Methods used to determine the constituents of the fibrous fraction, a review

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Abstract

The estimation of the chemical and bromatological composition of foods involves a series of studies that evaluate mainly the fiber fraction, since it presents great variability when compared to the other components. The growing number of publications on the analytical techniques available for determining the nutritional value of foods end up generalizing the use of ill-defined terms. There are several techniques for evaluating forage components, the detergent system being the most widely used, although there are more modern methods. However, the accessibility and cost of these modern methods are factors that limit their use in many laboratories. Furthermore, some of these methods are not recognized as official methods of analysis. In this context, the objectives of this review were: to highlight the most important concepts in the determination of the nutritional value of foods; to improve the use and the difficulties of interpretation of the analytical results. It was concluded that the use of analytical methods allows the estimation of the composition and the availability of the different fractions of the cell wall. But variability of the cell wall constituents requires knowledge of the different analytical methodologies available. The analytical methods, traditional or alternative, are still empirical since they present different results for the same analysis. These variations are generated most of the time by the differences between the steps of the analytical procedures. It is clear that the improvement of analytical methods is of paramount importance to estimate the nutritional value of foods.

Keywords: Alternative methods; Nutritive value; Van Soest; Source of variation

INTRODUCTION

The estimation of the nutritive value of foods for diet formulation involves a series of studies that evaluate mainly the fibrous fraction. Since this presents great variability and is directly related to energy availability and consumption (Jung, 1997; Berchielli et al., 2011).

In Brazil, where food resources are based on grasses, the knowledge of the nutritional value of food is of great importance for the understanding of physiological processes, since they are responsible for the transformation of food into animal products (Geron, et al. 2014).

Fiber is a nutritional term that can only be determined during the digestive process and its definition is linked to its origin and the analytical methodology used in its laboratory determination (Mertens, 2003; Gomes et al.,

2012). Resistant to enzymatic digestion, fiber ends up serving as a substrate for fermentative activity (Van Soest et al., 1991; Jung, 1997), by presenting variable digestibility, it occupies space in the gastrointestinal tract and can limit animal consumption (Jung, 1997; Mertens, 2003; Geron, et al. 2014). Thus, errors made in laboratory analysis for estimating the nutritive value of feedstuffs can compromise the balance of diets (Van Soet et al., 1991).

The traditional analytical method called detergent system, developed by Van Soest (1963b), for the quantification of insoluble fibrous compounds, underwent a series of modifications in certain stages of its process in an attempt to improve its use to cover a greater variability of foods. These modifications suggested the withdrawal of decalin used as an antifoaming agent; the withdrawal of sodium sulfite used to remove protein contaminations; the exclusion of sodium sulfite and the inclusion of α-amylase for amide removal (Van Soest & Robertson, 1985); the use of α-amylase, sodium sulfite and triethylene glycol without decalin (Van Soest et al., 1991).

Due to the various alterations made to the original method, in 1980 David Mertens, in order to reduce errors among laboratories, created only one single analytical method for all types of foods (Udén et al., 2005; Segura et al., 2007).

However, even if all researchers rigorously adopt the established reference method, there are still some practical shortcomings that may end up limiting its efficiency in terms of comparing results (Berchielli et al., 2001; Udén et al., 2005). There are many factors that cause variations in results between laboratories, such as, for example, adaptation of methods to increase throughput or even adaptation of equipment limitations or laboratory conditions. In addition, it is important to emphasize that the analytical results obtained generally indicate only the content of a feed and not the nutritional value that the animal will obtain by eating, digesting and metabolizing it (Jung, 1997; Mertens, 2003).

As a way to minimize these variations, Mertens (2003) cites that the different methods need to present inference and reproducibility. Inference refers to the way in which the method will provide correct information and promote an adequate description of the food and reproducibility refers to the ability of the method to be reproduced in duplicate in different laboratories, presenting similar results for the same analysis using the same food, thus ensuring accuracy and precision of the results.

In this context, even though the method for obtaining the fibrous components has been standardized by Mertens (2002) and indicated by the AOAC as a reference method. There are more current analytical methods, which differ from each other. The analysis of the fibrous components without the exclusion of possible errors associated with these differences can compromise their use, making it difficult to compare the results. According to Mertens (2003), the acceptance of an analytical method is based directly on its capacity to combine the concept of fiber and its reproducibility among laboratories.

The most current methods present a limiting factor for many laboratories, and it refers to their accessibility and cost of equipment, making their use as a method of routine analysis difficult. It is also important to point out that many of these methods are not officially recognized, making the detergent system proposed by Van Soest (1963b), even with its modifications, the most widely used, even though it does not determine exactly the composition of the cell wall components.

In this context, more research should be

carried out in order to eliminate possible errors in the analysis, to improve laboratory methods, and consequently to obtain results that present precision (Jung, 1997) and accuracy according to the citation of Mertens (2003).

DEFINITION AND CONSTITUENTS OF THE FIBER

Fiber is a nutritional term, which can only be determined during the digestive process and its definition is directly linked to its source and analytical methodology used in its laboratory determination (Mertens, 2003).

Chemical, enzymatic or gravimetric laboratory methods were developed to quantify a fraction resistant to digestion and not a plant substance recognizable in plants, in addition to the division of plant fractions based on their structures and chemical bonds. Thus, its accuracy and relevance is totally dependent on the analytical methodology used (Mertens, 2003).

Despite the divergences related to its definition, fiber originates from the cell wall of vegetative tissues, constituted by a set of highly complex and heterogeneous fractions (Jung, 2012), which are a mixture of polysaccharides frequently associated with other components, making them resistant to enzymatic digestion, but serving as a substrate for fermentative activity (Van Soest et al., 1991; Jung, 1997).

Fiber is classified according to its physiological state, water solubility and fermentation degree: insoluble fiber and soluble fiber, the insoluble fraction corresponds to the insoluble fractions in neutral detergent solution as cellulose and hemicellulose, which associated to lignin constitute the fiber in neutral detergent; the soluble fraction corresponds to the soluble fractions in neutral detergent, although more heterogeneous than the insoluble fraction, constituted by organic acids, simple sugars, amido and frutosans, plus pectic substances (Hall, 2007; Gern, et al. 2014; Da Silva, et al. 2018).

Cellulose is the most abundant polysaccharide in the cell wall of plants and its proportion varies from 20 to 40% on a dry basis (McDougall et al., 1993; Jung, 2012). Constituted by long linear chains of D-glycose, linked by β-1,4 bonds with a high degree of polymerization and molecular weight. With a fibrous structure of crystalline character, without branches and with rigid and inflexible conformation, it presents high resistance to breakage by different chemical substances (Giger-Reverdin, 1995; Jung, 2012). These linear chains are linked together by hydrogen bonds and Van der Waals forces, being called cellulose microfibrils. They are compacted and are responsible for the mechanical resistance of the tissues they compose, which is important in the evaluation of forage plants. Because this association can influence the sensitivity of the cellulose molecule to microbial enzymatic hydrolysis (Giger-Reverdin, 1995; Macedo Júnior et al., 2007).

Hemicellulose is associated with cellulose in the cell wall, in the intramolecular spaces of cellulose microfibrils, acting as a mass because of the way they connect with the other structural components. Hemicelluloses can be both linear and branched polymers, made up of amorphous polysaccharides with a lower degree of polymerization than cellulose (Giger-Reverdin, 1995). Plants in a more advanced stage of maturity are more closely associated to lignin by covalent bonds than to other polysaccharides, becoming unavailable for solubilization. These present great variation among the types of hemicelluloses and vegetative species, constituting about 10 to 25% of the dry base of forage plants (Giger-Reverdin, 1995). Hemicelluloses are divided into four subgroups,

also called glycans, which are linked to the microfibrils that make up the cellulose through hydrogen bridges, showing structural differences (Macedo Júnior et al., 2007).

Pectins are present in large quantities in cell walls and intracellular tissues of young tissues in the form of protopectins, contributing to rigidity, transport and water retention. Constituted by galacturonic acids that present covalent bonds of the α-1,4 type, intercalated with rhamnose, arabinose and galactose units with chain branches of pentose and hexose units, differing from each other in terms of esterification degree and number of methoxylations. These substances are divided into: pectic acids without methoxylations; pectinic acids with methoxylations that may or may not be water-soluble; and pectins, a subgroup of water-soluble acids (Grenet & Besle, 1991; Müller & Prado, 2004).

Lignins are complex polymers of little known structure and chemically linked to cellulose and hemicellulose (Geron et al., 2014). Constituted by condensed polymers, of different phenylpropanoid alkoxys whose precursors are p-coumaric, ferulic and sinapic acids, which will condense through an oxidative process forming cross-linked macromolecules, the lignins (Grenet & Besle, 1991; Jung, 1997; JUng 2012). Lignin is extremely resistant to chemical degradation, provides rigidity to the cell wall, structural support and physical resistance to plant tissues. It has a condensed structure, through carbon-carbon covalent bonds and ether or ester bonds. Due to these bonds, it becomes extremely resistant to the hydrolytic action of acids and bases; moreover, its composition, structure and quantity are variable in plant tissue (Grenet & Besle, 1991; Jung, 1997; Jung, 2012). According to Van Soest (1967b), this resistance delays the development of laboratory techniques for its quantification.

The fiber constitution is quite variable and depends on several factors. Mainly factors associated with the plant species, between varieties, within the same species and between organs or tissues, depending on the stage of development and/or maturity of the plant tissue (Jung, 1997; Knudsen, 2001; Segura et al., 2007).

Due to this variation, many analytical proposals are found in the literature for the determination of fibrous fractions, and they are divided into two groups: gravimetric and enzymatic-gravimetric (**Table I**).

DETERMINATION OF FIBROUS FRACTION, METHODOLOGICAL ALTERATIONS AND LIMITATIONS OF USE

According to Van Soest (1964), Einhoff in 1809 was the first researcher to try to separate the fibrous fraction of food, through the maceration of fibrous material in water, filtration and weighing of the residue. In 1859, the method known as proximal analysis system or Weende method for crude fiber determination, was standardized in the Experimental Station of Weende (Van Soest & Robertson, 1985). This method consists of two consecutive extractions and the food is treated with two solutions, one acidic and the other alkaline; in the first extraction with the use of a diluted acid solution there is removal of amide, simple sugars, part of the pectin and hemicellulose; and in the second extraction with the use of a diluted alkaline solution there is removal of proteins, pectins, part of the lignin and remanescent hemicellulose. The final residue is washed with quente water and alcohol, dried in an oven, weighed, finally incinerated and determined to cinza. The crude fiber is determined by subtraction of the cinza to the final residue (Jung, 1997; Knudsen, 2001).

Table I. Gravimetric and enzymatic-gravimetric methods**.** Methods Analysis Authors

Methods	Análises	Authors
Gravimetric	Crude fiber	Weende (1859)
	Fiber in neutral detergent and Fiber in acid detergent	Van Soest (1963)
	Acid detergent fiber	Goering & Van Soest (1970)
	Neutral detergent fiber	Van Soest & Wine (1967)
Enzymatic-gravimetric	Enzymatic neutral detergent fiber (eNDF)	Van Soest & Robertson (1985)
	Amylase-treated and amide-free fibrous organic material ("aFDNmo")	Merntes (2002)

Subsequently, the Wennde method was challenged, once after the analysis of the residues, it was verified that the crude fiber is constituted of cellulose with small amounts of lignin and hemicellulose. As a result, the method became limited, due to the solubilization of lignin, an indigestible component (Van Soest, 1964) and also of hemicellulose (Van Soest, 1967b), a fibrous carbohydrate. According to Van Soest (1967b) the amount of lignin solubilized in this method is removed in the second extraction by the alkaline solution together with hemicellulose, causing these fractions to become part of the non-nitrogenous extractive fraction. And the digestibility of the non-nitrogenous extractive becomes inferior to that of crude fiber mainly due to the presence of lignin (Van Soest, 1967b; Jung, 1997; Mertens, 2003).

Due to the above mentioned problem, Van Soest (1963a) developed the technique known as detergent system, with the intention of isolating the fibrous fraction and developing a more effective method to replace the crude fiber method. This was initially developed to isolate insoluble dietary fiber and plant cell wall constituents such as cellulose, hemicellulose and lignin (Van Soest & Wine 1967a; Van Soest & Robertson, 1985; Segura et al., 2007; Geron, et al. 2014).

Also in the 1960s, in the USA, the American organization AOAC (Association of Official Analytical Chemists) made official the Weende method for the determination of crude fiber, even with the above-mentioned limitations. In 1963 the AOAC recognized the method known as the Acid Detergent Method for the determination of fiber and lignin developed by Van Soest, since it was not difficult to standardize it among chemical analysis laboratories (Jung, 1997; Udén et al., 2005; Segura et al., 2007). This method quickly replaced the Weende method, since it was characterized as a simpler method and resulted in values similar to those found by the Weende method.

However, according to Mertens (2003) the acid detergent methodology is used to minimize lignin losses, thus it does not fit into the nutritional definition of dietary fiber. Because the hemicelluloses in acid detergent are removed and pectin, which is a rapidly fermentable carbohydrate, is not removed, it becomes an inadequate method for estimating the fibrous fraction. Possibly the precipitation of pectin in this method could be the reason why some foods with high amount of pectin show results of fiber in acid detergent higher than fiber in neutral detergent.

In 1967a, Van Soest & Wine developed the

Neutral Detergent method, where the results found for crude fiber were superior to those obtained until 1967, because it allowed greater isolation of the cell wall fractions, the acid detergent fiber methodology became less interesting for fiber determination.

According to Van Soeste (1967b) the acid detergent fiber methodology was developed as a preparatory analytical step for lignin determination and has never been used to measure fiber in food. In addition to assisting in the estimation of lignin, the acid detergent methodology allows the determination of celluloses, insoluble nitrogen in acid detergent and insoluble zinc in acid and silica (Udén et al., 2005). Fiber in acid detergent is determined by refluxing a sample in solution containing sulfuric acid (Van Soest, 1967b; MertenS, 2002).

The determination of neutral detergent fiber content is based on the solubilization of the cell wall of the food and through filtration the cell wall is separated from the cellular content. However, part of the pectin ends up being solubilized and quantified as cellular content, thus becoming a frequent routine in food chemical analysis laboratories (Berchielli et al., 2001).

Neutral detergent fiber is characterized as a fraction that occupies space in the gastrointestinal tract and has inconsistent digestibility (Mertens, 2002) and can affect consumption, presenting the need to reduce the size of its particles through mastication to facilitate digestive processes (Mertens, 2003). The soluble content in neutral detergent is highly digestible and totally fermentable in the rumen (Van Soest, 1964; 1967b), occupying little space in the treatment (Jung, 1997; Mertens, 2003). It is characterized as a standard fraction because it allows the separation of its fractions during ingestion and digestibility.

The system proposed by Van Soest used the concept developed by Lucas of ideal nutritional entities, in which the purpose was to identify the uniformity of the chemical fractions that constituted the food, true digestibility and constant endogenous loss. The same authors cited that if the food was analyzed by this concept, its nutritional value could be derived from the product's soma of each nutritional entity (Macedo Júnior et al., 2007).

However, Van Soeste (1967b) demonstrated that the cell wall of food does not behave in a uniform manner as the proposed concept, which makes it impossible to use a single fraction to predict the digestibility of dry matter. Even though the neutral detergent fiber is not uniformly presented according to the recommended concept, the soluble content in neutral detergent can be considered an ideal and uniform nutritional entity because it is almost completely available (98%) (Macedo Júnior et al., 2007). In addition, in this detergent system the food is divided into soluble fraction, which is quickly and completely available, and insoluble fraction, which is slow and completely unavailable.

In the original method of determination of fiber in neutral detergent, solutions with several reagents are used, each one having a specific function. Thus, a neutral detergent solution consisting of sodium tetraborate, ethylene diamine tetraacetic acid (EDTA), sodium hydrogen phosphate, sodium lauryl sulfate and triethylene glycol is used. Buffer solutions based on borate and phosphate are used to keep the pH close to 7.0 to avoid the solubilization of hemicellulose and lignin; sodium lauric sulfate and sodium sulfite to remove proteins; EDTA acid, being a chelating agent, helps in the solubilization of proteins and pectins and triethylene glycol for the solubilization of amides. Meanwhile, the method recovers cellulose, hemicellulose and lignin, with some

contamination by proteins, pectins and amide. Associated with the washing of the fibrous residue with quente water for removal of the non-fibrous matter, followed by the use of acetone for complete removal of lipids and pigments in most of the samples (Van Soest, 1967; Mertens, 2002; 2003).

Gradually, the original method developed by Van Soest & Wine underwent several modifications in one or another analytical step in order to open up a series of foods. Mainly those rich in amide and pectins, tannins and Maillard reaction products (VAN SOEST et al., 1991; Geron, et al. 2014). According to Van Soest et al. (1991) and Mertens (2002), the high values of neutral detergent fiber obtained in concentrated foods and forages could be associated with practical difficulties in the filtration stage, suggesting that the neutral detergent was not efficient in solubilizing mainly amide (Hall, 2007). Thus, in 1982 Mongeau & Brassard recommended an alteration of the original method, through the inclusion of α-amylase, in order to eliminate amide. By making it gelatinized and hydrolyzed with amyloglucosidase, amylases have been used until now in the determination of neutral detergent fiber (Van Soest et al., 1991; Hall, 2003; Valente et al., 2011b).

In the traditional method of Van Soest & Wine, monoethylene glycol ether was used for amide solubilization, but was replaced by triethylene glycol (Van Soest et al., 1991). Decalin, an antifoaming agent, was withdrawn because it caused an increase in neutral detergent fiber values by removing lignin. Sodium sulfite was used for removal of protein contaminations, but it was removed from the methodology because it did not remove them completely and did not degrade lignin. Van Soest & Robertson (1985) altered the original method by excluding sodium sulfite and including α-amylase for amide removal; the method of Van Soest et al. (1991) kept α-amylase and recommended the alternative use of sodium sulfite, without decalin and with the use of triethylene glycol. The method of Mertens (2002) recommends the use of sodium sulfite and α-amylase, in addition to the use of a reflux system with condensers for the extraction of cellular content, filtration and retention of insoluble residue in filtering chains.

David Mertens, in 1980 began to standardize the analysis of neutral detergent fiber among U.S. laboratories, concluding that the only way to reduce the errors among them would be through the analysis of all types of food from a single analytical method (Udén et al., 2005; Segura, et al., 2007). Thus, it was recommended that all foods should be analyzed using α-amylase, sodium sulfite and corrected for zinc. In addition, it was also determined that residual material such as amylase-treated and amide-free fibrous organic matter ("aFDNmo") should be defined as a nutritional entity (Mertens, 2002; 2003; Udén et al., 2005; Segura, et al., 2007).

Correction for blackheads allows elimination of errors arising from inadequate washing of the residues, and allows estimation of non-fibrous carbohydrates, by difference (Mertens, 2002). This allows the exclusion of cell wall constituents such as polysaccharides and pectin, which undergo rapid fermentation and digestion and are similar to the cellular content. Mertens (2002), also recommends that the aFDNmo, besides being conducted with standardized solutions of α-amylase, should be white, to correct possible errors related mainly to sample weighing (Da Silva et al., 2018).

The Mertens method (2002) was adopted as an official method by the Association of Official Analytical Chemists (AOAC, 2002), even with technical flaws, such as difficulties in filtration

and removal of amide, protein contamination, leading to the inclusion of an enzymatic digestion with the use of α-amylase. This was developed for all types of food, from forage plants, grains, oilseeds and animal agribusiness by-products. However, it presents methodological modifications when compared to the original and includes the addition of sodium sulfite to remove protein contaminations and α-amylase to remove amide contaminations during the extraction stage with neutral detergent. However, the use of α-amylase requires industrial standardization at each new analysis.

The methods developed and recommended by Van Soest et al. (1991) and Mertens (2002) are different. Van Soest et al. (1991) cite that the use of sodium sulfite produces undesirable reactions, which would damage cell wall proteins and could solubilize part of the lignin. Já Mertens (2002), cites that sodium sulfite does not solubilize lignin and its use is important for the removal of protein contamination.

Gomes et al. (2012), evaluated the effects of the use of sodium sulfite in a neutral detergent solution on the estimation of fibrous composts in tropical grasses and legumes. They observed that the NDF value decreased when sodium sulfite was used, with a greater reduction in legumes. In addition, sodium sulfite reduced the FDF value in both forages. Lignin content was reduced by sodium sulfite in legumes, but no effect was observed in grasses. The decrease in fiber content in legumes can be explained by the solubilization of lignin. However, the decrease of fibers in grasses cannot be explained only by the decrease of contaminant protein and lignin solubilization, probably losses of other fibrous compounds occurred.

In summary, the analytical methods are developed based on a standard method. In the case of the crude fiber method, there is no specific standard method, only a reference method recommended by the AOAC, and it was developed and improved gradually based on an empirical method. Portanto, the method for determining fibrous fractions still remains empirical, and all the analytical steps detailed by Mertens (2002) as well as any modification of the method may affect what is measured as fiber (Gomes et al., 2012; Da Silva et al., 2018). Thus, the results from the detergent system depend strictly on the recommendations described in the protocols.

Finally, it can be said that, although all researchers rigorously adopt the protocols, there are some practical limitations, which may end up limiting their efficiency in terms of comparing the results obtained (Berchielli et al., 2001; Udén et al., 2005).

In this context, the journal Animal Feed Science and Technology recommends the use of the article by Mertens (2002) as the main reference for neutral detergent fiber analysis. For the analysis of acid detergent fiber, the use of the AOAC manual with the identification number of the specified procedure is recommended; and for the analysis of lignin and cellulose, the sequential quantification from the oxidation of acid detergent fiber in potassium permanganate solution and through the burning of the residue in a muffle, respectively, is recommended (Jung, 1997; Udén et al., 2005; Segura et al., 2007).

Of the three methods used to quantify fiber (neutral detergent fiber, acid detergent fiber and dietary fiber), only neutral detergent fiber is able to estimate the three major indigestible or incompletely digestible components of plants: hemicellulose, cellulose and lignin.

Even following the recommendations of the Journal Animal Feed Science and Technology, the detergent system presents practical limitations, related to the excessive

work in the performance of the reflux and filtration stages of each evaluated sample, which ends up limiting its efficiency in the use of human, financial and even infrastructure resources within the laboratory (Berchielli et al., 2001; Udén et al., 2005). Thus, the choice of the analytical method to be used will depend on some criteria, such as the objective of the researcher or the laboratory, since each method has positive and negative points (Table II).

The conventional method, standardized by Mertens (2002), suggests the use of a refluxing apparatus with condensers and extraction carried out in tanks, and the final residue retained in individual filtering boxes. In addition, there are methodologies that are conducted in totally pressurized environments such as the Ankom® system, which is based on the digestion of conditioned samples in filter bags subjected to extraction with neutral detergent. Unlike the traditional method, this system is less laborious and allows the analysis of a large number of samples per day, where steps such as manual washing and filtering are eliminated. However, one of the drawbacks of using this system refers to the high cost of the bags used, which are obtained from the company that manufactures the equipment (F57 Ankom®) (Berchielli et al., 2001; Valente et al., 2011b).

Thus, in order to reduce the cost of analysis using Ankom® bags, many studies have already been conducted with the use of bags made of similar fabrics such as nylon and tecido-nylon-tetrafluoroethylene (TNT). In order to evaluate the efficiency of using nylon, F57 (Ankom®) and TNT bags in the analysis of indigestible neutral detergent fiber (INDF) of samples with particle size of 1 mm, Casali et al. (2009) observed that the INDF values found with F57 and TNT were higher than those obtained with nylon bags, due to particle loss. Valente et al. (2011b), with the same objective, but different foods, cited that the use of F57 and TNT bags resulted in accurate estimates of NDF content, different from nylon bags also due to the loss of fibrous particles, underestimating the result.

Based on the results presented, it is possible to conclude that TNT bags become an alternative to replace F57 in reducing the cost of analysis. Lanes et al. (2016) cites that possibly this particle loss is due to dilation of the nylon bag mesh during the reflux stage.

Berchielli et al. (2001), following the standardization of the conventional method of Van Soest, compared the values of neutral detergent fiber (NDF) and acid detergent fiber (ADF) of different foods from the Ankom® equipment with the use of four types of saquinhos, and concluded that the different types of saquinhos did not influence the NDF values, with the exception of bovine feces, obtaining lower NDF results. No difference was observed between NDF and FDF values in the results found by the Ankom® equipment and the conventional method, except for citrus pulp, where the FDF by Ankom® was lower than that obtained by the conventional method.

Ferreira & Mertens (2007) also followed the recommendations described by Ankom® and compared the results with the method of Merterns (2002). By evaluating the determination of NDF without the presence of sulfite, NDF with the use of α-amylase and aFDN (with the presence of sulfite and α-amylase) using two extraction methods: refluxing with filtering chains (Mertens, 2002) and Ankom®, in 33 samples of millet silage. They concluded that the absence of α-amylase in the Ankom® method overestimates the NDF values, possibly due to the gelatinization of the amide, which hinders the filtration of the residues. No differences were observed for aFDN concentrations in the two extraction methods, and the lower concentration of fibrous

residue for NDF could be attributed to a lower protein contamination of the fibrous residue.

Table II. Summary of the use and limitations of the main analytical methods used in the determination of fibrous fractions of forage plants. Adapted: SEGURA et al., 2007**.**

Gross Fiber	Part of the cell wall that survives digestion under acid and alkaline solubilization. Cellulose and lignin are recovered in large proportions.	Solubilization of lignin and hemicellulose. Overestimate or crude fiber value.	It consists of two consecutive extractions: in the first one, the food is subjected to an acid solution and the second to an alkaline solution. The residue is washed with hot water and alcohol, dry, heavy, by fim incinerated and determined to cinza. The crude fiber determined by subtraction of the mass of the cinza at mass of the final residue.
Neutral detergent fiber (NDF)	Fraction of food that is not completely digested, with almost complete recovery of the cell wall.	Partial recovery of pectins. Protein and amide soup.	Van Soest (1967): similar to Weende's method, only that is used chemical extractions with a detergent solution neutral over-refluxing. Mertens (2002): similar to the Van Soet method, with the inclusion of an enzymatic digestion through α-amylase.
Acid detergent fiber (ADF)	Cellular wall porpao.	Part of the lignin is solubilized. Pectin precipitation.	Van Soest (1967): similar to the neutral detergent method, only using chemical extractions with detergent solution acid over-reflux. Method of analysis approved by AOAC (2002).
FDN-FDA	Hemicellulose	Limitapóes dos métodos FDN e FDA.	FDN - FDA
Lignin	Lignin	Lignin solubilization in FDA methodology.	Sequential quantification from detergent fiber oxidation acid in potassium permanganate solution, and by means of the muffle residue burner. FDA methodology.
FDA - Lignin	Cellulose	Limitapóes dos métodos FDA e Lignina.	Sequential quantification from detergent fiber oxidation acid in potassium permanganate solution, and by means of the muffle residue burner.

The objective was to compare the values of the acid detergent fiber factor (FDA) of 12 foods using different analytical procedures: two of them using the Ankom® technique, one with direct treatment of the sample in acid detergent (D-ADF) and another sequential treatment with neutral and acid detergent solution (D-ANDF); the third procedure being the conventional methods of Goering and Van Soest (1970) (VS). Danelón et al. (2013), observed that the results found differed among themselves for all procedures (24.58, 27.83 and 28.01%). The FDA theory found in the procedure with direct treatment of the sample was higher than the sequential procedure, with the exception of the millet. Possibly due to the removal of food fractions by the neutral detergent, such as pectic substances.

Da Silva et al. (2018), by comparing alternative methods for NDF analysis with the official method recommended by the AOAC in reflux system and filter cartridges, using 20 foods handled by three analysts. The alternative methods were: refluxing in beakers with beakers, Ankom® system, Tecnal® system and Micro-FDN (Autoclave). Regarding the variability within the laboratory for aNDFom, they observed that the effect of the method-food-analyst component was not significant for the analyst variability component. With significant interaction between method-food for aNDFom. Therefore, the effect of in-feed methods and vice-versa revealed significant contrasts between alternative and reference methods for aNDFom. The refluxing and filtering methods showed no differences, independent of the analyzed food. Significant contrasts demonstrated the lack of quality of the alternative methods used to measure insoluble fiber compared to the reference methods. Barbosa et al. (2015), cite that the autoclave method can be substituted by the conventional method, because it generates accurate results.

In an experiment with the objective of comparing the results obtained by the FDN and FDA non-sequential and sequential analysis through the conventional method proposed by Van Soest et al. (1991) and the alternative method using the autoclave with different bags (Ankom®, TNT and filtering chains). Lourenço et al.(2017), observed that the accuracy of the analysis of NDF and FDA values did not show differences between non-sequential and sequential analysis, in all the foods and methods used, with the exception of the determination of FDA in millet silage, possibly due to the high amide content that was gelatinized inside the filter during the boiling process. Thus, the analytical precision of the alternative methods as well as their use, when compared to the conventional method, depends on the analyzed food.

When comparing the use of alternative equipment such as the fiber digester and the autoclave with nylon (50 μm) and TNT (100g/m²) bags in the determination of NDF and FDF to the conventional method, Farias et al. (2015) concluded that the NDF value obtained with TNT fabric in the two equipments did not differ from those found by the conventional method. However, the use of nylon fabric in both equipments was similar, indicating a possible alternative to the conventional method. There was no difference between the alternative methods of NDF analysis. The FDF theorems differed among the equipment and fabrics evaluated. However, the values obtained with the nylon fabric in the digester equipment were similar to the conventional method, which could be a practical alternative for FDF analysis. The NDF and FDF values obtained in the fiber digester equipment with nylon fabric were similar to the conventional method.

In this context, the modifications made to

the conventional method of Van Soeste and Van Soeste & Wine are different from each other and have advantages and disadvantages. In Brazil, the vast majority of published articles use the methodology described by Silva & Queiroz (2002), which is based on the methodology recommended by the AOAC. Lanes et al. (2016), evaluated the NDF and FDA values in bovine food and feed samples. Through the conventional method, following the recommendations of Silva & Queiroz (2002), and the automated method with TNT sachet (Tecnal® 149). They concluded that the automated method did not differ from the conventional method in terms of NDF concentration in tropical forage, fezes and millet silage samples. It was not efficient for samples with high amide content, even when using α-amylase.

Geron et al. (2014) evaluated the NDF and FDA values of four capins using three methods: the conventional method, the Ankom® method and the method adapted by EMBRAPA. The authors observed that the NDF and FDF values of the capins obtained by the different procedures did not differ from each other. They recommended the procedure adapted by EMBRAPA, since it did not differ in relation to the conventional and Ankom® methodologies, in addition to having lower reagent costs and consequently lower cost. It should be noted that the authors used the recommendations of Silva & Queiroz (2002).

The research presented here is aimed at obtaining alternative methods of analysis that are more precise and accurate, more economical and that reduce the work time when compared to the conventional method, which has a series of laborious steps. Thus, the neutral detergent system will continue to be empirical and its accuracy, reproducibility and inference of its results depend on the rigorous conduction of all the analytical steps, in order to produce comparable and acceptable results regarding the fiber quantity according to its definition.

ANALYTICAL PROBLEMS AND IMPLICATIONS OF THE METHODOLOGIES USED IN DETERMINING THE COMPONENTS OF CELL PARADE

According to Jaurena et al. (2012), the occurrence of errors in the interpretation of food chemical analysis results is becoming increasingly common. Thus, it is important to emphasize once again that there is a need for standardization of the terminologies used in analytical methods (Udén et al., 2005).

Mertens (2003) cites that before discussing any existing analytical variation, it is important to highlight some critical points, such as: all analytical results are only percussors of the real nutritional value of a large quantity of food; variability may be natural and unavoidable; variation can be divided into precision and exaggeration; reproducibility and statistical inference are necessary to detect differences and provide adequate confidence intervals for the results. However, it is important to note that currently one of the major problems related to analytical variation is the selection among the several existing possibilities of the best method to be used to determine the chemical-bromatological composition of the food (Hall, 2007).

The results from food evaluation are vulnerable to any variation, however, the intrinsic and extrinsic variation of the quality of the food used in the formulation of diets causes a greater variability of the analytical results. The sources of intrinsic variations refer to the food's own characteristics that differentiate it from others and are related to physical, chemical and nutritional properties. Extrinsic variations are

those foreign to the nature of the food and are associated, for example, with sampling, analytical procedures and the quality of the reagents used (Jaurena et al., 2012).

In this regard, it is important to consider the first critical point cited by Mertens (2003), that all the results obtained are only predictors of the real nutritional value of a large amount of food. Probably, the main problem consists in the fact of carrying out with precision a representative sample of the material to be analyzed. Associated with the sample, it is worth noting that the sample sent to the laboratory will be ground to obtain particles with a size of 1 mm and only a parcel of this (1g) will be analyzed (Mertens, 2002). However, even if more than one laboratory analyzes a plot of the main sample, it is possible that the results found will be different. Since the plots are not the same, the second critical point above can be established, where variation is normal and unavoidable. Due to the small amount of sample analyzed, it is likely that the result found is only an adequate estimate of the average composition of the whole, provided that the initial sample and the analysis are performed correctly. Thus, the sample of the material to be analyzed is probably the most important source of error in analytical methods (Undersander et al., 1993).

Valente et al. (2011a), in order to evaluate the influence of particle size (1 and 2 mm) on NDF values using nylon bags, F57 (Ankom®) and TNT. They concluded that the wetting should be carried out using 1 mm porosity pencils for proper extraction of the cellular content by neutral detergent and efficient action of the thermostable α-amylase enzyme, since the use of 2 mm particles led to an overestimation of the NDF values. Regarding the material used for sample conditioning, the results showed that F57 and TNT fabrics provided accurate estimates of NDF values. In turn, the accuracy of the results obtained with nylon fabric was compromised due to particle loss. Based on the results, it was expected that the samples wetted at 2 mm would overestimate the NDF values, because the surface area of the sample was so much greater for penetration of the neutral detergent when compared to the 1 mm samples. The opposite is also valid, wet samples with particle size smaller than 1 mm can be washed and lost in the filtration stage. In addition, particles larger than 2 mm can clog the filter membrane, making the filtration stage more difficult.

Even with the occurrence of possible errors associated with the stages of preparation of the sample to be analyzed, Mertens (2003) and Hall (2007), cite that variation can be divided into precision and accuracy, associated with reproducibility and statistical inference to detect differences and provide adequate confidence intervals for the results. Accuracy refers to the absence of variation between the results of the same analysis and the same food between laboratories. The accuracy is related to the result, which must be authentic or true (closer to the real).

In order to find accurate results, it is important that laboratories perform their analyses in duplicates, to obtain an average result closer to the real value. According to Undersander et al. (1993), there are standard deviation values that should be accepted when performing duplicate analyses, but if the values found are higher or lower than the real value, it will be necessary to perform new laboratory analyses.

However, there is an important factor that can affect the variation of the analysis and refers to the heterogeneity of forage plants and their fiber, as well as the heterogeneity of other foods used in the diet formulation of ruminant and non-ruminant animals. The constituents of

forage plants such as leaves and grass, for example, have different compositions independent of their physiological state, and a true result of the chemical-bromatological composition for comparison of the results will depend on a sample and a representative parcel of the whole. For this reason, analyses performed in duplicates or even triplicates allow additional information to be obtained to detect possible differences by calculating the mean and standard deviation of the sample.

Mertens (2003) cites that reproducibility is the ability of the method to be reproduced in duplicates in several laboratories, with similar results for the same analysis using the same food, ensuring accuracy and precision. The inference already refers to the way in which the method will provide correct information and promote an adequate description of the food. According to Jaurena et al. (2012), the variation coming from two laboratories has two specific causes: intra-laboratory variability, which refers to the variation existing within the same laboratory; and interlaboratory variability, which refers to the variations observed between different laboratories.

Probably, this variability may be related to small routine errors, differences between equipment and reagents used, lack of equipment calibration or even differences observed in the plots that are removed from the main sample sent for analysis that is not completely homogeneous. However, methodologies can be adapted to increase laboratory efficiency and save time. However, part of this variability can be reduced through the rigorous use of protocols, training of analysts and their familiarization with the methodology used.

It is also worth noting that, currently, this variability is easily observed through static analysis, where the errors related to the analytical methods are easily detectable by comparing the results found to those coming from the reference method.

Mertens (2002; 2003) cites that for a method to be considered ideal, the interlaboratorial variation must be equal to zero and the intra-laboratorial variation must be equal to the variation between analyses from duplicates or triplicates. These results are verified through voluntary collaborative studies conducted by AOAC and National Forage Testing (NFTA), with the aim of quantifying analytical variations and establishing acceptable reproducibility by generating reproducible and comparable analytical results for rotational analysis. It involves about 8 or more laboratories, which analyze about 5 samples in duplicate through the routine methodology used in them. The results are then subjected to three statistical analyses and verified by the organization responsible for the collaborative study.

Hristov et al. (2010), in a collaborative study to evaluate the variability in aFDN analysis, found a high variation in the analytical procedures used among laboratories, possibly due to the lack of rigorousness in following all the steps of the available protocol. They evaluated the variability for PDN analysis among 14 participating laboratories, through the use of the traditional reflux system and the Ankom®. They concluded that the results of aFDN did not differ among the 14 participating laboratories, but differences were observed among the laboratories within the same technique and a high incidence of outliers for the Ankom® method. They emphasized the need for laboratories to follow exactly the protocol of the official method, and recommended that laboratories carry out quality controls of the equipment and procedures used.

In this context, it is important to note that all available analytical methods for the determination of cell wall constituents are

empirical (Mertens, 2003). Fiber is defined by the method of analysis and its source, so any modification in the analytical method can determine a new fiber value that is not comparable with that coming from the conventional method (Hall, 2007). Thus, the results from laboratory analysis present some indeterminacy, due to errors associated with the critical points cited by Mertens (2003). These can be minimized by improving the aspects related to the methodologies and equipment used and personnel training, as well as the exaggeration in the use of the protocols so that the results present reproducibility and repeatability.

Associated with these improvements, it is important to carry out more research, with the aim of developing new analytical methods that provide reliable results and a more rigorous laboratory routine.

FINAL CONSIDERATIONS

The use of analytical methods allows estimating the composition and availability of the different fractions of the cell wall, but the incorrect interpretation of the results and the variability of the cell wall constituents require knowledge of the different analytical methodologies.

However, traditional or alternative analytical methods are still empirical. Considering that a negative point regarding the large number of analytical methodologies, which present different results for the same analysis, is the analysis and comparison of the results, since they are based on the variability of their own data to infer how much better or better is their result when compared to an intra- or interlaboratorial reference value or standard. In this context, this reference value or standard is derived from the traditional method, which ends up becoming "bad" when there is a very large variation of results between analytical methods. These can be minimized by the improvement of methodological procedures, equipment used and personnel training. In addition to the exaggeration in the use of the protocols so that the results present reproducibility and repeatability.

Alternative analytical methodologies that exhibit acuracy, reproducibility and repeatability when compared to the traditional method of Van Soest, Van Soest & Wine or the Mertens reference method appear to be viable in determining the constituents of the fibrous fractions.

Thus, the improvement of analytical methods is extremely important for the estimation of the nutritional value of foods. The limitations of analytical methods seem to be related to the inability to adequately solubilize the soluble fractions and part of the insoluble fractions, which end up being partially solubilized.

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Reference

1. Barbosa, MM, Detmann, E, Rocha, GC, Franco, MO, Valadares Filho, SC 2015, Evaluation of laboratory procedures to quantify the neutral detergent fiber content in forage, concentrate, and ruminant feces. Journal of AOAC International, vol. 98, no. 4, pp. 883-889.
2. Berchielli, TT, Sader, APO, Tonani, FL, Paziani, SF, & Andrade, P 2001, Avaliação da determinação da fibra em detergente neutro e da fibra em detergente ácido pelo sistema ANKOM. Revista Brasileira de Zootecnia, vol. 30, no. 5, pp. 1572-1578.

3. Berchieli, TT, Pires, AV, & Oliveira, SG, 2006, Nutrição de ruminantes. Jaboticabal: FUNEP, 2011. 583 p .
4. Casali, AO, Detmann, E, Valadares Filho, SC, Pereira, JC, Cunha, M. Detmann, KSC, & Paulinho, MF 2009, Estimação de teores de componentes fibrosos em alimentos para ruminantes em sacos de diferentes tecidos. Revista Brasileira de Zootecnia, vol.38, no. 1, pp.130-138.
5. Danelón, JL, Guaita, MS, Fay, P, Chifflet, S, Wawrzkiewicz, M, & Fernández, HM 2013, Technical note: comparison of three analytical procedures to estimate the acid detergent fiber concentration in feeds of widespread use in argentina. Archivos Latinoamericanos de Producción Animal, vol. 21, no 2. pp. 131-134.
6. Detmann, E, Paulino, MF, Mantovani, HC, Valadares Filho, SC, Sampaio, CB, Souza, MA, Lazarrini, I, & Detmann, KSC 2009, Parameterization of ruminal fibre degradation in low-quality tropical forage using michaelis-menten kinetics. Livestoc science, vol. 126, pp. 136-146.
7. Farias, JS, Queiroz, LO, Santos, GRA, Fagundes, JL, & Silva, MA 2015, Avaliação de tecidos e equipamentos alternativos na análise de fibra em detergente neutro e de fibra em detergente ácido. Boletim Industrial Animal, vol. 72, no 3. pp. 22933.
8. Ferreira, MB, & Mertens, DR 2007, Measuring detergent fibre and insoluble protein ir corn silage using crucibles or filter bags. Animal Feed Science and Techonology, vol. 133, pp. 335-340.
9. Giger-Reverdin, S, 1995, Review of the main methods of cell wall estimation: interest and limits for ruminants. Animal Feed Science Technology, vol. 55, pp. 295-334.
10. Gomes, DÍ, Detmann, E, Valadares Filho, SC, Mezzomo, R, Souza, NKP, Queiroz, AC, & Detmann, KSC, 2012, Evaluation of sodium sulfite and protein correction in analyses of fibrous compounds in tropical forages. Revista Brasileira de Zootecnia, vol. 41, no. 1, pp. 225-231.
11. Grenet, E, & Besle, JM, 1991, Microbes and fiber degradation. In: JOUANY, J.P. (Ed.), Rumen microbial metabolism and ruminant digestion. Paris: INRA, 1991. p. 107-129.
12. Gomes, DI, Detmann, E, Valente, TNP, Valadares Filho, SC, & Queiroz, AC 2011, Avaliação laboratorial de compostos fibrosos em alimentos e fezes bovinas sob diferentes ambientes físicos. Arquivo Brasileiro de Medicina Veterinária e Zootecnia, vol.63, no. 2, pp. 522-525.
13. Hall, MB 2003, Challenges with nonfiber carbohydrate methods, Journal of Animal Science, vol. pp. 3226 3232, 2003.
14. Hall, MB 2007, Methodological challenges in carbohydrate analyses. Revista Brasileira de Zootecnia, vol.36, special supplement, pp.359-367.
15. Hristov, A, Mertens, D, Zaman, S, Vander Pol, M, & Price, W 2010, Variability in feed and total mixed ration neutral detergent fiber and crude protein analyses among commercial laboratories. Journal of Dairy Science, vol. 93, no. 11,pp. 5348-5362.
16. Jaurena, G, Wawrzkiewicz, M, & Colombatto, D 2012, Proposed terminology for animal nutrition laboratory reports. Technical Note. Revista Argentina de Producción Animal, vol. 32, no. 2, pp. 135-147.
17. Jung, HG 1997, Jung Analysis of Forage Fiber and Cell Walls in Ruminant Nutrition. The Journal of Nutrition, vol. 127, pp. 810-813.
18. JUNG, HJG, 2012, Forage digestibility: the

intersection of cell wall lignification and plant tissue anatomy. Department of Agronomy and Plant Genetics, Department of Animal Sciences, University of Minnesota, USA, 2012, p. 162-173.
19. Knudsen, KEB 2001, The nutritional significance of "dietary fibre" analysis. Animal Feed Science and Technology, vol. 90, pp. 3-20.
20. Lanes, ÉCM, Lopes, FCF, Campos, NR, Oliveira, JS, & Morenz, MJF 2016, Frische-Neto, R 2016, Comparative efficacy of the conventional and automated methods for determining neutral and acid detergent fiber. Comunicata Scientiae, vol. 7, no. 1, pp. 30-37.
21. Lourenço, MS, Messana, JD, Sader, APO, Canesin, RC, Malheiros, EB, Castagnino, PS, & Berchielli, T. 2017, Comparison of laboratory methods to assess fiber contents in feedstuffs. Revista Colombiana de Ciencias Pecuarias, vol. 30, pp. 21-29.
22. Macedo Júnior, GL, Zanine, AM, Borges, I, & Pérez, JRO 2007, Qualidade da fibra para a dieta de ruminantes. Ciência Animal, vol.17, no. 1, pp. 7-17.
23. Mertens, DR 2002, Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. Journal of AOAC International, vol. 85, no. 6, pp. 1217-1240.
24. Mertens, DR 2003, Challenges in measuring insoluble dietary fiber. Journal of Animal Science, vol. 81, pp. 3233-3249.
25. Mongeau, R.; & Brassard, R 1982, Determination of Neutral Deteigent Fiber in Breakfast Cereals: Pentose, Hemicellulose, Cellulose and Lignin Content. Journal of Food Science, vol. 47, pp. 550-555.
26. Müller,M,& Prado,IN2004,Metabolismo da pectina em animais ruminantes uma revisão. Revista Varia Scientia, vol. 04, no. 8, pp. 45-56.
27. McDougall, GL, Morrison, IM, Stewart, D, Weyers, JDB, & Hillman, JR 1993, Plant Fibres: chemistry and processing for industrial use. Journal of the Science of Food Agriculture, vol. 62, pp.1-20.
28. Segura, SF, Echeverri, FR, Patiño LI, AC, & Mejía, GAI 2007, Description and discussion about methods of fiber analysis and nutritional value of forages and animal feeds. Journal of the Faculty of Pharmaceutical Chemistry, v. 14, p. 72-81.
29. SILVA, DJ, & QUEIROZ, AC, 2002, Análise de alimentos (métodos químicos e biológicos). 3.ed. Viçosa: Universidade Federal de Viçosa, 2002. 235 p.
30. Da Silva, RST, Fernandades, AM, Gomes, RS, Bendia, LCR, Silva, LC, Viera, RAM, 2018, On the specificity of different methods for neutral detergent fiber and related problems. Animal Feed Science and Technology, vol. 240, pp. 128-144.
31. Udén, P, Robinson, PH, & Wiseman, J 2005, Use of detergent system terminology and criteria for submission of manuscripts on new, or revised, analytical methods as well as descriptive information on feed analysis and/or variability. Animal Feed Science and Technology, vol. 118, pp. 181-186.
32. Undersander, D, Mertens, DR, & Thiex, N, 1993, Forage analyses procedures. Omaha: National Forage Testing Association, p. 139, 1993.
33. Valente, TNP, Detmann, E, Valadares Filho, SC, Queiroz, AC, Sampaio, CB, & Gomes, DI 2011, Avaliação dos teores de fibra em detergente neutro em forragens, concentrados e fezes bovinas moídas em diferentes tamanhos e em sacos de diferentes tecidos. Revista Brasileira de Zootecnia, vol 40, no. 5, pp.1148-1154a.

34. Valente, TNP, Detmann, E, Valadares Filho, SC, Paulino, MF, Figueiras, JF, & Souza, MA 2011, Simulation of variations in the composition of samples in the evaluation of neutral detergent fiber contents by using cellulose standard in filter bags made from different textiles. Revista Brasileira de Zootecnia, vol.40, no. 7, pp.1596-1602b.
35. Van Soest, PJ 1963, Use of Detergents in the Analysis of Fibrous Feeds. I. Preparation of Fiber Residues of Low Nitrogen Content. Journal of the Association of Official Analytical Chemists, vol. 46, no 5, pp. 825-829a.
36. Van Soest, PJ 1963, Use of detergents in the analysis of fibrous feeds. II a rapid method for determination of fiber and lignin. Journal of the Association of Official Analytical Chemists, vol. 46, no. 5, pp. 829-835b.
37. Van Soest, PJ 1964, Symposium on Nutrition and Forage and Pastures: New Chemical Procedures for Evaluating Forages. Journal of Animal Science, vol.23, no. 3, pp. 838-845.
38. Van Soest, PJ 1967, Development of a comprehensive system of feed analyses and its application to forages. Journal of Animal Science, vol. 26, pp. 119-128b.
39. Van Soest, PJ, Wine, RN 1967, Use of detergents in the analysis of fibrous feeds. IV. Determination of plant cell-wall constituents. Journal of the Association of Official Analytical Chemists, vol, 50, no. 1, pp. 50-55a.
40. Van Soest, PJ, Robertson, JB 1985, Analysis of forages and fibrous foods. AS 613 Manual, Dep. Animal Science, Cornell Univ., Ithaca, NY, 1985. Van Soest, PJ, Robertson, JB, & Lewis, B 1991, A Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. Journal of Dairy Science, vol. 74, no. 10, pp. 3583-3597.

ENVIRONMENTAL MONITORING OF HEAVY METALS IN AQUATICA MACROPHYTES BY ANALYZING ATOMIC ABSORPTION SPECTROMETRY - AAS IN THE CASCAVEL RIVER BASIN, GUARAPUAVA, PR

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Breno Henrique Marcondes de Oliveira2
Mariane Butik3

ABSTRACT

High concentrations of heavy metals in urban watersheds can have harmful effects on human health and have contributed to environmental contamination. This work aims to evaluate the total concentration of Zn (Zinc), Mg (Magnesium), Lead (Pb), Cr (Chrome), Manganese (Mn) and Ni (Nickel) analyzed according to FAAS "Flame Atomic Absorption Spectrometry". in samples of the aquatic macrophyte Egeria dense present in the urban stretch of the hydrographic basin of the river Cascavel, Guarapuava, PR; and specific objectives to identify potential sources of contamination by heavy metals and obtain physical parameters of the water. The elements zinc, magnesium, manganese and lead showed greater potential for upstream emissions, a fact associated with the proximity of the industrial zone of Guarapuava. It is likely that the increase in electrical conductivity values in the upstream points is related to the discharge of domestic effluents. The decrease in conductivity and total dissolved solids in the sampling points occurs downstream, corresponding to the lower topography of the study area and the end of the urban stretch of the Cascavel River basin. Despite the concentrations of heavy metals being considered critical, the plant showed efficiency in the bioaccumulation of these chemical elements, being an effective instrument for research and environmental assessment, and aquatic macrophytes can be the basis for biomonitoring studies of urban environments impacted by heavy metals.

keywords: biogeography, ecology, environmental analysis, analytical chemistry.

INTRODUCTION

Among the various aspects of environmental pollution resulting from industrial, agricultural and urban activities; pollution by toxic metals is important due to its high resistance to degradation,

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toxicity at low concentrations and potential for bioaccumulation in the aquatic system (Ahmad et al, 2014). The release of methane through human activities to the environment has increased over the years (Olowoyo et al, 2012), where its concentrations are gradually increased and consequently absorbed by organisms and/or sediment (Arai et al, 2007).

Heavy metals are inorganic pollutants, pollutants and can alter the physical, chemical or biological characteristics of natural waters, air, soil, plants and food (Karnitz Júnior, 2007), and can be absorbed in river sediments or accumulated in benthic organisms, sometimes in toxic levels (Arias et al, 2007), and contaminate the food chain by leaching to groundwater or by plant absorption and bioaccumulation (Batista, Freire, 2010), being associated with neurotoxicity, nephrotoxicity and hepatotoxicity in humans (Divan Junior, 2009).

In general, several plants have the ability to adapt and survive in contaminated environments (Pio et al, 2013), being able to absorb and/or accumulate phytotoxic heavy metals present in contaminated soils, waters or atmosphere, manifesting varied symptoms, usually specific to each type of contamination. Aquatic plants have proven to be one of the most apt bioindicators in the aquatic ecosystem, being able to accumulate metals in all tissues and transfer them to the food chain, being this accumulation one of the topics of environmental interest nowadays, either because of the phytotoxicity of many of these metals or because of the potentially harmful effects on animal and human health (Maiga et al, 2005). In addition, aquatic macrophytes can be used to assess the health of the water body, as remediators of ecosystems or even in constructed systems (wetlands), and can also be used to treat domestic and industrial effluents (Sipaúba-Tavares, 2012).

Among the various plant species that have proliferated in the urban stretch of the Cascavel river basin, in the municipality of Guarapuava, PR, stands out Egeria densa, also known as "Brazilian eloid", a submerged and rooted macrophyte of twelve submerged waters, with a limnetic and perennial environment (Oliveira et al, 2005), a plant native to the southeastern coast of Brazil (Alfasane et al, 2010), which multiplies mainly by fragmentation of the canopy, being reproduction by seeds very rare (Rodella et al, 2006), having a relatively high growth rate under ideal conditions.

In this work we consider the hypothesis that the entry of heavy metals resulting from urban pollution in the urban stretch of the hydrographic basin of the Cascavel River, municipality of Guarapuava, PR, may compromise the different environmental uses of this hydrographic basin in the medium and long term, since this hydrographic basin drains almost all the drainage of this city (Peres et al., 2008). Thus, it is considered that the analysis of the occurrence of heavy metals in dense Egeria along the urban basin of the Cascavel River, which presents different forms and intensities of degradation and degradation, with sources of pollution from residences located nearby, agricultural activities, industries, irregular waste disposal and urban sludge, could be an effective measure of environmental monitoring.

The general objective of this article is to evaluate the spatial variability and total concentration of Zn (zinc), Mg (magnesium), Chumbo (Pb), Cr (chromium), Manganese (Mn) and Ni (nickel) in the aquatic plant Egeria densa, in the urban stretch of the Cascavel River basin, with the specific objectives of identifying the potential sources of heavy metal contamination in the study area and obtaining physical parameters of the water. The total concentration of heavy metals in organic matter was analyzed according to FAAS "Flame Atomic Absorption Spectrometry", one of the most widely used techniques in the determination of elements in low concentrations, which are present in a variety

of samples, whether liquid, solid, suspended or even gaseous, and can be associated with flow analysis systems and allow speciation studies (Amorim et al, 2008).

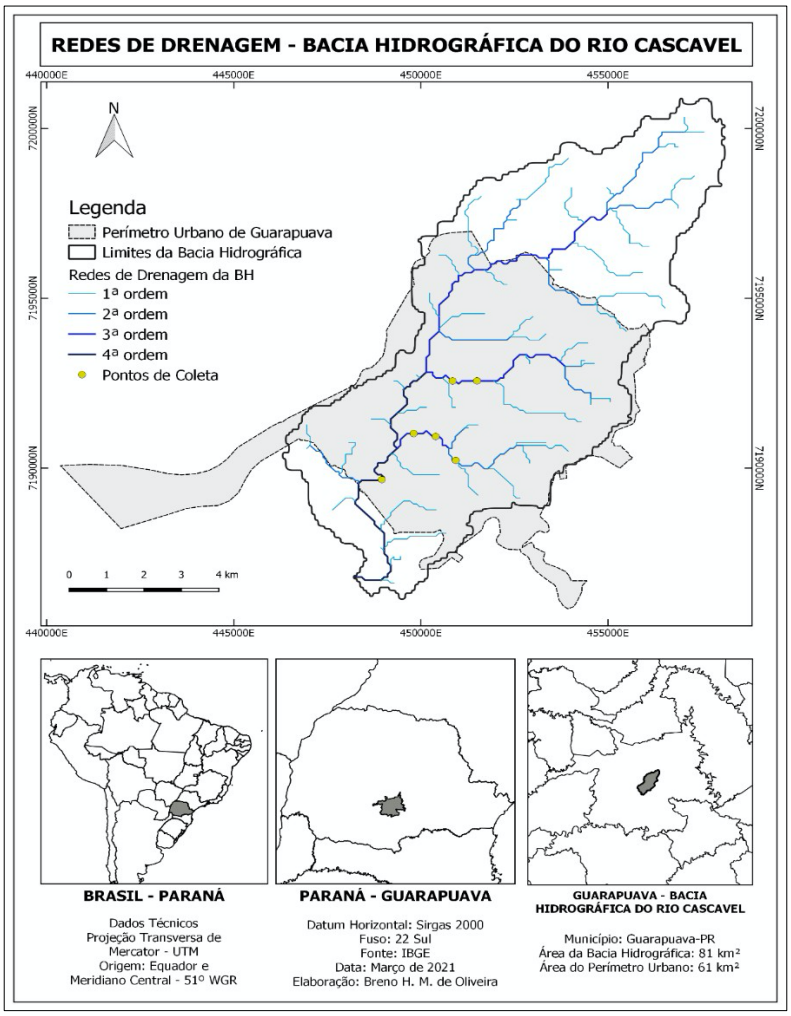
The collection of the determined tracts, identification, cataloguing, sample preparation and preliminary results took into consideration the seasons of the year and specific laboratory processes in the Hydrology Laboratory - LABHIDRO, of the Geography Department and the Trace Analysis and Instrumentation Laboratory - Chemistry Department, both located at the Midwest State University - UNICENTRO, Cedeteg campus, Guarapuava, PR.

Thus, it is considered the possibility of establishing an environmental monitoring program of heavy metals, for the analysis of the occurrence of these metals in aquatic macrophytes in the urban environment, aiming at the protection of human health, determination of spatial and temporal trends of pollution processes and their effects on ecosystems, as well as obtaining data for proper environmental management, contributing with information to governmental bodies and interested institutions for the implementation of strategies to control environmental pollution.

METHODOLOGY AND CHARACTERIZATION OF THE STUDY AREA

This work has as a spatial cutout the urban stretch of the Cascavel river basin, located in the center west of Paraná, in the municipality of Guarapuava - PR. The Cascavel River has a drainage area of 81.03 km², with a 4th order fluvial hierarchy, and has more than 40% of its basin occupied in the urban area of Guarapuava (67.86 km²). The municipality is located in the Center-South region of the state of Paraná, in the Third Paranaense Plateau or Guarapuava Plateau (Maack, 2002) and has a territorial area of 3,117.598 km². The total population of the municipality is 180,364 inhabitants (IPARDES, 2018), of which 152,993 (91.43%) reside in the urban area. The municipality, according to (Thomaz, Vestena, 2003), is an area of the extratropical zone, which favors temperatures with mesothermal character, predominating annual temperatures between 16º and 20ºC, with cold winter and summer enlivened by the altitudes. According to Köppen's classification, the climatic type is "Cfb", corresponding to the temperate climate, rainy and moderately hot summers (Ayoade, 1983).

Initially, a study of the urban stretch of the Cascavel River basin was carried out for the selection of collection points, evaluation of access to the site and logistics. Six aquatic macrophyte collection sites were chosen in the urban environment, strategically located considering the different geomorphological characteristics, as well as the use and occupation of the soil in the Vila Carli, Alto Cascavel and Olarias neighborhoods (Figure 1). The collection points were identified by their geographic coordinates.



Legenda
Drainage Networks of BH
Guarapuava Urban Perimeter
Limits of the Hydrographic Basin
1st order
2nd order
3rd order
4th order
Pontos de Coleta
DRAINAGE NETWORKS - HYDROGRAPHIC BASIN OF THE RIVER CASCAVEL
BRAZIL - PARANÁ
Technical Data Transverse Mercator Projection - UTM
Origin: Equator and
Central Meridian - 51st WGR
PARANA - GUARAPUAVA
Horizontal Datum: Sirgas 2000
Fuso: 22 Sul
Fonte: IBGE

Date: March 2021
Elaboração: Breno H. M. de Oliveira
GUARAPUAVA - HYDROGRAPHIC BASIN OF THE CASCAVEL RIVER
Municipality: Guarapuava-PR
Área da Bacia Hidrográfica:
Urban Perimeter Area:

Figure 1. Egeria densa collection points in the urban stretch of the Cascavel River Basin, Guarapuava-PR.
Source: Prepared by the authors (2021).

The identification of potential sources of contamination consisted of the survey of potential sources of heavy metals within the urban drainage area of the Cascavel river basin. Foram coletadas 48 amostras (sendo 12 amostras por campanha) da planta aquática Egeria densa em seis pontos ao longo do trecho urbano do Rio Cascavel, entre novembro de 2017, janeiro, abril e julho de 2018; sendo realizada com a utilização de luvas e material inerte para coleta do material. Samples were removed where the water level was sufficiently high to keep the plant submerged and prey to a stable substrate, in good physiological state. The physical and chemical parameters of water quality measured in the field were water temperature (C), specific conductivity (µs.cm-1), pH and total dissolved solids (mg/L), using a Hanna Multiparameter probe - Model HI 769828.

The samples were washed in the laboratory, directly in running water, in order to remove sediments and invertebrates, being later stored in plastic bags, catalogued and identified in the Hydrology Laboratory, UNICENTRO Geography Department. Before being subjected to analytical determinations, the samples were washed, dried in an oven with air circulation at a temperature of 30°C for 48 hours and manually wetted with the use of a gral and pistil. The biomass was weighed on an analytical balance (Bioprecisa, FA-2104N).

To analyze the presence of heavy metals, the procedures were performed using the FAAS technique "Flame Atomic Absorption Spectrometry", at the Laboratory of Trace Analysis and Instrumentation, linked to the Department of Chemistry - UNICENTRO, in duplicate. Samples of 0.1g of Egeria densa were prepared for wet digestion with nitric acid in a digestion block for subsequent quantification of the metabolites by flame atomization atomic absorption spectrometry (FAAS) in Varian equipment model SpectraAA220, equipped with an oxy-cathode lamp, in an air/acetylene flame, by the direct method and a melt corrector in flame analysis with oxy-cathode lamps, procedure carried out according to (SANTOS et al, 2006). The concentrations of these metals are expressed as a function of dry weight (mg/kg). The choice of the analysis of the heavy metals Zn (zinc), Mg (magnesium), Chumbo (Pb), Cr (chromium), Mn (manganese) and Ni (nickel) was based on studies by Dean (1972), Braile and Cavalcanti (1993) and Santos (2012), these metals being commonly present in industrial effluents.

The parameters used for the measurement of heavy metals in organic matter were in accordance with Kabata-Pendias, Pendias (2001), Brazil (1998), FAO (1992) and Malavolta (1994). The definition of water quality refers to the type of use to which it is destined, and stipulates the quality standards in resolution 357 of the National Council of the Environment (CONAMA, 2005) and its modifications in resolutions 410 of 2009 and 430 of 2011. The parameters are defined in acceptable limits of substances present according to water use.

RESULTS AND DISCUSSION

PHYSICAL PARAMETERS OF WATER

The hydrographic basin is understood as a fundamental geographic unit for the management of surface and groundwater resources and is also used for actions related to environmental planning (Gorayeb, Pereira, 2014). The use of the hydrographic basin as a unit of analysis of environmental systems, presents a more appropriate conception to work with the systemic approach, starting from the perspective of the tripod formed by the environmental, social and economic dimension (Albuquerque, 2015), making it possible to deal with the components and dynamics of the interrelationships necessary to the planning of land use and environmental conservation.

Because it is an open system with energy input (hydrological cycle) and material export (water, solutes, sediments, etc.), the importance of adopting hydrographic basins for urban planning has been discussed in areas such as hydrology, geology and other environmental areas, which began to discuss them during the nineteenth century. This advance is of great importance, as it serves as a basis for justifying the delimitation of these areas as "ideal" for urban planning purposes (Mirandal et al, 2017).

Table 1 shows the values obtained for the physical parameters of the water of the points chosen for sampling, by arithmetic mean and standard deviation. The pH and total dissolved solids were found to be adequate in relation to the values recommended by CONAMA, 2011; for Special Class 1, which establishes pH values between 6 to 9 and up to 500 mg/L for total dissolved solids. However, we emphasize that there was a decrease even lower in pH which, without oxygen, can influence the solubilization of heavy metals in sediments and cause their undesired introduction into the food chain (Gonçalvez, 2016).

Regarding conductivity, the sample points showed mean and standard deviation of 105.1+-15.2 µs.cm-1, being that natural waters show conductivity values in the range of 10 to 100 µs.cm-1. In environments polluted by domestic or industrial waste, the values can reach 1,000 µS/cm (FUNASA, 2014). It is probable that the increase in the values of the electrical conductivity in points 1 and 2 are related to the clearance of domestic effluents upstream of the analysis points. Philipi Júnior et al. (2004) affirm that as dissolved solids are added to a water body, the electrical conductivity of the water increases, as observed in the analysis points.

PONTO	Temperature C	conductivity µs.cm-1	PH	total dissolved solids (mg)
25°22'53.43 "S 51°28'56.54 "W	19.7	132.3	6.4	70.3
25°22'57.75 "S 51°29'14.40 "W	20.5	112.6	6.3	62.6
25°24'10.96 "S 51°29'17.48 "W	19.3	107.6	6.1	57.6
25°23'46.47 "S 51°29'34.57 "W	18.4	96.0	6.0	47.3
25°23'44.06 "S 51°29'56.36 "W	18.1	99.6	6.0	48.6
25°24'26.47 "S 51°30'29.98 "W	18.3	83.0	6.3	40.3
Arithmetic mean and standard deviation	19.5+-0.8	105.1+-15.2	6.1+0.2	54,4+-10.1

Table 1. Mean values of temperature (C), conductivity (µS/cm), pH and total dissolved solids (mg), arithmetic mean and standard deviation between sampling points in the urban stretch of the Cascavel River, municipality of Guarapuava, PR. Surveys conducted between November 2018 and

July 2019.

Source: Prepared by the authors (2021).

The decrease in conductivity and total dissolved solids in the sample points at the right, corresponding to the lower topography of the study area, at the end of the urban stretch of the Cascavel River basin towards the ETE-Estação de Tratamento de Esgoto, may indicate a process of natural dilution and accumulation of elements along the urban stretch. However, further research and more samples are needed to analyze this process.

QUANTIFICATION OF HEAVY METALS IN DENSE EGERIA BY SAMPLE POINT

According to Tuna et al, 2006, heavy metals are stable and persistent environmental pollutants, since they cannot be degraded and, depending on the physical and chemical characteristics of the aquatic environment, they reaggregate, disperse or are mobilized and deposited in sediments, constituting a potential hazard due to the bioavailability characteristics they can acquire. The choice of the evaluated targets was based on the identification of potential sources of pollution in the proposed study area.

The use and management of soils are indicators for the management of water resources and the health of a river basin, since the quality of water bodies is the result of the actions that occur along their streams. In relation to Table 2, the occurrence of zinc and nickel showed critical concentrations for plants. Magnesium showed values above the norm, however, Torres, 2005 affirms that magnesium does not cause significant problems for human health. In addition, this metal is commonly found in plants.

Ponto	Zn (mg/kg) media Teor normal 1-100 Critical Conc. 100-400	Ni (mg/kg) media Teor normal 0.02-5 Critical Conc. 10-100	Mg (mg/kg) média Teor normal 30-100* Mg (mg/kg) média Teor normal 30-100* Mg (mg/kg)
25°22'53.43 "S 51°28'56.54 "W	149 - 95 - 290- 207	160 36.561 - 27	554-382-242-285
25°22'57.75 "S 51°29'14.40 "W	183 -318 -482 -283	26 43.5 - 38 - 38	488-352-376-297
25°24'10.96 "S 51°29'17.48 "W	159 -68 -161- 166	11.5 - 59 - 20 - 49	390-379-271-289
25°23'46.47 "S 51°29'34.57 "W	191 -146 - 172-219	17.5 49.5 47 - 40	484-370-482-289
25°23'44.06 "S 51°29'56.36 "W	178 - 96 - 307-227	12.5 - 38 - 10 - 54	461-351-317-274
25°24'26.47 "S 51°30'29.98 "W	153-89 -227 - 95	68.5 - 71 - 55 - 36	420-415-327-229

Table 2. Average values found for Ni, Zn and Mg in Egeria densa, in the municipality of Guarapuava, PR and respective normal values and critical concentration (Kabata-Pendias & Pendias, 1992; *Knezek & Ellis, 1980). Without reference parameter. Surveys conducted between November 2018 and July 2019.

Source: Prepared by the authors (2021).

In relation to Table 3, the occurrence of chromium showed to be below the critical concentration for plants. However, the residues have a high power of contamination and chromium easily reaches the groundwater table or even reservoirs or rivers, which are the water supply sources of the cities (CETESB, 2005).

Ponto	Cr (mg/kg) median	Mn (mg/kg)media Teor	Pb (mg/kg)median Teor

	Theor normal nd Conc. critical 75-100	normal 0.02-5 Critical Conc. 10-100	normal 0.20-20 Critical Conc. 30-300
25°22'53.43 "S 51°28'56.54 "W	50 - 40 - 65 - 49	48 - 113 - 30 - 45	75.5 50.5 - 67 - 33
25°22'57.75 "S 51°29'14.40 "W	33 - 63 - 8 - 6	96 - 143 - 111 58	72.5 - 55 - 68 - 32
25°24'10.96 "S 51°29'17.48 "W	63.5 45.5 - 10 - 5	124 - 105 - 76 92	70 - 47 - 54 - 20
25°23'46.47 "S 51°29'34.57 "W	12 59.5 - 18 - 13	137 - 260 - 186 120	88.5 52.5 - 79 - 34
25°23'44.06 "S 51°29'56.36 "W	77 - 65 - 35 - 1	166 - 221 - 155 - 93	86.5 48.5 - 66 - 26
25°24'26.47 "S 51°30'29.98 "W	62 44.5 - 0 - 0	296 - 232 - 192 230	75 40 - 59 - 21

Table 3. Average values found for Cr, Mn and Pb in Egeria densa, in the municipality of Guarapuava, PR and respective standard values and critical concentration (Kabata-Pendias & Pendias, 1992). Without reference parameter. Surveys conducted between November 2018 and July 2019.

Source: Organized by the authors (2021).

Only points 4, 5 and 6 at the right are found with critical amount of manganese. The prickly pear cactus is within the critical limit of occurrence in all the points of analysis. In addition to natural weathering processes, the main sources of prickly pear are automobile exhaust, industrial flames, foundries, fertilizers, pesticides, pigments and atmospheric deposition in gasoline that contains it as an additive (Sharma, Bubey, 2005).

POLLUTING SOURCES IN THE URBAN STRETCH OF THE RIVER CASCAVEL, GUARAPUAVA, PR

The intensification of anthropic activities in the urban stretch of the river Cascavel, Guarapuava, PR occurred without planning or control, being associated with nutrient loading due to the contribution of domestic and industrial waste and chemical fertilizers used throughout the study area, leading to a condition of imbalance in the system. In addition, as well as the point pollution caused by clandestine sewage, the diffuse pollution from rainfall and surface runoff contributes as an important source of deterioration of the quality of urban drainage water (Song et al, 2017).

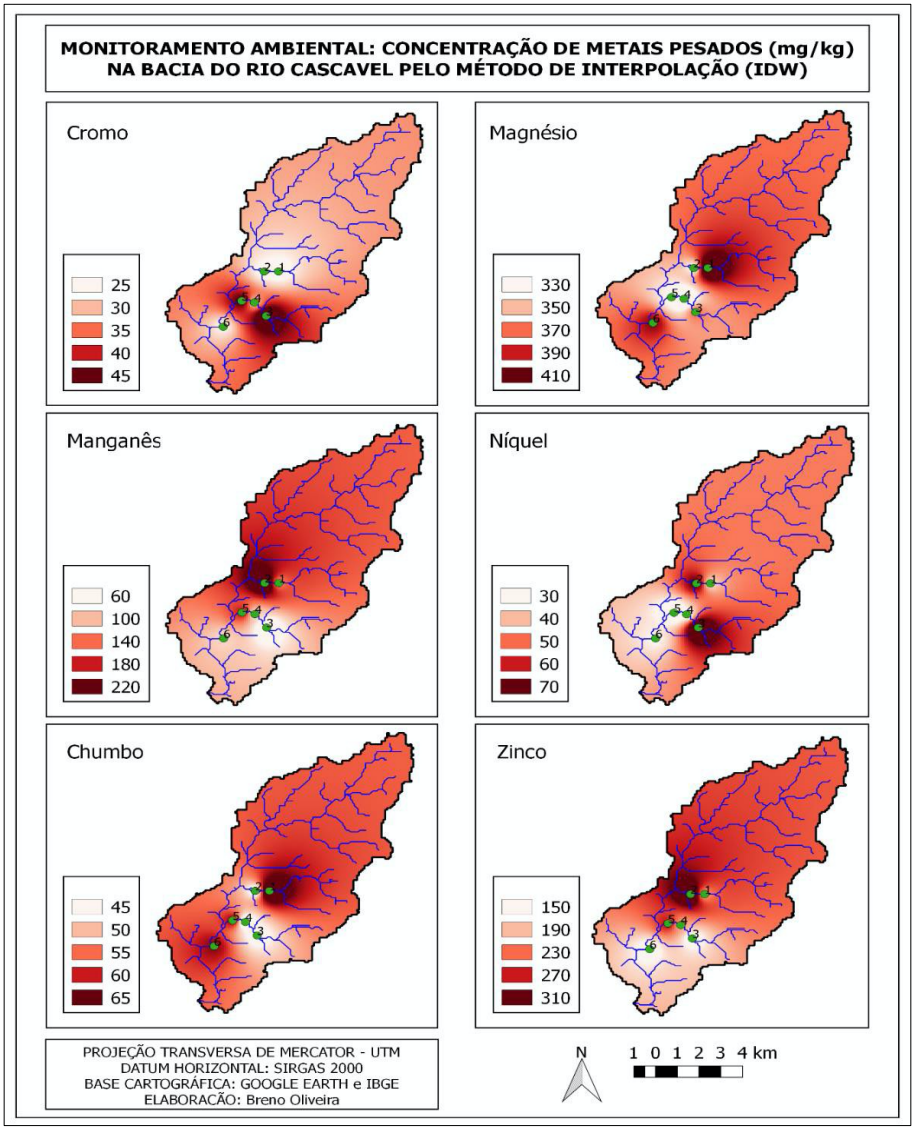
The anthropogenic sources of heavy metals in the study area are probably coming from industrial solid wastes (electroplating and metallurgy, foundries, welding, melting and modeling of alloys, incineration), being more evident in the southernmost points, to the east; and urban (wastewater from fuel and car washing plants, mechanical offices, sludge, sanitary landfills, urban and industrial waste, incineration), in the points chosen upstream; in addition to points with occurrence of pesticides, fertilizers and combustion of fossil fuels, found along the analyzed stretch.

Figure 2 shows that the dispersion of these elements in the urban environment may indicate their presence in the food chain, since these metals can reach and contaminate plants through water and soil (Mahmood et al., 2013). The geographic spatialization of the concentration of heavy metals, according to the average values obtained during the 4 samplings and analyses, using the interpolation complement of the QGIS software, aims to present the distribution of these concentrations in the entire length of the basin, including areas where they were not sampled.

According to the figure, the elements magnesium and prickly pear were found to have the highest concentrations in points 1 (montant) and 6 (justifying), while in the central points the contamination values are lower. For manganese and zinc, the highest concentrations are in points 1 and 2 (montante),

while in the other points these values decrease. The elements chromium and nickel presented the highest concentration values in point 3, while there is a variation between points 5 and 2, respectively, where the concentration appears accentuated.

The points with the highest concentration appear in the darkest red and as the value decreases, the red gets darker, until reaching white where the values are the lowest. Points 1 (montante), 2 and 3 correspond to stretches of the central region, with greater flow of people, vehicles and greater number of residences and consequently, greater generation of domestic effluents, while points 4 and 5 are located in neighborhoods more distant from the center, in areas with irregular occupations. Point 6 is located in the area just above the other points, a few meters from the municipal sewage treatment plant.



ENVIRONMENTAL MONITORING: CONCENTRAÇÃO DE METAIS PESADOS (mg/kg)
NA BACIA DO RIO CASCAVEL PELO MÉTODO DE INTERPOLAÇÃO (IDW)

Chrome

Magnésio

Nickel

Chumbo

Zinco

TRANSVERSE MERCATOR PROJECTION - UTM HORIZONTAL DATUM: SIRGAS 2000

CARTOGRAPHIC BASE: GOOGLE EARTH and IBGE

Concentration of heavy metals, generated by the interpolation of the data by the Inverse Distance Weighting (IDW) method.

Source: Organized by the authors.

In general terms, the geomorphological characteristics and use and occupation of the soil along the hydrographic basin of the Cascavel River have produced a cycle of contamination, generated by the effluents resulting from domestic-industrial and rainwater runoff, transported by surface runoff, being commonly found zinc, chromium, magnesium, prickly pear, nickel and manganese.

FINAL CONSIDERATIONS

The methodology proposed in this work, of working only with data on methane concentrations, partially meets the defined objective, since when assessing the level of contamination by heavy metals in water bodies and its variability of spatial and temporal concentration, the physical-chemical conditions of the environment and the environmental effects resulting from seasonality should also be taken into consideration.

Despite not having been determined more collection and analysis points distributed along the urban stretch of the hydrographic basin of the Cascavel river, in Guarapuava, PR, due to the scarcity of the aquatic macrophyte Egeria densa in the study area, it was possible to identify the heavy metals zinc, manganese, prickly pear, chromium, magnesium and nickel in its physiological structure. Although the concentrations of heavy metals are considered critical for organic matter according to Kabata-Pendias and Pendias (1992), the plant showed efficiency in the bioaccumulation of these chemical elements.

The methodology showed that the use of Egeria densa can be used for biomonitoring studies of heavy metals in polluted urban aquatic ecosystems. We emphasize that the Cascavel river is the main recipient of clandestine and industrial discharges in the municipality of Guarapuava, PR, and it is necessary to propose and implement an alternative monitoring program for its basin in emergencies.

REFERÊNCIAS

AHMAD, K.; AZIZULLAH, A.; SHAMA, S.; KHATTAK, M. N. K. Determination of heavy metal contents in water, sediments, and fish tissues of Shizothorax plagiostomus in river Panjkora at Lower Dir, Khyber Pakhtunkhwa, Pakistan. **Environmental Monitoring and Assessment**, v. 186, 2014. p. 7357-7366.

ALBUQUERQUE, E. L. S. **Avaliação das condições socioambientais em bacias hidrográficas costeiras: contribuição ao ordenamento territorial do setor leste da Região Metropolitana de Fortaleza, Ceará**. Tese (doutorado) Universidade Estadual do Ceará, Centro de Ciências e Tecnologia, Programa de Pós-Graduação em Geografia, Fortaleza, 2015. 258p.

ALFASANE, M.A. et al. Egeria densa Planchon: a new report for Hydrocharitaceae of Bangladesh. Bangladesh, **J. Plant Taxon**. 17(2), 2010. p. 209-213.

AMORIM, F. A. C. et al. Atomic absorption spectrometry: the road to multi-element determinations. **Nova Chem.** vol. 31 n.7, 2008.

ARAI, T.; OHJI, M.; HIRATA, T. Trace metal deposition in teleost fish otolith as an environmental indicator**. Water, Air and Soil Pollution**, v. 179, 2007. p. 255-263.

ARIAS, A. R. L.; et al. Use of bioindicators in the evaluation of impact in monitoring the contamination of rivers and streams by pesticides. **Science and Collective Health.** Associação Brasileira de Pós-Graduação em Saúde Coletiva, 2007.

AYOADE, J. O. **Introdução à climatologia para os trópicos.** Rio de Janeiro: Editora Bertrand Brasil, 1983.

BATISTA, F. G. A; FREIRE J. A. Avaliação de metais pesados no corpo aquático do Açude Velho: Campina Grande-Paraíba. **Engenharia Ambiental**, Espírito Santo do Pinhal, v. 7, n. 4, 2010. p. 166179.

BRAILE, P. M.; CAVALCANTI, J.E. W. A. **Manual de tratamento de águas residuárias industriais.** CETESB, 1993, apud HAYASHI, A. M. Remoção de cromo hexavalente através de processos de biossorção em algas marinhas. D. Thesis - Universidade Estadual de Campinas, CampinasSP, 2001.

BRAZIL. Conselho Nacional do Meio Ambiente. **Resolution No. 430, of 13 May 2011.** Dispõe sobre as condições e padrão de lançamento de efluentes complementa e altera a Resolução nº 357, de 17 de março de 2005, do Conselho Nacional do Meio Ambiente. CONAMA. Diário Oficial da União, n. 92, 16 May 2011, p. 89. Brasília, 2011.

BRAZIL: **Portaria nº 685, of August 27, 1998.** Princípios Gerais para o Estabelecimento de Níveis Máximos de Contaminantes Químicos em Alimentos. Disponível em:<https://www.univates.br/unianalises/media/imagens/Anexo_XI_61948_11.pdf> Acesso em 29 Apr 2018.

CETESB. **Guiding values for soil and groundwater**. São Paulo: CETESB, 2005.

DEAN, J.G.; BOSQUI, F.L.; LANOUETTE, V.H. Removing heavy metals from wastewater. **Environmental Science & Technology,** v. 6, 1972. p. 518-522.

DIVAN JUNIOR, A. M., et al. Using wild plant species as indicators for the accumulation of emissions from a thermal power plant, Candiota, South Brazil. **Ecological Indicators**, 9, 2009. p. 1156-1162.

FAO,. FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS. **Maize in human nutrition.** Rome, 1992. Disponível em: < http://www.fao.org/docrep/t0395e/T0395E00.htm>, Acesso em 28 abr 2018.

GORAYEB, A; PEREIRA, L. C. C. **Análise integrada das paisagens de Bacias Hidrográficas na Amazônia Oriental**. Fortaleza: Imprensa Universitária, 2014. 108p

IPARDES. **Caderno Estatístico Município de Guarapuava**. June 2018. Available at: http://www.ipardes.gov.br/cadernos/MontaCadPdf1.php?Municipio=85000. Acesso em 03 de junho de 2018.

JAKOB, A. A. E.; YOUNG, A. F. O uso de métodos de interpolação especial de dados nas análises sociodemográficas. In: Encontro Nacional de Estudos de População, 15, 2006, Caxambu. **Anais Eletrônicos...** Caxambu, MG: ABEP, 2006.

KABATA-PENDIAS, A., & PENDIAS, H. **Trace Elements in Soils and Plants**.3rd Edition, CRC New York: Press, 2001.

KARNITZ JÚNIOR, O. **Modificação química no bagaço de cana e celulose usando anidro**

de EDTA: uso destes materiais na adsorção de metais pesados em solução aquosa (Dissertação de Mestrado)-Universidade Federal de Ouro Preto - SP, 2007.

MAACK, Reinhard. **Geografia física do Estado do Paraná**. Ponta Grossa: Editora UEPG, 2002. 4.ed.

MAHMOOD, A., RASHID, S., MALIK, R. N. Determination of toxic heavy metals in indigenous medicinal plants used in Rawalpindi and Islamabad cities, Pakistan: **Journal of Ethnopharmacology**, , 148, 2013. p. 158-164.

MALAVOLTA, E. **Fertilizers and their environmental impact: micronutrients and heavy metals - myths, mystification and facts.** Piracicaba: Produquímica, 1994.

MIRANDAL, G. Aparecida; CAMILA, M. G.; MARTINS, C. S.; SILVA, R. G. **A bacia hidrográfica como unidade de planejamento urbano.** Florianópolis, SC: XXII SÍMPOSIO BRASILEIRO DE RECURSOS HÍDRICOS, 2017.

OLIVEIRA, N. M. B. et al. Capacidade de regeneração deEgeria densa nos reservatórios de Paulo Afonso, BA. **Planta Daninha,** v. 23, n. 2, 2005. p. 7-13.

OLOWOYO, J.O., et al. Uptake and translocation of heavy metals by medicinal plants growing around a waste dump site in Pretoria, South Africa. **South African Journal of Botany**, 78, 2012. p. 116-121.

PERES, C. K. et al. Diagnóstico da qualidade da água do Rio Cascavel, município de Guarapuava, Estado do Paraná. **Ambiência Guarapuava,** PR, v.4 n.1 2008. p.25-35.

PIO, M. C. S. S. da; SOUZA, K. S. dos; SANTANA, G. P. Capacidade da Lemna aequinoctialis para acumular metais pesados de água contaminada. **Acta amazônica**, v. 43, n. 2, 2013. p. 203-210.

RODELLA, R. A. et al. Differentiation between Egeria densa and Egeria najas by foliar anatomical characters. **Planta Daninha,** v. 24, n. 2, 2006. p. 211-220.

SANTOS, D.M., et al. Avaliação de Metais Pesados na Baía de Paranaguá, PR, Brasil, sob Influência das Atividades Antrópicas. **Journal Brazilian Society Ecotoxicology**, 1: 2006. p. 157-160.

SANTOS, Gustavo Henrique Fidelis. **Utilização da espectroscopia PIXE na avaliação do mecanismo de biossorção dos íons Cu2+, Zn2+, Cd2+ por la macrófitaEgeria densa.** (Dissertação de Mestrado) Universidade Estadual do Oeste do Paraná. Toledo Campus. Centro de Engenharias e Ciências Exatas. Toledo, PR: [s. n.], 2012.

SIPAÚBA-TAVARES, L. H.; BRAGA, F. M. S. Constructed wetland in wastewater treatment. **Acta Scientiarum. Biological Sciences**,v. 30, n. 3, 2012. p. 261-265.

SHARMA, P., DUBEY, R. S. Lead toxicity in plants. **Brazilian Journal Plant Physiol.** 17(1), 2005. p. 35-52.

SONG, C.; LIU, X.; SONG, Y.; LIU, R.; GAO, H.; HAN, L.; PENG, J. Key blackening and stinking pollutants in Dongsha River of Beijing: Spatial distribution and source identification. **Journal of Environmental Management, v. 200, p. 335-346.** <https://doi. org/10.1016/j.jenvman> 2017. p. 5-88.

THOMAZ, E. L. VESTENA, L. R: **Climatic Aspects of Guarapuava-PR.** Guarapuava: UNICENTRO, 2003.

TORRES, V. F. N. Engenharia ambiental subterrânea e aplicações. Rio de Janeiro: CETEMCYTED, 2005.

TUNA C., et al. Determination of nutritional value of some legume and grasses. Pakistan Journal of Biological Sciences, 7, 2004. p. 1750-1753.