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Antioxidant Potential Optimization of Mixed Vegetable Juice: A Chemometric Approach

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This study aimed to develop a healthy and antioxidant-rich mixed juice. The effect of the vegetables combination on the juice characteristics was evaluated and, to optimize the proportion components of the mixture, chemometric modeling was used. The maximum antioxidant activity was considered as a variable response. The mixed juice was evaluated for technological parameters (content of soluble solids, titratable acidity, density, viscosity, sedimentation, and color parameters). The phenolic compounds present in the vegetables were identified by ultra-high performance liquid chromatography-mass spectometry (UHPLC-MS) technique. The special cubic model was considered significant ($p < 0.05$), with low dispersion and homogeneous antioxidant activity data (coefficient of variation (C.V.) = 3.73%) and the most appropriate for statistical data analysis ($R^2 = 0.9937$). The optimal formulation consisted of a blend (m/m) with orange (73.37%), apple (20.45%), kale (5.58%), and ginger (0.59%) with greater desirability, 12534.2 µmol mL^{-1} of validated antioxidant activity and adequate physicochemical characteristics. The addition of orange juice resulted in a product with better functionality, due to the increased antioxidant capacity. This innovative study resulted in a functional formulation that involves the combination of low-cost vegetables with the maximum possible antioxidant action, capable of helping to protect against damage caused by free radicals.

Keywords: antioxidant activity, modeling chemometric, UHPLC-MS, mixed fruit, vegetable juice

Introduction

Fruits and vegetables are inherently detoxifying foods, as they contain biologically active antioxidants that aid in the elimination of potential mutagenic free radicals.[1] Consequently, various plant components offer additional beneficial functions to the body, contributing to the promotion of health and well-being and reducing the risk of chronic diseases.[2]

Orange (*Citrus sinensis*) stands out as a crucial citrus fruit, boasting a diverse array of phytochemicals and compounds. Among these are polyphenols such as flavonoids and hydroxycinnamic acids, which contribute to the flavor and overall properties of the fruits.[3] Kale (*Brassica oleracea* L. var. *acephala* L.), a globally consumed vegetable, exhibits an extract with notable antioxidant activity, antiulcerogenic properties, and antigenotoxic potential. Additionally, it contains a high content of flavonoids, primarily kaempferol derivatives and quercetin.[4] Apple (*Malus domestica*) serves as a significant source of nutrients and bioactive compounds for humans, commonly used as a juice filler due to its neutral taste.[5] Ginger (*Zingiber officinale Roscoe*) is a plant that contains a variety of nutrients, dietary fiber, and volatile compounds, known for its non-volatile biologically active substances with robust antioxidant effects.[6] The choice of the four specific ingredients (orange, apple, kale, and ginger) for the optimization of the mixed juice is a strategic proposal that considers not only the individual antioxidant potential but also the synergy among these ingredients, their availability, and low cost.[3-6] This not only makes the juice accessible but also paves the way for large-scale production.

In the beverage industry, fruit juices play a prominent role, serving as matrices for incorporating various bioactive constituents.[7] Mixed juices have been developed with diverse combinations such as pomegranate, amla and melon; spinach and other common vegetables as soursop,

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pineapple, mango and orange; orange, mango, kiwifruit, carrot and pineapple; among others.[8-11] Additionally, there are several commercial mixed juices. However, studies involving mixed juices with optimized formulations are still rare. In this context, researchers are exploring tools as viable alternatives for beverage development and optimization, aiming to preserve bioactive compounds and maintain the inherent characteristics of natural vegetables, including mixing modeling.

Mixture modeling, a mathematical-statistical approach, aims to optimize experimental processes by applying specific combinations of variable levels (forming an experimental matrix) to extract information about the studied system, in other words, a mixture design is useful in formula optimization.[12] Blend design is a useful tool for optimizing product formulations and investigating the role of ingredients and their interaction in the final formulation.[13] Through this technique, predictive mathematical models are generated, relating mixing factors and their responses.[13] In this sense, mixture design experiments are useful in product development and have been used in the optimization of food formulations.[12,14]

The ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) method, known for its sensitivity in detecting and characterizing constituent traits, has gained approval as a rapid technique for distinguishing phytochemicals and providing insights into food quality.[14] Hence, it was employed to determine the phenolic compounds present in the mixed juice and its components. The chemometric modeling introduction and the UHPLC-MS method as optimization and analysis tools, respectively, highlight a relative change in manufacturing processes and quality control in the production of antioxidant juices. By prioritizing low-cost ingredients, the study suggests that large-scale production of these optimized juices can maintain economic accessibility, making the health benefits provided by natural antioxidants available to a wide range of consumers. By employing chemometric modeling, the research not only seeks to optimize the formulation to ensure maximum antioxidant activity but also evaluates the technological characteristics of the mixed juice. [2,7]

To the best of the author's knowledge, no studies have explored the optimization of a potentially functional mixed juice combining orange, apple, kale, and ginger with a high antioxidant activity content. Therefore, this study aims to develop a healthful mixed juice, rich in antioxidants, using readily available and cost-effective ingredients, presenting adequate physicochemical characteristics. Additionally, it aims to optimize the proportion of components (fruits and vegetables) through chemometric modeling and evaluate the combined effects of vegetables on the characteristics of the juice.

Experimental

Materials

Orange Pera Rio (*Citrus sinensis*), kale (*Brassica oleracea* L. var. *acephala*), apple (*Malus domestica*), and ginger (*Zingiber officinale Roscoe*) were used to prepare the mixed juice. For further analysis, the following chemical reagents were used: 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Darmstadt, Germany), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Sigma-Aldrich, Darmstadt, Germany) and PA methanol (Synth, São Paulo, Brazil).

Extraction and formulation of mixed juices

Lots of oranges, apples, kale, and ginger (1 kg) were purchased from the local market in Maringá city (PR), Brazil. The ingredients were washed in running water, sanitized with a sodium hypochlorite solution (Merck, Darmstadt, Germany) (200 mg L^{-1}) for 15 min, rinsed in potable water, peeled, manually cut using stainless steel knives, and weighed. The oranges were extracted individually using a food processor (E10, Mondial, Brazil). The orange juice, kale, apple, and ginger were mixed in a domestic blender (L-550, Mondial, Brazil) for 3 min. The mixtures were sieved and reserved for further analysis with modifications.[2] Preliminary tests of "green juice" formulations from the literature were developed (Table 1). The juice was formulated to adjust the maximum antioxidant activity, which was measured as a response to the designed experiment. For 130 g of beverage (g g^{-1}), four main components varied. These components represented 100% by weight of the total formulation. A total of 20 formulations were proposed by the mixture model and used to produce the formulations, with 4 being the central point.

Table 1 presents important information from previous studies related to the development of mixed juices. In light of this, the present study proposes a new combination of vegetables within the broader scope of studies on mixed juice formulations.

Antioxidant activity (DPPH radical scavenging activity)

The antioxidant activity (DPPH method) of different mixed juice formulations was determined.[15] Data were evaluated using Design expert software.[22] For

Table 1. Summary of some fruits and vegetables mixed juices formulations in the literature

No.	Objective	Formulation	Reference
1	to optimize the mixing ratio of broccoli, cabbage, and carrot powders to develop juice powders with high amounts of diverse phenolic compounds, high antioxidant activity, and favorable sensory preference using a blending design	broccoli, cabbage and carrot powders as the main ingredients and using a fixed ratio of apple, tomato and radish powders as secondary ingredients	Kim *et al.*[1]
2	to describe willingness of kids to taste, like, and intake fruit-based smoothies containing some vegetables (for example, spinach, collards, kale), commonly referred to as "green smoothies," and explores individual differences in eating responses of children	spinach, collards, kale, strawberry, banana, 100% orange juice, soy milk	Rollins *et al.*[15]
3	to make a mixed fruit juice and compare it with a commercial juice that has the name "detox" on the label	25% pineapple juice, 20% carrot juice, 15% coconut water, 15% apple juice, 10% kale juice, 10% drinking water, added 0.1% ginger (m/v), 0.1% mint (m/v) and 1% fructose (m/v)	Machado *et al.*[16]
4	to evaluate the effect of single and multiple wavelengths on the microbial quality and physicochemical and phytochemical attributes of newly formulated mixed beverage	carrot, carob, ginger, grape, and lemon juice	Baykuş *et al.*[17]
5	to investigate quality changes of mixed juices during storage and to explore the potential to create shelf-stable mixed juice with fresh-like organoleptic quality through high-pressure processing	orange, mango, kiwifruit, carrot, and pineapple	Li *et al.*[11]
6	to evaluate the development of a mixture of "*Juçara*" and "*Ubá*" mango juice and the influence of pasteurization, high isostatic pressure (HIP), and the addition of a probiotic culture of *Lactobacillus rhamnoses* on the physical-chemical, microbiological, functional, and sensory characteristics of the products obtained	mixed "*Juçara*" mango and "*Ubá*" mango juice	Moreira *et al.*[2]
7	to make a comparison of the drying methods of potentially detoxifying pulps by lyophilization and by atomization to determine which method best preserves the characteristics of the original product	frozen pulps composed of pineapple, mint, and ginger	Ibiapina *et al*.[18]
8	to blend bitter orange juice with pineapple juice in appropriate proportion to contribute to the sufficient intake of natural antioxidants	orange and pineapple	Raji *et al*.[19]
9	to investigate the oxalate composition of green juice prepared using a high-speed blender compared with the juice prepared using a masticating juicer where the pulp fraction was discarded in the process	spinach and other common vegetables	Vanhanen *et al.*[9]
10	to evaluate consumer perception of the sensory characteristics of tropical mixed juice based on cashew apple, acerola, and melon obtained using different processing methods during cold storage at 4 °C	cashew apple, acerola, and melon	Martins *et al.*[20]
11	to test a green juice formulation and evaluate its properties, to promote the stability of this green juice, chia seed gel, and biosurfactant were evaluated; a kind of fat, two natural emulsifiers	416.70 g of pineapple (*Ananas comosus* L. Merr.), 0.70 g of carrot (*Daucus carota*), 200 mL of filtered water, 30.69 g of cabbage (*Brassica oleracea*), and 182.51 g of cucumber (*Cucumis sativus*)	Fraga *et al.*[21]
12	to optimize the formulation of a juice cocktail containing soursop, pineapple, mango, and orange, based on physical-chemical aspects and sensory attributes of the cocktail	soursop, pineapple, mango, and orange juice	Akonor *et al.*[10]
13	to develop a mixed fruit drink incorporating pomegranate, amla, and melon juices using sensory analysis and optimize the formulations through three methods viz; numerical optimization of the overall acceptability score, the ranking of formulations, and consumer acceptability	pomegranates, amla, and melon juices	Bhalerao *et al*.[8]

the DPPH standard, analytical curves were developed using the Trolox standard at concentrations from 100 to 1500 µmol L^{-1} and 50 to 2000 µmol L^{-1}. A stock solution of DPPH 6.25×10^{-5} mol L^{-1} in methanol was prepared. From the stock solution, a working solution was prepared using methanol as the diluent, where the absorbance of this solution at 517 nm was 0.700. 25 µL of the sample diluted in water at a ratio of 1:3 at a concentration of 1 mg mL^{-1} and 2 mL of the working solution were used. After preparing the samples, they were left for 30 min in the dark. Then, absorbance readings were taken at 517 nm and methanol was used to reset the instrument. The DPPH scavenging activity result was performed in triplicate and expressed as the equivalent concentration of Trolox in µmol L^{-1} g^{-1} sample.[15]

Data modeling and experimental design using D-optimization

An experimental design of four-component blending was used to optimize the proportions of ingredients (components) on the antioxidant activity of blended juices.[8] The antioxidant activity value for each formulation was calculated according to a calibration curve with Trolox standard against the DPPH radical (equation 1).

$$y = -0.0002x + 0.6956 \qquad (1)$$

The model was evaluated by analysis of variance (ANOVA) maintaining the terms that showed effects at a probability of $P < 0.05$. After analyzing the models, the special cubic model was considered more suitable for the experimental optimization of the antioxidant activity data.

The ideal juice mixing ratio was determined based on numerical and graphical optimizations.[16]

Physical and chemical characteristics of mixed juice

The apparent viscosity of the juice was evaluated in freshly prepared samples at 25 °C using a viscometer (Visco Star plus, Fungilab, USA) with a constant speed of 200.0 rpm.

Total acidity was evaluated according to Association of Official Agricultural Chemists (AOAC) by titration with 0.1 mol L^{-1} NaOH and expressed as percentage (method 947.05).[23] The pH was determined by potentiometric measurement using a pHmeter (MS Technopon, Piracicaba, Brazil).

The total soluble solids content was determined using a digital refractometer (Instrutherm, São Paulo, Brazil), and the results were expressed in °Brix. The color parameters (L*, a*, b*) were evaluated using a colorimeter (Konica Minolta®, CR-410 model, Tokyo, Japan) for individual vegetables and optimized juice.

The sedimentation index was measured using the method reported by Wu *et al*.[24] with some modifications. A graduated centrifuge tube was filled with 10 mL of juice sample, centrifuged at 3000 rpm for 10 min, and then kept in an oven at 40 °C for 24 h, after which the precipitate was weighed. The sedimentation index (%, m/m) was expressed as the weight ratio of the centrifuged sediment to the juice sample. The values were given by the average of three repetitions.

Quantification of phenolic compounds in mixed juice by UHPLC-MS/MS

Juice samples were prepared using a centrifuge (Daiki, DT 4500, Japan) for 10 min at 4000 rpm at 25 °C and the supernatant was used afterward. The supernatant was filtered through a polytetrafluoroethylene (PTFE) syringe (33 mm in diameter and 0.45 µm in pore size) (PES Membrane, Polyethersulfone, Filtril, Brazil) and stored in a 2.0 mL amber vial. Finally, 1.5 µL of the mobile phase was injected into the UHPLC-MS/MS for analysis. Extracts were stored in a freezer at −18 °C and analyzed within one day.

Analyzes were performed on an ACQUITY UHPLC® H-Class system (Milford, MA, USA) coupled to a Xevo TQD™ triple quadrupole mass spectrometer (Milford, MA, USA), equipped with a Waters Z Spray™ electrospray ionization source (ESI) (Milford, MA, USA). Samples were injected onto the ACQUITY UHPLC® C18 column (50 mm × 2.1 mm internal diameter, 1.7 µm) purchased from Waters (Milford, MA, USA). Mass spectrometry was operated in negative electrospray ionization (ESI–), optimized mode, and tuning parameters as follows: capillary voltage, 3.0 kV; cone voltage, 21 V; extractor voltage, 3.0 V. Source temperature and desolvation gas temperature were set to 130 and 550 °C, respectively. Nitrogen was used as cone gas and desolvation gas with flow rates of 50 and 700 L h^{-1}, respectively.

The mobile phase used was composed of ultrapure water acidified with 0.1% formic acid (eluent A) and methanol acidified with 0.1% formic acid (eluent B), and the column temperature was maintained at 30 ± 1 °C. The injection volume was adjusted to 1.5 µL. The mobile phase gradient program started at 90% A and 10% b until 0.01 min; up to 3 min the composition was 40% A and 60% B; between 4 and 5 min, the composition remained constant at 20% for A and B; from 5.5 min, the composition was 0% A and 100% B, and from 7 to 9 min, the composition of phase A increased from 0 to 40%, while phase B decreased from 100 to 60%; from 11.50 to 16 min, phase A remained constant at 90% and phase B constant at 10%.[19,25]

Statistical analysis

The optimal mix design for optimization and the effects of the variables were developed using Design Expert v-7.0 software (Stat-Ease, Minneapolis, USA).[22] Thus, the results were submitted to ANOVA and data were presented as mean ± standard deviation.

Results and Discussion

Antioxidant activity

The results varied considerably from one sample to another and were in the range of 1704 to 7789 µmol mL^{-1} (Table 2). The characteristics of a vegetable paste formulated by linear programming maximize the antioxidant activity and conclude that the effectiveness of the natural antioxidant depends on the chemical structure of the active compound and cannot be explained only by the total phenolic compounds but requires the characterization of the structure of the active compound.[20]

The interaction between the individual components of the beverage mixture was mathematically modeled about antioxidant activity responses. In addition to phenolic compounds and vitamin C, there are other components present in the mixed fruit and vegetable drink that can also interfere with the measurement of total antioxidant capacity, such as vitamin E present in kale and carotenoids present in kale and orange.[15] The coefficients of determination R^2 of the regression models were adjusted to the experimental data.

The model predictability for antioxidant activity was high (0.9937) and justified an excellent fit with significant interaction terms and less noise. The model's *F*-value of 73.13 and *p*-value ($p < 0.05$) showed that the model is significant, with low dispersion and homogeneous antioxidant activity data (coefficient of variation (C.V.) = 3.73%) and that there is only a 0.01% chance that an "*F*-value model" of this size could occur due to noise. A *p*-value less than 0.0500 indicates that the model terms are significant.

Table 2. Optimum mixing design for optimizing 20 mixed juice formulations based on 4 compounds with constraints

Sample number	Independent variables[a]				Physical-chemical attribute
	O / g	K / g	A / g	G / g	Antioxidant activity / (µmol mL^{-1})
1	89.617	7.384	32.000	0.999	6934 ± 0.04
2	90.405	9.033	30.262	0.300	6264 ± 0.03
3	100.000	9.855	19.145	1.000	6719 ± 0.03
4	90.921	10.000	28.082	0.998	7164 ± 0.02
5	95.498	10.000	23.503	0.999	6874 ± 0.01
6	100.000	9.855	19.145	1.000	6879 ± 0.01
7	94.409	8.908	25.683	1.000	6064 ± 0.08
8	100.000	8.713	20.973	0.313	7394 ± 0.03
9	91.570	7.00	31.130	0.300	7749 ± 0.00
10	88.694	8.656	32.000	0.650	7669 ± 0.01
11	89.617	7.384	32.000	0.999	6804 ± 0.01
12	99.997	7.000	22.214	0.789	1759 ± 0.03
13	87.664	10.000	31.992	0.344	6929 ± 0.00
14	97.167	8.046	23.834	0.953	5589 ± 0.06
15	87.009	10.000	31.997	0.994	6994 ± 0.04
16	88.694	8.656	32.000	0.650	7649 ± 0.01
17	98.278	10.000	21.200	0.522	6924 ± 0.03
18	100.000	8.713	20.973	0.313	7044 ± 0.01
19	94.081	7.000	28.619	0.300	7789 ± 0.03
20	99.997	7.000	22.214	0.789	1704 ± 0.02

[a]Independent variables: O (orange); K (kale); A (apple); G (ginger). Components are expressed in grams and response variable values are given as mean ± standard deviation. Mixing results were analyzed by a special cubic model by Design Expert v-7.0 software.[22]

In this case, linear mixture components, OK, OA, OG, KA, KG, AG, OKA, OKG, OAG, and KAG (where O: orange; K: kale; A: apple; G: ginger) are meaningful model terms. The value of 15.54 for "lack of fit *F*-value" implied that the lack of fit is not significant and that there is only a 1.09% chance that such a large "lack of fit" could be due to noise.

Diagnosis of statistical properties of models

The interaction between the components of the mixture produced a good correlation with the special cubic and quadratic model, with R^2 of 0.9937 and 0.9220 (Table 3), adjusted R^2 (0.9801) and (0.8517), lower standard deviation values (240.12) and (656.10), respectively. Therefore, we opted for the special cubic in terms of adjustment (C.V. = 3.73%), being the same adequate to estimate the antioxidant activity as a response in the present study.

Thus, the final equation in terms of L-pseudo components for the special cubic model (equation 2) was obtained.

$$\begin{aligned}&\text{Antioxidant activity} = -10471.23 \times O + 005\ K + \\ &2677.48 \times A + 6{,}739\ 10^{6}\ G + 005\ O \times K + \\ &43653.78\ O \times A + 006\ O \times G + 005\ K \times A + \\ &007\ K \times G + 006\ K \times G + 005\ O \times K \times A + \\ &007\ O \times K \times G + 006\ O \times A \times G + 007\ K \times A \times G \qquad (2)\end{aligned}$$

where O, A, K, and G represent the concentration of orange, apple, kale, and ginger, respectively; and the constants are coefficients, which signify the interaction effects of simultaneous variations in two or more independent variables on the antioxidant activity of the mixture.

Mixing design

Choice of ingredients

The color of fruits and vegetables is consistent with their antioxidant content and consequently their antioxidant capacity.[23]

The mixture of these vegetables results in drinks with maximized antioxidant activity since it is composed of fruits and vegetables, which are detoxifying foods by nature, as they contain biologically active antioxidants.[1] Furthermore, orange, kale, and ginger juice are the most used components to prepare mixed juices due to their availability and affordable price.[15]

These mixed juices are sensorially well approved, since dark green vegetables when mixed with sweeter fruits have great potential to result in more acceptable products, since the sweetness of these ingredients tends to minimize the bitter taste.[14]

Optimization of the mixed juice formulation

The general optimization process for beverage formulations is shown in Figure 1a. Therefore, the optimized formulation was orange: apple: kale: ginger = 95.383: 26.591:7.257:0.769 (m/m) (Figure 1b).

The generated plot of predicted values *versus* actual values is illustrated (Figure 1a).

Table 3. Statistics and analysis of variance (ANOVA) of the models

Parameter	Model			
	Linear	Quadratic	Special cubic	Cubic
Standard deviation	1419.51	656.10	240.12	129.78
Average			6444.75	
C.V. / %			3.73	
R^2	0.4155	0.9220	0.9937	0.9985
Adjusted R^2	0.3059	0.8517	0.9801	0.9942
Predicted R^2	0.0023	0.5411	–80.7967	
PRESS	5.503×10^{7}	2.531×10^{7}	4.512×10^{9}	
Adequate accuracy			30,000	
Lack of fit tests				
Sum of squares	3.216×10^{7}	4.221×10^{5}	2.617×10^{5}	0.000
Degrees of freedom	11	5	1	0
Mean square	2.923×10^{6}	8.441×10^{5}	2.617×10^{5}	
F-value	173.57	50.12	15.54	
p-value	< 0.0001	0.0003	0.0109	

C.V.: variation coefficient; R^2: regression coefficient; PRESS: predicted residual error sum of squares; *F*-value: the ratio of two variances; *p*-value: marginal significance level within a statistical hypothesis test.

The points presented in the graph indicated linearity between the data, which implied normality in the error term. Therefore, with this linear pattern, it was possible to state that the data are consistent and that there are no signs of problems, and thus the model was considered suitable for optimizing the formulation of mixed juice.

The diagnosis of predicted values *versus* actual values did not reveal statistical problems, which was also confirmed using the response surface plot (Figure 1a).

In graphical optimization, the overlapping region on the graph was represented as the highlighted optimal range, so the colored contour bands represented the range of adjusted antioxidant activity values (Figure 1b). The 2D contour plot appeared in graded color shading on the plot of antioxidant activity as a function of three components of the mixture. This slice included two centroids as indicated by the red dot and the number "2" in the middle of the contour plot.

The ternary diagram, or mixture triangle, was used to represent the optimization of component mixing as it is a simple and intuitive visual representation that allows graphically showing the relative proportion of three components in a mixture. This is useful since this work deals with three independent variables, where the optimal combination of orange, apple, and kale is determined. Any point in one of the vertices represents the expected response to the pure blend, the points located on the sides, binary mixtures, and any point in the inner region ternary mixtures.[12]

Figure 1b shows that there is an increase in the antioxidant potential of the mixed juice according to the interaction of the binary mixture of orange and apple, since the antioxidant potential (12534.2 µmol mL^{-1}) was optimized in this region of the graph.

The optimal mixing ratio determined from numerical optimization had the following ratio of ingredients: orange:apple:kale:ginger = 95.383:26.591:7.257:0.769 (m/m). The same values were obtained in the graphical optimization models (Figure 1b).

Desirability scores (Di) obtained after numerical optimization for antioxidant activity were between 0.306 and 1.00. The desirability score was used due to the ease of interpretation it provides, as a single desirability score simplifies the interpretation and communication of results, making it easier to understand and implement the optimized formulation.[8]

Numerical optimization was directed towards obtaining a maximum Di value with the highest importance assigned to the response. Being a potentially functional and detoxifying juice, it has been optimized to have greater antioxidant activity in the blend. Given the above, the values predicted by the model are those obtained in the research, and thus, it is demonstrated that the model predicts the results obtained.

Technological properties of juices

The juices had pH values ranging from 4.42 to 7.02, titratable acidity between 0.07 and 9.67%, TSS from 0.4 to 10.63 °Brix, density from 0.68 to 1.04, viscosity from 121517 to 292550 mPa s and pulp sedimentation index between 0.02 and 0.16% (Table 4).

The addition of fruits such as oranges and apples contributed in a greater proportion to the acidity intensity of the mixed juice (8.26%, Table 4) since the acidity intensity depends on the type of vegetable used. Orange promoted acidity in the mixed juice in a greater proportion (9.67%) and ginger contributed with a smaller proportion (0.07%) among fruits and vegetables. Citrus fruits are well known for their refreshing smell and ascorbic acid content, in addition, flavor balance (sugar/acid ratio), color, absence of bitter chemicals (limonin), amount of ascorbic acid,

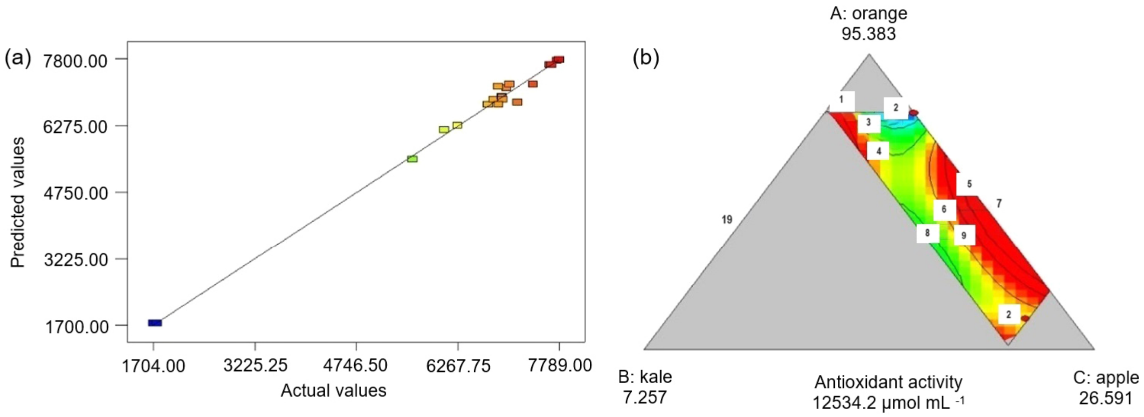

Figure 1. (a) Graph of predicted *versus* actual values for antioxidant activity. (b) Contour plots of the response surface (antioxidant activity) showing the relative interaction between the three constituents (x1 = A: orange), (x2 = B: kale), (x3 = C: apple) of the mixed vegetable juice; (1 = 7465.03), (2 = 2), (3 = 3234.98), (4 = 5350), (5 = 11695.1), (6 = 7465.03), (7 = 7), (8 = 5350), (9 = 9580.05), (19 = 19) µmol mL^{-1}. Data obtained using Design Expert v-7.0 software.[22]

Table 4. Technological properties of individual components and optimized mixed juice

Parameter	Formulations[a,b]				
	M.J.	O	K	A	G
L*[c]	33.29 ± 0.31	31.87 ± 0.46	26.05 ± 0.01	28.42 ± 0.01	30.51 ± 0.20
a*[c]	–6.22 ± 0.22	–1.90 ± 0.01	–2.59 ± 0.09	0.12 ± 0.02	–1.01 ± 0.10
b*[c]	11.43 ± 0.33	6.32 ± 0.08	4.85 ± 0.04	4.79 ± 0.03	2.61 ± 0.05
pH	4.52 ± 0.02	4.42 ± 0.03	6.75 ± 0.21	4.85 ± 0.11	7.02 ± 0.42
SSC[d] / ºBrix	8.57 ± 0.12	8.03 ± 0.12	0.4 ± 0.08	10.63 ± 0.12	0.15 ± 0.04
Titratable acidity / %	8.26 ± 0.10	9.67 ± 0.01	0.19 ± 0.02	0.87 ± 0.13	0.07 ± 0.02
Density / (g mL^{-1})	1.04 ± 0.01	1.03 ± 0.01	0.68 ± 0.49	1.00 ± 0.01	1.00 ± 0.00
Viscosity / (mPa s)	292550 ± 328.73	209214 ± 421.02	161519 ± 294.77	156134 ± 347.80	121517 ± 329.70
Sedimentation index / %	0.16 ± 0.04	0.09 ± 0.01	0.06 ± 0.00	0.05 ± 0.03	0.02 ± 0.00

[a]Means ± standard deviation in the same row accompanied by different letters are significantly different ($P < 0.05$). [b]Formulations: O (orange); A (apple); K (kale); G (ginger); M.J. (mixed juice). [c]L* ranging from 0 (black) to 100 (white); a* ranging from red (+a*) to green (–a*); and b* ranging from yellow (+b*) to blue (–b*). [d]SSC: soluble solids content.

degree of cloudiness, and amount of softened pulp are the main desired characteristics in oranges in terms of juice quality; besides that, orange and ginger have been used as natural flavoring agents, preservative, stabilizer during several food formulations and confectionaries because of their aroma, sugar or acidity.[8,18]

Higher content of soluble solids was observed in mixed juice (8.57 ºBrix) compared to ginger juice (0.15 ºBrix) and kale (0.4 ºBrix), which was already predicted since the solids content soluble solids are used to measure the approximate number of sugars, which are the predominant soluble solids in fruits such as oranges (8.03 ºBrix) and apples (10.63 ºBrix). The addition of fruits to foods can cause an increase in the total solids content since the presence of soluble fibers in the aqueous phase increases the total solids in this phase.[24] The results corroborate the data obtained by Machado *et al*.,[16] who obtained 8° Brix for mixed juice containing apple, kale, and ginger, among other ingredients.

As expected, the mixed juice had an acidic pH value (4.52) and there was no great influence of the addition of kale and ginger on the pH, titratable acidity, soluble solids content, and pulp sedimentation index, since the values of these parameters for the mixed juice they were closer to those found in the individual orange and apple components for pH, titratable acidity, soluble solids content, density and viscosity. Ibiapina *et al*.[18] found a slightly lower pH (3.15) for mixed pineapple, mint, and ginger juice. Also, the mixed juice formulation had the highest pulp sedimentation index (0.16%). Right after juice extraction, the coarse particles settle immediately by gravity, while the fine ones remain in suspension, and yet, sedimentation has been a major challenge for the beverage industry.[11]

The values of L*, a*, and b* for optimized mixed juice were (33.29; –6.22; 11.43), orange juice (31.87; –1.90; 6.32), orange juice of kale (26.05; –2.59; 4.85), apple juice (28.42; 0.12; 4.79) and ginger juice (30.51; –1.01; 2.61) respectively (Table 4).

In general, the luminosity (L*) in all juices is relatively low, as natural phenolic compounds can absorb light. In addition, the value of L* (33.29) for the optimized sample tends to be close to the luminosity of the orange, due to some compounds present in orange juice, such as polyphenols, carotenoids, and other pigments.[26] Similar results were obtained in mixed drink samples containing kale, ginger, coconut water, and orange, which presented an average value of 30.73 in lightness, indicating that the drink was dark. The presence of small suspended particles in the beverage samples may have influenced the low luminosity values and increased viscosity of the mixed juice compared to the vegetable juices used.[15]

The green color of the mixed juice is indicated by negative a* values (–6.22). A higher value for b* is assigned to orange juice. Degradation of the light green pigment of chlorophyll, due to kale, can be attributed to cellular damage caused during processing, which leads to the breakdown of chlorophyll molecules, which are associated with carotenoids, and under normal conditions mask the coloring of these. With cell injury after processing, a more yellowish coloration is highlighted, derived from biomolecules such as carotenoids, polyphenols, and flavonoids present in kale and orange.[26]

Profile of phenolic compounds of mixed juice

Ten phenolic compounds were identified using the UHPLC-MS/MS method, including 5 acids (caffeic, chlorogenic, gallic, ferulic, and *p*-coumaric), 1 alcohol (kaempferol), 1 polyphenol (catechin), and

3 flavonoids (epicatechin, quercetin, and naringenin) (Table 5). The antioxidant properties and bioactivity of the mixed juice come from the composition of the mixture, which is composed of vegetables such as orange, apple, kale, and ginger, rich in phenolic compounds.[27]

Polyphenols are metabolites present in all plant tissues, both in flowers and fruits, which are representative matrices because they contain a significant amount of polyphenols that can be used in the food industry.[28]

The use of orange in mixed juice resulted in a juice with great functional potential, since phenolics are the dominant bioactive compounds in this fruit and the profile of orange phenolic compounds (kaempferol, chlorogenic acid, gallic acid, quercetin, *p*-acid coumaric and naringenin) is like the optimized product. Similarly, a previous study[11] reported that chlorogenic acid and naringenin were the two dominant flavonoids in oranges.

The presence of several phenolic compounds and antioxidant activity was observed in the mixed juice formulation, an expected result because the native fruits used in the study have these compounds.[29] Other authors[29] have also identified phenolic compounds (limonin, nomilin, hesperidin, neohesperidin, naringin, chlorogenic acid, ascorbic acid, and total carotenoids, total phenolic content) and antioxidant activity in citrus juices. Phenolics were identified in the vegetable juice samples used and in the mixed juice (Table 5), so in the mixed juice, the presence of all compounds was obtained, which enhanced the functionality of the mixed juice.

The relationship between the phenolic compounds found in the individual juice samples showed that orange juice contributed the most phenolic compounds in the mixed juice (6 compounds) while ginger juice did not contribute. Orange is an underutilized source of antioxidants that may help curb degenerative diseases.[11]

Polyphenols were identified as the most relevant group of phytochemicals from fruit and vegetable processing residues as natural sources of extracts rich in antioxidants.[30] The results also corroborate the data obtained by He *et al*.[31] in which apple juice contained more phenolic compounds (hydroxycinnamic acids) than the corresponding ciders. Machado *et al*.[16] elaborated on a mixed juice with "detox" potential, containing pineapple, carrot, coconut water, apple, kale juice, ginger, and mint, and obtained total phenolic contents of 81.38 mg GAE 100 g^{-1}. The antioxidant activity of fruits and vegetables is attributed, in addition to other factors, to secondary metabolism compounds: phenolics. In short, the mixed juice under study is potentially a food rich in antioxidant compounds, as it presents a combination of phenolic compounds present in the individual juices.[31-33]

Table 5. Phenolic compounds identified by UHPLC-MS/MS in fresh mixed juice and juice components

Phenolic compound	Sample	Structural formula	t_R / min	Precursor ion (*m/z*)	Product ion (*m/z*)
Kaempferol	1,5	$C_{20}H_{18}O_{10}$	10.79	285	151 255
Catechin	2,5	$C_{15}H_{14}O_6$	8.59	289	179 245
Epicatechin	5	$C_{21}H_{24}O_{11}$	8.05	289	179 245
Caffeic acid	5	$C_9H_8O_4$	8.86	179	117 135
Chlorogenic acid	1,2,3,5	$C_{16}H_{18}O_9$	8.26	353	85 191
Gallic acid	1,3,5	$C_{13}H_{16}O_{10}$	6.51	168.9	78.9 124.9
Ferulic acid	5	$C_{10}H_{10}O_4$	9.42	193	134 178
Quercetin	1,5	$C_{15}H_{10}O_7$	10.11	301	121 151
p-Coumaric acid	1,3,5	$C_9H_8O_3$	9.26	163	119 92.9
Naringenin	1,5	$C_{15}H_{12}O_5$	10.09	271	119 151

1: orange juice sample, 2: apple juice sample, 3: kale juice sample, 4: ginger juice sample, 5: mixed juice sample; UHPLC-MS/MS: ultra-high performance liquid chromatography-MS; t_R: retention time; *m/z*: mass-to-charge ratio.

Conclusions

The present study showed the possibility of optimizing the formulation and allowing the maximization of the antioxidant activity. Based on chemometric modeling and the D-optimization criterion, the mixed juice was formulated and the final optimized blend (m/m) was orange juice (73.37%), apple (20.45%), kale (5.58%), and ginger (0.59%) with 12534.2 µmol mL^{-1} of validated antioxidant activity with the desirability of 1.00.

The formulated mixed juices showed an average antioxidant activity of around 6444.75 µmol mL^{-1}. Chlorogenic acid was the most predominant, accounting for phenolic compounds in 80% of the samples, and the color of fruits and vegetables was consistent with their antioxidant content and, consequently, their antioxidant capacity.

The mixing modeling approach proved to be a suitable method to optimize a mixed juice formulation. The results highlighted that the mixed juice is a source of antioxidant phenolics, with a combination of nutrients from low cost with maximum possible antioxidant action, capable of helping to protect against damage caused by free radicals. Finally, a healthy, attractive and inexpensive mixed juice formulation was developed, being a promising option for the food industry, as well as providing detoxifying benefits to consumers and meeting their expectations.

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Author Contributions

Jiuliane M. Silva was responsible for conceptualization, data curation, formal analysis, investigation, methodology, writing original draft; Zeinab El Hajj Hussein for formal analysis; Lucas Ulisses R. Chiavelli for methodology, software, writing review and editing; Oscar O. Santos for funding acquisition, project administration, resources, supervision, validation, visualization, writing review and editing.

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