Ingeniatrics
Nebulizers and Nozzles

Application Note

Selenium Speciation in Foods by External Calibration (MassNeb® nebulizer) and Isotopic Dilution Analysis (MultiNeb® nebulizer) using HPLC-ICP-MS

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Authors

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1. Introduction

Selenium (Se) is an essential trace element for both human and animals. Se is often found in the form of selenoproteins and is involved in various biochemical and physiological functions in mammalian systems, such as the enhancement of immunity and oxidation resistance. Knowledge about selenium content of foods containing selenium species is very important in terms of both nutrition and toxicity. The bioavailability of selenium species for human body varies. Hence, speciation of selenium is more important than total selenium determination. Although data on the total concentration of an analyte are necessary, they are not sufficient to provide a proper understanding of the behavior of the analyte relative to a system under study. This is because different species, despite having the same chemical element, do not possess the same physical, chemical, and toxicological characteristics.

Both the total content and the bioavailability of Se in food play a significant role in Se assimilation by humans and animals. Selenium species are frequently found in foods in both organic Se compounds (i.e., selenomethionine (SeMet), methyl selenocysteine (MeSeCys), selenocysteine (SeCys)) and inorganic compounds (i.e., selenite ($SeO_3^=$), selenate ($SeO_4^=$)). The bioavailability of selenium differs based on the chemical form of the element present in the food and is considerably greater for organic forms.

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In Europe, the use of feed additives is regulated through the European feed legislation. The current maximum limit for total Se in animal feeds including fish feed has been set at 0.5 mg/kg feed (Council Directive 70/524/EC and amendments). Recently, the European Food Safety Authority (EFSA) has issued several scientific opinions on the use of organic selenium-yeast forms as feed additives. Due to the apparent higher bioavailability of organic selenium compared to inorganic selenium, EFSA concluded that the supplementation level should be limited to a maximum of 0.2 mg/kg of feed to ensure consumer safety.

Therefore, the development of accurate and precise selenium speciation procedures is currently a prominent trend in analytical chemistry. In this context, the hyphenation of high-performance liquid chromatography (HPLC) with inductively coupled plasma mass spectrometry (ICP-MS) represents a versatile and powerful tool for selenium speciation analysis. However, while ICP-MS as a stand-alone instrument allows for high sample throughput for multi-element determinations, its coupling with a chromatographic separation system significantly reduces the sample throughput.

In this study, high-performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICP-MS) was optimized using an HPLC-ICP-MS to improve the analytical precision. For this purpose, we evaluate in this study the performance of MassNeb® inert, robustness and durability nebulizer and a specific connector for speciation analysis designed by Ingeniatrics Technologies S.L. for analytical methodologies based on the use of HPLC-ICP-MS using external calibration of 50 mm PEEK capillary (0.125 mm i.d.) to connect the exit of the chromatographic column directly to nebulizer (Figure 1A) and MultiNeb® nebulizer in case of HPLC-IDA-ICP-MS. Traditionally, for IDA quantification, the enriched standard required is mixed with the samples or chromatographic flow after HPLC separation using a Y connection. Recently, the novel MultiNeb® (Ingeniatrics Tecnologías S.L.) has been developed which allows a high mixing efficiency between two liquids, miscible or immiscible, since the mixing takes place under turbulent conditions of high pressure at the tip of the nebulizer.

In addition, in case of MultiNeb® nebulizer (two liquid inlets), the one-piece connectors designed for speciation analysis contains a 50 mm PEEK capillary (0.125 mm i.d.) to connect the exit of the chromatographic column directly to nebulizer and a second 50 mm PFA tubing (0.5 mm i.d.) (Figure 1B).

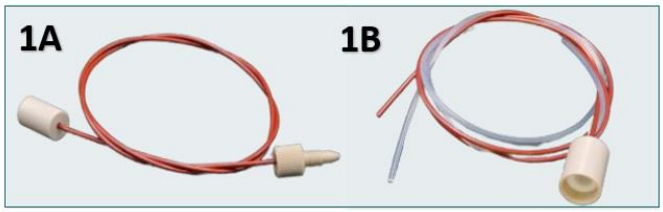

Figure 1. A) Speciation Analysis Connector for HPLC-ICP using MassNeb® Nebulizer (One Liquid Inlet) and B) Speciation Analysis Connector for HPLC-ICP using MultiNeb® Nebulizer (Two Liquid Inlets).

2. Experimental

Reagents and solutions

All reagents used for sample preparation were of the highest available purity. Milli-Q water (18.2 MΩ cm, EMD Millipore Corporation, MA, USA) was used for sample preparation and analysis. Seleno-DL-methionine (SeMet, ≥99% purity), sodium selenate (Se(VI), ≥98% purity), sodium selenite (Se(IV), 99% purity), seleno-DL-cystine (SeCys2, ≥98%), Se-methyl-seleno-L-cysteine (SeMetSeCys ≥ 98%), sodium citrate were all obtained from Sigma Aldrich (Oslo, Norway). Methanol (HPLC grade) pyridine, nitric acid (HNO_3, Trace select, ≥69.0% w/w), hydrochloric acid, ammonia solution, hydrogen peroxide (H_2O_2, Emsure ACS, ISO, 32% w/w) were purchased from Merck (Darmstadt, Germany).

Enriched ^{74}Se was obtained from Cambridge Isotope Laboratories (Andover, MA, USA) as elemental powder and it was dissolved in the minimum volume of nitric acid (Suprapur grade) and diluted to the appropriate volume with ultrapure water. The concentration of this solution was established by reverse isotope dilution analysis as described elsewhere[1]. Aqueous calibration standards for total selenium determination by ICP-MS were prepared by appropriate dilution of a mono-elemental stock solution of 1000 mg L^{-1} of Se (ICP CetriPUR, Merck, Darmstadt, Germany) in deionized water (18 MΩ cm resistivity). All aqueous solutions are acidified by adding up to 5% nitric acid. In addition, Yb, Rh and Re were investigated as internal standards, since they are unlikely to be contained in food samples. A solution containing internal standard is prepared by appropriate dilution of a 1000 mg L^{-1} of mono-elemental stock solutions of each internal standard element investigated in this work (high-purity mono-element standard solutions).

[1]L Hinojosa-Reyes, JM Marchante-Gayón, JI García-Alonso, A Sanz-Medel, J. Anal. At. Spectrom. 18 (2003) 1210.

Instrumentation

For total selenium determination a CEM Mars microwave system was employed for the mineralization of samples. Subsequently, an Agilent 7900 ICP-MS was used as sensitive and selective detector. The operational conditions are shown in Table I.

Conventionally, for total element determination bthrough y internal calibration or isotopic dilution analysis, internal or enriched standards are mixed with the calibration standards and samples using a Y connection. Recently, the novel MultiNeb® has been developed which allows a high mixing efficiency between two liquids, miscible or immiscible, since the mixing takes place under turbulent conditions of high pressure at the tip of the nebulizer. Instrumental

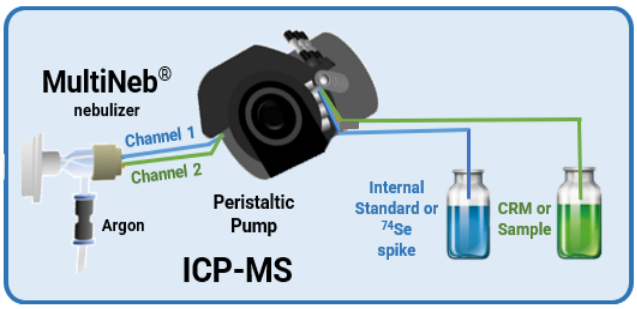

Figure 2. Schematic representation of MultiNeb®-based configuration for total element determination by internal standard calibration or isotopic dilution analysis techniques by ICP-MS.

On the other and, anion-exchange chromatography (AEC) analysis was carried on an Agilent 1260 HPLC system comprising a quaternary pump, autosampler and vacuum degasser was coupled to an Agilent 7900 ICP-MS. Selenium species were separated on a Hamilton PRP-X100 anion-exchange column (250 × 2.1 mm × 10 µm particle size). Samples and standard solutions were injected via a 100 µL sample loop. Temperature of the column was kept at 30°C during chromatographic separation. Selenium compounds were separated using 25 mM sodium citrate and 2% of methanol at pH 4 by isocratic elution. The flow rate of mobile phase was kept constant at 1.2 mL·min^{-1} during chromatographic separation. Total time for separating of selenium species was 15 min. Hamilton PRP-X100 is an anion exchange column that has strongly basic quaternary ammonium groups. The addition of a small amount of methanol to the mobile phase can change the elution capacity of the mobile phase and make it easier to control the retention time of the selenoamino acids.

7900 Agilent ICP-MS Operational Conditions	
RF Power (W)	1550
Plasma gas flow (L min^{-1})	15
Auxiliary gas flow (L min^{-1})	0.6
Nebulization gas flow (L min^{-1})	0.65
Sampling Depth (mm)	7.0
Sample introduction rate (rps)	0.1
Cell gas flow (mL min^{-1})	5.0 (He)
KED (V)	3.0
Isotopes monitored	**Internal Calibration** ^{78}Se, ^{80}Se and ^{103}Rh, ^{193}Ir and ^{185}Re (IS) **Isotopic Dilution Analysis** ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se, ^{82}Se, ^{79}Br, ^{81}Br, ^{83}Kr
Dead Time Detector	47 ns
Extract 2 (V)	-220
Omega Bias (V)	-90
Omega Lens (V)	7.2
OctP RF (V)	200
Dwell time (s)	0.3 per isotope

Table I. Operational conditions for 7900 ICP-MS optimized for total selenium determination by internal calibration and isotopic dilution analysis techniques.

Additionally, for speciation analysis based on HPLC-ICP-MS analytical approach, Ingeniatrics Tecnologias S.L. has released connectors designed to facilitate quick, reliable, and easy connection between your HPLC and your ICP (Figure 1). Speciation Analysis High Pressure Connectors for HPLC-ICP present some advantages, such as resists blockage, fast washout, minimize dead volume and peak broadening, principally. As mentioned earlier, in this study we compared the results obtained for selenium speciation using MassNeb® for external calibration and MultiNeb® for isotopic dilution analysis. The instrumental configurations are depicted in Figure 3."

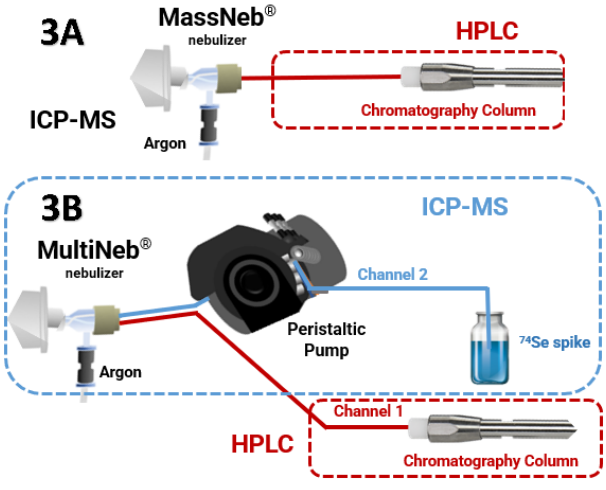

Figure 3. Schematic representation for selenium speciation analysis using: A) External calibration using MassNeb®-based configuration (One Liquid Inlet) and B) IDA using MultiNeb®-based configuration (Two Liquid Inlets).

Operating conditions for total speciation analysis by anion-exchange chromatography (AEC) combined with inductively coupled plasma mass spectrometry (ICP-MS) for selenium species quantification optimized are indicated in Table II.

HPLC-ICP-MS Operational Conditions		
7900 ICP-MS Parameters (Agilent Technologies)		
RF Power (W)	1550	
Plasma gas flow (L min^{-1})	15	
Auxiliary gas flow (L min^{-1})	0.6	
Nebulization gas flow (L min^{-1})	**MassNeb®**	0.65
	MultiNeb®	0.70
Sampling Depth (mm)	7.0	
Sample introduction rate (rps)	0.5	
Cell gas flow (mL min^{-1})	5.0 (He)	
KED (V)	3.0	
Isotopes monitored	^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se, ^{82}Se, ^{79}Br, ^{81}Br, ^{83}Kr	
Dead Time Detector	47 ns	
Extract 2 (V)	-220	
Omega Bias (V)	-90	
Omega Lens (V)	7.2	
OctP RF (V)	200	
Dwell time (s)	0.3 per isotope	
1260 HPLC Parameters (Agilent Technologies)		
Column	Hamilton PRP-X100 anion-exchange column (250 × 2.1 mm × 10 µm particle size)	
Temperature	30 °C	
Mobile phase	Isocratic elution: 25 mM sodium citrate, 2% Methanol (pH 4.0)	
Flow rate	1.2 mL min^{-1}	
Injection volume	100 µL	

Table II. Operational conditions for 7900 ICP-MS and 1260 HPLC optimized for selenium speciation using external calibration by HPLC-ICP-MS using MassNeb® nebulizer and isotopic dilution analysis by HPLC-ICP-MS using MultiNeb® nebulizer.

Samples and Certified Reference Materials (CRM)

Selenium enriched yeast certified reference material was used for quality control assays.

Additionally, commercially enriched selenium foods were analyzed, such as soybean, peanuts, broccoli, salmon, onion, garlic, egg and rice.

Commercial foods samples were lyophilized prior to analysis, as elemental analyses are commonly conducted on dry materials. Fresh samples were stored at -20 °C before lyophilization. The samples were then thawed and weighed, prior to lyophilization. After lyophilization, the samples were grounded, homogenized, and weighed again for moisture calculations of water content. The lyophilized samples were stored at room temperature until further analyses.

Sample preparation for total selenium determination in foods

For determination of total selenium concentration in all samples and CRM studied, approximately 0.2 g was microwave digested in 3.0 mL concentrated HNO_3 and 1.5 mL of H_2O_2 in XP1500 vessels in CEM Mars microwave system. The samples were diluted to the final volume of 25 mL with deionized water. Each sample was prepared in triplicate. The resulting solution was filtered through Iso-Disc poly(vinylidene difluoride) filters (25 mm diameter, 0.45 µm pore-size).

Sample preparation for selenium speciation in foods samples

On the other hand, for speciation analysis, the sample extraction and clean-up procedures are crucial steps when dealing with biota samples. This is because there is a possibility of analyte losses, changes in the species, or incomplete extraction of the selenium compounds, which can result in poor or erroneous results.

For selenium species extraction an extraction solution containing 50 mM ammonium phosphate monobasic was prepared and the pH was adjusted to 7.5. Samples were weighed to approximately 200 mg with 3 mL of the extraction solution. The extraction was performed with the assistance of an ultrasonic probe at 25% power for 2 minutes. Then, 30 mg of protease type XIV (from *Streptomyces griseus*, Sigma-Aldrich) and 10 mg of lipase (from *Candida rugose*, Sigma-Aldrich) dissolved in buffer solution were added to each sample and placed on a digestor Digiprep® (SCP Science, Canada, model MS-48) for 12 hours at 37 °C.

For speciation analysis each sample was prepared in duplicate and all extracts were filtered prior to analysis by HPLC-ICP-MS. In the absence of a certified reference material, a spiked sample (spiked with 10 µg/L mixed selenium species) was prepared to verify the analytical accuracy of the method.

3. Results and Discussion

Optimization of selenium speciation analysis by HPLC-ICP-MS

For arsenic speciation analysis, many studies used HPLC-ICP-MS as an effective method. Because of ionic compounds, either anion-exchange chromatography or ion pair chromatography has been employed for speciation of arsenic compounds. However, in most cases, mobile phases including phosphate buffer for anion-exchange chromatography or carbon-rich mobile phases for reversed-phase chromatography were used.

As a major drawback, carbon buildup and ion suppression commonly affect mobile phases in the case of ion pair chromatography or influence the physical properties of the sample introduction devices in the case of anion-exchange chromatography employing a high concentration of buffer, such as phosphate buffer. In this study, we used a mobile phase including sodium citrate and methanol adjusted to pH 4 for the separation of five selenium species.

Interconversion among selenium species was not observed under the detailed experimental conditions. Additionally, all five selenium species were successfully speciated under the operational and experimental conditions previously optimized and described (Figure 4)."

Signal stability and sensitivity

To evaluate the signal stability throughout the analysis sequence, a monitoring standard solution containing 50 ng mL-1 of each selenium species was prepared. This solution was analyzed once every five samples to assess the signal stability. The recoveries were required to fall within the range of 90-110%, as shown in Table III. Additionally, method detection limits (MDLs) were established by analyzing five replicate injections of the calibration blank and multiplying the obtained standard deviation by three. The results obtained are shown in Table III.

Selenium Speciation by HPLC-ICP-MS			
Quantification Method	**Se Species**	**Recoveries (%)**	**DLs (ng mL^{-1})**
External Calibration using MassNeb®	$SeCys_2$	91 – 108	0.04
	MeSeCys	93 – 99	0.06
	Se (IV)	90 – 109	0.04
	SeMet	93 – 104	0.03
	Se (VI)	91 - 109	0.08
Isotopic Dilution Analysis using MultiNeb®	$SeCys_2$	93 – 102	0.02
	MeSeCys	96 – 104	0.04
	Se (IV)	98 – 103	0.02
	SeMet	97 – 104	0.02
	Se (VI)	95 - 102	0.05

Table III. Experimental recoveries and LDs for selenium species concentrations using HPLC-ICP-MS by external calibration (MassNeb®) and isotopic dilution analysis (MultiNeb®).

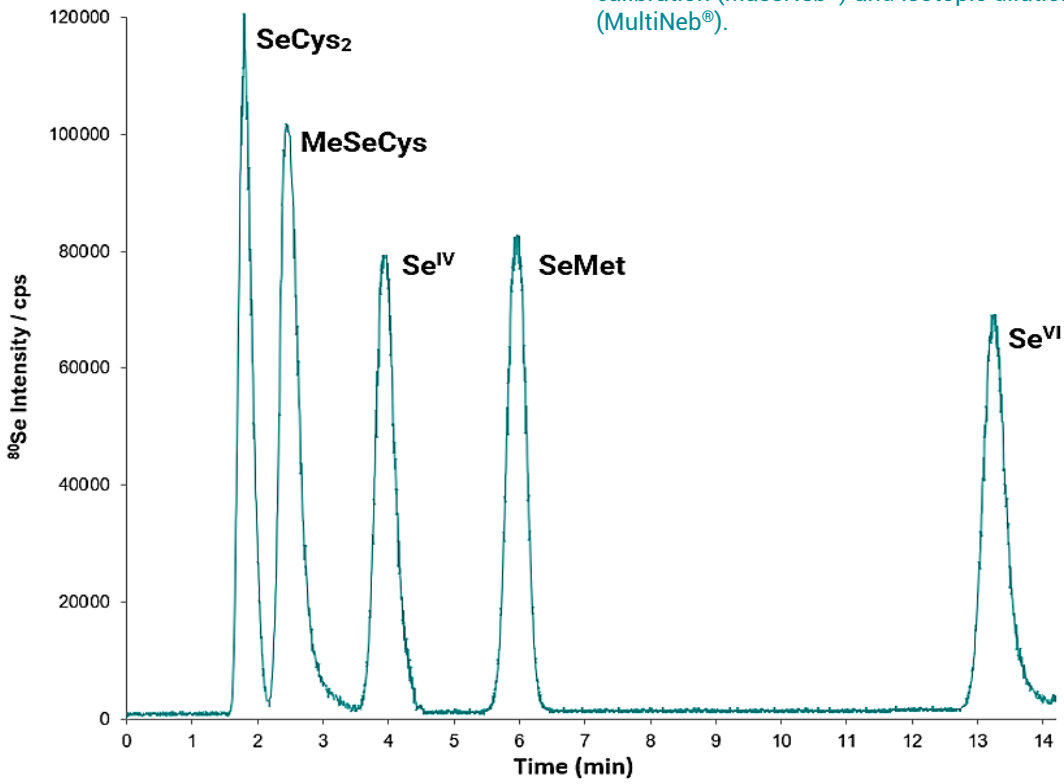

Figure 4. Chromatogram of five selenium species speciated using HPLC-ICP-MS (50 ng mL^{-1} according to Se for each form).

In Table III, we can observe better results in terms of recoveries and detection limits in all cases when quantifying selenium species by HPLC-ICP-MS using absolute quantification through isotopic dilution analysis. However, both techniques are acceptable for this purpose.

Additionally, the stability of the retention time was evaluated. The stability of retention time was achieved in the range of 0.06–1.24% (short term, n = 5) and 0.24–3.92% (long term, n=20) for all selenium species quantified.

Precision and reproducibility

Precision values were evaluated using seafood matrix certified reference materials (CRM) selenium enriched yeast SELM-1 (National Research Council Canada), after sample treatment previously described. Table IV summarizes the experimental registered values for each selenium species with both instrumental configuration and quantification method evaluated, as well as the RSD obtained for 3 replicates of each of the CRM or food used in the experimental development of this application note. Precision is expressed as the relative standard deviation percentage (RSD%).

In addition, in order to validate the method performance in real samples, a spike recovery test was performed using the mixed Se species standard solution. Post-digestion spikes of 10 µg kg^{-1} were prepared to check spike recoveries of selenium species. The recoveries obtained were 92 – 108 % in all cases for selenium speciation by HPLC-ICP-MS employing external calibration using MassNeb® nebulizer and 96 – 106 % for selenium speciation by HPLC-ICP-MS employing isotopic dilution analysis using MultiNeb® nebulizer.

Generally, the results obtained using the MultiNeb® nebulizer and isotopic dilution analysis showed better precision and reproducibility compared to the results obtained with the MassNeb® nebulizer and external calibration as a quantification method. In this context, the increased precision observed in the results using the MultiNeb® nebulizer is attributed to the use of isotopic dilution analysis as an absolute quantification method.

As species	Total Se (mg Kg^{1})	$SeCys_2$ (mg Kg^{1})	MeSeCys (mg Kg^{1})	Se (IV) (mg Kg^{1})	SeMet (mg Kg^{1})	Se (VI) (mg Kg^{1})
(CRM)	Mean ± RSD(%)	Mean ± RSD(%)	Mean ± RSD(%)	Mean ± RSD(%)	Mean ± RSD(%)	Mean ± RSD(%)
Se enriched yeast SELM-1 CRM	2031 ± 3.4	---	---	---	1284 ± 5.4	---
	1973 ± 2.6	---	---	---	1346 ± 5.5	---
	2012 ± 1.1	---	---	---	1306 ± 4.3	---
Salmon Muscle Tissue	0.381 ± 2.1	< LD	<LD	0.098 ± 3.9	0.277 ± 4.1	<LD
	0.402 ± 1.6	< LD	<LD	0.103± 2.6	0.282 ± 3.2	<LD
Soybean	0.123± 2.8	0.009 ± 4.4	<LD	0.022 ± 4.3	0.099 ± 5.1	<LD
	0.126 ± 2.0	0.011 ± 3.1	<LD	0.017 ± 3.4	0.101 ± 3.3	<LD
Peanuts	0.130 ± 2.4	0.010 ± 4.2	<LD	0.014 ± 4.5	0.111 ± 4.6	<LD
	0.133 ± 1.7	0.008 ± 3.4	<LD	0.013 ± 3.0	0.114 ± 4.2	<LD
Broccoli	0.237 ± 2.3	0.016 ± 4.1	<LD	0.055 ± 4.2	0.145 ± 3.9	<LD
	0.241 ± 1.2	0.016 ± 3.3	<LD	0.052 ± 2.9	0.151 ± 3.2	<LD
Onions	0.032 ± 2.4	<LD	<LD	0.010 ± 5.3	0.023 ± 6.1	<LD
	0.034 ± 1.7	<LD	<LD	0.012 ± 4.6	0.022 ± 5.3	<LD
Garlics	0.021 ± 2.6	0.007 ± 5.7	<LD	0.004 ± 4.9	0.010 ± 5.1	<LD
	0.022 ± 2.0	0.007 ± 4.2	<LD	0.005 ± 4.7	0.011 ± 4.2	<LD
Eggs	0.463 ± 2.2	0.023 ± 3.7	<LD	0.033 ± 3.6	0.392 ± 3.6	<LD
	0.471 ± 1.7	0.025 ± 3.3	<LD	0.032 ± 3.8	0.397 ± 2.0	<LD
rice	0.045 ± 2.9	0.004 ± 4.3	<LD	0.005 ± 4.2	0.038 ± 4.9	<LD
	0.047 ± 1.67	0.004 ± 4.0	<LD	0.005 ± 4.0	0.036 ± 4.4	<LD

Table IV. Experimental and certified mean values for total selenium and selenium species concentrations in the different selenium enriched foods analyzed, as well as the RSD obtained for 3 replicates using HPLC-ICP-MS and 5 replicates for ICP-MS determination using external calibration (green values) and isotopic dilution analysis (grey values).

4. Conclusions

In this study, two alternative strategies for selenium speciation in food samples were demonstrated in terms of precision, sensitivity, signal stability and reproducibility. Generally, the results obtained using MultiNeb® nebulizer and isotopic dilution analysis shown better precision and reproducibility compared to the results obtained with the MassNeb® nebulizer and external calibration as a quantification method.

Furthermore, the high-pressure connector designed by Ingeniatrics Tecnologias S.L. offers several advantages, such as resistance to blockage, fast washout, minimization of dead volume, and reduction of peak broadening, primarily. Additionally, this connector is simple to use, allowing for quick, cost-effective, reliable, and easy connection of your HPLC to your ICP instrument..

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