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ARTICLE

Optimization and Validation of Ultrasound-Assisted Extraction for Total Phosphorus Analysis in Cane Syrup

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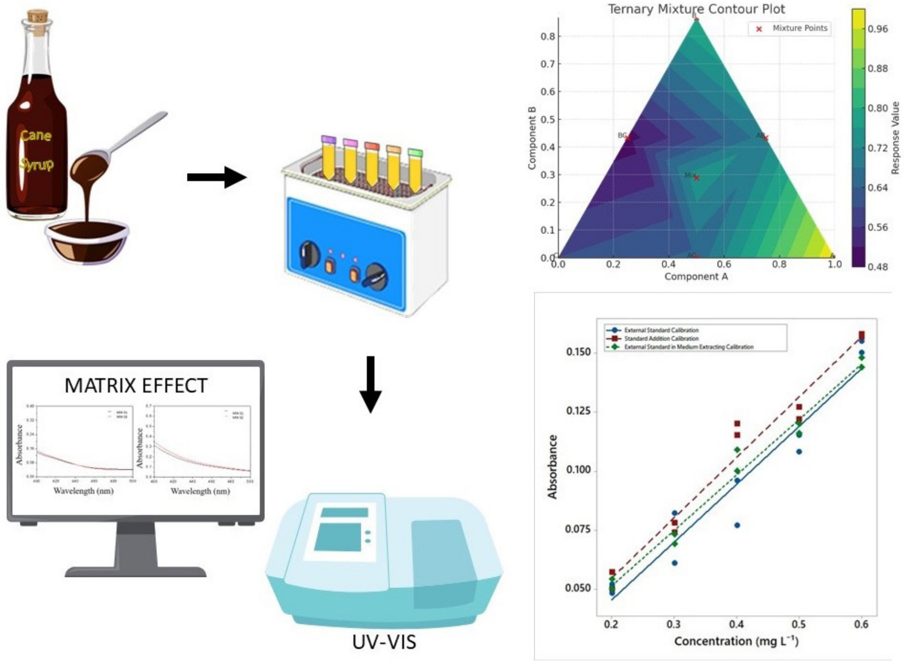

Cane syrup, a nutrient-rich by-product of sugarcane, is valued for its bioactive compounds and mineral content, including phosphorus, a vital macromineral essential for human bone health, enzyme activity, and plant metabolism. Conventional methods for total phosphorus analysis in such viscous matrices face challenges, such as matrix interference, high reagent consumption, and environmental impact. This study optimized and validated an ultrasound-assisted extraction (UAE) method combined with UV-Vis spectrophotometry for determining total phosphorus in cane syrup. UAE parameters were optimized using a simplex centroid mixture design to assess the effects of HNO_3, HCl, and ultrapure water as extraction solvents. UV-Vis spectra revealed that HCl-rich extraction solvents enhanced pigment production via the Maillard reaction, interfering with spectrophotometric detection. In contrast, ternary acid mixtures minimize these effects. The optimal conditions (1.67 mL HNO_3, 2.00 mL HCl, and 1.30 mL H_2O) achieved recovery rates of approximately 100%, without significant matrix interference. The validation of UAE combined with UV-Vis spectrophotometry demonstrated excellent selectivity and linearity ($R^2 > 98.0\%$), low limits of detection and quantification (0.296 μg g^{-1} and 0.898 μg g^{-1}, respectively), and good precision (RSD < 11%). The method's accuracy was confirmed through a paired *t*-test comparison with microwave-assisted digestion (MAD), showing no significant differences ($p > 0.05$). UAE proved to be more environmentally friendly than MAD, with lower energy consumption (4.17 vs. 62.50 Wh/sample) and reduced reagent usage, as indicated

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by the AGREEprep metrics (scores: 0.41 vs. 0.30). The total phosphorus content in cane syrup samples varied significantly (11.48–129.54 mg kg^{-1}), influenced by geographical origin and production processes. The validated UAE method provides a fast, cost-effective, and sustainable alternative for phosphorus analysis in complex food matrices, aligning with the principles of green chemistry.

Keywords: Ultrasound-assisted extraction, total phosphorus analysis, UV-Vis spectrophotometry, simplex-centroid design, method validation

INTRODUCTION

Sugar derived from sugarcane is a natural sweetener primarily composed of sucrose.[1] While sugarcane juice contains bioactive compounds such as phenolic acids, polyphenols, and flavonoids, these are significantly reduced during refining, resulting in a product with low nutritional content.[2] In contrast, less processed sugars, such as cane syrup, have gained consumer preference due to their higher nutrient retention and high energy value (about 300 kcal per 100 g).[3] Cane syrup is widely used in Brazilian cuisine and food industries as a sweetener and flavor enhancer.[4-6]

Cane syrup is a thick, viscous liquid composed of approximately 55% sugars, as well as flavonoid glycosides and phenolic acids.[7,8] Its composition varies according to plant variety, geographic location, and processing conditions.[9] Notably, cane syrup retains minerals naturally present in sugarcane juice, which play essential roles in human health.[10,11]

Phosphorus is a key macromineral, essential for the structure of bones, teeth, and certain muscle proteins.[12] It also participates in digestive enzyme activity and bone formation and interacts with other minerals through complexation reactions.[13] In plants, phosphorus is found in organic and inorganic forms, the latter being more soluble and bioavailable.[14,15] However, insoluble compounds may limit their bioavailability.[16] In sugarcane juice, most phosphorus is in soluble form, and around 10% is organic, with variations depending on cane maturity.[17]

Phosphorus bioavailability in plant-based foods is often reduced by the presence of phytates and phosphates, yet cane syrup shows relatively high bioaccessibility.[15] For laboratory analyses, total phosphorus (P_2O_5) is commonly determined via direct methods, where distinguishing between organic and inorganic forms is less relevant in routine quality control.[18]

Sample preparation for phosphorus determination typically involves techniques like microwave-assisted digestion, wet acid digestion, or dry ashing.[19-22] However, such methods may be inefficient or produce excessive waste when applied to complex matrices like cane syrup.[20,23] Therefore, optimized methods that are both accurate and environmentally sustainable are essential.

Ultrasound-assisted extraction (UAE) has proven effective for solid and semi-solid samples by enhancing dissolution, leaching, and analyte recovery through cavitation-induced disruption of the matrix.[24,25] While simple acid dilution may be sufficient for liquid matrices,[26] UAE offers advantages for viscous, sugar-rich products: (i) breaking macromolecular aggregates; (ii) reducing chromatic interferences; and (iii) minimizing reagent and energy consumption.[27-29]

Previous studies by our research group validated UAE for extracting elements such as iron, manganese, calcium, and magnesium from cane syrup and brown sugar, demonstrating excellent analytical performance.[30,31] However, variables like solvent composition, temperature, pH, ultrasound amplitude, and solid/solvent ratio influence efficiency, especially given cavitation inconsistencies in ultrasonic baths.[32-34]

Although UV-Vis molecular absorption spectrophotometry is widely used in quality control due to its speed and simplicity, no studies have applied it in combination with colorimetric methods and UAE for total phosphorus determination in sugar-rich food matrices, nor have matrix interferences been systematically evaluated.[35,36] This technique also provides structural information and is effective when paired with chemometric tools.[37-39] Additionally, colorimetric methods are widely employed in phosphorus analysis for their low cost and ease of use.[15,40] These methods typically involve the formation of a yellow phosphomolybdate complex, quantified via UV-Vis spectrophotometry.[18,41]

Analytical method development must consider equipment availability, simplicity, and efficiency.[42] However, validation is essential to ensure statistical reliability and regulatory compliance, particularly in food analysis, where it is linked to quality and safety.[43-45]

In this context, this study aimed to optimize and validate an ultrasound-assisted extraction methodology for the determination of total phosphorus in cane syrup, minimizing the influence of potential matrix interferents. Chemometric tools and UV-Vis spectrophotometry were employed for this purpose. The approach prioritized the principles of green chemistry, aiming to minimize reagent consumption, reduce waste generation, and ensure analytical efficiency.

MATERIALS AND METHODS

Cane syrups sampling

A total of 25 cane syrup samples were purchased from supermarkets and other commercial establishments (e-commerce), sourced from different producers and regions across Brazil. The samples were identified with the code "CS" followed by an Arabic number and were collected randomly. They represented the main sugarcane derivative-producing regions of the country: Rio Grande do Sul (RS): CS02, CS08, CS09, CS13, CS14, CS15, CS23, CS24, and CS25; Santa Catarina (SC): CS03, CS04, CS05, CS06, CS12, and CS16; Paraná (PR): CS01, CS17, CS20, and CS22; São Paulo (SP): CS10 and CS19; Minas Gerais (MG), Bahia (BA), and Rio de Janeiro (RJ): CS11, CS18, and CS21, respectively. The samples were stored in their original packaging in a dry, well-ventilated room at room temperature until the tests were performed.

Reagents and solutions

All reagents were of analytical grade. Ultrapure water (18.2 MΩ·cm), obtained from a Milli-Q® system (Millipore Corporation, USA), was used for the preparation of all solutions. For the determination of total phosphorus, a phosphate standard solution was prepared from a stock solution where 1.0 mL was equivalent to 0.2 mg of P_2O_5. Nitric acid (HNO_3, 65% w/v, Sigma-Aldrich, p.a.) and hydrochloric acid (HCl, 37% w/v, Fmaia, Brazil) were used in the ultrasound-assisted extraction and microwave-assisted digestion processes of the samples. To prevent contamination, all glassware was previously immersed in a 5.0% (v/v) nitric acid solution (Sigma-Aldrich, p.a.) for 24 hours and then rinsed with ultrapure water.

Determination of total phosphorus

Microwave-assisted digestion (MAD)

The total phosphorus digestion of the cane syrup matrix was performed using microwave-assisted digestion in a closed system (Anton Paar Multiwave GO Plus, Brazil). For the procedure, 1.0 g of cane syrup (wet basis) was weighed into a high-pressure-rated HVT50 vessel (PTFE-TFM, rated up to 100 bar), with the estimated maximum pressure during digestion ranging from 50 to 80 bar. Then, 5.0 mL of concentrated nitric acid (65%, v/v) and 1.0 mL of ultrapure water were added. The digestion process was conducted in two stages: (1) gradual heating (ramp) for 10 minutes up to 100 °C, followed by a 2-minute hold; (2) gradual heating for 20 minutes up to 200 °C, with an 8-minute hold, and a pressure release rate of 10 $bar \cdot min^{-1}$. The total duration of the procedure was 50 minutes. After cooling, the digested solution was transferred to a volumetric flask and diluted with ultrapure water to a final volume of 25.0 mL. The entire procedure was performed in duplicate. Additionally, for each digestion cycle, a blank was prepared in duplicate, containing only 5.0 mL of concentrated nitric acid.

Ultrasound-assisted extraction (UAE)

Cane syrup samples (0.5 g, wet basis) were directly placed into 15.0 mL Falcon tubes. The extraction solution, containing 1.70 mL of concentrated HNO_3, 2.0 mL of concentrated HCl, and 1.30 mL of ultrapure water, was added to the tube, totaling a volume of 5.0 mL. After manual homogenization, the tubes containing the samples were subjected to sonication in an Elmasonic P-30H ultrasonic bath (Analitica, Brazil) for 10 minutes. The process was carried out using an ultrasonic bath operating at a frequency of 37 kHz, with a power of 100 W and a controlled temperature of 25.0 ± 2.0 °C. After sonication, the samples were transferred to 10.0 mL volumetric flasks and filled up with ultrapure water. An analytical blank was prepared using the same procedure. All assays were performed in duplicate.

Total phosphorus analysis

The total phosphorus content in cane syrup was determined by its complexation in an acidic medium with molybdate and vanadate ions. This reaction results in the formation of a heteropolyacid, a yellow complex, as described by References No. 46 and No. 47, which absorbs visible radiation at 420 nm. For solution preparation, a 1.0 mL aliquot of the solution resulting from the processes of digestion (UAE and MAD) previously described was taken. This aliquot was transferred to a 10 mL volumetric flask, and 2.5 mL of the vanadomolybdate complexing agent was added. The volume was then completed with ultrapure water. After a 10-minute reaction time, the absorbance was measured using a UV-Vis spectrophotometer (Spectrum model SP-2000 UV) at 420 nm. The analyses were conducted in duplicate. The intensity of the radiation absorbed by the complex was proportional to the amount of phosphorus pentoxide (P_2O_5) present in the sample. Therefore, the total phosphorus concentration was calculated in mg P_2O_5 kg^{-1} and also expressed as the extraction rate, considering microwave-assisted digestion as the reference value, according to Equation 1.

$$Extraction\ (\%) = \frac{C_{UAE}}{C_{MAD}} \times 100 \qquad (1)$$

where:

- C_{UAE} is the total phosphorus concentration determined after ultrasound-assisted extraction, expressed in mg kg^{-1}.
- C_{MAD} is the total phosphorus concentration after microwave-assisted digestion, also expressed in mg kg^{-1}.

Multivariate optimization using Simplex-Centroid Mixture Design

The instrumental conditions for ultrasound-assisted extraction, such as frequency (37 and 80 kHz), power (100 and 300 W), sonication time (10 and 40 min), temperature (25 °C), tube position in the ultrasonic bath (erosion method with aluminum foil), mass-to-volume ratio (1:5 to 1:50 g mL^{-1}), and HNO_3:H_2O ratio (50:50% v/v), were optimized using a univariate strategy based on the independence of the instrumental factors, as indicated by preliminary tests. The detailed experimental conditions, including the optimized parameters, are described in Alves et al.[30]

To evaluate the total phosphorus extraction efficiency using the solvents HNO_3, HCl, and ultrapure water, a simplex-centroid mixture design was applied (Table I), following the methodology described by Barros Neto, Scarminio, and Bruns.[48] To adjust the mathematical models and assess experimental error, four replicates were performed at the central point, and additional replicates at pure components and binary mixtures to evaluate possible synergistic or antagonistic effects.[49] Additionally, to assess the influence of potential interferents in the total phosphorus analysis by spectrophotometry, arising from the composition of the cane syrup sample or the ultrasound-assisted extraction process, spectra were obtained from the scans of the experiments conducted in the mixture design (Table I) and from the solution resulting from microwave-assisted digestion. These scans were performed in the range of 400 to 500 nm, using a Shimadzu UV-Vis spectrophotometer, model UV-1280.

Subsequently, to determine the optimal extractor solvent conditions, the extraction rate values (Equation 1) for total phosphorus and the analysis of the scan spectrum profiles from the mixture design experiments (Table I) and microwave-assisted digestion were used. An analysis of variance (ANOVA) was applied to generate the adjusted statistical models. The lack of fit and the model significance, at a 95% confidence level, were also verified. Parameters such as the F_{reg} value, F_{lof} value, *p*-values, determination coefficients (R^2 and adjusted R^2), the square root of the residual mean square of the model, residual plots, and contour plots for the mixtures were also evaluated.[50] All analyses were performed using statistical software Minitab for Windows version 16.2.2.[51]

Method validation

The validation study was conducted by regulatory guidelines,[52-54] evaluating the figures of merit: selectivity, linearity, limits of detection (LOD) and quantification (LOQ), precision, and accuracy. Selectivity was investigated by comparing the confidence intervals of the slope, at the 95% confidence level, of the external standard (ES) and standard addition (SA) analytical curves in the concentration range of 0.2 to 1.8 mg L^{-1}, in order to identify possible matrix effects according to the recommendations of the References No. 55 and No. 56. Linearity was evaluated for the analytical curves obtained using three standardization methods: ES (external standard in aqueous solution), ES-ME (external standard in the extraction medium, and SA (standard added to the cane syrup matrix diluted at a 1:10 w/v ratio). All curves were subjected to sonication in an ultrasound bath in optimized UAE conditions.

The adequacy of the analytical curves was verified by linear regression and a lack-of-fit test, both at a 95% confidence level.[43] The limits of detection (LOD) and quantification (LOQ) were determined from the external standard analytical curve in the extraction medium (ES-ME), using the standard deviation (s) of ten analytical blanks. The LOD was calculated as 3 s/b and the LOQ as 10 s/b, where b is the slope of the analytical curve.[52,57] Precision was assessed by repeatability estimates (n = 5) and intermediate precision, which was evaluated over five consecutive days, with triplicate measurements performed each day (n = 3). The adequacy of the intermediate precision was evaluated by calculating the Horrat value.[54] Accuracy was validated by comparing the total phosphorus content determined by the proposed method (ultrasonic-assisted extraction) with that obtained using the reference method (microwave-assisted digestion) for five cane syrup samples (CS06, CS08, CS15, CS18, and CS19), analyzed in triplicate, using a paired *t*-test at a 95% confidence level.

RESULTS AND DISCUSSION

Optimization of ultrasound-assisted extraction of total phosphorus in cane syrup

The total phosphorus content was determined using a molecular absorption spectrophotometric method in the visible region by forming a yellow-colored complex between phosphorus and vanadate and molybdate ions, which absorb at 420 nm. Patnaik gives the complexation reaction by Equation 2.[58]

$$12MoO_3 + H_2PO_4 + HNO_3 \rightarrow [PMo_{12}O_{40}]^{3-} + H_2O \rightarrow [PMo_{12}O_{40}]^{3-} + VO_3^{-} \rightarrow [PMo_{11}VO_{40}]^{3-} \qquad (2)$$

This reaction results in the formation of a heteropolyacid of the Keggin type, responsible for the characteristic yellow color used in the quantification.

To determine the composition of the extraction solvent that would provide the best total phosphorus extraction rates in cane syrup, a simplex-centroid mixture design was applied (Table I). Acidic solvents were chosen as components of these mixtures because their efficiency in the extraction phase is well-documented in the literature, especially when combined, due to their oxidizing or complexing properties.[20,23,59] Additionally, the formation of more reactive products can occur, such as in the case of the mixture of hydrochloric acid and nitric acid (aqua regia), which accelerates the extraction process of elements from the matrix of interest.

Table I. The simplex-centroid mixture design applied to optimize the extraction solvent for total phosphorus analysis in cane syrup

	Extraction solvent composition (mL)			Extraction rate (%)
Experiments	HNO_3	HCl	H_2O	Total Phosphorus
1	5.00	0.00	0.00	77.38
2	0.00	5.00	0.00	131.17
3	0.00	0.00	5.00	121.02
4	2.50	2.50	0.00	78.94

(continues on next page)

Table I. The simplex-centroid mixture design applied to optimize the extraction solvent for total phosphorus analysis in cane syrup (continuation)

	Extraction solvent composition (mL)			**Extraction rate (%)**
Experiments	**HNO_3**	**HCl**	**H_2O**	**Total Phosphorus**
5	2.50	0.00	2.50	112.47
6	0.00	2.50	2.50	125.45
7	1.67	1.67	1.67	103.40
8	1.67	1.67	1.67	103.30
9	1.67	1.67	1.67	100.47
10	1.67	1.67	1.67	101.73

Color changes were observed in the mixture design experiments (Table I) before and after the ultrasonic-assisted extraction (UAE) (Figure 1). These visual alterations indicate potential matrix interferences in spectrophotometric analysis, as components present in cane syrup or formed during extraction may absorb at the same wavelength (420 nm) as the phosphorus–vanadomolybdate complex. This overlap can lead to overestimated total phosphorus levels, with extraction rates exceeding 100% in some experiments (Table I).

The standard spectrophotometric method employed in this study involves the complete oxidation of organic matter and dissolution of the remaining residue in hydrochloric acid, as in conventional sample preparation techniques (e.g., dry ashing, wet or microwave-assisted digestion).[19] These procedures remove potential interferents. In contrast, the UAE does not eliminate all organic content, depending on the solvent and sample composition, allowing matrix interferences to persist in the final solution.

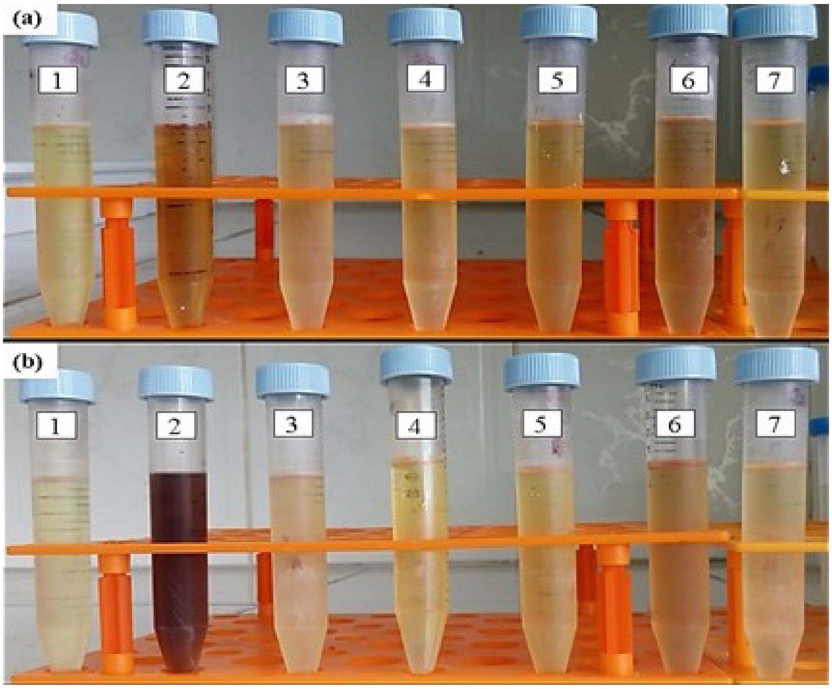


Figure 1. Color of solutions of experiments of simplex-centroid mixture design described in Table I: (a) before the sonication process and (b) after the sonication process.

Before sonication (Figure 1a), cane syrup solutions exhibited a natural yellow hue, attributed to intrinsic pigments such as chlorophylls, carotenoids, and anthocyanins.[58,59] However, processing and storage can lead to the formation of additional pigments, including Maillard reaction products (melanoidins) and oxidized phenolics, which also contribute to browning and may absorb near 420 nm.[27,28,60] After sonication, a reduction in color intensity was observed in experiments 1, 3, 4, 5, and 7, while experiments 2 and 6 showed evident darkening (Figure 1b). These two experiments used concentrated HCl or its binary mixture with water (50:50, v/v), suggesting that solvent composition strongly influences pigment formation and contributes to matrix interference.

To investigate this, visible spectra (400–500 nm) were recorded for the extracts after complexation (Figure S.1, Supplementary Material). Figure 2 presents the spectra for the most affected conditions (Experiments 2, 3, and 6). Extracts obtained using pure HCl showed the most pronounced absorbance at 420 nm and visual darkening, indicating significant matrix interference. This effect arises from the interaction between the solvent composition and ultrasonic cavitation, which enhances solvent–matrix interactions and promotes the release of compounds from the matrix.[61] In HCl-rich systems, cavitation may trigger Maillard-type reactions, leading to the formation of melanoidins that interfere with detection. In contrast, ternary solvent systems (Experiments 7–10) suppressed pigment formation despite being subjected to the same cavitation conditions, highlighting the critical role of solvent composition.

These ternary mixtures (HNO_3:HCl:H_2O) also achieved extraction rates closest to 100% (Table I), with minimal spectral interference (Figure S.1). The mildly acidic environment (pH ≈ 4) promotes solubilization and hydrolysis of organic phosphorus,[62] while cavitation enhances dispersion and fragmentation of macromolecular structures.[25,63,64]

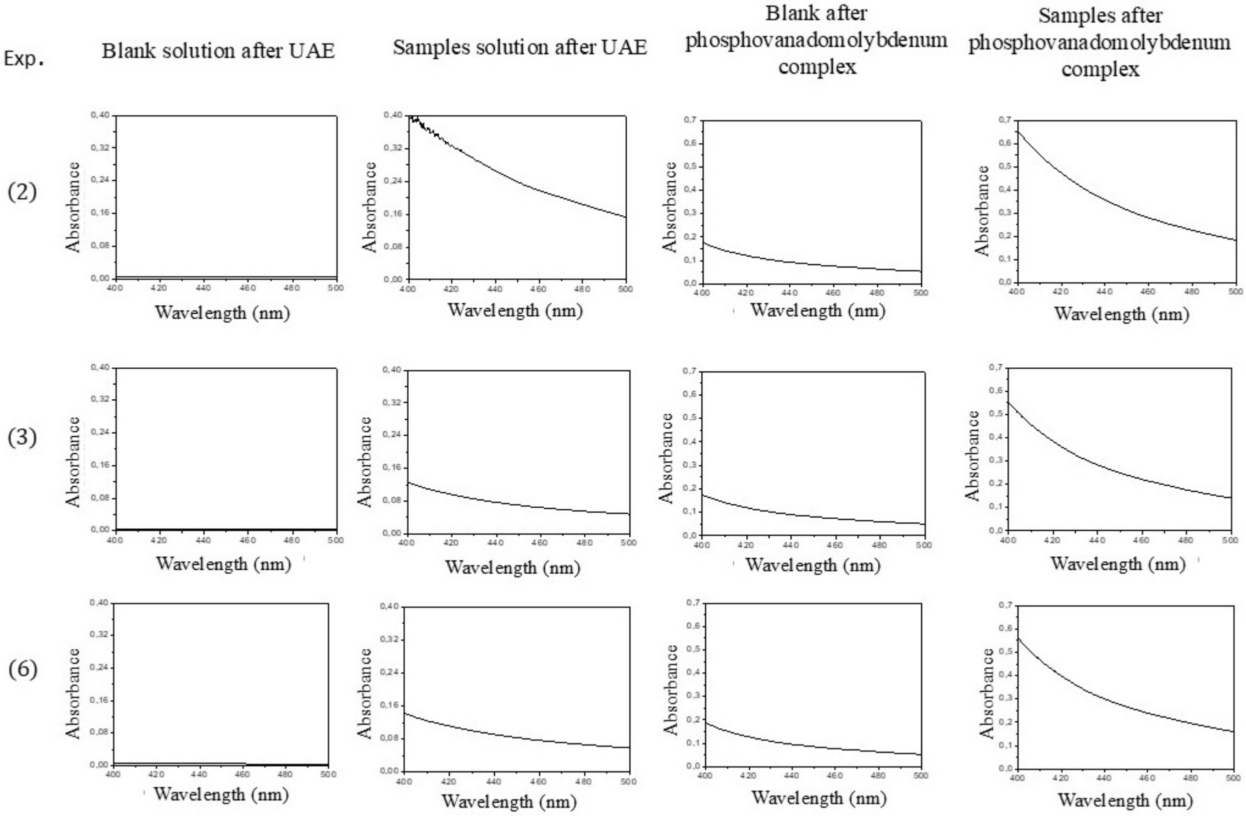


Figure 2. Overlap of UV–Vis Spectra of Experiments 2, 3, and 6 of Table I, considering blank solutions and sample solutions after UAE process and after phosphovanadomolybdenum complex formation.

Nonetheless, phosphorus species in cane syrup (such as phosphates, phytic acid, and phytates) may form insoluble metal complexes depending on pH,[65] which complicates extraction. Phytic acid can also chelate metals, suppress oxidative processes, and hinder phosphorus recovery.[66] These interactions contribute to matrix interference, whether through colored complexes or phosphorus-bound chromophores. Therefore, extraction in a moderately acidic medium is recommended, with conditions optimized to balance recovery efficiency and minimize spectral interference, considering the analytical method and matrix complexity.

To evaluate which mathematical model best fits the experimental data (Table I), an Analysis of Variance (ANOVA) characteristic of the simplex-centroid mixture design was performed. The results of the evaluated models are presented in Table II.

Table II. Analysis of Variance (ANOVA) for the optimization of the total phosphorus ultrasound-assisted extraction from cane syrup at a 95% confidence level

Models	**SS(1)**	**df(2)**	**MS$_{adj}$(3)**	$F_{regression}$	***p*-values**	**MS square root**	**R^2 (%)**	**R$^2_{adj}$ (%)**
Linear	2174.0	2	1087.0	1061[a]	0.008	10.12	75.19	68.10
Quadratic	2881.0	5	576.20	222.8[b]	0.000	1.60	99.64	99.20

Quadratic Model	**SS(1)**	**df(2)**	**MS$_{adj}$(3)**	**F$_{lof}$**	***p*-value**
Residual Error	10.34	4	2.585	–	–
Lack of Fit	4.49	1	4.487	2.30[c]	0.227
Pure error	5.85	3	1.951	–	–
Total	2891.35	9	–	–	–

(1)SS = Sum of square; (2)df = degrees of freedom; (3)MS$_{adj}$ = mean square adjusted;
[a]$F_{critical\ (0.05;2;7)}$ = 4.74; [b]$F_{critical\ (0.05;5;4)}$ = 6.26 and [c]$F_{lof\ (0.05;1;3)}$ = 10.1

The evaluated models showed statistical significance ($F_{regression} > F_{critical}$; $p < 0.05$) (Table II). However, the linear model presented a low coefficient of determination ($R^2 = 75.19\%$), indicating a significant influence of the residuals (Figure 3). On the other hand, the quadratic model exhibited high coefficients of determination (> 99%), demonstrating a better fit to the experimental data (Figure 3). Another parameter assessed was the square root of the mean squared residual, which showed a lower value for the quadratic model (1.60) compared to the linear model, again confirming a better fit of the quadratic model to the data of Table I. Additionally, it was verified that there was no lack of fit of this model, at 95% confidence level ($F_{lof} < F_{critical}$; $p > 0.05$). Thus, the quadratic model was considered the most suitable for describing the experimental data and determining the optimal conditions for ultrasound-assisted extraction.

The equation describing the fitted quadratic model was also determined, aiming to identify the optimal extraction conditions for the three components of the mixture. The adjusted model is represented by the polynomial described in Equation 3, where y represents the percentage of total phosphorus extraction, while the coefficients b_1, b_{12}, and b_{123} represent the parameters of the regression function.

$$y = b_1x_1 + b_2\,x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}\,x_1x_3 + b_{23}x_2x_3 \qquad (3)$$

The calculation of the regression equation showed that there are statistically significant interactions between the binary component systems. The generated quadratic model is represented in Equation 4.

$$\mathrm{y} = \mathbf{15.52}\ (\pm\mathbf{0.319})x_1 + \mathbf{26.28}\ (\pm\mathbf{0.319})x_2 + \mathbf{24.25}\ (\pm\mathbf{0.319})x_3 - \mathbf{4.253}\ (\pm\mathbf{0.276})x_1x_2 + \mathbf{1.923}\ (\pm\mathbf{0.276})x_1x_3 - 0.304\ (\pm 0.276)x_2x_3 \qquad (4)$$

The values for x_1, x_2, and x_3 represent the individual components of the mixture (HNO_3, HCl, and H_2O), while the values in parentheses indicate the confidence intervals of the coefficients. The significance of the components was evaluated using the *t*-test, with the critical points highlighted in bold. These critical points allow for the calculation of the predicted values for the tested models. The predicted values were then used in the linear regression technique to assess the suitability of the established model, based on the analysis of the plots of the experimental versus the adjusted values from the linear and quadratic models (Figure 3).

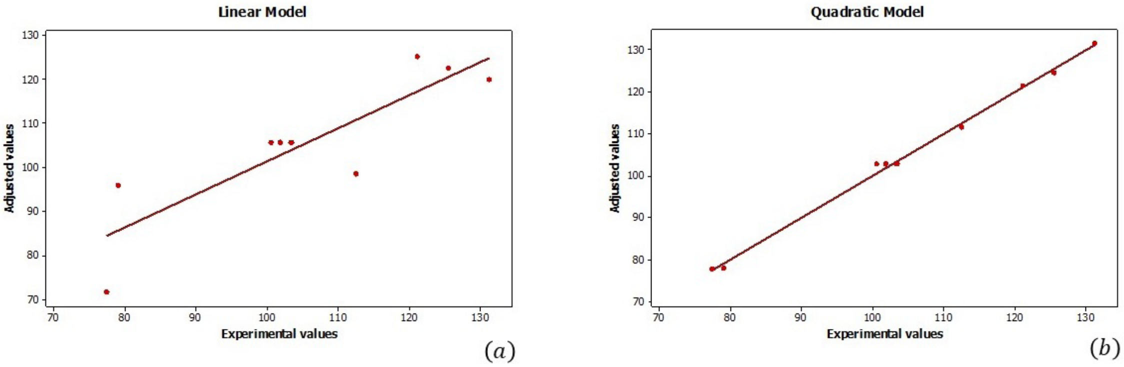


Figure 3. Plots for the correlation between the experimental and adjusted values (a) for the linear model and (b) quadratic model for optimization of the total phosphorus extraction from cane syrup using UAE.

After evaluating the adjusted mathematical model, a contour plot (Figure 4a) was generated to identify the solvent mixture composition with the highest efficiency in total phosphorus extraction rates. Additionally, the application of a *t*-test and the analysis of the Pareto chart (Figure 4b) allowed for the assessment of the individual effects of pure extraction solvents and solvent mixtures on the ultrasound-assisted extraction process.

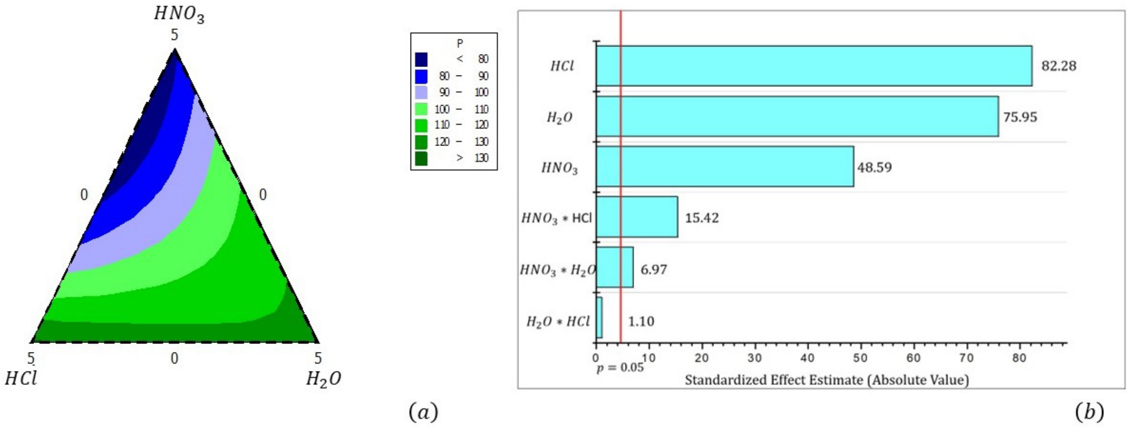


Figure 4. Contour plots (on the left) and Pareto charts (on the right) of the quadratic model adjusted from the simplex-centroid mixture design.

Figure 4a shows a significant interaction among the three extraction components, with total phosphorus extraction rates nearing 100% in the central region of the contour plot, corresponding to a ternary mixture. This indicates that phosphorus is strongly associated with the matrix, as it is a key component of macromolecules like phospholipids, nucleotides, and sugar phosphates.[65] In cane syrup, phosphorus exists mainly as phosphates, phytic acid, and phosphoproteins, which often form insoluble complexes.[66] Therefore, efficient extraction requires reactive solvent systems capable of disrupting these bonds.

The Pareto chart (Figure 4b) confirms that concentrated HCl had a significant positive effect on phosphorus extraction, likely due to its ability to form soluble complexes with phosphorus compounds. However, as previously discussed (Figure 1), pure HCl can also promote the formation of colored byproducts, which may compromise spectrophotometric analysis. Significant interactions were also observed between HNO_3 and H_2O, as well as between HNO_3 and HCl ($p < 0.05$), indicating a synergistic effect when these solvents are combined.

Mixtures of nitric and hydrochloric acid are known to enhance extraction efficiency, benefiting from the oxidative strength of HNO_3 and the complexing capacity of HCl.[23] Additionally, water contributes by lowering the viscosity of the mixture, promoting better cavitation during sonication and enhancing matrix disruption.

An individual desirability profile (Figure S.2, Supplementary Material) was used to identify the optimal solvent proportions. The condition that maximized total phosphorus extraction (desirability = 1.000) consisted of 1.67 mL of HNO_3, 2.00 mL of HCl, and 1.30 mL of ultrapure water.

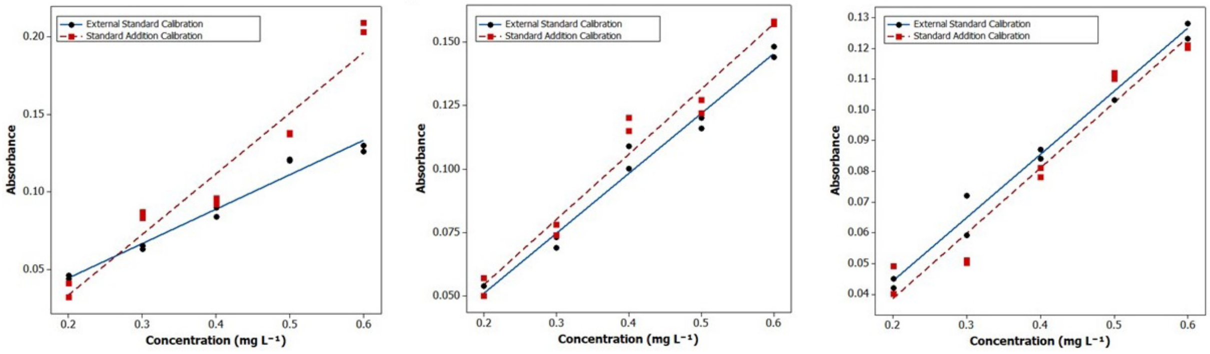
Method validation of UAE combined with UV-Vis spectrophotometry

Selectivity and linearity

The analysis of total phosphorus in complex matrices, such as cane syrup, poses a significant challenge for conventional analytical methods, mainly due to the matrix effect, which can compromise the accuracy and precision of the results. This issue becomes even more relevant when using ultrasound-assisted extraction, as sonication can amplify matrix-derived interferences, making it essential to assess the selectivity and efficiency of the extraction under different experimental conditions.

In this study, the selectivity of the methodology, combining ultrasound-assisted extraction with spectrophotometric analysis, was evaluated by comparing the confidence intervals of the slope of the external standard (ES) and standard addition (SA) analytical curves. These curves were constructed under the extraction conditions of experiments 2, 3, and 6 from Table I, which, as previously described, exhibited combined effects of matrix and the extraction process, evidenced by changes in the solution color before and after the sonication.

In light of this, the conditions for ultrasound-assisted extraction for total phosphorus quantification in cane syrup were investigated, with particular emphasis on analyzing the matrix effect induced by specific extraction solvents and their combinations. The analytical curves corresponding to experiments 2, 3, and 6 from Table I are illustrated in Figure 5, and the linear regression data, along with the confidence intervals of the slopes, are presented in Table III.



(*a*) (*b*) (*c*)

Figure 5. Analytical Curves corresponding to Experiments (a) 2, (b) 3, and (c) 6 of the Simplex-Centroid Mixture Design (Table I): External Standard in Medium Extracting (ES-ME) (●) vs. Standard Addition (■).

Table III. Linear Regression results for total phosphorus analysis using analytical curves of the External Standard in Medium Extracting (ES-ME) and Standard Addition (SA) corresponding to Experiments 2, 3, and 6 of the simplex-centroid mixture design

Extracting solvents[(2)]	Analytical Curves (0.2 – 1.8 mg L⁻¹)	Regression[(1)]				
		Slope ± Confidence Interval	r	R^2	*F*	*p*-value
HCl	ES-ME	0.223 (± 0.030)	0.975	96.8%	277.30	0.000
	SA	0.392 (± 0.079)	0.944	93.4%	129.13	0.000
H_2O	ES-ME	0.245 (± 0.054)	0.964	92.2%	107.50	0.000
	SA	0.230 (± 0.068)	0.887	86.9%	60.55	0.000
$HCl:H_2O$ (50:50%, v/v)	ES-ME	0.205 (± 0.021)	0.999	98.1%	468.09	0.000
	SA	0.212 (± 0.039)	0.955	94.5%	154.93	0.000

(1) $F_{critical\ (0.05;1;8)}$ = 5.32; (2) extracting solvents HCl, H_2O, and mixture of $HCl:H_2O$ (50:50%, v/v) corresponds to the experiments 2, 3, and 6 from Table I.

The results in Figure 5 and the confidence intervals in Table III corroborate the scan spectra from experiments 2, 3, and 6 (Figure 2), confirming matrix interference in the spectrophotometric quantification of total phosphorus under certain extraction conditions. This interference was most pronounced when concentrated HCl or its aqueous mixtures at ≥ 50% v/v were used (Experiments 2 and 3, Table III). According to SANTE guidelines,[55] matrix effects reached 75.9% with pure HCl and 3.40% with the HCl: H_2O mixture, reinforcing the impact of solvent composition on analytical reliability.

These findings highlight the importance of evaluating method selectivity, especially when using concentrated acids that may promote the formation of interfering compounds. Few studies have addressed matrix effects in ultrasound-assisted extraction or microwave-assisted digestion for phosphorus in complex matrices,[12,15] emphasizing the relevance of this investigation.

To assess linearity and method robustness, analytical curves were constructed using five phosphorus standards (0.2–1.8 mg L⁻¹) under three standardization approaches: (i) external standard (ES) in water; (ii) standard addition (SA) in diluted matrix (1:10, v/v); and (iii) external standard in the optimized extraction medium (ES-ME: 1.67 mL HNO_3, 2.00 mL HCl, 1.30 mL H_2O). Despite differences in solvent composition, the slope confidence intervals of the three analytical curves overlapped (Table IV), indicating no significant matrix interference under any condition. All models showed good linearity ($F_{reg} > F_{critical}$, $p < 0.05$) with no lack of fit ($F_{lof} < F_{critical}$, $p > 0.05$), confirming the method's suitability for total phosphorus determination in cane syrup. The ES-ME approach was selected for its analytical throughput advantage.

Detection and quantification limits

The limits of detection (LOD = 0.296 µg g⁻¹) and quantification (LOQ = 0.898 µg g⁻¹) for total phosphorus using UAE combined with UV-Vis spectrophotometry were approximately six times lower than those obtained by microwave-assisted digestion (LOD = 1.772 µg g⁻¹; LOQ = 5.379 µg g⁻¹). These results indicate higher sensitivity of the UAE method for analysing low concentrations of total phosphorus in cane syrup.

Gamela et al.[67] reported LOD and LOQ values of 2 µg g⁻¹ and 5 µg g⁻¹, respectively, for UAE in pepper samples, with slightly higher values for microwave-assisted digestion. Similarly, Liu et al.,[68] in a study on honey adulteration, reported a LOQ of 0.5 µg g⁻¹ for phosphorus using microwave digestion. Fuentes-Soriano et al.[15] achieved even lower detection limits (LOD = 0.0372 µg g⁻¹; LOQ = 0.1241 µg g⁻¹) in nuts, using optimized microwave-assisted digestion and blue molybdenum spectrophotometry.

Despite variations among matrices and detection systems, the results of this study confirm that UAE offers superior sensitivity for total phosphorus determination in complex matrices such as cane syrup, reinforcing its potential as a promising and efficient alternative to conventional digestion methods.

Precision and trueness

The repeatability estimate showed that the RSD (%) values were below 11%, meeting AOAC criteria,[54] and the Horrat values for intermediate precision were under 1.3, indicating excellent precision of the UAE combined with UV-Vis spectrophotometry (Table IV).

Trueness was evaluated by comparing total phosphorus concentrations in five cane syrup samples obtained using ultrasound-assisted extraction and microwave-assisted digestion, applying a paired *t*-test. The results showed no significant differences between the two methods ($t_{calculated} < t_{critical}$; $p > 0.05$). Previous studies, such as Gamela et al.,[67] demonstrated that UAE yields results consistent with certified reference materials. Similarly, Fuentes-Soriano et al.[15] confirmed the accuracy of a modified spectrophotometric method for phosphorus determination in nuts, with results comparable to ICP OES. These findings support the high accuracy and reliability of the proposed method in this work.

Table IV. Results of the in-house validation study

Analytical curves (0.2 – 1.8 mg L^{-1})	**Linear Regression$^{(1)}$**				
	Slope ± Confidence Interval	**r**	**R^2**	***F***	***p*-value**
ES	0.2450 ± (0.0545)	0.964	92.2	107.5	0.000
SA	0.2300 ± (0.0683)	0.886	86.9	60.6	0.000
(ES-ME)	0.2350 ± (0.0263)	0.987	97.9	422.6	0.000

Limits		**Precision**			**Trueness**	
LOD	**LOQ**	**Rep (RSD %)**	**IP**	**Horrat Value**	**paired *t*-test**	
					$t_{calculated}^{(2)}$	***p*-value**
0.296[a] 1.772[b]	0.898[a] 5.379[b]	1.06	0.87	0.07	1.51	0.166

Trueness of Extraction Methods in Cane Syrups		
Samples	**Total Phosphorus (mg kg^{-1})**	
	UAE	**MAD**
CS06	58.52±0.008	58.64±0.857
CS08	39.77±0.226	42.81±0.340
CS15	45.17±1.108	47.27±0.422
CS18	75.11±2.964	73.43±0.480
CS19	34.19±0.351	35.65±0.022

$^{(1)}F_{critical\ (0.05;1;8)} = 5.32$; $^{(2)}t_{critical\ (0.025;8)} = 2.31$; [a]Ultrasound assisted extraction and [b]Microwave-assisted digestion; LOD, detection limit (µg g^{-1}); LOQ, quantification limit (µg g^{-1}); Rep = repeatability; IP = intermediate precision. UAE = ultrasound-assisted extraction; MAD = microwave-assisted digestion.

Analysis of total phosphorus in cane syrup

The analysis of total phosphorus levels in different cane syrup samples is essential for understanding variations associated with the manufacturer or production region, contributing to ensuring the uniformity and quality of this sweetener. Additionally, this analysis helps identify patterns related to raw materials and production processes, including factors such as soil composition, element mobility in plants, agricultural practices, climatic conditions, cane species, and adopted management, that influence the concentrations of elements absorbed or introduced during processing.[30]

To classify or discriminate samples, the application of statistical tools, such as one-way ANOVA, combined with chemical analysis, is an efficient approach to detect significant differences between manufacturers or production regions.[68-70] Therefore, to evaluate the differences in the mean values of total phosphorus concentration in different cane syrup samples, a one-way ANOVA and a Tukey multiple comparison test were performed at 95% significance level.

The results of the ANOVA, followed by the Tukey post-hoc test, indicated that the cane syrup samples can be grouped based on the variability of total phosphorus content, according to their means, with the variations between region and manufacturer being statistically significant (Figure 6).

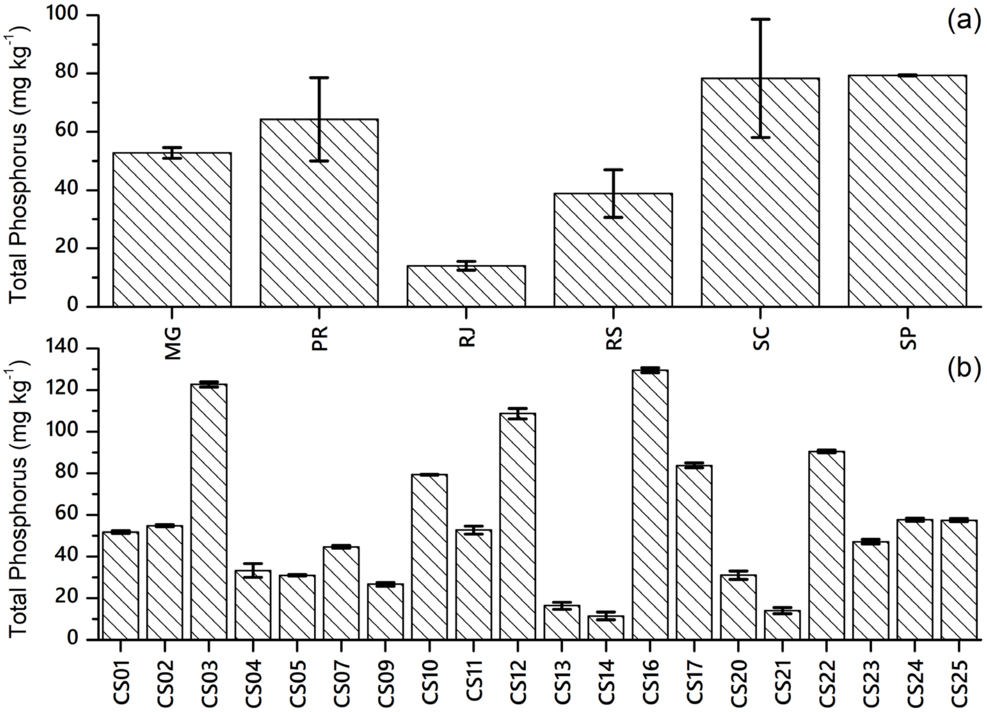


Figure 6. Total phosphorus content in cane syrup: comparison considering the variability between (a) producing regions and (b) among samples. MG = Minas Gerais; PR = Paraná; RJ = Rio de Janeiro; RS = Rio Grande do Sul; SC = Santa Catarina; SP = São Paulo.

Regarding the analysis of total phosphorus content (Figure 6), significant variations were observed between the cane syrup samples, suggesting the influence of several factors such as agricultural practices, soil composition, fertilization, and processing, which can vary both between regions and manufacturers.[30,71] Additionally, the biochemical competition between elements in the soil, such as calcium, magnesium, iron, manganese, and aluminum,[72,73] may affect phosphorus absorption, as exemplified by the differences observed in samples from the same manufacturer, (manufacturer A (CS01 and CS17) and manufacturer I (CS09 and CS13)) (Figure 6b), as well as regions with geographical proximity, such as the States of Santa Catarina (SC) and Rio Grande do Sul (RS) (Figure 6a).

Regarding geographic origin, the samples from Rio Grande do Sul, a Brazilian state recognized for its cane syrup production,[74] generally showed the lowest total phosphorus content. On the other hand, the samples from Santa Catarina (SC) exhibited considerable variability, with concentrations ranging from 30.98 mg kg^{-1} to 129.54 mg kg^{-1}. For instance, the CS16 and CS03 samples from Santa Catarina (SC) stood out with the highest levels (129.54 mg kg^{-1} and 122.65 mg kg^{-1}), while the CS13 and CS14 samples from Rio Grande do Sul (RS) showed the lowest phosphorus content, significantly differing from the other groups. This difference is particularly interesting because, given the geographical proximity, similar production practices would be expected. However, in southern Brazil, sugarcane is grown in a rainfed system, subject to climatic variations that influence the productivity and composition of its derivatives.[75]

Regarding the samples with intermediate concentrations, such as CS12 (108.60 mg kg^{-1}) and CS22 (90.49 mg kg^{-1}), these showed an overlap between groups, indicating a gradual transition between concentration ranges. Notably, the CS10 sample (79.34 mg kg^{-1}) from the State of São Paulo (SP) stood out for its low variability (SD ±0.175), suggesting standardization in its production. On the other hand, CS04 (33.26 mg kg^{-1}), also from Santa Catarina (SC), exhibited high variability (SD ± 2.951), indicating possible inconsistencies in processing.

This suggests that the region of cane syrup production directly influences its manufacturing process. In southern Brazil, the higher variability of total phosphorus content may be due to the involvement of small family-owned farms, which produce artisanal by-products using varied techniques. In contrast, regions with larger-scale production show greater standardization, likely associated with more industrialized processes.[75] Moreover, it is important to note that phosphorus is just one of several elements that can influence the product's composition. Therefore, while total phosphorus content allows for the discrimination of some samples, the inclusion of other chemical parameters or the use of multivariate analyses could generate clearer patterns, enhancing the reliability in identifying and discriminating cane syrup samples.

Evaluating the greenness of sample preparation procedures

The sample preparation stage is crucial in analytical procedures, as it helps minimize matrix interferences and ensures compatibility with instrumental techniques.[76] In total phosphorus analysis, methods such as microwave-assisted digestion and ultrasound-assisted extraction have shown high precision and sensitivity.[12,15,67,68] However, sample preparation can significantly increase the environmental impact of an analytical method due to high reagent consumption, high mass/solvent ratio, long analysis times, waste generation, and energy demand, among others. Therefore, it is essential to optimize this step to strike a balance between analytical efficiency and sustainability. In this study, the AGREEprep metric was applied, the first developed with a focus on sample preparation.[76,77] The pictograms for each of the sample preparation methods (UAE and MAD) are illustrated in Figure 7.

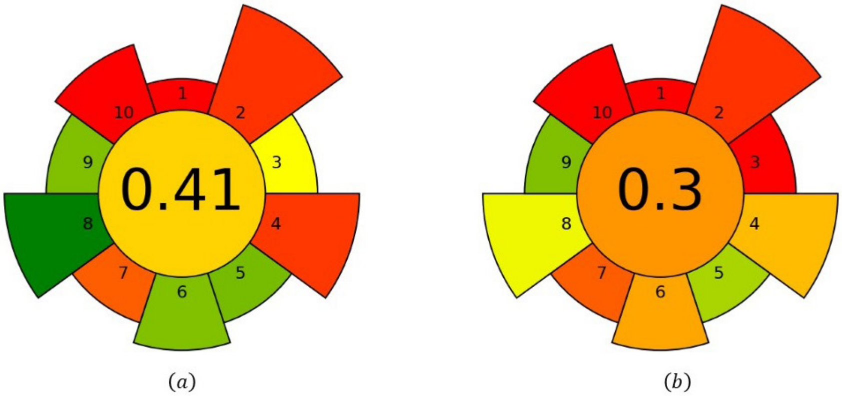


Figure 7. AGREEprep assessment results of sample preparation procedures: (a) UAE and (b) MAD.

The UAE procedure for total phosphorus extraction scores 0.41 on the AGREEprep metric (Figure 7a), making it more environmentally friendly compared to the MAD method, which received a score of 0.3 (Figure 7b). The strengths of the UAE include the reduced use of samples and reagents (criterion 5), the ability to perform simultaneous extractions in the ultrasonic bath, increasing analytical throughput (criterion 6), energy savings (criterion 8), and the use of efficient instrumental techniques after sample treatment (criterion 9). In contrast, the MAD method has lower environmental performance due to the use of concentrated inorganic acid (criterion 3), longer sample preparation times for series samples (criterion 6), and higher energy consumption. While MAD eliminates organics, consumes 62.50 Wh/sample, UAE only requires 4.17 Wh/sample (criterion 8).

CONCLUSIONS

The study highlights that the UAE is an effective method of sample preparation for total phosphorus analysis in cane syrup, particularly when conducted in a moderately acidic medium. The method's efficiency can be optimized while minimizing matrix interferences, as factors like sonication, syrup characteristics, and solvent composition may trigger secondary reactions, such as Maillard reactions, which affect the spectrophotometric analysis.

By combining specific reagents, UAE maximizes analyte extraction while reducing chemical interferences, underscoring the need to refine analytical methods for complex matrices. The study also demonstrates that the UAE offers high sensitivity, precision, and accuracy, with minimal environmental impact compared to MAD.

Additionally, the research indicates that the geographic origin and production practices of cane syrup samples contribute to significant variability in phosphorus levels, emphasizing the method's potential for quality control. The environmental assessment using the AGREEprep metric shows that the UAE is more sustainable than conventional methods, with a much lower energy consumption, reinforcing the importance of adopting greener, more efficient analytical practices.

Overall, the proposed methodology offers a faster, more sustainable approach for food analysis, balancing analytical performance with cost-effectiveness and the principles of green chemistry. It represents a viable alternative for quality control laboratories and the food industry.

Conflicts of interest

The authors confirm there are no financial or personal conflicts of interest related to this work.

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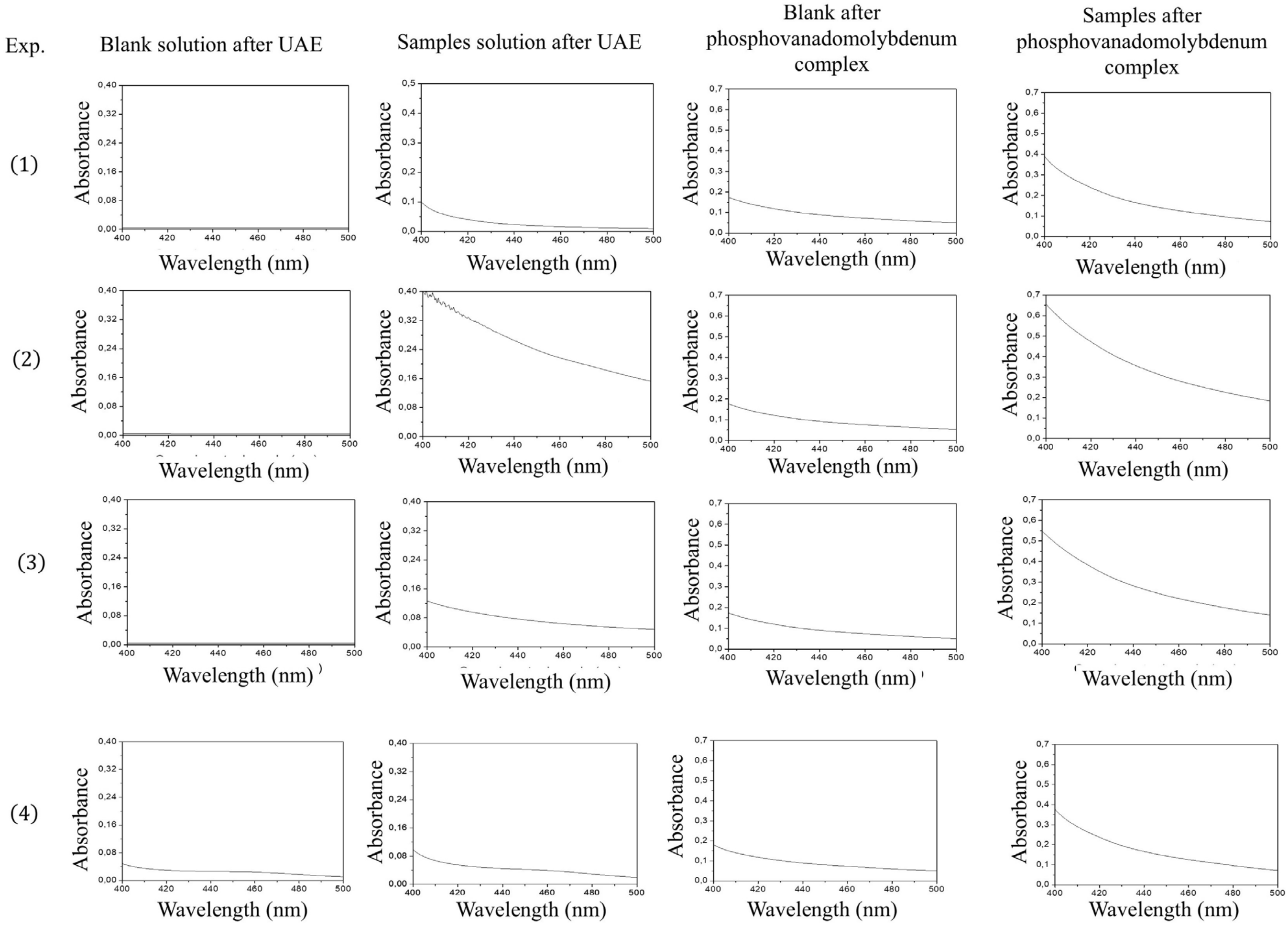
SUPPLEMENTARY MATERIAL



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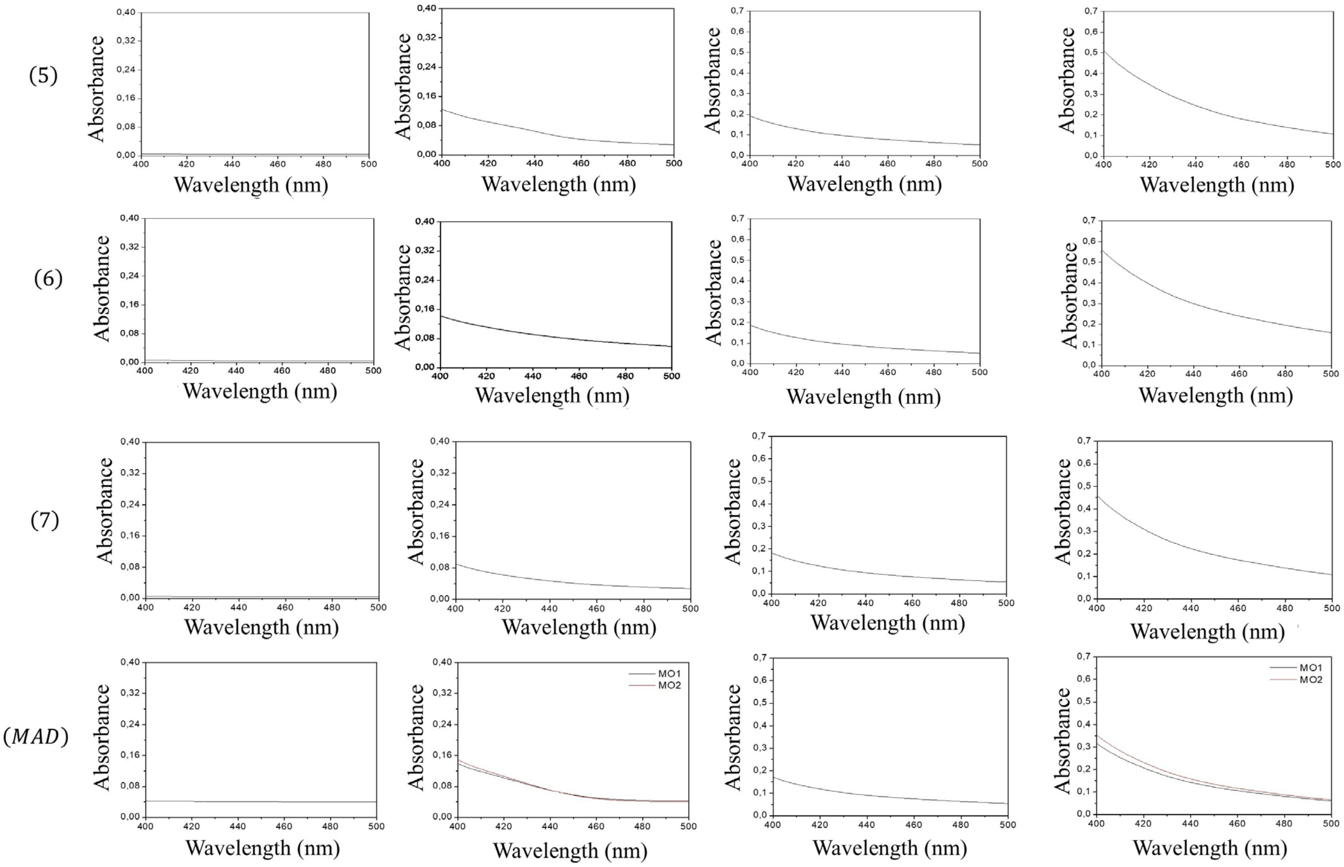

Figure S.1. Overlap of the UV–Vis spectra in the analysis of total phosphorus in the simplex centroid mixture design applied in the extraction solvent optimization.

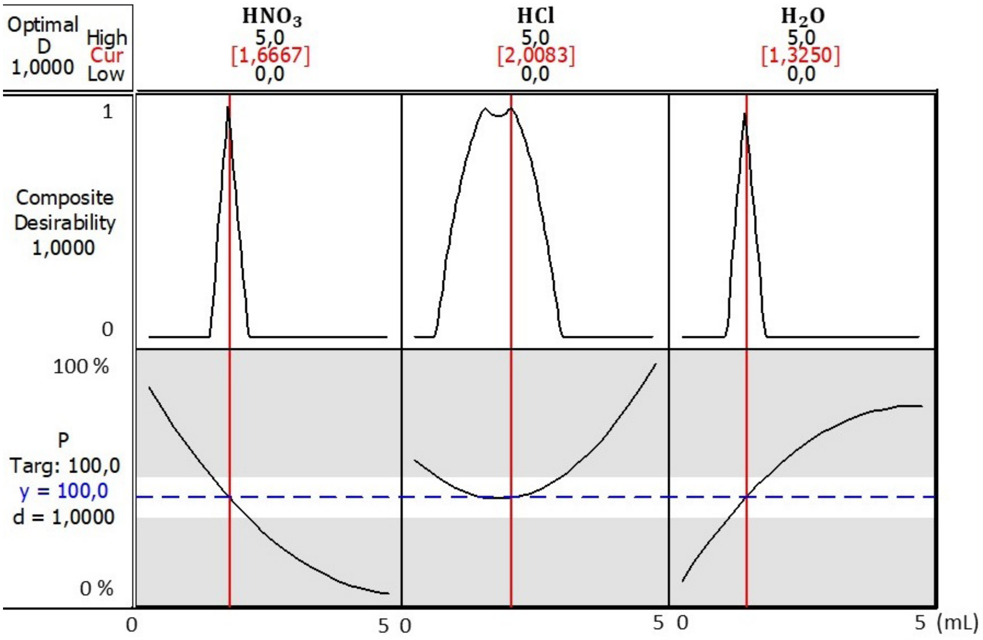

Figure S.2. Profiles for predictive values and individual desirability in optimizing the values of the extraction solvents.