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The spectrum model established for measuring the contents of Rebaudioside A and Stevioside quickly in the leaves of Stevia rebaudiana Bertoni

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Abstract

By the high-performance liquid chromatography (HPLC) technology, a near infrared (NIR) spectroscopy model is established to directly measure the stevioside glycosides—rebaudioside A (RA) and stevioside (STV) content in the leaves of Stevia rebaudiana Bertoni, this model can be directly applied to measure the content of RA and STV in the leaves of Stevia rebaudiana Bertoni, and resolved the problem of high cost and complex operation using the chemical method to measure the content of RA and STV in the laboratory in current.

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1. Introduction

Stevia originated in Paraguay of South America, is a perennial species of the Asteraceae, short-day plants, Stevia rebaudiana Bertoni contains glycosides and the glycoside can be found in all parts of the plants, the highest glycoside’s parts is the leaves in Stevia rebaudiana Bertoni, so the glycoside contents in Stevia rebaudiana Bertoni mainly depends on the contents of leaves. Glycoside has eight kinds of the main components [1] and the STV is one of the major components and the sweetness is 300 times more than sugar, followed by the rebaudioside A (RA) and the sweetness is 450 times more than sugar, which has the nearest sweetness for sweet sucrose, and other components are less, and the components of Stevioside have some pharmacological effects [2-3]. At present, the methods to determine the stevioside

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glycosides in leaves of Stevia rebaudiana Bertoni are complex, the former deal with the complexity of extraction methods and the extraction rate is not high, meanwhile, the high cost of the liquid chromatography can not fast and accurately measure the glycoside. Therefore, to establish a rapidly and accurately measuring method for glycosides content become the focus of this study.

Contemporary technology is the near-infrared chemical substances using near infrared spectroscopy in the optical properties of the region, rapid determination of the sample one or more of the content and characteristics of the chemical constituents of the physical measuring technology, currently in China's agricultural technology and food analysis has been a wide range of applications. It is a non-destructive, rapid and efficient technology for analysis, which can be overcome the disadvantage of chemical measurement in stevioside glycoside content, which is very suitable for rapidly and accurately measuring the glycoside content in Stevia rebaudiana Bertoni. This study is to establish a near infrared spectroscopy model to directly and rapidly analyze the glycoside content in Stevia rebaudiana Bertoni.

2. Materials and Methods

2.1 Experimental materials

This experiment selected 72 different strains of Stevia rebaudiana Bertoni from Shandong, Jiangsu and Anhui provinces in China. The leaves of Stevia rebaudiana Bertoni randomly selected from open field to verify the model. The standard sample of stevioside glycoside-- RA is 99% and STV is 95%, and the macroporous resin is D354-FD and made by Zhejiang Zhengguang Industrial Co., Ltd..

2.2 Experimental apparatus and equipment

Near infrared spectrometer--Germany Bruker near-infrared spectrometer (Matrix-1);
Liquid chromatograph--Agilent 1100;
Glass chromatography Column--30*290mm.

2.3 Experimental method

2.3.1 Spectra Acquisition

The instrument spectral region of the spectrum acquisition range is 12000-4000 cm⁻¹; The spectral resolution is 8cm⁻¹ The scanning speed is 210 KHZ; Modeling software: Opus 5.5 modeling software from Germany Bruker Corporation. The instrument is suitable for measuring the spectra acquisition [4].

2.3.2 Collected Spectra of fresh leaves

Before anthesis in Stevia rebaudiana Bertoni, the stevioside glycoside content of leaf is the highest [5] and collected 60 ~ 70 pieces in the middle and upper leaves, putting the fresh leaves into oven at 105 °C for deactivating the activity 30 minutes, keep the temperature at 70 °C for drying around 6 hours, using the grinder to crush into 30 ~ 40 mesh, and fully mixed, then using near infrared spectroscopy to collect the absorption spectrum, repeat three times for each sample, the absorption spectrum are saved in the computer.
2.4 Using the HPLC technology to measure the content of RA and STV in stevioside glycoside

Used the method of macroporous resin adsorption to extract the stevioside glycoside. Weighed 2g leaf powder (30 ~ 40 mesh, the absorption spectrum had been scanned), used the deionized water in 100ml, 50ml, 50ml respectively to soak and extract 2 ~ 4 hours by the concussion shaker and then getting the distilled liquid, put the distilled liquid together and then absorbed by macroporous resin. The adsorption will be passed into the 0.5% NaOH 200ml, used deionized water washing to neutral pH, passed into 0.5% HCl 200ml, then used deionized water washing to neutral pH, desorption by 70% ethanol 200ml, all analysis of liquid access 500ml flasks to dry, 60 ~ 80 degrees of negative pressure steam to 10ml then put into the dishes which had been weighed and keep the temperature 80°C to dry, collected the stevioside glycoside extract. Accurately weighed the check ( RA and STV respectively) and prepared the standard solution, then used the HPLC to measure.

HPLC conditions for choice:
- Column: phenomex 00G-4378-E0 Luna 5um NH2 100A 4.6 × 250mm;
- Mobile phase: acetonitrile : water = 78:22, Velocity: 1ml/min;
- Detector: DAD detector, Detection wavelength 210 nm;
- Pump: Agilent1100 pump System, auto sampler System.

3. Results and Analysis

3.1. Near-Infrared Spectroscopy

Fig. 1: The NIR spectra of dry leaves in Stevia rebaudiana Bertoni

The stevioside glycoside has good chemical stability and can not be decomposed in natural light condition, and the sweetness had little changes by heated at 95 °C for 8 hours [6]. From Fig. 1, the peak
wavelength are mainly concentrated in 4000~8000 cm\(^{-1}\), and the water vapor peak are showed in the band within 7000 ~ 8000 cm\(^{-1}\). Through optimizing selection the spectral region which is weak and effective information with samples of the correlation between the components of the spectral band has been removed, and modeling band range for interactive choice are in 4035–6934 cm\(^{-1}\). The existence of water vapor peak doesn’t affect the accuracy of the model.

3.2. Liquid spectrum

In Fig. 2, there is a different time for the peak appearance in RA and STV, RA at 11.250 minutes and STV at 7.887 minutes respectively. The content of RA and STV can be easily separated from the liquid spectrum. Using area normalization method to get the relative content of RA and STV in the leaves of Stevia rebaudiana Bertoni.

![Fig. 2: The liquid spectrum of stevioside glycoside](image)

3.3. Standard curve of RA and STV

In Fig. 3, the results show that the standard curve equation of RA is \(y=3.8056x-14.669\), the correlation coefficient reached 0.9998; and the standard curve of STV equation is \(y=4.3643x+85.731\), the correlation coefficient reached 0.9994, According to the standard curve, the content of RA and STV can be accurately calculated in leaves.

3.4. The establishment of spectral model

3.4.1 The internal cross-validation

In multi-component modeling system, only one group of samples used for modeling and testing. Prior to the beginning of modeling, one sample drawn from this group as a reused sample to check the sample in the group and established the model. Samples for testing must not appear in the modeling sample. For
example, selected compounds known content of n samples, from 1 to n in the arbitrary choice of a sample on the next, then the remaining n-1 samples used for modeling samples, and using this n-1 samples create a stoichiometric model, followed by the selected sample to test this model, such as a cycle, each sample should be used to test one time, quantitative analysis software OPUS 5.5 repeat this process until all samples have been used to test. Through cross-examination, the effects of the calibration model can be determined [7].

3.4.2 External tested set

External examination is used for two independent sample series, one is the model series, another for the test of this model. Both series contain the same number of samples, and are covered by the scope of the system's content. At the same time a sample should not include in the two sample sets. This method is fast and generally used in the calculation of a large number of samples. In a limited number of samples, the internal cross-examination is more superior to the external testing [8].

3.4.3 Data preprocessed

The data preprocessed for model established is a very important stage. First of all is to remove abnormal samples, if there is a big error of the existence of abnormal samples in the concentration of standard values or spectral data in the NIR model, it will seriously affect the grade of prediction model. Among them, the standard error of concentration resulted by many reasons, mainly due to mismanage methods and human factors; because the spectrometer itself has errors, the effects of sample pretreatment misconduct and environmental temperature and humidity will lead to the error of spectrum [9-10] action and the linear differential subtraction and the appropriate normalization and the smallest and the largest normalization and the one first derivative data preprocessed and the second derivative data preprocessed to do the data pretreatment.

3.4.4 Model Validation
Taken the fresh leaves in the open field and dried them and using the grinder to crush into 30 ~ 40 mesh in Lab. Then using the NIR spectrometer to scan and get the spectrum, then used the model to do the quantitative analysis, and at the same time measured the stevioside glycoside content of RA and STV by HPLC, compared the difference between the predicting value by model and real value by HPLC, the model will be verified.

4. Conclusion and discussion

4.1. Model Validation

4.1.1 Internal cross-examination of the effect of predicting model

![Graph showing NIR quantification model cross-validation chart in RA(a) and STV (b) (true value vs predictive value)](image)

In Fig. 4, there is a little difference between the predicting value by model and real value by HPLC on the model of cross-examination, and the x-axis express the data by HPLC, the y-axis express the prediction value by model via internal cross-confirmed. Because there is a linear relationship between the predicting value and the measuring data, so the quantitative model has a good predictive capability.

### Table 1: The difference of stevioside glycoside content in leaves by HPLC measuring and by spectral model

| Serial number | Composition | Measured value (%) | Spectral value (%) | Relative deviation (%) |
|---------------|-------------|--------------------|--------------------|------------------------|
| 1             | RA          | 67.92              | 66.87              | 1.55                   |
| 2             | RA          | 45.88              | 44.02              | 4.04                   |
| 3             | RA          | 63.20              | 60.92              | 3.61                   |
| 4             | RA          | 43.71              | 42.56              | 2.64                   |
| 1             | STV         | 31.92              | 30.87              | 3.30                   |
| 2             | STV         | 46.51              | 47.19              | 1.47                   |
| 3             | STV         | 59.46              | 60.66              | 2.01                   |
| 4             | STV         | 62.94              | 61.88              | 1.69                   |
In Table 1, there is a 1.55% ~ 4.04% difference of RA and 1.47% ~ 3.30% of STV in stevioside glycoside content in leaves between by HPLC measuring and by spectral model, there are no significant difference between predicting value by model and real value by HPLC, so the spectral model can be directly used to measure the stevioside glycoside content of RA and STV in leaves in Stevia rebaudiana Bertoni.

4.2. The impact of factors for setting up the spectral model

4.2.1 The type and quantity of samples

The more the number and types of samples [11], the more widely the acquisition of the spectral range, and the greater the scope of the model can be set up to use. Samples with strong representation, the model of leaves for measuring the stevioside glycosides content has stronger recognition in Stevia rebaudiana Bertoni. Although this experiment has a small number of samples, the sample has strong representative, among different species have a big difference in stevioside glycoside content, so the model has a widely measuring range, and it also was verified in Stevia rebaudiana Bertoni in open field.

4.2.2 Methods of spectral data preprocessing

There is a significant influence for choosing the preprocessing methods in setting up the spectral model. Pretreatment has a direct impact on modeling parameters[12]. The OPUS 5.5 software of this experimental comes with pre-treatment methods of choice, then shows the first derivative vector normalization and second derivative vector normalization method are relatively well pre-treatment methods.

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