized the application of HPLC to the determination of selenite, selenate, trimethylselenonium salts, and selenoamino-acids.[31] Shibata *et al.* in 1992 reviewed some HPLC methods, which were used for the identification of trimethylselenonium salts in human urine, of different chemical forms of selenium in human plasma and red blood cells and for the characterization of a selenium–mercury-containing complex isolated from dolphin liver.[32]

Selenite and selenate

Selenite and selenate are important species in the biogeochemical cycle of selenium. Because of the difference in oxidation state of selenium in selenite and selenate, these two species possess different chemical and biological properties. Liquid chromatography (ion chromatography) is frequently used for the separation of selenite and selenate. 'Selenite' and 'selenate' are used as generic terms and do not provide information about the degree of protonation of these species. The degree of dissociation of selenous acid ($pK_1 = 2.46$, $pK_2 = 7.31$) and selenic acid ($pK_2 = 1.92$) will be governed by the pH and composition of the medium.

Ion chromatography

Ion chromatography (based on exchange of anions in solution for anions on the stationary phase) was found to separate selenite from selenate with aqueous (basic) carbonate solutions as mobile phase in the suppressed-ion (SIC) mode or with an acidic or neutral mobile phase in the single-column mode (SCIC) (Tables 1 and 2).

Suppressed-ion chromatography (SIC). SIC is the classic version of ion chromatography. Anions are separated on a separator column with a low-capacity anion-exchange resin as the stationary phase. For the separation of cations a low-capacity cation exchanger is needed. Dilute solutions of a base (sodium carbonate/sodium bicarbonate or sodium hydroxide) are used as eluents. The eluent is passed through a suppressor column into the conductivity detector. The suppressor is necessary to maximize the difference between signals from the analyte ions and background signals from other ions in the mobile phase. Whereas older suppressor columns had to be regenerated periodically, modern instruments are equipped with continuously regenerating membrane suppressors. Polymer-based materials with functional groups on the surface (e.g. styrene–divinylbenzene copolymers functionalized with quaternary ammonium groups for the separation of anions) are preferred to silica-based sorbents as stationary phases in the separator column. The pH range over which the stationary phase is stable, is wider for polymer-based than for silica-based materials.

Several determinations of selenite and selenate in various matrices using suppressed-ion chromatography were reported[33–51] (Table 1). These methods have absolute detection limits in the low nanogram range and are, therefore, applicable to environmental analysis.[36,37] For example, Bar-Yosef used SIC to quantify selenite in clay fractions of different origin.[39,40] Zolotov and coworkers successfully separated and determined selenate, arsenate, molybdate and chromate.[41–43] Sarzanini *et al.* described the separation and determination of selenite, selenate and EDTA complexes of several metal ions.[36] Other publications report the determination of selenite and selenate in water,[47,48,52] soil solutions,[37,50] spent oil-shale leachates,[34] and copper electrolytes[44] or the determination of selenium as selenate in water[36] and drugs[38,49] after treatment of the samples with oxidizing agents, such as hydrogen peroxide and potassium permanganate.

Interferences in SIC determinations may arise from the presence of several inorganic anions with retention times similar to the selenium compounds. Selenite signals may be affected by chloride,[38,44,50,52] fluoride,[44] bromide,[52] carbonate,[38] phosphate[35,37,38,50,52] and nitrate.[35,37,48] With selenate, a strong interference was observed from sulfate.[35,37,44,48,52] Attempts to overcome these difficulties were made. Niss and Powers tried to determine selenite and selenate in the presence of excess nitrate, sulfate and phosphate.[34] The pH of the carbonate eluent was raised to 12 to eliminate the interference from phosphate. At this elevated pH, phosphate is present as PO_4^{3-}. This negative ion elutes after selenite and selenate. At this high pH, interferences from phosphate and also sulfate were successfully eliminated; however, the retention times

and peak areas for selenite and selenate suffered from poor repeatability. This problem was not reported when a 2 mM Na_2CO_3/1 mM NaOH mobile phase was used.[35] Interference problems from nitrate and sulfate were eliminated with a Dionex AG5 guard column, which separated the early-eluting chloride, nitrite, bromide and nitrate from selenite, and sulfate from selenate. The method was applied for the determination of selenite and selenate in extracts from coal fly-ash.

Hoover and Yager separated selenite and selenate from major anions such as chloride, nitrate and sulfate in environmental water samples by collecting the fractions containing selenite or selenate and re-chromatographing the fractions under the same conditions (multidimensional ion chromatography).[48] Murayama *et al.* used this shave-recycle technique for the ion chromatographic determination of selenium as selenate in sulfur-containing drugs.[49]

Single-column ion chromatography (SCIC). S-CIC with conductivity detection uses low-capacity anion-exchange columns and eluents with low

Table 1 Suppressed-ion chromatography (SIC) for the determination of selenite and selanate[a]

Analyte	Mobile Phase	Detection	Absolute detection limit (ng Se)	Sample	Ref.
Se(IV, VI)	2.4 mM Na_2CO_3/ 3 mM $NaHCO_3$	Conductivity	5,6	Water	33
Se(VI)	1 mM Na_2CO_3/ 10 mM $NaHCO_3$	Conductivity	NR	Water	33
Se(IV, VI)	1.0 mM Na_2CO_3 (pH 12)	Conductivity	NR	Spent oil-shale leachate	34
Se(IV, VI)	2.0 mM Na_2CO_3/ 1.0 mM NaOH	Conductivity	1.6, 1.4	Extracts of coal fly-ash	35
Se(VI)	2.5 mM Na_2CO_3/ 1.0 mM NaOH	Conductivity	0.96	Spiked river water	36
Se(IV, VI)	2 mM Na_2CO_3/ 1.7 mM $NaHCO_3$	Conductivity	1.3, 1.5	Spiked soil solutions	37
Se(VI)	4 mM Na_2CO_3/ 4 mM $NaHCO_3$	Conductivity	0.16	Bulk prednisolone (pharmaceutical)	38
Se(VI)	3 mM Na_2CO_3/ 5 mM KOH	Conductivity	1	River and drinking water	41
Se(IV, VI)	0.5 mM Na_2CO_3/ 1.5 mM $HaHCO_3$	Conductivity	2, 2	Water	45
Se(IV)	2.0 mM Na_2CO_3/ 3.0 mM $NaHCO_3$[b]	Conductivity	NR	Drinking, well and river water	48
Se(VI)	2.4 mM Na_2CO_3/ 3.0 mM $NaHCO_3$[b]	Conductivity	NR	Drinking, well and river water	48
Se(VI)	4 mM Na_2CO_3/ 4 mM $NaHCO_3$[b]	Conductivity	0.16	Drugs	49
Se(IV, VI)	4.5 mM Na_2CO_3/ 6.0 mM NaOH	Conductivity	1.2, 1.6	Soil solutions	50
Se(IV, VI)	2.2 mM Na_2CO_3/ 1.0 mM $NaHCO_3$	Conductivity	NR	Industrial sulfur	51
Se(IV, VI)	2.4 mM Na_2CO_3/ 3 mM $NaHCO_3$	UV 195	30, 800	[c]	61
Se(IV, VI)	2.25 mM Na_2CO_3/ 0.75 mM $NaHCO_3$	UV 195	6.2, 14	Water	63
Se(IV, VI)	2.4 mM Na_2CO_3/ 3.0 mM $NaHCO_3$	Conductivity–DCP AE	NR	[c]	64
Se(IV, VI)	8 mM Na_2CO_3	GF AA	20, 20	Synthetic river water	52

[a] Abbreviations: NR, not reported; UV, UV–VIS spectrometer, wavelength in nm; Se(IV), selenite; Se(VI), selenate. [b] Multidimensional chromatography. [c] The samples for these measurements are solutions of pure selenium compounds in distilled water.

Table 2 Single-column ion chromatography (SCIC) for the determination of selenite and selenate[a]

Analyte	Mobile phase	Detection	Absolute detection limit (ng Se)	Sample	Ref.
Se(IV)	1.5 mM phthalic acid (pH 2.7)	Conductivity	7.5	Soil extracts	53
Se(VI)	4 mM phthalic acid (pH 4.6)	Conductivity	32	Soil extracts	54
Se(IV, VI)	4 mM *p*-hydroxybenzoic acid (pH 8.0)	Conductivity	210, 105	Soil extracts	55
Se(IV, VI)	75 mM ammonium phosphate (pH 7.2)[b]	F (ex 380 em 520)	0.1, 0.1	Tap water	56
TMSe, Se(IV, VI)	80 mM ammonium citrate (pH 3.3)[c]	ICP AE	14, NR, 54	[d]	57
Se(IV, VI)	15 mM phosphate (pH 4.8)	UV 190	NR	[d]	62
Se(IV, VI)	3 mM KH phthalate (pH 7.4)	GF AA	23, 16	Supplementary solution	69
Se(IV, VI)	5 mM $(NH_4)OAc$/ 2 mM $(NH_4)H_2PO_4$ (pH 4.6)– 80 mM $(NH_4)H_2PO_4$ (pH 6.9)	ICP AE	140, 91	[d]	67
Se(IV, VI)	6 mM $(NH_4)_2SO_4$/ 0.01 mM $HClO_4$ (pH 9)	ICP MS	NR	Soil extracts	70

[a] Abbreviations: NR, Not reported; UV, UV–VIS spectrometer, wavelength in nm; F, fluorimetric detector, excitation and emission wavelengths in nm; Se(IV), selenite, Se(VI), selenate. [b] On-line reduction and post-column derivatization with 2,3-diaminonaphthalene. [c] Thermospray vaporizer. [d] The samples for these experiments are solutions of pure selenium compounds in distilled water.

conductivity that make a suppressor column unnecessary.

Karlson and Frankenberger were the first to describe an SCIC method for the determination of selenite[53] and selenate[54] in soil extracts. Aqueous solutions of phthalic acid with concentrations of 1.5 and 4 mM were used as mobile phases. Selenite and selenate were determined simultaneously with an aqueous solution of *p*-hydroxybenzoic acid (4 mM) as eluent.[55] Solutions of ammonium phosphate (75 mM)[56] and of ammonium citrate (80 mM)[57] were also reported to separate selenite and selenate. Liu *et al.* suggested the use of NaCl, NaBr or sodium tartrate solutions as eluents to separate selenite from tellurite in copper-anode mud or industrial wastewater.[58]

Besides conductivity detectors, other types of detectors were used to record SIC or SCIC chromatograms. Shibata *et al.* exploited the fluorimetric excitation of the piazselenole (from selenite and 2,3-diaminonaphthalene) generated by post-column derivatization for the detection of selenite.[56] Because selenate does not form a piazselenole, selenate must be reduced to selenite after the chromatographic separation. Amounts as low as 0.1 ng of selenium injected onto the column produce signals that can be quantified. Neutron activation generates radioactive nuclides (^{75}Se, ^{79m}Se, ^{81m}Se, ^{81g}Se, ^{83g}Se) that can be detected by radiometric methods after chromatographic separation of the selenium compounds.[59,60]

Several pre-treatments for samples were proposed. The interference from chloride in the determination of selenite was removed by passing the solution through a silver-ion-loaded cation-exchange resin;[53] interferences from sulfate were eliminated with a barium-ion-loaded column.[36,54] Because sulfate, in contrast to selenite and selenate, does not absorb above 190 nm, the selenium compounds were detected without sulfate inter-

ference by means of UV-detector set at 195 nm.[61] The detection limit for selenate in this system was rather poor (30 ng Se as selenite, 800 ng Se as selenate). Operating a UV-detector in this optical range requires a sufficiently transparent eluent. This condition can be met by using either single-column ion chromatography with a UV-inactive mobile phase[62] or by reducing the UV-absorbance of the effluent with a suppressed-ion chromatographic system.[63]

Element-specific detectors for ion chromatography. Interferences from other anions can largely be avoided with selenium-specific detectors that do not respond to other elements. The following instruments were used for the specific detection of selenium in column effluents: graphite furnace–atomic absorption (GF AA) spectrometer, direct-current plasma atomic emission (DCP AE) spectrometer, inductively coupled plasma atomic emission (ICP AE) spectrometer, and inductively coupled plasma mass spectrometer (ICP MS).

Urasa and Ferede detected selenite and selenate after separation on a suppressed-ion-chromatographic column with DCP AE and also with a conductivity detector.[64] The calibration curves for selenite and selenate were identical (same slope) with the DCP AE detector but different with the conductivity detector.

Fraley *et al.* showed that ICP AE can be used to detect selenium in liquid chromatography.[65] ICP AE detection occurs on-line and produces a continuous or almost continuous signal resulting in enhanced reproducibility of the measurements and low detection limits.[66]

McCarthy *et al.* employed a sequential, slew-scanning ICP AE spectrometer to detect selenite and selenate (also arsenite and arsenate after wavelength adjustment) in the column effluent after separation by anion exchange.[67] Because of the sequential operation of the ICP spectrometer, arsenic and selenium compounds could not be detected simultaneously and had to be separated. The detection limits for this sequential ICP AES HPLC system were too high to be applicable to biological samples.

Laborda *et al.* separated trimethylselenonium salts, selenite and selenate by anion-exchange chromatography, and quantified the selenium compounds with a sequential Perkin–Elmer Plasma-II ICP AE spectrometer.[57] With a thermospray vaporizer, the 3σ-detection limits were 14 ng Se for trimethylselenonium compounds and 54 ng Se for selenate. With a cross-flow nebulizer the detection limits were three times higher.

Irgolic and coworkers used a Hitachi–Zeeman GF AA spectrometer as detector for suppressed-ion chromatographic separations of selenite and selenate.[52,68] Concentrations of sulfate, phosphate, fluoride, chloride, bromide, nitrite or nitrate at least 1000 times higher than the concentrations of the selenium compounds did not reduce the GF AA signal by more than 5%. Although GF AA has excellent detection limits for selenium (10 pg to a few nanograms of Se), the dilution of the analytes during the chromatographic procedure and the limited number of absorption signals per chromatographic band increase the detection limit and may cause difficulties in quantifying the results. A computerized system for the collection and treatment of data produced by GF AA coupled to HPLC improves the quantification of the analytes.[69]

The ICP MS is also useful as a detector for the ion-chromatographic determination of selenite and selenate in mixtures with arsenite and arsenate.[70]

Reversed-phase chromatography

Reversed-phase chromatography (RPC) using hydrophobic stationary phases has found wide applications, because the stationary phases are stable from pH 1 to 13, the mobile phases can be varied almost at will, the separations show good reproducibility, and peaks usually do not 'tail'. For the reversed-phase chromatographic separation of ionic species, lipophilic ion-pairs must be formed. To achieve ion-pair formation, salts with lipophilic counterions are added to the mobile phase. The lipophilic ion-pairs partition between mobile and stationary phases.

Ion-pairing chromatography. Selenite and selenate were separated by ion-pairing chromatography employing selenium-specific detectors, radiometric detectors and electrochemical detectors. Table 3 summarizes the applications of ion-pairing chromatography to the determination of selenite and selenate.

Atomic emission or absorption spectrometers as detectors. Chakraborti and Irgolic used hexadecyltrimethylammonium bromide as counterion in the aqueous mobile phase to separate selenite and selenate as ion-pairs on a C_{18}

reversed-phase column.[71] After selenite had been eluted, methanol was used as mobile phase to elute selenate. A detection limit of 25 ng Se was achieved with a Zeeman GF AA detector. The same ion-pairing reagent, but in higher concentration, was used to retain selenite in the presence of arsenic compounds and phosphate on the column. Selenite was co-eluted with arsenate and phosphate by 1% aqueous acetic acid and detected with an ARL 34000 simultaneous ICP AE spectrometer as multi-element-specific detector.[66]

La Freniere *et al.* separated selenite and selenate by ion-pairing chromatography with tetrabutylammonium phosphate as ion-pairing reagent.[72] The ICP AE detector was operated with a micro-concentric nebulizer that transported all of the chromatographic effluent into the plasma and thus achieved detection limits (8 ng Se for selenite, 14 ng Se for selenate) much superior to those obtainable with a conventional nebulizer (nebulization efficiency approx. 5%).

An HPLC ICP AE system with ultrasonic nebulization and desolvation of the aerosol achieved 3σ-detection limits of 2–3 ng Se for selenite and selenate.[73] The separation was performed

Table 3 Reversed-phase ion-pairing HPLC determination of selenite and selenate[a]

Analyte	Phase		Detection	Absolute detection limit (ng Se)	Sample	Ref.
	Stationary	Mobile				
Se(IV, VI)	C_{18}	1 mM HTAB (pH 7)–CH_3OH	GF AA	25, 25	Spiked synthetic river water	71
Se(IV)	PB	2 mM HTAB (pH 9.6)–H_2O/CH_3COOH (99:1)	ICP AE	NR	[f]	66
Se(IV, VI)	C_{18}	5 mM TBAP in H_2O/CH_3OH (90:10)[b]	ICP AE	8, 14	[f]	72
Se(IV, VI)	C_{18}	5 mM TBAP in H_2O/CH_3OH (90:10)[c]	ICP AE	2–3, 2–3	[f]	73
Se(IV)	C_{18}	5 mM TBAP in H_2O/CH_3OH (90:10)[d]	ACP AE	4	Spiked river water	74
Se(IV)	C_{18}	Gradient of isopropanol with 5 mM SDS in phosphate buffer	Radiometry (^{75}Se)	0.001	Urine and plasma of rats	79
Se(IV)	TBP P-350	NaBr, NaCl, HCl, HBr at diff. concns.	Coulometry	NR	Anode mud, wastewater	80
Se(IV, VI)	C_{18}	2.5 mM TBAHS in 10 mM K_2HPO_4/ 10 mM KH_2PO_4 (pH 6.55)	UV 205	0.5, 5	Spiked water, animal feed premixes	76
Se(IV, VI)	C_{18}	2.5 mM TBAHS in 10 mM K_2HPO_4/ 10 mM KH_2PO_4 (pH 6.55)[e]	DCP AE	50, 50	Spiked water, animal feed premixes	76
Se(IV, VI)	C_{18}	5 mM TBAP in 5% CH_3OH	ICP MS	0.1, 0.1	Simulated practical samples	77

[a] Abbreviations: NR, Not reported; PB, polymer-based material; UV, UV–VIS spectrometer, wavelength in nm; HTAB, hexadecyltrimethylammonium bromide; TBAP, tetrabutylammonium phosphate; SDS, sodium dodecyl sulfate; TBAHS, tetrabutylammonium hydrogensulfate; TBP, tributyl phosphate; P 350, methyl bis(iso-octyl)phosphate; Se(IV), selenite; Se(VI), selenate. [b] Direct injection nebulizer. [c] Ultrasonic nebulizer. [d] Post-column hydride formation. [e] Direct introduction of eluent. [f] The samples for these experiments are solutions of pure selenium compounds in distilled water.

with an aqueous solution of 5 mM tetrabutylammonium phosphate containing 10% methanol as organic modifier on a C_{18} reversed-phase column. Under similar chromatographic conditions selenite was detected in a sample of river water spiked with selenite.[74] The selenite in the effluent was reduced by sodium borohydride in a post-column hydride generation system. The hydrogen selenide was swept by a stream of helium into the alternating-current helium-plasma. The selenium emission at 203.99 nm was monitored. Although a detection limit of 4 ng Se was achieved, the chromatograms are plagued by unstable baselines. An ion-chromatographic separation of selenite and selenate followed by reduction to hydrogen selenide with sodium borohydride and detection by atomic absorption spectrometry was patented.[75]

Childress *et al.* separated selenite and selenate on a reversed-phase C_{18} column using tetrabutylammonium hydrogen sulfate as ion-pairing reagent in a 10 mM phosphate buffer (pH 6.55).[76] A UV detector operated at 205 nm monitored the eluent during the optimization of the chromatographic conditions. For the determination of selenite and selenate in aqueous extracts from animal-feed premixes, the UV detector could not be used because of interfering components in the extract. A direct-current plasma (DCP) atomic emission detector with direct introduction of the eluent into the spray chamber allowed the determination of selenite and selenate without interference in these extracts. An absolute detection limit of 50 ng Se was achieved with this HPLC DCP system.

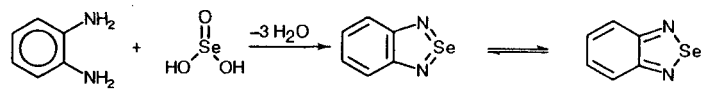
Plasma emission mass spectrometers as detectors. Thompson and Houk separated selenite from selenate with an aqueous solution of 5 mM tetrabutylammonium phosphate containing 5% methanol on a reversed-phase C_{18} column.[77] Monitoring the ^{78}Se isotope with an ICP MS allowed the selective detection of these selenium species in solutions which in addition contained several arsenic compounds. An absolute detection limit of 0.1 ng Se for selenite and selenate is reported. The method was found to be applicable for the identification of selenite and selenate in synthetic aqueous samples that contained a great excess of calcium or sodium to simulate practical samples.

Radiometric detectors. Gruebel *et al.* employed a radiometric detector combined with HPLC to study the adsorption of selenite and selenate on different minerals.[78] Solutions of selenite and selenate labelled with radioactive ^{75}Se were added to and equilibrated with suspensions of minerals in water containing various electrolytes. The concentrations of selenite and selenate in the resulting mixtures were measured by separating the selenium compounds on reversed-phase columns (ion-pairs with tetrabutylammonium ions), collecting fractions manually, and analyzing the fractions for ^{75}Se activity by crystal scintillation counting.

Baldew *et al.* used sodium dodecyl sulfate as ion-pairing reagent to explore the chromatographic behavior of ^{75}Se-labelled selenite and of the products obtained by incubation of [^{75}Se]selenite with reduced glutathione, cysteine and *N*-acetylcysteine.[79] The products might be bis(organothio) selenides, RS—Se—SR. The method was applied to check for selenium compounds in the urine and plasma of rats intravenously dosed with [^{75}Se]selenite. With an on-line Ge(Li) radiometric scintillation detector, an absolute detection limit of 1 pg Se was achieved.

Electrochemical detector. Silica gel impregnated with methyl bis(iso-octyl) phosphate or a tributyl phosphate extracting-resin was used to separate selenite from tellurite with halide-containing (HCl, HBr, NaCl, NaBr) aqueous mobile phases. Tellurite forming negatively charged pentahalo species (TeX_5^-) is retained through interaction with the phosphate esters, whereas selenite not forming halide complexes under these conditions moves with the solvent front. Selenite and tellurite were detected by coulometry. This method was applied to the determination of selenite and tellurite in wastewater and anode muds.[80]

Chromatography of compounds prepared from selenite. Selenite reacts with 1,2-diaminoarenes to produce fluorescent areno-2,1,3-selenadiazoles, with dithiocarbamates to yield UV-absorbing selenium bis(dithiocarbamates), with xanthates to form selenium dixanthates, and with thiols to render selenium dithiolates. The selenium compounds can be separated from excess reagents and products of other anions or cations with these reagents by high-performance liquid chromatography. The selenium compounds are detected with fluorescence spectrometers,



[1]

UV-absorption spectrometers or amperometric devices. Selenate does not react with these reagents. To become determinable by these methods, selenate must first be reduced to selenite. Other selenium compounds such as organic derivatives must be converted to selenite by appropriate methods. Mineralization procedures required for the conversion of organic selenium compounds to selenate and then to selenite destroy information about the chemical nature of the selenium compounds originally present in a sample. For this reason these methods can be used only for the identification of selenite and through controlled reduction of selenate.

Areno-2,1,3-selenadiazoles (piazselenoles). The reaction of selenite with aromatic 1,2-diamines to areno-2,1,3-selenadiazoles has found extensive application for the determination of selenium as selenite. The selenium heterocycles were quantified with UV–visible absorption or fluorescence spectroscopy.[81] As an example, the reaction of selenite with 1,2-diaminobenzene is shown (Eqn [1]).

2,3-Diaminonaphthalene is a powerful reagent for the fluorimetric determination of selenium because of the strong fluorescence of the corresponding piazselenole.[82]

Wheeler and Lott were the first to use the piazselenole derived from 2,3-diaminonaphthalene for the determination of selenium by HPLC.[30] Selenite-containing aqueous solutions of pH 1 were reacted with 2,3-diaminonaphthalene. Aliquots of the aqueous reaction mixture were chromatographed on a reversed-phase C_{18} column with ethanol/water (5:8) as the mobile phase to separate the piazselenole from the corresponding triazole (formed from nitrite), excess reagent and light-absorbing impurities. Because the piazselenole does not fluoresce in aqueous solutions, a UV-absorption detector was used, with which an absolute detection limit (estimated from the calibration curve) of 4 ng Se was achieved. This limit corresponds to a concentration of 0.5 mg Se dm^{-3} in the sample solution.

The high polarity of the ethanol/water solvent needed to elute the piazselenole formed from the reversed-phase column quenched the fluorescence completely.[83] Wheeler and Lott suggested that the piazselenole should be extracted into an organic solvent such as chloroform or 1,2-dichlorethane, and the extract chromatographed. The chromatographic separation of aliquots of the extract on a normal-phase column with chloroform as the mobile phase allowed the fluorimetric detection of the piazselenole with an absolute detection limit, calculated from the calibration curve, of 10 ng Se. This absolute detection limit corresponds under the experimental conditions used to a concentration of 10 μg Se dm^{-3} in the sample solution. This low 'concentration' detection limit (1/50th of the detection limit of 0.5 mg dm^{-3} for the direct injection and UV detection) was achieved through the preconcentration by extraction.

Shibata *et al.* also used an extractive procedure.[84,85] Selenite was reacted with 2,3-diaminonaphthalene in aqueous solution of pH 1.2, the reaction product was extracted into cyclohexane, and the extract chromatographed on a μ-Bondapak C_{18} reversed-phase column with acetonitrile as the mobile phase. With an optimized fluorescence detector (378 nm excitation, 557 nm emission) the absolute detection limit was lowered to 0.13 pg Se (6.5 ng Se dm^{-3}; one of the lowest detection limits thus far reported for selenium), even though acetonitrile decreased the fluorescence of the piazeselenole to 26%. The μ-Bondapak C_{18} column (10 μm particles) caused a high fluorescence in the blank. A Unisil 5C18 column (5 μm particles) gave a lower blank and better resolution than the Bondapak column. This method with the Unisil column was used to determine selenium in tissue samples, whole blood and plasma from lambs after administration of sodium selenite[86,87] and in effluent fractions of a chromatographic system used to separate different chemical forms of selenium in human plasma and erythrocyte lysate.[32]

Yamada *et al.* extracted the piazselenole from 2,3-diaminonaphthalene with cyclohexane, chromatographed the extract on a Cosmosil 5SL silicagel column, and eluted the piazselenole with cyclohexane/ethyl acetate (95:5).[89] With a μ-Porasil column (10 μm particles), cyclohexane/ethyl acetate (90:10) was used as the mobile phase.[83,90] These methods were used to determine selenium in standard soil samples (SO-1,

SO-2—Canadian Certified Reference Materials),[89] in Japanese soil reference materials,[91] in Japanese soils,[92–96] in soil extracts,[92–96] in human serum, human seminal plasma and human semen,[83,90] and in selenium-enriched yeast,[97] and to detect volatile selenium compounds from soils,[98,99] after appropriate digestion. Because oxidative digestions convert all selenium compounds into selenate, and only selenite reacts with aromatic 1,2-diamines to piazselenoles, the selenate must be reduced to selenite. This reduction was accomplished with hydrochloric acid alone[83] or in the presence of potassium bromide and copper ions.[100]

When 2,3-diaminonaphthalene that was not completely pure (95–98%) was used to produce piazselenoles, fluorescence peaks in addition to the signals from the piazselenole appeared in the chromatogram.[83] Commercially available pure (99%) 2,3-diaminonaphthalene did not produce these additional signals. With the pure reagent the chromatographic separation of the piazselenole from the impurities became unnecessary, and the extracts (cyclohexane) could be injected into an HPLC system without a column for the fluorimetric detection of the piazselenole.[101] This column-free method (absolute detection limit 60 pg Se) was used for the determination of selenium in protein fractions and biological samples.[101]

Selenite and selenate can be differentiated with the piazselenole methods. Because selenate is not converted to piazselenole, selenite can be determined in a mixture of selenite and selenate. After reduction of selenate to selenite (usually with hydrochloric acid), total inorganic selenium can be determined. The selenate concentration is obtained as the difference between the two selenium concentrations.[100] This method was used to determine selenite, selenate and organically bound selenium in extracts from soils[102] and environmental samples,[103] and to determine total selenium (after digestion and reduction) and selenite in blood and brain of rats given different selenium compounds.[104]

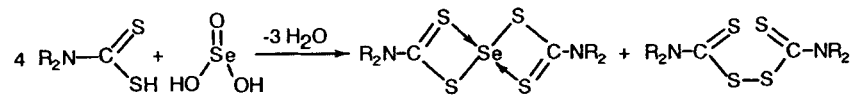
Handelman and co-workers added the fluorophore tetraphenylnaphthacene as an internal standard to the piazselenole-containing cyclohexane extract to avoid the need for rigorous control of volume during analysis (except pipetting of sample and internal standard).[107] The piazselenole and the fluorophore were separated on a reversed-phase C_{18} column with methanol as eluent. In this manner selenium was determined in whole blood and red blood cells with a detection limit of 0.15 ng Se.

Schwedt investigated other diamino derivatives, such as 1,2-diaminobenzene, 4-chloro-1,2-diaminobenzene and 4-nitro-1,2-diaminobenzene, as reagents to produce piazselenoles for UV detection and fluorescence measurements.[108] The piazselenoles were successfully separated from the reagents on reversed-phase columns with methanol or acetonitrile as mobile phases. The piazselenole from 2,3-diaminonaphthalene was chromatographed on a column with dimethylamino functional groups using chloroform as eluent to avoid quenching of the fluorescence. Selenium determinations with piazselenoles had detection limits of 1.6 ng Se (from 4-chloro-1,2-diaminobenzene) and 3.4 ng Se (from 1,2-diaminobenzene) with UV detection at 320 nm.[109] Khuhawar *et al.* examined the reagents 1,2-diaminobenzene, 4-nitro-1,2-diaminobenzene, 2,3-diaminonaphthalene, and 3,3′-diaminobenzidine for the determination of selenium using HPLC with UV detection.[110] The derivatization procedure with 4-nitro-1,2-diaminobenzene was used for the determination of selenium in shampoo and coal samples.

Piazselenoles are electroactive, can be reduced on dropping-mercury and glassy-carbon electrodes,[111] and thus can be identified in the effluent from a chromatographic column with an amperometric detector. Selenite was reacted with 4-nitro-1,2-diaminobenzene at pH 1. The piazselenole was extracted into hexane. The extract was chromatographed on a C_{18} column with methanol/citrate buffer, pH 4/THF as mobile phase. The amperometric detector with a glassy carbon electrode was set at −0.45 V vs. Ag/AgCl. This method (absolute detection limit 40 pg Se) was used to determine selenium in reference sera after digestion with nitric/sulfuric/perchloric acid.[112]

The determinations of selenite with HPLC using piazselenole formation are summarized in Table 4.

Selenium bis(dithiocarbamates). Dithiocarbamates behave as univalent, bidentate, anionic ligands, which in aqueous medium form colored precipitates with many elements.[113,114] Sodium dithiocarbamates combine also with selenite (selenous acid, selenium dioxide) in aqueous, acetate-buffered medium of pH 4 and produce in a redox reaction selenium bis(dithiocarbamates) (Eqn [2]).



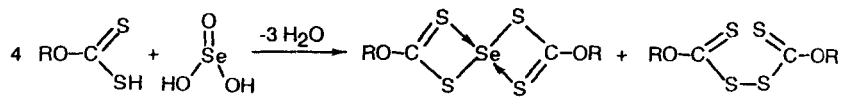
[2]

These selenium derivatives can be easily extracted into organic solvents such as chloroform and carbon tetrachloride. The extracts can be chromatographed on reversed-phase columns. Attempts to avoid the extraction step led to the deposition of red selenium.[115] The selenium diethyldithiocarbamate was separated in this manner from the nickel, lead and copper deriva-

Table 4 Reversed-phase HPLC of selenite as piazselenole[a]

Phase				Absolute detection limit		
Stationary	Mobile	Reagent	Detection	(ng Se)	Sample	Ref.
C_{18}	$EtOH/H_2O$ (5:8)	2,3-DAN	UV 254	~4	Industrial discharge solutions	30
NP	$CHCl_3$	2,3-DAN	F(ex?, em?)	~10	Industrial discharge solutions	30
C_{18}	CH_3CN	2,3-DAN	F(ex378, em557)	0.00013	[c]	84
C_{18}	CH_3CN	2,3-DAN	F(ex374, em555)	0.1	NR	88
NP	Cyclohexane/ EtOAc(95:5)	2,3-DAN	F(ex380, em525)	NR	Soil reference materials	89
NP	Cyclohexane/ EtOAc (90:10)	2,3-DAN	F(ex360, em500)	0.05	Human serum, seminal plasma, semen	83, 90
None	Cyclohexane	2,3-DAN	F(ex376, em520)	0.06	Serum, seminal plasma, semen, protein fractions	101
C_{18}	Cyclohexane/ THF (90:10)	2,3-DAN	F(ex375, em520)	NR	Atmospheric particulates	105
C_{18}	Cyclohexane/ THF (80:20)	2,3-DAN	F(ex380, em530)	NR	Atmospheric particulates, rainwater, water samples	106
NP	Cyclohexane/ THF (90:10)	2,3-DAN	F(ex376, em520)	NR	Drinking water, soil extracts	102
NP	?	2,3-DAN	F(ex?, em?)	0.0005	Environmental	103
C_{18}	CH_3OH[b]	2,3-DAN	F(ex480, em580)	0.15	Human whole blood	107
C_8, C_{18}	CH_3OH/H_2O (80:20)	1,2-DAB	UV 340, 320	3.4	Drinking, surface and waste-water	108, 109
C_8, C_{18}	CH_3OH/H_2O (80:20)	4-Cl-DAB	UV 313, 320, 340	1.6	Drinking, surface and waste-water	108, 109
C_{18}	CH_3OH/H_2O (80:20)	4-NO_2-DAB	UV 340	NR	[c]	108
C_{18}	CH_3OH/H_2O(70:30)	2,3-DAN	UV 254	NR	[c]	108
$-N(CH_3)_2$	$CHCl_3$	2,3-DAN	UV 254 F(ex350–400, em500)	NR	[c]	108
NP	Hexane/ $CHCl_3$ (90:10)	DAB	UV 332	0.13	[c]	110
		4-NO_2-DAB	UV 343	0.13	Shampoo, coal	110
		2,3-DAN	UV 262	0.05	[c]	110
NP	Hexane/ $CHCl_3$ (60:40)	DABZ	UV 340	NR	[c]	110
C_{18}	Citrate phosphate buffer (pH 4)/THF/ CH_3OH(68:2:30)	4-NO_2-DAB	Amperometry	0.04	Reference serum	112

[a] Abbreviations: NR, not reported; NP, Normal phase; UV, UV–VIS spectrometer, wavelength in nm; F, fluorimetric detector, excitation and emission wavelength in nm; 2,3-DAN, 2,3-diaminonaphthalene; DAB, 1,2-diaminobenzene; DABZ, 3,3'-diaminobenzidine. [b] Internal standard. [c] The samples for these experiments are solutions of pure selenite in distilled water.



R = C_2H_5, C_4H_9 [3]

tives (acetonitrile/water, 65:35)[108] and from the chromium, nickel, cobalt, lead, copper and mercury derivatives (methanol/water, 7:3).[116] A UV spectrometer set at 254 nm served as detector. Light absorption by substances derived from the reagent at the detection wavelength raised the detection limit to 25 ng Se. Chromatograms may show two signals assignable to selenium compounds [selenium bis(dithiocarbamate), selenium tetrakis(dithiocarbamate)?].[109] This method was used to determine selenium (present as selenite) in a synthetic electroplating solution containing sulfosalicylic acid, sulfanilamide, cobalt, chromium, copper, mercury, nickel, lead and tellurite.[117]

The extracts containing selenium diethyldithiocarbamates and the diethyldithiocarbamates of copper, nickel, cobalt, lead, mercury, cadmium and chromium were preconcentrated on a short silica-based C_{18} column. The dithiocarbamates were washed onto the analytical column with dichloromethane, separated with acetonitrile/methanol as mobile phase, and detected spectrophotometrically (254 nm) or amperometrically (glassy carbon, +1.20 V vs Ag/AgCl). Because of the preconcentration, a detection limit of 2 pg Se was achieved.[115]

The selenium tetramethylenedithiocarbamate was too unstable[115] and could not be separated from other metal dithiocarbamates.[118] *N*-Benzyl-*N*-(2-cyanoethyl)dithiocarbamate was successfully used to determine selenite in a NIST standard reference water (1643 b).[119]

Dibenzyldithiocarbamate was successfully used for the determination of selenium, as well as for the determination of cadmium, lead, chromium, nickel, antimony, mercury and copper, with HPLC using UV detection.[120] The metal dibenzyldithiocarbamates were extracted from acidic solution into chloroform and separated on a mixed-mode phase column, consisting of both C_{18} and cation-exchange functional groups bonded to the support material. Manganese, zinc and thallium must be extracted from pH 8 buffered solution. The method was proposed for the determination of these metals in water. The determinations of selenite with dithiocarbamates are summarized in Table 5.

Selenium bis(O-alkyl dithiocarbonates). Sodium *O*-alkyl dithiocarbonates (xanthates) react with selenous acid in acidic aqueous medium to produce selenium bis(*O*-alkyldithiocarbonates) in a redox reaction (Eqn [3]).

The selenium and other metal xanthates were extracted immediately into hexane, because the xanthates are decomposed by polar substances such as water, methanol, triethylamine and pyridine.[121] The separation of the *O*-butyl derivatives of selenium, tellurium, arsenic, antimony and bismuth was achieved on a C_{18} column with acetonitrile as mobile phase. The *O*-ethyl derivatives were separated on a Nucleosil CN column with n-hexane/t-butyl methyl ether as the mobile phase.[121] An RP C_{18} column with acetonitrile as mobile phase allowed the separation of the selenium bis(*O*-ethyldithiocarbonate) from the correspondig derivatives of arsenic, antimony, bismuth, tellurium and nickel.[122]

Selenium bis(alkanethiolates). Aoyama *et al.* developed a method for the determination of selenium based on the formation of a stable selenium bis(thiolate) (selenotrisulfide), that was subsequently tagged with a fluorescent dye.[123–126] Aqueous solutions (0.5 M HCl) of selenous acid were reacted with D-penicillamine (2-amino-3-methyl-3-mercaptobutyric acid (Eqn [4]).

$$4\,RSH + H_2SeO_3 \rightarrow RS{-}Se{-}SR + RS{-}SR + 3H_2O \quad [4]$$

The selenium bis(alkanethiolate) formed a fluorescent derivative with 7-fluoro-4-nitrobenz-2,1,3-oxadiazole, which was separated on a C_{18} column (acetonitrile/water/phosphoric acid, 400:600:1; 10 mM Li_2SO_4) from the reagents and other reaction products. With fluorimetric detection an absolute detection limit of 17 pg Se corresponding to 5 μg Se dm^{-3} was achieved.[124] This method was successfully applied to the determination of selenium in digested biological and environmental samples[124–126] and in water after preconcentration of selenite on a bismuthiol-II-sulfonic acid-loaded anion exchanger.[123]

Table 5 Reversed-phase chromatography of selenite after formation of bis(dithiocarbamates), bis(*O*-alkyldithiocarbamates) and bis(alkanethiolates)[a]

Phase		Reagent	Detector	Absolute detection limit (ng Se)	Sample	Ref.
Stationary	Mobile					
C_{18}	CH_3OH/ H_2O (80:20)	DDTC	UV 254, 320	25	Drinking, surface, and waste-water	108, 109
C_8	CH_3CN/H_2O (60:40) or CH_3OH/H_2O (68:32)	DDTC	UV 254	NR	[c]	116
C_{18}	CH_3OH/CH_3CN/ H_2O (40:35:25)	DDTC	UV 254	0.4 (?)	Synthetic electroplating solution	117
C_{18}	CH_3CN/20 mM acetate buffer (pH 5.5) (70:30)/ 10 mM $NaNO_3$	DDTC	UV 254, amperometry	0.005 0.002[b]	[c]	115
C_{18}	CH_3OH/CH_3CN/ $THF/CH_2Cl_2/H_2O$ (47.6:10.2:2.04:8.16:32.0) + 5 mM BPDTC and 10 mM acetate buffer (pH 4.9)	BPDTC	UV 260	4	Standard reference water	119
C_{18}/CE	CH_3OH/CH_3CN/ THF/100 mM acetate buffer (pH 5) (64:12:5:19)	DBDTC	UV 254	0.013	Standard reference material, leachates from a landfill	120
C_{18}	CH_3CN	Butyl xanthate	UV 250	NR	[c]	121
—CN	n-Hexane/t-butyl methyl ether (97:3)	Ethyl xanthate	UV 250	NR	[c]	121
C_{18}	CH_3CN	Ethyl xanthate	UV 250	NR	[c]	122
C_{18}	CH_3CN/H_2O/ H_3PO_4 (400:600:1)/ 10 mM Li_2SO_4 (pH 2.6)	Pen + NBD-F	F(ex470, em530)	0.016	Standard reference materials, water, human serum	124–126

[a] Abbreviations: NR, not reported; CE, cation-exchange; UV, UV–VIS spectrometer, wavelength in nm; F, fluorimetric detector, excitation and emission wavelength in nm; DAB, 1,2-diaminobenzene; DDTC, diethyldithiocarbamate; BPDTC, *N*-benzyl-*N*-(2-cyanoethyl)dithiocarbamate; DBDTC, dibenzyldithiocarbamate; Pen, D-penicillamine (2-amino-3-methyl-3-mercaptobutyric acid); NBD-F, 7-fluoro-4-nitrobenz-2,1,3-oxadiazole. [b] Preconcentration. [c] The samples for these experiments are solutions of pure selenite in distilled water.

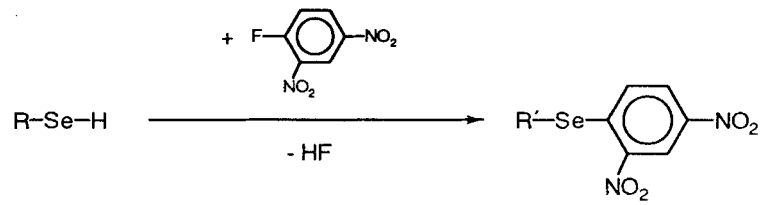
Hydrogen selenide and methaneselenol

Hydrogen selenide and methaneselenol are known to be intermediates in metabolic reactions of selenium. Hydrogen selenide is produced from selenocysteine in liver and other animal tissues by enzymic or non-enzymic reactions.[127] Hydrogen selenide may be an important intermediate in the synthesis of selenoproteins,[128] and may take part in the detoxification of selenium.

Ganther and Kraus described a method for the identification of hydrogen selenide and methaneselenol.[129,130] The compounds were reacted with 1-fluoro-2,4-dinitrobenzene (Sanger's reagent) to yield bis(2,4-dinitrophenyl) selenide and methyl 2,4-dinitrophenyl selenide (Eqn [5]).

The selenides are stable towards air (oxidation), are easily extacted into organic solvents, can be detected on TLC plates and in HPLC effluents because of the chromophoric groups, and can be characterized by mass spectrometry.

The method was developed with synthetic methaneselenol and with hydrogen selenide generated by reduction of selenite with zinc dust in hydrochloric acid medium (7 M). The hydrogen selenide was swept by a nitrogen stream into a

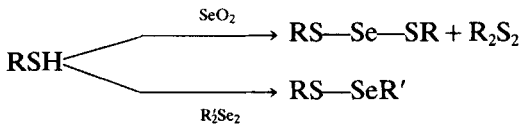


[5]

solution containing sodium hydrogen carbonate and 1-fluoro-2,4-dinitrobenzene in a dimethylformamide/water mixture.[130] The diaryl selenide formed was extracted into benzene. The extract was chromatographed on a Nucleosil NH_2-bonded column with n-heptane /chloroform[129, 130] or on an Alltech reversed-phase column with acetonitrile/water.[130] The effluent was monitored with a UV detector set at 254 nm. This method may be used for the determination of hydrogen selenide and methaneselenol, and for selenium compounds that are reducible to hydrogen selenide or methaneselenol.

Non-volatile selenols, diorgano-diselenides, alkyl organothioselenides and bis(organothio)selenides

Reactions of selenite or diselenides with reduced glutathione (a thiol, GSH) leading to bis(alkylthio)selenides or alkyl alkylthioselenides are important in biological systems (Eqn [6]).



[6]

Killa and Rabenstein obtained GS—SeR′ from reduced glutathione and bis(2-aminoethyl) diselenide and separated this compound from the 2-aminoethaneselenol and the corresponding diselenide on a reversed-phase C_{18} column with a mobile phase consisting of acetonitrile, sodium octylsulfate and phosphate buffer of pH 2.9.[131] The selenium compounds were detected electrochemically with two serial gold-amalgam electrodes. On the first electrode the diselenide and the selenide sulfide were reduced to selenols; the selenols were detected by oxidation on the second electrode with detection limits in the low nanogram range. The reaction products of selenite with reduced glutathione,[132, 133] penicillamine,[132, 133] cysteine,[79, 133] and *N*-acetylcysteine[79] were similarly separated. With a ^{75}Se-radiometric detector, a detection limit of 1 pg Se was achieved.[79] These methods were used to detect these selenium–sulfur compounds in spiked plasma samples[131] and in plasma and urine of rats treated with [^{75}Se]selenite.[79]

Jan *et al.* investigated the chromatographic behavior of 2-phenylseleno-2-phenylacetamides and diphenyl diselenide on a reversed-phase C_{18} column with methanol/water (7:3) as mobile phase and UV detection at 254 nm.[134] Electrochemical detection was not possible.

Vickrey *et al.* separated selenourea and dibenzyl selenide on a C_{18} column (Partisil-PXS-ODS) with methanol/water (2:1) as the mobile phase.[135] The selenium compounds in the effluent were detected with a UV detector set at 254 nm and a Hitachi–Zeeman graphite furnace atomic absorption spectrometer as selenium-specific detector.

Trimethylselenonium salts

The metabolism of selenium in higher animals produces two major excretory products: dimethyl selenide, which is exhaled and excreted through the skin, and the trimethylselenonium cation, which leaves the body in the urine. These methylation reactions are considered to be detoxification steps, because dimethyl selenide and trimethylselenonium salts are much less toxic than other selenium compounds.

Hoffman used ion chromatography on a strongly acidic cation-exchange resin with 4 mM HNO_3 as mobile phase to separate trimethylselenonium iodide from trimethylsulfonium iodide.[136] A conventional conductivity detector gave a response for both compounds similar to that for an inorganic cation such as potassium. Laborda *et al.* separated trimethylselenonium chloride from selenite and selenate on an anion-exchange column with 80 mM ammonium citrate (pH 3.3) as mobile phase.[57] The selenonium salt was eluted with the solvent front. Selenite and selenate had retention times of 3 and 7.5 min, respectively. An ICP AE detector provided an

absolute detection limit of 14 ng Se for the selenonium salt.

Because selenonium salts do not react with reducing agents to give volatile selenium compounds, a 'thermochemical hydride generation interface' was designed[137–139] to make the hydride-generation method applicable to selenonium salts and other non-reducible selenium compounds. Methanolic solutions of the analytes were nebulized by thermospray action, pyrolyzed in a methanol–oxygen flame in the presence of excess hydrogen, and atomized in a micro-diffusion flame maintained at the entrance to an unheated quartz T-tube. Trimethylselenonium iodide was separated from 2-hydroxyethyl dimethyl selenonium tetraphenylborate (selenocholine) on a cyanopropyl-bonded phase with a mixture of methanol, diethyl ether and acetic acid as eluent. The chromatographic separation was optimized by addition of two modifiers to the mobile phase. Triethylamine masked silanol groups on the surface of the stationary phase. The retention times of the selenonium salts decreased with increasing concentration of triethylamine in the mobile phase. The addition of trimethylsulfonium iodide to the mobile phase in small amounts sharpened the peaks, but influenced the retention times of the selenonium salts irreproducibly. A combination of triethylamine, acetic acid and trimethylsulfonium iodide in the methanolic phase seemed to offer an acceptable compromise between resolution and peak shape.[138] The method was used successfully to determine trimethylselenonium iodide in human urine spiked with this compound.[137]

Blotcky *et al.* investigated the chromatographic behavior of trimethylselenonium chloride, selenocystine, selenomethionine, selenoethionine and selenite on three different HPLC columns [Partisil-10-ODS-2 (C_{18} column), Partisil 10 (C_8 column), and Partisil 5 (silica gel)].[140] The mobile phase was water/acetonitrile (100:3). Fractions were collected, irradiated with neutrons, and counted for ^{77m}Se activity. Trimethylselenonium chloride moved with the solvent front on all three columns. For the determination of trimethylselenonium salts in urine, unprocessed urine was passed first through an anion- and then a cation-exchange column employing a 0.5 M $LiNO_3$ solution as mobile phase. The anion-exchange column trapped peptide-linked selenium and allowed trimethylselenonium salts to pass. The cation-exchange column captured trimethylselenonium salts selectively and, thus separated them from sodium ions (which would cause problems in the neutron activation analyses), chloride and selenite. The cation-exchange resin containing the trimethylselenonium ion was allowed to dry overnight. The dry resin was irradiated with neutrons and the ^{77m}Se activity determined.

Shibata *et al.* separated trimethylselenonium salts from selenite and selenate on a cation-exchange column with 200 mM tetramethylammonium chloride solutions as eluent or on a gel permeation column with 25 mM tetramethylammonium salt/25 mM malonic acid as eluent.[32] To determine trimethylselenonium salts in human urine, preconcentration was necessary when an ICP AE spectrometer was used as detector. With an ICP MS, determination of selenium compounds in human urine was possible without preconcentration. The chromatogram of human urine showed a large peak and trimethylselenonium was identified as a small shoulder of that peak by internal standard addition. The large peak was later identified as an interference caused by $^{81}BrH^+$, because this peak only appeared when ^{82}Se was monitored and not when ^{78}Se was monitored.

Kraus *et al.* studied methylation processes of selenium in higher organisms.[141] They developed a method for the separation of trimethylselenonium salts from other selenonium compounds and selenoamino acids on a strong cation-exchange resin. ^{75}Se-radiolabelled 2-amino-2-carboxy-1-ethyl methyl selenide (*Se*-methylselenocysteine) and 3-amino-3-carboxy-1-propyl dimethyl selenide (*Se*-methylselenomethionine) were injected into rats. The urine of these animals was chromatographed after a de-salting procedure. Chromatographic fractions were collected and γ-counted. The chromatograms showed that these two compounds are metabolized to trimethylselenonium salts and other unidentified cationic selenium metabolites. This method was also used to study the metabolic formation of trimethylselenonium salts in urine of rats after injection of dimethyl carboxymethyl selenonium bromide (selenobetaine),[142] with a modification of the chromatographic conditions[143] for the validation of a trimethylselenonium ion determination in human urine based on precipitation,[144] and, also under modified chromatographic conditions, to evaluate the inhibitory effect of periodate-oxidized adenosine on the formation of trimethylselenonium salts in mice treated with selenite.[145] A similar method was also described for the characterization of *S*-

adenosylmethionine:thioether *S*-methyltransferase.[146] This enzyme is responsible for the conversion of dimethyl selenide to trimethylselenonium ion in higher organisms with *S*-adenosylmethionine as methyl donor. Dimethyl selenide and [*methyl*-^{3}H]*S*-adenosylmethionine were allowed to react, and the products, trimethylselenonium ion and *S*-adenosylhomocysteine, were separated by cation-exchange HPLC. [^{3}H]Trimethylselenonium salts were quantified in collected fractions by liquid scintillation.

Selenoamino-acids and related compounds

Selenoamino-acids attract much interest, because these compounds take part in the biological selenium cycle and are incorported into proteins. Selenoamino-acids such as selenomethionine are used as selenium supplements for man and animals. Several selenoamino-acids and related selenium compounds were identified in tissues and body fluids. Selenocysteine is part of the active site of the enzyme glutathione peroxidase.

Kraus and co-workers identified selenocysteine in selenoproteins based on the formation of a 2,4-dinitrophenyl (DNP) derivative.[147,148] Pure erythrocyte glutathione peroxidase was denatured with guanidine and reduced with sodium borohydride. The reduced enzyme was then reacted with 1-fluoro-2,4-dinitrobenzene and hydrolyzed. The hydrolysate was extracted with benzene. The DNP-amino-acids were separated from other amino-acids by gel filtration. The pooled DNP-amino-acids were chromatographed on a semipreparative reversed-phase HPLC column with a methanol/water mixture as eluent. The chromatographic eluate was monitored at 254 nm. Additionally, fractions were collected and analyzed for selenium by GF AA. The selenium-containing fractions were further purified on an analytical column. Two selenium compounds were detected, one of which was identified as DNP-selenocysteine by mass spectrometry and by co-chromatography with a synthetic sample. The other compound was not identified.

Hawkes and Kutnink reacted selenocysteine with the fluorescent *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulphonic acid.[149] This reagent combines preferentially with selenocysteine at pH 6.6. The fluorescent derivatives of selenocysteine and of cysteine were chromatographed on a C_{18} reversed-phase column and detected by fluorimetry. The method was successfully used for the determination of selenocysteine in spiked plasma samples.

Hansen and Poulsen developed a method for the determination of selenomethionine, based on derivatization with 9-fluorenylmethyl chloroformate.[150] The chiral (+)-1-(9′-fluorenyl)-1-ethyl chloroformate allowed the separation of *R*- and *S*-enantiomers of selenomethionine and methionine. These methods were used to determine total selenomethionine and the optical purity of selenomethionine in tablets.[150] The same methodology was used for the identification of L-selenocysteine in selenoproteins isolated from selenium-enriched yeast.[97]

[^{75}Se]Selenomethionine was also derivatized with 5-dimethylamino-1-naphthalenesulfonyl (dansyl) chloride. The dansyl derivative and other related compounds were separated on a reversed-phase C_{18} column and detected by UV absorption or γ-counting.[151]

Wolf *et al.* separated selenomethionine from methionine, cystine, isoleucine and leucine on a cation-exchange column with a lithium buffer as mobile phase.[152] The amino-acids were detected fluorimetrically after post-column derivatization with *o*-phthalaldehyde in the presence of mercaptoethanol. Under these conditions selenomethionine and leucine were not separated. For this reason the sample solution was reacted with cyanogen bromide. Methionine reacted with cyanogen bromide giving homoserine and methylthiocyanate; while selenomethionine gave an unidentified reaction product with a higher elution time than leucine.

Kajander *et al.* investigated selenomethionine metabolites with ion-pairing HPLC.[153] Adenosylselenomethionine, adenosylselenohomocysteine, decarboxylated adenosylselenomethionine and methylselenoadenosine, and the corresponding sulfur compounds, were separated as ion-pairs with octanesulfonic acid on a reversed-phase column using a phosphate buffer/CH_3CN gradient. The chromatographic eluate was monitored at 257 nm. The method was used to study the metabolism of selenomethionine in cultured cells.[154] Kraus and co-workers successfully separated 3-amino-3-carboxy-1-propyl methyl selenide (selenomethionine), 2-amino-2-carboxy-1-ethyl methyl selenide (*Se*-methylselenocysteine), dimethyl carboxymethyl selenonium bromide (selenobetaine), dimethyl 2-amino-2-carboxy-1-ethyl selenonium iodide (*Se*,*Se*-dimethylselenocysteine), dimethyl 3-

amino-3-carboxy-1-propyl selenonium iodide (*Se*-methylselenomethionine), dimethyl ethoxycarbonylmethyl selenonium bromide (ethylselenobetaine), and trimethylselenonium salts.[141] The separation was achieved on a strong cation exchanger with a linear concentration gradient of $(NH_4)_2HPO_4$ as mobile phase. The method was used to determine the ^{75}Se-radiolabelled compounds in urine of rats.

Blotcky *et al.* investigated the chromatographic behavior of selenocystine, selenomethionine, selenoethionine, selenite, and trimethylselenonium chloride on a C_{18} column with water/acetonitrile as mobile phase, but the system showed insufficient separation of the compounds under investigation.[140]

A cyanopropyl-bonded phase was used for the separation of 2-hydroxyethyl dimethyl selenonium tetraphenylborate (selenocholine) from trimethylselenonium iodide[137–139] and from (2-acetoxyethyl) dimethyl selenonium tetraphenylborate (acetylselenocholine).[138]

Ogawa *et al.* suggested a new method for the biosynthesis of [^{73}Se]selenomethionine.[155] Micro-organisms were cultivated in a sulfur-deficient medium containing radioactive $^{73}SeO_2$. The hydrolysis of cellular protein of the micro-organisms yielded ^{73}Se-labelled selenomethionine. The separation and purification of selenomethionine from other hydrolysis products were performed with HPLC.

Selenium derivatives of carbohydrates

Although several selenium-containing carbohydrates have been synthesized,[156] only three selenocarbohydrates have been found in nature.[4, 157, 158]

A standard HPLC method for the separation of glucosinolates[159] was used to separate β-D-1-glucopyranosyl 1-(hydroxylimino)pent-4-en-1-yl selenide from its sulfur analogue.[4] The compounds could be separated to baseline. The selenocarbohydrate showed a slightly higher elution time than the sulfur compound.

Recently, an HPLC method was used for the characterization of a selenium-containing polysaccharide isolated from garlic (*Allium sativum*).[158]

Selenoproteins

The biological effects of selenium are generally believed to be exerted through selenoproteins; about 10 selenoproteins are now known to exist in mammalian tissues. The functions of some of these proteins are known.[160]

Vezina *et al.* used a C_8-bonded stationary phase to separate selenoproteins occurring in human seminal plasma with 0.1 M triethylammonium acetate/isopropanol (linear gradient, 0–40%).[101] Selenium was determined in the collected fractions by fluorimetry. Several unknown selenium-containing proteins were detected. A gel-permeation column was used to investigate the chemical form of selenium in human plasma and erythrocyte lysate.[32] Fractions were collected and analyzed for selenium with an HPLC–fluorimetric procedure.[84] The chromatograms showed that a large part of selenium in normal human erythrocytes is attached strongly to hemoglobin. A second, small peak was identified as the enzyme glutathione peroxidase. The distribution of selenium in human plasma is similar to the distribution of proteins. This fact may indicate that each protein contains selenium in low concentration. Brätter and co-workers coupled gel-permeation HPLC with ICP AE as detector for the determination of metal-binding proteins in body fluids.[161] To achieve lower detection limits for selenium, an on-line wet-digestion and hydride-generating system was developed. Proteins that contain selenium, zinc, iron, copper or manganese were identified in human serum, human milk and lysed erythrocytes.

Motchnik and Tappel characterized a selenoprotein P in rat plasma.[162, 163] [^{75}Se]Selenite was injected into rats. The blood was sampled after 3 h and centrifuged. Selenoprotein P was fractionated using salt precipitation, different kinds of chromatography and electrophoresis. The selenium-containing subunit of the protein was treated with trypsin[162, 163] or proteases.[163] Reversed-phase HPLC of the products with 0.05% trifluoroacetic acid and a gradient of acetonitrile produced three major ^{75}Se peaks. Fractions of these peaks were pooled for further analysis by gel-filtration chromatography. The results indicated that ^{75}Se was present at several locations in the selenium-containing subunit of selenoprotein P. Selenoprotein P was also digested with cyanogen bromide to cleave it at methionines.[164] The two resulting peptides were carboxymethylated, separated by reversed-phase HPLC and subjected to amino-acid analysis. These experiments suggest that selenoprotein P has one or more selenium-rich regions.

Reversed-phase HPLC was used to purify the enzyme glutathione peroxidase from rat liver

cytosol after application of several other chromatographic techniques.[165] The above-mentioned methodology (trypsin digestion and analysis with reversed-phase HPLC) was applied to characterize this enzyme.[166] The protein was purified from the liver of rats injected with ^{75}Se-radiolabelled selenite. Then the purified enzyme was carboxymethylated until no enzymic activity remained, treated with trypsin and the digest fractionated by reversed-phase HPLC. Collected fractions were counted for ^{75}Se. The chromatogram showed one peak, a fact that distinguishes glutathione peroxidase from selenoprotein P and a kidney selenoprotein. Another enzyme, thiolase, was explored in the same way.[167] The extent of incorporation of selenium into thiolase was found to be dependent on the form of selenium (selenite, selenomethionine) supplied. For the study of the location of the selenomethionine residues in the polypeptide chain of the enzyme, the ^{75}Se-labelled protein was treated with trypsin and the resulting peptides were isolated by reversed-phase HPLC. These studies led to the conclusion that selenium occurs in thiolase adventitiously and is not required for any biological function.

Ping *et al.* extracted and purified a new compound containing selenium and mercury from dolphin liver.[168,169] They wanted to characterize the substance by reversed-phase HPLC; however, it was not retained by the column.

Oikawa *et al.* synthesized metalloselenonein, the selenium analog of metallothionein.[170,171] The metalloselenonein synthesized was purified by preparative HPLC after reduction with $NaBH_4$. The chromatogram on an analytical C_{18} reversed-phase column with 0.1% trifluoroacetic acid and a gradient of acetonitrile showed a single symmetrical peak for metalloselenonein when absorbance at 220 nm was used for detection.

Selenium-containing tRNA

Selenium-modified nucleosides occur in tRNAs from several bacterial, mammalian and plant species; the precise biochemical role for selenium in these compounds is unknown.

Stadtman and co-workers investigated the incorporation of selenium into tRNAs of *Salmonella typhimurium*[172] and *Methanococcus vanielii*[173,174] with HPLC techniques. The cells were grown in a medium supplemented with ^{75}Se-radiolabelled selenite. Bulk tRNA was extracted, purified by chromatography, and completely hydrolyzed. The nucleosides in the hydrolyzate were separated on a C_{18} column. The column was eluted with a gradient of ammonium acetate buffer/methanol. The eluate was monitored at 313 nm; fractions were collected and their γ-activity determined. *Salmonella typhimurium* had produced three selenium-containing nucleosides and *Methanococcus vanielii* two. One of these compounds was identified as 5-methylaminomethyl-2-selenouridine by co-chromatography with a synthetic sample.

Miscellaneous

Selenotungstates, $WO_nSe_{4-n}^{2-}$, were separated on a non-derivatized divinylbenzene-based porous resin; tetrabutylammonium hydroxide served as ion-pairing reagent in a mobile phase containing Na_2CO_3 and CH_3CN.[175] The eluate was monitored at 254 mm. Efforts were made to explain the retention behavior of these anions as a function of the number of selenium atoms.

Optically active selenoxides were separated by HPLC using an optically active column consisting of (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine on aminopropylsilica.[176,177] The mobile phase was hexane containing 2–10% 2-propanol; the eluate was monitored at 265 nm. Gargaro *et al.* prepared a chiral stationary phase containing the 3,5-dinitrobenzoyl derivative of *R*,*R*(−)1,2-diaminocyclohexane for the enantiomeric separation of selenoxides.[178] An HPLC method was also used to separate the diastereomers of the $[Co(2,4\text{-pentanedionato})_2\text{-}(CH_3SeCH_2CH_2NH_2)]^+$ ion.[179]

Reversed-phase HPLC on a silica-based C_{18} material with methanol as mobile phase was found to be an appropriate method for the detection of the selenium cycles Se_6, Se_7 and Se_8 in solutions containing elemental selenium.[180] UV absorption at 254 nm was suitable for the detection of these species. The behavior of cyclic selenium sulfides of the type Se_xS_y was also investigated with this method.[181–188] Linear relations were found between chromatographic retention time and ring size, number of Se atoms and number of Se–S bonds per molecule, for 6-, 7-, 8- and 12-membered Se_xS_y rings.[189] Thus, it was possible to predict the retention times for all Se_xS_y cycles of these ring sizes. The results were applied to the qualitative analysis of mixed sulfur–selenium melts. Attempts were also made to determine these compounds in naturally occurring, selenium-containing sulfur, but no selenium sulfides were found.[190]

Similar investigations were carried out for the

diorganyl polyselenides (Et_2Se_n,[191,192] Bu_2Se_n[192] and Ph_2Se_n[193]) and for other organic polyselenides in which the selenium chains were bound to ethylthio-, butylthio- or phenylthio- groups at both ends.[193]

CRITICAL COMPARISON

An ideal chromatographic method would separate selenite, selenate, trimethylselenonium salts, selenium-containing carbohydrates, selenonucleosides, selenoproteins and several other organic selenium compounds in one run with absolute detection limits not higher than 1 ng Se, corresponding to a concentration of 10 μg dm^{-3}, when 100 μL are injected. This ideal method has not yet been developed. Such single-column separations of mixtures of inorganic and organic selenium compounds consisting of neutral, anionic and cationic species are not easy to achieve. Selenium-specific detectors simplify the chromatographic task, because only selenium compounds must be separated from each other. Compounds not containing selenium with the same retention time as a selenium compound will not interfere with the identification of the selenium compound. This characteristic of selenium-specific detectors is particularly helpful when selenium compounds must be identified and determined in complex matrices, e.g. in extracts from soils, sediments and biological samples.

Much of the chromatographic work with selenium compounds has been carried out with conventional (conductivity, UV-spectrometric) and not with selenium-specific detectors. However, during the past decade selenium-specific detectors gained popularity as more complex matrices were investigated and the attention shifted from selenite and selenate to organic selenium compounds. Most of the publications on the development and improvement of high-performance liquid-chromatographic methods for the identification and quantification of selenium compounds deal with the separation of selenite and selenate.

A widely used method for the separation of selenite and selenate is ion chromatography. Suppressed-ion chromatography (SIC) requires more complex instrumentation (two columns) and more time (regeneration) than single-column ion chromatography (SCIC), and is also restricted with respect to the composition of the mobile phase (carbonate-containing eluents). These disadvantages are only partly compensated by improved detection limits achievable with the conductivity detector customarily used in SIC. When analyzing complex matrices containing other ions such as sulfate, chloride and phosphate at higher concentrations, interference problems do occur with the conductivity detector. These impairments may be overcome by other detection systems, preferably selenium-specific; however, these consist of rather expensive instruments and require a coupling device between the HPLC and the detector. UV detection is inadequate because the absorption maxima for selenite and selenate are below 200 nm, where interferences from many UV-active compounds are very common.

Absolute detection limits for the ion-chromatographic determination of selenite and selenate are matrix- and detector-dependent. The literature reports absolute detection limits for suppressed-ion chromatography in the range 1–20 ng selenium and for single-column ion chromatography in the range from 0.1–200 ng. The detection limits for selenite and selenate generally are not the same but are of the same order of magnitude. Single-column ion chromatography with selenium-specific detection appears to be the method of choice for the determination of selenite and selenate in complex matrices.

Reversed-phase ion-pairing chromatography is also a good separating system for selenite and selenate, and allows, in principle, simultaneous separation of neutral selenium compounds. The column material is chemically rather inert, stable in the pH range 1–13, and usually gives better resolution of peaks than ion chromatography. Selenium-specific devices such as graphite furnace atomic absorption spectrometers, plasma atomic emission spectrometers, and plasma mass spectrometers may be used. GF AA spectrometers in general offer low detection limits, but the discontinuous sampling of the column effluent, allowing on average only one determination per minute, and the dilution of the injected aliquot of the sample increase the system detection limit by factors between 10 and 100. To define a chromatographic band appropriately through a series of GF AA signals, the peaks must be rather broad. This requirement leads to chromatographic runs of long duration (20–40 min). Detection limits of approximately 20 ng Se are reported for selenite and selenate under these conditions.

Continuously monitoring detectors surpass the performance of GF AA spectrometry by far. The

most powerful detector is an inductively coupled plasma mass spectrometer. This detector responds linearily to selenium concentration over several orders of magnitude with a detection limit of 1 μg Se dm^{-3} for ^{82}Se. Additionally, this instrument is able to monitor several elements at the same time. Limitations in the quantification may arise from drifts of the instrument response over a longer period of time. Detection limits as low as 0.1 ng Se were reported for the determination of selenite and selenate by reversed-phase HPLC with ICP MS as the selenium-specific detector.

Selenite—but not selenate and other organic selenium compounds—reacts with aromatic *o*-diamines to form piazselenoles that can be detected fluorimetrically. The piazselenoles can be detected directly after extraction from the reaction mixture or after separation by high-performance liquid chromatography from impurities and by-products. Absolute detection limits for selenite in the low picogram range were reported. This method is one of the best for the determination of very low concentrations of selenite and—after reduction of selenate to selenite—also for selenate.

Compared with the extensive investigations on selenite and selenate, very little work has been done with organic selenium compounds although the majority of selenium compounds in the natural selenium cycle have at least one C–Se bond. Methaneselenol (derivatized to a methyl aryl selenide), diorganyl diselenides, alkyl organothio selenides, bis(organothio) selenides, trimethylselenonium salts, selenoamino-acids, selenium-containing carbohydrates, selenium-containing nucleosides and selenoproteins were subjected to high-performance liquid chromatography. Systematic investigations and applications to 'real' samples are rare.

HPLC will become an indispensable tool for the separation, identification and quantification of various organic selenium species of biological relevance. The most essential problem to be overcome is the separation of homologous and analogous compounds in biological matrices. Compounds of selenium can be expected to be accompanied by a spectrum of similar sulfur compounds that often behave in a similar manner with respect to chromatographic separations.

Selenium-specific detectors make the separation of selenium compounds from sulfur compounds unnecessary and allow the detection of selenium compounds in complex matrices. Liquid chromatography with selenium-specific detectors is an indispensable tool for the quantification of different selenium species in various kinds of samples. For the determination of selenium compounds in environmental and biological matrices, HPLC will provide an adequate separation for selenium-containing metabolites, proteins, amino-acids and their derivatives. Of increasing interest is the action of selenium as a detoxifying agent. Chromatographic methods will be used to identify compounds that contain not only selenium, but also other trace elements such as mercury and arsenic. Element-specific detection systems that allow simultaneous monitoring of several elements will be of increasing importance. ICP MS and related instruments will find increasing use as detectors.

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