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Development of on-line spectrofluorimetric methodology for selenium monitoring in foods and biological fluids using Chrome azurol S quenching

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ABSTRACT

A novel, simple and accurate on-line green methodology for Se(IV) monitoring by molecular fluorescence has been developed. Because the analyte does not exhibit fluorescence, the organic dye Chrome azurol S (CAS) has been chosen to allow detection. The effect of metal-quenching on CAS excitation and emission conditions (λex=300 nm; λem=407 nm) was used as criterion for analyte quantification in presence of sodium cholate bile salt (NaC). The quenching mechanism was explored, and it can be classified as a collisional type with a Stern-Volmer constant value of 3.0×10^{7} mol L^{-1}. To improve the sampling rate, minimize the reagent consumption and generated wastes, an on-line configuration was designed. Experimental variables that affect the fluorimetric sensitivity were optimized using uni-variation assays. Under optimal experimental conditions, the limit of detection was 0.27 μg L^{-1} with a lineal range for Se(IV) concentration from 0.84 to 6.00 μg L^{-1}. The developed methodology is low cost and fast sampling, allowing Se(IV) quantification in the presence of other common ions. Bulbous vegetables and biological samples were successfully analyzed with an average recovery close to 100%.

1. Introduction

Selenium is considered an essential element for human beings because of its biological activity as an antioxidant by means of the glutathione peroxidase enzyme. Beneficial effects of selenium include antiviral capacity, cancer prevention, regulation of thyroid gland's metabolism and its role as an antagonist against the toxic effect of many metals such as arsenic, lead and cadmium [1].

This metal is ingested in the diet. Some vegetables especially garlic and onions accumulate high concentrations of selenium due to the presence of sulfur fractions and their derivatives [2]. Based on experiments performed on populations from China and New Zealand, a relationship between the amount of selenium and the activity of the enzyme glutathione peroxidase was determined and, the recommended amount of selenium was found to be 55 μg/day for adults [3].

It has been proven that a selenium deficiency could induce serious health problems, such as a decrease in immune and thyroid function, a high risk of some cancers including breast cancer and prostate cancer, and diseases such as Keshan and Kashin–Beck [4–7]. To prevent a deficiency, several states have undertaken initiatives such as increasing the selenium intake levels by incorporating it through dietary supplements [8].

The concentration range of this element to be considered deficient, essential or toxic for the human health is very narrow, therefore the study of the ingested dose is important. Although, it should be clarified that both acute and chronic poisonings involving selenium are unusual; the associated clinical manifestations are nausea, diarrhea, garlic breath, loss of hair and nails, nail changes, metabolic acidosis, fatigue, and impaired gastrointestinal and nervous system involvement [9].

For many years, several analytical methods for selenium determination have been developed, including spectrophotometry, spectrofluorimetry, high performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICP-MS), gas chromatography (GC), neutron activation analysis, electrochemical techniques, hydride generator atomic absorption spectroscopy (HG-AAS) and graphite furnace atomic absorption spectroscopy (ET-AAS) [10–18]. However, many times these methods involve the use of sophisticated and expensive instruments with limited sensitivity. Another frequent disadvantage is that to clean the sample, time consuming pre-treatments must be done using toxic and unstable reagents.

Quantification of trace elements represents a challenging task for analytical chemistry, mainly when samples with complex matrices must be analyzed. Accordingly, luminescent methods have been recognized by its high sensitivity, low cost, simplicity and versatility and have been used for the determination of a wide variety of analytes at trace levels in diverse matrices [19]. To detect and quantify inorganic species without native fluorescence by molecular spectroscopies, fluorescent reagents with com-

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plexation capacity are used [20–24]. Specifically, for selenium determination, o-diamines, 3,3-diaminobenzidine dithizone, o-phenylenediamine [21], 2,3-diaminonaphthalene [22,23] and 8-hydroxyquinoline [24] have been used as fluorophores.

The salts of bile acids ("bile salts") are the most important natural surfactants, and they are responsible for the solubilization of lipids, cholesterol, bilirubin, lecithin, and fat-soluble vitamins in living organisms. These biological compounds are synthesized from cholesterol in the liver; they are typically composed of a steroidal backbone with one or more α-oriented hydroxyl groups conjugated to an anionic chain or tail. Bile salts are self-assembled in aqueous solution, like classical amphiphiles. The presence of micellar aggregates leads to changes in spectral behaviors, often causing enhancement of fluorescent emission by increasing the microenvironmental rigidity or viscosity and decreasing competitive non-radiative de-excitation pathways [25,26].

The growing number of analytical tests in areas such as health, environment and food requires of the development of automated analytical processes. In this context, Flow injection (FI) methods allow greater frequency of analysis, with the consequent reduction of cost, generating lower volumes of waste, which provides benefits at the environmental level. In the present study, a new on-line methodology is proposed for Se (IV) determination using CAS as fluorimetric dye. Experimental variables that influence fluorescent complex formation and design of the FI (Flow Injection) system will be studied and optimized to enhance the analytical performance. Considering the frequent enhancement of fluorescence that bile salts micellar media cause, the effect of bile salt addition on selenium-dye system will be evaluated, and the obtained results will be compared with those acquired using conventional anionic and cationic surfactants. The developed methodology will be evaluated by analyte determination in complex samples, such as foods and biological fluids.

2. Material and methods

2.1. Instruments

Shimadzu RF-5301PC spectrofluorometer (Shimadzu Corporation, Analytical Instrument Division, Kyoto, Japan) equipped with a discharged Xenon lamp was used for recording the fluorescent measurements. A 1.0 cm quartz cell was employed for the batch assays and a 120 μL LC flow cell unit (Shimadzu Corporation, Analytical Instrument Division, Kyoto, Japan) for the flow measurements.

The FIA manifold consisted of a Rheodyne (Rohnert Park, CA) model-5020 six-port two-way rotary valve and two Gilson (Villiers, France) Minipuls 3 peristaltic pumps connected to 2.79 mm i.d. Tygon tubing (Middleton, WI, USA) for pumping sample solutions.

Adjustments of pH were carried out using Orion Expandable Ion Analyzer pH-meter (Orion Research, MA, USA) Model EA 940 with a combined glass electrode.

A Milestone STARTD microwave furnace (Italy) and Milestone polytetrafluoroethylene (PTFE) reactors hermetically sealed (internal volume 100 mL and 1 cm wall thickness) were used for microwave digestion.

2.2. Reagents

Standard solutions of 1000 mg L^{-1} Se (IV) and Se (VI) were prepared dissolving appropriate amounts of sodium selenite (Na_2SeO_3) and sodium selenate (Na_2SeO_4), respectively (Sigma–Aldrich, USA). The standard stock solutions were stored in glass bottles at 4 °C in the dark. Lower concentration standards were obtained weekly by dilution of the stock solutions.

Solutions of Chrome azurol S 1×10^{-3} mol L^{-1} (CAS, E-Merck, Darmstadt, Germany), dithizone (Fluka AG. Buchs, Suiza), 1,5-diphenylcarbazide (E-Merck), 8-hydroxyquinoleine (E-Merck) were prepared by dissolving appropriate amounts of this reagent in ethanol (Sigma Chemical Co., St. Louis, MO, United States) and were kept in the refrigerator (4 °C) for one week.

Surfactant solutions of sodium cholate (NaC, Sigma Chemical Co.), sodium taurodeoxycholate (NaTDC, Sigma Chemical Co), cetyltrimethylammonium chloride (CTAC, Tokyo Kasei Industries, Chuo-Ku, Tokyo, Japan) and sodium dodecylsulfate (SDS, Tokyo Kasei Industries) 2×10^{-2} mol L^{-1} were prepared using an adequate weight of each reagent and dissolving them in ultrapure water.

Buffer tris(hydroxymethyl)aminomethane (Tris, Fluka Chemie GmbH, Buchs, Switzerland) 1.0 mol L^{-1} solution was prepared. The desired pH was obtained by adding HCl (Merck, Darmstadt, Germany) or NaOH (Mallinckrodt Chemical Works) diluted solutions.

Masking solution of disodium ethylenediaminetetracetate (EDTA, Biopack, Buenos Aires, Argentina) 1% (w/v) was prepared using an adequate weight of the reagent by dissolving it in ultrapure water.

All chemicals used were analytical grade and ultrapure water was used throughout.

2.3. Sample collection and treatments

2.3.1. Biological samples

Per regulation, all participants of the present research signed a written informed consent.

The first urine in the morning was collected and stored in acid washed polyethylene bottles. The sample was centrifuged at 1500 *g* for 10 min. After that, supernatant was separated frozen at −18 °C, and reserved for analysis.

Blood samples from the same subject (10 mL each) was obtained by puncture of the forearm vein, and placed in two tubes; one with Li heparin (anticoagulant) and the other without it. The tubes with anticoagulant were homogenized and centrifuged (1500 *g*) for 15 min. The clear and transparent supernatant corresponding to plasma then was extracted and reserved at 4 °C for the Se (IV) assays. With the aim of accelerating the coagulation process and achieving serum separation, tubes containing blood without heparin were thermostatized at 37 °C for 30 min. After that, systems were centrifuged (1500 *g*) for 15 min, and the supernatant was placed in polypropylene tubes with a hermetic closing.

2.3.2. Bulbous vegetables

Dehydrated commercial onion and garlic were acquired in local stores. Entire products were homogenized and reserved for sample preparation.

2.3.3. Digestion treatment

A volume of 500 μL of each biological fluid and about 0.3 g of each solid sample of bulbous vegetables were introduced into Teflon microwave digestion vessels, and 5 mL HNO_3, 65% (w/v), and 1 mL H_2O_2 (30%) were added. The vessels were closed and placed into the microwave oven. The microwave digestion program was applied: 1000 W (10 min), 1000 W (20 min). Samples were heated to 200 °C to ensure reduction of Se(VI) to Se(IV). The resulting solutions were transferred to 25 mL volumetric flasks and brought to volume with ultrapure water. Dilutions of 1/10 mL digested samples were reserved for analyte determination.

2.4. General procedure

A 300 μL CAS solution (1×10^{-3} mol L^{-1}), Se(IV) sample/standard 0.84–6.0 μg L^{-1}), 1.0 mL Tris buffer (1×10^{-2} mol L^{-1}, pH 3.2), and 25 μL NaC (2×10^{-2} mol L^{-1}) were placed in a volumetric flask. The mixture was diluted to 10 mL with ultrapure water and then was injected into the continuous flow diagram shown in the Fig. 1b. In the "loading" position, the carrier consisted of an HCl solution (1×10^{-4} mol L^{-1}) and was propelled by the first peristaltic pump (PP)

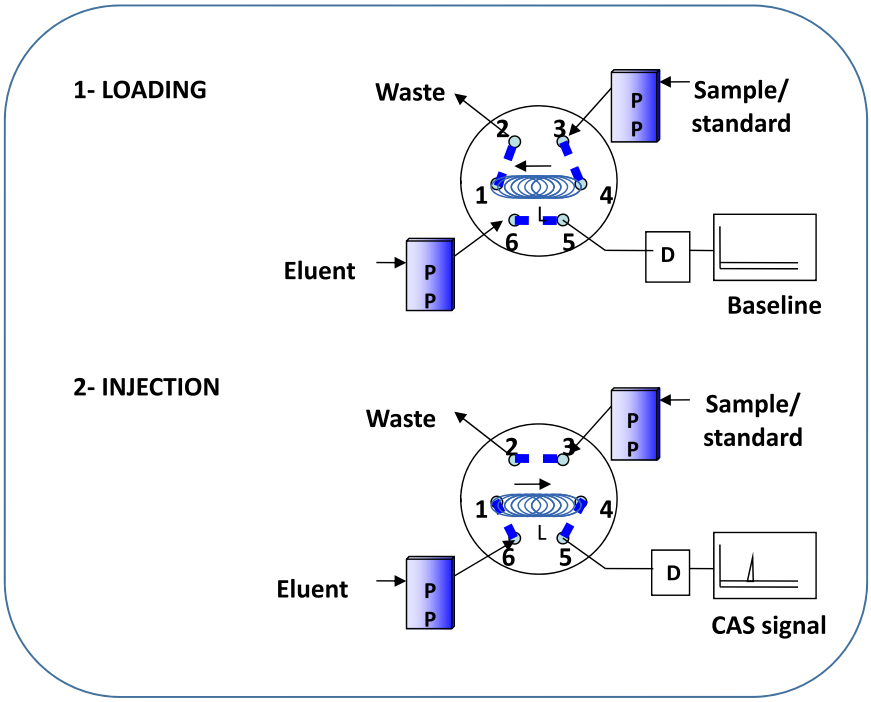


Fig. 1. Schematic representation of FI scheme of developed methodology.

to the fluorescence detector. The baseline was generated at 407 nm using λ_{exc}=300 nm (slits 10/5). At the same time, the second peristaltic pump aspirated the sample/standard, charging the sample loop (L) in the injection valve located among positions 1 and 4. The loading was carried out for 36 s, which was enough time to fill L with the Se(IV) sample/standard. In the "injection position" carrier flowed through the L and carried the analyte to the detector. The second PP then was turned off.

2.5. Interferences study

Different amounts of ions possibly present in studied samples (1/1, 1/10, 1/50 and 1/100 analyte/interferent ratio) were added to the test solution containing 4.00 μg L^{-1} Se (IV) and the general procedure was applied.

2.6. Recovery study

An adequate volume of samples was spiked with increasing amounts of Se (IV) (3.00 and 6.00 μg L^{-1}). Analyte concentrations were determined by proposed methodology.

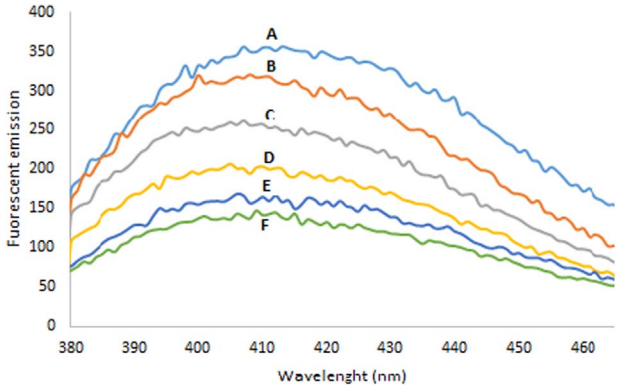
2.7. Precision study

The repeatability (within-day precision) of the method, replicate samples (n=3) containing Se(IV) 3.00 and 6.00 μg L^{-1}, respectively, were prepared and analyte contents were determined by proposed methodology. Also, the reproducibility (between-day precision) was also evaluated over 3 days for the same systems.

3. Results and discussions

To determine selenium by molecular fluorescence, a wide variety of fluorophores chelating reagents were assayed. Among all the tested reagents, Chrome azurol S (CAS) exhibited a strong quenching effect in presence of Se (IV) with suitable sensitivity (Fig. 2); so, it was chosen as the fluorophore reagent to continue the studies on Se (IV) determination.

The fluorescence property of the Se (IV)-CAS system showed to be



A: Blank solution CAS (C_{CAS} = 3x10^{-5} mol L^{-1})
B: Idem A with Se(IV) 0.499 μg L^{-1}.
C: Idem A with Se(IV) 0.998 μg L^{-1}.
D: Idem A with Se(IV) 1.99 μg L^{-1}.
E: Idem A with Se(IV) 3.99 μg L^{-1}.
F: Idem A with Se(IV) 5.99 μg L^{-1}.

Fig. 2. CAS emission in absence and presence of Se(IV). Conditions: λ_{exc}=300 nm; λ_{em}=407 nm; C_{CAS}=3×10^{-5} mol L^{-1}; C_{NaC}=2×10^{-4} mol L^{-1}; $C_{buffer\ Tris}$ =3×10^{-4} mol L^{-1} pH=3.24. Other experimental conditions are described under procedure.

sensitive to solution pH value. To obtain the higher quenching effect, systems were prepared using buffers of different nature and pH values, maintaining constant the Se (IV)-CAS ratio. Fig. 3 shows the obtained results, where the best quenching effect on CAS fluorescent signal in presence of Se(IV) was achieved at pH 3.2, using the buffer Tris.

Another experimental parameter studied was the excess of CAS fluorophore concentration for quantitative interaction between the metal and the dye. Systems were prepared by varying the ratio between Se (IV) and the organic reagent. The maximum quenching effect was obtained for the CAS concentration of 3×10^{-5} mol L^{-1} (Fig. 4). While the literature indicates that the ratio among metal and ligand (Me-CAS) should be 1:2, for practical purposes an excess of fluorometric dye was used with the aim of improving the sensitivity, reproducibility and selectivity [31].

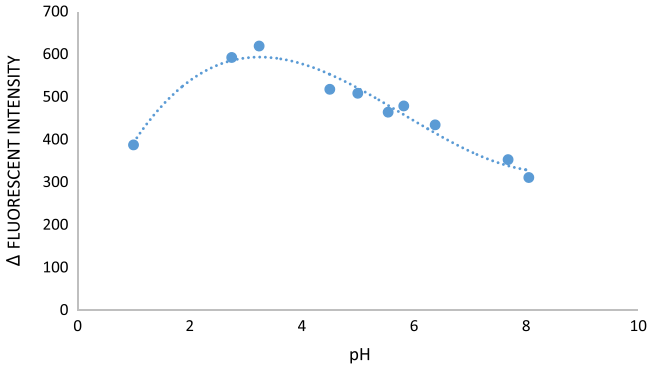


Fig. 3. Influence of pH on Se (IV) determination. Conditions: λ_{exc}=300 nm; λ_{em}=407 nm; $C_{CAS}=3\times10^{-5}$ mol L^{-1}; $C_{Se\ (IV)}$=3.99 μg L^{-1}; $C_{NaC}=2\times10^{-4}$ mol L^{-1}; $C_{buffer\ Tris}=3\times10^{-4}$ mol L^{-1}. Other experimental conditions are described under procedure.

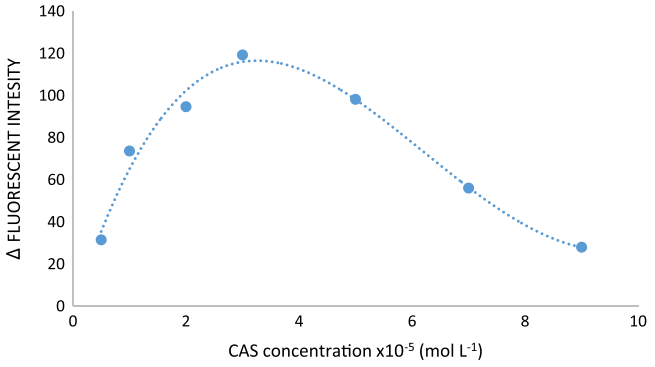


Fig. 4. Effect of CAS concentration on the Se(IV) determination. Conditions: λ_{exc}=300 nm; λ_{em}=407 nm; $C_{CAS}=3\times10^{-5}$ mol L^{-1}; $C_{Se(IV)}$=3.99 μg L^{-1}; $C_{NaC}=2\times10^{-4}$ mol L^{-1}; $C_{buffer\ Tris}=3\times10^{-4}$ mol L^{-1} pH=3.24. Other experimental conditions are described under procedure.

After the optimal conditions for the chemical interaction between Se(IV) and CAS were established, the influence of different surfactants on the quenching effect was tested. The results showed a relationship between the nature of the surfactant and the fluorescent emission of Se (IV)-CAS system, as follows:

- For systems with anionic surfactants (SDS, NaTDC, NaC) in a concentration of 2×10^{-4} mol L^{-1}, a quenching effect was observed.
- For systems with cationic surfactant (CTAC) in a concentration of 2×10^{-4} mol L^{-1}, an enhanced of CAS emission was observed.

According to the results obtained, bile salt NaC was selected as the optimal condition due to maximum quenching effect showed on CAS fluorescence signal.

Under optimized conditions, the luminescence quenching caused by Se (IV) was described by the Stern-Volmer equation:

$$I_0/I = K_{SV}(Q) + C$$

I_0 and I are the luminescence intensity in the absence and presence of the quencher (analyte), respectively, K_{SV} is the Stern–Volmer constant representing the affinity between CAS and Se (IV), Q is the analyte concentration, and C is a constant near to 1. The obtained K_{SV} value was 3×10^{7} mol L^{-1}.

To determine the nature of the quenching effect, studies were performed at different temperatures (1, 10, 20, 30 and 40 °C) and fluorescent emissions were determined. As K_{SV} increased with the temperature, it could be asseverated that a *collisional quenching* is occurring for Se(IV)-CAS system.

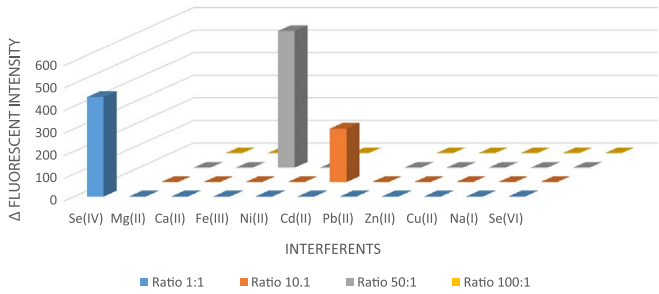


Fig. 5. Tolerance limits of interfering species in Se(IV) determination. Conditions: λ_{exc}=300 nm; λ_{em}=407 nm; $C_{CAS}=3\times10^{-5}$ mol L^{-1}; $C_{Se\ (IV)}$=3.99 μg L^{-1}; $C_{NaC}=2\times10^{-4}$ mol L^{-1}; $C_{bufferTris}=3\times10^{-4}$ mol L^{-1} pH=3.24. Other experimental conditions are described under procedure.

4. Interferences

When a methodology is selective, the fluorophore emission must not be influenced by the presence of others ions. So, the selectivity of the proposed method for Se (IV) determination was further evaluated with various common ions. A relative error of ± 5.0% was considered acceptable. Satisfactory tolerance was observed for inorganic compounds found in studied samples. The analyte quantification in presence of Se (VI) was likely a 1:1 ratio (Fig. 5).

A severe interference was found for Cd (II) and Fe (III) at 1:1 Se(IV): interferent ratio. The addition of 1% EDTA as a masking agent solved these problems. At optimal experimental conditions, the addition of the masking agent caused a 40% decrease in sensitivity of with the advantage of reducing the interference effects.

5. FI parameters optimization

Several experiments have been carried out in order to study the effect of the sample coil in the FI system on the fluorescent signal. The best results were obtained using a sample coil of 1 mL, guaranteeing the non-stationary state.

To generate the quenching effect, the interaction among Se (IV) and CAS dye must take place before injection into the FI system. If CAS conforms the carrier, the baseline would be higher than the signal generated by the Se (IV)-CAS system; therefore, the peaks after the analyte injection would be negative. With the proposed configuration, a reduced employment of reagents, mainly CAS, was achieved.

Carrier effects on the analytical response were studied using HCl ($0–6\times10^{-4}$ mol L^{-1}) at a flow rate between 1.0–3.0 mL min^{-1}. It was observed that the quenching effect increased with the carrier flow rate using HCl 1×10^{-4} mol L^{-1}. Despite the benefit of greater speed of analysis, flow rates greater than 2.25 mL min^{-1} generated undesirable pressures due to the high flow velocity, causing a decoupling of the FI configuration. Therefore, a carrier flow rate of 2.25 mL min^{-1} was selected as optimal.

Considering the enhancement of the quenching effect produced for bile salt NaC in the batch studies, assays were conducted preparing systems with different concentrations of bile salt NaC and injecting them into FI configuration. Beneficial effects were observed in the enhancement of quenching behaviour and minimization of sampling time with the addition of NaC at 2×10^{-4} mol L^{-1}. Under these optimal conditions, the sampling rate was 24 samples h^{-1}.

Table 1 shows experimental parameters, optimal values and quality analytical parameters for developed methodology. From the calibration curve, LOD (μg L^{-1}) and LOQ (μg L^{-1}) were calculated by three times the standard deviation of a blank solution divided by the slope value, and 10 times standard deviation of a blank solution divided by the slope value, respectively.

Table 1
Experimental conditions and analytical parameters for selenium (IV) determination.

Parameters	Studied range	Optimal conditions
pH	3.0–8.0	3.24
Tris	1×10^{-4}–1×10^{-3} mol L^{-1}	3×10^{-4} mol L^{-1}
CAS	5×10^{-6}–9×10^{-5} mol L^{-1}	3×10^{-5} mol L^{-1}
NaC	0–6×10^{-4} mol L^{-1}	2×10^{-4} mol L^{-1}
Carrier HCl	0–6×10^{-4} mol L^{-1}	1×10^{-4} mol L^{-1}
LOD	–	0.27 μg L^{-1}
LOQ	–	0.84 μg L^{-1}
LOL	–	0.84–6 μg L^{-1}
r^2	–	0.993

Table 2
Comparison of the published methods with the proposed method in this work.

Detection method	Comments	Reference
Spectrophotometry	LOD=4.4 ng mL^{-1} LOQ=14.65 ng mL^{-1} RSD=2.18% Applied to cosmetic and pharmaceutical preparations	[11]
Spectrofluorimetry	LOD=2.1 μg L^{-1} LOQ=7.0 μg L^{-1} RSD=5% Applied to vitamin tablets	[23]
HG-AFS	LOD=0.14 ng g^{-1} Applied to vegetables	[27]
HG-AFS	LOD=0.4 ng mL^{-1} LOQ=1.4 ng mL^{-1} RSD=4.2% Applied to nutritional supplements and shampoos	[28]
GF-AAS	LOD=0.08 ng mL^{-1} RSD=3.8% Applied to vegetable and fruit samples	[29]
ET-AAS	LOD=0.5–1.3 μg L^{-1} LOQ=1.7–3.3 μg L^{-1} Applied to onion and garlic	[30]
Spectrofluorimetry	LOD=0.27 μg L^{-1} LOQ=0.84 μg L^{-1} Applied to foods and biological fluids	This work

The robustness of the developed methodology was evaluated preparing systems varying each parameter as follows: the pH -from 3.0 to 4.5- and the CAS concentration -from 2.8 to 4.2×10^{-5} mol L^{-1}. In all studied cases, the percentual relative error (% RE) calculated for CAS quenching fluorescent signal was minor 2%. Results showed that the developed methodology has a suitable capacity to remain unaffected by the presence of scarce, but deliberate variations in selected experimental parameters, providing an indication of its reliability during normal usage.

Table 2 shows analytical quality parameters of the proposed methodology and those previously reported for Se (IV) determination.

6. Applications and validation

The usefulness of the proposed method was evaluated for Se(IV) determination in garlic, onion and biological samples. Fluorimetric methodologies require sample digestion to destroy organic matter and sample reduction to convert the total selenium to the Se (IV) oxidation state. All the garlic, onion and biological samples were digested with microwave assisted-wet digestion as was described in Digestion treatment item.

Table 3
Recovery studies by selenium (IV) determination in bulbs vegetables and biological samples.

Sample	Se (IV) added (μg L^{-1})	Proposed methodology		
		Se (IV) found ± RSD (μg L^{-1})	Recovery (%, n=3)	Real Se (IV) contents (μg L^{-1})[a]
1	–	0.11 ± 0.007	–	110.00
	2.99	3.10 ± 0.017	100.00	
	5.99	5.93 ± 0.009	97.16	
2	–	0.03 ± 0.014	–	35.37
	2.99	2.95 ± 0.017	97.65	
	5.99	6.00 ± 0.016	99.66	
3	–	0.17 ± 0.014	–	176.00
	2.99	3.16 ± 0.0005	100.00	
	5.99	5.90 ± 0.005	95.66	
4	–	0.19 ± 0.001	–	192.25
	2.99	3.37 ± 0.002	106.35	
	5.99	5.79 ± 0.0005	93.48	
5	–	0.16 ± 0.0002	–	169.20
	2.99	3.32 ± 0.004	105.00	
	5.99	5.82 ± 0.0002	94.49	

Real Al (III) contents (μg L^{-1})=Se (IV) found (μg L^{-1}) x fd
. Samples: 1- Plasma; 2- Serum; 3- Urine; 4- Onion; 5- Garlic.
[a:] Volume=0.1 mL.

The accuracy of the methodology was performed using the standard addition method. Diluted digested samples (100 μL, n=3) were spiked with increasing amounts of Se (IV). To solve undesirable effects due to concomitants present, 1 mL of 1% EDTA solution was added at each and treated as was described in the General procedure.

Obtained results showed satisfactory agreement with adequate precision and recovery between 93.48 and 106.35. The repeatability (within-day precision) of the method was evaluated carrying out the proposed methodology, 3 times for each sample. The reproducibility (between-day precision) was also define over 3 days by performing three determinations each day with a CV of 0.55. Table 3 shows the recovery results achieved for each sample. Obtained results indicate that the proposed methodology is suitable for determination of Se(IV) in studied samples.

7. Conclusions

In this work, a new on-line simple, inexpensive, fast methodology for Se (IV) quantification has been developed based on the quenching effect of Se (IV) on CAS dye fluorescent emission. The possible mechanism of collisional quenching has been proposed with a K_{SV} of 3×10^{7} mol L^{-1} value. Interference studies showed that Se (IV) could be quantified in the presence of many common ions in the studied samples by adding EDTA as a masking agent. The inherent sensitivity of fluorescent emission permitted analyte determination without a preconcentration step. Moreover, it was determined that the chemical interaction with CAS is selective for Se (IV), meanwhile in presence of Se(VI), the emission of CAS is not affected. Using the FI configuration under optimal work conditions, the sampling rate was 24 samples h^{-1}. The obtained analytical parameters such as sensitivity, accuracy and selectivity show the proposed methodology as a useful analytical tool, competitive with other conventional methods of selenium quantification.

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