Using UHPLC-Triple Quadrupole MS/MS to Detect the Presence of Bark Extract and Yohimbine Adulteration in Dietary Supplements and Botanicals

Application Note

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Abstract

A method using QuEChERS sample preparation and an Agilent 6490 Triple Quadrupole LC/MS has been developed that provides rapid and sub-parts per billion (ppb) detection of synthetic yohimbine and yohimbe bark extract in dietary supplements. Using the chromatographic separation of the diastereomers of yohimbine, the synthetic compound can be distinguished from the tree bark extract. An Agilent 6550 Q-TOF LC/MS system was used to confirm the presence of yohimbine and ajmalicine diastereomers, and the absence of any previously unidentified analogs.

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Introduction

Yohimbine is the primary alkaloid found in bark extracts of the *Pausinystalia johimbe* tree used in dietary supplements. These extracts are thought to enhance both sexual and athletic performance, although this claim is not fully supported by available clinical data. In fact, a risk assessment of the data on both yohimbe bark extracts and yohimbine HCl, which is an antagonist of the α2-adrenoreceptor, revealed a harmful effect to human health [1].

Yohimbine HCl has been associated with adverse health effects and death in Australia, and is banned in that country. It is a prescription drug in the USA, but when used in a dietary supplement it must originate from the bark when yohimbine or yohimbine extract is listed. The European Union also regulates yohimbe bark as a food additive. Robust and efficient analytical methods are needed to detect and characterize yohimbine and related alkaloids derived from bark extracts, and may be present at trace levels in complex dietary supplement matrices.

This application note summarizes the results of a published study [2] that used both liquid chromatography (LC)-triple quadrupole mass spectrometry (MS) and LC-quadrupole time-of-flight (Q-TOF) MS to quantitate and characterize trace levels of indole alkaloids present in yohimbe bark. The two major alkaloids, yohimbine and ajmalicine, were detected at levels as low as 0.1 parts per billion (ppb) using LC/MS with a run time of less than 15 minutes, and good chromatographic resolution of their diasteriomers. Qualitative LC-Q-TOF analysis revealed all known analogs of these alkaloids, and established the absence of any previously unidentified indole alkaloids in yohimbe bark extract.

Experimental

Samples

Samples were purchased from local dietary supplement stores as described [2]. Yohimbe bark was obtained from the botanical research collection at Flora laboratories.

Reagents and standards

Yohimbine HCl and ajmalicine standards were obtained from Sigma-Aldrich. Other reagents were obtained and used as described [2].

Instruments

This study was performed on an Agilent 1290 Infinity LC system equipped with:

- Agilent 4226A autosampler
- Agilent 4220A binary pump
- Agilent 1331C thermal column compartment

Quantitative analysis was performed using an Agilent 6490 Triple Quadrupole LC/MS with Agilent Jet Stream technology. Qualitative analysis to identify alkaloid analogs was done using an Agilent 6550 Q-TOF LC/MS system with the dual Jet Stream technology source. The second nebulizer was used to infuse internal reference compounds for accurate mass measurement better than 2 ppm. Table 1 shows the instrument conditions.

Sample preparation

Samples consisting of tablets, capsules, gel caps, and powders were homogenized and extracted using a QuEChERS protocol as described [2]. Briefly, homogenized 1 g samples were extracted by first adding water (9 mL), then acetonitrile (10 mL), and vortexing after each addition. Agilent QuEChERS salts (p/n 5982-5550) were added, followed by mechanical shaking for 3 minutes, and centrifugation at 8,500 x g for 5 minutes. Concentrated samples used a dispersive SPE (dSPE with C_{18}) to remove matrix as described [2]. Finally, 10 to 50,000 dilution was done with initial mobile phase, followed by filtration into an autosampler vial.

Acquisition parameters for Triple Quadrupole MS/MS

Yohimbine

m/z 355.2 → 211.9, CE = 24 eV
→ 144†, CE=32 eV

Ajmalicine

m/z 353.2 → 117.1, CE = 64 eV
→ 144†, CE =28 eV

†Quantitative transition

Dynamic multiple reaction monitoring (dMRM) was used across the entire chromatogram. The user needs only to set the cycle time (see Table 1), and the dwell time is set automatically.

Table 1. LC, Triple Quadrupole MS, and Q-TOF MS Run Conditions

LC Conditions	**Quantitative analysis**	**Qualitative analysis**
Column	Agilent Poroshell 120 EC-C18, 2.1 × 100 mm, 2.7 µm (p/n 695775-902)	Agilent SB-C18, 2.1 × 150 mm, 1.8 µm (p/n 859700-902)
Column temperature	Ambient	Ambient
Injection volume	2 µL	1 µL
Mobile phase	A = 0.1% formic acid in water B = 0.1% formic acid in methanol	A = 0.1% formic acid in water B = 0.1% formic acid in methanol
Flow rate	0.4 mL/min	0.35 mL/min
Linear gradient	Time (min) % B 0.00 25 hold 0.5 25 9.00 50 12.00 95	Time (min) % B 0.00 5 hold 2.0 5 14 90 15 100 17 100
Run time	12 minutes	17 minutes
MS Conditions		
Instrument	Agilent 6490 Triple Quadrupole LC/MS System	Agilent 6550 Q-TOF LC/MS System
Ionization mode	ESI in positive ion mode; Dynamic MRM	ESI in positive ion mode, same source as the 6490; Dynamic MRM
N_2 Drying gas temperature	150 °C	150 °C
N_2 Drying gas flow	15 L/min	17 L/min
Nebulizer pressure	30 psig	30 psig
Sheath gas temperature	350 °C	350 °C
Sheath gas flow	11 L/min	11 L/min
Capillary voltage	4,000 V	4,000 V
Nozzle voltage	1,000 V	500 V
High pressure Rf	150 V	Default value
Low pressure Rf	60 V	Default value
Acquisition rate	Cycle time = 500 ms	All Ions MS/MS: 2.5 spectra/sec for each collision energy Auto MS/MS: 4 spectra/sec for MS, and 3 spectra/sec for MS/MS
Collision energy (for MS/MS experiments)	See Experimental Acquisition Parameters [2]	All Ions MS/MS used 0 eV for pseudomolecular ions, and 30 eV for fragment generation. Auto MS/MS used 30 eV
Mass range	See Experimental Acquisition Parameters [2]	MS) *m/z* 100–1,000 Auto MS/MS) *m/z* 50–500
Reference mass correction		Protonated ions of purine (*m/z* 121.0508) and hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine (*m/z* 922.0098)

Results and Discussion

Sensitive, accurate, and reproducible quantitation using the Agilent 6490 Triple Quadrupole LC/MS

The calibration range for both yohimbine and ajmalicine was 0.1 to 100 ppb. The coefficient of linearity (R^2) was 0.9999 for both compounds (Figure 1). In addition, the signal-to-noise ratio (S/N) at 0.1 ppb was 420:1 for yohimbine, and 190:1 for ajmalicine. Using QuEChERS extraction and cleanup, this quantitative method is highly accurate and reproducible. Accuracy (recovery of spiked standard in a dietary supplement) varied from 99.7 to 103.3%. Reproducibility, expressed as percent relative standard deviation (%RSD) ranged from 2.0 to 3.6% across the concentration range of the calibration curve (Table 2). This is in contrast to accuracy ranging from 92.2 to 109.5%, and reproducibility as high as 6.4% using the dilute, filter, and shoot sample prep method.

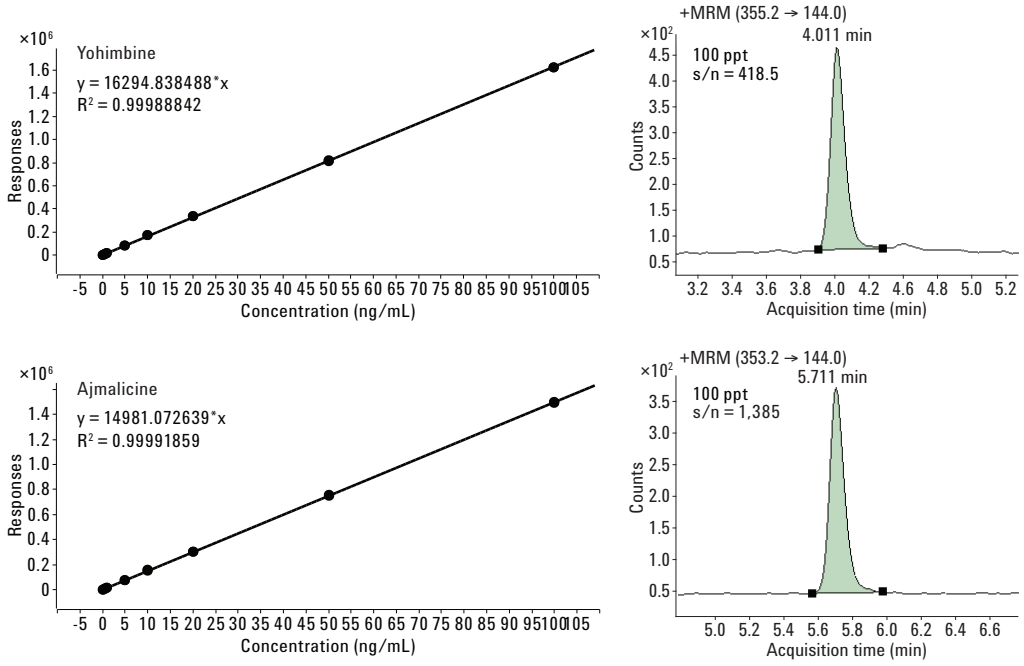

Figure 1. Calibration curves from 0.1 to 100 ppb for yohimbine and ajmalicine, using an Agilent 6490 LC-Triple Quadrupole MS.

*Table 2. Accuracy and Precision in a Spiked Dietary Supplement† Using an Agilent 6490 LC-Triple Quadrupole MS**

		10 ng/mL		50 ng/mL		100 ng/mL	
Sample prep	**Analyte**	**Recovery (%)**	**RSD (%)**	**Recovery (%)**	**RSD (%)**	**Recovery (%)**	**RSD (%)**
Dilute, filter, shoot	Yohimbine	109.5	4.2	100.9	2.2	95.3	3.5
	Ajmalicine	102.3	6.4	94.2	1.6	92.2	1.3
QuEChERS	Yohimbine	103.3	2.0	102.9	2.1	102.6	3.0
	Ajmalicine	102.4	2.4	99.7	3.5	100.3	3.6

†Product 1, Table 3
*n = 3

As a test of the utility of the method, 10 dietary supplements marketed for improved athletic and sexual performance were analyzed, six of which are shown in Table 3. Three of the 10 supplements did not contain any detectable yohimbine or ajmalicine, while one contained three times the concentration of yohimbine specified on the label [2].

Table 3. Dietary Supplement and Yohimbe Bark Analysis Using an Agilent 6490 LC-Triple Quadrupole MS

Sample	Yohimbine content (µg/g)	Ajmalicine content (µg/g)	Dilution factor
Product 1	ND	ND	100x
Product 2	0.0146	ND	100x
Product 3	ND	ND	100x
Product 4	72.544	0.107	1,000x
Product 5	9,910.8	74.900	50,000x
Product 6	29,474	580.25	500,000x
Wild yohimbe bark	10,752	59.905	500,000x

ND = Not detected

Detection of isomers

The Agilent 6400 Series Triple Quadrupole LC/MS also enabled detection of the isomers of yohimbine and ajmalicine. Using two transitions for each compound, analysis was done of both yohimbine and ajmalicine standards, as well as a yohimbe bark extract. The multiple reaction monitoring (MRM) chromatogram obtained for the standards reveals only the presence of the two compounds (Figure 2). However, analysis of the yohimbe bark reveals the presence of several other diastereomers of these two alkaloids that have the same MS/MS transitions as the yohimbine and ajmalicine standards. Diasteriomers are compounds with more than one chiral center, are not superimposable (mirror images), and can be separated chromatographically under appropriate conditions, as shown in Figure 2 of the yohimbe bark extract. Yohimbine is the predominant peak in the yohimbe bark, while ajmalicine is a very minor one. The US Federal Drug Administration (FDA) requires that the yohimbine in dietary extracts originate only from yohimbe bark when bark or extract are declared on the label. This LC-MS/MS method can be used to try to determine whether or not a supplement is compliant, since only yohimbe bark contains the diastereomer peaks.

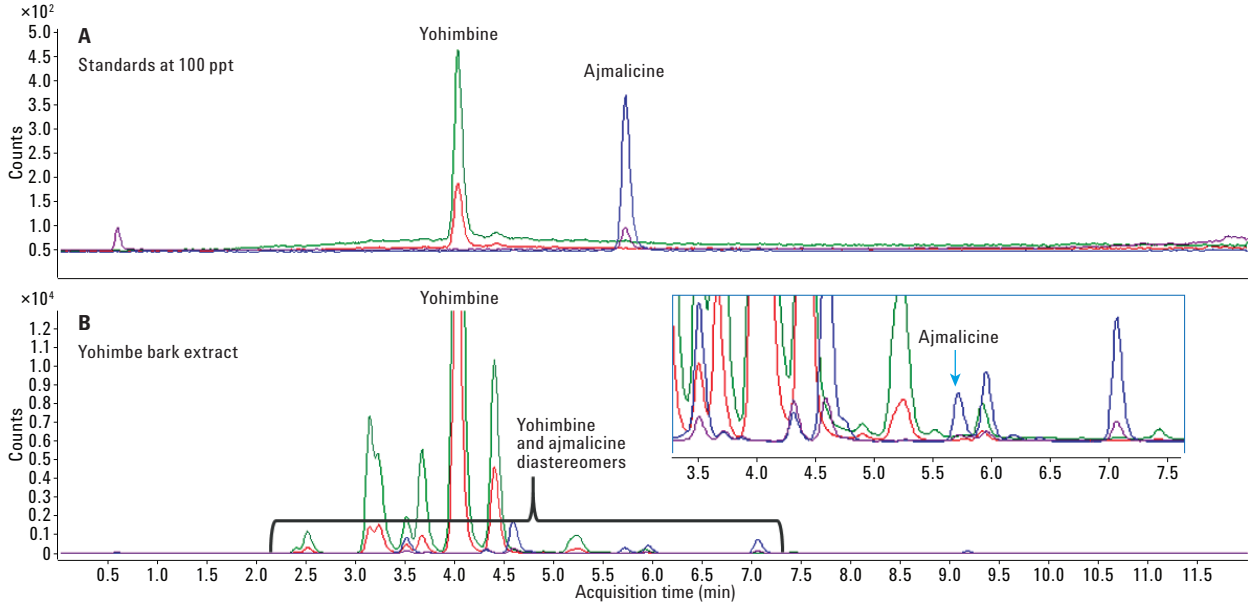

Figure 2. MRM chromatograms of yohimbine and ajmalicine standards (A) and yohimbe bark extract (B). The two transitions for each compound are overlaid. For the yohimbe bark extract, the region from about 3.3 to 7.7 minutes is expanded to show the ajmalicine peak, which is a minor component relative to yohimbine. The bracket encompasses the region in which the diastereomers of these indole alkaloids appear.

In fact, the difference in MRM chromatogram patterns between standards and yohimbe bark was subsequently used to attempt to determine the possible source of these compounds in the 10 dietary supplements [2]. The patterns obtained for two of these are shown in Figure 3 as examples. The results suggest that yohimbine and ajmalicine in Product 2 were not derived from yohimbe bark, and probably came from synthetic sources, since the peak patterns for yohimbine and ajmalicine transitions do not look very similar to those in the yohimbe bark.

Conversely, the peak patterns for both compounds in Product 6 are very similar to those in yohimbe bark, suggesting that the bark was the source of these compounds in this product. These results suggest that pattern analysis of LC-Triple Quadrupole MS/MS chromatograms of products claimed to contain yohimbine may be useful in determining whether the yohimbine and ajmalicine were derived from synthetic compounds or from yohimbe bark. However, more work is required to confirm the utility of this approach.

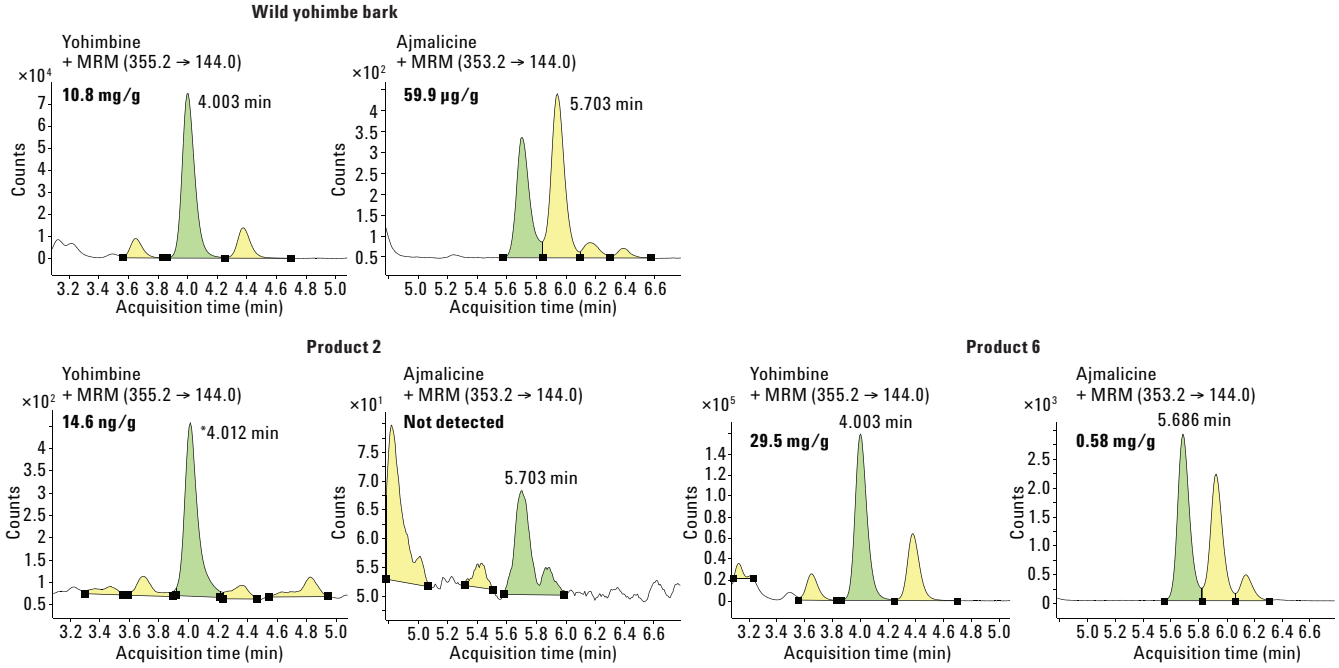

Figure 3. MRM chromatograms of yohimbe bark as well as two dietary supplement products. The peak patterns for yohimbine and ajmalicine transitions in Product 2 do not look very similar to those in the yohimbe bark, suggesting that bark was not the source of the yohimbine and ajmalicine in this product. However, the peak patterns for both compounds in Product 6 are very similar to those in yohimbe bark, suggesting that the bark was the source of these compounds in this product.

Identification of indole alkaloid analogs using LC-Q-TOF MS/MS

The extracted ions *m/z* 144.0807 and *m/z* 212.1287 were used to look for analogs, because the first ion is common to compounds with the ethyl-indole moiety, and the second represents the neutral loss of the ethenyl-indole moiety 143.0727. All Ions MS/MS was used to perform an all-inclusive search for these fragments. The same was done with Auto MS/MS, but All Ions MS/MS has the advantage of obtaining fragments for all ions being detected. In a complex matrix, Auto MS/MS may miss some coeluting ions in the single MS due to cycle time and the number of ions coeluting. However, in Auto MS/MS a neutral loss interrogation of the data set can be performed, and that is not available in All Ions MS/MS. No new analogs of the indole alkaloids were found using Q-TOF analysis with these two fragment ions in both All Ions MS/MS and Auto MS/MS modes. In addition, neutral loss of 143.0727 in Auto MS/MS mode revealed no new analogs. However, the Q-TOF MS/MS spectra did identify the chromatographic peaks appearing in the LC-Triple Quadrupole MS/MS analysis of yohimbe bark (Figure 2) as diastereomers of yohimbine and ajmalicine.

Conclusion

A rapid LC-Triple Quadrupole MS/MS method using QuEChERS sample preparation has been developed that produces the sensitivity, selectivity, and accurate quantitation required to provide the information needed to meet the diverse regulatory requirements for yohimbine around the world. In some regions, the yohimbe bark extract is allowed in dietary supplements, but the use of the synthetic compound yohimbine HCl is banned. This method is ideal for such regions, since it can distinguish bark extracts from synthetic yohimbine by the presence of diastereomers in the bark extract. The identity of these isomers was supported with full accurate mass MS/MS spectra using LC-Q-TOF MS/MS, which also demonstrated the absence of any previously unidentified analogs of yohimbine or ajmalicine.

References

1. E. Ernst, M. H. Pittler "Yohimbine for Erectile Dysfunction: A Systematic Review and Meta-Analysis of Randomized Clinical Trials" *J. Urol*. **159**, 433-436 (1998).
2. D. Lucas, J. Neal-Kababick, J. Zweigenbaum "Characterization and Quantitation of Yohimbine and Its Analogs in Botanicals and Dietary Supplements Using LC/QTOF-MS and LC/QQQ-MS for Determination of the Presence of Bark Extract and Yohimbine Adulteration" *J. AOAC Int*. **98**, 330-335 (2015).

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