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Short communication

Total anthocyanin content determination in intact açai (*Euterpe oleracea* Mart.) and palmitero-juçara (*Euterpe edulis* Mart.) fruit using near infrared spectroscopy (NIR) and multivariate calibrationMaria Raquel Cavalcanti Inácio^a, Kássio Michell Gomes de Lima^a, Valquiria Garcia Lopes^b, José Dalton Cruz Pessoa^b, Gustavo Henrique de Almeida Teixeira^{c,*}^aInstituto de Química, Universidade Federal do Rio Grande do Norte, Grupo de Pesquisa em Quimiometria Aplicada, CEP 59072-970 Natal, RN, Brazil^bEmpresa Brasileira de Pesquisa Agropecuária, Instrumentação Agropecuária, São Carlos, CEP 13560-970 São Paulo, Brazil^cUniversidade de São Paulo, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Av. do Café, s/n, Campus Universitário da USP, Ribeirão Preto, CEP 14040-903 São Paulo, Brazil

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ABSTRACT

The aim of this study was to evaluate near-infrared reflectance spectroscopy (NIR), and multivariate calibration potential as a rapid method to determinate anthocyanin content in intact fruit (açai and palmitero-juçara). Several multivariate calibration techniques, including partial least squares (PLS), interval partial least squares, genetic algorithm, successive projections algorithm, and net analyte signal were compared and validated by establishing figures of merit. Suitable results were obtained with the PLS model (four latent variables and 5-point smoothing) with a detection limit of 6.2 g kg⁻¹, limit of quantification of 20.7 g kg⁻¹, accuracy estimated as root mean square error of prediction of 4.8 g kg⁻¹, mean selectivity of 0.79 g kg⁻¹, sensitivity of 5.04 × 10⁻³ g kg⁻¹, precision of 27.8 g kg⁻¹, and signal-to-noise ratio of 1.04 × 10⁻³ g kg⁻¹. These results suggest NIR spectroscopy and multivariate calibration can be effectively used to determine anthocyanin content in intact açai and palmitero-juçara fruit.

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1. Introduction

The açai (*Euterpe oleracea* Mart.) and palmitero-juçara (*Euterpe edulis* Mart) palms are species native to Amazonia and the Atlantic Forest, respectively. The fruits are of economic importance in the Brazilian state of Pará. The fruits are considered a rich source of energy, and have been recognised for certain functional properties. In addition, the fruits are reported to be a substantial anthocyanin source, with high antioxidant activity (Lichtenthaler et al., 2005; Rosso & Mercadante, 2007; Rufino et al., 2010).

Several analytical methods have been reported to detect anthocyanins in fruits, including UV–Vis spectroscopy (Lee & Francis, 1972), nuclear magnetic resonance (NMR) (Missang, Guyot, & Renard, 2003), mass spectrometry (MS) (Williams et al., 2002), and capillary electrophoresis (CE) (Bednár et al., 2005). However, despite the reliability of these methods for anthocyanin detection, the approaches are destructive to the fruits, expensive, and generate chemical waste. In addition, some of the techniques, such as

NMR, require specialised reagents and personnel, which limit their application beyond the laboratory.

Due to the undesirable aspects of these analytical approaches, near infrared (NIR) spectroscopy has increased in acceptance in various analytical fields during the last decade (Geladi et al., 1999; Inácio, Moura, & Lima, 2011; Neves et al., 2012; Sakudo, Kato, Kuratsune, & Ikuta, 2009). NIR spectroscopy has the primary potential advantage of using intact samples presented directly to the instrument without sample preparation. This property constitutes an advantage over traditional destructive methods, and some NIR spectroscopy applications include anthocyanin detection from intact fruits (Dambergs, Cozzolino, Cynkar, Janik, & Gishen, 2007; Zude, Pflanz, Dosche, Spinelli, & Torricelli, 2011). Nevertheless, total anthocyanin content (TAC) has never been calibrated by NIR spectroscopy, or any other rapid technique with açai or palmitero-juçara fruits.

However, several complicating factors remain with the application of NIR spectroscopy. The primary difficulties in analysing anthocyanin in fruits by NIR include weak TAC signals from fruit compared to other components, particularly water, and the lack of resolution due to overlapping bands. Various chemometric algorithms applied to NIR spectroscopy data can serve to overcome

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these obstacles. Variable selection methods, such as iPLS (interval partial least squares) (Norgaard et al., 2000), GA (genetic algorithm) (Ferrand et al., 2011), and SPA (successive projections algorithm) (Araújo et al., 2001), result in improved multivariate models with a range of variables comprised of more relevant information. These algorithms identify and eliminate variables that do not directly correlate with the property of interest, including variables that add noise, nonlinearities, or irrelevant data. The algorithms also eliminate potential interferences and variables that generate a lower signal-to-noise ratio, which is indicative of low sensitivity.

The proposal and development of any new analytical procedure leads to an investigation and subsequent validation of the procedure's efficacy. The method is performed and observed under the same experimental conditions as will be used in future investigations. Validation occurs via determination of several parameters, known as the figures-of-merit (FOM) (Olivieri et al., 2006). The FOM number (selectivity, sensitivity, analytical sensitivity, precision, accuracy, limit of detection, limit of quantification, robustness, and linearity) to be determined, or the level to be reached in each validation, can vary depending on where the method is applied.

In this study, quantitative analyses of total anthocyanin content (TAC) in intact fruit (açai and palmitero-juçara) were carried out without sample preparation, using direct NIR spectroscopy absorption measurements. Several multivariate calibration techniques, including PLS, iPLS, SPA, GA, and outlier detection were conducted and compared to determine the best performing models. In addition, data pre-processing methods were evaluated to determine the method most suitable to analyse the data types. Finally, the best performing models were validated by the calculation of FOM obtained from the analyses, which included sensitivity, selectivity, and limit of detection.

2. Experimental

2.1. Plant material

Fruits were harvested at commercial maturity stage (fruits completely purple) from seven different genotypes each of *E. oleracea* (açai) and *E. edulis* (palmitero-juçara) species. Ten fruits were randomly selected from each of the 14 genotypes representing each species, totalling 139 fruit samples. Palmitero-juçara fruits were harvested at Viveiro Experimental de Plantas Ornamentais and Florestais, part of Faculdade de Ciências Agrárias e Veterinárias (FCAV), Universidade Estadual Paulista (UNESP), Jaboticabal Campus, Jaboticabal, São Paulo State (21° 15' S, 48° 15' W, 560 m a.s.l.). Fruit from five açai genotypes were harvested at Banco de Germoplasma of Instituto Agrônomo de Campinas (IAC), Ubatuba, São Paulo State (23° 27' S, 45° 04' W, 8 m a.s.l.), and fruit from the other two genotypes were harvested at FCAV-UNESP.

2.2. NIR spectroscopy

Spectral measurements were performed using an FT-IR Spectrum 100 N spectrophotometer (Perkin Elmer, Shelton, CT) equipped with a diffuse reflectance cell. NIR spectra were recorded over a range of 4000–10,000 cm⁻¹ (714–2500 nm) in triplicate with an 8 cm⁻¹ spectral resolution and co-addition of 64 scans. The average value from three different spectral measurement locations on each fruit was stored, and the mean spectrum was subsequently calculated for each sample. A polytetrafluoroethylene (PTFE) sample spectrum was used as background. Following NIR spectra acquisition from individual fruits, samples were rapidly frozen and stored at -18 °C.

2.3. Total anthocyanin content analysis

The pH differential method (AOAC method 2005-02) applicable to monomeric anthocyanin determination, expressed in fruit as cyanidin-3-glucoside, was used as the reference approach (AOAC, 2006). The method is suitable to determine total monomeric anthocyanin content based on structural changes in the anthocyanin chromophore between pH 1.0 and 4.5. Monomeric anthocyanins undergo a reversible structural transformation as a function of pH. Total anthocyanin extraction was conducted by separating the exocarp and mesocarp from the endocarp (stone) with a stainless steel knife, and the resulting material, approximately 0.2 g, was macerated using a porcelain mortar and pestle. Two macerated pulp portions were weighed to 0.05 g each. One portion was mixed with 0.025 M potassium chloride buffer (pH 1.0), and the other portion mixed with 0.4 M sodium acetate buffer pH 4.5. Following two hours of extraction at room temperature (~25 °C), samples were filtered through Whatman No. 1 filter paper, and absorbance recorded using a Shimadzu UV-1650 PC spectrophotometer (Shimadzu Corp., Kyoto, Japan) at wavelengths of 520 and 700 nm, for solutions at pH 1.0 and pH 4.5, respectively. TAC was expressed as cyanidin-3-glucoside (% w/w) equivalents, as follows:

Total anthocyanin content(%w/w)

$$= \frac{A}{\epsilon \times l} \times MW \times DF \times \frac{V}{W} \times 100\%$$

where, $A = (A_{520\text{nm}} - A_{700\text{nm}})_{\text{pH}1.0} - (A_{520\text{nm}} - A_{700\text{nm}})_{\text{pH}4.5}$; MW (molecular weight) = 449.2 g.mol⁻¹ for cyanidin-3-glucoside (cyd-3-glu); DF = dilution factor; W = sample weight (mg); l = path length in cm; $\epsilon = 26,900 \text{ M}$ extinction coefficient in L mol⁻¹ cm⁻¹ for cyd-3-glu; and 10³ = factor for conversion from g to mg. The total anthocyanin content (TAC) ranged from 1.5 to 82.0 g kg⁻¹.

2.4. Software and data analysis

Data loading, pre-processing (mean-centering, Savitzky-Golay smoothing with different windows [3,5,7], first polynomial order, derivatisation first and second derivatives), variable method selection (iPLS, GA, and SPA), chemometric regression model construction (Full-PLS, PLS-iPLS, PLS-GA, PLS-SPA, and MLR-SPA), and validation were performed in a MATLAB® version 7.10 environment (Math-Works, Natick, USA) with the PLS-toolbox version 4.0 (Eigenvector Research, Inc., Wenatchee, WA). Samples ($n = 139$) were divided into calibration ($n = 100$) and validation sets ($n = 39$) by applying the classic Kennard-Stone (KS) selection algorithm to the NIR spectra (Kennard & Stone, 1969). Calibration and validation sample numbers were, respectively selected at 100 (min. 1.50 g kg⁻¹; max. 82.0 g kg⁻¹; mean 28.6 g kg⁻¹; SD 20.8 g kg⁻¹) and 39 (min. 2.20 g kg⁻¹; max. 68.5 g kg⁻¹; mean 15.8 g kg⁻¹; SD 14.8 g kg⁻¹). Outlier detection was executed to improve model accuracy, remove samples with extreme values, which exhibit increased influence on the model, and eliminate unmodelled residues in the X and Y data responses. An elliptical joint confidence region (EJCR) was calculated to evaluate the slope and intercept for the reference regression, and predict values at a 95% confidence interval. Mathematics and applications for the EJCR can be addressed in the following references: González, Herrador, and Asuero (1999), and Goicoechea and Olivieri (2001). All calculations were performed using suitable MATLAB 7.10 approaches.

3. Results and discussion

3.1. Spectra overview

Açai and palmitero-juçara sample spectra (sample #1 and #80), with respective 3.3 and 36.9 g kg⁻¹ total anthocyanin content

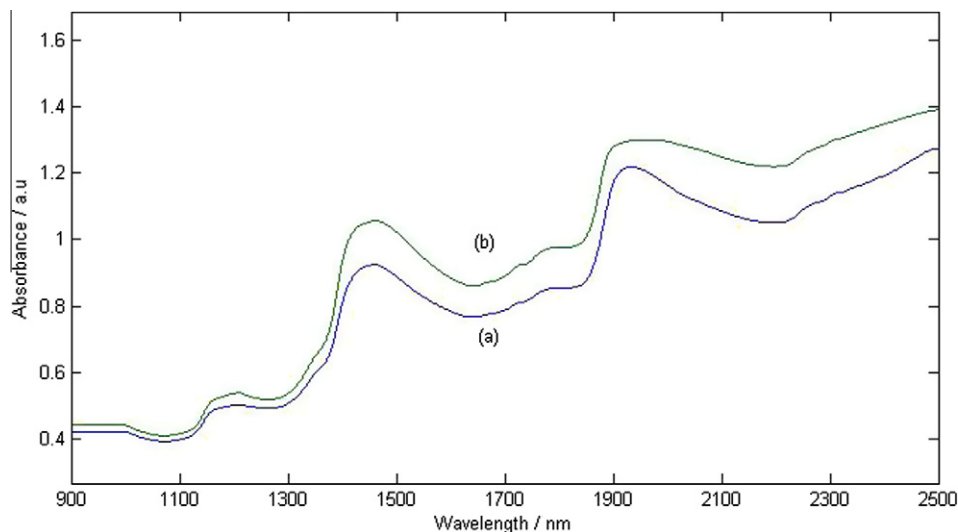


Fig. 1. Plot of raw NIR spectra for two samples from the full data set (a) sample #1 with 0.33 g kg^{-1} total anthocyanin concentration (TAC), (b) sample #80 with 3.69 g kg^{-1} TAC.

Table 1

Calibration and external validation set results for total anthocyanin content (TAC) (g kg^{-1}): root mean square error of cross validation (RMSECV) and prediction (RMSEP), correlation coefficient for calibration (r_c^2) and validation (r_p^2) sets, the number of spectral variables (Size), and the residual predictive deviation (RPD). The number of factors in the PLS, iPLS, PLS-SPA, and PLS-GA models are indicated in parentheses.

Models	Calibration		Prediction		Size	RPD
	r_c^2	RMSECV (g kg^{-1})	r_p^2	RMSEP (g kg^{-1})		
PLS(4)	0.75	14.6	0.75	9.3	2997	1.59
Smoothing 5 pts. ^a						
PLS(4)	0.97	14.2	0.85	8.1	2993	1.83
First deriv. ^b . 5 pts.						
PLS(4)	0.92	13.9	0.80	9.3	2995	1.59
First deriv. 7 pts.						
PLS(4)	0.99	21.3	0.32	19.4	2999	0.76
Sec. ^c . deriv. 3 pts.						
iPLS(5)	0.77	16.8	0.26	15.3	298	0.97
iPLS(4)	0.94	13.5	0.80	9.2	1497	1.61
PLS-SPA(4)	0.76	14.8	0.76	9.3	98	1.59
PLS-SPA(4)	0.83	13.6	0.75	9.9	99	1.49
PLS-GA(4)	0.77	13.8	0.75	9.1	899	1.63
PLS-GA(4)	0.98	9.3	0.84	8.5	774	1.74
PLS(4) ^d smoothing 5 pts.	0.97	13.8	0.90	4.8	2997	3.08

^a Pts.-points.

^b deriv.-derivative.

^c Sec.-second.

^d One application of outlier detection.

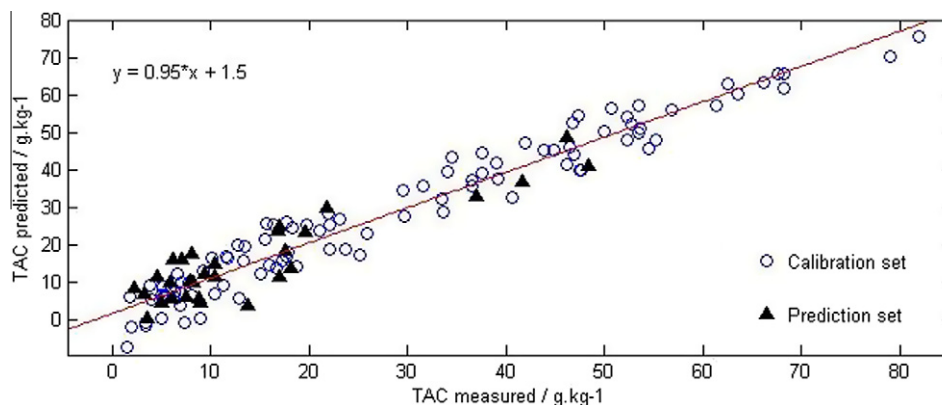


Fig. 2. Predicted versus measured anthocyanin content from calibration and validation reference samples for TAC using the PLS (4) smoothing (5 pts.) model. (○) calibration set; (▲) validation set.

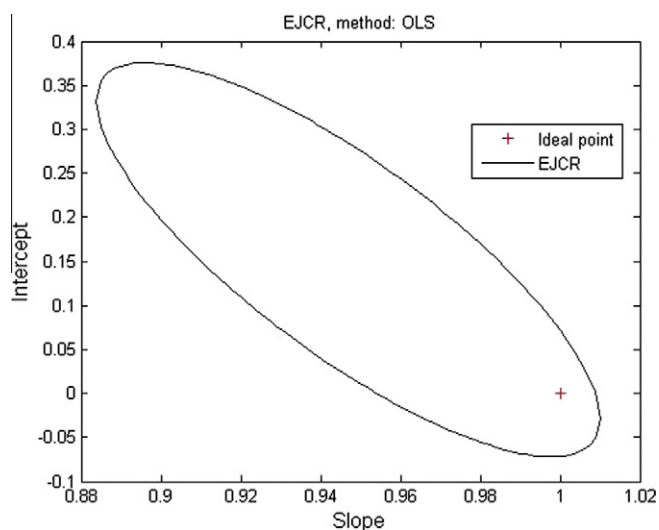


Fig. 3. Elliptical joint confidence region for the regression slope and intercept of predicted versus measured densities from the TAC external validation set using the PLS (4) smoothing (5 pts.) model.

Table 2
FOM (figures of merit) for the best performing models: TAC in g kg^{-1} .

FOM	Total anthocyanin content (g kg^{-1})
RMSEC	13.8
RMSEP	4.8
r_c^2	0.97
r_p^2	0.90
RPD ^c	3.08
Precision	27.8
SEN	5.04×10^{-3}
SEL	0.79
LOD	6.2
LOQ	20.7
S/N	1.04×10^{-3}

^a r_c^2 = calibration samples correlation coefficient.

^b r_p^2 = prediction samples correlation coefficient.

^c RPD = residual predictive deviation.

(TAC) are presented in Fig. 1. Fig. 1 depicts two spectra, (a) and (b), which characterise the following: a second overtone of the O–H stretch at 982 nm, the first overtone of the O–H stretch at 1456 nm, and combination bands of the asymmetric and scissor stretch O–H vibrations at 1940 nm. Sugar-related absorption bands were also observed at 926, 1208, 1728, 1762, 2308, and 2348 nm. The spectra were quite homogeneous, and no outliers were identified by *a priori* visual inspection.

The PLS method was initially performed on the entire original spectra wavelength range to develop the NIR model. In this way, TAC could be predicted non-destructively. Noise and systematic behaviour were undesirable features in the spectra, and therefore pre-processing was necessary. The original spectra were subjected to smoothing (first order), multiplicative scattering correction (MSC), and first, second order derivatisation (Savitzky–Golay).

3.2. Total anthocyanin content (TAC)

Calibration model results for the TAC NIR region in açai and palmitero-juçara fruits are shown in Table 1. In addition to the PLS models, PLS-SPA, PLS-GA, and iPLS model results are shown. The best performing model results from pre-processing tests (see methods) are provided. F-tests were performed for all models

using the prediction set. The results detected no significant differences (95% confidence level) between the best PLS model, the PLS (4) and other models, with the exception of PLS (4) smoothing (3 pts.), PLS (4) first derivative (7 pts.), PLS (4) second derivative (3 pts.), iPLS (5) using 298 spectral variables, and iPLS (4) using 1,497 spectral variables (data not shown). Therefore, variable selection was integral in this study, because it enabled the use of models that required a small number of spectral variables. The correlation coefficients (r^2) for the prediction set ranged from 0.75 to 0.90 for all models, with the exception of PLS (4) second derivative (3 pts.), and iPLS (5) (Fig. 2). Observations in the NIR spectral region for models with derivative data showed higher RMSEP values than models with raw or smoothed data, due to loss of some important spectral information when the derivative spectra were employed. Four or five latent variables were used for NIR spectra with PLS, iPLS, SPA, and GA models. The strategy for using GA models was the advantage of employing fewer variables (774) to build PLS models.

Two outliers were excluded from the calibration set, and the best PLS model for TAC was developed by applying a smoothing with five points. For this model, the lowest root mean square error of cross validation (RMSECV) and RMSEP were 13.8 and 4.8 g kg^{-1} , respectively. The correlation coefficient (r^2) for the validation set was 0.90, and was obtained using four latent variables. Fig. 2 depicts the correlation between measured and predicted values for TAC in açai and palmitero-juçara. The diagonal line represents ideal results; the closer the points plot to the diagonal, the better the fit to the model. Blue open circles represent calibration spectra, and solid triangles represent validation spectra.

An elliptic joint confidence region (EJCR) was constructed for the slope and intercept when plotting the predicted versus actual parameter values (at a 95% confidence interval) (Fig. 3). EJCR calculations are a convenient means to ascertain if bias exists in determination of both parameters when using the PLS (4) smoothing (5 pts.) model. The ellipse contained the expected theoretical value of 1.0 when built for TAC (Fig. 3).

The figures of merit results are provided in Table 2. Accuracy values represented by RMSEC (root mean square error of calibration) and RMSEP indicated the estimated multivariate model values exhibited acceptable agreement with the reference method. Precision, at level of repeatability, was assessed by analysing five samples/ten replicates per sample, with measurements recorded on the same day. Acceptable results were observed for sensitivity to the parameter evaluated, considering the analytical range of each model. A direct relationship with the prediction errors was not detected for the value of the signal-to-noise ratio, which was apparently low. This result suggested the estimated LD and LQ values might be optimistic (Table 2).

4. Conclusion

A rapid and non-destructive method to determine total anthocyanin content in intact açai and palmitero-juçara fruits using NIR spectroscopy and multivariate calibration was achieved in this study. The optimal combination of informative regions from the iPLS, GA, and SPA models directly improved predicted TAC values. The values for accuracy, precision, and other figures of merit exhibited promising results, indicating that the model developed by NIR spectroscopy for TAC can be used as an alternative to UV–Vis measurements.

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