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W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

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D. de Boer[¶,§], H. Venema[§], A. Castañera Ajenjo[¶], L.J.A.L.dos Reys[¶], and R.A.A. Maes[§]

Profiling cannabinoids; the significance of the detection of 11-nor-Δ^9-tetrahydrocannabivarin-9-carboxylic acid

[¶] Laboratório de Análises de Dopagem e Bioquímica, Lisboa, Portugal

[§] Department of Human Toxicology, Utrecht Institute of Pharmaceutical Sciences, Utrecht, The Netherlands

Introduction

Cannabis sativa L. contains the so-called cannabinoids that are unique to the *Cannabis* plant species (Figure 1). The 2-carboxylic analogues of cannabinol (CBN), Δ^9-tetrahydrocannabivarin (Δ^9-THCV) and of the isomers of tetrahydrocannabinol (THC) are among several other cannabinoids the main ones found in fresh plant material. These carboxylic compounds are the direct results of the biosynthesis, are not stable and easily loose their carboxy moiety [1]. Δ^9-Tetrahydrocannabinol (Δ^9-THC) is one of the isomers of THC that has lost its carboxy moiety and is the main cannabinoid responsible for the psychoactive effects of *Cannabis*. Another psychoactive isomer of THC is Δ^8-tetrahydrocannabinol (Δ^8-THC), which is supposed to be an artefact of Δ^9-THC and which is present in plant material in much lower concentrations than Δ^9-THC. CBN is a photochemical degradation product and is not psychoactive. Δ^9-THCV is a propyl analogue of Δ^9-THC, which is formed when in the biosynthesis process a polyketide is used with one ($CO-CH_2$)-subunit less than for Δ^9-THC, the pentyl analogue (Figure 1). Probably, Δ^9-THCV also contributes to the psychoactive active effects of *Cannabis*.

Pharmacological research has resulted in the development and marketing of dronabinol (Marinol®), a pharmaceutical product containing synthetic Δ^9-THC. It is used for the treatment of cancer, and to stimulate appetite in AIDS patients. Although the bulk active material of Marinol® is Δ^9-THC, side products of the synthetic process are Δ^8-THC and CBN. One of the future analytical toxicological challenges will be distinguish the misuse of *Cannabis* and the medical application of dronabinol.

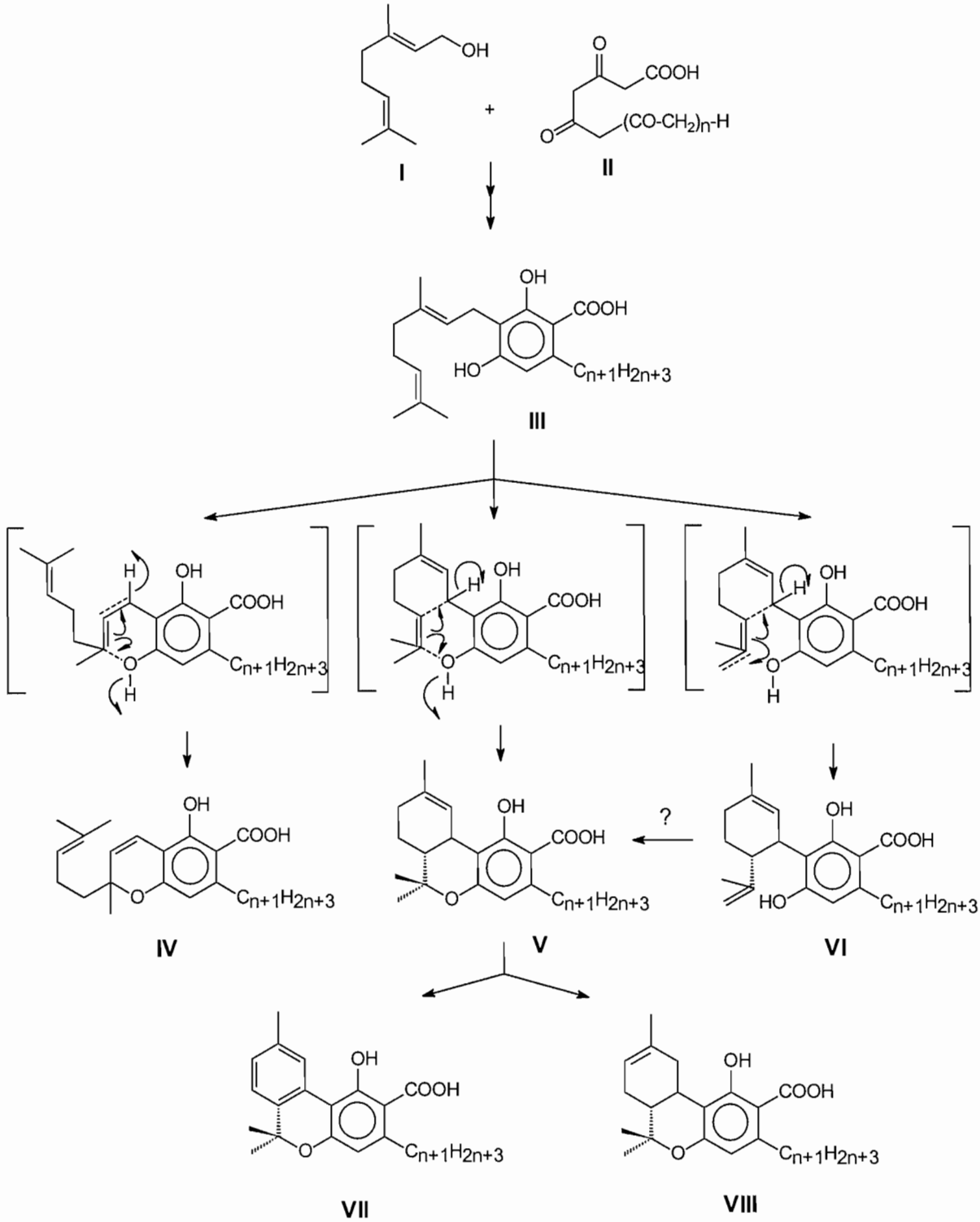

Figure 1 Biosynthesis of cannabinoids; I = geraniol; II = a polyketide; III = 2-carboxy-cannabigerol [n = 4]; IV = 2-carboxy-cannabichromene [n = 4]; V = 2-carboxy-Δ^9-tetrahydrocannabivarin [n = 2] or 2-carboxy-Δ^9-tetrahydrocannabinol [n = 4]; VI = 2-carboxy-cannabidiol [n = 4]; VII = 2-carboxy-cannabinol [n = 4]; VIII = 2-carboxy-Δ^8-tetrahydrocannabinol [n = 4]

The detection of the misuse of *Cannabis* using urine samples is in principal based on the determination of the main urinary metabolite of Δ^9-THC, namely 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (Δ^9-THCCOOH), conjugated or not with glucuronic acid (Figure 2). This metabolite is just one of the several metabolites of Δ^9-THC that, besides Δ^9-THC itself, can be found in urine. A less known urinary cannabinoid after *Cannabis* intake is 11-nor-Δ^9-tetrahydrocannabivarin-9-carboxylic acid (Δ^9-THCVCOOH), the main metabolite of Δ^9-THCV. It does not have a direct relationship to Δ^9-THC and thus also not with dronabinol. Therefore in order to make a possible distinction between the misuse of *Cannabis* and the application of dronabinol, the detection of Δ^9-THCVCOOH in urine could be of interest. This idea is encouraged by the fact that Δ^9-THCV is present in all subspecies of the *Cannabis* plant species, although sometimes in low amounts. Because of this perception, ElSohly and co-workers proposed that Δ^9-THCVCOOH is a reliable marker for the ingestion of *Cannabis* versus dronabinol [2,3]. We investigated in this study the potential relevance of this marker for sport drug testing using a method based on GC/MS/MS analysis.

Sample preparation

Introduce **1 mL of sample** into clean tubes

↓

Add **100 µL of 10 N KOH** and **1 µg of ketoprofen** (internal standard), vortex the tubes and incubate the sample at 60 °C for 15 min

↓

Add **3 mL of a mixture of n-hexane** and **ethyl acetate (9:1; v/v),** shake the tubes during 10 min on the shaker and centrifuge the tubes for 5 min at 2500 rpm and discard the organic phase

↓

Add **1 mL of 96% acetic acid** to the water phase and **3 mL of a mixture of n-hexane and ethyl acetate (9:1; v/v)** and shake the tubes during 10 min on the shaker and centrifuge the tubes for 5 min at 2500 rpm

↓

Separate the organic phase and introduce the organic phase in clean 25 mL tubes

↓

Repeat the n-hexane/ethyl acetate extraction step with 3 mL and combine the organic phases

↓

Remove the organic solvent with a rotating evaporator

↓

Add **80 µL** of the mixture **MSTFA/TMCS (100:5; v/v)** to the dry residue and incubate the mixture for 30 min at 80 °C

↓

Transfer the derivatised mixture to a 100 µL crimp vial, close it with a crimp cap and analyse the sample by GC/MS/MS

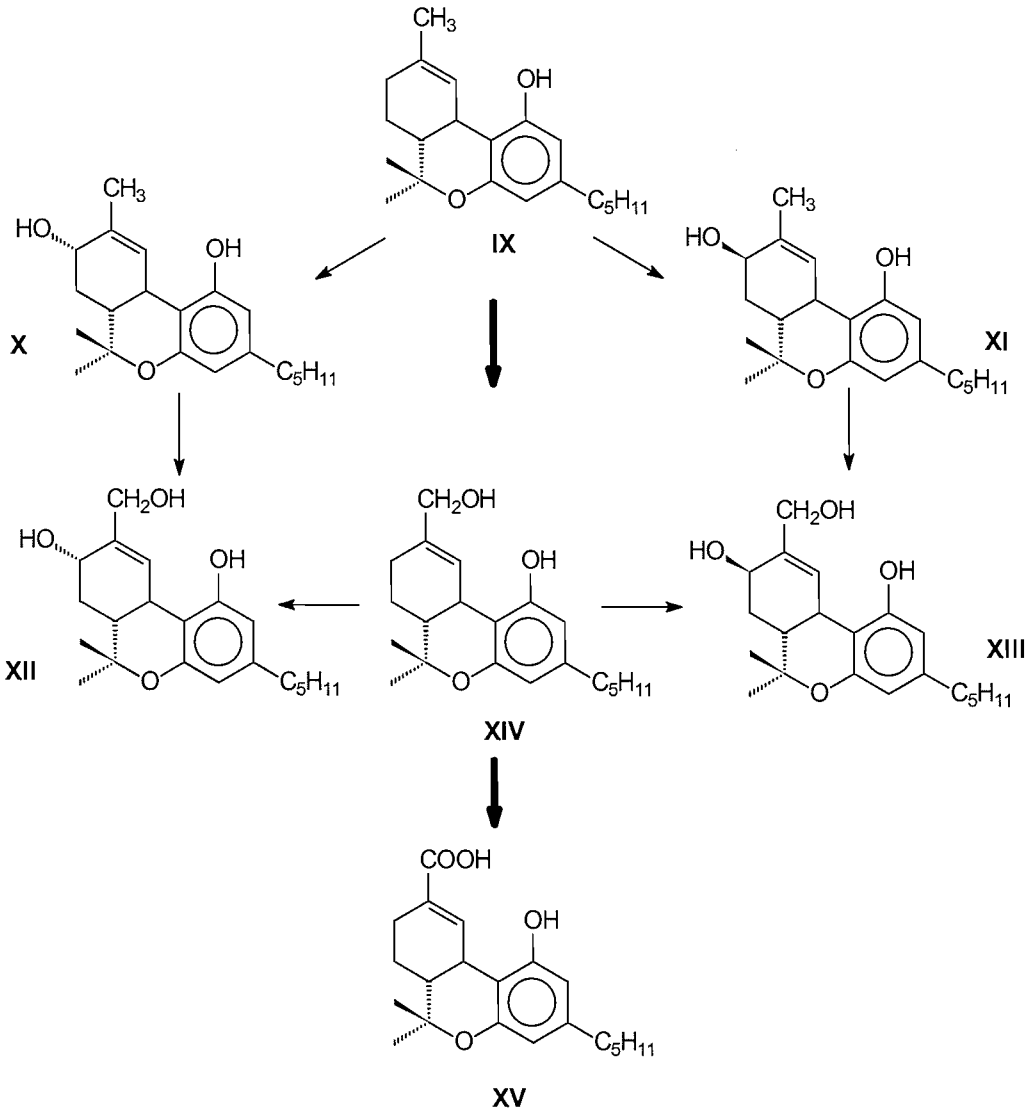

Figure 2 Metabolism of the cannabinoid Δ^9-tetrahydrocannabinol, IX = Δ^9-tetrahydrocannabinol (Δ^9-THC); X = 8α-hydroxy-Δ^9-tetrahydrocannabinol (8α-OH-Δ^9-THC); XI = 8β-hydroxy-Δ^9-tetrahydrocannabinol (8β-OH-Δ^9-THC); XII = 8α,11-dihydroxy-Δ^9-tetrahydrocannabinol (8α,11-OH-Δ^9-THC); XIII = 8β,11-dihydroxy-Δ^9-tetrahydrocannabinol (8β,11-OH-Δ^9-THC); XIV = 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-Δ^9-THC); XV = 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (Δ^9-THCCOOH)

Results and Discussion

Analytical validation of the GC/MS/MS method resulted in the following characteristics: limits of detection and of quantification of the equipment 0.4 and 1.3 ng/mL, respectively, extraction recovery 68%, limits of detection and of quantification of the total method 0.6 and 1.9 ng/mL, respectively, and the linearity range 5 – 80 ng/mL.

For the analysis of Δ^9-THCCOOH it is in principal not necessary to use GC/MS/MS in sport drug testing. After all, the decision limit for positive samples is 15 ng/mL. However, the initial reason to apply GC/MS/MS in this particular case was to simplify the sample preparation procedure and to utilise a specific detection methodology to overcome problems caused by the simplified sample preparation method. In such a sense, the described procedure is more than satisfactory and the overall analysis time for one sample was only 2 hours.

In the normal EI scan mode the mass spectrum of the obtained bis-*O*-TMS derivative of Δ^9-THCCOOH is very characteristic (Figure 3). The product ion mass spectrum of the selected product ion $[M - CH_3^{\bullet}]^+$ consists of only a limited number of ions (Figure 3), but the respective fragments are characteristic and their structures can be designated analogously to those obtained in the EI scan mode (Figure 3). The respective structures of the fragments either have been described or are being proposed here. The weak directing influence of the

GC/MS/MS conditions

Instrument	Varian MS SATURN 2000
Column	
brand	Hewlett Packard
type	HP-1
length	25 m
inner diameter	0.2 mm
film thickness	0.11 µm
Flow parameters	
carrier gas	helium
flow rate of carrier gas	0.8 mL/min
EPC mode	none
head pressure	18 psi
Injection parameters	
injection mode	splitless
splitless time	1.2 min
injection volume	2 µL
injector temperature	280 °C
Oven temperature program	
initial temperature	120 °C
initial time	1.4 min
rate1	10 °/min
final temperature	300 °C
final time	18 min
Mass spectrometric parameters	
ionisation mode	EI
acquisition mode	MS/MS
interface temperature	300 °C
ion trap temperature	220 °C
manifold temperature	40 °C
target	5000
multiplier voltage	autotune voltage + 200 eV
filament	80 µA

Ketoprofen mass spectrometric conditions	
precursor ion	*m/z* 311
PI fragmentation mode	CID
CID type	non- resonant
CID time	20 msec
CID amplitude	85 V
excitation storage level	*m/z* 140
Δ^9-THCVCOOH mass spectrometric conditions	
precursor ion	*m/z* 445
PI fragmentation mode	CID
CID type	non-resonant
CID time	20 msec
CID amplitude	90 V
excitation storage level	*m/z* 130
Δ^9-THCCOOH mass spectrometric conditions	
precursor ion	*m/z* 473
PI fragmentation mode	CID
CID type	non-resonant
CID time	20 msec
CID amplitude	90 V
excitation storage level	*m/z* 130

Abbreviations used: EPC = Electronic Pressure Control; EI = Electron Ionisation; PI = product ion; CID = Collision-Induced Dissociation

The **excitation storage level** is defined as the radio frequency storage level in *m/z* at which the dissociation waveform is applied following isolation

carboxylic group makes it possible to base the proposals on investigations of trimethylsilyl derivatives [4] as well as on non-derivatized or alternatively derivatized cannabinoids [5,6].

The "simple" product ion mass spectrum may be considered to have limited identification capacity and in order to compensate for this assumption, we decided to use Δ^9-THCVCOOH as an additional marker for the ingestion of *Cannabis*. As reference material Δ^9-THCVCOOH is not available and therefore we used a urine sample with a high concentration of Δ^9-THCCOOH to determine the retention time and the mass spectrum of the respective bis-*O*-TMS derivative of Δ^9-THCVCOOH. Figure 4 shows the spectrum as obtained in the normal EI scan mode as well as the product ion mass spectrum of the selected product ion $[M - CH_3^{\bullet}]^+$. Compared to those of Δ^9-THCCOOH, the spectra show identical fragmentation patterns.

During the last year we found a total number of 33 urine samples containing cannabinoids. Of these samples 32 samples were of Portuguese persons and 1 sample of a person with another nationality. The concentrations of Δ^9-THCCOOH were in the range of 3 – 80 ng/mL (25 samples) or > 80 ng/mL (8 samples). In all of these samples we detected Δ^9-THCVCOOH. Figure 5 presents two typical examples. We never detected traces of Δ^9-THCVCOOH in blank samples or in samples that were negative for the presence of Δ^9-THCCOOH. There was no correlation between the concentration of Δ^9-THCCOOH and response signals of Δ^9-THCVCOOH.

In urine samples of an excretion experiment with Marinol® also no traces of Δ^9-THCVCOOH were detected. This observation confirms the viewpoint of ElSohly and co-workers, who proposed that Δ^9-THCVCOOH is a reliable marker for the ingestion of *Cannabis* versus dronabinol [1,2]. However, we also analysed urine samples of an excretion experiment with so-called Dutch *Cannabis* and also in those samples no traces of Δ^9-THCVCOOH were detected. As indicated Δ^9-THCV is supposed to be present in all subspecies of the *Cannabis* plant species, although sometimes in low amounts. Probably in the studied Dutch *Cannabis* the amount of Δ^9-THCV was too low to result in detectable concentrations of Δ^9-THCVCOOH. Therefore, the usefulness of the presence of Δ^9-THCVCOOH in drug testing in general most likely seems to depend on the origin of the *Cannabis* ingested. However, if present it provides additional information about and prove of the ingestion of *Cannabis*. It is therefore that the Portuguese laboratory considers this type of cannabinoid profiling to be very useful.

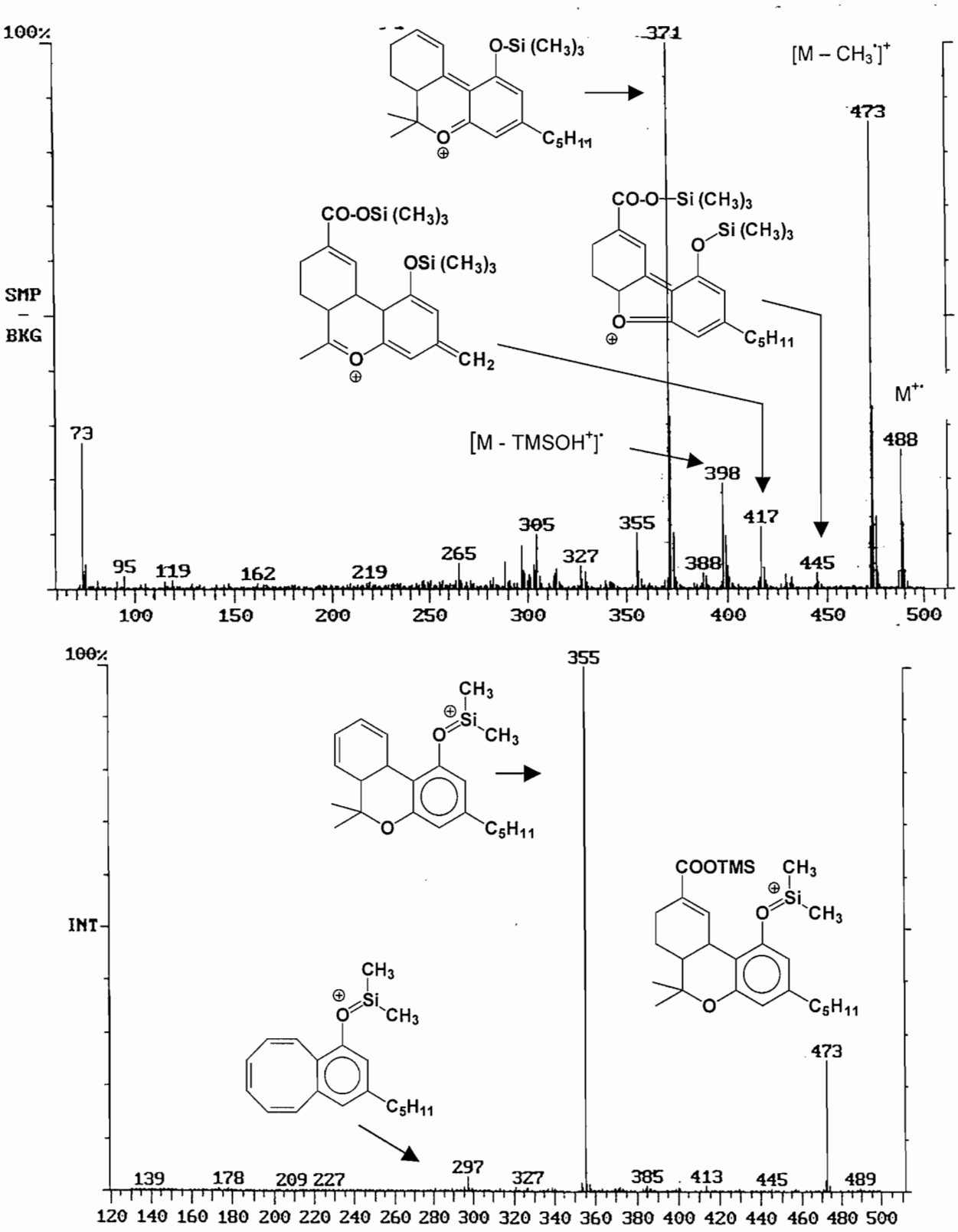

Figure 3 Mass spectrum of the bis-*O*-trimethylsilyl derivative of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (upper spectrum) and the product ion mass spectrum of the fragment $[M - CH_3^+]^\bullet$ of the bis-*O*-trimethylsilyl derivative of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (lower spectrum)

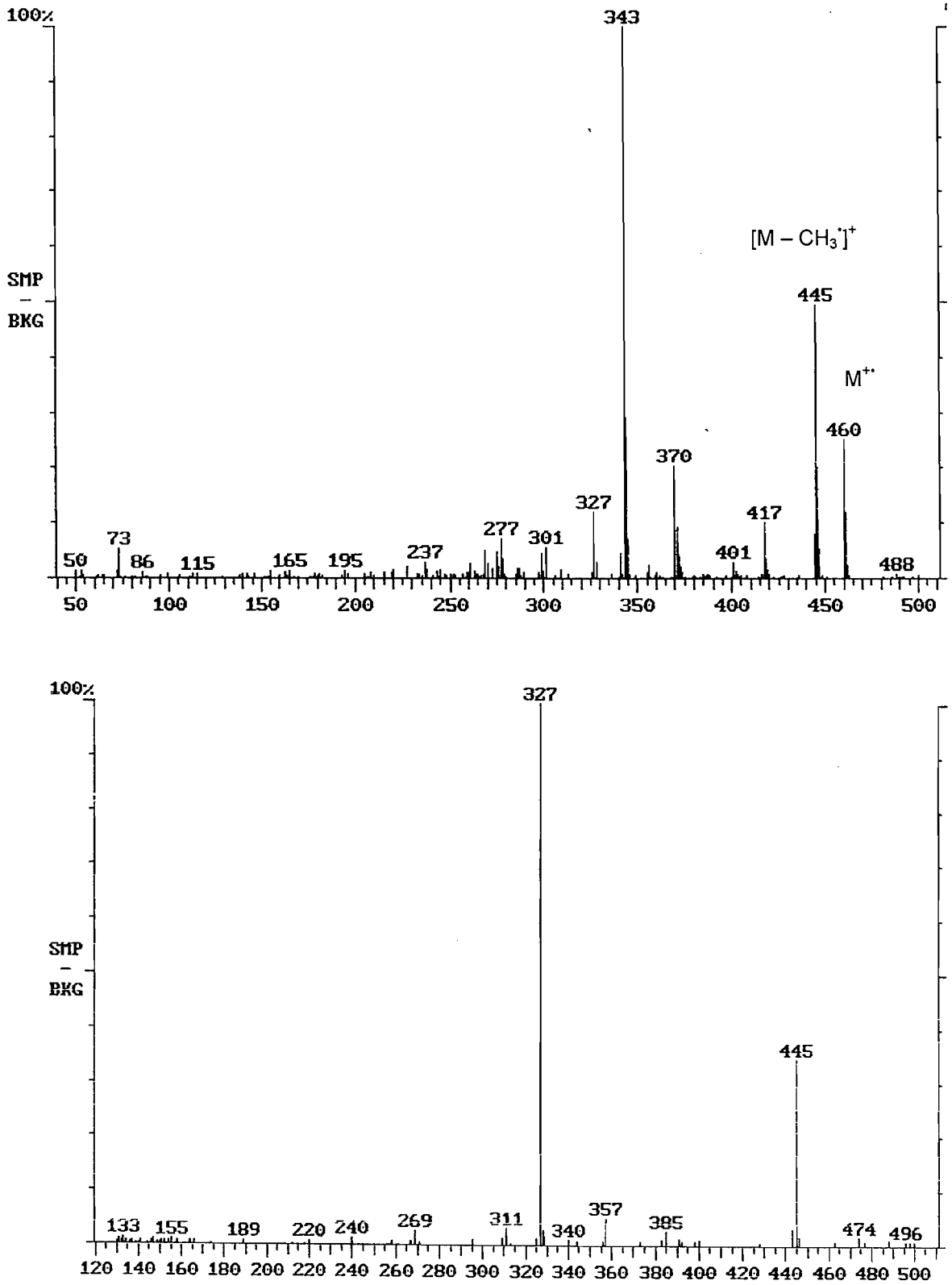

Figure 4 Mass spectrum of the bis-*O*-trimethylsilyl derivative of 11-nor-Δ^9-tetrahydrocannabivarin-9-carboxylic acid (upper spectrum) and the product ion mass spectrum of the fragment $[M - CH_3^+]^+$ of the bis-*O*-trimethylsilyl derivative of 11-nor-Δ^9-tetrahydro-cannabivarin-9-carboxylic acid (lower spectrum)

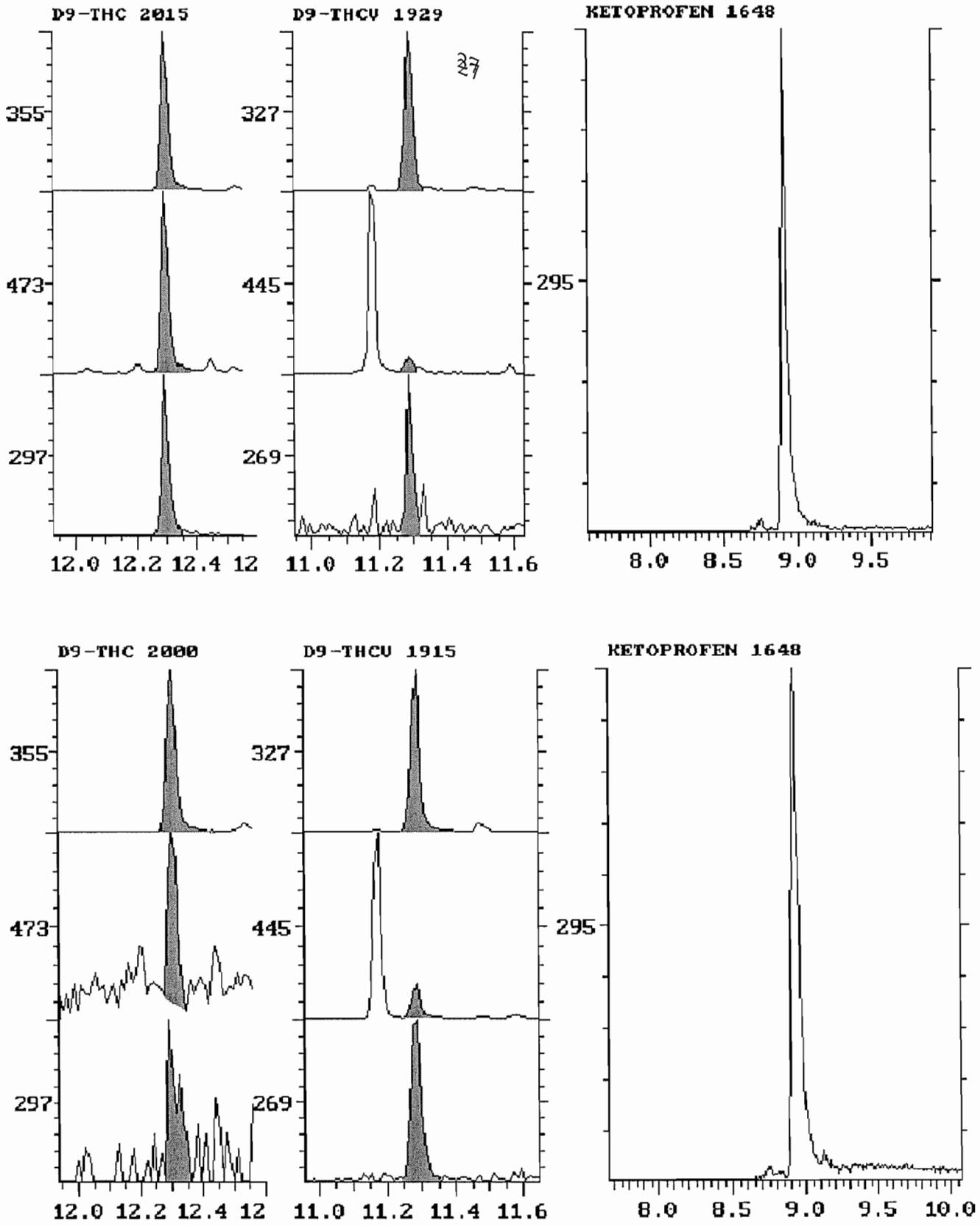

Figure 5 Typical chromatograms of urine samples containing Δ^9-THCCOOH as well as Δ^9-THCVCOOH; the upper chromatogram shows a sample with 35 ng/mL of Δ^9-THCCOOH and the lower chromatogram with 3 ng/ml of Δ^9-THCCOOH (numbers at the top are the respective scan numbers of the chromatographic peak)

Conclusions

1. The analysis of cannabinoids by GC/MS/MS is useful for the Lisbon laboratory, because that way additional information is obtained in an easy way

2. The presence of THCVCOOH indicates *Cannabis* use and excludes Marinol® application

3. The absence of THCVCOOH does not exclude *Cannabis* use and therefore does not distinguish *Cannabis* use from Marinol® application

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