An Improved Spectrofluorometric Determination of Selenium in Biological Materials After Microwave Digestion

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ABSTRACT

In the present work, a simple spectrofluorometric method for the determination of selenium in biological materials using microwave-assisted digestion was improved. The conditions of reduction of Se(VI) to Se(IV) and its complexation with DAN were studied as well as the excitation and emission wavelengths that provide higher fluorescence intensity. One of the goals in our research work was that no further adjustment of pH was required before solvent extraction since minor fluorescence background is obtained with the intrinsic H^+ concentration. The linear working range was found to be 0–48 ng Se/mL cyclohexane. Validation of the method was conducted using four certified reference materials: NIST CRM 1568a Rice Flour, NIST CRM 8415 Whole Egg Powder, NIST CRM 8418 Wheat Gluten, and NIST CRM 2781 Domestic Sludge. The recoveries obtained ranged between 90 and 110%. The limit of detection of our improved method was 11.0 ng of selenium per gram of biological matrix.

INTRODUCTION

In the last five decades, there has been an increasing interest in the trace determination of selenium (Se). This element has been recognized as an essential nutrient for humans based on its presence in the enzyme glutathione peroxidase (GSHPx), which affords cell protection against oxidative damage (1). Several research works have been published describing the beneficial role of Se in the treatment and prevention of some important cancers in humans (2–14), as well as in other pathologies such as infertility (15), hypothyroidism (16), and viral infections (17,18).

Selenium is a remarkable element in natural systems because of its dual role as both an essential nutrient at low concentrations (the recommended daily allowance is 55 μg day^{-1} for men and women) and a toxic substance at higher levels (more than 500 μg day^{-1}) (19, 20). The concentration range of Se as an essential element versus a toxic one is, moreover, rather narrow.

Because of its significance, several analytical techniques have been reported concerning the accurate determination of selenium in the environment, particularly in food products (21–24). Among these methods, spectrofluorometric analysis is a commonly used method for the analysis of biological materials using diamino compounds such as 3,3'-diaminobenzidine (DAB) (25-27) and 2,3-diaminonaphtalene (DAN) (28–35). Currently, the latter is the fluorescent reagent that gives the best results due to its versatility, sensitivity, and selectivity for Se(IV). The fluorometric method, based on the reaction of Se(IV) with DAN to form the fluorescent naphto-2-selena-1,3-diazole (4,5-benzopiazselenol), has been the most widely used chemical method for the determination of trace Se levels in biological materials.

A key aspect of Se determination in biological materials has been the need for decomposition of the sample, either to make Se available for the analytical measurement or to destroy the organic sample matrix to remove interferences. Conventional wet digestion in mineral acid mixtures (nitric acid, sulphuric acid, and perchloric acid) 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 fluorometric determinations of Se (29,31–34). In the wet digestion process, the samples are highly exposed to contamination, and volatile Se compounds can be lost causing large errors in the final determination. Recently, there has been an increasing interest in using microwave digestion methods to speed up the dissolution of a variety of biological materials. The merits of pressurized acid digestion in closed vessels with microwave heating, particularly the increased speed and reduced losses of volatile elements, are widely recognized. Additionally, sample dissolution can be achieved using microwave digestion with HNO_3 alone. Thus, the method can effectively avoid sample contamination resulting from the environment and from reagents (34,36).

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In many studies, Se quantification is performed using the spectrofluoromentric methodology with an adjustment of pH (28-33,35). But T. Koh and T.H. Benson (37) reported that the tedious step of pH adjustment is not only unnecessary but also has the disadvantage of producing a high blank reading. Yet, controversy exists regarding the pH adjustment and, at the present time, some researchers still include this step as fundamental for the correct Se determination.

Thus, in this paper, an improved approach to the spectrofluorometric determination of Se with DAN in biological materials is proposed. The approache includes a microwave preparation of biological materials and Se(IV) measurement by spectrofluorometry with no pH adjustment after the digestion process and the use of 375 nm as the excitation wavelength.

EXPERIMENTAL

Instrumentation

A Milestone microwave digestion system (Model MLS 1200 MEGA, Milestone, Italy) with a maximum power of 650 W was used in the experiment. The equipment has a turntable on which ten 100-mL tetrafluormethaxil (TFM) Teflon™ vessels with safety pressure-relief disks can be assembled. The system allows the programming of both pressure and time.

All fluorimetric measurements were performed on a PerkinElmer® Model LS-3 fluorescence spectrometer (PerkinElmer Life and Analytical Sciences, Shelton, CT, USA), equipped with a Xenon discharge lamp pulsed at line frequency (50 Hz) and an F/3 Monk-Gillieron (8.3 W) type monochromator.

Reagents and Solutions

All chemicals used in the sample preparation and analysis were of analytical grade or better. All beakers, funnels, and calibrated flasks, Teflon® vessels, together with other glassware used for the experiments were soaked in nitric acid (ca. 5M) for 24 hours, cleaned with non-ionic detergent, and then rinsed several times with ultrapure water before use.

All solutions were prepared with ultrapure water with a specific resistivity of 18 MΩ·cm obtained by filtering distilled water through a Milli-Q™ Plus purifier system (Millipore Corporation, Bedford, MA, USA) immediately before use.

Digestion of the matrix was carried out using concentrated nitric acid (65%) and for reduction purposes of Se(VI) to Se(IV), fuming hydrochloric acid 37% was employed, both purchased from E. Merck, Darmstadt, Germany. 2,3-diaminonaphtalene (DAN) 95-98% was supplied by Sigma-Aldrich (St.Louis, MO, USA). The disodium salt of ethylenediaminetetraacetic acid (EDTA) employed in this work was provided by Merck. Selenium stock solution 1000 mg L^{-1} was prepared in 0.5M nitric acid (Merck) and working standard solutions were prepared by suitable dilution of this stock solution. The certified reference materials NIST 1568a Rice Flour, NIST 8415 Whole Egg Powder, NIST 8418 Wheat Gluten, and NIST 2781 Domestic Sludge were obtained from the National Institute of Standards and Technology, Gaithersburg, MD, USA, and used to validate the analytical method and the instrument used in the selenium determination.

A sufficient amount of DAN was dissolved in 0.1M HCl to give a 0.1% solution (warming softly, if necessary, at a maximum of 30 °C). Once the solution achieved room temperature, it was extracted with 25 mL cyclohexane (Mallinckrodt of chromatographic purity). This procedure was repeated three times and the organic phase was separated and filtered (using Macherey-Nagel MN 640W paper).

Caution:

The reagent 2,3-diaminonaphthalene (DAN) is very toxic and a possible carcinogen. Contact with skin, eyes, and airways must be avoided. In case of accidental poisoning, medical advice must be sought immediately.

Procedure

Less than 1 g of biological material, in order to contain 400 to 500 ng of Se, was exactly weighed, 5 mL of nitric acid (65%) was added, and then the samples were digested according to conditions listed in Table I. After digestion with concentrated nitric acid, the reduction of Se(VI) to Se(IV) was achieved by adding an equal volume of concentrated hydrochloric acid and heating the Teflon vessels in a water bath (85-90°C) until the fumes of nitrogen dioxide disappeared (at least for one hour). This solution was cooled to room temperature and 25 mL of ultrapure water was added. A 5-mL volume of the sample solution was poured into a Pyrex® glass tube with 16 mL 0.014M EDTA solution and 2 mL of 0.1% DAN solution (freshly prepared). The tubes were warmed for a short time (30 minutes) at 60°C and, as soon as the solution reached room temperature, the piazselenol formed (4,5-benzopiazselenol) was extracted by 5 mL

TABLE I
Microwave Oven Program Used for the Digestion of the Biological Samples[a]

Step	Power (W)	Time (min)
1	250	1
2	0	5
3	250	5
4	450	5
5	650	5

[a] Caution: Digestion vessels must be cooled for an appropriate time before opening in order to avoid burns from hot and corrosive vapors.

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cyclohexane. The fluorescence in the cyclohexane layer was measured at excitation wavelengths of 366 and 375 nm and an emission wavelength of 520 nm.

Duplicate blanks were obtained by adding only nitric acid to the digestion vessel and the same procedure was performed to the final solution.

The calibration curve was obtained by adding from 0 to 0.24 ng Se (as selenite), just before the addition of the EDTA solution, to different 5-mL aliquots of the blank solution.

RESULTS AND DISCUSSION

Optimization of Procedure

Excitation and Emission Wavelengths

In order to study the influence of the excitation and emission wavelength over the fluorescence intensity of the piazselenol compound in cyclohexane, we registered such intensity by varying the excitation wavelength from 360 to 380 nm and the emission wavelength from 510 to 530 nm, as can be seen in Figure 1.

An interesting behavior was observed over the fluorescence intensity while varying both emission and excitation wavelengths. In accordance with the literature (33,34,37,38), we realized that when using the 520-nm emission wavelength, we were able to maximize the intensity measured. On the other hand, some controversy regarding the excitation wavelength still exits, since some report (32-34,37,38) using from 364 to 379 nm. We were able to attain the highest fluorescence intensity at 375 nm (as shown in Figure 1).

We also demonstrate that the best fluorescence conditions are achieved while using 375 nm as the excitation wavelength and 520 nm as the emission wavelength.

EDTA Concentration

A solution of EDTA is added to the system immediately after the reduction process takes place. Due to its high coordinating capacity, EDTA is used to complex all other ions (Ca, Cu, Fe, Mg, Pb, among others) (28,35) that can interfere in the formation of the piazselenol product. In order to determine the concentration of EDTA (which gives at the same time the highest sensitivity and the lowest blank intensity), Figure 2 shows the relation between the fluorescence intensity measured for the blank and the concentration of EDTA added.

As expected, the higher the concentration of EDTA, the higher the blank intensity obtained. It can be explained in terms of the intrinsic fluorescence of the higher concentration of reactants. The same performance was achieved for EDTA concentrations below 0.014M. In this case, the EDTA concentration is not enough to complex all metallic ions that leave traces of elements in the system (i.e., Cu) which probably catalyzes the oxidation of the fluorometric reagent DAN and thus provokes an increase in the fluorescence intensity (28). K.L. Cheng (39) reported an increase in the intensity of the blanks caused by the oxidation of the spectrofluorimetric reagent 3,3'-diaminobenzidine (DAB) (chemically related to DAN) by the presence of Cu(II) and Fe(III).

Therefore, the "U" shape in the curve established an optimum concentration of 0.014M that produces the best possible results. To confirm this, Figure 3 shows the relationship between the sensitivity of the method (in terms of calibration slope) and the concentration of EDTA at 366 and 375 nm.

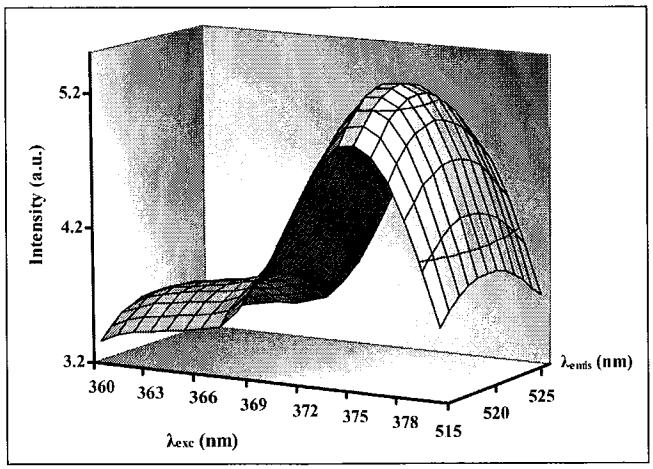

Fig. 1. Fluorescence intensity of the piazselenol in cyclohexane as a function of λ_{exc} and λ_{emis}.

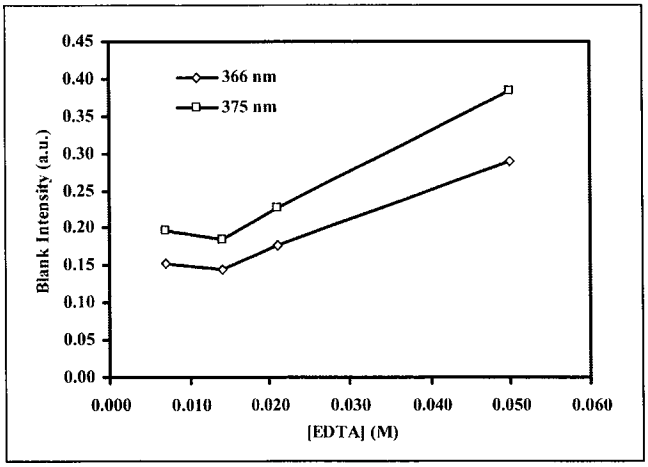

Fig. 2. Fluorescence intensity of the blank vs. EDTA concentration used.

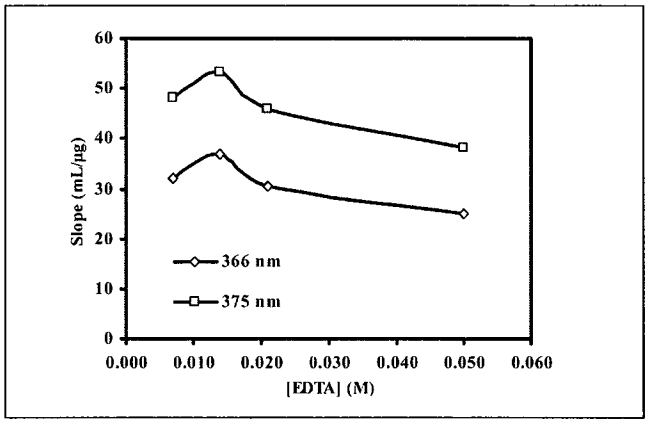

Fig. 3. Slope of the calibration curve vs. EDTA concentration used.

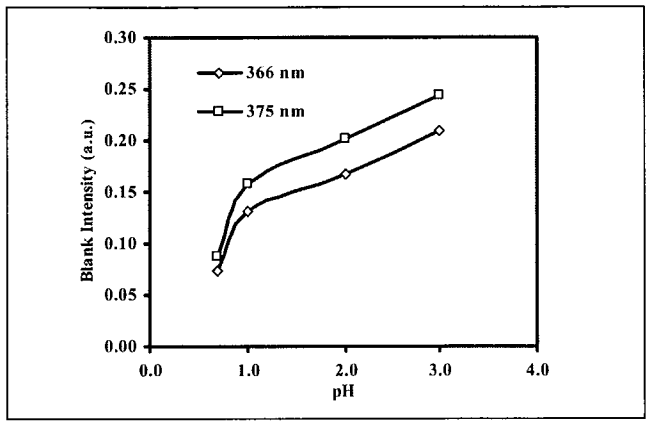

Fig. 4. Fluorescence intensity of the blank as a function of pH adjusted.

Figure 3 shows that a maximum of sensitivity is reached when using 0.014M EDTA at both excitation wavelengths, but 375 nm is the one that gives the highest sensitivity. Hence, the concentration of EDTA that optimizes the results of fluorescence intensity and blank interferences is 0.014M, which was used in this study.

pH Adjustment

In many studies reporting Se quantification by employing DAN as the fluorometric reagent (28-33), an adjustment of pH before solvent extraction was performed. But T. Koh et al. (37) described that the tedious step of pH adjustment is not only unnecessary and time-consuming but also has the disadvantage of producing a high blank reading. Yet, controversy exists regarding the pH adjustment and, at the present time, some researchers still include this step as fundamental for the correct Se determination.

Figure 4 shows the relationship between pH and the measured fluorescence intensity of the blank solution at 366 and 375 nm.

The H^+ concentration was adjusted just before the addition of the 0.1% DAN solution using 4M HCl and 15M NH_4OH. We observed that the higher the pH, the higher the fluorescence of the blank solution at the two excitation wavelengths studied. This behavior can be attributed to the introduction of foreign compounds producing non-specific fluorescence, which interferes with the intrinsic fluorescence of the solution and thus higher fluorescence was produced and measured. The same can be observed while working at a pH lower than naturally occurring, which causes a decrease in the sensitivity of the method (together with a higher blank intensity). This can be attributed to the protonation of DAN which reduces the concentration of piazselenol and consequently results in a reduction in the final sensitivity.

At this point it is worth noting that the sensitivity of the method is little affected by the concentration of H^+, and only a slight decrease in sensitivity is observed with an increase in pH. Contamination of the system and protonation of DAN are the main factors that contribute to an increase in the blank intensity while adjusting the pH. In this study, we found that working with the pH obtained from the process itself (pH 0.7) gives the best results, without use of extra reagents (with further contamination), in addition to being time saving since no pH adjustment is required.

Digestion, Reduction and Co-ordinating Conditions

Since the introduction of microwave energy for wet digestion in 1975, microwave-assisted decomposition has been widely employed and numerous applications have been described both for open-vessel and closed-vessel digestions. Closed-vessel digestion has several advantages over open-vessel digestion: smaller quantities of reagent (no evaporation), less contamination, and a higher reaction rate. The most commonly used acid digestion of biological materials is nitric acid, but various different acid mixtures have also been used (40,41). In our work, only nitric acid was employed, thus reducing contamination by supplementary reagents. This acid is the best alternative, because complete mineralization can be achieved at high temperature and pressure (34). Many authors suggest the inclusion of perchloric acid to facilitate the destruction of the matrix (even though a high risk of explosion exists when the mixture becomes dry) and to obtain higher percentage recoveries of the certified reference materials (CRMs) (29,31,32, 37). We found that satisfactory values were obtained for all of the CRMs analyzed in the present project when only nitric acid was used in the digestion process; thus,

avoiding higher fluorescence intensity of the blank and protecting the analyst from danger .

In addition, the heating program of the closed-vessel system maximizes the digestion efficiency, prevents losses of Se by reduction to volatile forms, and avoids cross-sample contamination.

A crucial step in the overall process is the reduction of Se(VI) to Se(IV), since only the latter species reacts with DAN. In comparison to previous research by other workers (26,29,32-34,37), we only used hydrochloric acid as the reductant agent. We also investigated the optimum conditions for temperature and time thus completely eliminating the fumes of nitrogen dioxide from the solution. We found that by keeping the vessels in a warm water bath at 85-90 °C for 60 minutes Se(VI) converts to Se(IV).

There is disagreement with regard to the preparation of the complexating agent DAN. Some authors suggest that the DAN solution can be stored in cool and light-proof conditions and used for a long time (28,34), while others recommend the use of a freshly prepared solution in order to avoid the formation of polymeric species which result in a high fluorescence background (29-31,37,38,42). We obtained outstanding results by using freshly prepared solutions of DAN even when it was prepared in the presence of light.

The conditions of complexation of Se(IV) with DAN are varied (28-33,37,38,42). We reached best results in terms of complete formation of 4,5-benzopiazselenol compound when working with 2 mL of the fluorescent agent of 0.1% DAN at 60 °C for 30 minutes.

Method Validation

Table II lists the linear range obtained for this method, the limit of detection (43), and the percentage recovery of different certified reference materials containing diverse concentration levels of Se:

NBS 1568a Rice Flour:
0.38 mg/Kg
NIST 8415 Whole Egg Powder:
1.39 mg/Kg
NIST 8418 Wheat Gluten:
2.58 mg/Kg
NIST 2781 Domestic Sludge:
16 mg/Kg)

The data obtained fitted a linear model within the range of 0-0.048 µg/mL Se cyclohexane with the equations:
$y = 40.158x + 0.0453$ ($r^2 = 0.9998$)
and
$y = 58.01x + 0.0502$ ($r^2 = 0.9999$)
for 366 and 375 nm, respectively. It should be noted that when working with Se concentrations above the range mentioned previously, a quadratic model should be applied. The sensitivity at 375 nm is about 44.5% higher than that at 366 nm; thus, the use of 375 nm as the excitation wavelength is strongly recommended.

To ensure a high level of analytical reliability and to study the matrix effect of the samples, analyte additions were performed. The samples were spiked with three different concentrations of a certified solution (Trace Metal Standard I, J.T. Baker Inc., Philipsburg, PA, USA) and then submitted to the digestion-extraction procedure. The difference between slopes (analyte addition and external calibration curve) was less than 7% for all samples (4.9% for 366 nm and 6.4% for 375 nm), so we can assume that the matrix effect is eliminated when using this procedure.

The limit of detection achieved for this method is higher than those previously reported (31-34). Although some research has been carried out to improve it, the results obtained were unsuccessful; therefore, further studies still need to be carried out to improve the limit of detection of this spectroflurometric method.

CONCLUSION

In this study, a conventional spectrofluorometric method for the determination of Se concentration in biological matrices has been improved to give a faster and more economical analysis in comparison to current procedures (avoiding the implication of sophisticated equipment). We have presented a fast, reliable, sensitive procedure that requires no pH adjustment and gives accurate and precise results.

TABLE II
Linear Range, Limit of Detection (LOD) (43), and Percentage Recovery of Certified Reference Material

	366 nm	375 nm
Linear Range (ng/mL)	0 - 48	0 - 48
Instrumental LOD (ng/mL)	0.61	0.48
Method LOD (ng/g)	14.0	11.0
Recovery (%)[a]		
Rice Flour NIST 1568	110.4±9.6	104.0±8.7
Whole Egg Powder NIST 8415	101.1±5.2	100.1±5.5
Wheat Gluten NIST 8418	96.8±3.9	96.9±4.0
Domestic Sludge NIST 2781	90.8±8.8	87.8±8.4

[a] Mean CRM recovery (Mean±1.96/√n) with a significance level of 0.05 (95% confidence level) and n=3.

The samples are completely destroyed by microwave oven digestion and the recovery test performed with four different certified reference materials ranged from 90 to 110%. The limit of detection of this improved method resulted in 11.0 ng of selenium per gram of biological matrix.

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