Application Note

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ePrep | Application 2022
Fully Automated Sample Preparation of Cannabinoids and Terpenoids from Cannabis Flowers

Pub No. 98-35028 Rev 01

SUMMARY

A fully automated robotic sample preparation method that includes extraction and filtering steps has been developed and validated for the analysis of cannabinoids and terpenoids from cannabis flowers. The ePrep robotic system extracts, filters and prepares the final solution directly into 2mL autosampler vials in an autosampler rack suitable for the chromatograph. The THCA was analysed by HPLC and the terpenoids were analysed by a GC-MS.

ePrep Sample Preparation Workstation

The procedure is described in detail thereby allowing any lab to modify steps in the workflow, to meet their laboratories' Standard Operating Procedures (SOPs) or local regulatory requirements. The analytical data from the automated ePrep procedure is compared to an Australian regulated (GMP) validated method. The average concentration of ten replicate analyses of Tetrahydro Cannabinoid acid (THCA) was found to be 25.4% (based on dried weight) which compared with 25.6% for the GMP validated method (99.1% recovery). The average recovery for a range of terpenoids was found to be 97.5%.

The average Relative Standard Deviation of ten replicate analyses was found to be 1.2% for both THCA and the terpenoids. This RSD included sampling of the cannabis flower. With the sampling excluded, the RSD of the ePrep sample preparation and the HPLC analytical method was found to be 0.3%.

INTRODUCTION

There has been progressive legalisation of recreational cannabis use throughout North America and an increased awareness of the medicinal properties of cannabis extract and targeted cannabinoids throughout the world. This use has accelerated the need for validated testing methods and automation of the sample preparation. The ePrep robotic sample preparation system is ideally suited to this task with extraction, filtering, precise make-up-to volume capabilities**.** These are coupled with features such as a wash station and automated syringe change technology. The analytical glass syringe used by the ePrep is fully suited to aliquoting volatile organic solvents and piercing container septa which is not possible with pipetting systems. The final filtered solution can be prepared into 2mL vials in any LC or GC autosampler rack, ready for the chromatograph instrument's autosampler.

In parallel, samples were prepared using a validated manual extraction and filtering procedure (Australian GMP validated method) to compare the results with the ePrep extraction based on sample vortexing and automated filtering. The validated manual extraction procedure involved accurately weighing 0.5g of cannabis flower and quantitatively transferring to 100mL volumetric flask and then making up to the mark with 96% ethanol. The volumetric flask was ultrasonicated for 10 minutes, the supernatant allowed to settle, before filtering directly into a 2mL autosampler vial.

Each step in the automated workflow will be fully discussed with parameters such as aspiration and dispense rates, pause times of the plunger, priming and washing steps. This will give insight to the technical requirements of the workflow thereby allowing modification to suit a laboratory's specific requirements.

INSTRUMENTATION

The LC/DAD analyses for THCA were performed using an Agilent Technologies Infinity II 1290 UHPLC. The UHPLC system consisted of a binary pump (G7120A), low-carryover multisampler fitted with multiwash and 100-µL loop and metering device options (G7167B), thermostatted column compartment (G7116B), and Agilent OpenLab CDS software.

HPLC Method Conditions	
Column	Agilent Poroshell C18 100 x 3mm ID x 2.7µm
Column Temperature	30
Injection Volume (µL)	5
Autosampler Temperature	8
Mobile Phase	Water / MeOH gradient 0.1% Formic Acid
Gradient flow rate	0.5 ml/min
Analysis and re-equilibration time	15 min / 5 min
Diode Array Detector Parameters	
Wavelength (THCA)	270 nm

Table 1.

The GC/MS analyses for the terpenoids were performed using an Agilent Technologies GC/MS system. The system consisted of a 7890GC coupled to a 5977 mass spectrometer with Agilent Masshunter software.

GC Method Conditions	
Column	Agilent J&W DB-624 (30 x 0.32mmID with a 1.8µm film)
Column Temperature	Temp Program (50°C hold 1 min) @ 8°C/min to 200°C (hold)
Injection Volume (µL)	1 uL
Injection Mode	Split (20:1)
MS Mode	SIM

Table 2.

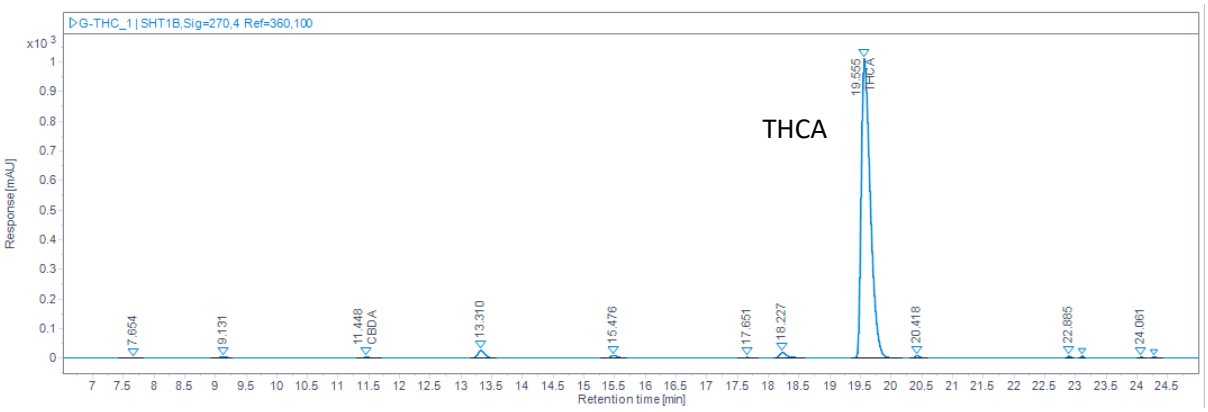


Figure 1. HPLC Chromatogram showing the elution of THCA

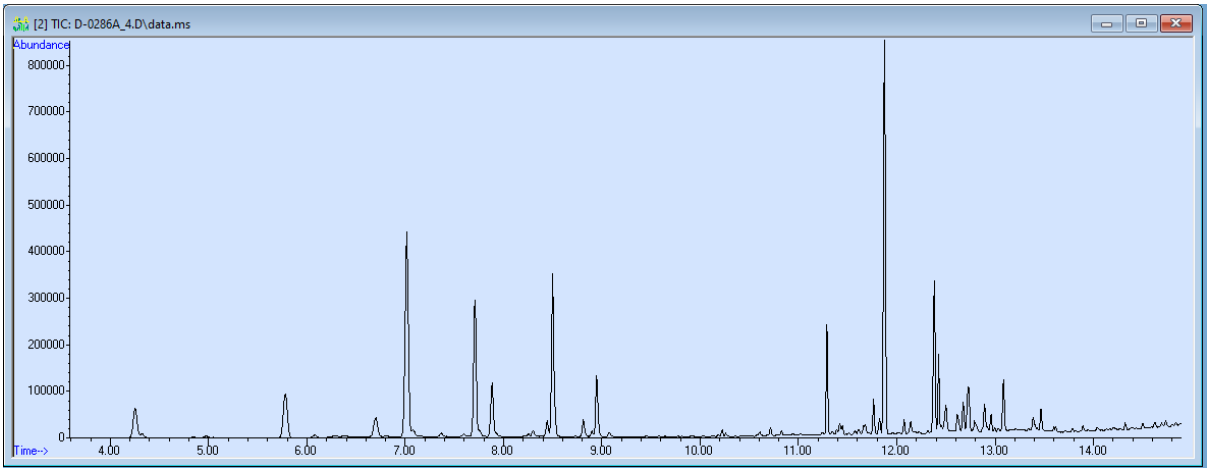


Figure 2. GC/MS Chromatogram showing the elution of Terpenoids (TIC of SIM ion windows)

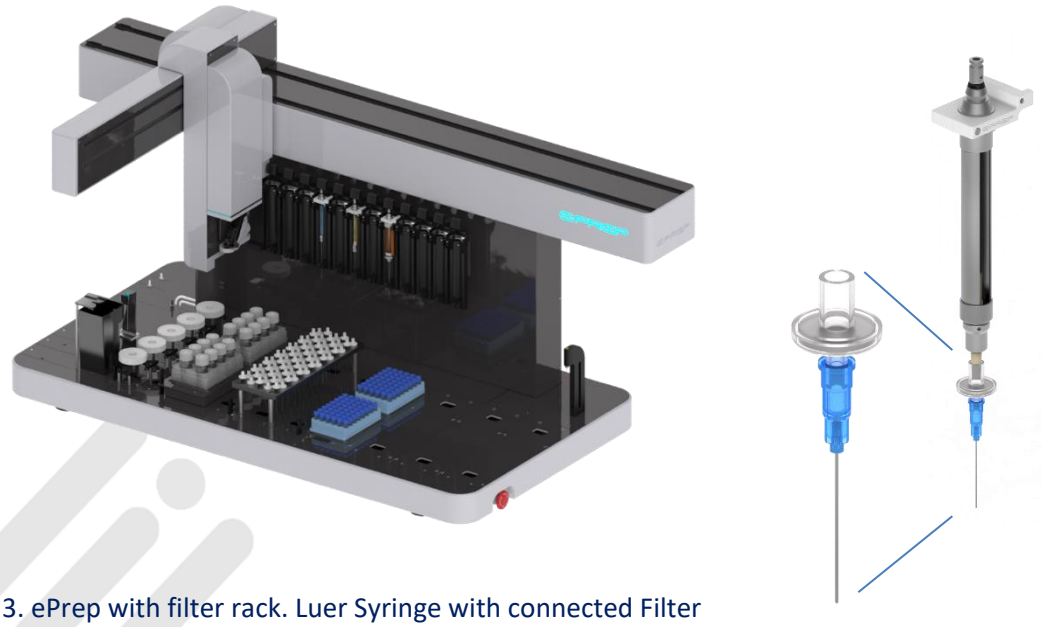
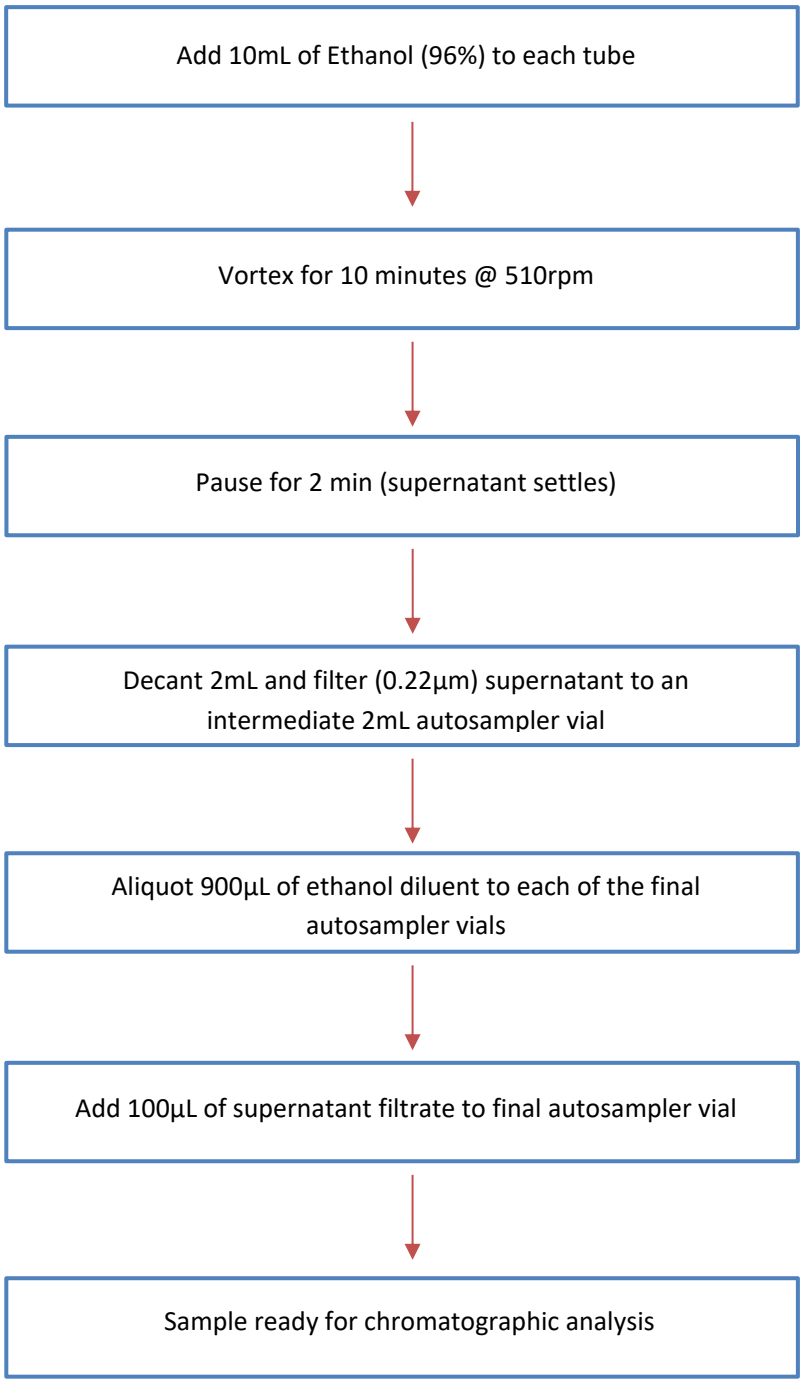


Figure 3. ePrep with filter rack. Luer Syringe with connected Filter

Summary of Automated Workflow

Note: samples were pre-weighed manually (approximately 0.5g on a four-figure balance) into 20mL enviro tubes prior to the ePrep workflow. The Workflow automates all liquid steps including extraction, filtering and preparation to the final vial.



Screen capture of the ePrep workflow

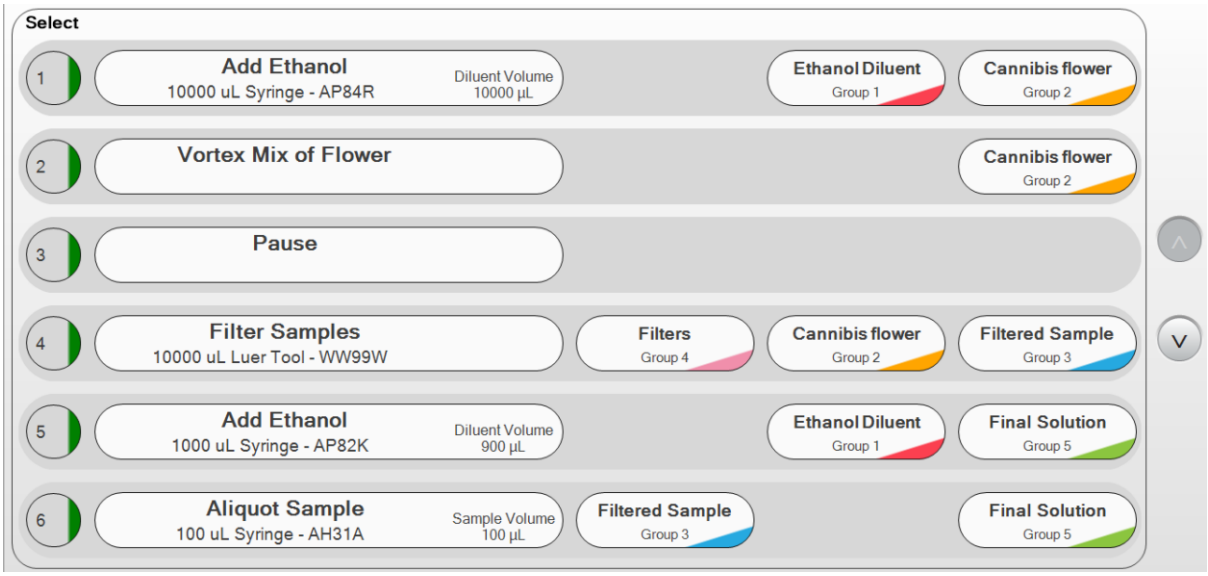


Figure 4. Screen capture of the ePrep deck for eleven cannabis samples (10 samples + 1 blank)

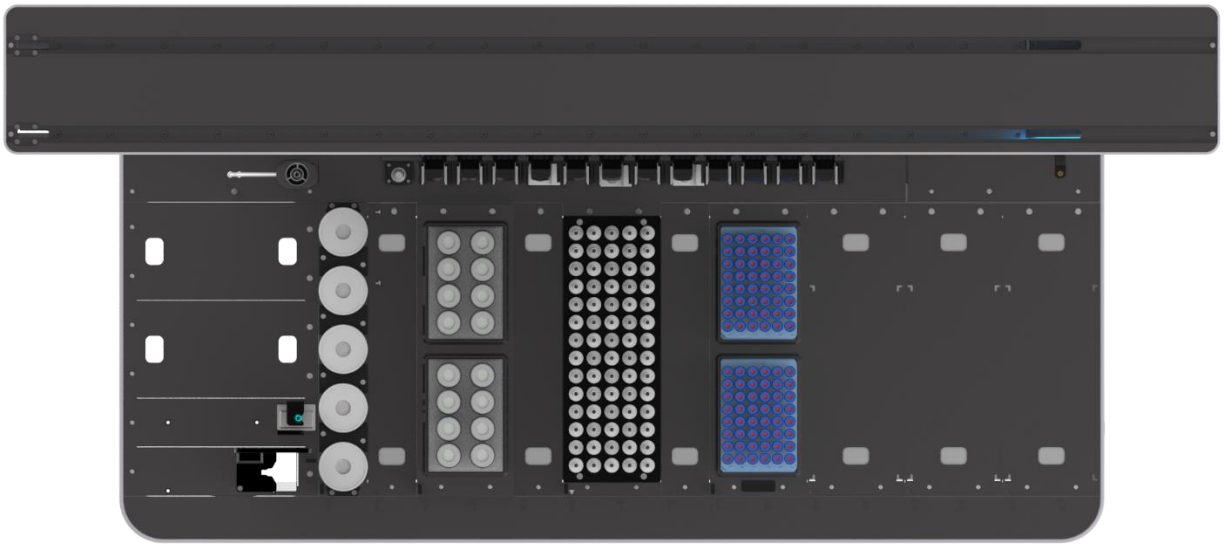


Figure 5. The layout of vials on the ePrep, the image is from the ePrep software.

DISCUSSION OF WORKFLOW

The settings of parameters at each stage of the workflow are very critical. The sample has been weighed into the 20mL tubes ready for the addition of the extraction solvent. In this workflow, it was decided to use 96% grade ethanol as the extracting solvent because the results will be compared directly against a TGA (Therapeutic Goods Administration Australia) GMP (Good Manufacturing Practice) validated method which also uses ethanol. The 10mL syringe used for the ethanol dispense is first washed at the wash station to ensure it is clean. The wash station contains ethanol / water (50/50). It is not necessary to wash the syringe between dispenses because only solvent is used. However, the dispense height of the needle into the tube is important as is the dispense rate of the ethanol. This is to ensure there is no splashing of the solvent from the flower back on to the needle which could cause cross-contamination. So, the needle was set as high as possible in the tube (10mm below cap) with a moderate dispense rate of 750µL/sec.

Once all tubes have received the extracting solvent, the tubes are vortexed for 10 minutes at a speed of 510rpm. The vortexing speed is important. If the speed is too low or too high, a vortex will not be produced, and this will affect extraction efficiency. If either the extraction solvent is changed or the volume of the solvent or the size of the extraction tube, the vortexing speed will need to be optimized. As a visual guide, once the vortex has reached the operating speed, the whole of the solid sample should appear in the vortex with little or no solid remaining on the floor of the tube.

It was found a pause time of 2 minutes was adequate for the flower sample to settle and the supernatant to be clear of particulates. This time, of course, is only critical for the first sample as the other samples will have much longer to settle as each sample gets filtered in turn. Too short a pause time for the first sample could cause blockage of the needle used to aspirate the supernatant into the filter syringe.

The software allows for setting of a filter hold-up volume and then the actual filtered volume. These two values were 700 and 1300µL, respectively. The hold-up volume could be dispensed into a waste tube but in our workflow, this was dispensed back (slowly) into the sample tube. It was observed the filters can have different hold up volumes so when choosing this parameter, it is best to set a higher filter hold-up volume to ensure the filter has been completely saturated. It also best practice to assume the filtered volume to a tube is not quantitative. So, for this reason rather than filter a defined volume into the final 2mL vial, the supernatant was first filtered into an intermediate vial and then a defined volume was aliquoted from the intermediate vial into the final vial.

The needle depth of the aspiration needle of the filter syringe into the supernatant and the aspiration flow rate are important. If the needle is too close to the top of the flower sediment or the aspiration rate is too high, then the flower bed could be disturbed causing flower sediment to also be aspirated. Depending on the severity of this, this could then cause blockage of the aspiration needle. The membrane used in the filter was 0.22µm PTFE. A pore size of 0.45µm could be used if the analytical technique is GC-MS but to fully protect a HPLC column and extend the column's longevity, a smaller pore size of 0.22µm is preferred.

To ensure no cross-contamination between filtered samples, the filter syringe coupled to the filter syringe was always washed in the wash station after a filtered sample. The wash station will both wash the interior of the syringe and needle and the exterior of the needle.

The make-up volume of 900µL of the ethanol diluent was first aliquoted to each of the final vials before transfer of the filtrate. As with the aliquoting of the extraction solvent, there was no need to wash the syringe between vials. For our workflow, we used a 1mL syringe for this task but if the time for the workflow needs to be reduced, a 10mL syringe could be used for this task and then the 10mL syringe would be filled for multi-dispense of 900µL volumes. Even though the 900µL is slightly lower than the recommended minimum volume of 10%, adequate accuracy and precision would still be achieved.

The ethanol diluent is added to the 2mL final vials first because it is good practice when aliquoting a sample, to dip the needle into the diluent on dispense to ensure complete transfer of the sample. If this is not done or the needle is above the surface of the liquid, there is a chance that a drop of the sample will be left hanging on the needle which will adversely affect the quantitative transfer. For smaller dispense volumes this variation can have a significant error so the default needle depth parameter in the software is 'Auto Low' for this reason.

The aspirate flow rate for the addition of 100µL filtrate to the final vial was set low at 10µL/sec with an aspirate pause time of 4sec. These are critical settings especially when using ethanol as a solvent. If the aspirate flow rate is set too high and / or the pause time is too short, cavitation can be seen in the syringe. This occurs because the plunger aspirate speed is too high for the viscosity of the liquid and the liquid will 'lag' behind the plunger tip. The pause time allows time for the liquid to 'catch up' to the plunger. By having a low aspiration rate and high pause time, there will be an extra degree of robustness in the aliquoting of the filtrate. These two settings are not as critical for priming and washing the syringe but are especially important when quantitative transfer is a must.

In our workflow, we were comparing the ePrep against a validated method which used 0.5g of cannabis flower weighed and transferred into a 100mL volumetric flask and then made up to the mark resulting in a dilution factor of 1:200. In our workflow we extracted 0.5g of flower into 10mL of solvent (1:20) and further diluted this in the final vial by taking 100µL of extract (after filtering) and diluting with 900µL of diluent (1:10) resulting in a final dilution of 1:200. These dilution factors can be changed possibly depending on the level of THC expected or the analytical technique used to measure the THC. In our case, the level of THC expected is very high (≈25%) as we were using a sample of medicinal cannabis. It is recommended as a starting point to change either the volume of the filtrate or the final volume (change the volume of diluent) if the dilution factor needs changing. As mentioned previously, changing the volume of the extraction solvent will affect the vortexing of the sample and the vortexing speed and time would need to be re-optimized compared with our workflow. Also, the amount of flower could be modified but with caution. For high THC-content flower which was used in our workflow, the flower is quite oily which can result in clumping and inhomogeneity and therefore lower sampling masses could result in sampling error.

This workflow is very adaptable and could include the addition of an internal standard step if required. The internal standard could be added directly to the ethanol diluent without any modification to the workflow. By using this method, the internal standard would be treated as a surrogate / internal standard as the standard would follow the extracted sample through each of the steps of filtering etc. Alternatively, an internal standard task could be added immediately after the 'add 900µL of diluent' step. If 100µL of internal standard solution was added, the diluent volume would need to be reduced to 800µL if the dilution factor was to remain unchanged. Adding the internal standard after the addition of the diluent would allow the syringe needle to be dipped into the diluent. Also, the inclusion of the internal standard at this stage of the workflow would save time as the syringe would only need to be washed and primed before the first addition. It would also make sense to dispense 100µL from a 1,000µL syringe so one aspirate would result in 10 dispenses and save workflow time.

RESULTS AND DISCUSSION

Potency Results

To test the accuracy and precision of the aliquoting and filtering in the workflow (independent of the cannabis flower sampling), into each of six tubes was aliquoted 10mL of a known concentration of CBD (cannabidiol) standard (400ppm) from a 50mL container and the standard solution was then filtered and made up to volume as described in the workflow. CBD was used in this case rather than THCA standard due to lower cost. A blank (Ethanol solvent only – no CBD) was analysed immediately after the six replicate analyses.

Samples (expecting 400 ppm)	Final Conc (ppm)
1	392.2
2	389.2
3	390.8
4	389.2
5	389.9
6	390.4
7 (blank)	1.3
SD	1.1
Average	390.3
RSD	0.3
Carryover (blank) %	0.3

Table 3. Results for six replicate analyses of standard THC A

From Table 3, the Relative Standard Deviation (RSD) of the six replicates was found to be 0.3% and the carryover was 0.3%. This RSD includes all steps of the ePrep workflow as well as the RSD of the HPLC analytical method.

For the sample preparation and analysis of THCA from cannabis flowers, ten samples were weighed and each sample was analysed in duplicate. The THCA results are contained in Table 4.

Sample #	%THCA	Extraction Efficiency (%)
1	25.333	99.0
2	25.393	99.2
3	25.901	101.2
4	25.289	98.8
5	25.312	98.9
6	25.079	98.0
7	25.285	98.8
8	25.754	100.6
9	24.784	96.8
10	25.530	99.7
11 (blank)	0.026	
Average	25.366	
SD	0.317	
RSD	1.2	
Average % Recovery		**99.1**

Table 4. Table showing extraction efficiency of THCA compared with a validated manual method

The average of the 10 replicate analyses was 25.4% and this compared to the validated GMP (Australian regulated method) value of 25.6%. This showed the extraction efficiency by vortexing the sample was excellent. The RSD was calculated at 1.2% which includes precision from sampling. The carryover of THCA from sample 10 to sample 11 (blank) was 0.1%.

Terpene Results

The same sample extracts were analysed for terpenoids by GC/MS and the results are contained in the table below. The extraction efficiency compared to the validated manual method was excellent with an average of 97.5% for 10 replicates averaged over all compounds.

Terpene	Average	Stdev	RSD %	GMP Result	% Extraction compared with GMP Result
alpha-Pinene	842.2	9.9	1.2	858	98.2
Camphene	42.4	0.5	1.2	43.5	97.5
beta-Pinene	474.3	5.6	1.2	483	98.2
3-Carene	202.2	2.4	1.2	207	97.7
beta-Myrcene	4276.9	50.5	1.2	4351	98.3
alpha-Terpinene	53.3	0.6	1.2	55.1	96.6
D-Limonene	1913.1	22.6	1.2	1948	98.2
Eucalyptol	< 10				
Z-beta-Ocimene	51.3	0.6	1.2	52.8	97.1
gamma-Terpinene	27.6	0.3	1.2	28.4	97.2
E-beta-Ocimene	642.0	7.6	1.2	658	97.6
p-Cymene	< 10				
Terpinolene	3132.0	37.0	1.2	3192	
(+/-) – Fenchone	< 10				
Linalool	661.7	7.8	1.2	681	97.2
Camphor	< 10				
Isopulegol	< 10				
Caryophyllene	2350.0	27.8	1.2	2401	97.9
Humulene	1822.4	21.5	1.2	1868	97.6
Terpineol	484.2	5.7	1.2	498	97.2
(+/-) – Borneol	63.1	0.7	1.2	65.1	96.9
Valencene	< 10				
Geraniol	< 10				
cis-Nerolidol	< 10				
trans-Nerolidol	181.5	2.1	1.2	187	97.0
Caryophyllene oxide	277.1	3.3	1.2	284	97.6

Guaiol	< 10				
alpha-Bisabolol	361.9	4.3	1.2	371	97.6
Average			**1.2**		**97.5**

Table 5. The extraction efficiency of various terpenoids compared with a validated manual method

CONCLUSION

The development of an automatic extraction / filtering / final sample preparation method using the ePrep automated sample preparation unit has been developed and described. This method is extremely flexible and allows for modification to suit the specific needs of a laboratory. Ways in which the method could be modified have been described herein. It was found that for the sample preparation of cannabis flower for the analysis of THCA and terpenoids, extraction efficiency was 99.1% and 97.5% against a manual validated method, respectively. The ePrep method is easily expandable to include dozens of samples which can all be prepared with unattended automatic operation.