# **Analysis of Cannabinoids:** Development and Validation of Methods for AOAC First Action Official Method Consideration

A EUROFINS WHITE PAPER

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**Hemp is increasingly used for medical purposes and still has many agricultural and industrial applications. Developers in the hemp industry often need support to test the safety of their product from seeds to finished products. This white paper discusses how developers can make use of hemp-specific analytical tests, including potency, to ensure their products are safe and deliver the highest quality, key factors to establishing and maintaining a role in today's competitive cannabinoids marketplace.**

# Table of Contents

## A Primer on Cannabis Sativa

Hemp and marijuana are members of the same plant species, Cannabis sativa. However, these plants are genetically distinct and unique in their chemical makeup. Hemp and marijuana differ in their cultivation requirements and areas of use.

Hemp derived cannabinoid usage is very popular and has been reported to aid in the treatment of various medical conditions. Many countries around the globe have adopted a more liberal view towards the use of this plant for medicinal purposes. Extensive research indicates the positive health effects of hemp derived cannabinoids result from interactions between cannabinoids, terpenes, phenolic compounds and other phytochemicals found in the plants. This phenomenon is known as the "entourage effect".

## The Properties of Cannabinoids

The phytochemicals produced in Cannabis plants are known as cannabinoids. More than 100 phytocannabinoids have been identified, but the major cannabinoids in hemp include cannabidiol (also known as CBD) and cannabidiolic acid (CBDA). On the other hand, tetrahydrocannabinol (THC) and its acidic form, tetrahydrocannabinolic acid (THCA) are dominant in marijuana. A few other examples of major cannabinoids include cannabidiolic acid (CBDA), cannabinol (CBN), cannabigerol (CBG) or cannabigerolic acid (CBGA).

Phytocannabinoids are decarboxylated into their corresponding neutral forms during cultivation and to a much larger extent, upon heating and harvesting. The concentration and relative abundance of cannabinoids depends on many factors such as humidity, light levels, growing conditions and nutrition.

Most of the biological properties related to cannabinoids rely on their interaction with the endocannabinoid system in humans. The endocannabinoid system includes two cannabinoid receptors, CB1 and CB2 as well as endocannabinoids and enzymes. The endocannabinoid system is thought to maintain homeostasis and play a regulatory role in a wide range of vital physiological processes, including appetite, pain, sensation, mood, memory, inflammation and the metabolism of fat.

THC activates cannabinoid receptor CB1, which is found in the areas of the brain involved in movement, stress and cognitive functions. The CB1 receptor appears to emit psychoactive properties of THC. Meanwhile, CBD binds to the second cannabinoid receptor, CB2, which is found in the peripheral nervous system.

Recent studies indicate that cannabinoids, especially CBD, may hold a wide range of potential benefits, such as neuroprotective effects, antioxidant properties, as well as reduction of epileptic seizures, inflammation, pain, anxiety, spasms, and nausea among many other conditions.[1]

## Testing of Cannabis and Related Products

Appropriate testing of Cannabis sativa and related products is of paramount importance. Developers can make use of testing to ensure products are compliant with legislation, are free of contaminants and are accurately labeled. In addition, testing can help to understand and optimize the composition of crops, which is particularly important to growers.

The test items may range from Cannabis sativa plants to various products including oils, concentrates, supplements, foods, cosmetics and other matrices. Cannabinoid analysis is important for many reasons, such as for legal compliance, as well as to assess suitability of the particular material in medical applications. Other commonly performed chemical tests include analysis of terpenes, pesticide residues, naturally occurring mycotoxins, as well as heavy metals. Items that are manufactured with the use of organic extraction solvents must be examined for these harmful substances. To cover the above analyses, various analytical techniques have to be used. The gold standard approaches are usually based on chromatographic separation coupled to either conventional or mass spectrometric detection.

## Development and Validation of LC-UV/MS Method

The analysis of CBD is most often performed with either gas chromatography (GC) with flame ionization detection, liquid chromatography (LC) with UV spectrophotometric or mass spectrometry detection. In general, LC-based analysis is preferred over GC based analysis, as the former approach allows obtaining more comprehensive cannabinoid profile. The technical challenges in this field relate to the need for testing of an increasing number of analytes occurring at largely different concentrations in highly complex matrices including microencapsulated materials. Additionally, until recently there was a lack of reliable and fully validated standard methods that could be adopted by the testing labs.

The gap in standard methodology was recognized by AOAC International, which is a nonprofit scientific association focusing on creation, validation and global publication of reliable standards. AOAC International issued a call for methods suitable for quantitation and identification of individual cannabinoids in Cannabis concentrates and plant material and published standard method performance requirements (SMPRs) to be met by the candidate methods. Eurofins decided to develop and validate LC-based methods for quantitative analysis of cannabinoids in dried plant materials, oils and concentrates that would meet the AOAC SMPR and submit the method to the AOAC expert review panel for first action official status consideration.

[1] Andre C.M., Hausman J.-F., Guerriero G. Cannabis Sativa: The Plant of the Thousand and One Molecules. Frontiers in Plant Science 2016, 7:19.

## Sample Preparation

The sample preparation procedure that Eurofins used differed for dried plant materials versus concentrates and oils (see below).

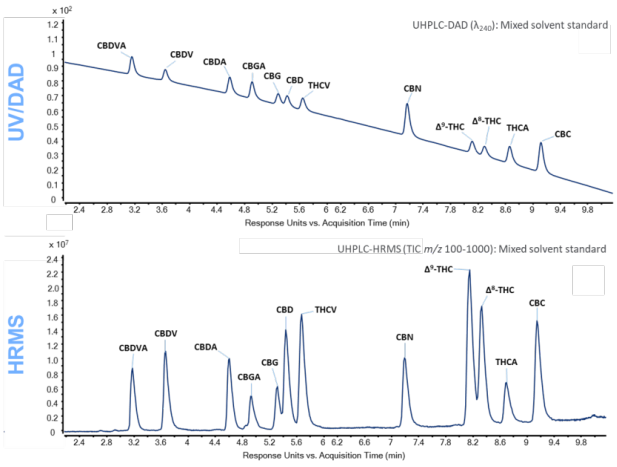
| Planet Material | Oils/Concentrates |
|---|---|
| Weigh 0.50 ± 0.01 of a thoroughly homogenized sample | Weigh 0.5/ 0.05 ± 0.01 g of a thoroughly homogenized sample |
| Add 20 mL of EtOH and shake for 30 min | Add 20 mL of EtOH and shake/sonicate |
| Centrifuge, collect supernatant and re-extract with 20 mL of EtOH | Dilute with EtOH to 25 mL with EtOH |
| Dilute the joint extracts to 50 mL with EtOH | Filter and perform dilution with MeOH |
| Filter and perform dilution with MeOH | Vial and analyze |
| Vial and analyze | |

## Instrumental Analysis

For instrumental analysis, ultra-high performance liquid chromatography (UHPLC) coupled to UV/DAD detector was used. UHPLC-UV/DAD data was employed for quantification and identification of target cannabinoids. During method development and validation, high-resolution quadrupole-time-of-flight mass spectrometer equipped with electrospray ionization was employed as a secondary detector and connected in series to the UV detector. Mass spectrometric detector was used for high-confidence identification of target analytes.

## Chromatographic Separation

Careful optimization of the chromatographic conditions was performed, in order to allow for efficient separation of the target cannabinoids. The LC separation step is critical for the UHPLC-UV/DAD analysis because this type of detection provides a lower level of selectivity as compared to mass spectrometry. In this respect, CBG and CBD as well as Δ^9-THC and Δ^8-THC represented two critical analyte pairs. Parameters that were optimized included chemistry of stationary phase, elution mode and composition of mobile phase components. The mobile phase composition had to be compatible with mass spectrometric detection so it could be used with both UV and MS instruments. The best results were obtained with Supelco Ascentis Express C18 (150 x 2.1 mm) column.



## Method Validation

Single laboratory validation of the method was performed in dried hemp plants, hemp concentrates and oils. Where possible, multiple materials differing in their cannabinoid profiles were employed in the validation. The performance characteristics evaluated in the study were selectivity, linearity, limit of quantification (LOQ), accuracy, extraction efficiency, precision and robustness.

### Selectivity

Selectivity of the method was evaluated during both method development and validation. This was tested in terms of analyte identification based on comparison of retention times, as well as UV and MS data collected in sample extracts and solvent standards.

### Linearity

Linearity was evaluated with each validation batch in terms of coefficient of determination and the residuals at five calibration levels. Excellent linearity was obtained during the method validation process.

### Accuracy

Accuracy was determined based on spiking experiments and by analysis of a proficiency test hemp oil sample. Spike recoveries were between 96.7 and 101.3% with relative standard deviations below 2.3%. These results fulfill the AOAC requirements. The analysis of proficiency test material gave results within the acceptable range. To ensure complete isolation of native cannabinoids from solid matrices, extraction efficiency was tested in dried hemp material via repeated extractions of test material and analysis of respective extracts.

### Precision

Repeatability and intermediate precision were determined based on repeated analyses of validation samples. Repeatability was expressed as relative standard deviation ($RSD_r$) and calculated based on results from five replicate extractions. To evaluate intermediate precision, the above experiment was repeated by a second analyst on a different day with a freshly prepared set of calibration standards. Intermediate precision ($RSD_{INT}$) was calculated based on the data generated by both first and second analysts using ten values in total. All precision data generated in this single laboratory validation study fulfilled the criteria in the SMPR documents.

### Other Performance Characteristics

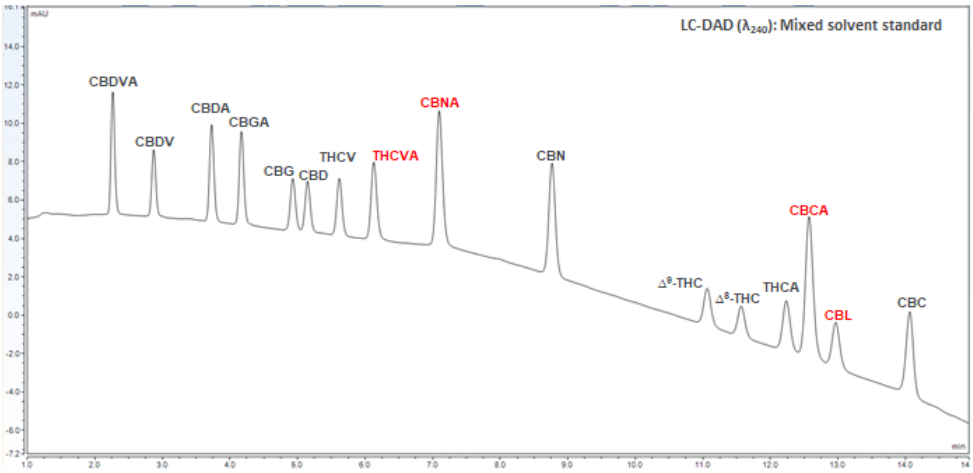
The method detection limit and limit of quantification were determined using the EPA Procedure for the Determination of the Method Detection Limit. This procedure can be used in cases where no blank matrix is available. Sufficient detectability was obtained for all analytes. Finally, robustness of the chromatographic separation was tested through small changes made to the pH value of the mobile phase, as this parameter affected retention of acidic cannabinoids.

## AOAC Review

The method validation report was submitted to the AOAC expert review panel for assessment. Following the review process, Eurofins method was granted AOAC First Action Official Method of Analysis status. The paper describing the single laboratory validation study was accepted for publication in the Journal of AOAC International.

## Recent Improvements

The hemp industry is rapidly changing and evolving, and a wide range of products are now available. To keep pace with the developments and meet the needs of our clients, some improvements have been introduced to our methods at Eurofins. We recently enlarged the matrix scope to include candies, gummies and cosmetics. Additionally, we extended the original analyte scope from 12 to 16 cannabinoids to be able to provide a more comprehensive cannabinoid profile. The inclusion of an additional four cannabinoids resulted in further improvement of chromatographic resolution between critical pairs of analytes.




Analysis of cannabinoids at low concentrations in complex matrices can be challenging; these hurdles can be dealt with either by employing extensive sample purification and concentration procedures or by utilization of a highly selective detection system. To address this challenge, we coupled the LC portion of the method to triple quadrupole tandem mass spectrometer detection to allow for higher selectivity and sensitivity.

## Summary

An innovative UHPLC-UV/DAD method has been developed and validated for analysis of major cannabinoids in various matrices. This method meets the requirements provided in AOAC Standard Method Performance Requirements 2017.001 and 2017.002 and was granted AOAC First Action OMA status. This method is fully compatible with MS detection, which allows for increased confidence in analyte identification and enhanced selectivity and sensitivity in complex matrices.

Eurofins plans to organize a collaborative study soon in order to obtain final action status and help developers in the hemp industry optimize how they analyze their cannabis and cannabinoid products.