

## Original Research Article

# Spectrum-effect relationships between high performance liquid chromatography fingerprints and bioactivities of charred areca nut

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### Abstract

**Purpose:** To investigate the spectrum-effect relationships between high performance liquid chromatography (HPLC) fingerprints and duodenum contractility of charred areca nut (CAN) on rats.

**Methods:** An HPLC method was used to establish the fingerprint of charred areca nut (CAN). The promoting effect on contractility of intestinal smooth was carried out to evaluate the duodenum contractility of CAN in vitro. In addition, the spectrum-effect relationships between HPLC fingerprints and bioactivities of CAN were investigated using multiple linear regression analysis (backward method).

**Results:** Fourteen common peaks were detected and peak 3 (5-Hydroxymethyl-2-furfural, 5-HMF) was selected as the reference peak to calculate the relative retention time of 13 other common peaks. In addition, the equation of spectrum-effect relationships  $\{Y = 3.818 - 1.126X_1 + 0.817X_2 - 0.045X_4 - 0.504X_5 + 0.728X_6 - 0.056X_8 + 1.122X_9 - 0.247X_{13} - 0.978X_{14} (p < 0.05, R^2 = 1)\}$  was established in the present study by the multiple linear regression analysis (backward method). According to the equation, the absolute value of the coefficient before  $X_1, X_2, X_4, X_5, X_6, X_8, X_9, X_{13}, X_{14}$  was the coefficient between the component and the parameter.

**Conclusion:** The model presented in this study successfully unraveled the spectrum-effect relationship of CAN, which provides a promising strategy for screening effective constituents of areca nut.

**Keywords:** Charred areca nut, Spectrum-effect relationships, HPLC fingerprints, Duodenum contractility

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## INTRODUCTION

The seed of *Areca catechu* (belonging to the family of Arecaceae), also called areca nut (AN), is a famous and widely used traditional Chinese medicine (TCM) in China [1]. Areca nut is commonly used to treat parasitic diseases, dyspepsia and abdominal distension, etc [1,2]. Furthermore, for clinical treatment of dyspepsia and abdominal distension, areca nut is commonly processed by stir-baking to dark

brown, resulting in charred areca nut (CAN) [3]. The aim of this process is mainly related to enhancing the effects of areca nut on gastrointestinal contraction [4]. In addition, previous investigation reported that arecoline is the major active constituent of areca nut, however, the quantity of arecoline is sharply decreased by this processing. Thus, the substance basis of the charred areca nut is still unclear [5,6].

It's well known that TCMs possess various active constituents and play lots of pharmacological activities via actions of multi-channels, multi-constituents, and multi-targets [7,8]. Additionally, the spectrum-effect relationships assay based on HPLC fingerprints and bioactivities is a good strategy for investigating the active compositions of TCMs [9,10]. Therefore, the present study was designed to investigate the spectrum-effect relationships between high performance liquid chromatography (HPLC) fingerprints and activity of charred areca nut on gastrointestinal contraction. This research would be beneficial for the quality control of charred areca nut and provide a promising strategy for screening effective constituents from this plant.

## EXPERIMENTAL

### Chemicals and reagents

Formic acid, acetonitrile, and methanol of HPLC grade were acquired from Sigma-Aldrich (Shanghai, China); the water used was purified by a Millipore water purification system (Millipore, Bedford, MA, USA); the reference standard agents including 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyranone (DDMP), 5-Hydroxymethyl-2-furfural (5-HMF) and 3-hydroxy-2-methyl- $\gamma$ -pyrone (maltol) were purchased from the China Institute of pharmaceutical and biological products (Beijing, China). All other chemicals and reagents used in the experiment were of analytical grade.

### Samples

The dried seeds of *Areca catechu* L. were obtained from Hehuachi Market of Traditional Chinese Herbs (Chengdu, China) in 2015, and identified by Professor Chun-Jie Wu [College of pharmacy, Chengdu University of Traditional Chinese Medicine, Chengdu, 610075, P.R. China]. They were processed by stir-baking to dark brown as previous report with an online-type and non-contact temperature measurement system (ONTMS, Haishan Pharmaceutical Equipment Co. Ltd., Hangzhou, China) [11,12]. A voucher specimen (sAN-20150903) has been deposited in College of Pharmacy, Chengdu University of Traditional Chinese Medicine (Chengdu, China).

### Animals

Sprague Dawley (SD) rats (weighing from 180 to 220 g) [Certificate No. SCXK (Chuan) 2008-24] were provided by Dashuo experimental animal Co. Ltd. (Chengdu, China). Total 16 SD rats

were used in our present duodenum contractility assay; eight were used for establishing the spectrum-effect relationships and the other eight rats were used for verification of the obtained spectrum-effect relationship equation. The animals were kept under controlled conditions of free access to chow and tap water, a 12 h light-dark cycle in a quarantine room and temperature range from 20 to 25 °C. They were fed for one week acclimatization period before experiments. All the experiments involving animals carried out were in accordance with the animal-use guidelines and approved by the Animal Ethics Committee of Chengdu University of Traditional Chinese Medicine (approval no. TCM20150903-AN-03).

### HPLC fingerprints assay

CAN was powdered and sieved through a 50 mesh sieve (inner diameter  $355 \pm 13 \mu\text{m}$ ). An amount of 0.3 g was accurately weighed and added into 50 mL grinding mouth Erlenmeyer flask, and extracted with 30 mL 40 % methanol (formic acid adjusted pH = 3.0) by ultrasonic extraction for 40 min. After filtering, the solution was prepared and then filtered through 0.45  $\mu\text{m}$  millipore film for HPLC analysis.

### Preparation of reference standard solution

The mixed standard solutions containing 0.5 mg/mL DDMP, 5-HMF, and Maltol were prepared by adding an accurately weighed amount of each standard stock into a volumetric flask and dissolving with 10 mL 40 % methanol and then filtered through 0.45  $\mu\text{m}$  millipore film to yield the mixed standard solution.

### Assay method

Method precision and reproducibility were evaluated by the successive analysis of six replicate injections of one sample and the injections of 6 samples prepared independently from S1 which had the best peak forms and peak resolution among the samples, respectively; meanwhile, to investigate sample stability, the sample was determined in different time points in a day (0, 2, 4, 8, 16 and 24 h).

### HPLC assay conditions

The HPLC chromatographic separation was performed using a gradient elution with a CAPCELL PAK MG II S5 C18 column (250 mm  $\times$  4.6 mm, 5  $\mu\text{m}$ ) (Shiseido, Japan) at 30 °C. The mobile phase was composed of solvent A (acetonitrile) and solvent B (water solution, the pH was adjusted to 3.0 with formic acid) working

with a linear gradient as follows: 0 – 10 min (95 % , B), 10 – 30 min (95 – 85 % , B), 30 – 60 min (85 – 70 % , B) with a mobile flow rate of 1.0 mL/min. The detection wavelength was set at 283 nm with the sample injection volume of 10.0  $\mu$ L.

### Tissue preparation and determination of the duodenum contractility

Samples preparation for contractility research were the same as that for HPLC analysis, except for the second step that the sample was extracted with sterilized Millipore water instead of 40 % methanol. According to the previous report, the final concentration of samples solution was dissolved at 40  $\mu$ g/mL [6]. The determination of duodenum contractility was carried out according to previously report [12]. Rats were fasted for 12 h before being sacrificed by cervical dislocation. Then the duodenum was excised from rats and stripped into approximately 2.0 cm segment. The contractility of intestinal smooth muscle was determined by using a 4 channel automatic organ bath system (Panlab S.L., Spain). One end of the smooth muscle segment was connected to a force-displacement transducer in longitudinal direction, and the other end was fastened to the bottom side of the organ bath chamber (20.0 mL solution). The initial load of resting tension was set at 2.0 g and before a dynamically equilibrated contractile state of duodenum segment was obtained, the buffer was changed every 10 min. Contractile response was recorded by using a physiological recording system of Panlab (Lab Chart 7 Chinese, Spain) with 8 independent assays.

### Statistical analysis

The spectrum-effect relationship between HPLC fingerprints and promoting contractility of charred areca nut was analyzed by the multiple linear regression analysis using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Data are expressed as mean  $\pm$  standard deviation (SD) or percentage. One-way analysis of variance (ANOVA) followed by Student's t-test was used to analyze the differences between the groups.  $P < 0.05$  was considered statistically significant.

## RESULTS

### HPLC fingerprints data

In our present study, results of methodology validation showed that the relative standard deviation (RSD) for reproducibility and precision were 0.1 - 0.3 % and 0.04 - 0.25 %, respectively. In addition, the R.S.D. value of the storage

stability was below 0.55 %. All results above indicated that the method of HPLC fingerprint analysis was valid and satisfactory. HPLC fingerprints of 40 % methanol extracts of CAN were obtained under the optimized processing condition and shown in Figure 1, and the reference standard fingerprint was generated in Figure 2. Peaks, which existed in all chromatograms of the 10 chromatograms of CAN samples from different charred degrees, with well segregation and large areas from subsequent peaks, were regarded as "common peaks", indicating similarity among diverse samples.

From the samples, fourteen common peaks were found by comparison of their HPLC retention time and ultraviolet spectra, and these 14 common peaks were marked in Figures 1 and 2. Peak 3, which was 5-HMF at the mean retention time 10.063 min by the comparison between the chromatogram of sample and the reference standard solution, was selected as the reference peak to calculate the relative retention time of other 13 common peaks. The average relative retention time, the coefficient of variance (CV %) and average peak area ( $n = 3$ ) of the peak area of 14 common characteristic peaks were shown in Table 1. The values of C.V. % of the peak area of common characteristic peak were all greater than 14.46 %, indicating that the content of each constituent in different samples diverse significantly, especially peaks 3 (5-HMF), 2 (DDMP), and 4 (Maltol).

### Results of the duodenal contractility determination

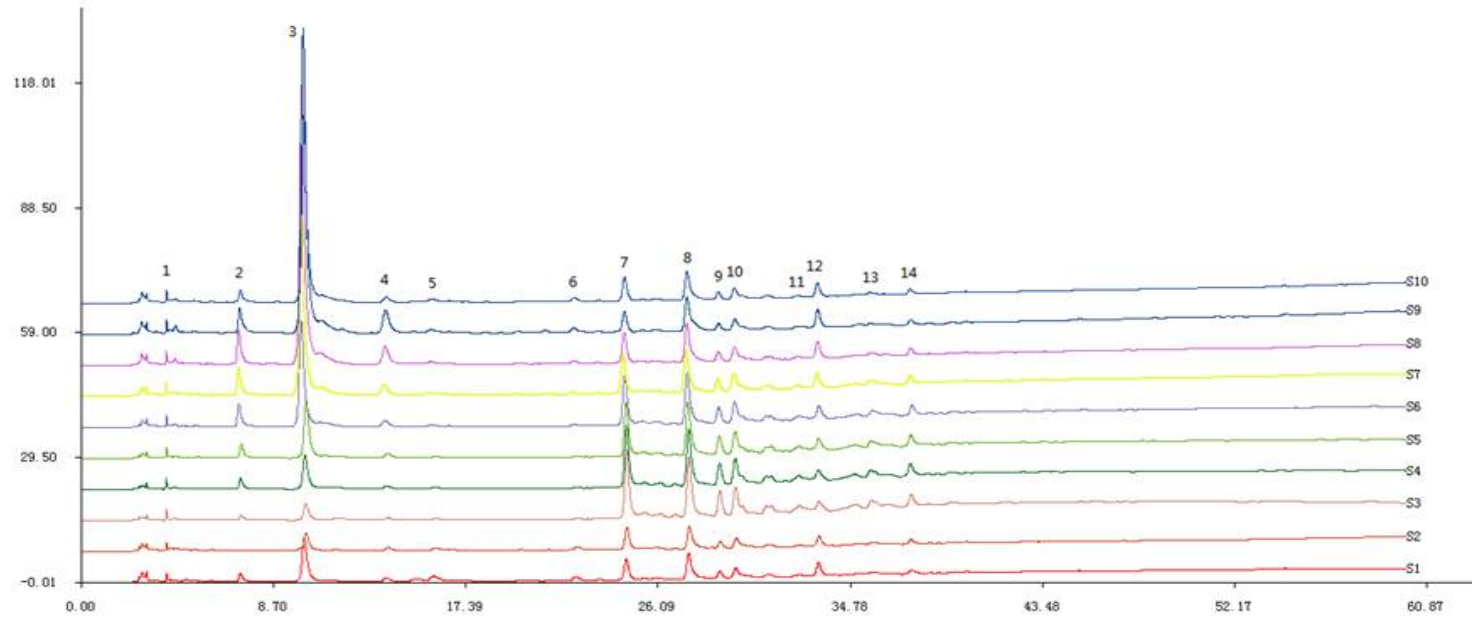
In the present study, the promoting effects of 10 batches of CAN on duodenum contractility of rats were evaluated, and the duodenum contractilities of different CAN samples (40  $\mu$ g/mL) are showed in Table 2. The duodenum contractilities of 10 batches of CAN (S1-S10) used to establish the spectrum-effect relationship were 3.27, 3.18, 3.21, 3.38, 3.41, 3.51, 3.73, 3.94, 4.12 and 3.54 g, respectively.

### Spectrum-effect relationship

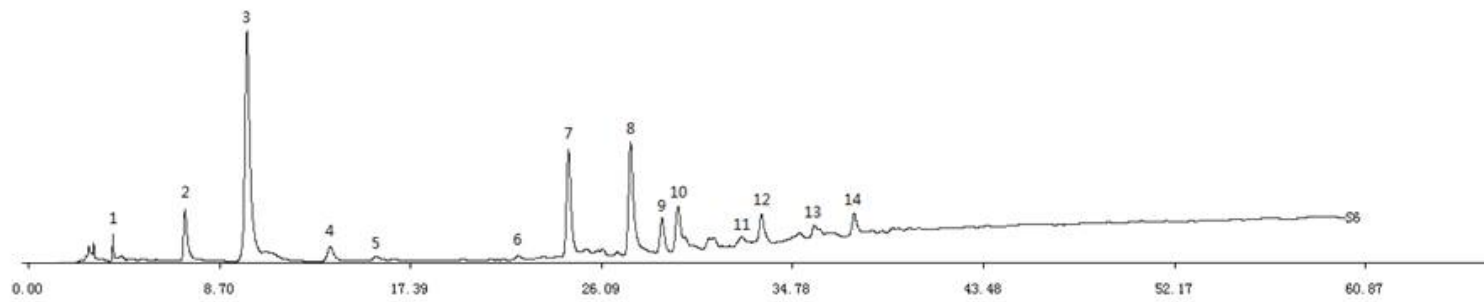
Each peak areas of the 10 batches of CAN were digitized by using the following equation (1), and processing into quantized characteristic peak data which is showed in Table 3.

$$X = P/P_0 \dots\dots\dots (1)$$

where P is the area value of  $X_i$  peak,  $P_0$  is the average area value of  $X_i$  peak ( $i = 1\sim 14$ ).



**Fig 1:** HPLC fingerprints of 40 % methanol extracts of 10 batches of CAN



**Fig 2:** Reference standard fingerprint from the fingerprints of 40 % methanol extracts of CAN

**Table 1:** Mean relative retention time and peak area of each common peak (n = 3)

Peak no.	RT	Mean peak area of every common peak										<sup>a</sup> CV %
		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	
1	3.8450	1620983	1444720	1410968	1423707	1495627	1676470	1881193	1964111	2059433	1876091	14.46
2	7.1780	4971547	6132960	3238037	5840122	7760747	1206465	1481262	1801562	1500028	8334268	62.85
3	10.063	2658838	1293980	1003163	1973377	3372302	5956217	1055787	1576848	1707463	1043630	86.35
4	13.792	4246557	3630409	2445982	3225801	4584464	5795542	9481434	1584323	1908460	5768235	76.79
5	15.895	7067924	3872580	2056001	2228217	2521081	2987165	3932856	4705142	5899905	5206508	41.26
6	22.336	6843220	5353678	1891368	2011853	2439004	2842301	3933950	4393752	7594086	6612659	48.28
7	24.609	1482350	1394322	3552219	3203930	2875943	2938013	2524979	1929944	1451638	1547696	35.9
8	27.433	2318101	1900528	4231754	4052075	3900579	3694982	3320766	3121298	3066490	2496138	24.41
9	28.850	8387943	6888612	1697603	1552067	1374652	1268919	1147693	9385148	8202029	8124308	31.24
10	29.576	1031001	8409775	1884940	1751786	1604501	1573332	1442981	1209311	1040264	1008951	26.85
11	32.453	8183145	6632139	1417405	1340004	1226646	1185242	1072379	9042442	8258291	7878221	25.34
12	33.332	1697884	1337623	2249039	2193492	2147400	2189334	2132028	1957028	1913088	1710860	15.03
13	35.710	6281129	5587332	1318042	1286392	1182803	2044393	1034033	8375734	6822650	7063608	44.08
14	37.525	20302790	1629151	3368387	3148495	3071903	2709838	2861189	2362805	1765471	2026535	24.75

RT = retention time; <sup>a</sup> CV% =  $\sigma/\mu \times 100$ , where  $\sigma$  was the standard deviation and  $\mu$  is the mean value of the peak area

**Table 2:** Results of contractility determination

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Tension (g)	3.27 ± 0.46	3.18 ± 0.59	3.21 ± 0.40	3.38 ± 0.57	3.41 ± 0.65	3.51 ± 0.56	3.73 ± 0.66	3.94 ± 0.61	4.12 ± 0.76	3.54 ± 0.76

**Table 3:** Quantization characteristic peak data for 10 batches of CAN

	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12
S1	0.9618191760	0.548426023	0.37931836	0.573036251	1.746141715	1.558256695	0.647285184	0.72208886	0.75297489	0.770090808	0.799049434	0.86947126
S2	0.8572325560	0.067654492	0.184603339	0.489892392	0.956726966	1.219075901	0.608846745	0.592014799	0.618381869	0.628155591	0.64760027	0.684984813
S3	0.8372056210	0.357197419	0.143114452	0.330064181	0.507938273	0.430679833	1.551117299	1.318192099	1.523916451	1.407927797	1.384035922	1.151712822
S4	0.8447643760	0.644241095	0.281528294	0.435294031	0.550484506	0.458115245	1.3990329	1.26222206	1.39327065	1.308470404	1.308457125	1.123267698
S5	0.8874385040	0.856110908	0.481103423	0.618633887	0.622837017	0.555380992	1.255813602	1.21503103	1.234007478	1.198458072	1.197767841	1.099664396
S6	0.9947426921	1.330887151	0.849733028	0.782058421	0.737983801	0.64721499	1.282917182	1.150987529	1.13909232	1.175176853	1.15733859	1.121138424
S7	1.11621621	1.634023832	1.506219609	1.279437765	0.971618246	0.895792321	1.102561133	1.03441918	1.030269294	1.077813119	1.047132653	1.091792532
S8	1.1654160611	1.987356216	2.24958195	2.137907281	1.162412715	1.000492965	0.842732254	0.972284864	0.842492705	0.9032768	0.882956145	1.002176592
S9	1.2219758941	1.654725161	2.4359215	2.575302214	1.457580789	1.729234973	0.633874436	0.955212163	0.736285629	0.777009667	0.806387123	0.97967531
S10	1.11318891	0.919377702	1.488876043	0.778373577	1.286275972	1.505756085	0.675819267	0.777547416	0.729308714	0.753620889	0.769274898	0.876116154

X = peak area value of  $X_i$  /  $X_i$ ; peak average area value

Quantization characteristic peak data was independent variables, intestinal contractile activity was the dependent variable, and the equation regarding spectrum – effect of CAN was established by using the multiple linear regression analysis (backward method).

$$Y = 3.818 - 1.126X_1 + 0.817X_2 - 0.045X_4 - 0.504X_5 + 0.728X_6 - 0.056X_8 + 1.122X_9 - 0.247X_{13} - 0.978X_{14} \quad (p < 0.05, R^2 = 1) \dots\dots\dots (2)$$

The absolute value of the coefficient before  $X_1, X_2, X_4, X_5, X_6, X_8, X_9, X_{13}, X_{14}$  was the coefficient between the component and the parameter. The bigger the coefficient of the parameter, the better correlation the component had with the parameter. The above equations showed that the values of Y were mainly decided by  $X_1, X_9$  and  $X_{14}$ , showing that  $X_1, X_9$  and  $X_{14}$  had the main influence on promoting effects of CAN on contraction activity.

In addition, in our present investigation, another five batches of CAN were used to verify the equation we obtained. The quantized HPLC characteristic peak data of the 5 batches of CAN were showed in Table 4. Furthermore, the contraction activities of the 5 batches of CAN were calculated by using the established spectrum – effect equation (2) which shown in Table 5. Subsequently, the deviation rate between the real determined and calculated values of contraction activities were calculated using Eq 3.

$$\text{Deviation rate} = \{(E/T) - 1\}100 \dots\dots\dots (3)$$

where E and T are estimated and true values, respectively.

As can be seen from the Table 5, all the deviation rate of 5 batches of CAN were less than 6 %, indicating that the equation (2) we established is successful.

## DISCUSSION

Nowadays, single or few components are commonly used as evaluating indicators to control TCMs' quality [13]. However, overall effect and synergistic effects of multi-components are characteristics of TCMs [14]. Therefore, the currently used quality controlling method cannot reflect the real bioactivities of TCM. In addition, increasing researches have demonstrated that spectrum-effect relationships assay possesses unique advantages for investigating the possible and potential active substances of TCMs [10,15].

In our present study, we investigated the spectrum-effect relationship between HPLC fingerprints and bioactivities of charred areca nut (CAN) for the first time, and obtained a possible formula to evaluate the duodenum contractility of CAN.

HPLC is the most commonly used method for investigating TCMs' fingerprint based on its inexpensive, rapid, and high sensitive characteristics [16]. Thus, in our study, we investigated the fingerprint spectrum of CAN using the HPLC assay, and we identified the chromatographic peaks of DDMP, 5-HMF, and maltol.

Based on traditional Chinese medicinal theory, areca nut is a known herbal medicine with the functions of killing parasites and promoting digestion. After processing by stir-baking to dark brown, charred areca nut's promoting effect on digestion are enhanced [1,2]. In addition, the related mechanism of promoting digestion is commonly based on promoting effects of CAN on contractility of digestive tract smooth muscle [17].

Thus, in the present study, we selected the duodenum contractility to represent the promoting effects of CAN on digestion. The results showed that the model presented in this study successfully discovered the spectrum-effect relationship of CAN. Based on the results of HPLC fingerprint spectrum and duodenum contractility, the following relationship was obtained:

$$Y = 3.818 - 1.126X_1 + 0.817X_2 - 0.045X_4 - 0.504X_5 + 0.728X_6 - 0.056X_8 + 1.122X_9 - 0.247X_{13} - 0.978X_{14} \quad (p < 0.05, R^2 = 1),$$

which can be used to evaluate the duodenum contractility of CAN samples. Besides, the verification test results also demonstrated the reliability of this formula.

## CONCLUSION

To the best of our knowledge, the present investigation is the first study on the spectrum-effect relationship between HPLC fingerprints and bioactivity of charred areca nut. The findings provide a rapid and valid method to evaluate the relationship between effective constituents and the biological activities of CAN.

## ACKNOWLEDGEMENT

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**Table 4:** Quantization characteristic peak data of 5 batches of CAN validation samples

	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12
<b>S11</b>	0.8973841	0.502615	2.8656372	1.1032793	1.1918665	1.6121335	0.5640454	0.6254286	0.5653417	0.5999679	0.6416603	0.7178775
<b>S12</b>	0.7110419	0.457019	0.3671131	0.8573902	0.8638959	0.7423007	1.1159408	1.0309411	1.0374559	0.9834744	1.0136566	1.0369899
<b>S13</b>	0.7399932	0.455930	0.6672485	1.1342406	1.0094542	0.9418542	0.9645850	0.9765881	0.9471784	0.9206100	0.9528716	0.9797360
<b>S14</b>	1.4077011	0.431979	0.3665238	1.2780824	1.8375569	1.8110669	0.3366238	0.4139448	0.4024503	0.4350145	0.4558331	0.5629355
<b>S15</b>	1.0549642	1.437738	0.6029791	0.7001731	0.5398503	0.4524948	1.4444515	1.3979462	1.4454113	1.4516432	1.3946468	1.2829012

**Table 5:** Results of verification on spectrum-effect relationship

Variable	Tension (g)		Deviation rate (%)
	Calculated value	Determined value	
S11	3.05	2.93 ± 0.51	4.246531523
S12	2.76	2.74 ± 0.68	0.916741618
S13	2.77	2.84 ± 0.39	-2.453354622
S14	2.34	2.34 ± 0.44	0.182353495
S15	3.21	3.03 ± 0.73	5.783675398

## CONFLICT OF INTEREST

No conflict of interest associated with this work.

## CONTRIBUTION OF AUTHORS

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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