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# Laboratory Protocols in Animal Physiology

## Part II: Clinical Biochemistry

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## Index

Introduction to the spectrophotometry	6
Carbohydrates	
Determination of glucose	8
Clinical relevance: Diabetes mellitus	12
Proteins	
Determination of total protein by spectrophotometric assay	14
Determination of total protein by refractometry	17
Serum protein electrophoresis	18
Clinical relevance: Intestinal protein loss (protein-losing enteropathy)	19
Lipids	
Determination of total cholesterol	20
Determination of triglycerides	25
Clinical relevance: Hyperlipidemia	28
Minerals	
Determination of calcium	29
Clinical relevance: Milk fever	32
Determination of phosphorus	34
Clinical relevance: Downer cow syndrome	37
Determination of magnesium	38
Clinical relevance: Grass tetany	40
Nitrogenous substances	
Nitrogenous metabolites in vertebrates	42
Determination of urea	44
Clinical relevance: Hypoadrenocorticism	46
Determination of uric acid	47
Clinical relevance: Gout	49
Enzymes	
Introduction to clinical enzymology	50
Determination of alanine aminotransferase (ALT) activity	52
Other enzymes of clinical relevance	55

List of acronyms	58
References	60
Appendixes	61

#### Introduction to the spectrophotometry

Spectrophotometry is a methodology that enables the quantitative determination of a substance, cell density or enzymatic activity in a sample solution. Spectrophotometric analyses are applied to biological, clinical, and chemical research. The principle of spectrophotometry is based on the capacity of a certain compound to absorb the light (i.e., photons) that crosses the solution in a given wavelength. Schematic representation of the spectrophotometer's components is shown in Figure 1. The term "spectrum" refers to the spectrum of light that is selected for performing the analysis since the ability of a substance to absorb photons varies depending on the wavelength. The photons that are able to cross the sample define the transmittance of the sample. Conversely, the absorbance of the sample is the inverse logarithm of the transmittance and corresponds to the numerical value provided by the spectrophotometer.



Figure 1. Schematic representation of the main components of a spectrophotometer.

As stated by the Beer-Lambert law, there is a linear relationship between the absorbance and the amount of the compound in the sample. Generally speaking, the higher is the absorbance, the higher is the amount of a compound in the solution. To transform the absorbance in a value that might be, for instance mM or mg ml<sup>-1</sup>, we have to perform a standard curve. The standard curve consists of the measurement of the absorbance of several concentrations (e.g., 12.5, 25, 50, 100, 200, 400  $\mu$ M) of a reference substance, which is usually very similar to the one we aim to determine by spectrophotometry (e.g., malondialdehyde for the assessment of the lipid peroxidation). Ideally, the standard curve has to be run in duplicate and with at least

5 different concentration values. Moreover, the correlation coefficient  $(r^2)$  of the standard curve should be as close as possible to 1, at least  $\geq 0.95$  (Figure 2). Thus, the concentration of the investigated substance is then inferred from the standard curve. Care should be given to avoid any factors that may interfere with the light transmission. Therefore, the cuvettes have to be handled with clean gloves and only touched on the sides that will not be crossed by the light beam. Moreover, the samples should be devoid of bubbles or particles. It is also important to bear in mind that before analyzing the standard curve and the samples, the spectrophotometer has to be calibrated against the blank, which is composed of the solution used for the analysis devoid of the sample.



Figure 2. Representative standard curve for spectrophotometric assays.

#### **Determination of glucose**

Glucose is the most abundant monosaccharide in the blood and many other biological fluids and also the main substrate used by cells for energy production (Figure 3).



Figure 3. Glucose molecule.

The levels of blood glucose or glycemia are regulated by two hormones: insulin and glucagon, which are both peptides and produced by the pancreatic islets (also named Langerhans islets in honor to Paul Langerhans, a German medical student who first described them in 1869). Insulin is produced by the  $\beta$ -cells, whereas glucagon is produced by  $\alpha$ -cells. The pancreatic islets represent the endocrine part of the pancreas because they produce hormones, which are substances that are released into the blood and exert their function on far target organs. Pancreas is also an exocrine gland because it releases the pancreatic juice into the gastrointestinal tract. The pancreatic juice is composed of enzymes (e.g., amylase, lipase, carboxypeptidase) and bicarbonate ions, which help the digestion of nutrients. Insulin and glucagon regulate the glycemia by an opposite way: insulin lowers blood glucose levels by stimulating cellular uptake and the synthesis of glycogen (glycogen synthesis or glycogenesis); on the other hand, glucagon raises blood glucose levels by stimulating the breakdown of glycogen in the liver (glycogenolysis). During and soon after each meal, blood glucose levels raise and stimulate the release of insulin, which favors the cellular uptake of glucose, thus reducing glycemia. At fasting or between meals, on the other hand, low blood

glucose levels stimulate the release of glucagon, which stimulates glycogenolysis, thus increasing blood glucose levels. The action of both hormones guarantees the homeostasis of blood glucose levels. Also, other hormones such as glucocorticoids and catecholamines (epinephrine and norepinephrine) participate in the regulation of blood glucose levels under conditions like prolonged stress or fasting.

Glucose concentration can be measured by reagent strips (urine samples), portable blood glucose meter (PBGM, blood sample), and by spectrophotometric assays (urine and blood samples). For spectrophotometric analysis, samples from blood serum or plasma (in EDTA or heparin) and urine can be used. For the determination of glycemia, it is recommended to collect blood plasma or serum in a tube containing fluoride, which is a glycolysis inhibitor. Otherwise, red blood cells (RBCs) would consume the glucose within 30 minutes.

In this seminar, glucose concentration will be determined by spectrophotometric assay. The intensity of the pink color observed following the reaction is proportional to the amount of glucose in the sample. Glucose concentration is expressed as mg dl<sup>-1</sup> in conventional units or mmol l<sup>-1</sup> in SI units (see the Appendix I for reference values in domestic animals).

#### **Equipment:**

- Samples (e.g., blood plasma or serum, urine)
- NB: The stability of glucose in blood samples without a glycolytic inhibitor is 8 hours if stored at room temperature (20-25 °C) or 3 days if stored in the fridge (4-8 °C). In urine samples stored in the fridge (4-8 °C), the stability of glucose is limited to 24 hours because bacteria commonly found in the urine collected by spontaneous micturition quickly decompose this carbohydrate.
- Distilled water (for blank)
- Glucose standard concentration (hereinafter standard; provided by the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic).)
- Glucose reagent solution (hereinafter R1; solution's composition is available in Appendix II; provided by the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic)
- Pipettes and tips
- Tubes

- Cuvettes
- Water bath set at 37 °C
- Spectrophotometer set at 500 nm (range 490-550 nm)

## **Procedure:**

- 1. This protocol is based on the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic.
- 2. Take 3 clean tubes and label them as blank, standard, or sample.
- 3. Place 1 ml of the glucose reagent solution in each tube as indicated in Table 1.
- 4. Add 10  $\mu$ l of distilled water in the blank tube, 10  $\mu$ l of standard in the standard tube, and 10  $\mu$ l of sample in the respective sample tube.
- 5. Mix it and incubate for 5-10 min at 37°C (alternatively 20 min at 20-25°C)
- 6. Measure the absorbance of the sample and the standard against the blank within 60 minutes.

Table 1. Determination of glucose concentration by spectrophotometric assay.

	Blank	Standard	Sample
Reagent 1	1.0 ml	1.0 ml	1.0 ml
Sample			0.01 ml
Standard		0.01 ml	
Distilled water	0.01 ml		

Glucose concentration (mmol l<sup>-1</sup>):

 $\frac{Sample \; absorbance}{Standard \; absorbance} \times Standard \; concentration$ 

Standard concentration: 5.55 mmol 1<sup>-1</sup>

**Unit conversion:** 1 mmol  $1^{-1} = 18 \text{ mg d}1^{-1}$ 

Horse	Cow	Sheep	Goat	Pig	Dog	Cat	Rabbit
62-134	40-100	50-80	50-75	85-150	70-120	80-120	75-155

**Table 2.** Reference values for blood glucose concentration (mg dl<sup>-1</sup>) in domestic animals.

The blood glucose levels are physiologically lower in ruminants than in other domestic animals.

## Main causes of hypoglycemia (low value of blood glucose concentration)

*Pathological:* Starvation, fasting, ketosis (it might be observed in dairy cows in the early lactation).

*Artifact:* Prolonged sample storage. Serum or plasma must be separated from red blood cells (RBCs) and white blood cells (WBCs) within 30 minutes after collection to minimize glucose consumption by cells. Also, expired reagents or inadequate calibration may lead to erroneous results.

*Iatrogenic (caused by medical intervention)*: inappropriate dosage of insulin during treatment of diabetes mellitus.

## Main causes of hyperglycemia (high value of blood glucose concentration)

*Physiological* (transitory condition): Postprandial (after a meal), stress (common in cats and rabbits because of excitement, fear, and aggression).

*Pathological*: Endocrine diseases (e.g., diabetes mellitus, hyperadrenocorticism), tumors (e.g., adrenal glands), inflammation (e.g. pancreas).

Whenever an animal shows hyperglycemia, it should be checked for glycosuria (presence of glucose in urine).

Under physiological conditions, the kidneys filter and reabsorb all blood glucose. However, the ability of the kidney to reabsorb the glucose is limited by the glucose renal threshold. This

means that over a certain limit, which varies across species, the kidneys cannot reabsorb the glucose, therefore the carbohydrate appears in the urine (glycosuria). Glycosuria often occurs because hyperglycemia (normal glycemia is 70-120 mg dl<sup>-1</sup> in dogs and 80-120 mg dl<sup>-1</sup> in cats) exceeds the renal threshold for glucose reabsorption (e.g., 180 mg dl<sup>-1</sup> in dogs and 280 mg dl<sup>-1</sup> in cats). Physiologically, urine from domestic animals must be negative for glucose.

#### Main causes of glycosuria (presence of glucose in the urine)

*Physiological:* puppies younger than 8 weeks old may show mild glycosuria due to immaturity of the kidneys. Note that also stress represents a frequent cause of transient glycosuria in cats and rabbits.

Pathological: Diabetes mellitus, renal diseases (e.g., Fanconi syndrome).

#### Clinical relevance: Diabetes mellitus

Diabetes mellitus is an endocrine disorder that mainly affects dogs and cats. Several factors may contribute to the appearance of this disease: genetic predisposition, obesity, physical inactivity, pancreatic infections, among others. Canine breeds such as Australian Terrier, Schnauzer, Bichon Frise, Miniature poodle, and Samoyed show a high risk of diabetes mellitus. In general, this pathological condition occurs at 4-14 years of age in dogs and at 10-13 years in cats. Both sexes are affected, although female dogs show a greater risk of developing this disease than males. In neutered cats, on the other hand, tomcats are twice at risk of developing diabetes than queens. In diabetic animals, hyperglycemia is associated with glycosuria since the blood glucose levels exceed the glucose renal threshold. The presence of glucose in the urine induces osmotic diuresis causing polyuria (increased volume of urine) and compensatory polydipsia (excessive thirst), which means that the animal drinks more than it usually does to avoid dehydration. Because insulin is also responsible for the feeling of satiety, its deficiency is associated with polyphagia (increased appetite). However, since the cells cannot uptake glucose, the animal losses weight. Therefore, the four clinical signs of diabetes mellitus include: polyuria, polydipsia, polyphagia, and weight loss. Hyperglycemia and glycosuria are the most frequent abnormalities observed by laboratory tests. Other

abnormalities that can be observed include high values of lipids (hyperlipidemia), typically high values of triglycerides, but also cholesterol (less frequently).

#### Determination of total protein by spectrophotometric assay

Proteins are the most diverse biological macromolecules both in terms of structure and function. Proteins are composed of amino acids: typically, the diversity and complexity of animal proteins relies on 20 different amino acids. Some animals are not able to synthesize *de novo* some amino acids, which therefore have to be introduced into the diet: for this reason, these amino acids are called essential. In humans, essential amino acids include histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Essential amino acids may slightly vary between species and stage of development: arginine, for instance, is an essential amino acid in growing rats but not in adult rats and humans.

Proteins participate in several functions such as transport (e.g., hemoglobin transports  $O_2$ , ceruloplasmin transports  $Cu^{2+}$ ), motion (e.g., actin and myosin are the main muscular proteins), defense (e.g., immunoglobulins protect the body against infections), regulation (e.g., insulin regulates blood glucose levels), or enzymatic catalysis (e.g., pepsin contributes to the protein digestion into the stomach). Blood plasma contains a complex variety of proteins, which are mainly represented by albumin (55% of total protein) and globulins (38%). Albumin is a protein (mw 69,000 Daltons) produced by the liver and responsible to maintain the colloid osmotic pressure of blood: albumin participates in the maintenance of the water balance between the blood and the interstitial fluid. When the albumin levels are lower than normal values, the blood is not able to retain most of the water that eventually persists in the interstitial space provoking edema.

Total protein concentration in biological fluids can be measured by refractometry or by spectrophotometry. Both methods only quantify the total protein concentration. Electrophoresis of blood serum proteins is useful (see the next chapter) to distinguish protein fractions and identify which one might be altered. In this seminar, the total protein concentration is determined by spectrophotometric assay (biuret method). Blood serum or plasma is placed in an alkaline solution rich in Cu<sup>2+</sup> ions. The blue color formed during the reaction is proportional to the protein concentration. Total protein concentration in blood is expressed as g dl<sup>-1</sup> or g l<sup>-1</sup> in SI units (see Appendix I for reference values in domestic animals) and usually ranges from 6 to 8 g dl<sup>-1</sup> (equivalent to 60-80 g l<sup>-1</sup>).

14

## **Equipment:**

- Sample (e.g., non hemolytic blood plasma or serum, urine).
- NB: Samples are stable 6 days if stored at room temperature (20-25 °C), 4 weeks if stored in the fridge (4-8 °C), or at least one year if stored in the freezer (-20 °C).
- Distilled water (for blank)
- Protein standard concentration (hereinafter standard; provided by the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic)
- Protein reagent solution (hereinafter R1; solution's composition is available in Appendix II; provided by the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic)
- Pipettes and tips
- Tubes
- Cuvettes
- Water bath set at 37 °C
- Spectrophotometer set at 546 nm (520-560 nm)

#### **Procedure:**

- 1. This protocol is based on the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic.
- 2. Take 3 clean tubes and mark them as blank, standard, or sample.
- 3. Place 1 ml of R1 in each tube as indicated in Table 3.
- Add 20 μl of distilled water in the blank tube, 20 μl of standard in the standard tube, and 20 μl of sample in the respective sample tube.
- 5. Mix it and incubate 10 min at 37°C in the dark.
- 6. Measure the absorbance of the sample and the standard against the blank within 30 minutes.

	Blank	Standard	Sample
Reagent 1	1.0 ml	1.0 ml	1.0 ml
Sample			0.02 ml
Standard		0.02 ml	
Distilled water	0.02 ml		

Table 3. Determination of protein concentration by spectrophotometric assay.

Protein concentration (g dl<sup>-1</sup>):

 $\frac{Sample \; absorbance}{Standard \; absorbance} \times Standard \; concentration$ 

Standard concentration: 5.06 g dl<sup>-1</sup>

<b>Table 4.</b> Reference values for blood total	protein concentration (g dl <sup>-1</sup>	) in domestic animals.
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Horse	Cow	Sheep	Goat	Pig	Dog	Cat	Rabbit
5.6-7.6	6.7-7.5	6.0-7.9	6.4-7.0	7.9-8.9	5.4-7.5	6.0-7.9	5.4-7.5

## Main causes of hypoproteinemia (low value of blood total protein concentration)

*Pathological:* dietary protein deficiency, hemorrhage, burns, as well as hepatic, intestinal, and renal diseases.

#### Main causes of hyperproteinemia (high value of blood total protein concentration)

Pathological: dehydration, fever, inflammation, immune-mediated diseases.

#### Determination of total protein concentration by refractometry

Refractometry is an analytical procedure that allows the assessment of fluid compositions based on their refractive index. In veterinary practice, the handheld refractometer is used to estimate the total protein concentration of blood serum and the urine specific gravity, which is proportional to the concentration of solutes in the solution.

To evaluate the protein concentration in the blood serum, place a drop of sample on the refractometer surface (Figure 4A). Then, observe the sample under the light: the blue line in the scale S.P. (serum proteins) will mark the value of total protein of your sample (Figure 4B). The readings have to be performed at room temperature.



**Figure 4.** Determination of total protein by refractometry. A) Refractometer; B) representative measurement of total protein by refractometry.

#### Serum protein electrophoresis

Electrophoresis is a quantitative method that allows the separation of protein fractions on agarose or polyacrylamide gels at pH 8.4-8.6 under electric current. Protein migration occurs thanks to the negative electric charge and is proportional to their molecular weight and size. The gel is then scanned by a densitometer to provide the characteristic pattern. Protein electrophoresis is generally performed on blood serum, but it can eventually be performed on urine. Samples can be fresh, refrigerated or frozen.

From the left to the right side, serum proteins are separated into four fractions: albumin,  $\alpha$ globulins,  $\beta$ -globulins, and  $\gamma$ -globulins, respectively. Canine and feline  $\alpha$ - and  $\beta$ -globulins are further classified into:  $\alpha 1$ ,  $\alpha 2$ ,  $\beta 1$ , and  $\beta 2$  (Figure 5). Given their abundance and homogeneity, albumin normally shows a narrow but very high peak; on the other hand,  $\gamma$ -globulins are very heterogeneous (there are different types of immunoglobulins: IgA, IgD, IgE, IgG, and IgM) and normally less abundant: their peak is therefore lower and wider than that of albumin.



Figure 5. Representative example of a normal serum protein electrophoretogram.

#### Clinical relevance: Intestinal protein loss (protein-losing enteropathy)

The condition is characterized by the loss of proteins from the intestinal tract, which results in hypoproteinemia. One of the most frequently observed symptoms is diarrhea together with weight loss and vomiting. In a few cases, however, diarrhea might be absent. Moreover, since albumin is mainly involved in the maintenance of the colloid osmotic pressure of the blood, their decrease is frequently associated with edema. In addition to hypoproteinemia (both albumin and globulins are usually decreased), hypocholesterolemia (low blood cholesterol levels), hypocalcemia (low blood Ca<sup>2+</sup> levels), hypomagnesemia (low blood Mg<sup>2+</sup> levels), and anemia (low RBC number, Hct and/or Hb concentration) might be also observed. The condition might be severe when total protein is lower than 2.5 g dl<sup>-1</sup>. Protein-losing enteropathy is mostly observed in dogs and usually associated with diseases of the gastrointestinal tract: heavy parasite infestation (e.g., Giardia), viral (e.g., parvovirus) and bacterial (e.g., salmonella) infections, ulcers, inflammation, and cancer. Overdose of some drugs (e.g., phenylbutazone, which is an analgesic and anti-inflammatory drug used to treat lameness) may also induce intestinal protein loss in horses. Some canine breeds like Yorkshire terrier, Rottweiler, German shepherd, and Shar Pei seem to have a higher risk of developing this disease than other breeds. Unfortunately, the survival rates of dogs affected by protein-losing enteropathy do not exceed 40%.

#### **Determination of total cholesterol**

Cholesterol is a lipid (more precisely a sterol, Figure 6) that can be synthesized by animal cells or introduced into the organism with the diet (mainly in carnivores and omnivores). As any other type of lipids such as wax, phospholipids, or fatty acids, cholesterol is a hydrophobic molecule. Cholesterol is a major component of the cell membrane to which it provides structural support and fluidity over a range of physiological temperatures.



Figure 6. Cholesterol molecule.

The behavior of cholesterol changes according to the temperature: it guarantees the fluidity of the cell membrane at low temperatures, while it provides rigidity at high temperatures. Therefore, the abundance of cholesterol helps to protect the cell membranes against injury provoked by temperature's changes, which occurs during artificial processes like refrigeration or cryopreservation of cells and tissues. Curiously, since porcine sperm cells show a reduced content of cholesterol compared to other domestic species, they are particularly sensitive to the process of cryopreservation. For this reason, in the porcine industry over 99% of artificial inseminations worldwide are performed with semen stored at 15-20 °C. The inconvenience of this type of storage is the limited sperm lifespan, which does not usually exceed 5 days. By contrast, sperm lifespan of cryopreserved samples is theoretically unlimited. Cholesterol is

also the precursor of steroid hormones, bile acids, and cholecalciferol, also known as vitamin  $D_3$ . Also, steroid hormones are synthesized from cholesterol: sexual steroids (e.g., progesterone, testosterone, and estrogens) and corticosteroids (e.g., cortisol and aldosterone). Approximately half of the cholesterol requirements are synthesized by the liver, while the remaining is produced by peripheral tissues or introduced with the diet.

Cholesterol and triglycerides mostly circulate in the blood bound to proteins (apolipoproteins) to form lipoproteins, which might be classified according to their size, lipid content, and electrophoretic mobility, among others. According to their density, lipoproteins are classified as: chylomicrons, very low-density lipoproteins (VLDLs), low-density lipoproteins (LDLs), and high-density lipoproteins (HDLs). The density of lipoproteins is negatively correlated with their content of triglycerides: therefore, the amount of triglycerides in chylomicrons and VLDLs is higher than that found in LDLs and HDLs. Conversely, HDLs and LDLs show a higher amount of cholesterol than VLDLs and chylomicrons. Lipoproteins are also functionally different: for instance, while VLDLs mainly transport triglycerides synthesized from the liver to the tissues, HDLs help to remove free cholesterol from the tissues transporting it back to the liver. Bile is the main route of cholesterol excretion. Given their low density, VLDLs and LDLs also tend to deposit into the blood vessels, typically arteries (atherosclerosis). Atherosclerosis is a condition characterized by the reduced diameter of arteries and is associated with an increased risk of cardiovascular disease in humans. For this reason, generally speaking, LDLs are called "bad cholesterol" while HDLs are called "good cholesterol". However, the association between high levels of LDL and increased risk of cardiovascular disease has not been found in domestic animals.

Cholesterol concentration can be determined by spectrophotometric, chromatographic, and enzymatic methods. In this seminar, you will perform the determination of total cholesterol in serum or plasma by spectrophotometry. Because of their bond to proteins, enzymatic digestion is required in order to determine the concentration of cholesterol and triglycerides in the blood. Cholesterol levels are expressed as mg dl<sup>-1</sup> in conventional units or as mmol l<sup>-1</sup> in SI units (see Appendix I for reference values in domestic animals).

## **Equipment:**

- Sample (blood serum or plasma, the latter with heparin or EDTA).
- NB: Samples are stable 7 days if stored at room temperature (20-25 °C) or in the fridge (4-8 °C) and up to 3 months if stored in the freezer (-20 °C).
- Distilled water (for blank)
- Cholesterol standard concentration (hereinafter standard; provided by the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic)
- Cholesterol reagent solution (hereinafter R1; solution's composition is available in Appendix II; provided by the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic)
- Pipettes and tips
- Tubes
- Cuvettes
- Water bath set at 37 °C
- Spectrophotometer set at 500 nm

## **Procedure:**

- 1. This protocol is based on the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic.
- 2. Take 3 clean tubes and mark them as blank, standard, or sample.
- 3. Place 1 ml of R1 in each tube as indicated in Table 5.
- Add 10 μl of distilled water in the blank tube, 10 μl of standard in the standard tube, and 10 μl of sample in the respective sample tube.
- 5. Mix it and incubate 10 min at 37°C in the dark.
- 6. Measure the absorbance of the sample and the standard against the blank within 1 hour.

	Blank	Standard	Sample
Reagent 1	1.0 ml	1.0 ml	1.0 ml
Sample			0.01 ml
Standard		0.01 ml	
Distilled water	0.01 ml		

Table 5. Determination of cholesterol concentration by spectrophotometric assay.

Total cholesterol concentration (g dl<sup>-1</sup>):

 $\frac{Sample \ absorbance}{Standard \ absorbance} \times Standard \ concentration$ 

Standard concentration: 4.14 mmol 1<sup>-1</sup>

**Unit conversion:** mg dl<sup>-1</sup> = mmol  $1^{-1} / 0.026$ 

Table 6. Reference values for total cholesterol concentration (mg dl<sup>-1</sup>) in domestic animals.

Horse	Cow	Sheep	Goat	Pig	Dog	Cat	Rabbit
75-150	80-180	52-76	80-130	36-54	135-278	71-156	10-80

#### Main causes of hypercholesterolemia (high values of blood cholesterol)

The condition is recognized only in dogs and cats. In herbivores, cholesterol levels are physiologically very low.

*Physiological:* Mild increase can be observed after a meal (not frequent since chylomicrons contain little amount of cholesterol)

*Pathological:* Liver and endocrine (e.g., diabetes mellitus, Cushing's disease, and hypothyroidism) diseases; inherited disorders have been reported in canine breeds like Briard, Rottweiler, Shetland sheepdog, and Doberman.

*Artifact:* severe hemolysis due to prolonged storage of blood samples may induce a false increase of cholesterol.

*Iatrogenic*: some drugs like corticosteroids can increase cholesterol concentration.

## Main causes of hypocholesterolemia (low values of blood cholesterol)

*Pathological:* Intestinal diseases (e.g., malabsorption), cancer, severe malnutrition, and sometimes paradoxically associated with liver diseases.

Artifact: severe jaundice may induce a false decrease of cholesterol.

#### **Determination of triglycerides**

Triglycerides, also named triacylglycerols, are lipids composed of a backbone molecule of glycerol bound to three esterified fatty acids (Figure 7). The main function of triglycerides is to store the excess of energy intake for later use. The three fatty acids that compose a molecule of triglyceride do not need to be identical; sometimes they are very different from one another.



Figure 7. Example of triglyceride molecule.

Fatty acids are classified as saturated (whether they have not double bonds in their molecule, e.g., palmitic acid) or unsaturated fatty acids (whether they show at least one double bond in their molecule, e.g., linolenic acid). Polyunsaturated fatty acids (PUFAs) contain at least two double bonds in their molecules. Animals generally synthesize saturated fatty acids, while plants generally synthesize unsaturated fatty acids. Because animals cannot synthesize PUFAs *de novo*, but they have to take them with the diet, PUFAs represent essential nutrients for animals.

The composition of fatty acids is expressed by the lipid number. For instance, the lipid number of linoleic acid is C18.2 $\omega$ 6, where: 18 indicates the number of carbon's atoms (C; numbered from  $\alpha$  to  $\omega$ , the latter corresponds to the methyl end -CH<sub>3</sub>); 2 indicates the number

of double bonds in the molecule; and 6 indicates the position of the first double bond encountered starting from the methyl end. Therefore, when we speak about omega-3 ( $\omega$ 3) or omega-6 fatty acids ( $\omega$ 6), we refer to PUFA in which the double bond is located in the third or sixth last atom of C within the molecule, respectively.

Triglycerides concentration can be determined by spectrophotometric assay and is expressed as mg dl<sup>-1</sup> in conventional units or as mmol l<sup>-1</sup> in SI units (see Appendix I for reference values in domestic animals).

## **Equipment:**

- Sample (blood serum or plasma, the latter with heparin or EDTA)
   NB: Samples are stable 7 days if stored in the fridge (4-8 °C) or at least one year if stored in the freezer (-20 °C)
- Distilled water (for blank)
- Triglycerides concentration (hereinafter standard; provided by the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic)
- Triglycerides reagent solution (hereinafter R1; solution's composition is available in Appendix II; provided by the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic)
- Pipettes and tips
- Tubes
- Cuvettes
- Water bath set at 37 °C
- Spectrophotometer set at 546 nm

## **Procedure:**

- 1. This protocol is based on the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic.
- 2. Take 3 clean tubes and mark them as blank, standard, or sample.
- 3. Place 1 ml of R1 in each tube as indicated in Table 7.

- 4. Add 10  $\mu$ l of distilled water in the blank tube, 10  $\mu$ l of standard in the standard tube, and 10  $\mu$ l of sample in the respective sample tube.
- 5. Mix it and incubate 10 minutes at 37  $^{\circ}$ C
- 6. Measure the absorbance of the sample and the standard against the blank within 1 hour.

**Table 7.** Determination of triglycerides concentration by spectrophotometric assay.

	Blank	Standard	Sample
Reagent 1	1.0 ml	1.0 ml	1.0 ml
Sample			0.01 ml
Standard		0.01 ml	
Distilled water	0.01 ml		

## Triglycerides concentration (mg dl<sup>-1</sup>):

 $\frac{Sample \; absorbance}{Standard \; absorbance} \times Standard \; concentration$ 

Standard concentration: 1.44 mmol 1<sup>-1</sup>

**Unit conversion:** mg dl<sup>-1</sup> = mmol  $l^{-1} \times 88.57$ 

Table 8. Reference values for	· blood triglycerides lev	vels (mg dl <sup>-1</sup> )	in domestic animals
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Horse	Cow	Sheep	Goat	Pig	Dog	Cat	Rabbit
14-65	10-19				29-291	25-160	

## Main causes of hypertriglyceridemia (high values of triglycerides in the blood)

*Physiological:* After a meal (postprandial) as a consequence of increased blood levels of chylomicrons. Usually, blood serum or plasma shows a milky appearance.

*Pathological:* Pancreas inflammation (i.e., pancreatitis) and endocrine (e.g., diabetes mellitus, hypothyroidism) diseases; prolonged dietary carbohydrate deficiency (starvation or anorexia): frequent in horses but rare in cattle.

*Iatrogenic (caused by medical intervention)*: some drugs like corticosteroids can increase levels of triglycerides.

#### Main causes of hypotriglyceridemia (low values of triglycerides in the blood)

The condition does not have clinical relevance.

#### Clinical relevance: Hyperlipidemia

Hyperlipidemia is a condition characterized by increased blood levels of cholesterol and/or Hyperlipidemia physiologically occurs after (postprandial triglycerides. a meal hyperlipidemia). High levels of lipids in the blood may also interfere with several analyses like the determination of glucose or calcium (Ca<sup>2+</sup>). For this reason, blood analysis should be better performed at fasting. Postprandial lipidemia generally disappears within 4-6 hours after a meal and is a common cause of high blood values of triglycerides in veterinary practice. In some cases, hyperlipidemia may also be an inherited issue in cats or in some dog breeds like Schnauzer, Shetland sheepdog, and Collie. Hyperlipidemia may also occur as a consequence of other diseases such as liver, renal, and endocrine diseases (e.g., diabetes mellitus, hypothyroidism, Cushing's disease). In pony mares and donkeys, hyperlipidemia arises as a consequence of impaired energy balance, which is frequently associated with obesity, stress, and late pregnancy. The condition might be fatal in 60% of the cases. In most of the cases, hyperlipidemia is asymptomatic. In dogs and cats, symptoms like ocular diseases, anorexia, abdominal pain, vomiting, and diarrhea might be observed. Unlike humans, the condition is not associated with an increased risk of cardiovascular diseases. On the other hand, dogs suffering from hyperlipidemia show an increased risk of pancreatitis (inflammation of the pancreas). Appropriate management of the hyperlipidemic patient includes fat-restricted diet with regular clinical and laboratory examinations.

#### **Determination of calcium**

Calcium  $(Ca^{2+})$  is a macromineral (i.e., a large amount in the body and dietary need, usually as mg dl<sup>-1</sup>) like potassium (K<sup>+</sup>) and sodium (Na<sup>+</sup>). Ca<sup>2+</sup> participates in several biological functions like muscular contraction, blood coagulation (clotting), neurotransmission, and reproductive function such as lactation in mammals and egg-shell formation in reptiles and birds. Together with phosphorus (P), Ca<sup>2+</sup> forms the skeleton and the teeth. Actually, around 98% of the Ca<sup>2+</sup> of the body is found in the skeleton. The remnant 2% is found in the extracellular fluids including the blood: half of Ca<sup>2+</sup> in the blood is found as a free ionized form, the remnant is mainly bound to albumin. Blood Ca<sup>2+</sup> levels are maintained within a strict range of 9-10 mg dl<sup>-1</sup> by a complex mechanism that involves the skeletal, digestive, urinary, and endocrine systems. The blood levels of Ca<sup>2+</sup> are regulated by two hormones: parathyroid hormone (PTH, produced by the parathyroid glands) and calcitonin (produced by the thyroid gland). Both thyroid and parathyroid glands are located in the neck, although the latter might vary in size and number among domestic animals. The PTH is released when the blood levels of Ca<sup>2+</sup> drop below the normal range. As a consequence, PTH stimulates the mechanism for renal reabsorption of Ca<sup>2+</sup> in order to minimize the urinary loss of this macroelement. When the losses of  $Ca^{2+}$  are larger, the PTH stimulates the absorption of  $Ca^{2+}$ from the digestive system and the release of  $Ca^{2+}$  from the skeleton (bone resorption). The absorption of  $Ca^{2+}$  in the digestive system may occur by passive transport when the concentration of  $Ca^{2+}$  is particularly high in the diet (e.g., milk) or by active transport thanks to the dihydroxyvitamin D or calcitriol, which is the active form of vitamin D synthesized by the kidney. However, hindgut fermenters (e.g., rabbits and horses) represent an exception since all available  $Ca^{2+}$  is absorbed in the digestive system independently of the vitamin D. For this reason, horses and rabbits show larger concentration of  $Ca^{2+}$  in the urine compared to other domestic animals. Moreover, to prevent the precipitation of Ca<sup>2+</sup>, the urine of these species is rich in mucus, which confers the urine a cloudy appearance. On the other hand, when the blood levels of  $Ca^{2+}$  are above normal values, the thyroid gland releases calcitonin, which inhibits the bone resorption and renal reabsorption, therefore stimulating urinary loss. Both reduced (hypocalcemia) or increased (hypercalcemia) levels of Ca2+ are critical conditions for animal health.

The levels of  $Ca^{2+}$  can be determined by spectrophotometric assay on blood plasma and urine. Blood  $Ca^{2+}$  concentration is conventionally expressed as mg dl<sup>-1</sup> or as mmol l<sup>-1</sup> in SI units (see Appendix I for reference values in domestic animals). Overall, the blood  $Ca^{2+}$  levels range from 9 to 12 mg dl<sup>-1</sup> or from 2.0 to 3.5 mmol l<sup>-1</sup> across domestic animals.

## **Equipment:**

- Samples (non-hemolytic blood plasma and urine).
- NB: Blood samples are stable 7 days if stored at room temperature (20-25 °C), 3 weeks if stored in the fridge (4-8 °C) or at least 8 months if stored in the freezer (-20 °C); Urine samples are stable 2 days if stored at room temperature (20-25 °C), 4 days if stored in the fridge (4-8 °C) or 3 weeks if stored in the freezer (-20 °C, samples can be frozen just once)
- Distilled water (for blank)
- Ca<sup>2+</sup> standard concentration (hereinafter standard; provided by the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic)
- Ca<sup>2+</sup> reagent solution (hereinafter R1; solution's composition is available in Appendix II; provided by the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic)
- Pipettes and tips
- Tubes
- Cuvettes
- Water bath set at 37 °C
- Spectrophotometer set at 650 nm (630-670 nm)

#### **Procedure:**

- 1. This protocol is based on the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic.
- 2. Take 3 clean tubes and mark them as blank, standard, or sample.
- 3. Place 1 ml of R1 in each tube as indicated in Table 9.
- 4. Add 10  $\mu$ l of distilled water in the blank tube, 10  $\mu$ l of standard in the standard tube, and 10  $\mu$ l of sample in the respective sample tube.
- 5. Mix it and incubate 1 min at 37 °C.

	Blank	Standard	Sample
Reagent 1	1.0 ml	1.0 ml	1.0 ml
Sample			0.01 ml
Standard		0.01 ml	
Distilled water	0.01 ml		

**Table 9.** Determination of  $Ca^{2+}$  concentration by spectrophotometric assay.

Ca<sup>2+</sup> concentration (mmol l<sup>-1</sup>):

 $\frac{Sample \; absorbance}{Standard \; absorbance} \times Standard \; concentration$ 

Standard concentration: 2.48 mmol 1-1

Unit conversion: mg dl<sup>-1</sup> = mmol l<sup>-1</sup> / 0.25

**Table 10.** Reference values for blood Ca<sup>2+</sup> concentration (mg dl<sup>-1</sup>) in domestic animals.

Horse	Cow	Sheep	Goat	Pig	Dog	Cat	Rabbit
10.2-13.4	8.0-11.4	11.5-12.8	8.9-11.7	7.1-11.6	9.1-11.7	8.7-11.7	11-14

## Main causes of hypercalcemia (high values of blood Ca<sup>2+</sup>):

The condition is overall less frequent than hypocalcemia.

Pathological: hyperparathyroidism and cancer.

*Iatrogenic (caused by medical intervention)*: due to excessive venous stasis during blood sample collection and prolonged administration of drugs like calcium borogluconate (frequent in cows).

#### Main causes of hypocalcemia (low values of blood Ca<sup>2+</sup>)

*Pathological*: hypoalbuminemia, periparturient hypocalcemia ("milk fever" in the dairy cow is frequent in the following days after parturition, see the next section for more details), pancreatitis, renal diseases, and oxalate poisoning (due to ingestion of some plants or antifreeze solution like ethylene glycol, which is unfortunately frequent in cats).

Artifact: excess of anticoagulant in the blood sample.

#### Clinical relevance: Milk fever

Milk fever, also known as periparturient paresis, is a condition that often affects cattle, sheep, and goats. Usually 5-10% of dairy cattle approaching or just after calving is affected by milk fever. Around 80% of the cases occur within 2 days after delivery. The condition may however arise in the weeks before or after the delivery. Cows of any age and breed are susceptible, although the milk fever seems to be more frequent in Jersey cows and in older individuals (usually at 5-10 years of age). Overall, blood serum Ca<sup>2+</sup> levels decline in all adult cows at calving due to the onset of lactation. However, first-year cows rarely develop milk fever because they can quickly respond to the physiologically increased demand of Ca<sup>2+</sup>. On the other hand, the adaptive mechanism becomes less efficient with increasing age, which explains the higher incidence of milk fever in older individuals. Both overweight and underweight conditions increase the risk of developing milk fever. Other major risk factors are: i) high milk yielding cow; ii) inappropriate nutrition during the dry period; iii) and housing. It is important to note that around calving the  $Ca^{2+}$  demand of the dairy cow is twice that required in the pregnant or in the non-lactating cow. Moreover, 10 kg of colostrum contains up to 9 times more  $Ca^{2+}$  than that found in the whole blood of cow. In addition to the remarkably increased demand of Ca<sup>2+</sup> that occurs at parturition, milk fever is often associated with an imbalance of the acid-base status that provokes reduced tissue responsiveness to the PTH. Often the hypocalcemia observed during milk fever is accompanied by hypophosphatemia. In the early stage of the disease the animal shows loss of appetite, nervousness, weakness, and shuffling of the hind feet. In the most severe phase, the animal shows trembling, tachycardia (increased heart rate), and lies on the ground with the head laid on the flank. Eventually, the cow can enter in a comatose status and die. The condition may

also increase the risk of other diseases like mastitis (inflammation of the mammary gland), obstructed labor (dystocia), retention of the fetal membranes, and uterine infections. Preventive measures include the administration of a low  $Ca^{2+}$  diet (less than 20 g per day per animal) in the weeks preceding calving to gradually prepare the organism to mobilize the  $Ca^{2+}$  for the increased demand requested during lactation. Intravenous administration of calcium borogluconate is effective in most cases.

#### **Determination of phosphorus**

Phosphorus (P) is another important macromineral and together with  $Ca^{2+}$  is a major component of bones and teeth. The majority of P is incorporated in molecules such as phospholipids, nucleic acids, coenzymes, and energy-transfer molecules like adenosine triphosphate (ATP) as organic phosphate (PO4<sup>3-</sup>). Only 30% of P is found free as inorganic mono- (HPO4<sup>2-</sup>) or diphosphate (H<sub>2</sub>PO4<sup>-</sup>), both of which are quantified by biochemical assays. Phosphorus is absorbed in the small intestine (i.e., jejunum) and mostly excreted in urine and saliva (the latter is a major route of excretion in adult ruminants). Other routes of excretion are feces and milk. The absorption of P is mediated by the dihydroxyvitamin D. Low dietary  $Ca^{2+}$  enhances the intestinal absorption of P, while high dietary  $Ca^{2+}$  decreases it. Unlike  $Ca^{2+}$ , the blood levels of P are not directly regulated by hormones. However, PTH stimulates the secretion of P in order to maintain the adequate ratio between  $Ca^{2+}$  and P levels. For this reason, hypocalcemia is usually associated with hypophosphatemia.

Low blood levels of P are usually associated with reduced growth rate (in young animals), reluctance to move, recumbency, and pica (abnormal appetite that may include for example ingestion of feces).

The concentration of P can be determined by spectrophotometric assay on blood plasma/serum and urine samples. It is important to note that spectrophotometric assays quantify the inorganic phosphate fractions. Blood P concentration is usually expressed as mg dl<sup>-1</sup> or mmol l<sup>-1</sup> in SI units (see Appendix I for reference values in domestic animals). Overall, the concentration of P in blood serum or plasma ranges from 4 to 9 mg dl<sup>-1</sup> or from 0.5 to 3.0 mmol l<sup>-1</sup> across domestic animals. Pigs may show high levels of P in the blood with no evidence of illness.

#### **Equipment:**

- Samples (urine and non-hemolytic blood plasma or serum).
- NB: Blood samples are stable 7 days if stored at room temperature (20-25 °C), or in the fridge (4-8 °C), and at least 3 months if stored in the freezer (-20 °C); Urine samples are stable 2 days if stored at room temperature (20-25 °C), 4 days if stored in the fridge (4-8 °C) or 3 weeks if stored in the freezer (-20 °C, samples can be frozen just

once). Moreover, heparin is the only suitable anticoagulant for the determination of P levels in blood plasma.

- Distilled water (for blank)
- Phosphate standard concentration (hereinafter standard; provided by the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic)
- Phosphate reagent solution (hereinafter R1; Solution's composition is available in Appendix II; provided by commercial kits from Erba Lachema s.r.o., Brno, Czech Republic)
- Pipettes and tips
- Tubes
- Cuvettes
- Water bath set at 37 °C
- Spectrophotometer set at 340 nm (334-365 nm)

## **Procedure:**

- 1. This protocol is based on the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic.
- 2. Take 3 clean tubes and mark them as blank, standard, or sample.
- 3. Place 1 ml of R1 in each tube as indicated in Table 11.
- 4. Add 10  $\mu$ l of distilled water in the blank tube, 10  $\mu$ l of standard in the standard tube, and 10  $\mu$ l of sample in the respective sample tube.
- 5. Mix it and incubate 5 min at 37 °C.

Table 11	. Determina	tion of phos	phate concentra	ation by spect	rophotometric assay.
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	Blank	Standard	Sample
Reagent 1	1.0 ml	1.0 ml	1.0 ml
Sample			0.01 ml
Standard		0.01 ml	
Distilled water	0.01 ml		

#### Phosphate concentration (mmol l<sup>-1</sup>):

 $\frac{Sample \ absorbance}{Standard \ absorbance} \times Standard \ concentration$ 

Standard concentration: 1.69 mmol 1<sup>-1</sup>

Unit conversion: mg dl<sup>-1</sup> = mmol l<sup>-1</sup> / 0.32

**Table 12.** Reference values for blood phosphate concentration (mg dl<sup>-1</sup>) in domestic animals.

Horse	Cow	Sheep	Goat	Pig	Dog	Cat	Rabbit
1.5-4.7	5.6-8.0	5.0-7.3	4.2-9.1	5.3-9.6	2.9-5.3	3.0-6.1	4.0-6.5

#### Main causes of hyperphosphatemia (high value of blood P):

*Pathological:* renal diseases (especially in small animals), endocrine disorders (e.g. hypoparathyroidism, hyperthyroidism, the latter especially in cats); excess of P intake (horse fed on diet rich in P like bran).

*Artifact:* hemolytic samples. During hemolysis, RBCs release large amounts of intracellular organic phosphate.

#### Main causes of hypophosphatemia (low value of blood P)

*Pathological:* Downer cow syndrome (see the next paragraph for an in-depth analysis); reduced dietary intake due to soil deficiency (e.g., deserts or arid regions); chronic renal diseases (especially in horses); endocrine disorders (e.g., hyperparathyroidism and Cushing's disease in dogs).

#### Clinical relevance: Downer cow syndrome

A downer cow is an animal that is not able to rise in a standing position for more than 12 hours. The condition frequently occurs around parturition from 2 days before till 10 days after the delivery. On average, 2-5% of the periparturient cows are affected. Twin pregnancy is a risk factor. In addition to prolonged recumbency, the animal is usually alert, ruminates, and shows normal appetite and thirst. The syndrome might be provoked by several factors including metabolic disorders (e.g., hypophosphatemia, hypocalcemia, hypomagnesemia), infectious diseases (e.g. infection of the mammary glands and reproductive tract), traumatic events (e.g. musculoskeletal injuries provoked by labor dystocia), and management mistakes (e.g. slippery floor, unbalanced diet). Often, the condition often arises as a complication of milk fever and is frequently associated with hypophosphatemia, which arises as a consequence of increased P loss due to fetal growth (at late gestation), milk production (during lactation), increased PTH (as a response to periparturient hypocalcemia), and increased cortisol levels (around parturition). Cows with blood phosphate levels <0.9 mmol 1<sup>-1</sup> are at high risk of developing the downer cow syndrome. Other common biochemical findings are: increased levels of creatine phosphokinase (CPK), lactate dehydrogenase (LDH), and aspartate aminotransferase (AST). Common complications are muscular ischemia (reduced blood flow due to the compression of soft tissues), necrosis, and nerve paralysis. Between 5 to 28% of cows showing milk fever become downers, with a fatality rate that ranges from 20 to 67%. Prognosis becomes unfavorable as the duration of recumbency exceeds 4 days. Therapeutic approach includes the pharmacological treatment of the underlying cause of the condition. Moreover, the animals should be placed in a clean and comfortable litter (e.g., sand provides better performance than sawdust or straw). Animal posture should be changed every hour (maximum every 3-4 hours) avoiding legs' traction. Massage therapy has shown beneficial effects. Cow has to be milked every 12 hours to avoid the onset of mastitis. Food and water should be easily accessible and available ad libitum. In some cases, hydrotherapy for 8-12 hours using warm water (38 °C) has shown beneficial effects in stimulating the peripheral circulation and in terms of animal welfare. Animals recumbent for less than 24 hours usually require 3 days for regaining the standing position, otherwise more than 5 days of treatment might be necessary. Hydrotherapy may reach up to 90% recovery's success rate.

#### **Determination of magnesium**

After Na<sup>+</sup>, magnesium (Mg<sup>2+</sup>) is a macromineral and the second most abundant cation within the cells. Mg<sup>2+</sup> participates in several enzymatic reactions and nerve conduction of electrical impulses. It is estimated that Mg<sup>2+</sup> contributes to the normal activity of hundreds of enzymes. Unlike Ca<sup>2+</sup>, there is no hormonal regulation of blood Mg<sup>2+</sup> levels, although increased levels of PTH partly contribute to the renal reabsorption of Mg<sup>2+</sup>. Therefore, the homeostasis of Mg<sup>2+</sup> in the body is the result of the balance between levels of the macromineral ingested and that excreted. Ileum and colon are the major sites for Mg<sup>2+</sup> absorption in monogastric animals, whereas the rumen and reticulum are the most important sites for the absorption in ruminants. Urine, feces, milk, and saliva (the latter mostly in ruminants) are the major routes of Mg<sup>2+</sup> excretion across animals. Mg<sup>2+</sup> is mainly stored in the bones; however, bone mobilization of Mg<sup>2+</sup> in the body. Therefore, the assessment of Mg<sup>2+</sup> levels in blood plasma or serum does not reflect the body Mg<sup>2+</sup> status.

The concentration of  $Mg^{2+}$  can be determined by spectrophotometric assay on blood plasma/serum and urine samples. The  $Mg^{2+}$  levels are expressed as mg dl<sup>-1</sup> or mmol l<sup>-1</sup> in SI units (see Appendix I for reference values in domestic animals). Overall, the concentration of  $Mg^{2+}$  in blood serum or plasma ranges from 1.5 to 3.5 mg dl<sup>-1</sup> or from 0.5 to 1.5 mmol l<sup>-1</sup> across domestic animals.

#### **Equipment:**

- Samples (urine and non-hemolytic blood plasma or serum).
- NB: Blood samples are stable 7 days if stored at room temperature (20-25 °C), or in the fridge (4-8 °C), and at least one year if stored in the freezer (-20 °C); do not use EDTA samples. Urine samples are stable 3 days if stored at room temperature (20-25 °C) or in the fridge (4-8 °C), and at least one year if stored in the freezer (-20 °C).
- Distilled water (for blank)
- Mg<sup>2+</sup> standard concentration (hereinafter standard; provided by commercial kits from Erba Lachema s.r.o., Brno, Czech Republic)

- Mg<sup>2+</sup> reagent solution (hereinafter R1; solution's composition is available in Appendix II; provided by commercial kits from Erba Lachema s.r.o., Brno, Czech Republic)
- Pipettes and tips
- Tubes
- Cuvettes
- Water bath set at 37 °C
- Spectrophotometer set at 520 nm (500-550 nm)

## **Procedure:**

- 1. This protocol is based on the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic.
- 2. Take 3 clean tubes and mark them as blank, standard, or sample.
- 3. Place 1 ml of R1 in each tube as indicated in Table 13.
- 4. Add 10  $\mu$ l of distilled water in the blank tube, 10  $\mu$ l of standard in the standard tube, and 10  $\mu$ l of sample in the respective sample tube.
- 5. Mix it and incubate 5 min at 37 °C.

Table 13. Determination of Mg <sup>2+</sup>	concentration by spectrophotometric assay.
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	Blank	Standard	Sample
Reagent 1	1.0 ml	1.0 ml	1.0 ml
Sample			0.01 ml
Standard		0.01 ml	
Distilled water	0.01 ml		

Mg<sup>2+</sup> concentration (mmol l<sup>-1</sup>):

# $\frac{Sample \; absorbance}{Standard \; absorbance} \times Standard \; concentration$

#### Standard concentration: 0.82 mmol 1-1

## **Unit conversion:** mg dl<sup>-1</sup> = mmol l<sup>-1</sup> / 0.4114

Table 14. Reference values for blood total Mg<sup>2+</sup> concentration (mg dl<sup>-1</sup>) in domestic animals.

Horse	Cow	Sheep	Goat	Pig	Dog	Cat	Rabbit
1.4-2.3	1.5-2.9	2.2-2.8	2.8-3.6	2.7-3.7	1.6-2.4	1.7-2.6	

#### Main causes of hypermagnesemia (high values of blood Mg<sup>2+</sup>):

*Pathological:* Rarely seen and usually associated with other abnormalities of the mineral balance. The most frequent causes of hypermagnesemia are renal diseases, which are common especially in dogs and cats. Some drugs (e.g., insulin, diuretics, and some antibiotics) may also induce hypermagnesemia.

Artifact: Hemolytic samples.

## Main causes of hypomagnesemia (low values of blood Mg<sup>2+</sup>)

*Pathological:* decreased dietary intake (e.g., grass tetany in ruminants, see the next paragraph for an in-depth analysis); increased gastrointestinal loss (e.g. in monogastric animals it frequently appears as a consequence of diarrhea) or urinary excretion (renal diseases).

#### Clinical relevance: Grass tetany

This condition is also named "spring tetany", "grass staggers" or "lactation tetany" and is frequently observed at the beginning of lactation in high milk-yielding cows and sheep. Although the amount of  $Mg^{2+}$  in the milk is relatively low (12 mg kg<sup>-1</sup>), it represents a large portion of the daily dietary intake. In the cow, the  $Mg^{2+}$  dietary requirement is estimated to be 1-1.3 g kg<sup>-1</sup> dry matter during pregnancy and 1.8-2.2 g kg<sup>-1</sup> during lactation. The terms

"spring -tetany" originates from the fact that the condition is frequently observed during the spring or autumn months when pasture growth is maximal but often Mg<sup>2+</sup> deficient. Moreover, the high content of K<sup>+</sup> and low content Na<sup>+</sup> in forage alter the ruminal absorption of Mg<sup>2+</sup>, which occurs by an active Na-linked ATPase-dependent transport system. Moreover, in forestomachs, the young grass (especially if it has been fertilized with nitrogenous fertilizers) is readily converted into ammonia leading to an increase in the ruminal pH above 6.5, which further inhibits the ruminal absorption of  $Mg^{2+}$ . For these reasons, pastures that contain K<sup>+</sup> concentration greater than 30 g kg<sup>-1</sup> dry matter and N concentration greater than 40 g kg<sup>-1</sup> dry matter are considered dangerous. Also,  $K^+$  / (Ca<sup>2+</sup> + Mg<sup>2+</sup>) ratio in the pasture greater than 2.2 mEq is considered a major risk factor. Frequent symptoms of grass tetany are incoordination, muscular spasms, and convulsions. As a consequence, the body temperature increases up to 40-40.5 °C. The animal frequently champs and froths at the mouth. In acute cases, death can occur within 1 to 5 hours after the appearance of symptoms. The most common clinical finding is hypomagnesemia, which sometimes is associated with hypocalcemia. The low levels of  $Mg^{2+}$  in the urine are also highly indicative of grass tetany. The therapy is based on administration based on  $Mg^{2+}$  alone or in association with  $Ca^{2+}$ . Preventive measures include dietary supplementation with Mg<sup>2+</sup> and avoidance of movement and food deprivation during the risk periods. However, one of the problems related to Mg<sup>2+</sup> supplements is their reduced palatability.

#### Nitrogenous metabolites in vertebrates

In mammals and amphibians, urea is a major waste product of nitrogenous metabolism (for this reason mammals are defined as ureotelic animals). Urea, whose chemical formula is  $CO(NH_2)_2$  (Figure 8), is produced by the liver during the amino acids breakdown by combining two molecules of ammonia (NH<sub>3</sub>, Figure 8) to one molecule of CO<sub>2</sub> using 3 molecules of ATP and one molecule of water (H<sub>2</sub>O). In most fish and aquatic invertebrates, ammonia represents the main waste of nitrogenous metabolism because their adaptation to aquatic environments ensures the possibility to quickly get rid of ammonia, urea is considerably less toxic. Moreover, while ammonia increases the pH, urea is neither acid nor alkaline; therefore, it does not impair the pH of body fluids. In reptiles and birds, uric acid (C<sub>5</sub>H<sub>4</sub>N<sub>4</sub>O<sub>3</sub>, (Figure 8) represents the major waste product of nitrogenous metabolism because part of their development occurs inside the eggshell, which is impermeable to water (uricotelic animals).



Figure 8. Nitrogenous end products in vertebrates.

The need to minimize water loss led birds and reptiles to develop an alternative way to excrete nitrogenous end-products. Once uric acid reaches a certain concentration in the renal tubules, it precipitates with extremely little amount of water. Unlike ammonia and urea, uric acid is not water soluble. It is also less toxic than ammonia and urea. However, the conversion of ammonia to uric acid is much more energetically expensive than conversion of ammonia into urea. Once excreted, uric acid is then converted to urate, which appears as a white coagulum in birds' and reptiles' feces. Interestingly, some amphibians like frogs excrete ammonia as tadpoles and then switch to urea after metamorphosis.

In mammals, urea is mainly excreted by the kidney as urine. Other routes of excretion are the colon (in horse), saliva, and sweat. Together with creatinine, urea is used as a marker of renal function. It is also important to note that in ruminants, urea is used by microorganisms that live in the forestomachs to synthesize new amino acids and proteins.

Urea can be quantified by spectrophotometric assay. However, there are different ways to express urea concentration, which we have to be aware of to avoid confusion. Urea concentration might be expressed as mg dl<sup>-1</sup> or as mg of urea nitrogen (N) dl<sup>-1</sup> (the latter is called blood urea nitrogen or BUN). According to the SI, the levels of urea and uric acid are expressed as mmol l<sup>-1</sup>.

#### **Determination of urea**

#### **Equipment:**

• Samples (non-hemolytic blood plasma and serum, urine). The use of heparin as an anticoagulant should be avoided.

NB: Stability of urea is 2 weeks in blood plasma/serum and 1 week in urine if samples are stored at 2-8 °C.

- Distilled water (for blank)
- Reagent solution (hereinafter R1, solution's composition is available in Appendix II; provided by commercial kits from Erba Lachema s.r.o., Brno, Czech Republic)
- Pipettes and tips
- Tubes
- Cuvettes
- Water bath set at 37 °C
- Spectrophotometer set at 340 nm (334-365 nm)

## **Procedure:**

- 1. This protocol is based on the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic.
- 2. Take 3 clean tubes and label each one as blank, standard, or ID sample.
- 3. Place 1 ml of R1 in the tubes as indicated in Table 15.
- 4. Add 10  $\mu$ l of distilled water, 10  $\mu$ l of standard or 10  $\mu$ l of sample in the respective sample tube.
- 5. Mix it and measure the absorbance of standard and sample at 30 sec (A<sub>1</sub>) and at 90 sec (A<sub>2</sub>) of incubation against the blank.
- 6. Calculate the absorbance change ( $\Delta A$  sample =  $A_2$ - $A_1$ /min)

	Blank	Standard	Sample
Reagent 1	1.0 ml	1.0 ml	1.0 ml
Sample			0.01 ml
Standard		0.01 ml	
Distilled water	0.01 ml		

Table 15. Determination of urea concentration by spectrophotometric assay.

Urea concentration (mmol l<sup>-1</sup>):

 $\frac{\Delta A \ sample / min}{\Delta A \ standard / min} \times Standard \ concentration$ 

## Standard concentration: 17.2 mmol 1<sup>-1</sup>

Table 16. Reference	values for urea	(mmol 1 <sup>-1</sup> )	) in domestic animals.
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Horse	Cow	Sheep	Goat	Pig	Dog	Cat	Rabbit
3.6-8.6	2.5-10.7	2.9-7.1	3.6-7.1	3.6-10.7	3.3-8.3	5.0-11.3	

Common causes of impaired blood urea levels are shown below. However, it is important to note that urea levels have to be considered together with urine analysis and with the determination of blood levels of creatinine, being the latter not affected by many extrarenal factors such as for urea.

#### Main causes of high blood levels of urea:

Physiological: increased protein intake, intense exercise.

*Pathological*: increased urea levels associated with increased blood levels of creatinine are indicative of reduce glomerular filtration rate (the latter might be a consequence of reduced

renal perfusion, renal diseases, or urethral obstruction); otherwise caused by extrarenal factors such as fever, burns, intestinal bleeding, and some drugs like corticosteroids.

Artifact: severe jaundice.

#### Main causes of low blood levels of urea:

*Pathological:* any cause of increased excretion (e.g., polyuria associated with diabetes mellitus), liver diseases, and enzymatic deficiencies in the urea cycle.

#### Clinical relevance: Hypoadrenocorticism

Hypoadrenocorticism, also known as Addison disease, is an endocrine disorder characterized by a deficiency of glucocorticoids or mineralocorticoids, or both. Glucocorticoids (e.g., cortisol) and mineralocorticoids (e.g., aldosterone) are hormones produced by the adrenal glands. The disease is frequently observed in dogs and occasionally in horses. The cause of the disease is unknown, but it is probably associated with autoimmune disorders in which the immune system attacks and destroys the cells of the body. Addison disease might affect dogs of any breed, age, or sex, although it seems more frequent in middle-aged female dogs and breeds such as Standard Poodles, West Highland White Terriers, Great Danes, and Bearded Collies. The symptoms are often unnoticed and vague, which make the diagnosis sometimes difficult. The animals may show vomiting, dehydration, weakness, weight loss, and bradycardia (low heart rate, which is expressed the number of heart beats per minute). Because of reduced aldosterone levels, the animal shows polyuria and polydipsia, hyperkalemia (increased blood K<sup>+</sup> levels), hyponatremia (decreased blood Na<sup>+</sup> levels), and hypochloremia (decreased blood Cl<sup>-</sup> levels), which may provoke cardio-circulatory collapse and renal failure (so called adrenal crisis, acute medical emergency). As a result of renal failure, the animal shows increased BUN and creatinine levels. For acute treatment during adrenal crisis consists of the restoration of blood volume and correction of electrolyte disorders. For long-term treatment, mineralocorticoid and glucocorticoid therapies are administered and electrolytes regularly checked.

#### **Determination of uric acid**

#### **Equipment:**

• Samples (non-hemolytic blood plasma and serum, urine).

NB: Stability of uric acid is 3 days if samples are stored at room temperature (20-25 °C), 7 days if samples are stored in the fridge (4-8 °C), and 6 months if samples are stored in the freezer (-20 °C). Stability of uric acid in the urine is 24 hours. Distilled water (for blank)

- Reagent solution (hereinafter R1; solution's composition is available in Appendix II; provided by commercial kits from Erba Lachema s.r.o., Brno, Czech Republic)
- Pipettes and tips
- Tubes
- Cuvettes
- Water bath set at 37 °C
- Spectrophotometer set at 505 nm

## **Procedure:**

- 1. This protocol is based on the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic.
- 2. Take 3 clean tubes and label each one as a blank, standard or ID sample.
- 3. Place 1 ml of R1 in the tubes as indicated in Table 17.
- 4. Add 25  $\mu$ l of distilled water, 25  $\mu$ l of standard or 25  $\mu$ l of sample in the respective sample tube.
- 5. Mix it and incubate 5 min at 37  $^{\circ}$ C
- 6. Measure the absorbance of standard and sample against the blank.

	Blank	Standard	Sample
Reagent 1	1.0 ml	1.0 ml	1.0 ml
Sample			0.025 ml
Standard		0.025 ml	
Distilled water	0.025 ml		

 Table 17. Determination of uric acid by spectrophotometric assay.

Uric acid concentration (µmol l<sup>-1</sup>):

 $\frac{Sample \ absorbance}{Standard \ absorbance} \times Standard \ concentration$ 

Standard concentration: 356.88 µmol 1-1

**Unit conversion:** mmol  $1^{-1} = \mu mol 1^{-1} / 1,000$ 

**Table 18.** Reference values for uric acid (mmol 1<sup>-1</sup>) in domestic animals.

Horse	Cow	Sheep	Goat	Pig	Dog	Cat	Rabbit
					<119	<59.5	

## Main causes of high blood levels of uric acid:

*Pathological*: gout (in reptiles and birds; see the next paragraph for an in-depth analysis); renal diseases; cancer; endocrine disorders (e.g., diabetes mellitus and hypothyroidism).

## Main causes of low blood levels of uric acid:

*Pathological:* Wilson's disease (inherited human disorder leading to copper accumulation in the body).

#### Clinical relevance: Gout

Gout is a disease associated with increased blood levels of uric acid (hyperuricemia) followed by their precipitation as urate salts in tissues and organs. The condition is mainly observed in captive birds and terrestrial reptiles raised under poor management of diet and limited access to water. The cause of gout is not clear, but it seems to be associated with increased dietary protein intake or inappropriate protein intake as predisposing factors. In birds, gout has been associated with diet rich in vitamin D and Ca<sup>2+</sup> and poor in vitamin A. Both in birds and reptiles, dehydration is a high-risk factor. Some nephrotoxic drugs may also induce gouts (iatrogenic gout). The animal is frequently reluctant to move due to the deposition of urate salts such as tophi in the joints, which appear swollen and painful (articular gout). Urate salts also deposit in the liver, kidney, heart, and other internal organs (visceral gout). Treatment consists of the proper dietary management (e.g., low protein intake) and hydration. Allopurinol treatment may reduce the production of uric acid; however, it does not diminish the urate salts already deposited in the tissues. Surgery might be required to "clean" the joints and relieve the pain. Amputation is sometimes performed as an extreme action. Irrespective of the treatments, the chances of success are unfortunately very low.

#### Introduction to clinical enzymology

Most of the chemical reactions that occur in the body are catalyzed by enzymes, which are mostly made of proteins (some RNA molecules also show catalytic activity). By lowering the activation energy required brake or form new bonds, enzymes make the chemical reaction to proceed more quickly than it would without the enzyme. To understand the importance of enzymes, we shall consider one of the most widespread enzymatic reactions that occur in our body:

$$CO_2 + H_2O = H_2CO_3$$

Without the intervention of any enzyme, the reaction occurs very slowly with perhaps 200 molecules formed in one hour. However, thanks to the enzymatic activity of carbonic anhydrase (which is abundant in many cells like red blood cells) around 600,000 molecules of carbonic acid ( $H_2CO_3$ ) are formed every second. Although all cells in the body share the same genes, their expression differs among cell types and contribute to their diversity and specialization. In this way, the abnormal levels of certain enzymes are suggestive of some damage or pathology in different tissues or organs. However, the majority of the enzyme is not organ specific; thus, in order to identify where the disease is localized, several biochemical parameters have to be considered together with clinical evidence. Moreover, it is important to bear in mind that the levels of enzyme in the blood are the result of the balance between the normal cell turnover and the rate of catabolism and excretion.

Moreover, the enzymatic activity might be evaluated by spectrophotometric assay. However, unlike proteins or lipids that are determined by endpoint measurement (i.e., one reading after the incubation period), enzymatic reactions are evaluated based on multipoints measurement to evaluate the velocity of chemical reactions. Because enzymatic activity is also sensitive to environmental conditions like pH or temperature or presence of inhibitors or activators, results may vary between laboratories based on the conditions or reagents composition used for the spectrophotometric assays. Overall, the optimal pH and temperature for enzymatic reactions range from 6 to 8 and from 35 to 40 °C, respectively.

Enzymatic activity is expressed as unit (U), which is defined as "the amount of enzyme that under given assay conditions catalyzes the conversion of 1  $\mu$ mol of substrate per minute". Although U is still commonly used, the SI encourages the use of  $\mu$ kat (katal), which is defined

as "the amount of enzyme that converts a mole of substrate in one second". One U is equal to 16.67 nkat (see Appendix I for reference values in domestic animals).

#### Determination of alanine aminotransferase activity

Alanine aminotransferase (ALT), previously named glutamate-pyruvate transaminase (GPT), is an intracellular enzyme that catalyses the transfer of the  $\alpha$ -amino group from L-alanine to  $\alpha$ -ketoglutarate, being the product of this reversible reaction L-glutamate and pyruvate.

L-alanine +  $\alpha$ -ketoglutarate  $\leftarrow \rightarrow$  L-glutamate + pyruvate

Since the enzyme is abundant in the cytoplasm, its increase is indicative of cell damage or necrosis. It is expressed in several tissues, although in dogs and cats it is predominantly expressed in the liver. For this reason, increased ALT levels in the blood are indicative of hepatocellular damage in dogs and cats. In large animals, on the other hand, ALT is predominantly expressed in the muscular tissue and is not considered to be of diagnostic significance for liver function. The ALT activity is expressed as U l<sup>-1</sup> in conventional units or as  $\mu$ kat l<sup>-1</sup> in SI units (see Appendix I for reference values in domestic animals).

#### **Equipment:**

- Samples (non-hemolytic blood plasma or serum).
   NB: Enzymatic loss of activity is lower than 10% if samples are stored at 2-8 °C and analyzed within 3 days. Samples are stable for at least 3 months if stored in the freezer (-20 °C).
- Distilled water (for blank)
- Buffer and substrate solution (hereinafter R1; solution's composition is available in Appendix II)
- Pipettes and tips
- Tubes
- Cuvettes
- Water bath set at 37 °C
- Spectrophotometer set at 340 nm

#### **Procedure:**

- 1. This protocol is based on the commercial kit from Erba Lachema s.r.o., Brno, Czech Republic.
- 2. Take 3 clean tubes and label each one as a blank, standard, or ID sample.
- 3. Place 1 ml of R1 in the tubes as indicated in Table 19.
- 4. Add 100  $\mu$ l of distilled water, 100  $\mu$ l of standard, or 100  $\mu$ l of sample in the respective sample tube.
- 5. Mix it and incubate 1 min at 37 °C.
- 6. Measure the absorbance of standard and sample after 1, 2, and 3 minutes of incubation against blank at 340 nm.
- 7. Calculate 1 minute absorbance change ( $\Delta A/min$ ) as shown in Table 20.

 Table 19. Determination of ALT activity by spectrophotometric assay.

	Blank	Standard	Sample
Reagent 1	1.0 ml	1.0 ml	1.0 ml
Sample			0.1 ml
Standard		0.1 ml	
Distilled water	0.1 ml		

Table 20. Representative calculation for the determination of ALT activity on blood samples.

Initial	After 1 min	After 2 min	After 3 min	
0.020	0.015	0.010	0.07	
0.020-0.015=0.005		0.010-00.07=0.003		
	(0.005+0.00)	03)/2=0.004		

The same type of calculation has to be performed on the standard solution.

ALT activity (U l<sup>-1</sup>):

$$\Delta A \ sample/min}{\Delta A \ standard/min} \times Standard \ concentration$$

#### Standard concentration: 94.2 U 1<sup>-1</sup>

**Unit conversion:**  $\mu$ kat  $1^{-1} = U 1^{-1} \times 0.017$ 

**Table 21.** Reference values for ALT activity (U l<sup>-1</sup>) in domestic animals.

Horse	Cow	Sheep	Goat	Pig	Dog	Cat	Rabbit
		26-34	6-19	31-58	10-109	25-97	45-80

#### Main causes of high blood levels of ALT:

*Pathological:* Highly indicative of liver diseases in dogs and cats. Also, pancreatitis may increase ALT levels because of the mechanical damage to the liver. Increased levels of ALT activity might also be associated with myopathy and hemolysis (the latter especially in pigs and cats).

## Main causes of low blood levels of ALT:

As most other enzymes decreased values have no clinical significance.

#### Other enzymes of clinical relevance:

#### Sorbitol dehydrogenase (SDH) or iditol dehydrogenase (ID)

SDH is a cytoplasmic enzyme that catalyzes the conversion of D-fructose to D-sorbitol. This enzyme is highly expressed in hepatocytes (hepatospecific in nearly all species) and is widely used to assess liver function in horses and cattle, similarly to ALT in small animals. Values above 5 U l<sup>-1</sup> are indicative of hepatocellular damage and might be detected within 24 hours after hepatic injury. Unfortunately, SDH is not a stable enzyme: the enzymatic activity is stable for less than 24 hours if samples are stored at room temperature, one week if stored in the fridge.

#### **Glutamate dehydrogenase (GDH)**

GDH is a mitochondrial enzyme that catalyzes the conversion of glutamate to 2-oxoglutarate. This enzyme is used to assess liver function in exotic (i.e., birds and reptiles) and in large animals together with SDH. However, it is important to bear in mind that GDH is also expressed in other organs such as the kidney and the salivary glands. The stability of GDH is superior to that of SDH. Therefore, it should be preferred to SDH when samples have to be posted. Values above 100 U 1<sup>-1</sup> are indicative of hepatocellular damage. In foals, GDH activity may be above the reference values established for adult individuals and may not be indicative of liver damage.

# Aspartate aminotransferase (AST), formerly known as glutamate oxaloacetate transaminase (GOT)

The AST catalyzes the transfer of the  $\alpha$ -amino group of aspartate to  $\alpha$ -ketoglutarate, which results in the synthesis of glutamate and oxaloacetate. The AST is most abundant in skeletal muscle and in the liver, but it is also found in cardiac muscle and in erythrocytes. Normal values are usually below 100 U l<sup>-1</sup> in domestic animals, except in horses (see Appendix I for reference values in domestic animals). Physiologically, AST may increase after exercise in horses. Common causes of abnormally high values of AST are related to hepatic and muscular diseases (i.e., myopathy). Prolonged recumbency may also cause an increase of

AST levels. In cats, hyperthyroidism may increase AST and ALT levels. Also, artifacts like hemolysis may cause a false increase of AST.

#### Gamma glutamyl transferase (γGT)

The  $\gamma$ GT catalyzes the transfer of  $\gamma$ -glutamyl functional groups from one molecule to another one. The enzyme occurs mainly in the liver and in the kidney. In large animals, high values are associated with chronic liver damage. In small animals, the  $\gamma$ GT pattern is usually parallel to ALP, but it is rarely requested in veterinary clinics. Half-life is long. Normal values are usually below 60 U l<sup>-1</sup> (see Appendix I for reference values in domestic animals). In cats, however,  $\gamma$ GT is normally undetectable. Physiologically, high values are observed in neonates (except horses). In addition, donkeys show physiologically higher values than horses.

#### Creatine kinase (CK), also named creatine phosphokinase (CPK)

The CK catalyzes the conversion of creatine to phosphocreatine. The enzyme is abundant in the muscular tissue. In domestic animals, CK levels are normally around 100 U l<sup>-1</sup> (see Appendix I for reference values in domestic animals). Increased CK levels in blood (up to 500,000 U l<sup>-1</sup>) can be used as a diagnostic marker of myocardial disease. Moreover, high CK levels are related to muscular injuries such as rhabdomyolysis in horses or provoked by iliac thrombosis in cats. High values might be observed after surgery, muscular injection, or if the animal is recumbent to a major muscle for some days as it occurs in ruminants affected by the "spring tetany". Half-life is quite short and usually ranges between 24-48 hours, which implies that its determination might not be useful in assessing muscular injuries that occurred in the previous 3 days.

#### Lactate dehydrogenase (LDH)

The LDH catalyzes the conversion of lactate to pyruvate and back. It also catalyzes the reversible reaction from NAD<sup>+</sup> to NADH. LDH is among the largest and most distributed enzymes in the body. It is particularly abundant in the muscle and liver, but also found in the kidneys and in the erythrocytes. Because of its wide distribution in the body, in veterinary medicine abnormally high values of LDH are often difficult to interpret. Common causes of abnormally high values of LDH are muscular injury, liver diseases, and cancer.

Physiologically, LDH levels may increase because of exercise. Artifacts like hemolysis may cause a false increase of LDH. Half-life is quite long, which makes the determination of LDH activity useful in retrospective diagnosis.

#### Alkaline phosphatase (ALP)

The ALP catalyzed reactions of dephosphorylation, which is the removal of phosphate groups from organic compounds. Its name comes from the fact that the highest activity is observed under high pH (i.e., alkaline). The ALP is one of the most abundant enzymes, which is distributed in bones, liver, intestine, and biological fluids like semen. Normal values are usually below 300 U l<sup>-1</sup> in several species except the horse where it may reach up to 500 U l<sup>-1</sup> (see Appendix I for reference values in domestic animals). Physiologically, high values of ALP are observed in young animals and in dogs under stress conditions. Abnormally high values are also associated with liver and endocrine diseases (e.g., hyperparathyroidism). Because its activity is strongly influenced by the pH, the buffer system used for its determination may affect the results.

#### List of acronyms

- ALP: alkaline phosphatase
- ALT: alanine aminotransferase
- ATP: adenosine-triphosphate
- AST: aspartate aminotransferase
- BUN: blood urea nitrogen
- CK: creatine kinase
- CPK: creatine phosphokinase
- EDTA: ethylenediaminetetraacetic acid
- GDH: glutamate dehydrogenase
- GOT: glutamate oxaloacetate transaminase
- GPT: glutamate pyruvate transaminase
- γGT: gamma glutamyl transferase
- Hb: hemoglobin
- Hct: hematocrit
- HDL: high-density lipoprotein
- ID: iditol dehydrogenase
- LDH: lactate dehydrogenase
- LDL: low-density lipoprotein
- PBGM: portable blood glucose meter
- PTH: parathyroid hormone
- PUFA: polyunsaturated fatty acid

RBC: red blood cell

SDH: sorbitol dehydrogenase

SI: International System of Units also known as Système International d'Unités (in French)

VLDL: very low-density lipoprotein

WBC: white blood cell

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# Appendixes

Variable	Horse	Cattle	Sheep	Goat	Pig	Dog	Cat
Glucose (mmol 1 <sup>-1</sup> )	4.2-6.4	2.5-4.1	2.8-4.4	2.8-4.2	4.7-8.3	3.1-6.7	3.1-6.9
Total proteins (g l <sup>-1</sup> )	55-75	60-80	60-90	55-80	65-90	55-75	57-95
Cholesterol (mmol l <sup>-1</sup> )	1.9-3.9	2-3.2	1.3-2.0	2.1-3.4	1-1.4	3.5-7.8	2-5.2
Triglycerides (mmol 1 <sup>-1</sup> )	0.10-0.50	0.00-0.35				0.30-3.90	0.5-2.6
Calcium (mmol l <sup>-1</sup> )	2.2-3.4	2.25-3.1	2.8-3.2	2.2-2.9	1.8-2.9	2.3-3	2.3-3
Phosphorus (mmol l <sup>-1</sup> )	1-1.8	1.8-2.1	1.6-2.6	1.6-3	1.7-3.1	1-2.1	0.9-2
Magnesium (mmol l <sup>-1</sup> )						0.7-1	
Uric acid (mmol 1 <sup>-1</sup> )						<119	<59.5
ALT (µkat l <sup>-1</sup> )					0.5-1	0.1-1	0.1-1
AST (µkat l <sup>-1</sup> )	3.7-6	1-2.2	1-4.6	2.8-8.5	0.5-1.5	0.1-1	0.1-1
γGT (µkat l <sup>-1</sup> )	0-0.2	0.1-0.6	0.3-0.9	0.3-0.9	0.1-1	0-0.16	0-0.16
CK (µkat l <sup>-1</sup> )	0.1-0.5	0.1-1.5	0.1-0.3	0-0.2	0.1-35	0.1-4	0.1-4
ALP (µkat l <sup>-1</sup> )	2.4-6.6	0.5-8	1.1-6.5	1.5-6.5	2-6.6	0.1-4	0.1-4

Appendix I. Hematological reference values according to SI units in domestic animals

## Appendix II. Reagents' composition

Solutions are based on commercial kits from Erba Lachema s.r.o. (Brno, Czech Republic).

Glucose reagent solution

Reagent	Concentration
Phosphate buffer	250 mmol 1 <sup>-1</sup>
Glucose oxidase	>25 U ml <sup>-1</sup>
Peroxidase	>2 U ml <sup>-1</sup>
Phenol	5 mmol 1 <sup>-1</sup>
4-aminoantipyrine	0.5 mmol 1 <sup>-1</sup>

Total protein reagent solution

Reagent	Concentration
Copper II sulphate	12 mmol l <sup>-1</sup>
Potassium sodium tartrate	31.9 mmol 1 <sup>-1</sup>
Potassium iodide	30.1 mmol 1 <sup>-1</sup>
Sodium hydroxide	0.6 mmol 1 <sup>-1</sup>

Cholesterol reagent solution

Reagent	Concentration
Good's buffer	50 mmol 1 <sup>-1</sup>
Phenol	5 mmol $l^{-1}$
4-aminoantipyrine	0.3 mmol l <sup>-1</sup>
Cholesterol esterase	$\geq 200 \text{ U} \text{ l}^{-1}$
Cholesterol oxidase	$\geq$ 50 U l <sup>-1</sup>
Peroxidase	$\geq$ 3 kU l <sup>-1</sup>

Triglycerides reagent solution

Reagent	Concentration
Good's buffer	50 mmol 1 <sup>-1</sup>
4-Chlorophenol	4 mmol 1 <sup>-1</sup>
$Mg^{2+}$	15 mmol l <sup>-1</sup>
ATP	2 mmol 1 <sup>-1</sup>
Glycerol kinase	$\geq$ 0.4 kU l <sup>-1</sup>
Peroxidase	$\geq$ 2 kU l <sup>-1</sup>
Lipoprotein lipase	$\geq$ 2 kU l <sup>-1</sup>
Glycerol-3-phosphate-oxidase	$\geq$ 0.5 kU l <sup>-1</sup>
4-aminoantipyrine	0.5 mmol l <sup>-1</sup>

Calcium reagent solution

Reagent	Concentration
Arsenazo III	0.1 mmol 1 <sup>-1</sup>
Phosphate buffer	50 mmol 1 <sup>-1</sup>

Phosphorus reagent solution

Reagent	Concentration
Ammonium molybdate	0.35 mmol 1 <sup>-1</sup>
Sulphuric acid	131 mmol l <sup>-1</sup>

Magnesium reagent solution

Reagent	Concentration
Xylidyl blue	110 μmol l <sup>-1</sup>
Ethanolamine	1 mol l <sup>-1</sup>
GEDTA	60 μmol l <sup>-1</sup>

Urea reagent solution

Reagent	Concentration
Tris buffer	96 mmol 1 <sup>-1</sup>
ADP	0.6 mmol l <sup>-1</sup>
Urease	$\geq$ 534 µkat l <sup>-1</sup>
GLDH	$\geq$ 5.4 µkat l <sup>-1</sup>
2-oxoglutarate	5 mmol l <sup>-1</sup>
NADP	0.24 mmol l <sup>-1</sup>

# Uric acid reagent solution

Reagent	Concentration
Pipes buffer	50 mmol 1 <sup>-1</sup>
DHBS	0.5 mmol l <sup>-1</sup>
Uricase	$\geq$ 0.32 kU l <sup>-1</sup>
Peroxidase	$\geq 1 \text{ kU } l^{-1}$
4-aminoantipyrine	0.31 mmol 1 <sup>-1</sup>

## ALT reagent solution

Reagent	Concentration
Tris buffer	137.5 mmol 1 <sup>-1</sup>
L-alanine	709 mmol 1 <sup>-1</sup>
LDH	$\geq$ 2000 U l <sup>-1</sup>
CAPSO	20 mmol 1 <sup>-1</sup>
2-oxoglutarate	85 mmol 1 <sup>-1</sup>
NADH	1.05 mmol 1 <sup>-1</sup>