CANNABIS

# Determination of Cannabinoids in *Cannabis sativa* Dried Flowers and Oils by LC-UV: Single-Laboratory Validation, First Action 2018.10

ELIZABETH M. MUDGE and PAULA N. BROWN[1]
British Columbia Institute of Technology, Natural Health and Food Products Research Group, 3700 Willingdon Ave, Burnaby, BC, Canada V5G 3H2

***Background:*** **Legalization of *Cannabis* across many U.S. states and in Canada had led to an urgent need for validated analytical methods for the quantitation of cannabinoids in *Cannabis sativa* L. flowers and finished products. The AOAC Stakeholder Panel on Strategic Food Analytical Methods *Cannabis* Expert Review Panel (ERP) approved an HPLC-diode-array detection (DAD) method for *First Action Official Methods*[SM] status.** ***Objective:*** **To present *Official Methods of Analysis*[SM] (OMA) 2018.10 method details, validation results, and additional method extension data as approved by the ERP and further requirements for *Final Action Official Methods*[SM] status.** ***Methods:*** **This previously published method used 80% aqueous methanol via sonication for extracting eight cannabinoids—tetrahydrocannabidiolic acid, tetrahydrocannabinol, cannabidiolic acid, cannabidiol, tetrahydrocannabivarin, cannabigerol, cannabinol, and cannabichromene—in dried flowers followed by reversed-phase chromatographic separation and UV detection.** ***Results:*** **The original method underwent extensive method optimization and a single-laboratory validation. Additional requirements requested by the *Standard Method Performance Requirement* (SMPR®) included a method extension, which was performed to collect repeatability data on two additional cannabinoids: cannabidivarinic acid and cannabigerolic acid. The methods performance was compared with the AOAC SMPR 2017.002 and 2017.001. $RSD_r$ ranged from 0.78 to 10.08% and recoveries from 90.7 to 99.2% in several different chemotypes.** ***Conclusions:*** **The ERP adopted the method and provided recommendations for achieving Final Action status.** ***Highlights:*** **After submission of additional validation data, an HPLC-DAD method for quantitation of cannabinoids in dried flowers and oils was accepted for *First Action Official Method* status (OMA 2018.10).**

Received June 26, 2019. Accepted by AH August 30, 2019.
This method was approved by the AOAC Expert Review Panel for *Cannabis* as First Action.

The Expert Review Panel 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 is critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.

[1]Corresponding author's e-mail: pbrown@bcit.ca

DOI: https://doi.org/10.5740/jaoacint.19-0197

In the summer of 2016, AOAC INTERNATIONAL announced partnership with industry to set voluntary consensus standards for *Cannabis*. An AOAC Stakeholder Panel on Strategic Food Analytical Methods working group began discussions in the fall of 2016 to develop *Standard Method Performance Requirements* (SMPRs®) for the quantitation of cannabinoids in dried flower and concentrates (SMPR 2017.002 and 2017.001, respectively). Through this process, several different cannabinoids were identified as requiring a valid quantitative analytical method for a consensus-based reference method.

With increased legalization within several U.S. states and in Canada, valid analytical methods are essential for this growing industry. SMPR 2017.002 was published on March 13, 2017, and required the quantitation of five cannabinoids: tetrahydrocannabidiolic acid (THCA), tetrahydrocannabinol (THC), cannabidiolic acid (CBDA), cannabidiol (CBD), and cannabinol (CBN) in *Cannabis* dried flowers; SMPR 2017.001, also published on March 13, 2017, required the quantitation of the same five cannabinoids in *Cannabis* concentrates or extracts (1, 2). The SMPRs specify nine additional cannabinoids, the quantitation of which are also desirable by the method.

A call for methods and AOAC Expert Review Panel (ERP) were formed in the fall of 2017 to evaluate quantitative *Cannabis* methods against the SMPRs. The method published by Mudge et al. (3) was submitted for consideration at the initial call for methods, where the original ERP submitted additional recommendations prior to adoption. These recommendations were taken into account and are summarized below. All additional method optimization and validation procedures can be found in the original publication. The method was adopted for First Action *Official Methods*[SM] status in a second ERP meeting that took place October 2018. Herein is a review of the method's performance in relation to SMPR 2017.001 and 2017.002.

## SMPR 2015.008 and LC-UV Method

This method for the quantitation of 10 cannabinoids in *Cannabis sativa* (marijuana) underwent extensive optimization using statistically guided protocols and was subjected to a single-laboratory validation according to AOAC guidelines for dietary supplements (3, 4). Briefly, 200 mg ground dried flowers was extracted with 25 mL 80% aqueous methanol for 15 min in an ultrasonicating bath with mixing on a vortex mixer every 5 min. Samples were centrifuged, filtered, and diluted prior to

HPLC-UV analysis. Oil samples (50 mg) were extracted with 10 mL methanol according to the same procedure (3). HPLC analysis separates the cannabinoids using gradient elution, and detection is conducted at 220 nm.

For dried *Cannabis* flowers, SMPR 2017.002 specified the minimum method performance requirements for the quantitation of individual cannabinoids. The method was assessed for 10 cannabinoids: THCA, THC, CBDA, CBD, CBN, tetrahydrocannabivarin (THCV), cannabidivarinic acid (CBDVA), cannabigerolic acid (CBGA), cannabigerol (CBG), and cannabichromene (CBC). The comparison between the SMPR and the LC-UV methods is summarized in Table 1. In many cases, the concentrations of the low-abundance cannabinoids were below the recommendations of the SMPR and are therefore not summarized in the table.

The LC-UV method accepted for First Action status was validated according to AOAC protocols for linearity, repeatability, intermediate precision, detection and quantitation limits, and recovery in nine different *Cannabis* chemotypes and one oil sample (3). Chromatographic separation yielded sufficient resolution and peak purity for all cannabinoids evaluated as shown in Figure 1. Because of the availability of additional cannabinoids after the original validation, two additional cannabinoids were assessed for repeatability: CBGA and CBDVA. Because of the low abundance of several cannabinoids in different chemotypes, repeatability data were only collected on samples with concentrations above the quantitation limits.

Linear responses were observed for each of the seven-point calibration curves for all 10 cannabinoids. The linear range evaluated for THCA and CBDA were from 5 to 250 µg/mL, THC and CBD from 1 to 50 µg/mL, and THCV, CBGA, CBDVA, CBG, CBC and CBN were linear from 0.5 to 25 µg/mL (3). The calibration ranges were designed to reflect the expected concentrations of the different cannabinoids naturally occurring in the flowers, although because of the high concentration of cannabinoids in most dried flowers, dilutions were necessary to maintain samples within the calibration range.

Recovery was performed for the most abundant cannabinoids—THCA, CBDA, THC, and CBD—by spiking a matrix blank composed of stinging nettle (*Urtica dioica*) with known concentrations of cannabinoids (2). Because of the cost of reference standards, the recovery studies were restricted to the major cannabinoids. The recoveries are summarized in Table 1. The ERP required additional recovery studies by performing spiking studies with *Cannabis* materials, which are summarized in Table 2, to assess recovery of CBDA and THC.

The original study determined repeatability and intermediate precision for eight cannabinoids in nine dried test materials and one oil, as summarized in the original publication (3). Repeatability for CBDVA and CBGA were evaluated in seven dried flowers and are summarized in Table 3. $RSD_r$ ranged from 1.61 to 7.24% for CBDA and from 0.78 to 7.64% for THCA, the two most abundant cannabinoids found in dried flowers. Because of the limited availability of strains available in the original study, four additional strains were used to evaluate the repeatability at higher concentrations (>15%). These data are summarized in Table 4.

**Table 1. SMPR 2017.002 requirements and Method 2018.10 results for dried flowers (3)**

| | | | Minimum acceptance criteria | HPLC method results | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Type of study | Parameter | | All cannabinoids | CDBA | CBD | CBN | THCA | THC | THCV | CBG | CBC | CBDVA | CBGA |
| Single-laboratory validation | LOQ, % (w/w) | | ≤0.1% | 0.03 | 0.04 | 0.03 | 0.04 | 0.02 | 0.03 | 0.05 | 0.06 | 0.05 | 0.05 |
| | Rec., % | 0.1–1% | 95–105% | 92.6–95.4 | 95.3–95.5 | ND[a] | 90.7–97.3 | 96.2–99.2 | ND | ND | ND | ND | ND |
| | | 1–25% | 97–103% | 97.7 | 91.3 | ND | 96.1 | 90.7–103.7 | ND | ND | ND | ND | ND |
| | | 25–50% | 98–102% | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| | Analytical range | | 0.1–50% | 0.04-18.1 | 0.22–0.70 | 0.02–0.15 | 0.11–20.7 | 0.03–3.31 | 0.03 | 0.02–0.24 | 0.03–0.09 | 0.04–0.13 | 0.57–1.4 |
| | $RSD_r$, % | 0.1–1% | ≤5% | 2.97–4.29 | 1.08–4.74 | 2.77 | 2.4–3.29 | 1.35–4.53 | ND | 1.06–4.77 | ND | 4.1 | 1.8–5.9 |
| | | 1–25% | ≤4% | 1.61–7.24 | ND | ND | 1.07–7.64 | 3.64 | ND | ND | ND | ND | 1.3–4.5 |
| | | 25–50% | ≤2% | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Multilaboratory validation | $RSD_R$, % | 0.1–1% | ≤7% | ND | | | | | | | | | |
| | | 1–25% | ≤5% | | | | | | | | | | |
| | | 25–50% | ≤3% | | | | | | | | | | |

[a] ND = Not determined.

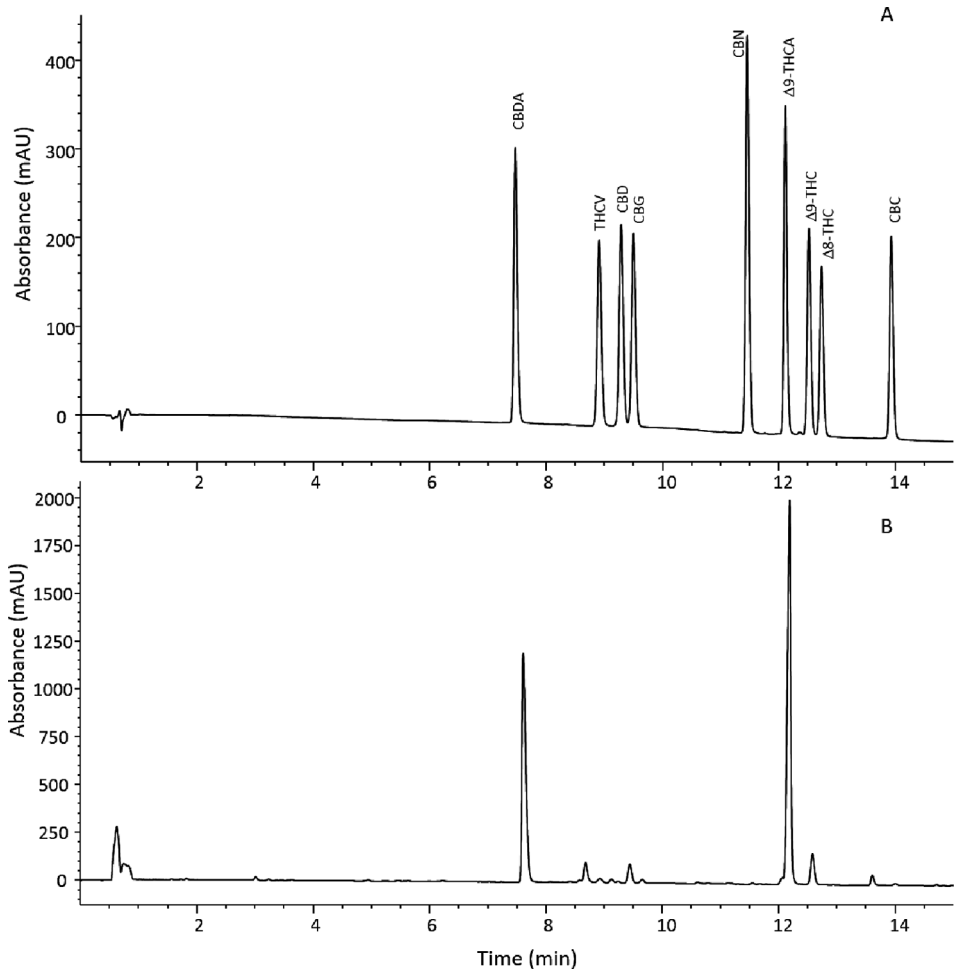


**Figure 1. Chromatographic separation of cannabinoids using a Kinetex C18 column (100×2.1 mm, 1.8 µm) with gradient elution at 220 nm. (A) Mixture of cannabinoids standards. (B) Authentic *Cannabis* extract.**

**Table 2. Recovery results for CBDA and THC using *Cannabis* as the matrix, as required by the ERP prior to OMA adoption**

| Cannabinoid | Spike concn, % (w/w) | Rec., % |
|---|---|---|
| CBDA | 0.006% | 108.2 |
| | 0.06% | 97.7 |
| THC | 0.48% | 97.6 |
| | 0.96% | 103.7 |

## AOAC Official Method 2018.10
### Determination of Cannabinoids in *Cannabis sativa* Dried Flowers and Oils Liquid Chromatography with UV Detection First Action 2018

### *A. Materials and Methods*

(**a**) *Reagents*.—HPLC grade methanol and acetonitrile were purchased from VWR International (Mississauga, ON, Canada). ACS grade chloroform was obtained from VWR International. Water was purified to 18 MΩ using a Barnstead Smart2Pure nanopure system (Thermo Scientific, Waltham, MA). Ammonium formate for HPLC (>99.0%) was purchased from Sigma Aldrich (Oakville, ON, Canada), and formic acid (98% pure) was purchased from Fisher Scientific (Ottawa, ON, Canada).

### *B. Calibration Standards*

Certified reference materials (CRMs) were purchased from Cerilliant Corp (Round Rock, TX) for nine cannabinoids: Δ9-THC, THCA, Δ8-THC, CBD, CBDA, CBG, CBN, CBC, and THCV. The individual cannabinoids were provided in solution at 1.0 mg/mL concentration certified by the supplier. The acidic cannabinoids were provided in acetonitrile and neutral cannabinoids in methanol. Fresh ampules were used for the validation study to ensure accurate quantitation of the individual constituents.

### *C. Test Materials*

Dried medical marijuana samples were purchased from several licensed producers within Canada. Nine products were selected for a variety of cannabinoid concentrations ranging from 0.2 to 17% total THC and 0.3 to 9% total CBD. As a result of the legal restrictions pertaining to these products, voucher specimens were not possible, but they were purchased directly from the source to ensure authenticity. A dried ethanol extract was dissolved in oil at a 1:10 dilution.

**Table 3. Repeatability for method extension to CBDVA and CBGA**

| Sample No. | Cannabinoid | Concn, % (w/w) | RSD, % |
|---|---|---|---|
| 1 | CBDVA | 0.13 | 4.1 |
| | CBGA | 0.57 | 4.2 |
| 2 | CBDVA | <DL[a] | |
| | CBGA | 1.13 | 4.5 |
| 3 | CBDVA | <DL | |
| | CBGA | 0.57 | 5.9 |
| 4 | CBDVA | 0.06 | 5.1 |
| | CBGA | 1.14 | 3.3 |
| 5 | CBDVA | 0.09 | 1.6 |
| | CBGA | 0.77 | 1.8 |
| 6 | CBDVA | <DL | |
| | CBGA | 1.3 | 2.7 |
| 7 | CBDVA | 0.04 | 4.2 |
| | CBGA | 1.4 | 1.3 |

[a] <DL = Below detection limit.

**Table 4. Repeatability for dried flowers with high concentrations of CBDA and THCA**

| | CBDA | | THCA | |
|---|---|---|---|---|
| Sample ID | Content, % (w/w) | RSD, % | Content, % (w/w) | RSD, % |
| A34-011 | 18.1 | 2.6 | 0.77 | 2.4 |
| W29-038 | 0.12 | 3.6 | 18.0 | 2.8 |
| W36-913 | <DL[a] | | 20.7 | 1.65 |
| AB18-775 | <QL[b] | | 19.4 | 1.07 |

[a] <DL = Below detection limit.

[b] <QL = Below quantitation limit.

### D. HPLC Analysis

An Agilent 1200 RRLC system equipped with a temperature-controlled autosampler, binary pump, and diode-array detector (Agilent Technologies, Mississauga, ON, Canada) was used to separate the cannabinoids. The separation was achieved on a Kinetex® C18, 1.7 µm, 100 × 3.0 mm i.d. column (Phenomenex, Torrance, CA). Mobile phase compositions were (A) 10 mM ammonium formate, pH 3.6 and (B) acetonitrile using gradient conditions at 0.6 mL/min. The separation was achieved according to the following gradient: 0–8 min, 52–66%B; 8–8.5 min, 66–70%B; 8.5–13 min, 70–80%B; 13–15 min, 80%B. A 7-min column equilibration was performed after each run. The injection volume was 5 µL and detection was at 220 nm. The autosampler was maintained at 4°C. Verification of analytes were performed by comparing UV spectra of samples with the standards in addition to peak purity of reference standards and samples.

### E. Preparation of Test Materials

(**a**) *Plant tissues*.—A minimum of 5 g dried flowers was ground together from each test sample to ensure sample homogeneity. Ground flowers were extracted by weighing 200.0 mg into a 50 mL amber centrifuge tube. Then, 25.00 mL 80% methanol was added and mixed on a vortex mixer for 30 s. Extraction took place using a sonicating bath for 15 min where samples were mixed on a vortex mixer every 5 min. Extracts were filtered with 0.22 µm Teflon filter, diluted 1:2, 1:5, or 1:10 using the extraction solvent into amber HPLC vials, and stored at 4°C until analysis.

(**b**) *Oil*.—*Cannabis* oil was mixed by inversion prior to sample preparation. Then, 50.0 mg oil was weighed into a 50 mL amber centrifuge tube to which 10.00 mL methanol was added and mixed on a vortex mixer for 30 s. Extracts were sonicated for 15 min with mixing on a vortex mixer every 5 min. Samples were filtered with 0.22 µm Teflon filters into amber HPLC vials and stored at 4°C until analysis.

### F. Method Optimization

(**a**) *Analyte stability*.—Mixed calibration standards were stored at –20°C, 4°C, and 22°C in the dark and tested at regular intervals to assess cannabinoid stability in solutions. Sample extracts were stored at 4°C and 22°C in light and dark conditions. A sample with greater than 5% loss from time zero was considered unstable.

(**b**) *Fractional factorial*.—The partial factorial design for method optimization and data analysis was completed using Minitab 16 (State College, PA). Individual cannabinoids were quantified as percentage weight per weight in *Cannabis* flowers and milligrams per gram in oil. Microsoft Excel (Richmond, WA) was used for quantitative calculations and statistical analysis of validation data.

### G. Single-Laboratory Validation Parameters

The optimized method was subjected to a single-laboratory validation according to AOAC guidelines for dietary supplements (4). Δ8-THC was not observed in any of the samples and therefore was not considered in the method validation.

(**a**) *Preparation of calibration solutions*.—Individual cannabinoid CRMs were used to prepare seven-point standard calibration curves for eight cannabinoids in concentrations ranging from 0.5 to 250 µg/mL. Dilutions of the CRMs were performed using the extraction solvent composed of 80% methanol. Concentration ranges were modified for each cannabinoid as summarized in Table 1. The calibration curves were plotted and the slope and *y*-intercept for each cannabinoid were used for linear regression analysis. Calibration curves were visually inspected and correlation coefficients were determined. An $r^2$ of at least 0.995 was deemed suitable for quantitation. Mixed standards were stored at 4°C and were stable for up to 3 days.

(**b**) *Selectivity*.—Selectivity was demonstrated by injecting the reference materials and raw flower extracts to evaluate the resolution between closely eluting peaks and potential interferences at 220 nm. Resolution of greater than 1.5 is deemed acceptable by AOAC guidelines (4). Peak purity was verified for all cannabinoids of interest.

(**c**) *Repeatability and intermediate precision*.—Quadruplicate samples of each test material were prepared on a single day to evaluate the repeatability as RSD % for the individual cannabinoids. Intermediate precision was determined by repeating the repeatability studies on 3 separate days. The within-day, between-day, and total SDs were calculated for each cannabinoid in each test material. HorRat values were calculated to assess the overall precision of the method (5).

(**d**) *Recovery*.—Recovery was determined at three concentration levels of the major cannabinoids: CBDA, CBD, THCA, and THC. Ground stinging nettle, used as the negative recovery material, was spiked with individual cannabinoids and prepared according to the sample preparation protocol.

(**e**) *LOD and LOQ*.—The LOD and LOQ were determined using the U.S. Environmental Protection Agency method detection limit (MDL) protocol (6). The MDL is defined as the minimum concentration of substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero. Extract solutions containing low concentrations of the cannabinoids were used to evaluate the method limits. Seven replicates were injected, and the calculation for MDL was determined as the SD of the calculated concentration between the seven replicates multiplied by the *t*-statistic at 99% confidence interval. LOQ was determined as 10 times the SD for the replicates to determine the MDL.

## Discussion

The comparison between the SMPR requirements and the validated method are summarized in Table 1. The LOQ and LOD for all cannabinoids are below the required specifications. A reassessment was performed on CBD from the original publication by spiking known concentrations into a CBD-free chemotype to obtain an accurate determination of LOQ for this closely eluting cannabinoid. The analytical range for THCA was evaluated up to 20.7%, which is not as high as the method requirements, as there are limited products with concentrations greater than 25% available. Repeatability was within range for almost all samples, with the exception of two strains obtained from the same supplier. These strains are thought to be inhomogeneous, as all other strains had repeatabilities within the specific ranged in the SMPR. Grinding samples with liquid nitrogen in a mortar and pestle reduced the issues of resin clumping and improved homogeneity of the sample. The ERP carefully evaluated the performance of the method and voted to adopt it on November 19, 2018, as a First Action *Official Method*.

Prior to consideration as a Final Action method, the method must undergo a multilaboratory validation to determine reproducibility.

## References


(1) Audino, S., Angerhofer, M., Atkins, P., Brauninger, R.M., Brown, P.N., Burdette, C.Q., Clifford, R., Cook, J.M., Donelson, J., George, K., Gibson, P., Harris, H.L., Hartlein, T., Hodgin, G., Inerowicz, D., Johnson, H.E., Joseph, G., Konings, E.J.M., Kowalski, J.A., Krogull, M.K., Kuzdzal, S., Lockerman, R., Ludwig, C., Marrongelli, C., Mastovska, K., Mudge, E., Niehaus, G.D., Nishimura, M., Phillips, M.M., Phinney, C.S., Reif, K., Rigdon, A., Rimmer, C.A., Ruthenburg, T., Solyom, A.M., Stenerson, K., Sudberg, S., Sutton, A., Szpylka, J., Weitzel, J., Winkler, P., Wong, S., Wurzer, J., You, H., & Coates, S.G. (2017) *J. AOAC Int.* **100**, 1204–1207. doi:10.5740/jaoacint.SMPR2017_002

(2) Audino, S., Angerhofer, M., Atkins, P., Brauninger, R.M., Brown, P.N., Burdette, C.Q., Clifford, R., Cook, J.M., Donelson, J., George, K., Gibson, P., Harris, H.L., Hartlein, T., Hodgin, G., Inerowicz, D., Johnson, H.E., Joseph, G., Konings, E.J.M., Kowalski, J.A., Krogull, M.K., Kuzdzal, S., Lockerman, R., Ludwig, C., Marrongelli, C., Mastovska, K., Mudge, E., Niehaus, G.D., Nishimura, M., Phillips, M.M., Phinney, C.S., Reif, K., Rigdon, A., Rimmer, C.A., Ruthenburg, T., Solyom, A.M., Stenerson, K., Sudberg, S., Sutton, A., Szpylka, J., Weitzel, J., Winkler, P., Wong, S., Wurzer, J., You, H., & Coates, S.G. (2017) *J. AOAC Int.* **100**, 1200–1203. doi:10.5740/jaoacint.SMPR2017_001.

(3) Mudge, E.M., Murch, S.J., & Brown, P.N. (2017) *Anal. Bioanal. Chem.* **409**, 3153–3163. doi:10.1007/s00216-017-0256-3

(4) *Official Methods of Analysis* (2013) Appendix K, AOAC INTERNATIONAL, Gaithersburg, MD

(5) Horwitz, W. (1982) *Anal. Chem.* **54**, 67–76. doi:10.1021/ac00238a002

(6) United States Environmental Protection Agency (1986) Definition and Procedure for the Determination of the Method Detection Limit, Revision 2, Appendix B, Part 136, https://www.epa.gov/sites/production/files/2016-12/documents/mdl-procedure_rev2_12-13-2016.pdf (accessed September 24, 2019)