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NATIONAL MEASUREMENT INSTITUTE
LABORATORY TEST METHOD

QUANTITATIVE DETERMINATION OF SELECTED CANNABINOIDS IN CANNABIS PRODUCTS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH DIODE ARRAY DETECTION (HPLC)

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PREFACE

This method has been prepared and validated by s22 for use as a quantitative analysis for 8 selected cannabinoids in cannabis products. It is approved and authorised by s22 Director of the Australian Forensic Drug Laboratory (AFDL). It has been developed, validated and had an uncertainty estimate applied in accordance with the current version of Method Development, Validation and Verification for Quantitative Analysis, ChemBio & Analytical Services, NMI.

Authorised on the _____ by,

_____

s22

METHOD REVISION HISTORY

Version	Date	Section	Page	Details	Authorised By:
1.0	June 2020	All	All	First Issue	s22

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FOREWORD

Cannabis sativa (Cannabis) (Figure 1) is a herbaceous flowing plant that sees widespread cultivation around the globe due to its long term use as a source of industrial fibre, seed oil, food, recreation, religious and spiritual involvement and medicine. It has commonly seen various stages of prohibition in western countries with a recent push in the west, including Australia, for its legalisation into medicinal usage. In 2017 the Therapeutic Goods Administration released Therapeutic Goods Order 93 – Standard for Medicinal Cannabis (TGO 93)[1] – within this requirement, Section 8 regulates the active ingredients in any medicinal cannabis product. A potency testing method for cannabinoid quantification in a variety of matrices is therefore required in order to both assist NMI pharmaceutical or other clients comply with Section 8 of TGO 93 and also to assist Australian Forensic Drug Laboratory (AFDL) law enforcement clients in determining the content of potentially illicit possessions of Cannabis and Cannabis products, referred to in this document as 'evidential Cannabis'.

The AFDL is approved to possess, hold and test prohibited substances as an Australian Commonwealth forensics drug testing facility and this enables analysts employed by AFDL-NMI to undertake these procedures.

Medicinal and recreational Cannabis can take many forms including the dried plant material (Flos), dried resin, liquid resin and finished product in an oil medium such as medium chain triglyceride (MCT). These above matrices are determined to be suitable for this methodology and validation has been completed using dried Cannabis Flos, liquid resin and finished product in MCT. Additional matrices could be validated and added to this methodology as Cannabis is also encountered as various edibles such as chocolates and gummies or in capsules and tablets.

A simple and accurate method for extracting 8 selected cannabinoids from certain Cannabis products and determining their content has been developed and validated based on 2 published methodologies. The extraction methodology is based on research article 'leaner and greener analysis of cannabionds'[2] and the instrument acquisition parameters are based on published Agilent Technologies paper 'Dedicated Cannabinoid Potency Testing Using the Agilent 1220 Infinity II LC System'[3] with deviations detailed below. This is achieved via a thorough methanolic extraction and dilution for liquid chromatography analysis. The 8 selected cannabinoids are listed below in Table 1 and their structures are shown in Figure 2. The cannabinoids are categorised into major and minor cannabinoids based on their expected concentration in dried plant material. These 8 cannabinoids were selected based on the Australian legislation, literaure[2, 3] and current NMI Pharmaceutical clients feedback however future cannabinoids could be added to the methodology after validation.

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Figure. 1 – *Cannabis Sativa*

Table 1 - Selected cannabinoids for determination

Major Cannabinoid	**Minor Cannabinoid**
Δ9-tetrahydrocannabinolic acid (THCA) (1)	Cannabinol (CBN) (5)
Δ9-tetrahydrocannabinol (THC) (2)	Cannabichromene (CBC) (6)
Cannabidiolic acid (CBDA) (3)	Cannabigerol (CBG) (7)
Cannabidiol (CBD) (4)	Tetrahydrocannabivarin (THCV) (8)

Stability of the cannabinoids in different mediums has been reported in literature and it is commonly agreed that THCA and CBDA decarboxylase into THC and CBD respectively over time. The breakdown of these acids is influenced by the heat and light conditions, and can occur quite rapidly (days to weeks)[4-6]. Conversion of cannabinoids to CBN occurs but at a slower rate. For these reasons it is acknowledged that the reported results are representative of the cannabinoids content as determine on the day of analysis and analysis of the same sample at a later date could produce differing results. Also there are certain 'dry weight' approaches to reporting cannabinoid content in cannabis products[7] and this is beyond the scope of the methodology and reporting.

For deviations to the published methods this analysis is based upon please see Appendix A.

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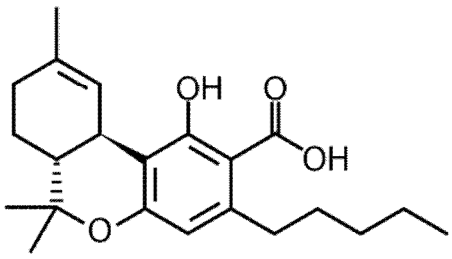
Figure 2 – Structures of selected cannabinoids for determination



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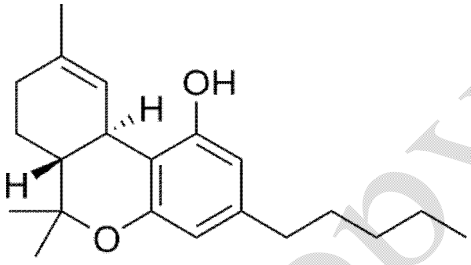

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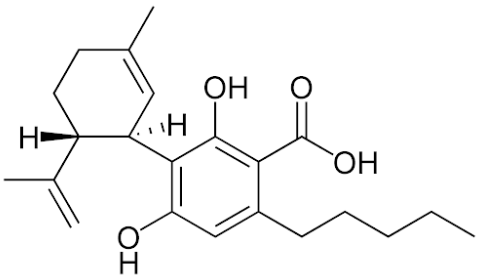

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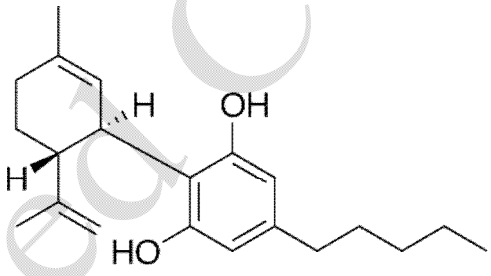

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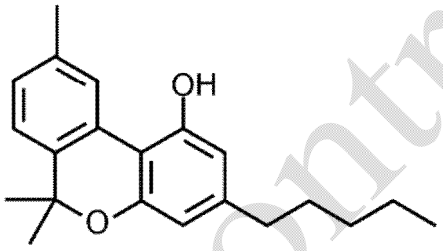

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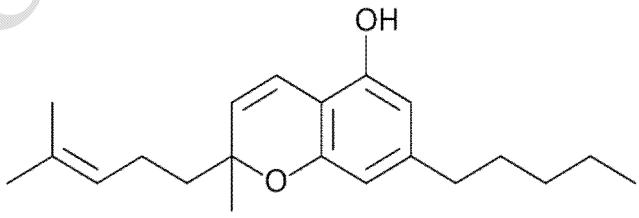

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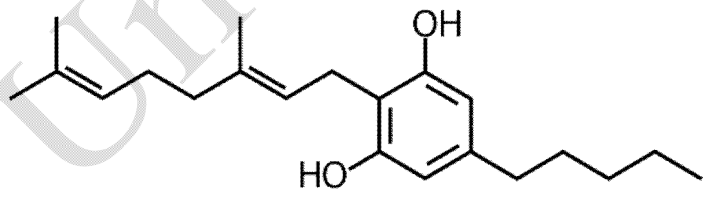

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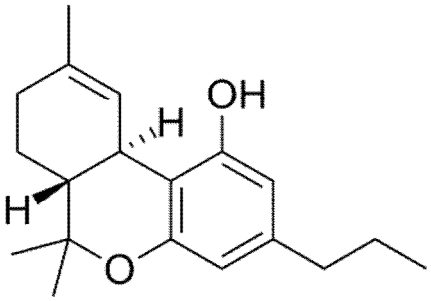

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CONTENTS

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1. SCOPE

This method describes the use of liquid chromatography with diode array detection (HPLC) to quantify 8 selected cannabinoids in Cannabis Flos, resin and finished product oil. This procedure is approved for use in testing medicinal Cannabis products and potential prohibited Cannabis products submitted to AFDL. It important to note that reporting of results for medicinal Cannabis products is different to the reporting of results for evidential analysis. This will be detailed under Reporting of Results section below.

1.1 Medicinal Cannabis

Medicinal Cannabis samples include Cannabis Flos, resin and finished oil product submitted for the purpose of compliance TGA potency testing using the HPLC method CaQ2. It should be noted that the tested materials are general Medicinal Cannabis matrices obtained by AFDL and are not product specific for any single client. Where a client might request a product specific validation for their own matrix this can be arranged. Initial screening of the samples will take place on GC/MS as per AFDL in house method 'CaMS_1.0' however this is non-reportable analysis and is simply used to indicate which cannabinoids are present in order to progress to CaQ2 potency testing.

1.2 Evidential Cannabis

At times cannabis samples may be submitted by law enforcement clients (example: AFP, ABF, NSWP) or prosecution or defence council for the purposes of determining the purity of any cannabinoid approved in this HPLC method CaQ2. Usually separated THC/THCA and CDB/CBDA results are requested and minor cannabinoids may not be analysed.

This method is complimentary to the laboratory's GC/MS identification method ID1 which will enable Cannabis specific cannabinoids such as THC and CBD and other significant cannabinoids to be detected within submitted samples.

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2. DEFINITIONS

For the purposes of this method, the following definitions apply:

ABF	Australian Border Force
AFDL	Australian Forensic Drug Laboratory
AFP	Australian Federal Police
ASB	Analytical Services Branch
CRM	Certified Reference Material
GC	Gas Chromatography
GMP	Good Manufacturing Practice
HPLC	High Performance Liquid Chromatography with Diode Array Detection
ISO	International Organisation for Standardization
MS	Mass Spectrometry
NSWP	New South Wales Police Force
NMI	National Measurement Institute
PPM	Parts per Million
TGA	Therapeutic Goods Administration

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3. PRINCIPLE

Submitted samples are registered in the laboratory database, stored in the AFDL Vault or AFDL freezer and assigned to an appropriately trained AFDL staff member for analysis. Refer to Storage section below for details.

1. Preliminary examination can include a physical description of the sample, e.g. "brown dry vegetable material" and if the sample appears to be a Cannabis product perform GC/MS analysis. Cannabis products presenting as an oil or resin should have their solubility in water, methanol, chloroform and toluene recorded.

2. For identification of cannabinoids in Medicinal Cannabis analyse using MSATS#3 and acquisition method CaMS_1.0. For Evidential Cannabis follow the GC/MS procedure as described within the Identification of Unknown Illicit Drug Substances (ID1) method. Note that cannabinolic acids degrade to the neutral species during GC/MS analysis and TMS derivatisation procedures are available if confirming the presence of acidic cannabinoids is required.
3. If the samples are found to contain cannabinoids in a suitable matrix, this methodology (CaQ2) can be used to determine up to 8 specific cannabinoids content.
4. Vegetable matter is ground and mixed. Oil and resin are mixed if possible. For Evidential Cannabis that is not of the described matrices, quantification may still be attempted using this methodology with some guidelines on reporting as outlined in the Reporting section below.

5. Cannabinoids are extracted from the Cannabis product through liquid extraction with hyper grade methanol after a series of vortex and sonication periods. The solution is filtered, centrifuged and diluted for analysis.

6. The solution is analysed by HPLC on the Agilent 1290 instrument (UPLC_01) for quantitative determination of the cannabinoid content using a 6 point calibration of desired cannabinoids via CRMs with a known concentration.

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4. REAGENTS AND STANDARDS

General requirements: Unless otherwise specified all reagents shall be of at least analytical reagent grade. Where water is used it shall be Milli-Q water. Larger or smaller volumes of reagents may be prepared provided the proportions of the components remain the same as outlined below.

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4.1. General reagents and solvents

4.1.1. Methanol

LCMS or Hyper Grade

WARNING! HIGLY FLAMMABLE LIQUID AND VAPOUR. TOXIC IF SWALLOWED. TOXIC IN CONTACT WITH SKIN. TOXIC IF INHALED. CAUSES DAMAGE TO ORGANS.

4.1.2. Formic Acid (98%)

Analytical Grade

WARNING! FLAMMABLE LIQUID AND VAPOUR. TOXIC IF INHALED. CAUSES SERVERE SKIN BURNS AND EYE DAMAGE. HARMFUL IF SWALLOWED. MAY BE CORROSIVE TO METALS.

4.2. Chemical standards

4.2.1. Cannabinoid Stock Solutions

1000ppm solutions of THCA, THC, CBDA, CBD, CBN, CBC, CBG and THCV.
(approved supplier for calibration is Cerilliant, through NovaChem)
(approved supplier for QA/QC spikes is Lipomed, through PM Separation)

4.2.2. JWH-302 Internal Standard

10mg aliquots of synthetic cannabinoid JWH-302 purchased from NMI Certified Reference Materials or equivalent CRM from alternate supplier.

4.3. Standard solutions

4.3.1. Cannabinoids Standard (CS)

Upon arrival in the laboratory the cannabinoids standards (CS) are registered into the AFDL standards database and stored as per their individual certificates of analysis (CoA) stated storage condition. For example THCA and CBDA are required to be stored at deep freeze temperature while THC requires freezer storage only. Before use CS must be allowed to come to room temperature and vortexed for 30 seconds. Note the expiry date of CS before opening and after opening transfer contents to an appropriately labelled amber GC vial and continue storing as per CoA for multiple uses in preparing the working standards.

4.3.2. Internal Standard (IS)

The internal standard solution is prepared by accurately weighing approximately 10mg (0.01mg accuracy) of JWH-302 (4.2.2) into a 25mL volumetric flask (~400ppm) and making to volume with methanol (4.1.1). The IS solution shall be kept in the refrigerator when not in use and allowed to come to room temperature and shaken before use. This solution is considered stable for six months.

4.3.3. Mixed CS for routine calibration

The mixed CS solution is prepared in methanol (4.1.1) and two temporary stocks are first made in order to create a 6 point calibration range using a minimal amount of each CRM. A procedure to prepare the calibration standards with a total volume of 1mL methanol in range of 0.5ppm to 50ppm is given in Figure 3 below. This involves combining the stated amounts

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of cannabinoid CRM in separate amber GC vials and adding an equivalent amount of IS (4.3.2) to a concentration of approximately 20ppm in each vial.

After each labelled vial is prepared and vortexed for 10 seconds, the 6 standards can be split into 3 sets of 6 using limited volume inserts filling each insert as much as possible so there is limited headspace. These 3 sets of labelled standards are stored in the freezer and can be used for calibration of a sample set on the UPLC_01. It is also important to record on the label the IS solution used, since future use of these standards require the same IS to be used for preparing the samples. These standards shall have an expiry duration of 1 month.

An excel worksheet (Figure 3) is used to calculate the working standard concentrations and can be found on V:\Securedata\AFDL\Systems-Documents\Standards\Quantitative\Cannabinoids\CaQ2-UPLC Ongoing.xls. The tab named 'Template 1 – 6 point' is copied and pasted into a new tab and the standards are named with format 'Cayymmdd' (for example Ca200514 Std1 – Std6). The Standard Reference (SR) number of each CS should be noted and the certified concertation recorded in the excel worksheet. This excel worksheet is then printed as a pdf and electronically signed by the preparing analyst and checked by an AFDL senior chemist.

It shall be noted that a set of mixed CS standards may be prepared where not all of the 8 cannabinoids are present. For example, an Evidential Cannabis sample may only require THC and CBD determination and so it's possible to adjust the standard preparation procedure appropriately and change accordingly the volume of methanol used to make up the working stocks and final standard vials. These changes must be clearly noted on the excel pdf printout and checked by an AFDL senior chemist.

Mixed standards have an established expiry of 1 week stored at 15°C or lower in the UPLC_01 chilled Autosampler and 1 month in the AFDL freezer at approximately -10°C.

4.3.4. Identification Only Standard

An identification only standard is used to monitor the retention times of the cannabinoids CBDV, CBG-A and 8-THC (see table 4 below). An approximate 20ppm of each in methanol with IS (4.3.2) is suitable and this vial can be left in the Autosampler or freezer for repeated use.

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Figure 3 – Example spreadsheet and 6 point standard calibration preparation

Australian Forensic Drug Laboratory

CALIBRATION STANDARD PREPARATION RECORD **AFDL**

Instrument: **UPLC** | Method: **CaQ2**

Preparation Date: Cayymmdd / Isyyddmm | Expiration Date:

Make sure to vortex each stock dilution before use

Standards have 6 week expiry; stored in full inserts in freezer Istd has 6 month expiry store in fridge

Please fill 1.0mL amounts of each standard into 3 sets of clearly labelled vials with full 250uL inserts, indicate the Istd used and store in freezer

SR No.	Id No. *Dilution in MeOH	Compound Dilution	Amount in stock (ppm)	Volume (mL)	Conc. ppm (µg/mL)	Conc. ppm 2nd Dil	Calib. Stds	Int Std 50uL	THCA	CBDA	THC	CBD	CBN	CBG	CBC	THCV	Make up with MeOH
	THCA	THCA	1000.0	1.0	1000.00												
SRXXXX	THCA(d)	THCA(d)	1000.0	0.5	100.00	10.00	Std 1	50 Isyyddmm	50.00 uL of Stock 2	←	←	←	←	←	←	←	900
	CBDA	CBDA	1000.0	1.0	1000.00			0.00	0.50 ppm	0.50 ppm	0.50 ppm	0.50 ppm	0.50 ppm	0.50 ppm	0.50 ppm	0.50 ppm	
SRXXXX	CBDA(d)	CBDA(d)	1000.0	0.5	100.00	10.00											
SRXXXX	THC	THC	1000.0	1.0	1000.00		Std2	50 Isyyddmm	100.00 uL of Stock 2	←	←	←	←	←	←	←	850
	THC(d)	THC(d)	1000.0	0.5	100.00	10.00		0.00	1.00 ppm	1.00 ppm	1.00 ppm	1.00 ppm	1.00 ppm	1.00 ppm	1.00 ppm	1.00 ppm	
SRXXXX	CBD	CBD	1000.0	1.0	1000.00												
	CBD(d)	CBD(d)	1000.0	0.5	100.00	10.00	Std3	50 Isyyddmm	50.00 uL of Stock 1	←	←	←	←	←	←	←	900
	CBN	CBN	1000.0	1.0	1000.00			0.00	5.00 ppm	5.00 ppm	5.00 ppm	5.00 ppm	5.00 ppm	5.00 ppm	5.00 ppm	5.00 ppm	
SRXXXX	CBN(d)	CBN(d)	1000.0	0.5	100.00	10.00											
	CBG	CBG	1000.0	1.0	1000.00		Std 4	50 Isyyddmm	100.00 uL of Stock 1	←	←	←	←	←	←	←	850
SRXXXX	CBG(d)	CBG(d)	1000.0	0.5	100.00	10.00		0.00	10.00 ppm	10.00 ppm	10.00 ppm	10.00 ppm	10.00 ppm	10.00 ppm	10.00 ppm	10.00 ppm	
	CBC	CBC	1000.0	1.0	1000.00												
SRXXXX	CBC(d)	CBC(d)	1000.0	0.5	100.00	10.00	Std 5	50 Isyyddmm	250.00 uL of Stock 1	←	←	←	←	←	←	←	700
	THCV	THCV	1000.0	1.0	1000.00			0.00	25.00 ppm	25.00 ppm	25.00 ppm	25.00 ppm	25.00 ppm	25.00 ppm	25.00 ppm	25.00 ppm	
SRXXXX	THCV(d)	THCV(d)	1000.0	0.5	100.00	10.00											Make up to 800uL
							Std 6	40 Isyyddmm	400.00 uL of Stock 1	←	←	←	←	←	←	←	360
								0.00	50.00 ppm	50.00 ppm	50.00 ppm	50.00 ppm	50.00 ppm	50.00 ppm	50.00 ppm	50.00 ppm	

Stock 1 **100ppm** = 100uL of each Cannabionoid into 1000uL (add 200uL MeOH)

Stock 2 **10ppm** = 50uL of above Stock 1 into 500uL (add 450uL MeOH)

Please BEWARE: Std 6 is made up using 40uL ISTD, 400uL Stock1 to total of 800uL (ensuring there is enough Stock 1)

	purity	amount	vol	mg/mL		ppm
Isyyddmm	99.1%			25	0.0000	0.00

JWH-302 SRXXXX in MeOH Vol Flask Ref:

LabX PDF in Stds Folder

I certify that the details on this form are correct.

Signature of Analyst Date:

Total Stocks Used uL	
THCA	100.0
CBDA	100.0
THC	100.0
CBD	100.0
CBN	100.0
CBG	100.0
CBC	100.0
THCV	100.0

5. APPARATUS

5.1. Grade A volumetric glassware (5-100mL), Grade B volumetric glassware or better for larger volumes. Volumetric flasks shall comply with ISO 1042. Use of volumetric glassware shall comply with AS 2162.

5.2. Diluter/dispenser station, Hamilton Microlab 500 series or equivalent.

5.3. Auto pipettes, positive displacement, 20-5000 µL capacity (Eppendorf Multipette XStream or equivalent).

5.4. Pasteur pipettes

5.5. A vortex

5.6. A sonicator

5.7. Analytical balance capable of weighing to a 0.00001 g (0.01 mg).

5.8. 2 mL amber sample vials.

5.9. 12 mL Pyrex tubes or similar.

5.10. 10 mL culture tubes

5.11. 0.22 µm PTFE syringe filters

5.12. A centrifuge for the 12mL Pyrex tubes

5.13. A rotary mixer.

5.14. Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm (or equivalent)

5.15. Agilent Poroshell 120, UHPLC Guard.EC-C18, 3.0 mm (guard column) (or equivalent)

5.16. A blender/grinder.

5.17. An Agilent 1290 Infinity II LC/DAD system with OpenLabs CDS software or equivalent TGA compliant system.

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6. SAMPLING, SAMPLE STORAGE, PREPARATION AND DISPOSAL

The sample is homogenised before commencing any analysis. For dry Cannabis products this involves blending approximately 5-10 grams of the dried vegetable matter to a fine powder to be extracted for analysis. If there is less than 5 grams total dried Cannabis in a sample, the whole sample should be homogenised. Liquid resin and oil should be thoroughly mixed where possible before sampling. NOTE: these are general procedures only and more specific arrangements for sampling may be required for some pharmaceutical clients.

6.1 Medicinal Cannabis

Medicinal Cannabis samples submitted to the laboratory will potentially vary in form. These may be pre-milled and so homogenisation is not required and storage will depend on the client's needs. Clear instruction as to storage and homogeneity must be sought in writing from the client in each instance.

6.2 Evidential Cannabis

Evidential Cannabis samples are submitted to the laboratory and have normally been sub-sampled from a bulk seizure by law enforcement officers. For large Cannabis seizures AFDL chemists may be required to select representative samples. For seizures that include a large quantity of samples a representative sampling technique should be employed. For a guide to subsampling see the United Nations Office on Drugs and Crime manuals.[8]

For legislative reasons it's important to determine the individual THCA and THC (or other cannabinoid) content within these samples and so based on literature[4-6] storage of samples in freezer condition will greatly slow the conversion of acidic cannabinoids to the neutral species.

It is important to maintain a chain of custody (COC) when storing Cannabis samples and so samples stored in the freezer (or locations other than individual analyst lockbox or AFDL vault) must be properly secured with NMI seals between sampling and the correct AFDL COC procedures followed.

All samples must be allowed to come to room temperature before sampling.

In some cases a pre-arranged procedure may be in place for disposal, return or destruction of samples that have completed analysis. In the instances when the procedure has not been pre-arranged, the disposal, return or destruction of samples must be agreed upon with the client in writing.

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