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Influence of haemolysis on blood biochemistry profiles in cattle

Belén Larrán [a,b], Marta López-Alonso [a,*], Marta Miranda [b,c], Almudena Graña [b], Lucas Rigueira [b,c], Inmaculada Orjales [b,c]

[a] Department of Animal Pathology, Faculty of Veterinary, Campus Terra, University of Santiago de Compostela, 27002 Lugo, Spain
[b] Rof-Codina Veterinary Teaching Hospital, Faculty of Veterinary, Campus Terra, University of Santiago de Compostela, 27002 Lugo, Spain
[c] Department of Anatomy, Animal Production and Clinical Veterinary Sciences, Faculty of Veterinary, Campus Terra, University of Santiago de Compostela, 27002 Lugo, Spain

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ABSTRACT

Although haemolysis is the most common source of preanalytical error in clinical laboratories, its influence on cattle biochemistry remains poorly understood. The effect of haemolysis and its clinical relevance were investigated in 70 samples in which haemolysis was artificially induced (by spiking with increasing amounts of haemolysate, yielding 0.0%, 0.2%, 0.5%, 1.0%, 2.5%, 5.0% and 10% haemolysis degree (HD)), focusing on key parameters for bovine metabolic health assessment, including albumin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), blood urea nitrogen (BUN), calcium (Ca), cholesterol, creatinine, creatine kinase (CK), gamma-glutamyl transferase (GGT), globulins, magnesium (Mg), phosphorus (P), total bilirubin (TBIL) and total proteins (TP). Preanalytical haemolysis significantly affected most (8 of 14) of the biochemical parameters analysed, leading to significant increases in concentrations of albumin (starting at 5% HD), cholesterol (at 5% HD) and P (at 10% HD) and to significant decreases in Ca (at 2.5% HD), creatinine (at 5% HD), globulins (at 10% HD), TBIL (at 2.5% HD) and TP (at 10% HD). Comparison of the present and previous data indicated that, for each parameter, the HD required to produce significant bias and the clinical relevance of over- and underestimation are variable and appear to depend on the analytical technique used. Therefore, different laboratories should evaluate the influence of haemolysis in their analytical results and provide advice to clinicians accordingly. Affected parameters should be interpreted together with clinical signs and other analytical data to minimize misinterpretations (false or masked variations). Finally, due to the significant impact on numerous parameters and the limited potential for correction, we recommend rejection of samples with >10% HD.

1. Introduction

Haemolysis is defined as the lysis of red blood cells, often accompanied by the breakdown of leukocytes and platelets. Haemolysis can either occur in vivo, due to pathological conditions, or in vitro, due to iatrogenic factors during sample collection and handling. Independently of its origin, haemolysis results in the release of intracellular contents into the surrounding fluid and turns the serum a reddish colour. However, visual and automated methods have been compared (Hawkins, 2002; Simundic et al., 2009) and the visual assessment of sample quality is discouraged (Lippi and Cadamuro, 2018). Consequently, the use of objective, automated methods for evaluating haemolysis in samples is strongly recommended (Lippi et al., 2018).

Haemolysis is the most prevalent source of preanalytical error and the leading cause of sample rejection in human medicine (Simundic et al., 2020). It can occur either in vivo, resulting from pathological conditions, or in vitro, caused by iatrogenic factors. While investigation of the preanalytical phase in veterinary medicine is limited (Braun et al., 2015), preanalytical errors are also very frequent in veterinary laboratories (Hooijberg et al., 2012; Whipple et al., 2020), with haemolysis being reported to occur in 16% of samples in a small animal teaching hospital (Whipple et al., 2020). It is worth noting that there are differences in erythrocyte fragility among distinct species(Ding et al., 2015), potentially leading to varying prevalence rates of haemolysis. The standards of human medicine for phlebotomy, sample transport preparation and storage, which are common risk factors for in vitro haemolysis (Simundic et al., 2020), are often challenging to adhere to in veterinary practice. This challenge is further compounded in large animal practice, which is typically conducted outdoors, under conditions that are very different from those in the hospital setting. For example,

* Corresponding author.
E-mail address: marta.lopez.alonso@usc.es (M. López-Alonso).

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venepuncture of the coccygeal vessels, a common practice in cattle herds, increases the risk of haemolytic samples (Slocombe and Colditz, 2012).

Haemolysis can bias analytical results through different mechanisms. These include spectrophotometric interference, chemical interference and altered concentrations of analytes, resulting in errors related to either concentration or dilution (Marques-Garcia, 2020). Haemoglobin has a broad absorbance spectrum, ranging from 340 to 440 nm and from 540 to 580 nm, with a prominent peak at 420 nm (Dolci and Panteghini, 2014), but it also can be detected at other wavelengths (480, 600, 630, 660, 700, 750 or 800 nm; Simundic et al., 2020). Through these mechanisms, haemolysis has the potential to affect a wide range of routine analyses. Furthermore, haemolysis-related interference may vary depending on the analyte, analytical method, instrument and species under consideration (Braun et al., 2015; Simundic et al., 2020). Consequently, the accurate extrapolation of results from published studies might be compromised if substantial differences exist among these factors compared to the conditions specific to each laboratory. Studies including these variables are necessary for correct result interpretation.

Several studies have reported the effects of haemolysis on biochemical parameters across different species (Almeida et al., 2011; Jacobs et al., 1992). Given the widespread use of basic biochemistry panels in assessing health status, investigating the impact of pre-analytical errors on the most frequently used parameters is important. However, haemolysis interference studies in cattle are scarce, and the existing studies often use different methods and yield inconsistent results (Almeida et al., 2011; Cincović et al., 2016; Jacobs et al., 1992; Larrán et al., 2021; Reimers et al., 1991; Stokol and Nydam, 2006). When analysing the same parameter, some studies may suggest no effect while others report a decrease or increase due to haemolysis.

This study aimed to investigate the influence of haemolysis on routine blood biochemistry in cattle and the clinical implications of the effects. The findings will provide laboratories and clinicians with valuable, up-to-date information, enabling determination of the reliability of results derived from haemolytic samples.

2. Materials and methods

2.1. Sample collection and preparation

Blood samples were obtained from 10 healthy Holstein-Friesian cows by jugular venepuncture. The cows were housed in a semi-intensive dairy farm located in northwestern Spain, and they were all in the third week of their third lactation. Sampling was carried out according to Directive 2010/63/EU on the protection of animals used for scientific purposes (European Parliament, 2010), and the trial complied with the Spanish legislation on animal care (Real Decreto 53/2013, n.d.). The procedures were supervised by the Bioethics Committee of the Rof-Codina Veterinary Teaching Hospital, University of Santiago de Compostela (Spain), protocol number AELU001/21/INVMED(02)/Animal (05)/MM/01.

Three types of blood sample were collected from each cow, to provide serum and whole blood. Blood samples were collected in 9 mL serum tubes (Vacuette®, CAT Serum Clot Activator; Greiner Bio-One, Kremsmünster, Austria) and centrifuged within 4 h of collection, at 1500 $\times g$ for 15 min, to yield serum to prepare the artificially-haemolysed samples for biochemical analysis. The tubes of serum were stored at −20 °C for further analysis. Whole blood samples were collected in 9 mL tubes containing sodium heparin (Vacuette®, NH sodium heparin, Greiner Bio-One, Kremsmünster, Austria) and used to produce the haemolysate by freezing (−20 °C). Finally, whole blood samples were also collected in 6 mL tubes containing ethylenediaminetetraacetic acid (EDTA) (Vacuette®, K2E EDTA K2, Greiner Bio-One, Kremsmünster, Austria) for haematological analysis to verify the health status of the cows.

To prepare the haemolysed samples, each serum sample was divided into seven subsamples to prepare a total of 70 artificially-haemolysed samples. These subsamples were spiked with increasing amounts of haemolysate, to produce 0.0%, 0.2%, 0.5%, 1.0%, 2.5%, 5.0% and 10% haemolysis degree (HD), as previously described (Larrán et al., 2021).

2.2. Biochemical analysis and statistics

An automated blood cell counter (ProCyte Dx, IDEXX Laboratories, Westbrook, Maine, USA) was used to produce complete blood counts in the EDTA-whole blood samples from each cow. A complete biochemical analysis of all of the haemolysed serum samples was carried out using a wet biochemistry analyser (MNCHIP PointCare V3, RAL, Sant Joan Despí, Barcelona, Spain). The analysis followed the manufacturer's instructions, incorporating automatic software updates, cleaning and quality control procedures. Reagent standardization, integrated into the QR code of the reagent disc, guarantees test accuracy and is achieved by the manufacturer. The analyser incorporates a quality control process (Built-in real-time Quality Control) executed during every analysis. Additionally, the Rof-Codina Veterinary Teaching Hospital laboratory conducts regular controls during clinician practice to ensure that its results consistently align with the manufacturer's specified acceptance ranges for both inter (≤10% variation) and intra-assay variation (≤5% variation, except for 6% in creatinine and 8% in aspartate aminotransferase (AST). The following 14 parameters were measured: albumin, alkaline phosphatase (ALP), AST, blood urea nitrogen (BUN), calcium (Ca), cholesterol, creatinine, creatine kinase (CK), gamma-glutamyl transferase (GGT), globulins, magnesium (Mg), phosphorus (P), total bilirubin (TBIL) and total proteins (TP). The haematological and biochemical parameters in the blank samples (0.0% HD) were within the reference range established in our laboratory for cattle (Table 1). Details of the analytical methods and the wavelength used in the biochemistry are summarised in Table 1.

The concentration of haemoglobin in the graded haemolysed

Table 1
Details of analytical methods used (including wavelengths) and laboratory reference intervals for the analysed biochemical parameters.

Analyte	Method / substrate	Wavelength (nm)	Reference intervals
Albumin	Green bromocresol	600, 700	2.2–4.4 (g/dL)
ALP	4-nitrophenylphosphate	405, 505	0–350 (UI/L)
AST	α-ketoglutarate/L-aspartate	340, 405	0.150 (UI/L)
BUN	Urease/glutamate dehydrogenase	340, 405	8–27 (mg/dL)
Ca	Arsenazo III	650	8.3–13.2 (mg/dL)
Cholesterol	Cholesterol esterase/cholesterol dehydrogenase	340, 405	27–337 (mg/dL)
CK	Creatine phosphate/adenosine diphosphate	340, 405	40–1250 (UI/L)
Creatinine	Creatinineamidohydrolase/ sarcosine oxidase/peroxidase	546, 700	0.6–2.2 (mg/dL)
GGT	L-γ-glutamyl-3-carboxy-4-nitroanilide/glycylglycine	405, 505	0–55 (UI/L)
Globulins	Determined by subtracting the albumin value from the TP measurement	–	2.2–4.4 (g/dL)
Mg	Hexokinase activation	340, 405	1.5–3.6 (mg/dL)
P	Maltose phosphorylase/ β-phosphoglucomutase/Glucose-6-phosphate dehydrogenase	340, 405	4.2–7.7 (mg/dL)
TBIL	Bilirubin oxidase	450, 546	0–0.9 (mg/dL)
TP	Biuret reaction	546, 800	4.8–9.8 (g/dL)

samples was measured by the cyanmethemoglobin method, with a laboratory assay kit (Spinreact, Girona, Spain) and a Thermo Scientific Genesys 6 spectrophotometer (Thermo Electron Corporation, Madison, USA).

The normality of data distribution was checked using the Kolmogorov–Smirnov test. The effect of artificially-induced haemolysis on the analytes was evaluated by one-way ANOVA and, when significant, a post hoc Tukey's multiple comparison test was applied. The percentage variation relative to the blank (0.0% HD) was also calculated (final/original x 100). The associations between haemoglobin concentration in the sample and the concentration of the parameters affected by haemolysis were evaluated by Pearson correlation analysis and regression analysis. In all cases, a significance level of $p < 0.05$ was applied. All statistical analyses were carried out with SPSS for Windows (vs. 25, Armonk, NY, USA). Graphical outputs were obtained from Microsoft Excel (vs. 2304, Microsoft Corporation, NM, USA).

3. Results

The mean values and standard deviations for the parameters analysed within each group of samples with different degrees of haemolysis are shown in Table 2. The addition of haemolysate had a statistically significant dose-dependent effect on 8 of the 14 analytes. Specifically, there was a statistically significant trend towards overestimation of albumin, cholesterol and P concentrations, while Ca, creatinine, globulins, TBIL and TP concentrations tended to decrease. These differences were observed at different HD: 2.5% for Ca and TBIL, 5.0% for albumin, cholesterol and creatinine, and 10% for globulins, P and TP. On the other hand, no statistically significant differences were observed in AST, BUN, CK, GGT or Mg. While a tendency towards underestimation was evident in the enzymatic activity of ALP, the difference relative to the 0.0% HD was not significant.

All of the biochemical parameters significantly affected by haemolysis were linearly related to the haemoglobin concentration in the serum (Table 3). However, highly statistically significant correlations ($|R| > 0.8$) were only observed for albumin, Ca, and cholesterol. These parameters are therefore suitable for accurate correction through the application of a regression model.

The difference relative to the samples with 0.0% HD is expressed as percentage change in Fig. 1 for the parameters that were statistically significantly altered by haemolysis. Analysis of whether the results of the artificially-haemolysed samples remained within the laboratory reference range (Table 1) showed that the concentrations of albumin, Ca, cholesterol and creatinine fell outside the established limits in some groups (Fig. 1). Specifically, albumin concentrations exceeded the reference range in 20% of the samples with 5% HD and in 70% of the samples with 10% HD. Calcium concentrations were below the reference range in 30% of samples with 2.5% HD, 80% with 5.0% HD and in all the analysed samples with 10% HD. Cholesterol levels exceeded the established limits in 30% of samples with 2.5% HD, 70% with 5.0% HD and in all samples with 10% HD. Finally, creatinine levels were below the physiological range in 30% of the samples with 0.2% HD, in 20% of the samples with 0.5% HD and in 40% of the samples with 5% HD and 10% HD.

Table 3
Summary of the associations between the haemoglobin concentration in serum and the parameters significantly biased by haemolysis as indicated by Pearson correlation analysis. For the parameters with a strong association ($|R| > 0.8$) a linear regression model was calculated.

Analyte	Pearson correlation analysis		Linear regression analysis	
	R	*p*	Regression line	*p*
Albumin	0.803	<0.001	Albumin = 3.859 + 0.063 Hb	<0.001
TBIL	−0.587	<0.001		
Ca	−0.934	<0.001	Ca = 10.206–0.6424 Hb	<0.001
Cholesterol	0.821	<0.001	Cholesterol = 235.12–23.534Hb	<0.001
Creatinine	−0.429	<0.001		
P	0.390	<0.001		
Globulins	−0.760	<0.001		
PT	−0.562	<0.001		

4. Discussion

The study findings indicate that the level of haemolysis in serum samples significantly affects most of the biochemical parameters commonly measured in metabolic profiles in cattle medicine. This is evident for 10% HD, which affected 8 out of the 14 parameters investigated. Notably, most of the parameters that were not affected were enzymes (AST, ALP, CK, GGT). This may be explained by the fact that the measurements are based on the rate of change in absorbance due to enzyme activity after the addition of specific substrates (Table 1). As both measurements are conducted under the same conditions of haemolysis, the potential spectrophotometric interference caused by haemoglobin can be ruled out. Moreover, the methods used also seem to be unaffected by interference by chemistry or concentration, resulting in no haemolysis bias in enzyme activities. However, in contrast to our findings, some prior studies have also indicated a positive or negative bias in measuring enzyme activity in haemolytic samples (Almeida et al., 2011; Cincović et al., 2016; Jacobs et al., 1992; Lippi et al., 2006).

The amount of haemolysis required to produce significant bias and the clinical relevance of an under/overestimation clearly differs among the studied parameters. Traditionally, a threshold of a 10% deviation from baseline values has been considered acceptable for haemolysis

Table 2
Effects of in vitro haemolysis on the values of 14 biochemical parameters in 70 healthy cattle serum samples (10 per each haemolysis degree group). The results are expressed as means ± standard deviation. Different letters indicate statistically significant differences for each haemolysis degree group.

Parameter	Degree of haemolysis (%)							*p*
	0.0	0.2	0.5	1.0	2.5	5.0	10	
Albumin (g/dL)	3.81 ± 0.23^{a}	3.92 ± 0.22^{a}	3.89 ± 0.11^{a}	3.92 ± 0.15^{a}	4.05 ± 0.18^{ab}	4.20 ± 0.19^{b}	4.53 ± 0.16^{c}	< 0.001
ALP (UI/L)	37.4 ± 11.7^{ab}	39.5 ± 13.4^{a}	35.4 ± 18.4^{ab}	31.5 ± 12.5^{ab}	31.7 ± 12.2^{ab}	22.0 ± 8.8^{b}	22.7 ± 7.0^{ab}	< 0.05
AST (UI/L)	104 ± 19	107 ± 20	104 ± 11	104 ± 11	108 ± 16	110 ± 16	117 ± 12	0.450
BUN (mg/dL)	12.0 ± 1.6	12.3 ± 1.5	11.6 ± 1.5	12.1 ± 1.5	11.9 ± 1.9	12.0 ± 1.5	11.9 ± 1.2	0.986
Ca (mg/dL)	10.29 ± 0.32^{ab}	10.49 ± 0.43^{a}	9.68 ± 0.53^{ab}	9.42 ± 0.52^{b}	8.10 ± 0.94^{c}	7.14 ± 1.00^{d}	3.00 ± 0.61^{e}	< 0.001
Cholesterol (mg/dL)	229 ± 51^{a}	238 ± 64^{a}	246 ± 63^{a}	257 ± 72^{a}	313 ± 60^{ab}	366 ± 71^{b}	485 ± 51^{c}	< 0.001
CK (UI/L)	165 ± 31	170 ± 26	170 ± 26	172 ± 31	172 ± 29	175 ± 26	172 ± 23	0.994
Creatinine (mg/dL)	0.85 ± 0.12^{a}	0.72 ± 0.15^{ab}	0.76 ± 0.20^{ab}	0.95 ± 0.25^{a}	0.85 ± 0.12^{a}	0.61 ± 0.15^{b}	0.57 ± 0.15^{b}	< 0.001
GGT (UI/L)	36 ± 13	37 ± 14	35 ± 14	34 ± 15	35 ± 11	37 ± 15	38 ± 12	0.999
Globulins (g/dL)	4.31 ± 0.41^{ab}	4.41 ± 0.49^{a}	4.26 ± 0.74^{ab}	4.20 ± 0.64^{ab}	4.01 ± 0.44^{ab}	3.64 ± 0.38^{b}	2.50 ± 0.36^{c}	< 0.001
Mg (mg/dL)	2.27 ± 0.22	2.35 ± 0.19	2.28 ± 0.23	2.28 ± 0.27	2.29 ± 0.20	2.30 ± 0.18	2.23 ± 0.25	0.953
P (mg/dL)	4.94 ± 0.91^{a}	5.02 ± 0.79^{ab}	4.94 ± 1.00^{a}	5.06 ± 1.00^{ab}	5.35 ± 0.83^{ab}	5.62 ± 0.83^{ab}	6.15 ± 0.77^{b}	< 0.05
TBIL (mg/dL)	0.31 ± 0.05^{a}	0.32 ± 0.09^{a}	0.32 ± 0.07^{a}	0.28 ± 0.07^{ab}	0.22 ± 0.05^{bc}	0.20 ± 0.05^{bc}	0.18 ± 0.04^{c}	< 0.001
PT (g/dL)	8.12 ± 0.40^{a}	8.33 ± 0.50^{a}	8.15 ± 0.81^{a}	8.12 ± 0.75^{a}	8.06 ± 0.42^{a}	7.84 ± 0.35^{a}	7.03 ± 0.40^{b}	< 0.001

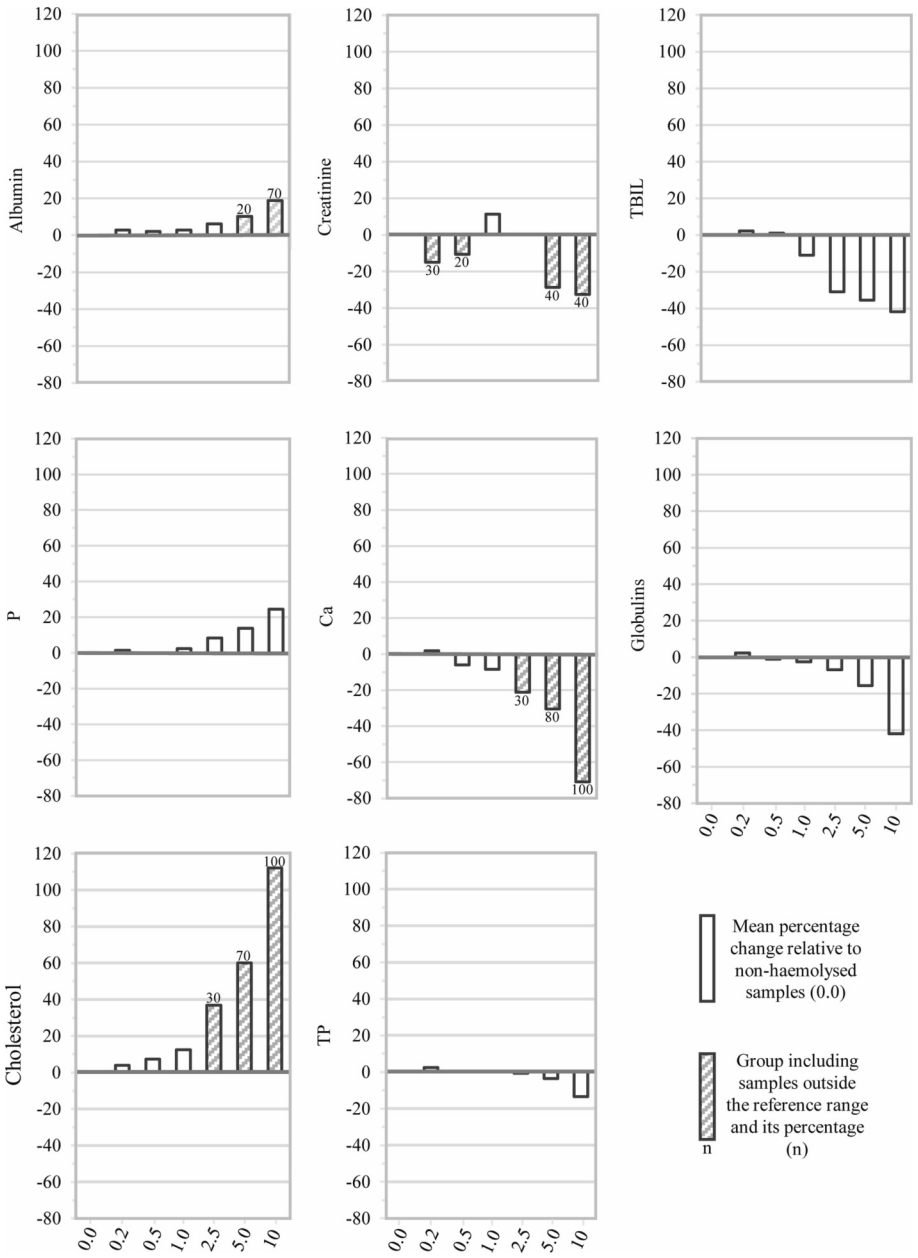

Fig. 1. Mean percentage change in parameter values induced by haemolysis. Only the parameters that showed statistically significant alterations are displayed. Each haemolysis degree (HD) is depicted on the X-axis and the mean percentage change relative to non-haemolysed samples (0.0% HD) is represented on the Y-axis. Bars with a striped pattern indicate groups that include samples outside the laboratory reference range (Table 1). TBIL = Total bilirubin; Ca = calcium; P = phosphorus; TP: total proteins.

interference (CLSI, 2005). In this study, the HD associated with statistically significant changes exceeded this threshold in all cases, reaching up to 120% in the groups of samples with 10% HD. Nevertheless, the recent guidelines of the American Society for Veterinary Clinical Pathology for principles of quality assurance and standards suggest an allowable total error grounded in species-specific clinical experience and discourages extrapolation of results for other species (Arnold et al., 2019). This approach suggests that a thorough evaluation of the clinical significance of the bias induced by haemolysis must be conducted when establishing haemolysis thresholds. Such assessment is also essential to avoid unnecessary rejection of samples. This is particularly important in animal production, as analyses are frequently performed in external laboratories and collection of new samples is not always feasible. Furthermore, this holds significant relevance in animal experimentation, ensuring adherence to the reduction principle.

In this approach, Ca is the parameter that would need greatest attention when handling haemolyzed blood samples in our laboratory. A low level of haemolysis (2.5% HD) results in a significant decrease (ca. 20%) in the real Ca concentration, falling below the adequate range in 30% of samples. Moreover, at 10% HD, the Ca concentration decreased by 70%, with values in all of the samples below the reference levels. In contrast, prior investigations into haemolysis effects on Ca measurement in cattle did not observe significant alterations (Jacobs et al., 1992; Larrán et al., 2021). Divergent results are also encountered in other species. Specifically, in studies involving pigs, haemolysis produced a positive bias in some instances (Dorner et al., 1983) and a negative bias in others (Di Martino et al., 2015). The observed Ca decrease can only be partly attributed to the difference in concentration between the serum and the intra-erythrocyte Ca concentration. In a previous study, evaluation of the influence of haemolysis in the cattle mineral profile by

inductively coupled plasma mass spectrometry (ICP-MS) revealed that the Ca concentrations in the blank samples were not significantly different from the artificially-induced haemolytic samples even at 10% HD (Larrán et al., 2021). ICP-MS is one of the most sensitive methods available for mineral quantification (Amais et al., 2020), closely reflecting the Ca release of erythrocytes content into serum. Consequently, the results observed in this study can mainly be attributed to analytical interference caused by haemolysis rather than to a genuine dilution of Ca in the serum. Furthermore, Ca levels were strongly negatively associated with the haemoglobin concentration of the haemolytic sample (Table 3), which would allow accurate correction of the measured Ca values by considering the haemoglobin concentration in the haemolysed serum. Spectrophotometric interference in Ca measurement has previously been reported in human serum. In a study similar to the present study, Janssen and Helbing (1991) used the Arsenazo III method, at a wavelength of 660 nm, and observed that haemolysis affected the analytical results. Conversely, Leary et al. (1992) noted haemolysis interference in Arsenazo III only when specific wavelength combinations were used (660/546 nm and 600/546 nm), but not when only 660 nm was used. Other Ca measurement methods appear to be less susceptible to the effects of haemolysis. Cattle serum Ca is not influenced by haemolysis in the cresolphthalein method (Jacobs et al., 1992) or the 5-nitro-5′-methyl-BAPTA method (Bach et al., 2020). Considering these findings, laboratories using the Arsenazo III method must report on the potential bias in Ca measurement when handling haemolytic samples. Whenever possible, Ca correction should be based on the haemoglobin concentration in the sample.

Accurate Ca measurement is essential in cattle practice. Hypocalcaemia, especially subclinical hypocalcaemia, is a prevalent disorder in dairy cattle, affecting up to 50% of postpartum cows (Goff, 2008). This condition has several implications for herd profitability, and multiple studies link it to an elevated risk of other peripartum diseases, lower milk production and poorer reproductive performance (Chapinal et al., 2012; Neves et al., 2018; Van Saun, 2023). In the present study, the mean serum Ca concentration (8.1 mg/dL) was below the reference range in samples with only 2.5% HD. This implies that subjects with this low level of haemolysis could already be inaccurately diagnosed as compatible with subclinical hypocalcaemia. Including a warning about the possible haemolysis-induced bias in Ca measurement, along with corrected Ca results based on the haemoglobin levels of the sample, would allow clinicians to weight the implication of hypocalcaemia in the animal clinical condition. Although correction of haemolysis-induced bias is generally discouraged (Lippi et al., 2018), the strong correlation observed between Ca and haemoglobin concentrations in the haemolysed samples, coupled with the clinical significance of hypocalcaemia and the challenges of a new sample collection, may justify this approach.

Total bilirubin was also affected by 2.5% HD, but its decrease was <0.1 mg/dL and even only 0.13 mg/dL in the 10% HD. Two previous cattle studies have shown that haemolysis negative biases TBIL (Cincović et al., 2016; Jacobs et al., 1992). Notably, in the study by Cincović et al. (2016), TBIL was the parameter most affected, with a more pronounced decrease than observed in the present study. This discrepancy can be attributed to differences in the methods used for TBIL analysis. Unlike Ca measurement, the slight reduction in TBIL caused by haemolysis in our study was probably not high enough to mask a case of hyperbilirubinemia. Moreover, in such cases other signs of haemolysis would typically be observed if a pre-hepatic process was involved, or an increase in hepatic enzyme activity if a hepatic or post-hepatic process were suspected. Thus, haemolysis bias in TBIL measurement in our laboratory can be considered clinically irrelevant.

At 5% HD, the concentrations of albumin, cholesterol and creatinine begin to exhibit significant changes. Of these parameters, cholesterol underwent the most remarkable increase (up to 120% at 10% HD), exceeding reference values for the species in most samples. Almeida et al. (2011) and Cincović et al. (2016) also reported a positive bias in cholesterol concentrations in haemolysed samples. However, Jacobs's study did not reveal any alterations in cholesterol concentrations. While decreased cholesterol levels can be associated with liver end-stage diseases (Cockcroft, 2015) or hepatic lipidosis (Peek and Divers, 2018), elevated levels are not commonly observed in metabolic profiles of cows. Consequently, the presence of haemolytic samples should be suspected when elevated cholesterol levels are detected. Furthermore, haemolysis could mask hypocholesterolaemia derived from a hepatic process. However, in this case clinical signs and other biochemical abnormalities, such as increased hepatic enzymes activity (e.g. AST, GGT and sorbitol dehydrogenase (SDH)) would be observed. The strong association between cholesterol and haemoglobin concentrations in the haemolysed serum would allow the correction of the cholesterol concentration in the sample, as with Ca.

Haemolysis had a weaker effect on albumin concentrations, which increased by 10.2% at 5% HD (equivalent to a 0.39-unit increase). Haemolysis also caused an increase in albumin concentrations in the study conducted by Cincović et al. (2016), whereas two other studies found no alterations in albumin levels (Almeida et al., 2011; Jacobs et al., 1992). By contrast, a negative bias was described in human samples by Lippi et al. (2008), attributed to a dilution effect. While hyperalbuminemia is indicative of dehydration, hypoalbuminemia is linked to end-stage liver disease or protein loss processes, such as enteropathies or nephropathies (Cockcroft, 2015), which would be accompanied by clinical signs and other biochemical anomalies. Although a high level of haemolysis could mask hypoalbuminemia, the overall fluctuation induced by haemolysis is unlikely to conceal underlying illness. Therefore, it is of little clinical relevance and its correction, although possible, may not be necessary.

Creatinine is a reliable indicator of the glomerular filtration rate in cows (Cockcroft, 2015). In the samples with 5% HD, an average decrease of 0.24 mg/dL in creatinine levels was observed. This negative bias aligns with Di Martino et al. (2015) findings in swine. By contrast, Jacobs et al. (1992) did not identify any haemolysis-related bias in creatinine levels. The observed change may be clinically significant, as increments of 0.3 mg/dL from a prior measurement or the reference range already suggest renal injury (Peek and Divers, 2018). When such an increase is encountered, especially in haemolytic samples, creatinine should be interpreted in conjunction with BUN and urine density. Despite the clinical relevance, creatinine levels should not be corrected, as suggested for cholesterol and Ca. This decision is based on the fact that creatinine was weakly correlated with haemoglobin (Table 3) and, as mentioned earlier, even minor alterations may be clinically significant. As observed in this study, non-linear correlations are occasionally encountered in haemolysis interference studies, frequently arising from multiple factors (Di Martino et al., 2015; Simundic et al., 2020).

Statistically significant effects of haemolysis on the concentration of globulins, P and TP were only detected in the samples with 10% HD. Total proteins and globulins exhibited a negative bias, with reductions of 1.09 and 1.81 units, respectively. Conversely, haemolysis led to a positive bias in P, resulting in an increase of 1.21 mg/dL. These findings are partly consistent with those of previous studies. In the study by Jacobs et al. (1992), haemolysis was reported to cause a decrease in globulins, and no changes were observed in PT or P. Conversely, Cincović et al. (2016) reported an increase in PT. Among these parameters, globulins seem to be the most affected by haemolysis. This predominant effect is only due to determination of globulins by subtracting the albumin concentration from the TP concentration, with albumin being more affected by haemolysis than TP. Regarding P, Lippi et al. (2008) also encountered a positive bias in human samples and hypothesized that it was due to erythrocyte release. However, no differences between cattle serum and whole blood P levels determined by ICP-MS were observed in a previous study (Larrán et al., 2021), indicating that the observed increment is due to spectrophotometric or chemical interference. Nevertheless, the alterations observed in these three parameters are minimal, requiring 10% HD to produce significant changes.

Consequently, we do not consider these alterations to be clinically significant.

Rejection of samples exhibiting pronounced haemolysis (> 10 mg/dL of haemoglobin) is recommended by the European Federation of Clinical Chemistry and Laboratory Medicine (Lippi et al., 2018), as samples with elevated levels of haemolysis will probably cause bias in most parameters under analysis. Our findings are consistent with this recommendation, as in samples with 10% HD (>10 mg/dL of free haemoglobin; Larrán et al., 2021) significant alterations occurred in 8 out of the 14 investigated parameters, with half of them falling outside the reference range for the species. Therefore, we also propose that highly haemolysed bovine blood samples should not be accepted in analytical laboratories.

5. Conclusions

Our findings clearly indicate that preanalytical haemolysis can significantly bias most of the biochemical parameters usually measured in cattle medicine. For each parameter, the amount of haemolysis needed to produce significant bias and the clinical relevance of over- or underestimation are variable and strongly depend on the analytical technique used. Therefore, each laboratory should assess and must advise clinicians on potential bias in analytical results. Affected parameters should be interpreted together with clinical signs and other analytical data to minimize misinterpretations (false or masked variations). Finally, considering the significant impact on numerous parameters and limited potential for correction (in our laboratory only albumin, Ca and cholesterol), we recommend rejection of samples containing >10% HD.

CRediT authorship contribution statement

Belén Larrán: Data curation, Formal analysis, Investigation, Writing – original draft. **Marta López-Alonso:** Formal analysis, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Marta Miranda:** Funding acquisition, Investigation, Writing – review & editing. **Almudena Graña:** Investigation. **Lucas Rigueira:** Investigation. **Inmaculada Orjales:** Conceptualization, Formal analysis, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

We declare that this research was partly supported by funding from RAL laboratory, which provided financial support for the procurement of specific materials essential for conducting the experiments outlined in this study. Specifically, RAL laboratory provided financial assistance for the acquisition of biochemistry panels crucial for the experimental procedures detailed in this study. However, RAL laboratory had no role in the study design, data collection, analysis, interpretation, or manuscript preparation. The authors declare no other conflicts of interest related to this work.

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