SUPPLEMENTARY MATERIAL

Optimisation of Pulsed Electric Fields Extraction of Anthocyanin from Beibinghong *Vitis Amurensis Rupr*

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Abstract: Beibinghong *Vitis amurensis Rupr* has wide plantation area, high productivity, and rich anthocyanidin. Common hot-extraction has poor deficiency and destroys anthocyanin severely. Beibinghong *Vitis amurensis Rupr* as materials, response surface optimized electric fields were used, the structure of Beibinghong was observed by SEM, antioxidant activity was measured by DPPH, ABTS and reducing force, the component of anthocyanin was analyzed by HPLC-MS. We found the content of total anthocyanin extracted by pulsed electric fields was 166.65±3.88 mg/100g.FW. Total anthocyanin from Beibinghong had high antioxidant activity, also contained multiple steady anthocyanin of delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, petunidin 3-O-glucoside, peonidin 3-O-glucoside, malvidin 3-O-glucoside, delphinidin-3-O-(6-O-acetyl) glucoside and delphinidin-3-O-(6-O-p-coumaroyl) glucoside et al. In conclusion, the optimized pulsed electric fields method can quickly and efficiently extract several kinds of anthocyanin from *Vitis amurensis Rupr*. This study promoted the

intensive processing of *Vitis amurensis Rupr* and widened the practical application of pulsed electric field technology.

Keywords: Beibinghong, anthocyanidin, pulsed electric fields, SEM, HPLC-MS

Experimental Details

Reagents

Concentrated Hydrochloric acid (36.0%) and absolute ethanol were bought from Beijing Chemical (China); delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, petunidin 3-O-glucoside, peonidin 3-O-glucoside, malvidin 3-O-glucoside, delphinidin, cyanidin, petunidin, peonidin and malvidin were purchased from PhytoLab GMbH & Co.KG (Germany); formic acid and methanol were obtained from Thermo Fisher (USA).

Materials and preprocessing

Vitis amurensis Rupr. cv. 'Beibinghong' were collected from Tonghua in China, at 110 d after blossom on 28, September, 2015, and identified by Professor Rungang Song, from the Chinese Academy of Agricultural Sciences Specialty Research Institute. A voucher specimen (95-2-482) was deposited at the Chinese Academy of Agricultural Sciences Specialty Research Institute.

Before experiment, a certain amount of frozen Beibinghong were collected, after unfrozen, fruit stems were removed, *Vitis amurensis Rupr* was beated by stamping machine, then we put it into colloid mill with 3000 r/min and grinded for 15 min for standby application.

Extraction by pulsed electric fields

According to the results of preprocessing, anthocyanin extracted by pulsed electric fields of LDT-200/10-20.1 (Huadi Biotechnology Development Co., Ltd., Changchun, China). Ratio of liquor (36.0% HCl-ethanol-distilled water, v/v/v = 1:65:34) to material （5, 7, 9 w/w), electric field intensity B（13, 15, 17 KV/cm), pulse numbers C (3, 4, 5) were designed by Box-Behnken Design (Table S1) (Celli et al. 2015; Wang et al. 2016; Yuan et al. 2015), and extracted solution was separated centrifugally of 4500 r/min for 10 min. The content of total anthocyanin was detected by pH-differential method in 2014 literature of Antonia Chiou.

Purification of anthocyanin of Vitis amurensis Rupr

Preprocessed D101 macroporous resin was packed column by wet method. Supernatant was concentrated under vacuum at 40-50 °C and loaded, standing for 20 min. Column and saccharide of water-soluble impurity were washed by deionized water with 4 times column volume for 2 mL/min. Anthocyanin was eluted by 75% ethanol solution (containing 0.01% hydrochloric acid) until the color of chromatographic column disappeared. The alcohol eluent was mixed and concentrated under vacuum, then we got refined solution of *Vitis amurensis Rupr*.

Scanning electron microscope (SEM)

After centrifugation, the centrifugal sediment of extracted solution was freeze-dried and spread uniformly on the conducting resin of metal object stage. The samples were observed under SSX-550 SEM (Shimadzu, Japan) after 100 s using vacuum spraying gold. A representative view was selected for microscopic imaging.

Antioxidant activity

DPPH assay

1 mL sample solution of Beibinghong anthocyanin with different concentrations were collected, then added into 2.0 mL 0.004% DPPH solution, reaction for 30 min under room temperature and away from light. Absorption value A was measured at 517 nm, zero setting of absolute ethanol. BHT was positive control.

$$\text{DPPH scavenging rate (\%)} = [A_0 - (A_1 - A_2)]/A_0 \times 100\% \quad (1)$$

In formula, A_0 - absorption value of 1.0 mL absolute ethanol + 2.0 mL DPPH solution; A_1 - absorption value of 1.0 mL sample solution + 2.0 mL DPPH solution; A_2 - absorption value of 1.0 mL sample solution+ 2.0 mL absolute ethanol.

ABTS assay

200 μL sample solution of Beibinghong anthocyanin with different concentrations were

collected, then added into 4 mL ABTS working solution (7 mmol/L ABTS mixed with 2.45 mmol/L potassium, persulfate at the ratio, reaction for 12 h to 16 h away from light, diluted by absolute ethanol, making absorption value was 0.7 ± 0.02 at 734 nm). After 10 minutes in the water bath at 37 °C, the absorption value at 734 nm was measured. BHT was positive control.

$$\text{ABTS scavenging rate (\%)} = [A_0 - (A_1 - A_2)]/A_0 \times 100\% \quad (2)$$

In formula, A_0 - absorption value of 200 μL absolute ethanol+ 4.0 mL ABTS solution; A_1 - absorption value of 200 μL sample solution+ 4.0 mL ABTS solution; A_2 - absorption value of 200 μL sample solution+ 4.0 mL absolute ethanol.

Reducing force

1.0 mL sample solution of Beibinghong anthocyanin with different concentrations were collected, then added into 2.5 mL of 0.2 mol/L phosphate buffer (pH = 6.6) and 2.5 mL of 1% potassium ferricyanide solution, then putting mixture into 50 °C water bath for keeping warm 20 min, then added into 2.5 mL 10%（w/v）trichloroacetic acid solution, mixed solution was centrifuged at 3000 r/min for 10 min. 2.5 mL supernatant was absorbed precisely, then added into 2.5 mL distilled water and 0.5 mL 0.1% ferric chloride solution, absorption value A was measured at 700 nm. Absolute ethanol replaced sample to measure A_0. BHT was positive control.

$$\text{Reducing force} = A/A_0 \quad (3)$$

Analysis of HPLC-MS

Mixed standard of delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, petunidin 3-O-glucoside, peonidin 3-O-glucoside, malvidin 3-O-glucoside, delphinidin, cyanidin, petunidin, peonidin and malvidin were prepared. Mixed standard of anthocyanin and sample solution of Beibinghong Anthocyanin after purification were analyzed for HPLC-MS.

Chromatographic condition: Agilent 1200-C18 column (2.1 mm × 150 mm, 3.5 μm); wave length was 530 nm; column temperature was 30 °C; sample size was 10 μL;

flow rate was 0.4 mL/min; moving phase A was 5% formic acid aqueous solution; moving phase B was methyl alcohol solution; condition of gradient elution was 0-5 min, 8-10% B; 5-23 min, 10% B; 23-25 min, 10-15% B; 25-28 min, 15-20% B; 28-30 min, 20-25% B; 30-32 min, 25-35% B; 32-42 min，35-45% B；42-47 min, 45-55% B; 47-50 min, 55-80% B.

Mass spectrometer condition: negative ionization mode (ESI^-) was performed; ion scanning mass range was 100-1500 m/z; nebulizer pressure was 35 psig; flow rate of drying gas was 8 L/min; gas temperature was 350 °C; VCap was 3.5 KV.

References

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Wang N, Zhang Y, Wang X, Huang X, Fei Y, Yu Y, Shou D. 2016. Antioxidant property of water-soluble polysaccharides from Poria cocos Wolf using different extraction methods. Int J Biol Macromol. 83:103-110.

Yuan J, Huang J, Wu G, Tong J, Xie G, Duan J-a, Qin M. 2015. Multiple responses optimization of ultrasonic-assisted extraction by response surface methodology (RSM) for rapid analysis of bioactive compounds in the flower head of Chrysanthemum morifolium Ramat. Ind Crop Prod.74:192-199.

Figure S1. SEM of untreated *Vitis amurensis Rupr*.

Figure S2. SEM of *Vitis amurensis Rupr* treated by pulsed electric fields.

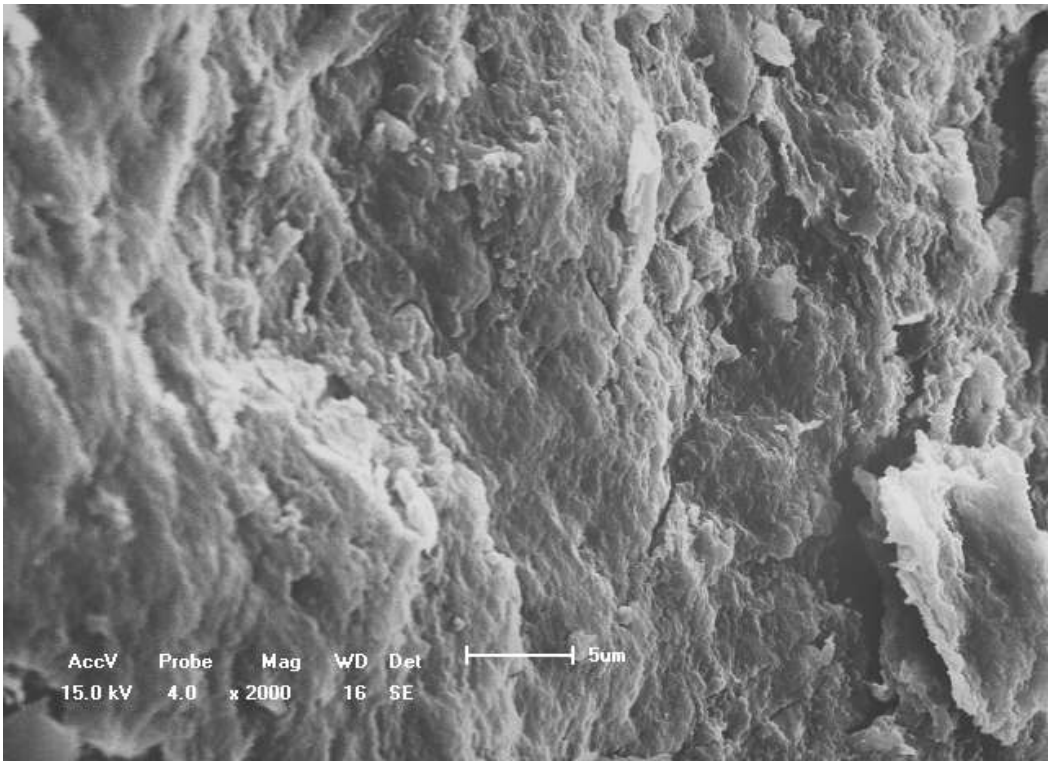
Figure S3. Total ion current diagram of anthocyaninof Beibinghong *Vitis amurensis Rupr* and its standards.

Figure S4. MS/MS spectra about peaks of Beibinghong anthocyanidin.

Table S1. Box-Behnken optimization design experiments and results.

Table S2. Variance analysis.

Table S3. Antioxidant ability of Beibinghong anthocyanidin.



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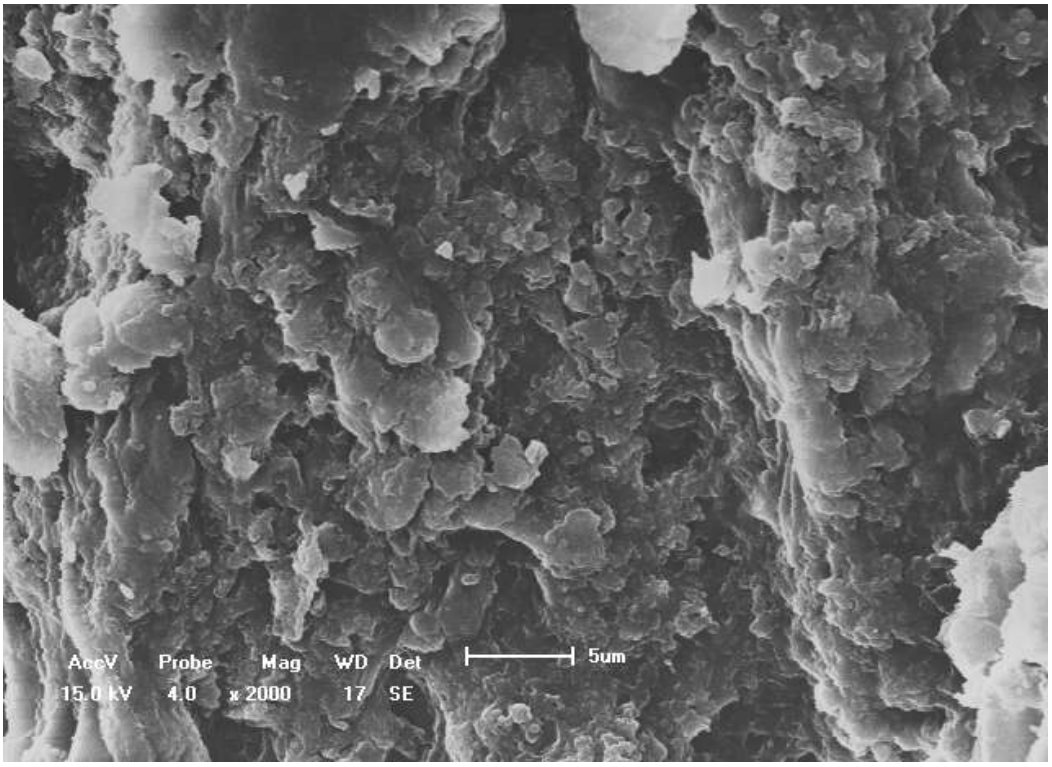


Figure S2. SEM of *Vitis amurensis Rupr* treated by pulsed electric fields.

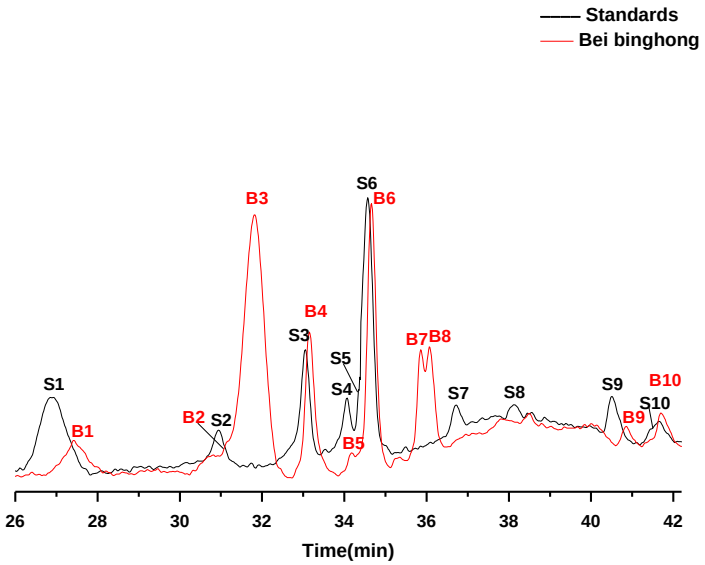


Figure S3. Total ion current diagram of anthocyaninof Beibinghong *Vitis amurensis Rupr* and its standards.

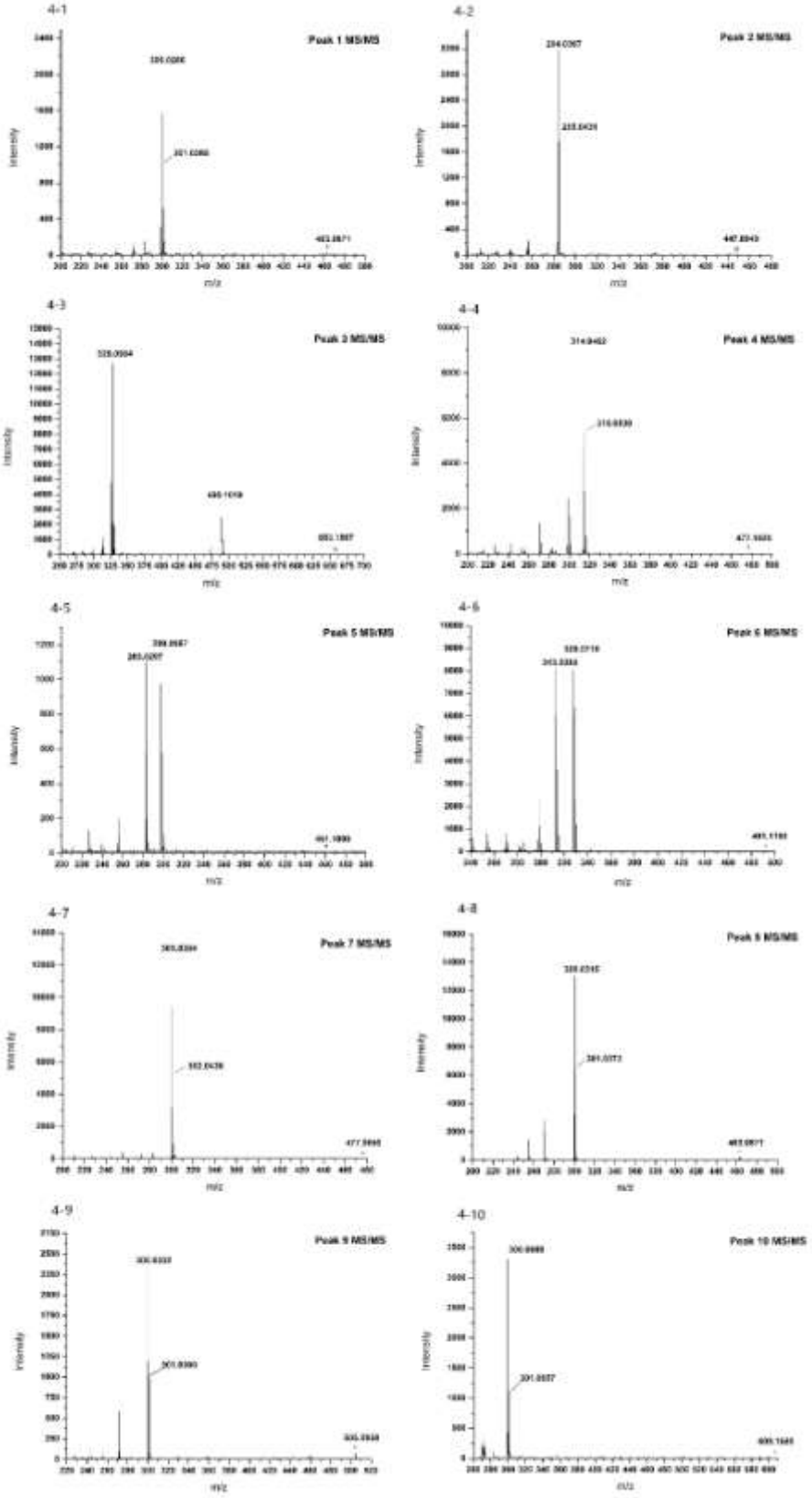


Figure S4. MS/MS spectra about peaks of Beibinghong anthocyanidin.

Table S1. Box-Behnken optimization design experiments and results.

Experiemntal number	A	B	C	The content of total anthocyanidin（mg/100g.FW）
1	5	15	5	145.91
2	7	13	3	137.89
3	9	15	3	148.25
4	9	15	5	153.93
5	7	15	4	159.94
6	7	17	5	147.25
7	7	15	4	161.94
8	5	15	3	133.22
9	7	17	3	139.9
10	5	13	4	147.91
11	5	17	4	148.92
12	7	15	4	160.94
13	7	15	4	162.61
14	9	17	4	158.27
15	7	15	4	160.27
16	7	13	5	148.92
17	9	13	4	156.6

Table S2. Variance analysis.

Source	Sum of Squares	df	Mean Square	F-Value	p-value
Model	1278.1272	9	142.0141	112.2036	< 0.0001
A-a ratio of material to solution	211.0485	1	211.0485	166.7469	< 0.0001
B-electric field intensity	1.1401	1	1.1401	0.9007	0.3742
C-pulse number	168.8203	1	168.8203	133.3829	< 0.0001
AB	0.1089	1	0.1089	0.0860	0.7778
AC	12.2850	1	12.2850	9.7062	0.0170
BC	3.3856	1	3.3856	2.6749	0.1460
A^2	42.8132	1	42.8132	33.8262	0.0007
B^2	106.3713	1	106.3713	84.0427	< 0.0001
C^2	670.9855	1	670.9855	530.1375	< 0.0001
Residual	8.8598	7	1.2657		
Lack of Fit	3.8220	3	1.2740	1.0115	0.4750
Pure Error	5.0378	4	1.2595		
Cor Total	1286.9870	16			

Table S3. Antioxidant ability of Beibinghong anthocyanidin.

Items	Concentration (mg/mL)	DPPH scavenging rate (%)	ABTS scavenging rate (%)	Reducing power
Beibinghong	0.002	18.34±0.67	15.67±1.67	5.67±0.34
	0.006	44.37±2.37	33.23±2.91	10.59±0.61
	0.010	60.70±3.00	42.49±2.60	20.32±1.80
	0.014	72.72±2.30	52.40±2.21	28.72±2.37
	0.018	83.40±2.30	72.70±2.96	33.2±2.07
BHT	0.060	37.29±2.41	71.66±3.13	11.35±1.98

ORIGINAL SOURCE

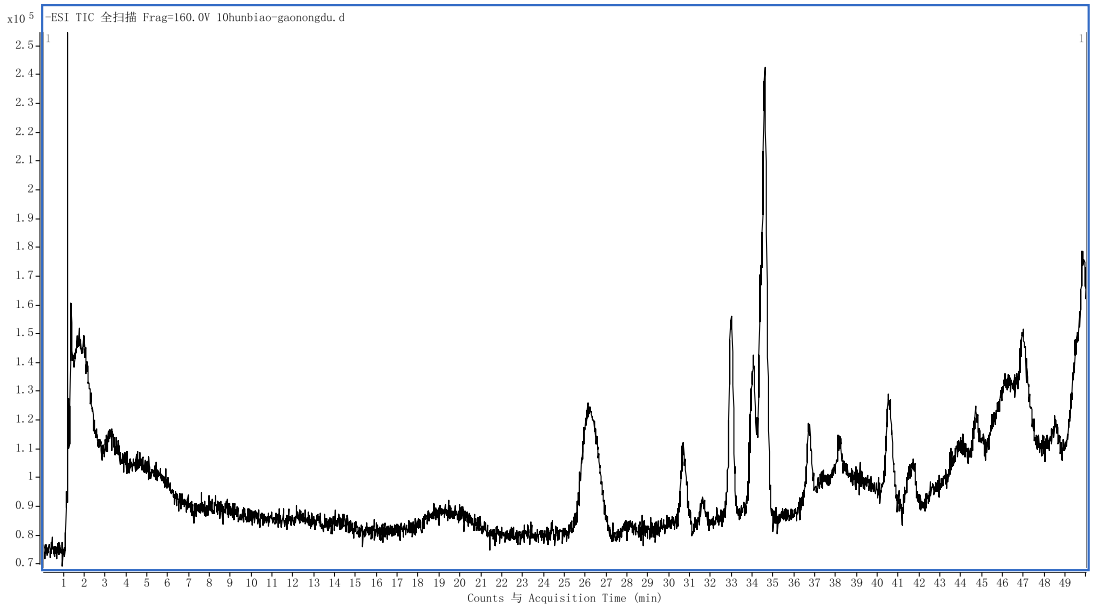


Figure S3------Original Graph (S)

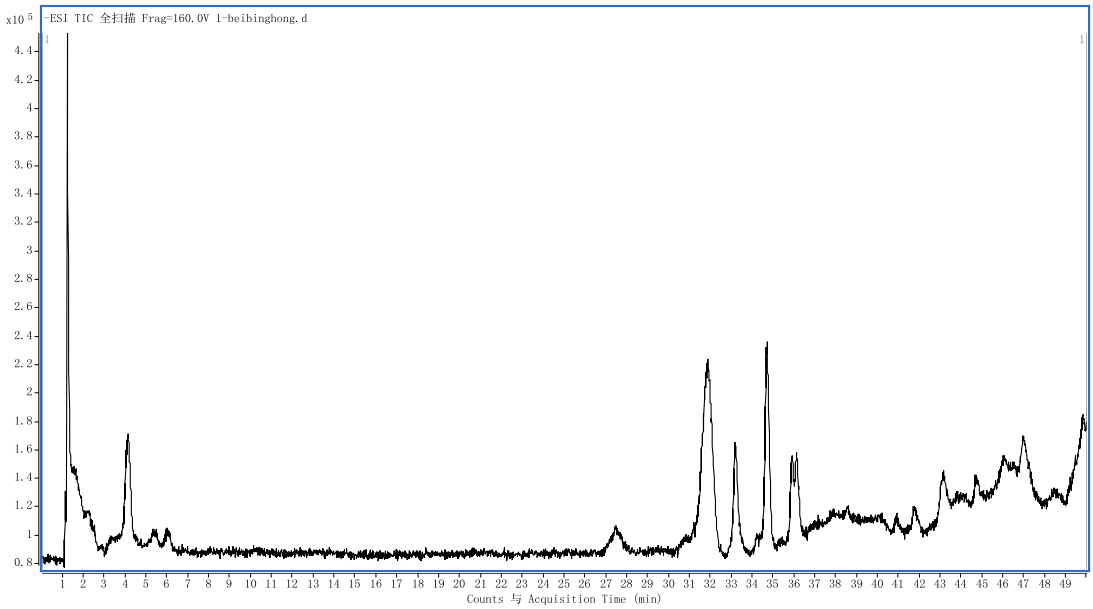


Figure S3------Original Graph (B)

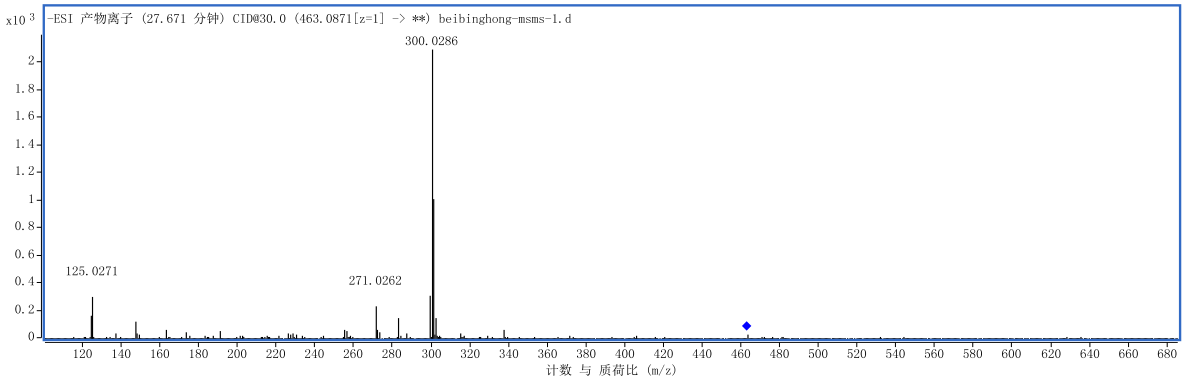


Figure S4------Original Graph (1)

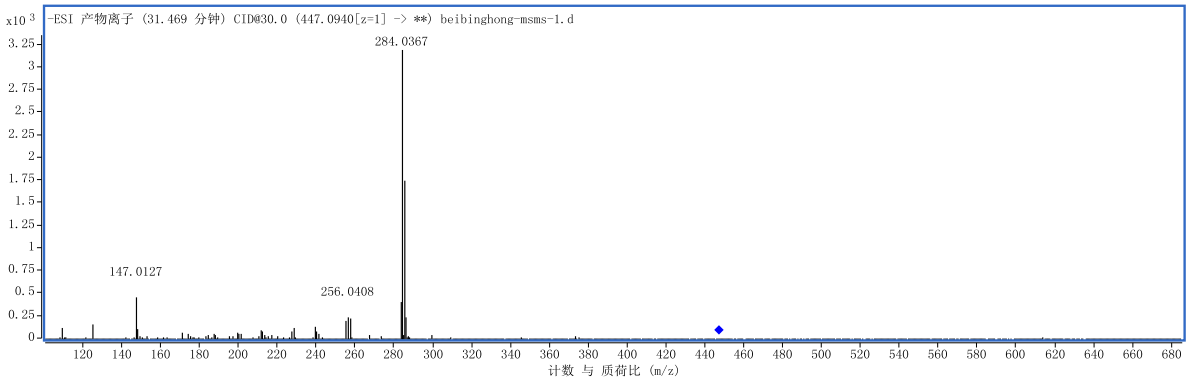


Figure S4------Original Graph (2)

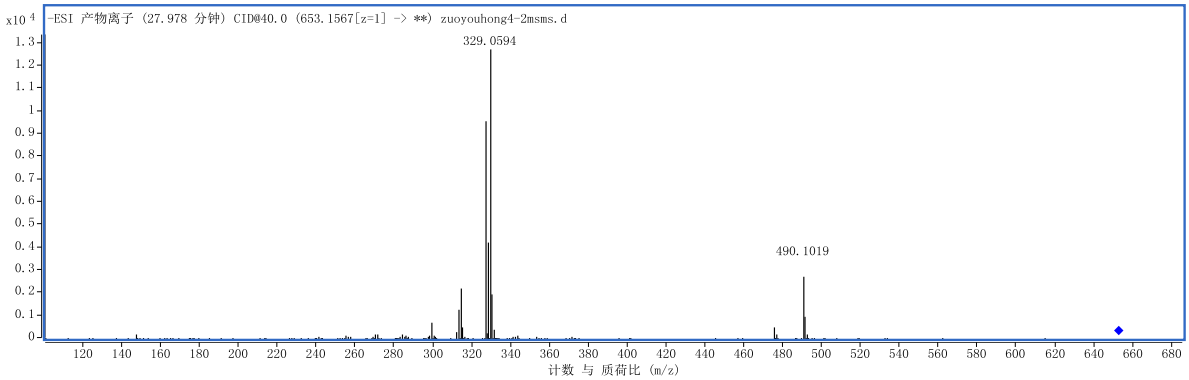


Figure S4------Original Graph (3)

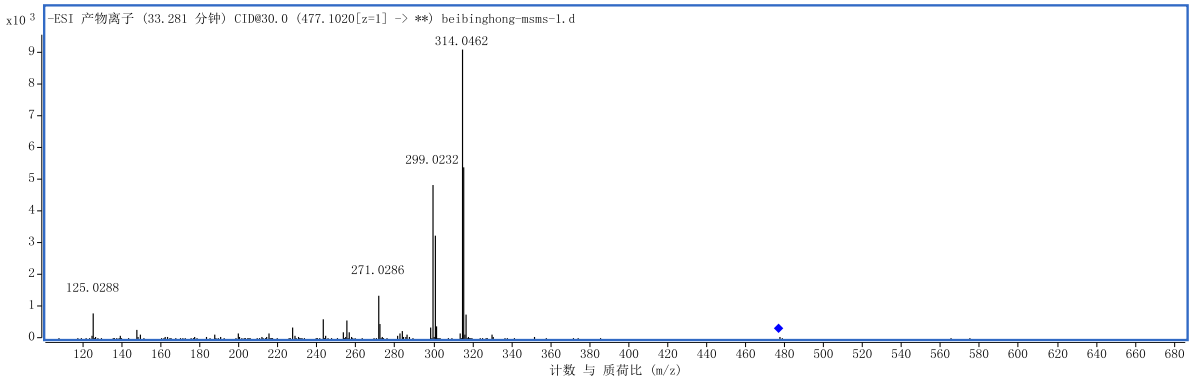
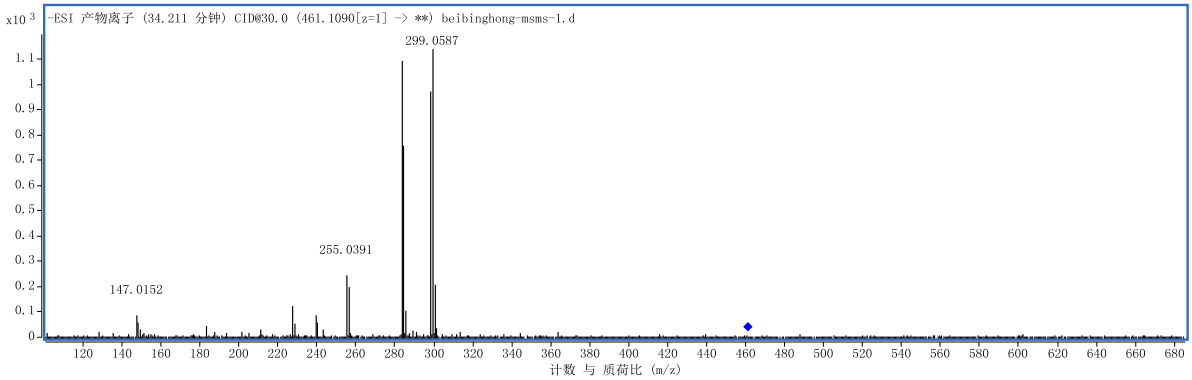


Figure S4------Original Graph (4)



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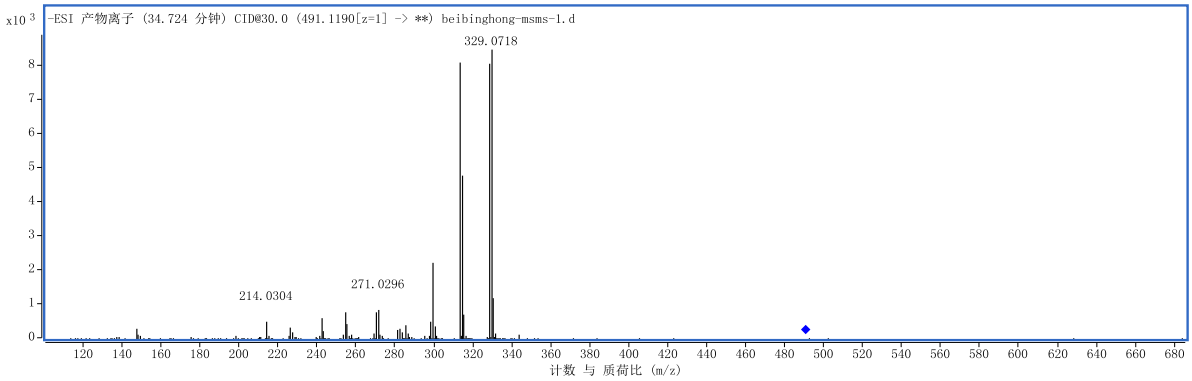

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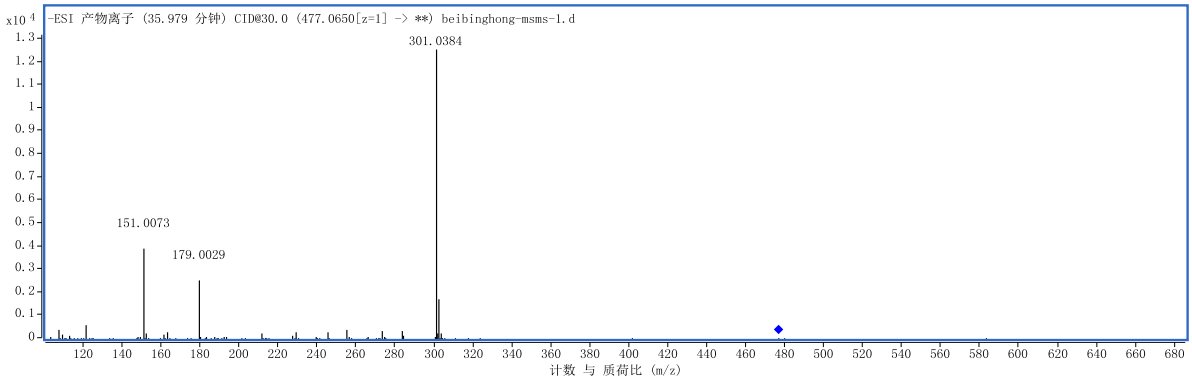

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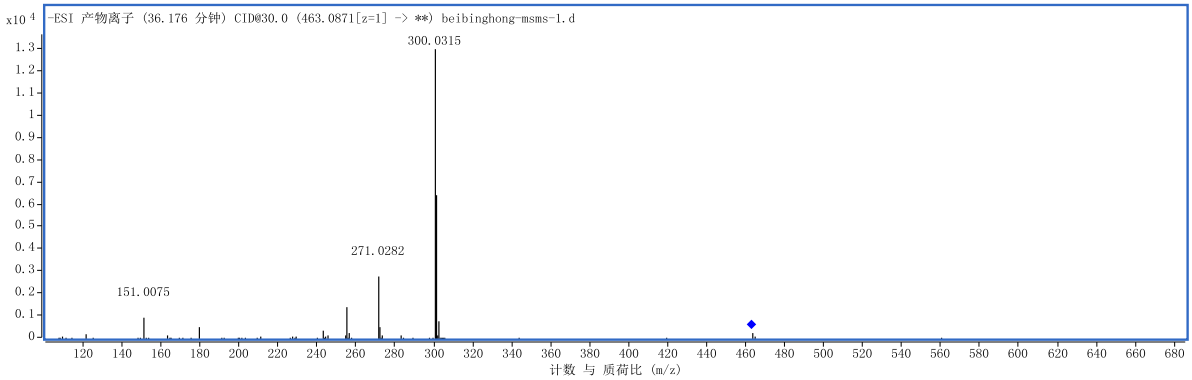

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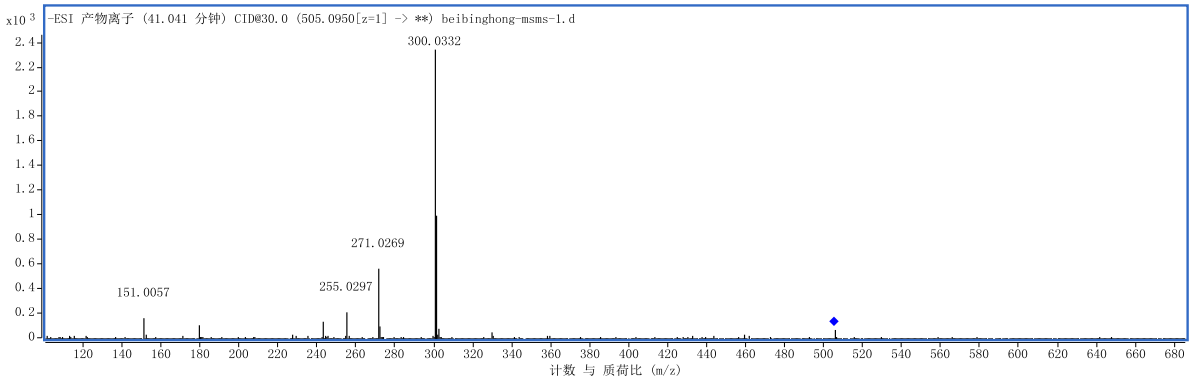

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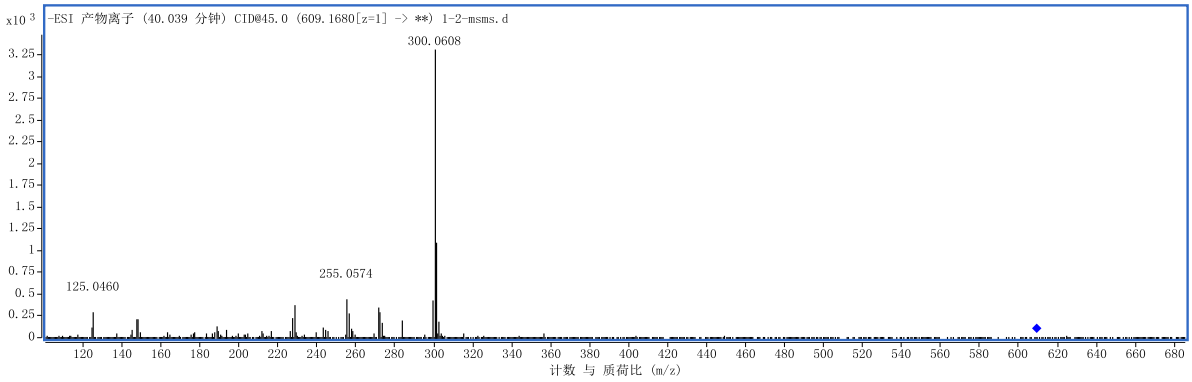

Figure S4------Original Graph (10)

	H1(Y)	A(X)	C1(Y)	D1(Y)	E1(Y)	F1(Y)	G1(Y)	B(Y)
Long Name	Items	Concentration	DPPH radical scavenging rate	Er	ABTS radical scavenging rate	Er	Reducing Power	Er
Units		mg/mL	%		%			
1	Bei binghong	0.002	18.34	0.67	15.67	1.67	5.67	0.34
2	Bei binghong	0.006	44.37	2.37	33.23	2.91	10.59	0.61
3	Bei binghong	0.01	60.7	3	42.49	2.6	20.32	1.8
4	Bei binghong	0.014	72.72	2.3	52.4	2.21	28.72	2.37
5	Bei binghong	0.018	83.4	2.3	72.7	2.96	33.2	2.07
6	BHT	0.06	37.29	2.41	71.66	3.13	11.35	1.98

Table S3------Original Graph