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Original Research Article

Rapid, Simple, Cost Effective and Validated Methodology for Total Selenium Determination in *Saccharomyces Cerevisiae* Using RP-HPLC-UV After Microwave Assisted Digestion

Mehdi Tavancheh[1]*, Saeedeh Molaee[2]

[1] *Tehran Chemie Pharmaceutical Co., 1378756411, Tehran, Iran*

[2] *Zist Takhmir Pharmaceutical Co., 1683848411, Tehran, Iran*

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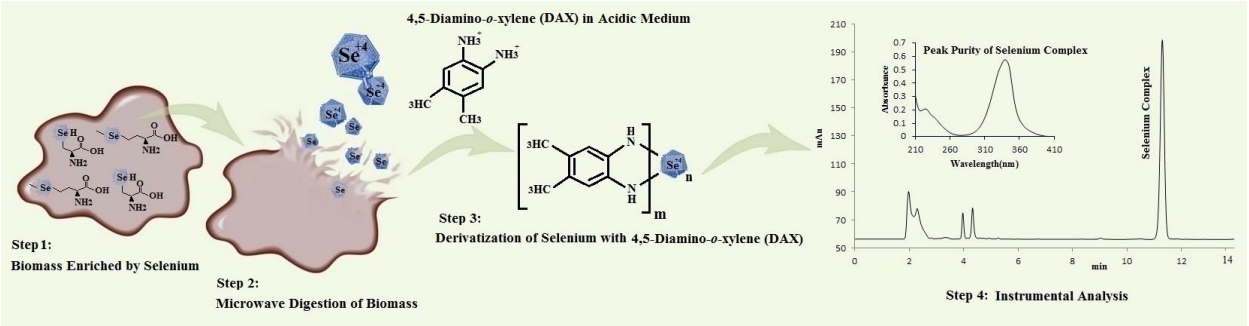
ABSTRACT

A facilitated, precise, specific methodology to determine the amount of total selenium in selenium-enriched biomass of Saccharomyces cerevisiae was designed and implemented using high-performance liquid chromatography technique and UV detection (HPLC-UV). In this research study, a novel chromogenic reagent of 4, 5-Diamino-o-xylene (DAX) was evaluated for off-line pre-column selenium (IV) complexation. The complex of Se (IV) was eluted isocratically on BRISA LC2 C18 (250×0.46 mm, 5 µm, Teknokroma) analytical column**.** The mobile phase used was a degassed solution of deionized water and acetonitrile solution in a ratio of 50:50 (v/v) at a flow rate of 1.0 mL/min. UV-Vis detector at 340nm was used for complex detection. The method parameters were validated according to the ICH Q2 (R1) requirements. The linearity was established over the dynamic range of 25-700 µg L^{-1} (r^2=0.9994). The relative standard deviations (RSDs) were 1.1% for within-day determination (n=6) and 1.8% for between-day determination (n=6). The detection limit (LOD) and quantitation limit (LOQ) were found to be 8.0 and 25 µg L^{-1}, respectively.

GRAPHICAL ABSTRACT



* Corresponding author: Tavancheh, Mehdi
✉ E-mail: mtavanchem@yahoo.com
☎ Tel number: +982177280522

Introduction

Selenium (Se) is the 34th element of the elements periodic table, respectively; was discovered in 1817 by J. Berzelius (1848-1779). It also exhibits metallic and non-metallic properties and is known as a metalloid. Its non-metallic properties are very similar to sulfur but selenium reacts much faster than sulfur [1]. This element is known as one of the micronutrients in human health [2, 3]. Therefore, it is important from the medicinal and pharmacological point of views [4, 5]. Generally, 55 micrograms of selenium in the diet is recommended daily [6]. Along with the benefits of this element, its toxic properties have always been an important feature in its recognition [7-10]. Potentially toxic dose for selenium is between 800 and 1000 µg Se per day [11]. The chemical form, bioavailability and dosage**,** determine the usefulness or harmfulness of selenium The bioavailability of selenium depends on its chemical form [12]. Two species of selenium in the form of selenite and selenate are two water-soluble minerals. It should be noted, however, that selenite is 5 to 10 times more bioavailable than selenate and is also more toxic. Organic form of selenium has much higher bioavailability and taken up than mineral species. This bioavailability reaches up to 1000 times [13]. Selenium protein compounds show much more bioavailability than other species of this element and microbial reduction methods are used to prepare these compounds [15-18]. Therefore, yeasts enriched with this element are an excellent source of nutrition [18]. Various methods have been proposed and published for total selenium and speciation determination in type of real samples such as atomic adsorption techniques (AAS) [19], atomic fluorescence techniques (AFS) [15, 16], flow injection spectroscopy [22], gas chromatography combined with mass detector (GC-MS) [23], high-performance liquid chromatography combined with inductively coupled plasma and following mass spectrometry (HPLC-ICP-MS) [24–28], inductively coupled plasma-optical emission spectrometry (ICP-OES) [29, 30], ICP-MS [31], [32], instrumental neutron activation [33] and HPLC-UV-hydride generation-atomic fluorescence spectrometry (HG-AFS) [34]. Most of which are very complex and or costly methods, limiting their application as a routine laboratory method. Numerous methods have been developed and published based on the molecular spectroscopy [35–44]. High detection limit and application of auxiliary techniques for pre-concentration of the analyte are the main drawbacks of molecular spectrophotometric methods, which in turn increases the variables that affect the accuracy of the results. Using simple but precise methods for routine analysis have always been a priority in research laboratories. HPLC-based methods are nowadays commonly used in research laboratories. HPLC techniques are interest for preconcentration effect on the analyte and separation of the analyte from the excipients, and we propose to determine the selenium species content. In this work, based on our previous published article [45], the novel chromogenic reagent of 4, 5-Diamino-o-xylene (DAX) has been examined for off-line pre-column selenium (IV) complexation and quantitated by reversed-phase HPLC using UV detection. Like other published works, in this study the sample pre-treatment procedure for selenium determination in real samples are generally based on microwave-assisted digestion [25, 46].

To the best of our knowledge, development of the fast, accurate, precise and validated method for analysis of selenium in biomass using reversed-phase HPLC-UV has not been studied yet. As a result, the proposed HPLC method is novel and has all the features necessary for routine testing of total selenium not only in biomass but also in most real samples.

Experimental

Instruments

An Agilent 1260 HPLC system a G4225A micro , vacuum degasser, a G7112B binary pump, a G1328C six-port two-position injection valve with a 20 µL sample loop, and an Agilent G1315C diode array detector (DAD) was carried out in the experiments. The output signal was processed using Chemstation software. The PerkinElmer® Optima™ 8000 ICP-OES instrument was used for selenium analysis. The operating condition for ICP-OES instrument are listed in Table 1. An ETHOS sealed-tube microwave digestion system (Milestone, Italy), to determine amount of total selenium, it was necessary for the yeast biomass complete digestion.

Table 1. ICP-OES configuration and applied experimental parameters for selenium determination

Spectrometer	ICP-OES(730-ES simultaneous CCD, Varian, USA)
RF generator	12 MHz
RF power (kW)	1.3
Nebulizer	Concentric
Spray chamber	Cyclonic
Plasma viewing	Axial
Argon gas flow in plasma (L/min)	8
Argon gas flow in auxiliary (L/min)	1.5
Argon gas flow in nebulizer (L/min)	0.94
Read delay (sec)	30
Rinse (sec)	30
Wavelength (nm)	206.27

Chromatographic conditions

The octadecylsilane (C_{18}) analytical column with dimension of 0.25 m (Length), 0.0046 m (Diameter), and 5 µm particle size from Teknokroma Company (Spain) at temperature of 30 °C was applied for separation. The mobile phase used was a degassed solution of deionized water and acetonitrile solution were prepared in a 50:50 ratio at a flow rate of 1 mL min^{-1} (Figure 1). Chromatographic runtime of the method was 25 min. The wavelength in the detector module was set to 340 nm and injection volume was 20 µL.

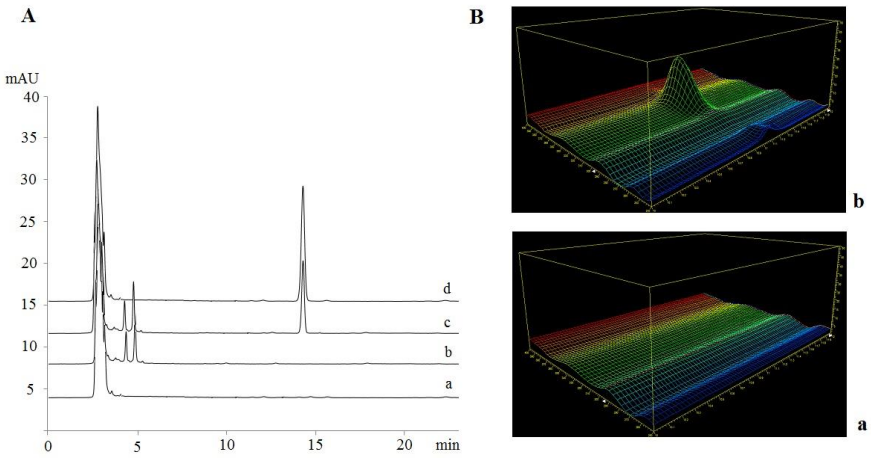


Figure 1. Representative chromatograms of blank (A-a), selenium-enriched biomass without DAX chelating agent (A-b), selenium-enriched biomass with DAX chelating agent (A-C) and Se(IV) standard solution (A-d) along with the blank (B-a) and three dimensional plot of peak purity of selenium standard obtained by the diode array detector software (B-b)

Reagents and solutions

Analytical grade chemical reagents were used for the solution preparation. Nitric acid (Suprapur®), hydrogen peroxide 30% (Suprapur®) and HPLC grade solvents like acetonitrile and methanol were provided from Merck-Millipore (Germany). 4, 5-Diamino-o-xylene was purchased from Sigma-Aldrich (USA). Ultra-pure water (18 MΩ Resistance) was obtained from Millipore, Direct-Q® 3, purification system (France). Selenium yeast (Selm-1) CRM was provioded from National Research Council Canada (NRCC, Ottawa, ON, Canada). The Se (IV), freshly prepared sodium selenite solution with appropriate concentration was used as stock standard solutions (Na_2SeO_3) (Sigma-Aldrich Co., St. Louis, USA) in ultra-pure water, and appropriate dilutions of stock solution were used for working standards. Freshly prepared 5-diamino-*o*-xylene in methanol with appropriate concentration (18 mM) was utilized as complexation reagent. Store the prepared solution in amber glass a 4 °C and use it during 12 h.

Selenium-enriched saccharomyces biomass

The Se-enriched Saccharomyces was provided in our research laboratory. Organisms, media and culture conditions were selected according to the published works with some modification. The Saccharomyces cerevisiae used in this study was obtained from the collection of the Industrial Microorganisms Laboratory of the Iranian Research Organization for Science and Technology (IROST)[14], [47, 48]. Agar plates for storing and maintaining the viability of yeast culture were prepared by addition of 15 g L^{-1} agar to the Sabouraud dextrose broth (SDB). The strain inclusion was performed at 10% of Saccharomyces cerevisiae in to medium with volume of 1.5 L. accordingly, the strains were fed with medium enriched in selenium nutrient (20.0 mg of Na_2SeO_3 per 1 L of medium). Cultivation was performed in a shaker agitated at 160 rpm at 28 °C for 72 h. The pH of the medium was initially adapted to 4.5-5 using HCl 0.1 M. In the course of cultivation, the yeast growth was investigated at 24-hour intervals (0, 24, 48, and 72 h) turbidimetrically, using measuring the suspension optical density at 600 nm wavelength (OD600). Based on the obtained OD600 results, maximum yeast growth was obtained at the cultivation time of 48 h and gradually decrease was observed thereafter. These findings were agreement with results obtained from pH, total count (cfu/mL) and dry biomass yield (dry weight l-1 medium) studies at 24-hour intervals. Once the process was completed, samples were centrifuged at 4000 rpm for 10 min at 20 °C. The supernatant was removed and the solid phase was rinsed with deionized water twice in order to remove residues of the medium and surface-bound selenium. In the following the strains was lyophilized. The lyophilized material was digested to determine the amount of selenium.

Analysis steps

Digestion using microwave technique

In order to determine the amount of total selenium, about 0.100 g of fine powder of selenium-enriched Saccharomyces was accurately weighed into a microwave Teflon screw bulb. Respectively, 2 mL of Nitric acid and 1 mL of hydrogen peroxide were added, and the sample was digested by sealed-tube microwave digestion. Digestion conditions were adjusted so that for 5 minutes at a pressure of 15 psi and power at 20%, 5 minutes at a pressure of 30 psi and power at 40%, 10 minutes at a pressure of 65psi and power at 65% and 20 minutes at a pressure of 140psi and power at 80%. It is necessary to reduce selenium(VI) to selenium(IV), after the digestion tubes have cooled, the amount of dilute hydrochloric acid (5 mL, 10% V/V) is added to the contents inside the tubes and with gentle heat we see the release of

white acid vapors [32]. The digested sample was transferred to 100 mL volumetric flask and diluted to volume with deionized water. 1.0 mL of obtained solution was pipet in to 5 mL volumetric flask and made up to volume with deionized water and used for analysis of total selenium.

HPLC-UV measurements

Se (IV)-DAX complexation in standards and real sample

10 µL of 4, 5-diamino-o-xylene Solution (18 mM) was added to 1.0 mL standard solutions or acid digests extract. The complexation procedure was completed after 15 min. The appropriate volume of solution (at least three times the injection volume) was introduced into the HPLC under the specified conditions.

Results and Discussion

Method development

Due to the fact that the important factors such as the complex formation time, chromogenic reagent concentration , stability of the complex and maximum wavelength of complex, 340 nm, as the detection wavelength, in our earlier research work were concluded [45], the method development steps here focused on the mobile phase optimization and selection of the appropriate analytical column.

Stationary and mobile phase optimization

For each of the parameters tested, data from three replicates (n = 3) were collected. The average retention time (t_R) and peak resolution (R_s) were used as monitoring responses.

First of all an octadecylsilane column from ACE Company (250*4.6 mm id, 1.5-10 µm) Selected as a Common stationary phase. What was important here as a powerful method was the separation of the complex chromatogram from the other impurities and the ligand did not react for this purpose other specific columns such as Phenyl groups, and other octadecylsilan columns with varying dimensions, particle size and carbon loading were screened. The properties of each column are shown in Table 2. Basically, the two-component mixture of water - methanol and water - acetonitrile were investigated as mobile phase.

Table 2. Results of preliminary stationary phase and their properties

HPLC column	Column properties	Column performance Comments
Altima Phenyl (150*3.0 mm; 3 µm, 110 A° pore size GRACE, Germany)	Less hydrophobic than C18 phase, 7% carbon loading, end capped, pH 1-10	No elution was done and no peak was observed
BRISA LC2 (150*4.6 mm; 5 µm, 120 A° pore size, Teknokoroma, Barcelona, Spain)	End capped using multifunctional endcapping deactivation (MED), metal free silica packing, 12% carbon loading, pH 2-11	Retention time was short and the peak purity test was not passed
ZORBAX SB-C18 (250*4.6 mm; 5 µm, 80 A° pore size, Agilent, USA)	Spherical ZOERBAX silica support designed to reduce or eliminate strong absorption of basic compounds, 10% carbon loading, pH 0.8-8.0	Peak fronting was observed in selenium(IV) complex
BRISA LC2 C18 (250×0.46 mm, 5 µm, Teknokroma)		Selenium(IV) complex eluted in a reasonable retention time , peak shape and peak purity

Although the Selenium-DAX complex dissolves readily in pure methanol and acetonitrile, the two-component water-methanol system as the mobile phase is looser in competition with the non-polar stationary phase, Octyldecylsilane, and practically no elution occurs. The two-component water-acetonitrile combination, although high in elution strength, had to be optimized to incorporate the acetonitrile percentage in the mobile phase to obtain the appropriate retention time to achieve non-interference and analysis time. Therefore, the mixture of water and acetonitrile (50% V/V) was selected as the mobile phase.

Selenium-DAX is a bulky complex without charge. Elution in this system does not require Counter ion; However, the pH effect has been investigated over a wide range, and research has shown that pH in the range of 1 to 12 has no effect on wash ability either in terms of retention time or in terms of surface area (Figure 2).

BRISA LC2 C18 (250×0.46 mm, 5 µm, Teknokroma) analytical column, was finally selected as the column.

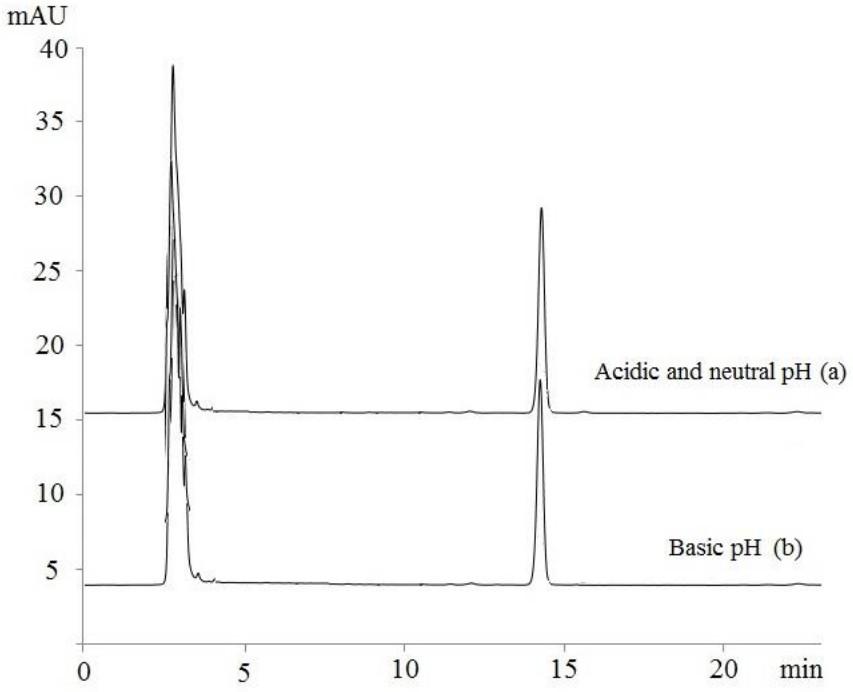


Figure 2. Representative chromatograms of Se(IV) standard solution in acidic and neutral pH (a) and basic pH (b)

Validation of analytical method

Selectivity

In the presence of a range of metal cations that were sometimes predicted as interfering cations or covalent cations in the periodic table**,** In the range of 10% of the selenium study range, the selectivity of selenium was investigated by chromatography and showed that a pure peak was obtained without any possible interference is depicted in Table 3.

Table 3. Tolerance limit of possible interfering ions at determination of 250 µg L^{-1} Se(IV) in model solutions

Foreign ions	Tolerance ratio	Recovery%
Cr(IV)	1000	97.2 ± 3
Co(II)	1000	99.8 ± 2
Fe(II)	1000	95.2 ± 4
K(I)	3000	98.4 ± 2
Cd(II)	3000	100.0 ± 3
Cu(II)	1000	95.9 ± 2
Pb(II)	1000	98.5 ± 4
Zn(II)	1000	95.3 ± 3
Ni(III)	1000	94.6 ± 2
Se(VI)	3000	97.7 ± 4
Ca(II)	3000	102.0 ± 2
Mg(II)	3000	99.5 ± 3
Mn(II)	3000	95.6 ± 3
Cl^-	5000	96.0 ± 4
CO_3^{2-}	5000	101.0 ± 4
NO_3^-	5000	100.3 ± 2
SO_4^{2-}	5000	100.0 ± 3
PO_4^{3-}	5000	95.8 ± 3

Specificity

Using photo diode array detector, the peak purity check was utilized for the study of the method specificity. Peak purity was carried out by comparison between selenium complex spectrum obtained from standard solution and the enriched biomass. Both spectra were in perfect agreement with each other. Chromatograms obtained from injection of blank, selenium-enriched biomass in the present and absent of complexing agent and also standard were showed that no other peak was observed at retention time of selenium complex chromatograms (Figure 1).

Detection limit (LOD) and quantification limit (LOQ)

The least detectable value(LOD) and the least quantitative determination value(LOQ) for Se(IV) determination in aqueous solutions was evaluated experimentally and the calibration line calculations. The results are depicted in Table 4. The detection and quantification limits, calculated at a signal-to-noise (S/N) ratio of 3 and 10 were 8.0 and 25 µg L^{-1}, respectively.

Linear range

The calibration line was obtained at the concentration range of 25-700 µg L^{-1} of Se(IV) with a correlation coefficient of 0.9994 and standard error of 2.5488 (Figure 3). According to obtained calibration equation, standard deviation of slope and intercept were 0.0253 and 2.1942, respectively. The results are demonstrated in Table 4.

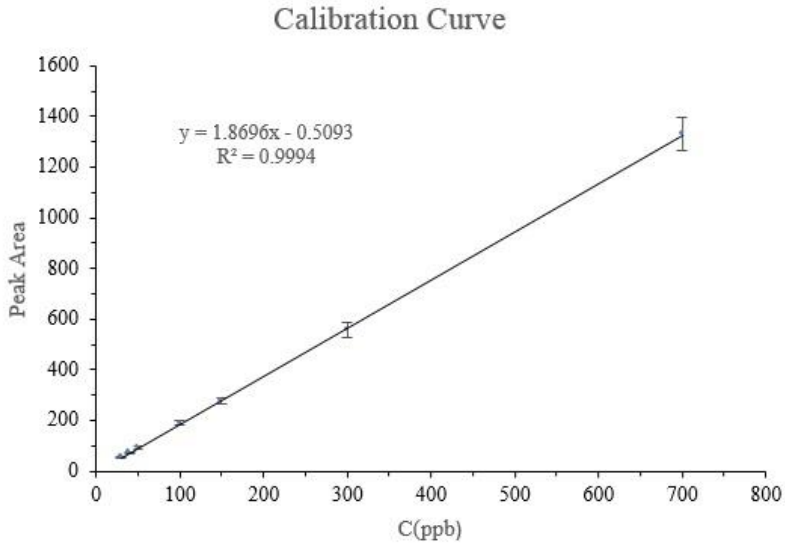


Figure 3. Calibration line at the concentration range of 25-700 µg L^{-1} of Se (IV)

Precision

The precision of the method was evaluated by examining the reproducibility or of repeatability in one day and between days with statistical studies. According to the obtained results, the relative standard deviations (RSDs) for 6 replicate measurements of Se(VI) were found to be 1.1% for within-day and 1.8% for between-day determinations (Table 4).

Table 4. Figures of merit for the proposed method

Calibration equation	**y= 1.8695x-0.5092**
Correlation coefficient	0.9994
Standard error	2.5488
Standard deviation of slope	0.0253
Standard deviation of intercept	2.1942
LR	25-700[a]
LOD	8.0
LOQ	25
Interday precision (n=6)	1.1%
Intraday precision (n=6)	1.8%

[a]All concentrations are in µg L^{-1}

Table 5. The parameters variations for robustness and roughness evaluations

Parameter	**Variable setting (Set point)**
Column	Two various columns with same stationary phases (C_{18}) from different manufacturers of Agilent and Restek
Instrument	Younglin 9100 and Agilent 1260 HPLC systems
Aqueous-organic ratio (v/v)	40-60 (50/50)
Flow rate (mL/min)	0.9-1.1 (1.0)
Column temperature (°C)	25-35 (30)
Detector wavelength (nm)	338-342 (340)
Analyst	Two analysts

Study of the ruggedness and robustness of HPLC parameters

For study of the Ruggedness and robustness of HPLC parameters, Minor changes according to Table 5 were applied to the factors affecting the results and the responses were evaluated. At the end, analysis of variance (ANOVA) has been applied for selenium determination results in biomass obtained by 2 analysts within 3 sequential days. The results in Table 6 showed that no significant variation was appears in the results.

Accuracy

In order to check the accuracy of the method, selenium-enriched biomass, selenium yeast CRM (SELM-1, NRCC, Canada) and commercial selenium yeast sample (Selenomax™, Tripharma Co., Germany) were analyzed for the total selenium determination (Table 7). The selenium yeast CRM and commercial samples have certified values of 2030±70 and 1200 µg g-1 for total selenium, respectively. Comparison of the results was obtained and their analysis with t-Student Test showed that there was no significant difference between the results in the 95% confidence range (p-value of 0.075 and 0.269 for Selenomax™ and CRM yeast samples, respectively). Quantitative measurements were validated using spiked recoveries at three concentration levels. The results obtained from the analysis of spike samples at different concentration levels showed that the analysis process covered the analysis of selenium content in the 95% confidence range have been done desirable. Finally, in order to more investigation of the accuracy of the proposed method, selenium content of the real samples was determined using ICP-OES. The significance test (student's t test) showed that there is no significant difference exists between the results obtained for selenium determination in the real samples by proposed HPLC-DAD and reference ICP-OES methods at a 95% confidence level (Table 7).

Finally, the correlation coefficient was calculated by analysis of total selenium of 50 biomass samples using HPLC-UV and ICP-OES methods (Figure 4). Obtained results show that they are properly correlated. As shown in Table 8, the comparable sensitivity of selenium detection with suitable linear range were obtained in contrast to the other techniques using sample pre-concentration procedures [43, 49].

Table 6. Analysis of variance (ANOVA) for selenium determination in biomass obtained by 2 analysts within 3 sequential days

	Day 1		**Day 2**		**Day 3**	
Analyst 1	1539		1548		1551	
	1565		1554		1549	
Analyst 2	1542		1551		1536	
	1557		1532		1559	
Source of Variation	***SS***	***df***	***MS***	***F***	***P*-value**	***F* critical**
Analyst	70.08333	1	70.08333	0.459312	0.523206	5.987378
Day	40.66667	2	20.33333	0.133261	0.877757	5.143253
Interaction	32.66667	2	16.33333	0.107045	0.900163	5.143253
Within	915.5	6	152.5833			
Total	1058.917	11				

Figure 4. The correlation graph obtained by the analysis of total selenium of 50 biomass samples using HPLC-DAD and ICP-OES.

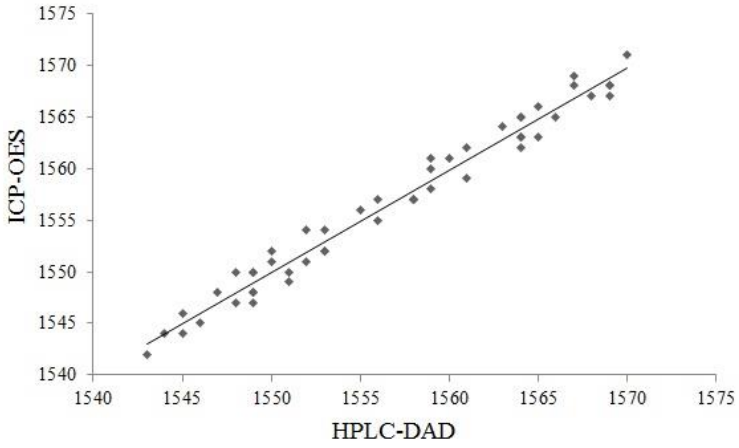


Table 7. The experimental results of selenium enriched yeast biomasses obtained from HPLC-DAD and ICP-OES methods

Sample	Total selenium (µg g^{-1})		Added Se(IV)(µg g^{-1})		Found (µg g^{-1})		Spiked recovery (%)	
	HPLC-DAD	ICP-OES	HPLC-DAD	ICP-OES	HPLC-DAD	ICP-OES	HPLC-DAD	ICP-OES
Selenium enriched yeast[a]	1540±27.3	1536±27.2	750.0	750.0	2286±41.1	2243±40.3	99.7±2.2	97.2±2.8
			1500	1500	3141±56.5	3119±56.1	106.5±1.9	105.4±2.1
			3000	3000	4652±83.7	4407±79.3	107.2±2.5	91.6±1.8
Commercial sample[b] (1200 µg Se/g)	1158±30.1[c]	1176±20.8[d]	750.0	750.0	1952±68.6	1959±59.3	103.8±2.1	102.8±2.5
			1500	1500	2589±31.5	2684±34.8	94.1±2.7	100.7±3.6
			3000	3000	4186±39.8	4211±42.5	102.4±1.8	102.9±2.6
SELM-1CRM[e] (2031 µg Se/g±70)	2021±13.5[f]	1998±23.4[g]	-	-	-	-	-	-

[a]Prepared by our laboratory.
[b]SelenomaxTM.
[c]p-value of 0.075 obtained by student's t test at 95% confidence level.
[d]p-value of 0.124 obtained by student's t test at 95% confidence level.
[e]Selenium enriched yeast certified reference material.
[f]p-value of 0.269 obtained by student's t test at 95% confidence level.
[g]p-value of 0.073 obtained by student's t test at 95% confidence level.

Table 8. Comparison of the proposed method with the other methods for determination of Selenium

Method of extraction	Detection system	LR (µg L^{-1})	LOD (µg L^{-1})	RSD%	Ref.
LLE[a]	UV-Vis spectrometry	1000-7000	166.5	n.a.	49
LPME[b]	GC-FID[c]	20-1000	0.9	3.2	50
-	DPP[d]	3.9-530.4	n.a.	5.5	51
DLLME-SFOD[e]	Photodiode array spectrophotometry	40-1000	16	2.1	43
HS-SPME[f]	Ion mobility spectrometry	20-320	12	<6	52
Utrasonic extraction	HPLC-ICP-MS[g]	0.10-200	0.033	2.5	53
SPE[h]	ETAAS[i]	0.50-200	0.18	<3.5	54
-	HPLC-DAD	25-700	8.0	1.8	This work

[a]Liquid liquid extraction.
[b]Liquid phase microextraction.

[c]Gas chromatography with flame ionization detector.
[d]Differential pulse polarography.
[e]Dispersive liquid liquid microextractio-solidified floating organic drop.
[f]Head space-solid phase microextraction.
[g]High performance liquid chromatography-inductivity coupled plasma-mass spectroscopy.
[h]Solid phase extraction.
[i]Electrothermal atomic absorption spectroscopy.

Conclusion

This research study introduced the high-performance liquid chromatography technique that used a uv-vis detector in determining the amount of selenium in selenium-enriched biomass of saccharomyces cerevisiae due to the demonstration of desirable performance parameters of the analysis method **.**The method is based on the complexation of Se(IV) with 4, 5-diamino-o-xylene as a novel complexing agent. The combination of two merits of using a selective reagent and using HPLC system, led to provide the high efficient determination of ultra-trace level of Se(IV) without utilization of any complicated sample pre-concentration or toxic organic solvents consumption. At the end, it should be noted that this method with suitable performance characteristics such as, specificity, linear range (25-700 ppb), low detection limit (8 ppb) is a powerful method in determining the amount of selenium.

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Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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