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COMPARATIVE ANALYSIS OF BETALAIN QUANTIFICATION: IMPLICATIONS FOR FOOD INDUSTRY APPLICATIONS AND HEALTH BENEFITS

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Abstract: Natural colorants, such as betalains, are crucial in food coloring and valued for their nutritional and therapeutic properties. However, their precise analysis is challenging due to the sensitivity and instability of these substances. Red beetroot, rich in betalains, is widely consumed and associated with various health benefits. There are different analytical methods for quantifying betalains, each with its advantages and limitations. This article conducted a literature review on betalains and applied analytical methodologies based on Nilsson's (1970) and Stintzing and Carle's (2008) absorption spectrophotometry. The results of the laboratory assays were compared using Tukey's test at 5% significance level, showing that both methods can effectively quantify betalains but may exhibit significant differences in betalain fractions. Betacyanins did not show significant differences, while betaxanthin and betalain fractions exhibited slight differences. Therefore, the choice between methods should consider the sample nature, the analysis objective, and the available technical limitations. In summary, ongoing research in betalain analysis is essential to develop and improve analytical methods that meet the specific needs of different samples, ensuring accurate and reliable quantification of these compounds.

Keywords: coloring; nitrogenous anthocyanins; beetroot; antioxidant.

Análise comparativa na quantificação de betalaínas: implicações para aplicações na indústria de alimentos e benefícios à saúde

Resumo: Os corantes naturais, como as betalaínas, são cruciais na coloração de alimentos e são valorizados por suas propriedades nutricionais e terapêuticas. No entanto, sua análise precisa ser desafiadora devido à sensibilidade e instabilidade dessas substâncias. A beterraba vermelha, rica em betalaínas, é amplamente consumida e associada a diversos benefícios à saúde. Existem diferentes métodos analíticos para quantificar betalaínas, cada um com suas vantagens e limitações. Neste artigo, foi realizada uma revisão bibliográfica sobre as betalaínas e foram aplicadas metodologias analíticas baseadas na espectrofotometria de absorção de Nilsson (1970) e de Stintzing e Carle (2008). Os resultados dos ensaios laboratoriais foram comparados pelo teste de Tukey a 5% e mostraram que ambos os métodos podem ser eficazes na quantificação de betalaínas, mas podem apresentar diferenças significativas nas frações de betalaínas. As betacianinas não apresentaram diferença significativa, enquanto as frações de betaxantinas e betalaínas mostraram uma leve diferença. Portanto, a escolha entre os métodos deve considerar a natureza da amostra, o objetivo da análise e as limitações técnicas disponíveis. Em resumo, a pesquisa contínua na análise de betalaínas é essencial para desenvolver e aprimorar métodos analíticos que atendam às necessidades específicas de diferentes amostras, garantindo uma quantificação precisa e confiável desses compostos.

Palavras-chave: coloração; antocianinas nitrogenadas; beterraba; antioxidante

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1 INTRODUCTION

Plant-derived compounds recognized by humans for their coloring are called pigments. The food and pharmaceutical industries frequently use dyes to enhance the appeal of products to consumers. The main plant-derived dyes belong to various categories of substances, such as anthocyanins, carotenoids, and betalains, with the latter group being less appreciated (Gonçalves, 2018).

The primary plant rich in betalains is red beetroot (Beta vulgaris L.), also known as red beet, a vegetable consumed worldwide. This pigment is also found in some cacti. Its composition includes various phenolic compounds that confer antioxidant and nutritional properties, benefits highlighted in studies associating it with reduced blood pressure, improved type 2 diabetes mellitus, oxidative stress, and inflammatory processes. Phenolic acids and flavonoids have even been identified in beet stems (Lorizola et al., 2018; Slimen, Najar, Abderrabba, 2017; Nascimento et al., 2022).

However, despite the growing recognition of the nutritional benefits of betalains, their analysis and quantification remain significant challenges due to their instability and sensitivity to certain analytical methods. The chemical nature of betalains, along with factors like pH, temperature, and oxygen presence, can influence their stability during extraction and analysis processes.

Therefore, different analytical methodologies must be tested to determine the best approach for precise analysis of betalains in plant samples, such as beetroot. It is important to emphasize that the choice of the appropriate analytical method will depend on the specific characteristics of the sample, the analysis objectives, and the available technical limitations.

Thus, this study aims to compare the effectiveness of Nilsson's (1970) and Stintzing and Carle's (2008) methods in analyzing and quantifying betalains in beetroot samples. Different analytical methods can offer variations in the accuracy and precision of results. Evaluating betalains using various methods helps ensure that the quantifications are reliable and accurate. This study aims to contribute to the improvement of analytical techniques used in the determination of betalains.

2 METHODOLOGY

2.1 Materials

All experiments were conducted in the Food Engineering course laboratories at UEPG, located on the Uvaranas Campus.

All reagents used were of analytical grade, and all solutions were prepared using ultrapure water (Millipore, São Paulo, Brazil).

The equipment used in the analyses included a High Speed refrigerated centrifuge model Himac CR21G II, a Shimadzu Visible UVmini 1240V 110 V spectrophotometer, a microplate reader (Epoch microplate spectrophotometer, Synergy-BioTek, Winooski, VT, USA), a benchtop pH meter (pH 21 meter, Hanna, Cotia, Brazil), and a colorimeter (Minolta CM-5, Minolta Co. Ltd., Osaka, Japan).

2.2 Methods

The beetroot sample acquired at the producer's market at Parque Ambiental in the municipality of Ponta Grossa/PR was subjected to proximate composition analysis, including pH (AOAC, 2007); moisture, ash, protein, and lipid content (IAL, 2008). Carbohydrate content was determined by difference. Additionally, a literature review was conducted to present information deemed relevant to understanding the value and importance of the predominant natural pigment in red beetroot. To retrieve relevant scientific production, various databases were consulted, including Google Scholar, CAPES Portal of Journals (Coordination for the Improvement of Higher Education Personnel), Web of Science, ScienceDirect, and SciELO.

A concise analysis was conducted regarding beetroot (*Beta vulgaris* L.) and its predominant coloring component, culminating in the presentation of the discrepancy in results between two distinct assay methods employed in the quantification of betalains.

For the laboratory assays, 1 kg of beetroots were purchased at the Producer's Fair at the Environmental Park in Ponta Grossa/PR. Subsequently, they were taken to the laboratory, thoroughly rinsed under running water for 30 seconds, and then immersed in a solution of peracetic acid (PAC 1g.L^{-1}) for 10 minutes. They were then rinsed with distilled water for 1 minute under running water. After rinsing, the beetroots were peeled and grated using a household grater, and then the quantification of betalains was performed using the following two analytical assays:

Assay 1: 20 g of beetroot were macerated with 50 mL of water at 4°C, and then this sample was centrifuged in a refrigerated centrifuge at 4°C with a force of 37742 g (15000 rpm) for 40 minutes. In a test tube, 400 µL of the supernatant were homogenized with 4 mL of distilled water. Readings of the solutions were performed in triplicate at 480 nm and 538 nm. Betacyanins and betaxanthins were calculated using equation 1. The sum of betacyanins and betaxanthins represents the content of betalains. The absorbance readings obtained were used to calculate the concentration of betalains in each sample, where A is absorbance, DF is the dilution factor, and l is the path length (1 cm). For the quantification of betacyanins and betaxanthins, the following molecular weights (MW) and molar extinction coefficients (e) were used: MW = 550 g moL^{-1}; e = 60000 L mol.cm in water and MW = 308 g moL^{-1}; e = 48000 L mol.cm in water (Stintzing, Carle; 2008).

$$BC\ (mg.L^{-1}) = (A \times DF \times MW \times 1000) \quad \text{equation 1}$$

Assay 2: 20 grams of beetroot were macerated in 50 mL of 0.05 M phosphate buffer pH 6.5, and the solution was transferred to 50 mL Falcon tubes and centrifuged using a refrigerated centrifuge at 4°C with a force of 37742 g for 40 minutes. In a test tube, 1 mL of the supernatant was homogenized with 24 mL of 0.05 M phosphate buffer pH 6.5. Readings of the samples were performed in triplicate at 476 nm, 538 nm, and 600 nm as determined according to the methodology described by Nilsson (1970). Calculations were performed using equations 2, 3, and 4.

$$x = 1.095(a - c) \quad \text{equation 2}$$

$$y = b - z - \frac{x}{3.1} \quad \text{equation 3}$$

$$z = a - x \quad \text{equation 4}$$

Given that a = absorbance reading at 538 nm; b = absorbance at 476 nm; c = absorbance at 600 nm; x = absorbance of betacyanin; y = absorbance of betaxanthins; z = absorbance of impurities. The total betalains value is the sum of x + y in mg.100 mL^{-1}, subsequently multiplied by 10 to convert to mg.L^{-1}.

For statistical analysis of the data, JAMOVI software version 2.3.28 solid was used, applying the Tukey test at 5% probability to detect differences between means. Absorbance values in both Assay 1 and Assay 2 were obtained from direct readings.

3 RESULTS AND DISCUSSION

The results of the proximate composition of the beetroot acquired at the open-air market in Ponta Grossa/PR are shown in Table 1 below:

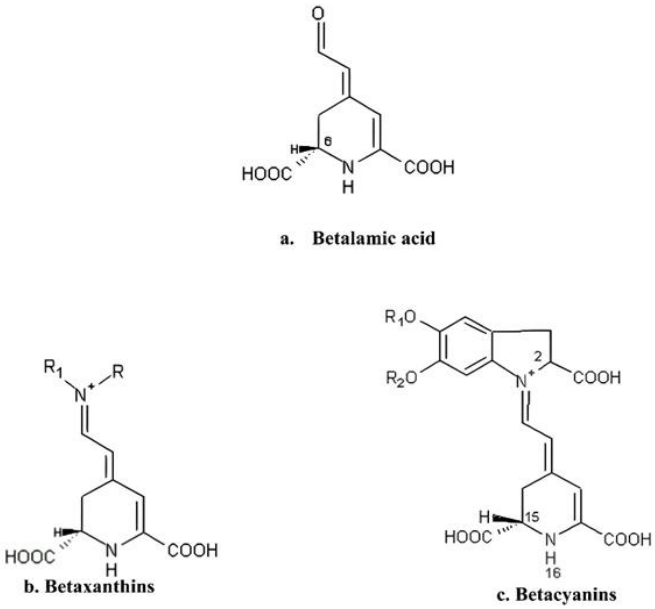
Table 1. Proximate composition of red beetroot.

Sample	pH	Moisture (%)	Ash (%)	Proteins (%)	Fat (%)	Carbohydrates (%)
Beetroot	5.10±0.01	86.30±0.20	0.72±0.01	0.12±0.01	0.10±0.01	12.76±0.12

When presenting the proximate composition, we consider it important in these discussions to further discuss red beetroot in order to promote its potential, highlighting its characteristics and the importance of its primary natural pigment, which are the fractions of betalains.

Red beetroot ranks among the top 10 vegetables with the highest concentration of antioxidants, largely due to betalains. The amount of betanin and phenolic compounds present in beetroot is higher in the peel than in the pulp and crown, indicating the potential for peel reuse. The peel also contains a high content of ferulic acid, which is characteristic of many species containing betalains (Figure 1) (Šeremet et al., 2020).

Figure 1. Structure of betalamic acid (a), betaxanthins (b), and betacyanins (c).



Source: Slimen et al. (2017)

The beetroot is an herbaceous plant belonging to the Chenopodiaceae family, extensively cultivated in temperate countries such as Germany, France, Turkey, Russia, among others, for sugar production. This process generates by-products such as beet pulp, which contains polysaccharides like cellulose, hemicellulose, and pectins. The amount of pectin present in beet pulp, associated with neutral sugars, allows them to form gels (Lara-Espinoza et al., 2021; Oliveira et al., 2023a; Oliveira et al., 2023b).

Purple beetroot is part of the Chenopodiaceae family. It is often referred to as a "root" tuber, but botanically categorized as a hypocotyl tuber, with true roots developing at the organ's end. Its origin dates back to temperate regions in Europe, North Africa, and the Mediterranean region. In Brazil, purple beetroot varieties are the most cultivated, used both for fresh consumption and in the industry (Kluge et al., 2005).

It stands out among vegetables for its nutritional composition, particularly its sugar and iron content, as well as the ways its root and leaves are consumed. Its commonly edible part is a globular tuberous root with a distinctly sweet flavor, and a reddish-purple coloration due to betalains. While the

root is more commonly consumed, its leaves are rich in provitamin A and ascorbic acid (Grangeiro et al., 2007; Zarate et al., 2010). Both white and purple beetroots, with the former being more prevalent in the sugar industry, contain approximately 17% sucrose by mass, on average, with 97% of the sucrose in beetroot being recoverable for fermentation (Manochio, 2017). According to Cosmo and Galeriani (2021), the proximate composition of beetroot as a percentage on a dry basis for the parameters of dry matter; ash; ether extract; crude protein; and fiber were: 100%; 14.18%; 1.48%; 15.97%; and 21.89%, respectively, which differ from the values found in our research. This variation can be explained by factors such as irrigation, soil, agricultural practices, and the harvest period, among others (Oliveira et al., 2023a).

Betalains, besides their color, are important as food ingredients. They lose color at pH levels below 3 through a poorly understood mechanism. In alkaline pH, betalains are cleaved into their biosynthetic precursors: betalamic acid and cyclo-dopa or amines, all of which are susceptible to degradation, thereby causing discoloration. As a general rule, betacyanins are considered more resistant to acidic conditions, while betaxanthins are more stable at neutral pH. Betalains degrade when exposed to light and oxygen. Temperature is the most critical factor affecting betalain stability, with degradation increasing above 30°C. Water activity also influences pigment stability, with low values (0.63) favoring their stability (Kluge and Preczenhak, 2016; Longaray, 2014).

It's important to highlight that pigments like betalains are essential in the food industry because they play a crucial role in imparting attractive visual characteristics to foods, ensuring uniformity that signals freshness and quality, and standardizing the color of processed foods. However, it's crucial to use safe pigments to ensure consumer health.

Betalains are pigments found in certain families within the Pentapetalae class of the Caryophyllales order. They are water-soluble nitrogenous compounds divided into two main groups: betacyanins (red-violet coloring) and betaxanthins (yellow-orange coloring). Betacyanins are further classified into four types based on their chemical structures: betanin, amaranthin, gomphrenin, and bougainvillein. They are of significant interest as food colorants in industrial applications and are relatively stable in a pH range of 3 to 7. They can enhance food characteristics by improving color, despite their low thermal stability, and possess antioxidant capacity 1.5 to 2.0 times greater than anthocyanins.The preservation of betalains in minimally processed beetroot is subject to detrimental factors such as enzymatic oxidation, temperature degradation, and exposure to light and oxygen (Kusznierewicz et al., 2021).

Betalains and anthocyanins are exclusive pigments in plants; they never occur together in the same species. For a long time, betaxanthins were mistakenly categorized as flavonoids, and betacyanins were termed "nitrogenous anthocyanins." Both types of pigments were first classified as betalains in 1968 by Mabry and Dreiding. Red beetroot has long been considered the sole source of betalains, which may explain the limited attention given to these pigments. However, in the past two decades, new sources have been reported, such as the genus Opuntia (cactus family), expanding the previously restricted perspectives on betalains as natural antioxidants and food colorants (Slimen; Najar; Abderrabba, 2017).

It is valid to emphasize that besides color, betalains have potential as functional food ingredients used in the food and medical industries due to their various health-promoting effects. Betalains, being non-toxic, can supplement therapies for conditions related to oxidative stress, inflammation, and dyslipidemia, such as arterial stenosis, atherosclerosis, hypertension, and cancer (Oliveira et al., 2023). Concerns about the toxicological safety and rigorous, costly toxicological tests of synthetic dyes, coupled with properties like safety, abundance, ease of extraction, bioaccessibility, and biodegradability of betalains, have encouraged the development and application of natural pigments as food ingredients. Based on Title 21 of the United States Code of Federal Regulations from the Food and Drug Administration (FDA), beetroot powder is considered an approved colorant, currently used to color a variety of fabrics, foods, and pharmaceutical products (Richhariya et al., 2017).

Oxidative enzymes such as polyphenol oxidase and peroxidase exhibit high activity in the region near the beetroot peel, working to minimize the action of reactive oxygen species and using phenols as reaction substrates and/or the betalains themselves, thereby causing their degradation (Kapadia and Rao, 2013). More than 70 betalains are known and divided into two groups based on their chemical structure (Kluge and Preczenhak, 2016).

The high antioxidant capacity confers nutraceutical properties to betalains; these pigments have the potential to eliminate approximately 75% of superoxide anion radicals. Betalains are also correlated with inhibition of DNA cleavage, providing protective effects by reducing damage caused by hydrogen peroxide to nucleic acids. They are known for inhibiting lipid peroxidation and neutralizing lipoperoxidases that can damage gastrointestinal cells during digestion. These pigments can modulate the intrinsic imbalance between oxidant species and the antioxidant defense system, creating a cellular environment favorable for neutralizing oxidative stress (Kluge and Preczenhak, 2016; Cai, Y., & Corke, H., 1999). The consumption of betalains contributes to a healthy dietary practice, as according to Pagliarino (2021) and Rodrigues and Koglin (2022), the adoption of a healthy dietary practice can assist in preventing the critical state of various diseases by aiding in the integrity of the body's immune barrier.

A literature review on the concentration of betalains in red beets, using both methods over the years, is presented in Table 2 below.

Table 2. Literature review of betalain analysis in red beet.

Sample	Fraction analyzed (Betalains, Betacyanins, or Betaxanthins)	Result and Unit	Method	References
Beetroot	Betalains	40 to 160 mg.100g^{-1}	Stintzing and Carle's (2008)	Stintzing e Carle (2008)
Beetroot	Betalains	288 mg.100g^{-1}	Stintzing and Carle's (2008)	Sardella (2016)
Beetroot	Betalains	40 to 77 mg.100g^{-1}	Stintzing and Carle's (2008)	Sanchez-Gonzalez et al.(2013)
Beetroot	Betalains	38.75 mg.100g^{-1}	Stintzing and Carle's (2008)	Melo et al.(2019)
Beetroot	Betalains	98.643 mg.g^{-1}	Stintzing and Carle's (2008)	Medeiros et al.(2022)
Beetroot	Betacyanins	88.04 mg.100g^{-1}	Stintzing and Carle's (2008)	Ferreira et al.(2017)
Beetroot	Betalains	183.61 mg.L^{-1}	Stintzing and Carle's (2008)	Hipólitto et al.(2022)
Lyophilized beetroot	Betacyanins	28.99 mg.100g^{-1}	Nilsson's (1970)	Oliveira et al.(2023b)
Beetroot	Betacyanins	93.67 mg.100g^{-1}	Nilsson's (1970)	Ferreira et al.(2017)
Beetroot	Betaxanthins	5.30 mg.100g^{-1}	Nilsson's (1970)	Ferreira et al.(2017)
Lyophilized beetroot	Betaxanthins	11.88 mg.100g^{-1}	Nilsson's (1970)	Oliveira et al.(2023b)
Beetroot	Betalains	135.75 mg.Kg^{-1}	Nilsson's (1970)	Ferreira et al.(2017)

Source: The author.

Regarding the results of the assays applied to quantify betalains in the beetroot sample, they are presented in Table 3.

Table 3. Betalain analysis

	Beetroot		
mg.L^{-1}	Assay 1	Assay 2	***p***
Betacyanins	4255.7±11.2^{a}	4236.92±41.3^{a}	>0.001
Betaxanthins	2316.8±2.9^{a}	1707.5±29.7^{b}	<0.001
Betalains	6572.6±14.0^{a}	5944.4±70.9^{b}	< 0.001
Impurities	-	4.89 ± 0.76^{a}	

^{ab} Note: Different letters in the same row indicate significant differences between samples, for p value <0.001.
Source: The author.

In Table 3, the mean results of different analytical methods analyzed using the Jamovi software are presented, demonstrating statistically significant differences observed between the methodologies. The beetroot sample used in these experiments had a pH of 5.1.

Among the methodologies studied, the assays showed similar results in the betacyanin subclass, with no significant difference despite methodological variations. However, significant differences were observed in the betaxanthin subclass, which impacted the significant difference in the total betalain quantification of the beetroot sample. In Nilsson's methodology (1970), impurities are components that are not betalains but remain in the sample after extraction and quantification. These impurities include substances such as phenolic compounds, proteins, sugars, organic acids, and other pigments or degradation products present in the beetroot matrix.

There are several approaches used to measure the amount of betalains, employing a variety of solvents, materials, and techniques. However, there is still no consensus on which method is superior. Therefore, it is crucial to conduct research to determine which methods are effective and which are not for a specific type of sample.

As observed in the review from Table 2, a study conducted in 2017 applied Nilsson's method to beetroot acquired from the Aracaju open-air market. The results indicated values of 88.04 to 93.67 $mg.100g^{-1}$ for the betacyanin fraction, 5.04 to 5.30 $mg.100g^{-1}$ for the betaxanthin fraction, and 9.42 to 9.62 $mg.100g^{-1}$ of impurities. Using the Stintzing and Carle methodology, values of 88.04 $mg.100g^{-1}$ were found solely for the betacyanin fraction (Ferreira et al., 2017). These values differ from those observed in this study, but it is important to consider that various cultivation factors may have influenced this variation, in addition to the author not detailing how the samples were treated.

Sanchez-Gonzalez et al. (2013) reported betalain levels of 40-77 $mg.100g^{-1}$ in commercial beet extracts obtained through different proportions of methanol, water, and acetic acid. Sardella (2016) reported a value of 288 $mg.100g^{-1}$ in their research. Conversely, Pitalua (2010) found 135.75 $mg.kg^{-1}$ in filtered beet extract. Stintzing and Carle (2008) verified that the normal range of betalain content typically found in red beets varies between 40 to 160 $mg.100g^{-1}$. Additionally, Oliveira et al. (2023b) found 28.99 $mg.100g^{-1}$ of betacyanins and 11.88 $mg.100g^{-1}$ of betaxanthins in freeze-dried beetroot, while Hipólito (2022) found 183.61 $mg.L^{-1}$ of betalains in beetroot.

The extraction conditions in these studies, such as the type of extraction solvent and adjustment in soluble solids content, a step which was not performed in the present study, as well as cultivation-related factors, may justify the differences between the results. The diversity of measurement units found in betalain quantification is justified by the need to adapt methodologies to sample specificities, ensure comparability across studies, and meet the specific objectives of each research endeavor.

4 CONCLUSION

Comparative analysis in the quantification of betalains in red beetroot revealed significant variations among the studied methods, reflecting the complexity and diversity of extraction and quantification processes. The literature review demonstrated a wide range of results found in different studies, with betalain levels varying considerably. Such differences can be attributed to variables such as the type of solvent used, cultivation conditions, and extraction techniques. The difference between quantification methods can significantly impact the results, and this variability highlights the importance of standardizing analytical methods to ensure consistent and comparable results.

Betalains, in addition to imparting vibrant color to foods, possess significant antioxidant properties, positioning them as promising ingredients for both the food industry and therapeutic applications. The use of betalains as natural colorants is an attractive alternative to synthetic dyes, given their safety, bioavailability, and functional properties. Future studies should focus on optimizing extraction techniques and understanding the factors that influence the stability and efficacy of betalains in different food matrices.

Therefore, through this study, it is feasible to examine both methodologies, considering their applicability characteristics. Furthermore, it is necessary to conduct analyses on different samples from

various regions with varied preparations to more deeply analyze the impact of methodologies on the results. This approach can not only improve the reliability of quantifications but also provide a solid foundation for future research and industrial applications of betalains, benefiting both science and the food industry.

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