

# Is Your LC Separation Sufficient Enough for Botanical Extracts Quantification? -Accurate LC/LC-MS/MS Quantification of 5 Polyphenolic Isomers

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## OVERVIEW

### NOVEL ASPECT

A good example of LC separation for a routine simultaneous quantification of 5 polyphenolic isomers in botanical extracts with an accurate, specific and sensitive LC-MS/MS method.

### PURPOSE

To develop an accurate and simple liquid chromatography-tandem mass spectrometry platform with sufficient separation for the simultaneous quantification of 5 polyphenolic isomers in botanical extracts.

To validate the methods in botanical extracts matrices following FDA guidelines, using LC-MS/MS.

To quantify multiple isomers in botanical extracts and production samples.

### METHODS

Using LC-MS/MS in combination with automated online sample preparation (LC/LC-MS/MS) to quantify trace amounts of 5 polyphenolic isomers (BNER1135, Schaftoside, Neoschaftoside, Vicenin-1, and Vicenin-3).

### RESULTS

The simultaneous quantification of 5 polyphenolic isomers extracted from Chinese herbs using a sensitive LC-MS/MS method was developed and validated in herbal matrix. A very sufficient separation for all isomers was achieved with the Phenomenex XB-C18 column.

The recoveries of extraction from herbal matrix were > 80%. All actives in the extraction solution were stable for at least 3 days when stored at 4°C and 24 h at room temperature, allowing for sufficient time for analysis without degradation. The method had a range of reliable response from 1.56 to 200 ng/mL for all analytes. The intra-day and inter-day accuracies for the method were 90.7-103.5%, and the inter-day precisions were < 5.0%.

## INTRODUCTION

Botanicals have been used as medicinal remedies for the treatment of disease for centuries across many civilizations. Large number of polyphenolic compounds have structurally been identified, including flavones, isoflavones, and chalcones from botanicals. Most analytical methods have been developed by LC-MS/MS, to date, focused on single target compounds and lacked good chromatographic separation. Botanical extracts contain multiple compounds, metabolites and isomers in a complex matrix containing mono and polysaccharides, lipids, proteins and waxes. Fast LC-MS/MS methods are always chosen for their high-throughput benefits; however, a proper LC separation with sufficient run time is critical for quantification of target compounds in an extract, especially when multiple isomers are present. Our goal was to develop an accurate and sensitive LC-MS/MS method for simultaneous quantification of 4 polyphenolic isomers with high specificity in botanical extracts.

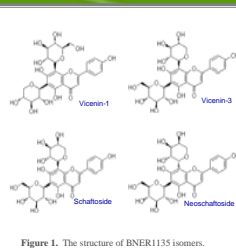


Figure 1. The structure of BNER1135 isomers.

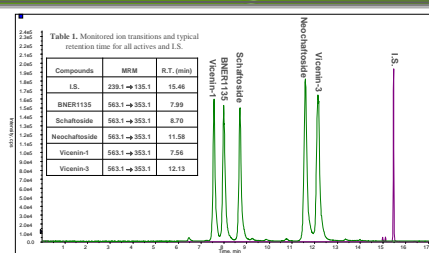


Figure 2. Chromatogram of standard mixture for all 5 isomers and internal standard (I.S.) separated with current LC-MS/MS method.

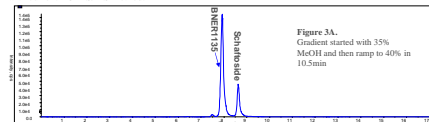


Figure 3A. Gradient started with 35% MeOH and then ramp to 40% in 10.5min

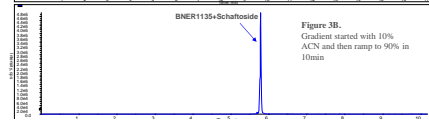


Figure 3B. Gradient started with 10% ACN and then ramp to 90% in 10min

Figure 3. Chromatograms of impure BNER1135 stock contained BNER1135 and Schaftoside eluted two different mobile phase systems. A: MeOH and B: ACN.

Table 2. Analysis linearity range, regression coefficient (r), LOD, and LLOQ for 5 isomers in herbal matrices (n = 3).

Compound ID	Linearity Range (LLOQ-ULOQ)	r			LLOQ (ng/ml)	S/N at LLOQ	LOD (ng/ml)	S/N at LOD
		Day 1	Day 2	Day 3				
BNER1135	1.56-200.0	0.9994	0.9996	0.9997	1.56	26.8	0.390	8.5
Schaftoside	1.56-200.0	0.9998	0.9998	0.9999	1.56	26.8	0.390	8.0
Neoschaftoside	1.56-200.0	0.9999	0.9999	0.9999	1.56	30.9	0.390	6.5
Vicenin-1	1.56-200.0	0.9995	0.9998	0.9998	1.56	25.9	0.390	4.2
Vicenin-3	1.56-200.0	0.9999	0.9998	1.0000	1.56	14.8	0.390	5.3

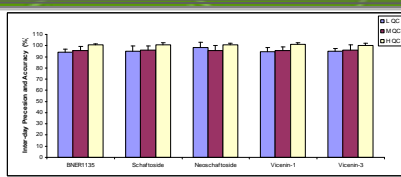


Figure 4. Inter-day (from 3 different days) precision and accuracy of 5 isomers at low, medium, and high QC concentration levels.

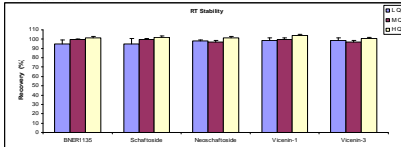


Figure 5. Stability of 5 isomers in extraction solution for 24 h (room temperature) at low, medium, and high QC concentration levels (n = 4).

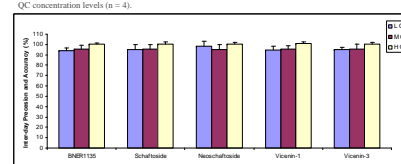


Figure 6. Stability of 5 isomers in extraction solution for 24 h (4°C) at low, medium, and high QC concentration levels (n = 4).

Table 3. The concentration of 5 isomers in herb (Luan Zi Xin, *Phanella Nelumbinis*) extracts from five provinces in China.

Sample Name	Final Conc. In Solid (mg/g)				
	BNER1135	Schaftoside	Neoschaftoside	Vicenin-1	Vicenin-3
LZSW	5.480	4.166	0.4073	2.259	1.501
LZSC	5.151	3.924	0.4507	2.153	1.576
LZHG	7.113	5.285	0.4008	2.810	1.918
LZST	5.510	4.033	0.4639	2.178	1.566
LZMC	4.216	3.255	0.2843	1.773	1.175

## EXPERIMENTAL

### Sample Preparation and Instrumentation:

Freeze dried botanical aqueous extracts were weighed out and mixed with an internal standard (2', 4'-dihydroxychalcone) solution (methanol/water/formic acid, 80/19.9/0.1, v/v) on a vortexer at room temperature for 15 min at 1,500 rpm. The above botanical solution (10 mg/mL) was then centrifuged for 15 min at 13,000 rpm. The appropriate dilutions were made from the supernatant and transferred to HPLC vials for analysis.

The calibrators and QCs were prepared from serial dilution of the standard working solution, which contained 5 isomer compounds (BNER1135, Schaftoside, Neoschaftoside, Vicenin-1, and Vicenin-3), with the same internal standard solution. Twenty microliters of sample was analyzed using a 2D-HPLC in combination with an API5000 system in ESI negative MRM mode. All analytes were chromatographed on a Phenomenex XB-C18 column (150 mm x 4.6 mm, 2.6 μm). Mobile phases were composed of aqueous formic acid (0.1%, v/v) and methanol with a flow rate of 0.8 mL/min. The linear gradient was started with 35% methanol and ramped to 40% methanol in 10.5 min, and then increased to 90% methanol in 1.5 min. The total run time was 17 min. An automated online sample extraction was achieved by loading samples on an Agilent Zorbax Eclipse XDB-C8 guard column (12.5 mm x 4.6 mm, 5 μm) and washing with 1% methanol for 0.5 min at a flow rate of 1.5 mL/min. The monitored ion transitions and typical retention times are shown in Table 1.

## RESULTS & CONCLUSIONS

For the five polyphenolic isomers (BNER1135, Schaftoside, Neoschaftoside, Vicenin-1, and Vicenin-3), the major interferences in our LC-MS/MS analysis, were the highly similar chemical structures of the compounds (Figure 1) and the fact that they have the same precursor ion ( $m/z = 563$ ) and product ion ( $m/z = 353, 383$ ) in negative ion mode. This made the LC separation very difficult. Therefore, the specific LC separation condition, for each of these compounds/isomers, is critical for accurate quantification of the compounds in the extracts. Methanol, methanol/acetonitrile (50/50, v/v), and acetonitrile were studied as the organic mobile phases. When we utilized the similar gradient, acetonitrile co-eluted Schaftoside and BNER1135 into one single peak; one split peak of schaftoside and BNER1135 was found when methanol/acetonitrile (50/50) was used; when pure methanol was used as the mobile phase, all five compounds separated distinctly under the conditions we developed. Using the Phenomenex Kinexet XB-C18 column with a slow linear gradient proved beneficial for the isomers' separation (Figure 3). Figure 2 is an LC-MS/MS chromatogram of all isomers and internal standard. All 5 isomers were separated from each other under condition we developed.

The simultaneous quantification of 5 polyphenolic isomers extracted from herb extracts using a sensitive LC-MS/MS method was developed and validated in the botanical matrix. The prepared QC (Low, Medium, and High) samples were stable at room temperature for at least 24 h with high accuracies (94.7-103.7%) (Figure 5). All analytes in the extraction solution were stable for at least 24 h when stored at 4°C (e.g., on an autosampler) (Figure 6); this allows for sufficient time for analysis without degradation. The extraction procedure was systematically developed and the conditions described above gave the best separation among the five analytes. No interfering peaks were found at the retention times of analyzed compounds. The LC-MS/MS methods we developed under the optimized conditions are highly selective.

The method was highly sensitive and had a linear range of reliable response from 1.56 to 200 ng/mL for all isomers. The lower limits of quantitation (LLOQ) was 1.56 ng/mL for all isomers. The linear range was determined by regression coefficients (r) of greater than 0.999. The typical standard curve parameters for all isomers in herbal matrices are shown in Table 2. The intra-day and inter-day accuracies for the method were 90.7-103.5%, with the precisions were <5.0% (Figure 4). The method was applied to the analysis of the concentration of the five isomers in the herb extracts from five provinces in China. (Table 3) The BNER1135 and Schaftoside had greater amount in the extract than the other three isomers.

In summary, the LC/LC-MS/MS assay for the separation and quantification of BNER1135, Schaftoside, Neoschaftoside, Vicenin-1, and Vicenin-3 in botanical extracts is specific and accurate for each of these compounds, and met all pre-defined acceptance criteria.