



Develop a Versatile Screening Method for the Discovery of Active Pharmaceutical Ingredients in Flowering Plants

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Introduction

Increase in popularity in herbal medicine (HM) means the requirement of evidence-based research. Phytochemicals are to be identified and characterized. Phytochemicals are secondary metabolites of plants that increase the herbs' survival fitness¹. **LC-MS/MS** is a potentially **accurate** and **efficient** tool to perform phytochemical fingerprinting for the extracts of botanical products². **Phytochemical fingerprinting** is crucial in development in commercial, technological, agronomical and ecological aspects of HM^{3,4}.

Aims

- Development of a versatile protocol for LC-MS/MS that allows the screening of phytochemicals in various plants through analytical quality by design (AQbD, Figure 1)

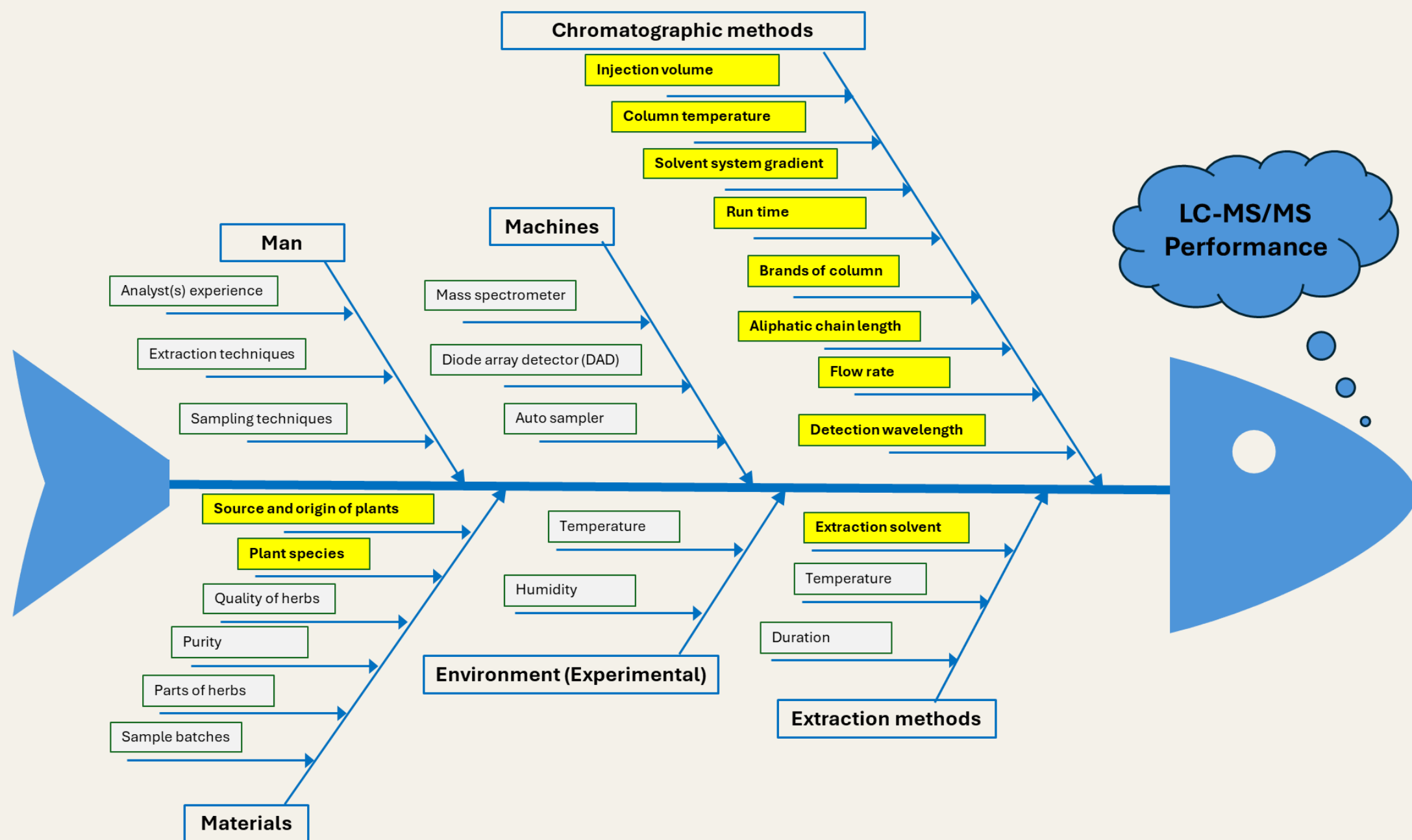


Figure 1: Ishikawa Fishbone Diagram on the LC-MS/MS Phytochemical Fingerprinting Performance
Highlighted cells represent parameters studied in this paper

Methods and Materials

Materials

- Dried flowers were purchased online from Mainland China and local Chinese medicine pharmacy
 - Bombax ceiba* (木棉花)
 - Chrysanthemum indicum* (菊花)
 - Styphnolobium japonicum* (槐花)
 - Lonicerae Japonicae* (金银花)
 - Plumeria rubra* (雞蛋花)

- Agilent 1260 HPLC with diode array detector (DAD) was used for the optimization of the versatile screening platform
 - SCIEX 5500+ QTRAP triple quadrupole (QqQ) tandem mass spectrometer (MS/MS) with electrospray ionization (ESI) in negative mode was used to identify phytochemicals through multiple reaction monitoring (MRM)

Methods

- Extracts were made with the modified method stated in Chinese Pharmacopoeia 2020⁵
- Design of Experiment (DoE) was employed to study the interactions between critical quality attributes (CQAs), including **sum of resolution (Y_{sum})** and **count (Y_n) of significant peaks** (Peak area > 1% of total chromatogram) and critical processing parameters (CPPs) with Box-Behnken Design (BBD, Table 1) based on the *B. ceiba* extract
- Respond surface analysis was done using Analysis of Variance (ANOVA) to frame the two polynomial equations followed by the evaluation of the fit of the models

Standard order	Flow rate (mL/min)	Run time (min)	Column temperature (°C)	Mean Y_{sum} (SD)	Mean Y_n (SD)
1	0.5	10	35	19.4 (0.555)	14 (0.00)
2	1	10	25	18.7 (1.18)	17 (1.00)
3	1	10	45	19.8 (1.08)	16 (0.00)
4	1.5	10	35	17.3 (0.384)	16 (0.577)
5	0.5	25	25	24.5 (0.210)	18 (0.00)
6	0.5	25	45	23.1 (1.00)	16 (0.00)
7	1	25	35	23.2 (1.11)	17 (0.577)
8	1	25	35	23.0 (2.68)	16 (1.15)
9	1	25	35	24.2 (0.714)	17 (0.577)
10	1.5	25	25	17.6 (1.43)	16 (0.577)
11	1.5	25	45	16.8 (0.798)	14 (0.577)
12	0.5	40	35	23.4 (2.98)	17 (1.15)
13	1	40	25	21.8 (0.180)	18 (0.00)
14	1	40	45	18.8 (4.81)	14 (3.79)
15	1.5	40	35	19.3 (1.46)	17 (1.00)
Levels of factors studied		Range Levels			
Flow rate (mL/min)		Low (-1)	Central (0)	High (+1)	
Run time (min)		0.5	1.0	1.5	
Column temperature (°C)		10	25	40	
		25	35	45	

Table 1: BBD Matrix for LC Method Optimization and Studied Responses

Results

- The regression models created for the CQAs cannot explain for the variation of data and predict future data (Table 2 & 3)
- Post hoc analysis revealed significant differences when using different columns, detection wavelength and extraction solvent, but not so with different aliphatic chain length (Figure 2)
- The diverse and complex nature of botanical extracts were demonstrated through the chromatograms (Figure 3). MRM reveals phytochemicals such as quinic acid, gallic acid, protocatechuic acid and chlorogenic acids were seen across the flowers sampled (Table 4)

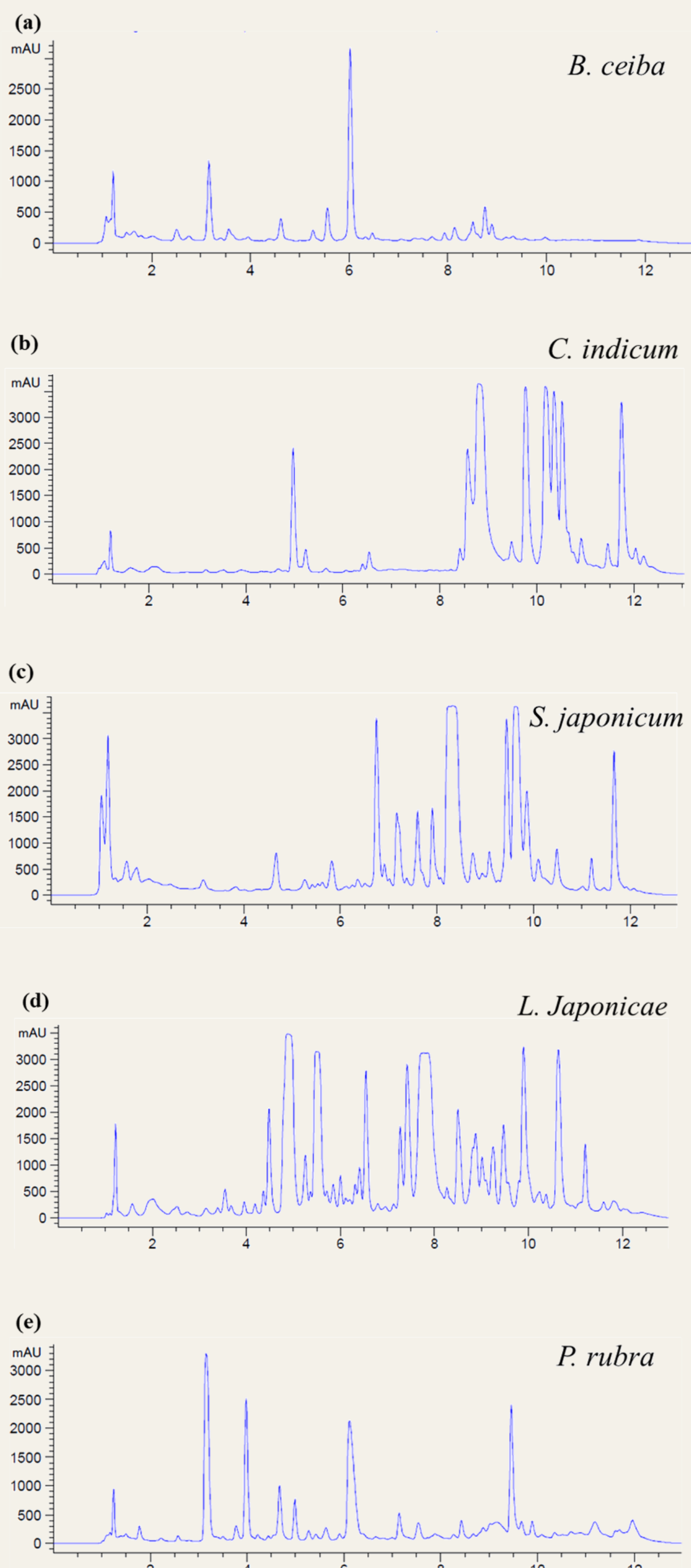


Figure 3: Chromatograms of Extracts from the Flowers of Five-Flowered Tea Using C18 Column*
*Phenomenex® HyperClone™ 4.6 x 100mm 3 µm C18 column

Source	Degree of freedom	Adj Sum of squares	Adj Mean squares	F-Value	P-Value
ANOVA result for response Y_{sum}					
Model	9	136.408	15.1564	1.99	0.071
Linear	3	53.272	17.7572	2.33	0.092
Flow rate (mL/min)	1	1.921	1.9210	0.25	0.619
Run time (min)	1	44.309	44.3088	5.80	0.021
Column temp (°C)	1	7.042	7.0417	0.92	0.343
Square	3	8.853	2.9511	0.39	0.763
Flow rate (mL/min)*Flow rate (mL/min)	1	5.577	5.5773	0.73	0.399
Run time (min)*Run time (min)	1	3.469	3.4686	0.45	0.505
Column temp (°C)*Column temp (°C)	1	0.125	0.1250	0.02	0.899
2-Way Interaction	3	74.283	24.7610	3.24	0.034
Flow rate (mL/min)*Run time (min)	1	61.427	61.4269	8.05	0.008
Flow rate (mL/min)*Column temp (°C)	1	1.527	1.5265	0.20	0.658
Run time (min)*Column temp (°C)	1	11.330	11.3296	1.48	0.231
Error	35	267.232	7.6352		
Lack-of-Fit	3	26.224	8.7413	1.16	0.340
Pure Error	32	241.008	7.5315		
Total	44	403.640			
ANOVA result for response Y_n					
Model	9	56.589	6.2877	3.57	0.003
Linear	3	12.333	4.1111	2.33	0.091
Flow rate (mL/min)	1	1.500	1.5000	0.85	0.363
Run time (min)	1	8.167	8.1667	4.63	0.038
Column temp (°C)	1	2.667	2.6667	1.51	0.227
Square	3	13.339	4.4463	2.52	0.074
Flow rate (mL/min)*Flow rate (mL/min)	1	9.028	9.0278	5.12	0.030
Run time (min)*Run time (min)	1	3.592	3.5919	2.04	0.162
Column temp (°C)*Column temp (°C)	1	0.771	0.7714	0.44	0.513
2-Way Interaction	3	30.917	10.3056	5.84	0.002
Flow rate (mL/min)*Run time (min)	1	30.083	30.0833	17.06	0.000
Flow rate (mL/min)*Column temp (°C)	1	0.083	0.0833	0.05	0.829
Run time (min)*Column temp (°C)	1	0.750	0.7500	0.43	0.519
Error	35	61.722	1.7635		
Lack-of-Fit	3	0.167	0.0556	0.03	0.993
Pure Error	32	61.556	1.9236		
Total	44	118.311			

Table 2: ANOVA results for Respond Y_{sum} and Y_n Obtained from BBD
Highlighted means significant ($p < 0.05$)

Y_{sum}			
S	R ²	R ² (adjusted)	R ² (predicted)
2.76319	33.79%	16.77%	0.00%
Y_n			
S	R ²	R ² (adjusted)	R ² (predicted)
1.32797	47.83%	34.42%	14.57%

Table 3: Model Summaries of the Respond Surface Analysis of Y_{sum} and Y_n

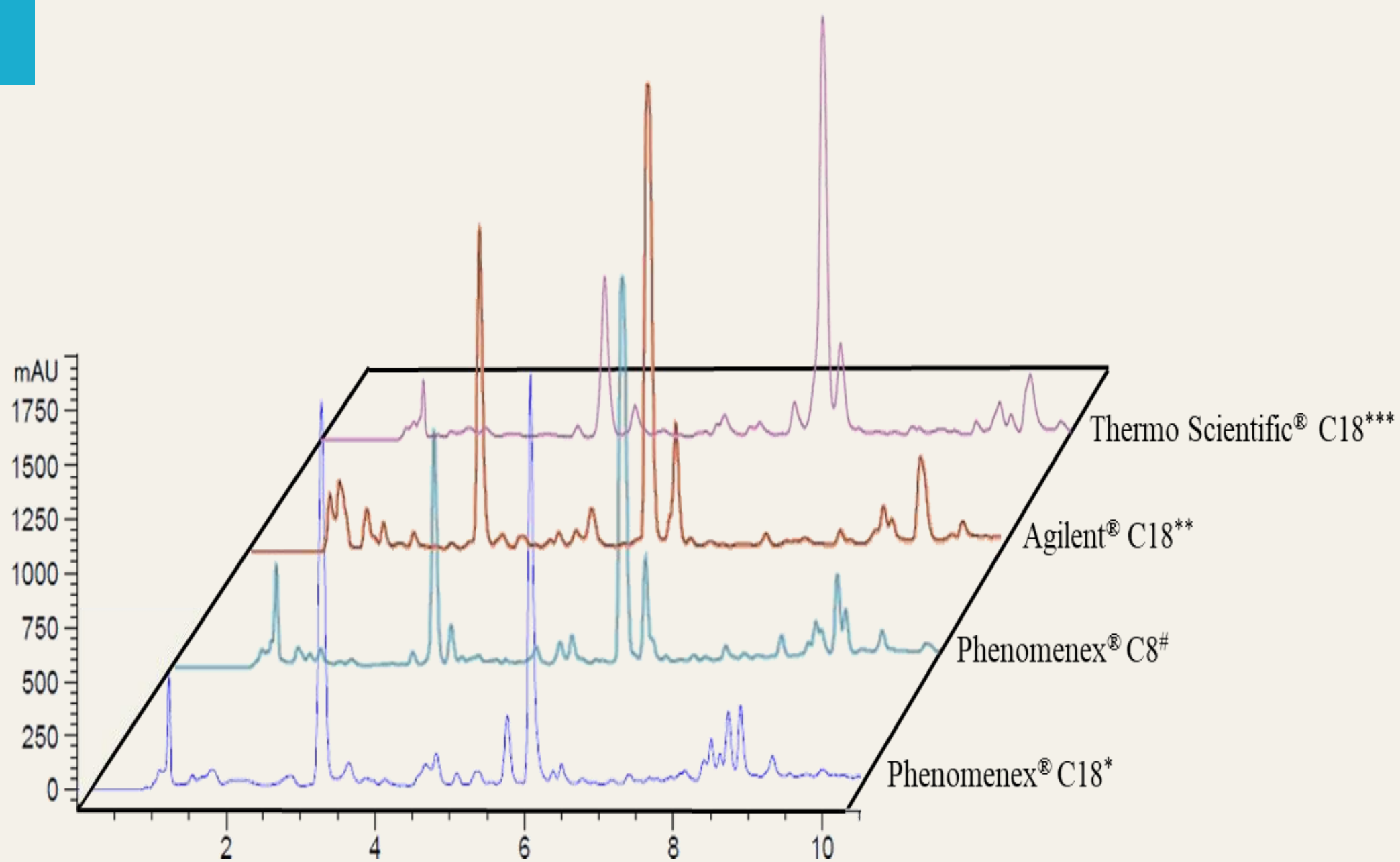


Figure 2: Chromatograms of *B. ceiba* Extract Using Different Columns
*Phenomenex® HyperClone™ 4.6 x 100mm 3 µm C18 column
#Phenomenex® HyperClone™ 4.6 x 100mm 3 µm C8 column
**Agilent® ZORBAX Eclipse Plus 4.6 x 100mm 3.5 µm C18 column
***Thermo Scientific® ODS HYPERSIL 4.6 x 100mm 3 µm C18 column

Phytochemicals	Molecular formula	B ceiba	C indicum	S japonicum	L Japonicae	P rubra	MRM transition (m/z)	Ref
Gallic acid	C ₇ H ₆ O ₅	Y		Y		Y	168.945→125.100 168.945→79.100	Reference standard
(-)-Epicatechin	C ₁₅ H ₁₀ O ₆						289.028→123.000 289.028→108.900	Reference standard
(-)-Epicatechin gallate	C ₂₂ H ₁₈ O ₁₀						441.220→96.800 441.220→80.000	Reference standard
Luteolin	C ₁₅ H ₁₀ O ₆						284.968→133.100 284.968→131.900	Reference standard
Quercetin	C ₁₅ H ₁₀ O ₇	Y					300.964→151.000 300.964→65.000	Reference standard
Chlorogenic acid	C ₁₆ H ₁₀ O ₉	Y	Y		Y	Y	353→191	PubChem ⁶
Quinic acid	C ₇ H ₁₂ O ₆	Y	Y		Y		191→127	Yin et al 2022 ⁷
Malic acid	C ₄ H ₆ O ₅						133→115	Yin et al 2022 ⁷
Ellagic acid	C ₁₄ H ₆ O ₈						301→284	Chernomsonov et al 2017 ⁸
Myricetin	C ₁₅ H ₁₀ O ₈						317→151	PubChem ⁹
Protocatechuic acid	C ₇ H ₆ O ₄	Y		Y		Y	137→93	Yin et al 2022 ⁷
Apigenin	C ₁₅ H ₁₀ O ₅						269→117	PubChem ¹⁰
Mangiferin	C ₁₉ H ₁₈ O ₁₁	Y	Y				421→259	Yin et al 2022 ⁷

Table 4: Characterization of Phytochemicals in the Flowers Using LC-MS/MS
Reference standard: MRM transitions were selected by the software using the standards
Y: Phytochemical present in the extract

Discussion

- Phytochemical fingerprinting of crude extract was difficult to be optimized, based on:
 - Complexity of crude extracts→ change in CPPs will not lead to significant changes on CQAs
 - Diversity of structures of phytochemicals (e.g. isomerism)→ inability to accurately identify phytochemicals despite use of LC-MS/MS
- Improvement can be made by:
 - Identify, understand and quantify all possible processing parameters from raw materials to final process^{11,12} (Figure 1)
 - Refining CQAs through selection of crucial phytochemicals only instead of full spectrum

Conclusion

- AQbD is an effective technique for development of methods for phytochemical fingerprinting
- However, greater investigation in understanding all possible parameters are needed
- Diversity of phytochemicals and complexity of crude extract maybe barriers to full phytochemical characterization

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