Pergamon

0039-9140(94)E0060-5

Talanta, Vol. 41, No. 12, pp. 2025–2031, 1994

0039-9140/94 $7.00 + 0.00

CRITICAL STUDY OF FLUORIMETRIC DETERMINATION OF SELENIUM IN URINE

E. M. RODRIGUEZ, M. T. SANZ and C. DIAZ ROMERO*

Department of Analytical Chemistry, Food Science and Toxicology, 38071-La Laguna (Tenerife), Spain

(*Received* 26 *November* 1993. *Revised* 25 *February* 1994. *Accepted* 25 *February* 1994)

Summary—Different steps for the fluorimetric determination of Se in urine have been investigated. A HNO_3–$HClO_4$ (4:1) mixture is useful for urine digestion, and reduction of Se(VI) to Se(IV) is effectively carried out with HCl (6*M*). Selenium(VI) present after the digestion process constitutes 14.5–36.6% of total Se. An optimum pH of 1.80 ± 0.05 and the addition of 1 ml of 2,3-diaminonaphthalene (DAN) (0.1%, w/v) are established in the formation of Se–DAN complex. Heating to 60°C, a time of incubation of 15 min is recommended to assure the complete formation of Se–DAN complex. A volume of 5 ml of cyclohexane and vigorous shaking for 45 sec is necessary for the extraction process. With this optimized method, the detection limit of selenium was 0.82 μg/l., within-day precision for a 50.0 μg/l. standard solution and urine (27.3 μg/l.) were 2.4 and 2.7% and between-day for the urine was 3.9% (33.9 μg/l.). Analytical recovery of 0.5 ml of Se standard (250 μg/l.) added to 1 ml of urine was $99.9 \pm 2.9\%$ (95.8–104.4, $n = 12$). Normal levels of selenium excretion in urine obtained from healthy people were 27.9 ± 8.7 μg/day (13.2–44.1), not observing significant differences ($P < 0.05$) between sexes.

There is a rather narrow range of adequacy of several essential elements, such as Se, in human beings. Although it is firmly established that Se is an essential element,[1,2] initially interest in Se was caused by its potential toxicity.[3] Thus, there has been an increasing interest in the determination of selenium, in a wide variety of matrices for establishing appropriate Se intake and/or supplementation guidelines, and for the monitoring of environmental and occupational exposure.[2,4]

Selenium is somewhat unique among trace elements in that several good and independent analytical methods have been developed. Some authors have reviewed the analytical procedures for Se determination in urine[5] and several human fluids.[6,7] Three analytical methods currently in use depend, respectively, on neutron activation analysis, atomic-absorption spectrometry and spectrofluorimetry.

Spectrofluorimetry is very useful in Se determination in urine and other body fluids because it is not too expensive and has a good sensitivity and precision.[5,7] However, sample treatment is normally long and tedious. So, spectrofluorimetric measurements utilize the fluorescence of the Se–DAN complex (piazselenols) derived from selenite. All original species of Se present in the sample must be converted to selenite. Thus, an acid-digestion step is initially required, previous to a reduction step to reduce Se(VI) to Se(IV).[5,7] Few papers[8] have been published to determine the trimethylselenonium ion ($TMSe^+$) and other Se species from urine by spectrofluorimetry. Analytical methodology for separation of $TMSe^+$ and other Se compounds by cation exchange chromatography and fluorimetric determination have been proposed.[8]

In the present paper, all the steps for the fluorimetric determination of Se in urine have been critically studied in order to improve this determination.

EXPERIMENTAL

Apparatus

Fluorescence Spectrophotometer Perkin–Elmer model MFP-44A with automatic recorder, arc of xenon Osram XBO, differential corrected spectra unit DCSU-1, digital lector VDR-3, and heating water bath Selecta Frigitherm S-382.

Reagents and solutions

Selenium standard solution (1 mg/ml) Fisher for AAS. Working standards (0–250 μg/l.) were

*Author to whom correspondence should be addressed.

prepared for dilution of the latter solution with milli-Q water.

2,3-Diaminonaphthalene (DAN) solution (0.1%, w/v): DAN (0.1 g, previously crystallized) was weighed into a 250 ml conical flask. HCl (100 ml, 0.1*M*) with 0.5 g of hydroxylammonium chloride was added and the solution placed in a 60°C water bath for 15 min. The cold solution was transferred to a separatory funnel and the impurities of DAN extracted with 10 ml of cyclohexane. This washing procedure was repeated three more times, discarding the cyclohexane layer each time. The aqueous phase was filtered through filter porous no. 4 and stored in darkness at 4°C.

Crystallization of DAN: 1 g of DAN was dissolved into 60 ml ethanol, heated to 50°C in a dark room, the solution cooled to −20°C and the brown crystals filtered through filter porous no. 4. The crystals obtained were dried and stored at −20°C.

Disodium ethylenediaminetetracetic acid ($Na_2EDTA \cdot 2H_2O$) solution (0.04*M*): 7.44 g of $Na_2EDTA \cdot 2H_2O$ was dissolved and diluted to 1 l. with milli-Q water.

Nitric, perchloric, sulfuric, hydrochloric acids, cyclohexane and other reagents used were of analytical quality.

Samples

Urinary samples were collected over a 24-hr period since Se can exhibit diurnal variations as a result of variation in drinking patterns.[5] The samples were stored frozen (−20°C) in containers of polyethylene without conservants. These containers were previously cleaned with detergent, water soaked overnight with HNO_3 (7.2*M*), and rinsed several times with milli-Q water.

If sedimentation occurred, a suspension was produced by vigorous shaking, before taking the aliquot for analysis.

Optimized procedure

One ml of urine perfectly measured and homogenized and 5 ml of acid mixture HNO_3–$HClO_4$ (4:1) were placed in a beaker closed with a coquille and left overnight. Next morning, the acid mixture was progressively heated, in order to avoid the possible losses of volatile Se compounds, according to the following sequence: 110°C/25 min; 140°C/30 min; 180°C/30 min. After fumes of $HClO_4$ appeared, the acid digest was heated for another 5 min. Then, 1 ml of HCl (6*M*) was added to the digest and heated to 180°C/5 min in order to reduce Se(VI) to Se(IV). Consecutively, 2 ml of Na_2EDTA and 1 ml of DAN were added to the pyrex tubes with the solution obtained above. Later, the pH was adjusted to 1.80 ± 0.05 with NH_3 (13.3*M*) and HCl (7.7*M*) and this solution was heated in 60°C water bath for 15 min. After cooling, the Se–DAN complex was shaken and extracted with 5 ml of cyclohexane for 45 sec. The organic phase was separated by centrifugation at 2000 rpm and the fluorescence of this phase measured at $\lambda_{ex} = 377$ nm and $\lambda_{em} = 516$ nm.

RESULTS AND DISCUSSION

The fluorimetric determination of Se can be divided into four steps: (1) digestion; (2) reduction; (3) formation of Se–DAN complex; (4) extraction.

1. *Digestion of urine samples*

The effect of previous drying temperature of urine samples on Se losses was studied. Previously to fluorimetric Se determination, several urine samples were dried at 75°C and 100°C. The results obtained were compared with those found without a previous drying process. No significant differences ($P < 0.05$) were observed between dried samples and undried samples.

There is a controversy about the need for previous mineralization of urine samples for the fluorimetric determination of Se. Some authors[9,10] point out that the digestion of urine and most biological materials do not require the use of $HClO_4$. However, the majority of authors[11,12] indicate that complete oxidation of urinary Se compounds requires the use of HNO_3–$HClO_4$ and other mixtures such as HNO_3–H_2O_2, H_2SO_4–H_2O_2 or HNO_3–H_2SO_4, indicating also that continuous combustion and oxygen flask methods are not suitable. The presence of $TMSe^+$ and other Se compounds in the urine can resist the oxidation except with HNO_3–$HClO_4$.[13,14]

Some of the acid mixtures used in the literature have been assayed for digestion of urine samples. The behavior of the urine samples with respect to this step of digestion depends on the amount of organic matter present. The acid mixtures assayed were 5 ml of: HNO_3–$HClO_4$ (4:1); HNO_3–H_2SO_4 (2:1); HNO_3–H_3PO_4 (4:1); and HNO_3–H_2SO_4–$HClO_4$ (4:2:1). Aliquots of 1 ml of a urine sample were treated with 5 ml

of the acid mixture, and the experimental procedure was followed afterwards. Figure 1a shows the fluorescent spectra of the organic extracts obtained with each acid mixture. Except for the HNO_3–$HClO_4$ mixture, all acid mixtures had a yellow colour after the digestion process due to incomplete oxidation of the urine sample. Also, a good resolution of fluorescent peak was only obtained when the acid mixture HNO_3–$HClO_4$ was used (Figure 1a).

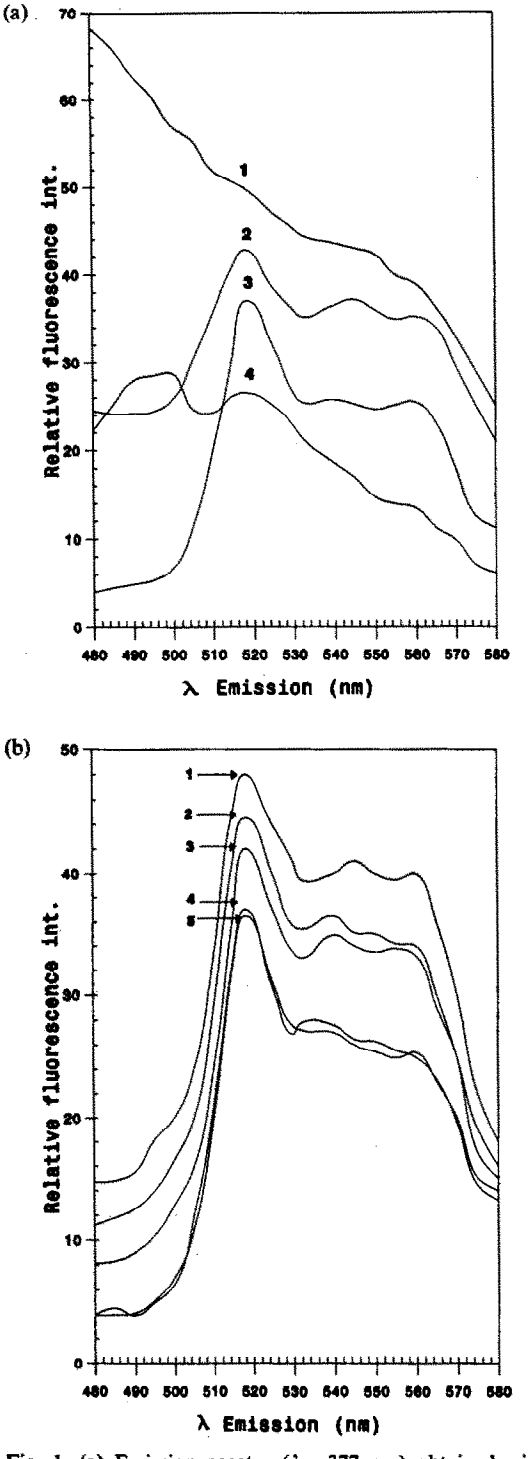

Fig. 1. (a) Emission spectra ($\lambda = 377$ nm) obtained with different acid mixture: (1) HNO_3–H_3PO_4, (2) HNO_3–H_2SO_4–$HClO_4$, (3) HNO_3–$HClO_4$ and (4) HNO_3–H_2SO_4. (b) Emission spectra ($\lambda = 377$ nm) obtained with different proportions of the acid mixture HNO_3–$HClO_4$: (1) 1:1, (2) 2:1, (3) 3:1, (4) 4:1 and (5) 5:1.

Table 1. Influence of the concentration of HCl and temperature/time in the reduction step

	Rel. fluorescence intensity				
	Sample			Temperature (°C)	
HCl concentration	20 μg Se/l.	Urine	Time (min)	100	180
0.0	21.0	22.1	2	40.5	46.3
1.0	21.4	27.1	5	41.3	51.1
3.0	20.0	27.0	10	44.6	50.9
6.0	20.7	26.0	20	50.9	45.3
7.7	20.5	27.7	30	51.8	46.6

In order to determine the optimum ratio of the selected acid mixture, several ratios between both acids were applied to aliquots of a urine sample (Figure 1b). After a 4:1 proportion (HNO_3–$HClO_4$), the digestion of sample was complete because no colour was observed in the extract of cyclohexane, and the height of the peak of the Se–DAN complex was constant.

2. *Reduction of* Se(VI) *to* Se(IV)

After digestion, it must be ensured that all selenium in the sample is in the tetravalent state. This is the only form of selenium that reacts with diaminonaphthalene to give the derived fluorescent species. Generally, in this step, HCl (4–6*M*) is the reducing agent used but some laboratories have reported the use of H_2O_2[15,16] or hydroxylamine.[17–19]

Table 1 presents the influence of the HCl concentration and temperature (100°C and 180°C) on the reduction process. The described procedure was applied to several aliquots of a urine sample, using different concentrations of HCl. It can be concluded that the reduction step

Table 2. Percentage of Se(VI) with respect to total selenium after the digestion step*

	Se (μg/l.)		
Urine	Without reduction	With reduction	Percentage Se(VI)
1	18.305	25.250	27.5
2	21.450	28.489	24.7
3	22.836	34.895	14.5
4	9.499	14.976	36.6
5	8.373	10.675	21.6
6	24.764	32.299	23.5
7	20.441	29.625	31.0

*Average of two determinations.

is independent of the concentration of HCl. However, it must be considered that factors such as type of urine, amounts of organic matter, digestion process, *etc.*, can influence the amount of Se(VI) present after the digestion process. Thus, a concentration 6*M* was selected to ensure that all possible Se(VI) present after digestion was reduced to Se(IV). Using this concentration, the influence of temperature on the fluorescence of reaction was studied. Twenty-five minutes was necessary to achieve the total reduction of the Se(VI) when heating to 100°C and 180°C (Table 1). In contrast with some authors,[11,20] no losses of Se were found when the temperature of reaction was 100°C. However, if the temperature was maintained at 180°C, losses of 9.7–12.3% were observed after 20–30 min.

Selenium concentrations in urine samples and standards, with and without the reduction step, are shown in Table 2. The results obtained with the Se(IV) standard indicate that there are significant differences ($P < 0.05$) between the means obtained with and without the reduction step. Thus, Se(IV) is not oxidized to Se(VI) with the digestion method proposed. The results observed with reduction treatment were higher than those obtained without reduction step, when urine samples were used. It can be deduced that Se(VI) present after the digestion process if variable, constituting $27.0 \pm 7.1\%$ (14.5–36.6%) of the total Se.

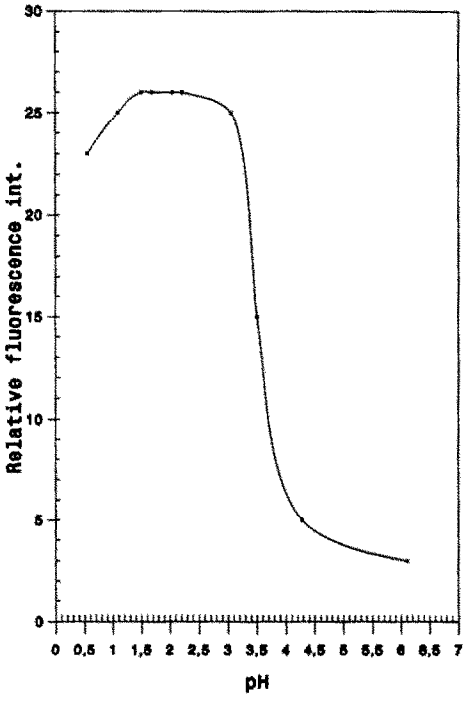

Fig. 2. Variation of the fluorescence intensity of the Se–DAN complex with the pH of reaction mixture.

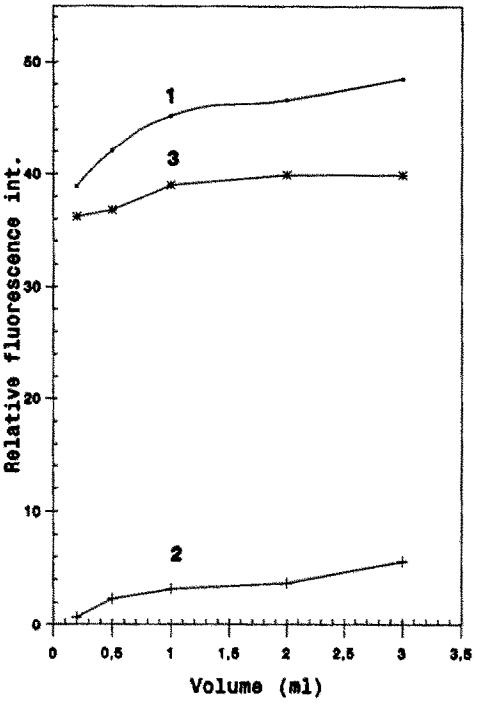

Fig. 3. Variation of the fluorescence intensity of the Se–DAN complex with the volume of DAN (0.1%, w/v) added. (1) Piazselenol complex, (2) blank, (3) difference 1–2.

3. *Formation of* Se–*DAN complex*

There is no agreement about the optimal conditions such as: pH, DAN concentration and temperature/time of incubation, for the formation of Se–DAN complex.

*Influence of p*H. Many authors have indicated that the optimum pH in the formation of piazselenol is between 1 and 2.[11,13,19,21–32]

In the present study, 1 ml of standard of Se (250 μg/l.), 2 ml of DAN, and 2 ml of EDTA were mixed in pyrex tube. Then, a buffer of glycine/HCl of pH 2.4 was added and the pH was adjusted to a value between 0.5 and 6 with NH_3 or HCl. The formation of complex and its extraction were carried out according to the described method.

Maximum fluorescent signal of the Se–DAN complex was observed in the range of pH 1–3 (Fig. 2) which agrees with the results obtained by Bayfield and Romalis.[33] However, we recommend a pH 1.80 ± 0.05 in order to ensure a maximum extraction. The adjustment of pH must be carried out before the addition of DAN, because it can be degraded in a strong acid medium.

Table 3. Influence of the relation temperature/time in formation of the piazselenol complex

Time (min)	Rel. fluorescence intensity (% recovery)* Temperature (°C) 25	40	60	80
2		20.0 (51.0%)	31.0 (79.1%)	36.6 (93.4%)
5		25.7 (65.7%)	38.0 (96.9%)	38.9 (99.3%)
10	24.8 (63.3%)	33.1 (84.5%)	39.0 (99.5%)	40.8 (104.1%)
15		37.3 (95.2%)	38.9 (99.3%)	40.4 (103.1%)
20	27.0 (68.9%)	38.5 (98.3%)	38.9 (99.3%)	39.0 (99.53%)
30	32.0 (81.7%)	38.8 (99.0%)	39.1 (99.7%)	38.6 (98.5%)
45	34.0 (86.8%)			
60	35.3 (90.1%)			

*Percentage with respect to the mean fluorescence of the added standard of 40 μg/l.: $x \pm SD = 39.2 \pm 0.6$ ($n = 10$).

Influence of DAN concentration. Some authors[21] have studied the effects of temperature and concentration of DAN on the reaction of DAN and Se(IV). They selected a concentration of DAN dihydrochloride of 0.5% (w/v) and a reaction temperature of 40°C.

Solutions prepared with 1 ml Se(IV) (50 μg/l.), 2 ml EDTA, 2 ml water milli-Q, and variable amounts (0.2–3 ml) of DAN, were treated with the described procedure without the digestion and reduction steps. For each solution, a blank was also prepared. When the concentration of DAN was increased, so did the fluorescent signal, but it had the disadvantage of producing a high blank reading. The percentage of fluorescence of all the considered solutions as well as the difference between the sample and blank are shown in Fig. 3. A plateau in the difference of fluorescent signal and the corresponding blanks were reached when the added volume of DAN was higher than 1 ml. One millilitre of DAN was chosen as optimal, since volumes higher than 1 ml produce the same difference between the fluorescent signal of standard and blank.

Influence of temperature/time of incubation. There is no agreement about the optimal temperature/time relation for the formation of Se–DAN complex: 110°C/30 min,[34] 75°C/10 min,[19] 60°C/30 min[31] or 20 min,[35] 50°C/30 min[36,37] or 15 min,[30] among others, have been proposed.

Table 3 shows the fluorescence of the cyclohexane extracts relative with respect to the mean value of the standard of Se and percentage of recovery obtained of Se standards (1 ml 40 μg Se/l.) which were undertaken with 2 ml of EDTA, 2 ml water milli-Q and 1 ml of DAN to pH 1.8, in different relations temperature/time. Incomplete recovery, 90.1%, was reached at room temperature after 1 hr. However, complete formation of the Se–DAN complex was obtained at 40°C/20 min, 60°C/10 min, 80°C/5 min. The relation 60°C/15 min was adopted in order to ensure greater tolerance margin for the total formation of Se–DAN complex as well as decreasing the DAN degradation risk.

4. *Extraction of* Se–*DAN complex*

The cyclohexane extraction allows preconcentration of Se and eliminates the non-soluble interferences in cyclohexane. For this study, 1 ml of Se standard (250 μg/l.), 2 ml of EDTA solution and 2 ml of DAN were placed in a pyrex tube. After adjustment of pH (1.80 ± 0.05), formation of the Se–DAN complex was carried out following the experimental procedure. The complex was successively extracted with different volumes (5 and 10 ml) of cyclohexane. All Se was extracted with three extractions for both volumes of the used extractants, giving only values of 1.1% of Se in the third extraction (Table 4). In the first extraction, levels higher than 90% were reached, using 5 and 10 ml of cyclohexane. A volume of 5 ml was

Table 4. Percentage of the extracted Se–DAN complex with the shaking time

Time (sec)	Rel. fluorescence int. (% recovery)*
0	1.65 (7.3)
5	18.03 (79.4)
15	20.83 (91.8)
30	21.73 (95.7)
45	22.63 (99.7)
60	22.73 (100.1)
90	22.63 (99.7)

*Percentage with respect to the mean fluorescence of added standard 20 μg Se/l.: $x \pm SD = 22.66 \pm 0.06$ ($n = 4$).

Table 5. Urinary Se concentration (μg/l.) and daily Se excretion (μg/day) in healthy people

Sample	n	Se ($\mu g/day$) mean $\pm$ SD	Range	Se ($\mu g/l.$) mean $\pm$ SD	Range
Females	10	27.193 $\pm$ 8.896	(13.161–42.528)	29.910 $\pm$ 10.243	(17.231–55.446)
Males	10	28.734 $\pm$ 8.949	(13.371–44.109)	25.009 $\pm$ 4.792	(16.791–33.930)
Total	20	27.964 $\pm$ 8.820	(13.161–44.109)	27.459 $\pm$ 8.179	(16.791–55.446)

proposed to improve the preconcentration of Se.

The vigorous shaking time was also studied (Table 4). The conditions of this study were the same as above but using 5 ml of cyclohexane throughout. A 7% Se–DAN complex was extracted without the shaking process and a time of 45 sec was considered optimum for complete extraction of the complex.

Validation and applicability of the optimized method

The optimized method was assayed on normal and Se spiked urinary samples. The obtained recovery (99.9 $\pm$ 2.9%) was almost complete, which agrees with most authors. The within-day precision of the method was 3.2% (11.9 μg Se/l. $n = 5$) or 2.7% (27.3 μg Se/l., $n = 5$) for urine samples, and 2.4% (50.0 μg Se/l., $n = 8$) or 0.8% (260.0 μg Se/l., $n = 5$) for Se standard solutions. While between-day precision was 4.4% (24.8 μg/l., $n = 6$) or 3.9% (33.9 μg/l., $n = 6$) for urine samples. A calibration curve to determine the concentration of selenium in a sample solution, was obtained by treating a standard selenium solution with the same procedure as the urinary samples:

$$F = 0.812 \times [\text{Se}(\mu\text{g/l.})] - 0.371 \quad (r = 0.9997;\ n = 10).$$

The linear interval was confirmed between 0 and 250 μg/l. and the optimum interval for measurement was 10–50 μg/l. The detection limit of this fluorimetric procedure (mean + 3SD of the blank fluorescence value), was 0.82 μg/l. (0.82 ng of Se).

This method optimized was applied to 20 samples of urine of health people, the results are summarized in Table 5. All samples contained a Se concentration of no more than 100 μg/l, which represents the maximum allowable concentration recommended by Glover.[38,29]

The urinary Se concentrations obtained in this work were higher than those reported in low Se areas where Keshan disease was found.[40–42] On the other hand, our data were lower than the average urine concentrations of residents in seleniferous areas of China,[40–42] Wyoming[43,44] and South Dakota[45] of the U.S.A. or Venezuela.[46,47] Similarly to most regions in Europe,[48,49] the average Se is normally considered as a reference value.[50,51]

No significant differences ($P < 0.05$) were found between the mean concentrations observed in males and females, which agrees with Oster and Prellwitz.[49] In contrast, some authors[48] have reported that male Greeks excrete less ($0.011 < P < 0.05$) Se than female Greeks.

REFERENCES

1. H. A. Schroeder, D. V. Frost and J. J. Balassa, *J. Chron. Dis.*, 1970, **23**, 227.
2. O. A. Levander, *Ann. Rev. Nutr.*, 1987, **7**, 227.
3. C. Wilber, *Clin. Toxicol.*, 1980, **17**, 171.
4. O. A. Levander, *Fed. Proc.*, 1985, **44**, 2579.
5. H. J. Robberecht and H. A. Deelstra, *Talanta*, 1984, **31**, 497.
6. L. Fishbein, *Int. J. Environ. Anal. Chem.*, 1984, **17**, 113.
7. M. Sanz Alaejos and C. Díaz Romero, *Clin. Chem. (Winston-Salem, N. C.)*, 1993, **39**, 2040.
8. R. Hasunuma, T. Ogawa and Y. Kawanishi, *Bull. Environ. Contam. Toxicol.*, 1993, **50**, 19.
9. V. W. Bunker and H. T. Delves, *Anal. Chim. Acta*, 1987, **201**, 331.
10. V. W. Bunker, M. S. Lawson, M. F. Stansfield and B. E. Clayton, *Br. J. Nutr.*, 1988, **59**, 171.
11. Analytical Methods Committee, *Analyst (London)*, 1979, **104**, 778.
12. M. Janghorbani, B. T. G. Ting, A. Nahapetian and V. R. Young, *Anal. Chem.*, 1982, **54**, 1188.
13. O. E. Olson, I. S. Palmer and E. E. Cary, *J. Assoc. Off. Anal. Chem.*, 1975, **58**, 117.
14. J. Nève, M. Hanocq, L. Molle and G. Lefebvre, *Analyst (London)*, 1982, **107**, 934.
15. N. D. Michie, E. J. Dixon and N. G. Bunton, *J. Assoc. Off. Anal. Chem.*, 1978, **61**, 48.
16. G. Norheim, K. Saeed and Y. Thomassen, *Atmos. Spectrosc.*, 1983, **4**, 99.
17. B. Holynska and K. Lipinska-Kalita, *Radiochem. Radioanal. Lett.*, 1977, **30**, 241.
18. H. J. Peters and H. Koehler, *Zentralbl. Pharm. Pharmakother. Laboratoriumdiagn.*, 1982, **121**, 127.
19. R. Yang, J. Huang, G. Feng and Z. Mei, *Med. Lab. Sci.*, 1986, **43**, 331.
20. A. I. Vogel, *A Text-book of Macro and Semimicro Qualitative Inorganic Analysis*, 4th Edn., p. 584. Longmans, London, 1954.
21. M. W. Brown and J. H. Watkinson, *Anal. Chim. Acta*, 1977, **89**, 29.

22. A. Geahchan and P. Chambon, *Clin. Chem. (Winston-Salem, N.C.)*, 1980, **26**, 1272.
23. S. Y. Chen, P. J. Collipp, L. H. Boasi, D. S. Isenschmid, R. J. Verolla, G. A. San Román and J. K. Yeh, *Ann. Nutr. Metab.*, 1982, **26**, 186.
24. J. H. Watkinson, *Anal. Chem.*, 1966, **38**, 92.
25. P. R. Haddad and L. E. Smythe, *Talanta*, 1974, **21**, 859.
26. C. C. Y. Chan, *Anal. Chim. Acta*, 1976, **82**, 213.
27. J. E. Spallholz, G. F. Collins and K. Schwarz, *Bioinorg. Chem.*, 1978, **9**, 453.
28. L. Lalonde, Y. Jean, K. D. Roberts, A. Chapdelaine and G. Bleau, *Clin. Chem. (Winston-Salem, N.C.)*, 1982, **28**, 172.
29. G. Alfthan, *Anal. Chim. Acta*, 1984, **165**, 187.
30. Y. Tamari, S. Ohmori and K. Hiraki, *Clin. Chem. (Winston-Salem, N.C.)*, 1986, **32**, 1464.
31. J. Pettersson, L. Hansson, U. Örnemark and A. Olin, *Clin. Chem. (Winston-Salem, N.C.)*, 1988, **34**, 1908.
32. Z. Szilvassy-Vamos, J. Lazar, M. Horvath and A. Kertai-Simon, *J. Anal. Atmos. Spectrom.*, 1990, **5**, 705.
33. R. F. Bayfield and L. F. Romalis, *Anal. Biochem.*, 1985, **144**, 569.
34. J. A. Cocho, C. Parrado, J. R. Cervilla, J. R. Alonso-Fernández and J. M. Fraga, *Quim. Clín.*, 1984, **3**, 19.
35. H. W. Lane, S. Dudrick and D. C. Warren, *Proc. Soc. Exp. Biol. Med.*, 1981, **167**, 383.
36. P. A. Whetter and D. E. Ullrey, *J. Assoc. Off. Anal. Chem.*, 1978, **61**, 927.
37. E. B. Thorling, K. Overvad, A. Heerfordt and A. Foldspang, *Biol. Trace Elem. Res.*, 1985, **8**, 65.
38. J. R. Glover, *Ann. Occup. Hyg.*, 1967, **10**, 3.
39. S. Amin, S. Y. Chen, P. J. Collipp, M. Castro-Magana, V. T. Maddaiah and S. Klein, *Nutr. Metab.*, 1980, **24**, 331.
40. G. Yang, S. Wang, R. Zhou and S. Sun, *Am. J. Clin. Nutr.*, 1983, **37**, 872.
41. G. Yang, R. Zhou, S. Yin, L. Gu, B. Yan, Y. Liu, Y. Liu and X. Li, *J. Trace Elem. Electrolytes Hlth Dis.*, 1989, **3**, 77.
42. G. Yang, S. Yin, R. Zhou, L. Gu, B. Yan, Y. Liu and Y. Liu, *J. Trace Elem. Electrolytes Hlth Dis.*, 1989, **3**, 123.
43. J. L. Valentine, H. K. Kang, Ph.-M. Dang and M. Schluchter, *J. Toxicol. Environ. Hlth*, 1980, **6**, 731.
44. J. L. Valentine, B. Faraji and H. K. Kang, *Environ. Res.*, 1988, **45**, 16.
45. I. S. Palmer, O. E. Olson, L. Ketterling and C. E. Shank, *J. Am. Diet. Assoc.*, 1983, **82**, 511.
46. M. C. de Mondragón and W. G. Jaffé, *Arch. Latinoam. Nutr.*, 1971, **21**, 185.
47. W. G. Jaffé, M. D. Ruphael, M. C. de Mondragón and M. A. Cuevas, *Arch. Latinoam. Nutr.*, 1972, **22**, 595.
48. M. S. Bratakos, H. C. Kanaki, A. Vasiliou-Waite and P.V. Ioannou, *Sci. Total Environ.*, 1990, **91**, 161.
49. O. Oster and W. Prellwitz, *Biol. Trace Elem. Res.*, 1990, **24**, 119.
50. J. H. Watkinson, *N. Z. Med. J.*, 1974, **523**, 202.
51. V. Iyengar and J. Woittiez, *Clin. Chem. (Winston-Salem, N.C.)*, 1988, **34**, 474.