APPLICATION NOTE

Identification and quantification of different cannabinoids in *Cannabis sativa*

A-108.1

Keywords

Cannabinoids, HPTLC-MS, hemp, marihuana, hashish, CBD, CBN, CBC, CBG, THCV, CBDA, limit test, cannabinol, tetrahydrocannabinol, cannabidiol

Introduction

Cannabis sativa, the hemp plant, is native to Central Asia and the Indian subcontinent. Throughout history, hemp has widely been used for a broad range of purposes, from production of textile fibers to relief of pain. Cannabis resin (hashish) has been in disrepute because of its intoxicating effect and it is prohibited in most countries. Currently, discussions about the legalization of cannabis for medicinal use have started worldwide. Some countries allow cannabis for the treatment of various diseases such as multiple sclerosis, cancer, epilepsy, etc. [1]. The effect is based on the cannabinoids, of which cannabidiol (CBD), Δ9-tetrahydrocannabinol (THC), and cannabinol (CBN) are studied best. Through years of research, different genetic strains of *Cannabis sativa* have been developed, in which the content of cannabinoids varies according to the intended usage. In Europe industrial hemp may not contain more than 0.2% of THC whereas in the US and Switzerland the limit is set to 1%. Medical cannabis can contain varying amounts of THC, CBD, CBN, and other cannabinoids used for different medical applications. For illicit use as a drug, there are varieties with a high content of THC. In response to the increasing interest on the use of *Cannabis sativa* extracts in industry and health sciences, we have developed a fast, simple, and reproducible HPTLC method for identification of *Cannabis sativa* strains. Quantitative evaluation is done by scanning densitometry and confirmation by HPTLC-MS and UV spectra.

Scope

The methods described below are suitable for the quantification of THC and THCA (and other cannabinoids) in *Cannabis sativa* and to check THC-free Cannabis for compliance. The same methodology can be used for identity testing of sample extracts and comparison with reference materials. The CAMAG TLC-MS Interface 2 is used to directly elute target zones from the HPTLC plate into the Waters ACQUITY QDa® for mass detection. A second confirmation can be achieved by recording the UV spectra with the TLC Scanner.

Required or recommended devices

Automatic TLC Sampler 4, Automatic Developing Chamber ADC 2, Chromatogram Immersion Device 3 or Derivatizer, TLC Plate Heater 3, TLC Visualizer 2, visionCATS, TLC Scanner 4, UV Cabinet 4, TLC-MS Interface 2, Waters ACQUITY QDa Detector (Performance), Empower® or MassLynx® software

Sample

500 mg of each dry and homogenized herbal Cannabis sample are extracted with 5 mL of methanol - hexane 9:1 (v/v) by the following procedure:

10 seconds on a vortex, 15 min ultrasonic bath including again vortex after 5, 10 and 15 minutes, then centrifugation.
Decarboxylation (if required): In an auto sampler vial evaporate 1 mL of extract with nitrogen to dryness, heat the vials in an oven for 15 minutes at 210°C, after cooling dissolve in 1 mL of methanol - hexane 9:1 (*v/v*)
2 µL of samples are applied for identity testing. Prior to quantification and limit test samples are diluted 1:10 with methanol - hexane 9:1, then 2 µL are applied

Other samples: cannabis tincture and cannabis oil are diluted with methanol (1:10 resp. 1:20); 200 mg of cream are extracted with 2 mL of methanol – hexane 9:1; 0.4 g of the intermediate from CBD extraction (mother liquor) are dissolved in 2 mL of methanol – hexane 9:1, 0.2 g of CO_2-extracted CBD are dissolved in 2 mL of methanol – hexane 9:1

Standards

For quantification and limit test: each standard is dissolved in methanol at a concentration of 10 ng/µL (THCA and CBDA with 100 ng/ µL)
Standards were provided by Lipomed AG (Arlesheim, Switzerland).
THCV was purchased from Sigma.
For System Suitability Test (SST): mixture of CBD, THC, CBN (each 100 ng/µL) in methanol

Chromatography

Stationary phase	HPTLC Si 60 F_{254}, 20 x 10 cm (Merck) alternative: HPTLC Si 60 RP-18 F_{254}, 20 x 10 cm (Merck)
Sample application	Bandwise application with ATS 4, 15 tracks, band length 8 mm, track distance 11.4 mm, distance from left edge 20 mm, distance from lower edge 8 mm, application volume 2 µL for sample and between 2-10 µL for standard solutions.
Developing solvent	(1) *n*-heptane - diethyl ether - formic acid 75:25:0.3 (or UN B method: cyclohexane - di-isopropyl ether - diethylamine 52:40:8 [3]) (2) RP-18: methanol, water, acetic acid 70:15:15
Development	In the ADC 2 with chamber saturation (with filter paper) 20 min and after conditioning at 33% relative humidity for 10 min using a saturated solution of magnesium chloride.
Developing distance	70 mm (from the lower edge)
Plate drying	Drying 5 min in the ADC 2
Documentation	With the TLC Visualizer under UV 254 and UV 366 nm prior derivatization and white light after derivatization
Densitometry	Densitometric analyses are performed with the TLC Scanner at 210 and 285 nm for quantification (multi-wavelength scan), slit dimension 5.0 x 0.2 mm, scanning speed 20 mm/s, spectra recording 190 to 450 nm
Derivatization	Reagent name: Fast Blue Salt B (FBS) *Note: not suitable for reversed-phase* Reagent preparation (dipping): Weigh 1 g of FBS (o-dianisidine bis(diazotized) zinc double salt) into a glass bottle and dissolve it in 200 mL of water.

Reagent use: The plate is immersed into freshly-prepared FBS reagent using the Chromatogram Immersion Device, immersion time 5 s and immersion speed 3 cm/s and then dried in flow of cold air in the fume hood for 5 minutes.

Reagent preparation (spraying): Dissolve 250 mg of FBS in 10 mL of H_2O and mix with 25 mL of methanol and 15 ml of dichloromethane.
Reagent use: The plate is sprayed with 2 mL of freshly-prepared FBS reagent with the Derivatizer, green nozzle, spraying level 3.

Alternative derivatization reagents suitable for straight-phase and RP-18:

a) Reagent name: Ehrlich reagent

Reagent preparation: 0.5 g of 4-dimethylaminobenzaldehyde are dissolved in 150 mL of methanol and 50 mL of hydrochloric acid (37%) are added
Reagent use: The plate is sprayed with 2 mL of Ehrlich reagent with the Derivatizer, yellow nozzle, spraying level 5 and heated for 5 min at 100°C on the TLC Plate Heater

b) Reagent name: Vanillin-sulfuric acid reagent (Anisaldehyde reagent can also be used)

Reagent preparation: Carefully add, dropwise, 2 mL of sulfuric acid to 100 mL of vanillin solution (10 g/L in ethanol (96%))
Reagent use: The plate is sprayed 2 mL of Vanillin-sulfuric acid reagent with the Derivatizer, yellow nozzle, spraying level 3 and heated for 3 min at 100°C on the TLC Plate Heater

Note: reagent transfer for Ehrlich (reagent for dipping requires 4 g of 4-dimethylaminobenzaldehyde instead of 0.5 g) and Vanillin-sulfuric acid can also be used for immersion of the plate into 200 mL of the respective reagent using the Chromatogram Immersion Device

MS confirmation

The zones to be eluted are marked with a soft pencil under UV 254 nm using the UV Cabinet or TLC Visualizer 2. For non-UV-active compounds (or low response at UV 254 nm): standards or samples are applied twice. One part of the plate is derivatized for localizing the corresponding zones on the non-derivatized part of the plate.
Target zones are directly eluted using the TLC-MS Interface 2 with oval elution head into the ACQUITY QDa Detector at a flow rate of 0.5 mL/min with methanol (with 0.1% ammonium hydroxide). For a full scan spectrum it is recommended to first elute a blank, which can be subtracted from the spectra of the target zones. For confirmation of substances between 50 and 500 ng per zone are required.

MS parameter

The ACQUITY QDa Detector is operated in ESI^- mode using default parameters. The ESI capillary is set to 0.8 kV, cone voltage to 15 V, and desolvation temperature at 600 °C. A full scan mass spectrum between m/z 50 and 650 is acquired at a sampling rate of 10.0 points/sec (continuum). Data processing and evaluation of mass spectra are performed with Empower. For routine use in quality control Single Ion Recording (SIR) can be performed.

Results

Qualitative results (HPTLC Fingerprint):
(1) SST on silica gel 60 F_{254} under white light after derivatization with FBS:
CBD shows an orange-brownish zone at RF ~ 0.49
THC shows a red-pinkish zone at RF ~ 0.45
CBN shows a purple zone at RF ~ 0.40

NOTE: The presented results are to be regarded as examples only!

(2) SST on reversed-phase under white light after derivatization with Ehrlich reagent (Vanillin-sulfuric acid reagent leads to dark purple zones):
CBD shows a purple zone at R_F ~ 0.34
CBN shows a red-pinkish zone at R_F ~ 0.23
THC shows a purple zone at R_F ~ 0.18

(3) SST on silica gel 60 F_{254} (UN B method) under white light after derivatization with FBS:
CBD shows an orange-brownish zone at R_F ~ 0.45
THC shows a red-pinkish zone at R_F ~ 0.41
CBN shows a purple zone at R_F ~ 0.29

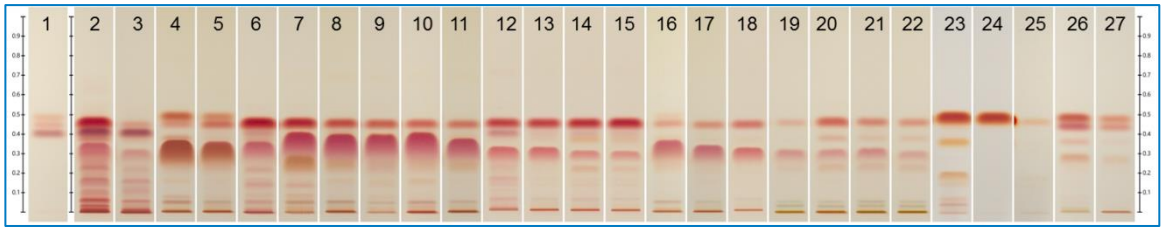

Fig. 1 Comparison of HPTLC fingerprints (chromatograms) under white light after derivatization with FBS reagent (method 1); track 1: SST (CBN, THC, and CBD with increasing RF), track 2: hashish sample, tracks 3-18: C. sativa samples, tracks 19-22: C. sativa samples (young plants), track 23: intermediate from CBD extraction (mother liquor, 1:10 diluted), track 24: CO2-extracted CBD (1:10 diluted), track 25: CBD cream, track 26: Cannabis oil (1:20 diluted), track 27: Cannabis tincture (1:10 diluted);

Note: C. sativa extracts have been applied undiluted, high concentration of the cannabinoid acids leads to shifted RF values (higher RF through matrix effects)

Comparison of the 3 methods:

Silica gel 60 F_{254}		**RP-18**		**UN B (Si 60 F_{254})**	
Cannabinoid	hR_F	Cannabinoid	hR_F	Cannabinoid	hR_F
CBDA	23	THCA	8	CBDA	0
THCA	24	CBC	13	THCA	0
CBC	36	THC	18	CBC	17
CBG	36	CBN	23	CBN	29
CBN	40	THCV	30	CBG	32
THCV	41	CBDA	31	THCV	38
THC	45	CBD	34	THC	41
CBD	49	CBG	36	CBD	45

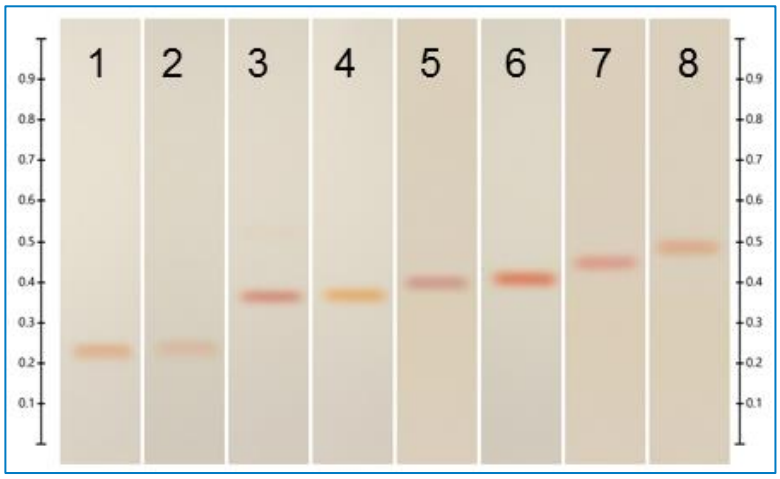

Fig. 2 HPTLC chromatograms after derivatization with FBS (method 1) under white light; tracks 1-8: standards (CBDA, THCA, CBC, CBG, CBN, THCV, THC, CBD with increasing R_F values)

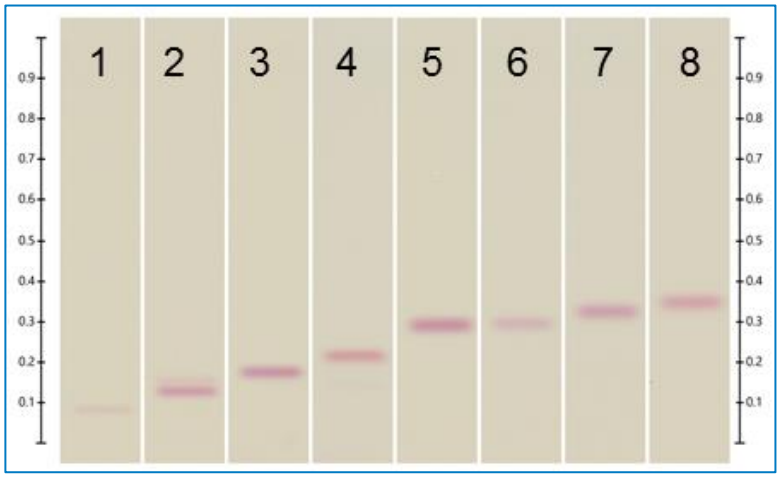

Fig. 3 HPTLC chromatograms after derivatization with Ehrlich (method 2) under white light; tracks 1-8: standards (THCA, CBC, THC, CBN, THCV, CBDA, CBD, CBG with increasing R_F values)

	Silica gel 60 F_{254}	**RP-18 F_{254}**	**UN B (Si 60 F_{254})**
Separation capacity	Three co-eluting substances	+	No migration of the cannabinoid acids (THCA, CBDA), decarboxylation recommended
Fast Blue salt B (color-differences of the zones)	+	-	+
Cost of the plates	+	-	+
Derivatization with Ehrlich and Vanillin reagent	+	+	+

Non-decarboxylated samples *versus* decarboxylated samples

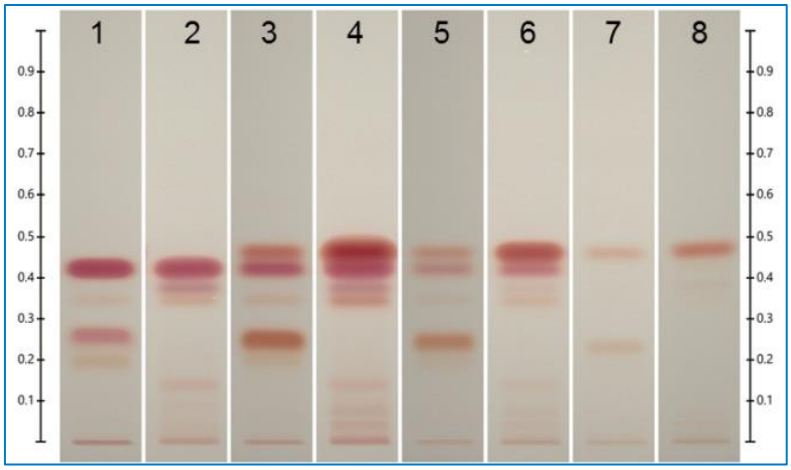

Fig. 4 HPTLC chromatograms under white light after derivatization with FBS; track 1: C. sativa type 1 (THC-rich, non-decarboxylated), track 2: C. sativa type 1 (THC-rich, decarboxylated), track 3: C. sativa type 2 (THC ≈ CBD, non-decarboxylated), track 4: C. sativa type 2 (THC ≈ CBD, decarboxylated), track 5: C. sativa type 3 (CBD-rich, non-decarboxylated), track 6: C. sativa type 3 (CBD-rich, decarboxylated), track 7: C. sativa THC-free (non-decarboxylated), tracks 8: C. sativa THC-free (decarboxylated); of all sample extracts 2 µL were applied 1:10 diluted

Note: $THC_{total} < 1\%_{CH, USA}$ and $< 0.2\%_{EU}$ (colors of the zones differ from Fig.1 because of different reagent preparation, here: 0.5% FBS in water)

Quantitative results:

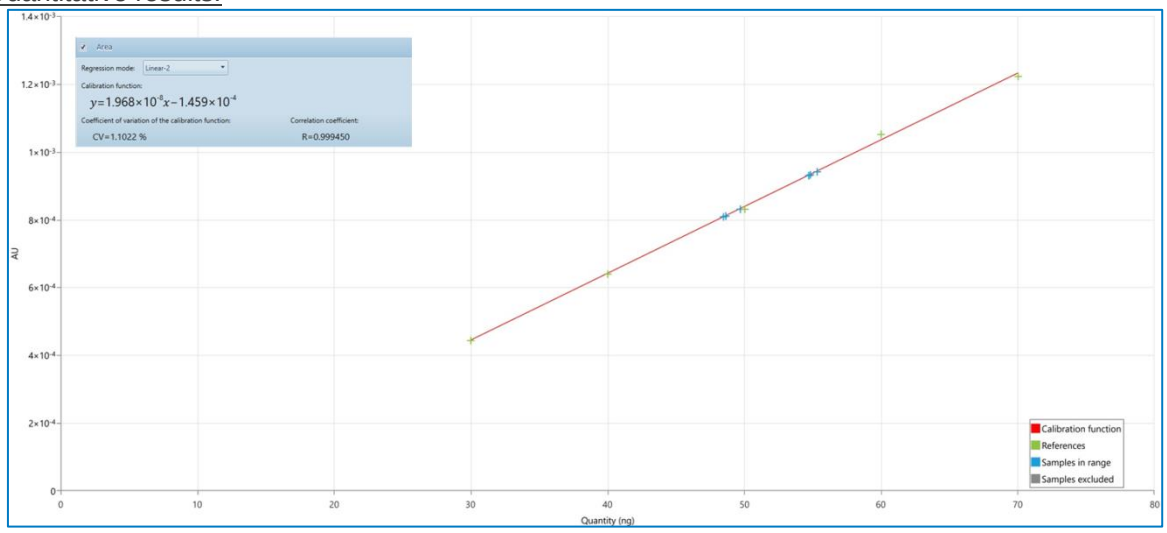

Fig. 5 Calibration curve of THC and quantification of THC in 2 samples (green standards and blue replicates of the samples), scanned at 210 nm, linear working range from 30-70 ng

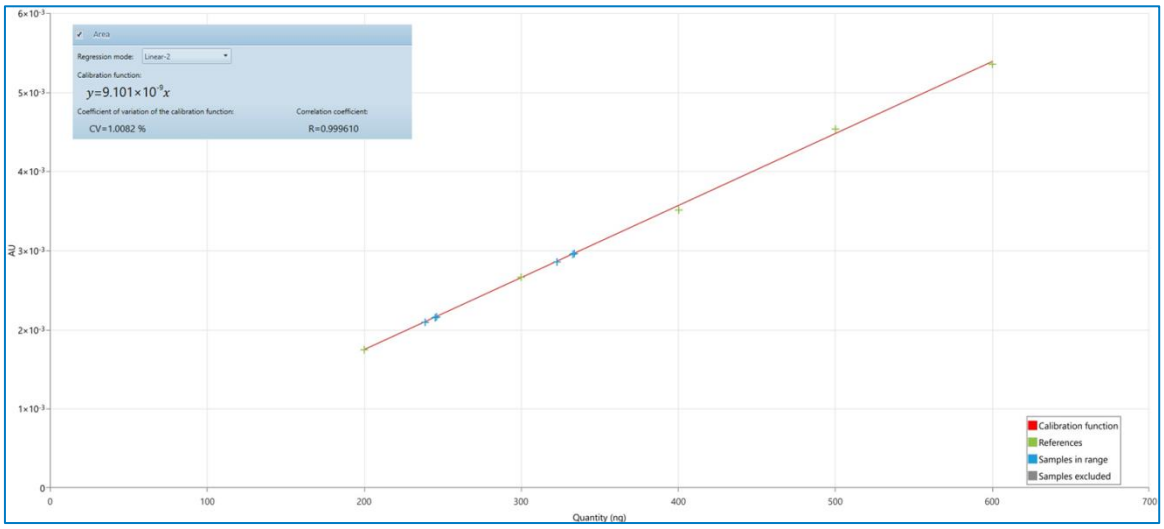

Fig. 6 Calibration curve of THCA and quantification of THCA in 2 samples (green standards and blue replicated of the samples), scanned at 285 nm, linear working range from 200-600 ng

For quantitation of THC in non-decarboxylated sample extracts the total THC content is calculated:

THC_{total} = (% THCA) x 0.877 + (% THC) (for CBD equivalent)

Quantitative results:

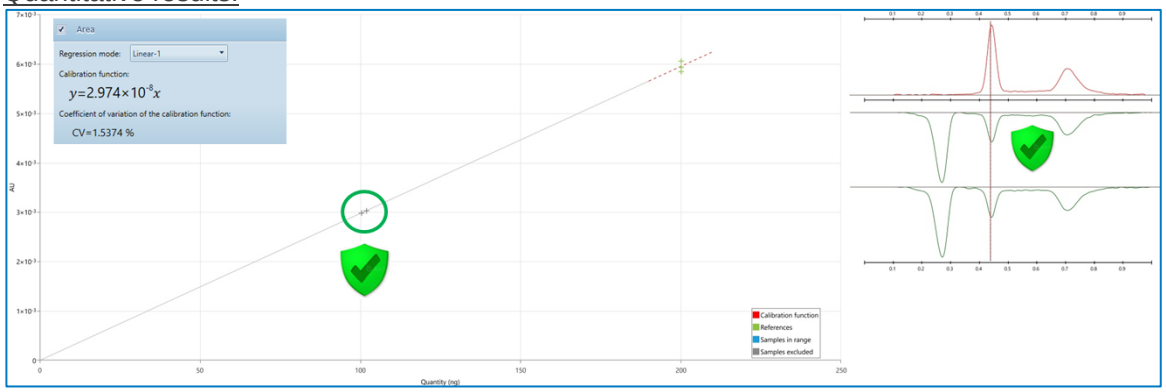

Fig. 7 Limit test with a sample (applied in duplicate) which pass the USA and CH limit (<1 % THC)

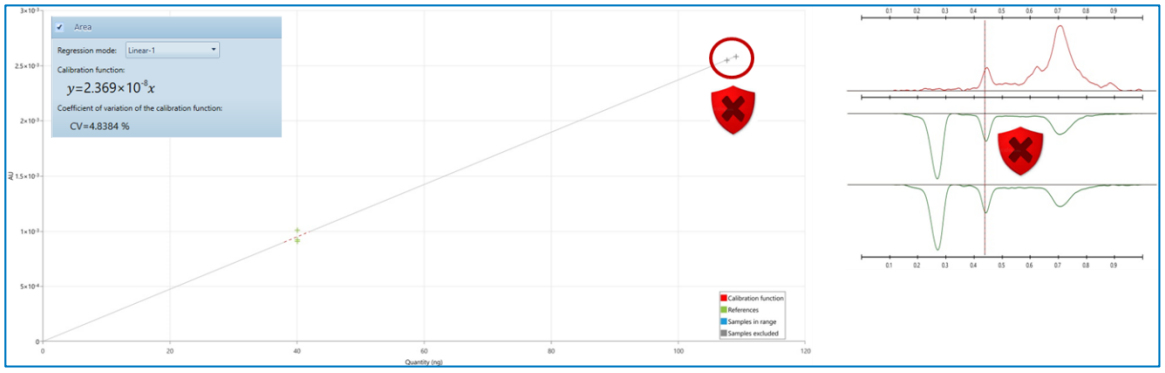

Fig. 8 Limit test with a sample (applied in duplicate) which fail the EU limit (<0.2 % THC)

HPTLC-MS:

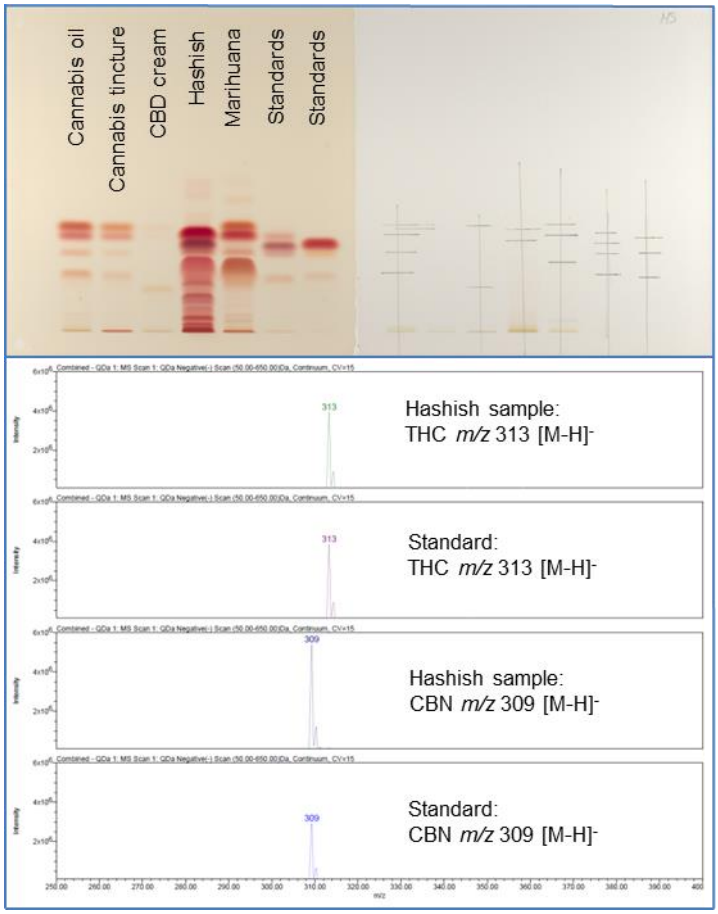

Fig. 9 Plate under white light; (left) plate derivatized with FBS reagent, (right) plate with marked zones of interest; bottom: HPTLC-MS spectra of THC and CBN (Hashish sample compared to standards), displayed range m/z 250 to 400

Table 2

Cannabinoid	***m/z* [M-H]⁻**
CBDA	357
THCA	357
CBC	313
CBG	315
CBN	309
THCV	285
THC (Δ8/Δ9)	313
CBD	313

Discussion

The presented methods are suitable for Cannabis testing. Depending on the analytical task each of them provides certain advantages. Method 1 is the best for the identification of *C. sativa* samples without decarboxylation. The entire information on the sample composition is obtained. Furthermore it can be used for the limit test (THC-free samples) and for quantification of THC and THCA in type 1 (THC-rich) and for CBD and CBDA in type 3 (CBD-rich) Cannabis samples. For type 2 Cannabis samples (THC ≈ CBD) a decarboxylation would be recommended because of the co-elution of THCA and CBDA. The application of decarboxylated samples has also the advantage to directly measure CBD and THC (no calculation of the total content required). Also the mobile phase from [3] can then be used, with a better separation (less co-eluting cannabinoids). RP-18 plates (method 2) can be used for identification and quantitation of cannabinoids as well. Main disadvantages are the higher costs of the plates and the incompatibility with FBS. Color-differences obtained with FBS can help to distinguish cannabinoids for a quick visual evaluation.

Acknowledgement

We are grateful to Jörg-Peter Gütlein, Daniele Schibano from AiFame (Switzerland), Matthias Grill from Lipomed AG (Switzerland), Claire Thouminot from Faculté de Pharmacie, Université Paris-Sud (France), and Alexandre Maciuk from Fédération Nationale des Producteurs de Chanvre (France) for the excellent collaboration.

Literature

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