

# High-Performance Liquid Chromatographic–Fluorescence Determination of Traces of Selenium in Biological Materials

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Received February 20, 1996

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**An improved method for the determination of selenium in biological materials has been developed. This work both extends and validates the procedure of Vézina and Bleau (*J. Chromatog.* 426, 385–391, 1988) which is based on high-performance liquid chromatographic determination of the fluorophore formed by reaction of Se(IV) with 2,3-diaminonaphthalene. The mass detection limit is 48 pg selenium ( $3\sigma$ ) and the concentration detection limits are 48 parts per trillion in biological fluids and 120 to 480 parts per trillion in dried biological materials. The linear dynamic range of the method has been extended up to approximately 800 ng. Relative standard deviations of 9.4 to 2.7% were observed in repeated analyses of standards in the range of 0.5 to 500 ng. The proposed method was validated with respect to 23 biological reference materials spanning an 1800-fold range of selenium concentrations and was found to be free of significant constant or proportional biases despite greatly different matrix compositions. This method offers an unsurpassed combination of sensitivity, accuracy, linear dynamic range, and freedom from matrix interferences and may be considered a reference method for the reliable determination of selenium in biological materials.**

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Selenium (Se)<sup>2</sup> is a ubiquitous trace element that is essential to life at sub-parts-per-million (ppm) levels. Se is also very toxic, showing adverse effects in many species at levels of around 10 ppm in the diet. The

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<sup>2</sup> Abbreviations used: Se, selenium; ppm, parts per million; ppb, parts per billion; ppt, parts per trillion; AAS, atomic absorption spectrometry.

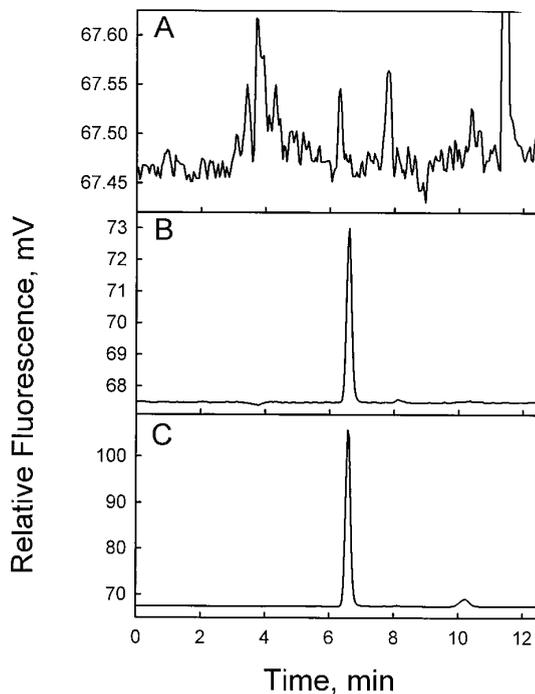
toxicity of Se is also of environmental concern because parts-per-billion (ppb) levels of inorganic Se in water can, through bioaccumulation and bioconcentration in the food chain, lead to lethal consequences for wildlife (1, 2). Se in biological samples occurs primarily as selenoamino acids such as selenocysteine and selenomethionine, which are homologous to the sulfur amino acids, cysteine and methionine, and are incorporated into proteins as part of the peptide backbone. Plant materials may contain other selenoamino acids, such as Se-methylselenocysteine, selenocystathionine, and selenohomocysteine. Se also occurs in biological specimens as complexes with metals such as mercury, cadmium, and zinc.

Because of the wide range of biologically relevant Se concentrations and the many chemical forms of Se, the determination of Se in biological samples has been problematic, leading to many specialized methods suitable for specific sample types. The fluorometric method, based on reaction of Se(IV) with 2,3-diaminonaphthalene to form the fluorescent naphtho-2-selena-1,3-diazole (4,5-benzopiazselenol) (3), has been the most widely used chemical method for the determination of trace levels of selenium in biological materials. However, it is a tedious procedure requiring individual sample digestions and a difficult pH adjustment, and the sensitivity is not adequate for trace analysis in many biological materials. Although some instrumental methods require less sample preparation or have greater sensitivity, none have demonstrated better reliability or more general applicability than the classical fluorometric method (4, 5) and few have been validated with biological reference materials. Atomic absorption spectrometry (AAS) has been applied to Se determinations. Hydride-generation AAS is the most sensitive AAS technique for Se and has been extensively applied to biological materials, although some metals can inter-

ferre with formation of the  $H_2Se$  gas. The sensitivity and accuracy of graphite furnace AAS is adequate for Se determinations in biological fluids and the method requires little sample preparation. However, graphite furnace AAS is very sensitive to matrix interferences and reliable methods have been validated only for blood plasma and serum.

A key aspect of Se determinations in biological materials has been the need for decomposition of the sample, either to make the Se available for derivatization or atomic spectroscopic measurement or to destroy the organic sample matrix to remove interferences. Digestion in mineral acid mixtures destroys the organic matrix and releases the Se as a mixture of inorganic oxyanions. Treatment with hydrochloric acid converts these oxyanions to Se(IV), the chemical form required for hydride generation AAS, fluorometric, and gas chromatographic determinations of Se. Although numerous attempts have been made to eliminate perchloric acid for safety reasons, quantitative recoveries of Se in the +4 oxidation state and mineralization of the matrix are improved by its presence in the mineral acid mixture.

An interesting modification of Watkinson's fluorometric procedure (3) using double-distilled acids for sample digestion and liquid chromatography to isolate the piarselenol fluorophore was reported by Vézina and Bleau (6, 7). These authors reported vastly improved detection limits due to lowered blanks and removal of fluorescent impurities. However, these modified methods were not validated with biological reference materials nor against a reference method, and have not been widely adopted. We built on the work of Vézina and Bleau to optimize this methodology for the determination of Se in real biological samples. We have devised an unattended acid digestion procedure with a much larger sample capacity, greatly decreased the blank signal from reagent impurities, and buffered the derivatization reaction pH at 1.75 to improve reproducibility. The sample sizes and acid digestion conditions were adjusted to achieve adequate mineralization of all the sample types without compromising sensitivity. The contribution of each reagent to the blank's Se signal was systematically studied and minimized to decrease the magnitude and variability of the Se signal in the blank. The precision and linearity of the assay were characterized over 4 orders of magnitude of Se concentrations. We demonstrated that the method is free of matrix interferences and systematic errors by validation with a suite of 23 biological reference materials. This improved method uses streamlined chemical procedures that allow batch sizes of up to 96 samples and standards, can quantitate much lower concentrations of Se than previous adaptations of the fluorometric method, and yields accurate results over an 1800-fold range of Se concentrations despite greatly different matrix compositions.

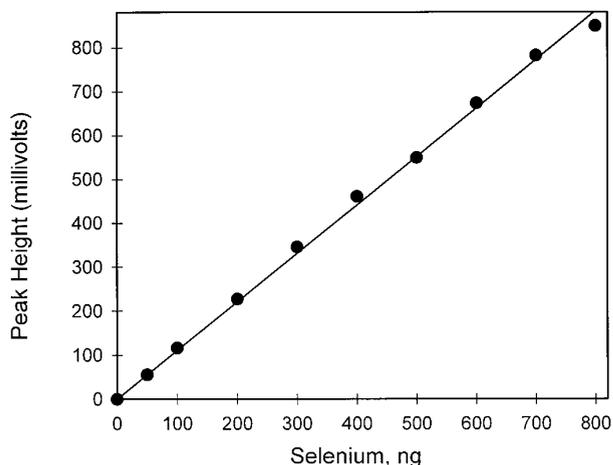


**FIG. 1.** Representative chromatograms of the piarselenol derivative from the complete proposed procedure for (A) a blank, (B) a 5-ng Se standard, and (C) a human blood sample (0.2 ml) as described under Materials and Methods. All chromatograms are plotted relative to the same arbitrary vertical voltage scale.

## MATERIALS AND METHODS

**Chemicals.** Double-distilled nitric acid (70%, Ultrex II) was from J. T. Baker (Phillipsburg, NJ). Redistilled ammonium hydroxide (30%) was from GFS Chemicals (Columbus, OH). Perchloric acid (70%, AR Select grade) and hydrochloric acid (35%, AR Select grade) were from Mallinckrodt (Chesterfield, MO). Glycine base (ACS grade) was from Fisher (Pittsburgh, PA). Cyclohexane and ethyl acetate (Omnisolv) were from E. M. Merck (Gibbstown, NJ). 2,3-Diaminonaphthalene hydrochloride (99%) was from Aldrich (Milwaukee, WI). All other chemicals used were of reagent grade or the highest purity available.

**Sample digestion.** Se standards were prepared from National Institute of Standards and Technology Standard Reference Material 3149, selenium aqueous standard solution. Up to 1 ml of biological fluids or 0.06–0.4 g of dry powdered material was mixed with 2.5 ml concentrated double-distilled nitric acid (70%) and 1 ml concentrated perchloric acid (70%) in 16 × 125-mm graduated tubes with two or three Teflon boiling chips and held at room temperature until foaming and evolution of brown fumes subsided. (Caution: Dry samples may react violently when first heated! This may be avoided by holding at room temperature overnight or longer. Hot perchloric acid must be used in a



**FIG. 2.** A typical calibration graph for the proposed method. A blank and standards containing the indicated amounts of Se were taken through the complete procedure as described under Materials and Methods. The relative fluorescence peak heights were regressed against the amounts of Se to construct the linear calibration graph with the intercept forced through zero.

properly functioning and maintained, dedicated perchloric acid hood.) Samples were digested in a preheated heating block for 90 min at  $140 \pm 2^\circ\text{C}$  and then for 75 min at  $200 \pm 2^\circ\text{C}$ , with the block taking approximately 40 min to reach  $200^\circ\text{C}$ . After cooling, 1 ml of 4 M HCl was added to the digested samples and the tubes were returned to the heating block for 15 min at  $150^\circ\text{C}$  to reduce Se(VI) to Se(IV).

**Adjustment of pH and derivatization.** One milliliter of 2 M glycine base, 0.09 M  $\text{Na}_4\text{EDTA}$ , and 4 drops of 0.02% cresol red were added and the pH was adjusted

to pH 1.5–2.0 (amber–orange) with 7 M  $\text{NH}_4\text{OH}$ . The glycine base was added to dampen swings in pH while approaching the endpoint. One and one-half milliliters of 2 M glycine, pH 1.75, was added and the samples were diluted to 8 ml with distilled water. The glycine buffer was added to minimize the tube-to-tube variation in final pH. One milliliter of 0.1% (w/v) 2,3-diaminonaphthalene hydrochloride in 0.1 M HCl (extracted once with cyclohexane just before use) was added and the mixtures were heated for 45 min at  $50^\circ\text{C}$ . (Caution: 2,3-diaminonaphthalene is a suspected human carcinogen.) From this point on, samples were protected from photodegradation by working under a dim yellow light. After cooling, 3 ml cyclohexane was added, polyethylene stoppers were placed on the tubes, and they were shaken for 15 min on a mechanical shaker to extract the fluorescent selenol. After each use, the digestion tubes were cleaned with detergent and dried, then soaked for at least 1 h in hot ( $100^\circ\text{C}$ ) 7 M nitric acid to remove residual traces of Se.

**Chromatography.** Chromatography was by a modification of the method of Vézina and Bleau (6). The HPLC system consisted of a Perkin Elmer ISS-100 autosampler and Series 4 solvent delivery system. The column was water-deactivated  $\mu\text{Porasil}$ , 10  $\mu\text{m}$ , 30 cm  $\times$  3.9 mm i.d. (Whatman, Clifton, NJ) with a 10  $\times$  4.6-mm i.d. silica guard column (Direct-Connect, Alltech, Deerfield, IL). Prior to its first use, the column was washed with 100 ml methanol, 100 ml water, 10 ml methanol, and finally 30 ml ethyl acetate, and then equilibrated with mobile phase. The cyclohexane extracts were placed directly into 2-ml autosampler vials without filtration. Four hundred microliters of cyclohexane extract was injected from each sample, using a

**TABLE 1**  
Within-Batch Repeatability and Between-Batch Reproducibility

Amount taken, ng	Mean amount found, ng ( $N^b$ )	Standard deviation			
		Within-batch		Between-batch <sup>a</sup>	
		ng	RSD <sup>c</sup> (%)	ng	RSD (%)
Blank	0.11 (13)	0.016	13.8	NA <sup>d</sup>	NA
0.5	0.60 (22)	0.057	9.4	0.046	7.7
5	5.21 (28)	0.147	2.8	0.095	1.8
50	50.5 (22)	1.35	2.7	1.50	3.0
500	508 (24)	21.3	4.2	NS <sup>e</sup>	NS

*Note.* Replicate samples of each standard were digested, derivatized, and chromatographed within each batch. This was repeated in five separate batches, on different days. The contribution of within-batch and between-batch sources of error were separated and estimated using one-way analysis of variance.

<sup>a</sup> Between-batch standard deviation does not include within-batch sources of error.

<sup>b</sup> Total number of replicate samples analyzed (some samples lost due to injector malfunction).

<sup>c</sup> RSD, relative standard deviation, (SD/mean)  $\times$  100%.

<sup>d</sup> NA, not applicable.

<sup>e</sup> NS, not significantly different from within-batch standard deviation in analysis of variance.

TABLE 2  
Validation with Biological Reference Materials

Reference material	Source	Sample size used, g or ml	Certified concentration $\pm$ uncertainty <sup>a</sup> , n/g or ng/ml	Mean concentration found $\pm$ SD ( <i>N</i> <sup>b</sup> ), ng/g or ng/ml
Corn kernel, RM 8413	NIST <sup>c</sup>	0.4	4 $\pm$ 2	4.99 $\pm$ 0.63 (4)
Fresh water, SRM 1643b	NIST	1.0	9.9 $\pm$ 0.5	10.0 $\pm$ 0.3 (4)
Corn stalk, RM 8412	NIST	0.4	16 $\pm$ 8	14.9 $\pm$ 5.9 (4)
Citrus leaves, SRM 1572	NIST	0.4	25 <sup>d</sup>	32.0 $\pm$ 1.4 (5)
Human urine (normal), SRM 2670	NIST	1.0	30 $\pm$ 8	26.2 $\pm$ 2.2 (5)
Wheat flour	ARC-CL <sup>e</sup>	0.4	58.5 $\pm$ 9.2	52.3 $\pm$ 3.5 (5)
Bovine muscle, RM 8414	NIST	0.2	76 $\pm$ 10	69.0 $\pm$ 5.0 (3)
Milk powder (2% fat)	ARC-CL	0.4	82 $\pm$ 7.7	75.1 $\pm$ 3.6 (5)
Milk powder, nonfat, SRM 1549	NIST	0.4	110 $\pm$ 10	110 $\pm$ 6.1 (5)
Mixed human diet, RM 8431	NIST	0.25	242 $\pm$ 30	246 $\pm$ 19 (5)
Porcine muscle	ARC-CL	0.2	394 $\pm$ 31	401 $\pm$ 28 (3)
Rice flour, SRM 1568	NIST	0.2	400 $\pm$ 100	369 $\pm$ 16 (2)
Human urine (elevated), SRM 2670	NIST	1.0	460 $\pm$ 30	488 $\pm$ 15 (5)
Bovine liver, SRM 1577a	NIST	0.25	710 $\pm$ 70	674 $\pm$ 61 (3)
Bovine liver, SRM 1577b	NIST	0.25	730 $\pm$ 60	712 $\pm$ 23 (6)
Bovine liver, SRM 1577	NIST	0.25	1100 $\pm$ 100	1092 $\pm$ 47 (6)
Wheat flour, SRM 1567a	NIST	0.4	1100 $\pm$ 200	1162 $\pm$ 55 (5)
Whole egg powder, RM 8415	NIST	0.1	1390 $\pm$ 170	1319 $\pm$ 49 (4)
Dogfish muscle, DORM-1	NRC <sup>f</sup>	0.12	1620 $\pm$ 120	1368 $\pm$ 189 <sup>g</sup> (4)
Human hair, CRM 397	BCR <sup>h</sup>	0.1	2000 $\pm$ 80	1889 $\pm$ 143 <sup>g</sup> (5)
Albacore tuna, RM 50	NIST	0.1	3600 $\pm$ 400	3663 $\pm$ 45 (2)
Lobster hepatopancreas, TORT-1	NRC	0.06	6880 $\pm$ 470	6570 $\pm$ 238 (2)
Dogfish liver, DOLT-1	NRC	0.1	7340 $\pm$ 420	6467 $\pm$ 486 <sup>g</sup> (3)

Note. Se concentrations in reference materials were determined two to six times each, using the complete proposed procedure as described under Materials and Methods.

<sup>a</sup> Various methods were used by the certifying agencies to set an uncertainty range.

<sup>b</sup> *N*, number of independent determinations.

<sup>c</sup> NIST, National Institute of Standards and Technology.

<sup>d</sup> Not certified for Se concentration, information value only.

<sup>e</sup> ARC-CL, Agricultural Research Centre, Central Laboratory, Finland.

<sup>f</sup> NRC, National Research Council, Canada.

<sup>g</sup> Mean Se concentration found was not within certified uncertainty range.

<sup>h</sup> BCR, Community Bureau of Reference, Commission of the European Communities.

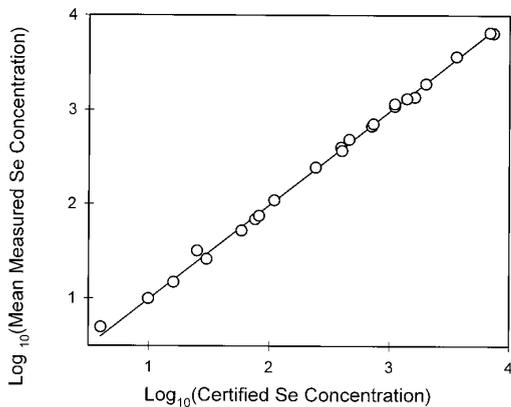
2-ml sample loop. Elution was isocratic with a mobile phase of 90% cyclohexane/10% ethyl acetate, at a flow rate of 1 ml/min. The detector was a Hewlett–Packard 1046A fluorescence detector (Hewlett–Packard Co., Palo Alto, CA) at excitation wavelength of 378 nm and emission wavelength of 530 nm, with slits adjusted for maximum sensitivity. The fluorescent derivative, naphtho-2-selena-1,3-diazole (4,5-benzopiazselenol), eluted at 6.5 min and was quantitated by peak height measurement using a Perkin Elmer Omega 4 chromatography data system (Perkin Elmer Corp., Norwalk, CT) using a linear external standard calibration curve with an intercept forced through zero. Samples were injected every 15 min.

## RESULTS

**Chromatography.** Representative chromatograms of the procedure blank, a 5-ng standard, and human

blood are shown in Figs. 1A–1C. As first reported by Vézina and Bleau (6), water deactivation of the silica column was necessary to allow the elution of the piazselenol at a low ethyl acetate concentration, which was in turn necessary to avoid strongly quenching the fluorescence of the piazselenol (6). After several hundred samples, the retention time of the piazselenol gradually increased by up to 1.5 min, with no loss of resolution. The normal retention time was restored by repeating the water-deactivation procedure described under Materials and Methods.

**Calibration, precision, and detection limit.** The procedure displayed a linear response up to approximately 800 ng Se. Consequently, the sensitivity of the fluorescence detector was increased until the detector's non-linearity began to become significant at around 800 ng Se, so that the detector's linear dynamic range coincided with that of the method. The detector's response



**FIG. 3.** Log-log regression of analyzed Se concentrations against certified Se concentrations for 23 biological reference materials. Se was determined in each reference material multiple times and the logarithms of the mean values were regressed against the logarithms of the certified values. The correlation coefficient ( $r$ ) was 0.999, with a slope of  $0.983 \pm 0.020$  (95% CI) and an intercept of  $0.032 \pm 0.053$  (95% CI).

time was then adjusted to minimize the baseline noise without degrading the chromatographic resolution. The detector conditions thus obtained provided the widest possible linear dynamic range while optimizing the detectability of the Se peak in blanks. The accuracy of determinations of  $<50$  ng Se was improved by forcing the intercept of the calibration graph through zero. Calibration graphs were typically linear up to 800 ng Se ( $r > 0.99$ ) (Fig. 2). However, for reasons we were not able to determine, some calibration graphs were occasionally linear up to only around 600 ng.

The within-batch and between-batch imprecision of the method was separated and estimated by one-way analyses of variance of the data obtained by analyzing five replicates of several levels of Se within each of five separate analytical batches (8, 9). As is typical of an analytical system with such a wide dynamic range, the within-batch standard deviation increased with increasing Se. The within-batch standard deviation of the blank was used to estimate the mass detection limit of the complete procedure as 48 pg ( $3\sigma$ ). Because no more than 0.4 g of dry material was compatible with the digestion conditions and some sample types (whole egg powder, dogfish muscle, and human hair, Table 2) only gave good recoveries of Se at sample masses of about 0.1 g, this corresponded to concentration detection limits of 48 parts-per-trillion (ppt) for biological fluids, 120 ppt for 0.4 g dry samples, and about 480 ppt for 0.1 g dry samples.

In addition to sample size, the other main determinant of the detection limit in this method was the size of the piarselenol peak from blanks, due to traces of Se in the reagents. Nitric acid was found to be the main contributor of Se in the blanks. Reagent-grade nitric

acid designated as suitable for trace metals analysis (AR Select, Mallinckrodt) contributed about 5 ng of Se to the blank. The AR Select reagent-grade perchloric and hydrochloric acids were not significant contributors of Se in the blank. Although the contribution to the blank's Se from reagent-grade ammonium hydroxide was small (about 50 pg), redistilled product was used to minimize the variability of the blank because each tube received different quantities of ammonium hydroxide during the pH adjustment. Application of these measures to minimize Se contamination from the reagents resulted in an average peak height in the blanks equivalent to 0.11 ng Se (Table 1).

*Validation with biological reference materials.* To assess the accuracy of the proposed method, 23 biological reference materials, certified as to their Se concentrations, were analyzed repeatedly. The results are shown in Table 2. In all but 3 cases, the mean value determined by the proposed method was within the uncertainty range established by the certifying agency. Dogfish muscle, dogfish liver, and human hair mean measured values were outside the uncertainty ranges and were an average of 11% below their certified values. However, the 95% confidence intervals of the Se concentrations we measured in these 3 materials each included the respective certified value, so none of the measured Se concentrations differed significantly from their certified values. Regression of the logarithms of the 23 mean Se concentrations against the logarithms of the certified values indicated a very close agreement ( $r = 0.999$ ) with no evidence of systematic constant (intercept =  $0.032 \pm 0.053$  [95% CI]) or proportional (slope =  $0.983 \pm 0.020$  [95% CI]) errors (Fig. 3). The data were transformed to logarithms to reduce the inherent weighting of larger values that occurs when regressing values that cover an 1800-fold range.

## DISCUSSION

The absolute mass detection limit of the proposed method (48 pg) is not significantly different from that reported for the method upon which it is based (50 pg) (6). However, due primarily to the larger capacity of the sample digestion and the larger volume of cyclohexane injected onto the column in the proposed method, the concentration detection limits were improved 26-fold compared to earlier chromatographic methods for Se (48 ppt vs 1250 ppt) (6). Minimization of Se contamination from the reagents and optimization of the fluorescence detector also contributed to the improved detection limits. The concentration detection limit of the proposed method in biological fluids is comparable to the most sensitive Se determination methods available (5, 10) and should be adequate for Se determinations in any biological material.

One factor that has limited the application of fluo-

rometric Se assays has been the need either to tailor the digestion conditions for each sample type or to digest each sample individually to a visual endpoint (11–13). The digestion conditions in the proposed method are suitable for all of the sample types tested, allowing a mixture of different sample types to be digested in one uniform batch without constant attention. The wide linear dynamic range precludes any need to re-assay or dilute samples to stay within the method's linear range. The main limitation of this digestion method is that the recovery of Se in some sample types decreased as sample mass was increased toward the upper limit of 0.4 g dry material. Because we did not systematically study the effects of sample mass on Se recoveries for all sample types, the listed sample sizes should be considered the maximum recommended sizes. The effect of sample size on Se recovery should be determined before applying this method to new sample matrices not listed in Table 2. However, the sensitivity of the method more than compensates for any limitations imposed by sample size restrictions, allowing a detection limit of <0.5 ppb, even in the most difficult samples.

As is the case for the original fluorometric method upon which it is based (3), the proposed method is free from interferences from other elements, due to mineralization of the organic sample matrix, complexation of metals by EDTA, selective reaction between Se(IV) and 2,3-diaminonaphthalene, chemical separation by solvent extraction, and the specific fluorescence of the piaszelenol. The proposed method offers an even greater selectivity for Se due to the chromatographic purification of the piaszelenol. The proposed method's accuracy and freedom from matrix interferences are demonstrated by the excellent agreement between the analyzed Se contents and the certified values of this very diverse group of biological reference materials covering an 1800-fold range of Se concentrations.

This improved method for Se determinations offers a combination of sensitivity, accuracy, linear dynamic range, and freedom from matrix interferences that is unsurpassed by any chemical or instrumental method.

The method is applicable to a very wide range of biological sample types. Up to 96 samples and standards of widely varying matrices and Se concentrations may be digested without close supervision and analyzed together in a single run. The proposed method exhibits the characteristics required of a reference method for the determination of Se in biological samples.

#### ACKNOWLEDGMENTS

This work was supported by the U.S. Department of Agriculture. Mention of trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture or the Agricultural Research Service, nor does it imply approval to the exclusion of other products that may be suitable. Opinions expressed herein represent those of the authors and do not necessarily represent those of the U.S. Department of Agriculture or the Agricultural Research Service.

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