Determination of Selenium in Biological Materials

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A fluorometric method for determination of submicrogram quantities of selenium combines the wet digestion of organic samples, coprecipitation of selenium with arsenic and measurement of fluorescence with 2,3-diaminonaphthalene. The recovery of organically bound selenium-75 was 95% when carried through the complete procedure. When selenite was added to plant materials, recoveries were 103%. Satisfactory agreement was obtained between other analytical methods and this method, which allows estimation of the selenium content of samples containing 0.01 p.p.m. The coefficient of variation was less than 7% when the samples contained more than 0.1 p.p.m. of selenium. This method has the combined advantage of relative speed and accuracy.

Microgram quantities of supplemental selenium have been found to exert desirable effects on the growth and development of lambs (Muth *et al.*, 1958), chicks (Patterson *et al.*, 1957), and rats (Schwarz and Foltz, 1957). In some areas of the United States, the supplementation of livestock rations may be desirable, since the ingredients produced locally are low in selenium (less than 0.1 p.p.m.). Before selenium is added to these diets, a knowledge of the distribution and retention of selenium by the animal is necessary. Studies designed to follow the metabolism of selenium require a method of analysis sufficiently sensitive to measure less than microgram quantities accurately, yet rapid enough to allow analysis of large numbers of tissue samples in a reasonable length of time.

A number of methods reported in the past few years partially meet the requirements mentioned above (Allaway and Cary, 1964; Cousins, 1960; Cummins *et al.*, 1964; Dye *et al.*, 1963; Watkinson 1960, 1966). Allaway and Cary (1964) described a method that uses a Schöniger flask to oxidize the organic matter before isolating the selenium by coprecipitation with arsenic and measuring the fluorescence of the 4,5-benzopiazselenol. This procedure is sensitive and gives reproducible results that agree with neutron activation analysis. However, the individual oxidation of samples in the Schöniger flasks limits the number of samples and the sample size that can be analyzed.

Cummins *et al.* (1965) reported a technique for determining selenium directly in the wet digest of biological materials. The wet digestion reagent was efficient in rapid oxidation of organic matter. However, the method had been used only with samples containing several parts per million of selenium. The wet digestion method of Cummins *et al.* (1965) and the coprecipitation technique of Allaway and Cary (1964) have been combined in the method described here to allow rapid determination of submicrogram amounts of selenium in rat, lamb, and plant tissue.

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REAGENTS

Selenium-Free Sulfuric Acid. Reagent grade concentrated sulfuric acid was diluted with an equal volume of water, and 15 ml. of reagent grade 48% hydrobromic acid were added per 200 ml. of the mixture. The solution was heated strongly until dense white fumes appeared. The remaining acid, which was clear or lightly straw colored, was designated as selenium-free concentrated sulfuric acid.

Digestion Reagent. Sodium molybdate (10 grams) was dissolved in 150 ml. of deionized water, and 150 ml. of selenium-free concentrated sulfuric acid were added. After cooling, 200 ml. of 70% perchloric acid were added.

Arsenic Solution. Arsenic trioxide (primary standard, 0.315 gram) and 10 pellets of sodium hydroxide (ca. 1.5 grams) were placed in a 50-ml. flask. Approximately 20 ml. of deionized water were added, and the flask was shaken until the solids were dissolved. The solution was diluted to volume with deionized water.

Reducing Solution. Concentrated hydrochloric acid (1500 ml.) was diluted with 500 ml. of deionized water. After mixing well, 500 ml. of purified 50% hypophosphorus acid were added, and the solution was mixed. Any selenium present in the reagent was removed by adding 8 ml. of arsenic solution per liter of reducing solution, followed by boiling for 5 minutes. The solution was cooled and filtered through Whatman No. 42 filter paper and was then ready for use.

Stabilizing Solution. This solution was prepared by dissolving 29.2 grams of (ethylenedinitrilo)tetraacetic acid and 100 grams of hydroxylamine hydrochloride in deionized water and diluting to 1000 ml.

Diaminonaphthalene Solution. As recommended by Allaway and Cary (1964), this reagent was prepared in dim or yellow light, and was made fresh for each set of concurrently analyzed samples. The diaminonaphthalene (2,3-diaminonaphthalene, K and K Laboratories, Plainview, N. Y.; 0.1 gram) was dissolved in 100 ml. of 0.1*N* hydrochloric acid containing 0.5 gram of hydroxylamine hydrochloride. The solution was extracted by shaking with 15 to 20 ml. of Decalin (decahydronaphthalene, Aldrich Chemical Co., Milwaukee, Wis.) in a separatory funnel. The aqueous layer was retained and was extracted again with 15 to 20 ml of Decalin. The layers were allowed to separate and the aqueous layer was withdrawn

and centrifuged to remove the last traces of Decalin.

Selenium Standard. Selenium dioxide was sublimed and 0.1398 gram placed in a 100-ml. volumetric flask. The selenium dioxide was dissolved in 0.1*N* hydrochloric acid and made to volume. One milliliter of this solution was diluted to 100 ml. with 0.1*N* hydrochloric acid to give an intermediate standard. The working standard (0.1 μg. of selenium per ml.) was prepared by diluting 1 ml. of the intermediate standard to 100 ml. with 0.1*N* hydrochloric acid.

PROCEDURE

A sample containing less than 0.2 μg. of selenium was accurately weighed and placed in a 100-ml. Kjeldahl flask. For samples of high selenium content, at least 0.1 gram was used, and an aliquot of the digested sample was used for the fluorometric determination. To each flask 10 ml. of digestion reagent were added. For samples larger than 1.0 gram, a 300-ml. digestion flask was used, and 30 ml. of the digestion reagent were added. The flask was heated gently, and as the mixture cleared (15 to 20 minutes), the flask was swirled to remove the particles of the sample adhering to the sides of the flask. Heating was continued until the contents became greenish yellow. The flask was removed from the heat and allowed to cool. If necessary, the digest was transferred to a volumetric flask and diluted to the mark.

The digest or an aliquot of the digest was transferred to a beaker, and 1.0 ml. of the arsenic solution was added. The reducing solution (5 ml. for each ml. of digestion reagent present, but not less than 25 ml.) was added and the beaker was covered with a watch glass. Occasionally when the reducing solution is added a yellow color may appear rather than the normal green color. After boiling, this color disappears and has not been observed to interfere with the isolation of the coprecipitate. The solution was boiled gently for 5 minutes. Excessive boiling must be avoided, since the possibility of an explosion exists if the mixture of the strong reducing reagent and perchloric acid becomes too concentrated. The volume of the solution after boiling should be 20 ml. or more. After cooling, the clear green supernate was drawn off through an immersion fritted glass filter (medium porosity) by applying vacuum. After the supernate was removed, the sides of the beaker were washed with 5*N* hydrochloric acid. The hydrochloric acid was also drawn off through the filter. The beaker was then washed with deionized water to remove the last traces of reducing solution, and the water was removed through the filter.

The precipitate was dissolved by washing down the sides of the beaker with 2 ml. of concentrated nitric acid, and adding 1 ml. of concentrated nitric acid to the inside of the filter. After standing for 10 minutes, the nitric acid was forced through the filter with 8 to 10 pounds of air pressure. Nitric acid (2 ml., 8*N*) was added in the inside of the filter, and after 2 minutes, it was forced out with pressure. This was repeated with 2 ml. of 8*N* nitric acid, and finally with two 5-ml. aliquots of deionized water. The filter was rinsed thoroughly with deionized water and removed from the beaker. The solution (at least 20 to 25 ml.) was covered with a watch glass and boiled for 5 minutes to remove any nitrite formed and to dissolve any of the precipitate adhering to the sides of the beaker.

After cooling, the following were added with mixing in the order indicated: 2.5 ml. of 5*N* hydrochloric acid, 5 ml. of dilute formic acid (1 part 88% formic acid and 1 part deionized water), and 2.5 ml. of stabilizing solution. The pH was adjusted to 2 with ammonium hydroxide (2 parts of concentrated base to 1 part of water) and 5 ml. of the diaminonaphthalene solution were added. The volume was adjusted to 75 ml. and after mixing the solution was incubated at 50° C. for 45 minutes.

The contents of the beaker were quantitatively transferred to a 250-ml. separatory funnel (Teflon stopcock) and adjusted to a constant final volume of approximately 125 ml. Exactly 10 ml. of Decalin were added, and the separatory funnel was shaken for 7 minutes on a Burrell wrist-action shaker adapted for separatory funnels. The aqueous layer was removed, and 50 ml. of 0.1*N* hydrochloric acid were added. The funnels were shaken again for 7 minutes, and the layers were allowed to separate. The aqueous layer was removed, and the organic layer was transferred to a cuvette.

A Coleman Model 12C electronic photofluorometer with Coleman 12-221 activation filter (modified by removal of the intensity reduction disk) and 14-212 filter was used. The Decalin solution from the 0.2-μg. standard was used to establish the maximum reading and the Decalin layer from a reagent blank was used to set the zero value. There was a linear relationship between fluorescence and the amount of selenium within this range. The fluorescence (% F) of the unknown was compared to the fluorescence of the standard, and the parts per million of selenium in the sample were calculated.

$$\frac{\%\text{ F unknown}}{\%\text{ F standard}} \times \frac{\mu\text{g. of selenium in the standard}}{\text{grams of sample}} = \text{p.p.m. of selenium}$$

To avoid contamination from traces of selenium, all glassware was washed in detergent (Alconox), rinsed in tap water, soaked in concentrated nitric acid for at least 10 minutes, rinsed in deionized water, and dried in an oven before use in the analysis. The filter sticks were placed in concentrated nitric acid and brought to a boil. The acid was allowed to cool, and the filter sticks were removed and at least 150 ml. of deionized water were drawn through the filter before drying in the oven. Deionized water was used in all reagents and all steps of the analysis.

The method of Cousins (1960) can also be used for the isolation of the coprecipitate of arsenic and selenium. In this modification, the coprecipitate was isolated by filtration through Whatman No. 42 filter paper, and after rinsing with 5*N* hydrochloric acid, the filter paper and the precipitate were digested by a mixture of nitric and perchloric acids (15 ml., composed of 6 parts of concentrated nitric acid, 2 parts of red fuming nitric acid, and 2 parts of perchloric acid). Extreme caution must be taken in handling this digestion reagent since it is a very strong oxidizing agent. Particular caution should be taken to prevent the digest from evaporating to dryness, since many perchloric salts are explosive. The resulting digest was then treated

with hydrochloric acid as suggested by Dye *et al.* (1963) to ensure that any selenate was converted to selenite (12 ml. of water, followed by 8 ml. of concentrated hydrochloric acid, and boiling for 10 minutes). The selenium was complexed with diaminonaphthalene after the addition of formic acid, EDTA, and hydroxylamine as outlined above.

RESULTS AND DISCUSSION

The digestion reagent originally proposed by Bolin and Stramberg (1944) and used for the determination of selenium by Cummins *et al.* (1964) was very efficient. Small samples (0.1 to 0.2 gram) were digested in approximately 20 to 30 minutes, while larger samples required approximately 30 to 60 minutes to oxidize completely. The recoveries of added selenite and organic radioactive selenium (Table I) were good and indicated that there were no losses of selenium during this step.

The efficiency of the isolation of selenium from the digest was evaluated with both medium and fine porosity immersion filters. Radioactive selenite (approximately 0.004 μg. and 70,000 c.p.m.) was coprecipitated with arsenic isolated on immersion filters, and dissolved with nitric acid. In all cases, at least 95% of the selenium was recovered after the precipitate was isolated and redissolved. When concentrated rather than 8*N* nitric was used to dissolve the precipitate initially, the recovery of selenium was improved to more than 98%. Medium porosity filters recovered nearly as much selenium (98.2%) as did fine porosity filters (98.7%), and were preferred, since the rate of filtration was much faster.

Table I shows the recoveries obtained from a series of samples that contained organically bound radioactive selenium. These samples were obtained from male weanling rats which were fed purified diets 2 weeks prior to injection with 1 μg. of radioactive selenite (Ewan *et al.*, 1967). Then, at 7 and 35 days after injection, rats were sacrificed and samples of blood and liver were taken for analysis. The radioactivity of the samples was determined with a Nuclear-Chicago well-type scintillation counter (Model 132B) with an automatic sample changer (Model C120-1) and a time interval printer (Model C111B) prior to digestion. The radioactivity of aliquots of the digest and of the Decalin phase was determined, and recoveries for the various steps were calculated.

The selenium in these samples should represent as nearly as possible the forms of selenium normally occurring in animal tissues. The injected selenite is bound to protein, and remains part of a relatively stable pool at 7 and 35 days after injection (Ewan *et al.*, 1967). Under these conditions, there was no difference between the recoveries obtained with liver or blood in the digestion step or in the preparation for fluorometric reading. The pooled data indicate that there was a loss of about 1 to 2% during the digestion and an additional loss of 3 to 4% during the manipulation prior to fluorometry. This loss would appear to be due primarily to selenium that was retained on the immersion filters. Since standards were precipitated and isolated in the same manner as the samples in each series, this loss would not affect the accuracy of the method. The combined data indicate that 95% of the radioactive selenium was recovered. Dye *et al.* (1963) have reported recoveries of about 90% for selenium-75 from samples of alfalfa grown in a nutrient solution containing radioactive selenium.

The recoveries obtained when selenite was added to various samples of plant materials were also evaluated. Of 15 samples, 12 contained less than 0.058 p.p.m., while three samples contained less than 0.265 p.p.m. of selenium. The average recovery of 0.1 μg. of selenium added to these samples was 103.6 ± 3.4% (mean ± standard error). The published recoveries (Allaway and Cary, 1964; Cousins, 1960; Kelleher and Johnson, 1961; and Watkinson, 1960, 1966) range from 90.5 to 104.7%, indicating that the present method is at least equal in this respect to other existing methods.

Table II compares the values obtained when the same samples were analyzed by the present fluorometric method, and by the much more laborious method of Kelleher and Johnson (1961). All of the samples except two were within the standard deviation of the averages. When pellets and chow were analyzed, the values obtained by the fluorometric method were higher.

One possible explanation for the higher values obtained with the fluorometric method is that the form of selenium in the sample influenced its stability under the conditions of the analysis. Kelleher and Johnson (1961) obtained recoveries of 70 to 75% with their method and were forced to include isotope dilution into the method to correct for these

Table I. Recovery of Organic Selenuim-75

Tissue[a]	Step	No. of Samples	% Recovery
Blood	Digestion	20	99.4 ± 2.6[b]
	Fluorometric	49	95.6 ± 1.6
	Total	49	94.2 ± 1.9
Liver	Digestion	15	98.7 ± 2.4
	Fluorometric	35	96.0 ± 0.8
	Total	49	95.1 ± 1.3
Combined data	Digestion	35	99.1 ± 1.4
	Fluorometric	84	95.8 ± 1.0
	Total	84	94.6 ± 1.0

[a] Sample of liver and blood obtained 7 and 35 days after injection with 1 μg. of selenium containing 0.1 μc. of selenium-75. Details of the experimental procedure have been described (Ewan *et al.*, 1967). [b] Average ± standard error.

Table II. Comparison of Selenium Content of Samples Analyzed by Different Analytical Methods

Sample Type	Present Method, P.P.M.	Kelleher and Johnson, P.P.M.
Hay	0.020 ± 0.004[a]	0.030 ± 0.006
	0.017 ± 0.004	0.026 ± 0.005
	0.023 ± 0.005	0.020 ± 0.005
Oats	0.026 ± 0.005	0.022 ± 0.006
Pellets	0.102 ± 0.040	0.072 ± 0.002
Torula yeast	0.009 ± 0.001	0.013 ± 0.004
Purina Chow	0.585 ± 0.014	0.422 ± 0.012

[a] Average = standard deviation.

losses. If inorganic radioactive selenite was more stable than organic selenium under the conditions of the assay, the values reported would be lower than the true selenium content of the sample. The essentially quantitative recoveries of selenium obtained in this modified fluorometric method suggest that it is the more accurate of the two procedures.

The standard deviation and the coefficient of variation were calculated for each of 158 samples that had been analyzed by this method. The samples were grouped according to selenium content and the type of tissue and the selenium content and coefficients of variation were averaged within groups. Table III summarizes the results of these groupings. The coefficient of variation was high when the selenium content of the sample was less than 0.01 p.p.m., but when all samples above 0.1 p.p.m. were compared, it was less than 7.0%. The values for blood appeared to be more variable than those for liver or muscle.

The values reported for blood, liver, and muscle were obtained from determinations of aliquots of a single digest, and as such should represent the expected error for the manipulations beyond the digestion steps. The values for feeds represent different samples in each determination, and show approximately 80% greater deviations. These deviations not only represent the entire procedure but sampling error as well.

The major objectives in the development of the procedures described were to shorten the time required for the assay without sacrifice in accuracy. These seem to have been achieved. The method utilizes a wet digestion which, as pointed out by Cummins *et al.* (1964), is very rapid, and results in quantitative recoveries of selenium. A wet digestion (in contrast to ignition in oxygen) is suitable for fresh tissues such as blood, liver, and muscle without prior sample preparation. The wet digestion method can be used with a much wider range of sample sizes than can the combustion techniques.

In the isolation step, both selenite and selenate which result from the digestion of organic selenium by this method (Watkinson, 1966) are quantitatively reduced to elemental selenium, and then oxidized to selenite by nitric acid. Thus, all the selenium in the sample is available to form the fluorescent piazselenol.

The range of selenium levels measured in the present procedure was from 0 to 0.2 μg. of selenium and allowed detection of less than 0.01 μg. This sensitivity is not as good as reported by Watkinson (1966), but is comparable with the method described by Allaway and Cary (1964), and is more sensitive than the other methods available. The coefficient of variation is large, however, at these low levels of selenium. The error could possibly be reduced by increasing the sample size.

The proposed procedure requires approximately 6 hours to complete an analysis of 10 samples, a blank, and a standard. This is an improvement over most existing methods (Allaway and Cary, 1964; Cousins, 1960; Dye *et al.*, 1963; Kelleher and Johnson, 1961; and Watkinson, 1966). Several authors (Cummins *et al.*, 1965; Dye *et al.*, 1963; Watkinson, 1966) have used procedures that do not require isolation of the selenium from the mixture prior to fluorometric measurement. This would shorten the time required to complete a series of determinations. However, in our hands, a direct determination after acid digestion has not given values comparable to the proposed method with liver and kidney samples from swine.

Table III. Estimate of Reproducibility

Material	Range of Selenium Content, P.P.M.	No. of Samples	Av. Selenium Content, P.P.M.	Av. Coefficient[a] of Variation
Muscle	<0.01	6	0.005	57.2
	0.01–0.1	43	0.044	18.7
	0.1–0.3	1	0.114	5.0
Liver	<0.1	5	0.047	22.7
	0.1–0.3	25	0.222	5.4
	0.3–0.8	6	0.593	5.7
	>0.8	26	1.111	4.5
Blood	<0.3	10	0.224	10.2
	0.3–0.8	15	0.559	3.3
Feed	<0.01	9	0.004	98.5
	0.01–0.1	10	0.028	32.0
	0.10–.3	2	0.115	10.4
Combined data	<0.01	15	0.004	82.0
	0.01–0.1	58	0.042	21.4
	0.1–0.3	38	0.214	6.9
	0.3–0.8	21	0.569	4.0
	>0.8	26	1.111	4.5

[a] Coefficient of variation = $\frac{\text{standard deviation}}{\text{mean}} \times 100$.

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