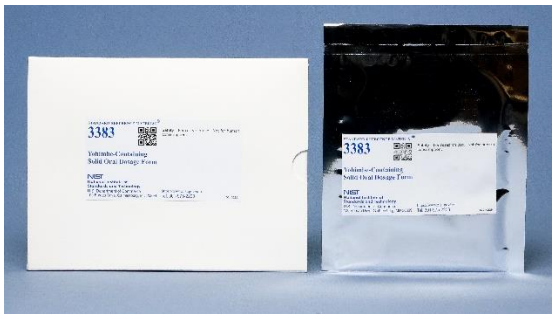
NIST Special Publication 260
NIST SP 260-240

Certification of Standard Reference Material® 3383

Yohimbe-Containing Solid Oral Dosage Form

Walter B. Wilson
Sanem Hosbas Coskun
Jerome Mulloor
Michael A. Nelson
James H. Yen



This publication is available free of charge from:
https://doi.org/10.6028/NIST.SP.260-240

NIST NATIONAL INSTITUTE OF STANDARDS AND TECHNOLOGY
U.S. DEPARTMENT OF COMMERCE

This page intentionally blank.

NIST Special Publication 260
NIST SP 260-240

Certification of Standard Reference Material® 3383

Yohimbe-Containing Solid Oral Dosage Form

Walter B. Wilson, Sanem Hosbas Coskun (Associate)
Jerome Mulloor, Michael A. Nelson
Chemical Sciences Division
Material Measurement Laboratory

James H. Yen
Statistical Engineering Division
Information Technology Laboratory

This publication is available free of charge from:
https://doi.org/10.6028/NIST.SP.260-240

April 2024

U.S. Department of Commerce
Gina M. Raimondo, Secretary

National Institute of Standards and Technology
Laurie E. Locascio, NIST Director and Under Secretary of Commerce for Standards and Technology

NIST SP 260-240
April 2024

NIST Technical Series Policies
Copyright, Fair Use, and Licensing Statements
NIST Technical Series Publication Identifier Syntax

Publication History
Approved by the NIST Editorial Review Board on 2023-10-05

How to Cite this NIST Technical Series Publication
Wilson WB, Hosbas Coskun S, Mulloor J, Nelson MA, Yen JH (2024) Certification of Standard Reference Material® 3383: Yohimbe-Containing Solid Oral Dosage Form. (National Institute of Standards and Technology, Gaithersburg, MD), NIST Special Publication 260 (SP) NIST SP 260-240. https://doi.org/10.6028/NIST.SP.260-240

NIST Author ORCID iDs
Wilson WB: 0000-0003-1763-781X
Hosbas Coskun S: 0000-0002-0413-8934
Mulloor J: 0000 0002 7516 2165
Nelson MA: 0000-0003-0503-4501
Yen JH: 0000-0001-5242-7629

Contact Information
Please address technical questions you may have about this SRM to srms@nist.gov where they will be assigned to the appropriate Technical Contact responsible for support of this material. For sales and customer service inquiries, please contact srminfo@nist.gov

Abstract

Standard Reference Material® (SRM®) 3383 Yohimbe-Containing Solid Oral Dosage Form was developed as part of a collaborative effort between the National Institute for Standards and Technology (NIST) and the National Institutes of Health Office of Dietary Supplements (NIH-ODS). SRM 3383 was produced from multiple commercial dietary supplements containing yohimbe to replicate typical analytical challenges associated with the measurement of yohimbine in a finished product sample matrix. The material was prepared at NIST and packaged by High Purity Standards, an experienced contract manufacturer. A certified value for yohimbine has been assigned based upon data obtained from NIST. A description of the material, sample preparations, results, and data analysis are discussed in the following report.

Key words

Dietary Supplement;
Rauhimbine;
Solid Oral Dosage Form;
Yohimbe;
Yohimbine.

Table of Contents

List of Tables

List of Figures

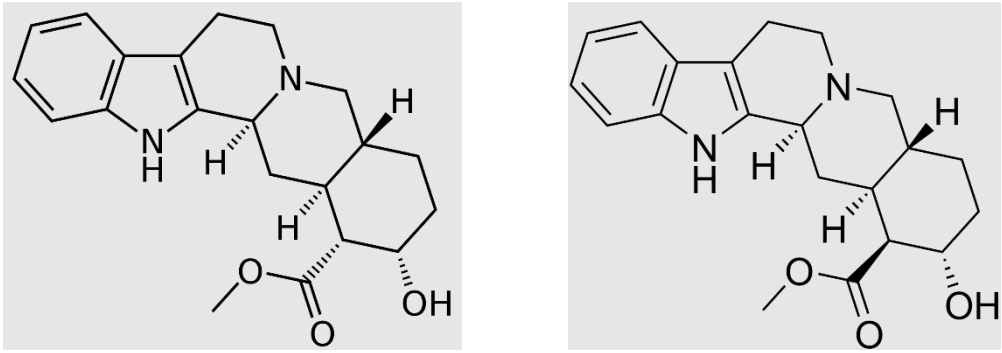
Acknowledgments

Brandi Benford and Katherine E. Sharpless are acknowledged for their work on material acquisition and preparation.

Lisa Steinhauser, Melissa M. Phillips, and Catherine A. Rimmer are acknowledged for their exploratory work on extraction and chromatographic approaches for yohimbine in dietary supplements.

1. Introduction

Standard Reference Material® (SRM®) 3383 Yohimbe-Containing Solid Oral Dosage Form is part of a continuing collaboration with NIH-ODS to develop dietary supplement reference materials (RMs). SRM 3383 is a finely ground powdered prepared from a mixture of four commercially available yohimbine containing finished products. SRM 3383 has a certified value for 17α-hydroxy-yohimban-16α-carboxylic acid methyl ester hydrochloride (yohimbine) and a non-certified value for 17α -hydroxy-yohimban-16β -carboxylic acid methyl ester (rauhimbine) shown in Fig. 1. Yohimbe bark and its extract are derived from *Pausinystalia yohimbe* [K. Schumann] Pierre ex Beille, a tree native to tropical West Africa. These materials are popular herbal supplements used as a general tonic, performance enhancer, and as an aphrodisiac. In 2017, the American Botanical Council reported that the mainstream market of herbal supplements in the United States (US) in 2016 was 7.45 billion dollars [1]. Yohimbe represents almost 21 million dollars of these sales and ranks 13th in their listing of the top 40 bestselling herbs/supplements in the US. Yohimbine is the major and most active alkaloid present in yohimbe bark and yohimbine HCl is available in the United States as a prescription drug. Yohimbine is a phosphodiesterase type 5 (PDE5) inhibitor that can produce side effects such as paresthesia, incoordination, tremulousness, dissociative states, antidiuresis, central nervous system excitation, dizziness, headache, skin flushing, and orthostatic hypotension. The detection and accurate quantification of yohimbine in commercially available supplements is important for evaluating potential health and safety hazards. Quantitation of rauhimbine, a naturally occurring stereoisomer of yohimbine, can help identify supplements that have been adulterated with synthetic PDE5 inhibitors.



yohimbine rauhimbine

Fig. 1. Chemical structures of Yohimbine and Rauhimbine.

A schematic diagram is shown in Fig. 2 for the preparation and value assignment plan of SRM 3383. The preparation and packing of the SRM 3383 material are described in Section 2. The characterization of the reference standards is described in Section 3. The value assignment measurements involve the use of two analytical methods. The “Analysis #1” method described in Section 4 consists of the combination of pressurized fluid extraction (PFE) with liquid chromatography coupled to an electrospray ionization mass spectrometer (LC-ESI-MS). The “Analysis #2” method described in Section 5 consists of solvent extraction using a methanol basic

solution for analysis by LC coupled with atmospheric pressure chemical ionization mass spectrometry (LC-APCI-MS). The statistical analysis of the value assignment measurements is described in Section 7.

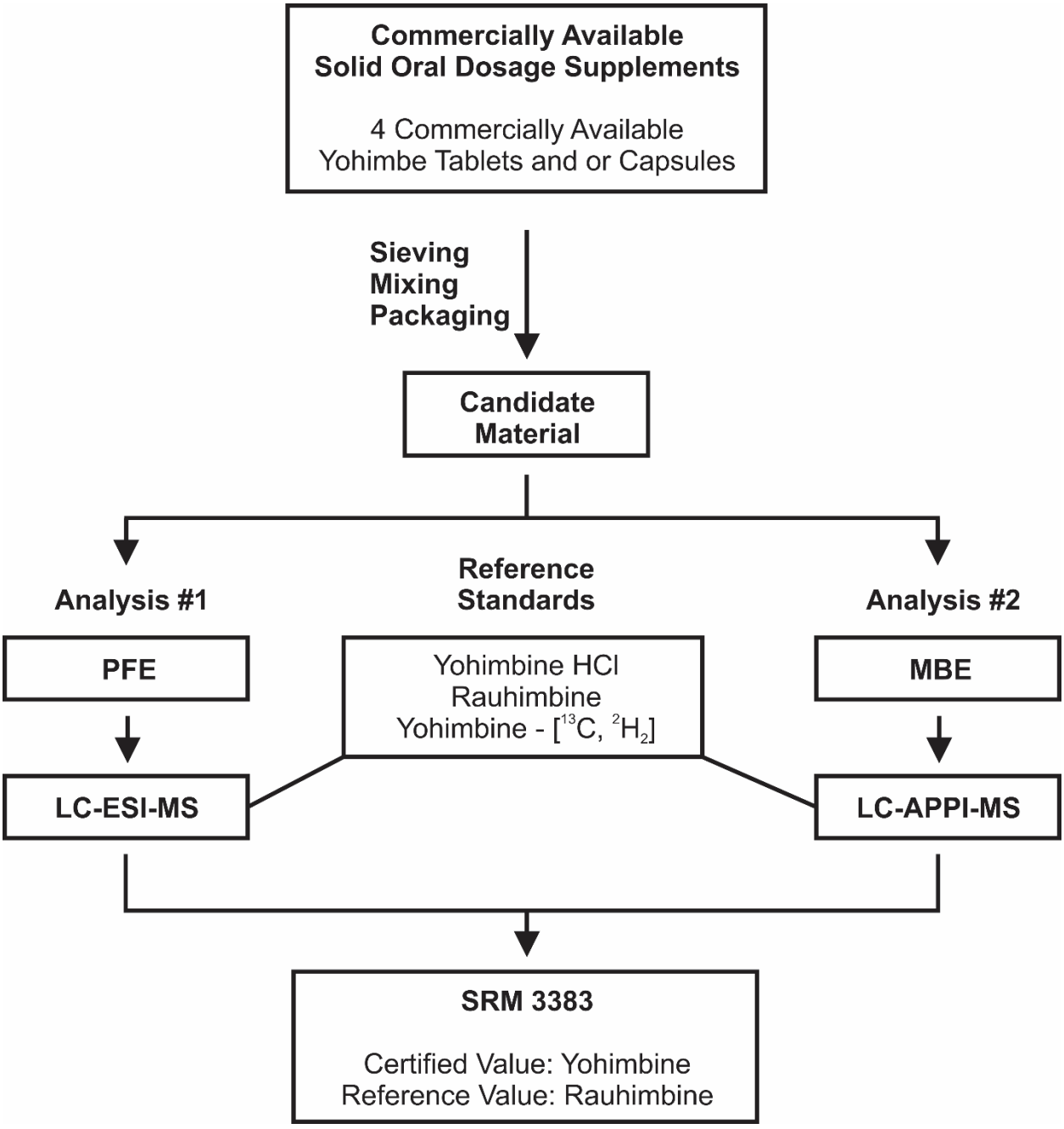


Fig. 2. Schematic Diagram for the Preparation and Value Assignment of SRM 3383.

2. Material

2.1. Dosage Forms and Their Nominal Compositions

The SRM 3383 material is a blend of four over-the-counter yohimbe-containing solid oral dosage forms including the following information from the product labels:

- Material 1 contains niacin, taurine, raw testicular extract, yohimbe extract, eleuthero root, saw palmetto, guava extract, oat extract, and ginkgo extract.
- Material 2 contains vitamin B_1, vitamin B_2, vitamin B_6, niacin, pantothenic acid, vitamin E, zinc gluconate, chromium GTF, phenylalanine, raw testicular concentrate, yohimbe extract, *tribulus terrestris*, Korean ginseng root, damiana leaf, *muira puama* extract, saw palmetto, suma root, and schisandra.
- Material 3 contains yohimbe extract, Korean ginseng, eleuthero, horny goat weed, damiana leaf, *muira puama* root, nettle leaf extract, catuaba bark, saw palmetto, oatstraw extract, and kola nut extract.
- Material 4 contains vitamin B_6, pantothenic acid, vitamin E, choline, zinc picolinate, L-arginine, L-tyrosine, yohimbe extract, saw palmetto, ginkgo extract, and phytosterols.

2.2. Preparation

Materials 1 and 2 were tablets ground following an established cryogenic homogenization procedure [2], except that these materials were homogenized at room temperature. The materials were arranged around the inside of a room temperature Teflon disk mill containing a concentric Teflon ring and puck. After shaking, the mill was opened and the resulting powder was transferred into a Teflon bag designated for pooling that material. The materials were processed separately.

- Material 1 was derived from one lot. The contents of five bottles were poured into the Teflon mill and ground for 4 min to 5 min. A total of 4 millings were done resulting in 0.759 kg of powder.
- The bottles of Material 2 did not have lot numbers. The contents of five bottles were poured into the Teflon mill for grinding. Two millings of 4 min to 5 min each were performed resulting in 0.538 kg of powder.

Materials 3 and 4 were contained in gelatin capsules that were opened and the powder they contained transferred into a Teflon bag. The materials were processed separately.

- Material 3 was derived from two lots. A total of 0.604 kg of powder was obtained.
- Material 4 was derived from one lot. A total of 0.500 kg of powder was obtained.

All material was sieved thorough 45 mesh, 60 mesh, and 80 mesh screens. The stack of sieves was placed on a rotary shaker for 5 min. Approximately 300 g to 400 g was sieved in each batch. The initial sieving following the Teflon millings was done for 5 min with each fraction pooled in

separate Teflon bags. The material that did not pass though the 80 mesh sieve was ground in a Vitamix blender for 1 min and sieved again. At the completion of grinding and sieving, a total of 2.202 kg of material from the four source materials was available for packaging. The final materials were stored in four Teflon bags, each in a “screw top storage container.

2.3. Packaging

The four containers were shipped to High-Purity Standards, North Charleston, SC for blending and packaging. The materials in the containers were combined in a mixing vessel and rolled for one hour to ensure homogeneity. After blending, in 250 mL beaker aliquots, samples of 1.1 g ± 0.1 g of the yohimbe solid oral dosage powder were weighed out using a static-free container on a platform balance with accuracy of ± 0.1 g. Each sample was immediately transferred into a 4 mil polyethylene bag. The bag was flushed with dry nitrogen and immediately heat-sealed. This bag was overpacked in aluminized packet with two packets of SORB-IT silica gel while being swept with dry nitrogen before double sealing. A total of 1955 packets, each containing 1.1 g ± 0.1 g of yohimbe solid oral dosage powder were prepared. These packets were packed sequentially into four boxes, three containing 600 packets each and one of 155 packets. The boxes were shipped to NIST and stored at controlled room temperature. SRM 3383 was irradiated by Neutron Products, Inc. (Dickerson, MD) in the cardboard boxes from High-Purity Standards. The target for the absorbed dose was 6.0 kGy to 10.0 kGy. The actual absorbed doses measured by Neutron Products were 7.1 kGy to 8.5 kGy. Each SRM sales unit consists of five packets of material for a total of about approximately 350 sales units.

3. Calibration Standards

Reference standards of yohimbine hydrochloride and rauhimbine were obtained from the United States Pharmacopeia (USP, Rockville, MD, Lot F) and ChromaDex (Irvine, CA, Lot # 00003812-315), respectively. These materials were used to prepare calibration solutions as part of the certification measurements of the yohimbine and rauhimbine content of SRM 3383. The chromatographic purity of the yohimbine reference standard is stated by USP to be in the range 98.0 % to 100.0 %, based on liquid chromatography with ultraviolet (UV) absorbance detection (LC-UV) measurements with detection at 229 nm [3]. The containers of the USP Lot F standard indicate an assayed purity of 99.1 %, but no uncertainty estimate nor further documentation was provided. The rauhimbine material is a reagent grade (RG) chemical that is not guaranteed as a quantitative standard.

3.1. Chromatographic Purity

The chromatographic purity for the primary reference standard yohimbine hydrochloride was confirmed and rauhimbine was determined by LC coupled to a photodiode-array detector (PDA).

3.1.1. Materials

All LC mobile phase solvents were LC-MS grade solvents from Fisher Scientific.

3.1.2. Sample Preparation

Small quantities of each reference standard were dissolved in 90 % methanol/10 % ammonium formate buffer, which is the same solvent used during the optimal PFE method described in Section 4. The pH of the ammonium formate buffer solution was adjusted to approximately 8.42 through the addition of ≈ 500 µL of ammonium hydroxide. These solutions were evaluated one day after they were prepared.

3.1.3. LC-PDA Analysis

The LC-PDA and LC-ESI-MS measurements were performed on an Agilent 1290 LC system equipped with a binary pump, degasser, autosampler, column compartment, variable wavelength absorbance detector, fluorescence detector, and a 6410 Triple Quad MS. The instrument was computer controlled using commercial software (Mass Hunter, Agilent). Separations were carried out on an Ascentis Express RP-Amide column purchased from Supelco (MillaporeSigma, Bellefonte, PA) with the following characteristics: 15 cm length, 3.0 mm diameter, and 2.7 µm average particle diameters. The separation conditions are detailed in Section 4. The absorbance spectra obtained from reference standards at the apex of the yohimbine and rauhimbine chromatographic peaks are displayed in Fig. 3. Both have broad spectral bands at 200 nm, 220 nm, and 275 nm that will be used for purity measurements. Chromatographic purity assessments of the yohimbine and rauhimbine reference standards are summarized in Table 1 from triplicate LC-PDA measurements. LC-PDA permitted the total

wavelength chromatogram (TWC) and extracted wavelength chromatograms at 200 nm, 220 nm, and 275 nm. The 200 nm has the advantage that almost all organic compounds will absorb somewhat at this wavelength. A longer wavelength of 320 nm was evaluated but no chromatographic peaks were observed.

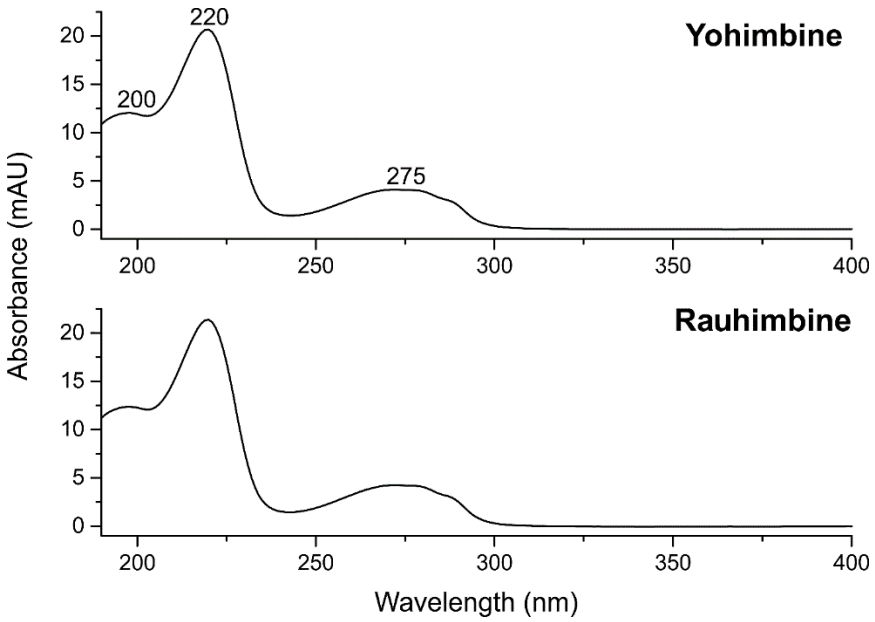


Fig. 3. UV Spectra Collected at the Apex of the Chromatographic Peaks.

Table 1. Chromatographic Purity Values by LC-PDA.

		TWC	200 nm	220 nm	275 nm
Yohimbine	Injection 1	99.46	97.94	98.78	99.11
	Injection 2	97.27	96.90	98.23	99.28
	Injection 3	97.90	96.30	97.91	99.24
	Mean ± SD	98.21 ± 1.13	97.05 ± 0.83	98.31 ± 0.44	99.21 ± 0.09
Rauhimbine	Injection 1	98.23	97.68	98.20	100.00
	Injection 2	99.50	100.00	99.70	100.00
	Injection 3	95.92	95.25	97.76	100.00
	Mean ± SD	97.88 ± 1.82	97.64 ± 2.38	98.55 ± 1.02	100.00 ± 0.0

To determine percent impurity, the area of the chromatographic peak of interest is divided by the total area for all detected peaks and multiplied by 100. Blanks of the 90 % methanol/10 % ammonium formate sample solvents were measured to ensure that there were no relevant impurities. The chromatographic purity values for yohimbine and rauhimbine obtained at 200 nm and 222 nm are similar to those using TWC chromatograms, suggesting that the TWC approach is valid. The USP monograph states their yohimbine hydrochloride reference material contains “not less than 98.0 % and not more than 102.0 % of $C_{21}H_{26}N_2O_3 \cdot HCl$”. The TWC

chromatographic purity value for yohimbine lies within this range. A stoichiometric correction of 0.9067 should be included in the chromatographic purity values for the yohimbine hydrochloride standard, which was calculated by dividing the mass of yohimbine (354.45 g/mol) by the mass of yohimbine hydrochloride (390.91 g/mol). The purity of yohimbine itself in the USP reference standard is thus (98.21 ± 1.13)(354.45/390.91) = 89.05 % ± 1.02 %. For additional confirmation of no significant impurities, both materials were evaluated by LC-ESI-MS.

3.2. Structure Verification and Impurity Assessment

Nuclear magnetic resonance (NMR) spectroscopic experiments were performed to determine, without ambiguity, the natural stereo-specific organic chemical structure of the species in the yohimbine and rauhimbine reference materials and elucidate whether significant structurally-related or organic chemical impurities are present. A single quantitative NMR (qNMR) measurement using an internal standard was performed to determine the mass fraction of yohimbine in the yohimbine HCl reference standard and confirm the stoichiometric ratio of these components. The water and volatile content of these reference standards were also assessed via thermogravimetric analysis (TGA) to confirm that these impurity components are a small mass fraction of the reference materials.

3.2.1. Materials

Maleic acid (Lot # BCBM8127V) was used as an internal standard for the qNMR measurement. Deuterated solvents with ≥ 99.8 % D-atom purity is typically used for qNMR applications. For the NMR samples, the neat chemical materials were diluted with Cambridge Isotope Laboratories DMSO-d_6 (D, 99.8 %) and D_2O (“100 %” D atom Purity).

3.2.2. Sample Preparation

Clean Bruker 600 MHz NMR tubes (5 mm internal diameter, 178 mm length) were stored in a desiccator prior to use. Sample mass determinations and preparation for ^1H NMR analysis were performed in accordance with established balance use and sample preparation procedures. Neat material masses were determined using an ultra-microbalance (Mettler Toledo XPR2U). For qualitative NMR measurements, 4.2541 mg of yohimbine HCl and 0.8063 mg of rauhimbine were weighed. For the qNMR measurement, 0.7205 mg of yohimbine and 3.5814 mg of maleic acid were weighed. Approximately 0.7 mL of solvent was used to dilute the samples. To facilitate total dissolution, samples were sonicated and vortexed. Care was taken to ensure complete dissolution and that no crystals of the neat materials adhered to the weigh bottle walls. Samples for TGA were weighed as is on tared platinum crucibles using the ultra-microbalance. The crucible was then placed on the hangdown wire on the TGA instrument. The recorded masses were 3.2049 mg and 1.2776 mg for yohimbine HCl and rauhimbine, respectively.

3.2.3. NMR Evaluations

Experimental NMR data was acquired by a Bruker Avance II 600 MHz spectrometer equipped with a 5-mm broadband inverse detection probe and operating with Topspin (Version 3.2) software. One dimensional ^{1}H, multiplicity-edited ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC), and ^{1}H-^{1}H correlation (COSY) NMR spectroscopy experiments were performed for determination of chemical structure. All experiments were conducted at 298 K. ^{1}H experiments were conducted with 128 scans, 30 s to 45 s recycle delays, 20.0276 ppm spectral sweep width, and 6.175 ppm transmitter frequency offset (O1). 90-degree excitation pulse widths were used without ^{13}C decoupling during FID acquisition. Data acquisition time was 5.4525952 s for each scan to generate an FID with 131072 data points. ^{1}H-^{13}C HSQC was conducted using the following parameters: 1024 data points and spectral width of 13.0179 ppm was collected for the F2 axis (^{1}H); 256 data points and 185 ppm spectral width was collected for the F1 (^{13}C) axis; 8 scans and 16 dummy scans were performed; 64 µs dwell time; 6.012 ppm F2 frequency offset; 90 ppm F1 frequency offset. The multiplicity-edited ^{1}H-^{13}C HSQC spectra of yohimbine HCl and rauhimbine are displayed in Fig. 4 with chemical structure assignments of peaks.

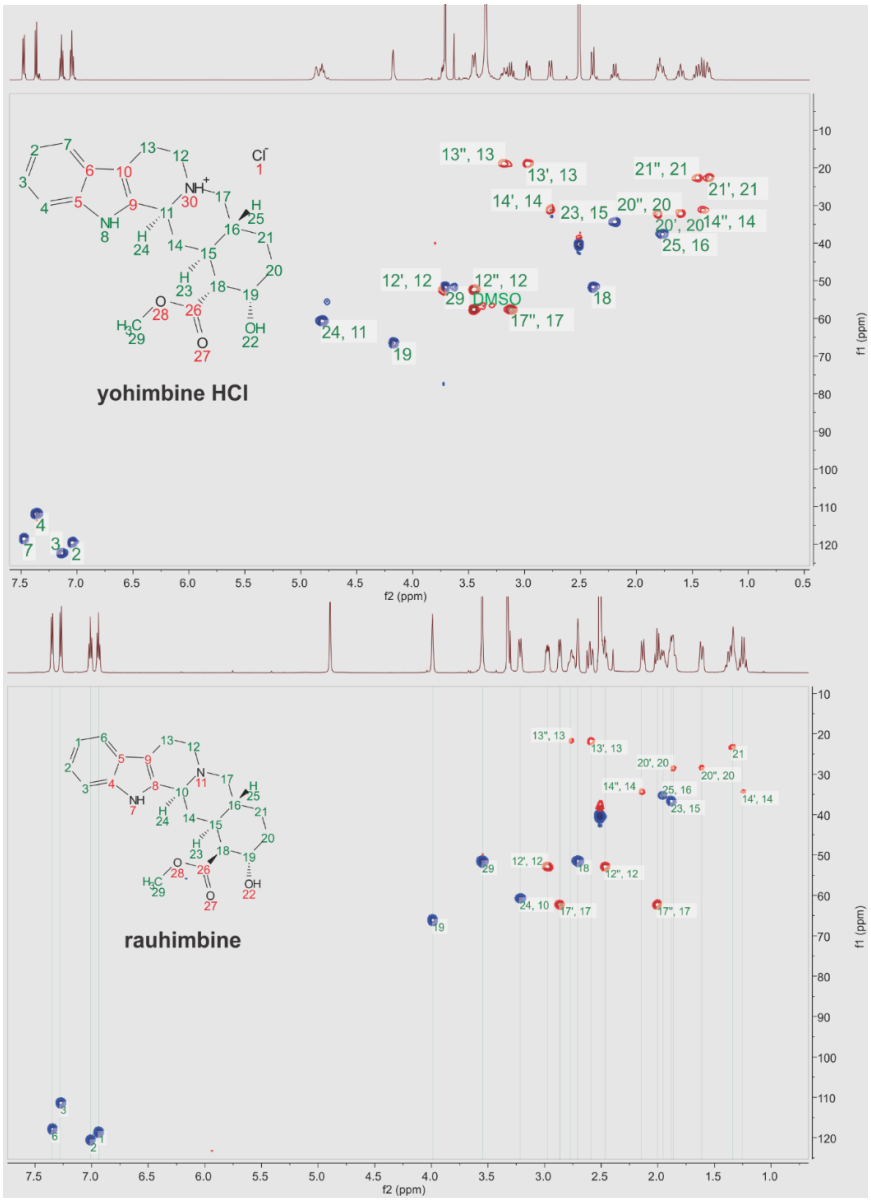


Fig. 4. Multiplicity-Edited ^{1}H-^{13}C HSQC Spectra of Yohimbine HCl and Rauhimbine.

^{1}H-^{1}H COSY was conducted using the following parameters: 2048 data points and spectral width of 13.3517 was collected for the F2 dimension; 128 data points and 13.3517 ppm spectral width was collected for the F1 dimension; 4 scans and 8 dummy scans were performed; 62.4 µs dwell time; 6.012 ppm F2 frequency offset for both F2 and F1 axes. The ^{1}H and ^{1}H-^{1}H COSY spectra of yohimbine HCl and rauhimbine in DMSO-d_6 are displayed in Fig. 5.

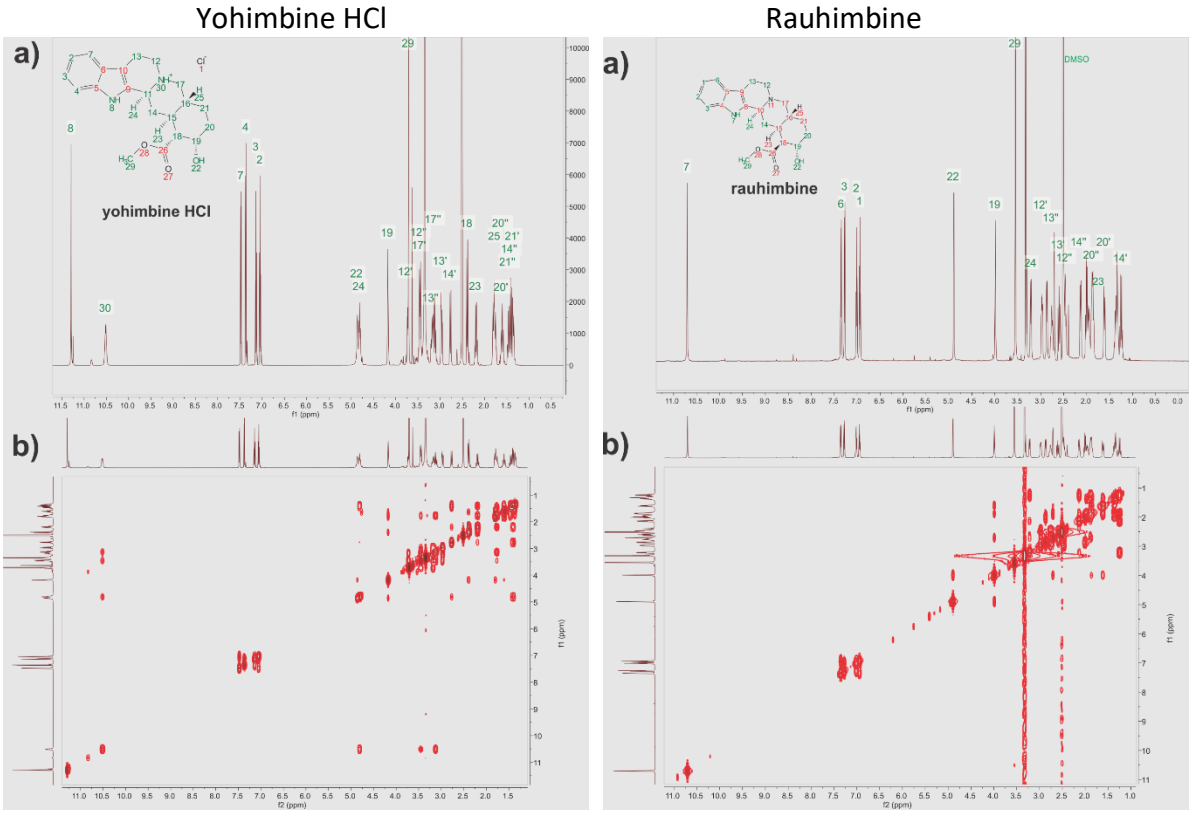


Fig. 5. Yohimbine HCl and Rauhimbine Spectra in DMSO-d_6.

^{1}H spectra are displayed in the (a) panels. ^{1}H-^{1}H COSY spectra are displayed in the (b) panels.

The mass fraction (g/g) purity (P) of yohimbine in a single sample of yohimbine HCl was determined using ^{1}H-qNMR via the following:

$$P = \left(\frac{N_I}{N_P}\right) \times \left(\frac{M_C}{M_I}\right) \times \left(\frac{A_P}{A_I}\right) \times \left(\frac{m_I}{m_C}\right) \times P_I$$

where:
N_P multiplicity (# H/peak) of the primary chemical component spectral peak,
N_I multiplicity (# H/peak) of the internal standard peak,
M_P relative molar mass (molecular weight, g/mol) of primary chemical component,
M_I relative molar mass (molecular weight, g/mol) of the internal standard,
A_P integrated area of the primary component peak,
A_I integrated area of the internal standard peak,
m_C mass (g) of the composite yohimbine HCl material,
m_I mass (g) of the internal standard,
P_I purity (g/g) of the maleic acid internal standard.

At least two forms of yohimbine were observable in samples of yohimbine HCl in DMSO-d_6, the major form being yohimbine$^+$. This material was dissolved in D_2O to circumvent the observable effects of H^+ exchange with the yohimbine species and confirm that it is observable in DMSO-d_6 as multiple forms rather than in the presence of a large relative amount of related impurity. The ^{1}H spectra in D_2O and DMSO-d_6 are compared in Fig. 6. Yohimbine was measured by NMR

primarily as freebase form in D_2O and large quantities of related impurities were not observed. From a single qNMR measurement using maleic acid internal standard (0.9999 g/g), the mass fraction of yohimbine in the yohimbine HCl reference material is 0.904 g/g.

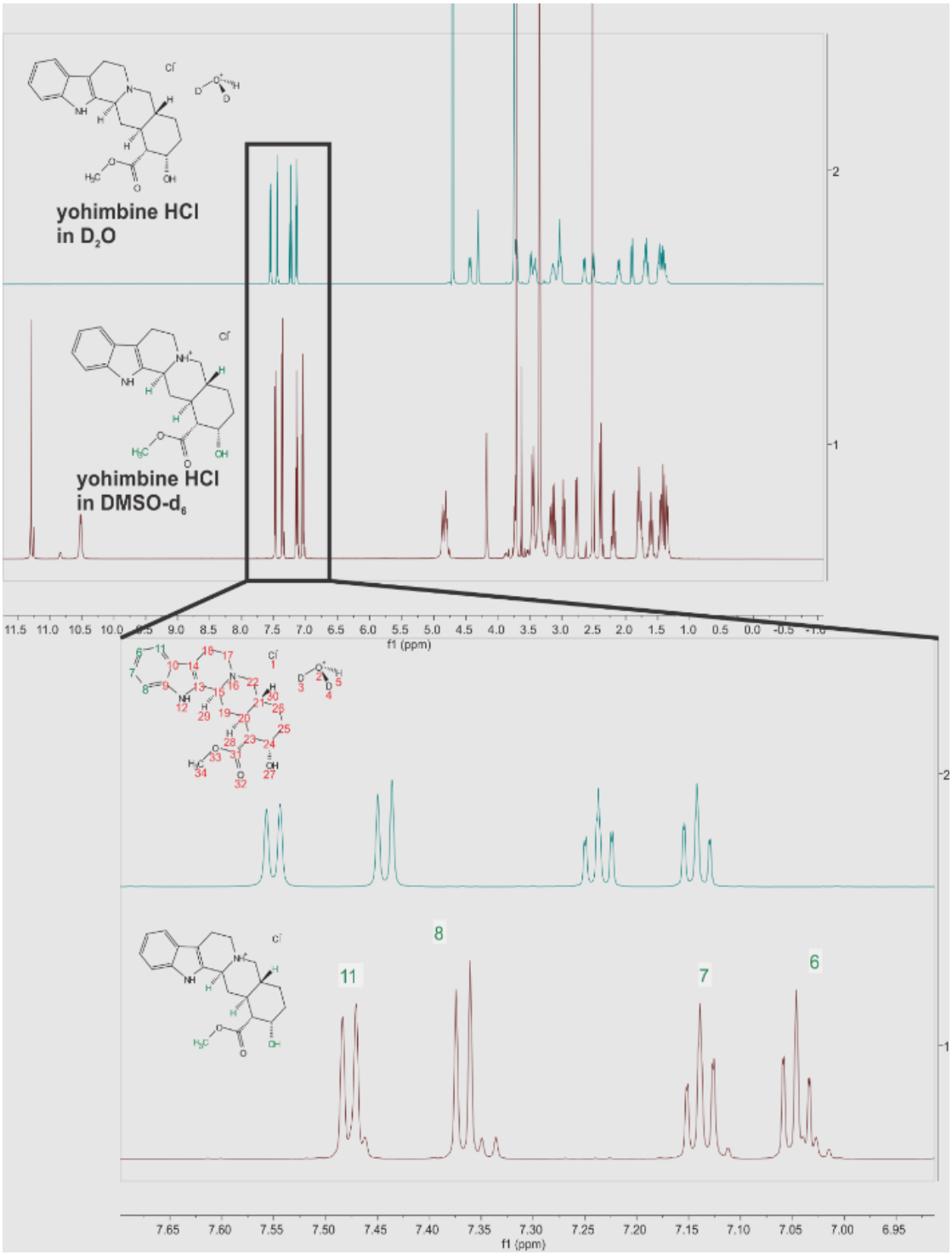


Fig. 6. ^{1}H NMR Spectra of Yohimbine HCl in D_2O and DMSO-d_6.

The ^{1}H-NMR spectrum of rauhimbine displayed in Fig. 7 has several resonances that are suspected to be impurity components. The cumulative peak area of these resonances are > 5 % relative to that of the yohimbine species. This is indicative of amount fraction (mol/mol) of impurity ^{1}H but is not necessarily indicative of impurity chemical mass fraction since the structural specificity (^{1}H multiplicity) and associated molecular weight of the impurities giving rise to these resonances was not determined.

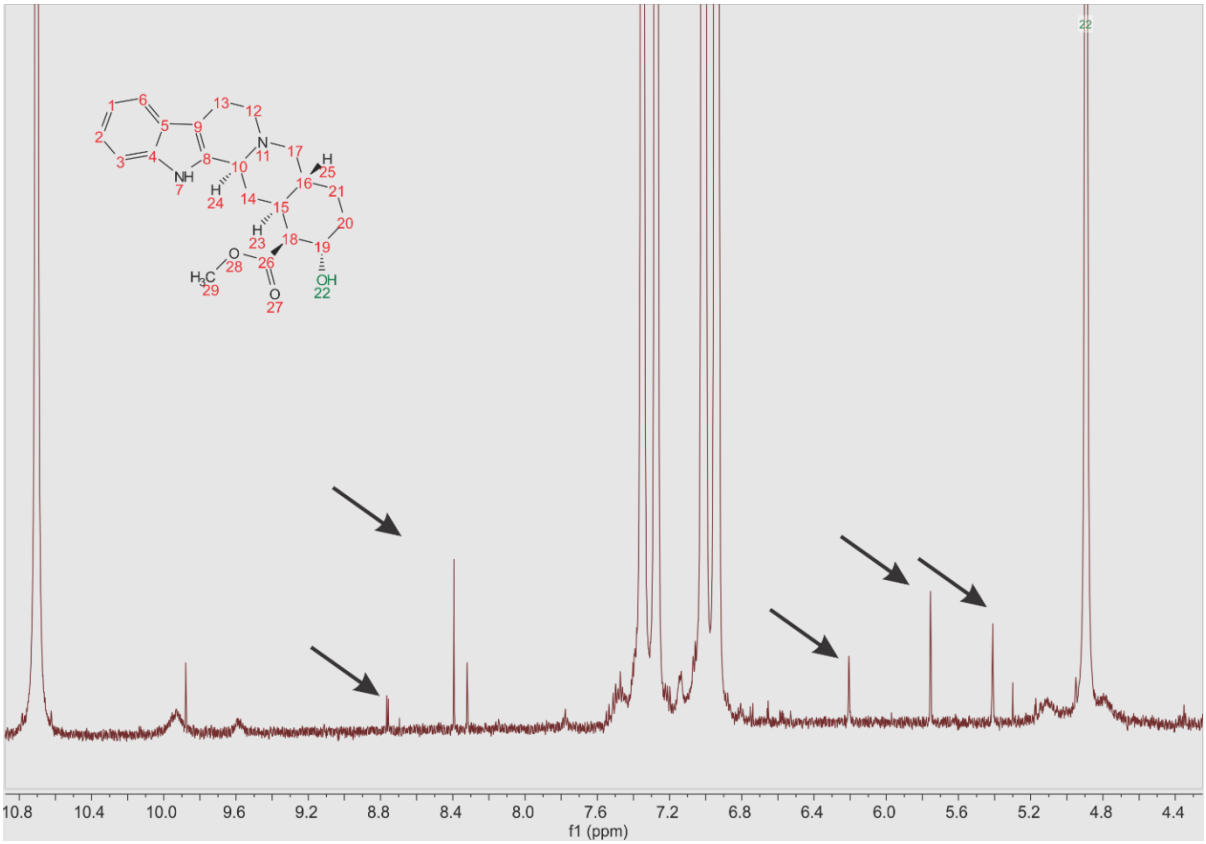


Fig. 7. ^{1}H-NMR Spectrum of Rauhimbine in DMSO-d_6.

The arrows indicate suspected impurity ^{1}H resonances.

The stereo-specificity of organic chemical structure that substantiates the distinction between yohimbine (16α-carboxylic acid methyl ester) and rauhimbine (16β -carboxylic acid methyl ester) is distinguishable by NMR. Panel a of Fig. 8 presents an overlay of the ^{1}H-^{13}C HSQC spectra of yohimbine and rauhimbine in DMSO-d_6. Features of these spectra indicative of different relative spatial position of the methyl ester groups of these stereoisomers, resulting from differences of electron density symmetry that influence the respective resonances, are accentuated in panel b of Fig. 8.

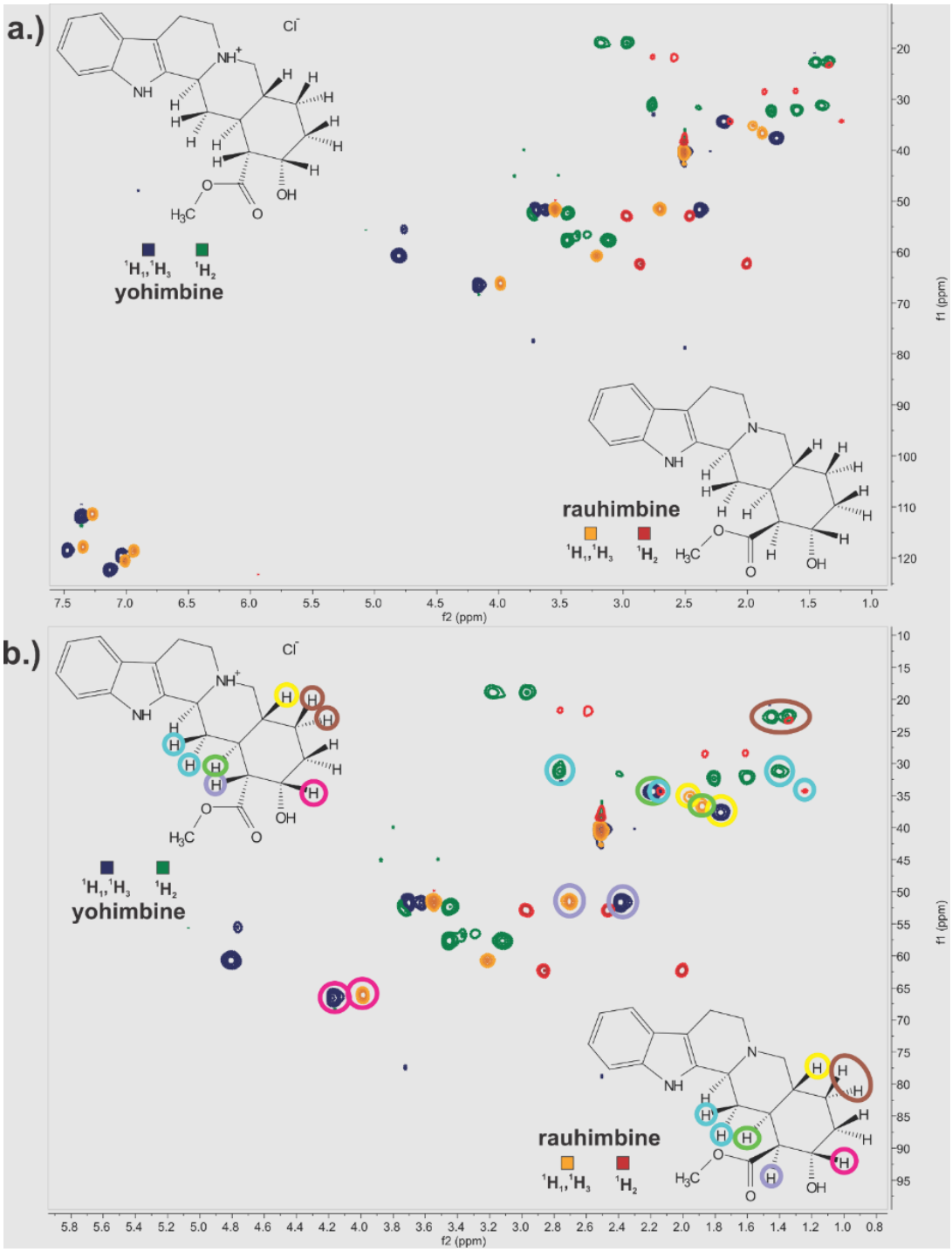


Fig. 8. Overlay of Multiplicity-Edited ^{1}H-^{13}C HSQC Spectra.

Panel (a) is an overlay of the yohimbine and rauhimbine ^{1}H-^{13}C HSQC spectra in DMSO-d_6. Panel (b) assigns the resonances that distinguish the relative spatial positioning of the methyl ester groups of the yohimbine and rauhimbine stereoisomers.

3.2.4. TGA Evaluations

TGA was performed using an Instrument Specialists Incorporated TGAi 1000. The TGA oven was purged with dry nitrogen gas at a 30 mL/min flow rate. The TGA oven ramp (for all samples, including the baseline) started at 21 °C and the temperature increased at 10°C/min to a final temperature of 200 °C. An initial baseline run was analyzed to correct for weight fluctuations due to variations in the sample chamber caused by the purge gas and oven temperature changes. The baseline was smoothed and subtracted from the subsequent sample runs. Only one TGA measurement each for the yohimbine HCl and rauhimbine materials was performed due to limited sample availability. The initial mass of the analytes as recorded on the balance was subtracted from the mass recorded by the instrument at 130 °C to determine the mass loss. By these criteria, the mass losses were 0.23 % for yohimbine HCl and 0.67 % for rauhimbine.

3.3. Yohimbine HCl Purity Assignment

NMR measurements confirm that the primary component of the USP yohimbine HCl calibration standard is yohimbine. The yohimbine content of this material was measured by qNMR using an internal standard of known purity with metrological traceability to the International System of Units (SI). The measurement indicated a mass fraction of yohimbine in the yohimbine HCl material of 0.904 g/g, for an effective purity of 99.7 % for yohimbine HCl. This is consistent with the USP's stated 98.0 % to 102.0 % $C_{21}H_{26}N_2O_3 \cdot HCl$ dry-basis purity interval [3].

A 0.7 % to 2.9 % proportion of impurities with structures similar to yohimbine was measured by LC-PDA at NIST, suggesting that additional related impurities should be included in the qNMR assay. The fraction of impurity mass loss by TGA was 0.23 %. Based on all complementary evidence from NIST measurements, the true value of yohimbine mass fraction in the yohimbine HCl calibration standard, is expected to lie within the symmetric interval 89.9 % ± 0.9 % with a confidence level of about 95 %.

The yohimbine result is metrologically traceable to the SI through a series of qNMR comparisons linking the purity of the yohimbine calibration standard to that of the NIST PS1 Primary Standard for qNMR (Benzoic Acid) [4]. It is fit for use in providing a certified result for yohimbine in the SRM 3383 material.

3.4. Rauhimbine Purity Assignment

NMR measurements confirm that the primary component of the ChromaDex rauhimbine material is rauhimbine. Chromatographic purity assessment indicates that the rauhimbine stereoisomer is 97.9 % with a standard uncertainty of 1.82/√3 = 1.1 %. The 0.7 % mass fraction volatile losses combined with the NMR observation of several unaccounted-for ^1H resonances suggest a rauhimbine purity of about 97 % with an asymmetrical uncertainty interval from 94 % to 99 %. This rauhimbine result is fit for use in providing a non-certified value for rauhimbine in the SRM 3383 material.

4. Development of Analysis Method #1: PFE-LC-ESI-MS

4.1. Pressurized Fluid Extraction (PFE) Studies

Solvent extraction with aid of sonication or mixing is the most common alkaloid extraction method reported in the literature [5-15]. The typical procedure includes adding a strong base (*i.e.,* ammonium hydroxide) and/or organic solvent mixture (*i.e.,* methanol and water). Sample is sonicated for 20 min to 60 min and removed for analysis after centrifugation. Recently, Zhang *et al*. [22] published a new quick, easy, cheap, effective, rugged, and safe (QuEChERS) method for the extraction of residual levels of yohimbine in porcine muscle for determination by LC-MS.

Multiple studies have been published using PFE for the extraction of plant materials or supplements [16-22]; however, none have been reported for yohimbine in yohimbe bark or supplements. PFE permits an increase in extraction speed and performance compared to traditional methods of extraction (*i.e.,* sonication or Soxhlet) through enhanced solubility and mass transfer above the atmospheric boiling points of the extraction solvents.

4.1.1. Experimental

4.1.1.1. Materials

SRM 3383 samples are described in detail in Section 2. The reference standard of yohimbine used in this study is described in Section 3.

4.1.1.2. Instrumental Methods

The PFE extractions were performed on a Dionex Accelerated Solvent Extractor 350 equipped with an ASE 350 solvent controller and integrated collection unit with 24 collection vial positions. Stainless steel (22 mL) extraction cells and 60 mL amber collection vials were used. Specific extraction conditions such as extraction time and extraction cycles will be summarized below. The PFE extracts produced in the different extraction studies were measured using two LC-ESI-MS instruments. The PFE studies for sample size and extraction temperature were performed on an Agilent 1200 LC system coupled to a 6130 Single Quad MS. The PFE studies for the number of cycles were performed on an Agilent 1100 LC system coupled to a G1956B Single Quad MS. Both instruments were computer controlled using commercial software (ChemStation, Agilent). Separations were carried out on a HALO C_{18} column purchased from Advanced Materials Technology (Wilmington, DE). Column specifics and separation conditions are listed as Method 1 in Table 2.

Table 2. Operating Conditions for the LC-ESI-MS Methods.

Parameter	Method 1			Method 2			Method 3		
Injection Volume	2 µL			2 µL			1 µL		
Columns	HALO C_{18} 150 x 4.6 mm id 2.7 µm particle size			Ascentis Express RP-Amide 150 x 3.0 mm id 2.7 µm particle size			Ascentis Express RP-Amide 150 x 3.0 mm id 2.7 µm particle size		
Column Temperature	40 °C			25 °C			25 °C		
Flow rate	0.7 mL/min			0.7 mL/min			0.7 mL/min		
Mobile Phase A	25 mmol/L NH_4HCO_2 in 5 % ACN			0.1 % FA in H_2O			0.1 % FA in H_2O		
Mobile Phase B	ACN			0.1 % FA in ACN			0.1 % FA in ACN		
Mobile Phase Program	Time (min)	A (%)	B (%)	Time (min)	A (%)	B (%)	Time (min)	A (%)	B (%)
	0	90	10	0	90	10	0	86	14
	20	60	40	10	90	10	20	86	14
	30	10	90	30	80	20	35	0	100
	31	10	90	45	0	100	37	86	14
	32	90	10	50	90	10	43	86	14
	35	90	10						
Capillary Potential	3.0 kV			3.0 kV			4.0 kV		
Source Temperature	200 °C			200 °C			200 °C		
Gas Temperature	250 °C			250 °C			250 °C		
SIM Mode	*m/z* 355 and *m/z* 359			*m/z* 355 and *m/z* 359			*m/z* 355 and *m/z* 359		
Cone Voltage	70 V			70 V			70 V		

4.1.2. Results and Discussion

The Chemical Sciences Division at the NIST has used PFE for analyte extraction from multiple sample matrixes such as yerba mate leaves (SRM 3253), whole egg powder (SRM 1845), bitter orange (SRM 3258), and ginkgo biloba (SRM 3246). In the present study, the influence of samples size, extraction temperature, and number of cycles were evaluated for the extractions of yohimbine from the SRM 3383 material. In all studies, the extraction solvent consisted of 90:10 vol/vol methanol:25 mmol/L NH_4CO_2. Each extraction was evaporated to ≈ 500 µL and loaded into a LC sample vial. The composition of the extraction solvent was selected based on published solvent extraction methods [7]. The 10 % of 25 mmol/L NH_4CO_2 was selected because of its presence in the mobile phase of the LC-ESI-MS method.

4.1.2.1. Extraction Sample Size

Three sets of five samples of SRM 3383 were weighed at 1.14 mg, 5.33 mg, 10.4 mg, 21.3 mg, and 42.3 mg and extracted with three extraction cycles at a temperature of 40 °C. The average chromatographic peak areas for yohimbine for the different masses are displayed in Fig. 9. The yohimbine peak areas for the first extraction linearly increase from the 1.14 mg to 10.4 mg samples. The areas are relatively the same for the 10.4 mg, 21.3 mg, and 42.3 mg sample sizes. The chromatographic peak areas for the second and third extractions were relatively small for 1.14 mg to 10.4 mg. The yohimbine peak area drastically increased in the second extraction of the 21.3 mg and 42.3 mg samples. Overlay chromatograms at *m/z* 355 for the three extractions of the 5.33 mg and 42.3 mg samples are displayed in Fig. 10. The estimated concentration of ≈ 0.60 mg/mL for yohimbine in the PFE extract using 42.3 mg of SRM 3383 material is well below the reported solubility values in water (8 mg/L) and ethanol (2.5 mg/L) [23]. The presence of yohimbine in the second extraction process in even the 5 mg sample suggests that samples may need additional extraction cycles for partitioning into the extraction solvent. A sample size of approximately 5 mg was selected for all remaining studies.

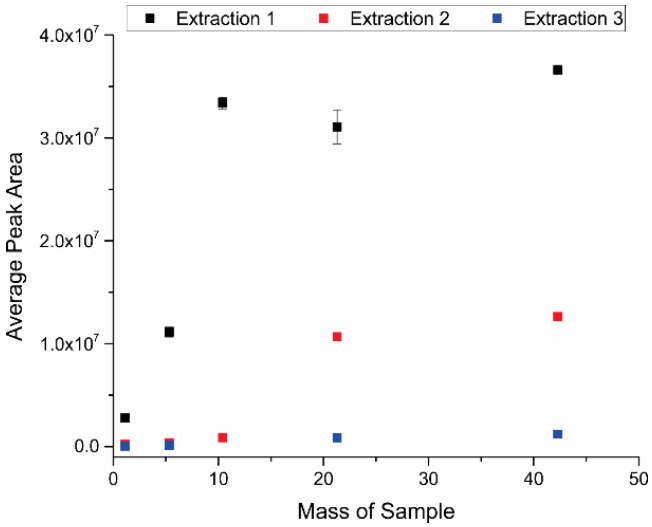


Fig. 9. Average LC-ESI-MS Yohimbine Peak Areas as a Function of Sample Mass.

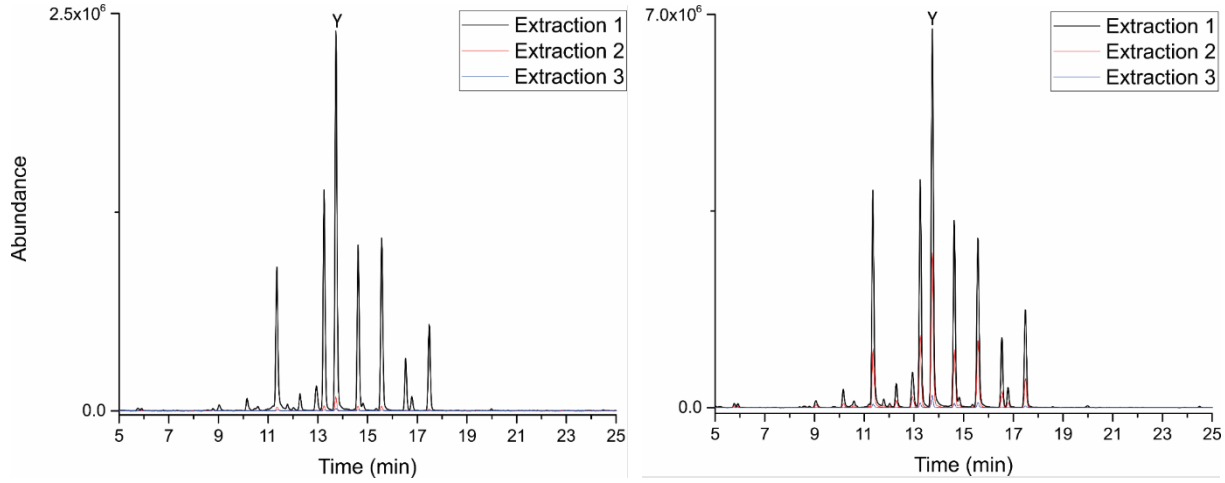


Fig. 10. LC-ESI-MS SIM Chromatograms for Sample Sizes of 5 mg and 42 mg.

The panel to the left depicts the SIM chromatograms at *m/z* 355 for sample size of 5 mg; the panel to the right depicts the chromatograms a sample size of 42 mg. The "Y" denotes the yohimbine peak.

4.1.2.2. Extraction Temperature

Four samples (≈ 5 mg) of the SRM 3383 material were extracted at temperatures of 40 °C, 60 °C, 80 °C, or 100 °C. The ACE 350 PFE instrument can reach temperatures up to 200 °C but to reduce potential degradation these temperatures were not evaluated. Three extraction cycles at a constant pressure of 1500 psi were used. The yohimbine chromatographic peak area percentages are summarized in Fig. 11 for the three sequential extractions at the four temperatures. The percentage of yohimbine present from the second and third extraction decreased from ≈ 3 % at 40 °C to less than 0.5 % at 100 °C temperatures. The extraction temperature was set to 100 °C for all remaining studies.

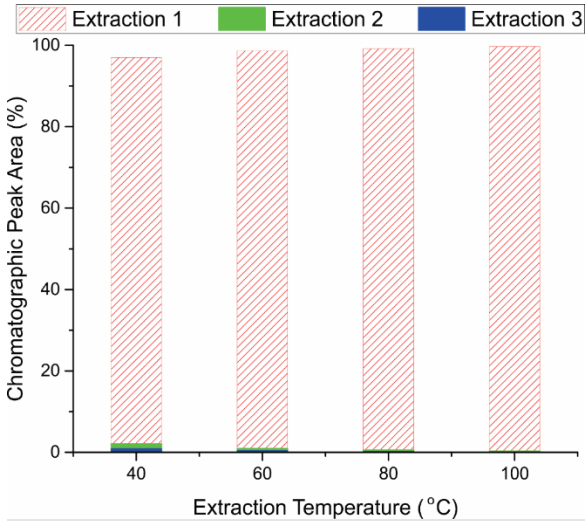


Fig. 11. Yohimbine Peak Area Percentages at Four Extraction Temperatures.

4.1.2.3. Number of Cycles

Five samples (≈ 5 mg) of SRM 3383 were extracted using one, two, three, four, or five cycles a constant pressure of 1500 psi and a temperature of 100 °C. An extraction cycle refers to the number of times the extraction solvent is removed from the sample and a fresh aliquot of solvent is added. The chromatographic peak area percentages for the five cycle numbers are summarized in Fig. 12. The percentage of yohimbine detected from the second extraction using 1 – 4 cycles was less than 0.5 %. Although the percentage of yohimbine detected with five cycles slightly increased to 1.7 %, three cycles were selected for all future studies.

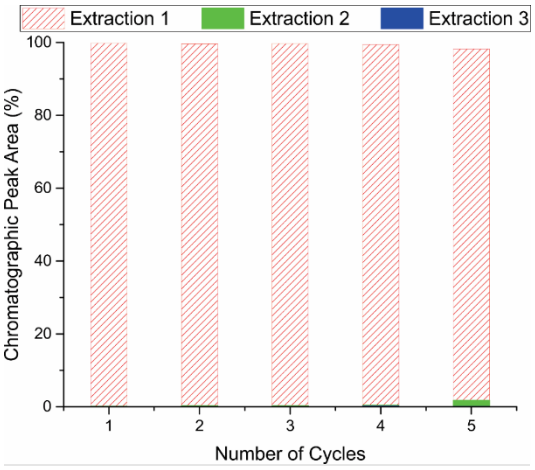


Fig. 12. Yohimbine Peak Area Percentages at Five Extraction Cycles.

4.2. Liquid Chromatography – Electrospray Mass Spectrometry (LC-ESI-MS)

LC is the most prevalent separation technique for the analysis of yohimbe products because of its speed and selectivity. LC has the additional advantage of compatibility with various detection techniques such as UV [5,12,13,15], MS [12,13,15,**Error! Bookmark not defined.**], and tandem M S [22].

4.2.1. Experimental

4.2.1.1. Materials

SRM 3383 samples are described in detail in Section 2. The reference standard of yohimbine used in this study are described in Section 3.

4.2.1.2. Extraction method

Yohimbe samples were extracted by the optimized PFE conditions summarized in Section 4.1.

4.2.1.3. Chromatographic method

The LC-ESI-MS measurements were collected using three chromatographic methods summarized in Table 2. Method 1 and Method 2 were performed on an Agilent LC-ESI-MS instrument with an 1100 LC system coupled to a G1956B Single Quad MS. This instrument was computer controlled using commercial software (ChemStation, Agilent). The LC-ESI-MS measurements used for Method 3 was performed on an Agilent LC-ESI-MS with a 1290 LC system equipped with a binary pump, degasser, autosampler, column compartment, variable wavelength absorbance detector, fluorescence detector, and a 6410 Triple Quad MS. The instrument was computer controlled using commercial software (Mass Hunter, Agilent). Separations conditions are discussed in detail in later sections.

4.2.2. Results and Discussion

Previous studies in the literature have demonstrated the effectiveness of separating yohimbine and rauhimbine in yohimbe extracts using various commercially available C_{18} stationary phases [5,12,13,15,**Error! Bookmark not defined.**,22,24] using H_2O:ACN or H_2O:methanol. The H_2O solvent normally consisted of 0.05 % to 0.2 % FA, NH_4HCO_2 buffer, or trimethylamine buffer. Method 1 in Table 2 is a legacy LC-ESI-MS method developed to achieve the separation of yohimbine and rauhimbine in SRM 3383 using a C_{18} stationary phase and NH_4HCO_2:ACN mobile phase program. The SIM chromatogram (*m/z* 355) for this separation is shown in panel A of Fig. 13 providing a baseline separation for yohimbine and rauhimbine from other potential co-eluting analytes.

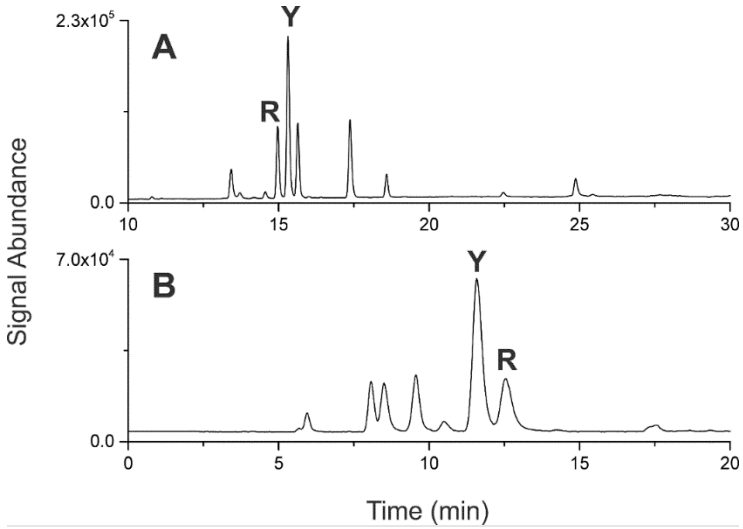


Fig. 13. LC-ESI-MS Chromatograms Using Methods 1 and 2.

Panel A depicts the SIM Mode chromatogram of a PFE extract of SRM 3383 at *m/z* 355 using Method 1; panel B depicts the SIM Mode chromatogram using Method 2. The yohimbine peak is denoted Y; the rauhimbine peak is denoted R.

To minimize measurement biases for the certification of yohimbine and rauhimbine in SRM 3383, a second LC-ESI-MS method was developed using an alternative separation mode employing a

RP-amide stationary phase and 0.1 % FA in H_2O:ACN mobile phase program (Method 2, Table 2). The SIM chromatogram (*m/z* 355) for the initial separation is shown in panel B of Fig. 13 providing a possible baseline separation; however, additional optimization studies discussed below provided an improved separation method (Method 3, Table 2). The LC-ESI-MS SIM chromatogram using Method 3 is shown in Fig. 14.

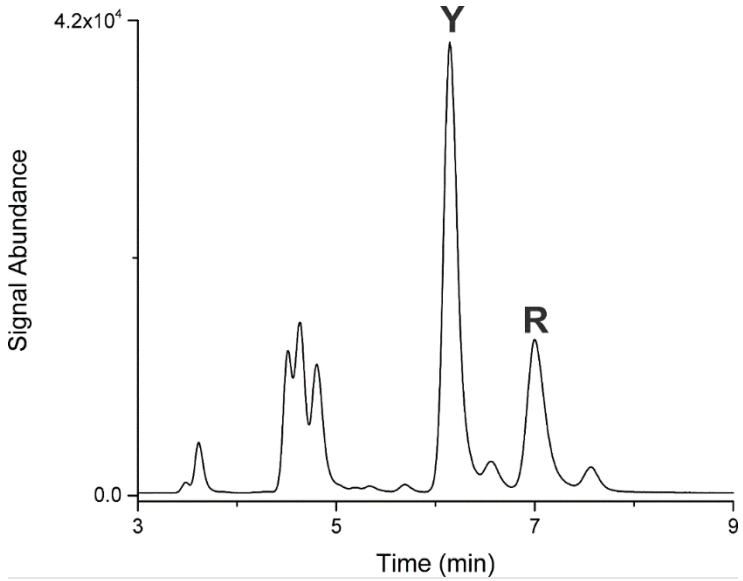


Fig. 14. LC-ESI-MS Chromatogram Using Method 3.

The figure depicts the SIM Mode chromatogram of a PFE extract of SRM 3383 at *m/z* 355. The yohimbine peak is denoted Y; the rauhimbine peak is denoted R.

4.2.2.1. Selection of Ionization Source, Modes, and Internal Standard

In LC-MS, the choice between ESI, atmospheric pressure photo-ionization (APPI), and atmospheric pressure chemical ionization (APCI) sources have been shown to play a significant role in the detection of yohimbine. Based on previous work by Bortolini et al. [24], positive ion mode was reported to provide a better signal-to-noise ratio and was selected in the present study. ESI was selected for the current method (Method 3). The maximum signal abundance observed in a full scan mass spectra of yohimbine and rauhimbine is the protonated molecule $[M+H]^+$ and its corresponding *m/z* 355 was selected for all quantitative measurements in SIM mode.

The LC-ESI-MS method developed here will be used for quantitative measurements of yohimbine and rauhimbine in SRM 3383 based on the internal standard calibration method. The appropriate internal standard will account for variations in detection response and injection irreproducibility. Internal standard should exhibit similar characteristics to analyte such as chemical properties, similar detector response, and chromatographically resolved form potential interferences [27]. Isotopically labeled internal standards should be selected when available and for the current study yohimbine-[^{13}C, $^{2}H_3$] is available. ESI positive mass spectra were collected for yohimbine-[^{13}C, $^{2}H_3$] identified that *m/z* 359 ($[M+H]^+$) was the appropriate SIM ion for this internal standard.

4.2.2.2. Column Temperature

The effect of column temperature on the separation of yohimbine and rauhimbine in SRM 3383 is shown in Fig. 15. Separations were carried out over the interval 15 °C to 40 °C, with a constant flow rate and initial mobile phase composition of 0.70 mL/min and 10 % (vol/vol) ACN. The was no significant resolution changes for yohimbine and rauhimbine at the different column temperatures; however, a couple of the other eluting analytes had some changes in their retention behaviors at 35 °C and 40 °C. A column temperature of 25 °C was selected for all further studies.

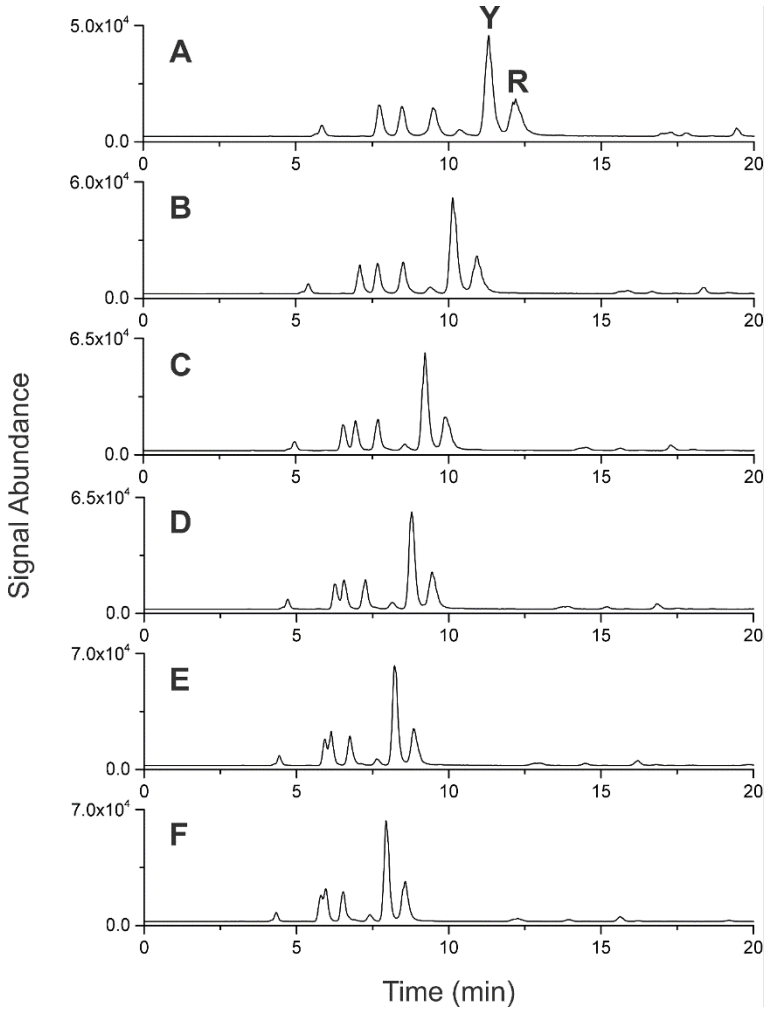


Fig. 15. LC-ESI-MS Chromatograms at Five Column Temperatures.

SIM Mode chromatograms of a PFE extract of SRM 3383 at m/z 355 using Method 2. Panel A depicts the chromatogram with a column temperature of 15 °C, panel B at 20 °C, panel C at 25 °C, panel D at 30 °C, panel E at 35 °C, and panel F at 40 °C. The location of the yohimbine peak is denoted Y; the rauhimbine peak is denoted R.

4.2.2.3. Initial Mobile Phase Composition

The separation of yohimbine and rauhimbine in the PFE extracts of SRM 3383 was sensitive to the initial mobile phase composition of the gradient program. To evaluate the influence of mobile phase composition and selectivity, the initial conditions were varied at 75 %, 80 %, 85 %, and 90 % H_2O for 20 min followed by a linear gradient to 0 % H_2O over 15 min. The flow rate and column temperature were held constant at 0.70 mL/min and 25 °C, respectively. The chromatograms under these conditions are displayed in Fig. 16. The best chromatographic resolution was obtained with an initial mobile phase composition of 90 % H_2O; however, several other interfering chromatographic peaks were not observed at these conditions suggesting they were co-eluting with yohimbine and rauhimbine. The initial mobile phase condition was further evaluated by varying from 86 % to 89 % H_2O; the chromatograms under these conditions are displayed in Fig. 17. The best chromatographic separation of the interfering peaks from yohimbine and rauhimbine was observed with 86 % H_2O in the initial mobile phase.

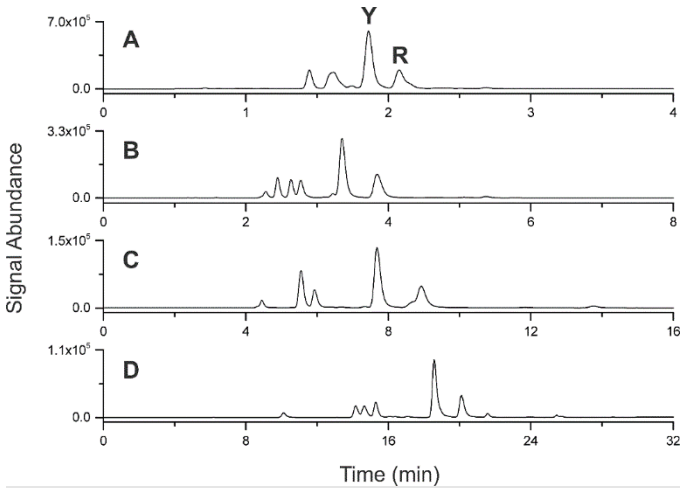


Fig. 16. LC-ESI-MS Chromatograms at Four Initial Mobile Phase Compositions.

SIM Mode chromatograms of a PFE extract of SRM 3383 at m/z 355 using Method 2. Panel A depicts the chromatogram with mobile phase H_2O percentage of 75 %, panel B 80 %, panel C 85 %, and panel D 90 %. The location of the yohimbine peak is denoted Y; the rauhimbine peak is denoted R.

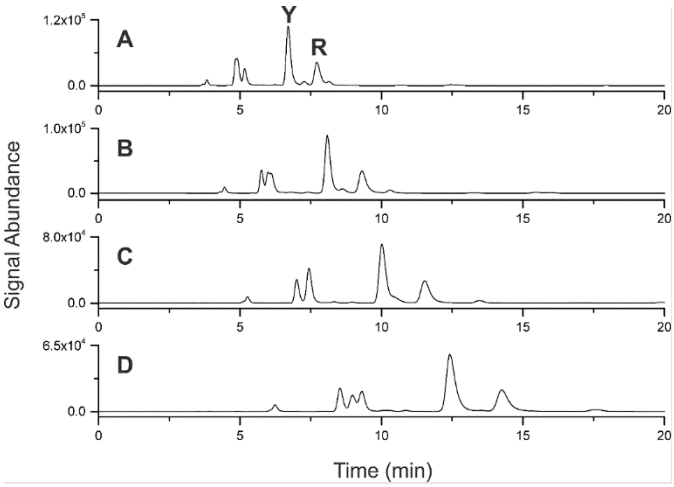


Fig. 17. LC-ESI-MS Chromatograms at an Additional Four Initial Mobile Phase Compositions.

SIM Mode chromatograms of a PFE extract of SRM 3383 at m/z 355 using Method 2. Panel A depicts the chromatogram with mobile phase H_2O percentage of 86 %, panel B 87 %, panel C 88 %, and panel D 89 %. The location of the yohimbine peak is denoted Y; the rauhimbine peak is denoted R.

4.2.2.4. Flow rate

The flow rate of the mobile phase normally influences separation efficiency and detection sensitivity; however, previous studies at NIST have demonstrate its potential to influence the column selectivity for the separation of 12 ginsenosides. Multiple flow rates were investigated for the separation of yohimbine and rauhimbine in the PFE extract of SRM 3383; however, no influence was observed for the separation selectivity. The flow rate of 0.70 mL/min was selected for all future studies.

4.3. Conclusions

A new LC-MS method using ESI ionization was developed to value assign values for yohimbine and rauhimbine in SRM 3383 Yohimbe-Containing Solid Oral Dosage Form. The PFE extraction conditions were optimized to include 5 mg of sample, 100 ºC extraction temperature, and three extraction cycle. The LC-ESI-MS method used a Ascentis Express RP-Amide column with the following characteristics: 15.0 cm length, 3.0 mm i.d., and 2.7 µm average particle diameter. Separations were optimized to have a flow rate of 0.7 mL/min, column temperature of 25 ºC, and the following mobile phase gradient: isocratic elution with 86/14 H_2O/ACN in 0.1 % FA for 20 min, linear gradient to 100% ACN in 0.1 % FA over 15 min, equilibrated to initial conditions after 2 min, and isocratic elution for 6 min (Method 3, Table 2).

5. Development of Analysis Method #2: MBE-LC-APCI-MS

Previous studies discussed in Section 4 highlighted three different chromatographic methods that were evaluated for the separation and identification of yohimbine and rauhimbine in SRM 3383 using ESI-MS. Method 1 in Table 2 was selected for Analysis Method #2 because it provides a different type of chromatographic profile with a HALO C_{18} column in-comparison to Analysis Method #1 (Ascentis Express RP-Amide Column). The soft ionization technique APCI was used instead of ESI to provide additional selectivity difference in the LC-MS methods.

The most common alkaloid extraction method reported in the literature is solvent extraction with the addition of a strong base (*i.e.,* ammonium hydroxide) and/or organic solvent mixture (*i.e.,* methanol and water). [5-15] Samples are normally sonicated or vigorously mixed for 20 min to 60 min and removed for analysis after centrifugation. Preliminary studies at NIST evaluated chloroform, methanol, acetone, and ethyl acetate as potential extraction solvents for yohimbine in the two dietary supplements (data not provided). Methanol was selected as the best extraction solvent and was used in the current study. In the present study, the influence of extraction methods and duration times for yohimbine from SRM 3383 were evaluated.

5.1. Experimental

5.1.1. Materials

SRM 3383 samples are described in detail in Section 2. Isotopically labeled yohimbine (Yohimbine-[^{13}C, $^{2}H_3$]) was obtained from IsoSciences.

5.1.2. Internal Standard Preparation

The internal stock solution was prepared by dissolving 13.6068 mg of Yohimbine-[^{13}C, $^{2}H_3$] in 11.16749 g (13.61 mL) of methanol.

5.1.3. Instrumental Methods

The methanol basic extraction (MBE) samples produced in the different extraction studies were measured on an Agilent 1200 LC system coupled to a 6130 Single Quad MS. The instrument was computer controlled using commercial software (ChemStation, Agilent). Separations were carried out on a HALO C_{18} column (150 × 4.6 mm i.d., 2.7 µm particles) using the chromatographic conditions from Method 1 in Table 2. The 25 mmol/L ammonium acetate buffer in water must have its pH adjusted to approximately 8.3 with ammonium hydroxide. LC-MS measurements were performed using an APCI source in positive mode. The APCI parameters include gas temperature of 250 °C, Gas Flow of 12 L/min, nebulizer pressure of 35 psi, and capillary voltages of 3000 V for positive polarity. Yohimbine, rauhimbine, and yohimbine-[^{13}C, $^{2}H_3$] were selectively detected by single ion monitoring (SIM) mode for their respective $[M+H]^+$ ions (*m/z* 355.2, 355.2, and 359.2, respectively).

5.2. Results and Discussion

Following thorough mixing, samples of SRM 3383 were prepared in triplicate by weighing approximately 0.05 g into 15 mL polypropylene centrifuge tubes. Massed aliquot (≈ 0.2 mL) of yohimbine-[^{13}C, $^{2}H_3$] solution (0.1 mg/mL in methanol) was added to each sample. Afterwards 1 mL of 14.8 mol/L ammonium hydroxide was added, the final volume adjusted to 10 mL with methanol for each sample, and vortexed for 15 s. After sonication or rotational mixing, the samples were centrifuged at 3800 rpm for 15 min and a portion of the extract was analyzed by LC-APCI-MS. The effects of extraction times for sonication (heat controlled at 35 °C) or rotational mixing for (0, 5, 15, 30, 60, and 120) min are summarized in Fig. 18. The mean and standard deviations of the response factors were calculated using the peak area ratio of yohimbine/yohimbine-[^{13}C, $^{2}H_3$]. No significant difference was observed between the extraction times in this study except for 120 min of rotational mixing; however, the results are not statistically different from the other time points.

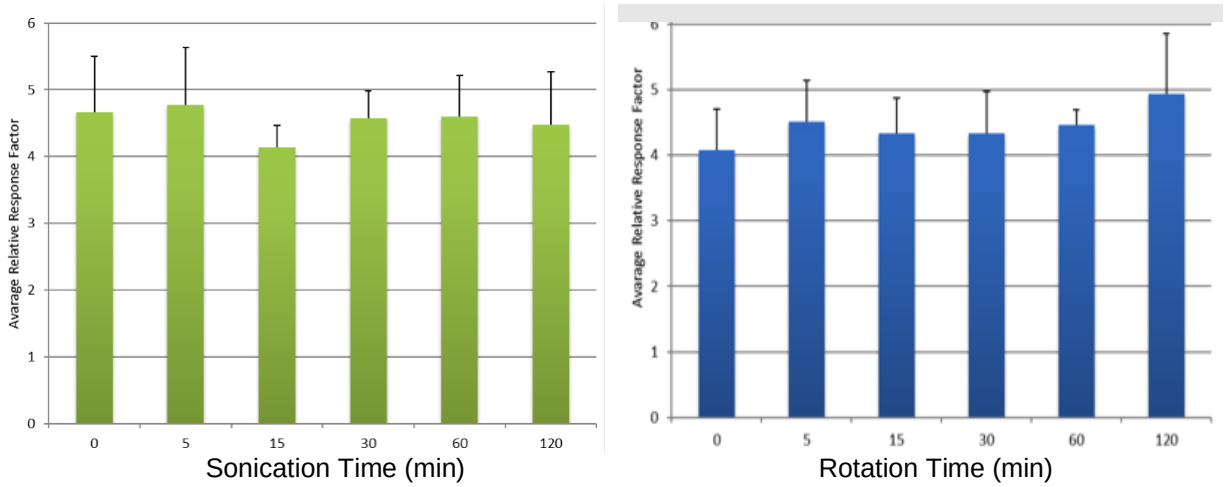


Fig. 18. Effect of Sonication and Rotational Mixing Times on Extraction Efficiency.

Error bars represent the standard deviation of three replicates.

5.3. Conclusion

A new LC-MS method (Method 1, Table 2) using APCI ionization was developed to value assign yohimbine and rauhimbine in SRM 3383 Yohimbe-Containing Solid Oral Dosage Form. The effectiveness of the sample extraction using rotational mixing and sonication was evaluated with no significant difference observed between methods. The length of extraction times did not have a significant influence, but an intermediate extraction time of 30 min was selected for value assignment measurements.

6. Value Assignment Measurements

The value assignment measurements involve the use of two analytical methods: 1) the combination of PFE with LC-ESI-MS described Section 4 and 2) MBE combined with LC-APCI-MS described in Section 5. The use of two chromatographic methods with different stationary phases (C_{18} vs Amide), organic modifiers (25 mmol/L NH_4HCO_2 vs 0.1 formic acid (FA), and column temperatures (40 °C vs 25 °C) minimizes the potential for chromatographic bias from incompletely resolved interferents. The LC-ESI-MS quantitation measurements were based on the internal standard calibration method. The values for the LC-APCI-MS method are based on the response factor ratios between yohimbine or rauhimbine with the isotopically labeled yohimbine – [^{13}C, $^{2}H_3$] internal standard (IS):

$$RF = \frac{A_{\text{analyte}}}{A_{\text{IS}}} \frac{w_{\text{IS}}}{w_{\text{analyte}}}$$

where *RF* is the response factor, A_{analyte} is the area of the analyte (yohimbine or rauhimbine) peak, A_{IS} is the area of the IS peak, w_{IS} is the mass fraction of IS in the sample, and w_{analyte} is the mass fraction of the analyte in the sample. The mean *RF* is determined by linear regression from measurements of calibrants of known composition using the model:

$$\frac{A_{\text{analyte}}}{A_{\text{IS}}} = RF \frac{w_{\text{analyte}}}{w_{\text{IS}}}.$$

The mass fraction analyte in a sample where only w_{IS} is known is then:

$$w_{\text{analyte}} = \frac{w_{\text{IS}}}{RF} \frac{A_{\text{analyte}}}{A_{\text{IS}}}.$$

Since the samples of SRM material are created by combining a known mass of SRM, m_{SRM}, with a (much smaller) known mass of an IS working solution, m_{IS}, the mass fraction of analyte in the SRM is then:

$$w_{\text{analyte in SRM}} = w_{\text{analyte in sample}} \frac{m_{\text{SRM}} + m_{\text{IS}}}{m_{\text{SRM}}}$$

6.1. Analysis 1 Method: PFE-LC-ESI-MS

6.1.1. Experimental

6.1.1.1. Materials

SRM 3383 samples are described in detail in Section 2. The reference standard of yohimbine and rauhimbine used in this study are described in Section 3. Isotopically labeled yohimbine (Yohimbine-[^{13}C, $^{2}H_3$]) was obtained from IsoSciences. From Section 3.3, the mass fraction of yohimbine, $w_{\text{yohimbine}}$ in the yohimbine HCl standard is $w_{\text{yohimbine}}$ = 0.899 g/g ± 0.009 g/g. From Section 3.4, the mass fraction of rauhimbine, $w_{\text{rauhimbine,}}$ in the rauhimbine RG material is $w_{\text{rauhimbine}}$ = 0.979 g/g ± 0.011 g/g.

6.1.1.2. Stock and Calibration Solutions

Four yohimbine and rauhimbine calibration stock solutions (Cal Stock 1, 2, 3, and 4) and one IS stock solution were prepared using the reference standards described in Section 3. The masses used to prepare these stock solutions are listed in Table 3. Two calibrants were gravimetrically prepared from each of the Cal Stock solutions. Table 4 details the compositions of the eight calibration solutions. An IS working solution was also prepared, combining 0.24677 mg of the IS Stock with 3398.8 mg methanol, producing a working solution having a 0.0821 mg/g mass fraction of the IS.

Table 3. Calibrant and IS Stock Masses and Mass Fractions for PFE-LC-ESI-MS

	Weighed Masses *m*, mg				Mass Fractions *w*, mg/g			*w*(Purity Corrected) *w*, mg/g	
Stock Solutions	Yohimbine HCl	Rauhimbine RG	IS	Methanol	Yohimbine HCl	Rauhimbine RG	IS	Yohimbine	Rauhimbine
Cal Stock 1	6.2951	0.2711		2422.81	2.5912	0.1116		2.3295	0.1092
Cal Stock 2	6.4049	0.5262		2317.46	2.7555	0.2264		2.4772	0.2216
Cal Stock 3	5.3642	0.3551		2356.09	2.2712	0.1504		2.0418	0.1472
Cal Stock 4	4.4282	0.8185		2334.87	1.8923	0.3498		1.7012	0.3424
IS Stock			2.4745	2186.18			1.1306		

Table 4. Calibration Solution Masses and Mass Fractions for PFE-LC-ESI-MS

	Weighed Masses *m*, mg					Mass Fractions *w*, mg/g		
	Cal Stock 1	Cal Stock 2	Cal Stock 3	Cal Stock 4	IS Stock	Yohimbine	Rauhimbine	IS
Calibrant 1	0.07771				0.21104	0.6269	0.0294	0.8263
Calibrant 2		0.39434			0.07819	2.0673	0.1850	0.1871
Calibrant 3			0.07654		0.22551	0.5174	0.0373	0.8441
Calibrant 4				0.19576	0.19709	0.8477	0.1706	0.5672
Calibrant 5	0.06515				0.06635	1.1541	0.0541	0.5705
Calibrant 6		0.07122			0.07959	1.1699	0.1047	0.5967
Calibrant 7			0.09714		0.06879	1.1953	0.0862	0.4687
Calibrant 8				0.0698	0.07053	0.8462	0.1703	0.5682

6.1.1.3. Sample Preparation

Ten SRM 3383 samples were randomly selected from the four boxes and were labeled based on the box number and packet location (top, bottom, left, right) within the box, *e.g.*, the label 4TR represents box 4 top right. Calibration solutions were stored in the freezer (-20 °C). Small quantities of the internal standard stock solution (≈ 0.1 mg) and SRM 3383 samples (≈ 5 mg) were weighed out and mixed in the PFE cells after addition of hydromatrix. Duplicate samples were prepared from each of the ten packets on in two sessions a week apart. The masses of these samples are listed in Table 5.

Table 5. SRM 3383 Sample Composition for PFE-LC-ESI-MS.

	Measurement Set 1				Measurement Set 2		
Sample	SRM *m*, mg	IS *m*, mg	IS *w*, mg/g	Sample	SRM *m*, mg	IS *m*, mg	IS *w*, mg/g
1BL-1	6.2857	0.08333	1.07392	1BL-2	5.9928	0.08686	1.17270
1BR-1	5.3977	0.08383	1.25529	1BR-2	6.4655	0.06947	0.87257
1TL-1	6.0552	0.08666	1.15815	1TL-2	7.2902	0.08045	0.89591
2BL-1	6.2351	0.11024	1.42603	2BL-2	7.3105	0.10173	1.12654
2TL-1	6.832	0.09044	1.07238	2TL-2	6.9194	0.12002	1.39947
2TR-1	4.6867	0.09459	1.62385	2TR-2	6.5306	0.08396	1.04188
3BL-1	5.7996	0.1086	1.50876	3BL-2	6.5647	0.12833	1.57380
3BR-1	6.0383	0.07975	1.06995	3BR-2	6.4491	0.09044	1.13516
3TL-1	5.2991	0.09235	1.40597	3TL-2	5.4767	0.09384	1.38273
4BL-1	5.5077	0.10261	1.50123	4BL-2	4.8503	0.08228	1.36920

6.1.1.4. Instrumental Methods

The PFE extractions were performed on a Dionex Accelerated Solvent Extractor 350 equipped with an ASE 350 solvent controller and integrated collection unit with 24 collection vial positions. Stainless steel (22 mL) extraction cells and 60 mL amber collection vials were used for the measurements. The LC-ESI-MS measurements were performed on an Agilent 1290 LC system equipped with a binary pump, degasser, autosampler, column compartment, variable wavelength absorbance detector, fluorescence detector, and a 6410 Triple Quad MS. The instrument was computer controlled using commercial software (Mass Hunter, Agilent). The optimized extraction procedure and analytical method is described in Section 4. The chromatographic peak areas are summarized in Table 6.

Table 6. Calibrant and Sample PFE-LC-ESI-MS Peak Areas.

	Measurement Set 1			Measurement Set 2		
Calibrant/ Sample	Yohimbine Area, au	Rauhimbine Area, au	IS Area, au	Yohimbine Area, au	Rauhimbine Area, au	IS Area, au
Calibrant 1	6198849	580522	7090150	33146	2540	38238
Calibrant 2	18845082	2723830	1524357	101500	14400	8306
Calibrant 3	5064429	654204	7203749	25821	3220	36315
Calibrant 4	8058741	2493869	4764716	46846	14441	27867
Calibrant 5	5344995	461860	2420309	346154	26961	155302
Calibrant 6	6599080	1013740	3047722	281579	39254	130261
Calibrant 7	10014928	1281388	3705724	244646	28256	88503
Calibrant 8	6249359	1982346	3823321	172910	56207	104016
1BL	571163	212326	155653	316634	162132	110850
1BR	498789	194014	165639	343814	176467	87888
1TL	497627	207650	179708	372543	188886	95940
2BL	640043	260556	244279	368190	188287	122568
2TL	451571	197230	121302	343687	173390	141210
2TR	460652	212911	180722	339571	177254	102669
3BL	419465	185095	156543	336384	166610	159229
3BR	494822	200420	138266	326802	172960	109847
3TL	497042	187537	227643	267220	130978	112643
4BL	546806	266515	212032	233145	132952	102760

6.1.2. Results and Discussion

The LC-ESI-MS quantitation measurements were based on the internal standard calibration method. Small quantities of the IS were mixed with the calibrant solutions and SRM 3383 samples. The eight calibrants, ten samples of set 1, and ten samples of set 2 were measured one time to provide the graphs shown in Fig. 19. There was no difference observed in the chromatographic profile for calibrants or SRM 3383 extracts through both sets of LC-ESI-MS measurements indicating no instrumental or sample degradation issues during the measurements. The LC-ESI-MS method provide excellent linearity with R^2 values greater than or equal to 0.9992. Except for two rauhimbine measurements for set 2, the chromatographic responses for yohimbine and rauhimbine are within the data points of the calibration curves. The individual mass fraction values for each PFE-LC-ESI-MS measurement and their combined results are shown in Table 7. The potential trends in the mass-fraction yohimbine and rauhimbine content of the SRM 3383 samples evaluated with the PFE-LC-ESI-MS method are displayed in Fig. 20. There are no obvious production order, extraction order, or analysis order trends.

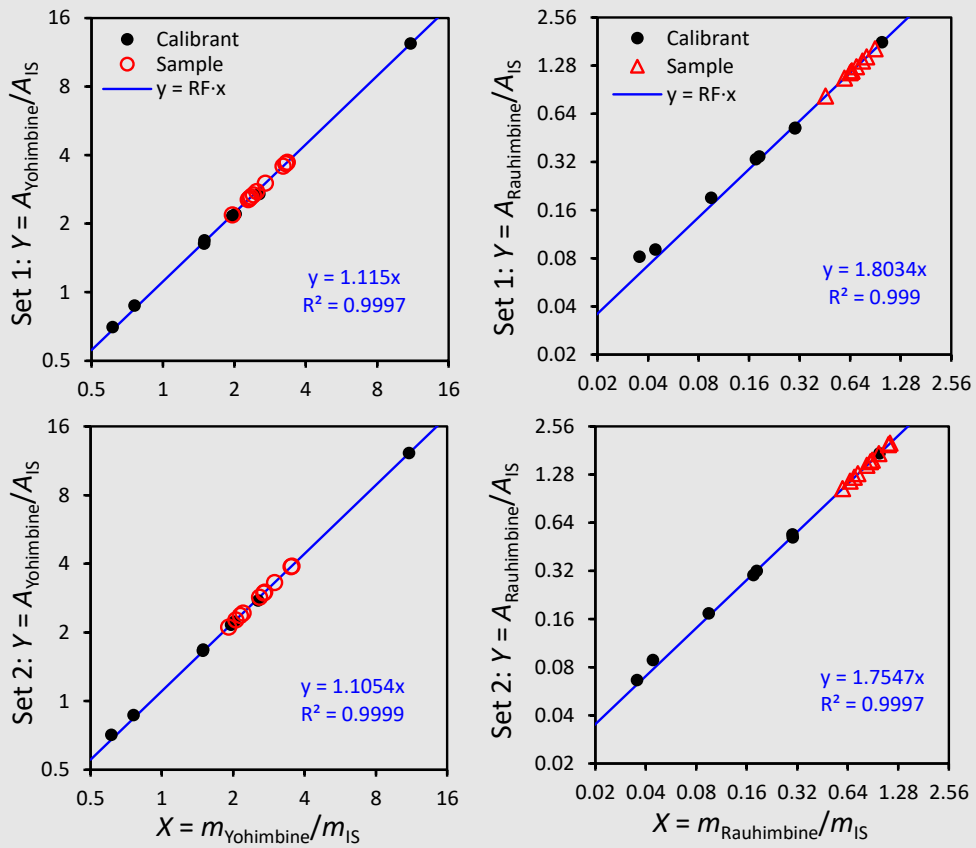


Fig. 19. PFE-LC-ESI-MS Calibration Curves.

Table 7. PFE-LC-ESI-MS Estimates of Yohimbine and Rauhimbine in SRM 3383.

Sample	Yohimbine mg/g	Rauhimbine mg/g	Sample	Yohimbine mg/g	Rauhimbine mg/g
1BL-1	3.581	0.823	1BL-2	3.074	0.978
1BR-1	3.443	0.828	1BR-2	3.121	0.998
1TL-1	2.917	0.753	1TL-2	3.182	1.005
2BL-1	3.410	0.858	2BL-2	3.104	0.986
2TL-1	3.628	0.980	2TL-2	3.135	0.979
2TR-1	3.787	1.082	2TR-2	3.157	1.025
3BL-1	3.694	1.008	3BL-2	3.067	0.939
3BR-1	3.480	0.871	3BR-2	3.098	1.019
3TL-1	2.801	0.653	3TL-2	3.018	0.916
4BL-1	3.537	1.066	4BL-2	2.858	1.010
Number	10	10	Number	10	10
Mean	3.428	0.892	Mean	3.081	0.986
SD	0.322	0.139	SD	0.091	0.035

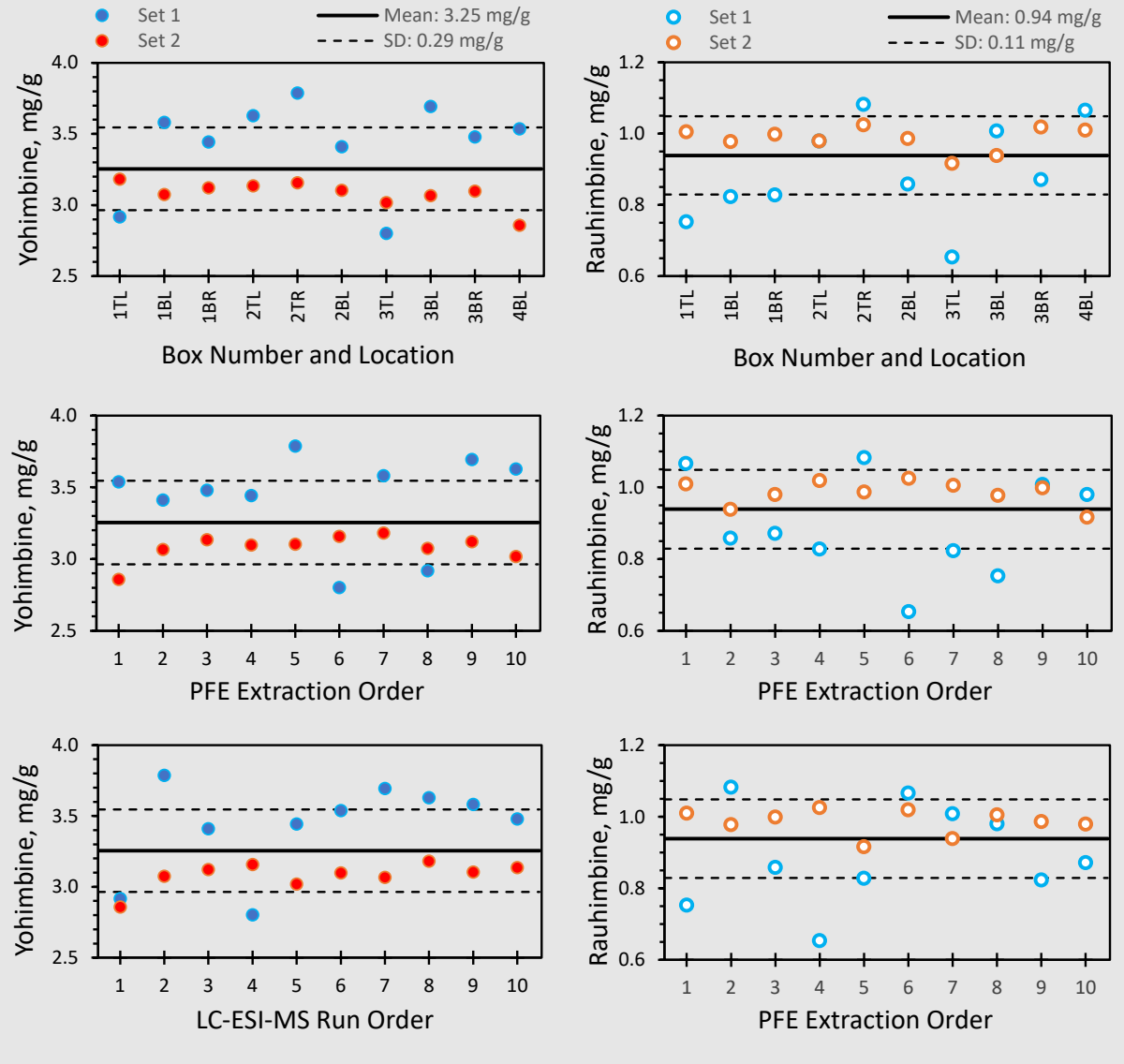


Fig. 20. PFE-LC-ESI-MS Evaluation of Production, Extraction, and Analysis Order.

6.2. Analysis 2 Method: MBE-LC-APCI-MS

6.2.1. Experimental

6.2.1.1. Materials

SRM 3383 samples are described in detail in Section 2. The reference standard of yohimbine and rauhimbine used in this study are described in Section 3. Isotopically labeled yohimbine (Yohimbine-[^{13}C, $^{2}H_3$]) was obtained from IsoSciences.

6.2.1.2. Stock and Calibration Solutions

One yohimbine calibration stock solution, one rauhimbine calibration stock solution, and two IS stock solution were prepared using the reference standards described in Section 3. The masses used to prepare these stock solutions are listed in Table 8. Calibrants 1 to 4 for measurement set 1 and Calibrants 1 to 3 for measurement set 2 were gravimetrically prepared from stock solutions and are summarized in Table 9. Calibration solutions were stored in the freezer (–20 °C).

Table 8. Calibrant and IS Stock Masses and Mass Fractions for MBE-LC-APCI-MS.

	Weighed Masses *m*, mg				Mass Fractions *w*, mg/g			*w*(Purity Corrected) mg/g	
Stock Solutions	Yohimbine HCl	Rauhimbine RG	IS	Methanol	Yohimbine HCl	Rauhimbine RG	IS	Yohimbine	Rauhimbine
Cal Stock 1	16.650	2.13		5988.57	2.780	0.908		2.519	0.889
IS Stock 1			6.60	2749.26			2.40		
IS Stock 2			14.771	11529.4			1.2811		

Table 9. Calibration Solution Masses and Mass Fractions for MBE-LC-APCI-MS.

		Weighed Masses *m*, mg					Mass Fractions *w*, mg/g		
		Yohimbine	Rauhimbine	IS Stock 1	IS Stock 2	Methanol	Yohimbine	Rauhimbine	IS
Set 1	Calibrant 1	0.06620	0.06798	0.21104		0.66764	0.165	0.060	0.500
	Calibrant 2	0.07204	0.06852	0.06913		0.64600	0.212	0.071	0.194
	Calibrant 3	0.07005	0.06868	0.06985		0.67672	0.199	0.069	0.101
	Calibrant 4	0.06619	0.07075	0.06920		0.61742	0.202	0.076	0.108
Set 2	Calibrant 1	0.01198	0.01387		0.03088	1.38363	0.011	0.012	0.027
	Calibrant 2	0.01314	0.01425		0.02734	1.38448	0.012	0.013	0.024
	Calibrant 3	0.01093	0.01093		0.02834	1.44556	0.012	0.009	0.024

6.2.1.3. Sample Preparation

Ten SRM 3383 samples were randomly selected from the four boxes and were labeled based on the box number and packet location (top, bottom, left, right) within the box, *e.g.*, the label 4TR represents box 4 top right. Small quantities of the internal standard stock solution (≈ 0.15 g) and SRM 3383 samples (≈ 50 mg) were weighed out and mixed in the centrifuge tubes. Duplicate

samples were prepared from each of the ten packets two months apart. The masses of these samples are listed in Table 10.

Table 10. SRM 3383 Sample Composition for MBE-LC-APCI-MS.

	Measurement Set 1				Measurement Set 2		
Sample	SRM *m*, mg	IS *m*, mg	IS *w*, mg/g	Sample	SRM *m*, mg	IS *m*, mg	IS *w*, mg/g
3TL-1	0.05088	0.16552	0.73775	3TL-2	0.05075	0.1924	0.33792
2TR-1	0.05083	0.16032	0.76093	2TR-2	0.0528	0.1568	0.43139
1BR-1	0.05304	0.15657	0.81303	1BR-2	0.05292	0.1973	0.34362
1TR-1	0.05044	0.16186	0.74791	1TR-2	0.05118	0.2019	0.32475
1TL-1	0.05098	0.15779	0.77541	1TL-2	0.05424	0.1955	0.35543
3BR-1	0.04981	0.15503	0.77110	3BR-2	0.05044	0.2047	0.31568
3TR-1	0.05346	0.15535	0.82590	3TR-2	0.05308	0.1987	0.34223
4BL-1	0.05215	0.14989	0.83501	4BL-2	0.05039	0.1976	0.32669
2BR-1	0.05079	0.14910	0.81755	2BR-2	0.05234	0.1961	0.34193
2BL-1	0.05262	0.15654	0.80675	2BL-2	0.05158	0.1947	0.33939

An MBE procedure was applied as a 1 mL aliquot of 14.8 mol/L ammonium hydroxide added to the sample, with the final volume adjusted to 10 mL with methanol. The samples were vortexed briefly prior to being shaken for 30 min and centrifuged at 3800 rpm for 15 min. The supernatants were decanted into clean 15 mL polypropylene centrifuge tubes and filtered into LC vials with nylon filters. These extraction conditions were previously optimized in Section 5. The chromatographic peak areas are summarized in Table 11.

Table 11. Calibrant and Sample MBE-LC-APCI-MS Peak Areas.

	Measurement Set 1			Measurement Set 2		
Calibrant/ Sample	Yohimbine Area, a.u..	Rauhimbine Area, a.u..	IS Area, a.u..	Yohimbine Area, a.u..	Rauhimbine Area, a.u..	IS Area, a.u..
Calibrant 1	6580282	2724870	6856425	1221169	251359	657479
	12723021	3375332	11526398	944377	199708	547613
	11691580	2671934	10336663	997487	184887	541740
Calibrant 2	7590050	3265408	6413769	1850594	364559	893840
	-	-	-	1024118	214568	447671
	8160626	3068186	8712046	1025881	191035	471397
Calibrant 3	1493973	340607	1129402	1473735	172668	659060
	1534575	300894	1238775	1019027	120527	456375
	1151697	252848	805509	829519	95083	363638
Calibrant 4	545576	103121	531415			
	1269927	184860	957926			
	613433	178111	587872			
1BR	946814	184032	922869	2031390	288572	981207
1TR	769795	145331	692720	924464	124406	478043
1TL	1063566	363140	1215598	997265	132224	453501
2BL	1352166	273641	921983	885288	117137	395707
2TR	1664392	184688	1855515	869268	134622	311123
3BR	1104732	222584	1017243	876538	96358	492043
3TL	1437293	286845	1958969	964620	129627	475923
3TR	995312	241741	895528	2028791	287951	1026312
4BL	817129	239063	865375	1417207	177635	708575
4BR	640920	269256	647612	1289213	172900	624140

6.2.1.4. Instrumental Methods

The LC-APCI-MS measurements were performed on an Agilent 1200 LC system (Palo Alto, CA) equipped with a binary pump, degasser, autosampler, column compartment, variable wavelength absorbance detector, and a 6130 Single Quad MS. The instrument was computer controlled using commercial software (ChemStation, Agilent). The optimized analytical method is described in Section 4.

6.2.2. Results and Discussion

The LC-APCI-MS quantitation measurements were based on the average response factor ratios between yohimbine or rauhimbine with the isotopically labeled yohimbine – [^{13}C, $^{2}H_3$] summarized in Table 12. Small quantities of the internal standard were mixed with the calibrant solutions and SRM 3383 samples. There was no difference observed in the chromatographic profile for calibrants or SRM 3383 extracts through both sets of LC-APCI-MS measurements indicating no instrumental or sample degradation issues during the measurements. All calibrants were measured in triplicate and the sample extracts were measured one time to provide the chromatographic peak areas in Table 11. A summary of the determined mass fractions for yohimbine and rauhimbine by LC-APCI-MS for both Measurement Sets is provided in Table 13.

Table 12. Calibrant and Sample MBE-LC-APCI-MS Response Factors.

	Measurement Set 1		Measurement Set 2	
Calibrant/ Sample	Yohimbine	Rauhimbine	Yohimbine	Rauhimbine
Calibrant 1	0.9738	0.8615	2.3698	1.1638
Calibrant 2	0.7577	1.5677	2.3049	1.1910
Calibrant 3	1.2207	0.7841	2.3741	0.9809
Calibrant 4	1.0719	0.6302		
1TL	0.1646	0.0722	0.1829	0.0512
1TR	0.2145	0.0520	0.1661	0.0472
1BR	0.1915	0.0478	0.1737	0.0521
2TR	0.1715	0.0244	0.1864	0.0610
2BL	0.2738	0.0712	0.1853	0.0518
2BR	0.1760	0.0950	0.1723	0.0488
3TL	0.1448	0.0371	0.1659	0.0471
3TR	0.2059	0.0642	0.1671	0.0501
3BR	0.2008	0.0519	0.1552	0.0360
4BL	0.1688	0.0634	0.1682	0.0445

Table 13. MBE-LC-APCI-MS Estimates of Yohimbine and Rauhimbine in SRM 3383.

Sample	Yohimbine mg/g	Rauhimbine mg/g	Sample	Yohimbine mg/g	Rauhimbine mg/g
3TL-1	3.107	0.750	3TL-2	3.271	0.929
2TR-1	3.683	0.494	2TR-2	3.532	1.156
1BR-1	3.943	0.927	1BR-2	3.284	0.986
1TR-1	4.642	1.060	1TR-2	3.247	0.923
1TL-1	3.526	1.456	1TL-2	3.373	0.945
3BR-1	4.400	1.072	3BR-2	3.078	0.715
3TR-1	4.205	1.235	3TR-2	3.149	0.945
4BL-1	3.533	1.250	4BL-2	3.338	0.884
2BR-1	3.782	1.922	2BR-2	3.293	0.933
2BL-1	5.680	1.390	2BL-2	3.593	1.005
Number	10	10	Number	10	10
Mean	4.050	1.156	Mean	3.316	0.942
SD	0.731	0.397	SD	0.156	0.109

6.3. Conclusions

The analytical data used for the final mass fraction (mg/g) values for yohimbine and rauhimbine in SRM 3383 are summarized in Table 14 by PFE-LC-ESI-MS (Analysis Method #1) and MBE-LC-APCI-MS (Analysis Method #2) methods. The mass fraction values obtained by both analytical methods were in good agreement. The results from the first measurement sets by both methods were not included here because the measurement precision was significantly improved in the second measurement set. These improvements are attributed to analyst error in initially performing the quantitative measurements.

Table 14. Summary of Yohimbine and Rauhimbine Mass Fractions (mg/g) in SRM 3383.

	Yohimbine		Rauhimbine	
	Method 1	Method 2	Method 1	Method 2
	3.074	3.271	0.978	0.929
	3.121	3.532	0.998	1.156
	3.182	3.284	1.005	0.986
	3.104	3.247	0.986	0.923
	3.135	3.373	0.979	0.945
	3.157	3.078	1.025	0.715
	3.067	3.149	0.939	0.945
	3.098	3.338	1.019	0.884
	3.018	3.293	0.916	0.933
	2.858	3.593	1.010	1.005
Mean	3.196		0.972	
SD	0.176		0.084	
%RSD	5.50		8.67	

7. Statistical Assessment

7.1. Assignment of values and uncertainties:

For each analyte, the certified or non-certified value is the mean of the method estimates available for that analyte. For an analyte, the method estimate is the mean of the measurements available for that analyte. The uncertainty of each method mean is the standard error of that mean. The uncertainty of the combined mean mass fraction values are estimated using a bootstrap procedure based on a Gaussian random effects model for the between method effects [34,35,36]. The estimated certified and non-certified values with their expanded uncertainties for yohimbine and rauhimbine, respectively, in SRM 3383 are listed in Table 15.

Table 15. Yohimbe-Containing Solid Oral Dosage Powder.

	Mass Fraction (mg/g)	$U_{k=2}$ (mg/g)
Yohimbine	3.20	0.24
Rauhimbine	0.972	0.060

7.2. Potential Uncertainty Statement

The uncertainty provided with each value is an expanded uncertainty about the mean to cover the ginsenosides with approximately 95 % confidence. The expanded uncertainty is calculated as $U_k = k\, u_c$, where the combined uncertainty u_c incorporates the observed difference between the results from the methods and their respective uncertainties, consistently with the ISO Guide and with its Supplement 1, and k = 2 is a coverage factor corresponding to approximately 95 % confidence [34,35,36].

7.3. Homogeneity Assessment

To address issues of possible inhomogeneity of the SRM, duplicate analysis was performed by two analytical methods. There was no evidence of significant packet effects.

7.4. Analysis Results

The results of the statistical analyses are presented in the Certificate of Analysis for SRM 3383. For the most current version of this document, please visit:
https://shop.nist.gov/ccrz__ProductDetails?sku=3383.

References

1 Smith T, Kawa K, Eckl V, Morton C, Stredney R (2017) Herbal Supplement Sales in US Increase 7.7% in 2016. HerbalGram, American Botanical Council 115:56-65. https://www.herbalgram.org/media/15603/hg115-mktrpt.pdf (Accessed 12/20/2022)

2 Zeisler R, Langland JK, Harrison SA (1983) Cryogenic Homogenization of Biological Tissues. Anal Chem 55(14):2431-2434. https://doi.org/10.1021/ac00264a055

3 USP 35 Official Monograph: Yohimbine 5053.

4 Nelson MA, Waters JF, Toman B, Lang BE, Rück A, Breitruck K, Obkircher M, Windust A, Lippa KA (2018) A New Realization of SI for Organic Chemical Measurement: NIST PS1 Primary Standard for Quantitative NMR (Benzoic Acid). Anal Chem 90(17):10510-10517. https://doi.org/10.1021/acs.analchem.8b02575

5 Zanolari B, Ndjoko K, Ioset J-R, Marston A, Hostettmann K (2003) Qualitative and quantitative determination of yohimbine in authentic yohimbe bark and in commercial aphrodisiacs by HPLC-UV-API/MS methods. Phytochem Anal 14(4):193-201. https://doi.org/10.1002/pca.699

6 Chen Q, Li P, Zhang Z, Li K, Liu K, Li Q (2008)Analysis of yohimbine alkaloid from *Pausinystalia yohimbe* by non-aqueous capillary electrophoresis and gas chromatography-mass spectrometry. J Sep Sci 31(12):2211-2218. https://doi.org/10.1002/jssc.200800055

7 Sun J, Baker A, Chen P (2011) Profiling the indole alkaloids in yohimbe bark with ultra-performance liquid chromatography coupled with ion mobility quadrupole time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 25(18):2591-2602. https://doi.org/10.1002/rcm.5158

8 Sun J, Chen P (2012) Chromatographic fingerprint analysis of yohimbe bark and related dietary supplements using UHPLC/UV/MS. J Pharm Biomed Anal 61:142-149. https://doi.org/10.1016/j.jpba.2011.11.013

9 Gupta S, Shanker K, Srivastava SK (2012) HPTLC method for the simultaneous determination of four indole alkaloids in *Rauwolfia tetraphylla*: A study of organic/green solvent and continuous/pulse sonication, J Pharm Biomed Analysis 66:33-39. https://doi.org/10.1016/j.jpba.2012.02.018

10 Ren Y, Wu C, Zhang J (2012). Simultaneous screening and determination of 18 illegal adulterants in herbal medicines and health foods for male sexual potency by ultra-fast liquid chromatography-electrospray ionization tandem mass spectrometry. J Sep Sci 35(21):2847-2857. https://doi.org/10.1002/jssc.201200280

11 Chen Q, Chen J, Li P, Xiong L, Liu Q (2013) Simple and fast determination of reserpine and yohimbine from *Rauvolfia yunnanensis* by nonaqueous capillary electrophoresis. Anal Methods 5:3347-3352. https://doi.org/10.1039/C3AY40492G

12 Raman V, Avula B, Galal AM, Wang Y-H, Khan IA (2013) Microscopic and UPLC-UV-MS analyses of authentic and commercial yohimbe (*Pausinystalia johimbe*) bark samples. J Nat Med 67(1):42-50. https://doi.org/10.1007/s11418-012-0642-2

13 Hong B, Li W, Song A, Zhao C (2013) Determination of indole alkaloids and highly volatile compounds in *Rauvolfia verticillata* by HPLC-UV and GC-MS. J Chromatogr Sci 51(10):926-930. https://doi.org/10.1093/chromsci/bms191

14 Cohen PA, Wang Y-H, Maller G, DeSouza R, Khan IA (2016) Pharmaceutical quantities of yohimbine found in dietary supplements in the USA. Drug Test Anal 8(3-4):357-369. https://doi.org/10.1002/dta.1849

15 Sagi S, Avula B, Wang Y-H, Khan IA (2016) Quantification and characterization of alkaloids from roots of *Rauwolfia serpentina* using ultra-high performance liquid chromatogrpahy-photo diode array-mass spectrometry. Anal Bioanal Chem 408:177-190. https://doi.org/10.1007/s00216-015-9093-4

16 Carabias R, Rodriguez-Gonozalo E, Revilla-Ruiz P, Hernandez-Mendez J (2005) Pressurized liquid extraction in the analysis of food and biological samples. J Chromatogr A 1089(1-2):1-17. https://doi.org/10.1016/j.chroma.2005.06.072

17 Williams FB, Sander LC, Wise SA, Girard J (2006) Development and evaluation of methods for determination of naphthodianthrones and flavonoids in St. John's wort. J Chromatogr A 1115(1-2):93-102. https://doi.org/10.1016/j.chroma.2006.02.078

18 Putzbach K, Rimmer CA, Sharpless KE, Wise SA, Sander LC (2007) Determination of bitter orange alkaloids in dietary supplement Standard Reference Materials by liquid chromatography with atmospheric-pressure ionization mass spectrometry. Anal Bioanal Chem 389(1):197-205. https://doi.org/10.1007/s00216-007-1409-6

19 Ramandi NF, Najafi NM, Raofie F, Ghasemi E (2011) Central composite design for the optimization of supercritical carbon dioxide fluid extraction of fatty acids from *Borago officinalis* L. Flower. J Food Sci 76:C1262-C1266. https://doi.org/10.1111/j.1750-3841.2011.02394.x

20 Mustafa A, Turner C (2011) Pressurized liquid extraction as a green approach in food and herbal plants extraction: a review. Anal Chim Acta 703(1):8-18. https://doi.org/10.1016/j.aca.2011.07.018

21 Heng MY, Tan SN, Yong JWH, Ong ES (2013) Emerging green technologies for the chemical standardization of botanicals and herbal preparations. TrAC 50:1-10. https://doi.org/10.1016/j.trac.2013.03.012

22 Zhang D, Park J-A, Kim S-K, Cho S-H, Cho S-M, Yi H, Shim J-H, Kim J-S, El-Aty AMA, Shin HC (2016) Determination of residual levels of naloxone, yohimbine, thiophanate, and altrenogest in porcine muscle using QuEChERS with liquid chromatography and triple quadrupole mass spectrometry. J Sep Sci 39(5):835-841. https://doi.org/10.1002/jssc.201501206

23 Sigma Product Information Sheet for Yohimbine hydrochloride, Product Number Y 3125. https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product_Information_Sheet/y3125pis.pdf (Accessed 12/20/2022)

24 Bortolini C, Pivato A, Bogialli S, Pastore P (2015) "One-shot" analysis of PDE-5 inhibitors and analogues in counterfeit herbal natural products using an LC-DAD-QTOF system. Anal Bioanl Chem 407(20):6207-6216. https://doi.org/10.1007/s00216-015-8801-4

25 Chen Q, Li P, Zhang Z, Li K, Liu K, Li Q (2008) Analysis of yohimbine alkaloid from *Pausinystalia yohimbe* by non-aqueous capillary electrophoresis and gas chromatography-mass spectrometry. J Sep Sci 31(12):2211-2218. https://doi.org/10.1002/jssc.200800055

26 Sun J, Baker A, Chen P (2011) Profiling the indole alkaloids in yohimbe bark with ultra-performance liquid chromatography coupled with ion mobility quadrupole time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 25(18):2591-2602. https://doi.org/10.1002/rcm.5158

27 Sun J, Chen P (2012) Chromatographic fingerprint analysis of yohimbe bark and related dietary supplements using UHPLC/UV/MS. J Pharm Biomed Anal 61:142-149. https://doi.org/10.1016/j.jpba.2011.11.013

28 Gupta S, Shanker K, Srivastava SK (2012) HPTLC method for the simultaneous determination of four indole alkaloids in *Rauwolfia tetraphylla*: A study of organic/green solvent and continuous/pulse sonication, J Pharm Biomed Analysis 66:33-39. https://doi.org/10.1016/j.jpba.2012.02.018

29 Ren Y, Wu C, Zhang J (2012) Simultaneous screening and determination of 18 illegal adulterants in herbal medicines and health foods for male sexual potency by ultra-fast liquid chromatography-electrospray ionization tandem mass spectrometry. J Sep Sci 35(21):2847-2857. https://doi.org/10.1002/jssc.201200280

30 Chen Q, Chen J, Li P, Xiong L, Liu Q (2013) Simple and fast determination of reserpine and yohimbine from *Rauvolfia yunnanensis* by nonaqueous capillary electrophoresis. Anal Methods 5:3347-3352. https://doi.org/10.1039/C3AY40492G

31 Raman V, Avula B, Galal AM, Wang Y-H, Khan IA (2013) Microscopic and UPLC-UV-MS analyses of authentic and commercial yohimbe (*Pausinystalia johimbe*) bark samples. J Nat Med 67(1):42-50. https://doi.org/10.1007/s11418-012-0642-2

32 Hong B, Li W, Song A, Zhao C (2013) Determination of indole alkaloids and highly volatile compounds in *Rauvolfia verticillata* by HPLC-UV and GC-MS. J Chromatogr Sci 51(10):926-930. https://doi.org/10.1093/chromsci/bms191

33 Cohen PA, Wang Y-H, Maller G, DeSouza R, Khan IA (2016) Pharmaceutical quantities of yohimbine found in dietary supplements in the USA. Drug Test Anal 8(3-4):357-369. https://doi.org/10.1002/dta.1849

34 Efron B, Tibshirani JR (1993) An Introduction to the Bootstrap, Chapman and Hall, UK.

35 Taylor BN, Kuyatt CE (1994) Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results, NIST Technical Note 1297, U.S. Government Printing Office: Washington, DC. http://www.nist.gov/pml/pubs/tn1297/index.cfm

36 JCGM-100:2008 (2008) Evaluation of Measurement Data – Guide to the Expression of Uncertainty in Measurement. Joint Committee for Guides in Metrology. http://www.bipm.org/utils/common/documents/jcgm/JCGM_100_2008_E.pdf

Appendix A. List of Acronyms

ACN	acetonitrile
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
au	arbitrary unit
COSY	1H-1H correlation (COSY) NMR spectroscopy
D	deuterium
DMSO	dimethyl sulfone
ESI	electrospray ionization
FA	formic acid
H_2O	water
HSQC	heteronuclear single quantum coherence
IS	internal standard
LC	liquid chromatography
LC-APCI-MS	liquid chromatography atmospheric pressure chemical ionization mass spectrometry
LC-ESI-MS	liquid chromatography electrospray mass spectrometry
LC-PDA	liquid chromatography photodiode array detection
LC-UV	liquid chromatography ultraviolet absorbance detection
MeOH	methanol
MBE	methanol basic extraction
NIH-ODS	National Institutes of Health Office of Dietary Supplements
NIST	National Institute of Standards and Technology
NMR	nuclear magnetic resonance spectroscopy
^{1}H-NMR	proton NMR
PDA	photodiode-array detector
PDE5	phosphodiesterase type 5
PFE	pressurized fluid extraction
qNMR	quantitative ^{1}H-NMR
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
RG	reagent grade
RM	reference material
RP	reverse phase
RSD	relative standard deviation
SD	standard deviation
SI	International System of Units
SIM	single ion monitoring
SRM	Standard Reference Material
TGA	thermogravimetric analysis
TWC	total wavelength chromatogram
US	United States
USP	United States Pharmacopeia
UV	ultraviolet absorbance detection