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Determination of uncertainty in the measurement of Aflatoxins B1 in pistachio nuts by the HPLC-FLD method

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ABSTRACT

The present study defined uncertainties during Aflatoxin B1 measurements in pistachio nuts using HPLC equipped with fluorescence detector and derivatizing cell (Kobra® Cell) to increase the detector signal. The uncertainty of a method is one of the parameters required by EURACHEM/CITAC Guide, and forms part of the validation of analytical methods. Six variables affected the final value of the measured concentration, which were included in the mathematical model. The contribution index of each variable to the combined uncertainty (u_c) was also calculated and analysis of the Pareto diagram showed that 80.47% of the variability was the result of the calculation and execution of the calibration curve of the Aflatoxin B1 standard, 19% was determined by the recovery rate of the pistachio sample and volumetric measurements affected the concentration to a very lesser extent. The value of expanded uncertainty in the determination of Aflatoxin B1 for pistachios was Ucx = 0.83 μg/kg.

1. Introduction

The main world producers of pistachio nuts are United States, Iran, Syria, Turkey, China, and Greece. Argentina has about 1000 ha planted with pistachio nuts and it an important agro-industrial activity in the central west region [1]. Italy and Brazil are the most important buyers of Argentine pistachio.

Pistachio nuts are commercialized in a salty, roasted, and shelled form for direct consumption as a snack or for the preparation of bakery and pastry products [2].

Aflatoxins are among the most harmful carcinogenic natural toxins present in nuts such as walnuts, peanuts, pistachios or dried figs, which are their main reservoirs [3]. Aflatoxins are synthesized by *Aspergillus flavus* and *Aspergillus parasiticus, and* these moulds can develop toxins on a wide variety of substrates, as they are able to survive under harsh storage conditions. Among the different aflatoxins, Aflatoxin B1 is considered the most toxic and carcinogenic. Because of its high toxicity, the concentration of aflatoxins in nuts is regulated in many countries in the world.

Codex Alimentarius [4] has established a level of 15 μg of total aflatoxin/kg of almonds, hazelnuts and pistachios intended for further processing and a level of 10 μg total aflatoxin/kg of "ready-to-eat" almonds, hazelnuts and pistachios. The Quality Protocol for dried pistachios for the Argentine food certificate [5] has set the maximum limit of Aflatoxin B1 in pistachios at twelve parts per billion (12 μg/kg).

To market food products, the exporter needs to have internationally accepted Certificates of Analysis, to guarantee that the product meets the agreed quality levels.

Issuing Certificates of Analysis that are internationally accepted implies that a testing and calibration laboratory has the accreditation of the ISO 17025 Standard, granted by an organization authorized by the government of each country. The Argentine Accreditation Organization (OAA) is the only entity authorized to accredit the IRAM-ISO/IEC 17025 Standard in the Argentine Republic [6] (2018).

The School of Chemical Engineering of the National University of San Juan has its own analysis laboratory: LAPRIQ (Laboratory of Analysis for Regional Chemical Engineering Products). This laboratory is accredited by the IRAM-ISO/IEC 17025:2017 Standard since 2013, and it is specialized in determination of Ochratoxin A in raisins, wine and grape juice. It is important for the mentioned laboratory, to ask the OAA to extend the scope of accreditation to Aflatoxin B1 in pistachio nuts.

One of the requisites of the ISO 17025 is method validation. The method uncertainty is one of the parameters requested for quality assurance of analysis results, together with other parameters such as selectivity, working range and linearity and detection or quantification limit [7] (2016). The result of an accredited analysis must

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be expressed with its uncertainty value, according to the LAPRIQ protocol.

The objective of this study was to determine the uncertainty of measurement of Aflatoxin B1 in pistachio nuts using High Performance Liquid chromatography equipped with fluorescence detector and derivatizing cell (Kobra® Cell) to increase the detector signal.

The method described in the present study can be used by any laboratory that performs this analysis, modifying the corresponding values according to the individual uncertainties of lab technicians and instruments used at each laboratory.

2. Materials and methods

2.1. Materials

All reagents used in the laboratory have a certificate to the SI System. All measurements were carried out with instruments calibrated with standards traceable to the SI system and with certified standards.

It is important to mention that all the equipment and instruments used in LAPRIQ are calibrated and/or verified as established in their respective operating procedures, which regulate the work procedure and its frequency. These calibrations are carried out in organizations accredited under the IRAM-ISO/IEC 17025 Standard for calibration laboratories.

2.2. Method

In order to estimate the uncertainty of a chemical analysis process there exist two general methods: *top-down* and *bottom-up* [8] (2006). The first one is based on the processing of long-term recorded data, derived from proficiency test results, laboratory control samples, published bibliographic data, etc. The *bottom-up* procedure used in the present report is based on an exhaustive study of errors arising from each of the analytical operations broken down into primary activities.

Our uncertainty calculation procedure was based on EURACHEM/CITAC Guides (2012) [9].

Determination of the final concentration $\boldsymbol{C_x}$ of a chemical species in a matrix involves calculation of a function which depends on the calculation variables (extraction volume, equipment response, etc.). All possible sources of error identified and defined in that function are represented in an Ishikawa Diagram, also called Fishbone.

For each component v_i that contributes to the uncertainty of the process, the standard deviation was determined according to Eq. (1) in which u_{mi} is the individual uncertainty of each factor involved in a specific measurement (linearity, temperature, sensitivity, etc.), according to the propagation of uncertainty law.

$$u_i = \sqrt{\sum_i u_{mi}^2} \tag{1}$$

To determine the standard uncertainty of each measurement, all the necessary tests were carried out, even chemical analysis or verification of mass/volume measurements.

The Combined Uncertainty was calculated with Eq. (2), in which each standard uncertainty was multiplied by a sensitivity coefficient $\boldsymbol{c_i}$ to weigh its contribution to the overall uncertainty.

$$u_c = \sqrt{\sum_i \left(c_i \times u_i\right)^2} \tag{2}$$

The sensitivity coefficients were determined with Eq. (3), and C_x with respect to v_i was derived as follows:

$$c_i = \frac{dC_x}{d_{v_i}} \tag{3}$$

The uncertainty of the method, called the **Expanded Uncertainty**, is the interval within which the true value of the concentration of the compound is assured to lie, with a specified degree of confidence. This was calculated as the combined uncertainty multiplied by the coverage factor *K*, according to Eq. (4). $K = 2$ ensured a 0.9545% confidence for the measurement.

$$U_{C_x} = K \times u_c \tag{4}$$

Finally, the contribution of each variable to the total uncertainty of the process was determined through the contribution index of each of them, given in Eq. (5). This enabled to establish the critical points that can be improved to reduce the uncertainty of the method. It was defined as the contribution of the uncertainty of each variable to the total uncertainty. Consequently, critical points could be established to reduce the total method uncertainty.

$$Ind\% = \frac{\left(c_i \times u_i\right) \times 100}{\sum_i \left(c_i \times u_i\right)} \tag{5}$$

3. Experiments

Samples of pistachio nuts were directly collected from a production field in San Juan province (31°32′15″ S 68°32.183′ O) during February 2021.

After harvesting, samples were ground to achieve homogenization and subsequently diluted in a solution of methanol: water (60/40).

Aflatoxins B1 were extracted by cleaning up, using EASY-EXTRACT ® AFLATOXIN immunoaffinity columns [11], and the concentration was quantified using High Performance Liquid Chromatography (HPLC, Perkin Elmer) equipped with a derivatization cell and fluorescence detector (FLD).

The equipment consisted of an autosampler, a pump, a TeknoKroma C18 column, Series UV/VIS fluorescence detector, and Total Chrom software. Derivatization was carried out with an electrochemical cell (Kobra® Cell R-Biopharm Rhóne Ltd.) [12] to improve the Aflatoxin B1 signal.

Details of procedures and the HPLC operation conditions are described in protocols and instruction documents of the LAPRIQ Quality Management System.

Assay standard solutions were prepared from certified standard solutions. The analytical scales (with resolution ± 0.001 g) and the volumetric elements were frequently calibrated and verified according to LAPRIQ protocol, and the calibration certificate was traceable to the SI system. The ambient temperature was maintained stable and any variation was recorded with a calibrated thermometer also traceable to the SI system. The entire methodological framework conforms to what is required by the norm IRAM-ISO/IEC 17025 (2018) [6].

4. Results and discussion

The presented results follow the order established in the Ref [10].

4.1. Definition of the measurand

The measurand defined for the present study was the Aflatoxin B1 content in pistachio nuts for export, symbolized by $\mathbf{c_x}$ in μg/**kg** units.

Eq. (6) was used to calculate the concentration of Aflatoxin B1 (AFB1). The definition of all its terms and their value are presented in this section.

$$AFB1\ Concentration = C_x = \frac{C_{HPLC} \times V_1 \times V_2}{V_3 \times M \times R} \tag{6}$$

4.2. Identification of the variables that influence the measurement

According to the procedure described in the methodology, the variables that affect the measurement were grouped into four: mass, volume, recovery and concentration of Aflatoxin B1 in the standard solution.

The variables considered in the uncertainty of the process were: M, V_1, V_2, V_3, R and C_{HPLC}. The values and components used for the calculation of the sensitivity coefficients are shown in Table 1.

Table 1
Variables considered to the uncertainty of the process.

Symbol	Description	Value
M	Amount of fruit analysed	42 g
V_1	Elution volume (1.5 ml of HPLC methanol + 1.5 ml of HPLC water)	3ml
V_2	Extraction volume (200 ml of methanol p.a. + 50 ml of HPLC water)	250 ml
V_3	Aliquot of the analysed elution volume	10 ml
R	AFB1 recovery	0.8380
C_{HPLC}	Concentration measured in HPLC for the maximum value of the coefficient of variation.	$8.0^{*}10^{-10}$ g/ml

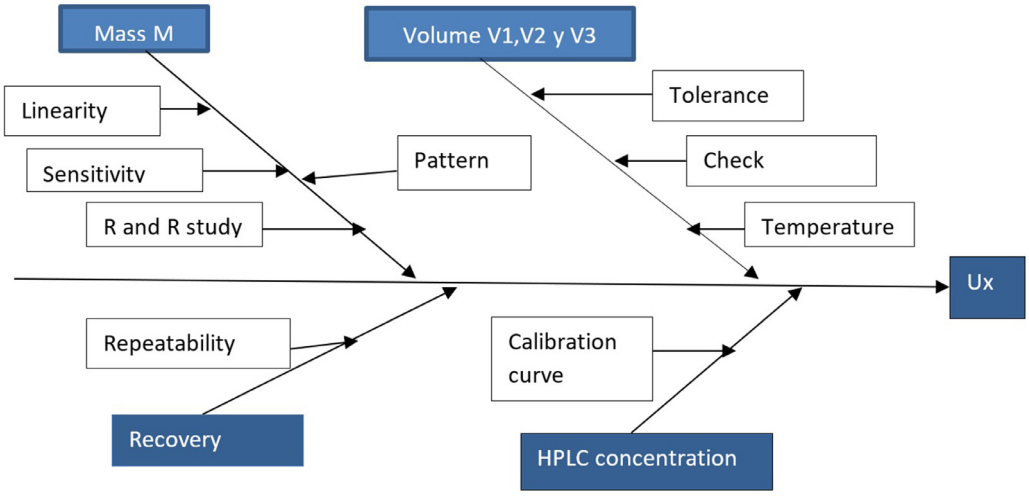

Fig. 1. Ishikawa diagram for the analyzed variables.

Fig. 1 shows the Ishikawa diagram defined for the determination of the uncertainty of measurement.

4.3. Quantifying standard uncertainty

The standard uncertainties that contributed to a combined uncertainty were defined as: u_M, u_{V1}, u_{V2}, u_{V3}, u_R, and u_{CHPLC}. The units of all dimensional measurements are presented in g and ml.

Only equations that were used and their results are presented in the present study, and all mentioned individual contributions of error were defined following LAPRIQ procedures according to the Quality Management System. The information about the assays and procedures carried out are duly registered and protected at the laboratory.

4.4. Calculation of individual uncertainties

4.4.1. Determination of u_M

A Mettler Toledo AL204 analytical balance was used to measure the weight of the pistachio samples. The standard uncertainty given in Eq. (1) and reported in g was calculated as the sum of the uncertainties contributed to the weight standards used, linearity, sensitivity and the study of R & R (allowing simultaneous evaluation of repeatability and reproducibility).

$$u_M = \sqrt{u_{sens}^2 + u_{lin}^2 + u_{RyR}^2 + u_{pat}^2}$$

$$u_M = \sqrt{(2.352 * 10^{-4}) + \left(8.665 * 10^{-5}\right)^2 + \left(3.235 * 10^{-5}\right)^2 + \left(2.752 * 10^{-5}\right)^2}$$

$$u_M = 2.5422^{*}10^{-4} g$$

4.4.2. Determination of u_{V1}

The elution volume (V_1) of the mixture was measured with a 1.0 ml Glassco micropipette. This micropipette was used four times to have the required 3 ml total measurement: two measurements of 0.750 ml of methanol and two measurements of 0.750 ml of HPLC-quality water. The uncertainty of each measurement consists of three components: micropipette tolerance, variation in temperature (methanol and water) and deviation verification, according to following equation.

$$u_{V1} = \sqrt{4\left(u_{tol}\right)^2 + 2\left(u_{\text{methanol}}\right)^2 + 2\left(u_{water}\right)^2 + 4\left(u_{verif}\right)^2}$$

This was repeated for all volume measurements with a micropipette measured in ml.

The manufacturer´s tolerance was calculated as:

$$u_{tol} = \frac{\left(Manufacturer's\ tolerance\right)}{\sqrt{3}}$$

$$u_{tol} = \frac{(0,0005)}{\sqrt{3}} = 0.00028867\ ml$$

The uncertainty of temperature variation was calculated according to Eq. (7), where α is the volumetric expansion coefficient corresponding to the fluid that is measured in $^{\circ}\ K^{-1}$. The calculation is not described because this would be too extensive.

$$u_{T methanol} = \frac{Variation\ in\ volume\ caused\ by\ temperature\ range}{\sqrt{3}}$$

$$u_T = \frac{\Delta T \times\ \alpha \times V nominal}{\sqrt{3}} \quad (7)$$

$$u_{T methanol} = 3.91010\ \times 10^{-3} ml$$

$$u_{T water} = 6.36528\ \times 10^{-4} ml$$

To determine the verification uncertainty, a type A evaluation method [13] (2009) was adopted Eq. (8). was used, where "s" is the standard deviation of the measurements made in the instrument verification and "n" is the number of measurements.

$$u_{verif} = \frac{(s)}{\sqrt{n}} \quad (8)$$

$$u_{verif} = 5.48800 \times 10^{-5} ml$$

$$u_{V1} = \sqrt{4\left(2.90074 * 10^{-4}\right)^2 + 2\left(3.91010 * 10^{-3}\right)^2 + 2\left(6.36528 * 10^{-4}\right)^2 + 4\left(5.48800 * 10^{-5}\right)^2}$$

$$u_{V1} = 5.63353 \times 10^{-3} ml$$

4.4.3. Determination of u_{V2}

Volume V_2 is the mixture of methanol and water in which the Aflatoxin B1 was extracted from the pistachio sample, it was measured with two calibrated flasks of 200 and 50 ml, respectively. The individual uncertainties of the methanol and water measurement were determined as follows.

$$u_{V2} = \sqrt{u_{methanol} + u_{water}}$$

Calculation of the measurement uncertainties for methanol and water is obtained with the following expression.

$$u_{metanol} = \sqrt{(u_{tol})^2 + (u_{Tmethanol})^2 + (u_{verif})^2}$$

The tolerance uncertainty of the flask was determined as:

$$u_{tol} = \sqrt{\left(\frac{Tolerance}{\sqrt{6}}\right)^2}$$

According to the equation above, determination of the corresponding measurement uncertainty of the volume V2 is as follows:

$$u_{water} = \sqrt{(0.024494897)^2 + (0.0424652)^2 + (0.001908522)^2}$$

$$u_{water} = 0.049062022 ml$$

$$u_{methanol} = \sqrt{(0.061237243)^2 + (1.04269)^2 + (0.062)^2}$$

$$u_{methanol} = 0.753855 ml$$

$$u_{V2} = 0.7554864\ ml$$

4.4.4. Determination of u_{V3}

After extraction, the pistachio sample was centrifuged to remove the supernatant and V_3 was the aliquot taken during the clean-up step. 10 ml of mixture was measured with a calibrated micropipette. Calculation was carried out according to Eq. (10).

$$u_{met+water} = \sqrt{\left(u_{tolerance}\right)^2 + \left(u_{Tmix}\right)^2 + \left(u_{verif}\right)^2} \quad (10)$$

$$u_{met+water} = \sqrt{(0.000816497)^2 + (0.029705)^2 + (0.001271224)^2}$$

$$u_{V3} = 0.029743398\ ml$$

4.4.5. Determination of u_R

Eq. (8) was used to calculate the accuracy uncertainty of the method. This required 20 determinations (n) with fortified samples with known concentrations. The standard deviation of the within-laboratory reproducibility (s) was taken using the following type A uncertainty equation.

$$u_{R=} \frac{0.261085913}{\sqrt{20}}$$

$$u_{R=} 0.058380585$$

4.4.6. Determination of u_{HPLC}

The corresponding calibration curve required five tests with three different analysts on different days, using calibration solutions of 0, 0.8, 2, 4 and 8 mg/l of Aflatoxin B1. A Trilogy® standard was used with a concentration of 5000 μg/l total toxins and a ratio AFB1:AFB2:AFG1:AFG2 of 4:1:4:1, respectively. The uncertainty of AFB1 in g/ml was determined from the calibration line obtained in accordance with the following equation, where S_c is the standard deviation.

$$u_{HPLC} = \frac{Sc}{\sqrt{3}}$$

$$u_{HPLC} = 1.87583 \times 10^{-10} ml$$

The results of the obtained standard uncertainties are presented in Table 2.

4.5. Quantifying combined and extended uncertainties

Once the individual uncertainty was defined, the corresponding sensitivity coefficients were calculated Eq. (6). was applied to calculate the Aflatoxin B1 concentration in microgram/kilogram.

The sensitivity coefficients $\boldsymbol{c_i}$ were calculated taking into account Eq. (3) and the values given in Table 1. The results are shown in Table 3 together with the derived equations for their calculation.

It is very important for the final calculation to note that c_i and C_{HPLC} are in g/ml but C_x, u_c and U_{Cx} are given in g/kg.

The combined standard uncertainty is calculated as indicated by Eq. (2),

$$u_c = 0.417099249\ \mu g/kg$$

and the expanded uncertainty U_{Cx} was determined with Eq. (4) as follows:

$$U_{cx} = K \times u_c = 2 \times 0.417099249\ \mu g/kg$$

$$U_{cx} = 0.83\ \mu g/kg$$

4.6. Contribution index of uncertainty components

Eq. (5) was used to calculate the percentage of the contribution index Table 4. shows the percentage of the contribution index of each variable. It can be observed that 74.87% of the variability is a result of the calculation of the calibration curve, followed by the recovery of Aflatoxin B1 in pistachio nuts. Three decimal digits had to be considered to visualize the contribution of the mass measurement, which was extremely small.

Fig. 2 shows the Pareto chart with a graphical contribution, the percentage and accumulated index.

5. Conclusions

According the objective of this study, the uncertainty of the measurement (U_{CX}) of Aflatoxin B1 in pistachio nuts was 0.83 microgram/kilogram.

The Pareto chart graphically exhibits the percentage of the contribution indices of each individual uncertainty and total uncertainty. The percentage of C_{HPLC} was the highest, which means that determination of the HPLC calibration curves is the variable that most contributes to the total uncertainty with 74.87%, followed by the recovery rate with 22.24%. In fact, to simplify the calculation, measurements of the smaller volumes (V_1 *and* V_3) could be ignored and the mass measurement (M) is absolutely insignificant and can be discarded.

Obtaining calibration curves and the recovery index of the pistachio samples constitute an arduous task of laboratory and statistical calculations. Nevertheless, it is very important to work on both to reduce the overall uncertainty.

Table 2
Calculated standard uncertainties.

Standard uncertainty	Value	Standard uncertainty	Value
u_M	$2.5422^{*}10^{-4}$ g	u_{V3}	0.029743398 ml
u_{V1}	$5.63353^{*}10^{-3}$ ml	u_R	$5.8380585^{*}10^{-2}$
u_{V2}	1.046607485 ml	u_{HPLC}	$1.87583^{*}10^{-10}$ g/ml

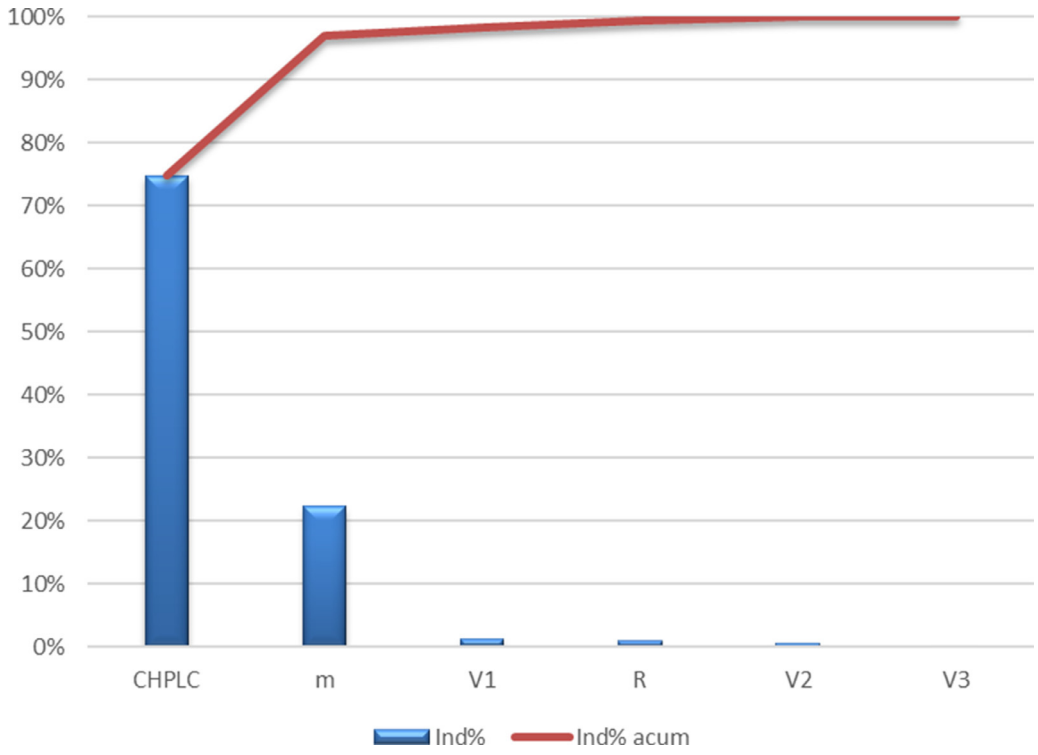

Fig. 2. Pareto diagram of the contributions of the analysis variables.

Table 3
Value of the sensitivity coefficients (C_i).

Variable	Corresponding equation	c_i
M	$c_M = \frac{dCx}{dM} = \frac{C_{HPLC}\times V_1\times V_2}{V_3\times M^2} \times \frac{1}{R}$	$4.0589^{*}10^{-11}$ g
V_1	$c_{V_1} = \frac{dC_x}{dV_1} = \frac{C_{HPLC}\times V_2}{V_3\times M} \times \frac{1}{R}$	$5.68246^{*}10^{-10}$ ml
V_2	$c_{V_2} = \frac{dC_x}{dV_2} = \frac{C_{HPLC}\times V_1}{V_3\times M} \times \frac{1}{R}$	$6.81896^{*}10^{-12}$ ml
V_3	$c_{V_3} = \frac{dC_x}{dV_3} = \frac{C_{HPLC}\times V_1\times V_2}{V_3^2\times M} \times \frac{1}{R}$	$1.70474^{*}10^{-10}$ ml
R	$c_R = \frac{dC_x}{dR} = \frac{c_{HPLC}\times V_1\times V_2}{V_3\times M} \times \frac{1}{R^2}$	$2.03429^{*}10^{-9}$
C_{HPLC}	$c_{HPLC} = \frac{dC_x}{dC_{HPLC}} = \frac{V_1\times V_2}{V_3\times M} \times \frac{1}{R}$	2.130923969 g/ml

Table 4
Percentage of the contribution index of uncertainties and total uncertainty.

Variable	Index%	Accumulated index %
C_{HPLC}	74.868	74.868
R	22.244	97.112
V_2	1.337	98.449
V_3	0.950	99.398
V_1	0.600	99.998
m	0.002	100.00

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Rodríguez Ana María: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft. **Gutiérrez Aida:** Formal analysis, Funding acquisition, Investigation, Software, Visualization, Writing – review & editing. **Giménez Myriam:** Methodology, Validation. **Martínez Nora:** Funding acquisition, Supervision, Validation, Visualization, Writing – original draft. **Mamaní Arminda:** Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Writing – review & editing.

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