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Chemotaxonomic classification of closed-related sweet oranges (*Citrus sinensis* L.) using principal component and cluster analysis of color and bioactive compounds in the orange peels

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INFO

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orange
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ABSTRACT

Chemotaxonomy is a systematic approach to plant classification based on discernible variations and similarities in their biochemical compositions, with a particular emphasis on secondary metabolites that often exhibit specificity within taxonomically related plant species. In the context of our investigation, we conducted a comprehensive chemotaxonomic analysis of sweet oranges utilized in concentrated juice production. This involved a thorough examination of antioxidant activities, characterization of flavonoid and phenolic profiles, UV-visible spectra scanning, and the implementation of color analysis. The results from cluster analysis demonstrated the superior efficacy of color analysis in discerning subtle distinctions among orange varieties. Further refinement in the analytical process requires the application of supplementary techniques after an exhaustive and precise extraction facilitated by organic solvents. Notably, all employed methodologies successfully discriminated between distinct orange types. While the compilation of extensive datasets presents inherent challenges, the incorporation of Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) enhances the interpretability of the obtained results. Considering that existing methodologies for assessing fresh fruit quality rely on subjective human visual inspection or orange juice analysis by ratio, which may lack precision in quantifying chemical constituents, there exists a growing demand for expedited techniques that can reliably ensure the quality of orange juice within the industry.

Palavras-chaves
laranja
classificação
composição química
metabolismo secundário
quimiometria

RESUMO

Classificação quimiotaxonômica de laranjas doces relacionadas (Citrus sinensis L.) utilizando análise de componentes principais e análise de agrupamento de cor e compostos bioativos nas cascas das laranjas

A quimiotaxonomia é uma abordagem sistemática para a classificação de plantas com base em variações e semelhanças discerníveis em suas composições bioquímicas, com ênfase especial em metabólitos secundários que frequentemente apresentam especificidade dentro de espécies de plantas taxonomicamente relacionadas. No contexto de nossa pesquisa, conduzimos uma análise quimiotaxonômica abrangente de laranjas doces utilizadas na produção de suco concentrado. Isso envolveu uma examinação minuciosa das atividades antioxidantes, caracterização dos perfis de flavonoides e fenólicos, varredura dos espectros UV-visíveis e a implementação da análise de cor. Os resultados da análise de agrupamento demonstraram a eficácia superior da análise de cor em discernir distinções sutis entre as variedades de laranja. Um refinamento adicional no processo analítico requer a aplicação de técnicas suplementares após uma extração exaustiva e precisa facilitada por solventes orgânicos. Notavelmente, todas as metodologias empregadas discriminaram com sucesso entre diferentes tipos de laranja. Embora a compilação de conjuntos de dados extensos apresente desafios inerentes, a incorporação da Análise de Componentes Principais (PCA) e Análise de Agrupamento Hierárquico (HCA) aprimora a interpretabilidade dos resultados obtidos. Considerando que as metodologias existentes para avaliar a qualidade de frutas frescas dependem de inspeção visual subjetiva humana ou análise de suco de laranja por meio de proporção, o que pode carecer de precisão na quantificação de constituintes químicos, existe uma demanda crescente por técnicas rápidas que possam garantir de maneira confiável a qualidade do suco de laranja na indústria.

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INTRODUCTION

Brazilian orange juice holds a prominent position as the largest industry globally (Blauer, 2023). Due to the extensive scale and cost-effectiveness of the final product, there is a pressing need to reduce production costs, particularly those linked to sorting fruits based on type and quality. In response to emerging market concepts, the orange juice industry has been fostering collaborations with academia, facilitating opportunities for open innovation (Granato et al., 2018; Saguy and Sirotinskaya, 2016). To comprehend the impact of chemical composition on color changes, it is essential to first understand orange trees (*Citrus sinensis* (L.) Osbeck), members of the Rutaceae family and prolific producers of the world's most cultivated fruits (Bayer et al., 2009; Pfeil and Crisp, 2008). While numerous orange varieties exist, sweet oranges dominate in Brazilian citrus belts, particularly for juice extraction (Rodrigues et al., 2019). The fruit comprises juice vesicles containing sugars, organic acids, vitamins, minerals, pectins, and pigments, shielded by a peel with a wax film responsible for the fruit's color and aroma compounds (Matheyambath et al., 2016). The peel is notably rich in phenolic compounds, flavonoids, carotenoids, and terpenes (Saini et al., 2022), This will significantly contribute to the theme of the present study.

Phenolic compounds are secondary metabolisms produced by plants, usually to protect them from environmental adversities or even as an attraction for pollinators, and which have in their structure the phenol group, an aromatic ring with a hydroxyl group (Chen et al., 2020a; Malta and Liu, 2014). These compounds are classified according to their main skeleton, having as a basic structure a benzene ring (C6). The main classes are phenolic acids (C6-C1), flavonoids (C6-C3-C6), coumarins (C6-C3), tannins (C6-C3-C6n), lignins (C6-C3n), among others (Vuolo et al., 2019; Tsimogiannis and Oreopoulou, 2019). The polyphenol content is higher in the citrus peel than in the pulp, such as caffeic, ferulic, synaptic, and p-cumaric acids (Bouhafsoun et al., 2018). Flavonoids are the largest class of plant phenolics, whose basic structure contains two aromatic rings connected by a bridge with three carbons. They can be divided into six different groups: flavones, flavanones, flavonols, isoflavones, anthocyanidins, and catechins. Some studies suggest that flavonoids represent about 2–3% (w/v) in citrus peels (Denaro et al., 2021; Nieto et al., 2021). Naringin, neoeriocitrin, and neohesperidine are the main flavanones found in the bergamot (400–1,000 mg/100 g), lemon (400–600 mg/100 g) and sour orange (380–1,100 mg/100 g) peels (Wang et al., 2021). Hesperidin and narirutin are most abundant in sweet oranges (270–350 mg/100 g of dry peel) (Sawalha et al., 2009). Flavonoids have aromatic chromophores indicating absorption in the UV region (250 nm), which can undergo π, π* excitation and react from excited states π, π*. They may also contain carbonyl chromophores and absorb UV in the region of 300 nm (Krishnan et al., 2021). This characteristic allows us to analyze the flavonoids by scanning in the UV region from 200 to 360 nm, and this diversity in composition and its chemical structures will contribute to the establishment of rapid analysis protocols for distinguishing between types of sweet oranges.

Some chemical structures of the main citrus peel flavonoids are shown in Figure 1.

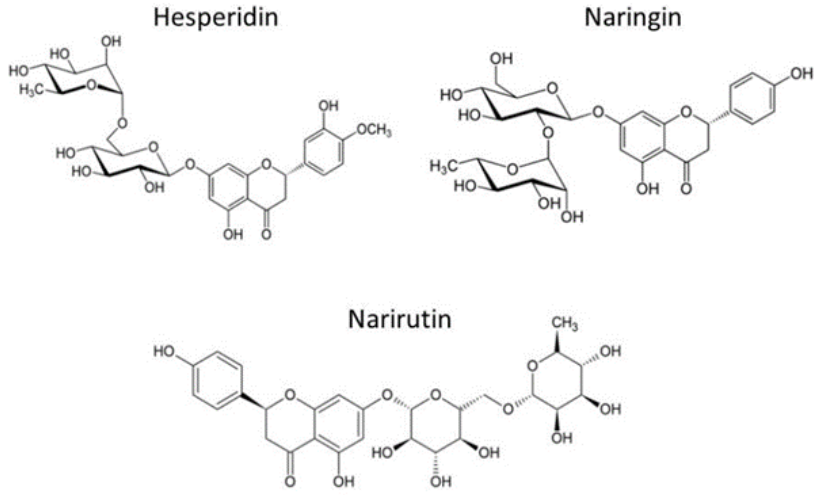

Figure 1 - Chemical structure of the main citrus peel flavonoids: hesperidin, naringin, and narirutin

Sweet oranges also contain natural pigments known as carotenoids, displaying colors ranging from yellow to red. Classified as tetraterpenes (C40), these pigments are composed of isoprene units (C5). Although generally insoluble in water, carotenoids can dissolve in organic solvents like

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ethanol (Kultys and Kurck, 2022; Honda et al., 2019; Tiwari et al., 2019). Carotenoids fall into two categories: (a) carotenes, identified by a linear hydrocarbon chain with one or two cyclic structures at the ends, and (b) xanthophylls, oxygenated compounds derived from carotenes. Among the most prevalent in citrus peels are α-carotene, β-carotene, lutein, zeaxanthin, and β-cryptoxanthin (Figure 2) (Maoka, 2020; Meléndez-Martínez et al., 2019)

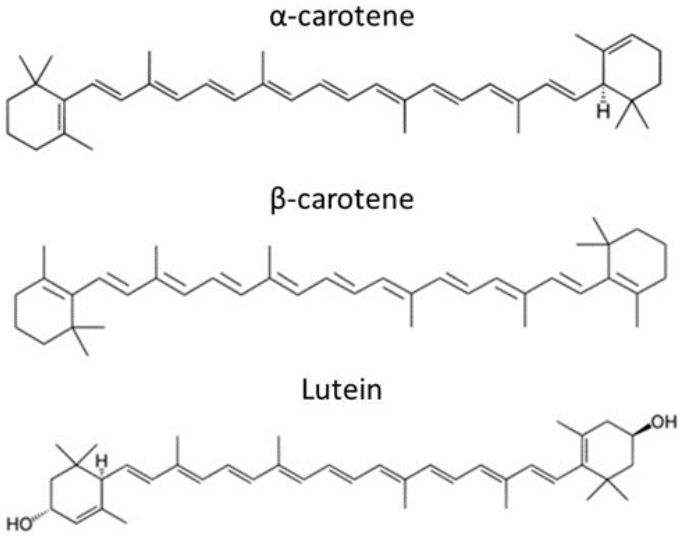

Figure 2 - Chemical structures of carotenoids present in orange peels

Utilizing instrumental measurements to assess the color of orange juice through its carotenoids offers a valuable tool for quality control. Carotenoids (along with chlorophylls) primarily absorb light in the visible region, and their *in vivo* measurement is feasible when exposed to UV light (Pérez-Gálvez and Fontecha, 2020; Virtanen et al., 2020). As indicated by Flores-Hidalgo et al. (2017), electronic properties can be examined through energy representation in boundary orbitals, as demonstrated in UV-visible absorption spectroscopy. Certainly, light is undeniably a primary environmental factor influencing the color of fruits, significantly impacting the absorption spectrum by their carotenoid composition (Lado et al., 2019). The color holds undeniable importance as a pivotal parameter for quality control, especially in the context of fresh fruits (Pigni et al., 2020; Murador et al., 2019).

The significance of phenolic compounds in orange peel is apparent, yet there is no consensus on their quantification and identification methods; while many authors recommend liquid phase chromatographic methods (with or without coupling with mass spectrometers) for pigment quantification and identification, the extraction protocols for these compounds are widely debated due to high costs or lengthy sample preparation times (Saini and Keum, 2018). The pursuit of fast analytical and exploratory data methods that facilitate recognizing similarities between samples is crucial for the fruit and its derivatives industries (Baqueta et al., 2020; Quelal-Vásconez et al., 2020). Granato et al. (2018) propose that principal component analysis (PCA) reduces dimensionality, visualizing intricate connections between bioactive compounds and functional properties, while hierarchical cluster analysis (HCA) categorizes similar items, revealing patterns; their comprehensive multivariate approach concurrently examines these components, unveiling subtle connections often overlooked in univariate analyses, with a primary focus on bioactive compounds, exploring their correlation with sensory, nutritional, and processing attributes, and conducted with a discerning perspective, critically evaluating the limitations, challenges, and implications of using PCA and HCA, providing a nuanced exploration of intricate relationships in foods, like fruits. Additionally, Granato et al. (2017) underscored the pivotal role of experimental design and statistical data analysis in understanding interconnections among nutrition, biology, pharmacology, functional properties, and chemical components in foods and their extracts; in the evaluation of diverse food extracts and bioactivities, the application of chemometric tools and statistical methodologies gains heightened relevance, as discussed by Granato et al. (2014). Spagolla et al. (2009) also reported an intercorrelation among phenolic compounds in *Vaccinium ashei*, extracted in hydroalcoholic solutions, and their effects on in vitro biological activity; the same classes of compounds that have been associated with the coloring of *V. ashei* fruits.

The implementation of intelligent systems

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capable of automatically sorting based on predetermined standards (Pérez-Gálvez et al., 2020) or utilizing various measurable variables reflecting similarities in the physical and chemical properties holds significant promise for the typification of orange types in the industry (Basri et al., 2019). Given the intricacies involved in human visual perception for orange quality control or distinguishing between varieties, this study assesses the discriminative capacity among three commonly found sweet orange varieties in juice industries. It proposes a straightforward method for extracting and analyzing the chemical composition of orange peel, coupled with chemometric analysis.

MATERIAL AND METHODS

Harvesting of orange samples in the industry

Sweet oranges (*Citrus sinensis* L.) were carefully harvested and sourced from Citrosuco Agroindustry S/A, Brazil. The harvesting of the Hamlin, Pêra-Rio, and Valencia cultivars occurred in June, July-August, and September 2019, respectively, at a farm situated in the São Paulo state within the Bebedouro orange belt, Brazil. The harvest timing was meticulously determined based on fruit ripeness, guided by the fruit ratio, a measure encompassing the ratio between total soluble solids and acidity. For this study, a specific ratio of 14 was rigorously established during the selection of fruits, ensuring a precise and standardized approach for the present research.

Preparation of methanolic extracts from orange peel

Peels from three varieties of oranges (Hamlin, Pêra-Rio, and Valencia) were finely grated and combined with analytical-grade methanol at a ratio of 1:5 (w/v). The mixture underwent ultrasonication in a water bath (Unique model T28220; frequency of 25 kHz and power of 120 W) at 35 °C for 1 hour. Subsequently, the liquid phase was separated by filtration using a qualitative filter (0.8 mm diameter pore) to completely isolate the fibrous components. The methanolic extracts underwent distillation to partially remove the solvent, and the concentrated extracts were stored at 4 °C until compositional analyses were conducted. The samples were designated as Hamlin orange peel, Pêra-Rio orange peel, and Valencia orange peel methanolic extracts.

Colorimetric analysis of total phenolic compounds

The quantitative analysis of total phenolic compounds in methanolic extracts of orange peel was conducted utilizing the Folin-Ciocalteu spectrophotometric method, following the procedure outlined by Mazen and Nashat (2019). Tannic acid served as a known standard for constructing the calibration curve. The ethanolic extracts were appropriately diluted, and a 1 mL aliquot of each sample was transferred to a test tube containing 0.5 mL of Folin-Ciocalteu reagent (diluted in water at a 1:10 ratio, v/v). After shaking, the test tubes were allowed to rest for 40 minutes. Subsequently, 2.5 mL of 20% sodium carbonate (Na_2CO_3) was added to the mixture. Absorbance readings were taken at 725 nm using a UV-Mini 1240 spectrophotometer (Shimadzu, Japan). A blank sample using ultrapure water was prepared under the same conditions. The results were expressed in tannic acid equivalents as the mean±standard deviation (in mg tannic acid equivalent per g of sample).

Colorimetric analysis of total flavonoids

The total flavonoid analysis of methanolic extracts from orange peel was carried out following the spectrophotometric method proposed by Sartori et al. (2013), with rutin serving as a known standard for constructing the calibration curve. In test tubes, 0.5 mL of methanolic extracts were combined with 4.3 mL of 70% ethanol, 0.1 mL of 2% aluminum chloride, and 0.1 mL of sodium acetate at 1 mol L^{-1}. After shaking, the test tubes were left undisturbed for 40 min, and absorbance readings were taken at 415 nm using a UV-Mini 1240 spectrophotometer (Shimadzu, Japan). A blank sample using ultrapure water was prepared under identical conditions. The results were expressed in rutin equivalents as the mean±standard deviation (in mg rutin equivalent per g of sample).

High-performance liquid chromatography (HPLC) profiles of orange peel methanolic extracts

Flavonoid analysis of methanolic extracts from orange peel was conducted following the method outlined by Aguiar et al. (2012), employing a high-performance liquid chromatography model LC-20AT with a photodiode array detector (model SPD-M20A; Shimadzu, Japan). The elution of compounds occurred on a C18 column model Shim-pack CLC ODS(M) using a mobile phase consisting of solvent A (0.2% formic acid in ultrapure water) and solvent B (acetonitrile), with an elution flow of 1.0 mL min^{-1} and the column maintained at 40 °C. The gradient system was as

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follows: 0 to 8 min: 10 to 13% B; 8 to 25 min: 13 to 20% B; 25 to 40 min: 20 to 40% B; 40 to 45 min: 40 to 60% B; 45 to 50 min: 60 to 10% B; 50 to 65 min: 10% B. A 10 µL injection volume was used, and the concentration of naringin and hesperidin was determined against a calibration curve utilizing authentic flavonoid standards (HPLC grade) at 270 nm.

Color analysis directly on orange peels by CIELab system

The color of the orange peel was assessed using the CIELab system, employing the Minolta colorimeter (model CR-300, Japan), with guidance provided by Dr. A. P. Jacomino from the University of São Paulo. Three readings were captured at equidistant points in the equatorial region of 15 whole fruits for each variety. The results were presented in terms of luminosity (L*), a* coordinate, b* coordinate, hue angle (*h*), and chromaticity (c).

Spectral scanning of orange peel methanolic extracts

The determination of the UV-visible spectral absorption maximum was conducted by combining 10 µL of each methanolic extract from the orange peel with 5 mL of analytical-grade methanol. The spectra were swiftly acquired using a UV-Mini 1240 spectrophotometer (Shimadzu Co., Japan) across the range of 200 to 600 nm.

***In vitro* antioxidant activity by DPPH radical scavenging**

According to Mensor et al. (2001), each methanolic extract from orange peels (0.5 mL) was placed in test tubes containing 3 mL of 95% ethanol (v/v) and 0.3 mL of DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical at a concentration of 0.3 mmol mL^{-1} in methanol. The solutions were thoroughly mixed and left undisturbed for 40 min at room temperature in a dark container. The negative control was prepared by replacing the extracts with ethanol (used as a diluent). The color change was measured using a spectrophotometer at 517 nm, and the DPPH radical scavenging activity was determined as the antioxidant activity percentage (AA%):

$$AA\% = 100 - \frac{(Aa - Ab) \times 100}{Ac}$$

Where: Aa = absorbance of orange peels methanolic extracts; Ab = absorbance measured of methanol; Ac = absorbance of negative control.

Total antioxidant capacity by phosphomolybdenum method

Following the procedure outlined by Prieto et al. (1999), 0.1 mL of ethanolic extracts from orange peels was introduced into test tubes containing 1 mL of a reactive solution (comprising sulfuric acid at a final concentration of 3 mol L^{-1}, monobasic sodium phosphate at 0.1 mol L^{-1}, and ammonium molybdate at 30 mmol L^{-1}). The test tubes were maintained at 95 °C for 90 min. After cooling to room temperature, the absorbance of the samples was measured using a spectrophotometer at 625 nm. A blank control was prepared by substituting the extracts with ultrapure water. A calibration curve was established by diluting ascorbic acid (HPLC grade) at 200 µmol L^{-1} and adding quantitative ultrapure water to achieve concentrations ranging from 10 to 200 µmol L^{-1}. The antioxidant capacity of the methanolic extracts was expressed as ascorbic acid equivalent, specifically in micrograms of ascorbic acid per milliliter of orange peel methanolic extract.

Statistical data analysis

In this analysis, the study delved into the statistical differences in key components of orange peels, namely total phenolics, total flavonoids, antioxidant activities, and the content of narigin and hesperidin, determined using high-performance liquid chromatography (HPLC). To scrutinize these differences, a one-way analysis of variance (ANOVA) and Tukey's means test were employed, revealing insights into the variability among the samples. The results, crucial for understanding the overall trends, were presented as the mean value±standard deviation based on a sample size of n=9. For a more in-depth exploration, the study extended its analysis to include Pearson correlation, hierarchical clustering analysis (HCA), and principal component analysis (PCA) using the statistical package MetaboAnalyst v. 5.0 (Xia et al., 2009). These sophisticated statistical techniques provided a nuanced understanding of the interrelationships between the various parameters studied. Furthermore, the study incorporated data from UV-visible spectra of orange peel ethanolic extracts and direct color analysis on the orange peel surface using the CIELab system into the PCA and HCA analyses. These additional datasets were processed with metabolomics data analysis software, and the data were normalized by the sum, with scaling performed using Pareto, without undergoing any data transformations. This comprehensive statistical approach aimed to untangle and decipher the intricate relationships

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among the diverse parameters, offering a robust and nuanced comprehension of the bioactive compounds and functional properties present in orange peels.

RESULTS AND DISCUSSION

Chemical profiles and antioxidant activities from methanolic extracts of orange peels

The peel of citrus fruits is a reservoir of essential oils and volatile substances, contributing to the characteristic aroma of these fruits, which includes terpenes and other secondary metabolites serving as protective agents against abiotic factors. However, this study serves as a foundation for distinguishing between types of oranges based on the chemical composition of their peels. Monoterpene hydrocarbons, notably limonene (87.9%), myrcene (2.4%), and α-pinene (0.5%), along with monoterpenoid alcohols such as linalool (1.2%), and aliphatic aldehydes, including octanal (1.3%) and decanal (0.2%), have been identified as predominant in sweet oranges by Njoroge et al. (2009). These compounds exhibit antioxidant properties, as demonstrated by Singh et al. (2010), highlighting D-limonene's remarkable capacity to inhibit free radicals. Despite the availability of various analytical techniques for characterizing orange peel composition, the challenge arises on an industrial scale due to the massive volumes of oranges processed. Consequently, there is a demand for rapid, non-destructive methodologies that can effectively differentiate orange types based on their quality. Colorimetric techniques, widely used in the food industry, including orange juice, proved effective in discriminating between orange types through the assessment of total phenolic and flavonoid contents, along with in vitro antioxidant activities. Principal component analysis revealed significant variability, with PC1 explaining 99.1% of the differences between clusters. Unlike color analysis, PCA exhibited no overlap between Pêra-Rio and Valência oranges, indicating the potential for differentiation among orange types such as Hamlin, Pêra-Rio, and Valencia. The HeatMap diagram further illustrated substantial differences between orange types, highlighting Valencia's prominence across various evaluated parameters, except for antioxidant activity against free DPPH radicals.

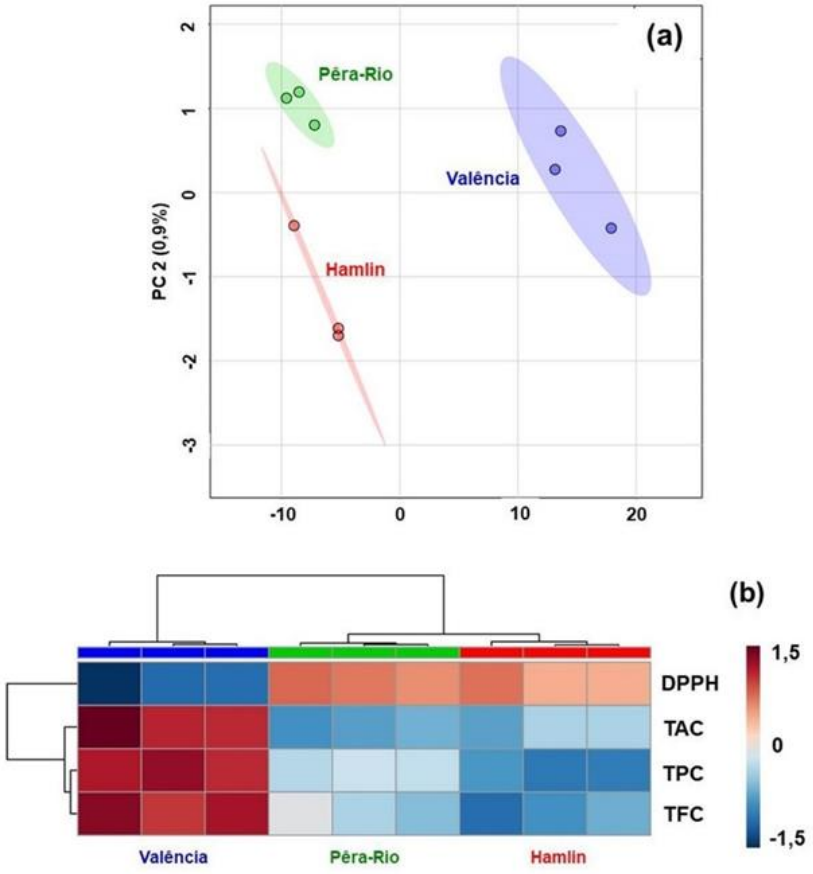

Figure 3 - (a) Principal Component Analysis (PCA) and (b) HeatMap of total phenolic (TPC) and total flavonoid (TFC) contents and *in vitro* antioxidant activities from methanolic peel extracts of Hamlin, Pêra-Rio and Valencia orange types. Where: DPPH, antioxidant activity by free DPPH radical scavenging; and TAC, total antioxidant capacity by phosphomolybdenum method

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While the Hamlin and Pêra-Rio stood out from the Valência type for phenolic compounds, total flavonoids, and total antioxidant capacity, both are similar one each other. Valencia had greater prominence in all data. Box Plot data analyses (Figure 4) allow us to observe what variables most influenced each orange type. The antioxidant activity by DPPH was more important to Pêra-Rio and Hamlin while antioxidant activity by phosphomolybdenum method was to Valência**.**

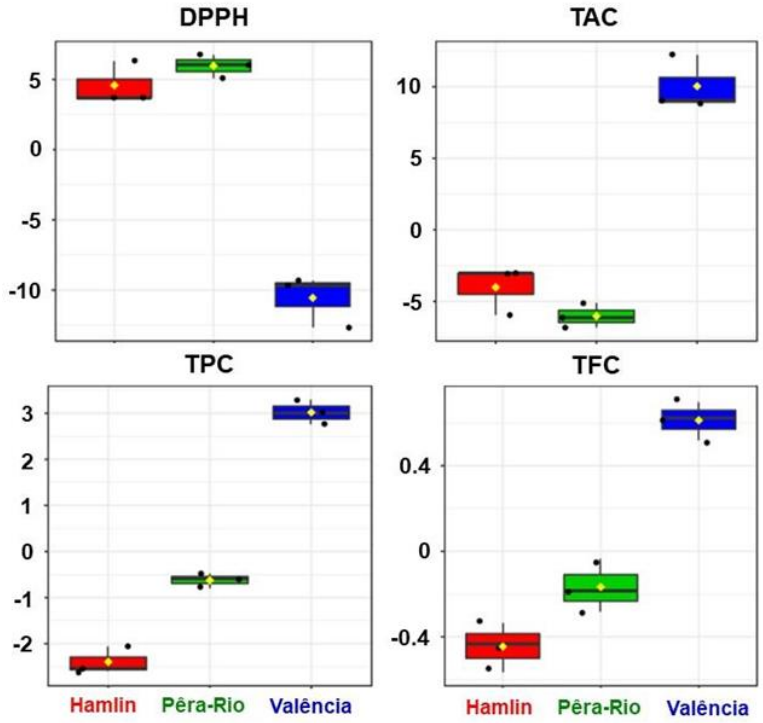

Figure 4 - Box Plot of total phenolic, total flavonoid contents, and *in vitro* antioxidant activities of methanolic extracts of peel from Hamlin (*red box*), Pêra-Rio (*green box*), and Valência (*blue box*) oranges. Where: DPPH, antioxidant activity by free DPPH radical scavenging; TAC, antioxidant activity by phosphomolybdenum method; TPC, total phenolic compounds and TFC, total flavonoid compounds

Liew et al. (2018) determined the total phenolic and total flavonoids in the peel of *Citrus sinensis* using methanol as solvent and found total phenolic contents of 36.1±2.9 mg TAE/gram dried peel and total flavonoids contents of 4.6±0.1 mg RE/gram dried peel. Melo et al. (2014) using ethanol as a solvent to extract the phenolic compounds from sweet orange bagasse found 8.1±0.5 mg TAE/gram of freeze-dried peel. For the antioxidant activity by DPPH free radical scavenging, Pereira et al. (2017) found similar values to this work with antioxidant activity above 60% for methanolic extracts of Hamlin, Pêra-Rio, and Valencia cultivars. Hegazy and Ibrahim (2012) analyzed the methanolic extract of sweet orange peel extracted by the Soxhlet technique and the antioxidant activity for DPPH radical scavenging of 73.4% was found. Sagar et al. (2020) and Seelam et al. (2016) reported that the activity for DPPH radical scavenging of 89% in *Malpighia emarginata* fruits.

Table 1 - Total phenolic compounds, total flavonoids, activity for DPPH radical scavenging, and total antioxidant capacity of methanolic extracts of orange peel from Hamlin, Pêra-Rio, and Valência cultivars (n = 15)

Orange types	Total phenolics (mg/g)[a]	Total flavonoids (mg/g)[b]	DPPH radical scavenging (%)	Total antioxidant capacity (µg/mL)[c]
Hamlin	5.92	0.23	78.16	34.07
Pêra-Rio	4.71	0.18	58.20	23.61
Valência	7.19	0.28	61.40	45.16

[a]mg TAE/g peel (mg of total phenolic compounds in tannic acid equivalent per gram of orange peel); [b]mg RE/g peel (mg of total flavonoid in rutin equivalent per gram of orange peel); and [c]µg AAE/mL extract (µg of ascorbic acid equivalent per mL of methanolic extract of orange peel)

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The antioxidant activity by DPPH radical scavenging showed that there is a moderate negative interaction between total flavonoids and weakly negative with total phenolic compounds. Thus, the higher concentration of phenolic compounds and total flavonoids do not have a direct relationship with the increase in antioxidant activity by DPPH radical scavenging (Figure 5). Aguiar et al. (2012), and Aguiar et al. (2009) have shown that soybean flavonoids are widely found as conjugate forms, and they present low antioxidant activity. Thus, the active site of flavonoids could not be able to scavenge the free radicals during the in vitro antioxidant tests. Besides, orange peels have other compounds that may have antioxidant activity, such as terpenes and carotenoids. Carotenoids are known as vitamin A precursors (such as retinol, retinal or retinoic acid) and they are associated with cellular protection against free radicals, due to their antioxidant activities, either through the reaction with singlet oxygen and peroxyl radical scavenging (Rufino et al., 2010). While, total antioxidant capacity by the phosphomolybdenum method, which has a broad spectrum of antioxidant activity evaluation or is a non-specific method, presented a moderate positive correlation with the total phenolic and total flavonoid contents. According to Kaska et al. (2019), the phosphomolybdenum method as total antioxidant capacity was used to ascertain the antioxidant properties in plant extracts. According to Prieto et al. (1999) and Nur-Alam et al. (2013), this methodology had been associated with the reductones presence once those compounds present reducing capacity on Mo+4 to Mo+5 and development of green phosphate/ Mo+5 complex.

Wan et al. (2011) analyzed total phenolic and total flavonoid contents no significant difference was noted for two different extraction temperatures (90 and 100 °C). The extracts obtained at a higher temperature exhibited a significant antioxidant activity by free DPPH radical scavenging compared with extraction at lower temperatures. As our group showed (Aguiar et al., 2012), under high heating (121°C/30 min) the malonyl isoflavones present in soybean flour were converted into glycosyl forms and increased in vitro antioxidant activity.

Between total phenolic compounds and total flavonoids, the correlation was strongly positive (Figure 3), since flavonoids belong to the chemical group of phenolic compounds. Many authors report that quercetin, hesperetin, and naringin and its conjugate forms, rutin, hesperidin, and naringenin, were widely found as flavonoids in citrus fruits (Singh et al., 2020; Bento et al., 2017).

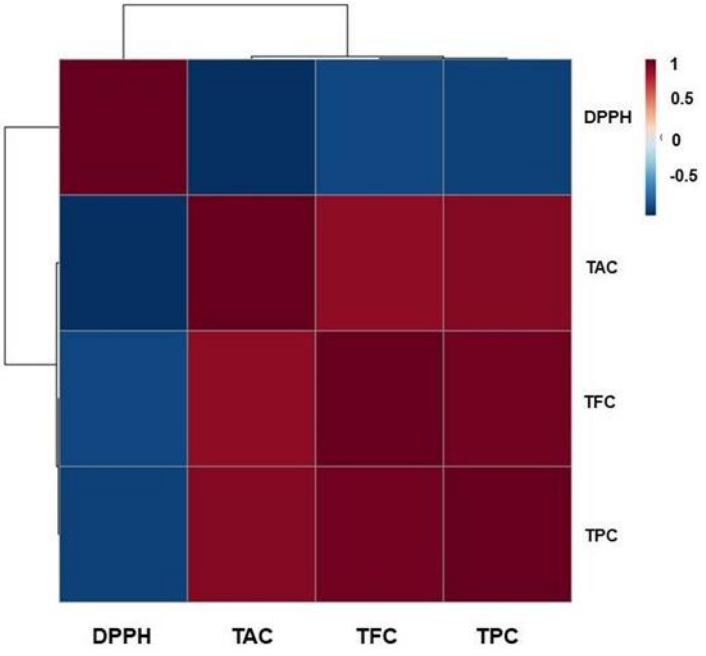

Figure 5 - Pearson correlation for total phenolics (TPC) and total flavonoids contents (TFC), antioxidant activity by DPPH free radical scavenging (DPPH) and total antioxidant capacity by phosphomolybdenum method (TAC)

High-efficiency liquid chromatography and co-chromatography analyses were carried out against flavonoid authentic patterns (HPLC grade) and made it possible to note the presence of major flavonoids (hesperidin and naringin) in methanolic extracts of orange peels. Hesperidin was present in all methanolic extracts of orange peel analyzed. Its concentrations vary between the orange types, with

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the highest content present in the Hamlin orange, followed by Valencia and Pêra-Rio, respectively. Naringin was not detected in the extracts from the Pêra-Rio and Valencia orange types. Whereas, the methanolic extract of Hamlin peel presents low concentration. Like Gómez-Mejía et al. (2019), Chen et al. (2020b), and Singh et al. (2020), low naringin concentrations were found when compared to hesperidin. Although the RP-HPLC analysis showed the highest concentrations of hesperidin and naringin were found in the Hamlin extracts, for the other analysis (total phenolic and total flavonoid) the results were more expressive for the methanolic extracts from Valência orange (Table 2 and Figure 6). Probably, other flavonoids were present in the analyzed orange peel samples, such as catechin and rutin (Singh et al., 2010), neoerocitrin, lutein, narirutin, apigenin, neohesperidin, or diosmin (Manthey and Grohmann, 2001).

Table 2 - Concentrations of hesperidin and naringin in the methanolic extracts of Hamlin, Pêra-Rio and Valencia orange peels

Orange types	t_R (min)	UV $\lambda_{máx}$ (nm)[a]	Compounds[b]	Concentration (µg/mL)[c]
Hamlin[d]	31.9	220 and 280	Naringin	382.7 ± 2.3
	33.6	224 and 276	Hesperidin	2,076.8 ± 2.7[A]
Pêra-Rio	33.4	224 and 276	Hesperidin	1,026.7 ± 5.8[C]
Valência	33.6	224 and 276	Hesperidin	1,562.9 ± 2.3[B]

[a]UV-visible spectra were carried out with the methanolic extracts from orange peels and the Figure 7 shows these spectra of corresponding peaks to hesperidin and naringin in the samples. [b]The authenticity of each flavonoid was checked by co-chromatography, retention time and UV-visible spectra from photodiode-array detector against authentic standards (HPLC grade). [c]Concentrations of each flavonoid were presented as mean±standard deviation and the same capital letter within a column (for hesperidin concentrations) are not significantly different according to the Tukey test at $p<0.01$.

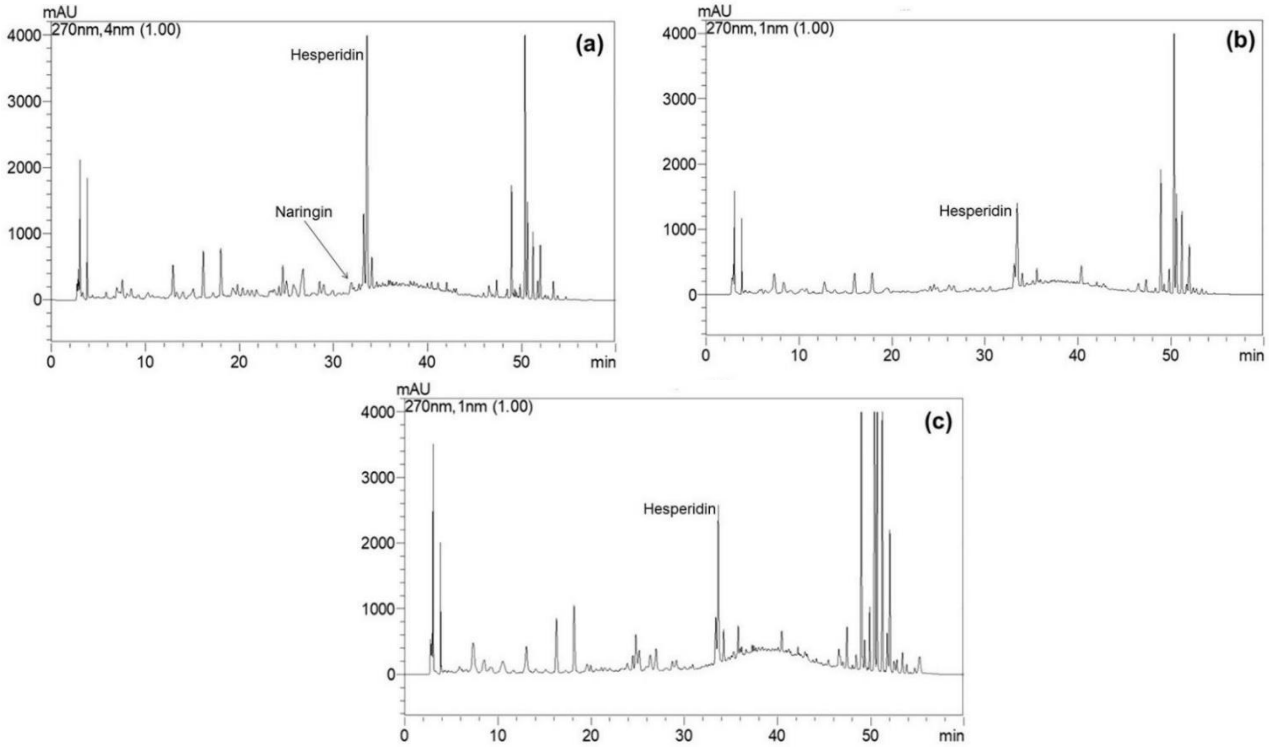

Figure 6 - RP-HPLC chromatograms of hesperidin and naringin from methanolic extracts from three orange peel types: (a) Hamlin; (b) Pêra-Rio; and (c) Valência

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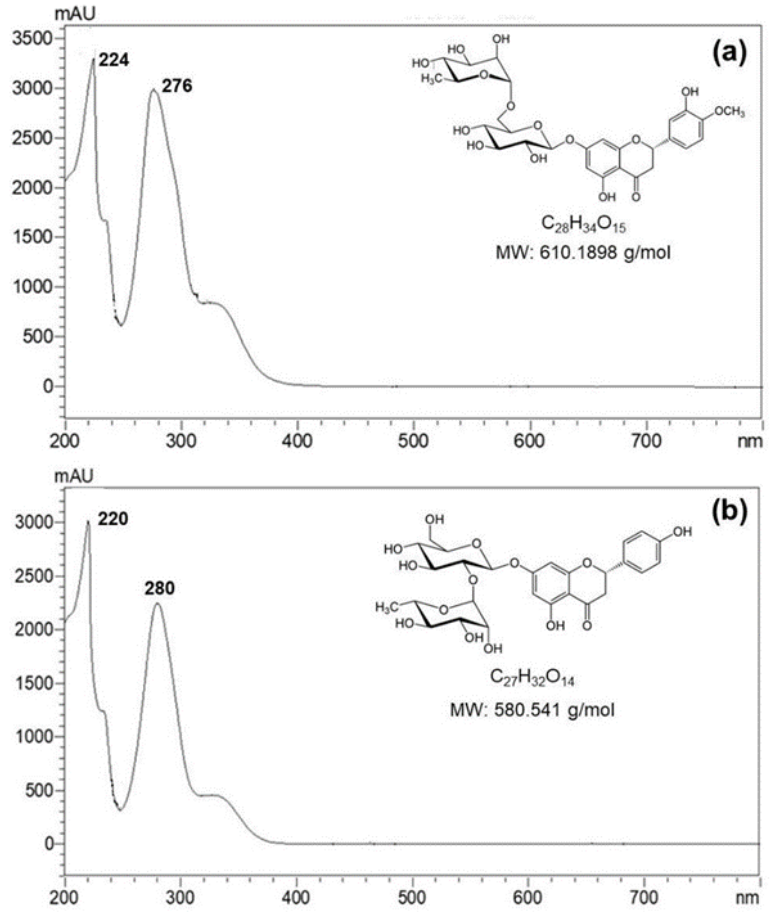

Figure 7 - UV-visible spectra of hesperidin and naringin from methanolic orange peel extracts

Color analysis from methanolic extracts of orange peels

By analyzing the samples by CIELab colorimetry it was possible to notice a statistical difference between the orange types studied. PC1 explained 96.9% (Figure 8a) of the data variability, and the Hamlin samples stood out from the other orange types since there was an overlap between the data of the Pêra-Rio and Valencia types. The same small overlap of some data was noticed in the HeatMap hierarchical analysis (Figure 8b).

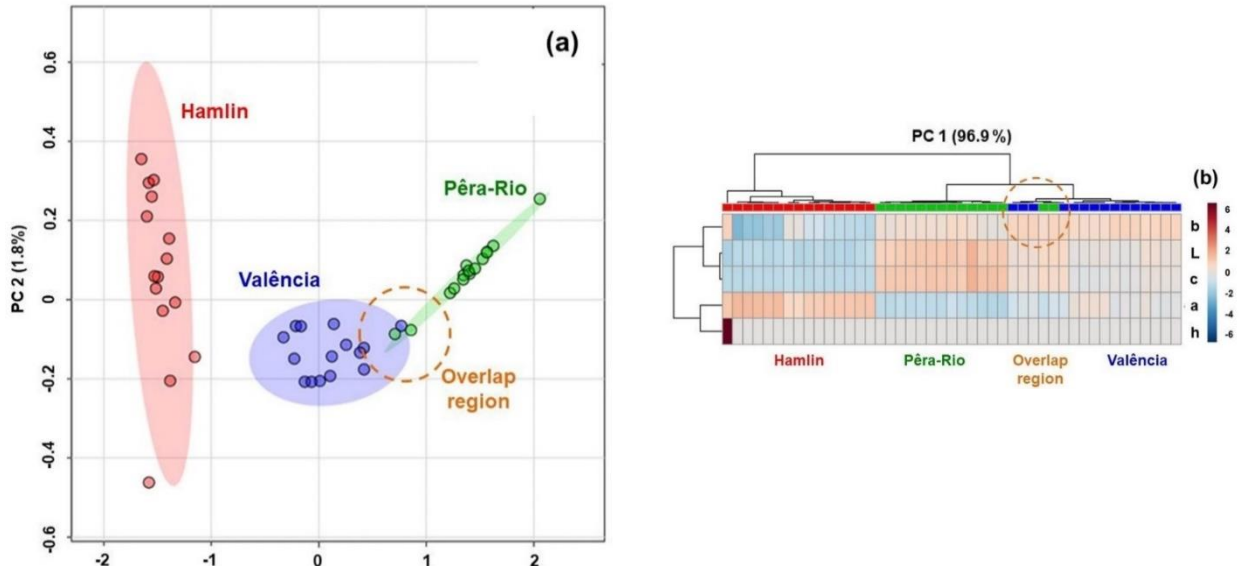

Figure 8 - (a) Principal Component and (b) HeatMap analysis for CIELab colorimetry for direct and fast analysis in peels' surface of Hamlin, Pêra-Rio, and Valência orange types

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It was noted by HeatMap analysis in Hamlin orange, that the a* coordinate was more significant than others, while for Pêra-Rio were L* and c*, in opposite to Valência that the b* coordinate was significant. These differences between parameters and orange types are important to discrimination by CIELab analysis, promoting the use of this technique in industrial processing scale. CIELab analysis is fast, cheap, and friendly to use. In addition, the dendrogram (results not shown) analysis confirmed the high difference between Hamlin and other orange types. The dissimilarity was upper than 30% between Hamlin and others, and between Pêra-Rio and Valência was 15%.

On the colorimetric analyses by Space Color from the CIELab system (Figure 9), the Hamlin was far from other orange types by plan distances. According to Pozzan and Triboni (2005), the Hamlin fruits are perfectly colored, intense orange and without spots, while Pêra-Rio presents fruits with a greenish-yellow color and Valencia accentuates intense yellow. Color nuances are a result of the thermal amplitude. In regions with mild temperatures, the peel color becomes more orange. Another factor is associated with the long periods of rain and sunshine that activate the chlorophyll synthesis and leave the fruits greenish.

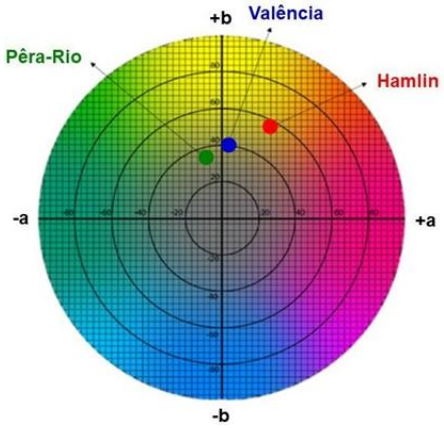
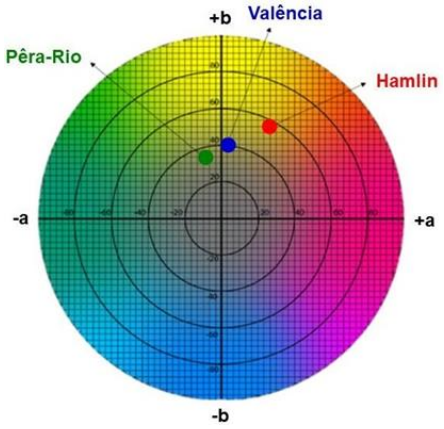


Figure 9 - Coordinates obtained by colorimetric analysis from Space Color of the CIELab system on Hamlin, Pêra-Rio and Valencia orange surfaces

UV-visible spectral scanning analysis from methanolic extracts of orange peels

UV-visible maximum absorption scanning from 200 to 600 nm was carried out with methanolic extracts from orange peels of Hamlin, Pêra-Rio, and Valência. The PCA did not show efficiency in distinguishing the orange types. Thus, we chose to analyze the UV-Vis data by PLS-DA (Figure 10a), which uses a multivariate curve, which was more successful in discriminating the orange types, although this differentiation was not entirely clear.

Component 1 (PLS-DA) explained 71.3% of the variability, in particular, between Valencia and the other orange types. However, Hamlin and Pêra-Rio did not show a great difference in the analysis of PLS-DA. It was verified that the analysis by scanning the UV-visible maximum spectrum did not prove to be a technique capable of differentiating the peel compositions from the orange types.



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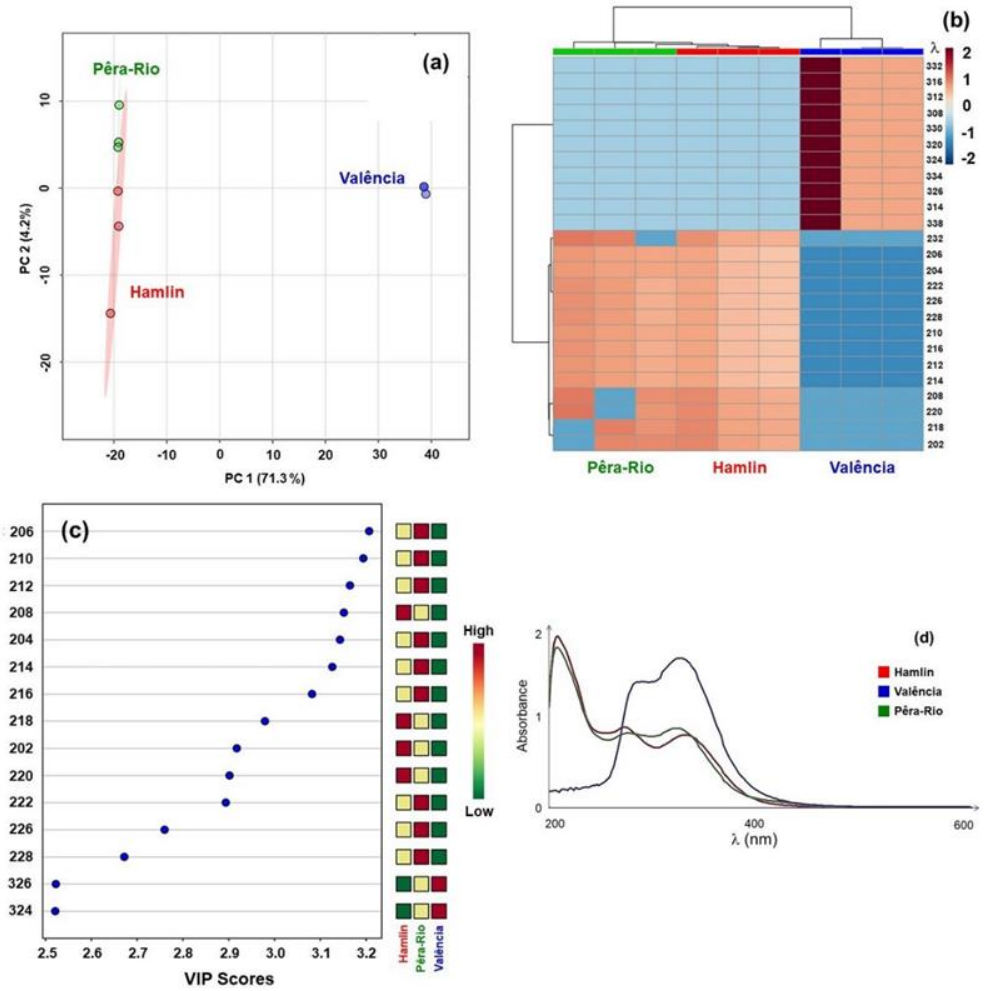

Figure 10 - (a) PLS-DA; (b) HeatMap; (c) VIP Score; and (d) overlapped UV-VIS spectra of methanolic extracts of peels from Hamlin (red); Valência (blue) and Pêra-Rio (green) orange types

However, a more detailed treatment of the data by HeatMap and VIP Score was effective in showing the difference between the characteristic wavelengths of each methanolic extract. It was noted that Valencia presented characteristic wavelengths in the range of 308 to 338 nm, with the wavelengths 324 and 326 nm being the most pronounced. According to Holser (2012), ferulic acid has a maximum absorbance at 215 nm with additional absorbances at 287 and 312 nm. In contrast, *p*-coumaric acid exhibits maximum absorption at 286 nm with additional absorbances at 209 and 220 nm. Harnly et al. (2007) reported that flavonoids can be identified and quantified based on UV-Vis absorption between 200 and 600 nm. The extracts from Hamlin and Pêra-Rio presented characteristic wavelengths in the range of 202 to 232. However, the wavelengths 204, 206, 210, 212, 214, 216, 222, 226, and 228 nm were more pronounced for Pêra-Rio, and 202, 208, 218, and 220 nm were more pronounced for Hamlin. Borello and Domenici (2019) reported that between

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390 and 560 nm, carotenoid and chlorophyll derivatives contribute to the spectrum, while the region between 630 and 700 nm can be safely attributed to the contribution of chlorophyll-exclusive derivatives. According to Makarska-Bialokoz and Kaczor (2014), chlorophyll forms have the maximum absorption at different wavelengths. Many organisms capable of conducting photosynthesis have chlorophyll which absorbs red light at approximately 662 nm and under violet light at around 430 nm. Chlorophyll *b* strongly absorbs light at 642 nm and blue light at 453 nm. Plants with more complex chlorophyll systems absorb light in the greatest range. During the photosynthesis process, the light is absorbed by chlorophylls below 480 nm and from 550 to 700 nm. Light between 480 and 550 nm (i.e., green) is not absorbed by chlorophylls, but they are reflected.

CONCLUSIONS

The cluster analysis showed that the color analysis was more effective, as its analysis was performed directly on the surface of the orange peels. The other techniques must be performed after extensive and careful extraction by organic solvents. However, all the applied techniques are capable of distinguishing the types of orange analyzed in this work. In addition, large datasets must be prepared, often difficult to interpret, but the use of PCA and HCA increases interpretability during orange differentiation. Optical methods coupled with chemometrics would greatly benefit from accessible, up-to-date tools by automatization process of orange identification.

AUTHOR CONTRIBUTIONS

Conceptualization: C.L. and I.B.C.; funding acquisition: C.L.; investigation: I.B.C., G.A.G.C., and C.L.; methodology: I.B.C., G.A.G.C., and C.L.; supervision: C.L.; writing (original draft preparation): I.B.C., G.A.G.C.; writing (preparation, review, and editing): C.L. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest regarding this article.

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