

Article

Direct *in vivo* Analysis of CBD- and THC-Acid Type Cannabinoids and Classification of *Cannabis* Cultivars by SpiderMass

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Abstract: In the recent years, *Cannabis* and hemp-based products have become increasingly popular for various applications ranging from recreational use, edibles, beverages to health care products and medicines. The rapid detection and differentiation of phytocannabinoids is, therefore, essential to assess the potency, therapeutic and nutritional values of cannabis cultivars. Here, we implemented the SpiderMass technology for the *in vivo* detection of cannabidiol acid (CBDA) and tetrahydrocannabinol acid (THCA) and other endogenous organic plant compounds to access distribution gradients within the plants and differentiate cultivars. The SpiderMass system is composed of an IR- laser handheld microsampling probe connected to the mass spectrometer through a transfer tube. The analysis was performed *in situ* on different plant organs from freshly cultivated *Cannabis* plants in only a few seconds. SpiderMass analysis easily discriminated the two acid phytocannabinoid isomers by MS/MS and the built statistical models differentiated between four *Cannabis* cultivars. Different abundances of acid phytocannabinoids were also found along the plant as well as between different cultivars. All together, these results introduce the direct analysis by SpiderMass as a compelling analytical alternative for forensic and hemp industrial analysis.

Keywords: Water-Assisted Laser Desorption/Ionization; SpiderMass; cannabinoids; mass spectrometry; plants; *in vivo*

1. Introduction

The hemp plant or cannabis (*Cannabis sativa* L. and subspecies *indica*) has been cultivated for recreational, medicinal, and industrial purposes since ancient history and remains the most widely cultivated plant. The popularity of cannabis has increased over the last few years due to its unparalleled versatility of use, particularly for medicinal purposes.

Cannabinoids are synthesized from geranyl pyrophosphate and olivetolic acid and they are always present in the "acid" form in the plants, the most important being Δ^9 -tetrahydrocannabinol acid (THCA) and cannabidiol acid (CBDA)[1]. Out of many, "potency" remains the most frequently measured feature in *Cannabis* and is referring to the percentage of THC and CBD, respectively the decarboxylated forms of THCA and CBDA. Together THC, CBD and CBN (THC degradation product, cannabinol) are often monitored for legal or medicinal reasons. These cannabinoids display remarkable similarities as structural isomers but differ in their pharmacological properties. Indeed, only THC shows psychotropic effects while CBD and CBN have anti-inflammatory effects [2,3]. In addition, potency can also be reflected as a function of other plant constituents mainly terpenes and flavonoids present in different ratios in various cannabis strains or "cultivars". It is, therefore, important to also consider additional chemical characterization of

the cultivar composition in order to better decipher the pharmacological effects of cannabis products considering their growing medical applications [2,4].

Mass spectrometry (MS) has long been playing an important role as a sensitive and selective analytical technique in cannabinoids and other plant component analysis for the hemp industry, cosmetics and forensics [2]. Most frequently, cannabinoids are analyzed by gas chromatography (GC)[5] and liquid chromatography (LC) coupled to MS (GC-MS and LC-MS)[6,7]. For GC-MS, chemical derivatization is often necessary to make acid cannabinoids volatile enough and preserve their structure. However, the analysis of plant samples can be particularly time-consuming with these techniques since they require a multi-step sample preparation (drying, grinding, homogenizing, extraction etc.)[8].

Ambient Ionization Mass Spectrometry techniques, therefore, pose a great alternative since they enable for direct sample analysis while do not requiring any sample preparation. For example, desorption atmospheric pressure photoionization-mass spectrometry (DAPPI-MS) was used for direct analysis of THC and CBD in marijuana samples confiscated by the police. Thanks to the formation of molecular ions, the discrimination of THC and CBD was made possible by MS/MS analysis [9]. Alternatively, paper spray MS was used for the detection of THC and CBD in commercial oils through the use of an Ag(I)-impregnated paper [10]. Direct analysis or “swabbing” of cannabis leaves was also evaluated by DESI-MS [11] and atmospheric solids analysis probe (ASAP) using positive ion mode [2]. However, most of these techniques can only be foreseen as preliminary screening techniques for targeted compounds in hemp and marijuana samples [2]. Still, the clear differentiation and quantification of THC and CBD- acid derivatives in combination with other plant molecules remains challenging and incomplete. Recent reports suggest it is possible to differentiate between the cannabinoid isomers by accurate MS/MS fragmentation patterns in negative ion mode as a guideline for chemical characterization [12].

In this work, we present an analytical alternative, the Water-Assisted Laser Desorption Ionization Mass Spectrometry (WALDI-MS), for the direct detection from the plants without any sample preparation of tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA), and other plant components. The WALDI-MS (or SpiderMass) is designed for *in vivo* and real time analysis under minimally invasive conditions of biological organisms [13,14]. The instrument achieves real-time metabolomic analysis through the combination of an IR-laser microprobe and a transfer tube on a MS instrument. Though the SpiderMass system has predominantly been developed for *in vivo* and real-time applications in oncology[15], it can be applied to a variety of different research areas such as dermatology, cosmetics, food safety, drug pharmacokinetic, toxicology and forensics. Here, we apply the SpiderMass technology in combination with real-time MS/MS to detect and discriminate THCA and CBDA directly in plants. We further show the different level of cannabinoids across the plant and the possibility to classify different *cannabis* cultivars based on their metabo-lipidomic profiles.

2. Result

2.1. Analysis of cannabinoid standards

To verify the detection of cannabinoids with SpiderMass we first performed direct analysis of Δ^9 -tetrahydrocannabinolic acid (Δ^9 -THCA) and cannabidiolic acid (CBDA) standards. These were analyzed in methanol with the addition of 1 μ L glycerol to stabilize the droplet by lowering the volatility and increase the ionization efficiency since the standards do not contain water. The MS spectra of the two standards in negative ion mode are presented in **Figure 1**. The MS spectra all contain the deprotonated Δ^9 -THCA (**Figure 1a**) and CBDA (**Figure 1b**) peak at m/z 357.21 as base peak. Since the two bioactive compounds are constitutional isomers, their molecular ions appear at the same m/z . Thereof, the only way to distinguish between the two of them is through MS/MS analysis where each compound displays a specific fragmentation pattern. The MS² spectra of the 2 standards are shown in **Figure 1c** and **1d**. The fragmentation pattern of CBDA revealed a base peak at m/z 245.16. For Δ^9 -THCA the most intense peak corresponds to the m/z 313.22

fragment which is attributed to the neutral loss of 44 Da (CO_2) resulting in the deprotonated Δ^9 -THC molecule $[\text{C}_{21}\text{H}_{30}\text{O}_2\text{-H}]^-$. However, Δ^9 -THCA also displays a peak at m/z 245.16 about 4-5 less intense though in comparison to CBDA. Both fragmentation spectra also indicate the presence of m/z 339.20 fragment corresponding to $[\text{M-H-H}_2\text{O}]^-$ and a subsequent loss of 28 Da (CO) generating the $[\text{C}_{21}\text{H}_{28}\text{O}_2\text{-H}]^-$ ion at m/z 311.21 (to $[\text{M-H-H}_2\text{O-CO}]^-$). There is also a subsequent transition from m/z 313.22 to the m/z 245.16 by a neutral loss of 68 Da (C_5H_8) due to double hemolytic cleavage from the six membered rings. It was previously demonstrated in the literature, that THCA leads to a MS/MS spectrum dominated by a m/z 313.22 ion while CBDA base peak is the m/z 245.16. Besides, for THCA it was shown that the m/z ratio $339/313 < 1$ and the m/z ratio $313/311 > 1$ and that for CBDA these are inversed (the m/z ratio $339/313 > 1$ and $313/311 < 1$) [12]. Here, we indeed observe the predominance of the m/z 313.22 for THCA and the m/z 245.16 for CBDA though the m/z 339/313 ratio is $\ll 1$ and the m/z 313/311 ratio $\gg 1$ for THCA and these are close to 1 for CBDA. This is attributed to the difference in the ion internal energy of the parent ion due to the WALDI process and the used collision energy. However, there are still marked difference between the MS/MS spectra of the 2 isomers and this offers a way to assess the predominance of THCA or CBDA in situ from the plant while not using any separative step.

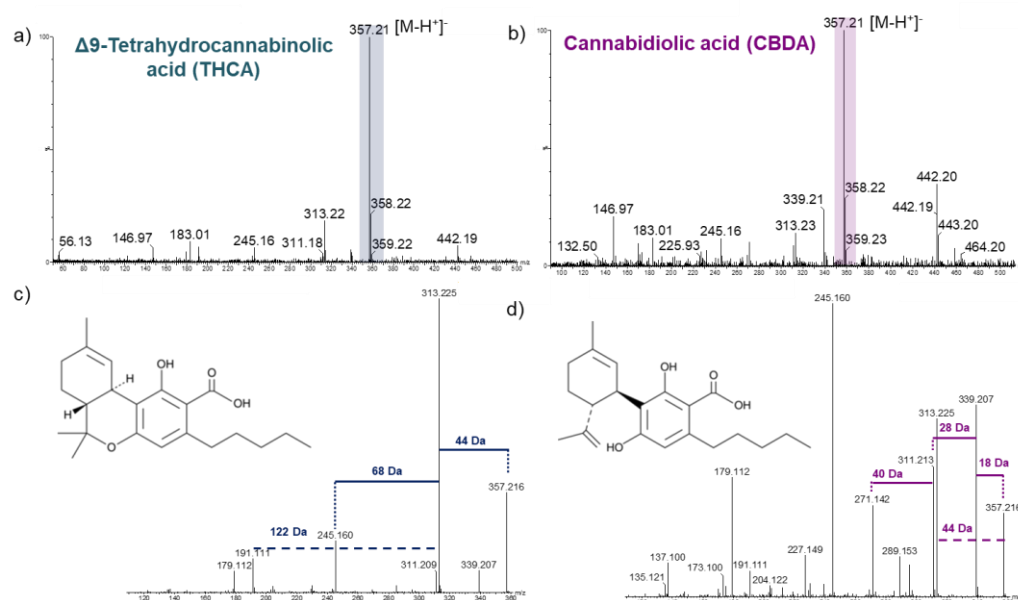


Figure 1. Cannabinoid mass and fragmentation negative ion mode spectra. A) Δ^9 -Tetrahydrocannabinolic acid (Δ^9 -THCA) and b) Cannabidiolic acid (CBDA). There is a clear molecular peak at m/z 357.21 corresponding to the $[\text{M-H}]^-$. The fragmentation spectra of m/z 357.21 precursor ion for each cannabinoid are shown in c) and d) respectively

2.2. Detection of acid cannabinoids across the plants

The SpiderMass analyses were performed on four different plant organs (**Figure 2**): flower, bulb (the outermost whorl of parts that form the flower), sugar leaf (leaves developing around the flower) and three spots from the fan leaf (**Figure 2a**). In this way, we captured the molecular profiles across parts of the plant known to have the highest cannabinoid concentrations. Molecular differences were clearly visualized for each analyzed part. An example is shown in **Figure 2b and c**. The flower, bulb and sugar leaf show relatively similar profiles in contrary to the fan leaf. In the MS spectra of the sugar leaf, bulb, and flower there is a very noticeable peak of high intensity detected at m/z 357.22. This peak corresponds to the deprotonated THCA and CBDA isomers. On the other hand, only a low intense m/z 357.22 is observed across the fan leaf. The fan leaves, however, do display rich metabolic spectra within the m/z 200-900 range compared to the other parts of the plant. Additional principal component analysis (PCA) confirmed the spectral

observations. The fan leaves ($n = 39$) show a clear separation from the flower ($n = 42$), bulbs ($n = 28$) and sugar leaves ($n = 37$) (**Figure 3a**). The PCA 1 displays 60.8% of variance mainly governed by the m/z 357.22 and the m/z 245.15 ions in the loading plot (**Figure 3b**) and loading-mass plot (**Figure 3c**). Further statistical tests (**Figure 3d**) showed a significant difference in relative intensities comparing flowers ($p < 0.0001$), bulbs ($p < 0.0001$), and sugar leaves ($p = 0.0005$) to fan leaves. The significant difference is also observed between bulb and sugar leaves ($p = 0.0341$).

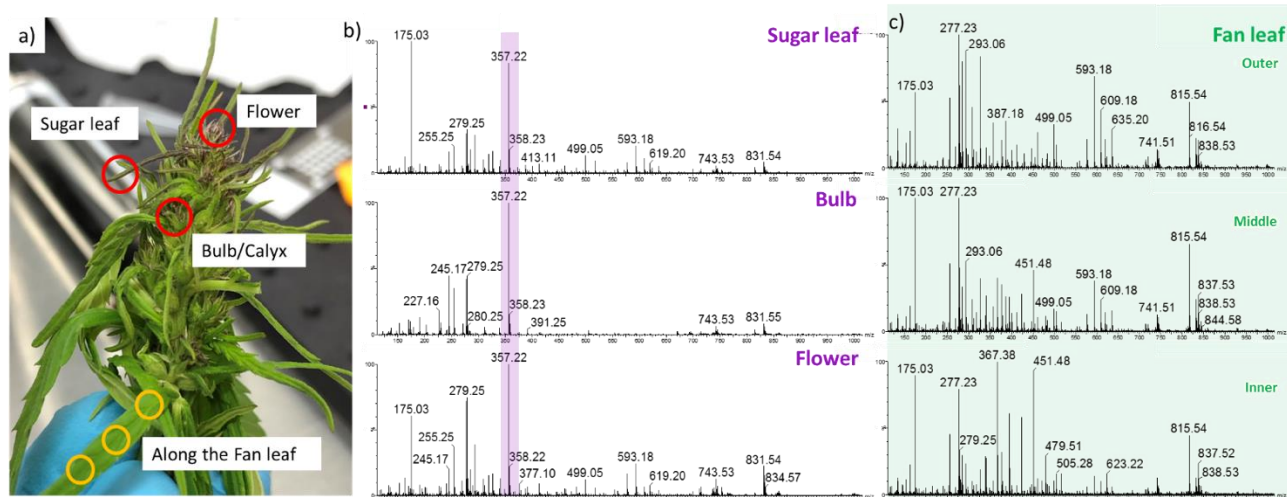


Figure 2. *in vivo* SpiderMass analysis on cannabis plants. (a) Photo of a cannabis plant with indicated plant organs and measurement points (red and yellow circles). (b) Examples of negative ion mode mass spectra from the sugar leaf, bulb, and flower, respectively. The highlighted area shows the m/z 357.21 ion that corresponds to the mixed signal of THCA/CBDA. (c) Examples of negative ion mode spectra recorded along the fan leaf (outer, middle, and inner).

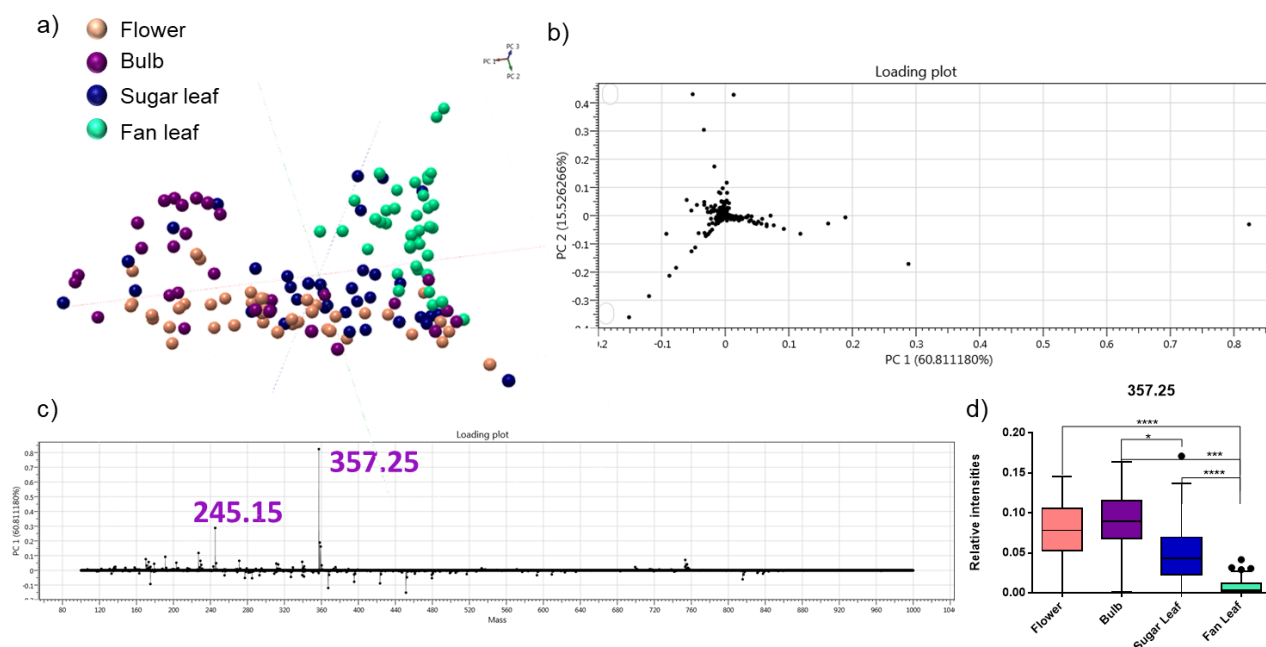


Figure 3. Multivariate statistical analysis of the molecular profiles from different plant organs combining all cultivars. (a) PCA plot and (b) PCA loading plot of 4 plant organs (flowers, bulbs, sugar leaves and fan leaves) from all cultivars. (c) PC1 loading plot showing the highest variance between the flower (rose), bulb (purple), sugar leaf (blue) and fan leaf (green) due to the cannabinoid acid peak at m/z 357.25 and m/z 245.15. (d) Relative intensity boxplots of the selected peaks showing the highest contribution to the variance of PC1. The intensities were

normalized to the total ion chromatogram and represented as boxplots for each year with Tukey method whisker definition. There was a significant difference for m/z 357 between the flower ($***p = < 0.0001$), bulb ($***p = < 0.0001$), sugar leaves ($***p = 0.0005$) to fan leaves. The significant difference is also observed between bulb and sugar leaves ($*p = 0.0341$)

2.3. Discrimination of Cannabis cultivars

Four cannabis subtypes were selected to create metabolic/lipid-based classifiers. The raw spectra of all four subtypes (Felina (n = 4), Fedora (n = 4), Finola (n = 4) and USO (n=4)) and all regions (flower, bulb, sugar leaf and fan leaf) were imported into a single classification model. The 3D LDA representation of the 4-class PCA-LDA analysis of both positive and negative ion mode is shown in **Figure 4a and b**, respectively. A substantial discrimination was observed in LD1 for Finola compared to the remaining subtypes. The second component (LD2) discriminates Fedora in positive and Felina in negative ion mode. The loadings plot of LD1 are shown in **Supplementary data Figure S1**.

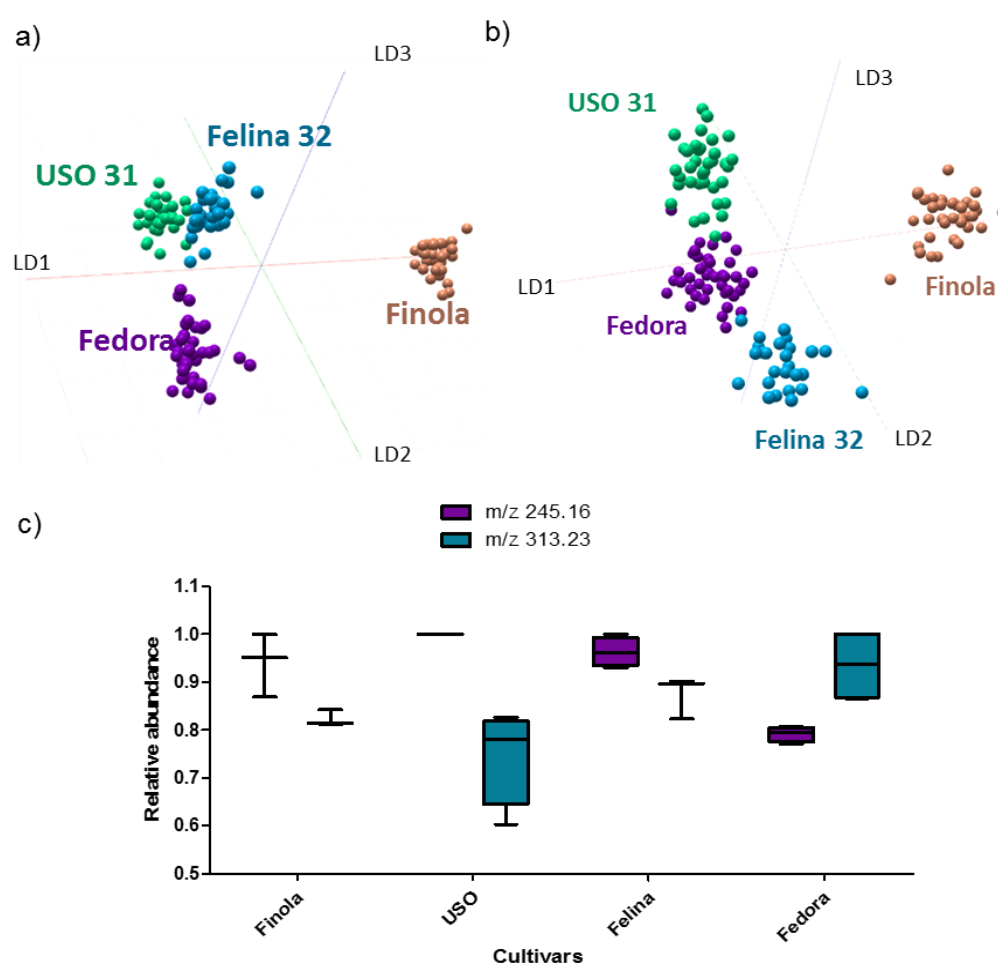


Figure 4. Multivariate statistical analysis of molecular profiles from different cannabis plant cultivars. LDA Representation of the 4-class PCA-LDA including Finola, USO31, Felina 32 and Fedora in (a) positive and (b) negative ion modes. In both polarities the Finola subtype is well discriminated in LD1 (c) Relative abundances of the MS/MS fragments m/z 245.16 and m/z 313.23 for each cultivar.

Several ions such as fatty acids and lipids are contributing to the discrimination between the different cultivars and the weight of the m/z 357.22 was not found to be important. We further investigated the MS/MS fragmentation spectra from each flower to evaluate if they do present different levels of the acid phytocannabinoid isomers THCA and CBDA (**Supplemental Figure 2**). This was achieved using the rapid and straightforward workflow that is based on the evaluation of the diagnostic ions, m/z 339.21, 313.23

and 311.21, as previously discussed for the standard molecules [12]. Diagnostic ions relative intensity and, m/z 339/ 313 and m/z 313/ 311 ratios are provided in **Supplemental Table S1**. The relative abundances of indicative CBDA base peak ion m/z 245.16 and the indicative THCA base peak m/z 313.23 are shown in **Figure 4c**. For Finola and Fenola, the m/z 313/ 311 ratio is close to 1 while it is much above 1 for USO and Fedora. Regarding the m/z 339/ 313 ratio is about 1.2 for Finola, USO and Felina though it is about 1 for Fedora. m/z 313 is the predominant ion only for Fedora while for Finalo, Fenola and USO the m/z 245 is prevalent with a peculiarly marked difference with the m/z 313 ion for USO. This indicates that both THCA and CBDA are present in the different cultivars though at different levels for each of them.

3. Discussion

We have demonstrated the possibility to analyze cannabis plants in situ using the SpiderMass. Since the analysis is mini-invasive it can be directly performed on plant organs. Similarly, to mammalian tissue the plants can retain between 98% in fresh and up to 30-40% of water in severely dehydrated leaves which is necessary to induce the Water-Assisted Laser Desorption Ionization process by SpiderMass [14]. The analysis of each plant organ (flower, bulb, sugar leaf and fan leaf) resulted in a metabolic-rich spectrum. In the first instance all the spectra from different cultivars were used to assess the presence of the cannabinoids. The raw spectra and further multivariate statistical analysis immediately revealed the presence of the m/z 357.22 attributed to THCA and CBDA in all cultivars but with a much higher intensity in the flowers, bulbs, and sugar leaves. There was a significant difference found in relative intensities when comparing flower ($p < 0.0001$), bulb ($p < 0.0001$), sugar leaves ($p = 0.0005$) to fan leaves. This demonstrates the change of abundances of cannabinoids along the cannabis plant and confirms that the highest phytocannabinoid concentrations are found in the flowers [18].

Regarding the different cultivars, we were able to discriminate them through their molecular profiles and assess the relative abundances of tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA) directly *in vivo* in the negative ion mode. If both isomers cannot be separated in MS where they share the deprotonated molecular ion at m/z 357.22; they can be discriminated by their fragmentation pattern since Δ^9 -THCA shows a base peak at m/z 313.21, while for CBDA it is at m/z 245.15 as expected from previous works [12,16]. Piccolella et al. found that the most useful information to discriminate the isomers is obtained when the parent ion fragmentation reaches about 70–75 % (at 35–45 V CE using nitrogen gas) in our case the same effect was observed when using CE=25 V with Argon gas. Indeed, the observation of diagnostic fragment ions with specific intensities is linked to their center-of-mass energy which depends on the parent ion mass, the mass of the target gas for CID and the used collision energy (inter quadrupole voltage difference) [17]. In addition, Piccolella et al. also described the ratios of m/z 339.20 $[M-H-H_2O]^-$ / m/z 313.22 $[M-H-CO_2]^-$ to be <1 for THCA and >1 for CBDA while the m/z ratio 313/ 311 is >1 for THCA and <1 for CBDA. We first examined these ratios from the standard molecules and found that in our experimental condition (CE=25V with Ar from ions formed by WALDI) the m/z ratios 339/313 and 313/ 311 are close to 1 for CBDA. Then we extracted these ratios to evaluate the presence of CBDA and THCA in each of the four cannabis cultivars (USO32, Felina, Fedora and Finola) that were directly analyzed by SpiderMass. Because they do not use any separative technique, the diagnostic ions can be both issued from THCA and CBDA and the calculated ratios are composites. Still, we were able to observe difference between the different cultivars and Fedora (predominant m/z 313 ion, m/z ratio 313/ 311 close to 1.5 and m/z ratio 339/ 313 close to 1) is the subtype presenting the highest content of THCA while more limited content of CBDA. Finola and Felina are quite similar while USO appears to have both high content of CBDA and THCA. On the other hand, the comparison of the cultivar based on their global molecular profiles shows that Finola demonstrates the higher discrimination out of all the cultivars in both

positive and negative ion mode. The most discriminative peaks correspond to either small metabolite species or lipids present throughout the whole plant. It has already previously been demonstrated that the presence of certain terpenes, flavones and other organic molecules influence the production and ratios of THCA and CBDA present in different varieties and are also responsible for the distinctive scents [2,4,19,20]. Therefore, it is not unusual to find different abundances of THCA and CBDA in varieties of cannabis plants, where for example USO showed a higher relative abundance of the CBDA base peak m/z 245.17, while Fedora display a higher abundance of the THCA base peak m/z 313.23. Since the PCA-LDA plots also show a high degree of separation for each cultivar they can be adapted for *in vivo* analysis of plant cultivars in hemp industry and for discrimination of cultivars in forensic analysis. Although the SpiderMass technology was primarily developed for intraoperative analysis it can also be used in nutritional, forensics, and food industry. Several intraoperative techniques have already been applied for example for the determination of food authenticity [21–23]. The results clearly demonstrate the sensitivity and versatility of the SpiderMass technology.

To conclude, this first demonstration of the SpiderMass *in situ* on plants has shown the possibility to unambiguously differentiate between THC- and CBD- acid cannabinoids and *Cannabis* cultivars. Very interestingly, the method is rapid and doesn't require any sample preparation while providing spatial information. The SpiderMass is, therefore, a sensitive and selective screening technique for targeted and untargeted analysis in cannabis samples to be used in the future for hemp industry and forensic analysis.

4. Materials and Methods

Cannabinoid standards

Δ^9 -Tetrahydrocannabinolic acid (Δ^9 -THCA) and Cannabidiolic acid (CBDA) standards at 1mg/ml were purchased from LGC (Molsheim, France). The samples were stored at -20°C .

Cultivation of cannabis

Four *cannabis* plants of each subtype or "cultivar" (USO, Finola, Fedora and Felina) were provided by the local Luxemburg hemp producer. All plants were cultivated outdoors and harvested on 11 July 2021. Samples were cooled at 5°C to be preserved until analysis which was achieved within less than 48H after the samples were harvested.

SpiderMass and data analysis. The basic design of the instrument setup is described in detail elsewhere [13,14]. For the standards, the analysis was conducted from 1 μL of standard deposited with 1 μL of glycerol onto a Prosolia Omni TM glass slide. For the plants, different regions were analyzed, including the flower, the bulb, the sugar leaves and along the fan leaf, by directly placing the laser at the right focal distance from the laser probe. For any sample, the acquisition was composed of 10 laser shots consecutively fired (i.e. 1s) repeated 3 times with a 10 s pause between each series. Ten laser shots result in one individual MS spectrum. The laser energy was fixed at 4 mJ/pulse and the spot size was 500 μm . The data was acquired in both polarities in sensitivity mode in the m/z 100-2000 mass range. Four independent biological repetitions were realized for each *cannabis* plant subtypes. The raw data were then imported into "Abstract Model Builder" - AMX (version 1.0 1972.0, Waters, Hungary). The classification model was built using individual spectra (on average 3 per sample) and by subjecting them to principal component analysis (PCA) and linear discriminant analysis (LDA). The classification model was built using a mass range of 100-1000 m/z with a 0.1 binning, $1e^3$ threshold intensity, applied normalization to the TIC and background subtraction. An isopropanol solution of 1ng/ μL Leu-enkephaline peptide was used as lock mass correction. Non-parametrical one-way ANOVA (Kruskal-Wallis) followed by Dunn's test was performed to calculate significant differences between normalized intensities for discriminative ions using GraphPad Prism 5. The cannabinoids peaks were selected for MS/MS analysis with a 0.1 Da isolation window. MS/MS was performed with collision-induced dissociation (CID) using Argon as collision gas and a

collision energy set to 25 V. The fragmentation spectra were then further used to evaluate the presence of CBDA and THCA based on the ratios of neutral losses 18 Da/44 Da and 44 Da/46 Da. Relative abundances were calculated for the m/z 245.26 and 313.23 fragment ions for each cultivar based on the base peak from the fragmentation spectra.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: LD1 loading mass spectra showing the discrimination between the Finola and the rest of the cultivars. Figure S2: MS/MS fragmentation spectra from the flowers of each Cannabis subtype.

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Data Availability Statement: In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Please refer to suggested Data Availability Statements in section “MDPI Research Data Policies” at <https://www.mdpi.com/ethics>. You might choose to exclude this statement if the study did not report any data.

Conflicts of Interest: NO, SS and AB declare no conflict of interest. M.S. and I.F. are inventors of the patent (priority number WO2015IB57301 20150922) related to part of the described protocol.

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