

Selenium determination in tissue samples of Nile tilapia using ultrasound-assisted extraction

Research Article

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Abstract: This paper proposes a method to determine selenium in samples of fish muscle and liver tissue using ultrasound assisted extraction process, and analysed by graphite furnace atomic absorption spectrometry (GFAAS). The selenium content was extracted by 0.10 M HCl at the optimal extraction conditions which were established as follows: sample mass of 100 mg; granulometry of the sample <math>< 60 \mu\text{m}</math>; sonication time of five 40 s cycles; and sonication power of 136 W. The selenium determinations were performed by GFAAS, at a drying temperature of 120°C/250°C, pyrolysis temperature of 1300°C, atomization temperature of 2300°C, and cleaning temperature of 2800°C. Palladium nitrate was used as a chemical modifier coinjected with the samples, and tungsten as a permanent modifier. The concentration of selenium determined in the pool of fish muscle and liver tissue were 280.4 ± 4.2 e $592.3 \pm 6.7 \mu\text{g kg}^{-1}$, respectively. The accuracy and precision of the proposed extraction method were evaluated using certified standard Bovine Muscle – NIST 8414. The results obtained by the ultrasonic extraction method were equivalent to those obtained by the method of acid mineralization of samples in a microwave oven

Keywords: Selenium extraction • Graphite furnace atomic absorption spectrometry • Essentiality of Selenium • Fish nutrition • Nile tilapia

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1. Introduction

Due to essential and non-essential role of potentially toxic elements for living organisms, research on the content of these elements in foods are one of the main objects of toxicology studies. Selenium is an essential element

for our body and also an essential component of the diet of mammals. Selenium gives protection against several heart diseases, prevents the effects of potentially toxic metals and has anti-carcinogenic activity. Selenium is promising as an anti-proliferative, anti-inflammatory, antiviral and immune modifying agent [1-4].

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In animals, the margin between the nutritive requirements and the toxic limit of the micronutrient selenium is very narrow. This element is an integral part of the enzyme glutathione peroxidase that acts in the cell cytosol, converting hydrogen peroxide into nontoxic compounds, and the level of activity of this enzyme in the liver or plasma indicates the body's supply of selenium. The main biochemical indicator of selenium deficiency in animals is the reduction of glutathione peroxidase activity. Adequate monitoring of the concentration of this element in animal tissues and blood is important in studies of oxidative stress [4-6].

The determination of selenium is problematic due to very low concentrations of selenium in animal tissues (in the range of $\mu\text{g kg}^{-1}$). Normally, this extraction is performed by acid mineralization of the samples under slow heating in block digesters, using a nitric-perchloric mixture. The resulting extracts are acid solutions containing the inorganic nutrients. This procedure is not only time-consuming but may also lead to loss of selenium through volatilization [7,8]. Ultrasound-assisted extraction of inorganic analytes has proved to be a very robust alternative technique [9]. Ultrasounds occur due to the phenomenon of acoustic cavitation, which is a process of nucleation, growth and collapse of transient bubbles in liquids exposed to low-frequency ultrasonic waves (<1 MHz). In the preparation of samples, the collapse of microbubbles favors the extraction of chemical species from solid materials, as well as their dissolution or complete mineralization in the case of organic compounds. Therefore, the use of ultrasound-assisted extraction of inorganic compounds and/or ions from biological samples can replace the acid mineralization [10-14].

This paper describes the development of a method for the determination of selenium in muscle and liver tissue of Nile tilapia (*Oreochromis niloticus*) by GFAAS, using ultrasound-assisted extraction, instead of acid digestion of samples.

2. Experimental Procedure

2.1. Reagents and solutions

Ultrapure water ($18.2 \text{ M}\Omega \text{ cm}^{-1}$) obtained with an Elga PURELAB Ultra Ionic system (Technology Drive Lowell, MA, USA), and spectroscopic grade hydrochloric acid Merck (Darmstadt, Germany) were used throughout. The stock solutions of selenium and concomitants were prepared by dilution of titrisol standards Merck (Darmstadt, Germany). The remaining solutions, including the extraction solutions, were prepared from Merck reagents. All the solutions were stored in

polypropylene flasks.

All the sample storage flasks and standard solutions, glassware and the sampler flasks of the atomic absorption spectrometer were washed with nitric acid 10% v/v for 24 hours, rinsed with ultrapure water and air-jet dried prior to the injection of standards and/or samples.

2.2. Collection and preparation of samples

Samples of muscle and liver tissue were collected after digestibility experiments involving Nile tilapia juveniles, which were subjected to a diet with different sources of food. These experiments were conducted in the Fish Nutrition Laboratory of the Faculty of Veterinary Medicine and Zootechny/UNESP at Botucatu. At the end of the food management program (90 days after the start of fish diet), six fishes from each aquarium (with an average size of 15 ± 2 cm and weighing 100 ± 10 g) were anesthetized with a benzocaine solution (100 mg L^{-1}) and euthanized for removal the samples of muscle and liver, which were placed in 15 mL polypropylene flasks and stored at -20°C [15-18].

In preparation for the selenium extraction stage, was prepared five pool of each tissue samples (muscle and liver) which were dehydrated in a forced air circulation oven at 40°C for 48 h, then were ground in a porcelain mortar and pestle in the presence of liquid nitrogen until they were reduced to particles with a granulometry of less than $60 \mu\text{m}$ [19].

2.3. Extraction of metal nutrients

After each drying and grinding step, 100.25 mg of sample and 10 mL of 0.10 M hydrochloric acid were placed in 50-mL Teflon flasks for the extraction process. The sample/solution mixture was then shaken ultrasonically to extract the selenium. Following this procedure, different sonication times and ultrasound power were evaluated in the analyte extraction process. The acid extracts obtained were separated from the remaining solid phase by centrifugation – 13000 rpm at 4°C by 5 minutes.

Part of the samples was also mineralized in microwave oven. To this end, quantities of 100.15 mg of samples were transferred directly to the Teflon flasks of the microwave oven, and 2.5 mL of suprapure nitric acid 14 mol L^{-1} plus 0.50 mL of hydrogen peroxide 30% m/m were added. Thereafter, the following power/timer program was run: step 1 – 300 W/10 min; step 2 (ventilation) – 0 W/5 min; step 3 – 450 W/5 min; step 4 – 550 W/10 min; step 5 – 650 W/10 min; step 6 (ventilation) – 0 W/5 min. After cooling, the resulting acid digests were diluted to 50 mL with ultrapure water [7,8].

2.4. Equipment

TECNAL model TE – 394/1 forced air circulation oven (Campinas, SP, Brazil).

UNIQUE ultrasonic cell disruptor (Campinas, SP, Brazil) equipped with titanium macro- and micro-tips was used in the selenium extraction process.

Provecto Analítica model DGT 100 *plus* microwave oven (Campinas, SP, Brazil) was used to mineralize the samples whenever necessary.

The selenium was determined using a SHIMADZU AA-6800 atomic absorption spectrometer (Tokyo, Japan) equipped with background absorption correction with a deuterium lamp and self-reverse (SR) system, pyrolytic graphite tube with integrated platform and an ASC-6100 automatic sampler. A SHIMADZU hollow cathode selenium lamp was used, operating with 23 mA of current. The wavelength applied was 196.0 nm and the spectral resolution was 0.5 nm. Argon was used as inert gas, maintaining a constant flow of 1 L min⁻¹ throughout the heating program, except in the atomization stage, when the gas flow was interrupted. The absorbance signals were measured in the peak area.

2.5. Preparation of the graphite tube coated internally with tungsten carbide

The inner walls of the pyrolytic graphite tubes with integrated platform used for the selenium determinations were coated with tungsten. This was done by injecting aliquots of 25 µL of solution containing 1000 mg L⁻¹ of sodium tungstate modifier into the atomizer, which was then subjected to the stages of the heating program described in Table 1. This procedure was repeated 20 times. When heated to 500°C, tungsten ions are deposited on the platform of the graphite tube, forming a layer of tungsten carbide that acts as a chemical modifier [7]. The mass of tungsten deposited upon the platform of the graphite tube was 500 µg.

2.6. Preparation of the calibration curve

The calibration curve was prepared with a standard selenium stock solution (Merck) and the absorbance values were read by GFAAS. The order of preparation

of the calibration solutions were as follows: volumes of 4, 6, 8, 16, 32 and 40 µL of standard solution containing 500 µg L⁻¹ of selenium; 10 µL of suprapure hydrochloric acid 10 mol L⁻¹; 50 µL of Triton X-100 at 1% v/v; 100 µL of solution 1000 mg L⁻¹ of Pd(II); and ultrapure water until the final volume of solutions was adjusted to 1000 µL, were transferred to the spectrometer's autosampler cups so that the selenium concentrations remained in the range of 2 to 20 µg L⁻¹.

2.7. Preparation of acid extracts for injection into the graphite tube

After the ultrasound-assisted extraction, 500 µL of the resulting acid extracts were transferred directly into the autosampler cups of the atomic absorption spectrophotometer, followed by the addition of 5 µL of suprapure 10 M hydrochloric acid (Merck), 50 µL of Triton X-100 at 1% v/v, 100 µL of solution 1000 mg L⁻¹ of Pd(II) and 335 µL of ultrapure water.

2.8. Analytic procedures

After preparation of the samples (acid extracts) and standard solutions directly in the autosampler's cups, a volume of 20 µL of standard and/or sample was injected into the graphite tube (coated internally with tungsten carbide), using the autosampler's micropipette. The measurements were taken with five repetitions. The heating program of the graphite tube was optimized for the determination of selenium, as described in Table 2.

3. Results and Discussion

3.1. Optimization of sonication time in the metal analyte extraction process

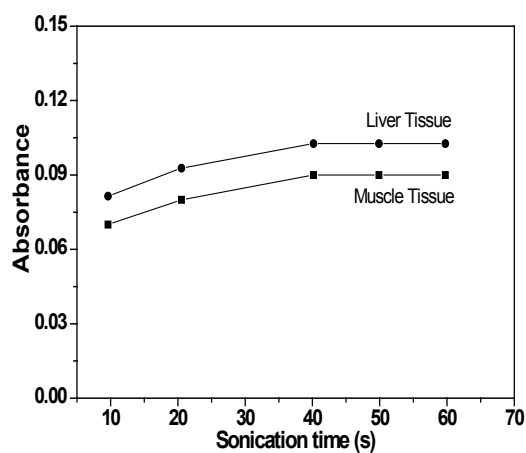
The efficiency of the process of ultrasound-assisted extraction of selenium from samples of fish muscle and liver tissue was evaluated by varying the extraction time and ultrasonic power. In the sample grinding process, care was taken to achieve the smallest possible granulometry, which favors the homogeneity of the analyte in the solid material and prevents possible errors associated with the samples representativeness. Excessive handling of

Table 1. Heating program applied to coat the inner wall of the graphite tube with tungsten carbide.

Stage	Temperature (°C)	Stages		Argon Flow (L min ⁻¹)
		Ramp (s)	Heating (s)	
Drying	110	10	25	0.30
Drying	150	10	25	0.30
Reduction	600	10	20	0.30
Reduction	1200	10	20	0.30
Cleaning	2600	0	5	0.30

Table 2. Heating program of the graphite tube optimized for the determination of selenium in the acid extracts obtained from samples of Nile tilapia muscle and liver tissue.

Stages	Temperature (°C)	Stages		Argon flow (L min ⁻¹)
		Ramp (s)	Heating (s)	
Drying	90	10	0	1
Drying	150	10	5	1
Drying	250	10	5	1
Pyrolysis	1300	5	10	1
Atomization	2300	1	5	0
Cleaning	2800	5	0	1

**Figure 1.** Influence of sonication time in the extraction of selenium from samples of Nile tilapia muscle and liver tissue. Experimental conditions: sonication time – 5 cycles of 10, 20, 40, 50 and 60 s; power – 136 W; extraction solution – Hydrochloric Acid 0.10 mol L⁻¹.

the samples was also avoided in this stage in order to avoid possible sources of contamination. As discussed in the literature, ultrasonic waves promote the extraction of analytes from the matrix by their action upon the organic molecules in the sample, destroying them and breaking down the metal bonds with these molecules [9,14,20]. Therefore, sonication time optimization experiments were carried out initially for the extraction of the analytes from the samples. Fig. 1 depicts the results obtained in these experiments.

Increasing the sonication time caused an increase of the temperature of the extraction medium. A temperature about 50°C normally favors the extraction efficiency because it increases the number of acoustic cavitation nuclei formed near the surface of the solid phase of the suspension (solid-liquid system) [20]. However, when the temperature of the extraction medium approaches the ebullition temperature of the liquid phase, the efficiency of sonication declines because the surface tension of the medium decreases and the water vapor inside the microbubbles increases, which may cause a reduction of the shock waves [10,14]. Therefore, there

is a sonication time considered optimal in the process of metal extraction from solid samples. Acoustic cavitation, or the cycle of formation, growth and collapse of the microbubbles formed by wave propagation, occurs during this time. After the cavitation bubbles collapse, a large amount of energy is released in the microregion close to the surface of the solid phase, thus causing extraction of the metal ions and, in some cases, even dissolution of the solid material [14,20].

Analyzing the graphs in Fig. 1, notes that starting from five cycles of 40 s of ultrasonic shaking (3.33 min), the absorbance signals measured by GFAAS remained practically constant and the temperature measured in the solid-liquid system was 62°C. Therefore, this time was considered efficient for the selenium extraction process.

3.2. Optimization of the ultrasound power in the selenium extraction process

The intensity of the ultrasound transmitted to the medium is directly related to the amplitude of the vibration of the sonotrode tip. Usually, increasing the power intensity favors an increase of the chemical effects caused by sonication. The influence of the ultrasound amplitude on a scale of 20-60% of the total work power (340 W) is shown in the graphs of Fig. 2.

When very high vibrational amplitude is used, a large number of cavitation bubbles are generated in the solution, which may be unfavorable for the growth and collapse of these bubbles, resulting in a decrease of the energy released through the liquid phase. For the metal under study, the extraction efficiency (see Fig. 2) increased with growing amplitude of 20 to 40% of total power (corresponding to 68-136 W), remaining constant at the highest values of amplitude. The results obtained indicated that the maximum efficiency of the extraction process was achieved on an amplitude scale of 40-60% of power (136-204 W). The amplitude of 40% (136 W) was therefore chosen for all the remaining experiments, since this amplitude causes less increase of the temperature of 62°C of the

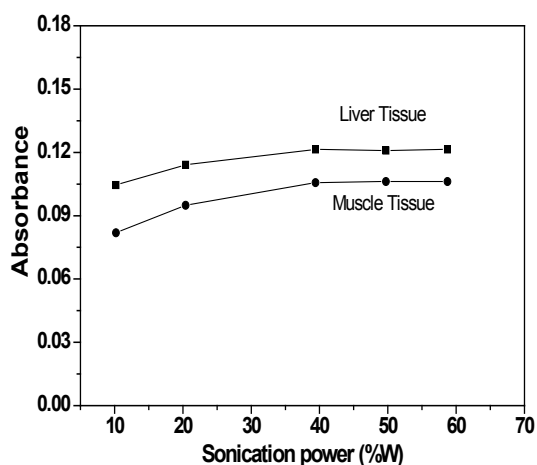


Figure 2. Influence of sonication power in the process of selenium extraction from samples of Nile tilapia muscle and liver tissue. Experimental conditions: sonication time – 5 cycles of 40 s; extraction solution – Hydrochloric Acid 0.10 mol L⁻¹.

extraction solution, when compared with the 76 and 84°C that result from the use of amplitudes of 50% (170 W) and 60% (204 W) of power [8-14,20-22].

3.3. Optimization of the experimental conditions for the GFAAS determination of selenium in the acid extracts obtained from the extraction process

Pyrolysis and atomization curves were drawn to determine the optimal temperatures of pyrolysis and atomization of selenium in the standard solutions and acid extracts from the Nile tilapia muscle and liver tissues obtained in the ultrasound-assisted extraction process using the graphite tube coated internally with tungsten carbide. Figs. 3 and 4 illustrate the influence of the pyrolysis and atomization temperatures on the absorbance signal obtained for selenium in a standard containing 10 µg L⁻¹ and in the acid extracts of the biological materials.

The pyrolysis temperature of 1300°C was chosen, as indicated in Fig. 3, because the absorbance signals obtained for Se remained constant starting at 1000°C, undergoing a rapid decline from 1300°C. All the other experiments were carried out at this temperature in the pyrolysis stage, because the selenium remained thermally stable even at this temperature. With regard to the atomization temperature (Fig. 4), it was found that the absorbance signals obtained for selenium became constant starting at 2000°C. In view of this finding, an atomization temperature of 2300°C was selected for all the remaining experiments. The good thermal stability of selenium achieved in these experiments demonstrates

the efficiency of the chemical modifier Pd(II) coinjected with the sample and of the tungsten carbide which acted as a permanent modifier [19,23].

3.4. Construction of the analytic curve

Based on the optimized physicochemical parameters (pyrolysis and atomization temperatures), the calibration curve of selenium was constructed in the range of concentration of 2 to 20 µg L⁻¹ (as described under section Preparation of the analytic curve). The straight-line equation obtained for the calibration curve of selenium was: $C_{Se} = Ab. - 0.00522/0.01063$ ($r = 0.9999$). The values obtained from the absorbance signals of the selenium standards ($n=5$) showed good repeatability and reproducibility (relative standard deviations lower than 2%), which reinforces the efficiency of the chemical modifiers, Pd(II) and tungsten carbide in the thermal stabilization of selenium during the pyrolysis and atomization stage of the heating program employed. The characteristic mass (m_0) in relation to the standard of 10 µg L⁻¹ of selenium was 45 pg [26]. The limits of detection (LOD) and of quantification (LOQ), calculated considering the standard deviation of 20 readings obtained from the blank of the standard solutions and from the slope of the analytic curve ($LOD = 3\sigma/slope$ and $LOQ = 10\sigma/slope$), were, respectively, 0.28 and 0.93 µg L⁻¹ of selenium [26,27]. The life time of the graphite tube was equivalent to 962 firings, which, considering the complexity of biological matrices, is acceptable when compared with other methods described in the literature [19,23-25,28-31].

3.5. Figures of merit of the ultrasound-assisted extraction method

After the optimization of the physicochemical parameters of the ultrasound-assisted extraction process and the GFAAS quantification of selenium, the proposed method was applied for the determination of the analyte in five pools of samples of muscle and liver tissue of Nile tilapia. The results obtained were compared with those obtained from the acid mineralization of the samples in a microwave oven (Table 3). The precision and accuracy of the method were evaluated by the determination of selenium in standard certified Bovine Muscle – NIST RM 8414, as also indicated in Table 3.

An analysis of the results obtained by ultrasound-assisted extraction confirms that they are consistent with the results obtained by acid mineralization of the samples in a microwave oven. The calculated relative standard deviations were lower than 2% (compared to 6% in acid mineralization), demonstrating that the proposed method provides accurate measurements.

Table 3. Results obtained in the determinations of selenium from the pool of samples of Nile tilapia muscle and liver tissue and from standard certified Bovine Muscle – NIST RM 8414.

Samples	Ultrasound-assisted extraction ^a ($\mu\text{g kg}^{-1}$)	Acid mineralization ^b ($\mu\text{g kg}^{-1}$)
Pool of muscle tissue	280.4±4.2	284.2±17.1
Pool of liver tissue	592.3±6.7	589.5±35.2
Certified standard * NIST 8414	75.6±1.4	74.1±3.7

^{a,b}Confidence interval at a 95% confidence level (t -student = 2.78); Results expressed as mean value ± standard deviation ($n = 5$); *Certified Value – 0.076±0.010 mg kg⁻¹ or 76±10 $\mu\text{g kg}^{-1}$

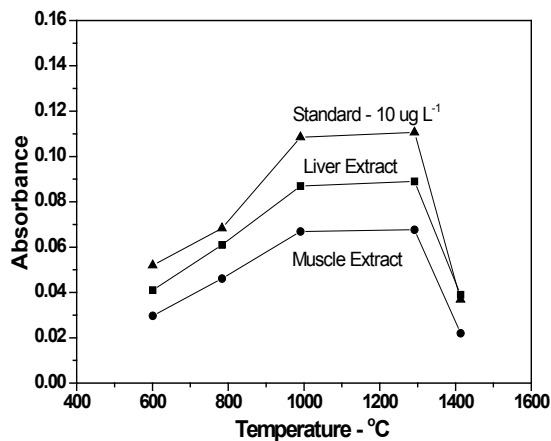


Figure 3. Pyrolysis temperature curves of the acid extracts from Nile tilapia muscle and liver tissue samples and of the standard containing 10 $\mu\text{g L}^{-1}$ of Se.

With regard to the results obtained for the certified standard, not only did the values present relative standard deviations of less than 2% (against the 5% in acid mineralization), characterizing good repeatability among the measurements, but also these results were much closer to the certified value than the results obtained using acid mineralization in a microwave oven, thus confirming the greater accuracy of the proposed extraction method. Moreover, it was observed that the accuracy of the results was not statistically different at a 95% confidence level (t -student = 2.78).

4. Conclusions

The results obtained by the proposed method for the determination of selenium in samples of muscle and liver tissue of Nile tilapia using ultrasound-assisted

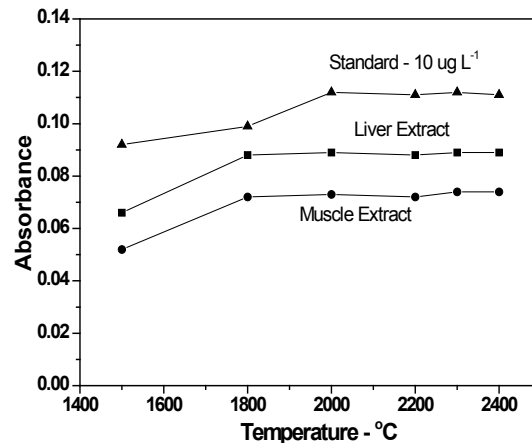


Figure 4. Atomization temperature curves of the acid extracts from Nile tilapia muscle and liver tissue samples and of the standard containing 10 $\mu\text{g L}^{-1}$ of Se.

extraction were equivalent to those obtained by the method of acid mineralization of samples in a microwave oven. However, it should be noted that the proposed method reduces considerably the analysis time, thus favoring the analytical speed. Moreover, the accuracy and precision of the values of selenium determinations were superior to those obtained by acid mineralization of the samples.

Acknowledgments

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