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Research Article
Determination of Stevioside and Rebaudioside A from Simulated Stevia Beverages Using FTIR Spectroscopy in Combination with Multivariate Calibration

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Abstract

Background and Objective: Simulated stevia beverages contain major diterpene glycoside, stevioside and rebaudioside A from *Stevia rebaudiana*. **Methodology:** The objective of this study was to develop FTIR spectroscopy in combination with multivariate analysis of Partial Least Square (PLS) regression for determination of stevioside and rebaudioside A in simulated stevia beverages. The PLS calibration was optimized by selecting wave number region capable provided the highest coefficient determination, R^2 and lowest Root Mean Square Error Calibration (RMSEC). **Results:** Finally, the combined wave number range of 1161-2773 and 868-1041 cm^{-1} using Multiplicative Scattering Correction (MSC) followed by offset correction transformation spectra was chosen for stevioside determination with R^2 and RMSEC value of 0.9954 and 3.40%, respectively. Meanwhile, selected wave number region at 791-1839 cm^{-1} without spectra transformation revealed optimal PLS regression for rebaudioside A determination with R^2 and RMSEC of 0.9820 and 2.91%, respectively. **Conclusion:** The FTIR spectroscopy in combination with multivariate analysis of PLS regression could be used an alternative method for determining stevioside and rebaudioside A in simulated stevia beverages.

Key words: FTIR spectroscopy, partial least square regression, stevioside, rebaudioside A, stevia beverages

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Stevia rebaudiana is herbal plant from Paraguay. *Stevia rebaudiana* contains diterpene glycosides as active constituent. Major diterpene glycosides contained in *S. rebaudiana* are stevioside (4-13%) and rebaudioside A (2-4%)[1]. Stevioside and rebaudioside A have several bioactivities such as antidiabetes[2,3], antihypertensive[4], immunomodulator[5], anti-diarrhea[6], antioxidant[7] and anticancer[8]. The previous study has simulated *S. rebaudiana* as beverages[9]. Simulated Stevia Beverages (SSB) showed antioxidant[10] and antihyperglycemic activity[9]. Bioactivities of SSB come from its bioactive compounds of stevioside and rebaudioside A. Therefore, it is necessary to quantify the levels of bioactive compounds, particularly stevioside and rebaudioside A in SSB.

Several methods have developed to determine stevioside and rebaudioside A. Most of these method are HPLC method[1,11-17]. However, it is necessary to develop method analysis which is more practical in use, efficient in cost, simple in sample preparation and non-destructive for sample analysis. The FTIR spectroscopy in combination with multivariate calibration using PLS was potential to be developed for determining bioactive compounds from natural sources[18-20] due to its property as fingerprint technique.

It is challenging to develop FTIR spectroscopy method for determining bioactive compounds in beverages. The challenge is due to hydrogen bonding which absorb infrared radiation strongly. The hydrogen bonding peak will shield other peaks and make limited peak information. However, there are no exactly similar spectra due to interactions between infrared radiations with molecules in sample[19]. The purpose of this study was to develop FTIR spectroscopy method in combination with multivariate calibration PLS for determining stevioside and rebaudioside A in SSB.

MATERIALS AND METHODS

Materials: *Stevia rebaudiana* leaves were obtained from P.T. Java Sakti Niaga, Indonesia from three different areas of hill in Bandungan, Tajuk and Poloboga, Central Java, Indonesia. Reference standards of stevioside and rebaudioside A were obtained from WAKO, Japan with purity>99.0%. Acetonitrile (HPLC grade), methanol (HPLC grade), trifluorocetic acid (TFA, pro analysis grade) and ethanol (pro analysis grade) were purchased from E-Merck (Darmstat, Germany). Millipore filter (0.45 μm) with diameter 2.5 cm was obtained from Whatman (United Kingdom).

Methods

Simulated stevia beverages production: The SSB production was done according to Martono and Dewi[9]. *Stevia rebaudiana* leaves with various ages and planting area from several hills in Central Java, Indonesia (total 5 variations) were dried using cabinet dryer at 50°C at night. Dried leaves were grinded into powdered. A number of 4.0 g dried leaves powdered was macerated using 40 mL boiling water for 60 min at 80°C in water bath. The solution was filtered, filtrate was collected and the residue was re-extracted with the same way. Extraction was repeated 3 times. The filtrate collected was adjusted until 120 mL with water. The filtrate pH was adjusted until pH 3.0 with citric acid 50% (w/v). Subsequently, the solution was filtered and the pH filtrate was adjusted into pH 10.0 with saturated calcium carbonate solution. After pH adjustment, the solution was filtered and the filtrate was adjusted in pH 7.0 with citric acid 50% (w/v). The final solution was diluted with dilution factor of 4, 10 and 15 times to obtain 15 different sample solutions. These solutions were kept in refrigerator until being used for next analysis.

Measurement of FTIR spectra: Sample solution was placed on Horizontal Attenuated Total Reflectance (HATR) equipment at room temperature (25°C). The FTIR spectra of all samples were scanned using a FTIR spectrophotometer ABB MB3000 (Clairet Scientific, Northampton, UK), equipped with deuteratedtriglycinesulphate (DTGS) detector andbeam splitter of germanium. Spectra of FTIR were scanned in wavenumber region of 4000-650 cm^{-1} with resolution of 4 cm^{-1} and number of scanning of 32. All spectra were calibrated using background of air spectrum as reference. After every scan, a new reference air background spectrum was taken. These spectra were recorded asabsorbance values at each data point in triplicate.

Determination of stevioside and rebaudioside A by HPLC: The PLS calibration model was established by plotting the actual value of stevioside and rebaudioside A vs. the FTIR predicted value. The determination of actual value of stevioside and rebaudioside A was performed using HPLC as reference method. Determination of stevioside and rebaudioside A using HPLC was carried out according to Martono *et al.*[21]. Stationary phase of HPLC system used Eurosphere RP-18 (250×4.6 mm, 5 μm) column with guard column and kept at 30°C in thermostat. A mixture of water: methanol (90: 10 v/v, adjusted to pH of 3.0 by phosphoric acid), acetonitrile and trifluoroacetic acid was used as mobile phase with the ratio of 65:35:0.1 (v/v/v). Flow rate applied

was 0.6 mL min^{-1} and detection was performed using UV detector at 210 nm. Sample volume injection was 20 µL. Concentration of stevioside and rebaudioside A were determined by plotting area into external calibration standard curve.

Chemomtrics analysis: Multivariate analysis of PLS was established using Horizon MB FTIR software version 3.0.13.1 (ABB, Canada) included in FTIR spectrophotometer. The PLS will correlate actual value from stevioside and rebaudioside A content obtained from HPLC determination and predicted value established from FTIR spectra-partial least square. Validation was performed using the leave one out technique. The PLS regression was optimized using coefficient correlation (R^2) and Root Mean Square Error of Calibration (RMSEC) from the established model. Root Mean Square Error of Predicted (RMSEP) and coefficient determination (R^2) indicate the predictive ability of PLS calibration model to calculate the validation samples[19].

RESULTS AND DISCUSSION

Determination stevioside and rebaudioside A by RP- HPLC: To perform multivariate calibration of PLS, analyte(s) of interest with various concentration must be developed. In order to meet variation data of analyte level, SSB was made from *S. rebaudiana* leaves which were obtained from various ages, planting area and dilution factor. Rebaudioside A and stevioside content in SSB can be seen in Table 1. Chromatogram of sample solution was shown in Fig. 1. The content of stevioside and rebaudioside A based on HPLC determination will be used as actual value during PLS regression modeling.

Analysis of stevioside and rebaudioside A using FTIR spectra and PLS calibration: The FTIR spectra of SSB various solutions are presented in Fig. 2. Wide and intense absorption at 3303 cm^{-1} corresponds to the stretching vibration of the OH bond (¾OH stretching) and is associated with the

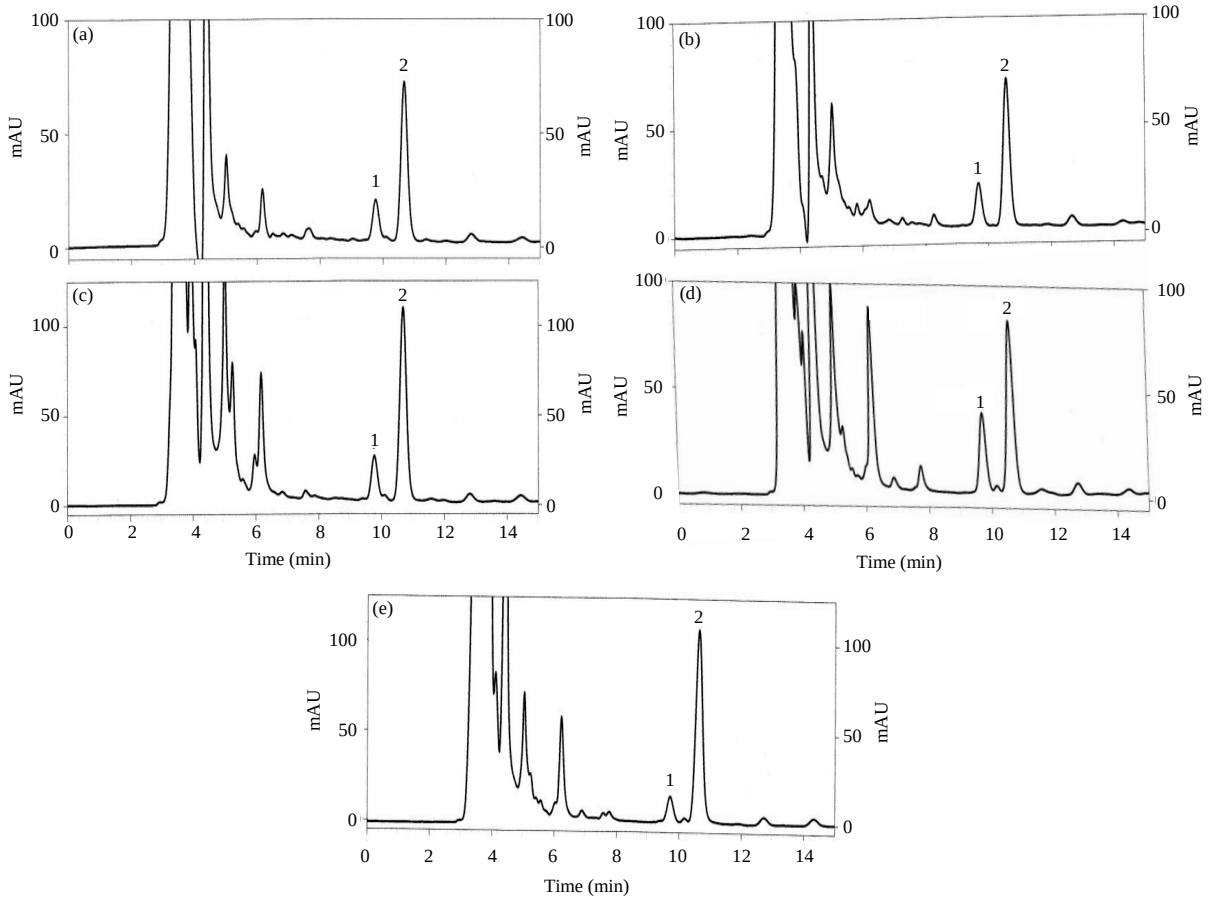

Fig. 1(a-e): HPLC chromatogram profile of simulated stevia beverages which were produced using *S. rebaudiana* leaves from (a) Bandungan, (b) Poloboga, (c) Tajuk (BPBP seed), (d) Tajuk (Gedongsongo seed) dan and (e) Tajuk (Tigra seed). Peak 1: Rebaudioside A and 2: Stevioside

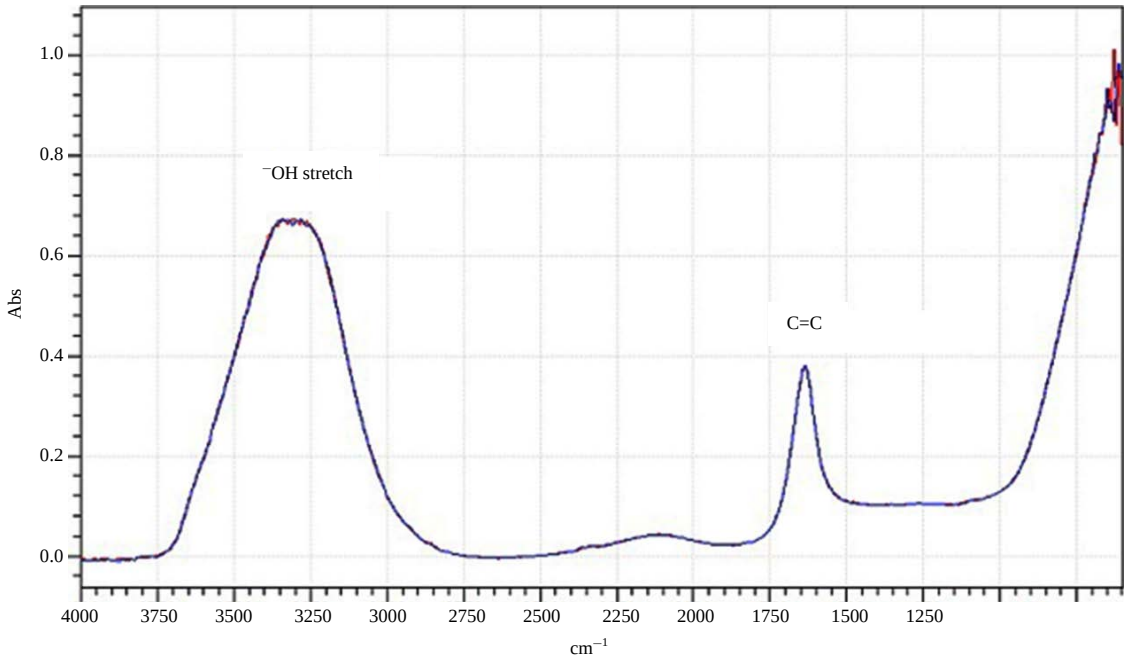

Fig. 2: FTIR spectra of simulated stevia beverages scanned at mid infrared region (4000-650 cm^{-1}). X-axis: Wavenumbers and Y-axis: Response (absorbance)

Table 1: Stevioside (μg mL^{-1}) and rebaudioside A (μg mL^{-1}) content in Simulated Stevia Beverages (SSB) by HPLC determination

	Rebaudioside A level (μg mL^{-1}) dilution factor			Stevioside level (μg mL^{-1}) dilution factor		
Sample planting area (leaves ages)	4	10	15	4	10	15
Bandungan (1 month)	95.42	27.94	15.89	362.26	133.20	77.96
Tajuk (Tigra seed, 4 months)	71.66	27.30	13.13	564.69	209.54	134.45
Tajuk (BPBP seed, 4 months)	103.93	33.24	24.92	564.37	158.56	109.70
Tajuk (Gedongsongo seed, 1 month)	182.97	79.14	44.18	418.66	173.01	100.35
Poloboga (2 months)	98.57	33.65	14.43	355.04	128.20	91.77

presence of hydrogen bond. Absorption at 1635 cm^{-1} are characteristic of stretching C=C bond. As well as hypothesized, hydrogen bonding absorption will generate broad and strong peak and shield other peak of FTIR spectra. There was only two major peak information from SSB FTIR spectra as shown in Fig. 2. However, there are not exactly same spectra in FTIR spectroscopy due to interaction between molecules contained in sample with infrared radiation, neither in absorption nor intensity value from infrared frequency ranged[19]. Furthermore, FTIR was considered as fingerprint technique which correlated important information at fingerprint frequency region to sample functional group for establishing PLS calibration[22].

Wave number or frequency range selection was critical point during developing PLS calibration. Therefore, the initial step to establish PLS calibration was carried out by selecting spectra range used for calibration optimization based on the highest R^2 and the lowest RMSEC revealed by PLS model. Optimal spectra region selected considerably refined the performing of PLS model[23,24]. Optimal frequency range was screening from full spectra range. The frequency of 1412-2369 cm^{-1} revealed better PLS for stevioside analysis, while frequency of 791-1839 cm^{-1} offers best PLS model for rebaudioside A determination. The next optimization is done by treating FTIR spectra into several transformations such as first and second derivatives, Multiplicative Scattering Correction (MSC), normalization and Standard Normal Variate (SNV). Calculation was done in several factor of 1-10 to obtain better PLS model based on lower Prediction Residual Error Sum of Square (PRESS). The optimal factor calculation will reveal highest R^2 and lowest RMSEC. The result of optimization PLS modeling was shown in Table 2 and 3.

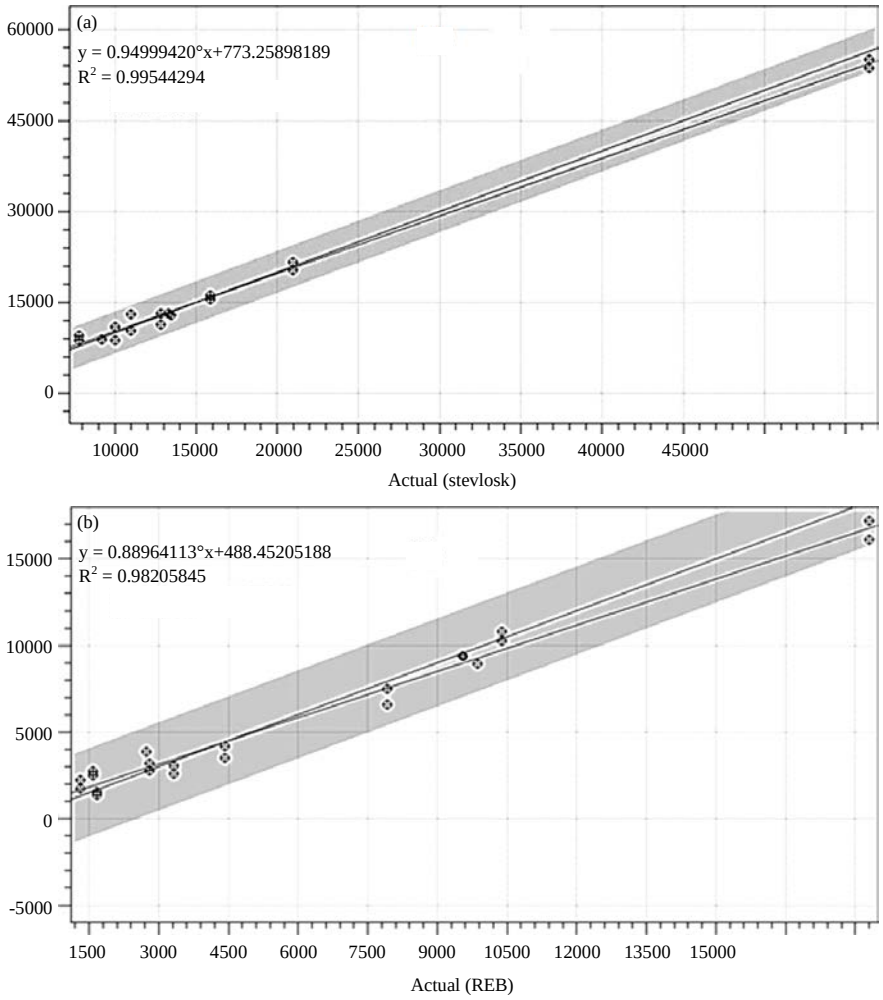

Fig. 3(a-b): Correlation between actual values of (a) Stevioside and (b) Rebaudioside A in simulated stevia beverages determined by HPLC method as actual value (x-axis) and predicted values using FTIR spectroscopy combined with PLS (y-axis) at frequency of 1161-2773, 868-1041 cm^{-1} for stevioside, (a) Determination and 791-1839 cm^{-1} for rebaudioside A and (b) determination

Table 2: Spectra treatments and PLS regression model for stevioside determination at frequency 1412-2369 cm^{-1}

Treatments	R^2	RMSEC	Factor
Original	0.9858	4.34	10
First derivative	0.8757	7.45	6
Second derivative	0.8563	7.71	6
MSC	0.9889	3.93	9
Normalization	0.9499	5.92	6
SNV	0.9775	4.85	4

Original: FTIR spectra without transformation, SNV: Standard normal variate, MSC: Multiplicative scatter (or signal) correction, RMSEC: Root mean square error calibration

The MSC transformation gave better PLS for stevioside determination. Therefore, the optimization was continued by selecting new spectra range with MSC transformation of FTIR spectra. The PLS model was improved by calculating again PLS model established with offset reduction transformation. Optimized combination of spectra region selection was considered to improve PLS model capacity, producing higher R^2 and lower RMSEC[25]. The combined frequency region of 1161-2773 and 868-1041 cm^{-1} was employed to establish PLS model for stevioside determination in SSB (Table 4).

The similar optimization of combined spectra region selection was applied to establish PLS model for rebaudioside A determination in SSB. Unfortunately, the optimization did not achieve better PLS model than original spectra used. Optimal PLS model was validated using leave one out validation technique to reveal RMSEP. Both PLS model for stevioside and rebaudioside A calibration revealed lower RMSEP as shown in Table 5. Internal cross examination which correlated actual value of HPLC determination and predicted value from spectra FTIR calibration for stevioside and rebaudioside a determination in SSB were shown in Fig. 3.

Table 3: Spectra treatments and PLS regression model for rebaudioside A determination at frequency 791-1839 cm^{-1}

Treatment spektra	R^2	RMSEC	Factor
Original	0.9820	2.91	10
Normalization	0.9342	3.79	9
First derivative	0.8809	4.38	5
Second derivative	0.8415	4.52	6
MSC	0.9507	3.60	8
Offset correction	0.9682	3.16	8
SNV	0.9574	3.47	8

Original: FTIR spectra without transformation, SNV: Standard normal variate, MSC: Multiplicative scatter (or signal) correction, RMSEC: Root mean square error calibration

Table 4: Continued optimization of PLS calibration model for stevioside determination in simulated stevia beverages

Treatments	Frequency (cm^{-1})	R^2	RMSEC	Factor
MSC-offset correction	1238-2368	0.9895	3.84	7
MSC-offset correction-interval combination	1161-2773, 868-1041	0.9954	3.40	7

MSC: Multiplicative scatter (or signal) correction, RMSEC: Root mean square error calibration

Table 5: Validation performance of PLS regression for prediction of stevioside and rebaudioside a in simulated stevia beverages

			Validation parameter	
Analytes	Spectra treatment	Linear regression	R^2	RMSEP
Steviosida	MSC-offset correction-interval combination	y = 1.0057x-0.6637	0.9999	1.07
Rebaudiosida A	Original	y = 0.9972x+0.3348	0.9999	0.4124

Original: FTIR spectra without transformation, MSC: Multiplicative scatter (or signal) correction, RMSEP: Root mean square error of predicted

CONCLUSION

The FTIR spectroscopy in combination with multivariate analysis of PLS regression could be used an alternative method for determining stevioside and rebaudioside A in simulated stevia beverages.

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