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Methodological performance of selenium determination in ruminal fluid by hydride generation atomic absorption spectroscopy

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ABSTRACT

Selenium (Se) is a trace element used by multiple enzymes. In ruminants, Se present in food is released to ruminal fluid allowing its absorption in the small intestine. Se release may be analysed by hydride generation atomic absorption spectroscopy (HG-AAS). According to the Association Official of Analytical Chemists (AOAC), analysing the methodological performances is important to support the results of a methodology. The aim of this study was to develop and verify the analytical methodology of Se determination in ruminal fluid by HG-AAS. According to the AOAC guide, the parameters to be analyzed, and their respective acceptance criteria (AC) are: applicable range (AC: 0.220 - 9.68 µg/l), bias (AC: 70-125%), precision by repeatability (RSDr%) (AC: < 21%), intermediate precision (RSDip%) (AC: < 21%), recovery (AC: 80-110%), limit of detection (LOD) (AC: < 0.22 µg/l), and relative uncertainty (AC: <15%). The technique used is based on a wet digestion of ruminal fluid with $HClO_4$, H_2SO_4 and HNO_3 concentrated at temperatures ranging from 120°C to 180°C. Then the digest was pre-reduced in HCl at 50 % and was quantified in the HG-AAS. The results obtained were: LOD 0.196 µg/l, bias 96.8% (1.25 µg/l); 98.3% (2.50 µg/l); 94.8% (5.00 µg/l), precision by repeatability 8.61% (3.28 µg/l); 8.90 % (3.52 µg/l); 9.80% (1.85 µg/l), intermediate precision RDSip 10.2%, recovery 109.2% (10 µg/l); 96.7% (25 µg/l); 96.8% (50 µg/l) and applicable range 0.196-62.5 µg/l. The values obtained are within the requirements stated by the AC.

Keywords: selenium, ruminants, bovine, hydride generation atomic absorption spectrometry.

RESUMEN

El selenio (Se) es un elemento traza utilizado por múltiples enzimas. En rumiantes, el Se de los alimentos es liberado hacia el licor ruminal y esto permite su absorción en el intestino delgado. El Se liberado puede ser analizado por espectrofotometría de absorción atómica con plataforma de hidruros (HG-AAS) De acuerdo con la asociación oficial de química analítica (AOAC) analizar la performance metodológica es importante para respaldar los resultados de una metodología. El objetivo de este estudio fue el desarrollo y verificación de la metodología analítica de la determinación de Se en fluido ruminal mediante un HG-AAS. De acuerdo con la guía de la AOAC, los parámetros para analizar para esto, y sus respectivos criterios de aceptación (CA), son: rango aplicable (CA: 0,22-9,68 µg/l), bias (CA: 70-125%), precisión por repetibilidad (CA: RSDr <21%), precisión intermedia (CA: RSDR <21%), recuperación (CA: 80-110%) y límite de detección (LOD) (CA: <0,22

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µg/l), incertidumbre relativa expandida (CA: <15%). La técnica que se utilizó se basó en una digestión húmeda del fluido ruminal con $HClO_4$ (c), H_2SO_4 (c) y HNO_3 (c) a temperaturas que van de los 120 °C a 180 °C. Luego se realiza una prerreducción del equis con HCl al 50% y se cuantificó en el HG-AAS. Los resultados obtenidos fueron los siguientes: LOD 0,196 µg/l, Bias 96,8% (1,25 µg/l); 98,3% (2,50 µg/l); 94,8% (5,00 µg/l), equisite por repetibilidad 8,61% (3,28 µg/l); 8,90% (3,52 µg/l); 9,80% (1,85 µg/l), equisite intermedia RDSR 10,2%, recuperación 109, 2% (10 µg/l); 96,7% (25 µg/l); 96,8% (50 µg/l) y rango equisites 0.196-62,5 µg/l. Los valores obtenidos están dentro de los equisites mínimos indicados por los CA.

Palabras clave: *selenio, ruminates, bovinos, espectrofotómetro de absorción atómica con plataforma de hidruros.*

INTRODUCTION

Biological interest

Nowadays, it is known that in mammals, Se is a trace element used by more than 30 Se-proteins in various metabolic pathways, in which it behaves as a cofactor. They incorporate the mineral co-transporting it as a cysteine residue, an amino acid that contains a sulphur atom which is replaced by Se to form Se-cysteine (Suttle, 2010; Schomburg, 2011; Combs, 2015).

Many Se-proteins have antioxidant functions. This is essential for mammals, since they protect the tissues from the attack of reactive oxygen species (ROS) which are secondary products of energy metabolism. An imbalance between the production of ROS and antioxidants can initiate oxidative chain reactions and peroxidation on lipids, proteins and DNA, causing serious cellular damage (Jaramillo *et al*., 2005; Chauhan *et al*., 2014).

In mammals, Se is incorporated into the organism primarily from food, but with differences in absorption efficiency (Se absorbed/Se in food). In non-ruminants and pre ruminants, efficiency ranges from 0.85 to 0.90, while in ruminants it is less, with values between 0.31-0.59, due to reduction of dietary Se to insoluble forms, such as elemental Se or selenide in the ruminal environment (Spears, 2003; Suttle, 2010).

Se absorption mainly occurs in the first portion of the small intestine, but for this to happen, it must be solubilised from food. In ruminants, Se release from food to the ruminal fluid is essential for its posterior absorption. So, It is crucial to clarify the release of the mineral from the forage to the ruminal liquor (Suttle, 2010; Combs, 2015).

Selenium quantification in ruminal fluid

The quantitative analysis of Se in biological samples has always been a challenge for researchers due to the low concentration present in this type of material and its high volatilization during the decomposition of the sample. There are a large number of methodologies for quantifying this mineral, such as: graphite furnace coupled to an atomic absorption spectrophotometer, spectrofluorometry, inductive coupling plasma to mass spectrometer, HG-AAS, among others. Spectrofluorometry and HG-EAA are the most frequently used techniques for the determination of Se in biological samples, due to their relatively low cost and minimal interference. The other techniques mentioned, although they allow the detection of Se at low concentrations and have more sensitivity, have more interference and are more expensive (Combs, 2015; Tinggi *et al*., 1992; Smrkolj *et al*., 2004).

The determination of Se by HG-EAA is a methodology that allows readings of low concentrations, of the order of 0.1 µg/l. To quantify a sample by this technique, it must be previously mineralized by wet digestion with concentrated acids and adequate heating can be carried out (Welz *et al*., 1985).

Performance verification

As mentioned above, it was important to develop a methodology to quantify Se in ruminal fluid. The methodology developed uses hydride generation atomic absorption spectroscopy (HG-AAS) to quantify Se.

To verify the reliability of the technique, the performance was evaluated. Such verification is extremely important. It is required on quality management systems, particularly the ISO 17025/IRAM 301 (IRAM, 2005), as a general condition for testing in calibration laboratories. This allows to know the functional characteristics of the method, providing a high level of confidence on it (AOAC, 2016).

To check the performance of the determination, Appendix F of the AOAC guide (AOAC, 2016) was used. It contains a detailed description of the minimum requirements of analytical performance to be analysed, according to the type of technique, in order to evaluate the performance. As Se quantification in ruminal bovine fluid by HG-AAS is categorized as a quantitative determination of a trace element, the analytical parameters necessary to evaluate are: applicable range, bias, precision by repeatability, intermediate precision, recovery, limit of detection (LOD) and relative uncertainty, all of which will be defined later.

Prior to the analysis of the above-mentioned parameters, acceptance criteria (AC) were established. These are the

values that the method should fulfil in order to be reliably used for the required purposes. Some acceptance criteria were established by the AOAC guide (AOAC, 2016), while others were fixed by the professional in charge of the validation. Both are indicated below.

Objective

To develop and verify a methodological analytical performance of Se determination in ruminal fluid by HG-AAS.

MATERIALS AND METHODS

Sample preparation

The ruminal fluid used was extracted from a fistulous animal, which was fed with a stipulated and constant diet. This guaranteed approximately the same microbial population in the fluid over time. The ruminal fluid was wrung out and filtered through a cheesecloth to separate traces of forage. It was maintained in isothermal and anaerobic conditions (39 ºC) until its analysis to maintain the microbial environment.

Analytical technique

An HG-AAS was used where the reaction of acidified aqueous samples with a reducing agent, such as sodium borohydride ($NaBH_4$). This reaction generates volatile selenium hydride (SeH_2) which was transported to a quartz cell by means of an argon carrier gas. This cell was at 900ºC, which promotes the conversion to gaseous metal atoms (Se^0). In the quartz cell, the generated analyte metal atoms are contained in the path of a light emitted by an EDL lamp (electrodeless discharge lamp). Thus, the atoms absorb part of the beam, and this is used by the computer to quantify the Se of the sample (PERKINELMER. 2000).

The method used is based on wet digestion of 5 ml of ruminal fluid with $HClO_4$, H_2SO_4 and HNO_3 concentrated (2:1:3). The sample was put in a thermostatic aluminium block programmed with ramps of temperatures ranging from 120 ºC to 180 ºC. The digest obtained must be transparent or slightly yellow (Alfthan. 1984; Raptis, *et al.,* 1983).

Once digestion was completed, the digest was resuspended in 25 ml with doubly distilled water. In these conditions, all species of Se present in the fluid were in a state of oxidation +4 or +6. Since only the species +4 generate hydrides, it was necessary to pre-reduce the digest before reading it in the HG-AAS. For pre-reduction 10 ml of digest were put with 5 ml of HCl (c) in a water bath at 90º C for 20 minutes. Later, such mixture was taken to 25 ml with doubly distilled water.

Then, the generation of hydrides with a FIAS 100 generator took place, using HCl 10% (v/v) as a carrier solution, $NaBH_4$ 0.2% (w/v) in NaOH 0.05% (P/V) as reducing solution and Ar as a carrier gas at a flow rate of 80 ml/min (Perkinelmer, 2000).

Verification of the methodological performance

Acceptance criteria

They are boundary values established to compare with the results of the performance analysis and verify the applicability of the method. They were adjusted by the person responsible for the test, in a reliable and scientific manner (table 1).

Parameters analysed-Calculations

The AOAC, in its guide, prescribes the minimum requirements for analytical performance. These parameters are calculated as follows (AOAC. 2016):

- Limit of detection (LOD): is the lowest concentration of analyte that can be quantified, with a 99% probability that it is not an equipment noise. It is considered that a quantification below LOD is no longer reliable, because the uncertainty related to the measurement would be larger than the measurement value itself.

  $$LOD=Bco+3\times Sbco$$

  Bco: Average signal of 10 calibration blanks.
  Sbco: Standard deviation of the mean of 10 targets

  The AC established for this parameter was adjusted based on the lowest concentration found in the bibliography (Serra *et al.,* 1994; Ruggieri *et al.,* 2016).

- Applicable range: is the Se concentration range that the method can measure without additional dilutions. It is characterized by the range that goes from the LOD until the last standard in the calibration curve, multiplied by 12.5, which is the dilution factor of the sample. The AC established had at least the lowest concentration (0.220 µg/l) and at most the highest concentration found in the bibliography (9.68 µg/l) (AOAC, 2016; Serra et al., 1994).

- Bias (Bias %): is the analyte concentration that is recovered from certified standard when the sample is carried out throughout the whole method (or the maximum possible part). In our case, it was not possible to have a certified ruminal liquor standard. Then, as it is suggested by AOAC (2016), samples fortified with a certified standard solution of 980 ppm of Se in 1% HNO3 were used. We analyzed 5 independent replicates of 3 levels of fortification. The AC established corresponded to that determined by the Shah conference reports et al. (1992; 2000), widely accepted in bioanalytical analyses.The calculation was:

  $$Bias(\%)=[(Co/Ca)-1]\times 100$$

  Co: Obtained concentration
  Ca: Added concentration

- Precision: is the degree of concordance between the obtained results from replicated measurements over the same object under specific conditions. It is a parameter only related to the random error. The specific conditions may be:

  - Repeatability (RSDr): is the degree of concordance between the results of independent analyses done

with the same method, over the same sample, in the same lab, by the same operator, on the same day. Seven independent replicates of three samples with different concentration levels were analysed. For this parameter, the AC is the one suggested by the AOAC in appendix F (2016); which is indicated according to the native concentration of the sample. The RSDr % was characterized for each level as:

$$RSDr\ (\%) = \frac{Sm}{Xm} \times 100$$

Sm: Standard deviation of the mean of the replicates in each concentration.
Xm: Average of the replicates in each concentration.

- Intermediate Precision (RSDip): is the degree of concordance between the results of independent analyses done with the same method, over the same sample, in the same lab, by the same operator, on different days, with different calibration curves. The established AC is the one suggested by AOAC in appendix F (2016) for the precision by repeatability, assuming that the variation between days is not significant. Seven replicates of a sample were analysed on three different days, and the total RSDip was characterized as:

$$RSDip\ (\%) = \frac{SM}{XM} \times 100$$

SM: Standard deviation of the mean of the replicates throughout the days.
XM: Mean of the replicates throughout the days.

- Recovery (Recup.f %): is the fraction or percentage of the analyte that is quantified in a fortified sample. Seven replicates at three fortification levels (50, 25 and 10 µg/l) were analysed over the same sample of ruminal liquor.

  The established AC is that suggested by AOAC in appendix F (2016), which is indicated according to the native concentration of the sample. The average percentage of recovery for each level of concentration was characterized as:

$$Recup.f\ (\%) = \left(\frac{Lf\text{-}Ls}{Cf}\right) \times 100$$

Lf: Concentration read in the fortified ruminal fluid.
Ls: Concentration read in the not fortified ruminal fluid.
Cf: Fortification concentration.

- Expanded relative uncertainty (UREL %): is a parameter associated to the result of a measurement which characterizes the dispersion of the values that might be reasonably attributed to the measure. The established AC is suggested by the United Nations Office on Drugs and Crime (UNODC) (2009) in its guide for methods validation (OAA, 2013; AOAC, 2013; Ruggieri *et al.,* 2016).

  The UREL % can be calculated through the "black box" model. In this model, proposed by Reyes and Cerezo (2009), only the Bias and the intermediate precision are considered for the calculation of the uncertainty, as can be seen in the following equation:

$$UREL(\%) = \sqrt{2x\left(\frac{RSDip\%}{n}\right)^2 + \left(\frac{Bias}{\sqrt{3}}\right)^2}$$

UREL %: expanded relative uncertainty.
RSDip%: coefficient of variation of intermediate precision.
n: number of days in which the intermediate precision was analysed.

RESULTS AND DISCUSSION

The results obtained by analysing the parameters required to evaluate the technique performance are shown in table 1:

Parameter	CA	Value obtained
Limit of detection (LOD)	< 0.22 µg/l	0.196 µg/l
Applicable range	0.220 – 9.68 µg/l	0.196-62.5 µg/l
Bias %	15%	10 µg/l: 6.8%
		25 µg/l: 10.9%
		50 µg/l: 6.0%
Precision by repeatability (RSDr)	< 21%	3.28 µg/l: 6.2%
		3.52 µg/l: 9.8%
		1.85 µg/l: 9.8%
Intermediate Precision (RSDip)	< 21%	3.52 µg/l: 10.2%
Recovery (Recup.f %)	80 - 110%	10 µg/l: 109.2%
		25 µg/l: 96.7%
		50 µg/l: 96.8%
Expanded relative uncertainty (UREL %)	< 15%	12.5%

Table 1. Results of the analysed parameters.

The methodology was developed based on the available equipment and capabilities in the laboratory, following the guidelines established by Alfthan (1984); Tinggi *et al.* (1992) and Raptis *et al.* (1983).

As table 1 shows, all the parameters studied are within the limits of the AC. In the case of LOD, it was not possible to find references to discuss data for this parameter in ruminal fluid.

In the case of the applicable range, this exceeds what is required in the AC, which allows to loosely cover the range of possible Se concentrations in ruminal fluid (Serra *et al*., 1994; Koening *et al*., 1997).

For Bias, it is observed that the differences obtained among the three concentrations of fortification that were used and the theoretical value are both in agreement with the AC established. The values coincided with the ones obtained by Tinggi *et al*. (1992), who informed Bias % with fortified biological samples that fluctuated between 0-8.3%; but they were higher to the ones obtained by Navarro *et al.* (1995), which indicated up to 3.84%.

The precision by repeatability was within the terms of the AOAC (2016) guide, which takes into account the possible repeatability value based on the range of analyte concentrations. A limit of 21% is suggested for RSDr. Kadrabova *et al.* (1997) and Surai *et al.* (2008) obtained RSDr of 1.1-3.8% and 1-5%, respectively.

The intermediate precision obtained is below the established AC. Alfthan (1984) obtained RSDip between 3.6-5.7% for concentrations of 49.4; 129 and 1025 µg/l of Se, respectively. The RSDip mentioned are inferior to those obtained in this work, but the authors worked with higher Se concentrations (3.5 µg/l of Se).

The recoveries with fortified samples are included within the limits suggested by the AOAC (2016), according to the range of Se concentrations, of 60-115%. They were also similar to those obtained by other authors. Maher (1987) obtained recoveries of 97% in biological samples, Tinggi *et al.* (1992) of 104%, Alfthan (1984) of 101% and Kadravoba *et al.* (1997) from 93.9 to 101%, all of them in diverse concentrations and biological samples.

The expanded relative uncertainty was also below the AC for the purposes required. It was not possible to get references to compare this parameter for ruminal fluid.

CONCLUSIONS

The obtained values are within the minimum requirements indicated by the AC. In this way, it is concluded that the performance of the technique is suitable for the quantification of Se in ruminal fluid.

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