



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

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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 in consequence of exposure to contaminated environments. In this context, many analytical techniques are used for the quantitative determination of these and other potentially toxic elements. Among the most used methods, Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-OES), Atomic Absorption Spectroscopy (AAS), Neutron Activation Analysis (AAN), among others stand out. The techniques mentioned have broadly satisfactory sensitivity and reliability [1] and are internationally recognized. However, in terms of disadvantages, the first two require prior sample preparation: the use of chemical reagents is essential, thus producing residues [2]. In addition, the last method needs a nuclear reactor, which is usually inaccessible for routine laboratories. X-ray fluorescence (XRF) is an analytical tool used to detect heavy metals in several sample types. 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 and preserve the core since it is non-destructive. [4]. Its main disadvantages are related to the method's sensitivity. It is insufficient to guarantee probable contamination due to interelement effects (matrix and/or overlap), depending on the specimen analyzed.

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 in addition to 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 essentials when quick results and actions 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, As, and Hg 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 simultaneous detection of minor and trace elements in the blood serum of dairy cattle (*Bos taurus*) by WDXRF.

### 2. Methodology

This research was conducted under the Ethics Committee in the use of animals of 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 puncture, 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 -20°C until further processing.

The analysis of Al, Cr, Co, Fe, Cu, As, and Hg in serum samples was analyzed using the “Droplet

Method” [4] using a WDXRF spectrometer (Supermini200, Rigaku Co, Japan). Briefly, 50  $\mu$ l 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 palladium anode and a maximum acceleration voltage of 50 kV. Parameters such as emission line, the diffraction crystal, detector, and Bragg’s positions are described in table 1.

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

Emission Line	Bragg’s Position			Diffraction Crystal	Detector	PHA
	Peak (°)	BG1(°)	BG2(°)			
<b>Al-K<math>\alpha</math></b>	144.610	***	***	PET	FPC	100-300
<b>Cr-K<math>\alpha</math></b>	69.330	***	***			
<b>Fe-K<math>\alpha</math></b>	57.500	***	***			
<b>Co-K<math>\alpha</math></b>	52.770	***	***			
<b>Cu-K<math>\alpha</math></b>	45.010	***	***	LiF (200)	SC	
<b>As-K<math>\beta</math>1</b>	30.430	30.180	30.660			
<b>Hg-L<math>\alpha</math></b>	35.890	35.600	36.160			

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

A standard curve was built by analysis of four different volumes (10, 20, 30 e 50  $\mu$ l, corresponding to 10, 20, 30, and 50 mg/L, respectively) of each standard solution of Al, Cr, Co, Fe, Cu, As, and Hg (1000 mgL<sup>-1</sup>; Merck, Germany) using the “Droplet Method”. Each concentration was tested in triplicates, and 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. Limit of detection (LD) and limit of quantification (LQ) for WDXRF were calculated. LD and LQ were estimated as three times and ten times the standard deviation, respectively.

The same elements in the same samples were also detected using the Spectro ARCOS-ICP optical emission spectrometer (Spectro Analytical Instruments Co, Kleve, Germany), equipped with an axially viewed plasma. The sample introduction system was composed of a cross-flow nebulizer and a Scott double-pass. Instrumental parameters selected for determination were: 1400 W RF power, 12 L min<sup>-1</sup> plasma flow, 1 L min<sup>-1</sup> auxiliary flow, 1 L min<sup>-1</sup> nebulizer flow, 3 replicates.

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>). All analyses were performed using RStudio v. 4.0.4 [6].

### 3. Results and Discussion

WDXRF and ICP-OES results were equivalent to all analyzed elements, except for Fe and Al, which are still comparable. 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 comparable since they are in the same order of magnitude. We hypothesize that there may be contamination by red blood cells while obtaining the serum. A pre-concentration of Fe may be occurred due to the greater sample volume used in the ICP protocol. The sensitivity of the WDXRF technique to light elements and the fact that the determined value was close to the LD may explain the Al\_WD result.

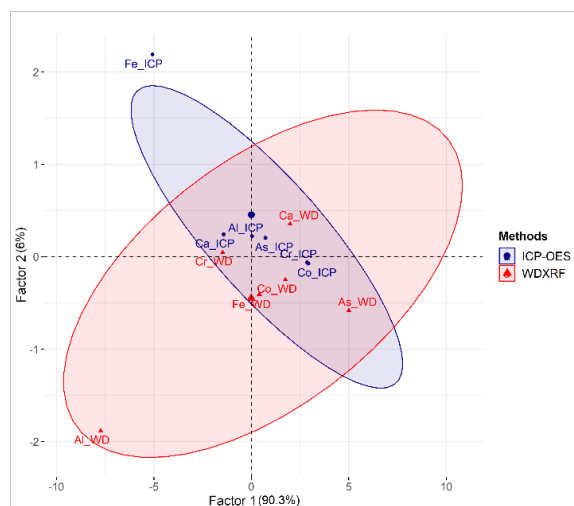


Figure 1: Principal Component Analysis (PCA) showing the intersection of WDXRF and ICP-OES results. The data are mainly explained by factor 1, and Al\_WD and Fe\_ICP are probably outliers.

Concentrations of iron, copper, and cobalt are commonly evaluated in ruminants due to their clinical importance. Normal iron and copper serum levels in cattle are 17.9 to 35.8  $\mu\text{mol/L}$  [7] and 9.4  $\mu\text{mol/L}$  - 11  $\mu\text{mol/L}$  [8], respectively. Serum cobalt concentrations of healthy sheep range from 0.17 to 0.51  $\mu\text{mol/L}$  [7]. 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.

Table 2. Mean determined value (mg/l), percentage relative standard deviation (RSD%), limit of detection (LD), and limit of quantification (LQ) for each element by WDXRF and ICP-OES analysis. Mercury (Hg) analysis by ICP-OES was not possible.

Element	WDXRF	RSD%	LD	LQ	ICP-OES	RSD%	LQ	LD
Al	1.7	26	1.4	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	0.4	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
As	-3.6	-38	4.1	13.8	0.33	9	0.09	0.3
Cu	0.1	76	0.3	1.1	0.7	26	0.5	1.7
Hg	0.1	91	0.3	0.9	-	-	-	-

Less used for medical purposes (Durak et al., 2010; Singh et al., 2018), WDXRF is generally applied for geological analysis. This study WDXRF has its first application in the veterinary area. WDXRF is a good tool available as an alternative for ICP-based techniques because it does not involve sample destruction and waste production, generates quick results, and generally evaluates samples without prior treatment [9, 10]. In addition, the low sample volume required for WDXRF makes it possible to use it widely in veterinary medicine, given the insufficient sample volume of many species.

#### 4. Conclusions

WDXRF proved to be a suitable alternative methodology for heavy metal detection in animal blood serum samples, mainly due to the low sample volume. The results of this study enable a new and fast method for veterinary internal medicine, which requires a technique that quickly detects animals with a high concentration of toxic elements to assure the correct protocols to prevent the animal's death.

#### Acknowledgments

The authors are thankful to Coordination for the Improvement of Higher Education Personnel (CAPES) for support and the farm owners for agreeing to participate in this research. NCG and LJ and his laboratory were supported by São Paulo Research Foundation (FAPESP) [grant numbers 2016/23204-9, 2011/22018-3, respectively]. L.G. is a research fellow of CNPq [312249/2017-9].

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