SPECTROFLUORIMETRIC DETERMINATION OF ORGANIC AND INORGANIC SELENIUM IN VITAMIN SUPPLEMENTS AFTER CLOUD POINT EXTRACTION

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ABSTRACT

SPECTROFLUORIMETRIC DETERMINATION OF ORGANIC AND INORGANIC SELENIUM IN VITAMIN SUPPLEMENTS AFTER CLOUD POINT EXTRACTION

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Selenium is a trace and essential element for good health but required only in very narrow range. Hence, determination of selenium in trace level in any matrix related with human health is important. A preconcentration method is performed to obtain a low detection limit for analyte. In this study, the methodology of cloud point extraction (CPE) was used as the preconcentration method for speciation of selenium in vitamin tablets. Non-ionic surfactant Triton X-114 and a fluorimetric ligand, 2,3-diaminonaphtalene (DAN) were used for the extraction of trace levels of organic and inorganic selenium species as a prior step to their determination by spectrofluorimetry. The aqueous solutions of nonionic and zwitterionic surfactant materials become cloudy when temperature reaches the cloud point temperature and analyte collapses with surface active material. The volume of surfactant rich phase is smaller than the solution volume and by this way high preconcentration factor was obtained. Optimization of the CPE parameters affecting complexation and phase separation was performed. Standard addition method was used in

the quantitative measurements. Spectrofluorimetric determination of selenium was done using excitation and emission wavelegths of 380 nm end 570 nm respectively. The detection limit, established as 3s /slope where s is the standard deviation of 12 measurements of 0.02 mg/L Se-DAN complex after 10 fold preconcentration was 2.3 μg/L. Accuracy of the method was checked using EnviroMat Waste Water, EU-L-2 as SRM and the result was in good agreement with certified value.Besides, selenium rich vegetables (dill, watercress herb and garlic) were grown in a pot at a controlled atmosphere.Selenium in plants (dill, watercress gerb and garlic), both control and enriched groups was determined by ICP-MS.

Keywords: Cloud Point Extraction, Preconcentration, Selenium, Spectroflorometry, 2,3-diaminonaphthalene (DAN), Vitamin, Dill, Watercress Herb, Garlic.

ÖZ

BULUTLANMA NOKTASI EKSTRAKSİYONUNDAN SONRA ORGANİK VE İNORGANİK SELENYUMUN VİTAMİN DESTEKLERİNDE SPEKTROFLUORİMETRİK OLARAK TAYİNİ

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Selenyum sağlık için önemli temel elementlerden biri olmasına rağmen sadece çok az miktarına ihtiyaç duyulur. Bu nedenle insan sağlığı ile ilgili her hangi bir matriksteki selenyum tayini çok önemlidir. Önzenginleştirme işlemi, analit için düşük bir gözlenebilme sınırı elde ermek için uygulanır. Bu çalışmada, vitamin tabletlerindeki selenyum türlendirmesinde önzenginleştirme yöntemi olarak bulutlanma noktası ekstraksiyonu (CPE) kullanılmıştır. Eser düzeydeki organik ve inorganik selenyum türlerinin ekstraksiyonunun spektroflorometri ile tayininde ön basamak için yüzey aktif madde olarak Triton X-114 ve fluorimetrik bir ligant olan 2,3-diaminonaftalin (DAN) kullanılmıştır. İyonik olmayan ve zwitteriyonik yüzey aktif maddelerin sulu çözeltileri, bulutlanma noktası sıcaklığının üzerinde bulanıklaşır ve analit yüzey aktif madde ile birlikte çöker. Yüzey aktif madde bakımından zengin olan fazın hacmi, toplam hacimden çok daha düşüktür ve böylece yüksek bir önzenginleştirme

oranı elde edilir. Araştırmada, kompleksleşme süresi ve faz ayırımını etkileyen bulutlanma noktası ekstraksiyonunun optimizasyon parametreleri uygulanmıştır. Standart ekleme kantitatif ölçümler için kullanılmıştır. Selenyumun spektroflorimetrik tayini sırasıyla 380 nm ve 570 nm uyarma ve emisyon dalga boylarında yapılmıştır. Yöntemin 3s/eğim olarak tarif edilen gözlenebilme sınırı 2.3 μg/L. Burada s, 10 kat önzenginleştirilmiş 0.02 mg/L Se-DAN bileşiğinin 12 kez ölçümünden elde edilen standart sapma değeridir. Yöntemin doğruluğu standart referans madde olarak EnviroMat Waste Water, EU-L-2 analizi ile teyit edilmiştir ve sonuç sertifika değeri ile uyumlu bulunmuştur. Selenyum ile zenginleştirilmiş bitkiler (dere otu, tere otu, ve sarımsak) kontrollü bir ortamda saksılarda yetiştirilmiştir. Ayrıca, hem kontrol hem de zenginleştirilmiş bitkilerde (dere otu, tere otu, ve sarımsak) selenyum derişimi ICP-MS ile tayin edilmiştir.

Anahtar Kelimeler: Bulutlanma noktası ekstraksiyonu, Önzenginleştirme, Selenyum, Spektroflorometri, 2,3-diaminonaftalin (DAN), Vitamin, Dere otu, Tere otu, Sarımsak.

To My Parents and My Angel Sister İrem

I love you so much from the bottom of my heart

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INTRODUCTION

1.1 Selenium

Selenium was discovered in 1817 by J. J. Berzelius (1779-1848) and J. G. Gahn (1745-1818) who were studying the chemicals used in making sulfuric acid at a plant in Sweden. They reported that tellurium was present in sulphuric acid. However, tellurium had been discovered some 30 years earlier, mixed with some gold deposits in Hungary. Therefore, Berzelius studied on it again in his laboratory in Stockholm and he found that he and Gahn had been mistaking. They decided that the impurity was not tellurium but another closely related element that he subsequently identified as selenium. Berzelius suggested naming the element selenium; from the Greek word *selene*, for "moon" [1-3].

Selenium mainly occurs in the earth's crust associated with sulfide minerals or as selenides of silver, copper, lead, mercury and nickel or other metals. Its original source was probably volcanic activity. Directly being in the same column with sulfur and tellurium in the periodic table, selenium shows similar chemical characteristics with both of these elements. It combines with both metals and non-metals, directly and hydrochemically by forming organic and inorganic compounds. It is also an oxidant and a reductant as being an important factor in soil formation [2, 4].

Selenium has many allotropic forms having different physical and chemical properties. An amorphous red powder without crystalline shape is one of the allotropes of selenium. A second allotrope of selenium has a bluish, metallic appearance. A number of other allotropes have properties somewhere between these two forms. The most important physical characteristics of selenium are its electrical properties as being semiconductor and photoelectric. It is often used in the manufacture of transistors for computers, cellular phones, hand-held electronic games, and photoelectric cells. Furthermore, selenium becomes better at making the conversion of light energy into electrical energy as the light intensity or brightness increases.

Selenium also is used as pigments (coloring agents) for paints, plastics, ceramics, and glazes. Depending on the form of selenium used, the color ranges from deep red to light orange [3, 5].

1.2 Selenium Species

The species of selenium are classified into three main groups; that are i) inorganic selenium species, ii) organic selenium species, and iii) amino acids. Selenite, Se(IV) and selenate, Se(VI) are the most abundant inorganic species of selenium in environmental matrices, because these oxidation states are the most environmentally mobile and geochemically important forms of this element [6]. Naturally occurring organic selenium compounds are selenocysteine, selenocystine, selenohomocystine, Se-methylselenocysteine, selenocystathionine, selenomethionine, Se-methylselenomethionine, dimethylselenide, dimethyldiselenide, trimethyl

selenonium, selenotaurine [7]. The main species of selenium found in environmental and biological systems are given in the Table 1.1.

Table 1.1 Selenium species in environmental and biological systems [8].

Selenium Forms	**Species**
Inorganic Selenium Species	Se [element], Selenide [Se(−II)] Selenite [Se(IV), (SeO_3^{2-})], Selenate [Se(VI), (SeO_4^{2-})]
Organic Selenium Species	Methylselenol (MeSeH) Dimethylselenide (Me_2Se) Dimethyldiselenide (Me_2Se^2) Trimethylselenonium Cation (Me_3Se^+) Dimethylselenone (Me_2SeO^2) Dimethylselenoxide (Me_2SeO) Methylseleninic Acid Anion ($MeSe(O)O^-$ Dimethylselenosulfide (MeSSeMe) Selenocyanate ($SeCN^-$) Selenourea ($Se=C(NH_2)_2$
Amino Acids and Low Molecular Mass Selenium Species	Selenomethionine, Selenocysteine Selenocystine, Se-methylselenocysteine Selenocysteic acid Se-methylselenomethionine *S*-(methylseleno)cysteine, Selenoxide hydrate, Selenohomocysteine Gama-glutamyl-Se-methylselenocysteine Se-adenosylselenohomocysteine Selenocholine Selenobetaine, Selenoglutathione
Other Compounds	Selenopeptides, Selenoproteins Selenoenzymes, Selenosugars Se-metal metallothionines

1.3 Selenium and Health

Selenium occurs in all the cells and tissues of the body at levels that vary with the tissue as well as the level of selenium in the diet. The kidney, and especially the kidney cortex, has by far the greatest selenium concentration, followed by the glandular tissues, especially the pancreas, and the pituitary, and the liver. Muscles, bones, and blood are relatively low and adipose tissue is very low in selenium. Cardiac muscle is consistently higher in selenium than skeletal muscle. The kidney and the liver are the most sensitive indicators of the selenium status of the animal, and the selenium concentrations in these organs can provide valuable diagnostic information [4]. Selenium is an essential nutritional factor with a chemopreventive potential. It is the component of the enzyme glutathione peroxide (GSHPx) which is an antioxidant enzyme that affords cell protection against free radical damage and inhibits the toxicity of some metals such as lead and mercury [9- 11].

Supranutritional intakes of this essential element apparently reduce the cancer risk as being an antioxidant. Especially, bladder [12], colon [13], prostate [14], breast [15], ovary [16], pancreas [17], thyroid [18], stomach [19] and lung cancer [20] have an inverse relationship with the amount of Se intake [15]. However, this prevention depends on the diet including the proper amount of selenium. The toxicity and bioavailability of Se depend on its chemical form, its concentration level and the amount of food containing selenium taken in the diet [21-23]. In a balanced diet the amount of selenium should be at least 40 μg/ day and perhaps as much as 300 μg/ day to prevent the cancer risk. The recommended dietary allowances (RDA) for selenium are 55 μg/ day (0.7 μmol/ day) for both

women and men [7,22, 24]. Detailed information about the proper amount of selenium according to the ages can be seen in the Table 1.2.

Table 1.2 Recommended Dietary Allowances (RDA) for Selenium [24].

Age (years & months)	**Males and Females (µg/day)**	**Pregnancy (µg/day)**	**Lactation (µg/day)**
0-6 m	15	N/A	N/A
7-12 m	20	N/A	N/A
1-3 y	20	N/A	N/A
4-8 y	30	N/A	N/A
9-13 y	40	N/A	N/A
14-18 y	55	60	70
19 y +	55	60	70

The amount of selenium in the soils varies from high to low concentrations regionally in the world. The USSR, Canada, Venezuela, some regions in China and the USA (especially southwest) have the soils contain naturally high concentration of Se [25-28]. However, besides these regions having high Se levels in soils, there are certain regions that are poor in Se in Australia, northeast and south central China, New Zealand, East Siberia, North Korea, Nepal, Tibet and also many parts of Europe [26-29]. In the USA, the estimated daily Se intake varies between 60 and 160 µg [30], and in Canada the amount is much higher with the range 98-224 µg/day [31] whereas the daily intake of selenium in China(especially in Keshan region) is between 3 and 11 µg/ day [32]. In Turkey, the amount of Se intake is 30 µg/day, which is low according to RDA value, 55 µg/ day [32]. This low amount of selenium taken in Turkey is almost the limit of Se deficiency.

Although selenium is an essential nutrient for humans, the range of concentration between deficiency and toxicity is very narrow and mainly depends on the chemical form of selenium. [33] Se deficiency may appear at intake below 20 μg/ day and the toxicity at more than 800 μg/ day. [34] Food and Nutrition Board Institute of Medicine (2000) established the tolerable upper Se intake level as 400 μg/day in 2000 [7].

Low selenium intake can cause serious negative effects on human health. China is the most affected country a Se deficiency in the world [35]. Especially Keshan County in China is low Se area where is severed from Keshan disease. It is disease where a congestive cardiomyopathy is caused by dietary deficiency of selenium [29, 35-37]. The soil contains only 0.125 μg/g Se in Keshan area, therefore food products have very low Se concentration in diet [38]. Besides Keshan disease (heart enlargement) and Kashin beck disease (osteoarthropathy) [27,29], multiple sclerosis, endemic cretinism, cancer [35], miscarriages [39], Alzheimer's disease [40], and hypothyroidism [41] are the main disorders caused by Se deficiency.

Se toxicity depends on many factors like the form of Se compound, method of administration, animal species, exposure time and interaction with other metals. Loss of hair and nails [42], gastrointestinal problems [43], skin rash [30], garlic breath [44], abnormal functioning of the nervous system, nausea [45], and dizziness are the main symptoms of selenium toxicity.

1.4 Determination of Selenium

Being an essential ingredient in the daily diet, determination of the chemical form and the amount of selenium has a great importance for human life. A number of methods are available for the determination of selenium in variety of matrices. Hydride generation atomic absorption spectrometry (HGAAS), [46, 50] graphite furnace atomic absorption spectrometry (GFAAS) [9, 51-53], stripping voltammeter [54, 55], inductively-coupled plasma-mass spectrometry (ICP-MS) [56, 57], atomic fluorescence spectrometry (AFS) [58, 59] and molecular luminescence spectrometry are some of the techniques that have been used to determine the selenium.

The determination of selenium in biological samples is challenging because of its low concentration and the losses that occur owing to volatilization during the sample decomposition [60]. The hydride generation technique (HG-AAS) commonly used can easily detect low Se concentrations. This method is based on hydride generation from analyte which is first reduced to hydride in the liquid phase, and consequently converted into the vapor phase. HG-AAS minimizes the problem of spectral interferences [61].

Hydride generation coupled with atomic fluorescence spectrometry (HG-AFS) is another convenient method for determining low concentrations of selenium, owing to less interference and due to its high sensitivity and low detection limits. Moreover, the instrumentation is less expensive in contrast to the other alternative instruments [62].

Neutron activation analysis (NAA) is also used to determine selenium in biological samples. For determining trace levels, NAA based on the principles of cyclic activation analysis is most suitable. In this method, the signal-to-background ratio can be improved significantly by lowering the detection limits [63, 64]. In the radiochemical neutron activation anaysis (RNAA), a higher sensitivity is achieved by separating the desired radionuclide according to experimental conditions [65]. However, there is a disadvantage about RNAA that it is time-consuming, making it unsuitable for routine analysis [66].

Inductively coupled plasma mass spectrometry (ICP-MS) coupled with chromatographic systems is widely used for selenium determination in speciation analysis. This method has been mostly preferred because of its low detection limits, high sensitivity, wide dynamic linear range, and multi-element capability [67, 68].

Stripping voltammetry has attracted considerable attention for the determination of trace and ultratrace amounts of Se due to its simplicity, rapidity with the added advantages of multielement capabilities, low instrumental and maintenance costs. Moreover, stripping voltammetry needs small volumes of analysis samples and can be readily automated for monitoring purposes [69].

Fluorimetric method is one of the simplest, least expensive and most versatile of all the methods to determine Selenium. Fluorescence technique is not a direct method so it requires analyte derivatization. Fluorimetry has a low detection limit, high sensitivity and precision, but

less widely applicable than the other techniques based upon absorption, because many species do not fluoresce [70, 71].

Table 1.3 Selected studies with different methods for Se determination in several matrices.

Year	**Analyte**	**Sample**	**Method**	**LOD**	**Ref**
2002	Se	River water samples	CSV	4×10^{-10} mol l^{-1}	72
2004	As, Se	Phosphoric acid extracts of sediment samples	HPLC-ICP-MS	2 and 40 ng g^{-1} for As and Se, respectively	73
2005	Se	Garlic and onion	ET-AAS	1.3 and 0.5 µg l^{-1} for garlic and onion, respectively	74
2007	As, Se	Soil and sludge	ICP-MS	0.03 and 0.02 mg kg^{-1} for As and Se, respectively	75
2009	Se	Non-fat milk powder, human hair, whole egg powder, oyster tissue, and lyophilised pig kidney	ICP-OES	0.10 µg g^{-1}	76
2010	As, Sb, Se, Te and Bi	Vegetables, pulses and cereals	ET-AAS	1001, 47, 270, 104 and 48 ng g^{-1} for As, Sb, Se, Te and Bi, respectively.	77

1.5 Speciation of Selenium

Selenium can be presented in both inorganic (selenite, selenate, hydrogen selenide etc) and organic forms (selenomethionine, selenocysteine and selenoproteins etc). Among them, hydrogen selenide is extremely toxic while selenomethionine is essential and nutritional to the human body. The forms and the concentrations of selenium define its role in the environment and in the living systems. In order to obtain more specific information about deficiency or toxicity of element in environment both of its different chemical forms and total concentration of element should be determined [78].

The first step in speciation analysis of selenium is determination of the total metal content. For this purpose, several analytical techniques have been applied like fluorometry, polarography, voltammetry, GF-AAS, HG-AAS, MS and NAA [79, 80, 81]. The inorganic selenium species mainly found in water and soils; selenite and selenate, organoselenium compounds frequently found in biological samples; selenoamino acids, and dimethylselenide (DMSe), dimethyldiselenide (DMDSe), and the trimethylselenonium (TMSe) ion as the major product of selenium metabolism are the Se compounds which are determined by the speciation methods mentioned above. [21] These selenium species and their formulas are given in the Table 1.4.

Generally, selenium species related to human health have been determined in literature some seen in Table 1.4.

Table 1.4 Some examples of speciation studies for Se.

Analyte	**Sample**	**Method**	**LOD**	**Ref**
Se(IV), Se(VI)	Animal Supplementation	FAAS	31 and 51 ng for Se(IV) and Se(VI), respectively	82
TMSe, Se(IV), Se(VI)	Spiked water; urine	GFAAS	1.67, 1.27, and 0.76 ng for TMSe, Se(IV) and Se(VI), respectively	83
Se(IV), Se(VI), Se-Met	Diet supplements	HPLC-MS	27.7, 5.1, and 3.4 μg L^{-1} for Se(IV), Se(VI), and Se-Met respectively	7
Se(IV), Se(VI), Se-Cys, Se-Met	Yeast	GFAAS	0.80, 1.50, 1.70, and 1.20 for Se-Cys, Se-Met, Se(IV), and Se(VI) respectively	84
Se(VI), Se(IV)	Environmental samples	ETAAS	1.0 μg L^{-1} for Se(VI)	22
Se(IV), Sb(III)	Water samples	ICP-MS	0.05 and 0.03 μg L^{-1} for Se(IV) and Sb(III) respectively	6
Se-Cys, Se(IV), Se-Met, Se(VI)	Standards	ICP-AES	380, 700, 760, and 440 ng for Se-Cys, Se(IV), Se-Met, and Se(VI) respectively	85

Table 1.5 Some selenium species and their formulas [7, 86]

Selenium Compound	**Molecular Formula**
Selenite	SeO_3^{-2}
Selenate	SeO_4^{-2}
Trimethylselenonium ion	Me_3Se^+
Dimethylselenide	Me_2Se
Selenocystine	H_3N^+–CH(COO–)–CH_2–Se–Se–CH_2–CH(COO–)–NH_3^+
Selenomethionine	$CH_3SeCH_2CH_2CH(NH_2)COOH$
S-(methylseleno)cysteine	$CH_3SeSCH_2CH(NH_2)COOH$
Selenodiglutathione	$C_{20}H_{32}N_6O_{12}S_2Se$
Selenomethionine Se-oxide hydrate	$CH_3Se(OH)_2CH_2CH_2CH(NH_2)COOH$

1.6 Selenium Supplementation

The amount of selenium in food is directly related to the amount of selenium in the soil where the food was grown. Selenium enters the food chain through plants as amino acids L-selenocysteine and L-selenomethionine from the soil where the plant is grown. Selenium, like most trace elements and minerals found in the soil, is not found in the same concentrations in all regions of the world. Because of the uneven concentrations of selenium, disorders of both selenium deficiency and selenium excess are common [23, 87].

Plant foods, such as vegetables, are the most common dietary sources of selenium. How much selenium is in the vegetables you eat depends on how much of the mineral was in the soil where the plants grew. Fish, shellfish, red meat, grains, eggs, chicken, liver, and garlic are all good

sources of selenium. Meats produced from animals that ate grains or plants found in selenium-rich soil have higher levels of selenium [88].

Se-enriched vegetables represent an important source of this element in the human diet. Vegetables like garlic, dill, broccoli, mushroom, wheat germ, whole grains are consumed everywhere and sufficient enrichment of these plants with selenium could provide a good source of supplementation, using them as seasoning in our diet. However, the degree of enrichment with selenium in terms of the total selenium and the individual species must be investigated in order to provide the necessary knowledge to evaluate their disease-preventive potential [89].

Like most minerals, selenium is present in many different forms in food, and can vary greatly in its response to cooking and processing. In some foods, where a greater concentration of selenium is found in water-soluble form and contact with water is great, high losses of selenium can occur. For example, when navy beans are cooked, 50% of the original selenium is lost. In the case of animal foods, loss of selenium from cooking appears minimal. For example, when beef is broiled, virtually none of the selenium is lost. Dill and garlic as vegetables can be eaten without cooking, that is no selenium lost. Therefore; in this study, vegetables (dill and garlic) for determination of Se were preferred. Also, vitamin tablets as good source of selenium especially for daily intake were analyzed for selenium speciation. In our laboratory, the amount of selenium in chicken's egg, and farm animals like chicken determined in previous studies [90].

1.7 Selenium in Supplement Tablets

Antioxidants are essential for the growth of the body and for preserving the general health of the person. These antioxidants are available in supplements such as vitamins. Phytochemical, lycopene, selenium, vitamin A, and vitamin E are some the main antioxidants. Selenium is an antioxidant which in combination with vitamin C has the ability to prevent cancer and many other ailments. In fact, it develops a type of defense system in the body which fights these diseases [1, 23]. Garlic, fish, chicken, meat, egg, and some vegetables are rich in selenium. For example, if one does not like the odor of garlic, they can use the vitamin tablets.

There are many studies about selenium speciation in vitamin tablets. Seleno-yeast and selenate in 50, 100 and 200 µg /tablet dosages (seleno-yeast) and 25 and 200 µg/tablet were determined by Veatch et al. [91]. Arsenite [As(III)], arsenate [As(V)], monomethylarsonic acid, dimethylarsinic acid, selenate [Se(VI)], selenite [Se(IV)], selenocystine, selenomethionine and selenocystamine were determined by Sun et al. through UV detection [92].

1.8 Surfactants

1.8.1 Types of Surfactants

A surfactant (an abbreviation for surface active agent means active at surface) has the characteristic of tendency to absorb at surfaces and interfaces, boundaries between any two immiscible phases. Another property of surface active agents is to lower the interfacial free energies of

surfaces. It is the minimum amount of required energy to create the interface. This term is also used as interfacial tension. When surfactant molecules cover the phase boundary, the surface tension tends to decrease [93].

There are three main types of surfactant which are ionic**,** zwitterionic and nonionic. Ionic surfactants are separated two parts as anionic and cationic surfactants [94].

Carboxylate, sulfate, sulfonate, and phosphate having negative charge are anionic surfactants. Generally, anionic surfactants are sensitive to hard water by decreasing in the order of carboxylate, phosphate, sulfate and sulfonate. A short polyoxyethylene chain between the polar group and the hydrocarbon tail improves salt tolerance and solubility in organic solvents [95].

Many cationics are based on the nitrogen atom carrying the cationic charge. The typical cationic surfactants are made with amine salts such as cetyl trimethyl ammonium bromide and cetyl pyridinium chloride [94].

Non ionic surfactants contain uncharged hydrophilic head groups that consist of either polyether or a polyhydroxyl unit. The main type of non ionic surfactant is that with an oxyethylene group as the polar head. Ethoxylation is usually carried out under alkaline conditions. Any material containing active hydrogen can be ethoxylated. Ethoxylates are referred to as C_mE_n with m being the number of carbon atoms in the alkyl chain and n being the number of oxyethylene units. A non-ionic micelle has a thick interfacial layer of polar head groups rather than the quite

sharp transition from the hydrophobic micellar interior to the aqueous bulk of ionics. They have the additional property that they may be combined for use with either anionic or cationic surfactants. Igepals, Tritons, Tergitols, Suronics, and Plurafacs are the examples of the commonly used nonionic surfactants [96].

Zwitterionic surfactants contain both two charged group of different sign. Whereas the positive charge is almost invariably ammonium, the source of negative charge may vary, although carboxylate is by far the most common. Common types of zwitterionic surfactants are N-alkyl derivatives of simple amino acids such as glycine, betaine and amino propionic acid [96].

1.8.2 Formation of Micelles

Surfactants are amphiphilic molecules that contain both hydrophilic and hydrophobic groups, that is, a part of surfactant would be soluble in the liquid and another part would be insoluble in it. The insoluble parts have a more favorable free energy when they are away from the liquid; thus these molecules tend to concentrate at the liquid boundary [97]. The aqueous solution properties of some surfactants are given in Table 1.6 [98].

Table 1.6 The aqueous solution properties of some surfactants [98].

Surfactant	CMC[a], mM	Aggregation Number(N)	Cloud point
Triton X-100 (TX-100)	0.17-0.30	120-140	64 – 65
Triton X-114 (TX-114)	0.20-0.35	–	23 – 25
PONPE-7.5	0.085	–	5 – 20
PONPE-10	0.07-0.085	100	62 – 65
Igepal CO-630	–	–	48 – 52
C_8E_3	5.9-7.5	–	10.6
$C_{10}E_4$	0.6-0.8	30	19 – 21
Genapol X-80	0.05	–	42
Brij-30($C_{12}E_4$)	0.02-0.06	40	2 – 7
Brij-56($C_{16}E_{10}$)	0.0006	–	64 – 69
octylβ-D-Thioglucoside(OTG)	9.0	–	10 – 20
octylβ-D-Glucoside(OG)	20.0-25.0	84	2 – 20
C_8-lecithin	–	500	45
C_9-$APSO_4$	45.0	–	65

[a]CMC: Critical micelle concentration

The dual nature of amphiphilicity of surfactant molecules provides a thermodynamic driving force for adsorption and aggregation of surfactant molecules. In aqueous media the hydrophobic sections of surfactant molecules are attracted to the hydrophobic section of adjacent surfactant molecules. The association of adjacent hydrophobic sections of surfactant molecules reduces the less favorable interactions between water molecules and individual hydrophobic sections of surfactant, thereby reducing system free energy. The effect of association between adjacent hydrophobic sections of surfactant molecules are enhanced in aggregate structures such as adsorbed layers of surfactant and solution micelles.

In the solution, micelles constitute a peculiar donor of monomers. If the number of monomers is reduced, their loss may be compensated by decomposition of the micelles. The main cause of monomer self-aggregation is called hydrophobic effect, whose driving force is the unfavorable interactions between the hydrophobic chains and water. As a result, the amphiphilic molecules 'migrate' to the interface of form micelles. The micelles do not constitute stationary systems, but undergo constant changes. The changes are connected with the size, shape, and number of aggregates. A state of dynamic equilibrium between micelles and monomers occurs, and its position can be shifted as a result of changes in external factors [94].

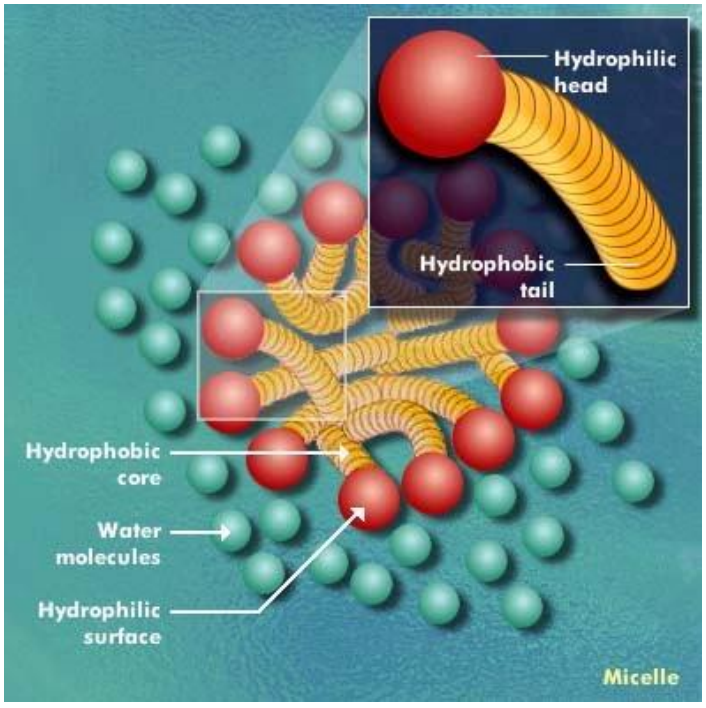

Figure 1.1 The schematic representation of a micelle.

The formation of a micelle is represented in Figure 1.1. The micelles are in equilibrium with a fixed volume fraction of dispersed surfactant. Since adding surfactants cannot increase the dispersed concentration above this amount, all the added surfactants must be in the form of micelles. Likewise, removing surfactants reduces the number of micelles without changing the dispersed number. This continues until there is no excess surfactant available to form micelles.

The formation of micelles from the constituent monomers involves a rapid, dynamic, association-dissociation equilibrium. Micelles are undetectable in dilute solutions of the monomers, and become detectable over a narrow range of concentrations. The concentration at which the micelles become first detectable is called the critical micelle concentration (CMC) [97]. Approximately 40 to 100 ions aggregate to form roughly spherical particles with hydrophobic tail oriented toward the center, and the hydrophilic head oriented toward the outside of the micellar particle as could be seen in the Figure 1.1. In this way, a second phase is formed, and uncharged species may be solubilized into the micelle. The metallic species has different polarities partitioned between aqueous phase, stationary phase, micellar hydrophobic phase, and the micellar hydrophilic phase. Therefore both ionic and uncharged species may be separated easily.

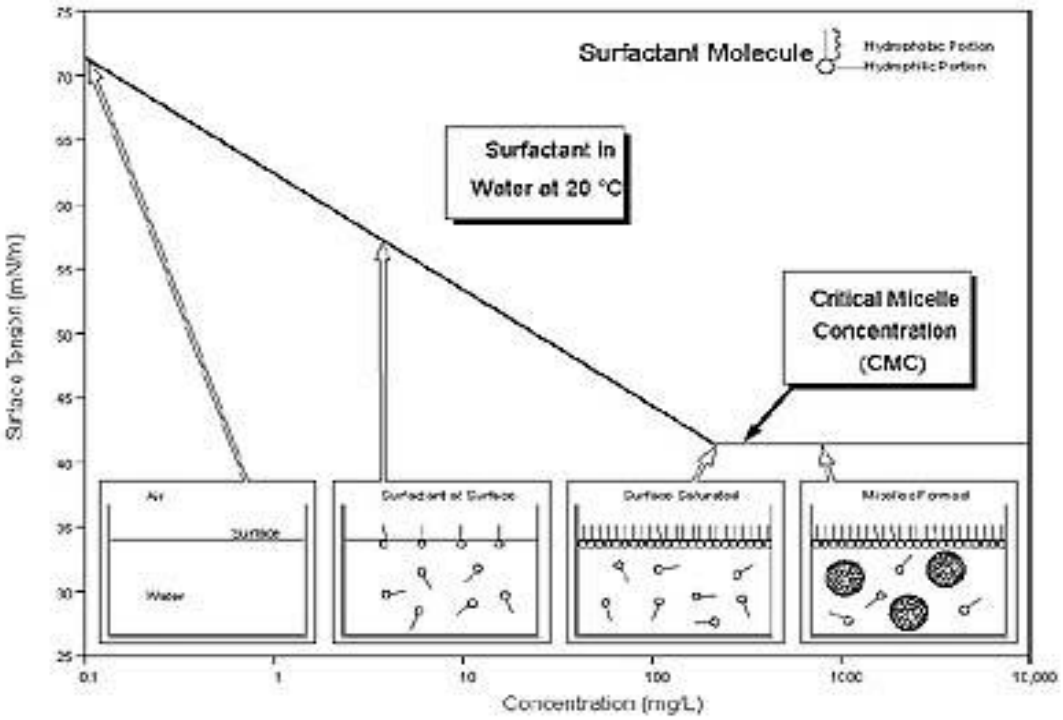

Figure 1.2 Schematic represanation of micelle formation [97].

The geometry of micelles and the number of aggregates depend on the structure of the ethoxylates. Spherical micelles are formed by alcohols with short alkyl chains and at lower temperatures. Longer chain surfactants at higher temperatures form cylindrical micelles. From geometric considerations, the aggregation numbers of micelles in aqueous media should increase rapidly with increase in the length of the hydrophobic group of the surfactant molecule and decrease with increase in the cross-sectional area of the hydrophilic group or the volume of the hydrophobic group.[93].

The structure of the surfactant, the presence of added electrolyte in the solution, temperature of the solution, and the presence of a second liquid phase are the factors that affect the CMC [93].

The solubility of oxyethylated fatty alcohols (nonionic surfactants) results from the formation of hydrogen bonds between water and a free electron pair on the oxygen atom of the ether group. The dehydration process connected with an increase in the temperature of the solution is important from the point of view of application. Above a certain temperature, called the cloud point, hydrogen bonds are broken, and the compound loses its amphiphilic properties. As a result of desolvation, alcohol relatively increases its hydrophobic properties. These observations can be confirmed by the decrease in the CMC values with increasing temperature. The dehydration process is reversible and with a decrease in temperature, repeated hydration of molecules may occur at a certain temperature, called clear point. Both the cloud point and the clear point depend on the structure of the surfactant, particularly on a relative proportion of the hydrophilic and hydrophobic parts [94].

1.9 Cloud Point Extraction

Determination of trace element in samples is a challenge analytical task, mostly due to the complexity of the matrix and the low concentrations of the analyte. Hence, determination of trace metals generally requires preconcentration processes. There are many separataion/preconcentration techniques such as liquid-liquid extraction (LLE), solid phase extraction (SPE), solid phase micro-extraction (SPME), and supercritical extraction (SE). Some of these methods require a large volume of organic solvents and are time consuming or high cost [2, 99]. Cloud point extraction is an

alternative preconcentration method to traditional extraction systems due to its efficiency, simplicity, low cost, commercially available surfactant, rapidity and safety [100]. The aqueous solutions of nonionic surfactant materials become cloudy when their temperature reaches the cloud point temperature and analyte collapses with a small volume by a surface active material. The cloud point extraction can also be termed as temperature induced phase separation or micelle-mediated extraction [101-103]. Thus, CPE can lead to high recovery efficiency and a large preconcentration factor due to a very small volume of micellar phase binding the analyte that was dispersed in the original matrix. [104] CPE is not only preconcentration but also a separation method, and this method has been applied for the determination of many elements including Au [105,106], Tl [107,108], Co [109,110], Mn [111,112], Cr [113,114], Zn [115,116], Ni [117,118], Pb [119,120], Cd [121,122], Cu [123,124], Ag [125,126], As [127,128], Se [129,130], and Sb [131,132] in trace levels.

1.10 Aim of This Study

One of the aims of this study is the determination of selenium in the vitamin tablets at trace level. Cloud point extraction technique will be used for the determination of Se(IV), Se(VI) and total organic selenium species in commercially available selenium supplemented vitamin tablets. In the cloud point extraction process, Se(IV) is taken into a hydrophobic complex with 2, 3-diaminonaphthalene (DAN) and then solubilized within the nonionic micelle triton X-114. The hydrophobic complex, 4, 5-benzopiazselenol, preconcentrated in the surfactant rich phase of Triton X-114 will be determined spectrofluorometrically.

Besides, selenium rich vegetables (Dill, watercress herb and garlic) will be grown in a pot at a controlled atmosphere. The total selenium contents of the vegetables and also vitamin tablets will be investigated utilizing microwave nitric acid digestion and ICP-MS detection.

CHAPTER 2

EXPERIMENTAL

2.1 Chemical and Reagents

i) **Se (IV) stock solution (1000 mg L^{-1}):** Prepared by dissolving proper amount (0.219 g) of sodium selenite, Na_2SeO_3 (Ventron), in 100 mL de-ionized water.

ii) **Se (IV) std solution (1 mg L^{-1}):** Prepared from Se (IV) stock solution (1000 mg L^{-1}) by dilution with de-ionized water.

iii) **Se (VI) stock solution (1000 mg L^{-1}):** Prepared by dissolving proper amount of (0.239 g) sodium selenite; Na_2SeO_4 (Ventron), in 100 mL de-ionized water.

iv) **Se (VI) std solution (1 mg L^{-1}):** Prepared from Se (VI) stock solution (1000 mg L^{-1}) by dilution with de-ionized water.

v) **2, 3-diaminonaphthalene (DAN) solution (0.1%, w/v):** Prepared by dissolving 0.1 g of 2,3- diaminonaphtalene (Sigma Aldrich) and 5.0 g hydroxylamine hydrochloride; $NH_2OH.HCI$ (Carlo Erba reagent) in 100 mL of 0.10 M HCI. To provide dissolution the contents were heated at 50 °C for 25 min in a water bath with using magnetic stirrer. All of the working solutions were freshly prepared each day and stored in the dark due to its sensitivity to the light.

vi) **0.25 % (w/v) Triton X-114 (octylphenoxypolyethoxyethanol):** Prepared by diluting 0.0625 g of Triton X-114 (Sigma) in 25.0 mL of cold de-ionized water. Cloud point temperature of Triton X-114 in aqueous solution is 22- 24 °C.

vii) **Hydrochloric acid, 37%, (w/v)** was purchased from Merck.

viii) **6 M Hydrochloric acid:** Prepared by diluting concentrated HCl 1:1(v/v) with deionized water.

ix) **Nitric acid, 65%,** was purchased from Merck.

All other reagents were of analytical-reagent grade. For sample and standard preparations, de-ionized water was used. All the glassware and plasticware were cleaned by soaking them in 10% HNO_3 for at least 24 hours and then rinsing three times with distilled water then rinsing with deionized water.

2.2 Instrumentation

De-ionized water obtained from a Millipore water purification system was used in all standard solution preparations and dilution studies. AF-2500 FL Hitachi Spectrofluorometer equipped with a xenon lamp was used for fluorescence measurements. Instrument excitation and emission slits were adjusted to 20 nm. A glass cell with dimensioıns of 10x10 mm was used. Excitation and emission wavelengths were set to 380 nm and 530 nm, respectively. Spectra were obtained by scanning. PMT Voltage was 400 V. Thermo X series ICP-MS was used for the determination of total

selenium. Elma, Elmasonic S 40 H brand sonication instrument and shaker were used in the extraction studies. Sigma 2-16 (D-37520, Germany) brand ultracentrifuge instrument was used to separate supernatant. Milestone Ethos Plus microwave oven was used for the reduction of selenate to selenite in vitamin tablets and digestion of vegetables. For lyophilization of vegetables, Heto-Holtan model Maxi Dry Lyo was used as freeze dryer. Before freeze drying, vegetables were kept at – 80 °C in the freezer overnight. Then, samples in the lio flasks were frozen at – 110 °C in vacuum.

Table 2.1 Microwave program used to reduce Se(VI) to Se(IV) in vitamin tablets.

Time, min	**Temperature, ^{0}C**
5	0-100
10	100
5	100-150
10	150
5	Ventilation

Table 2.2 Microwave program used to digest vegetables.

Time, min	Temperature, ^{o}C
5	0-100
15	100
5	100-150
20	150
5	150-180
15	180
10	Ventilation

2.3 Total Selenium Determination Utilizing ICP-MS

Supplement tablets were digested using concentrated nitric acid for the quantification of total selenium. The weight of tablets had a range of 1.0-1.5 g. Each tablet was weighed into a 100 mL PTFE vial and dissolved in 30 mL of conc. HNO_3. Samples were heated to boiling on a hot-plate for 2.0 h. At the end of digestion, mixtures were filtered using 0.45 μm nylon filters. The final solutions were made up to 100.0 mL with deionized water. Three points standard addition technique was applied to sample solutions to eliminate the possible matrix interferences. In the standard addition technique, selenate, Se(VI), was used because all the selenium species should be converted to highest oxidation state after HNO_3 digestion.

For dill, watercress herb and garlic, 0.2 g of lyophilized samples was powdered in the mortar and 3 mL of concentrated HNO_3 and 3 mL of concentrated H_2O_2 were added for digestion step. After digestion in

microwave oven, the solutions completed to 10 mL with de-ionized water and total selenium was determined by ICP-MS.

2.4 Determination of Selenium in Vitamin Tablets

In this study for the cloud point extraction of selenium reduced by HCl, a fluorimetric ligand, 2,3- diaminonaphtalene was used and the hydrophobic Se(IV)- DAN complex formed (4.5-benzopiazselenol) was extracted with Triton X-114 as the non-ionic surfactant. 2,3-diaminonaphtalene was chosen as a complexing reagent due to its selectivity and sensitivity for selenite Se (IV) in the fluorescence measurements after the reduction of selenate to selenite form.

2.4.1 Cloud Point Extraction Procedure

In this study, Se(IV) reacts with 2,3-diaminonaphthalene (DAN) yielding the complex 4,5-benzopiazselenol (Se–DAN complex) that allows the determination of selenium in trace amounts either by spectrophotometry or fluorometry [133, 135]. At the initial step of the cloud point extraction, pH of the 9 mL solutions was adjusted to 1.50 by dropwise addition of 1.0 M NaOH solution and 1.0 M HCl and using a pH meter. In the second step, 0.10% (w/v) of DAN solution was added to the solutions. The molar concentration ratio of DAN to Se(IV) was equal to 25 in the final solution. Contents were heated at 70 °C in a water bath for 45 min in order to form Se(IV)-DAN complex. Then, 1.0 mL of 0.25 % (w/v) of Triton X-114 was added to the solutions and the contents were kept at the refrigerator for 30 min to solubilize the hydrophobic complex in micellar medium. After this step, the mixture was again heated to 50 °C in water and kept there for 10 min in order to form the separated surfactant phase. Resulting

mixture was centrifuged for 5 min at 4500 rpm and surfactant rich phase was separated from aqueous phase, then its volume was completed to the 1.0 mL with de- ionized water; finally fluorescence emission was measured.

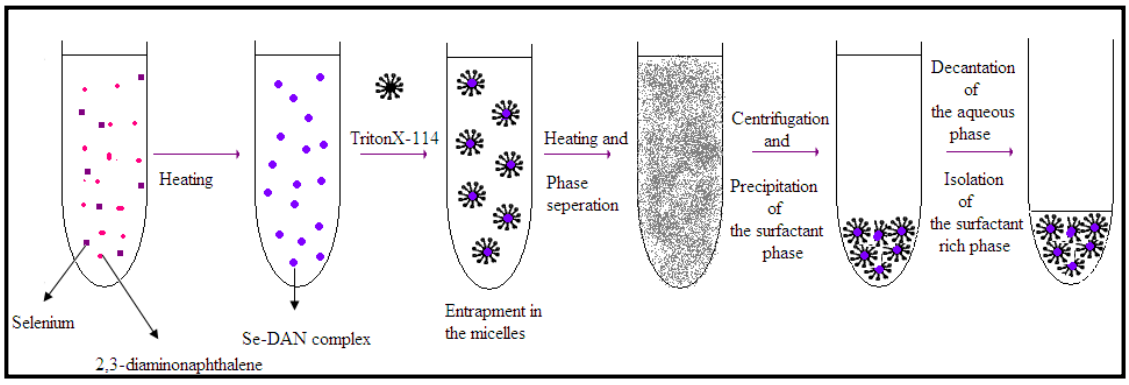

Figure 2.1 Procedure steps of complex formation and Preconcentration of complex by surfactant.

2.4.2 Preparation of Vitamin Tablets

2.4.2.1 Sample Preparation for Selenite, Se(IV) Containing Samples, "Selenite Procedure"

Tablets were removed from the pack and crushed using a mortar and pestle. The resulting powder was accurately weighed into glass round bottom flasks and 30 mL of water were added. The extracts obtained after 25 min of sonication were centrifuged at 4500 rpm for 5.0 min, filtered

and separated into three portions for 3-point standard addition measurement. After spiking of Se(IV) standard solutions, cloud point extraction procedure given above was applied to each portion.

2.4.2.2 Sample Preparation for Selenate, Se(VI) Containing Samples, "Selenate Procedure"

A tablet of vitamin containing selenate was dissolved in 30 mL of 12.06 M HCI in the ultrasonic bath for 25 minutes. After the sonication vitamin solution was filtered by a pump of filtration and solid part was discarded. Then it was seperated into three 9 mL of volume and they were put in to the microwave for the reduction of Se(VI) to Se(IV) by applying microwave program given in the Table 1. After microwave program the solutions were kept in the cold water to cool them for not to lose any amount of samples. If they are opened while they are stil hot, gas vapor goes out, thus, there can be loss of sample with the vapor. Therefore the solutions were cooled, and then they were filtered again since the components (silicate) in the vitamin formed solid particles when they react with HCI. After filtration cloud point extraction was applied. At the initial step, pH of the solutions was adjusted to 2.0 by dropwise addition of concentrated NaOH solution and 1.0 M HCI by using a pH meter. Then, 1 mL of water, 1 mL of 5 mg L^{-1} Se(IV), and 1 mL 10 mg L^{-1} Se(IV) were added to the solutions respectively. In the second step DAN solution was added to the solutions to form the complex. For the third step, 1 mL of 0.25 % (w/v) of Triton X-114 was added to the solutions after formation of complex and they were kept at the refrigerator for 30 min for micelle formation. In the last step, solutions were centrifuged for 5 min at 4500 rpm and surfactant rich phase was seperated from aqueous phase, then the volume of

surfactant rich phase was completed to the 1.0 mL with deionize water and the fluorescence emission was measured.

2.4.2.3 Sample Preparation for Organic Selenium Species Containing Samples, "Organic Selenium Procedure"

The "Organic Selenium Procedure" was developed for the determination of the organometalic selenium species. In this method, the tablet containing selenomethionine was dissolved in 30.0 mL of 14.3 M HNO_3 in the ultrasonic bath for 30 minutes. After the sonication it was heated for 30 min and completed to 100 mL with de-ionized water. All of the selenomethionine should be oxidized to Se(VI) after HNO_3 digestion. Solution was filtrated using a filtration pump and solid part was discarded. 30 mL were taken from the vitamin solution and it was seperated into 3 parts of 10.0 mL each for further standard addition. At the beginning, each solution was heated up till getting 2.0 mL solution was left and 8.0 mL of water was added. Solution was heated up again until 2.0 mL solution was left. pH of solutions were checked. 0.05 g $NaBH_4$ prepared in 10.0% (w/v) of NaOH was added to solution to make not only the neutralization but also the reduction of the oxidized selenium to Se(IV). pH of the solution was controlled to make sure that neutralization was completed. After neutralization part, 6.0 mL of 12.06 M HCl were added and solution was put into microwave to reduce the all oxidized selenium. Microwave program given in the Table 1 was applied. After the reduction step using microwave, cloud point extraction was applied for all solutions. At the end of cloud point extraction, volume of surfactant rich phase was completed to the 1.0 mL and the fluorescence emission was measured.

2.4.3 Optimization of the Parameters for Cloud Point Extraction of the Complex

All of the parameters in the CPE method, pH, reaction period, and reaction temperature for the formation of Se-DAN complex, ligand concentration, effect of pH to CPE and percentage of surfactant were optimized to improve the sensitivity for the determination of the selenium in vitamin tablets.

2.4.3.1 Effect of pH to Extraction Efficiency

To investigate the effect of pH to CPE a set of experiment containing 2.0 mg/ L Se (IV) standard and 500 µL 0.1% of DAN were prepared, before adding DAN pH of the solutions were adjusted by using NaOH solution or dilute HCl. The pH values of the resulting solutions were 1.0, 1.5, 2.0, 2.5 and 3.0. After adjusting the pH of the solutions, the cloud point extraction method was applied to each solution. Then, the fluorescence intensity of surfactant rich phase was measured. Conditions: λ_{ex}= 380nm, slit width= 20.

2.4.3.2 Optimization of Molar DAN/Selenium Ratio

In order to find the proper amount of DAN, a set of experiments containing various concentrations of DAN was performed. It was observed that fluorescence intensity increases as the DAN to selenium molar ratio increases from 20 to 50, becomes steady when the ratio is varied from 50 to 500 and decreases slightly afterwards. When the ratio of DAN to

selenium was increased above 25, blank readings were also increased. Optimum DAN to selenium ratio was found to be 25.

2.4.3.3 The Effect of Triton X-114 Concentration on the CPE Efficiency of Selenite

The effect of surfactant percentage was determined by a set of experiments. 2.0 mgL^{-1} Se (IV) was reacted with 500 μL 0.1% (w/v) DAN solution ([DAN]/ [Se (IV)] = 25) at pH 1.5. After complexation part, Triton X-114 solution was added to the solution that surfactant concentration was varied from 0.1% (w/v) to 0.5% (w/v) and mixed. For formation of micelles, each solution was kept at refrigerator for 30 min. After micellization, samples were heated for 10 min at 50 °C and centrifuge part of cloud point extraction for separation surfactant phase from aqueous phase. The volume of surfactant rich phase was diluted to 1 mL. Then, the emission signals of each solution including different concentration of surfactant were measured. Conditions: λ_{ex}= 380 nm, slit width= 20.

Se (IV) standards with concentrations varied from 0.1 mg L^{-1} to 1.0 mg L^{-1} were used for the calibration curve. First their DAN complexes were formed and than cloud point extraction method was applied as decribed before.

The volume of the surfactant rich phase remaining in the centrifuge tube was diluted to 1 mL with de-ionized water and the fluorescence emission signals of the surfactant rich phases were measured.

2.4.4 Analysis of Real Sample (SRM)

Accuracy of the method was checked using EnviroMat Waste Water, EU-L-2 as SRM. Selenate Procedure using HCI was applied in the extraction step. Proper amount of SRM was taken before reduction step and these were completed to 9.0 mL with 12.06 M HCI. Standard addition was applied to eliminate any possible interferences coming from matrix. Microwave program given in the Table 2.1 was used in the reduction. Quantitative determination of the selenium in SRM was done using CPE method. At the end of the CPE method described in the experimental part, surfactant rich phase is separated from aqueous phase, then the volume of surfactant rich phase is completed to the 1.0 mL with de- ionize water and the fluorescence emission is measured. It was observed that Se(IV)- DAN complex prepared from SRM EU-L2 gave the signal around 570 nm where we are expected the signal of complex appears normally. There is no shift of the signal wavelength on the spectrum unlike vitamin measurements.

2.5 Determination of Selenium in Vegetables

In this part of the study, total selenium was determined in dill, watercress herb and garlic samples. The samples were digested in microwave oven. For the determination of total selenium in vegetables; ICP-MS was used.

2.5.1 Materials and Sample Pretreatment

Dill, watercress herb and garlic seeds (Taşköprü region of Kastamonu) were collected from a local market. The plants irrigated with selenium rich water were grown in a pot at METU Biology Department's greenhouse. The samples were irrigated with de-ionized water till

germination of seeds. The number of days of irrigation is given in the Table 2.3. Afterwards the plants were supplemented with 250 mL of 10 mg L^{-1} and 20 mg L^{-1} selenite solutions for 14 days and 9 days respectively. Also control groups were grown under the same controlled environment but irrigated with de-ionized water only. When the plants just started to turn yellow as seen in Figure 2.2 and 2.3, while they were still edible, they were cut from the roots with a stainless-steel scissors. After harvesting the stem and leaf parts of the plants, they were weighed to be analyzed for moisture before lyophilization. Then, plants were placed in a 500 mL of volumetric flasks and deepfreezed overnight at -80 °C. The frozen samples were lyophilized at -110 °C with vacuum freeze drier. The durations of lyophilization were 6 and 10 days for dill and garlic in the vacuum freeze drier respectively. After lyophilization process, the dry samples were powdered in a mortar to get them ready for digestion step and they were kept at the refrigerator till being digested.

Table 2.3 The number of days of irrigation of plants using indicated solutions; de-ionized water[a], 10 mg L^{-1} Se(IV)[b], and 20 mg L^{-1} Se(IV)[c]

Plants	**# of pots of plants**	**Control group[a]**	**Enriched group[b]**	**Enriched group[c]**
Dill	2	20	14	7
Watercress herb	2	20	14	7
Garlic	2	20	16	8

Figure 2.2 Watercress herb

Figure 2.3 Anethum graveolens (Dill)

2.5.2 Digestion of Samples

Dill, watercress herb and garlic samples were digested with microwave assisted-wet digestion method. The microwave program applied is given in Table 2.2. 0.2 g of dried dill and garlic samples was weighed and placed in the Teflon pots. They were digested with an acid mixture of 3 mL of HNO_3 (65%)w/v and 3 mL of H_20_2 (35%)w/v.

CHAPTER 3

RESULTS AND DISCUSSION

In this study, cloud point extraction technique was used for the determination of Se(IV), Se(VI) and total organic selenium species in commercially available selenium supplemented vitamin tablets. In the cloud point extraction process, Se(IV) is taken into a hydrophobic complex with 2, 3-diaminonaphthalene (DAN) and then solubilized within the nonionic micelle Triton X-114. The hydrophobic complex, 4,5-benzopiazselenol, preconcentrated in the surfactant rich phase of Triton X-114 was determined spectrofluorometrically. The total selenium contents of the samples were determined utilizing microwave nitric acid digestion and ICP-MS detection. Furthermore selenium rich dill, watercress herb and garlic samples were grown in pot under a controlled atmosphere. Their total selenium determinations were done utilizing ICP-MS.

3.1 Fluorescence Spectra of 2, 3-diaminonaphthalene (DAN) and Se-DAN Complex

In the cloud point extraction the extracted complex should be in the hydrophobic form. 2, 3-diaminonaphtalene was a fluorescent material used. The reaction of selenite with DAN produces the hydrophobic compound 4, 5-benzopiazselenol (Se(IV)-DAN). It is stable to air-drying and does not decompose below 290 °C. The emission spectra of DAN and its piazselenol in water are given in Figure 3.1. After the formation of

Se(IV)-DAN complex the emission wavelength shifts from 440 nm (the emission wavelength of DAN) to 570 nm.

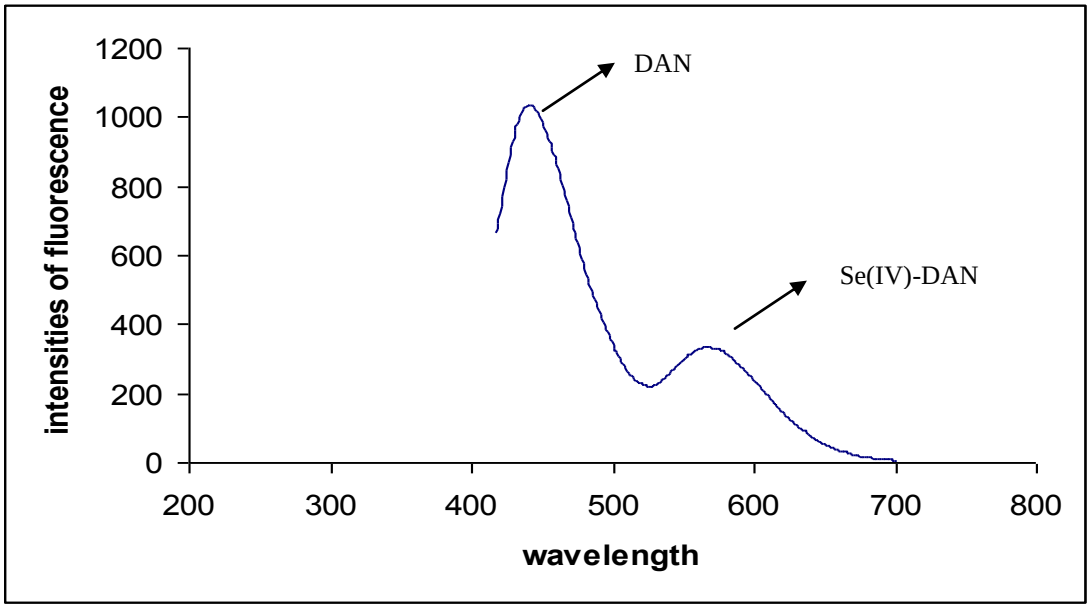

Figure 3.1 Fluorescence emission signals of DAN and Se(IV)-DAN complex in water (λ_{em} = 440 nm for DAN and λ_{em} =570 nm for Se(IV)-DAN complex).

3.2 Optimization of the Parameters for Cloud Point Extraction of the Complex

All of the parameters in the cloud point extraction method were optimized to get high sensitivity for the determination of the selenium content of tablets.

3.2.1 Effect of pH of the Medium on Complex Formation

To find the optimum pH for the complex formation The pH values of these solutions were adjusted in the range of 1.0-3.0. DAN was added after pH adjustment since it is not stable in strong acid medium. Fluorescence measurements were taken after cloud point extraction As can be seen from Figure 3.2, fluoresecence intensity reaches a maxium at pH 2.0.

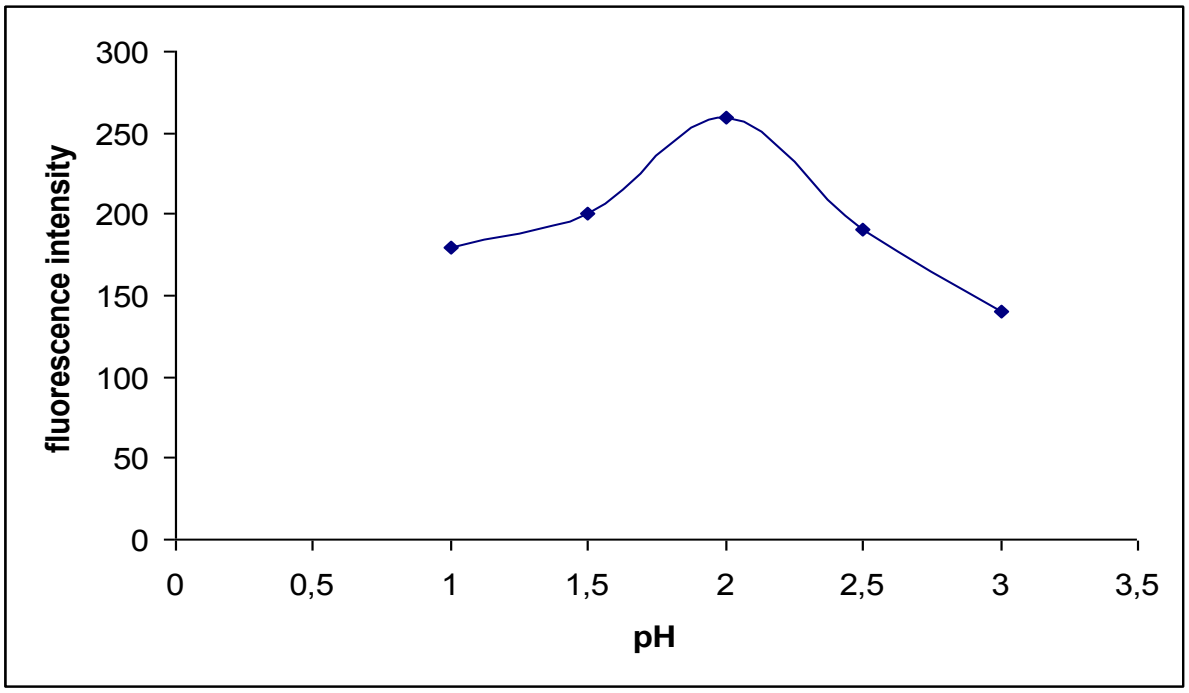

Figure 3.2 Effect of pH on the formation of Se(IV)-DAN complex. (λ_{em}= 570 nm for Se(IV)-DAN complex).

3.2.2 The Effect of Triton X-114 Concentration on the CPE Efficiency of the Complex

The concentration of the surfactant used in CPE is a very critical parameter. Because the volume of the extracted layer and associated with it the preconcentration factor depend on the nature and the total amount of the surfactant employed. In most extraction experiments given in literature [136, 142], the surfactant concentration is kept in the range of 0.1 to 2.0 % w/v, with a corresponding volume of the extraction layer being between 2 and 10 % of the initial solution volume.

To see the effect of surfactant concentration on the extraction efficiency and the fluorescence intensity of the Se-DAN complex, 0.15 mg/L Se (IV) standard was reacted with appropriate amount of DAN and CPE was performed with different surfactant concentration in between 0.1% and 0.5% Triton X-114. As can be seen in the Figure 3.3, the fluorescence signal reaches a plateau after the usage of % 0.25 (w/v) Triton X-114. However as the surfactant concentration increases, volume of the surfactant rich phase also increases. For example; when we used % 0.1 Triton X-114, the volume of the surfactant rich phase was about 30-40 μL (or 40 mg) whereas the volume of the surfactant rich phase was approximately 100 μL (90 mg) when % 0.25 Triton X-114 was used. Hence, at low surfactant concentrations the preconcentration factor increases. In general, the volume of the surfactant rich phase is in between 50-400 μL. In this study 0.25 % surfactant concentration was decided to be used due to the high extraction efficiency and the preconcentration factor obtained.

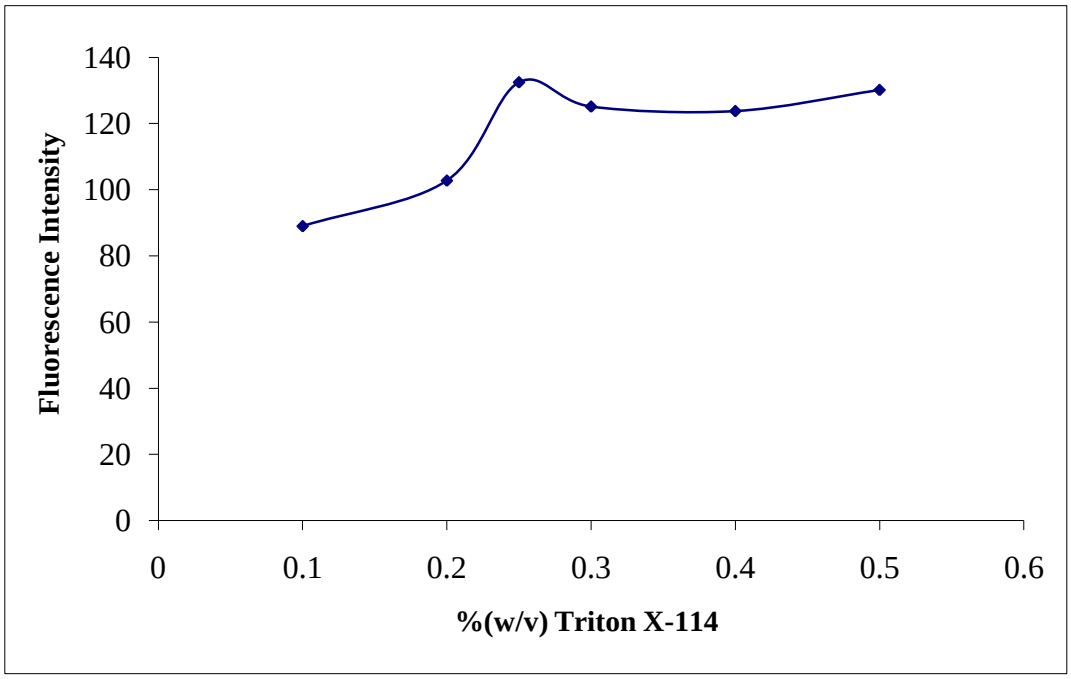

Figure 3.3 Effect of surfactant concentration on the fluorescence intensity of Se-DAN complex. (λ_{em}= 570 nm for Se(IV)-DAN complex)

3.2.3 Effect of DAN Amount on the Complex Formation

In order find the proper amount of DAN solution used in the experiment, several experiments were carried out using various DAN to Se(IV) ratio. As can be seen in Figure 3.4, the fluorescence intensity is maximum at the DAN to Se(IV) molar ratio of 12.5. However it is not possible to predict the amount of selenium in a real sample. Therefore, to be in the safe site, we used DAN / Se(IV) molar ratio of 25.

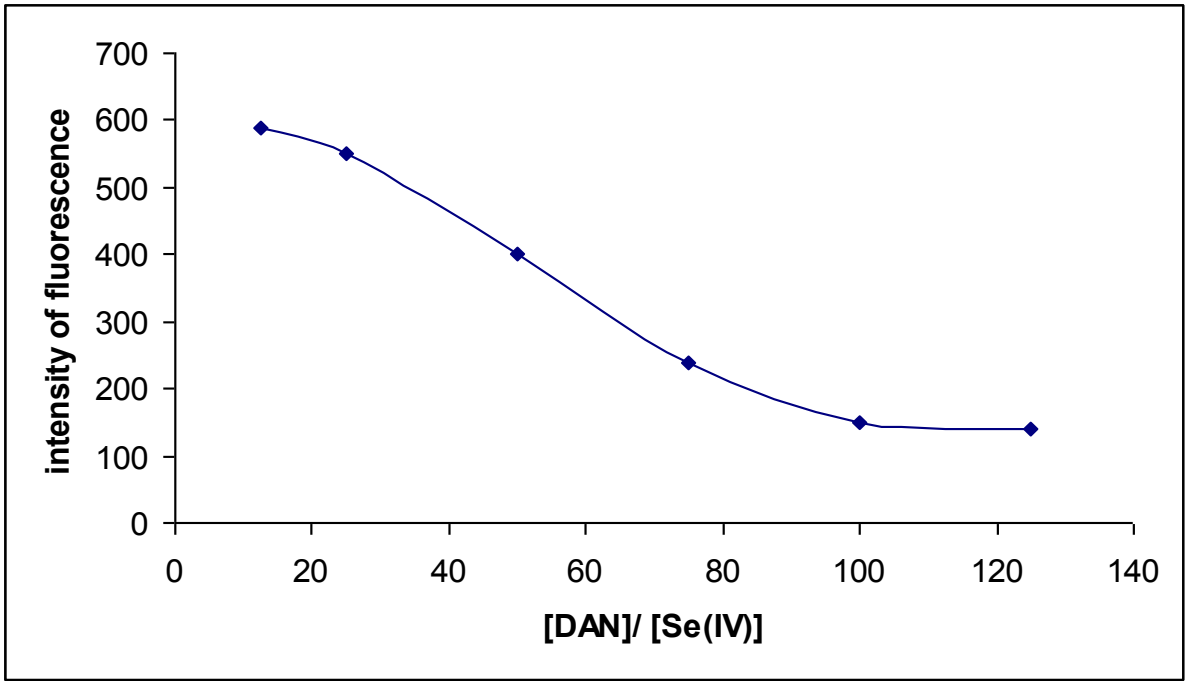

Figure 3.4 Effect of DAN / Se(IV) molar ratio on fluorescence intensity of Se(IV)-DAN complex. (λ_{em}= 570 nm for Se(IV)-DAN complex)

3.3 Analytical Performance Characteristics for CPE and Fluorescence Determination of Selenite

The calibration plot obtained after the cloud point extractions of 0.1 mg/L to 1.0 mg/L selenite as Se-DAN complex. Standard deviation of 12 replicates of 0.02 mg/L Se-DAN complex after 10 fold preconcentration was calculated. The detection limit, established as 3*s* /slope was found as 2.3 µg/L Se.

It has to be point out that, in this study fluorescence measurements were done in a regular cell (4 mL) for which at least one mL solution was

required for the measurement. For that reason preconcetration factor (PF) was restricted to 10 (10/1).

However PF and relatedly detection limit can be improved either by using smaller volume cell or applying CPE to a large volume of solution having low selenite concentration. In our studies the size of the centrifuge tubes used was too small for a practical separation of sample volumes larger than 10 mL. Therefore, our CPE studies were limited to 10 mL volume of the original solution.

3.4 Accuracy Check

Evaluation of accuracy was performed using EnviroMat Waste Water, EU-L-2 certified reference material. The chemical form of the selenium was not stated for this CRM. The sample preparation procedure for selenate (also considered as total inorganic selenium) was applied. Calibration curve is given in the Figure 3.5 and results are given in Table 3.1. The selenium concentration was found as 2.5 ± 0.3 mg/L. The certified value of the standard was reported as 2.7 ± 0.1 mg/L. According to student t-test at 95% confidence level calculated and reported values are the same.

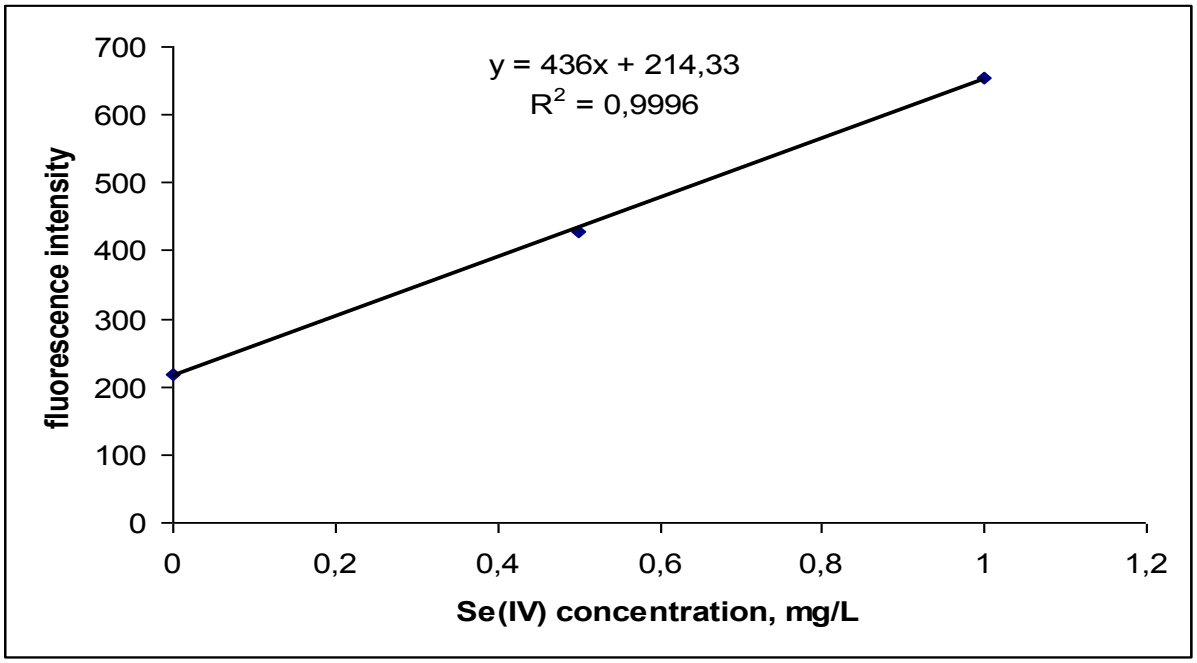

Figure 3.5 Calibration curve of Se(IV)-DAN obtained from SRM after cloud point extraction

Table 3.1 Determination of Se in Standard Reference Material using Selenate Procedure.

Sample	Se concentration (µg/L)
Se(found)	2.5 ± 0.3
Se (certified value)	2.7 ± 0.1

The result given in Table 3.1 was in good agreement with the certified values which confirmed the accuracy of the fluorometric selenite determination after CPE. Furthermore, the closeness of the result to the

reported value was also confirmed that the proposed method for selenite determination was not affected from the other ions present in the waste water standard at their reported concentrations.

3.5 Determination of Selenium Species in Vitamin Tablets

Various selenium forms are mixed with excipients normally incorporated into commercially available pharmaceutical tablets; thus the sample matrix is complex and consequently an appropriate sample preparation procedure should be chosen for measuring the concentration of selenium species in vitamin tablets. The liquid extraction and enzymatic hydrolysis procedures have been used in literature for the extraction of selenium species from various matrices [143]. Several solvents, such as aqueous HCl; hot water; methanesulfonic acid; tetramethylammonium hydroxide (TMAH) and sodium dodecyl sulphate (SDS) are used in liquid extraction, whereas for enzymatic hydrolysis, Proteinase K and Protease XIV are usually prefered [145,146]. Enzymatic hydrolysis is particularly important when selenium is attached to peptides or proteins as in the case of yeast. Enzyme cleaves proteins at preferred sites to remove selenium species from yeast or yeast containing food or vitamin supplements.

Selenium should be in selenite form for fluorescence detection. CPE-fluorescence detection methodology was applied to the extracts of the selenium supplemented vitamin tablets to investigate both the identity and concentration of selenium species present in the samples.

Among the inorganic and organic selenium species, only selenite can form complex with DAN. This fact was explored for the speciation of the selenium in terms of selenite, selenate and organic selenium by modifying

sample preparation procedure slightly prior to CPE. Detailed procedures specific to selenite, selenate and organic selenium were given in the experimental section. As a summary, if the form of the selenium in the tablet was selenite, CPE can be directly applied to the sample extracts. If selenate was present in the tablet, CPE should be applied to the sample extract after a reduction of selenate to selenite using 12 M HCI. In case of organic selenium containing tablets, the sample extract should be first oxidized to convert all the organic selenium into selenate. Then solution should be neutralized and again selenate should be reduced to selenite. Afterwards CPE was employed to find the total selenium. Thus, if the label stated that the content of the tablet was inorganic (Brand A), CPE procedures for selenite and selenate were employed. If organic selenium was claimed (Brand B&C) CPE procedures for selenite, selenate and organic selenium were employed. In general, using the procedure for organic selenium, total Se content is determined. The procedure for Se(IV) gives the total inorganic Se content. Since Se(IV) procedure provides Se(IV) content, concentration of Se(IV) and organic Se are found by taking appropriate differences. The results were compared with the total selenium content of the tablets acquired by using an independent method, wet digestion-ICP-MS, HPLC-ICP-MS was used for comparison.

The quantification of Se species in selenium supplemented vitamin utilizing CPE- Fluorescence methodology was based on three point standard addition method. Preconcentration factor was 10. The fluorescence signals for spiked and unspiked sample extracts of Brand A , B and C are given in Figure 3.6, Figure 3.8 and Figure 3.10, respectively.

3.5.1 Brand A

The vitamin tablet of Brand A contains inorganic form of selenium; hence the 'selenite procedure' and the 'selenate procedure' were applied. The fluorescence signals for spiked and unspiked sample extract of Brand A are given in Figure 3.6. The standard addition plot is presented in Figure 3.7.

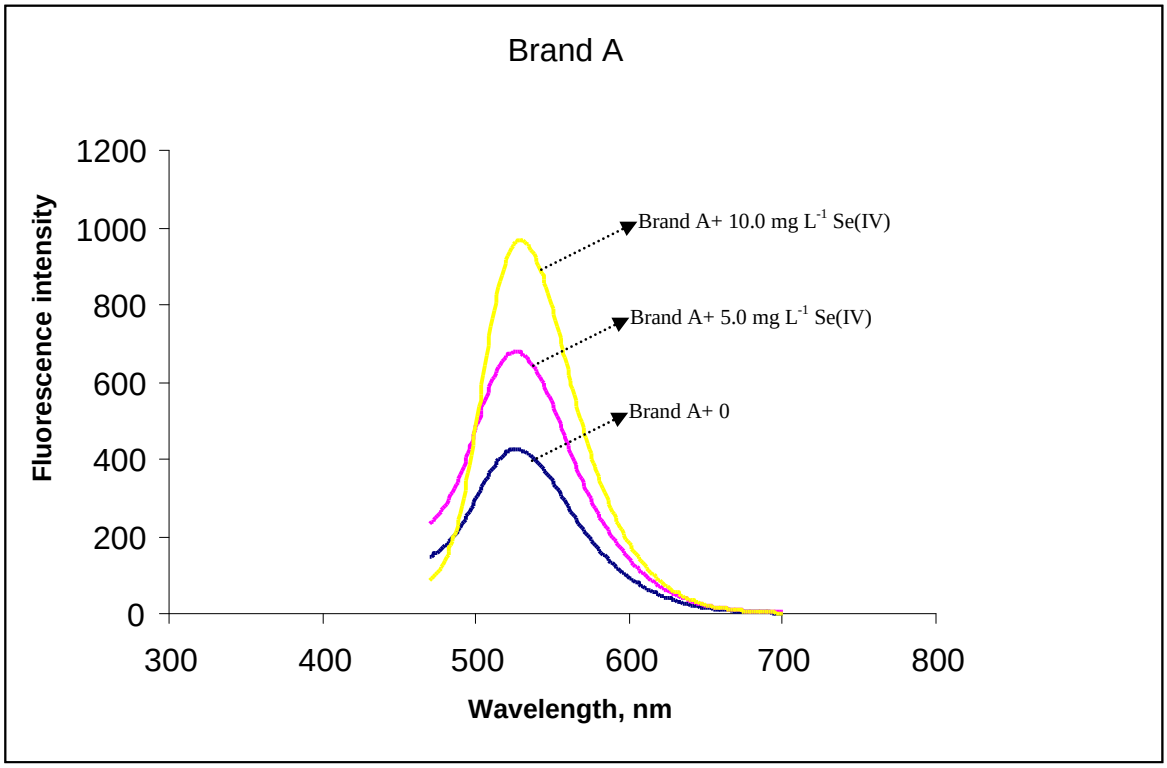

Figure 3.6 Fluorescence spectra of Se(IV)-DAN obtained from Brand A after cloud point extraction using the optimum parameters.

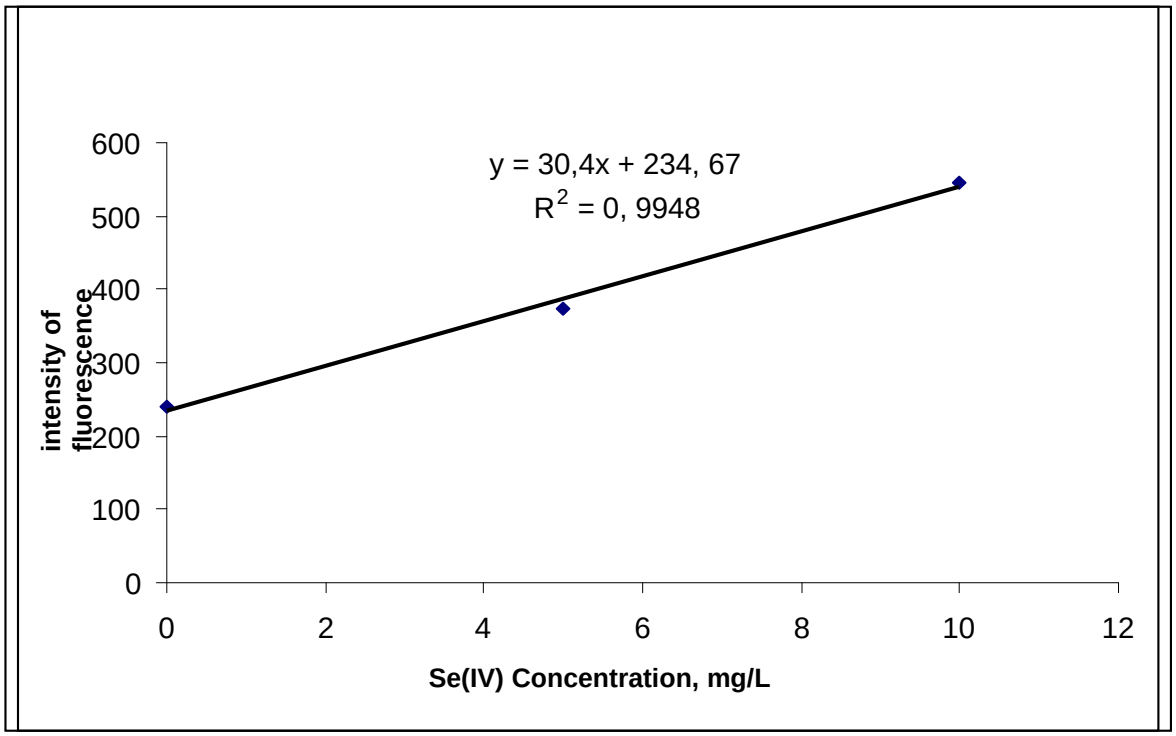

Figure 3.7 Calibration curve of Se(IV)-DAN obtained from Brand A after cloud point extraction.

3.5.2 Brand B

The vitamin tablet of Brand B contains organometallic form of selenium; hence the 'organic selenium procedure' was applied. The fluorescence signals for spiked and unspiked sample extract of Brand B are given in Figure 3.8. The standard addition plot is presented in Figure 3.9.

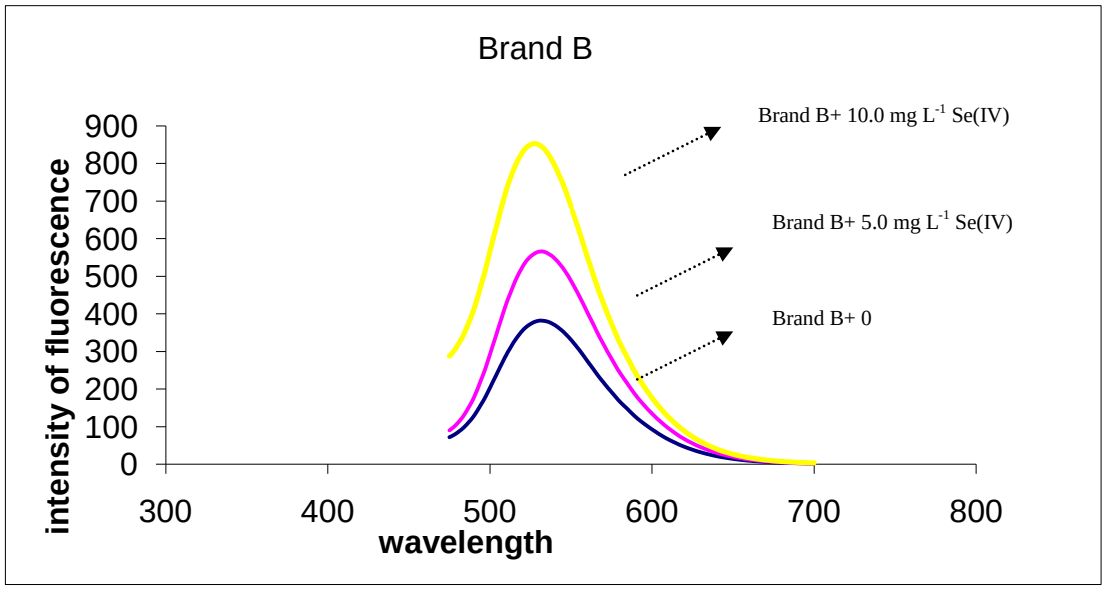

Figure 3.8 Fluorescence spectra of Se(IV)-DAN obtained from Brand B after cloud point extraction.

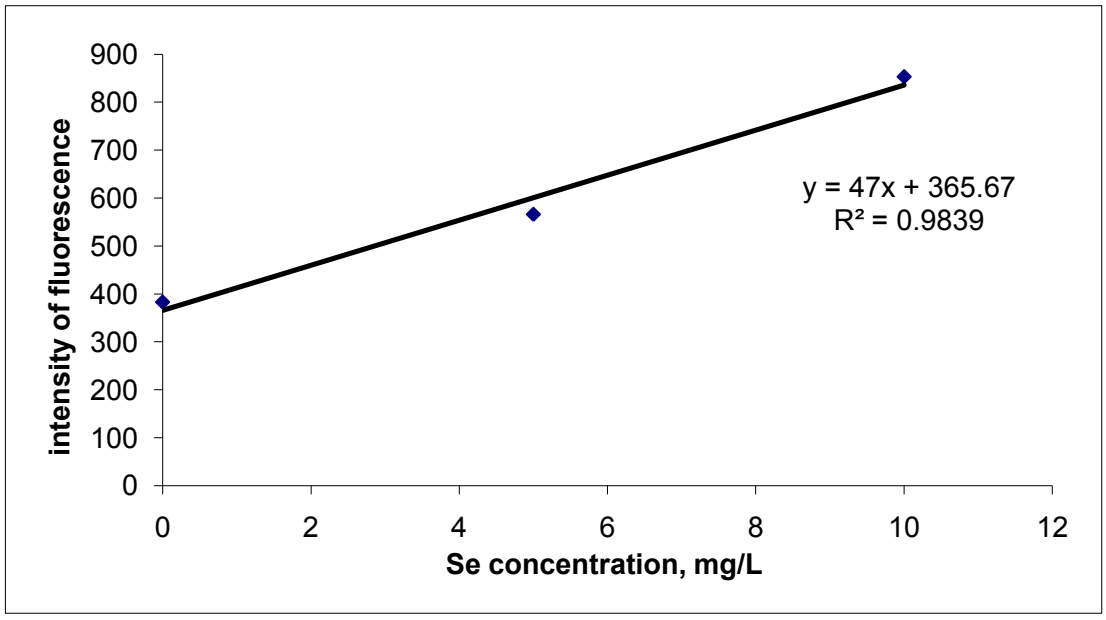

Figure 3.9 Calibration curve of Se(IV)-DAN obtained from Brand B after cloud point extraction

3.4.3 Brand C

The vitamin tablet of Brand C contains inorganic form of selenium; hence the 'selenite procedure' and the 'selenate procedure' were applied. The fluorescence signals for spiked and unspiked sample extract of Brand C are given in Figure 3.10. The standard addition plot is presentedin Figure 3.11.

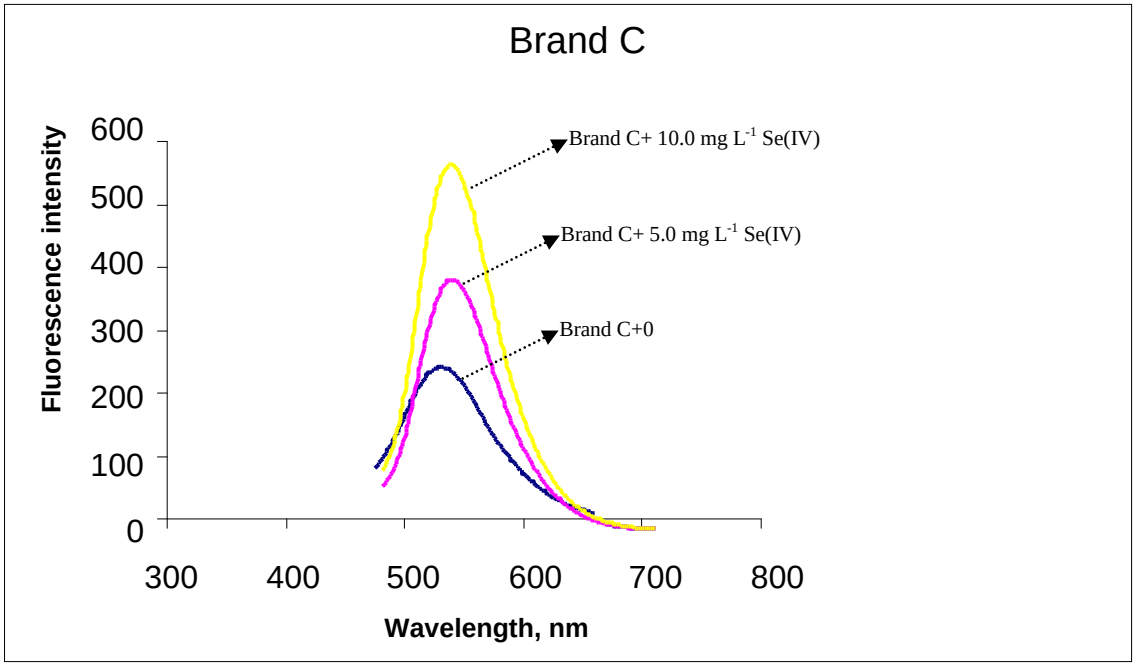

Figure 3.10 Fluorescence spectra of Se(IV)-DAN obtained from Brand C after cloud point extraction

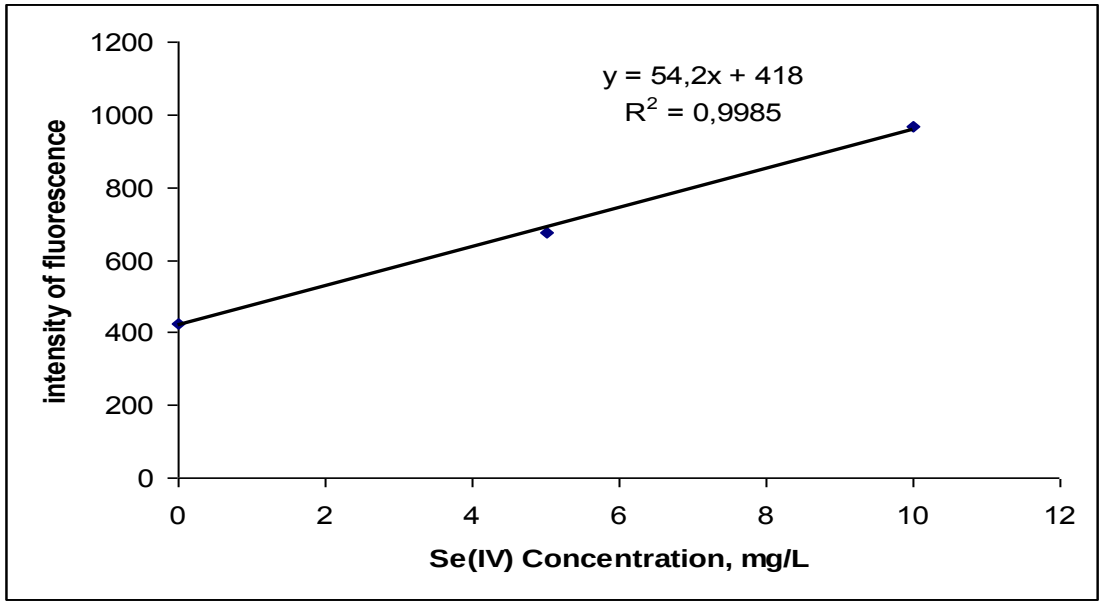

Figure 3.11 Calibration curve of Se(IV)-DAN obtained from Brand C after cloud point extraction.

As can be seen from Figure 3.6; 3.8 and 3.10, the wavelength maximum of the fluorescence emission signals for the sample extracts (both spiked and unspiked) was slightly shifted (ca 30 nm) compared to that of the aqueous standard solutions. This was probably due to the matrix effect. The magnitude of selenium per vitamin tablet determined by CPE-Fluorescence and ICP-MS methods are given in Table 3.2 together with the selenium amounts claimed by the producers. The four replicates vitamin tablet CPE-Fluorescence assay gave results reasonably close to the label value of the supplement.

Table 3.2 Selenium results given on selenium supplement tablets and results obtained by using both CPE-Fluorescence and Wet digestion- ICP-MS methods.

Samples	Total Se (µg)/Tablet Labeled amount	Se (µg)/Tablet Found by CPE-Fluorescence Method	Total Se (µg)/Tablet Found by Wet digestion- ICP- MS method
Brand A	25 µg Se	25. 8 ± 3.3 µg Se N=4	26.0 ± 0.8 µg
Brand B	100 µg Se	103. 4 ± 14.8 µg Se N=4	109.1 ± 1.5 µg
Brand C	25 µg Se	27. 2 ± 3.6 µg Se N=4	32.8 ± 1.6 µg

The 25 µg Se per tablet Brands A and C, and 100 µg Se per tablet Brand B had selenium levels of 25.8, 27.2, 103.4 µg respectively. The amounts of total selenium per tablet acquired by wet digestion-ICP-MS method were 26.0 µg for Brand A, 109.1 µg for Brand B and 32.8 µg for Brand C. The results of CPE-fluorescence method for Brand A and B are closely matching with that of the wet digestion ICP-MS method. Only the result of Brand C by ICP-MS stays as an outlier. However, comparison with wet digestion ICP-MS method should be considered as the accuracy check of the total analysis in terms of both CPE-fluorescence methodology and the acid extraction of the selenium supplemented vitamin tablets. Although practical, low selenium recoveries have been reported for the analysis of food supplements utilizing extraction method instead of total digestion

[147,148].The possible reasons were stated as inefficient removal of the selenium from the matrix and loss of selenium through adsorption onto the insoluble tablet excipients during filtering process. Besides, tablet to tablet variations of selenium content during manufacturing may also influence the outcome variability of the selenium supplemented food product analysis. Considering these facts, CPE-Fluorescence methodology can be considered as a suitable method for the quantitative determination of selenium in the food supplements.

The chemical form of the selenium in the tablets was identified by applying three different sample preparation procedures specific to selenite, selenate and organic selenium and the fluorescence signal of the solutions were followed. Here, organic selenium corresponds to all organic selenium species (selenomethionine, selenocysteine, etc.) in which selenium is in (-II) oxidation state. The results are shown in Table 3.3.

Table 3.3 Selenium species in selenium supplement tablets. All amounts refer to Se.

	CPE procedures applied			
Sample name and Selenium Identity Claimed	**CPE procedure for Selenite**	**CPE procedure for Selanate**	**CPE procedure for organic Se**	**Identity of Selenium Found**
Brand A; as selenate	n.d.*	25. 8 ± 3.3 µg	Not applied	Selenate
Brand B; as selenomethionine	n.d.	n.d.	103. 4 ± 14.8 µg Se	Organic selenium
Brand C; as selenomethionine	n.d.	27. 2 ± 3.6 µg Se	26.4± 3.1 µg Se	Selenate

*n.d. : not detected

3.6 Determination of Selenium in Plants

0.2 g of lyophilized watercress herb and dill were powdered in the mortar. Then, both control groups and samples enriched with 10 and 20 ppm were digested with the acid mixture (1:1) of HNO_3 and H_2O_2 in the microwave. After digestion of samples, total selenium in plants was determined by ICP-MS with two replicates in Central Laboratory. The results given in the Table 3.4, indicated that enriched samples contain 70 times higher amount of selenium than control groups. Also, it can be seen from the results that feeding with 20 ppm Se(IV) did not make much more difference in the amount of selenium in the plants than feeding with 10 mg L^{-1} Se(IV). That is, feeding plants with 10 mg L^{-1} Se(IV) is enough for getting recommended amount of selenium intake.

Table 3.4 Selenium results obtained by using Wet digestion- ICP- MS methods for plants.

Watercress herb	**Se(µg/g)**
Control group	0.81±0.026
+ 10 ppm Se(IV)	60.86±0.702
+ 20 ppm Se(IV)	69.07±0.204

CONCLUSION

In this study, we have successfully used the cloud point extraction as a preconcentration and separation method to find the amount of organometallic and inorganic selenium in vitamin tablets. To the best of our knowledge, this report is the first one where a simple CPE procedure is used for Se speciation. Several studies have shown that [149] organic selenium is more bioavailable and less toxic than inorganic forms of selenium such as sodium selenate and selenite. Hence, quality control measurements should be able to identify the form of the selenium as well as the amount of selenium present in the tablet. As investigated in this study, CPE-fluorescence methodology proposed herein is potent enough to determine both the amount and the identity (selenate, selenite and organic selenium) of the selenium present in selenium supplemented vitamin tablets. Furthermore, the proposed methodology has the advantages of the simple operation, good sensitivity and reasonable reproducibility; in addition, the method is safer as compared to some other solvent extraction procedures since there is no exposure to organic compound vapors. All these factors have shown the potentiality and versatility of CPE-fluorescence method for quality control of selenium supplemented food products in terms of both quantity and identity. Selenium Hrich dill, watercress herb and garlic were grown in pots by watering with 10 and 20 μg mL^{-1} selenite solutions. Their total selenium contents were determined by ICP-MS. Se supplemented samples contain 70 fold higher amount of selenium compared to that of control groups. It was found that, feeding plants with 10 mg L^{-1} Se(IV) was enough for getting recommended amount of selenium intake.

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