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Quantitative analysis of minor and trace elements in blood serum of dairy cattle (*Bos taurus*) by WDXRF

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ABSTRACT

Assessment of heavy metal content in biological fluids is essential to determine human and animal health due to exposure to contaminated environments, food and water. WDXRF is a non-destructive method that involves no chemical treatments. It is possible to perform fast chemical analysis without producing hazardous waste and uses a small sample volume (± 50 μL), which is helpful for veterinary usage. Therefore, this work aimed to implement an alternative method for minor and trace elements in blood serum samples of dairy cattle (*Bos taurus*) using WDXRF. It was implemented to determine Al, Cr, Co, Fe, Cu, and As. The method was evaluated by comparing WDXRF and ICP-OES results of a set of 14 blood serum samples of dairy cattle using principal component analysis (PCA). Results of WDXRF and ICP-OES were statistically equivalent to all analyzed elements, except for Fe and Al, which were still comparable. WDXRF proved to be a suitable alternative methodology for heavy metal detection in animal blood serum samples due to the low sample volume. The results of this study enable a new and fast method for veterinary internal medicine, which detects elevated levels of toxic elements in animal blood samples to ensure the correct protocols to prevent the animal's death.

Keywords: **X-ray, Heavy metal, Bovine, Detection.**

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1. INTRODUCTION

Elements content in biological fluids, especially heavy metals (such as Al, Cr, Co, Fe, Cu, As, Hg, Pb, etc.), are an indicator that enables evaluating animal and human health as a consequence of exposure to contaminated environments. Quantitatively, many analytical techniques are used to determine these and other potentially toxic elements in this context. Among the most used methods, Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-OES), Atomic Absorption Spectroscopy (AAS), and Neutron Activation Analysis (AAN), among others, stand out. The techniques mentioned have broadly satisfactory sensitivity and reliability [1] and are internationally recognized. However, regarding disadvantages, the first two require prior sample preparation: chemical reagents are essential, thus producing residues [2]. In addition, the last method needs a nuclear reactor, which is usually inaccessible to routine laboratories. X-ray fluorescence (XRF) is an analytical tool that detects heavy metals in several sample types, including human blood [15,16].

Wavelength Dispersion X-ray Fluorescence (WDXRF) is one of the most used techniques [3], and it is an alternative. It applies procedures that do not require prior chemical treatments to prepare samples, thus preserving the integrity of the samples [4]. Its main disadvantages are related to the method's sensitivity. Depending on the specimen analyzed, it is insufficient to guarantee possible interference due to interelement effects (matrix and/or overlap).

Veterinary diagnosis often suffers due to the low sample volume of rare or tiny species. Moreover, quick and easy-to-get samples, such as blood serum and clinical manifestations, are essential for diagnosing acute poisoning in animals and monitoring their health after environmental contamination. Given the background, fast, reliable, and non-destructive diagnostic tools are crucial when readiness for results and quick response are necessary.

Here, we present a procedure that minimizes the matrix effects and reaches quantification limits sufficiently satisfactory for determining Al, Cr, Co, Fe, Cu, and As in the blood serum of dairy cattle. The results obtained by the proposed methodology were in agreement with those obtained by ICP-OES for a set of 14 samples, demonstrating the reliability and validity of the method. Therefore, this research aimed to implement an alternative methodology for the simultaneous detection of minor and trace elements in the blood serum of dairy cattle (*Bos taurus*) by WDXRF to be introduced in the veterinary diagnostic routine.

2. MATERIALS AND METHODS

This research was conducted under the Ethics Committee on the use of animals at the School of Veterinary Medicine and Animal Science, the University of São Paulo (protocol # 6424070217). Nine milliliters of blood from 14 dairy cattle were collected by brachial or coccygeal vein punction, respectively, using a dry tube (Trace Metals; BD, USA). Serum samples were obtained according to the Early Detection Research Network [5]. The serum was transferred to microtubes and stored at -20ºC until further processing.

2.1. WDXRF

The analysis of Al, Cr, Co, Fe, Cu, and As in serum samples was analyzed using the "Droplet Method" [4] using a WDXRF spectrometer (Supermini200, Rigaku Co, Japan). Briefly, 50 µl of each element was deposited in the center of a paper filter (MicroCarry, Rigaku, Japan) and dried at room temperature. The MicroCarry filters are essential to standardize the matrix for any liquid sample, preventing the interelement effect. The X-ray equipment had an X-ray tube with a Pd anode and a maximum acceleration of 50 kV. Parameters such as the emission line, the diffraction crystal, the detector, and Bragg's positions are described in Table 1.

Table 1: Instrumental conditions for analyzing minor and trace elements in the WDXRF equipment, model Supermini200 (Rigaku, Japan).

Emission Line	**Bragg's Position**			**Diffraction Crystal**	**Detector**	**PHA**
	Peak (º)	BG1(º)	BG2(º)			
Al-Kα	144.610	***	***	PET	FPC	100-300
Cr-Kα	69.330	***	***			
Fe-Kα	57.500	***	***			
Co-Kα	52.770	***	***			
Cu-Kα	45.010	***	***	LiF (200)	SC	
As-Kβ1	30.430	30.180	30.660			

BG: Background; PET: Pentaerythritol; LiF: Lithium Fluoride; FPC: Gas-flow Proportional Counter; SC: Scintillation Detector; PHA: Pulse Height Analyzer

A calibration curve was established by analysis of four different volumes (10, 20, 30 e 50 µl, corresponding to 10, 20, 30, and 50 mg/L, respectively) of each standard solution of Al, Cr, Co, Fe, Cu, and As (1000 mgL^{-1}; Merck, Germany) using the "Droplet Method". Each concentration was tested in triplicates. The standard curves for each element were constructed in terms of intensity versus concentration using the linear regression test and adjusted by the least-squares regression. Each curve was used for calibration and sample measurement. Fifty microliters of Milli Q water were also analyzed using the "Droplet Method," and values were used as blank. A paper filter was also used as a blank to help analyze the results, whose values were subtracted from the determined values to ensure the correct results. The limit of detection (LD) and quantification (LQ) for WDXRF was calculated. LD and LQ were estimated as three times and ten times the standard deviation, respectively.

2.2. ICP-OES

The analysis of Al, Cr, Co, Fe, Cu, and As was performed using a Spectro ARCOS-ICP optical emission spectrometer (Spectro Analytical Instruments Co, Kleve, Germany); equipped with an axially viewed plasma. Instrumental parameters selected were as follows: 1400 W RF power, 12 L min^{-1} plasma flow, 1 L min^{-1} auxiliary flow, and 1 L min^{-1} nebulizer flow. The analysis was performed using three replicates. The sample introduction system occurred by a cross-flow nebulizer and a Scott double-pass. The calibration curves were constructed using a standard solution of 1000 mg/L: AccuStandard® (Al and Cu) and Merk® (Cr, Co, Fe, and As). Five elements' concentrations were specified: 0.05, 0.10, 0.20, 0.50, 1.0 and 2.0 mg/L. The serum samples were analyzed directly, i.e., without previous chemical treatments. In instrumental conditions established, about two milliliters of each specimen were measured. The concentration of the mentioned elements was obtained by interpolating the count rate (cps) in the respective calibration curves. The procedure followed the ISO 17025:2017 standard protocol.

2.3. Data analysis

Results from WDXRF were compared with the ICP-OES using principal component analysis (PCA). A matrix was constructed considering 14 cattle serum samples (C1-C14) as variables and the analytical results obtained by WDXRF and ICP-OES as cases. Results were plotted in a 2D-graph with the more significant weigh factors using the factoextra R package (v. 1.0.7) (https://cran.r-project.org/web/packages/factoextra/index.html). RSD was compared using the t-test. All analyses were performed using RStudio v. 4.0.4 [6].

3. RESULTS AND DISCUSSION

WDXRF and ICP-OES results were statistically equal to all analyzed elements except Fe and Al. The PCA analysis showed two factors describing the correlation between WDXRF and ICP-OES (factor 1: 90.3%; factor 2: 6.0%). The ICP-OES set intersected with the WDXRF set (Figure 1), demonstrating homogeneity between the sets. In addition, two samples were excluded from the crossed groups (Al_WD and Fe_ICP), indicating probable outliers. Nevertheless, Al and Fe results are still comparable since they are in the same order of magnitude.

Fe and Al may be easily found in the environment. Still, this interference was eliminated by cleaning the laboratory bench using 70% alcohol. In addition, a blank (paper filter with no reagent) was analyzed by WDXRF, revealing the following determined values in mg/L (Al <1.4, Cr <0.4, Fe <0.5, Co <0.2, Cu = <0,3 and As <4.1). Thus, we hypothesize that there may be contamination by red blood cells while obtaining the serum, which increases the Fe concentration. A pre-concentration of Fe may be occurred due to the greater sample volume used in the ICP protocol. At the same time, Al is also part of the paper filter, which may interfere with the results. In addition, the sensitivity of the WDXRF technique to light elements and the fact that the determined value was close to the LD may also explain the Al_WD result.

Due to their clinical importance, iron, copper, and cobalt concentrations are commonly evaluated in ruminants. In cattle, normal iron and copper serum levels are 2.06 to 4.12 mg/L [7] and 1.08 to 1.26 mg/L [8], respectively. The cobalt concentrations in the serum of healthy sheep range from 0.01 to 0.05 mg/L [7]. In addition, average serum concentrations of healthy cattle have been determined for Cr (0.035 to 0.04 µg/L) [17]. No ranges are available for arsenic and aluminum in the blood serum of ruminants, but studies have been conducted to determine the concentration of both elements in

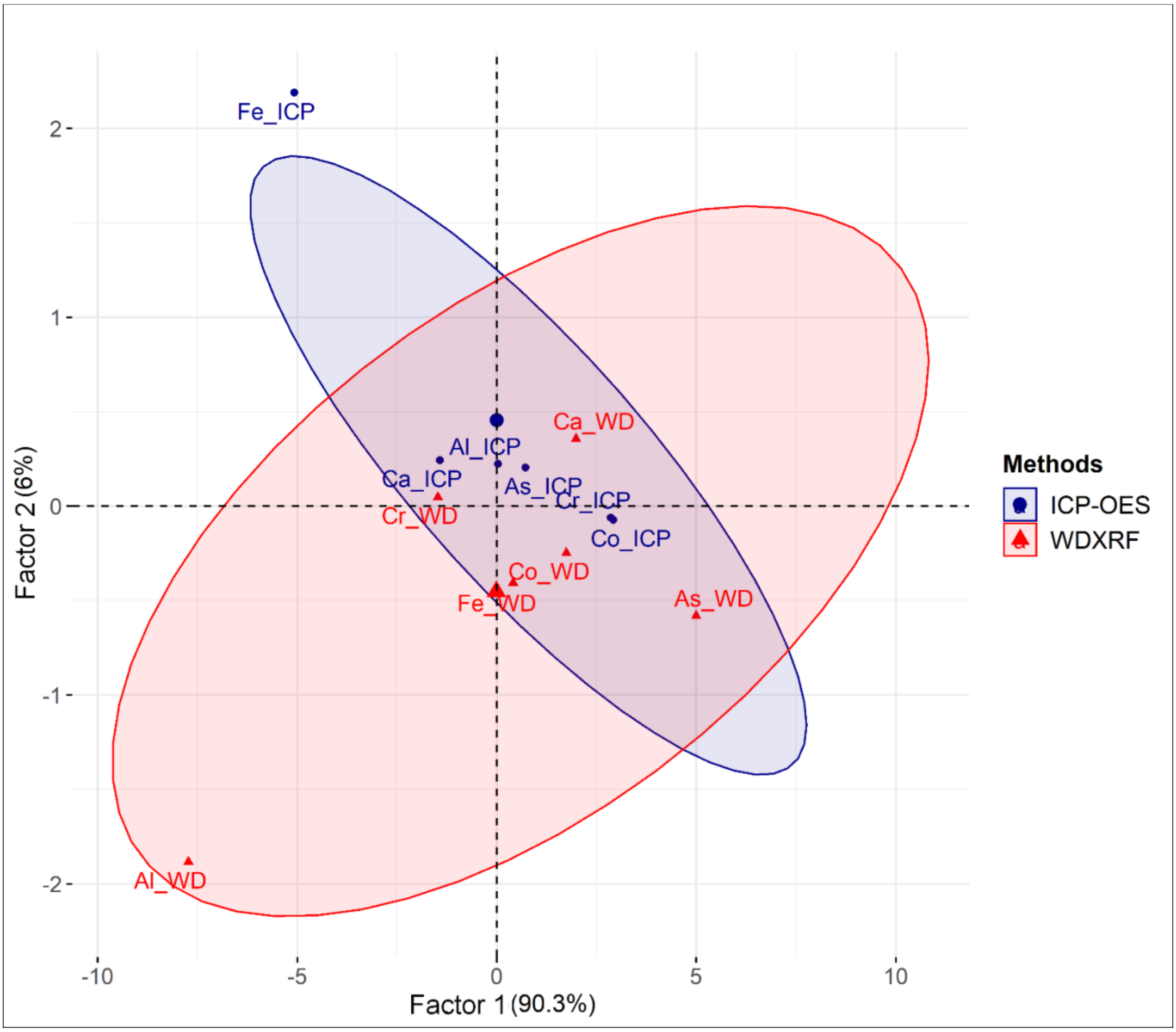

Figure 1: *Principal Component Analysis (PCA) showing the intersection of WDXRF and ICP-OES results. The data are mainly explained by factor 1, and Al_WD and Fe_ICP are probably outliers.*

these species. Healthy cattle showed serum concentrations of arsenic and aluminum between 0.002±0.001 mg/L and 0.005±0.003 mg/L and 0.277 and 1.567 mg/L, respectively, in Poland [18]. The detection limits of WDXRF compared to the ICP-OES were calculated (Table 2), and the WDXRF was trustworthy to be used in daily diagnosis, particularly in detecting a high concentration of these elements, which is usually associated with animal toxicosis [12, 13].

Table 2: Mean determined value (mg/L), percentage relative standard deviation (RSD), the limit of detection (LD), and limit of quantification (LQ) for each element by WDXRF and ICP-OES analysis.

Element	WDXRF	RSD	LD	LQ	ICP-OES	RSD	LD	LQ
Al	1.7	26	1.5	4.6	0.44	10	0.14	0.5
Cr	0.7	20	0.4	1.4	0.007	19	0.004	0.01
Fe	< 0.5	43	0.5	1.7	1.2	21	0.8	2.6
Co	0.2	41	0.2	0.7	0.002	42	0.002	0.01
Cu	< 0.4	76	0.4	1.1	0.7	26	0.5	1.7
As	< 3.8	---	3.8	13.8	0.33	9	0.09	0.3

The WDXRF technique has been extensively used for geological, metallic, and other sample analyses but less for biological purposes, where its usage has been expanded and grown substantially [11, 19-20]. The WDXRF has been lowly used for diagnostics in veterinary medicine, but fortunately, this study showed that it is possible to use this technique in this area successfully. In terms of precision, the WDXRF_RSD and ICP_RSD were compared by the t-Student test (95% confidence level). The results showed statistical equality for the determination of Al, Cr, Fe, Co, Cu, and As, then $t_{\text{critical}} < t_{\text{calculated}}$ ($t_{\text{calculated}} = 1.83$; $t_{\text{critical}} = 2.45$). Regarding LD and LQ, the results were also statistically equal according to the t-Student test when LD: ($t_{\text{calculated}} = 1.42$ and $t_{\text{critical}} = 2.57$), and LQ: ($t_{\text{calculated}} = 1.44$ and $t_{\text{critical}} = 2.57$) ($P < 0.05$). Therefore, the results demonstrated that the WDXRF is an alternative analytical technique that is not destructive and produces little chemical waste [9, 10]. In addition, the low sample volume required for WDXRF allows its wide use in veterinary medicine, given the insufficient sample volume of many species, such as birds, bats and other small mammals.

4. CONCLUSION

The intersection of both WDXRF and ICP-OES data and the statistically comparable results, in terms of precision, enabled WDXRF to be a suitable alternative for detecting Al, Cr, Fe, Co, Cu, and As in animal blood serum samples. It is a new method for veterinary internal medicine that should be extensively used, mainly when there is low input and fast results are necessary.

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