## ARTICLE

Comparison of Ultrasound-assisted Methods for Copper Determination in Bovine and Ovine Liver as Strategies for Food Surveillance and Animal Status Monitoring

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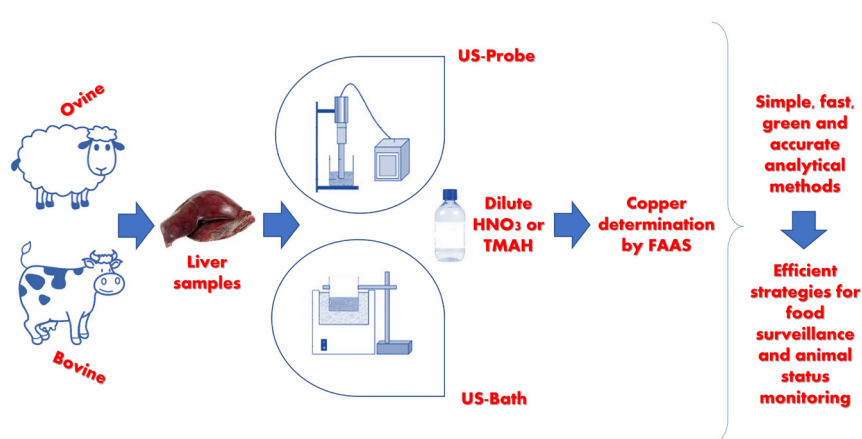
Four methods for the efficient extraction of copper from bovine and ovine liver were optimized. Sample preparation consisted of extractions with dilute nitric acid or dilute tetramethyl ammonium hydroxide, assisted by an ultrasonic bath or an ultrasonic probe. Copper was determined by flame atomic absorption spectrometry. The experimental conditions were optimized using multivariate experiments. All methods were considered adequate for copper extraction, however, the two methods involving the probe turned out to be more efficient and faster, so they were selected for subsequent validation. Trueness was verified after the analysis of a certified reference material and the performance of a microwave-assisted extraction. Results were statistically equivalent, at the 95% significance level, to the values declared on the certificate. Precision (expressed as relative standard deviation) was better than 5% for all methods. Samples obtained from Uruguayan animals were analyzed. Obtained results agreed with previous results from sheep and cattle abroad. The proposed methods are simple alternatives for food surveillance and animal status monitoring, being straightforward and aligned with Green Chemistry principles, as it was demonstrated by performing the analytical Eco-Scale comprehensive approach. A discussion related to the particle size distribution obtained during the multivariate experiments was also included, to give some deeper insight into ultrasound effect on the biological tissue in different media. In addition, ultrasound-assisted extraction was compared to magnetic stirring to prove the effect of ultrasound.

INTRODUCTION

One of the main trends in sample preparation is to seek for procedures that involve mild conditions and short times of extraction.[1] In this regard, many efficient techniques have been developed, particularly ultrasound-assisted extraction (UAE), that permit fast, reproducible, and quantitative analysis of trace elements from complex samples. UAE methods are very efficient alternatives for biological samples analysis that allow significant shortening of treatments with subsequent saving in energy, reduce solvents consumption, simplify manipulation, and involve safer procedures, thus being ecofriendly and low-cost strategies aligned with Green Chemistry principles.[2-5]

Ultrasonication can induce a physical phenomenon called cavitation, which accelerates extraction procedures, while inducing cell rupture and subsequently releasing encapsulated analytes.[4] Moreover, ultrasonication promotes several reactions that include the generation of oxidizing species and reactive radicals such as $OH^{\bullet}$ and H_2O_2 that favor the oxidation of organic matter.[6]

When cavitation bubbles implode on the surface of a given sample, micro-jets are generated, giving rise to different effects such as erosion, surface peeling, and particle breakdown. This fragmentation of the matrix results in a better contact with the extractant and an enhanced extraction yield of the analyte,[3] which is also favored by the vigorous mixing generated by the propagation of the ultrasound waves.[7]

Copper (Cu) is essential for the activity of several enzymes, cofactors, and reactive proteins in cattle. It is involved in reproduction, pigmentation, connective tissue development, cellular respiration, protection from oxidants, and iron transport.[8] Numerous naturally occurring diseases reported in grazing animals can be attributed to Cu deficiency. A broad range of Cu-responsive disorders are associated with low Cu status known as 'hypocuprosis', showing the importance of performing Cu assessments. When a given organ is responsible for the accumulation of a certain element, its weight is predictable, and it can be then biopsied. This occurs with the liver of ruminants, where Cu retention can be estimated from the rates of increase of the element concentration in this organ. Thus, enhanced Cu levels in the liver can give an estimation of the true absorption of this element in both cattle and sheep.[9] On the other hand, chronic Cu accumulation may occur, depending on its absorption rate from a supplement or feed, which also depends on the interactions with other constituents of the ration, especially sulfur (S), molybdenum (Mo) and iron (Fe).[9]

When it comes to the food science field, meat and its derivatives constitute a very important part of the human diet, providing essential nutrients that cannot be easily obtained from vegetable sources. Furthermore, organs such as liver, are even richer in minerals and vitamins when compared to other muscular tissues, being more adequate sources to meet the nutritional requirements of human beings.[10] In this context, the evaluation of an essential element such as Cu, constitutes a highly valuable input to assess the food quality of this product.

Most conventional UAE-based methods involve extractions that use dilute acids with oxidizing properties such as nitric acid (HNO_3).[11] However, an alternative approach for sample preparation of biological tissues is the use of strong alkaline reagents such as tetramethylammonium hydroxide (TMAH). This compound has been used as a 'tissue solubilizer' for various biological samples prior to analysis of Cu by flame atomic absorption spectrometry (FAAS).[12,13] Working with TMAH offers an efficient approach to the preparation of homogenized biological sample solutions, under mild conditions. Besides, only a small amount of TMAH is used, becoming an attractive alternative for the treatment of protein-rich samples. Additionally, its efficiency can be increased by using ultrasound energy.[14,15]

In this context, the aim of this work was the optimization and validation of simple and fast analytical methods for Cu extraction from bovine and ovine liver by means of UAE, using a bath or a probe and employing either dilute HNO_3 or dilute TMAH as extraction solvents, with subsequent determination by FAAS. Also, to compare the efficiency of the selected UAE methods against a microwave-assisted extraction (MAE) method in terms of greenness, reassuring the advantage of ultrasound over standard methods. Furthermore, a discussion related to the particle size distribution was also included, to give some

deeper insight into ultrasound effect over the sample in the different media assayed. The novelty of the work is related to the development of green, straightforward, and accurate strategies that can be easily implemented for both food surveillance and animal status monitoring. To the best of our knowledge, scarce information is available on the use of ultrasound technology for Cu extraction from bovine and ovine tissue samples, specially employing TMAH solubilization.

MATERIALS AND METHODS
Reagents

For alkaline extractions, dilute TMAH was prepared from 25% w w^{-1} TMAH in methanol (Sigma Aldrich, St. Louis, MO, USA). For acid extractions, dilute HNO_3 was prepared from 67% w w^{-1} HNO_3 (Merck, Darmstadt, Germany). Ultrapure water (18.2 MΩ cm resistivity) was obtained from a Millipore™ DirectQ3 UV water purification system (Bedford, MA, USA). Standard solutions for calibration curves were prepared by serial dilution of a 1000 mg L^{-1} commercial atomic absorption solution of Cu (Fluka, Hauppauge, NY, USA), using the same final HNO_3 or TMAH concentration as in the samples.

A bovine liver reference material (RM) RM-Agro E3001a (Embrapa, São Carlos, SP, Brazil) was used for optimization experiments while a bovine liver certified reference material (CRM) NIST 1577c (Gaithersburg, MD, USA) was employed for trueness and precision evaluation of the selected analytical method.

All other reagents were of analytical grade. All glassware was decontaminated with 1.5 mol L^{-1} HNO_3 before its use.

Samples

Bovine and ovine liver samples were provided by a local slaughterhouse, all coming from young animals (12 – 24 months). Samples were washed with deionized water, triturated, and homogenized using a food blender with stainless steel blades, and finally stored in polypropylene tubes at -18 °C until analysis.

Sample preparation procedures
Microwave-assisted extraction

For the total determinations of Cu, a microwave-assisted acid digestion (MAE) was performed, using a CEM Mars 6 microwave digestor (Matthews, NC, USA) equipped with 12 Easy Prep Plus™ vessels. Each vessel was charged with 0.25 g of sample and 10.0 mL of 2.8 mol L^{-1} HNO_3. The power of the digestor varied between 400 – 1800 W. After reaching 200 °C in 15 min, the program was maintained at this temperature for 10 min. The maximum pressure was set at 500 psi. Obtained solutions were used for analytical determinations without further dilution. Treatments were run in triplicate. Reagent blanks were carried out.

UAE with probe (UAE-probe)

The treatment was performed using a Sonics® Vibra-Cell™ VC505 (Newtown, CT, USA) ultrasonic homogenizer equipped with a 13-mm titanium alloy probe (750 W, 20 kHz, 230 V$_{AC}$). A 50-mL flask was charged with 0.25 g of sample and 10.0 mL of 2.8 mol L^{-1} HNO_3 or 0.36 mol L^{-1} TMAH, and the probe was immersed 2 cm inside the mixture for 10 min at a sonication amplitude of 35%. The obtained suspension was filtered through a 0.45 µm pore size nitrocellulose filter (Millipore™, Bedford, MA, USA) and transferred to a 50-mL polypropylene tube. The filtrate solution was used for analytical determinations without further dilution. The procedure was run in triplicate. Reagent blanks were carried out.

UAE with bath (UAE-bath)

Samples were prepared in a Cole-Parmer® 8893 (Vernon Hills, IL, USA) ultrasonic bath (47 kHz, 230 V$_{AC}$). A 50-mL flask was charged with 0.25 g of sample and 10.0 mL of 2.8 mol L^{-1} HNO_3 or 0.36 mol L^{-1} TMAH, and then the vessel was immersed in the bath for 20 min. The obtained suspension was filtered through a 0.45 µm pore size nitrocellulose filter (Millipore™, Bedford, MA, USA) and transferred to a 50-mL

polypropylene tube. The filtrate solution was used for analytical determinations without further dilution. The procedure was run in triplicate. Reagent blanks were carried out. Samples were located in the middle part of the bath (12 cm from the front and 15 cm from the sides) since for baths fitted with a single transducer, the maximum ultrasonic intensity is found above the transducer.

Magnetic stirring extraction

A silent condition using magnetic stirring was performed to establish whether the observed effects were in fact due to ultrasound energy and not due to other parameters. Samples were prepared using a Thermolyne Cimarec® 2 (Dubuque, IA, USA) magnetic stirrer. A 50-mL flask was charged with 0.25 g of sample and 10.0 mL of 2.8 mol L^{-1} HNO$_3$ or 0.36 mol L^{-1} TMAH, and the vessel was put over the stirrer for 10 min (HNO$_3$) or 20 min (TMAH) and stirred at 500 rpm. The obtained suspension was filtered through a 0.45 µm pore size nitrocellulose filter (Millipore™, Bedford, MA, USA) and used for analytical determinations without further dilution. The procedure was carried out in triplicate.

Analytical determinations

Copper was determined by FAAS using a Perkin Elmer AAnalyst 200 spectrometer (Norwalk, CT, USA) with a 10-cm burner and operated at 324.75 nm. A Lumina™ lamp from Perkin Elmer (Norwalk, CT, USA) was used. The flame was composed by acetylene (2.8 L min^{-1}) and air (10.2 L min^{-1}). Acetylene 99.5% was purchased from Praxair (Montevideo, Uruguay). Calibration curves were prepared at the same TMAH or HNO$_3$ concentration used in the samples.

Optimization of extraction conditions

Multivariate experiments were used for the optimization of UAE-based methods. A two-factor three-level (3^2) factorial design was employed to determine the best extraction conditions.[16] The two variables studied were HNO$_3$ or TMAH concentration and sonication time. The evaluated response was the average recovery R (%), by comparison with the informed value of the bovine liver RM (Table I). Additionally, it was verified by using analysis of variance (ANOVA).

Table I. Experimental conditions of the multivariate experimental design applied to UAE-based methods, using the bovine liver RM

Experiment	HNO$_3$ Concentration (mol L^{-1})	TMAH Concentration (mol L^{-1})	Time (min) Probe & Bath
1	2.8	0.36	15
2	1.4	0.24	10
3	4.2	0.48	10
4	1.4	0.24	15
5	2.8	0.36	10
6	4.2	0.48	15
7	2.8	0.36	20
8	1.4	0.24	20
9	4.2	0.48	20

Method validation

After the critical variables were optimized, the validation was performed according to the recommendations of the Eurachem Guide.[17] The figures of merit evaluated were precision, trueness, linear range, limit of detection (LOD), and limit of quantification (LOQ).

Particle size analysis

The particle size distribution of residues obtained after different sonication procedures was evaluated with a Coulter™ LS230 (Brea, CA, USA) laser diffraction analyzer, in the range 0.04 – 2000 μm, using a laser light with a wavelength of 750 nm. Samples were dispersed in distilled water prior to its introduction into the fluid module. The Fraunhofer mathematical model was selected to calculate the particle size based on the concentric ring pattern of light-scatter from the laser beam. The amount of sample needed was determined by the obscuration percentage of the laser beam passing through the sample cell, between 8 and 12%.

RESULTS AND DISCUSSION

Optimization experiments

The optimal conditions for UAE-based methods were selected after the evaluation of the per cent recovery estimated as R (%) = [obtained concentration (mg kg^{-1})/informed concentration (mg kg^{-1})] × 100, for each experiment. Simplest and fastest conditions with an R (%) statistically equal to 100% were chosen for subsequent validation.

Table II shows the R (%) values obtained for each experiment. The best results for UAE-probe, either for the acid or the alkaline medium, were those obtained under conditions of Experiment 5, allowing a faster, with less amount of concentrated acid and quantitative extraction procedure. However, for the UAE-bath, longer sonication times were needed using the same concentration of HNO$_3$ or TMAH employed in Experiment 5, for a quantitative extraction, as shown in recovery values obtained for Experiment 7.

Additionally, an ANOVA analysis was performed. Since the calculated F values were much greater (>20) than the critical F value of 4.066 (P = 0.05), the null hypothesis was rejected, so, the sample means differed significantly. Means were arranged in increasing order and the difference between adjacent values compared to the least significant difference. Results showed that the means obtained in experiments (5 and 7) did not differ significantly from the informed value of the RM, also demonstrated by a Student's *t*-test, being the experimental *t*-value = 1.67, below the theoretical *t* (0.05, 5) = 2.57.

Table II. Recovery results of the multivariate experimental design applied to UAE-based methods, using the bovine liver RM

Experiment	R (%) – HNO$_3$		R (%) – TMAH	
	Probe	**Bath**	**Probe**	**Bath**
1	99 ± 3	90 ± 2	99 ± 2	91 ± 4
2	93 ± 4	84 ± 4	92 ± 5	80 ± 3
3	98 ± 3	89 ± 5	93 ± 2	85 ± 3
4	95 ± 2	88 ± 3	93 ± 4	81 ± 4
5	100 ± 3	89 ± 4	101 ± 2	86 ± 5
6	99 ± 2	93 ± 3	100 ± 2	90 ± 4
7	101 ± 1	99 ± 2	100 ± 1	99 ± 3
8	95 ± 2	89 ± 3	94 ± 1	89 ± 2
9	100 ± 2	99 ± 1	100 ± 3	99 ± 2

Expressed as: Mean ± standard deviation (n = 3).

Finally, the optimum conditions for the proposed UAE methods turned out to be: 0.25 g of sample and 10.0 mL of 2.8 mol L^{-1} HNO$_3$ or 0.36 mol L^{-1} TMAH placed in a flask, and then sonicated for 10 min (probe) or 20 min (bath). Afterwards, the suspensions were filtered using a 0.45 μm pore membrane, and the obtained solutions used without further dilution for Cu determinations.

The addition of water when performing the alkaline extraction method was necessary to moisturize the biological tissue and promote the efficient penetration of TMAH. The hydration allowed this reagent to easily penetrate the protein matrix, dissolve it, and release Cu to the soluble phase.[13,18]

Considering that the purpose of the work was to develop green alternatives for the rapid diagnosis of animal status, the most straightforward methods able to give answers in a short period of time were highly desired. Thus, UAE-probe methods were selected for subsequent validation, according to the much shorter sonication time required for Cu quantitative extraction.

Effect of ultrasound on particle size

The impact of fragmentation was studied using the probe (20 kHz) and the bath (47 kHz). A fast fragmentation of liver samples was noticed after a few minutes of probe-based sonication, while this effect was less pronounced when the ultrasonic bath was used. As a matter of fact, a quantitative extraction was achieved in 10 min when using the probe, whereas the double of time was needed when using the bath.

Residues were collected after filtration and the particle size distribution was measured. When the probe was used, a remarkable decrease in particle size with increasing sonication time was observed, changing from 250 µm in the control sample to 130 µm (2.8 mol L^{-1} HNO_3)/90 µm (0.36 mol L^{-1} TMAH) after 10 min, leading to nearly complete solubilization of the sample after 20 min. This phenomenon is associated to inter-particle collisions and shockwaves created by collapsing cavitation bubbles in the liquid, as mentioned before. A direct consequence of this reduction in particle size is the increase of the surface area of the solid, which leads to a more pronounced mass transfer and a higher extraction yield.[1] When the bath was used, a slower decrease in particle size with the increment of sonication time was observed, changing from 250 µm in the control sample to 190 µm (2.8 mol L^{-1} HNO_3)/135 µm (0.36 mol L^{-1} TMAH) after 20 min. The differences found between UAE-probe and UAE-bath are probably due to a faster reduction of the particle size caused by a more intimate contact when using the probe. Additionally, the differences found between the acid and the alkaline medium agrees with the 'tissue solubilizer' capacity assigned to TMAH.

UAE of trace elements from biological matrices involves several effects such as fragmentation, erosion, and detexturation, most likely acting in combination. Furthermore, the transfer of elements to the liquid phase is favored by the intense mixing caused by the propagation of ultrasound waves. These physical effects lead to the higher efficiency achieved with UAE.

All UAE-based methods described here in this work were suitable for the quantitative extraction of Cu from bovine and ovine liver samples. The difference in optimum sonication times between the probe and the bath is logical, considering the higher output power of the probe, that provides more energy in the sinus of the suspension when compared to the bath. The larger remaining amount of residue and the bigger particle size obtained when working with the ultrasonic bath, leaded to a more difficult filtration process. This fact was also considered when selecting UAE-probe methods for subsequent validation.

In addition, to prove the real effect of the ultrasound over the extraction, it was necessary to perform another round of experiments using magnetic stirring. These experiments were important to assure that ultrasound energy truly influenced the proposed process. Recovery results obtained for Cu after performing the sample preparation procedure described as 'silent condition' were 27 ± 1% (HNO_3) and 24 ± 2% (TMAH), expressed as: mean ± standard deviation (n = 3). This shows that, although magnetic stirring can extract part of the analyte from the matrix, this extraction is far from being quantitative. So, the effect of ultrasound over the extraction was assured, after comparing with the quantitative recoveries observed for experiment 5 and experiment 7 in Table II.

Methods validation: MAE vs. UAE-probe

MAE was employed for total sample digestion. This method was validated for Cu determinations following the suggestions of the Eurachem Guide.[17] Results are shown in Table III. A Student's *t*-test was performed for trueness evaluation, to prove if there were statistical differences between the experimental values and the certified value of the CRM, which showed no differences at the 95 % confidence level.[19]

Repeatability expressed as RSD (%) after the analysis of the CRM (n = 6), was less than 5%. Thus, the method was considered accurate. The LOQ expressed as 10σ (n = 10, fresh weight) was 1.1 mg kg^{-1}.

Table III. Figures of merit obtained for the MAE and the developed UAE methods

Parameter	MAE	UAE-Probe	
		HNO$_3$	TMAH
Linear range (mg kg^{-1})	0.03 – 6.0	0.03 – 6.0	0.04 – 6.0
LOD (mg kg^{-1})	0.01[a]/0.3[b]	0.01[a]/0.9[b]	0.01[a]/1.3[b]
LOQ (mg kg^{-1})	0.03[a]/1.1[b]	0,03[a]/1.1[b]	0.04[a]/1.6[b]
Precision, RSD (%) [c]	3.8	4.5	4.0
Trueness, R (%)[c]	100	102	101
$t_{experimental}$ [c]	0.31	0.96	0.46
t (0.05, 5)	2.57	2.57	2.57

(a) Instrumental LOD and LOQ; (b) LOD and LOQ expressed in sample, fresh weight; (c) CRM NIST 1577c.

UAE-probe methods were also validated following Eurachem Guide recommendations.[17] Obtained figures of merit are shown in Table III. Six-point calibration curves were constructed to evaluate linearity. Good linearity was observed with determination coefficients (R^2) better than 0.9999. Linear range was up to 6.0 mg kg^{-1}. Individual residuals were studied, and their random distribution verified. The obtained LOQ expressed as 10σ (n = 10, fresh weight) were 1.1 and 1.6 mg kg^{-1}, respectively, being one to two orders below the expected Cu levels in liver samples. Repeatability expressed as RSD (%) after the analysis of the CRM (n = 6) was less than 5%, which was considered suitable for this application, according to the Horwitz theory related to variability at trace levels (RSD < 30%).[20] A Student's t test was performed for trueness evaluation, to compare the obtained values with the certified one. Results are shown in Table III. Experimental t values were below the theoretical t (0.05, 5) = 2.57, indicating that concentrations did not differ significantly from the certified value at the 95% confidence level. These figures of merit demonstrate that the developed methods were accurate for Cu determination in liver samples.

Since MAE is widely employed for elemental determinations in complex matrices, and prior validation confirmed its reliability, the values obtained using this method were considered as reference values for the analyzed real samples. Thus, the performance of the newly developed UAE methods was statistically evaluated by comparing the results obtained after the analysis of real bovine and ovine liver samples with the results obtained by MAE. A Student's t test was employed for this task as shown in Table IV. Experimental t values were below the respective theoretical t (0.05, 5) = 2.57 demonstrating that, at the 95% significance level, the results obtained using either UAE-probe or MAE were statistically equivalent. Thus, the proposed methods were considered adequate for the analysis of Cu in bovine and ovine liver samples.

Table IV. Comparison between the developed UAE and MAE methods by Student's *t* test

	MAE		UAE-Probe (HNO$_3$)		UAE-Probe (TMAH)	
Sample	Bovine	Ovine	Bovine	Ovine	Bovine	Ovine
Average	62.5 ± 8.1	105 ± 12	60.9 ± 7.9	108 ± 14	65.8 ± 7.5	104 ± 11
t experimental	---	---	-0.37	0.20	0.77	-0.12
t (0.05, 5)	---	---	2.57	2.57	2.57	2.57

Expressed as: Mean ± standard deviation (n = 6) (mg kg^{-1}).

It is important to highlight once again at this point what has been previously described in the specialized literature on UAE methods that clearly proved its efficiency, allowing a significant decrease in extraction solvent concentration, operation time and harsh reaction conditions.[1-5] UAE makes possible the use of less acid concentration, when compared to MAE, while allowing extractions at room temperature under atmospheric pressure, instead of working at high pressure in closed reaction vessels, thus involving safer procedures.

Application to bovine and ovine liver samples

A total of 6 bovine and 6 ovine liver samples from different animals were analyzed. Copper concentrations found in bovine liver samples ranged from 58.8 ± 3.3 to 82.7 ± 3.9 mg kg^{-1} (mean ± standard deviation, fresh weight), while those found in ovine liver samples ranged from 66.2 ± 3.1 to 159.8 ± 4.3 mg kg^{-1} (mean ± standard deviation, fresh weight). The obtained Cu levels agree with those previously reported in Australian[21] and Norwegian[22] sheep, and Spanish cows[23].

The presented information is relevant, since Cu is key to the enzymatic systems, cofactors, and reactive proteins, while its deficiency is related to the development of several disorders as previously stated. Copper levels in the liver can be used to estimate deficiencies and/or poisoning to sheep and cattle. None of the animals studied in this work presented Cu concentrations in the liver below 2 mg kg^{-1} (fresh weight), a level considered indicative of Cu responsive disorder in ruminants.[9] Also, none of the samples exceeded 200 mg kg^{-1} (fresh weight), a level indicative of toxicity. Based on liver Cu concentrations found, the results suggest that sheep and cattle in Uruguay are well-managed with respect to Cu supplementation. However, it is important to perform this sort of studies, since some isolated cases of chronic Cu poisoning have been reported in sheep in our country.[24]

Taking into consideration the contribution of Cu to human diet, an intake of 50 g of liver would provide between 2.9 – 4.1 mg (bovine) and 3.3 – 8.0 mg (ovine). These values would broadly cover the recommended dietary allowance (RDA) of this essential element, that has been established by the National Institutes of Health (NIH) in 0.9 mg/day for adults over 19 years, without reaching the tolerable upper intake level (UL) of 10 mg/day, a value based on protection from human liver damage as the most critical adverse effect.[25]

Analytical Eco-Scale

To evaluate the greenness of the developed UAE methods, the analytical Eco-Scale comprehensive approach was performed, which is a good semi-quantitative tool that can be considered as an alternative to traditional Green Chemistry metrics.[26] It is based on assigning penalty points to certain parameters of the analytical process, namely, amount of reagents, hazards, energy and waste, that do not agree with an ideal green analysis. The sum of these points for the whole procedure must be included in the Eco-Scale calculation, according to the following formula: Analytical Eco-Scale score = 100 – total penalty points. The result of the calculation is then ranked on a scale. If the score is >75 it represents an excellent green analysis, if the score is >50 it represents an acceptable green analysis, and if the score is <50 it represents an inadequate green analysis.[26]

Results of the analytical Eco-Scale applied to the methods developed here in this work are shown in Table V. As it can be observed, the three methods are equivalent in terms of greenness and all of them pose a score over 75, representing excellent green strategies according to this approach. However, the UAE-probe method using dilute HNO_3 can be considered a slightly greener alternative.

Table V. Penalty points for Cu determination by developed UAE-probe and MAE methods and Eco-Scale total scores

	Method		
	UAE-Probe (TMAH)	**UAE-Probe (HNO$_3$)**	**MAE (HNO$_3$)**
	Penalty points		
Reagents			
TMAH or HNO$_3$ (Amount × Hazard)	6	4	4
Cu standard solutions	3	3	3
Acetylene	3	3	3
Instruments			
Probe or MW (energy)	1	1	2
Spectrometer (energy)	2	2	2
Occupational hazard (vapors)	3	3	3
Waste (Amount + Treatment)	6	6	6
Total penalty points	24	22	23
Analytical Eco-Scale score	76	78	77

CONCLUSIONS

Four efficient methods for the extraction and subsequent determination of Cu in bovine and ovine liver samples were optimized. Ultrasound-assisted methods were performed using dilute HNO_3 and dilute TMAH and without the need of external heating, thus being aligned with the postulates of Green Chemistry and can accordingly be proposed as simple and economical methods for animal monitoring, as well as for food control purposes. However, the probe-based procedures were selected for validation since they were more efficient alternatives. The greenness of the newly developed methods was assured by performing the analytical Eco-Scale approach, that showed the excellency of green analysis.

Also, it was once again highlighted the efficiency of UAE, as well as its main advantages, such as decrease in acid concentration, operation time and harsh reaction conditions, when compared to MAE. The research group will continue working on the decrease of solvent concentration, working under milder and greener conditions.

Regarding Cu levels in Uruguayan samples, results agreed with previous values informed in sheep and cattle abroad. Besides, Cu concentrations lied between the minimum level indicative of Cu responsive disorder and the maximum level indicative of Cu toxicity, suggesting that sheep and cattle in Uruguay are well-managed with respect to Cu supplementation. Furthermore, the intake of 50 g of liver would completely cover the RDA of Cu for adults over 19 years, according to data provided by the NIH. This constitutes novel information since there is scarce data available on this matter in academic search engines.

Conflict of interest
The authors declare that there is no conflict of interest regarding the publication of this article.

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