

## Research Article

### Rapid Simultaneous Analysis for Three Sugars Fraction in Enzymatic Degradation of Stachyose

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**Abstract:** The objective of the present study was to develop an effective method of High Performance Liquid Chromatography-Refractive Index Detector (HPLC-RID) for the simultaneous quantification of stachyose, raffinose and galactose in enzymatic degradation of stachyose. The three sugars were separated on an Agilent zorbax carbohydrate column with acetonitrile water (75:25 v/v). The calibration curves of the three standards exhibited good linearity ( $R^2 > 0.9989$ ). The Limits of Detection (LOD) values of the stachyose, raffinose and galactose were 0.137, 0.301 and 0.386  $\mu\text{g/mL}$ , respectively and their Limits of Quantification (LOQ) values were 0.452, 0.993 and 1.25  $\mu\text{g/mL}$ , respectively. The Relative Standard Deviations (RSDs) of the data of the intra- and inter-day experiments were less than 2.68, 2.42 and 3.70%, respectively. The results of the recovery test were 97.71-102.61% with an RSD value of 0.92-2.41%. In conclusion, the results of validation showed that the proposed HPLC-RID method was useful for the quantification of the three sugars in enzymatic degradation of stachyose and similar reaction.

**Keywords:** Galactose, HPLC-RID, raffinose, stachyose

## INTRODUCTION

Stachyose [galactopyranosyl ( $\alpha 1 \rightarrow 6$ ) - galactopyranosyl- ( $\alpha 1 \rightarrow 6$ ) - glucopyranosyl- ( $\alpha 1 \leftrightarrow 2\beta$ ) - fructofuranosil], a tetrasaccharide of Raffinose Family Oligosaccharides (RFOs), is naturally found in Leguminosae, Cucurbitaceae and other plant families (Peterbauer *et al.*, 1999). Although RFOs as prebiotics for human health have attracted an attention by researchers recently (Sangwan *et al.*, 2011), they are also the flatulence factors in legumes associated with a discomfort of gastrointestinal tract (Frias *et al.*, 2003).

Soybean, a worldwide legume crop, is an important source of protein-rich food matrix and meal for livestock feed formulations (Singh and Kayastha, 2013). But the presence of non-digestible oligosaccharides restricts the popularization of soybean and diminishes the nutritive value. Especially in feed industry, the non-oil fraction of soybean contains approximately 12% soluble carbohydrates, principally sucrose, raffinose and stachyose. Only sucrose is positive for Metabolizable Energy (ME), both raffinose and stachyose lower the ME efficiency of feed because these sugars are not digested in the small intestine of monogastric animals due to the lack of  $\alpha$ -galactosidase ( $\alpha$ -Gal, E. C.3.2.1.22) (Hagely *et al.*, 2013; Kumar *et al.*, 2010; Viana *et al.*, 2005). Previous research has

demonstrated that removal of stachyose and raffinose from soybean meal increases ME in poultry (Perryman and Dozier III, 2012).  $\alpha$ -Gal catalyzes cleavage of terminal galactosyl residues from  $\alpha$ -D-galactose containing oligo- and polysaccharides. The enzymatic degradation of non-digestible oligosaccharide was found to be most effective in comparison to traditional methods, soaking and heating for legumes products (Singh and Kayastha, 2013). In the enzyme reaction, it usually contains three or four kind of sugars. For estimating the enzyme activity and monitoring the reaction proceeding, a rapid, simultaneous, easy-to-handle and reliable analytical methods need to be established.

The present work is principally concerned with determination of deferent sugar fraction in enzymatic degradation of stachyose. In the reaction, stachyose was hydrolyzed into raffinose and galactose by  $\alpha$ -Gal. To analyze the varieties of the sugars in the enzymatic degradation by one step, an applicable analytical method for fast and efficient determination of these carbohydrates is necessary. Currently, there are a variety of means to detect stachyose, raffinose and galactose, the commonly used methods include Thin Layer Chromatography (TLC) scanning (Gulewicz *et al.*, 2000; Liao *et al.*, 2010), High Performance Liquid Chromatography-Ultraviolet detector (HPLC-

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UV) (Giannoccaro *et al.*, 2008), High-Performance Capillary Electrophoresis (HPCE) (Andersen *et al.*, 2003), High Performance Anion Exchange Chromatography-Pulsed Amperometric Detector (HPAEC-PAD) (Bansleben *et al.*, 2008; Cataldi *et al.*, 2003) and so on. TLC is a simple method with lack of sensitivity and automaticity. For HPLC-UV detection, precolumn derivatization is necessary and it can't be explored in multi sugars sample. Although HPCE has low detection limits and high efficiency of column, sample preparation for analysis is complex and reproducibility of results is slightly lower than others. HPAEC-PAD is novel HPLC with powerful separating ability, essential of sample desalination restricts the method to be directly used in an enzymatic reaction. In this study, a rapid, easy, direct and reproducible HPLC-RID method for sugars determination of the enzymatic degradation of stachyose was developed. Using this method, stachyose, raffinose and galactose could be simultaneously determined without complex preparation of sample e.g., desalination, precolumn derivatization, etc.

## MATERIALS AND METHODS

**Chemicals and reagents:** Sample of stachyose product (purity $\geq$ 80%) was purchased from Shanghai Kayon Biological Technology Co. Ltd.  $\alpha$ -Gal was provided by Food Biotechnology lab of Shenyang normal University. HPLC standards of stachyose, raffinose and galactose were purchased from Sigma Chemical Co. Ltd. U.S.A. The other reagents were HPLC grade obtained from Sinopharm Group Chemical Reagent Co., Ltd. China. Deionized water was purified with a Milli-Q water system (Millipore, Bedford, MA). All working solvents were degassed under vacuum and filtered through a 0.45  $\mu$ m nylon membrane prior to use in HPLC analysis.

**Sample preparation:** To determine the fraction varieties in the enzymatic degradation of stachyose, reaction mixtures (100  $\mu$ L) individually contained 50  $\mu$ L of  $\alpha$ -Gal (0.1 U/mL) in McIlvaine buffer (pH 5.0) and 50  $\mu$ L of stachyose solution, after 0, 2 h incubation at 30°C, the reaction was stopped by boiling for 5 min. Then the change of stachyose, raffinose and galactose in the reaction mixtures were determined using HPLC-RID.

**HPLC-RID analysis:** The determination was carried out by using an Agilent 1200 series HPLC system consisting of G1311A quaternionic pump, a G1316A column oven, a G1314B Refractive Index Detector (RID) and an LC Ver1.23 workstation (Agilent Technologies). The separation was completed using Agilent zorbax carbohydrate column (150 $\times$ 4.6 mm, 5  $\mu$ m). The mobile phase was used and consisted of acetonitrile-deionized water at ratio of 60/40, 65/35, 70/30, 75/25 and 80/20 (v/v). The elution was run at 1

mL/min flow rate with an oven temperature set at 30°C and the injection volume was 10  $\mu$ L.

Sugars of the enzymatic degradation were identified by comparing the retention time of the unknown peaks with that of serious standards. Quantification was obtained using calibration curves established from authentic external standard. Each sample was injected in six replicates and the unknown concentrations were determined from the calibration curves regression equations.

**Validation of HPLC-RID analysis:** HPLC-RID validation tests were carried out using calibration curves and tests of precision, selectivity, recovery, Limit of Detection (LOD) and Limit of Quantification (LOQ). Calibration curves were constructed from the mean peak areas of six replicates HPLC-RID analyses of five concentrations (12, 24, 36, 60, 192 mg/mL for stachyose; 3, 6, 12, 24, 48 mg/mL for raffinose; and 3, 6, 12, 24, 48 mg/mL for galactose, respectively) of the standards. The linear regression equation was calculated from the calibration curve as  $y = ax + b$ , where  $a$  is the slope of the calibration curve,  $b$  is the intercept of the calibration curve,  $x$  is the concentration of the compounds and  $y$  is the peak area. The determination coefficient ( $R^2$ ) values indicated linearity. Precision (reproducibility) of the method was determined by calculating the Relative Standard Deviation (RSD) from repeated injections of different sugars standards, respectively, in the intra- and inter-day tests. RSDs were calibrated using the formula  $RSD (\%) = (\text{standard deviation}/\text{mean measured amount}) \times 100\%$ . The intra-day precision was evaluated by determining six replicates analysis of standards and samples on the same day. For inter-day same practice was done on different days. Selectivity of the method was investigated against compounds in the enzymatic reaction solution. Blanks of the reaction mixtures were analyzed under the described chromatographic conditions to determine whether any endogenous compounds might interfere at the retention time of each analyte. The recovery test was validated by a standard addition method. Samples of the reaction mixtures with known content were mixed with 3 different concentrations of stachyose, raffinose and galactose and then the content was confirmed according to the calibration curve for the verification of accuracy. The recovery of the added standard was calculated by the equation:  $\text{Recovery} (\%) = (\text{Found amount} - \text{Original amount}) / \text{Spiked amount} \times 100$ . The LOD and LOQ were calculated at Signal-to-Noise ratios (S/N) of 3 and 10, respectively.

## RESULTS AND DISCUSSION

**Optimization of HPLC condition:** Optimization of mobile phase is crucial for improving various

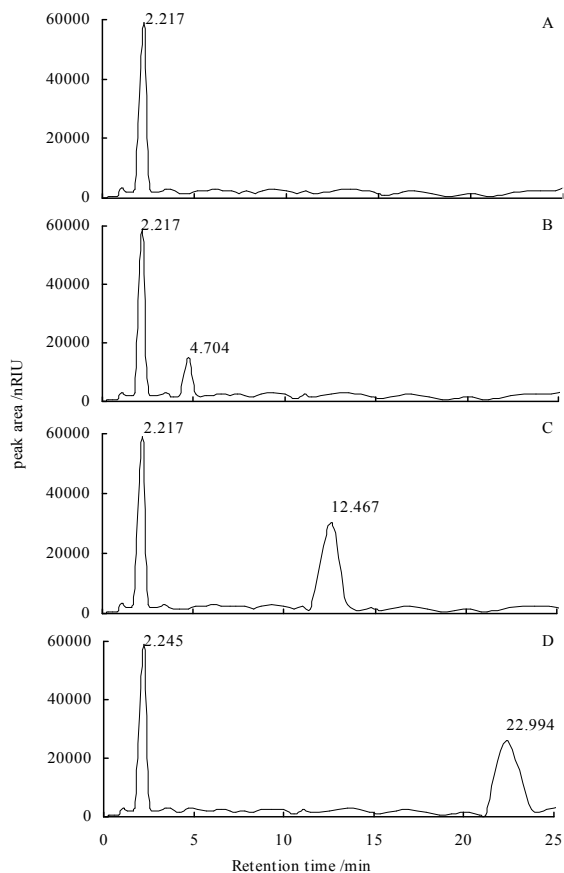


Fig. 1: HPLC chromatograms of sugar standards, (A) blank, (B) galactose, (C) raffinose, (D) stachyose

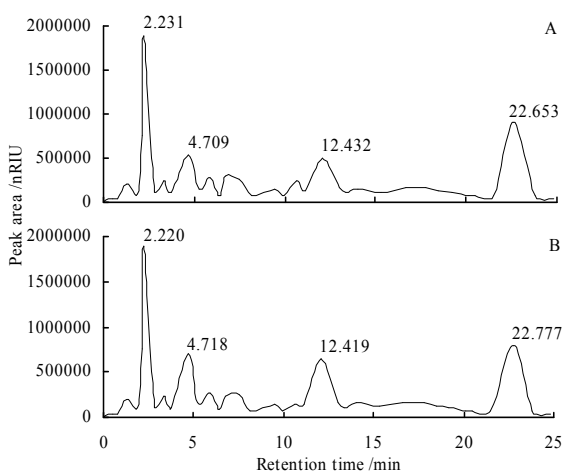


Fig. 2: Chromatograms of stachyose hydrolysis, (A) 0 h of the enzymatic reaction and (B) 2 h of the enzymatic reaction

chromatographic parameters; peak shapes, detection, sensitivity and retention time. Aqueous acetonitrile in various proportions having 20 to 40% water were used for analysis and the best chromatographic separation was achieved with acetonitrile water (75:25 v/v). The

HPLC system was equilibrated with the initial mobile phase; followed consecutive injections of the same standard were used to evaluate the system suitability of method validation. Representative chromatogram is shown in Fig. 1. All parameters (capacity factor, resolution and asymmetric factor) were found satisfactory with good specificity for the stability assessment of stachyose, raffinose and galactose. Selectivity was determined by comparing the chromatograms of the blank with the corresponding spiked standards. No interference from endogenous substance was observed at the retention time of galactose, raffinose and stachyose.

#### Method validation:

**Linearity, LOD and LOQ:** To establish the calibration curve, mixed standard solutions of five different concentrations were analyzed six times. The determination coefficient values of the sugar standards showed good linearity ( $R^2 > 0.9989$ ). The LOD values of the stachyose, raffinose and galactose were 0.137, 0.301 and 0.386  $\mu\text{g/mL}$  and their LOQ values were 0.452, 0.993 and 1.25  $\mu\text{g/mL}$ , respectively (Table 1).

**Precision:** The precision of the method was investigated with respect to repeatability. Three concentrations of sugar standards were analyzed for intra- and inter-day precision. Data are summarized in Table 2. Generally acceptable repeatability of the results within one day and day-to-day was observed. The RSD values of the intra-day and inter-day tests for stachyose were 0.81-2.68% and 0.76-3.23%; for raffinose were 1.08-2.42% and 0.96-2.75%; for galactose were 0.56-3.70% and 1.42-2.64%, respectively.

**Recovery:** In the result of recovery (Table 3), stachyose ranged from 98.38 to 100.39%, RSD 0.92-2.04%; raffinose ranged from 97.71 to 102.61%, RSD 1.27-2.41%; and galactose ranged from 97.79 to 101.51%, RSD 1.18-2.22%; all values were within acceptable limits, the analysis method used in the sugar standards showed excellent accuracy.

#### Analysis of stachyose, raffinose and galactose in the enzymatic degradation of stachyose:

The reaction mixtures were analyzed after incubated 0 and 2 h using the HPLC-RID method (Fig. 2). As shown in the results, the peaks for stachyose, raffinose and galactose showed good resolution without interference from other components at the same retention time. The contents of the sugars were calculated from the calibration curve of the standards. Table 4 showed the varieties of the sugars before and after the reaction. The contents of stachyose, raffinose and galactose in assayed samples were found to be significantly different. In 2 h, the content of raffinose and galactose were increase

Table 1: Calibration curve, LOD and LOQ of the sugar standards

Compounds	Regression equation	Correlation coefficient (R <sup>2</sup> )	LOD (µg/mL)	LOQ (µg/mL)
Stachyose	y = 8452.5x+49953	0.9915	0.137	0.452
Raffinose	y = 77196x+168646	0.9994	0.301	0.993
Galactose	y = 49830x+7631	0.9989	0.386	1.276

Table 2: Precision data of the sugar standards

Compound	Concentration (mg/mL)	Intra-day		Inter-day	
		Mean±S.D. (mg/mL)	RSD (%)	Mean±S.D. (mg/mL)	RSD (%)
Stachyose	48	48.09±0.39	0.81	47.78±0.72	1.51
	24	24.22±0.65	2.68	24.13±0.78	3.23
	12	12.09±0.13	1.07	11.86±0.09	0.76
Raffinose	48	48.11±0.52	1.08	47.88±1.32	2.75
	24	23.91±0.58	2.42	23.86±0.23	0.96
	3	3.06±0.06	1.96	2.94±0.04	1.36
Galactose	24	24.13±0.14	0.56	23.91±0.42	1.76
	12	11.96±0.23	1.88	11.89±0.17	1.42
	3	2.97±0.11	3.70	3.03±0.08	2.64

S.D.: Standard deviation

Table 3: Recoveries for the assay of three investigated sugars

Sugars	Original amount (mg/mL)	Spiked amount (mg/mL)	Found amount (mg/mL)	Recovery (%)	RSD (%)
Stachyose	75.96	80	156.58	100.39±0.92	0.92
		40	114.09	98.38±1.62	1.65
		20	94.81	98.80±2.02	2.04
Raffinose	9.94	15	24.37	97.71±1.24	1.27
		10	19.53	97.94±2.37	2.41
		5	15.33	102.61±1.81	1.76
Galactose	4.62	8	12.91	102.29±1.21	1.18
		4	8.43	97.79±1.59	1.63
		2	6.72	101.51±2.25	2.22

Table 4: Varieties of sugars in stachyose hydrolysis by α-gal

Reaction time (h)	Stachyose (mg/mL)	Raffinose (mg/mL)	Galactose (mg/mL)
0 (as control)	76.8	9.8	4.6
2	64.8	18.5	14.9

correspondingly to stachyose decrease. These results suggest that stachyose were hydrolyzed to raffinose and galactose efficiently by α-Gal.

### CONCLUSION

In this study, a HPLC-RID method has been developed that offers a feasible, reliable, direct and simultaneous means of measuring stachyose, raffinose and galactose without desalination and precolumn derivatization of sample. Validation statistics showed that this method possesses good sensitivity, precision and repeatability. Linearity was confirmed over a wide calibration range with regression coefficients higher than 0.9989. The assay method was applied successfully to quantify the stachyose, raffinose and galactose in the enzymatic degradation of stachyose. Therefore, the simultaneous quantification of sugars can be recommended for the effective quality evaluation of similar enzymatic hydrolysis.

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