



REVIEW ARTICLE

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Relevance of the study of metabolic profiles in sheep and goat flock. Present and future: A review

Joaquín Hernández (Hernández, J), José L. Benedito (Benedito, JL) and Cristina Castillo (Castillo, C)

Universidade de Santiago de Compostela, Facultad de Veterinaria de Lugo, Departamento de Patología Animal. Campus Universitario, 27002 Lugo, Spain.

Abstract

Current adoption of technical methods of the production systems and the genetic improvement of flocks' productivity have led to the emergence of the well-known metabolic diseases or diseases linked to production. These disorders affect the health status of the flock, thereby generating strong economic losses in the livestock sector. The solution goes through the assessment of the ration, the characteristics of the facilities, the physiological state and the health of the flock, but also, assessing the health condition which is not always reflected in their body condition or feed intake. In field conditions, metabolic profiles could be considered as possible intermediate monitoring tool between animal production and nutrition, because they are able to express a(n) (im)balance between production requirements and feed intake. This information can be accessed by performing measurements and interpreting different blood parameters in a clinical context. Thus, the aim of this review is to offer current information about biochemical metabolic parameters in small ruminants, covering some influencing aspects related to sampling procedure, management and interpretation of results.

Additional key words: laboratory; biochemical parameters; small ruminants.

Abbreviations used: AG (anion gap); ALP (alkaline phosphatase); AST (aspartate aminotransferase); ALT (alanine aminotransferase); BHB (beta-hydroxybutyrate); CK (creatin kinase); ECF (extracellular fluid); EDTA (ethylenediamine tetraacetic acid); GGT (gamma-glutamyl transferase); GLP (good laboratory practice); LDH (lactate dehydrogenase); LMDB (livestock metabolome data base); MUN (milk urea nitrogen); NEB (negative energy balance); NEFA (non-esterified fatty acids); NMR (nuclear magnetic resonance); OS (oxidative stress); OSi (oxidative stress index); ppCO₂ (partial pressure of carbon dioxide); ROS (reactive oxygen substances); RI (reference interval); SUN (serum urea nitrogen)

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Correspondence should be addressed to Joaquín Hernández: joaquin.hernandez@usc.es

Introduction

The emergence of the well-known metabolic diseases or diseases linked to production has been caused by the increase in intensification in the production system and the improvement of productivity by genetic selection (Castillo & Hernández, 2016).

Metabolites have been referred as detectors of the genome: so, like others sentinels, they can be perfectly sensitive indicators of problems in the genome (Pearson, 2007). Metabolites are the final products of complex interactions that occur within the cell and events that occur outside the cell or the organism (Goldansaz *et al.*, 2017). Biochemical composition of blood plasma faithfully re-

flects the metabolic status of animal tissues in order to assess tissue damage, disorders of organ function, adaptation of the animal to nutritional and physiological challenges, and specific metabolic or nutritional imbalances (González, 2018).

Metabolic disorders affect the health status of the flock and generate strong economic losses. In the last decades, most of the attention has been focused on studying calcium and phosphorus homeostasis in milk fever, magnesium homeostasis relative to grass tetany, blood glucose and ketones relative to ketosis and potassium relative to hyperkalemia on cereal grazing, but likely other metabolic imbalances not yet identified could be of great importance (Radostits *et al.*, 2006).

Under this scenario, it is clear that the solution goes through the monitoring of all factors that could have an influence in the metabolism such as the ration, the characteristics of facilities, the health and sanitary status of the flock, but also, the general body condition and the feed intake. This information can be accessed by measuring and interpreting some blood parameters in a clinical context.

The metabolic profile test was coined in the late 1960s as a means of diagnosis for various forms of production disease in dairy cows (Payne *et al.*, 1970), but it was quickly extended to all production animals, suffering many modifications from the original proposed test along the time. Payne *et al.* (1970) established the *Compton Metabolic Profile Test*, pointing out that its main purpose was to indicate whether a herd is liable to suffering from production disease. The test was based on a statistical evaluation of blood chemistry that reveals early signs of abnormality, constituting a pre-symptomatic diagnostic tool capable of giving early warning of certain types of metabolic derangement. In fact, metabolic profiles are very useful to evaluate disease risk. When we use these profiles in association with animal, diet and management assessments, we can get highly valuable information about both health and welfare status (Calamari *et al.*, 2016).

In essence, the metabolic profiles represent the way to know what animals are feeling about their diet composition (Macrae *et al.*, 2006) and they make it possible to identify possible nutritional disorders even before the productivity of the herd is affected. If used properly, metabolic profiles could answer several important questions such as whether something is, or about to go, wrong or what is the best practical and economical solution.

According to the potential of monitoring metabolic disorders, the aim of this review is to offer information about biochemical metabolic parameters in small ruminants, covering some aspects related to sample processing, management and interpreting the results obtained.

Optimal time-point to establish a metabolic profile

The frequency and timing of clinical pathology testing are dependent upon study duration, study objectives, the biological activity of the test material, and the species tested (Evans, 2009). Most sheep and goat flocks are managed to enable the conversion of forage and cereal crops into marketable products, such as meat, milk, wool and pelts. Such management is challenged by both economics of the production and the need to ensure good welfare standards. One of the principles of flock health management, or veterinary flock health planning in commercial flocks includes the use of a rational diagnostic approach

to identify any constraints to productivity (Sargison & Scott, 2010).

Several critical situations proposed in which metabolic profiles could be of great clinical interest have been proposed (Castillo & Hernández, 2016):

— 1. *The periparturient period*. This status, that covers late pregnancy and early lactation, occurs from 2-3 weeks pre-calving to 2-3 weeks post-calving. The dairy cow transition philosophy can be applied to the prolific ewe or doe, as this period is certainly at the frontier of flock nutrition. There are many similarities to the transition dairy cow, except timelines shift ahead by 1 week-10 days. Within this very short period the animal is forced to deal with radical changes such as final difficulties of gestation, intake restriction, parturition itself, onset of lactation, intake and appetite fluctuations, diet transitions (from a gestation diet to a lactation diet), and fluctuations in hormone concentrations (Castillo & Hernández, 2013; González, 2018). A mismatch of energy supply and demand at this time results in a net energy deficiency or Negative Energy Balance (NEB). On the contrary, the excess of energy with the ration is a common feature in several flocks, leading to endocrine imbalances, which affect animal health through failure in the insulin response by the peripheral tissues (Castillo *et al.*, 1999). Currently, the most common metabolic diseases of ewes around the periparturient period, namely hypocalcemia, hypomagnesaemia and pregnancy toxemia, are caused by the failure of meeting the nutritional requirements of the animals during late pregnancy and/or early lactation. Transition from pregnancy to lactation triggers an imbalance between pro-oxidants (derived from the metabolic action of pregnant) and the defensive barrier of antioxidants (those minerals, vitamins and enzymes, with exogenous or endogenous origin). When the first predominate over the second, the animal goes through a state of oxidative stress (OS) that has been shown to be linked to the appearance of inflammatory processes (such as mastitis) and reproductive disorders, as well as failures in embryo implantation, abortions or delays in the appearance of heat (Celi *et al.*, 2010). Nowadays, it is debated whether oxidative stress is itself a metabolic disease or the consequence of the typical imbalances of the transition phase (Castillo & Hernández, 2016). In addition, it has been concluded that the presence of oxidative stress plays a key role in the regulation of insulin levels, causing greater resistance from peripheral tissues towards this hormone and preventing the correct glucose metabolism, predisposing the animal to future ketosis processes, especially in dairy breeds (Abuelo *et al.*, 2016). Therefore, it is necessary to provide useful OS markers that help to analyse it with accuracy to define protective nutritional strategies on the basis of antioxidant supplementation (Abuelo *et al.*, 2013). It is important to evaluate not

only concentrations of oxidants and antioxidants separately, but also the relationship between them (Castillo *et al.*, 2005). There are different methodologies for OS determination (oxidants and antioxidants) and differences between models and methodologies make difficult to make meaningful comparisons (Celi, 2011) with practical conclusions. In general, it has been described that an increase in the OSI ratio (based on oxidants/antioxidants quotient), indicates a risk of OS due to the increase in oxidant production and/or defensive antioxidant consumption (Abuelo *et al.*, 2013).

— 2. *Ration modifications.* In situations when a new component in the ration is introduced, either changes in the quality of ration itself or an additive or supplement, it must be considered whether or not the new diet is correctly adjusted to suit the metabolic requirements of the destination animal. In the introduction of a new component or supplement, it is interesting to know if it is well metabolized, if it interferes with other nutrients or if it affects their health. An unbalanced diet leads to nutritional deficiencies or overweight. Frequently, dairy sheep and goats ingest more crude protein than necessary producing an excess of nitrogen that harms animals by overloading the renal function. In addition, excessive nitrogen is released into the environment causing respiratory problems in closed stables with poor ventilation (Sevi *et al.*, 2009). Also, N excretion in milk is associated with an increase in somatic cell count attributable to a higher bacterial load (Sevi *et al.*, 2006). It is known that urea levels in milk higher than 40 mg/dL (6.6 mmol/L) are associated with lower reproductive efficiency (Cannas, 2002). At this point, it is time to incorporate the concept of 'nitrosative stress' (Dalle-Donne *et al.*, 2005). While we pointed out that oxygen is a great producer of free radicals or pro-oxidants, generating oxidative stress, in recent years it is known that N plays a fundamental role in the damage to the different biological structures through the generation of reactive N species that negatively affect the smooth muscles, cardiomyocytes, platelets, nerves and renal cells, causing imbalances in the organs.

— 3. *Changing new farming models from intensive to organic system.* Blood tests were firstly used for monitoring the health status in intensive breeding systems. But changes to new models carry changes in nutritional protocols, leading to nutritional disorders associated with undernutrition (Abuelo *et al.*, 2014), mainly in energy and minerals. This fact is especially important in ewes and does with high milk production that are changed to organic farming. Therefore, in this new farming system it will be mandatory to establish the best blood test in order to verify the health of the flock. Probably, pregnancy toxemia and hypocalcaemia will be present as common metabolic diseases if preventive measures are not adopted.

Selecting the proper parameters for different profiles

When the clinical pathology is used contemporarily to the anamnesis, the physical examination and even other laboratory tests, (*e.g.* urine tests) it could be useful in establishing the initial reference range for a patient. In addition, it would help to formulate a list of problems, to rule out or confirm a diagnosis, to determine the prognosis, to plan treatment options and to monitor the response to treatment.

Usually, a ruminant chemistry panel includes, among others, several analytes and enzymes (Russell & Rousel, 2007): glucose, lactate, serum urea nitrogen (SUN), creatinine, electrolytes (sodium, chloride, and potassium), total carbon dioxide (TCO₂), hepatic enzymes (ALP –alanine aminotransferase– and GGT –gamma-glutamyl transferase–), bilirubin, minerals (calcium, phosphorous, and magnesium), serum proteins (total protein and albumin), and muscle enzymes (CK –creatin kinase– and AST –aspartate aminotransferase–). In addition, AG (anion gap) and globulin concentration, can be determined, if not provided, by the calculation $AG = (Na^+ + K^+) - (Cl^- + HCO_3^-)$ and the differences between total protein and albumin, respectively.

Most of these parameters have been incorporated in the so-called 'General Panel' (Smith, 2015), giving information about not only the health of the animal but also about many medical conditions.

For sheep and goat flocks, it has been pointed out that a metabolic panel should include the serum determination of the following metabolites: glucose, SUN, creatinine, and enzymes like AST, GGT, ALP and CK; total proteins (including their fractions albumin and globulin), sodium, potassium, calcium, phosphorus and magnesium (Castillo & Hernández, 2016). In general, it is also suggested to measure the concentrations of non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB) in ruminants (Braun *et al.*, 2010). Moreover, for a better interpretation of the results, this panel could be divided into three main groups: 1) *energetic* (glucose, NEFA and BHB); 2) *protein* (urea and total proteins) and 3) *mineral* (Ca, P and Mg).

We present here different clinical metabolic panels (see Table 1), connecting their changes with common metabolic diseases:

— *Hepatic panel:* Taking into account that hepatocytes contain high activity of the enzymes AST, GDH (glutamate dehydrogenase) and LDH, and they are referred to as 'leakage enzymes', these parameters would need to be measured. Other metabolites to consider in this panel should be SUN, bilirubin, glucose, total proteins and albumin (Braun *et al.*, 2010; Castillo *et al.*, 2011).

Connected with this panel appears the concept 'clinical enzymology'. So, with acute or chronic hepatocellular

Table 1. Biological parameters proposed to be measured in different profiles in small ruminants

Profile ^[1]		Recommended parameters to be measured ^{[2][3]}
Chemistry profile	(S)	AG , bilirubin , creatinine, electrolytes (sodium, chloride , and potassium), glucose, hepatic (ALP and GGT) and muscle enzymes (CK and AST), lactate , minerals (calcium, phosphorus, and magnesium), serum proteins (total protein, albumin and globulin), SUN and TCO₂ .
Metabolic profile	(S)	BHB, creatinine, electrolytes (sodium and potassium), enzymes like AST, GGT, ALP and CK enzymes; glucose, minerals (calcium, phosphorus and magnesium), NEFA, SUN and total proteins (including their fractions albumin and globulin).
Clinical metabolic profile		Considering the specific organ/system to be investigated.
	Hepatic panel (S)	Bilirubin, enzymes AST, GDH and LDH, glucose, SUN, total proteins and albumin.
	Renal panel (S)	Creatinine, serum micro minerals (calcium and phosphorus) and total proteins and albumin.
	Digestive panel (S)	Acid-base balance assessment: blood pH, ppCO ₂ , base excess, HCO ₃ ⁻ and Ca ⁺² , ions (sodium, potassium, and chlorine) and total proteins (including their fractions albumin and globulin). D and L lactate.
	Muscular panel (S)	Enzymes AST, ALT, CK and LDH.
	Energy panel (S)	BHB and NEFA.
	Protein panel (S), (U), (M)	Albumin, milk urea, SUN and urinary N.
	Mineral panel (S)	Minerals (calcium, phosphorus and magnesium).
	Acid-base panel (B), (U)	Blood and urine pH, ppCO ₂ , base excess, HCO ₃ ⁻ and ionized Ca ⁺² . AG.
	Electrolyte panel (S)	Chloride, potassium and sodium.

^[1] S: serum/plasma determination. U: urine determination. M: milk determination. B: blood determination. ^[2] Parameters in bold mean that they appear in chemistry and not in metabolic profile. ^[3] AG: anion gap; (Ca⁺²): ionized calcium, ALP: alkaline phosphatase; AST: aspartate aminotransferase; CK: creatin kinase; GDH: glutamate dehydrogenase; GGT: gamma-glutamyl transferase; HCO₃⁻: blood bicarbonate; LDH: lactate dehydrogenase; ppCO₂: partial pressure of carbon dioxide; SUN: serum urea nitrogen; TCO₂: total carbon dioxide.

injury or necrosis, the serum activity of these enzymes increases because they pass from the intracellular space to the plasma. The hepatocyte activity ALT (alanine aminotransferase), used to detect hepatocellular injury in small animals, is low in ruminants, and therefore, it is not useful to assess liver disease (Russell & Roussel, 2007; Kaneko *et al.*, 2008). Increased hepatocyte production of GGT and ALP occurs with cholestasis. Pathological conditions like fascioliasis or cholelithiasis that promote biliary obstruction, will produce an increase in the activity of these enzymes, being the increase higher in chronic hepatic damage in relation to acute damage (Smith, 2015).

— *Renal panel*: The chemistry profile and urinalysis are used to evaluate these functions and are very useful to diagnose kidney disease (Russell & Roussel, 2007). Determinations of both serum protein compounds (creatinine, total proteins and albumin) and serum micro minerals (calcium and phosphorus) will facilitate the diagnosis of renal diseases (Castillo & Hernández, 2016). From a practical point of view, it should be taken into account that serum creatinine concentration is influenced by muscle mass but not so much by nutrition or protein catabolism.

In ruminants, urea is recycled through salivary incorporation into the rumen, a process that could reduce the utility of SUN in the diagnosis of kidney disease. Considering the external factors that can modify both parameters, it has been pointed out that creatinine is the gold standard for evaluating the renal function in ruminants (Russell & Roussel, 2007; Castillo *et al.*, 2011).

‘Azotemia’ is a medical condition recognized by the increase of SUN and serum creatinine concentrations in the renal panel. Azotemia can be classified as *pre-renal*, *renal*, or *post-renal*. Common causes of pre-renal azotemia include: reduced renal perfusion from dehydration, hypovolemia, or shock. Renal azotemia is associated with acute or chronic renal failure. Postrenal azotemia is associated with urolithiasis secondary to renal, ureteral or urethral calculi, or uroabdomen (Milne & Scott, 2006).

— *Digestive panel*: In ruminants, this panel should be considered useful to diagnose not only ruminal diseases, but also in situations associated with nutrition problems. In both situations, the determination of total proteins (including their fractions albumin and globulin), ions (sodium, potassium and chlorine) and acid-base balance

assessment: blood pH, ppCO_2 , base excess, bicarbonate (HCO_3^-) and ionized calcium (Ca^{+2}) is useful (Castillo *et al.*, 2011). When possible, measurements of both serum enantiomers of lactate (L and D) are recommended (Castillo *et al.*, 2009; Abuelo *et al.*, 2015). Microbial fermentation in the gastrointestinal tract may result in the production of both the D and L forms of lactate. Different studies have shown that, in milk-fed kids, the accumulation of D Lactate is a major factor in the profound acidosis associated with certain digestive disorders (Smith, 2015), producing the called ‘floppy kid syndrome’.

— *Muscular panel*: The increase of enzymatic activity of serum AST and LDH can be promoted as a result of muscle injury, albeit they are not muscle-specific enzymes. Meanwhile, serum CK is a sensitive and specific indicator of muscular damage. Extremely high activities of CK in the absence of recumbency suggest a primary myopathy, such as white muscle disease or ‘Senna toxicity’ (Russell & Roussel, 2007). Considering that half-life of AST in serum is much longer than CK, we can assume that an increase and a decrease in AST activity will be slower than in CK, and it can be used in combination with CK to stage muscle injury. In relation to ALT activity, an increase has been pointed out in muscular diseases (Bender, 2003).

— *Energy panel*: NEFAs are sensitive indicators of energy balance. It has been reported that they could be used to monitor energy status of small ruminants in the last period of gestation (Castillo *et al.*, 1999, 2001). High values of NEFA indicate a NEB status which occurs in animals that are inappetent for any illness. Under this condition the body uses the glycogen stores in the liver to produce glucose, so then switches to mobilize high amounts of body fat reserves to make up the deficit, resulting in the production of ketone bodies. However, when ketone concentrations increase in the body, glucose concentrations fall. Another less specific marker of energy balance is serum BHB with high values associated with reduced milk production, increased clinical ketosis and reduced fertility. Moreover, the gold standard test for subclinical ketosis diagnosis is blood BHB, with serum concentrations above 1000 $\mu\text{mol/L}$, and is considered more stable than other ketones such as acetone or acetoacetate (Radostits *et al.*, 2006). It has been reported that a good strategy for energy balance monitoring in transition females could be to search for NEFA in the last week before calving and BHB in the first week after calving (Castillo *et al.*, 1999; Celi *et al.*, 2010). In fact, BHB should be measured in pregnant goats in late stages of gestation with hypoglycemia or with elevated urea and total protein concentrations as a tool to differentiate potential subclinical pregnancy toxemia (Bani-Ismail *et al.*, 2008).

— *Protein panel*: To monitor protein status, SUN testing to evaluate milk urea N (MUN) can be used as a tool to improve dairy flock nutrition and monitor the nutri-

tional status of lactating dairy ewes or goats. Urinary N (UN) excretion has been shown to have a positive linear relationship with MUN. Elevated MUN indicates that an excess of protein has been fed to the animal. An increase in SUN concentration and ammonia occur primarily as a result of inefficient nitrogen utilization. An excess of rumen degradable protein results in an increase in the concentration of rumen ammonia, which is absorbed through the rumen wall and transported to the liver, where it is converted into urea (Radostits *et al.*, 2006; Braun *et al.*, 2010). Urea moves passively from the blood into the milk and there is a close relationship between its concentrations in the two fluids. Thus, milk urea has been used as a non-invasive substitute for the measurement of the protein status and protein intake of ruminants (Castillo & Hernández, 2016). Nevertheless, an excess of urea is closely connected with the development of a nitrosative stress status (Dalle-Donne *et al.*, 2005), with detrimental consequences in the animal health.

Serum albumin is also related to the protein status of the animal as being the most abundant protein in serum. Changes in blood volume cause changes in total protein concentration. Hemorrhage or massive exudation with large external losses of plasma is followed by a rapid movement of interstitial fluid (without protein) into the plasma compartment, to induce an acute hypoproteinaemia. Conversely, dehydration leads to hemoconcentration through a reduction in fluid volume and consequent hyperproteinemia. Hyperproteinemia may be seen in dehydration due to inadequate water intake or to excessive water loss (*i.e.*, severe diarrhoea, and rumen acidosis) or as a result of increased production of proteins. Increased polyclonal protein production is seen in reactive, inflammatory processes. Mild hyperproteinemia may be caused by an increase in the concentration of specific proteins that are normally present in relatively low concentration, for example, increases in acute phase reactants produced in inflammatory states, late-stage liver disease, and infections. Hypoproteinemia may be due to decreased production (*e.g.*, chronic liver disease, malnutrition) or increased protein loss (*e.g.*, nephrotic syndrome, protein-losing enteropathy) (Kaneko *et al.*, 2008).

— *Mineral panel*: Taking into account that serum microminerals (calcium, phosphorus, and magnesium) have the same homeostatic mechanisms, we can assume that changes in their concentrations could be connected. The distribution of total serum calcium can be presented as follows: 50% ionized calcium (the biologically active form), 40% unionized calcium (most of which is albumin-bound), and 10% complexed with anions (Russell & Roussel, 2007). Changes in ionized calcium (Ca^{+2}) are associated with changes in blood pH with increases in acidosis and decrease in alkalosis (Castillo *et al.*, 1997). In relation to total serum magnesium, 55% will be present as an ionized, 30% is present as a protein-bound fraction,

and 15% is complexed with anions. Serum phosphorus is present as dissociated phosphoric acid (H_2PO_4^-) (Russell & Roussel, 2007).

Hypocalcemia is usually present in several gastrointestinal diseases (e.g., left abomasal displacement, Hoflund syndrome). Calcium is extremely important for proper smooth muscle contractility and neuromuscular transmission. Hence, low-serum or plasma calcium concentration has been identified as among the main metabolic factors associated with the decreased abomasal motility preceding DA (abomasal displacement) development. Hypocalcemia and hypophosphatemia in sheep usually appear in the last month of pregnancy rather than lactation period (Russell & Roussel, 2007), due to the fact that the foetal calcium requirement is higher than that in lactation in sheep (Allen & Sansom, 1986; Castillo *et al.*, 1997).

Both medical conditions, hypomagnesemia with hypocalcemia can promote the development of grass tetany (goats) or 'grass staggers' (sheep) (Russell & Roussel, 2007). Hyperkalemia could be an important factor in the development of hypomagnesemia. In fact, several studies have demonstrated that ruminants consuming diets with high concentrations of potassium experience a significant reduction in magnesium absorption from the gastrointestinal tract. This effect appears to be dose dependent (Castillo *et al.*, 2015).

— *Acid-base panel*: The pH of body fluids is kept within narrow limits, which is necessary to maintain the structure and function of proteins essential for normal progression of metabolic events. Most enzymatic reactions have a narrowly defined optimum pH range, and changes in hydrogen ion concentration have direct effects on the rates of reaction and, thus, resulting in many basic biological processes (Kaneko *et al.*, 2008). Variations on acid-base balance parameters indicate the need for improving goats and kids' rations in traditional production systems according to the literature (Antunovic *et al.*, 2017).

Using the traditional approach to acid-base balance, the four primary imbalances and their compensatory mechanisms are explained as follows. Acidosis is associated with an increase in the hydrogen ion concentration (decreasing blood pH), whereas, alkalosis is caused by a decrease in the hydrogen ion concentration (increasing blood pH). When the primary imbalance is associated with a change in the bicarbonate concentration the acid-base imbalance is called a 'metabolic acid-base imbalance'. The compensatory response to a metabolic acid-base imbalance is mediated by the respiratory tract, which alters the partial pressure of carbon dioxide (ppCO_2), trying to restore to the normal pH. Primary respiratory imbalances are related to changes in alveolar ventilation which result in an increased ppCO_2 (hypoventilation or respiratory acidosis), or a decreased ppCO_2 (hyperventilation or respiratory alkalosis). The compensatory response for such processes is mediated by the kidneys through presumed

alterations in the excretion or retention of hydrogen ions and bicarbonate, producing aciduria in acidosis or alcaluria in alkalosis (Kaneko *et al.*, 2008; Braun *et al.*, 2010).

Historically the AG was used to determine the type of metabolic acidosis: 1) increased acid production; 2) bicarbonate loss; and 3) decreased renal acid excretion. Hypoproteinemia and hyperchloremic metabolic acidosis are the most common causes of a decrease in the AG. The cause of normal or low AG hyperchloremic metabolic acidosis often can be differentiated on the basis of the serum K concentrations (Smith, 2015). Animals with hyperchloremic metabolic acidosis associated with gastrointestinal fluid losses or renal tubular acidosis most often manifest hypokalemia, whereas hyperkalemia generally is seen in patients with renal failure with renal shutdown. A decrease in the AG also results from over-hydration caused by decreases in the protein concentration and changes in the relative concentration of blood Na^+ and Cl^- .

Most commonly, high AG acidosis is associated with accumulation of metabolizable acid, most commonly lactic acid associated with grain overload, acute gastrointestinal accidents or hypovolemic shock (e.g., diarrhoea in lambs and kids). Ketoacidosis, uremic acidosis, and poisoning with a variety of anionic poisons result in increases in non-metabolizable acids that also cause an increased AG.

— *Electrolyte panel*: Serum ions included in a chemistry profile are cations (sodium, Na^+ and potassium K^+) and anions like chloride (Cl^-). Na^+ is the main extracellular cation being responsible of the osmotic force which maintains the extracellular fluid compartment. Serum Na^+ concentration reflects total body sodium content because this cation is basically confined to the extracellular compartment. It is important to take into account that the assessment of the serum Na^+ concentration should be done considering the animal's hydration status (Russell & Roussel, 2007). K^+ is the major intracellular cation, which is present in the ECF (extracellular fluid) but is strictly regulated, because small changes can have marked effects on organ function. Severe changes can be life threatening. Hyperkalemia or hypokalemia cause muscle weakness affecting the skeletal, cardiac, and smooth muscle. Chloride is the major anion in ECF. Chloride and HCO_3^- often maintain a reciprocal relationship in the disorders, causing in the case of an increase in chloride a decrease in bicarbonate, and vice versa, attending to Anion gap and electroneutrality principle (Russell & Roussel, 2007).

Hypernatremia and concurrent hyperchloremia are secondary to loss of water or sodium retention (e.g. salt toxicity). Albeit in ruminant fluid loss typically occurs with concurrent loss of electrolytes, these abnormalities are rare in dehydration. Hyponatremia and concurrent hypochloremia occur with excessive loss of sodium-rich fluids (diarrhea), renal failure, or an obstructed or ruptured urinary tract (Russell & Roussel, 2007).

Depending on the duration and severity, sheep and goats with urolithiasis may show hyperkalemia; uroabdomen in sheep or goats is generally characterized by hyponatremia, hypochloremia, hyperkalemia, and hyperphosphatemia (Belknap & Pugh, 2002). Several imbalances of serum K^+ are associated with acid-base disturbances, anorexia, gastrointestinal and renal losses (Kaneko *et al.*, 2008; Castillo *et al.*, 2009).

Reference intervals in sheeps and goats

The concept of reference interval represents the normal values that should be found from a healthy animal (reference population) and is needed to interpret patients' results (Friedrichs *et al.*, 2012).

It has been reported that reference intervals should be evaluated every 3–5 years (Ceriotti *et al.*, 2009). In addition, evaluation is recommended when excessive false-positive and false-negative results are noted by clinicians and whenever there are significant changes in animal patient populations, preanalytical techniques, or analytical quality.

In a very interesting review, consensus guidelines performed by the American Society for Veterinary Clinical Pathology (ASVCP) for determination of de novo reference intervals in veterinary species with language and examples specific to veterinary species have been presented (Friedrichs *et al.*, 2012). In addition, laboratories performing regulatory studies must ensure that the studies comply with the principles of 'good laboratory practice' (GLP) as interpreted by national and international regulatory authorities and their inspectorates (Evans, 2009). Among their different regulations, GLP includes general laboratory facilities, calibration and maintenance of equipment, reagent preparation and quality control procedures for assays, the characterization of other experimental variables and authorized and documented standard operating procedures (European GLP is available in ECI, 2015).

In this scenario, we should provide general reference intervals (Table 2) for the commonly used blood biochemical analytes in ovine and caprine obtained from different reference books (Kaneko *et al.*, 2008; Smith, 2015) combined with our clinical experience during more than 20 years (Castillo *et al.*, 2000; 2015; Castillo & Hernández, 2016).

Before the interpretation of data, it is important to consider some of the variables that can affect the data but are unrelated to the compound being tested because changes can occur in plasma and urine values due to normal biological variations (Evans, 2009). Some of them have been established by different authors:

— *Species*: Although there are no significant variations in reference range in most blood parameters in ruminants, it is highly recommended to compare our results with tho-

se specified for each species (Radostits *et al.*, 2006; Friedrichs *et al.*, 2012; Castillo *et al.*, 2016).

— *Breed*: Generally, breed factor is closely connected with productive characteristics: meat, milk or wool. For this reason, productive demands determine specific endocrine profiles that will condition specific metabolic parameters, such as glucose, NEFA, SUN, creatinine or electrolytes and acid-base balance (Castillo *et al.*, 2000, 2001) and should be considered for reference range interval establishment.

— *Time of sampling*: The influence of season is often difficult to separate from confusion factors, such as feed supply, and/or reproductive status in female animals. For some analytes, temporal changes can be related to seasonal influences, such as formation/resorption of bone and corresponding changes in bone turnover markers. In other cases, large variations are observed and no seasonal and/or physiological cause may be found. For instance, mean plasma lactate concentration was reported to range from 1.7 to 2.9 mmol/L in a group of 20 ewes sampled monthly for one year (Allison *et al.*, 2008). In some cases, circadian variations of analytes cannot be unambiguously linked to an endogenous rhythm but may also result from the pattern of feed administration, as observed for plasma and salivary urea concentrations (Piccione *et al.*, 2006; González, 2018).

— *Age*: Several important differences in clinical chemistry exist between neonatal and adult animals within species. Generally, and in comparison with adults, suckling neonatal animals tend to have lower SUN, slightly lower total protein and globulin, moderate higher GGT and phosphate, and markedly greater ALP (Radostits *et al.*, 2006; Braun *et al.*, 2010; Smith, 2015; González, 2018).

— *Gender*: With the obvious exception of sex hormone concentration, there are few recognized differences in clinical chemistry values (Humann-Ziehank & Ganter, 2012).

— *Physiological stage*: Pregnancy and lactation determine significant changes in several metabolites, especially those connected with energy, mineral and acid-base balances. In sheep and goats, we have also included the influence of double/triple pregnancies, increasing nutrient partitioning in the organism (Castillo *et al.*, 1999, 2000, 2001).

Analytical process

Once the clinical profiles have been established and the practical situations in which is highly recommended to establish a clinical profile, the next step is to consider how to obtain good samples for good results and proper interpretation. In this sense, three different periods should be considered: *pre-analytical*, *analytical* and *post-analytical*. We refer again to the GLP pointed out in previous

Table 2. Clinical chemistry: reference intervals for ovine and caprine (Castillo *et al.*, 1997, 1999, 2000, 2001; Allison *et al.*, 2008; Kaneko *et al.*, 2008)

Parameter ^[1]	Units	Ovine	Caprine
Metabolites			
Total bilirubin	mg/dL	0.1-0.5	0-0.1
• Direct (conjugated)	mg/dL	0-0.27	0-0.1
• Indirect (unconjugated)	mg/dL	0-0.12	0-0.1
Cholesterol	mg/dL	52-76	80-130
Creatinine	mg/dL	1.2-1.9	1.0-1.8
Glucose	mg/dL	50-80	50-75
Total proteins	g/dL	6.0-7.9	6.4-7.0
Albumin	g/dL	2.4-3.0	2.7-3.9
Globulins	g/dL	3.5-5.7	2.7-4.1
SUN	mg/dL	8.0-20	10-20
NEFA	mmol/L	0.14-0.28	0.46-0.52
BHB	mmol/L	0.30-0.38	0.41-0.45
Enzymes			
ALP	IU/L	68-380	93-387
AST	IU/L	60-280	167-513
CK	IU/L	64-158	104-219
GGT	IU/L	40-79	20-56
LDH	IU/L	238-440	123-392
Minerals and electrolytes			
Sodium	mmol/L	139-152	142-155
Potassium	mmol/L	3.9-5.4	3.5-6.7
Chlorine	mmol/L	95-103	99-110
Calcium	mg/dL	11.5-12.8	8.9-11.7
Phosphorus	mg/dL	5.0-7.3	6.5
Magnesium	mg/dL	2.2-2.8	2.8-3.6
Acid-base (venous blood)			
pH	---	7.32-7.54	7.37-7.4
ppCO ₂	mmHg	37-46	44.4-46
HCO ₃ ⁻	mmol/L	20-25	24.5-28
AG	mmol/L	12-24	18-20.5
OS balance			
ROS	Carr U	57.72-120.53	53.50-195.50
SAC	μmol HClO/mL	248.5-368.1	595.6-756.1
OSi (ROS/SAC)	---	0.2-0.43	0.08-0.3

^[1] SUN: serum urea nitrogen; NEFA: non-esterified fatty acid; BHB: beta-hydroxybutyrate; ALP: alkaline phosphatase; AST: aspartate aminotransferase; CK: creatin kinase; GGT: gamma-glutamyl transferase; LDH: lactate dehydrogenase; ppCO₂: partial pressure of carbon dioxide; HCO₃⁻: blood bicarbonate; AG: anion gap; ROS: reactive oxygen substances; SAC: serum antioxidant capacity; OSi (ROS/SAC): oxidative stress index.

studies (Evans, 2009): adherence to the principles of GLP is essential for those laboratories that perform regulatory studies, and it provides a good framework for laboratories that do not do so.

- The *pre-analytical* period is considered as important as the rest of the periods. In fact, several problems are related to sample handling or procedural misconceptions (Vap & Weiser, 2007). Therefore, this first step includes

not only sample collection but also sample conservation and transport to the lab, because some parameters are not usually measured in field conditions (Tóthóva *et al.*, 2012). This problem could be solved by using a handheld portable instrument (like a point of care testing, an analytical test performed in situ and with immediate availability of results).

It is very interesting to control this phase in relation to determine acid-base balance, due to physiological properties of blood. Several errors can occur in the pre-analytical phase, from handling to transportation, forcing in many cases to measure these samples using a handheld portable analyzer, avoiding a great number of problems that usually appear using traditional techniques. This device requires only specific humidity and temperature conditions (at least 90% and it runs between 16°C to 31°C, respectively).

One important question is when to perform the sample collection. In this sense is critical to take into account the time of feeding because they may influence some metabolite concentrations, which could provide wrong conclusions. Nevertheless, if we need to repeat sampling as a monitoring tool, venepuncture should be performed at approximately the same time of the day to minimize diurnal and prandial variations (Castillo & Hernández, 2016).

In relation to the type of sample, the use of serum has been recommended (Braun *et al.*, 2010), although the majority of the parameters can be measured using both plasma and serum. The blood sample (with EDTA or heparin as an anticoagulant) is placed in a centrifuge, which spins and separates the cells from the liquid part of the blood before sent to the laboratory, because they can go straight to the centrifuge without delay and therefore the separation occurs more easily (Castillo & Hernández, 2016). Clotted blood, in contrast, must be left at room temperature until the clot has fully formed (can be around 2 hours) before any attempt is made to separate it. If clotted samples are centrifuged too soon the serum itself will clot solid, and this can often result in the entire sample

being wasted. It is important to note also that the clotted blood must be collected into glass vials, or plastic vials which have been specially coated to be suitable for the purpose. Whole blood will stick to uncoated plastic as it clots, the clot will not retract, and the resulting serum will be haemolysed and of very poor quality. Also it has been recommended to store blood or serum samples in plastic vials (Russell & Roussel, 2007).

Once samples are separated and stored, these should be sent to the laboratory as soon as possible, on ice or with cold packs, avoiding interferences, due to an inadequate biological sample storage, because they may markedly affect the stability of many biochemical variables (Tóthóva *et al.*, 2012). It has been recommended that whenever possible, prolonged sample storage should occur at -70 °C. If a -70 °C freezer is not available, -20 °C storage for as long as 90 days is acceptable for common analytes used in sheep and goats medicine (Castillo & Hernández, 2016).

Analytical interference by endogenous substances on laboratory test is a common problem in laboratory medicine. These altered results may lead to repeat tests, incorrect interpretation, wrong diagnosis, and potentially inappropriate intervention and unfavourable outcome for the patients. Hemolysis, icterus, and lipemia commonly interfere with spectrophotometric methods with hemolysis being the most common (Ji & Meng, 2011).

Haemolyses should be avoided because of potential interference with test methodology modifying the obtained results in many analytes (*e.g.*, K⁺, phosphorus, cytosolic enzymes). The presence of lipemia or icterus can also potentially affect test results (false lectures in serum Na⁺, glucose, cholesterol or creatinine). Table 3 shows the effects of each type of altered serum on different parameters.

In relation to the *analytical procedure*, bibliography is extensive about the different methods to be used (colorimetric, enzymatic, etc.) and instrumentation. In a brief summary, and a general idea, it has been pointed out that a lack of information on techniques and procedures in the literature can often lead to a misleading situation, many of

Table 3. Effects of hemolysis, lipemia and icterus on tests results

Hemolysis ^[1]	Lipemia	Icterus
Increase in serum concentration of K, Mg, Fe and enzymatic activities of LDH, AST, CK.	Interferences in water content, causing false hyponatremia.	Interferences in measurement of serum glucose, creatinine and cholesterol.
Interferences in measurement with ultraviolet techniques (wavelengths at 340 nm) giving unpredictable values.	Interferences in measurement of serum total proteins, bilirubin, glucose, phosphorus and cholesterol.	
Interference with chemical reactions used for the determinations of glucose, cholesterol and LDH.		

^[1] LDH: lactate dehydrogenase; AST: aspartate aminotransferase; CK: creatin kinase.

which are difficult, or sometimes impossible, to draw conclusions from, based on a comparison of our results with the values published in the literature (Braun *et al.*, 2010). However, what it has been globally accepted is that complete analytical validation of the techniques is necessary before the routine use of any assay. For this analytical validation, the minimal parameters that should be included are intra-assay and inter-assay precision, accuracy and limit of detection. Nevertheless, the European Commission, in 2015, established for the member states functioning national GLP compliance monitoring programmes (EC, 2015).

— The *post-analytical phase* is the final step of the overall testing process and involves processing of results, issuing of reports that include their evaluation in the context of established reference values, together with the recommendation of a decision to carry out new trials, to assist decision-making in a clinical context.

Sampling theory

The interpretation of flock-based tests is different from interpreting laboratory tests for metabolites from individual animals (Oetzel, 2003). A review describes different theories (establishment of cut-off values) and methodologies (*i.e.* sample size needed) to detect a specific disease (Castillo & Hernández, 2016). In general terms, most of the suggestions are made for bovine, thus the number of samplings significantly varies in comparison with ovine and caprine flocks, with a greater population. Nevertheless, there are some questions that, independently of the species, should be taken into account if we want to obtain reliable and reproducible data (Elbers *et al.*, 1995):

— *Population size*: It is important to keep in mind that the sample size needed to estimate the prevalence of a process increases as the size of the population increases.

— *Allowable error in the estimated prevalence*: Usually, a smaller allowable error is accompanied by an increase in sample size.

— *Desired level of confidence*: Usually, a confidence level of 95% is used; the greater the confidence level desired, the larger the sample size needed. A 95% level of confidence means that when the survey is repeated 100 times, 95 times the observed prevalence will be within the stated confidence interval (Castillo *et al.*, 2016). We can reduce required sample numbers by reducing the level of confidence needed in the results. Clinical decision making does not require 95% confidence in a conclusion as for research, but 75% confidence in a result may be reasonable (Oetzel, 2003).

— *Between-herd and within-herd variance*: Most of our production animals are kept in clusters (pen, compartment, flock, herd), rather than individually (Elbers *et al.*, 1995). Animals within a cluster share common charac-

teristics such as nutrition, housing or environment. As a consequence, differences in prevalence between clusters are larger than differences between animals within clusters. Therefore, it is important to sample relatively more clusters and fewer animals within a cluster (two-stage sampling design) than in a situation without clustering of disease events. The two-stage random sampling is defined as a process in which clusters are first randomly selected and then individuals are selected from each cluster (Fraenkel *et al.*, 2012). Previously, a measure for agreement in status between animals within a cluster was given by the intra-cluster correlation coefficient (Donald & Donner, 1987). This coefficient can be used in the sample size calculations to account for the within and between cluster variance in prevalence.

Sampling includes selecting a representative part of a population in order to determine the parameters or characteristics of the entire population, and drawing conclusions about the populations. A significant reduction in costs is achieved (Elbers *et al.*, 1995) avoiding a complete population sampling.

On the other hand, the concept of population-based reference values (RV) or reference intervals (RI) was introduced in human medicine in 1969 and subsequently applied to veterinary science (Friedrichs *et al.*, 2012). Since their introduction, population-based RI has become one of the most popular and useful tools used in clinical decision-making processes. Previously, some authors (Radostits *et al.*, 2006) considered that a RI is based on a large number of samples obtained from the reference population and that should be calculated theoretically to include 95% of the healthy population.

In this scenario for a flock is generally recommended that at least 60 clinically healthy animals should be used to establish a reference interval. The new trends establish that a minimum of 120 reference individuals is recommended in order to determine reference limits by nonparametric methods with 90% confidence intervals (CI). Additional samples should be collected to allow for possible rejection of outliers (Friedrichs *et al.*, 2012).

Independently of the number and costs, it is important to point out that when we decide to establish our own reference interval group, all animals have to be in the same conditions (age, breed, physiological stage) avoiding interferences derived from biological variability (Castillo *et al.*, 2016).

Looking to the future: the use of other techniques for measuring metabolic profiles with deeper information

Metabolomics uses advanced analytical chemistry techniques to comprehensively measure large numbers of small molecule metabolites in cells, tissues and biofluids.

The ability to rapidly detect and quantify hundreds or even thousands of metabolites within a single sample is helping scientists paint a far more complete picture of system-wide metabolism and biology (Goldansaz *et al.*, 2017). This provides a platform for the investigation of drug toxicity, disease processes, and altered gene function (Nicholson *et al.*, 1999). Common metabolomics technologies include *top-down proteomics* using advanced ¹H-nuclear magnetic resonance (NMR), or metabolites chip (Nicholson *et al.*, 2002). Of these techniques, NMR has important advantages, including its ability to provide unbiased and rapid results on a wide range of small metabolites and leading the detection of altered metabolic pathways (Sun *et al.*, 2014, 2017). Mass spectrometry (MS), with or without gas or liquid chromatography, is one of the most common analytical techniques in metabolomics, being used to assess the metabolic profile in cows in the last years (Zhang *et al.*, 2013). To conduct such measurements plasma or serum samples are used. The first documented studies using NMR were performed on ketosis in dairy cattle (Klein *et al.*, 2012). It has been demonstrated that plasma NMR-based metabolomics, coupled with pattern recognition analytical methods, not only has the sensitivity and specificity to distinguish cows with clinical and subclinical ketosis from healthy controls, but also has the potential to be developed into a clinically useful diagnostic tool that could contribute to a further understanding of the disease mechanisms (Sun *et al.*, 2014). In addition, it requires little sample preparation and offers high sample throughput efficacy and reproducibility. Furthermore, the acquired spectral NMR profile from an individual reflects the metabolic imprints and evaluates metabolic changes associated with nutrition, and has been extensively applied in the area of nutrition science (Klein *et al.*, 2012).

The use of Fourier Transform mid-infrared spectroscopy as an analytical tool in farm animals has been proposed and offers fairly accurate measurement of various plasma biomarkers of great importance for the evaluation of the metabolism and inflammatory status of dairy cows (Calamari *et al.*, 2016).

Nevertheless, little is known about its application to sheep and goats. A recent study performed by Sun *et al.*, (2017) in pregnant ewes carrying twin fetuses demonstrates that the metabolomics approach has value for evaluating metabolism in pregnancy with advancing gestation. Thus, during normal pregnancy in sheep, related metabolites play an important role in amino acid and lipid metabolism for meeting the nutritional demands of pregnant ewes. Previous studies have demonstrated that undernutrition in pregnant ewes leads to alterations in foetal thyroid development, muscle mitochondrial function, and hepatic and renal gluconeogenic enzyme activity (Oliver *et al.*, 2001; Bloomfield *et al.*, 2004).

However, in our opinion although this line of determination of metabolic profiles is exciting, it can only be applied at the research level, providing relevant information about the pathogenesis of many metabolic disorders. However, there is still a long way to go before they can be applied in field conditions. Likewise, these methodologies are not yet widely available in many livestock research facilities. Furthermore, there continues to be a significant shortage of data resources that could facilitate the interpretation of livestock metabolomics data. A recent review describes that majority of metabolomics studies among all livestock categories that have been conducted in cattle focusing on various fields of bovine research (Goldansaz *et al.*, 2017). Metabolomics studies on sheep came third with 12% of the selected articles. Reports about metabolomics in goats are scarce. Most studies appear to be directed towards disease detection, production and bioproduct assessment, feed efficiency determination and reproduction. Data for livestock metabolome (LMDB) are available at a public database <http://lmdb.ca/> which reports concentrations that were transformed into a standardized concentration unit (micromolar; μ M) and each entry is associated with an abbreviated description of the experimental context, the sample type, and the methodologies used for the metabolomic analyses.

Conclusions

By monitoring and collecting the data through the use of metabolic profile tests the professional and farmer can obtain good information about the balance between the quality of the diet and the production requirements in the flock. In this way, different nutritional imbalances can be detected and corrected earlier than production, reproduction and health performances could be affected.

We must ensure that we apply the best suited procedures to our working conditions for both sampling and analysis, in order to ensure the accuracy of our results.

It must be remembered that metabolic profiles are almost useless without being coupled with animal and housing evaluations, body condition scoring and ration evaluation.

The published reference intervals in medium-large flocks are not always applicable in our flock conditions, so the best option would be to establish our own reference values for sheep or goat flocks. Nevertheless, several biological and mathematical factors have to be taken into consideration for the planning of a metabolic profile test as well as for its interpretation in order to obtain reliable data. Special care has to be taken in choosing statistical methods as well as for establishing reference values, otherwise the impact of extreme values or outliers could lead to false interpretation.

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