APPLICATION NOTE

CRM Mixtures Improve Quantitation Accuracy

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Key Features

- High-quality reference materials with established metrological traceability are an essential element for measurement accuracy.
- Reproducibility errors that arise during standard curve preparations using certified reference materials (CRMs) may stem from inherent variability around pipetting procedures.
- We compare two different methods to generate a standard for the quantitation of ten prevalent phytocannabinoids against a calibration curve.
- Pre-made, multi-component CRM mixtures save time and consumable cost in the preparation of standard curves and improve quantitation accuracy during routine analytical checks as well as *Cannabis* product quality testing and profiling.

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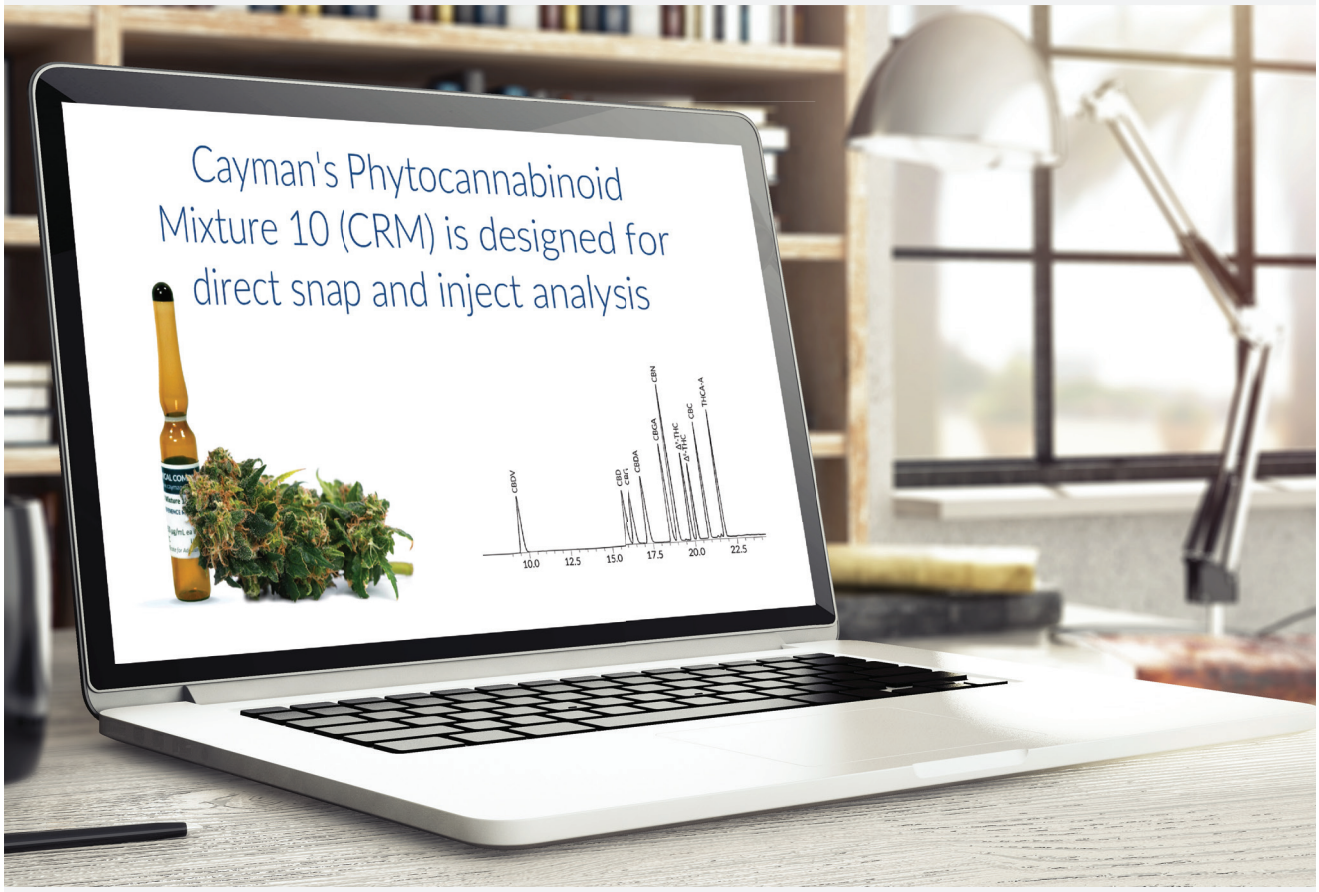

See why testing labs are implementing Cayman's pre-made, multi-component CRM mixtures into their workflows.

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Introduction

With the emergence of *Cannabis* testing laboratories, various stakeholders are moving toward standardizing this growing industry. To ensure the competency of the *Cannabis* testing laboratories, stakeholders are strongly urging, if not requiring, these laboratories to become ISO/IEC 17025 accredited. Under an ISO/IEC 17025 quality system, laboratories are required to maintain metrological traceability of measurement results. Metrological traceability is defined as the property of a measurement result whereby the result can be related to a reference through a documented, unbroken chain of calibrations, each contributing to the measurement uncertainty,[1] thus providing confidence in the accuracy of analytical measurements.

Laboratories using certified reference materials (CRMs), produced under an ISO 17034 quality system, are able to meet this metrological traceability requirement. The benefits of using ISO 17034 CRMs are highlighted by Franckowski.[2] This information is documented on an accompanying certificate of analysis, which includes the certified property value and the associated uncertainty of the material making CRMs ideal for quantitative analysis.

Historically, single analyte CRMs have been the default option for quality control testing, mainly due to the lack of availability of multi-component CRMs. Now that multi-component CRMs are available, they provide an advantage in many applications. Cayman Chemical designed the Phytocannabinoid Mixture 10 (CRM) (Cayman Item No. 21305) to confirm the ten most prevalent phytocannabinoids found in *Cannabis* samples. This mixture, in acetonitrile, contains 250 µg/ml of each of the following: cannabidivarin (CBDV), cannabidiolic acid (CBDA), cannabigerolic acid (CBGA), cannabigerol (CBG), cannabidiol (CBD), cannabinol (CBN), tetrahydrocannabinolic acid A (THCA-A), Δ^9-tetrahydrocannabinol (Δ^9-THC), Δ^8-tetrahydrocannabinol (Δ^8-THC), and (±)-cannabichromene (CBC). A pre-made, multi-component CRM bypasses the additional steps required in the preparation of a stock mixture from individual CRMs.

The data provided in this application note compares the reproducibility, or precision, and accuracy of a stock solution prepared using several single-component CRMs (**Method A**) to that of a pre-made, multi-component CRM (**Method B**).

Methods

A total of eight solutions were prepared by two separate analysts using each method on two separate days. For Method A, ampules of the ten single-component CRMs (each at 1 mg/ml in 1 ml of either acetonitrile or methanol) were used to prepare the stock solution by snapping the neck of each ampule and pouring the contents of the CRMs into separate HPLC vials. Then, 1 ml of each individual CRM compound was pipetted into a scintillation vial to create a mixture at a concentration of 100 µg/ml (**Figure 1**).

Method A

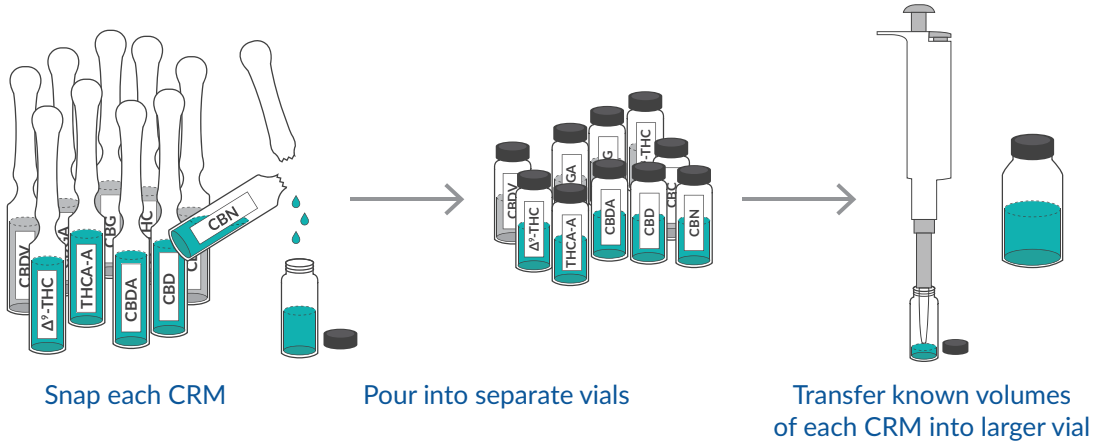


Figure 1. Each of the ten individual 1 mg/ml CRMs was snapped open and poured into a separate HPLC vial. Then, 1 ml of each individual CRM compound was pipetted into a scintillation vial.

For Method B, a batch of Cayman's pre-made Phytocannabinoid Mixture 10 (CRM) (Cayman Item No. 21305) served as the commercially formulated multi-component CRM. The neck of the single ampule was snapped and the contents were poured into a separate HPLC vial, then a 400 µl aliquot was transferred to a 1 ml volumetric flask and brought to volume with methanol to create the 100 µg/ml solution (**Figure 2**). Appropriate dilutions were performed to yield the 1 µg/ml sample concentration for each method. All pipetting techniques were performed using air displacement Eppendorf pipettes.

Method B

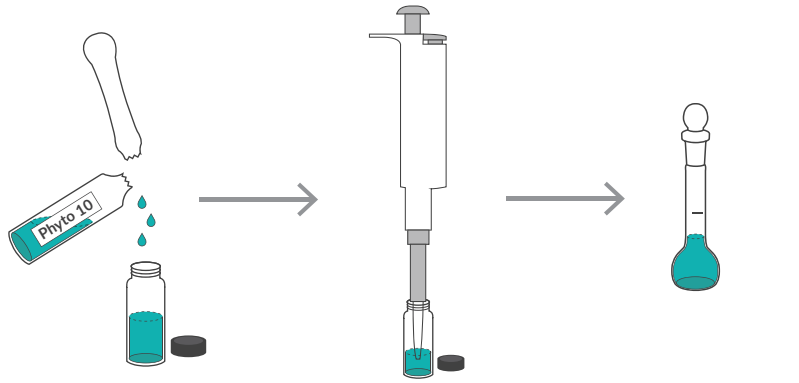


Figure 2. The Phytocannabinoid Mixture 10 (CRM) (250 µg/ml per component) from Cayman Chemical did not require preparation. The ampule was snapped open and transferred to a larger HPLC vial where 400 µl was pipetted into a volumetric flask and brought to a total volume of 1 ml of methanol.

The mixtures from each of these preparation methods were injected on the Cannabis Analyzer for Potency™ HPLC model LC-2030C Plus from Shimadzu Scientific Instruments. The calibration curve was developed using an independent batch of Cayman's Phytocannabinoid Mixture 10 (CRM) in accordance with the "Cannabis Analyzer for Potency™ Quick Guide" provided by Shimadzu (**Appendix A**).[3] The High-Resolution Method from Shimadzu was used in the analysis of this calibration curve as well as the analysis of the sample solutions.[4] A linear dynamic range of 0.5 µg/ml to 250 µg/ml was established for each of the ten analytes in the Phytocannabinoid Mixture 10 (CRM). A weighted regression of $1/[X]^2$ was generated for each curve to provide a more accurate representation of compound concentration at both the low and high ends of the curve.

Concentrations of 1 µg/ml and 100 µg/ml were analyzed against the weighted calibration curve to evaluate differences between the two methods. The concentrations selected for this experiment were based on ease of dilution and represented low and high reference points to be analyzed against the weighted calibration curve. Accuracy of the mixtures was determined by comparing the experimental concentrations to the theoretical concentrations of each component.

Results and Discussion

The Shimadzu HPLC generated a weighted linear regression model for each component in the Phytocannabinoid Mixture 10 (CRM). The R^2 values are reported in **Table 1**. The concentrations used for the curve were obtained from the certificate of analysis provided by Cayman Chemical for each respective component. A weighted linear regression model with linearity yielding $R^2 \geq 0.9980$ was observed for all ten of the analytes. These curves were then used to determine the concentration of the samples in both Method A and Method B. For both methods, a sample set of injections (n = 8) was analyzed against the calibration curve. Each of the sample concentrations were averaged across all injections for each component.

Table 1 - R^2 values from Shimadzu calibration curve for each component.

Compound	R^2 Values	Compound	R^2 Values
CBDV	0.9989	CBN	0.9993
CBDA	0.9993	THCA-A	0.9980
CBGA	0.9993	Δ^9-THC	0.9980
CBG	0.9990	Δ^8-THC	0.9988
CBD	0.9990	CBC	0.9990

Method Comparison

Both methods were evaluated at 1 µg/ml and 100 µg/ml and were compared against the theoretical concentrations derived from the respective certificate of analyses. The average concentrations were plotted with their respective standard deviations (**Figures 3 and 4**). Method A at 1 µg/ml showed relatively higher concentrations when compared to the theoretical value, whereas Method B showed values that were much closer to the theoretical concentration.

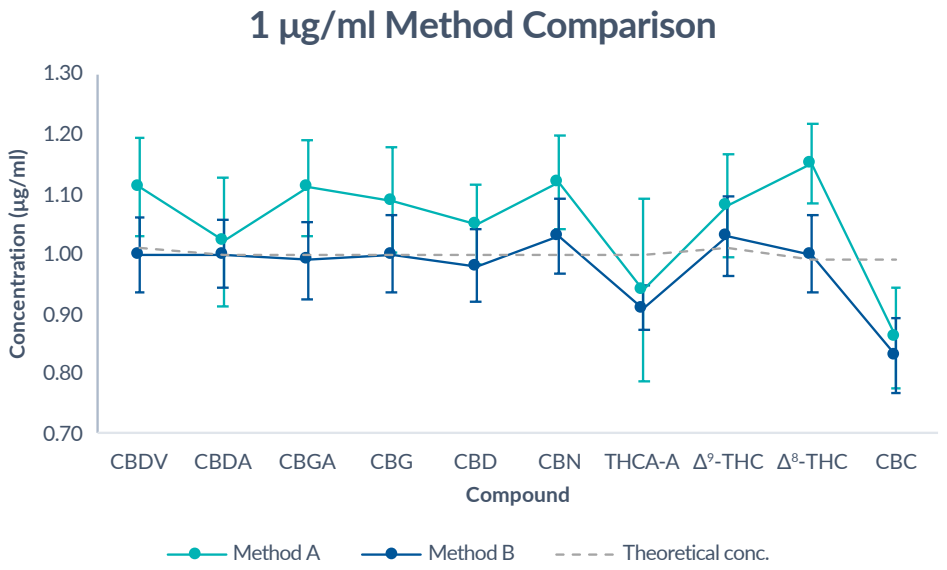


Figure 3. 1 µg/ml concentrations compared between Method A and Method B.

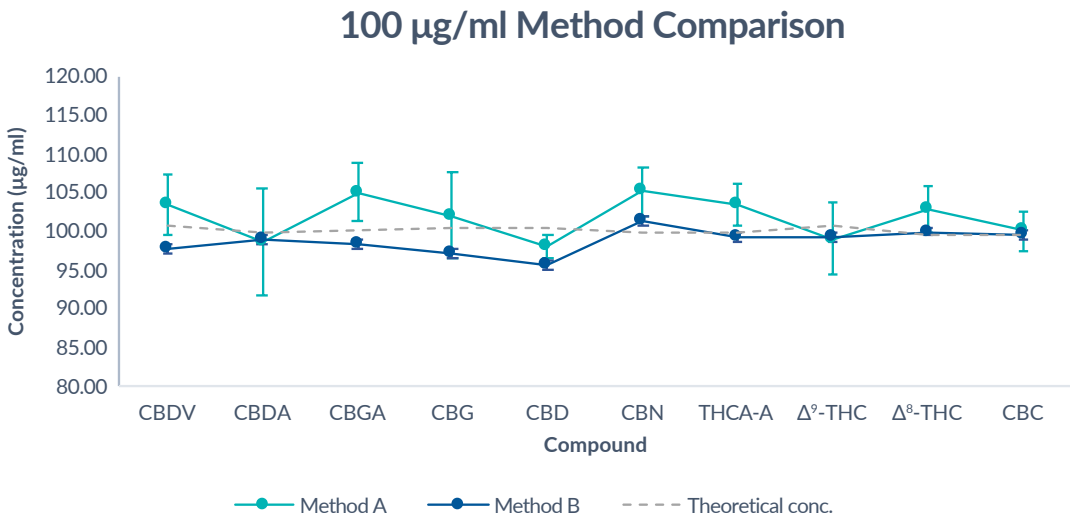


Figure 4. 100 µg/ml concentrations compared between Method A and Method B.

The accuracy, relative error, and precision results were tabulated for each compound (**Table 2 and 3**). The accuracy and relative error of the experimental concentrations for each of the sample components were calculated against the respective theoretical concentrations. Greater deviation of the concentrations was observed with Method A than with Method B. Some variations in both Method A and B may be attributed to the loss of solution during the transfer of the CRM to the larger HPLC vial, altering the concentration. A relative error of ±10% of the verified concentration is an acceptable criterion in analytical testing.[5] This criteria was used to determine acceptable results for the purposes of this publication with the caveat that acceptance criteria may be defined differently elsewhere. Reproducibility, or precision, is defined as the coefficient of variation in percent form. As shown in **Table 2 and 3**, the precision observed by Method B is better across most components compared to Method A. The results were reported for both the 1 µg/ml and 100 µg/ml concentrations.

Table 2 - 1 µg/ml Accuracy, Relative Error, and Precision (n=8)

1 µg/ml	Accuracy (%)		Relative Error (%)		Precision (CV, %)	
Compound	Method A	Method B	Method A	Method B	Method A	Method B
CBDV	1.11	1.00	10.17	-1.04	7.40	6.25
CBDA	1.02	1.00	1.66	0.26	10.72	5.82
CBGA	1.11	0.99	10.94	-0.94	7.09	6.48
CBG	1.09	1.00	8.84	-0.09	8.06	6.54
CBD	1.05	0.98	4.68	-2.24	6.27	6.19
CBN	1.12	1.03	11.80	2.73	7.00	6.18
THCA-A	0.94	0.91	-5.68	-9.33	16.06	4.04
Δ^9-THC	1.08	1.03	7.12	1.61	8.05	6.52
Δ^8-THC	1.15	1.00	16.39	1.46	5.85	6.49
CBC	0.86	0.83	-12.90	-16.04	9.79	7.56

Table 3 - 100 µg/ml Accuracy, Relative Error, and Precision (n=8)

100 µg/ml	Accuracy (%)		Relative Error (%)		Precision (CV, %)	
Compound	Method A	Method B	Method A	Method B	Method A	Method B
CBDV	102.77	97.14	2.77	-2.86	3.72	0.60
CBDA	98.71	99.10	-1.29	-0.90	7.14	0.63
CBGA	104.94	98.24	4.94	-1.76	3.58	0.66
CBG	101.42	96.55	1.42	-3.45	5.44	0.64
CBD	97.72	95.32	-2.28	-4.68	1.49	0.61
CBN	105.31	101.43	5.31	1.43	2.99	0.62
THCA-A	103.45	99.21	3.45	-0.79	2.53	0.64
Δ^9-THC	98.24	98.40	-1.76	-1.60	4.80	0.61
Δ^8-THC	103.40	100.46	3.40	0.46	2.85	0.58
CBC	100.52	100.02	0.52	0.02	2.64	0.64

Two outliers were observed in both methods when evaluating CBC and THCA-A at 1 µg/ml. It will take further investigation to elucidate the cause of these anomalies. The standard deviation for Method A was larger than that of Method B across almost all injections for the 1 µg/ml concentration. In terms of accuracy, there were only two compounds in Method B that showed a strong deviation from the theoretical concentration at 1 µg/ml. With Method A, all compounds evaluated at 1 µg/ml demonstrated greater deviations.

When evaluating the 100 µg/ml concentration, a large difference in precision can be observed between the two methods. Although Method A showed acceptable accuracy, it was not as precise as Method B, which showed both acceptable accuracy and precision. The standard deviation across all injections made in Method A was much greater than that observed for Method B. Though there was a slight deviation from the theoretical concentration in both methods, Method B was the more accurate and precise of the analyzed methods.

Using the relative error criterion of ±10%, Method A was within the acceptable criteria at the higher concentration, but five compounds fell outside that criteria at the lower concentration. For Method B, all but one compound (CBC) met the acceptable criteria at the lower concentration, but all compounds were within acceptable criteria at the higher concentration. The data provided from Method B shows that utilizing Cayman's Phytocannabinoid Mixture 10 (CRM) standard will provide the most accurate and precise data.

Conclusion

There are multiple approaches to create working solutions of a phytocannabinoid mixture. Complications can arise from preparing a stock standard using single CRMs. Because all CRM materials used in this experiment were produced under ISO 17034 standards and, therefore, have established metrological traceability, the data variability seen in Method A is likely related to the preparation of the stock mixture. Variability arises from repeated pipetting steps for multiple components, which may affect the actual concentration of the stock solution. The approach utilized in Method A is a seemingly practical method seen in industry standards but has shown inconsistencies in reproducibility. Phytocannabinoid mixtures provide an easier approach to collecting accurate data while saving time, increasing efficiency, and reducing cost.

Cayman's CRM Mixtures

Cayman offers a suite of ISO 17034-produced multi-component CRM mixtures designed and engineered to the highest standards to give you confidence in your analytical data and products. While offering simplicity in their use, these mixtures provide highly accurate and precise data when used with proper methodology.

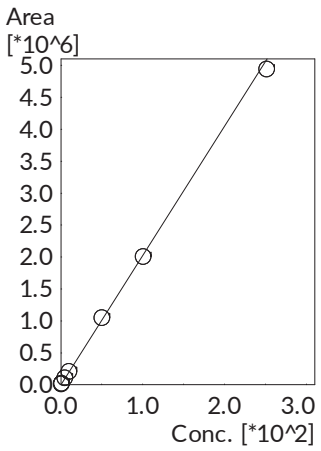
Item No.	Product Name
23251	Phytocannabinoid Mixture 3 (CRM)
25076	Phytocannabinoid Mixture 5 (CRM)
25077	Phytocannabinoid Mixture 6 (CRM)
21305	Phytocannabinoid Mixture 10 (CRM)

References

1. International Standard ISO/IEC 17025. *General requirements for the competence of testing and calibration laboratories.* (2017).
2. Franckowski, R. Precision testing. *Grow Opportunity* **3(4)**, 18 (2019).
3. Shimadzu. *Cannabis analyzer for potency quick guide.* (2018).
4. Shimadzu. *Potency testing in cannabis extracts using a high resolution method with the cannabis analyzer for potency.* (2017).
5. DeSilva, B., Smith, W., Weiner, R.M., *et al.* Recommendations for the bioanalytical method validation of ligand-binding assays to support pharmacokinetic assessments of macromolecules. *Pharm. Res.* **20(11)**, 1885-1900 (2003).

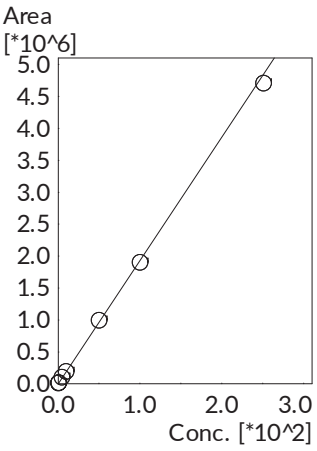
Appendix A. Calibration Curve

Name : CBDV
Quantitative Method : External Standard
Function : f(x)=20253.4*x+663.860
R^2 =0.9989167
FitType : Linear
Weighted Regression : 1/[X]^2
Detector Name : Detector A



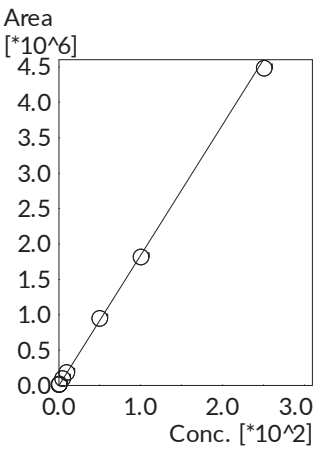
Conc.	MeanArea
0.5	10798
1.01	20910
5	107273
10.1	200748
50.3	1045394
100.6	2004344
251.6	4941982

Name : CBD
Quantitative Method : External Standard
Function : f(x)=19307.2*x+892.436
R^2 =0.9990103
FitType : Linear
Weighted Regression : 1/[X]^2
Detector Name : Detector A



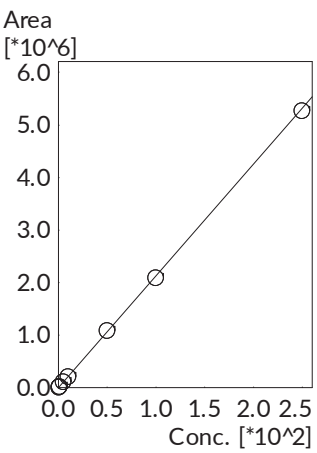
Conc.	MeanArea
0.5	10573
1	19920
5	102333
10	191156
50.2	992651
100.4	1903399
251	4705007

Name : CBG
Quantitative Method : External Standard
Function : f(x)=18391.7*x+707.400
R^2 =0.9990319
FitType : Linear
Weighted Regression : 1/[X]^2
Detector Name : Detector A



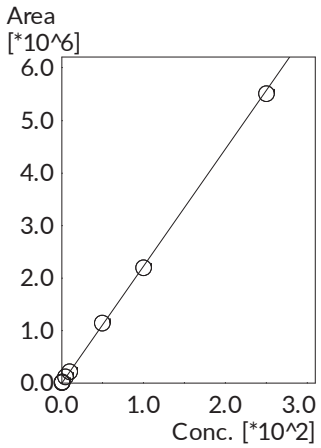
Conc.	MeanArea
0.5	9925
1	18855
5	97139
10	182118
50.2	947246
100.5	1814015
251.2	4480409

ID# : 5
Name : CBDA
Quantitative Method : External Standard
Function : f(x)=21310.9*x+302.233
R^2 =0.9993381
FitType : Linear
Weighted Regression : 1/[X]^2
Detector Name : Detector A



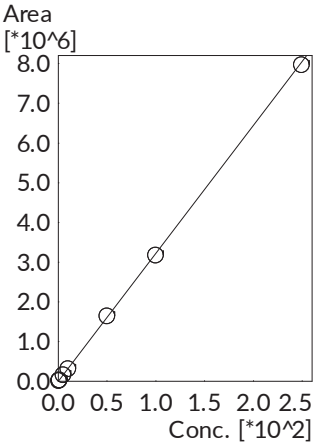
Conc.	MeanArea
0.5	11009
1	21298
5	110797
10	208058
49.9	1089298
99.9	2094482
249.7	5270543

ID# : 6
Name : CBGA
Quantitative Method : External Standard
Function : f(x)=22266.8*x+577.436
R^2 =0.9992538
FitType : Linear
Weighted Regression : $1/[X]^2$
Detector Name : Detector A



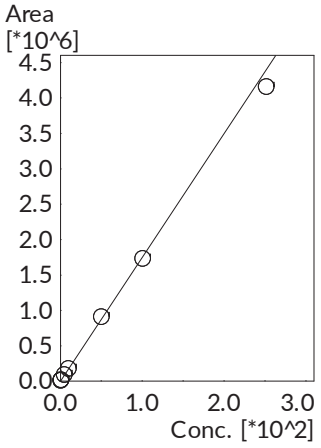
Conc.	MeanArea
0.5	11813
1	22302
5	116271
10	218434
50	1140246
100.1	2195732
250.2	5511016

Name : CBN
Quantitative Method : External Standard
Function : f(x)=32201.0*x+835.860
R^2 =0.9993274
FitType : Linear
Weighted Regression : $1/[X]^2$
Detector Name : Detector A



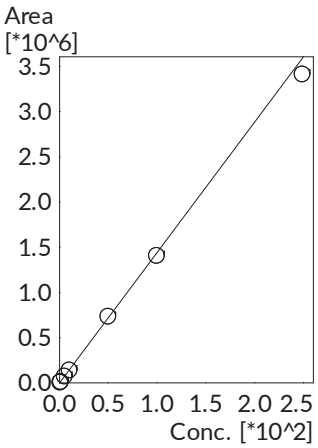
Conc.	MeanArea
0.5	17100
1	32233
5	167174
10	315112
49.9	1648026
99.8	3176288
249.4	7971020

Name : d9-THC
Quantitative Method : External Standard
Function : f(x)=17503.7*x+882.347
R^2 =0.9980092
FitType : Linear
Weighted Regression : $1/[X]^2$
Detector Name : Detector A



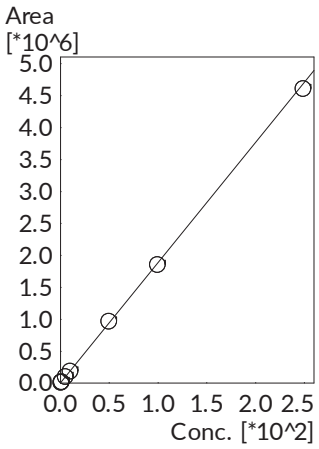
Conc.	MeanArea
0.5	9673
1.01	18174
5	94131
10.1	176765
50.4	912536
100.8	1732020
252	4158339

Name : d8-THC
Quantitative Method : External Standard
Function : f(x)=14394.3*x+1061.13
R^2 =0.9987547
FitType : Linear
Weighted Regression : $1/[X]^2$
Detector Name : Detector A



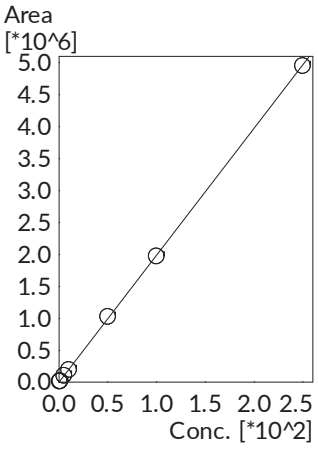
Conc.	MeanArea
0.5	8271
0.99	15129
5	76520
9.9	142793
49.7	738946
99.5	1408929
248.7	3414733

Name : CBC
Quantitative Method : External Standard
Function : f(x)=18800.1*x+5391.86
R^2 =0.9989982
FitType : Linear
Weighted Regression : 1/[X]^2
Detector Name : Detector A



Conc.	MeanArea
0.5	14966
0.99	23209
5	102704
9.9	188908
49.7	966994
99.5	1854691
248.7	4611612

Name : THCA-A
Quantitative Method : External Standard
Function : f(x)=19857.1*x+4902.98
R^2 =0.9979649
FitType : Linear
Weighted Regression : 1/[X]^2
Detector Name : Detector A



Conc.	MeanArea
0.5	15183
1	23235
5	107810
10	199814
50	1028103
99.9	1978315
249.8	4955188