THE APPLICATION OF ISOTOPIC DILUTION ANALYSIS TO THE FLUORIMETRIC DETERMINATION OF SELENIUM IN PLANT MATERIAL

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Interest in the determination of selenium in plant material stems from the work of SCHWARZ AND FOLTZ[1] which showed that trace amounts of selenium are of nutritional benefit. The nutritional aspects of selenium in biological material were recently reviewed by KING[2] who also pointed out the need for a better method of analysis. Difficulties in the analysis of selenium in plant material come about from loss of selenium in the first stages of the analysis in which the sample is decomposed, and from interference effects by foreign ions in the sample which prevent proper color development.

TAUSSKY and co-workers[3] as well as GUTENMANN AND LISK[4] used an oxygen-flask combustion to prepare biological samples for selenium analysis and then determined selenium spectrophotometrically by extracting the piazselenol formed with selenium and the aromatic *o*-diamine, 3,3'-diaminobenzidine. WATKINSON[5] employed this same reagent for a fluorimetric determination of selenium. The use of this reagent in selenium analysis has been recently reviewed[6]. Studies of other *o*-aromatic diamines[7,8] have shown that 2,3-diaminonaphthalene (DAN) is a more sensitive reagent. GORSUCH[9] incorporated radioactive selenium in a study of the "wet oxidation" of cocoa and showed the great dependency on the reaction conditions for the recovery of selenium in this method of sample preparation.

Reported herein is a study on the determination of micro amounts of selenium in plant material in which the samples are prepared by oxygen-flask combustion and their selenium content is determined fluorimetrically with DAN. Compensation for loss of selenium throughout the analytical procedure is made by the incorporation of radioactive selenium into the analytical scheme. Also included is further information relating to the reaction between DAN and selenium, the structure of piazselenols, and the reaction of additional aromatic *o*-diamines with selenium.

EXPERIMENTAL

Apparatus and reagents

Optical measurements were performed with a Coleman Model 12 C Photofluorimeter using as primary filters the combination of Corning Glass Co. 7-60 and 0-52 filters, and an Eastman Kodak Wratten K2 secondary filter.

Radioactivity measurements were made with a Nuclear Chicago Model 151A

scaler using a 3/8″ well-type scintillation probe with a sodium iodide crystal.

2,3-Diaminonaphthalene solution was prepared by dissolving 50 mg of purified material in 100 ml of 0.10 *N* hydrochloric acid. The commercial 2,3-diaminonaphthalene (Aldrich Chemical Co., Milwaukee, Wisconsin) was purified by forming a saturated solution of the amine in 5 *N* hydrochloric acid and then adding saturated sodium hydroxide solution dropwise to reform the amine. The amine was filtered and washed with water until the pH of the filtrate was between 5–7; the amine was then dried at 100°. Observed melting points 190–191°; literature 191–194°. The amine was stored under refrigeration and was repurified whenever the crystals were yellowish in appearance.

Standard selenium solution containing 1.00 mg of selenium per ml was prepared by dissolving 1.6336 g of selenous acid (H_2SeO_3) in 1 l of water. Other solutions were prepared by appropriate dilution of this stock solution.

Radioactive selenium-75 solution was prepared by adding 100 μl of the original 1 mC/2 ml selenous acid solution (Oak Ridge National Laboratory) to 100 ml of 1.2 *N* hydrochloric acid.

Spectrograde cyclohexane was used.

PROCEDURE

For the determination of selenium in plant material, combustion sample holders were cut from No. 589 black ribbon Schleicher and Schüll filter paper, using a Fisher Scientific Co. # 13–304 combustion sample holder as a pattern. A series of sample holders were placed on watchglasses and 50 μl of radioactive selenium solution were placed upon each sample holder using a 0.25-ml tuberculin syringe with a 26-gauge needle. A pipetting stop was fastened to the syringe. The sample holders were allowed to dry overnight in air and then variable amounts (from 0 to 100 μl) of 5 p.p.m. selenium solution were placed upon the sample holders respectively and the sample holders were again allowed to dry. A 100-mg sample of plant material was wrapped into the treated sample holder, which was then inserted into the combustion head. To the combustion flask were added 30 ml of water, and prior to ignition the outside of the filter paper was wetted with 2 drops of cyclohexane. After combustion, the flask was shaken manually for 5 min to dissolve the products from the combustion; the contents of the flask were transferred to a 150-ml beaker and the combustion flask was rinsed with 10 ml of water which was then added to the previous contents of the flask. Then 1 ml of freshly prepared potassium persulfate solution (0.1 g/l) was added, and the contents of the beaker were boiled for 5 min. After the solution had cooled to room temperature, 1 ml of 0.1 *M* EDTA solution was added and the acidity was adjusted to pH 2.0 using either hydrochloric acid or sodium hydroxide solution; then 0.5 ml of DAN solution was added and the mixture was allowed to stand for 2 h. A 3/8″ × 3″ Lusteroid tube (Atomic Accessories, Valley Stream, N.Y.) was filled with a portion of the sample and a count was made for 4000 counts. The contents of the beaker and the Lusteroid tube were transferred to a 120-ml separatory funnel and the sample was extracted with 10 ml of cyclohexane by shaking for 5 min. The layers were separated, and the aqueous layer was re-extracted with 5 ml of cyclohexane, by shaking again for 5 min. The organic extracts were combined and the fluorescence of the organic layer was measured. Then a portion of the organic layer was transferred to another Lusteroid tube and counted for 1000 counts.

Two calibration curves were prepared by adding to a series of beakers which contained 40 ml of water and 50 μl of radioactive selenium solution, from 0 to 100 μl of 5 p.p.m. selenium solution respectively; 1 ml of potassium persulfate solution was added, and then the procedure was continued with the boiling of the samples and all steps described above. A sample containing no selenium was used to set the zero reading of the fluorimeter and the sample of highest concentration containing either 0.3 or 0.5 μg of selenium was used to set the instrument at 85% fluorescence. This was done to prepare calibration curves to cover the range of selenium concentration present in actual samples. Dilute solutions of quinine sulfate were prepared to cover this same range of fluorescence intensities, and these quinine sulfate solutions were then employed as "reference" standards for daily use in retaining the instrument settings.

The average specific activity of the aqueous phase and of the organic phase of the standards which were used to prepare the calibration curve was calculated. To correct for loss of selenium in an unknown sample, the value of the concentration of selenium measured from the calibration curve was multiplied by the correction factor obtained as follows:

$$\text{Correction factor} = \frac{\text{activity of sample in aqueous phase}}{\text{activity of standard in aqueous phase}} \times \frac{\text{activity of standard in organic phase}}{\text{activity of sample in organic phase}}$$

(The average specific activity of the standard sample in the aqueous phase was 50 counts/min/ml and the average specific activity of the organic phase after extraction was 80 counts/min/ml.)

Results on the analysis of the plant samples are reported in Table I.

TABLE I

RESULTS OF SELENIUM ANALYSIS

Sample	*Selenium*			*Average Se content (p.p.m.)*
	Added (μg)	*Found (μg)*[a]	*Present (μg)*	
Oats	None	0.047	0.063	
(0.100 g)	0.10	0.234	0.034	None
	0.20	0.276	0.024	
Cornflakes	None	0.118	0.018	
(0.100 g)	0.10	0.248	0.048	0.44
	0.30	0.342	0.068	
	0.50	0.780	0.180	
Rice	None	0.15	0.05	
(0.100 g)	0.10	0.22	0.02	0.20
	0.20	0.31	0.01	
	0.30	0.40	0.00	
Grass-seeds	None	0.305	0.205	
(0.050 g)	0.10	0.335	0.135	0.60
	0.20	0.880	0.580	
	0.30	0.770	0.370	

[a] Before subtracting 0.10 μg for the average selenium content of the filter paper.

RESULTS AND DISCUSSION

Extraction conditions and interference study

Previous work[8] has shown that the maximum color development takes place at pH 2 after standing for 2 h and that in toluene the maximum intensity for fluorescent radiation is at an excitation wavelength of 390 mμ and fluorescent wavelength of 540 mμ. In the course of the previous work measurements were made with an Amin-co–Bowman Spectrophotofluorimeter. As that instrument was not available for present work, measurements were limited to the Coleman 12 C photofluorimeter. This instrument showed a very high blank reading with toluene and impure DAN. Consequently, other solvents were tested as extracting agents by measuring the difference in the fluorescence between the reagent (DAN) and its piazselenol. Values for the per cent piazselenol extracted were obtained by measuring the specific activity of the isotope in the organic phase and aqueous phase after extraction. The data are presented in Table II.

TABLE II

STUDY OF DIFFERENT SOLVENTS AS EXTRACTING AGENTS[a]

Solvent	*Se added (μg)*	*Fluorescence*	*% Extracted*
Carbon tetrachloride	0.0	5	
	5.0	71	19.4
Chloroform	0.0	49	
	5.0	51	20.0
Ethylenedichloride	0.0	35	
	5.0	62	20.0
Toluene	0.0	20	
	0.25	25	
	0.50	53	
	1.00	100	22.2
Cyclohexane	0.0	6	
	0.25	30	
	0.50	55	
	1.00	100	23.8

[a] 40 ml of aqueous solution at pH 2 which contained the indicated amount of selenium was extracted for 1 min with 10 ml of solvent.

The above results confirmed the work of PARKER AND HARVEY[7] that cyclohexane is a better extracting solvent than toluene and that purified DAN is necessary to minimize the blank reading. Because of the availability of spectrograde cyclohexane it was chosen in preference to decalin.

The effect of the pH of the aqueous layer prior to extraction with cyclohexane was measured and the results are presented in Fig. 1. Data on per cent extraction as a function of extracting time are reported in Table III.

The use of ion-exchange resins in the procedure was impossible with the photofluorimeter used in this study. Trace amounts of resin monomer were carried along in the procedure and the fluorescence of the monomer could not be filtered out.

Consequently a study of the effect of foreign ions in the procedure was made in which the radioactive selenium isotope was used to correct for interferences. For this study 2 ml of 0.01 *M* foreign ion solution was added to 40 ml of water containing 0.2 μg of selenium and 50 μl of the radioactive selenium solution. The results are listed in

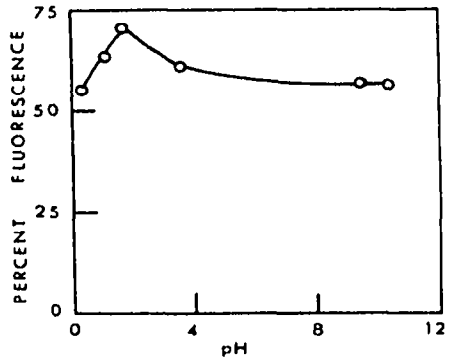


Fig. 1. Effect of pH on extraction.

TABLE III

PER CENT PIAZSELENOL EXTRACTED AS FUNCTION OF EXTRACTION TIME

First extraction: 10 ml cyclohexane					*Second extraction: 5 ml cyclohexane*	*Combined 15 ml cyclohexane extract*
Time of extraction (min)	1	3	5	10	5	—
% Extracted	23.8	28.2	29.0	29.0	5.2	34.0

Table IV. Of 20 metallic ions tested, major interference was observed from chromium(III), antimony(III) and tin(IV). Radioactivity measurements showed that the interference of tin was due to a reduction of selenium, while antimony and chromium enhanced the fluorescence.

Combustion conditions

GUTENMANN AND LISK[4] employed a spectrophotometric determination to detect the amount of selenium in oats after oxygen-flask combustion. Because of the lower sensitivity of this method compared to a fluorimetric determination, they required 1-g samples compared to 0.1 g in our procedure. Smaller sample concentrations are desirable as in larger samples complete combustion is more difficult. We observed that the conditions of combustion were not reproducible. This was ascertained by using as combustion samples filter paper which contained only radioactive selenium and standard selenium solution. A comparison of the specific activity of the aqueous layer measured directly after combustion against the specific activity of the aqueous layer of standard solutions, showed that the recovery was random and at times only 65% of the selenium added was recovered. This variability can be corrected by isotopic dilution techniques. Measurements of the ratios of the specific activity of the

TABLE IV

FOREIGN ION EFFECT

(Selenium concentration 0.20 μg)

Ion	Specific activity of aqueous phase prior to extraction (counts/min/ml)	Specific activity of organic phase after extraction (counts/min/ml)	Correction factor	Selenium concentration (μg)	
				Calibration curve	Corrected by factor
Al(III)	53.2	82.5	1.03	0.24	0.25
Ba(II)	36.2	88.5	0.65	0.43	0.28
Bi(III)	42.5	73.5	0.93	0.26	0.24
Ca(II)	40.0	73.5	0.88	0.22	0.19
Cd(II)	53.2	82.5	1.03	0.24	0.25
Co(II)	44.5	109.0	0.65	0.40	0.26
Cr(III)	39.4	105.0	1.40	0.38	0.53
Cu(II)	36.7	98.0	1.18	0.08	0.09
Fe(III)	47.6	57.2	1.34	0.14	0.18
Hg(II)	52.0	73.1	1.13	0.24	0.27
K(I)	53.2	82.5	1.03	0.24	0.25
Mg(II)	39.0	88.5	0.71	0.48	0.34
Mn(II)	40.0	73.2	0.89	0.23	0.20
Na(I)	53.2	82.5	1.03	0.24	0.25
Ni(II)	71.5	103.0	1.11	0.24	0.27
Pb(II)	53.2	82.5	1.03	0.24	0.25
Sn(IV)	41.0	—	—	0.15	—
Sr(II)	37.7	63.0	0.96	0.22	0.21
Sb(III)	35.2	72.0	0.79	0.55	0.44
Zn(II)	53.2	82.5	1.03	0.24	0.25

organic and aqueous layers showed that the extraction step is more reproducible than the combustion step (Table V).

A variable introduced into the procedure is the selenium content of the filter paper used to prepare the sample holders for the combustion. The selenium content of the filter paper was about 0.1 μg, which necessitated a high blank subtraction in the combustion analysis. Different combustion materials were tried such as dialysis casing, acetate paper, gelatin capsules and different makes of filter paper. The Schleicher and Schüll 589 paper appeared most uniform and was used throughout for the combustion. This paper showed an average selenium content of 0.1 μg which

TABLE V

REPRODUCIBILITY OF COMBUSTION AND EXTRACTION

Selenium added (μg)	% Recovered in aqueous solution after combustion	% Extracted from the aqueous solution
None	65	18.5
0.10	75	19.5
0.20	80	16.7
0.30	100	19.2
0.50	79	21.2

was subtracted from all samples. An exhaustive study on the selenium content of filter paper was recently reported by WEST AND CIMERMAN[10].

At one time diluted solutions of the isotope were stored in Lusteroid tubes. After several days of storage, the specific activity of the organic layer of samples prepared in the same manner as those of the calibration curve, was lower than the specific activity of the organic layer of similarly treated samples which went through the flask combustion. Yet, the specific activity of the aqueous solution was at its normal value in both cases. This was probably due to a partial reduction of selenium(IV) in the Lusteroid tube. Only selenium(IV) reacts with aromatic *o*-diamines to form the piazselenols, and during the combustion any reduced selenium was reoxidized to the selenium(IV) state. Reduction of the isotope solution was not observed if the isotope was stored in glass containers; consequently, all solutions of the isotope were stored in glass bottles.

EDTA was employed in the procedure as a masking agent. Potassium persulfate was added as an oxidizing agent to assure that all selenium was in the tetravalent state and that any traces of nitrite were oxidized to nitrate. The interference of nitrite but not nitrate has been reported previously[8].

Accuracy and precision

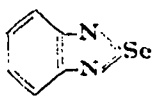
The recovery of selenium in the samples tested was satisfactory. Based upon the interference tests in Table IV, the standard deviation for the determination of selenium was 0.04 μg. The precision and accuracy of the method does not appear to be limited by the chemical steps in the procedure, but rather upon the quality of the fluorescent readings. With the instrument which was available, measurements could not be made with sufficiently monochromatic radiation to attain ultimate accuracy.

Reaction of selenium with aromatic o-diamines

The reaction of selenium with 2,3-diaminonaphthalene[8] and 3,3'-diaminobenzidine[11] is quite pH-dependent. Preliminary kinetic investigations showed that the rate of reaction is dependent upon the amine being protonated (a favorable condition at low pH) and upon the biselenite ion concentration (a favorable effect at high pH). Other aromatic *o*-diamines were prepared; for these amines the optimum rate of reaction also occurred in the acid range (pH 1–3). The fluorescent and absorption spectra were measured for those compounds which showed the greatest promise as analytical reagents. The molar absorptivities and relative degree of fluorescence are summarized in Table VI. The fluorescent spectra are presented in Fig. 2.

The reaction of selenium with aromatic *o*-diamines (*e.g.* 1,2-diaminobenzene) could produce either a benzenoid structure or a quinoid structure for the piazselenol.

Benzenoid structure



Quinoid structure

The quinoid structure was proposed by LUZZATI[12], from X-ray crystallographic measurements. Although nuclear magnetic resonance spectra of the piazselenols of 2,3-diaminonaphthalene and 1,2-diamino-4,5-dichlorobenzene were not conclusive in

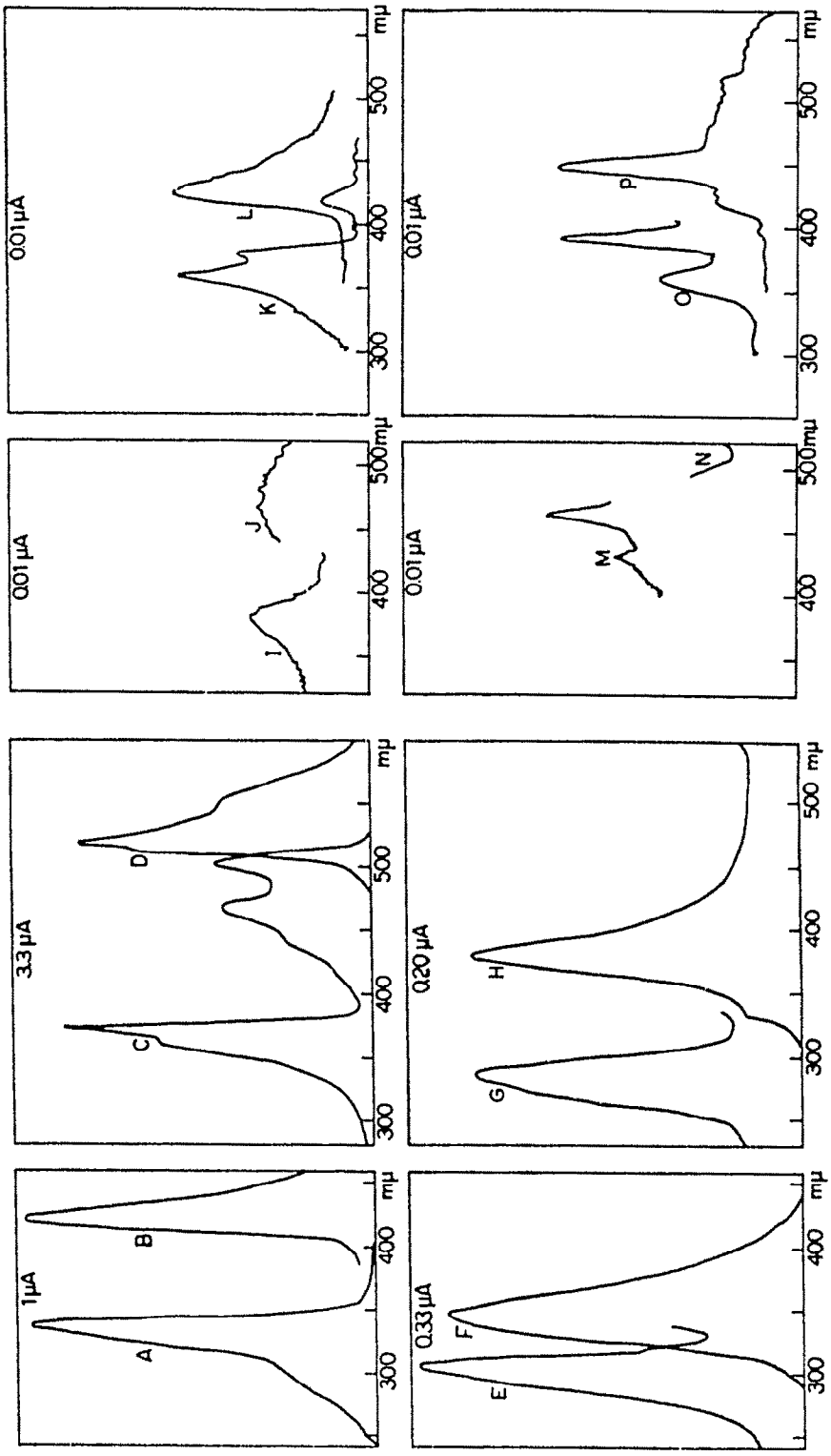
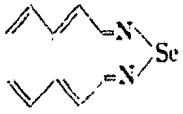
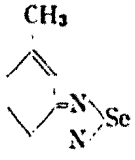
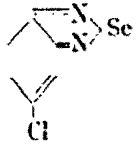
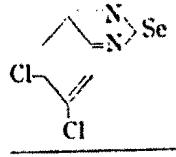


Fig. 2. Fluorescence spectra; wavelength (mμ) *vs.* fluorescence, arbitrary units, full scale, μA. (A) Excitation spectrum, 2,3-diaminonaphthalene; (B) fluorescence spectrum, 2,3-diaminonaphthalene; (C) excitation spectrum, 2,1,3-naphtho(2,3-c)selenadiazole; (D) fluorescence spectrum, 2,1,3-naphtho(2,3-c)selenadiazole; (E) excitation spectrum, 1-methyl-3,4-diaminobenzene; (F) fluorescence spectrum, 1-methyl-3,4-diaminobenzene; (G) excitation spectrum, 6-methyl-2,1,3-benzo(c)selenadiazole; (H) fluorescence spectrum, 6-methyl-2,1,3-benzo(c)-selenadiazole; (I) excitation spectrum, 1,2-diamino-4,5-dichlorobenzene; (J) fluorescence spectrum, 1,2-diamino-4,5-dichlorobenzene; (K) excitation spectrum, 5,6-dichloro-2,1,3-benzo(c)-selenadiazole; (L) fluorescence spectrum, 5,6-dichloro-2,1,3-benzo(c)selenadiazole; (M) excitation spectrum, 4-chloro-1,2-diaminobenzene; (N) fluorescence spectrum, 4-chloro-1,2-diaminobenzene; (O) excitation spectrum, 6-chloro-2,1,3-benzo(c)selenadiazole; (P) fluorescence spectrum, 6-chloro-2,1 3-benzo(c)selenadiazole.

Compound	Purification procedure	Melting point (°)		Molar absorptivity[a]	Fluorescence		
		Observed	Literature		Relative intensity	Fluorescence wavelength	Excitation wavelength
2,3-Diaminonaphthalene	b	190–191	190–194	135000 (245 mμ)	88	423	336
2,1,3-Naphtho(2,3-c)-selenadiazole 	c	270 dec.	290 dec.	41000 (378 mμ)	250.8	520	377
1-Methyl-3,4-diaminobenzene	b	82–83	88	52000 (220 mμ)	30.4	342	305
6-Methyl-2,1,3-benzo(c)selan-diazole 	c	64	—	25000 (335 mμ)	1.7	383	291
4-Chloro-1,2-diaminobenzene	b	69–70	72	140000 (280 mμ)	None	492	492
6-Chloro-2,1,3-benzo(c)-selenadiazole 	c	117–119	—	49000 (340 mμ)	0.62	448	400
1,2-Diamino-4,5-dichloro-benzene	b	159–161	162–164	123000 (218 mμ)	0.28	478	375
5,6-Dichloro-2,1,3-benzo(c)-selenadiazole 	c	212–214	210	55000 (350 mμ)	0.51	430	360

[a] Compound dissolved in cyclohexane
[b] Purified by forming the hydrochloride of the compound and re-precipitating the compound with sodium hydroxide
[c] Recrystallized from petroleum ether

showing that in these compounds the piazselenol also exists in the quinoid form, the quinoid structure was confirmed by infrared spectra.

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SUMMARY

A method has been developed for the fluorimetric determination of selenium in plant material using the reagent 2,3-diaminonaphthalene. Oxygen-flask combustion is used to oxidize plant material. Isotopic dilution techniques are incorporated to account for loss in different stages of the procedure. Measurements are also included on the suitability of several other amines as reagents for selenium.

RÉSUMÉ

Les auteurs ont développé une méthode pour le dosage fluorométrique du sélénium dans les plantes, après destruction de la substance dans l'oxygène, en utilisant le diamino-2,3-naphtalène comme réactif. Ils ont appliqué des techniques par dilution isotopique pour évaluer les pertes dans les différents stades du procédé. Plusieurs autres amines ont été également examinées, en tant que réactifs du sélénium.

ZUSAMMENFASSUNG

Es wurde eine Methode für die fluorimetrische Bestimmung von Selen im Pflanzenmaterial entwickelt. Die Substanz wurde im Schöniger-Kolben verbrannt und 2,3-Diaminonaphthalin als Reagenz benutzt. Mit Hilfe der Isotopenverdünnungstechnik wurde der Verlust bei den verschiedenen Verfahrensstufen festgestellt. Verschiedene andere Amine wurden auf ihre Brauchbarkeit als Reagenz für Selen geprüft.

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