2017 AOAC OFFICIAL METHODS BOARD AWARDS

2014 - 2016 SPDS METHODS TO BE REVIEWED FOR

2017 METHOD OF THE YEAR

OFFICIAL METHODS OF ANALYSIS OF AOAC INTERNATIONAL

METHOD OF THE YEAR
OMB may select more than one method in this category each year.

Selection Criteria
The minimum criteria for selection are:

a. The method must have been approved for first or final action within the last three years.
b. Generally, some unique or particularly noteworthy aspect of the method is highlighted as making it worthy of the award, such as innovative technology or application, breadth of applicability, critical need, difficult analysis, and/or range of collaborators.
c. The method demonstrates significant merit in scope or is an innovative approach to an analytical problem.

Selection Process:

a. AOAC staff lists all eligible methods for consideration and forwards that list with supporting documentation (e.g. ERP chair recommendation(s)) to the Chair of the Official Methods Board (OMB).
b. The Chair forwards the list along with any supporting information to the members of the OMB.
c. The OMB selects the Method of the Year. The winner is selected by 2/3 vote. If necessary, the OMB chair may cast tie-breaking vote.

Award
An appropriate letter of appreciation and thanks will be sent to the author(s) of the winning method. The corresponding author will be announced at the appropriate session of the AOAC INTERNATIONAL annual meeting, with presentation of an award. All authors will be acknowledged at the annual meeting, will receive an award and a letter of appreciation. The name of the winner(s), with supporting story, will be carried in the announcement in the *ILM*.

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DIETARY SUPPLEMENTS

Quantitative Analysis of Aloins and Aloin-Emodin in Aloe Vera Raw Materials and Finished Products Using High-Performance Liquid Chromatography: Single-Laboratory Validation, First Action 2016.09

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A single-laboratory validation study is described for a method of quantitative analysis of aloins (aloins A and B) and aloe-emodin in aloe vera raw materials and finished products. This method used HPLC coupled with UV detection at 380 nm for the aloins and 430 nm for aloe-emodin. The advantage of this test method is that the target analytes are concentrated from the sample matrix (either liquid or solid form) using stepwise liquid–liquid extraction (water–ethyl acetate–methanol), followed by solvent evaporation and reconstitution. This sample preparation process is suitable for different forms of products. The concentrating step for aloins and aloe-emodin has enhanced the method quantitation level to 20 parts per billion (ppb). Reversed-phase chromatography using a 250 × 4.6 mm column under gradient elution conditions was used. Mobile phase A is 0.1% acetic acid in water and mobile phase B is 0.1% acetic acid in acetonitrile. The HPLC run starts with a 20% mobile phase B that reaches 35% at 13 min. From 13 to 30 min, mobile phase B is increased from 35 to 100%. From 30 to 40 min, mobile phase B is changed from 100% back to the initial condition of 20% for re-equilibration. The flow rate is 1 mL/min, with a 100 µL injection volume. Baseline separation (R_s > 2.0) for aloins A and B and aloe-emodin was observed under this chromatographic condition. This test method was validated with raw materials of aloe vera 5× (liquid) and aloe vera 200× (powder) and finished products of aloe concentrate (liquid) and aloe (powder). The linearity of the method was studied from 10 to 500 ppb for aloins A and B and aloe-emodin, with correlation coefficients of 0.999964, 0.999957, and 0.999980, respectively. The test method was proven to be specific, precise, accurate, rugged, and suitable for the intended quantitative analysis of aloins and aloe-emodin in raw materials and finished products. The S/N for aloins A and B and aloe-emodin at 10 ppb level were 12, 10, and 8, respectively, indicating our conservative LOD level at 10 ppb (the typical LOD level S/N is about 3). The S/N for aloins A and B and aloe-emodin at the 20 ppb level were 17, 14, and 16, respectively, indicating our conservative LOQ level at 20 ppb (the typical LOQ level S/N is about 10). The stock standard solution of a mixture of aloins and aloe-emodin and a working standard solution were found to be stable for at least 19 days when stored refrigerated at 2–8°C, with a recovery of 100 ± 5%.

Aloe vera has long been used in health foods and dietary supplements (1, 2). Aloin A (barbaloin), aloin B (isobarbaloin), and aloe-emodin are natural components in aloe vera and are referred to as anthraquinones (3). These compounds are removed during the raw material manufacturing process because recent literature indicates adverse effects if the compounds are consumed in sufficient quantities (4). In 2011, the International Aloe Science Council provided an industry guideline limiting the amount of aloins present in aloe products for oral consumption to less than 10 parts per million (ppm; Figure 1; 4).

In 2015, AOAC *Standard Method Performance Requirements* called for "Methods for the Determination of Aloin A and Aloin B in Dietary Supplement Products and Ingredients" (5) with a low parts-per-billion (ppb) level quantitation limit. To support this AOAC initiative and to QC raw materials and finished products of aloe vera, it was essential to develop and validate a method to quantitate aloins A and B. Several analytical methods are published in the literature for the detection and quantitation of aloins; however, these lack the required performance characteristics of accuracy, precision, specificity, linearity, and LOD and LOQ (6–11). Our test method had conservative LOQ levels at 20 ppb with nearly twice the response (S/N) of what is usually required using a conventional-sized HPLC column (250 × 4.6 mm) and HPLC instrument. In using an ultra-performance LC system and smaller particle-size columns, it is possible that the LOQ level may reach single-digit ppb levels.

Received November 16, 2016. Accepted by AP January 17, 2017.
This method was approved by the AOAC Expert Review Panel for Aloin as First Action.
The Expert Review Panel for Aloin Methods invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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DOI: 10.5740/jaoacint.16-0387

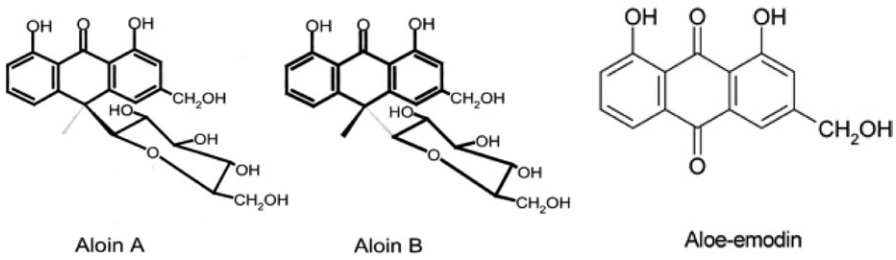

Figure 1. Chemical structures of aloins A and B and aloe-emodin.

The current test method has been used in multiple laboratories for many years, indicating the robustness of the method in an industrial QC test environment.

AOAC Official Method 2016.09
Quantitative Analysis of Aloins and Aloin-Emodin in Aloe Vera Raw Material and Finished Product
HPLC
First Action 2016

A. Principle

This test method uses a reversed-phase HPLC system for the separation of aloins A and B and aloe-emodin with an external standard for the quantitation of raw materials and finished products. A dual-wavelength UV detector or photodiode array (PDA) detector can be used. For sample preparation, stepwise liquid–liquid extraction of target analytes (aloins and aloe-modin) was used, followed by the evaporation of extraction media, then reconstitution in solvents that concentrated the target analytes by volume reduction. UV wavelengths were optimized for sensitivity and to reduce interference from sample matrixes.

This method is validated for linearity, accuracy, precision, ruggedness, specificity, standard solution stability, and system suitability. The details of the test procedure and method validation are described below.

B. Reagents and Samples

(a) *Aloin A reference standard*.—Chromadex Cat. No. 00001625 (stored in a refrigerator).

(b) *Aloin B reference standard*.—Chromadex Cat. No. 00001626 (stored in a freezer).

(c) *Aloe-emodin reference standard*.—Sigma Cat. No. A7687 (stored in a refrigerator).

(d) *Purified water or equivalent*.

(e) *Reagent alcohol*.—Spectrum Cat. No. A1040 or equivalent.

(f) *Acetonitrile*.—HPLC grade.

(g) *Methanol*.—HPLC grade.

(h) *Glacial acetic acid*.—ACS grade.

(i) *Sodium chloride crystal*.—Reagent, ACS grade, Spectrum Cat. No. S1240 or equivalent.

(j) *Ethyl acetate*.—HPLC grade, Spectrum Cat. No. HP602 or equivalent.

(k) *Aloe raw materials*.—Obtained from raw material suppliers.

(l) *Aloe concentrate liquid product and aloe powder products*.—Obtained from Herbalife.

C. Apparatus

(a) *Analytical balance*.—Capable of reading ±0.01 mg, Mettler Toledo or equivalent.

(b) *Disposable syringe filters*.—17 mm, 0.2 µm, PVDF, Thermo Scientific Cat. No. 42213-PV or equivalent.

(c) *Vortex mixer*.—Fisher Scientific Part No. 1978331 or equivalent.

(d) *Centrifuge*.—Thermo Scientific Sorvall ST 16R or equivalent.

(e) *Sonicator*.—Fisher Scientific Model 110 or equivalent.

(f) *Nitrogen evaporator with water bath*.—Organomation Associates, Inc. or equivalent.

(g) *Automatic pipet (range of 100–1000 µL and 0.5–5 mL)*.—Eppendorf Research plus or Class A volumetric pipet or equivalent.

D. HPLC System

(a) *HPLC system*.—Waters 2695 Alliance Separations Module (Milford, MA), consisting of a pump and an autosampler.

(b) *PDA detector or any variable wavelength UV detector*.—Waters Corp.

(c) *HPLC column*.—Phenomenex Synergi Hydro-RP, 250 × 4.6 mm, Part No. 00G-4375-E0.

(d) *Guard column*.—Phenomenex C18, 4 × 3.0 mm, Cat. No. AJO-4287.

(e) *HPLC conditions*.—(*1*) Mobile phase A consists of 0.1% acetic acid in water and B consists of 0.1% acetic acid in acetonitrile (*see* Table **2016.09**).

(*2*) *Flow rate*.—1.0 mL/min.

(*3*) *Column and sample solution temperature*.—Ambient (20–25°C).

(*4*) *Wavelength*.—380 nm for aloins A and B and 430 nm for aloe-emodin.

(*5*) *Injection volume*.—100 µL.

(*6*) *Run time*.—40 min: aloin A, ~11.3 min; aloin B, ~10.4 min; and aloe-emodin ~23.2 min.

Table 2016.09. Gradient table

Time, min	Flow, mL/min	A, %	B, %
0	1	80	20
13	1	65	35
30	1	0	100
31	1	80	20
40	1	80	20

E. Preparation of Extraction Solutions

(a) *Saturated sodium chloride solution.*—Add 50 g sodium chloride to 100 mL freshly boiled purified water.

(b) *Ethyl acetate–methanol (90 + 10) solution.*—Combine 900 mL ethyl acetate with 100 mL methanol. Mix well.

(c) *Methanol–water (60 + 40) solution.*—Combine 600 mL methanol with 400 mL purified water. Mix well.

F. Preparation of Standards

(a) *Stock standard solutions*.—(*1*) *Aloin A and B stock standard solution.*—Accurately weigh about 5 mg each of aloin A and B reference standards into the same 50 mL volumetric flask. Dissolve and dilute to volume with methanol. Store the stock standard solution in a refrigerator [2–8°C, concentration of ~100 parts per million (ppm)].

(*2*) *Aloe-emodin stock standard solution*.—Accurately weigh about 5 mg aloe-emodin reference standard into a 50 mL volumetric flask. Dissolve and dilute to volume with methanol. Store the stock standard solution in a refrigerator (2–8°C, concentration of ~100 ppm).

(*3*) *Aloin A and B mid-standard solution*.—Pipet 100 µL aloin A and B stock standard solution into a 10 mL volumetric flask and dilute to volume with methanol–water (60 + 40). Mix well (concentration of ~1 ppm).

(*4*) *Aloe-emodin mid-standard solution*.—Pipet 100 µL aloe-emodin stock standard solution into a 10 mL volumetric flask and dilute to volume with methanol–water (60 + 40). Mix well (concentration of ~1 ppm).

(b) *Working standard solution*.—(*1*) *Standard [300 parts per billion (ppb)]*.—Pipet 3 mL mid-standard solution into a 10 mL volumetric flask and dilute to volume with methanol–water (60 + 40). Mix well.

(*2*) *Standard (80 ppb)*.—Pipet 2 mL mid-standard solution into a 25 mL volumetric flask and dilute to volume with methanol–water (60 + 40). Mix well.

(*3*) *Standard (20 ppb)*.—Pipet 200 µL mid-standard solution into a 10 mL volumetric flask and dilute to volume with methanol–water (60 + 40). Mix well.

G. Preparation of Samples

(a) *Raw material (powder and liquid form)*.—(*1*) Accurately weigh about 0.1 g powder raw material sample into a 15 mL screw-cap test tube; pipet 1 mL purified water and mix the sample well on a vortex mixer to dissolve the powder. For the liquid raw material, weigh ~1 g raw material sample into a 15 mL screw-cap test tube.

(*2*) Pipet 1 mL reagent alcohol and 2 mL saturated sodium chloride solution into the test tube. Mix the sample well on the vortex mixer.

(*3*) Pipet 4 mL ethyl acetate–methanol (90 + 10) solution into the test tube.

(*4*) Cap the test tube and mix the sample on the vortex mixer for about 60 s at maximum speed.

(*5*) If necessary, centrifuge the sample at 2000 rpm for 5 min to aid separation of layers.

(*6*) Transfer the top-most organic layer into a second 15 mL screw-cap test tube. Extract the sample from the original test tube one more time using a 4 mL portion of the ethyl acetate–methanol (90 + 10) solution. Mix the sample on the vortex mixer for about 30 s. If necessary, centrifuge the sample at 2000 rpm for 5 min to separate layers. Transfer the top-most organic layer into the second test tube.

(*7*) Evaporate the combined sample extract of the second test tube to dryness using an N_2 purge in a 50°C water bath.

(*8*) Pipet 0.5 mL methanol–water (60 + 40) into the second test tube containing the dried sample residue. Mix the sample on a vortex mixer for 15 s.

(*9*) Filter the sample through a 0.2 µm PVDF filter into a glass HPLC vial with an insert or a microvial.

(b) *Finished product (powder and liquid form)*.—(*1*) *Powder product*.—Accurately weigh about 0.5 g powder product into a 15 mL screw-cap test tube, pipet 1 mL purified water, and mix the sample well on the vortex mixer to dissolve the powder.

(*2*) *Liquid product*.—Accurately weigh about 1 g liquid product into a 15 mL screw-cap test tube.

(*3*) Pipet 1 mL reagent alcohol and 2 mL saturated sodium chloride solution into the test tube. Mix the sample well on the vortex mixer.

(*4*) Pipet 4 mL ethyl acetate–methanol (90 + 10) solution into the test tube.

(*5*) Cap the test tube and mix the sample on the vortex mixer for 60 s at maximum speed.

(*6*) If necessary, centrifuge the sample at 2000 rpm for 5 min to separate layers.

(*7*) Transfer the top-most organic layer into a second 15 mL screw-cap test tube.

(*8*) Extract the sample from the original test tube one more time using a 4 mL portion of the ethyl acetate–methanol (90 + 10) solution. Mix the sample on a vortex mixer for 30 s. If necessary, centrifuge the sample at 2000 rpm for 5 min to separate layers. Transfer the top-most organic layer into the second test tube.

(*9*) Evaporate the combined sample extract of the second test tube to dryness using an N_2 purge in a 50°C water bath.

(*10*) Pipet 0.5 mL methanol–water (60 + 40) into the second test tube containing the dried sample residue.

(*11*) Mix the sample on a vortex mixer for 15 s.

(*12*) Filter the sample through a 0.2 µm PVDF filter into a glass HPLC vial with the insert or a microvial.

H. System Suitability

(a) In chromatographing the standards, the correlation coefficient (R) for the aloin A and B (and aloe-emodin, if necessary) curves should not be less than 0.998.

(b) Run a standard check (80 ppb standard) after every six sample injections and at the end of the run. The peak area of each check standard should be within 90–110% of the peak area of the working standard from the calibration curve.

(c) The theoretical plate count should not be less than 10 000 for aloins A and B and aloe-emodin (if required).

(d) The tailing factor should not be more than 2.0 for aloins A and B and aloe-emodin (if required).

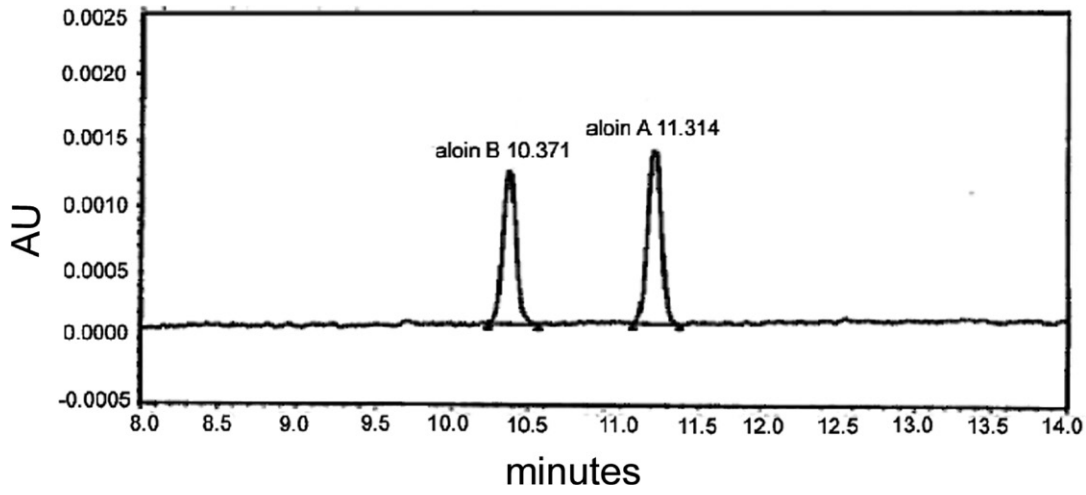

Figure 2016.09A. HPLC chromatogram of an 80 ppb standard mixture injection. A refers to aloins A and B at 380 nm.

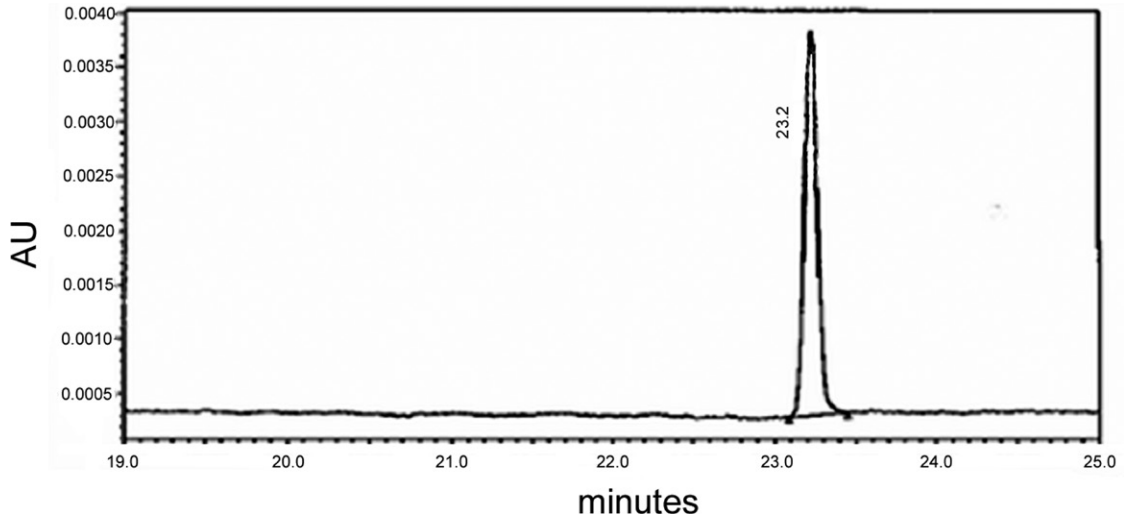

Figure 2016.09B. HPLC chromatogram of an 80 ppb standard mixture injection. B refers to aloe-emodin at 430 nm.

I. Calculations

Obtain the standard curves for aloins A and B (and aloe-emodin, if necessary) by plotting the standard concentrations of aloins A and B (and aloe-emodin, if necessary) versus the peak areas of aloins A and B (and aloe-emodin, if necessary). Calculate the amount of aloin A and B (and aloe-emodin, if necessary) in the sample according to the formulas below for the raw materials and finished products:

$$\frac{(\text{aloin A}/\text{aloin B}/\text{aloe-emodin})}{(\mu\text{g}/\text{g or ppm})} = \frac{\text{sample concn}_{(\mu\text{g/mL})} \times \text{DF}_{(\text{mL})}}{\text{sample wt}_{(\text{g})}}$$

where sample concn = the sample concentration from the standard curve and DF = the dilution factor for the sample. A chromatogram of the aloin A and B standard (80 ppb) at 380 nm is shown in Figure **2016.09A**. A chromatogram of the aloe-emodin standard (80 ppb) at 430 nm is shown in Figure **2016.09B**.

Results and Discussion

Single-Laboratory Validation Parameters

This method validation work was conducted following the guidelines of AOAC INTERNATIONAL criteria for single-laboratory validation (12).

Specificity

Chromatograms from the blank and placebo runs were overlaid with the chromatograms from the standard and sample to show that there was no significant interference at the retention times (RTs) of the peaks of aloins A and B and aloe-emodin (Figure 2). No significant interfering peaks were present at the RTs of aloins A and B and aloe-emodin from the placebo and blank solution. Two aloin compound peaks from the standard injection were observed at about 10.4 and 11.3 min. No interference peaks were observed for the injections (from the bottom upwards) of solvent blank, placebo of powder product,

and placebo of liquid concentrate product. When 20 ppb aloins were spiked into the powder placebo or into the placebo of liquid concentrate product, aloin peaks were clearly observed (matching the RTs of the aloin peaks in the standard injection).

For aloe-emodin, the specificity study results clearly showed that there was no significant interference from the placebos of either the powder product or liquid concentrate product (Figure 3).

When 20 ppb aloe-emodin were spiked into the powder placebo or into the placebo of the liquid concentrate product, aloe-emodin peaks were clearly observed (matching the RTs of the aloe-emodin peaks in the standard injection).

Precision

Six replicate samples from each of the four products were prepared according to the previously described test method. Samples were analyzed against a freshly prepared standard solution. The amounts of aloin A and B and aloe-emodin recovered from each sample were then calculated.

Tables 1–4 show that the RSDs of six test results for aloin A and B and aloe-emodin for each of the four products were less than 10%.

Accuracy

Liquid and powder placebo samples were spiked in triplicate with 10, 20, and 30 ppb spiking solutions of aloins A and B and aloe-emodin at the 50, 100, and 150% level. The spiked samples (three concentrations and three replicates of each concentration) were analyzed according to the internal test method. The amount of aloins A and B and aloe-emodin in the spiked samples were calculated as the percentage recovery.

Tables 5–10 show that average recoveries for the spiked samples were 82.9–100.1% for aloin A, 85.5–89.5% for aloin B, and 94.2–109.1% for aloe-emodin, which were all within the acceptable limit range of 80–120%.

Linearity/Range

A standard solution containing 1 ppm of aloins A and B and aloe-emodin was prepared. Dilutions from the 1 ppm standard were made to obtain standard solutions containing 10, 20, 40, 80, 160, and 500 ppb of aloins A and B and aloe-emodin (*see* Table 11).

Three replicate injections were made for each of the six solutions prepared above. The peak areas for aloins A and B and aloe-emodin that were obtained for each solution were plotted against their corresponding concentrations. Linear regression analyses on the six coordinates were performed.

Tables 12–17 and Figures 4–6 show that the linearity of detector response for aloins A and B and aloe-emodin in the range of 10–500 ppb yielded linear correlation coefficients (R) of 0.9999, which were within the acceptable limit of >0.998.

Ruggedness

The same four products were analyzed (in duplicate) by a second analyst on a different day, using a different HPLC system and a different Phenomenex Synergi Hydro-RP HPLC column. Results were compared with the average results from the precision test for aloins A and B and aloe-emodin.

Table 18 shows that there was a difference of <10% in the test results obtained by the two analysts for aloins A and B. The difference in the test results for aloe-emodin in aloe vera 5×, aloe concentrate, and aloe powder was <10% and in the aloe vera gel 200×, the difference was slightly higher, at 15.7%. The obtained 15.7% difference was considered justifiable given the method accuracy requirement was 80–120%. Therefore, 15.7% was within the method accuracy requirement of 20% variability from 100%.

System Suitability

System suitability parameters for working standards of aloins A and B and aloe-emodin were calculated using Waters

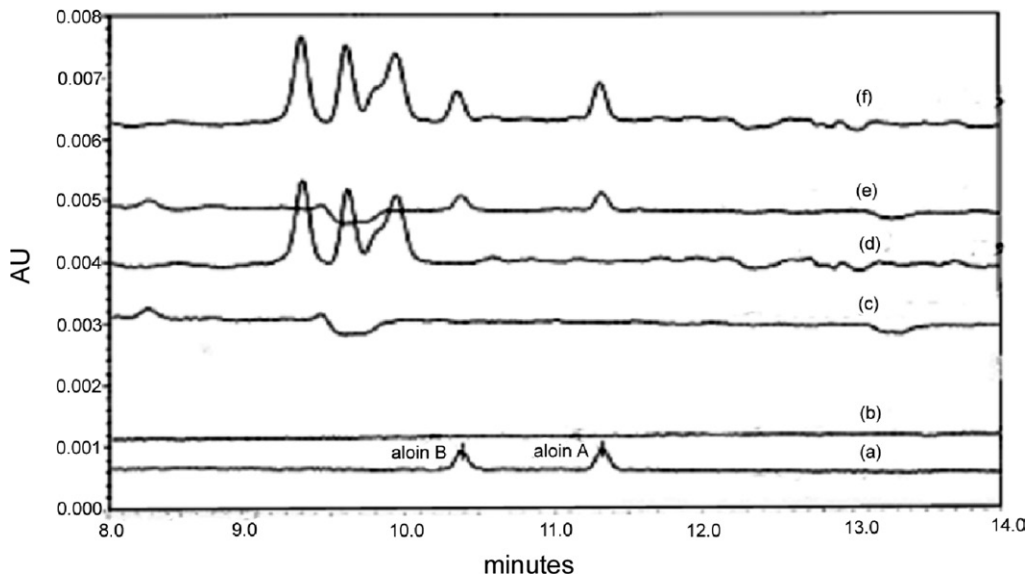

Figure 2. Aloin region overlay HPLC chromatograms of (a) 20 ppb standard mixture, (b) solvent blank, (c) placebo of the powder product, (d) placebo of the liquid concentrate product, (e) 20 ppb standard mixture-spiked placebo of the powder product, and (f) 20 ppb standard-spiked placebo of the liquid concentrate product.

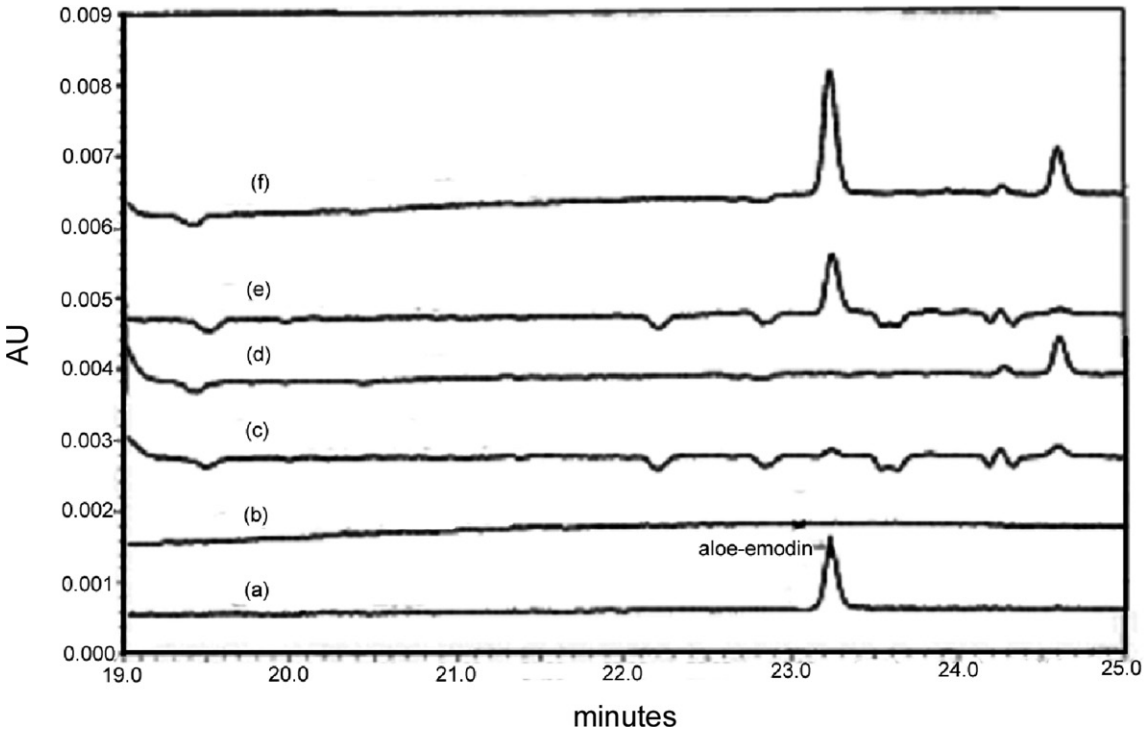

Figure 3. Aloe-emodin region overlay HPLC chromatograms of (a) 20 ppb standard mixture, (b) solvent blank, (c) placebo of the powder product, (d) placebo of the liquid concentrate product, (e) 20 ppb standard mixture-spiked placebo of the powder product, and (f) 20 ppb standard-spiked placebo of the liquid concentrate product.

Table 1. Precision test for a typical lot of aloe vera 5× raw material

Lot No.	Aloin A, ppb	Aloin B, ppb	Aloe-emodin, ppb
1	ND[a]	ND	ND
2	ND	ND	ND
3	ND	ND	ND
4	ND	ND	ND
5	ND	ND	ND
6	ND	ND	ND
Avg.	NA[b]	NA	NA
RSD, %	NA	NA	NA

[a] ND = None detected.

[b] NA = not available.

Table 2. Precision test for a typical lot of aloe vera 200× raw material

Lot No.	Aloin A, ppb	Aloin B, ppb	Aloe-emodin, ppb
1	345.9	325.5	22.9
2	355.1	351.5	26.6
3	340.6	327.9	23.7
4	349.1	330.1	22.1
5	348.8	330.1	24.1
6	341.4	333.9	21.6
Avg.	346.8	333.2	23.5
RSD, %	1.6	2.8	7.5

Table 3. Precision test for a typical lot of aloe concentrate product

Lot No.	Aloin A, ppb	Aloin B, ppb	Aloe-emodin, ppb
1	ND[a]	ND	ND
2	ND	ND	ND
3	ND	ND	ND
4	ND	ND	ND
5	ND	ND	ND
6	ND	ND	ND
Avg.	NA[b]	NA	NA
RSD, %	NA	NA	NA

[a] ND = None detected.

[b] NA = not available.

Table 4. Precision test for a typical lot of aloe powder

Lot No.	Aloin A, ppb	Aloin B, ppb	Aloe-emodin, ppb
1	197.0	198.8	<20
2	198.7	202.7	<20
3	206.2	205.4	<20
4	201.4	206.6	<20
5	202.8	208.7	<20
6	201.3	207.7	<20
Avg.	201.2	205.0	NA[a]
RSD, %	1.6	1.8	NA

[a] NA = not available.

Table 5. Accuracy test for aloin A in aloe concentrate

Spike level, ppb	Sample wt, g	Aloin A added, ng	Expected amount of aloin A, ppb	Aloin A found, ppb	Recovery, %	Avg. recovery, %
10	1.0066	11.07	11.00	9.17	83.4	82.9
	1.0229	11.07	10.82	9.28	85.7	
	1.0294	11.07	10.75	8.57	79.7	
20	1.0060	22.14	22.01	18.96	86.2	86.7
	1.0205	22.14	21.70	19.02	87.7	
	1.0256	22.14	21.59	18.64	86.3	
30	1.0164	33.21	32.67	29.13	89.2	88.1
	1.0228	33.21	32.47	28.13	86.6	
	1.0014	33.21	33.16	29.31	88.4	

Table 6. Accuracy test for aloin B in aloe concentrate

Spike level, ppb	Sample wt, g	Aloin B added, ng	Expected amount of aloin B, ppb	Aloin B found, ppb	Recovery, %	Avg. recovery, %
10	1.0066	10.23	10.16	8.31	81.8	87.1
	1.0229	10.23	10.00	8.99	89.9	
	1.0294	10.23	9.94	8.90	89.6	
20	1.0060	20.46	20.34	19.14	94.1	89.5
	1.0205	20.46	20.05	18.19	90.7	
	1.0256	20.46	19.95	16.68	83.6	
30	1.0164	30.69	30.19	25.15	83.3	86.1
	1.0228	30.69	30.01	25.34	84.5	
	1.0014	30.69	30.65	27.71	90.4	

Table 7. Accuracy test for aloe-emodin in aloe concentrate

Spike level, ppb	Sample wt, g	Aloe-emodin added, ng	Expected amount of aloe-emodin, ppb	Aloe-emodin found, ppb	Recovery, %	Avg. recovery, %
10	1.0066	11.14	11.07	11.05	99.8	99.1
	1.0229	11.14	10.89	10.57	97.1	
	1.0294	11.14	10.82	10.85	100.3	
20	1.0060	22.28	22.15	21.25	95.9	95.0
	1.0205	22.28	21.83	20.27	92.8	
	1.0256	22.28	21.72	20.94	96.4	
30	1.0164	33.42	32.88	31.29	95.2	94.2
	1.0228	33.42	32.68	30.38	93.0	
	1.0014	33.42	33.37	31.55	94.5	

Table 8. Accuracy test for aloin A in aloe powder

Spike level, ppb	Sample wt, g	Aloin A added, ng	Expected amount of aloin A, ppb	Aloin A found, ppb	Recovery, %	Avg. recovery, %
10	0.5046	5.535	10.97	11.07	100.9	100.1
	0.5087	5.535	10.88	9.74	89.5	
	0.5105	5.535	10.84	11.93	110.0	
20	0.5194	11.07	21.31	18.88	88.6	85.1
	0.5059	11.07	21.88	18.45	84.3	
	0.5059	11.07	21.88	18.04	82.4	
30	0.5134	16.61	32.34	26.01	80.4	84.5
	0.5106	16.61	32.52	26.90	82.7	
	0.5018	16.61	33.09	29.89	90.3	

Table 9. Accuracy test for aloin B in aloe powder

Spike level, ppb	Sample wt, g	Aloin B added, ng	Expected amount of aloin B, ppb	Aloin B found, ppb	Recovery, %	Avg. recovery, %
10	0.5046	5.115	10.14	9.56	94.3	89.1
	0.5087	5.115	10.06	9.06	90.1	
	0.5105	5.115	10.02	8.31	82.9	
20	0.5194	10.23	19.70	15.86	80.5	85.5
	0.5059	10.23	20.22	18.96	93.8	
	0.5059	10.23	20.22	16.60	82.1	
30	0.5134	15.35	29.89	25.95	86.8	88.1
	0.5106	15.35	30.05	26.34	87.6	
	0.5018	15.35	30.58	27.48	89.9	

Table 10. Accuracy test for aloe-emodin in aloe powder

Spike level, ppb	Sample wt, g	Aloe-emodin added, ng	Expected amount of aloe-emodin, ppb	Aloe-emodin found, ppb	Recovery, %	Avg. recovery, %
10	0.5046	5.570	11.04	11.72	106.2	109.1
	0.5087	5.570	10.95	12.14	110.9	
	0.5105	5.570	10.91	12.04	110.3	
20	0.5194	11.14	21.45	20.08	93.6	95.6
	0.5059	11.14	22.02	21.28	96.6	
	0.5059	11.14	22.02	21.29	96.7	
30	0.5134	16.71	32.55	30.49	93.7	94.9
	0.5106	16.71	32.73	31.02	94.8	
	0.5018	16.71	33.30	32.01	96.1	

Table 11. Linearity and range standard solutions

Linearity standards concn, ppb	1 ppm aloin A and B and aloe-emodin standard, mL	Final volume, mL
10	2.0	200
20	2.0	100
40	2.0	50
80	2.0	25
160	4.0	25
500	5.0	10

Table 12. Linearity test for aloin A

Concn, ppb	Peak area
10.10	845
20.20	1947
40.39	3829
80.79	7451
161.57	14641
504.92	46321

Table 13. Regression analysis for aloin A

R	0.999964
y-Intercept	14.03195
Slope	91.62991

Table 14. Linearity test for aloin B

Concn, ppb	Peak area
10.04	760
20.09	1584
40.17	3143
80.34	6348
160.69	12947
502.15	40416

Table 15. Regression analysis for aloin B

R	0.999957
y-Intercept	–65.10327
Slope	80.62814

Table 16. Linearity test for aloe-emodin

Concn, ppb	Peak area
10.37	2386
20.74	4896
41.47	9774
82.94	19977
165.89	40316
518.40	128405

Table 17. Regression analysis for aloe-emodin

R	0.999980
y-Intercept	–461.6635
Slope	248.2991

Empower System Suitability software. The curves for aloins A and B and aloe-emodin had an R of >0.998. Tailing factors for the aloin A and B and aloe-emodin peaks were <2.0. The theoretical plates of the peaks for aloins A and B and aloe-emodin were >10 000 (Table 19).

LOD

The LOD of the method was determined by measuring S/N from a spiked placebo sample from herbal aloe concentrate mango at 10 ppb level.

The LOD met acceptance criteria of not more than 10 ppb. The S/N for aloins A and B and aloe-emodin were 11.8, 9.8, and 7.8, respectively. The LOD of this method was 10 ppb. (This approach was very conservative because a typical LOD level requires a S/N of about 3.)

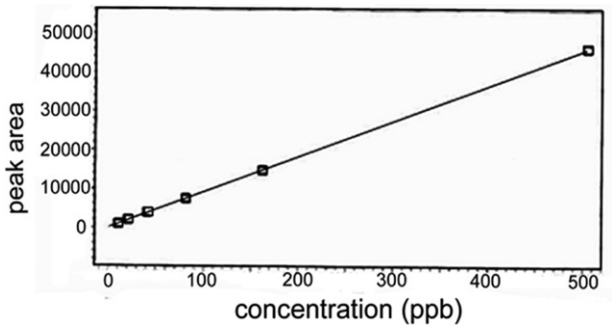

Figure 4. Linearity plot of aloin A.

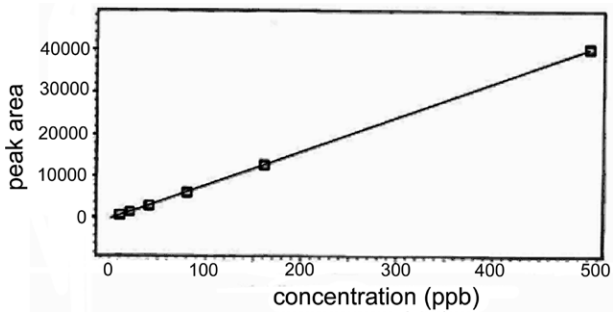

Figure 5. Linearity plot of aloin B.

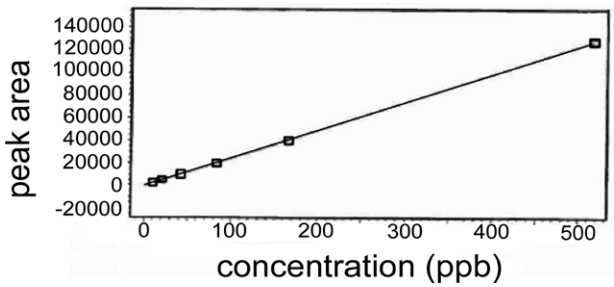

Figure 6. Linearity plot for aloe-emodin.

Table 18. Ruggedness test for aloins A and B and aloe-emodin

Analyst[a,b]	Product	Aloin A, ppb	Aloin B, ppb	Aloe-emodin, ppb	Analysis date	Difference
Analyst 1	Aloe vera WL 5×, lot 1	ND[c]	ND	ND	December 10, 2010	NA[d]
Analyst 2	Aloe vera WL 5×, lot 1	ND	ND	ND	January 12, 2011	
Analyst 1	Aloe vera gel 200×, lot 2	346.8	333.2	23.5	December 10, 2010	4.7% for aloin A
Analyst 2	Aloe vera gel 200×, lot 2	363.5	362.6	27.5	January 12, 2011	8.5% for aloin B
						15.7% for aloe-emodin
Analyst 1	Herbal aloe concentrate mango, lot 3	ND	ND	ND	December 10, 2010	NA
Analyst 2	Herbal aloe concentrate mango, lot 3	ND	ND	ND	January 12, 2011	
Analyst 1	Herbal aloe powder mango, lot 4	201.2	205.0	<20	December 10, 2010	2.5% for aloin A
Analyst 2	Herbal aloe powder mango, lot 4	206.3	217.5	<20	January 12, 2011	5.9% for aloin B
						NA for aloe-emodin

[a] Analyst 1 used the following equipment: HPLC No. 4, Waters Alliance System, Model 2695, Serial No. J07SM4025A and a Phenomenex Synergi Hydro-RP column, 80 A, 4 µ, 250 × 4.6 mm (Part No. 00G-4375-E0), Serial No. 553344-13.

[b] Analyst 2 used the following equipment: HPLC No. 3, Waters Alliance System, Model 2695, Serial No. J07SM4030A and a Phenomenex Synergi Hydro-RP column, 80 A, 4 µ, 250 × 4.6 mm (Part No. 00G-4375-E0), Serial No. 524451-39.

[c] ND = None detected.

[d] NA = not available.

LOQ

The LOQ of the method was determined by measuring S/N from a spiked placebo sample of herbal aloe concentrate mango at the 20 ppb level.

The LOQ met acceptance criteria of not more than 20 ppb. The S/N for aloins A and B and aloe-emodin were 16.7, 14.3, and 16, respectively. The LOQ of this method was at 20 ppb. (This approach was very conservative because a typical LOQ level requires a S/N of about 10.)

Aloin A and B and Aloe-Emodin Stock Standard Stability

The stock standard solution of aloins A and B and aloe-emodin that was initially prepared was stored in a refrigerator (2–8°C). After 19 days, the stock standard solution was removed from the refrigerator and a fresh stock standard solution was prepared. Both stock standard solutions were diluted and tested. Table 20 shows the recovery of the refrigerated standard calculated against the fresh standard to determine the stability of the stock standard solution of aloins A and B and aloe-emodin.

Based on the above test results, the stock standard solution of aloins A and B and aloe-emodin was stable for 19 days when stored in the refrigerator at 2–8°C. An expiration date of 2 weeks (14 days) will be assigned to the stock standard when stored refrigerated at 2–8°C.

Table 19. Typical system suitability results (precision test)

Analyte	R	Tailing factor	Plate count
Aloin A	0.999990	1.0	81176
Aloin B	0.999843	1.0	67627
Aloe-emodin	0.999964	1.1	417385

Table 20. Aloin A and B and aloe-emodin stock standard solution stability

80 ppb working standard	Concn expected, ppb	Concn found, ppb	Recovery, %
Aloin A	83.89	81.73	97.4
Aloin B	75.96	75.03	98.8
Aloe-emodin	76.80	80.52	104.8

Conclusions

The linearity, specificity, precision, accuracy, ruggedness, and LOD and LOQ test results demonstrated that this test method for the determination of aloins A and B and aloe-emodin by HPLC in raw materials and finished products is suitable for its intended use.

Acknowledgments

We would like to acknowledge Steven Dentali and Andrew Shao for helpful discussions.

References

(1) Pugh, N., Ross, S.A., Elsohly, M.A., & Pasco, D.S. (2001) *J. Agric. Food Chem.* **49**, 1030–1034. doi:10.1021/jf001036d

(2) Aysan, E., Bektas, H., & Ersoz, F. (2010) *Eur. J. Obstet. Gynecol. Reprod. Biol.* **149**, 195–198. doi:10.1016/j.ejogrb.2009.11.019

(3) Dagne, E. (1996) *Bull. Chem. Soc. Ethiop.* **10**, 89–103

(4) International Aloe Science Council (2017) Version 1.0, (search term: "aloin limit"), http://www.iasc.org/SearchResults.aspx?Search=aloin+limit

(5) *J. AOAC Int.* **99**, 318(2016). doi: 10.5740/jaoacint.SMPR2015.016

(6) Dell'Agli, M., Giavarini, F., Ferraboschi, P., Galli, G., & Bosisio, E. (2007) *J. Agric. Food Chem.* **55**, 3363–3367. doi:10.1021/jf070182h

(7) El Sohly, M., Gul, W., Avula, B., & Khan, I.A. (2007) *J. AOAC Int.* **90**, 28–42

(8) Fanali, S., Aturki, Z., D'Orazio, G., Rocco, A., Ferranti, A., Mercolini, L., & Raggi, M.A. (2010) *J. Sep. Sci.* **33**, 2663–2670. doi:10.1002/jssc.201000408

(9) Zahn, M., Trinh, T., Jeong, M.L., Wang, D., Abeysinghe, P., Jia, Q., & Ma, W.A. (2008) *Phytochem. Anal.* **19**, 122–126. doi:10.1002/pca.1024

(10) Zonta, F., Bogoni, P., Masotti, P., & Micali, G. (1995) *J. Chromatogr. A* **718**, 99–106. doi:10.1016/0021-9673(95)00637-0

(11) Brown, P., Yu, R., Kuan, C., Finley, J., Mudge, E., & Dentali, S. (2014) *J. AOAC Int.* **97**, 1323–1328. doi:10.5740/jaoacint.13-028

(12) Official Methods of Analysis of AOAC INTERNATIONAL (2016) 20th Ed., Appendix K: Guidelines for Dietary Supplements and Botanicals, AOAC INTERNATIONAL, Rockville, MD. www.eoma.aoac.org

DIETARY SUPPLEMENTS

Analysis of Theanine in Tea (*Camellia sinensis*) Dietary Ingredients and Supplements by High-Performance Liquid Chromatography with Postcolumn Derivatization: Single-Laboratory Validation, First Action 2016.10

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An HPLC method with postcolumn derivatization was developed and validated for the determination of theanine content in tea dietary ingredients and supplements. A variety of common commercially available supplement forms such as powders, liquid tinctures, tablets, softgels, and gelcaps, as well as three National Institute of Standards and Technology *Camellia sinensis* Standard Reference Materials were investigated in the study. A simple extraction procedure using citrate buffer at pH 2.2 allowed for the analysis of theanine without additional cleanup or concentration steps, even at low ppm levels. Theanine was separated from other naturally occurring amino acids using a cation-exchange column and detected using a UV-Vis detector after derivatization with ninhydrin reagent. A single-laboratory validation demonstrated that specificity, accuracy, precision, and other method performance parameters have met the requirements set for theanine analysis by the AOAC Stakeholder Panel on Dietary Supplements.

Tea has been consumed all over the world throughout human history and its positive effects on mood, cognitive functions, and overall health is well-recognized. The leaves of the tea plant (*Camellia sinensis*) contain a number of biologically active compounds, such as caffeine and polyphenol antioxidants, and a unique nonproteinogenic amino acid, theanine. Theanine content generally accounts for 1–4% of the dry weight of tea leaves and depends on growing conditions, tea variety, grade, and degree of fermentation (1, 2).

Studies have found that theanine promotes relaxation and alertness, decreases anxiety, may protect from environmental neurotoxins, and may even enhance the activity of certain antitumor medications (1, 3–7). It has also been noticed that many of theanine's health effects are more pronounced at higher levels of intake than made possible by drinking brewed tea alone.

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This method was approved by the Expert Review Panel for Dietary Supplements as First Action.
The Expert Review Panel for Dietary Supplements invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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Dietary supplements containing green tea have gained popularity as sources of antioxidants, weight-loss agents, and a means to improve energy level and alertness. Currently, most supplement manufacturers list polyphenol content and the amount of green tea extract, but not the amount of theanine present in the formulation. As awareness of theanine health benefits grows, consumers and manufacturers alike are looking to expand label claims to include theanine. Because the quality of starting materials, as well as manufacturing processes, affects the amino acid profile of tea-containing products, it is expected that the amount of theanine varies greatly from supplement to supplement. To support label claims and ensure the integrity of the supplement market, it is important for the industry to have reliable methods for theanine analysis in dietary ingredients and final products.

AOAC stakeholder panels comprise representatives from industry and regulatory organizations, contract laboratories, and academic institutions who are tasked with determining the need for methods, as well as method evaluation parameters. In 2015, the AOAC Stakeholder Panel on Dietary Supplements (SPDS) developed and adopted a *Standard Method Performance Requirements* (SMPRs®) for several compounds, including theanine, in tea dietary ingredients and supplements (8). The SMPRs specify the matrixes the method should be applicable to as well as accuracy, precision, and other parameters.

Analyzing amino acids in natural products comes with a unique set of challenges. Most amino acids, including theanine, do not exhibit strong light absorption or fluorescence, making them difficult to detect, especially in complex plant matrixes. Reported methods for analyzing theanine in teas mostly use chromatographic techniques such as HPLC, capillary electrophoresis, and micellar electrokinetic capillary chromatography (9–13). Theanine is then detected with or without derivatization using UV or fluorescence detection, amperometric detection, or MS (9, 14–17). Matrix effects frequently challenge these methods, potentially having a negative affect on the sensitivity and precision of the analysis and requiring additional sample cleanup steps or method adjustments for different matrixes.

Cation-exchange chromatography with postcolumn ninhydrin derivatization has long been a trusted technique for amino acid analysis in foods, animal feeds, pharmaceuticals, and clinical samples. A selective retention mechanism allows the separation of free amino acids from other matrix components, so no extensive sample cleanup is required. And because the derivatization reaction occurs after the compounds are chromatographically separated, there are no matrix effects to affect the reaction rate and signal intensity, thus ensuring that the same method and detection parameters could be used for analyzing a wide variety of complex matrixes.

Green tea-containing supplements are available in a variety of forms, such as tablets, liquid and dry capsules, tinctures, softgels, and gelcaps. They often also contain other active and inactive ingredients—including vitamins, minerals, and oils, and other plant extracts—making them exceptionally challenging and diverse samples to work with. The presented method for theanine analysis uses a simple citrate buffer extraction with no sample cleanup, followed by cation-exchange chromatography, postcolumn reaction with ninhydrin reagent, and UV-Vis detection. This method was developed in response to a call for methods issued by the SPDS and successfully validated against the requirements listed in AOAC SMPR 2015.014 (8). In August 2016, the "*Analysis of Theanine in Tea (Camellia sinensis) Dietary Ingredients and Supplements by High-Performance Liquid Chromatography with Postcolumn Derivatization*" method was approved by the AOAC Expert Review Panel and adopted as First Action *Official Methods of Analysis*SM (OMA) **2016.10**.

AOAC Official Method 2016.10
Theanine in Tea (*Camellia sinensis*) Dietary Ingredients and Supplements
High-Performance Liquid Chromatography with Postcolumn Derivatization
First Action 2016

[Applicable to the determination of L-theanine in tea (*Camellia sinensis*) dietary ingredients and supplements in the form of powders, liquids, tablets, capsules, softgels, and gelcaps.]

A. Principle

Theanine was extracted from samples with lithium citrate buffer (pH 2.2) using an ultrasonic water bath. L-Norleucine was used as the internal standard (IS). The extract was filtered and injected into a lithium cation-exchange HPLC column and theanine was separated from other free amino acids using lithium citrate buffers with different pH and concentrations as mobile phases. All amino acids, including L-theanine, react with ninhydrin reagent in the postcolumn derivatization system at 130°C and are converted to a colored derivative. Detection was performed at 570 nm using a UV-Vis detector.

B. Apparatus

(a) *HPLC system.*—Ternary or quaternary LC pump capable of delivering a pulse-free flow of 0.1–2 mL/min. An autosampler with an injection loop suitable for injections of 10–50 µL. UV-Vis or diode-array detector capable of monitoring signals at 570 nm. (Agilent Technologies 1290 or equivalent.)

(b) *Postcolumn derivatization system.*—Single-pump postcolumn derivatization system equipped with a pulse-free pump capable of delivering a flow rate of 0.3 mL/min, 0.5 mL reaction coil capable of maintaining a temperature of 130±0.5°C, and a column oven capable of controlling the temperature to between 30 and 75°C. (Pinnacle PCX, Pickering Laboratories, Inc.; or equivalent.)

(c) *Postcolumn reagent bottles.*—1 L safety-coated glass bottles, pressure resistant up to 10 psi (Part No. 3107-0137, Pickering Laboratories, Inc.; or equivalent).

(d) *HPLC columns and guards.*—Lithium cation-exchange analytical column 4×100 mm (Part No. 0354100T; Pickering Laboratories, Inc.). Cation-exchange GARD (Part No. 1700-3102; Pickering Laboratories, Inc.).

(e) *Ultrasonic water bath.*—Fisher Scientific Model FS30 or equivalent.

(f) *Centrifuge.*—Capable of accepting 50 mL centrifuge tubes (Thermo IEC Centra CL2 or equivalent).

(g) *Centrifuge tubes.*—Plastic, 50 mL, with screw cap (Fisher Scientific).

(h) *Analytical balance.*—With a readability of 0.1 mg, maximum capacity of 120 g (Fisher Scientific Accu-124, or equivalent).

(i) *Pipets.*—Various sizes, adjustable (Eppendorf or equivalent).

(j) *Pipet tips.*—Various sizes.

(k) *Syringe filters.*—Nylon, 0.45 µm, 13 mm (Whatman or equivalent).

(l) *Disposable syringes.*—Plastic 1 mL with lure connection (BD Luer-Lok or equivalent).

C. Reagents

(a) *Deionized water.*—HPLC grade water (Millipore or equivalent).

(b) *LC mobile phases.*—Lithium citrate buffer solutions for the cation-exchange separation of amino acids, pH 2.8–13 (Part Nos. Li275, Li750, and RG003; Pickering Laboratories, Inc.).

(c) *Postcolumn derivatization reagent.*—Ninhydrin reagent for amino acid analysis (Trione reagent, Part Nos. T100C or T200; Pickering Laboratories, Inc.).

(d) *Extraction solution.*—Lithium citrate buffer, pH 2.2 (Part No. Li220; Pickering Laboratories, Inc.).

(e) *L-Theanine reference standard.*—L-Theanine, CAS 3081-61-6, purity ≥98% (Sigma-Aldrich).

(f) *L-Norleucine reference standard.*—L-Norleucine, CAS 327-57-1, purity ≥98% (Sigma-Aldrich).

(g) *National Institute of Standards and Technology (NIST) Standard Reference Materials (SRMs).*—SRM 3254 *C. sinensis* (green tea) leaves, SRM 3255 *C. sinensis* (green tea) extract, SRM 3256 green tea-containing solid oral dosage form (all from NIST, Gaithersburg, MD).

(h) *Tea supplements (C. sinensis).*—The five tea supplements used in this study are listed below. Supplements were purchased from local vitamin and supplement stores. Content information was taken from the product label and not independently verified.—*(1) Liquid green tea leaf extract.*—Organic green tea leaf extract prepared in water–grain alcohol (United States Pharmacopeia grade; 35–45%), 500 mg/mL dry herb equivalent.

(*2*) *Capsules with dry green tea extract.*—Water-extracted green tea leaf extract (5:1), 500 mg extract per capsule. Dry extract containing ~50% polyphenols (30% catechins). This supplement also contained magnesium stearate, cellulose, and silicone dioxide. Capsules were made of gelatin.

(*3*) *Green tea extract gelcaps.*—Each gelcap contained 350 mg green tea extract in vegetable glycerin. Approximately 150 mg polyphenols per gelcap. The gelcap shell was made of vegetable cellulose.

(*4*) *Green tea softgels.*—Each softgel contained green tea extract (200 mg with 50% polyphenol content), fish oil (425 mg with 30% omega-3 fatty acids content), a mixture of black pepper and ginger extract (3 mg), chromium (63 µg), gelatin, glycerin, soy lecithin, titanium dioxide, and copper chlorophyllin. The softgel shell was beeswax-based.

(*5*) *Green tea extract tablets.*—Each tablet contained 500 mg green tea standardized extract (175 mg epigallocatechin gallate), calcium phosphate (47 mg calcium), stearic acid, modified cellulose gum, and silica.

D. Preparation of Standard Solutions

(a) *L-Theanine stock solution (500 µg/mL).*—L-Theanine stock solution was prepared by weighing 50 mg L-theanine in a 100 mL volumetric flask and diluting to volume with extraction solution. The final concentration was corrected for purity stated in the certificate of analysis. The solution was stored refrigerated for up to 8 weeks.

(b) *IS stock solution (500 µg/mL).*—L-Norleucine stock solution was prepared by weighing 50 mg L-norleucine in a 100 mL volumetric flask and diluting to volume with extraction solution. The final concentration was corrected to the purity stated in Certificate of Analysis. The solution was stored refrigerated for up to 8 weeks.

(c) *L-Theanine intermediate stock solution (50 µg/mL).*—L-Theanine intermediate stock solution was prepared by pipetting 2.5 mL L-theanine stock solution into a 25 mL volumetric flask and diluting to volume with extraction solution.

(d) *Mixed working calibration solutions.*—Mixed working calibration solutions were prepared by diluting stock solutions of L-theanine and L-norleucine with extraction solution according to Table **2016.10A**. All working calibration solutions were prepared on the day of analysis.

E. Sample Preparation and Extraction

Sample size and volume of extraction solution were chosen based on sample availability, sample type, and expected theanine concentration.

(a) *For samples in tablet form.*—At least 20 tablets were finely ground and the resulting sample thoroughly mixed before separating out the test portion. A 0.1–0.5 g portion was accurately weighed in a 10 or 25 mL volumetric flask. IS stock solution (500 µL) and extraction solution (20 mL) were added to the 25 mL volumetric flask and mixed well. IS stock solution (200 µL) and extraction solution (8 mL) were added to the 10 mL volumetric flask and mixed well. The flask was placed in an ultrasonic water bath for 2 h. After cooling the flask to room temperature, the solution was diluted to volume with extraction solution and then mixed and transferred to a 50 mL centrifuge tube. The extract was centrifuged for 20 min at 3800 rpm, filtered through 0.45 µm syringe filter, and placed in an HPLC autosampler vial for analysis.

Table 2016.10A. Preparation of mixed working calibration solutions

Volume of L-theanine stock solution, mL	Volume of L-norleucine stock solution, mL	Total volume of calibration solution, mL	L-Theanine concn, µg/mL	L-Norleucine concn, µg/mL
2.00	0.5	25	40	10
1.250	0.5	25	25	10
0.500	0.5	25	10	10
0.374	0.5	25	7.48	10
0.250	0.5	25	5	10
0.125	0.5	25	2.5	10
0.050	0.5	25	1	10

(b) *For samples in powder form.*—The sample was thoroughly mixed before separating out the test portion. A 0.1–0.5 g portion was accurately weighed in a 10 or 25 mL volumetric flask. IS stock solution (500 µL) and extraction solution (20 mL) were added to the 25 mL volumetric flask and mixed well. IS stock solution (200 µL) and extraction solution (8 mL) were added to the 10 mL volumetric flask and mixed well. The flask was placed in an ultrasonic water bath for 2 h. After cooling the flask to room temperature, the solution was diluted to volume with extraction solution and then mixed and transferred to a 50 mL centrifuge tube. The extract was centrifuged for 20 min at 3800 rpm, filtered through 0.45 µm syringe filter, and placed in an HPLC autosampler vial for analysis.

(c) *For samples in liquid form.*—The sample was thoroughly mixed before separating out the test portion. A 0.1–0.5 g portion was accurately weighed in a 10 or 25 mL volumetric flask. IS stock solution (500 µL) and extraction solution (20 mL) were added to the 25 mL volumetric flask and mixed well. IS stock solution (200 µL) and extraction solution (8 mL) were added to the 10 mL volumetric flask and mixed well. The flask was placed in an ultrasonic water bath for 2 h. After cooling the flask to room temperature, the solution was diluted to volume with extraction solution and then mixed and transferred to a 50 mL centrifuge tube. The extract was centrifuged for 20 min at 3800 rpm, filtered through 0.45 µm syringe filter, and placed in an HPLC autosampler vial for analysis.

(d) *For softgels, gelcaps, or encapsulated dry supplement samples.*—The contents of at least 15 capsules were removed and thoroughly mixed before separating out the test portion. A 0.1–0.5 g portion was accurately weighed in a 10 or 25 mL volumetric flask. IS stock solution (500 µL) and extraction solution (20 mL) were added to the 25 mL volumetric flask and mixed well. IS stock solution (200 µL) and extraction solution (8 mL) were added to the 10 mL volumetric flask and mixed well. The flask was placed in an ultrasonic water bath for 2 h. After cooling the flask to room temperature, the solution was diluted to volume with extraction solution and then mixed and transferred to a 50 mL centrifuge tube. The extract was centrifuged for 20 min at 3800 rpm, filtered through 0.45 µm syringe filter, and placed in an HPLC autosampler vial for analysis.

(e) *For SRMs.*—A 0.1 g sample was accurately weighed in a 10 or 25 mL volumetric flask. IS stock solution (500 µL) and extraction solution (20 mL) were added to the 25 mL volumetric flask and mixed well. IS stock solution (200 µL) and extraction solution (8 mL) were added to the 10 mL volumetric flask and mixed well. The flask was placed in an ultrasonic water bath for 2 h. After cooling the flask to room temperature, the solution was diluted to volume with extraction solution and then mixed and transferred to a 50 mL centrifuge tube. The extract was centrifuged for 20 min at 3800 rpm, filtered through 0.45 µm syringe filter, and placed in an HPLC autosampler vial for analysis.

F. Safety

Postcolumn ninhydrin reagent (Trione) is sensitive to oxidation, so reagent bottles were pressurized with nitrogen at 5 psi. Safety-coated bottles were used to hold the postcolumn reagent.

A 100 psi back-pressure regulator was installed on the detector outlet line to prevent any liquid from boiling in the postcolumn reactor.

G. HPLC Conditions

The equipment was connected in the following order: HPLC pump, autosampler, guard column, analytical column, postcolumn derivatization system, and UV-Vis detector. A Lithium cation-exchange column and lithium-based buffer solutions were used to separate L-theanine. The HPLC pump flow rate was set to 0.35 mL/min and the postcolumn reagent pump flow rate was set to 0.3 mL/min. The column oven temperature was set to 37°C and the postcolumn reactor temperature was set to 130°C. The HPLC pump gradient conditions that were used for the analysis are listed in Table **2016.10B**. The UV-Vis detector signal was monitored at 570 nm with the reference wavelength set at 630 nm. An injection volume of 10 µL was used.

Before starting the analysis, the system was equilibrated for at least 30 min until all temperatures and pressures were stable. At least one reagent blank was injected to equilibrate the column before injecting the working calibration solutions, control samples, samples extracts, and reagent blank. A mid-range calibration solution was run every 10 injections to confirm the stability of the calibration curve.

H. System Suitability

(a) Retention times for L-theanine in sample extracts and calibration solutions were within 0.5 min.

(b) Retention times for L-norleucine in sample extracts and calibration solutions were within 0.5 min.

(c) The correlation coefficient R^2 for the weighted linear regression calibration curve was ≥0.9998.

(d) Relative error for the back-calculated concentration for the mid-range calibration standard was within ±4%.

I. Calculations

The response ratio for the calibration standards ($Area_{L\text{-}theanine}/Area_{IS}$) vs its corresponding ratio for the concentrations ($Concn_{L\text{-}theanine}/Concn_{IS}$) was plotted to obtain a weighted linear regression calibration curve.

The concentration of L-theanine (µg/mL) in the sample extracts was calculated by interpolating the calibration curve.

The amount of L-theanine in the sample was calculated by using the following formula:

$$\text{Concn sample}\left(\text{mg/g}\right)=\frac{\text{Concn extract}\left(\frac{\mu\text{g}}{\text{mL}}\right)\times\text{Volume extract}\left(\text{mL}\right)}{\text{Mass sample}\left(\text{g}\right)\times 1000}$$

J. Precision Testing

Each matrix was analyzed in triplicate over 4 days. Working calibration solutions were prepared on each day of the analysis. Repeatability precision was assessed by calculating s_r and RSD_r (%) for same-day replicates measured under the same conditions. To determine intermediate precision, the conditions of analysis were intentionally varied by performing the analysis on different days by two different analysts using different lots of reagents and different calibration curves. In addition, samples SRM 3254, SRM 3255, and SRM 3256 were analyzed using two different HPLC systems.

The Grubbs' outlier test for a 95% confidence interval was applied to the results with no outliers detected.

Table 2016.10B. HPLC pump gradient conditions

Time, min	Li275, %	Li750, %	RG003, %
0	100	0	0
12	100	0	0
45	66	34	0
45.1	0	0	100
50	0	0	100
50.1	100	0	0
62	100	0	0

K. Accuracy Testing

Method accuracy was evaluated by analyzing SRM 3254, SRM 3255, and SRM 3256, as well as by conducting spike recovery studies for seven matrixes.

SRMs were analyzed in triplicate over 4 days by two different analysts using two different HPLC systems and different lots of reagents and columns.

For spike recovery studies, each matrix was spiked at two levels and samples analyzed in duplicate over 3 days by two different analysts using different lots of reagents. L-Theanine stock solution and L-theanine intermediate stock solution were used to spike the samples. The overall mean for unspiked samples determined during the course of the precision study was used to calculate the recoveries.

L. Ruggedness Testing

The effect of seven factors (*see* Table **2016.10C** for a list) was evaluated using the Youden ruggedness trial design (18). In each experiment, the values of four factors were modified as shown in

Table 2016.10C. Ruggedness trial experimental design

Factor	Value 1	Value 2
Formulation of ninhydrin reagent	T100, 1-part ninhydrin reagent (A)	T200, 2-part ninhydrin reagent (a)
HPLC flow rate	0.35 mL/min (B)	0.38 mL/min (b)
Extraction volume	25 mL (C)	10 mL (c)
Analyst	Analyst 1 (D)	Analyst 2 (d)
Extraction time	2 h (E)	1.5 h (e)
Extraction solution Li220	Lot 1 (F)	Lot 2 (f)
Reactor temperature	130 °C (G)	125 °C (g)

Experiment No.	Combination of factors
1	ABCDEFG
2	ABcDefg
3	AbCdEfg
4	AbcdeFG
5	aBCdeFG
6	aBcdEfG
7	abCDefG
8	abcDEFg

Table **2016.10C**. The effect of any specific factor was evaluated by comparing the difference between the averages of two subsets of four experiments with $\sqrt{2} \times$ SD, where SD represents the SD between the replicates performed under the same conditions.

Dry green tea extract capsules were used for the ruggedness trial and each experiment was performed in duplicate.

M. Calibration Curves

Sample analysis throughout the validation study was performed using a seven-point working calibration curve covering a range from 1 to 40 µg/mL (Table **2016.10A**).

Additionally, the linearity of the method was evaluated by building extended calibration curves consisting of 13 mixed calibration standards that ranged from 0.5 to 100 µg/mL.

Extended calibration curves were obtained on 6 separate days by two different analysts using different lots of reagents. Calibration standards for each curve were freshly prepared on the day of analysis.

Results and Discussion

Validation Study

The single-laboratory validation (SLV) study was conducted to compare performance characteristics of this method with the characteristics of AOAC SMPR 2015.014, "*Standard Method Performance Requirements* for Determination of Catechins, Methyl Xanthines, Theaflavins, and Theanine in Tea (*Camellia sinensis*) Dietary Ingredients and Supplements" (8), which are listed in Table 1.

Table 1. SMPR for the determination of L-theanine in tea dietary ingredients and supplements (8)

Analytical range, ppm	10–100000		
LOQ, ppm	≤ 5		
Method performance parameters by range			
	10–50 ppm	51–500 ppm	>500 ppm
Recovery, %	80–110	90–107	95–105
RSD_r, %	≤7	≤5	≤5
RSD_R, %	≤10	≤8	≤8

Matrixes

Eight matrixes were used in the validation study: five green tea-containing dietary supplements and three NIST SRMs.

The dietary supplements included tablets, dry capsules, liquid formulation, softgels, and gelcaps. According to label claims, all dietary supplements contained green tea extract. The liquid formulation contained up to 45% alcohol, whereas the tablets and dry capsules contained calcium and magnesium salts, as well as common inactive ingredients. The gelcaps contained glycerin and the softgels contained fish oil, caffeine, lecithin, glycerin, and several plant extracts. None of the dietary supplements had label claims indicating theanine content.

NIST SRMs included SRM 3254 *C. sinensis* (green tea) leaves, SRM 3255 *C. sinensis* (green tea) extract, and SRM 3256 green tea-containing solid oral dosage form. Only reference (noncertified) mass fraction values for L-theanine were available from NIST. L-Theanine reference values represented data from a single laboratory using an LC-MS method.

Selectivity

Performing a postcolumn reaction with ninhydrin reagent is specific for primary amino groups and allows for the selective detection of amino acids in complex matrixes. Lithium cation-exchange columns and lithium citrate buffers represent a chromatographic system designed to separate free amino acids. Only free amino acids and a very limited number of organic amines are retained in a lithium cation-exchange column under the analytical conditions used for this analysis, and therefore could be detected after reaction with ninhydrin postcolumn reagent.

L-Theanine peak identity was confirmed by comparing the HPLC elution profiles of L-theanine standard solution with the profiles of the samples by using two types of cation-exchange columns and different sets of buffers as mobile phases. The peaks of L-theanine and L-norleucine (IS) were fully resolved from the other peaks present in the chromatograms with a resolution $R_S \geq 1.5$.

The chromatograms of dietary supplements analyzed during the course of this study are shown in Figures 1–3.

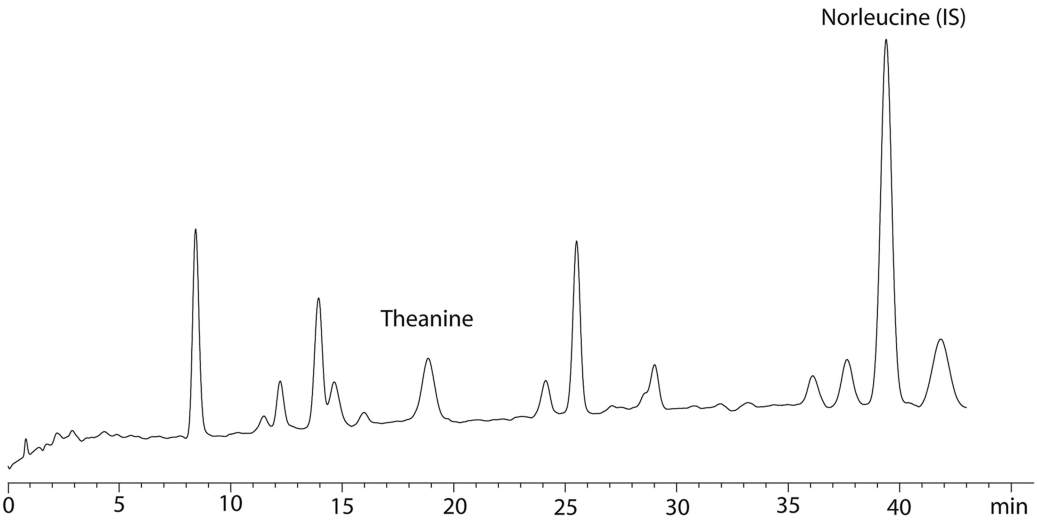


Figure 1. Chromatogram of a green tea softgel sample. The concentration of theanine in the sample was 0.1432 mg/g.

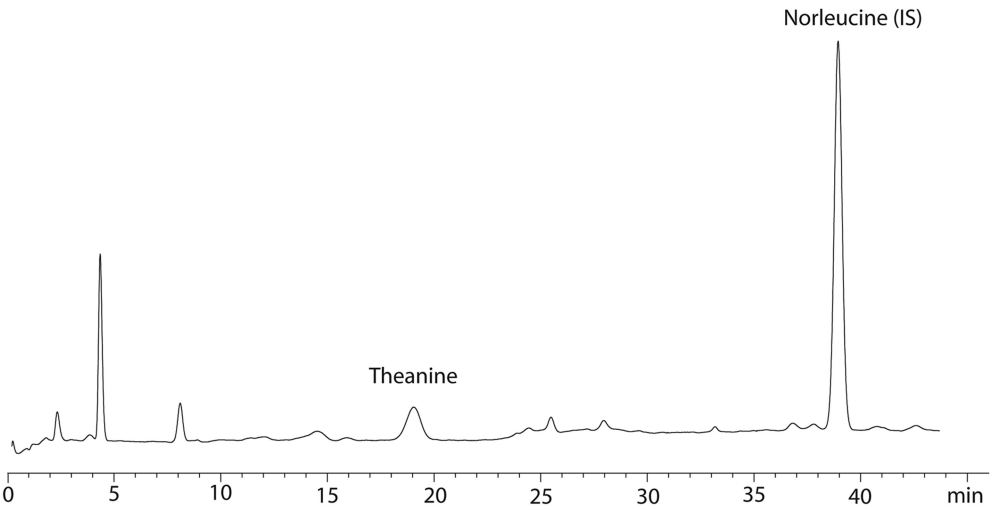


Figure 2. Chromatogram of green tea extract tablet sample. The concentration of theanine in the sample was 0.0410 mg/g.

Precision Results

Method precision was evaluated using the eight matrixes discussed earlier. The chosen samples represented common forms of green tea dietary ingredients and supplements and were found to cover a wide range of L-theanine concentrations (from 0.04 to 4 mg/g).

Repeatability and intermediate precision data for L-theanine analysis are presented in Table 2.

The SMPR 2015.014 document approved by the SPDS (8) sets repeatability and reproducibility requirements for different levels of L-theanine in samples with upper limits for RSD_r and RSD_R being 5–7% and 8–10%, respectively. The RSD_r for this method ranged from 0.76 to 2.95% and the intermediate precision (RSD_{iR}) ranged from 1.81 to 5.33%, thereby meeting the method precision requirements for all the studied matrixes.

Calculated Horwitz ratio values ranged between 0.32 and 0.62 and met the acceptance criteria for within-laboratory precision with values of 0.3–1.3 (19).

Accuracy Results

The results of the analysis of NIST SRM 3254, SRM 3255, and SRM 3256 are presented in Table 3 and are in close agreement with the reference values for L-theanine obtained by the NIST laboratory using an LC-MS method.

Spike recovery studies were completed for a total of seven matrixes—including SRM 3254, SRM 3255, and SRM 3256—to cover dietary ingredients, such as green tea leaves and pure green tea extract, in addition to different supplement formulations.

Spike concentrations varied from 0.02 to 3.6 mg/g and for most matrixes represented a 50 and 100% overspike of the native levels. Data for total and marginal recoveries are presented in Tables 4 and 5, respectively.

Total recoveries ranged between 98.8 and 102.1%, with a maximum RSD of 3.3% for liquid gelcap samples. Marginal recoveries ranged between 97.6 and 108.7%, with the highest SD at 6.4%, again obtained for liquid gelcaps.

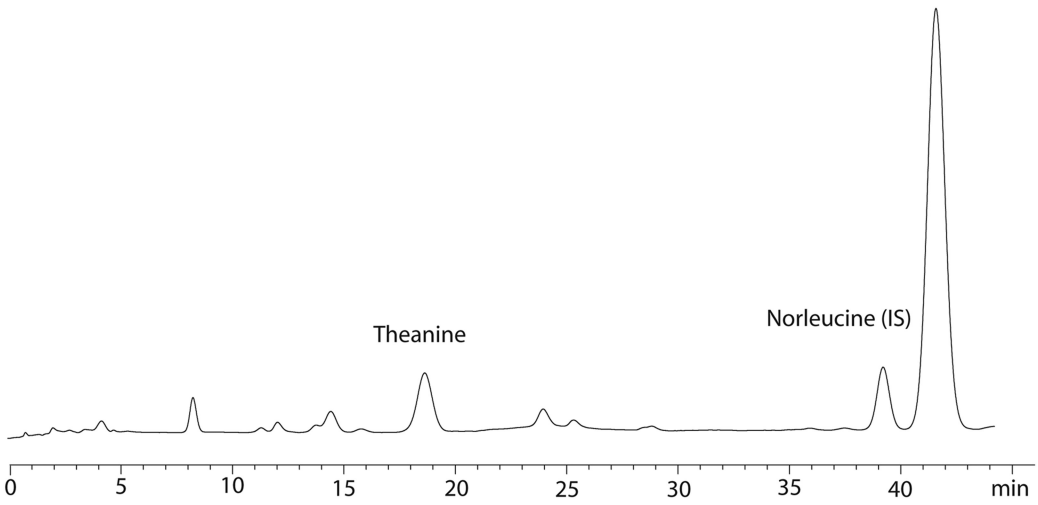


Figure 3. Chromatogram of a sample of SRM 3256 (green tea-containing solid oral dosage form). Concentration of theanine in the sample 3.949 mg/g.

Table 2. Repeatability and intermediate precision data for L-theanine analysis in green tea-containing matrixes[a]

Sample	Concn level, mg/g	SD_r	RSD_r, %	SD_{iR},	RSD_{iR}, %
Liquid green tea extract formulation	0.575	0.017	2.95	0.022	3.79
Dry green tea extract capsules	3.959	0.058	1.46	0.074	1.88
Green tea liquid gelcaps	0.1897	0.0047	2.47	0.0063	3.31
Green tea softgels	0.1432	0.0042	2.92	0.0044	3.06
Green tea extract tablets	0.0410	0.00096	2.39	0.0022	5.33
SRM 3254	2.051	0.037	1.78	0.059	2.89
SRM 3255	0.3168	0.0053	1.66	0.0068	2.16
SRM 3256	3.949	0.030	0.76	0.071	1.81

[a] Number of replicates (3 replicates × 4 days).

RSDs >6% for marginal recoveries were observed for the lowest level of L-theanine and for samples with nonuniform distribution of material, such as in the liquid extract formulation and liquid gelcaps. The liquid green tea extract formulation contained insoluble materials, and the liquid gelcap content varied considerably in density, most likely as a result of partial evaporation.

These findings highlight the challenges in obtaining a uniform and representative sample for the analysis of formulations containing natural products.

SMPR 2015.014 specifies the ranges for acceptable recoveries for L-theanine concentrations as 80–110% for 10–50 ppm, 90–107% for –500 ppm, and 95–105% for >501 ppm. These recovery specifications are more restrictive than those listed in AOAC OMA "*Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals*" (20).

Table 3. Analysis of the NIST SRMs

SRM No.	Description	Results, mg/g	RSD_{IR}, %; $n = 12$	L-Theanine mass fraction reference value, mg/g[a]
SRM 3254	*C. sinensis* (green tea) leaves	2.051	2.89	2.130 ± 0.054
SRM 3255	*C. sinensis* (green tea) extract	0.3168	2.16	0.340 ± 0.008
SRM 3256	Green tea-containing solid oral dosage form	3.949	1.81	3.7 ± 1.2

[a] Only reference mass fraction values are available from NIST for L-theanine. Reference values are noncertified values that are the best estimate of the true values based on available data. Reference values for L-theanine represent data from a single laboratory using an LC-MS method.

Table 4. Total recoveries for L-theanine

Sample	Level in the sample, mg/g	Spike level 1, mg/g	Rec., %	RSD, %	Spike level 2, mg/g	Rec., %	RSD, %
Liquid green tea extract formulation	0.575	0.285	102.8	2.1	0.570	100.7	1.8
Green tea liquid gelcaps	0.1897	0.1025	101.9	1.4	0.2050	101.8	3.3
Green tea softgels	0.1432	0.707	99.6	1.3	1.414	99.5	1.3
Green tea extract tablets	0.0410	0.0200	100.1	0.7	0.0401	100.3	3.0
SRM 3254	2.051	1.002	100.3	1.2	2.004	98.8	1.5
SRM 3255	0.3176	0.1515	99.5	1.8	0.3030	101.1	1.8
SRM 3256	3.949	1.804	100.7	0.9	3.607	102.1	1.0

Total recoveries for all spiked levels of L-theanine in all study samples met the specifications outlined in SMPR 2015.014.

The marginal recoveries of 108.7% obtained for the 0.285 mg/g spike of liquid green tea extract formulation exceeded SMPRs of 107%. For all other spike levels in all the studied matrixes, marginal recoveries met the specifications listed in SMPR 2015.014.

Linearity

For 13-point calibration curves covering the concentration range from 0.5 to 100 µg/mL, the correlation coefficient R^2 for weighted linear regressions fell between 0.99993 and 0.99998. The relative errors of back-calculated concentrations are presented in Table 6.

For all calibration curves, the back-calculated errors for standards at concentrations ≥1 µg/mL were <5%, with most calibrators falling within 2%. For the lowest calibration level

Table 5. Marginal recoveries for L-theanine

Sample	Level in the sample, mg/g	Spike level 1, mg/g	Rec., %	RSD, %	Spike level 2, mg/g	Rec., %	RSD, %
Liquid green tea extract formulation	0.575	0.285	108.7	6.2	0.570	101.4	3.6
Green tea liquid gelcaps	0.1897	0.1025	105.7	4.2	0.2050	103.6	6.4
Green tea softgels	0.1432	0.707	99.5	1.6	1.414	99.4	1.4
Green tea extract tablets	0.0410	0.0200	100.2	2.3	0.0401	100.7	6.1
SRM 3254	2.051	1.002	101.0	3.6	2.004	97.6	3.0
SRM 3255	0.3176	0.1515	98.4	5.5	0.3030	102.2	3.6
SRM 3256	3.949	1.804	102.1	2.9	3.607	104.4	2.0

Table 6. Relative errors for back-calculated concentrations for calibration standards

Standard	L-Theanine concn, µg/mL	L-Norleucine concn, µg/mL	Relative back-calculated errors, %					
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
1	100	10	0.37	0.51	−0.31	0.17	0.69	0.29
2	80	10	0.02	1.21	0.28	−0.11	−0.05	−0.88
3	60	10	0.09	0.26	0.22	0.82	−0.06	0.36
4	50	10	0.14	−0.41	0.88	0.01	−0.55	1.08
5	40	10	−0.78	−1.01	−0.14	−0.73	0.02	0.24
6	25	10	−0.67	−0.73	−0.49	−0.49	0.14	−0.49
7	20	10	−1.35	−2.13	−0.63	0.16	−0.71	−1.45
8	10	10	1.35	−1.99	0.68	−0.69	0.02	−0.25
9	7.48	10	1.05	−1.91	−1.03	−1.36	0.01	−2.03
10	5	10	−0.02	−3.33	−1.65	−0.30	−0.48	1.69
11	2	10	3.74	3.49	−3.55	−1.75	0.82	2.13
12	1	10	4.19	−0.79	1.09	−0.17	−0.33	4.24
13	0.5	10	1.70	6.82	14.9	4.43	0.88	−4.95

of 0.5 µg/mL, the back-calculated error was >5%, with 2 out of 6 days having errors of 6.82 and 14.9%, respectively.

Ruggedness Test

The following seven factors were studied during this trial: different formulations of ninhydrin postcolumn reagent, postcolumn reactor temperature, different lots of extraction solution, HPLC flow rate, sample–extraction solution ratio, extraction time, and different analysts. The results of the ruggedness trial are presented in Table 7.

For five out of seven factors, the differences between two subsets of four experiments were below $\sqrt{2} \times SD$, indicating that expected differences in ninhydrin formulation, extraction solutions, extraction time, HPLC flow rate, and the analysts' way of performing the analysis did not affect the final results.

Table 7. Results of the ruggedness trial

The effect of changing factors calculated as described (20)	
$\sqrt{2} \times SD = 0.0639$	
A-a	$0.0467 < \sqrt{2} \times SD$
B-b	$0.0002 < \sqrt{2} \times SD$
C-c	$-0.0656 > \sqrt{2} \times SD$
D-d	$0.0431 < \sqrt{2} \times SD$
E-e	$0.0156 < \sqrt{2} \times SD$
F-f	$0.0078 < \sqrt{2} \times SD$
G-g	$-0.0658 > \sqrt{2} \times SD$

For factors such as the sample–extraction solution ratio and postcolumn reactor temperature, the calculated differences were slightly above a $\sqrt{2} \times SD$ of 0.0639 (0.0656 and 0.0658, respectively). Though observed differences were small, the results underline the importance of performing theanine extraction using a sufficient volume of extraction solution and performing regular calibration of postcolumn reactor temperature.

LOQ and LOD

Ten low-level L-theanine standards (0.7 µg/mL) were prepared and analyzed as samples using a 10 µL injection volume. Up to 50 µL extract can be injected for analysis if detection of even lower levels of L-theanine is required.

The LOD was calculated as $3 \times SD$ and the LOQ was calculated as $10 \times SD$.

$$LOD = 0.09 \text{ µg/mL}$$

$$LOQ = 0.30 \text{ µg/mL}$$

LODs and LOQs for the samples (µg/g) were calculated for 1 g sample extracted with 10 mL extraction solution:

$$LOD = 0.91 \text{ µg/g}$$

$$LOQ = 3.05 \text{ µg/g}$$

The LODs and LOQs met the requirements outlined in SMPR 2015.014 for L-theanine.

Conclusions

The presented method allows for the analysis of theanine in green tea dietary supplements and ingredients. The method is based on a proven methodology for detecting amino acids in native samples and is rugged, sensitive, and easy to implement. Easy extraction with no additional cleanup steps is suitable for a wide array of matrixes without the need for additional optimization. Results of the SLV showed that this method meets the SMPR approved by the SPDS and supports the First Action status of the method, and that therefore, this method is well-suited for laboratories tasked with testing theanine in green tea-containing samples.

References

(1) Vuong, Q.V., Bowyer, M.C., & Roach, P.D. (2011) *J. Sci. Food Agric.* **91**, 1931–1939. doi:10.1002/jsfa.4373

(2) Ying, Y., Ho, J.W., Chen, Z.Y., & Wang, J. (2005) *J. Liq. Chromatogr. R.T.* **28**, 727–737. doi:10.1081/JLC-200048894

(3) Haskell, C.F., Kennedy, D.O., Milne, A.L., Wesnes, K.A., & Scholey, A.B. (2008) *Biol. Psychol.* **77**, 113–122. doi:10.1016/j.biopsycho.2007.09.008

(4) Dimpfel, W., Kler, A., Kriesl, E., Lehnfield, R., & Keplinger-Dimpfel, I.K. (2007) *Nutr. Neurosci.* **10**, 169–180. doi:10.1080/03093640701580610

(5) Camfield, D., Stough, C., Farrimond, J., & Scholey, A.B. (2014) *Nutr. Rev.* **72**, 507–522. doi:10.1111/nure.12120
(6) Cho, H., Kim, S., Lee, S., Park, J.A., Kim, S.J., & Chun, H.S. (2008) *Neurotoxicology* **29**, 656–662. doi:10.1016/j.neuro.2008.03.004
(7) Sugiyama, T., Sadzuka, Y., Tanaka, K., & Sonobe, T. (2001) *Toxicol. Lett.* **121**, 89–96. doi:10.1016/S0378-4274(01)00317-4
(8) *Official Methods of Analysis* (2016) 20th Ed., AOAC INTERNATIONAL, Rockville, MD, AOAC SMPR 2015.014
(9) Wang, L., Xu, R., Hu, B., Sun, Y., Tu, Y., & Zeng, X. (2010) *Food Chem.* **123**, 1259–1266. doi:10.1016/j.foodchem.2010.05.063
(10) Zhu, X., Ma, M., Luo, X., Zhang, F., Yao, S., Wan, Z., Yang, D., & Hang, H. (2004) *J. Pharm. Biomed. Anal.* **34**, 695–704. doi:10.1016/S0731-7085(03)00605-8
(11) Aucamp, J.P., Hara, Y., & Apostolides, Z. (2000) *J. Chromatogr. A* **876**, 235–242. doi:10.1016/S0021-9673(00)00145-X
(12) Yashin, A.Y., Nemzer, B.V., Combet, E., & Yashin, Y.I. (2015) *J. Food Res.* **4**, 56–87. doi:10.5539/jfr.v4n3p56
(13) Horie, H., & Kohata, K. (2000) *J. Chromatogr. A* **881**, 425–438. doi:10.1016/S0021-9673(99)01345-X
(14) Ding, Y., Yu, H., & Mou, S. (2002) *J. Chromatogr. A* **982**, 237–244. doi:10.1016/S0021-9673(02)01650-3
(15) Desai, M.J., & Armstrong, D.W. (2004) *Rapid Commun. Mass Spectrom.* **18**, 251–256. doi:10.1002/rcm.1319
(16) Peng, L., Song, X., Shi, X., Li, J., & Ye, C.J. (2008) *Food Comp. Anal.* **21**, 559–563. doi:10.1016/j.jfca.2008.05.002
(17) Thippeswamy, R., Gouda, K.G., Rao, D.H., Martin, A., & Gowda, L.R. (2006) *J. Agric. Good Chem.* **54**, 7014–7019. doi:10.1021/jf061715+
(18) Youden, W.J., & Steiner, E.H. (1984) *Statistical Manual of the Association of the Official Analytical Chemists*, AOAC INTERNATIONAL, Gaithersburg, MD
(19) *Official Methods of Analysis* (2012) AOAC INTERNATIONAL, Gaithersburg, MD, Appendix F
(20) *Official Methods of Analysis* (2013) 19th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Appendix K

AOAC Official Method 2016.16
Curcuminoids in Turmeric
Liquid Chromatography with UV-Vis Detection
First Action 2016

(Applicable to the determination of curcumin, demethoxycurcumin and bisdemethoxycurcumin in turmeric (*Curcuma longa* Linn) rhizomes, dietary ingredients and dietary supplement products containing turmeric alone or in combination with other dietary ingredients.)

Caution: Solvents utilized are common-use solvents. Refer to adequate manuals or Material Safety Data Sheets (MSDS) to ensure that the safety guidelines are applied before using chemicals. Store in a flammable liquid storage cabinet. Harmful if inhaled, swallowed, or absorbed through the skin. Use appropriate personal protective equipment such as a laboratory coat, safety glasses, rubber gloves, and a fume hood. Dispose of all materials according to federal, state, and local regulations.

See Table **2016.16A** for method performance data supporting acceptance of the method.

A. Principle

Samples are extracted with methanol using a wrist-action shaker. Dilution of highly concentrated samples may be required prior to LC analysis. Quantitation is performed with external calibration against reference standards for curcumin, demethoxycurcumin and bisdemethoxycurcumin.

B. Apparatus

(**a**) *Analytical balance.*-Sensitive to at least 0.0001 g.

(**b**) *Grinder.*-Retsch or equivalent centrifugal mill fitted with a 0.25 mm screen.

(**c**) *Volumetric pipet.*-Class A, 25 mL.

(**d**) *Centrifuge tubes.*-Conical polypropylene tubes, 50 mL.

(**e**) *Vortex mixer.*

(**f**) *Centrifuge.*-Benchtop with rotor for 50 mL conical tubes, maximum 21,000 x *g*.

(**g**) *Shaker.*-Wrist action.

(**h**) *Micropipettors.*-Capable of pipetting 200 and 1000 µL.

(**i**) *LC vials with caps.*

(**j**) *Volumetric flasks.*-Class A, 10 mL.

(**k**) *LC system.*-Agilent HP 1200 (Agilent Technologies, Santa Clara, CA, USA) with binary pump operating at 400 bar, temperature controlled column, autosampler, and UV-Vis diode array detector.

(**l**) *Chromatographic column.*-Kinetex C18, 2.6 µm, 2.1 x 30 mm (Phenomenex, Torrance, CA, USA).

(**m**) *Syringes.*-Disposable luer-lok, 3 mL.

(**n**) *Syringe filters.*-Polytetrafluoroethylene (PTFE) membrane, 0.2 µm.

(**o**) *Bottles.*-LC solvent bottles, 1 L.

C. Materials and Reagents

(**a**) *Curcumin (CUR) primary standard.*-Product No. ASB-00003926 (ChromaDex, Irvine, CA, USA).

(**b**) *Demethoxycurcumin (DMC) primary standard.*-Product No. ASB-00004230 (ChromaDex).

(**c**) *Bisdemethoxycurcumin (BDMC) primary standard.*-Product No. ASB-00004231 (ChromaDex).

(**d**) *Water.*-Nanopure deionized.

(**e**) *Methanol.*-HPLC grade.

(**f**) *Acetonitrile.*-HPLC grade.

(**g**) *Formic acid.*

D. Preparation of Solutions and Standards

(**a**) *Curcumin stock solution, 1000 µg/mL (CUR-1000).*-Weigh an amount of curcumin primary standard equivalent to 10.0 mg after correction for purity and place in a 10 mL volumetric flask. Fill to volume with methanol, mix, and store in a screw top vial at -20°C for up to 4 weeks.

(**b**) *Demethoxycurcumin stock solution, 1000 µg/mL (DMC-1000).*-Weigh an amount of demethoxycurcumin primary standard equivalent to 10.0 mg after correction for purity and place in a 10 mL volumetric flask. Fill to volume with methanol, mix, and store in a screw top vial at -20°C for up to 4 weeks.

(**c**) *Bisdemethoxycurcumin stock solution, 1000 µg/mL (BDMC-1000).*-Weigh an amount of bisdemethoxycurcumin primary standard equivalent to 10.0 mg after correction for purity and place in a 10 mL volumetric flask. Fill to volume with methanol, mix, and store in a screw top vial at -20°C for up to 4 weeks.

(**d**) *Mixed calibration standards.*-Prepare the mixed calibration standards daily. Std 1 to Std 5 are described in Table **2016.16B** by dilution of CUR-1000, DMC-1000 and BDMC-1000 in methanol

as specified in Table **2016.16C**. Prepare Std 6 by diluting 100 µL Std 4 with 900 µL methanol. Prepare Std 7 by diluting 100 µL Std 5 with 900 µL methanol.

(**e**) *LC mobile phase A.*-Dilute 1 mL formic acid with 999 mL water to make 0.1% formic acid in water.

(**f**) *LC mobile phase B.*-Dilute 1 mL formic acid with 999 mL acetonitrile to make 0.1% formic acid in acetonitrile.

E. Preparation of Samples

(**a**) *Homogenization of solid samples.*-(*1*) Grind dried turmeric rhizome in a centrifugal mill with 0.25 mm screen.

(*2*) Combine 20 dosage units of bulk extract, capsules, or tablets and grind. Tablets can be ground using a coffee grinder.

(*3*) Combine the contents of 20 dosage units of softgel capsules and grind.

(*4*) Store ground samples in polypropylene tubes at room temperature protected from light. Mix well before removing test portions.

(**b**) *Extraction of solid test portions.*-Prepare all test portions in triplicate.

(*1*) For ground dried rhizomes, bulk extracts, capsules, or tablets, accurately weigh 75 mg into a 50 mL centrifuge tube.

(*2*) For ground softgel capsule contents, accurately weigh 200-300 mg into a 50 mL centrifuge tube.

(*3*) Add 25 mL methanol and vortex mix for 30 s.

(*4*) Shake on a wrist-action shaker for 15 min at room temperature.

(*5*) Centrifuge at 4500 *g* for 5 min.

(*6*) Filter 1 mL aliquot through a syringe filter into an LC vial.

(*7*) Store LC vial at 4°C protected from light until analysis.

(**c**) *Preparation of tinctures.*-Prepare all test portions in triplicate.

(*1*) Mix tincture by inversion several times.

(*2*) Dilute 100 µL tincture with 900 µL methanol and vortex mix.

(*3*) Filter through a syringe filter into an LC vial.

(*4*) Store LC vial at 4°C protected from light until analysis.

F. LC Analysis

(**a**) *Setup.*-(*1*) Set detector to 425 nm; column temperature to 55°C; injection volume to 0.8 µL; and flow rate to 1.4 mL/min.

(*2*) Program the gradient shown in Table **2016.16D**.

(*3*) Equilibrate the column with mobile phase A.

(**b**) *Procedure.*-(*1*) Make single injections of one set of calibration standards (Std 1-Std 7).

(*2*) Make single injections of each LC sample.

(*3*) After approximately every 10 sample injections, and at the end of the run, re-inject one of the calibration standards for quality control purposes.

G. Calculations

(**a**) *Calibration standards.*-(*1*) Measure peak areas for CUR, DMC, and BDMC in the set of calibration standards and the re-injected calibration standards.

(*2*) Ensure that the re-injected calibration standard peak areas are within 5% of the initial calibration standard peak areas.

(*3*) Construct a plot of analyte concentration (x-axis) versus individual peak area (y-axis) for CUR, DMC, and BDMC. Use least squares analysis to determine the slope, intercept, and correlation coefficient (r^2) of the best-fit line for each analyte.

(**b**) *Unknown samples.*-(*1*) From the standard curves and the peak areas of each analyte in the samples, calculate the concentration of CUR, DMC, and BDMC in each sample solution. If the peak area of any analyte is above the standard curve for that analyte, dilute the extract 1/10 or 1/20 in methanol, filter, and repeat the analysis for that extract.

$$\text{Curcuminoid Concentration } \left(\frac{\text{mg}}{\text{L}}\right) = \frac{\text{curcuminoid peak area} - \text{intercept of linear regression}}{\text{slope of linear regression}}$$

(*2*) Calculate the amount of curcuminoid in the original sample as:

$$Curcuminoid\ in\ original\ sample\ \left(\frac{mg}{g}\right) = \frac{Concentration\ (C) \times Volume(V)}{Mass\ (W)} \times D$$

where C is the concentration of analyte from the standard curve (mg/L); V is the extract volume (0.025 L); W is the weight of the test portion (g); and D is the dilution factor.

(*3*) Report the curcuminoid concentrations in the original samples as the mean concentrations with 95% confidence intervals as follows:

Reported amount (mg/g) = $\overline{X} \pm t_{\alpha,\nu} \cdot s_{\bar{x}}$

where $\overline{X}$ is the mean of the triplicate values; $t_{\alpha,\nu}$ is the *t* critical value; and $s_{\bar{x}}$ is the size of the standard error [$s_{\bar{x}} = s/\sqrt{n}$, where *s* is the standard deviation and *n* is the number of replicates]. For triplicate measurements and α=0.05, *t* critical value is 4.303.

H. Typical Chromatograms

Typical chromatograms are shown in Figure **2016.16** for (a) a mixed calibration standard solution and (b) a turmeric rhizome sample.

References: *J. AOAC Int*. (future issue)

AOAC SMPR 2016.003
J. AOAC Int. **99**, 1102(2016)
Posted: March 1, 2017

Table 2016.16A. SMPR 2016.003 and First Action *Official Method*SM 2016.16 results

Type of study	Parameter	Requirement		LC method results		
Single-laboratory validation	LOQ, %	≤0.1 (w/w)		CUR[a]	0.16	
				DMC[b]	0.03	
				BDMC[c]	0.03	
	Recovery, %	95-110		CUR	100.9	
				DMC	99.8	
				BDMC	99.3	
	Analytical range, %	≤0.1-50 (w/w)	>50 (w/w)		≤0.1-50 (w/w)	>50 (w/w)
	Repeatability (RSD_r), %	≤5	≤3	CUR	0.4-4.0	0.3-3.2
				DMC	0.9-4.1	ND[d]
				BDMC	1.2-5.5	ND
Multi-laboratory validation	Reproducibility (RSD_R), %	≤8	≤5	CUR	ND	ND
				DMC	ND	ND
				BDMC	ND	ND

[a] CUR = Curcumin.
[b] DMC = Demethoxycurcumin.
[c] BDMC = Bisdemethoxycurcumin.
[d] ND = Not determined.

Table 2016.16B. Approximate concentrations of curcuminoids in mixed calibration standards

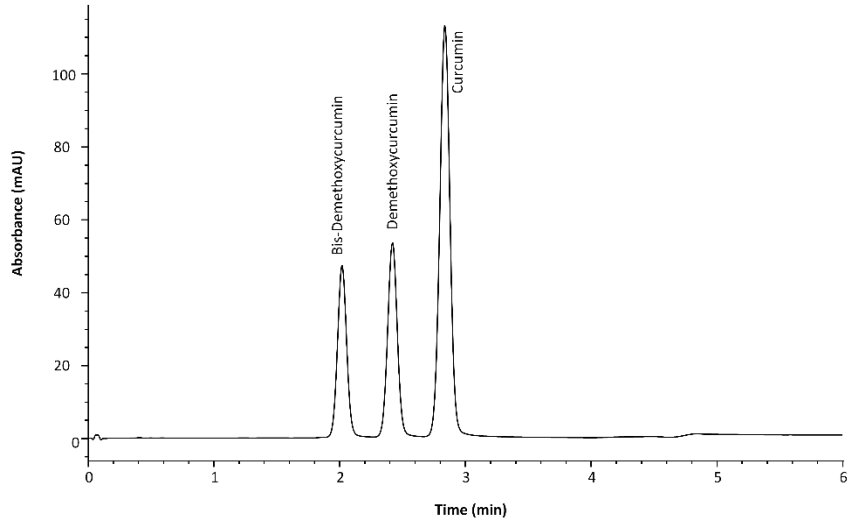
Curcuminoid	Approximate concentration, µg/mL						
	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
Curcumin (CUR)	300	225	150	100	50	10	5
Demethoxycurcumin (DMC)	100	80	60	25	10	2.5	1
Bisdemethoxycurcumin (BDMC)	120	75	40	25	10	2.5	1

Table 2016.16C. Preparation of mixed calibration standards

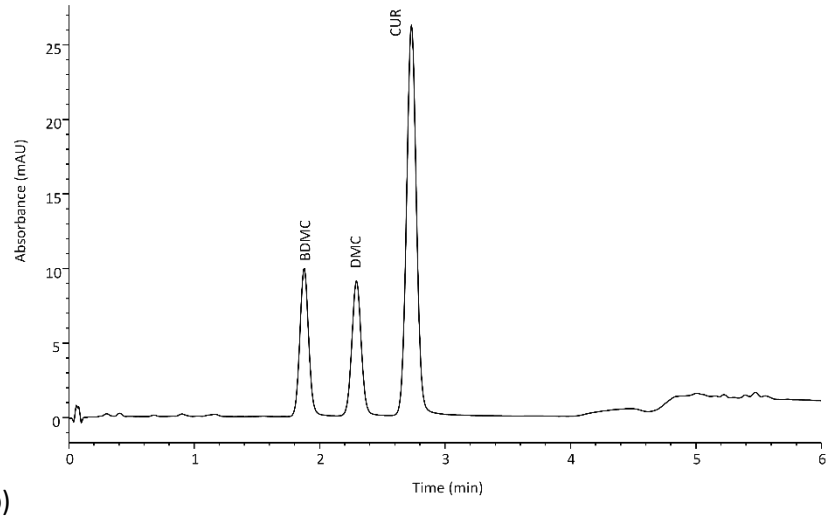
Solution	Volume, µL						
	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
C-1000	300	225	150	100	50		
DMC-1000	100	80	60	25	10		
BDMC-1000	120	75	40	25	10		
Std 4						100	
Std 5							100
Methanol	480	620	750	850	930	900	900

Table 2016.16D. LC gradient

Time (min)	% Mobile phase B	Flow rate (mL/min)
0	28	1.40
1.0	28	1.40
2.0	30	1.40
4.0	30	1.40
4.1	50	1.75
6.0	50	1.75
8.5	28	1.40



(**a**)



(**b**)

Figure 2016.16. Typical chromatograms from (a) a mixed calibration standard solution and (b) a turmeric rhizome sample.

DIETARY SUPPLEMENTS

Determination of Chondroitin Sulfate Content in Raw Materials and Dietary Supplements by High-Performance Liquid Chromatography with UV Detection After Enzymatic Hydrolysis: Single-Laboratory Validation First Action 2015.11

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A previously validated method for determination of chondroitin sulfate in raw materials and dietary supplements was submitted to the AOAC Expert Review Panel (ERP) for Stakeholder Panel on Dietary Supplements Set 1 Ingredients (Anthocyanins, Chondroitin, and PDE5 Inhibitors) for consideration of First Action *Official Methods*^{SM} status. The ERP evaluated the single-laboratory validation results against AOAC *Standard Method Performance Requirements* 2014.009. With recoveries of 100.8–101.6% in raw materials and 105.4–105.8% in finished products and precision of 0.25–1.8% RSD_r within-day and 1.6–4.72% RSD_r overall, the ERP adopted the method for First Action *Official Methods* status and provided recommendations for achieving Final Action status.

Under a contract from the National Institutes of Health Office of Dietary Supplements, AOAC INTERNATIONAL created the Stakeholder Panel on Dietary Supplements (SPDS), whose mission is to establish voluntary consensus standards for 25 high-priority ingredients. The high-priority ingredients are those for which scientifically valid methods were lacking at the time. As with all stakeholder panels, AOAC engaged industry, government, and academic experts to populate the panel and drive consensus.

In September 2014, SPDS finalized and approved *Standard Method Performance Requirements* (SMPR®) for determination of total chondroitin sulfate (CS) in dietary ingredients and supplements, SMPR 2014.009 (1). This SMPR was intended to outline the minimum recommended performance characteristics for a reference method for routine analysis or dispute resolution. SMPRs are used by AOAC Expert Review Panels (ERPs) as a basis for evaluating candidate methods.

A call for methods was issued by AOAC on January 13, 2015, to select and evaluate methods for CS in dietary supplements according to SMPR 2014.009. The ERP for SPDS Set 1 Ingredients (Anthocyanins, Chondroitin, and PDE5 Inhibitors) considered four methods for CS, and only the method developed by Ji et al. (2) was adopted for *Official Methods* First Action status.

SMPR 2014.009 and LC Method

The LC method and its single-laboratory validation (SLV) were first described in 2007 (2) and in 2015 were submitted to the ERP for SPDS Set 1 Ingredients in response to the call for methods. Briefly, 200 mg of raw material, ground tablets, or ground capsule contents is dissolved in 100 mL water with sonication and filtered if needed. Liquid formulations (200 mg) are diluted to 100 mL with water. The resulting test solution is subjected to hydrolysis with chondroitinase AC II to produce un-, mono-, di-, and trisulfated unsaturated disaccharides. Samples are then analyzed by ion-pairing reverse-phase LC with UV detection, and total CS is determined by summing the amounts of individual disaccharides.

SMPR 2014.009 (1) describes the minimum method performance requirements established by SPDS as summarized in Table 1. In a single-laboratory evaluation, methods must have an LOQ of 1% (w/w); relative SD of repeatability of ≤3% in the low analytical range of 1–10% and ≤2% in the high analytical range of >10–100%; and recovery of 92–105% in the low range and 98–102% in the high range. The matrices to be included in the validation are tablets, capsules, softgels, gel caps, gummies, chewables, liquids, and powders.

The LC method was validated for raw material, capsules, chewables, tablets, softgels, and liquid supplements. Selectivity was evaluated by analyzing CS in the presence of other common dietary supplement ingredients including calcium sulfate, magnesium chloride, zinc chloride, cupric sulfate, glucosamine HCl, methyl sulfonylmethane, chromium(III) chloride, dermatan sulfate, and carrageenan to look for interference with the method, either by deactivating the enzyme or interfering with the LC. Recoveries of CS varied from 97.3 to 102%, demonstrating no interference from the ingredients tested. In addition, hyaluronic acid (HA) was analyzed by the method in the absence of CS and, as expected, produced a signal for ΔDi-0S. HA is hydrolyzed by chondroitinase AC II to generate $\Delta Di\text{-}0S_{HA}$, a diastereomer of ΔDi-0S that cannot be

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This method was approved by the Expert Review Panel for Dietary Supplements as First Action.
The Expert Review Panel for Dietary Supplements invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
This work was supported by National Institutes of Health Office of Dietary Supplements Contract HHSN263201300015C.
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Table 1. SMPR 2014.009 requirements and Method 2015.11 results

Type of study	Parameter	Minimum acceptable criteria		LC method results
Single-laboratory validation	LOQ	1% (w/w)		NR[a]
	Analytical range	1–10% (w/w)	>10–100% (w/w)	5–100%
	Repeatability (RSD_r)	≤3%	≤2%	0.25–1.8% within-day; 1.60–4.72% total
	Recovery	92–105%	98–102%	100.8–101.6% at 33–60% (w/w) RM[b] 105.4–105.8% at 16.7–37.5% (w/w) FP[c]
Multilaboratory validation	Reproducibility (RSD_R)	≤6%	≤4%	ND[d]

[a] NR = Not reported.

[b] RM = Raw material.

[c] FP = Finished product.

[d] ND = Not determined.

resolved under the LC conditions. Because HA is considerably more expensive than CS, however, it is unlikely to be used as an adulterant.

The linearity of the 5-point calibration curves was demonstrated over a range of 0.2–10 µg/mL ΔDi-0S, 1.4–70 µg/mL ΔDi-4S, and 2–100 µg/mL ΔDi-6S by showing no trend in the residual plots. Values for the coefficient of determination (r) were all >0.999.

Recovery was determined by spiking CS into raw material (heparin was used as a control raw material) and a non-CS commercial tablet product containing glucosamine HCl and methyl sulfonylmethane. Spiked raw material contained 33, 50, and 60% CS by weight, corresponding to 50, 100, and 200% of typical CS amounts in dietary supplements. Spiked finished product contained 16.7, 28.6, and 37.5% CS by weight, corresponding to 50, 100, and 150% of typical CS amounts in dietary supplements. Samples were analyzed in triplicate on 3 days. The method yielded recoveries of 100.8–101.6% over the three levels in raw material and 105.4–105.8% over the three levels in finished product. Repeatability from the spiked samples was 0.98–2.8% RSD_r in raw material and 2.0–3.5% RSD_r in finished product.

Repeatability was determined in three raw materials, two tablets, capsules, chewables, softgels, and liquid supplements by testing four replicate preparations on either 1 or 3 days. Within-day repeatability ranged from 0.25 to 1.8% RSD_r, between-day repeatability ranged from 1.32 to 4.66% RSD_r, and total repeatability ranged from 1.60 to 4.72% RSD_r. Interestingly, the liquid supplement was found not to contain CS, but, when spiked, yielded 99.6% recovery, demonstrating the applicability of the method to liquid supplements.

Finally, a Youden ruggedness trial demonstrated no effect from variation of seven parameters including sample sonication time, sample weight, enzyme hydrolysis time, enzyme concentration, enzyme buffer pH, injection volume, and detector wavelength.

AOAC Official Method 2015.11
Chondroitin Sulfate Content in Raw Materials and Dietary Supplements
High-Performance Liquid Chromatography with UV Detection After Enzymatic Hydrolysis
First Action 2015

Refer to the published method for further details (2).

Discussion

Table 1 provides a comparison of the SMPR and the LC method SLV results. The validation study demonstrated acceptable results for within-day repeatability, although the range of total repeatability (within-day + between-day) exceeded the limit. Recovery was within the SMPR allowable range when spiked surrogate raw material was tested, but recovery from spiked finished product was slightly higher than the allowable range. After careful consideration, the ERP voted on August 3, 2015, to adopt the LC method for First Action *Official Methods* status.

Before obtaining Final Action status, the ERP recommended the following actions: (*1*) optimize and control the moisture in the CS including appropriate vessels and glassware; (*2*) investigate alternate LC columns; (*3*) optimize the LC conditions; (*4*) review lessons learned from the U.S. Pharmacopeia; (*5*) include a potency evaluation of the enzyme used; (*6*) investigate use of the currently available U.S. Pharmacopeia standard; and (*7*) use a certified reference material.

References

(1) AOAC SMPR 2014.009 (2014) *J. AOAC Int.* **98**, 1058–1059

(2) Ji, D., Roman, M., Zhou, J., & Hildreth, J. (2007) *J. AOAC Int.* **90**, 659–669

DIETARY SUPPLEMENT

Single-Laboratory Validation Study of a Method for Screening and Identification of Phosphodiesterase Type 5 Inhibitors in Dietary Ingredients and Supplements Using Liquid Chromatography/Quadrupole–Orbital Ion Trap Mass Spectrometry: First Action 2015.12

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A single-laboratory validation study of a method for screening and identification of phosphodiesterase type 5 (PDE5) inhibitors in dietary ingredients and supplements is described. PDE5 inhibitors were extracted from the samples using a 50:50 (v/v) mixture of acetonitrile and water and centrifuged. Supernatant was diluted, filtered, and analyzed by LC–high-resolution MS. Data were collected in MS acquisition mode that combined full-scan MS experiment with all-ion fragmentation and data-dependent MS/MS product from the ion scan experiment. This approach enabled collection of MS and tandem MS (MS/MS) data for both targeted and nontargeted PDE5 inhibitors in a single chromatographic run. Software-facilitated identification of targeted analytes was performed based on the retention time, accurate mass, and isotopic pattern of pseudomolecular ions, and accurate masses of fragment ions using an in-house compound database. Detection and identification of other PDE5 inhibitors and novel analogs were performed by retrospective evaluation of MS and MS/MS experimental data. The method validation results obtained for evaluated matrixes fulfilled the probability of identification requirements and probability of detection requirements (for the pooled data) set at 90% (95% confidence interval) in the respective AOAC *Standard Method Performance Requirements* for identification and screening methods for PDE5 inhibitors. Limited data demonstrating the quantification capability of the method were also generated. Mean recovery and repeatability obtained for the evaluated PDE5 inhibitors were in the range 69–90% and 0.4–1.8%, respectively.

Deliberate addition of active pharmaceutical ingredients to dietary supplements is a profit-driven practice that aims to develop or intensify the claimed biological effect of the product (1, 2). Phosphodiesterase type 5 (PDE5) inhibitors, such as avanafil, lodenafil carbonate, mirodenafil, sildenafil, tadalafil, udenafil, or vardenafil and their unapproved designer analogs, represent an important class of pharmaceuticals that are frequently used to adulterate products advertised to provide an enhancement to sexual performance and ingredients used in their manufacturing (3, 4). Considering that PDE5 inhibitors can negatively interact with certain prescription drugs and that limited knowledge is available on safety and efficacy of the designer analogs, the presence of such compounds in dietary supplements may represent a serious health risk to consumers (2). Therefore, reliable analytical methods are needed for detection, identification, and quantification of PDE5 inhibitors in relevant dietary supplement raw materials and finished products.

To address this problem, AOAC INTERNATIONAL issued a call for methods for screening, identification, and determination of PDE5 inhibitors in dietary ingredients and supplements based on *Standard Method Performance Requirements* (SMPRs®) developed by a working group of the AOAC INTERNATIONAL Stakeholder Panel on Dietary Supplements (5–7). Single-laboratory validation (SLV) requirements provided in AOAC SMPR 2014.010 for identification of PDE5 inhibitors are summarized in Table 1.

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The Expert Review Panel for Dietary Supplements invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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Table 1. Method performance requirements (AOAC SMPR 2014.010)

Type of study	Study	Parameter	Parameter requirements	Target test concn	Minimum acceptable results
SLV	Matrix study	POI at low concn	Minimum of 33 replicates representing all target compounds in Annex I and ideally all matrix types listed in Annex II, spiked at or below the designated low level target test concentration	100 ppm	90% POI[a] of the pooled data for all target compounds and matrixes
		POI at high concn	Minimum of 5 replicates per matrix type spiked at 10× the designated low level target test concentration	10× low concn	100% correct analyses are expected[b]
		POI at 0 concn	Minimum of 5 replicates per matrix type	0 ppm	

[a] 95% Confidence interval.

[b] 100% Correct analyses are expected. Some aberrations may be acceptable if the aberrations are investigated, and acceptable explanations can be determined and communicated to method users.

SLV Study

This validation study evaluated probability of identification (POI) for 15 target panel PDE5 inhibitors provided in the AOAC SMPR 2014.010 (*see* Table 2). The evaluation was performed at concentrations of 0, 100, and 1000 mg/kg. Considering the availability and cost of the reference standards and amounts needed to obtain the above target concentrations in the samples, postextraction spiking of blank matrix extracts with target panel compounds was performed at 250 and 2500 ng/mL to obtain concentrations corresponding to 100 and 1000 mg/kg in the samples, respectively. Five samples were prepared for each concentration level in each of the seven evaluated matrixes. This experimental design resulted in 35 samples per concentration level and a final set of 105 samples, which fulfilled requirements provided in AOAC SMPR 2014.010. The samples were analyzed using LC–high-resolution MS (LC-HRMS) with a Q-Exactive Plus instrument (Thermo Fisher Scientific, San Jose, CA), followed by raw data processing with TraceFinder software (Thermo Fisher Scientific, San Jose, CA) that allowed for the automatic identification of the target PDE5 inhibitors using the identification criteria discussed below.

To demonstrate the ability of the method to extract PDE5 inhibitors from the samples, a homogenized capsule dietary supplement (M5 in Table 3) was spiked in triplicate with the target panel compounds at 50 mg/kg and extracted according to the method sample preparation protocol. Analyte recoveries were calculated using matrix-matched standards.

The evaluated matrixes covered the dietary ingredient and supplement matrix types provided in Annex II of AOAC SMPR 2014.010: tablets, capsules (both content and capsule shells), softgels, liquid drink, herbal tincture, botanical powder, and botanical extract. Representative samples of each matrix type were selected to cover the variety of typical ingredients used in the manufacture of sexual enhancement supplements. Table 3 lists the samples and ingredients declared by the vendor on the label of the respective product.

AOAC Official Method 2015.12 Screening and Identification of Phosphodiesterase Type 5 Inhibitors in Dietary Ingredients and Supplements Using Liquid Chromatography/ Quadrupole–Orbital Ion Trap Mass Spectrometry First Action 2015

[Applicable to the screening and identification of acetaminotadalafil, acetildenafil, avanafil, homosildenafil, hydroxyacetildenafil, hydroxyhomosildenafil, hydroxythiohomosildenafil, lodenafil carbonate, mirodenafil, propoxyphenyl homohydroxysildenafil, sildenafil, tadalafil, thiohomosildenafil, udenafil, vardenafil, and other known and novel analogs of the above PDE5 inhibitors.]

Caution: See AOAC Official Methods of Analysis℠ Appendix B: Laboratory Safety (8). Use appropriate personal protective equipment such as a laboratory coat, safety glasses, rubber gloves, and a fume hood. Dispose of solvents and solutions according to federal, state, and local regulations.

A. Apparatus

(a) *LC-MS system.*—UltiMate 3000 LC system (Thermo Fisher Scientific, San Jose, CA) (or an equivalent LC system) with Q-Exactive Plus mass spectrometer equipped with electrospray ionization [or equivalent high-resolution tandem MS (MS/MS)] instrument.

(b) *Analytical balances.*—Accurate to two and four decimal places.

(c) *Gilson positive displacements pipets*.—Assorted for 100–1000 µL.

(d) *Repeater pipet.*—For 10 µL to 50 mL size tips.

(e) *Horizontal shaker.*—Shaking speed at least 250 rpm.

(f) *Centrifuge.*—Relative centrifugal force of at least 3000 × *g*.

(g) *Volumetric flasks*.—Class A, glass, assorted sizes.

(h) *Laboratory glassware.*—Class A, various.

(i) *Disposable polypropylene centrifuge tubes*.—15 and 50 mL.

(j) *Disposable plastic syringes*.—3 mL.

(k) *Syringe filters.*—PTFE, 0.22 µm.

(l) *LC vials and caps*.

(m) *Chromatographic column.*—Thermo Fisher Scientific Accucore aQ C18 (Part No. 17326-102130), 2.6 µm, 100 × 2.1 mm.

(n) *Guard column.*—Thermo Fisher Scientific Accucore aQ C18 (Part No. 17326-012105), 2.6 µm, 10 × 2.1 mm.

B. Materials and Reagents

(a) *Methanol (MeOH).*—LC-MS and HPLC grade.

(b) *Water (H_2O).*—LC-MS grade or deionized.

(c) *Acetonitrile (ACN).*—LC-MS and HPLC grade.

(d) *Chloroform.*—HPLC grade.

(e) *Ammonium formate (NH_4OFor).*—LC-MS grade.

(f) *Formic acid (FA).*—LC-MS grade.

C. Reference Standards

The reference standards (purity ≥95%) listed in Table 2 were purchased from Toronto Research Chemicals (Toronto,

Table 2. Overview of PDE5 inhibitors analyzed in the study

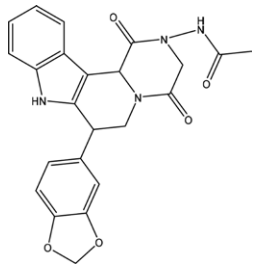
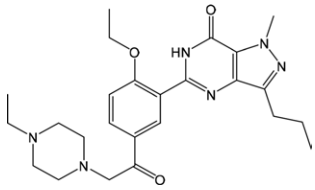
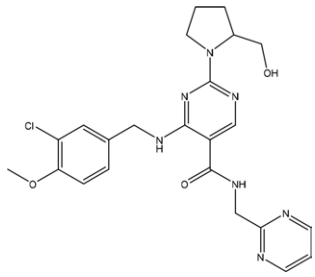
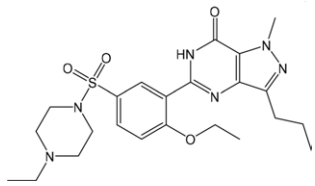
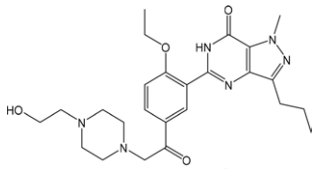
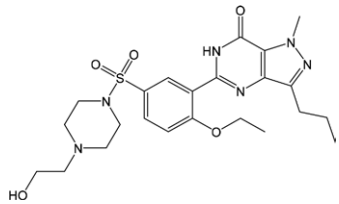
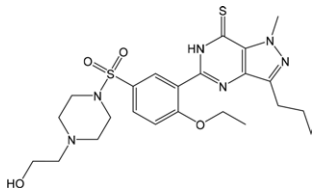
Analyte	Chemical Abstracts Service No.	Formula	Note	Structure
Acetaminotadalafil	1446144-71-3	$C_{23}H_{20}N_4O_5$	Target panel	
Acetildenafil	831217-01-7	$C_{25}H_{34}N_6O_3$	Target panel	
Avanafil	330784-47-9	$C_{23}H_{26}ClN_7O_3$	Target panel	
Homosildenafil	642928-07-2	$C_{23}H_{32}N_6O_4S$	Target panel	
Hydroxyacetildenafil	147676-56-0	$C_{25}H_{34}N_6O_4$	Target panel	
Hydroxyhomosildenafil	139755-85-4	$C_{23}H_{32}N_6O_5S$	Target panel	
Hydroxythiohomo sildenafil	479073-82-0	$C_{23}H_{32}N_6O_4S_2$	Target panel	

Table 2. (*continued*)

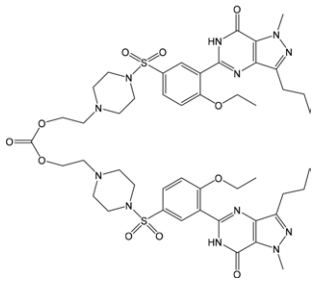
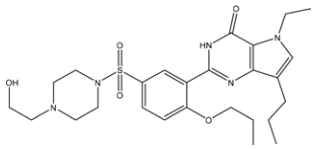
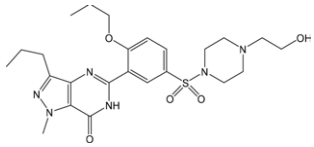
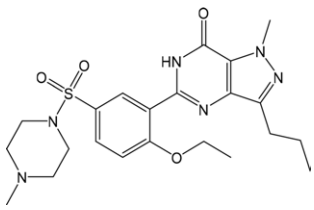
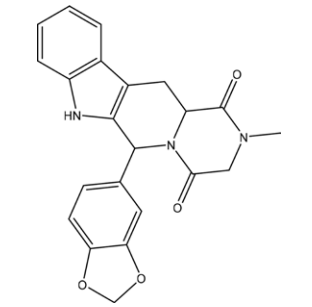
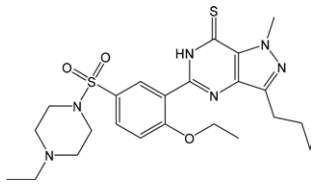
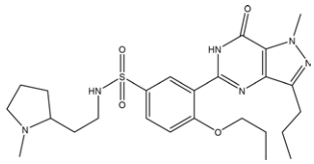
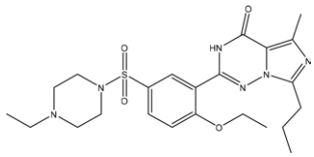
Analyte	Chemical Abstracts Service No.	Formula	Note	Structure
Lodenafil carbonate	398507-55-6	$C_{47}H_{62}N_{12}O_{11}S_2$	Target panel	
Mirodenafil	862189-95-5	$C_{26}H_{37}N_5O_5S$	Target panel	
Propoxyphenyl homohydroxysildenafil	139755-87-6	$C_{24}H_{34}N_6O_5S$	Target panel	
Sildenafil	139755-83-2	$C_{22}H_{30}N_6O_4S$	Target panel	
Tadalafil	171596-29-5	$C_{22}H_{19}N_3O_4$	Target panel	
Thiohomosildenafil	479073-80-8	$C_{23}H_{32}N_6O_3S_2$	Target panel	
Udenafil	268203-93-6	$C_{25}H_{36}N_6O_4S$	Target panel	
Vardenafil	224785-90-4	$C_{23}H_{32}N_6O_4S$	Target panel	

Table 2. (***continued***)

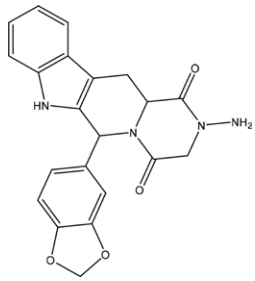
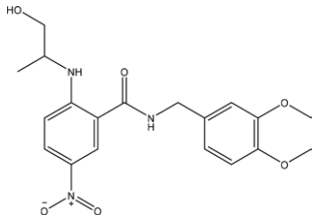
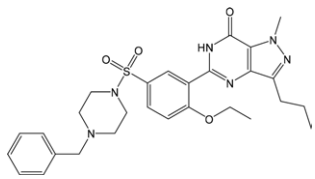
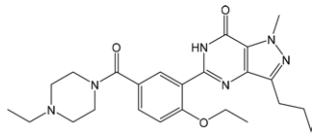
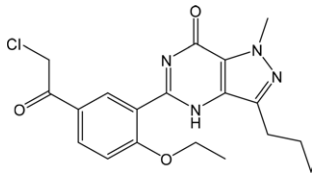
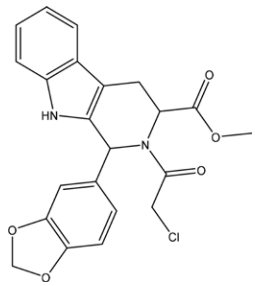
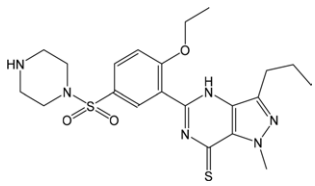
Analyte	Chemical Abstracts Service No.	Formula	Note	Structure
Aminotadalafil	385769-84-6	$C_{21}H_{18}N_4O_4$	—[a]	
Benzamidenafil	1020251-53-9	$C_{19}H_{23}N_3O_6$	—	
Benzylsildenafil	1446089-89-2	$C_{28}H_{34}N_6O_4S$	—	
Carbodenafil	Not available	$C_{24}H_{32}N_6O_3$	—	
Chlorodenafil	1058653-74-9	$C_{19}H_{21}ClN_4O_3$	—	
Chloropretadalafil	171489-59-1	$C_{22}H_{19}ClN_2O_5$	—	
Desmethylthiosildenafil	479073-86-4	$C_{21}H_{28}N_6O_3S_2$	—	

Table 2. (*continued*)

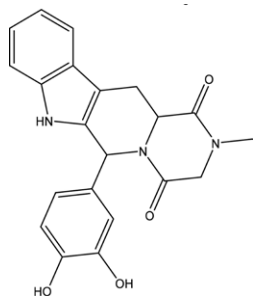
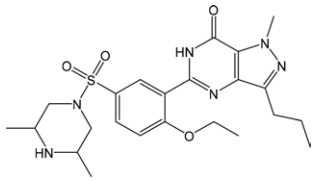
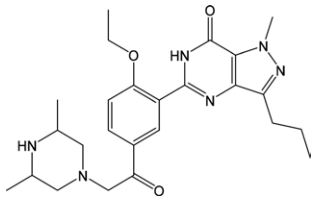
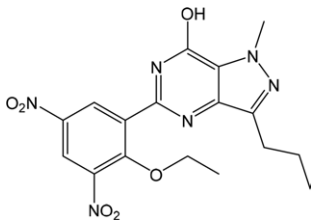
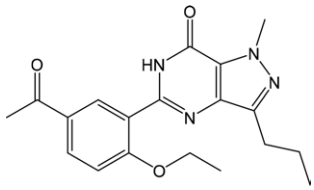
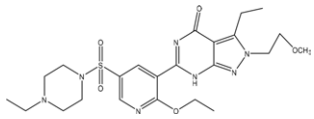
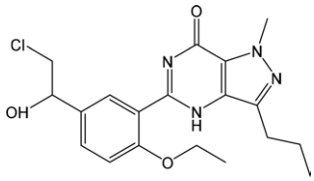
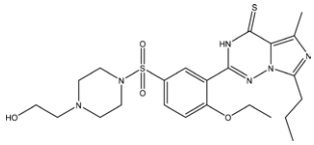
Analyte	Chemical Abstracts Service No.	Formula	Note	Structure
Desmethylenetadalafil	171489-03-5	$C_{21}H_{19}N_3O_4$	—	
Dimethylsildenafil	1416130-63-6	$C_{23}H_{32}N_6O_4S$	—	
Dimethylacetildenafil	Not available	$C_{25}H_{34}N_6O_3$	—	
Dinitrodenafil	Not available	$C_{17}H_{18}N_6O_6$	—	
Gendenafil	147676-66-2	$C_{19}H_{22}N_4O_3$	—	
Gisadenafil	334826-98-1	$C_{23}H_{33}N_7O_5S$	—	
Hydroxychlorodenafil	1391054-00-4	$C_{19}H_{23}ClN_4O_3$	—	
Hydroxythiovardenafil	912576-30-8	$C_{23}H_{32}N_6O_4S_2$	—	

Table 2. (*continued*)

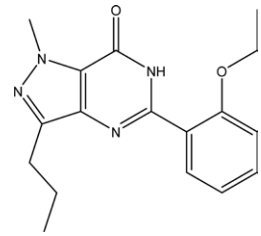
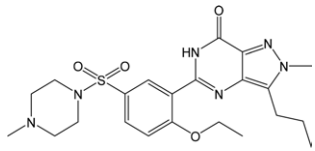
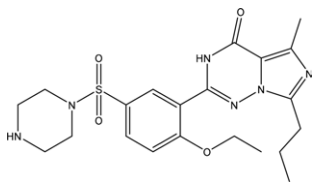
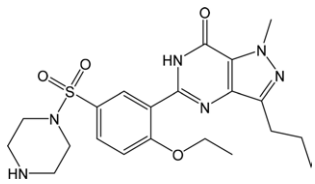
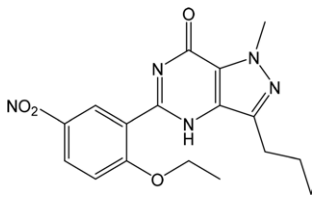
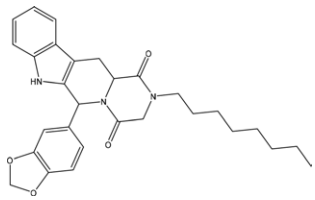
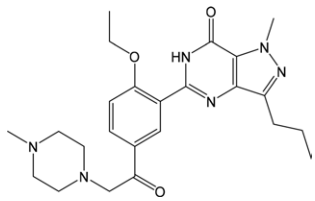
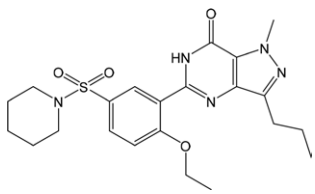
Analyte	Chemical Abstracts Service No.	Formula	Note	Structure
Imidazosagatriazinone	139756-21-1	$C_{17}H_{20}N_4O_2$	—	
Isosildenafil	253178-46-0	$C_{22}H_{30}N_6O_4S$	—	
N-Desethyl vardenafil	448184-46-1	$C_{21}H_{28}N_6O_4S$	—	
N-Desmethyl sildenafil	139755-82-1	$C_{21}H_{28}N_6O_4S$	—	
Nitrodenafil	147676-99-1	$C_{17}H_{19}N_5O_4$	—	
N-Octyl nortadalafil	1173706-35-8	$C_{29}H_{33}N_3O_4$	—	
Noracetildenafil	949091-38-7	$C_{24}H_{32}N_6O_3$	—	
Norneosildenafil	371959-09-0	$C_{22}H_{29}N_5O_4S$	—	

Table 2. (*continued*)

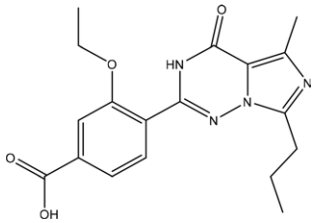
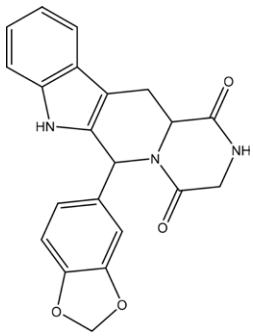
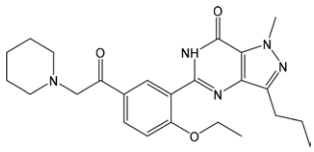
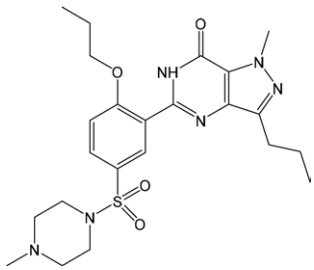
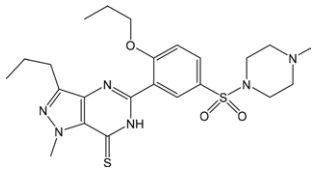
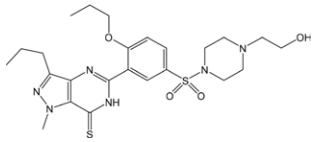
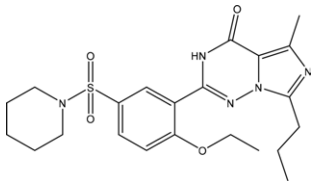
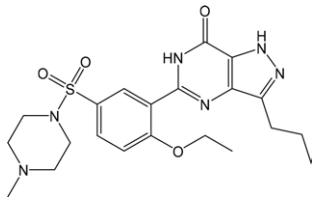
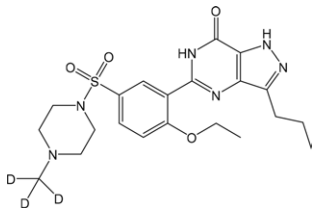
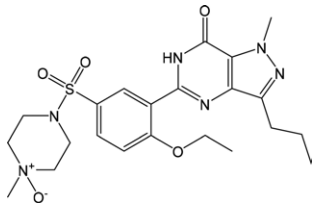
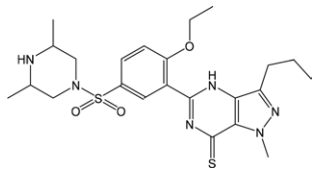
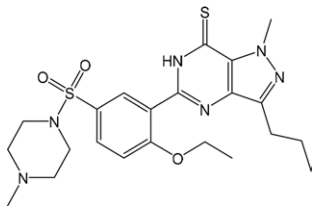
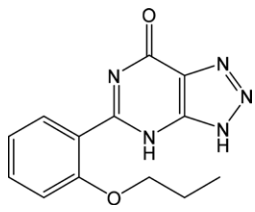
Analyte	Chemical Abstracts Service No.	Formula	Note	Structure
Norneovardenafil	358390-39-3	$C_{18}H_{20}N_4O_4$	—	
Nortadalafil	171596-36-4	$C_{21}H_{17}N_3O_4$	—	
Piperiacetildenafil	147676-50-4	$C_{24}H_{31}N_5O_3$	—	
Propoxyphenyl sildenafil	877777-10-1	$C_{23}H_{32}N_6O_4S$	—	
Propoxyphenyl thiosildenafil	479073-87-5	$C_{23}H_{32}N_6O_3S_2$	—	
Propoxyphenyl thiohydroxyhomosildenafil	479073-90-0	$C_{24}H_{34}N_6O_4S_2$	—	
Pseudovardenafil	224788-34-5	$C_{22}H_{29}N_5O_4S$	—	

Table 2. (*continued*)

Analyte	Chemical Abstracts Service No.	Formula	Note	Structure
Pyrazole *N*-demethyl sildenafil	139755-95-6	$C_{21}H_{28}N_6O_4S$	—	
Pyrazole *N*-demethyl sildenafil-d_3	Not available	$C_{21}H_{25}D_3N_6O_4S$	IS	
Sildenafil *N*-oxide	1094598-75-0	$C_{22}H_{30}N_6O_5S$	—	
Thioaildenafil	856190-47-1	$C_{23}H_{32}N_6O_3S_2$	—	
Thiosildenafil	479073-79-5	$C_{22}H_{30}N_6O_3S_2$	—	
Zaprinast	37762-06-4	$C_{13}H_{13}N_5O_2$	—	

[a] — = Additional evaluated analytes.

Table 3. Matrixes evaluated in the SLV study

Code	Form	Active ingredients	Other ingredients
M1	Powder	*Tribulus terrestris*	Not available
M2	Extract	*Epimedium*	Not available
M3	Softgel	Maca root powder, *Ashwagandha* powder, *Epimedium* extract, *Tribulus* extract, Yohimbe bark extract, ginger root extract, long pepper fruit extract, black pepper fruit extract	Soybean oil, gelatin, glycerin, purified water, beeswax, Soy lecithin, caramel color
M4	Liquid	Damiana leaf extract, ginseng root extract, saw palmetto, *Tribulus terrestris* fruit extract, *Avena sativa* extract, bee pollen extract, guarana seed extract, Yohimbe bark extract, royal jelly	Distilled water, glycerin
M5	Capsule	Maca powder, Horny goat weed extract, *Tribulus* extract, Yohimbe extract, cayenne extract, Asian ginseng extract, ginger extract, long pepper extract, black pepper extract	Gelatin, silica, vegetable stearate
M6	Tablet	*Pinus pinaster* bark extract, *Epimedium sagittatum* extract	Corn starch, maltodextrin, cellulose, vegetable stearate, silica, glycerin, purified water
M7	Liquid extract (tincture)	*Epimedium grandiflorum* dried leaves	Glycerine, alcohol 60%, distilled water

Canada), Cachesyn (Mississagua, Canada), TLC Pharmachem (Vaughan, Canada), and Sigma-Aldrich (St. Louis, MO).

D. Preparation of Reagent Solutions and Standards

(a) *50:50 (v/v) ACN:H_2O.*—Combine 500 mL HPLC grade ACN and 500 mL deionized H_2O. Sonicate for 2 min.

(b) *70:30 (v/v) H_2O:ACN.*—Combine 700 mL deionized H_2O and 300 mL HPLC grade ACN. Sonicate for 2 min.

(c) *LC mobile phase A.*—Weigh 0.63±0.01 g NH_4OFor in an appropriate reservoir and add 1000 mL H_2O and 1 mL FA. Mix thoroughly.

(d) *LC mobile phase B.*—Weigh 0.63±0.01 g NH_4OFor in an appropriate reservoir and add 500 mL MeOH. Sonicate for approximately 3 min. Add 500 mL ACN and 1 mL FA. Mix thoroughly.

(e) *Individual stock solutions.*—Prepare individual solutions of PDE5 inhibitors at concentrations ranging from 1500 to 4000 µg/mL. For aminotadalafil, benzyl sildenafil, chloropretadalafil, desmethylene tadalafil, lodenafil carbonate, tadalafil, and thioaildenafil use a mixture of MeOH and chloroform (2:1, v/v). For the remaining analytes, use MeOH. If needed, sonicate at approximately 30°C to allow for complete dissolution of the solid standard.

(f) *Mixed stock standard solution.*—Combine individual analyte stock solutions to prepare a composite solution at 20 µg/mL in MeOH.

(g) *Internal standard (IS) solution.*—Prepare a solution at 20 µg/mL in MeOH using a stock solution of pyrazole *N*-demethyl sildenafil-d_3.

(h) *QC solvent standard.*—Accurately transfer 125 µL of the mixed stock standard solution and 125 µL the IS solution into a 10 mL volumetric flask. Dilute to volume with 70:30 (v/v) H_2O:ACN solution.

E. Sample Preparation

(a) *Homogenization and storage of samples.*—Solid samples such as botanical powders, extracts, and tablets were blended to obtain homogeneity and stored at –4°C. Softgels, gelcaps, and capsules were homogenized using cryogenic grinding with liquid nitrogen and stored at –70°C. Liquid samples were briefly shaken and stored at –4°C.

(b) *Extraction procedure.*—(*1*) Weigh 1.00±0.02 g thoroughly homogenized sample in a 50 mL centrifuge tube.

(*2*) Add 20 mL 50:50 (v/v) ACN:H_2O solution, briefly hand shake/vortex, and then shake for 15 min using a horizontal shaker set at approximately 250 rpm.

(*3*) Centrifuge the tube at >3000 × *g* for 5 min.

(*4*) Transfer 1 mL supernatant to another 50 mL centrifuge tube.

Note: When transferring extract aliquots obtained for softgels, avoid the upper lipophilic layer that forms during the centrifugation step.

(*5*) Add 19 mL 70:30 (v/v) H_2O:ACN solution and briefly vortex mix.

(*6*) Filter approximately 3 mL diluted extract using a plastic syringe fitted with a 0.22 µm PFTE syringe filter into a 15 mL centrifuge tube.

(*7*) Transfer 1 mL filtrate to a 2 mL autosampler vial and add 12.5 µL IS solution.

(*8*) Cap the vial and briefly vortex mix.

(*9*) Perform LC-HRMS analysis.

F. LC-HRMS Analysis

(a) *LC operating conditions.*—(*1*) *Column.*—Thermo Scientific Accucore aQ, 2.6 µm, 100×2.1 mm.

(*2*) *Column temperature.*—30°C.

(*3*) *Mobile phase A.*—10 mM NH_4OFor and 0.1% FA in H_2O.

(*4*) *Mobile phase B.*—10 mM NH_4OFor and 0.1% FA in ACN–MeOH (50:50, v/v).

(*5*) *Flow rate.*—0.3 mL/min.

(*6*) *Elution gradient.*—*See* Table **2015.12A**.

(*7*) *Injection volume.*—3 µL.

(*8*) *Autosampler temperature.*—15°C.

(*9*) *Run time.*—25 min.

(b) *MS data acquisition and operating conditions.*—MS data acquisition is performed in full MS–data-dependent product ion scan (dd-MS^2) and all-ion fragmentation (AIF) modes using the parameter settings provided below. Data-dependent product ion scan experiment is initiated if a mass (*m/z*) specified in an inclusion list (*see* Table **2015.12A**) is detected in the correct retention time (RT) window within a mass error of 10 ppm and at an intensity above the set threshold level. The ion

Table 2015.12A. Gradient elution program

Time, min	A, %	B, %
0.00	98	2
0.50	98	2
2.00	60	40
20.00	5	95
23.00	5	95
23.01	98	2
24.00	98	2

fragmentation in AIF and dd-MS^2 modes is performed at three discrete normalized collision energy (NCE) values.

(*1*) *Ionization mode.*—positive ESI.
(*2*) *Sheath gas flow.*—35 arb.
(*3*) *Auxiliary gas flow.*—10 arb.
(*4*) *Sweep gas flow.*—1 arb.
(*5*) *Spray voltage.*—3.5 kV.
(*6*) *Capillary temperature.*—350°C.
(*7*) *S-lens RF level.*—50 V.
(*8*) *Auxiliary gas heater temperature.*—350°C.
(*9*) *Full MS resolution.*—70 000 full width at half-maximum (FWHM).
(*10*) *Full MS automatic gain control AGC target.*—1e6.
(*11*) *Full MS maximum injection time (IT).*—100 ms.
(*12*) *Full MS scan range.—m/z* 200–1100.
(*13*) *dd-MS2 resolution.*—17 500 FWHM.
(*14*) *dd-MS2 AGC target.*—1e5.
(*15*) *dd-MS2 isolation window.*—1.0 Da.
(*16*) *dd-MS2 stepped NCE.*—40, 70, 100%.
(*17*) *Intensity threshold.*—2.0e4.
(*18*) *Apex trigger.*—1 to 6 s
(*19*) *Dynamic exclusion.*—6 s
(*20*) *AIF resolution.*—70 000 FWHM.
(*21*) *AIF AGC target.*—1e6.
(*22*) *AIF maximum IT.*—100 ms.
(*23*) *AIF stepped NCE.*—40, 70, 100%.
(*24*) *AIF scan range.—m/z* 50–750.

(c) *Inclusion list.—See* Table **2015.12B**.

(d) *Positive and negative control.*—Analyze a reagent blank (a negative control) with each sample set. Inject the QC solvent standard (a positive control) at the beginning of the LC-HRMS sequence, after every 10 samples, and again at the end of the LC-HRMS sequence. The IS response in samples should be within 40–140% of its average response in the QC solvent standards.

G. Data Processing

(a) *Workflow and detection/identification criteria.*—Detection and identification of analytes was performed with TraceFinder software and the settings indicated below. Detection of targeted PDE5 inhibitors was based on the automatic comparison of peak RTs extracted the from full MS record and the accurate mass of respective pseudomolecular ions $[M+H]^+$ with information from the TraceFinder compound database (*see* Table **2015.12C**). An RT of 30 s and mass tolerances of 5 ppm were used. To identify an analyte, additional criteria must be fulfilled. These include mass accuracy ($\Delta\, m/z \leq 5$ ppm) and relative responses (10% tolerance) of pseudomolecular ion isotopes, as well as criteria for fragment ions detected in appropriate dd-MS^2 records. For positive identification, one or more fragment ions listed in the TraceFinder compound database must be detected above the intensity threshold with a mass error of ≤5 ppm. The detection/identification workflow for targeted compounds is provided in Figure **2015.12**. PDE5 inhibitors not included in the TraceFinder compound database can be detected and identified by extracting the respective pseudomolecular ions from the full MS records and evaluating fragment ions in AIF records. A search using common PDE5 inhibitor fragments can be used to highlight components with structures similar to known PDE5 inhibitors.

(b) *TraceFinder software settings.*—(*1*) *RT range.*—1–23 min.
(*2*) *Peak area threshold.*—100 000.
(*3*) *Signal-to-noise threshold.*—10.
(*4*) *Mass tolerance (parent ion).*—5 ppm.

Table 2015.12B. Inclusion list used in the dd-MS^2 experiment for the target compound panel

Mass, *m/z*	Chemical formula	Species	Charge state	Polarity	Start, min	End, min
483.27143	$C_{25}H_{34}N_6O_4$	+H	1	Positive	4.06	4.36
467.27652	$C_{25}H_{34}N_6O_3$	+H	1	Positive	4.35	4.65
489.22785	$C_{23}H_{32}N_6O_4S$	+H	1	Positive	4.72	5.02
505.22277	$C_{23}H_{32}N_6O_5S$	+H	1	Positive	4.86	5.16
484.18584	$C_{23}H_{26}ClN_7O_3$	+H	1	Positive	4.88	5.18
475.21220	$C_{22}H_{30}N_6O_4S$	+H	1	Positive	4.92	5.22
489.22785	$C_{23}H_{32}N_6O_4S$	+H	1	Positive	5.08	5.38
433.15065	$C_{23}H_{20}N_4O_5$	+H	1	Positive	5.18	5.48
517.25915	$C_{25}H_{36}N_6O_4S$	+H	1	Positive	5.73	6.03
519.23842	$C_{24}H_{34}N_6O_5S$	+H	1	Positive	5.80	6.10
390.14483	$C_{22}H_{19}N_3O_4$	+H	1	Positive	5.97	6.27
532.25882	$C_{26}H_{37}N_5O_5S$	+H	1	Positive	7.78	8.08
521.19992	$C_{23}H_{32}N_6O_4S_2$	+H	1	Positive	8.60	8.90
505.20501	$C_{23}H_{32}N_6O_3S_2$	+H	1	Positive	8.92	9.22
1035.41752	$C_{47}H_{62}N_{12}O_{11}S_2$	+H	1	Positive	12.78	13.08

Table 2015.12C. TraceFinder software compound database for the target compound panel

Compound name	Chemical formula	Extracted mass	Adduct	RT	Fragment ions, *m/z*
Acetaminotadalafil	$C_{23}H_{20}N_4O_5$	433.15065	M+H	5.33	204.08078; 262.08626; 135.04406; 205.08860; 233.08352; 232.07569; 169.07602; 191.07295; 263.09408; 250.08626
Acetildenafil	$C_{25}H_{34}N_6O_3$	467.27652	M+H	4.50	111.09167; 97.07602; 70.06513; 84.08078; 72.08078; 127.12297; 112.09950; 297.13460; 56.04948; 166.09749
Avanafil	$C_{23}H_{26}ClN_7O_3$	484.18584	M+H	5.03	155.02582; 375.12184; 105.03349; 77.03858; 95.04914; 53.03858; 357.11128; 233.10330; 67.05423; 221.10330
Homosildenafil	$C_{23}H_{32}N_6O_4S$	489.22785	M+H	5.23	72.08078; 58.06513; 99.09167; 113.10732; 70.06513; 283.11895; 84.08078; 71.07295; 114.11515; 311.15025
Hydroxyacetildenafil	$C_{25}H_{34}N_6O_4$	483.27143	M+H	4.21	97.07602; 70.06513; 127.08659; 143.11789; 100.07569; 297.13460; 88.07569; 166.09749; 112.09950; 128.09441
Hydroxyhomosildenafil	$C_{23}H_{32}N_6O_5S$	505.22277	M+H	5.01	99.09167; 70.06513; 58.06513; 84.06820; 97.07602; 283.11895; 88.07569; 129.10224; 112.0995; 311.15025
Hydroxythiohomo-sildenafil	$C_{23}H_{32}N_6O_4S_2$	521.19992	M+H	8.75	99.09167; 70.06513; 58.06513; 84.06820; 299.09611; 129.10224; 97.07602; 88.07569; 327.12741; 112.09950
Lodenafil carbonate	$C_{47}H_{62}N_{12}O_{11}S_2$	1035.41752	M+H	12.93	112.09950; 82.06513; 97.07602; 111.09167; 487.21220; 83.06037; 84.08078; 283.11895
Mirodenafil	$C_{26}H_{37}N_5O_5S$	532.25882	M+H	7.93	99.09167; 296.13935; 312.13427; 70.06513; 56.04948;84.06820; 210.06619; 129.10224; 88.07569; 121.03964
Propoxyphenyl homohydroxysildenafil	$C_{24}H_{34}N_6O_5S$	519.23842	M+H	5.95	99.09167; 70.06513; 283.11895; 84.06820; 97.07602; 299.11387; 129.10224; 88.07569; 112.09950; 255.12404
Sildenafil	$C_{22}H_{30}N_6O_4S$	475.2122	M+H	5.07	58.06513; 100.09950; 99.09167; 56.04948; 283.11895; 70.06513; 311.15025; 225.07709; 299.11387
Tadalafil	$C_{22}H_{19}N_3O_4$	390.14483	M+H	6.12	204.08078; 135.04406; 262.08626; 169.07602; 205.08860; 232.07569; 233.08352; 240.11314; 268.10805; 250.08626
Thiohomosildenafil	$C_{23}H_{32}N_6O_3S_2$	505.20501	M+H	9.07	72.08078; 99.09167; 113.10732; 56.04948; 299.09611; 70.06513; 84.08078; 327.12741; 71.07295; 355.15806
Udenafil	$C_{25}H_{36}N_6O_4S$	517.25915	M+H	5.88	84.08078; 112.11208; 283.11895; 58.06513; 325.16590; 299.11387; 81.06988; 255.124037; 79.05423; 82.06513
Vardenafil	$C_{23}H_{32}N_6O_4S$	489.22785	M+H	4.87	169.09715; 344.14791; 110.06004; 299.11387; 72.08078; 123.09167; 70.06513; 376.10740; 68.01309; 113.10732

(*5*) *RT tolerance.*—30 s.

(*6*) *Minimum No. of fragments.*—1.

(*7*) *Intensity threshold.*—1000.

(*8*) *Mass tolerance (fragment ion).*—5 ppm.

(*9*) *Isotope pattern fit threshold.*—95%.

(*10*) *Mass tolerance (isotope).*—5 ppm.

(*11*) *Intensity tolerance (isotope).*—10%.

(c) *TraceFinder compound database.*—The compound database (*see* Table **2015.12C**) comprises information on the exact mass of pseudomolecular ions, molecular formulas, and RTs and the exact masses for 8–10 fragment ions for each analyte. The *m/z* values of fragments in the compound database represent exact masses that were calculated using experimental data obtained by HRMS analysis of reference standards and elucidation of fragment ions in Mass Frontier (Thermo Fisher Scientific, San Jose, CA) spectral interpretation software or based on information available in mzCloud database (Thermo Fisher Scientific, San Jose, CA) and scientific literature.

Results and Discussion

Chromatographic Separation

PDE5 inhibitors have multiple basic nitrogen groups in their molecules, which makes them prone to pH-dependent chromatographic issues, such as tailing or poor peak shape caused by the presence of analytes in both neutral and ionized forms. The mobile phase composition was optimized to minimize/eliminate these problems by using 10 mM ammonium formate and 0.1% FA in both mobile phases A and B. Addition of the acid to the mobile phase was essential to obtaining a good peak shape for norneovardenafil, which has an acidic carboxyl group in its molecule.

The composition of the organic mobile phase component had a significant impact on the chromatographic resolution between several isobaric compounds. Because some of these analytes cannot be differentiated based on their MS fragmentation patterns, their sufficient chromatographic separation is critical for reliable identification. Best results were obtained when a

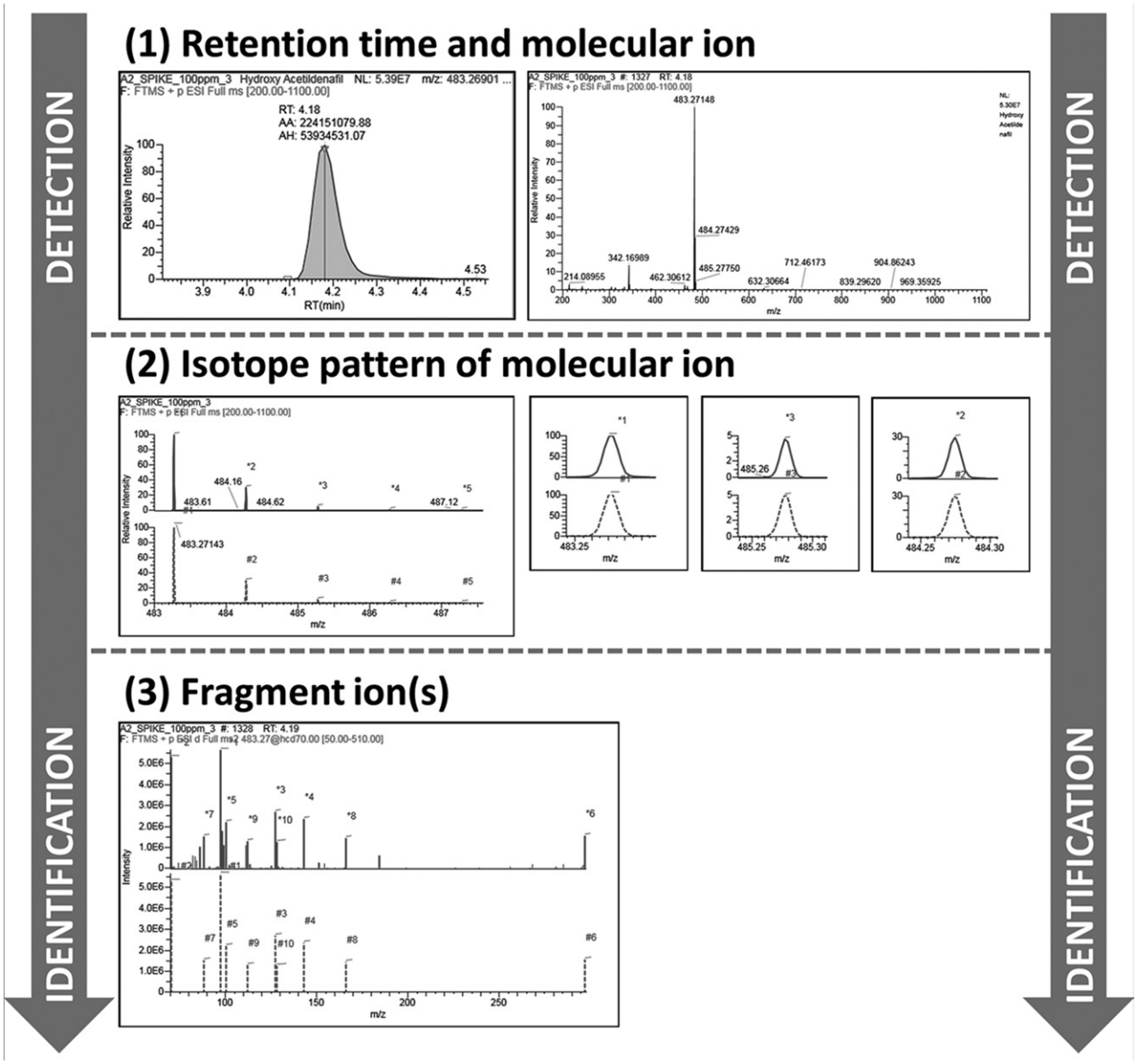


Figure 2015.12. Detection/identification workflow for targeted analytes.

mixture containing equal amounts of MeOH and ACN was used as the organic component of the mobile phase (*see* Figure 1). Under optimized conditions, analytes eluted between 3 and 15 min of the run with typical at-base peak widths ranging from 12 to 18 s. Of eight isobaric analyte groups, each containing two to four compounds, all analytes could be chromatographically resolved.

MS/MS Spectra

The availability of MS/MS data are crucial for reliable screening and identification of both known PDE5 inhibitors and their novel analogs. The MS/MS spectra of analytes were recorded in data-dependent product ion scan mode through the isolation and fragmentation of their respective pseudomolecular ions and in AIF mode. Rather than performing fragmentation at a single NCE setting, three discrete values of 40, 70, and 100% were used. This stepped NCE approach allowed obtaining fragments stable under different collision energies in a single MS experiment and resulted in information-rich MS/MS spectra.

Based on the review of the MS/MS spectra of all analytes, product ions frequently occurring in records of parent PDE5 inhibitors and their analogs were found. For example, fragment ion exact masses *m/z* 377.12780, 311.15025, 299.09611, 285.13460, 283.11895, and 99.09167 were frequently present in fragmentation spectra of sildenafil and its analogs, fragment *m/z* 204.08078 was characteristic of tadalafil and its analogs, and fragment ions *m/z* 123.09167 and 110.06004 were characteristic of vardenafil and its analogs. A combined

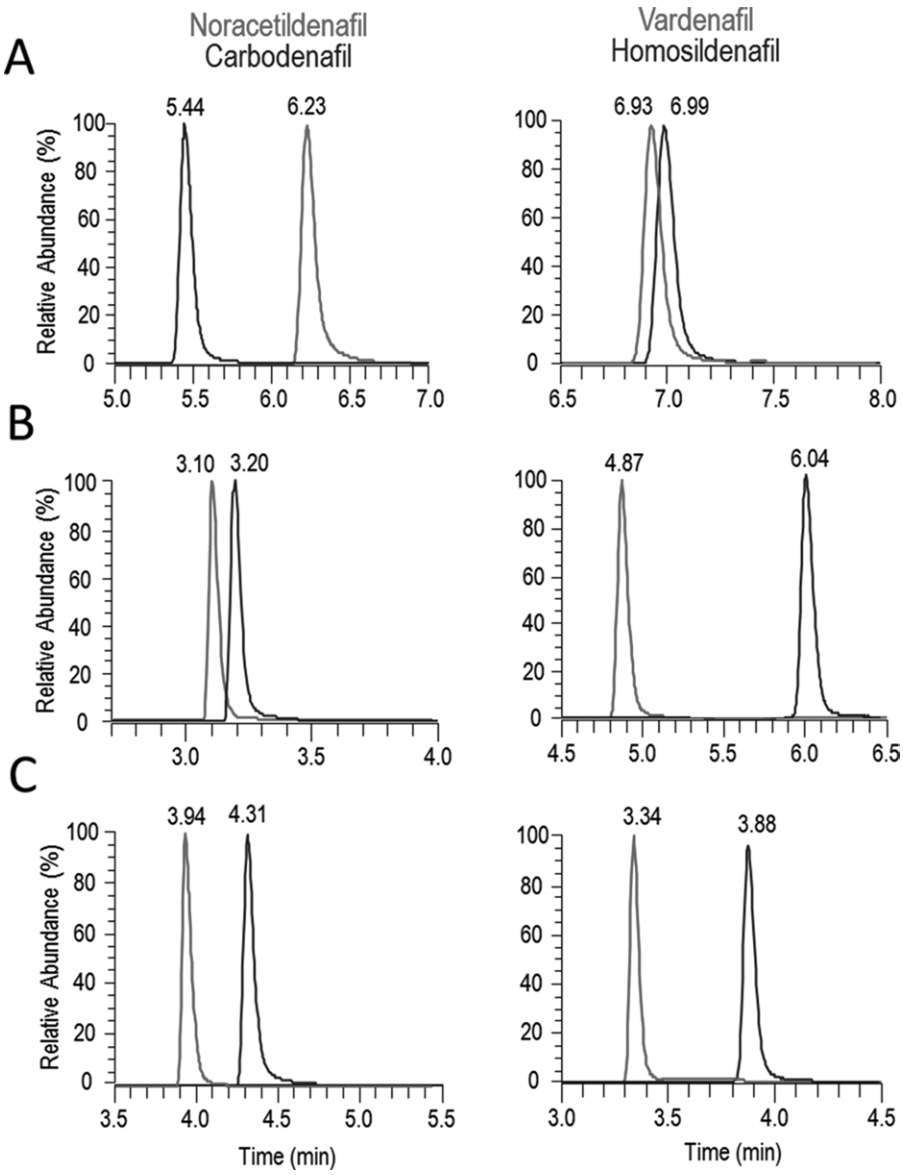


Figure 1. Impact of the mobile phase composition on peak shape and chromatographic resolution between isobaric analytes. (A) Mobile phase A/B: 0.1% FA in H_2O/0.1% FA in MeOH. (B) Mobile phase A/B: 5 mM ammonium formate in H_2O/5 mM ammonium formate in ACN. (C) Mobile phase A/B: 10 mM ammonium formate and 0.1% FA in H_2O/10 mM ammonium formate and 0.1% FA in ACN:MeOH (1:1, v/v).

search of these *m/z* values in AIF records can be used to detect nontargeted, novel PDE5 inhibitor adulterants based on their structural similarity to known PDE5 inhibitors.

Recovery and Repeatability

Results of recovery experiments conducted in triplicate at 50 mg/kg in a capsule sample in M5 are presented in Table 4. The test level of 50 mg/kg was selected for this evaluation to demonstrate the method performance at the target LOQ of AOAC SMPR 2014.011 for the determination of PDE5 inhibitors (6). The mean recoveries ranged from 69 to 90%. Only one target compound (thiohomosildenafil) was slightly below the recovery range of 70–120% provided in AOAC SMPR 2014.011. This method showed excellent repeatability with RSD_r values of 0.4–1.8%, well below the repeatability criteria of ≤20% in AOAC SMPR 2014.011.

POI

Detection and identification results are summarized in Table 5. In total, 1575 data points were evaluated to demonstrate POI and also probability of detection (POD) for detection/screening of PDE5 inhibitors. Correct detection/identification results compliant with identification requirements provided in the European Commission Decision 2002/657/EC (9) were obtained for all evaluated analytes at all concentration levels and in all matrixes. The method validation results fulfilled the

Table 4. Analyte recoveries and RSD_r obtained for the target compound panel in matrix M5 (capsule) at a spiking level of 50 mg/kg (n = 3)

Analyte	Mean recovery, %	RSD_r, %
Acetaminotadalafil	90	0.7
Acetildenafil	78	0.7
Avanafil	85	0.7
Homosildenafil	87	1.5
Hydroxyacetildenafil	79	1.8
Hydroxyhomosildenafil	88	1.1
Hydroxythiohomosildenafil	71	1.8
Lodenafil carbonate	83	1.6
Mirodenafil	85	0.8
Propoxyphenyl homohydroxysildenafil	85	0.7
Sildenafil	86	1.3
Tadalafil	90	0.4
Thiohomosildenafil	69	1.7
Udenafil	89	1.2
Vardenafil	83	2.2

POI requirements listed in AOAC SMPR 2014.010 and POD requirements (for the pooled data) listed in AOAC SMPR 2014.012.

Depending on the analyte and matrix type, 3–7 isotopic ions and 8–10 fragment ions in the raw data were typically matched with the information in the TraceFinder compound database. Excellent mass accuracy was obtained for pseudomolecular, isotopic, and fragment ions over a period of nearly 3 days of measurements with typical mass errors <1 ppm. Such stability of mass measurement was achieved with single-mass axis calibration of the instrument performed prior to starting the data acquisition. An example of chromatogram and product ion mass spectra obtained for sildenafil and tadalafil at 100 mg/kg (low test concentration) in matrix M1 (botanical powder) and their comparison with product ion spectra of reference standards are provided in Figure 2.

Figure 3 shows examples of chromatographic and mass spectral data obtained in matrix M1 at 100 mg/kg for two nontarget PDE5 inhibitors (*N*-octyl sildenafil and aminosildenafil) that were not included in the inclusion list or compound database. This demonstrates the method's ability to collect data for both targeted and nontargeted PDE5 inhibitors in a single chromatographic run. Detection and identification of nontarget PDE5 inhibitors and novel analogs are performed through the retrospective evaluation of MS and MS/MS experimental data. Common PDE5 inhibitor MS fragments can be used to detect compounds with structures similar to known PDE5 inhibitors and provide at least class identification in the cases of novel PDE5 inhibitor analogs, for which reference standards are not available.

Time-to-Result

Time-to-result for the analysis of one sample and detection/identification of PDE5 inhibitors included in the compound database were less than 1 h.

Conclusions

The SLV data demonstrate the acceptable performance of the presented method for screening and identification of both target and nontarget PDE5 inhibitors in dietary ingredients and supplements, meeting the requirements provided in AOAC SMPR **2014.010** and **2014.012**. The obtained recovery and repeatability results indicate that the method can be also used for quantification of PDE5 inhibitors. As discussed previously, only a reserved quantification evaluation was performed due to the limited availability and high cost of the reference standards required to spike samples at the high ppm levels.

Acknowledgments

The financial support through the Leeds City Region Enterprise Partnership Grant (BGP151COV) is gratefully acknowledged. This study was conducted in collaboration with Thermo Fisher Scientific. In particular, the authors thank the following people from Thermo Fisher Scientific in the United States and Europe: Charles Yang, Michael Hauer, Daniel Quinn, Frans Schoutsen, Michal Godula, Richard Fussell, and Dipankar Ghosh.

Table 5. Summary of the SLV results

Parameter	Test design	Target test concn, ppm	Correct detection results, %[a]	Correct identification results, %
POI at low concn	525 pooled data points, including evaluation of all target panel compounds in 7 matrix types with 5 replicates per matrix (35 samples and 15 analytes)	100	100	100
POI at high concn	525 pooled data points, including evaluation of all target panel compounds in 7 matrix types with 5 replicates per matrix (35 samples and 15 analytes)	1000	100	100
POI at 0 concn	525 pooled data points, including evaluation of all target panel compounds in 7 matrix types with 5 replicates per matrix (35 samples and 15 analytes)	0	100	100

[a] POD applies (AOAC SMPR 2014.012).

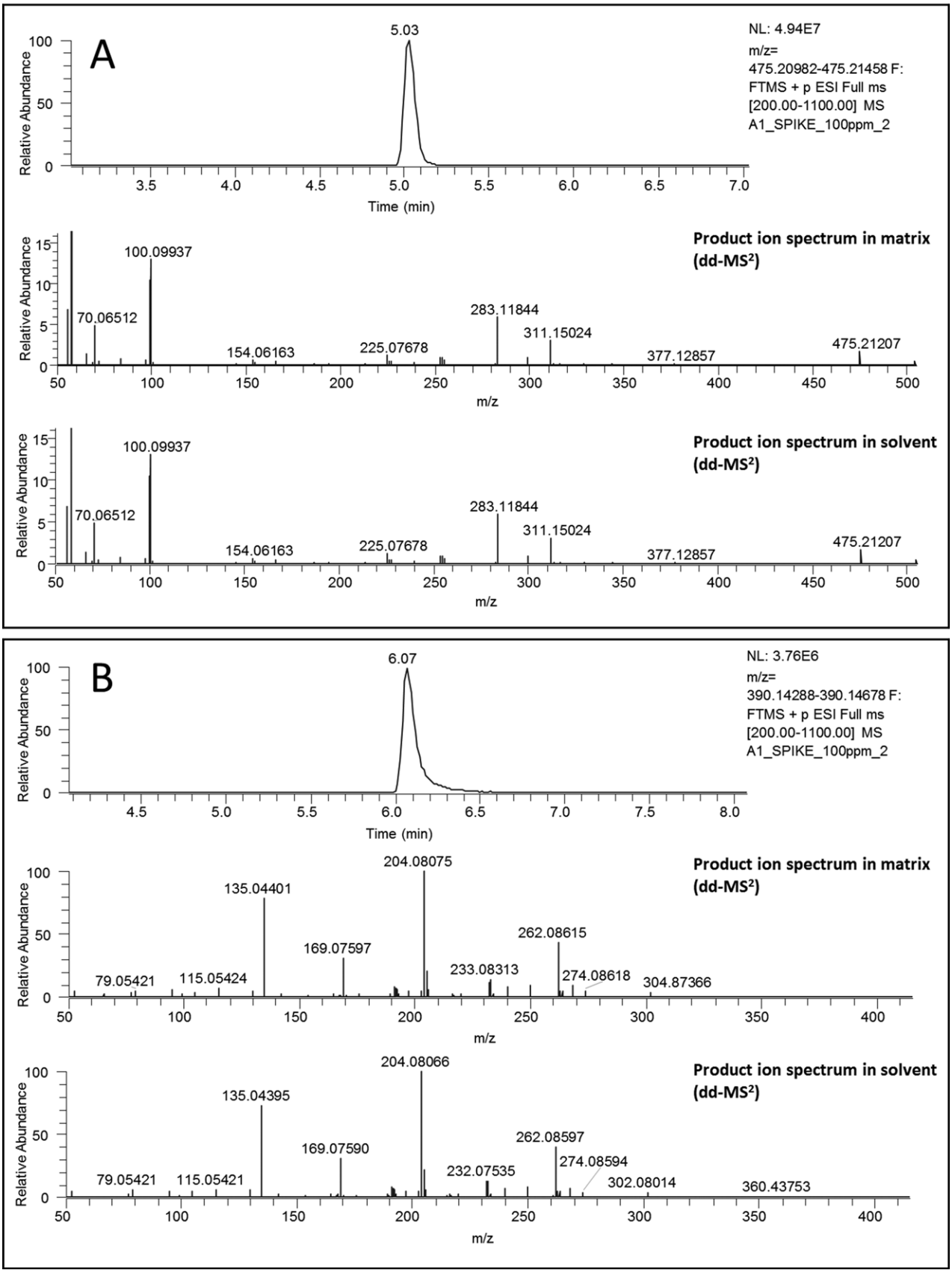


Figure 2. Chromatograms and mass spectra at 100 mg/kg (low test concentration) in matrix M1 (powder). (A) Sildenafil and (B) tadalafil.

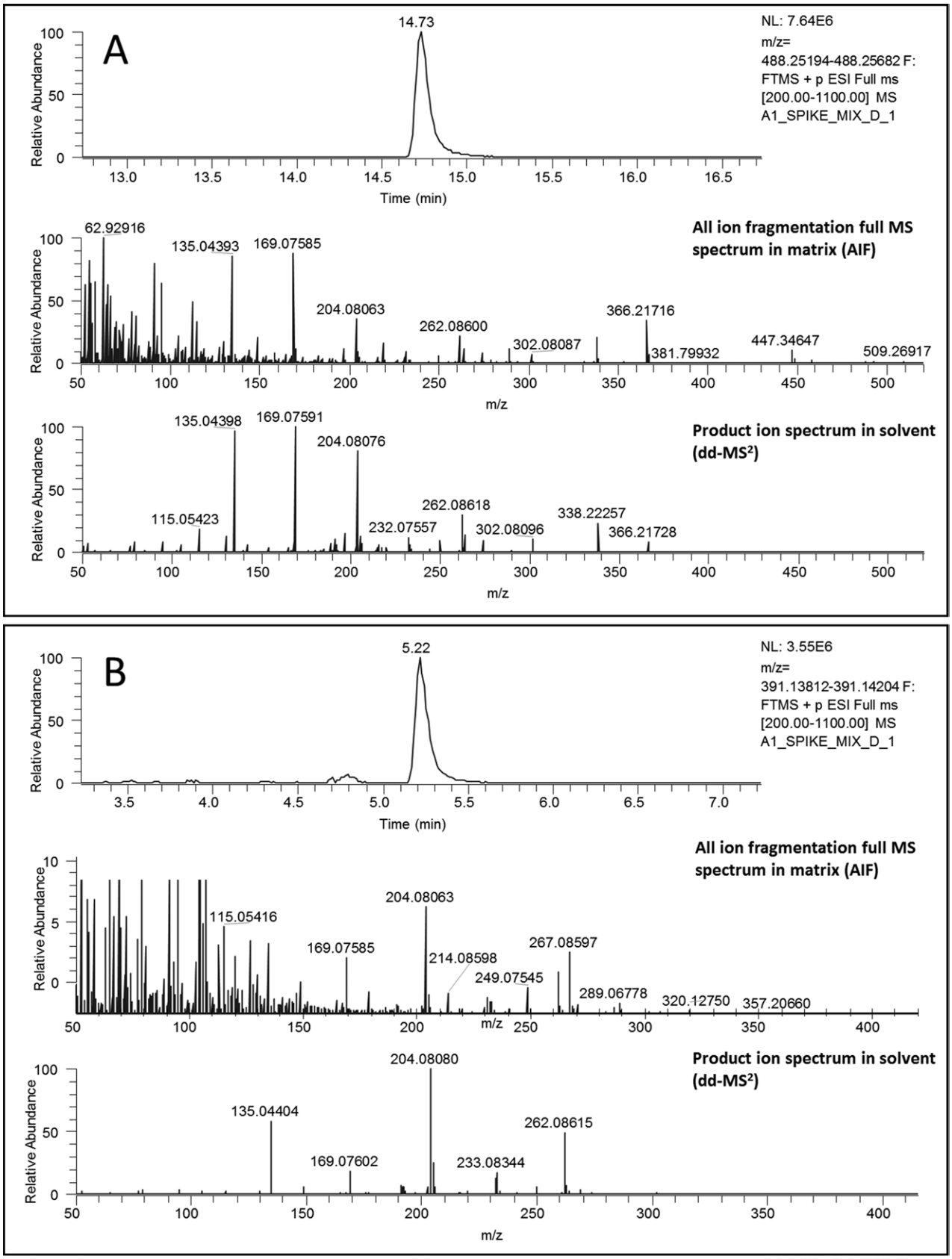


Figure 3. Examples of chromatograms and mass spectra obtained for selected nontarget analytes (not included in the inclusion list or compound database) at 100 mg/kg. (A) *N*-Octyl sildenafil and (B) aminotadalafil.

References

(1) Cole, M.R. & Fetrow, C.W. (2003) *Am. J. Health-System Pharm.* **60**, 1576–1580

(2) Vaclavik, L., Krynitsky, A.J., & Rader, J.I. (2014) *Anal. Bioanal. Chem.* **406**, 6767–6790

(3) Venhuis, B.J. & de Kaste, D. (2012) *J. Pharm. Biomed. Anal.* **69**, 196–208

(4) Patel, D.N., Li, L., Kee, C.L., Ge, X., Low, M.Y., & Koh, H.L. (2014) *J. Pharm. Biomed. Anal.* **87**, 176–190

(5) *Standard Method Performance Requirements* 2014.010 2014.011 (2015) *J. AOAC Int.* **98**, 1060.doi: 10.5740/jaoac.int.SMPR2014.010

(6) *Standard Method Performance Requirements* 2014.011 (2015) *J. AOAC Int.* **98**, 1064.doi: 10.5740/jaoac.int.SMPR2014.011

(7) *Standard Method Performance Requirements* 2014.012 (2015) *J. AOAC Int.* **98**, 1068.doi: 10.5740/jaoac.int.SMPR2014.012

(8) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Appendix B: Laboratory Safety

(9) Commission of the European Communities (2002) European Commission Decision 2002/657/EC of August 12, 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, *Off. J. Eur. Communities*. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:221:0008:0036:EN:PDF

DIETARY SUPPLEMENTS

Determination of Withanolides in *Withania somnifera* by Liquid Chromatography: Single-Laboratory Validation, First Action 2015.17

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An LC method was developed and validated in 2007 for analyzing *Withania somnifera* raw material (root) and dried extracts for withanolide content, including withanoside IV, withanoside V, withaferin A, 12-deoxywithastromonolide, withanolide A, and withanolide B. The method involved the extraction of the analytes with methanol, their subsequent filtration, and then analysis on a C18 column with an acetonitrile gradient and UV detection. Single-laboratory validation yielded linearity generally in the range of 20 to 200 µg/mL for each analyte, with a repeatability precision of RSD < 3% in most cases, and recovery in the range of 90 to 105%. These results compare well with the performance criteria recently detailed in AOAC *Standard Method Performance Requirement* 2015.007. The method was shown to be rugged with respect to different analysts, equipment, and days of analysis, and the sample solution was shown to be stable for 24 h at room temperature after extraction. The method was reviewed by the AOAC Expert Review Panel on Dietary Supplements (Set 2 Ingredients) and approved for First Action *Official Method*[SM] status.

Withania somnifera, commonly known as ashwagandha or Indian ginseng, is a berry-producing plant from the nightshade family used in Ayurvedic medicine to treat a wide variety of ailments and diseases. Various parts of the plant, including leaves, roots, and berries, are harvested for use as herbal remedies. The plant produces a variety of steroidal lactones called withanolides (1), which have been shown to have biological activity (2). Plant extracts are used in dietary supplements to reduce stress, improve memory, and reduce immunosuppression in patients undergoing chemotherapy.

In 2015, the AOAC Stakeholder Panel on Dietary Supplements published *Standard Method Performance Requirement* (SMPR®) 2015.007 (3), outlining the requirements for quantitative methods for withanolide glycosides and aglycones of ashwagandha (*W. somnifera*). These methods must target the following analytes: withanoside IV, physagulin D, 27-hydroxywithanone, withanoside V, withanoside VI, withaferin A, withastromonolide, withanolide A, withanone, and withanolide B. The relevant dietary supplement matrixes include tablets, capsules, liquids, powders, extracts, and plant products. SMPR 2015.007 includes single-laboratory and collaborative validation requirements for analytical range, LOQ, recovery, and repeatability and reproducibility of total glycosides and aglycones as shown in Tables 1 and 2.

Natural Remedies Private Ltd developed and validated in a single laboratory an LC method for six withanolides, including withanoside IV (W-IV), withanoside V (W-V), withaferin A (WF-A), 12-deoxywithastramonolide (12-D), withanolide A (W-A), and withanolide B (W-B) in 2007. The method is intended for the determination of withanolide content in *W. somnifera* raw material (roots) and extracts used as ingredients for dietary supplements. The method uses hot methanol to extract withanolides, filtration to clean up the sample, and C18 RPLC with an acetonitrile gradient for analysis. The single-laboratory validation (SLV) data from 2007 were submitted to the AOAC Expert Review Panel (ERP) on Dietary Supplements (Set 2 Ingredients) for consideration. The ERP approved the method for First Action *Official Method*[SM] status on December 10, 2015. This paper presents the SLV study.

Single-Laboratory Validation

Natural Remedies Private Ltd validated the LC method prior to the development of AOAC *Standard Method Performance Requirement* (SMPR®) 2015.007, entitled *Standard Method Performance Requirements* for Withanolide Glycosides and Aglycones of Ashwagandha (*W. somnifera*) (3), and the validation guidelines for dietary supplements (4).

Natural Remedies Private Ltd supplied the reference standards for the six target withanolides. Purities were all >90%. In addition, Natural Remedies Private Ltd supplied two raw materials and six dried ashwagandha extract materials as described in Table 3.

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This method was approved by the Expert Review Panel for Dietary Supplements as First Action.
The Expert Review Panel for Dietary Supplements invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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DOI: 10.5740/jaoacint.16-0202

Table 1. Performance requirements for analytical range and LOQ

Parameter	Total glycosides	Aglycones
Analytical range, ppm	10–250000	10–20000
LOQ, ppm	10	

Table 2. Performance requirements for recovery, repeatability, and reproducibility

	Range, ppm			
Parameter	10–100	>100–1000	>1000–10000	>10000
Recovery, %	80–110	90–107	95–105	97–103
Repeatability, %	≤7	≤6	≤4	≤1
Reproducibility, %	≤10	≤9	≤6	≤2

Table 3. Sample materials used in the validation study

Sample	Standardized to	Batch No.
Raw material	Total withanolides >0.2% by HPLC	RD/1162
Raw material	Total withanolides >0.2% by HPLC	ERH-046
Water extract	Total withanolides >0.15% by HPLC	WS/05Lot20
Hydroalcoholic extract	Total withanolides >1.0% by HPLC	WS/05Lot21
Methanolic extract	Total withanolides >1.5% by HPLC	RD/1045
Methanolic extract	Total withanolides >2.5% by HPLC	WS/06Lot08
Methanolic extract	Total withanolides >2.5% by HPLC	WS/06Lot10
Methanolic extract	Total withanolides >2.5% by HPLC	RD/1170

AOAC Official Method 2015.17
Withanolides (Withanoside IV, Withanoside V, Withaferin A, 12-Deoxywithastromonolide, Withanolide A, and Withanolide B) in *Withania somnifera*
LC
First Action 2015

[Applicable to the determination of withanoside IV, withanoside V, withaferin A, 12-deoxywithastromonolide, withanolide A, and withanolide B content in raw material (roots) and dried extracts.]

Caution: This method uses common-use solvents and reagents. Refer to an adequate manual or Material Safety Data Sheets (MSDSs) to ensure that safety guidelines are applied before using chemicals. Store solvents in a flammable-liquid storage cabinet. The solvents and reagents used herein are harmful if inhaled, swallowed, or absorbed through the skin. Use appropriate personal protective equipment, such as a laboratory coat, safety glasses, rubber gloves, and a fume hood. Dispose of all materials according to federal, state, and local regulations.

A. Principle

Withanolides are compounds specific to *Withania somnifera*. This method comprises extraction of withanolides from the sample matrix using methanol and separation of the compounds using gradient LC on a C18 column with UV detection at 227 nm.

B. Apparatus

(a) *LC system*.—Shimadzu HPLC system equipped with an LC10A pump with an SPD-M 10Avp photodiode array (PDA) or UV detector in combination with CLASS-VP software (Shimadzu) or an LC-2010A and LC-2010HT integrated system (Shimadzu) equipped with a quaternary gradient and autoinjector in combination with laboratory solution software; or any other suitable HPLC system with a similar configuration.

(b) *Column*.—Phenomenex Luna C18(2), 250×4.6 mm, with 5 µm particle size (Part No. 00G-4252- E0; Phenomenex, Torrance, CA; http://www.phenomenex.com); or equivalent.

(c) *Analytical balance*.—Accuracy to 0.1 mg.

(d) *Filtration apparatus*.—0.45 µm nylon filter.

(e) *Ultrasonic bath*.

(f) *Syringe filter*.—0.45 µm polyethersulfone filter.

C. Reagents

(a) *Degassed mobile phase*.—(*1*) *Solvent A*.—Dissolve 0.136 g anhydrous potassium dihydrogen orthophosphate (KH_2PO_4) in 900 mL HPLC grade water (Milli-Q Water purification system; Millipore) and add 0.5 mL orthophosphoric acid. Dilute to 1000 mL volume with water, filter through 0.45 µm membrane, and degas in a sonicator for 3 min (solvent A).

(*2*) *Solvent B*.—Acetonitrile.

(b) *Diluent*.—Methanol.

(c) *Individual withanolide standards*.—Natural Remedies Private Ltd (Bangalore, India; www.naturalremedy.com), or other suppliers.—(*1*) *Withanoside IV*.—CAS No. 362472-81-9.

(*2*) *Withanoside V*.—CAS No. 256520-90-8.

(*3*) *Withaferin A*.—CAS No. 5119-48-2.

(*4*) *12-Deoxywithastromonolide*.—CAS No. 60124-17-6.

(*5*) *Withanolide A*.—CAS No. 32911-62-9.

(*6*) *Withanolide B*.—CAS No. 56973-41-2.

D. Preparation of Mixed Standards

Accurately weigh 10 mg each of withanoside IV, withanoside V, withaferin A, 12-deoxywithastromonolide, withanolide A, and withanolide B reference standards in a 50 mL volumetric flask, dissolve in 10 mL methanol with the aid of gentle heating, cool, and dilute to a 50 mL volume with methanol to yield 200 µg/mL of each standard. Suitably prepare three additional concentrations of withanolides to obtain concentrations of 150, 100, and 50 µg/mL.

E. Preparation of Test Solutions

(a) *Raw material*.—Accurately weigh a sample quantity of *W. somnifera* raw material equivalent to 5 mg (~2.5 g is sufficient) of withanoside IV, withanoside V, withaferin A, 12-deoxywithastromonolide, withanolide A, and withanolide B in a 250 mL beaker. Extract raw material with 100 mL methanol on a boiling water bath for 10–15 min, and repeat this procedure three to four times until the raw material has been completely extracted or the extracts become colorless. Combine all extracts and evaporate the methanol on a water bath or by using a vacuum

rotary evaporator until the volume is less than 40 mL. Cool the solution, transfer quantitatively to a 50 mL volumetric flask and bring to 50 mL with methanol. Filter through a 0.45 micron membrane filter.

(b) *Standardized (common) extract*.—Accurately weigh a sample quantity of *W. somnifera* extract equivalent to 5 mg (~0.5 g is sufficient) of withanoside IV, withanoside V, withaferin A, 12-deoxywithastromonolide, withanolide A, and withanolide B in a 250 mL beaker. Extract the standardized extract with 100 mL methanol, boil in a water bath for 10–15 min, and repeat this procedure three to four times until the raw material has been completely extracted or the extracts become colorless. Combine all extracts and evaporate the methanol on a water bath or by using a vacuum rotary evaporator until the volume is less than 40 mL. Cool the solution, transfer quantitatively to a 50 mL volumetric flask and bring to 50 mL with methanol. Filter through a 0.45 micron membrane filter.

F. Analysis

(a) *Chromatographic conditions*.—(*1*) *Column*.—Phenomenex Luna C18(2), 250 × 4.6 mm, 5 µm particle size (Part No. 00G-4252- E0).

(*2*) *Temperature*.—Maintained constant between 20 and 30°C (preferably 27°C).

(*3*) *Detector*.—SPD-M 10Avp PDA or UV detector.

(*4*) *Wavelength*.—227 nm.

(*5*) *Flow rate*.—1.5 mL/min.

(*6*) *Run time*.—45 min.

(*7*) *Injection volume*.—20 µL.

(*8*) *Peak integration*.—Base-to-base.

(*9*) *Gradient*.—*See* Table **2015.17**.

(b) *Procedure*.—(*1*) Inject 20 µL mixed standard preparations in triplicate at three different concentrations: 50, 100, and 150 µg/mL.

(*2*) Inject 20 µL of each test solution in duplicate.

(c) *System suitability*.—Verify that the following system suitability requirements are met with each run. If the system suitability requirements are not met, adjust the composition of the mobile phase or use a new LC column to meet system suitability before analyzing samples.—(*1*) *Repeatability*.—The RSD of the peak areas from the triplicate injections of the 50 µg/mL mixed standard preparation must be ≤2.0% for each withanolide.

(*2*) *Retention times*.—The relative retention times of the standards should be 0.70 for withanoside IV, 0.89 for withanoside V, 0.92 for withaferin A, 0.96 for 12-deoxywithastramonolide, 1.0 for withanolide A, and 1.15 for withanolide B.

Table 2015.17. Gradient

Time, min	Solvent A %	Solvent B %
0.01	95.0	5.0
18.0	55.0	45.0
25.0	20.0	80.0
28.0	20.0	80.0
35.0	55.0	45.0
40.0	95.0	5.0
45.0	95.0	5.0

(*3*) *Resolution*.—Calculate the resolution between withanoside V and withaferin A peaks in the 50 µg/mL mixed standard preparation as follows:

$$R = 2 \times \frac{T2 - T1}{W1 + W2}$$

where T1 and T2 are the retention times of withanoside V and withaferin A, respectively; and W1 and W2 are their peak widths measured at the baseline between tangents drawn to the sides of the peak. The resolution between withanoside V and withaferin A should be ≥3.0.

(*4*) *Tailing*.—Calculate the tailing factor (F) for each withanolide in the 50 µg/mL mixed standard preparation as follows:

$$F = \frac{L + R}{2L}$$

where L is the width (measured at 5% maximum peak height) from the front slope of the peak to the center line and R is the width (measured at 5% maximum peak height) from the center line to the back slope of the peak. The tailing factor must be ≤1.5 for all individual withanolides.

(*5*) *Coefficient of determination*.—Plot peak area versus concentration for the mixed standard preparations in the range of 50 to 150 µg/mL. The r^2 for the regression line of peak area versus concentration for each withanolide must be ≥0.998.

(d) *Calculation of withanolide content*.—(*1*) Calculate the percentage of withanoside IV, withanoside V, withaferin A, 12-deoxywithastramonolide, withanolide A, and withanolide B content from the mean peak areas of the duplicate test solution injections and the triplicate mixed standard preparation injections producing the most similar peak areas using the formula:

$$\text{Individual withanolide } (\%\,w/w) = \frac{\text{Mean peak area of sample}}{\text{Mean peak area of standard}} \times \frac{\text{Weight of standard (mg)}}{\text{Final standard preparation volume (mL)}} \times \frac{\text{Final test solution volume (mL)}}{\text{Sample weight (mg)}} \times \text{Purity of standard (\%)}$$

(*2*) Alternatively, calculate the percentage of each withanolide from the mean peak areas of the duplicate test solution injections and the plots of peak area versus concentration from Section **F(c)(*5*)** using the formula:

$$\text{Individual withanolide } (\%\,w/w) = \frac{\text{Mean peak area of sample} - b}{m} \times \frac{\text{Final test solution volume (mL)}}{\text{Sample weight (mg)}} \times \text{Purity of standard (\%)}$$

where m is the slope of the plot and b is the y-intercept.

Specificity

Specificity was assessed by spectral similarity, by determining the resolution between each peak, and by checking peak purity using a PDA detector. Reference standard solutions of withanoside IV, withanoside V, withaferin A, 12-deoxywithastramonolide,

withanolide A, and withanolide B were prepared in methanol at the concentrations shown in Table 4.

Dietary ingredient samples, including raw material (plant root, Batch No. ERH-046, 4091 mg) and a standardized dried methanolic extract (Batch No. WS/06Lot10, 750 or 1520 mg) were extracted with hot methanol according to the method. Each reference standard, a mixed standard solution, and the sample solutions were analyzed according to the method on two different LC systems—a Shimadzu LC 2010A with a UV-Vis detector and a Shimadzu LC 10A with a PDA detector—on different days.

Linearity

The reference stock standard solution was prepared in methanol at ~1.2–1.5 mg/mL after correction for purity. Six 2-fold serial dilutions of the stock standard solution were then prepared in methanol. Five replicates of the stock and each dilution were analyzed.

Precision

Peak areas and retention times—The data from the stock standard and serial dilutions for the linearity study were analyzed for repeatability based on the peak areas and retention times for each analyte. A standardized dried methanolic extract of *W. somnifera* (Batch No. WS/06Lot10, >2.5% total withanolides by the candidate LC method) was tested at three levels—754, 1503, and 2096 mg—each extracted and analyzed in duplicate according to the method.

Precision

Content—Standards were prepared at ~0.2–0.4 mg/mL in methanol and analyzed in triplicate. Eight materials (1552 mg WS/06Lot08, 1536 mg WS/06Lot10, 5048 mg WS/05Lot20, 1527 mg WS/05Lot21, 1503 mg RD/1170, 1653 mg RD/1045, 4336 mg RD/1162, and 4229 mg ERH-46) were extracted and analyzed in triplicate. Withanolide content was calculated for each analyte.

Recovery

Four materials (2613 mg WS/05Lot21, 1520 mg WS/06Lot10, 1633 mg RD/1170, and 4004 mg RD/1162) were spiked with pure standards (W-IV, WF-A, 12-D, W-A, and W-B) using the third serial dilution of the stock standard solution from the linearity study. A sample material containing 36% W-V was used for the W-V spike. The spiked materials were extracted and analyzed for total (endogenous + spike) withanolide content according to the candidate method.

In a separate experiment, four materials (1714 mg WS/06Lot08, 5030 mg WS/05Lot20, 1640 mg RD/1045, and 4091 mg ERH-46) were extracted according to the candidate method. The extracts were spiked with pure standards using the third serial dilution of the stock standard solution from the linearity study. The spiked extracts were analyzed for total (endogenous + spike) withanolide content according to the candidate method.

Table 4. Reference standard concentrations

	Concn, µg/mL	
Reference standard	UV detector	PDA detector
Withanoside IV	330.30	310.0
Withanoside V	300.15	360.0
Withaferin A	368.78	210.0
12-Deoxywithastramonolide	355.30	300.0
Withanolide A	345.51	240.0
Withanolide B	337.59	350.0

Ruggedness

The ruggedness evaluation was conducted as an evaluation of intermediate precision. All eight materials were tested under two sets of conditions, including different analysts on different days using different LC equipment, column temperatures, and concentrations of H_3PO_4 in the LC mobile phase (Table 5). Each material was extracted, and the extracts analyzed under each of the two conditions without replication.

Stability of Sample Solution

Material WS/05Lot21 (2613 mg) was extracted and filtered according to the method and placed into an autoinjector vial. The LC system and autosampler were in a room maintained at 25±2°C. The extracted sample and linearity standards were analyzed immediately and after 24 h. The difference in results between 0 and 24 h was determined.

System Suitability

In addition to the standards and samples injected with every run, a QC-check sample was developed for use during validation. Batch No. WS/06Lot10 was sampled (~500 g), homogenized, and stored in an airtight container for use throughout the validation study. An initial test portion was extracted according to the method and tested in duplicate by two analysts on different days on multiple days by multiple analysts to establish a "true" value. If the RSD of the obtained results was <2.5%, then the mean value of the obtained results was accepted as the true value. The QC-check sample was then included with every run during the validation study.

Table 5. Conditions for ruggedness evaluation

Parameter	Condition 1	Condition 2
Analyst	A	B
Day	1	2
HPLC	Shimadzu LC-2010A	Shimadzu LC-10A
Detector	UV-Vis	PDA
C18 column	Merck LiChrospher 100[a]	Phenomenex Luna
	RP-18e (5 µm) Hibar RT	5 µm C18(2), 100 Å
	250×4.6 mm	250×4.6 mm
Column temperature, °C	25	30
H_3PO_4 concn	0.5 mL in 1000 mL	1.5 mL in 1000 mL

[a] EMD Millipore, Billerica, MA.

Results and Discussion

Specificity

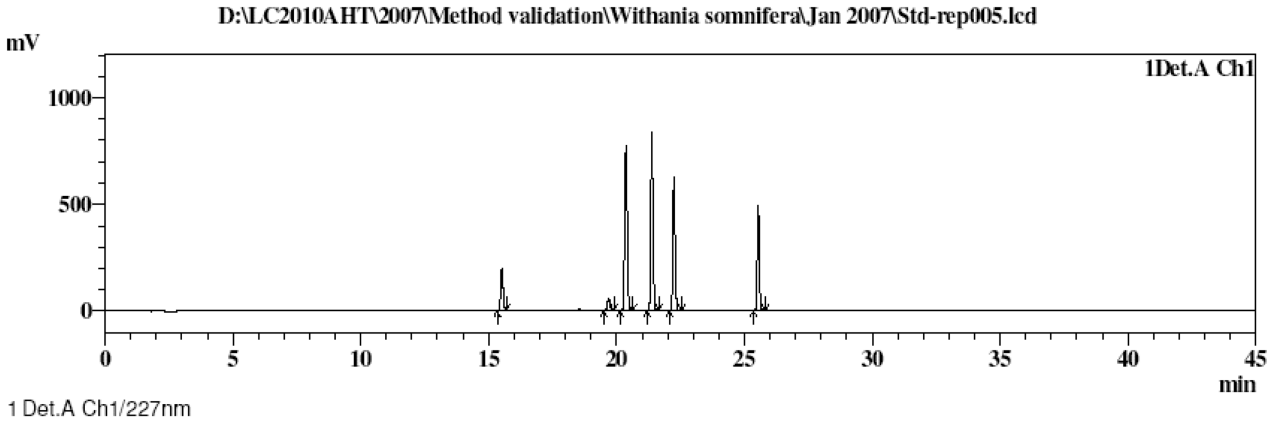
A representative chromatogram of the mixed standard with UV detection is shown in Figure 1. The peaks are well-resolved and the retention times are very similar to injections of the individual standards (data not shown). Figures 2 and 3 show chromatograms of a methanolic extract sample and a raw material sample using a UV detector and a PDA detector. Although more peaks are observed in the samples compared with the mixed standard, the six withanolide peaks are still well-resolved and the retention times and relative retention times of the six withanolide peaks in the samples are very similar to those in the mixed standard (Table 6). Furthermore, the PDA spectra of the withanolide peaks in the samples are identical to the spectra of the corresponding standards, and the peak purities based on PDA spectral analysis are >0.99 in the standards and samples (data not shown). These results indicate no matrix interference in the LC method. The relative retention times of the analyte peaks were 0.70 for withanoside IV, 0.89 for withanoside V, 0.92 for withaferin A, 0.96 for 12-deoxywithastramonolide, 1.0 for withanolide A, and 1.15 for withanolide B. These relative retention times agree with the values from the *United States Pharmacopeia* LC method as reported in SMPR 2015.007 (1).

Linearity

The mean peak areas of the linearity standards were plotted against concentration and are presented in Figures 4–9. Table 7 summarizes the data. After performing linear regression, linearity was assessed by determining goodness-of-fit (square of the correlation coefficient, r^2), by examining residuals over the concentration range, and by determining the response factor (peak area/concentration) at each concentration examined. Over the full range of concentrations examined, r^2 varied from 0.76 for withanolide B to 0.99 for withanoside IV and V. A plot of the residuals by concentration revealed a convex pattern with significant residuals as high as 78%. The pattern indicates a lack of linearity and suggests that alternative regression analyses (e.g., weighted regression or polynomial regression) should be evaluated for a better fit to the data over the large concentration range. Alternatively, the concentration range can be narrowed to allow for a better approximation of linearity. Using the latter approach, the data for each analyte were analyzed over a narrower concentration range until linearity was more closely approximated. As can be seen in the nonrandom residual plots of Figures 4–9, even over a narrower concentration range, the data are not strictly linear. The residuals, however, are much smaller and tolerable at ≤6.25%. In the linear regression plots, all r^2 are >0.999 and the *y*-intercept has been greatly reduced in most cases. Over the narrower concentration ranges, the response factors, defined as mean peak area divided by concentration, vary by <5% from the mean response factor. The ranges for each analyte that approximate linearity are as follows: withanoside IV 20–330 µg/mL, withanoside V 19–300 µg/mL, withaferin A 23–184 µg/mL, 12-deoxywithastramonolide 22–178 µg/mL, withanolide A 22–173 µg/mL, and withanolide B 21–169 µg/mL. For routine analyses, the method includes three concentrations of standards within the linear range.

Precision

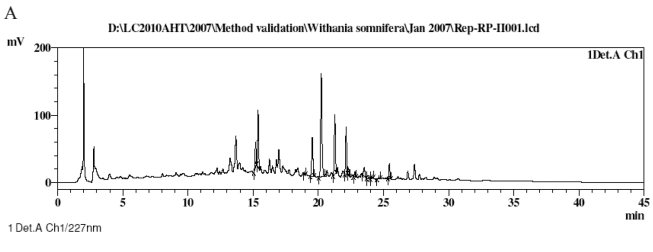
Peak areas and retention times.—Repeatability precision of standards in methanol based on the measurement of the peak area is presented in Table 7 and retention time in Table 8. The RSDs from five replicate injections at each concentration were all <1%. When a sample, WS/06Lot10, was analyzed



Detector A Ch1 227nm

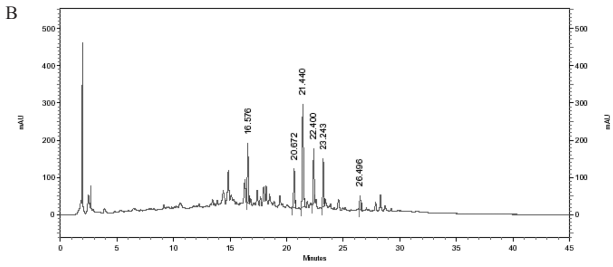
Peak #	Ret. Time	Name	Area	Area %	Resolution
1	15.516	Withanoside IV	1234072	6.645	0.000
2	19.692	Withanoside V	391438	2.108	22.163
3	20.366	Withaferin A	5318748	28.640	3.454
4	21.379	12- Deoxy withastramonolide	5001196	26.930	5.576
5	22.242	Withanollide A	3786871	20.391	5.030
6	25.539	Withanolide B	2839005	15.287	19.518
Total			18571330	100.000	

Figure 1. Representative chromatogram of mixed standard solution.



Detector A Ch1 227nm

Peak #	Ret. Time	Name	Area	Area %	Resolution
1	15.187	RT15.187	225004	6.435	0.000
2	15.376	Withanoside	478462	13.685	1.135
3	18.909	RT18.909	3921	0.112	23.432
4	19.537	Withanoside V	400277	11.448	3.776
5	20.226	Withaferin A	1097206	31.381	3.516
6	21.263	Withastramonolide	548663	15.692	5.550
7	22.128	Withanolide A	398098	11.386	5.034
8	22.311	RT22.311	43911	1.256	1.219
9	22.804	RT22.804	36992	1.058	3.406
10	23.524	RT23.524	100800	2.883	3.854
11	23.891	RT23.891	15874	0.454	1.951
12	24.112	RT24.112	17872	0.511	1.423
13	24.625	RT24.625	6589	0.188	3.252
14	25.447	Withanolide B	122702	3.509	5.045
Total			3496369	100.000	



Detector A-227 nm

Retention Time	Area	Area Percent	Resolution	Asymmetry	Theoretical plates	Capacity factor	Width	USP Width	Height
16.576	998797	17.55	0.00	1.04	137409.30	-0.04	0.24	0.18	159064
20.672	745691	13.11	21.84	1.02	177685.13	0.20	0.30	0.20	104250
21.440	2024836	35.59	3.87	1.13	182807.92	0.24	0.34	0.20	275305
22.400	940711	16.53	5.02	1.04	243337.77	0.30	0.25	0.18	146645
23.243	757964	13.32	4.78	1.02	296866.34	0.35	0.22	0.17	127311
26.496	221905	3.90	19.32	1.19	406992.84	0.54	0.22	0.17	37559
Totals	5689904	100.00							850134

Figure 2. Chromatograms of methanolic extract (WS/06Lot10) sample using (A) UV detection and (B) PDA detection.

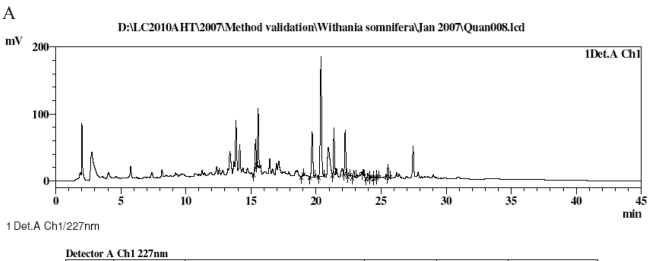
in duplicate at three extraction weights, the repeatability was <2.2% for peak area and <1% for retention time (Table 9).

Content.—Repeatability precision based on the determination of content (% w/w) of each analyte was evaluated in two experiments. In the first experiment, each of the eight study samples was extracted and the resulting sample solution analyzed in triplicate. The results are shown in Table 10. Seven of eight samples demonstrated a repeatability of <3% for each of the six analytes, as well as the sum of withanolides, meeting the acceptance criteria of SMPR 2015.007. In two cases, WS/06Lot08 and RD/1170, the sum of withanolides was >1% w/w and the repeatability slightly exceeded the acceptance criterion of ≤1%. The eighth sample, WS/05Lot20, yielded RSD_r values as high as 10.8% for withanolide B and <7% for the remaining five analytes and the sum of analytes. This sample is the only water extract sample tested, and the withanolide content is the lowest of all samples tested, including the raw materials. Compared with the SMPR requirements, the repeatability values of withanolide B and 12-deoxywithastramonolide are greater than the allowed variability, but the four other analytes were within the acceptance criteria.

In the second experiment, the full method including methanol extraction was replicated three to four times at five sample weights, and each sample extract was analyzed once (Table 11). Not surprisingly, the RSD_r (%) of the full method, including all sample weights, was somewhat higher than the repeatability of replicate injections of a single extract in the first experiment. RSD_r values varied from 0.83 to 2.01% across all analytes and the sum of the analytes, meeting the acceptance criteria of $RSD_r \leq 1\%$ at >1% w/w, ≤4% at >0.1–1% w/w, and ≤6% at >0.01–0.1% w/w.

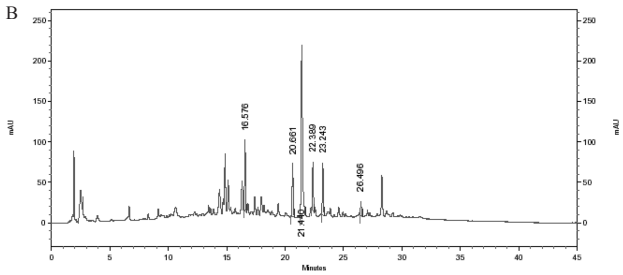
Recovery

Recovery of total withanolide content after spiking is shown in Table 12. Materials spiked prior to extraction (WS/05Lot21, WS/06Lot10, RD/1170, and RD/1162) demonstrated recoveries ranging from 90.5 to 104.7%. With total content of each



Detector A Ch1 227nm

Peak #	Ret. Time	Name	Area	Area %	Resolution
1	15.352	RT15.352	265649	7.501	0.000
2	15.547	Withanoside-IV	491157	13.869	1.158
3	18.980	RT18.980	8341	0.236	21.729
4	19.698	Withanoside V	473414	13.368	4.128
5	20.372	Withaferin A	1250895	35.323	3.455
6	21.377	Withastramonolide	410796	11.600	5.561
7	22.239	Withanolide A	384409	10.855	5.143
8	22.491	RT22.491	1440	0.041	1.589
9	22.903	RT22.903	54369	1.535	2.662
10	23.619	RT23.619	36688	1.036	4.654
11	23.987	RT23.987	38306	1.082	2.373
12	24.208	RT24.208	14767	0.417	1.282
13	24.567	RT24.567	4931	0.139	2.088
14	24.709	RT24.709	4074	0.115	0.994
15	25.523	Withanolide B	102080	2.883	5.790
Total			3541316	100.000	



Detector A-227 nm

Retention Time	Area	Area Percent	Resolution	Asymmetry	Theoretical plates	Capacity factor	Width	USP Width	Height
16.576	537308	15.13	0.00	1.08	145898.22	-0.04	0.24	0.17	87496
20.661	453713	12.78	22.24	1.01	181706.16	0.20	0.29	0.19	65400
21.440	1723297	48.54	3.82	1.39	161359.55	0.24	0.48	0.21	211277
22.389	357061	10.06	5.00	1.02	291271.69	0.30	0.24	0.17	59849
23.243	380726	10.72	5.14	1.04	314259.59	0.35	0.26	0.17	64237
26.496	98366	2.77	20.08	1.23	449097.84	0.54	0.21	0.16	17761
Totals	3550471	100.00							506020

Figure 3. Chromatograms of raw material (ERH-046) sample using (A) UV detection and (B) PDA detection.

Table 6. Retention times of analytes in standards and samples

		UV detector		PDA detector	
Substance	Compound	Retention time, min	Relative retention time	Retention time, min	Relative retention time
Mixed standard	W-IV	15.516	0.698	16.373	0.705
	W-V	19.692	0.885	20.565	0.886
	WF-A	20.366	0.916	21.376	0.921
	12-D	21.379	0.961	22.368	0.964
	W-A	22.242	1.0	23.211	1.0
	W-B	25.539	1.148	26.475	1.141
Methanolic extract WS/06Lot10	W-IV	15.376	0.695	16.576	0.713
	W-V	19.537	0.883	20.672	0.889
	WF-A	20.226	0.914	21.440	0.922
	12-D	21.263	0.961	22.400	0.964
	W-A	22.128	1.0	23.243	1.0
	W-B	25.447	1.149	26.496	1.140
Raw material ERH-046	W-IV	15.547	0.699	16.576	0.713
	W-V	19.698	0.886	20.661	0.889
	WF-A	20.372	0.916	21.440	0.922
	12-D	21.377	0.961	22.389	0.963
	W-A	22.239	1.0	23.243	1.0
	W-B	25.523	1.148	26.496	1.140

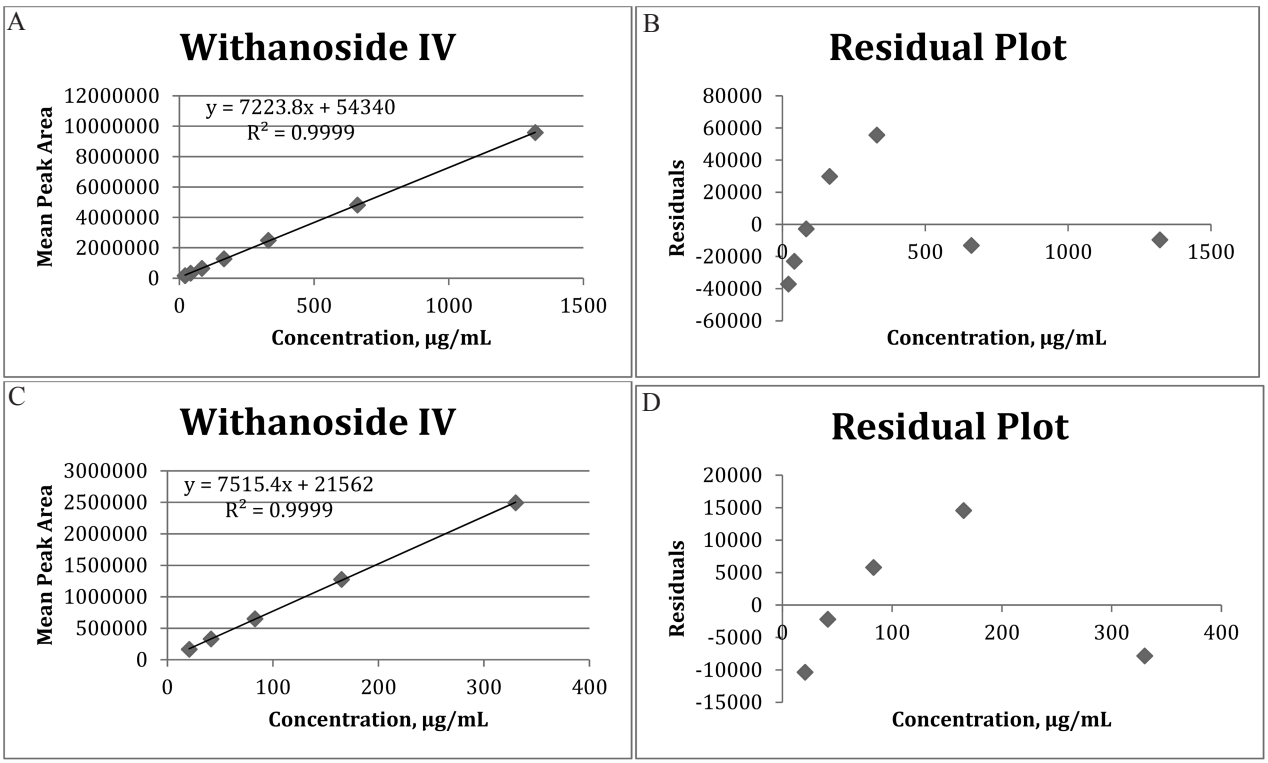


Figure 4. Linearity of response for withanoside IV over the full concentration range by (A) linear regression and (B) residual analysis and over a truncated concentration range by (C) linear regression and (D) residual analysis.

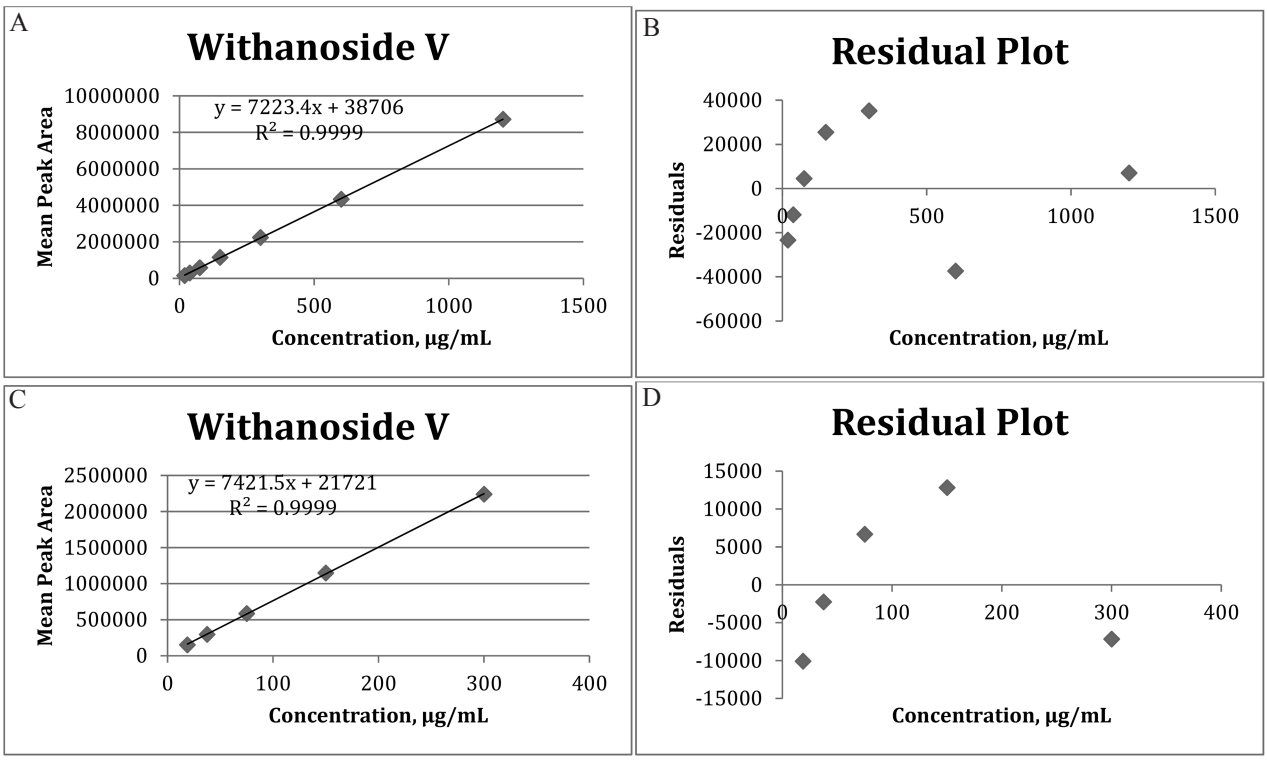


Figure 5. Linearity of response for withanoside V over the full concentration range by (A) linear regression and (B) residual analysis and over a truncated concentration range by (C) linear regression and (D) residual analysis.

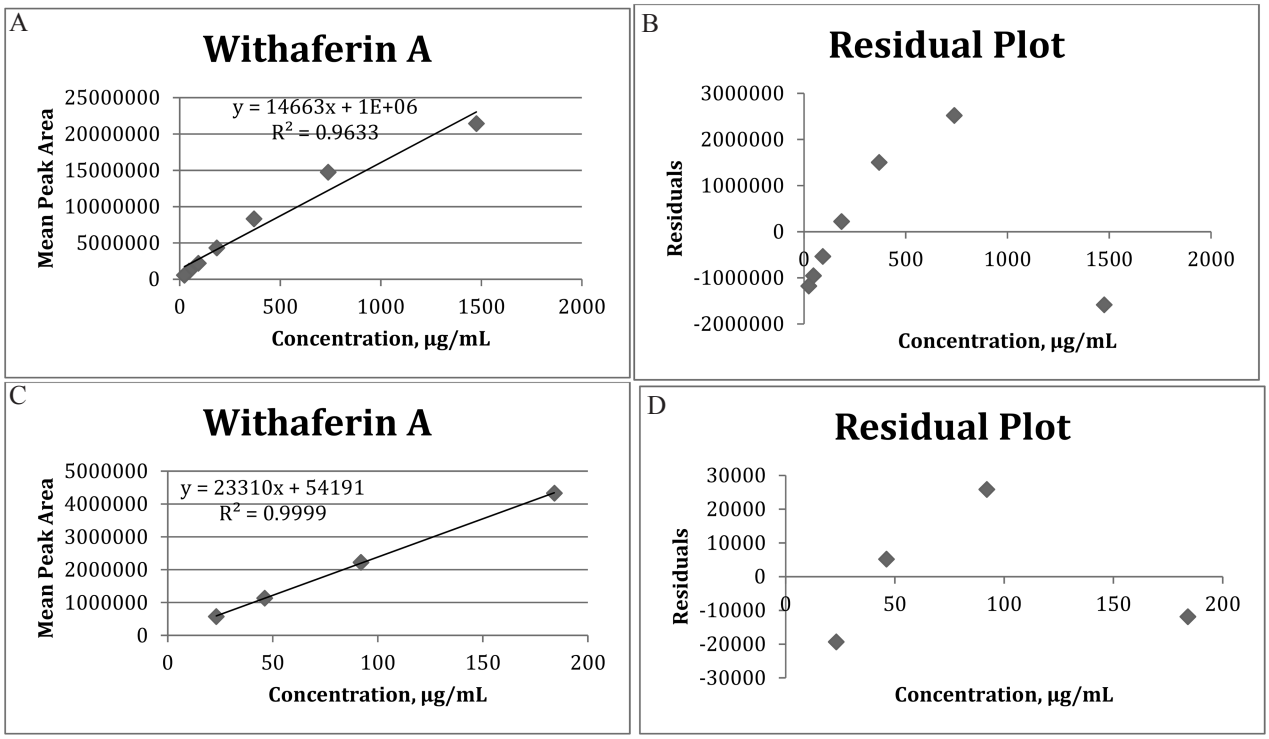


Figure 6. Linearity of response for withaferin A over the full concentration range by (A) linear regression and (B) residual analysis and over a truncated concentration range by (C) linear regression and (D) residual analysis.

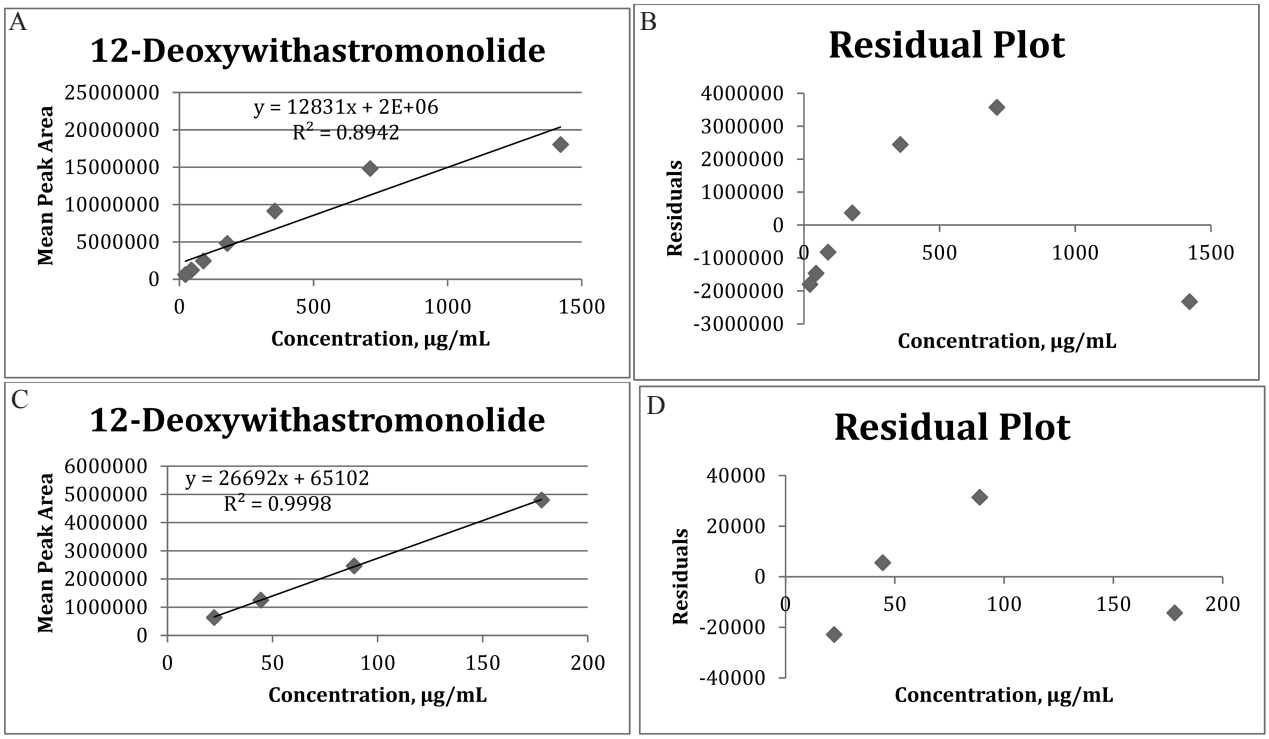


Figure 7. Linearity of response for 12-deoxywithastramonolide over the full concentration range by (A) linear regression and (B) residual analysis and over a truncated concentration range by (C) linear regression and (D) residual analysis.

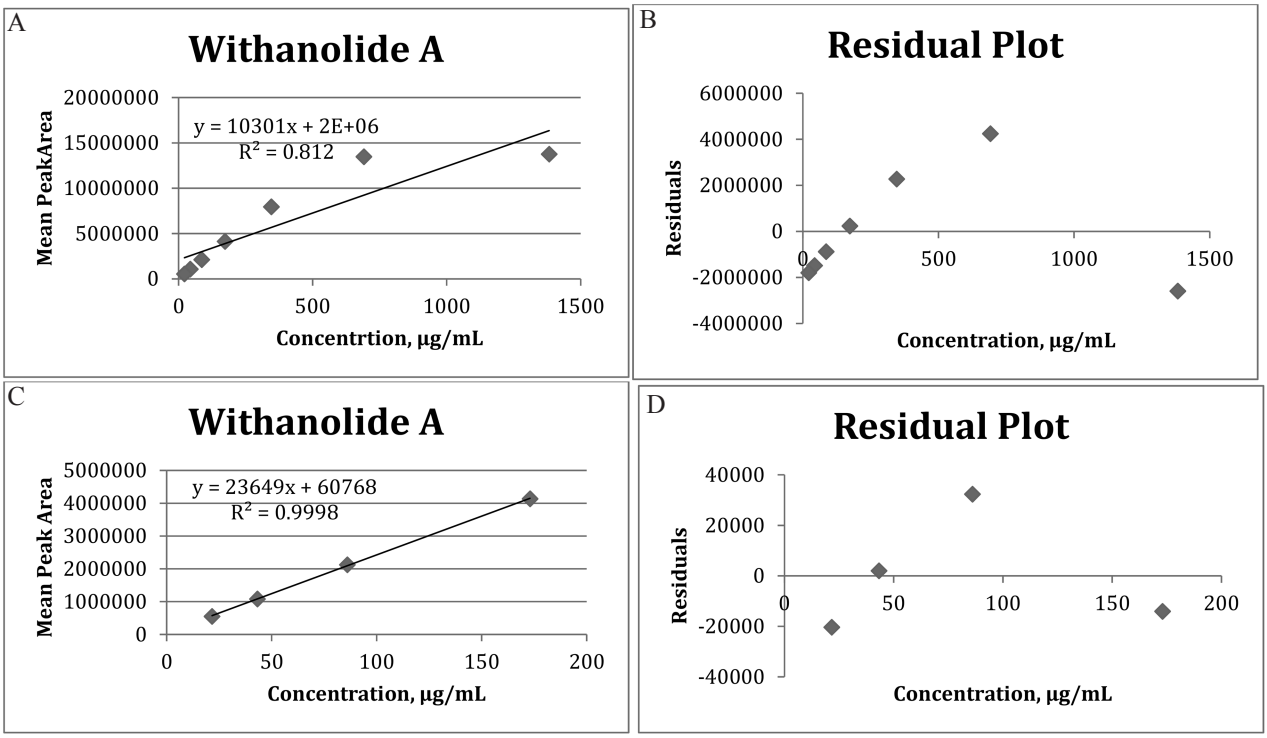


Figure 8. Linearity of response for withanolide A over the full concentration range by (A) linear regression and (B) residual analysis and over a truncated concentration range by (C) linear regression and (D) residual analysis.

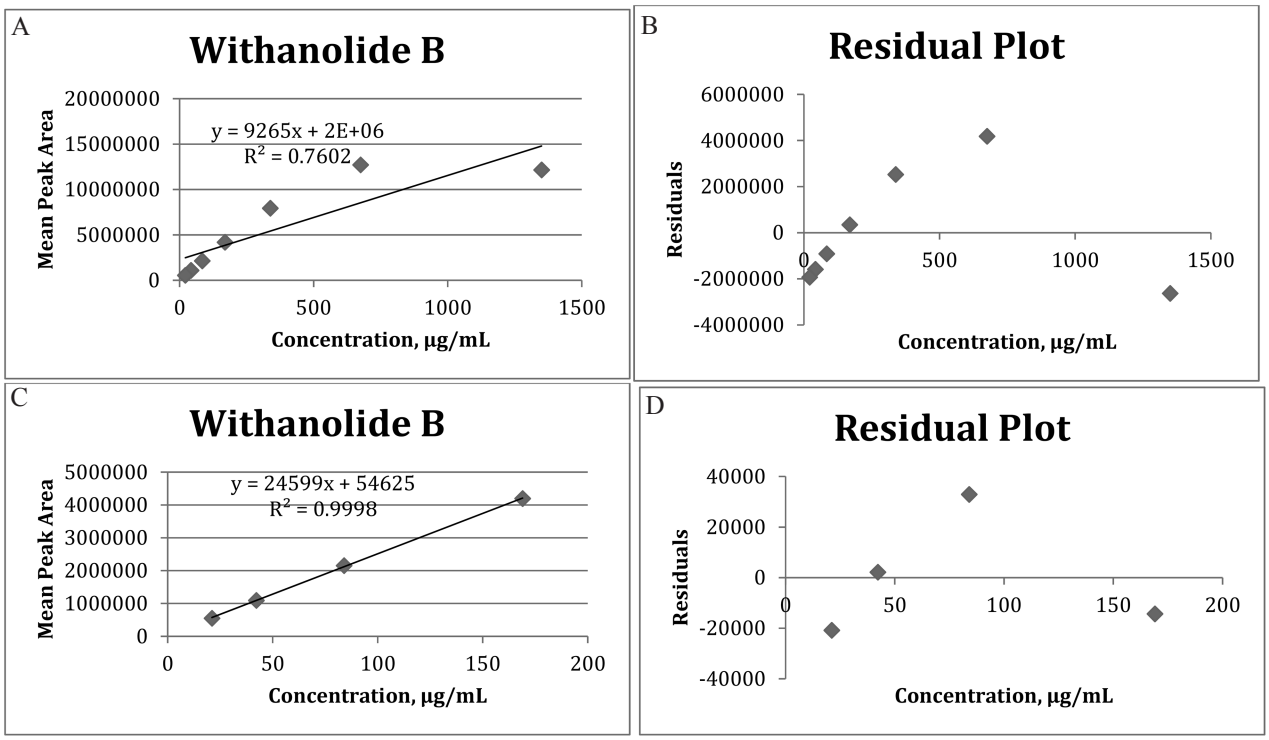


Figure 9. Linearity of response for withanolide B over the full concentration range by (A) linear regression and (B) residual analysis and over a truncated concentration range by (C) linear regression and (D) residual analysis.

withanolide in each material varying from 0.15 to 1.10% w/w (1500–11 000 ppm), the acceptable range of recovery is 95–105% (Table 2). Five of the six withanolide analytes were <95% recovery in at least one of the four materials, but none of the withanolide analytes was <95% recovery in all four materials.

Recovery of total withanolide content from materials spiked after extraction (WS/06Lot08, WS/05Lot20, RD/1045, and ERH-46) varied from 91.0 to 99.6% for W-IV, W-V, WF-A, 12-D, and W-A. These results are very similar to the recoveries when the materials are spiked prior to extraction, demonstrating that the extraction process is efficient. The results for W-B were unexpectedly low in this experiment, at 70.7–72.2% recovery. A review of the experimental details did not uncover a likely reason for this low recovery, however it is noted that these values are not typical for this analyte. If the extraction of W-B were inefficient, one would expect lower recoveries for the materials spiked before extraction compared with those spiked after. The observed lower recoveries of W-B when materials were spiked after extraction suggests an error in the amount of W-B added, but this could not be confirmed.

Ruggedness

Table 13 shows the results of ruggedness testing. The intermediate precision on the sum of withanolides varied from 0.91 to 6.17%. Because the experiment was not performed as a Youden-type ruggedness trial, the primary source(s) of error cannot be determined, though the sum of withanolides is generally higher under condition 2 compared with condition 1. The differences are small enough, however, that the method is considered rugged based on intermediate precision.

Stability of Sample Solution

Stability data are presented in Table 14. There was no significant decrease in withanolide content for any of the analytes or the sum of withanolides in the sample extract over a 24 h period at 25±2°C.

System Suitability

System suitability parameters were determined throughout method development and tested during the validation study. The following suitability parameters define the optimal performance of the method: (*1*) Injection of mixed standard preparations in triplicate with every run; (*2*) peak resolution between withanoside V and withaferin A is >3; (*3*) the tailing factor for each analyte is <1.5; (*4*) the relative retention times compared with withanolide A are ~0.70 for withanoside IV, ~0.89 for withanoside V, ~0.92 for withaferin A, ~0.96 for 12-deoxywithastromonolide, and ~1.15 for withanolide B; (*5*)–the repeatability precision is <2.0% RSD for standards and samples; (*6*) the retention time window is within 3σ of the mean retention time of the standard or QC-check sample; and (*7*) the QC-check sample result is within 10% of the label value. It is

Table 7. Results from linearity of standards

					Full range		Partial range		
Compound	Concn, µg/mL	*N*	Mean peak area	RSD_r, %	r^2	Residual, %	r^2	Response factor, peak area/concn	Residual, %
Withanoside IV	1321	5	9587357	0.07	0.9999	−0.10	—	—	—
	661	5	4816148	0.07		−0.27	—	—	—
	330	5	2493821	0.10		2.28	0.9999	7550	−0.31
	165	5	1276177	0.33		2.40		7727	1.16
	83	5	651132	0.13		−0.43		7885	0.90
	41.3	5	329761	0.11		−6.50		7987	−0.66
	20.6	5	166024	0.01		−18.28		8042	−5.87
Withanoside V	1201	5	8721109	0.07	0.9999	0.08	—	—	—
	600	5	4335433	0.16		−0.85	—	—	—
	300	5	2241004	0.21		1.60	0.9999	7466	−0.32
	150	5	1147767	0.42		2.28		7648	1.13
	75	5	585013	0.33		0.78		7796	1.15
	37.5	5	297773	0.11		−3.82		7937	−0.75
	18.8	5	151167	0.08		−13.37		8058	−6.25
Withaferin A	1475	5	21454279	0.10	0.9633	−6.88	—	—	—
	738	5	14752690	0.34		20.61	—	—	—
	369	5	8326374	0.09		22.06	—	—	—
	184	5	4331341	0.28		5.42	0.9999	23490	−0.27
	92	5	2224593	0.35		−19.39		24130	1.18
	46.1	5	1134003	0.12		−45.66		24600	0.46
	23.1	5	573348	0.07		−67.23		24876	−3.26
12-Deoxy withastramonolide	1421	5	18055804	0.20	0.8942	−11.40	—	—	—
	711	5	14844246	0.17		31.74	—	—	—
	355	5	9146389	0.08		36.51	—	—	—
	178	5	4802093	0.29		8.42	0.9998	27031	−0.30
	88.8	5	2466826	0.41		−24.90		27772	1.29
	44.4	5	1255825	0.08		−53.75		28276	0.45
	22.2	5	634878	0.06		−73.88		28590	−3.47
Withanolide A	1382	5	13768071	0.60	0.8120	−15.84	—	—	—
	691	5	13486375	0.11		45.93	—	—	—
	346	5	7961571	0.09		39.99	—	—	—
	173	5	4138065	0.29		5.96	0.9998	23953	−0.34
	86	5	2127011	0.39		−29.31		24625	1.55
	43.2	5	1084444	0.08		−57.77		25109	0.19
	21.6	5	551256	0.13		−76.50		25528	−3.56
Withanolide B	1350	5	12163116	0.66	0.7602	−17.79	—	—	—
	675	5	12726145	0.05		48.99	—	—	—
	338	5	7949510	0.12		46.69	—	—	—
	169	5	4197521	0.32		8.93	0.9998	24868	−0.34
	84	5	2153855	0.36		−29.75		25520	1.55
	42.2	5	1094892	0.09		−59.13		25946	0.20
	21.1	5	552858	0.08		−77.74		26203	−3.63

— = data not included in partial range analysis

Table 8. Precision of retention time of standards

			Retention time	
Compound	Concn, µg/mL	*N*	Mean, min	RSD_r, %
W-IV	1321	5	15.51	0.02
	661	5	15.56	0.62
	330	5	15.53	0.54
	165	5	15.52	0.12
	83	5	15.52	0.05
	41.3	5	15.50	0.08
	20.6	5	15.47	0.05
W-V	1201	5	19.70	0.04
	600	5	19.73	0.55
	300	5	19.72	0.46
	150	5	19.70	0.07
	75	5	19.72	0.40
	37.5	5	19.66	0.03
	18.8	5	19.65	0.06
WF-A	1475	5	20.35	0.04
	738	5	20.39	0.49
	369	5	20.38	0.41
	184	5	20.36	0.06
	92	5	20.36	0.06
	46.1	5	20.34	0.03
	23.1	5	20.32	0.05
12-D	1421	5	21.37	0.03
	711	5	21.40	0.38
	355	5	21.39	0.32
	178	5	21.38	0.05
	88.8	5	21.37	0.05
	44.4	5	21.36	0.02
	22.2	5	21.35	0.04
W-A	1382	5	22.22	0.02
	691	5	22.26	0.34
	346	5	22.25	0.29
	173	5	22.24	0.05
	86	5	22.24	0.05
	43.2	5	22.22	0.02
	21.6	5	22.22	0.04
W-B	1350	5	25.52	0.02
	675	5	25.54	0.21
	338	5	25.54	0.19
	169	5	25.53	0.03
	84	5	25.53	0.04
	42.2	5	25.52	0.03
	21.1	5	25.51	0.01

Table 9. Precision of peak areas and retention times for sample WS/06Lot10

Compound	Weight of sample extracted, mg	*N*	Peak area		Retention time	
			Mean, XXX	RSD_r, %	Mean, min	RSD_r, %
W-IV	2096	2	1327044	0.70	15.43	0.33
	1503	2	949877	0.01	15.39	0.02
	754	2	478702	0.20	15.45	0.72
W-V	2096	2	1096086	0.77	19.60	0.25
	1503	2	788419	0.88	19.54	0.02
	754	2	398268	1.22	19.61	0.55
WF-A	2096	2	3021467	0.71	20.28	0.21
	1503	2	2163270	0.69	20.23	0.01
	754	2	1091661	0.04	20.29	0.48
12-D	2096	2	1512283	0.05	21.31	0.15
	1503	2	1081855	0.84	21.26	0.00
	754	2	543382	0.45	21.32	0.39
W-A	2096	2	1110624	1.20	22.17	0.14
	1503	2	790581	1.17	22.13	0.01
	754	2	387811	2.12	22.18	0.34
W-B	2096	2	337372	0.54	25.49	0.06
	1503	2	240698	0.88	25.45	0.00
	754	2	121812	1.27	25.49	0.23

recommended that the following system suitability checks be performed with each run: (*1*) the repeatability (RSD) of peak areas from triplicate injections of mixed standard preparation at 50 µg/mL; (*2*) the relative retention times of standards; (*3*) the resolution between withanoside V and withaferin A peaks; (*4*) the tailing factor for each analyte; and (*5*) the r^2 from the plot of peak area versus concentration for each standard analyte (must be ≥0.998). Note that the QC-check sample is not commercially available, however if desired, laboratories can develop their own QC-check sample following the procedure described above. If the system suitability requirements are not met, the composition of the mobile phase can be adjusted or a new LC column used to meet system suitability before analyzing samples.

Table 10. Repeatability precision of withanolide content in samples with replicate injections of the sample solution

Sample	Weight extracted, mg	*N*	Parameter	W-IV	W-V	WF-A	12-D	W-A	W-B	Sum
WS/06Lot08	1552	3	Mean, % w/w	0.92	0.65	0.66	0.28	0.23	0.07	2.80
			RSD_r, %	0.82	1.63	0.92	0.93	0.92	0.87	1.04
WS/06Lot10	1536	3	Mean, % w/w	0.91	0.62	0.62	0.27	0.21	0.06	2.68
			RSD_r, %	0.17	0.97	0.09	0.22	0.28	0.93	0.32
WS/05Lot20	5048	3	Mean, % w/w	0.07	0.04	0.08	0.02	0.02	0.01	0.24
			RSD_r, %	4.48	4.55	5.01	6.84	4.76	10.83	4.62
WS/05Lot21	1527	3	Mean, % w/w	0.48	0.31	0.41	0.12	0.10	0.03	1.45
			RSD_r, %	0.21	0.75	0.14	0.46	0.00	0.00	0.22
RD/1170	1503	3	Mean, % w/w	0.85	0.59	0.70	0.33	0.23	0.08	2.78
			RSD_r, %	1.36	1.65	1.00	1.22	1.51	0.76	1.30
RD/1045	1653	3	Mean, % w/w	0.61	0.36	0.40	0.25	0.13	0.04	1.79
			RSDr, %	0.67	0.73	1.08	0.93	0.88	1.36	0.79
RD/1162	4336	3	Mean, % w/w	0.15	0.09	0.10	0.02	0.02	0.00	0.39
			RSD_r, %	1.01	0.64	1.00	2.79	2.34	0.00	0.92
ERH-46	4229	3	Mean, % w/w	0.18	0.15	0.15	0.04	0.04	0.01	0.57
			RSD_r, %	1.13	1.03	0.79	1.40	1.40	0.00	1.07

Table 11. Repeatability precision of withanolide content in sample WS/06Lot10 with replicate extractions of the sample at five sample weights

			Withanolide content, % w/w						
Level	Trial	Amount extracted, mg	W-IV	W-V	WF-A	12-D	W-A	W-B	Sum
A	1	751	0.802	0.681	0.605	0.263	0.209	0.064	2.625
	2	754	0.821	0.697	0.617	0.268	0.212	0.066	2.680
	3	747	0.828	0.696	0.626	0.272	0.221	0.066	2.709
	4	755	0.821	0.684	0.615	0.265	0.218	0.064	2.667
B	1	1032	0.808	0.684	0.609	0.265	0.211	0.065	2.642
	2	936	0.827	0.703	0.621	0.270	0.215	0.065	2.701
	3	1073	0.818	0.684	0.612	0.265	0.217	0.064	2.661
	4	1011	0.826	0.687	0.618	0.268	0.220	0.065	2.684
C	1	1660	0.813	0.685	0.608	0.265	0.214	0.064	2.649
	2	1628	0.818	0.691	0.618	0.269	0.216	0.065	2.678
	3	1503	0.818	0.690	0.616	0.268	0.221	0.065	2.677
	4	1497	0.821	0.684	0.612	0.266	0.219	0.064	2.666
D	1	1959	0.806	0.686	0.607	0.265	0.210	0.065	2.638
	2	2159	0.811	0.685	0.612	0.267	0.212	0.065	2.651
	3	2100	0.822	0.686	0.616	0.266	0.223	0.065	2.677
	4	2096	0.815	0.680	0.611	0.267	0.219	0.064	2.657
E	1	2762	0.800	0.677	0.601	0.264	0.211	0.064	2.617
	2	2449	0.817	0.679	0.61	0.267	0.218	0.065	2.656
	3	2509	0.818	0.679	0.612	0.268	0.221	0.065	2.663
Mean, % w/w			0.816	0.686	0.613	0.267	0.216	0.065	2.663
RSD_r, %			0.98	0.98	0.96	0.83	2.01	1.01	0.88

Conclusions

The LC method demonstrated acceptable specificity, precision, and recovery, meeting the recently established SLV criteria of SMPR 2015.007 within the linear range of the method. The AOAC ERP on Dietary Supplements ERP approved the method for First Action *Official Method* status on December 10, 2015. The ERP recommendations prior to moving to Final Action status include improving baseline separation of peak 1, optimizing the pH of the phosphate buffer, optimizing the column temperature, investigating the heat stability of the analytes during extraction, simplifying sample preparation, reducing the number of reference standards to two (one withanolide and one withanoside) with the use of relative

Table 12. Recovery of withanolides after spiking samples prior to or after sample extraction

		Recovery, %					
Batch No.	Spiking	Withanoside IV	Withanoside V	Withaferin A	12-Deoxy withastramonolide	Withanolide A	Withanolide B
WS/05Lot21	Prior to sample extraction	94.9	95.0	97.5	92.2	92.2	104.7
WS/06Lot10		93.5	95.4	94.5	91.6	96.5	97.3
RD/1170		93.6	93.6	95.1	95.0	94.1	95.8
RD/1162		97.7	94.4	97.6	90.5	93.2	96.5
WS/06Lot08	After sample extraction	95.2	94.6	94.6	95.5	92.1	71.1
WS/05Lot20		99.6	98.5	97.8	99.2	96.4	72.4
RD/1045		94.3	94.7	94.8	95.8	91.0	70.7
ERH-46		99.5	99.0	98.4	99.3	97.2	72.7

Table 13. Analysis of withanolide content under intermediate precision conditions

Sample No.	Condition	Content, % w/w							Mean of sum, % w/w	RSD of sum, %
		W-IV	W-V	WF-A	12-D	W-A	W-B	Sum		
WS/06Lot08	1	0.917	0.650	0.659	0.284	0.225	0.066	2.802	2.888	4.21
	2	1.071	0.629	0.608	0.304	0.290	0.071	2.974		
WS/06Lot10	1	0.911	0.621	0.616	0.267	0.205	0.062	2.681	2.749	3.50
	2	1.047	0.601	0.561	0.282	0.258	0.067	2.817		
WS/05Lot20	1	0.067	0.044	0.081	0.022	0.021	0.005	0.241	0.252	6.17
	2	0.080	0.044	0.083	0.024	0.027	0.001	0.263		
WS/05Lot21	1	0.478	0.310	0.410	0.125	0.102	0.029	1.453	1.495	3.97
	2	0.538	0.298	0.415	0.130	0.128	0.027	1.537		
RD/1170	1	0.848	0.594	0.700	0.332	0.229	0.076	2.779	2.900	5.88
	2	0.995	0.599	0.740	0.257	0.348	0.080	3.020		
RD/1045	1	0.606	0.364	0.402	0.248	0.131	0.042	1.793	1.850	4.32
	2	0.744	0.357	0.422	0.140	0.201	0.043	1.906		
RD/1162	1	0.152	0.090	0.100	0.021	0.025	0.004	0.391	0.389	0.91
	2	0.162	0.083	0.090	0.022	0.025	0.000	0.386		
ERH-46	1	0.184	0.148	0.145	0.041	0.041	0.010	0.570	0.586	3.74
	2	0.213	0.147	0.141	0.046	0.043	0.011	0.601		

Table 14. Stability of withanolide content in the sample solution over 24 h at room temperature

Time	Withanolide content, % w/w						
	W-IV	W-V	WF-A	12-D	W-A	W-B	Sum
0 h	0.399	0.319	0.413	0.123	0.107	0.030	1.390
24 h	0.409	0.350	0.415	0.122	0.110	0.030	1.435
Difference	0.010	0.031	0.002	−0.001	0.003	0.000	0.045
% Difference from $t=0$	2.51	9.72	0.48	−0.81	2.80	0.00	3.24

response factors, providing data on finished products, clarifying the discard volume in the filtration step, and determining the LOQ of the method.

References

(1) Mirjalili, M.H., Moyano, E., Bonfill, M., Cusido, R.M., & Palazón, J. (2009) *Molecules* **14**, 2373–2393. doi:10.3390/molecules14072373

(2) Ichikawa, H., Takada, Y., Shishodia, S., Jayaprakasam, B., Nair, M.G., & Aggarwal, B.B. (2006) *Mol. Cancer Ther.* **5**, 1434–1445. doi:10.1158/1535-7163.MCT-06-0096

(3) *Official Methods of Analysis* (2015) AOAC INTERNATIONAL, Rockville, MD, SMPR 2015.007

(4) *Official Methods of Analysis* (2012) AOAC INTERNATIONAL, Rockville, MD, Appendix K