Profiles of Δ^9-Tetrahydrocannabinol Metabolites in Urine of Marijuana Users: Preliminary Observations by High Performance Liquid Chromatography-Radioimmunoassay

by

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ABSTRACT

Metabolic profiles of 11-nor-9-carboxylic acid-Δ^9-THC (COOH-THC) and other THC metabolites were determined in an infrequent and a frequent marijuana user by HPLC-RIA. In the infrequent user no unconjugated COOH-THC was detected in urine samples for the first eight hours following smoking whereas this metabolite was detected in the urine samples from a frequent user. A metabolite was also detected in the frequent user, which was not present in the urine sample from the infrequent user.

INTRODUCTION

The major metabolic pathway of Δ^9-tetrahydrocannabinol (Δ^9-THC) involves cytochrome P450-mediated hydroxylation of the allylic methyl group (position C-11 based on dibenzopyran numbering system) followed by conversion to 11-nor-9-carboxylic acid-Δ^9-THC (COOH-THC). Glucuronide conjugates of THC and its metabolites can be formed at the carboxylic acid group (position C-11) [1], or the phenolic group (position C-1) [2]. In the case of COOH-THC conjugation may occur at both sites. The major metabolites in both blood and urine have been reported to be COOH-THC and it's glucuronide conjugates [3,4].

A variety of analytical techniques have been used to measure Δ^9-THC and its metabolites in biological fluids, such as immunoassay [5], thin layer chromatography (TLC) [6], gas chromatography (GC) [7,8], gas chromatography-mass spectrometry (GC-MS) [9], high performance liquid chromatography (HPLC) [10,11] and HPLC- radioimmunoassay (HPLC-RIA) [12,13]. This last procedure has two advantages, firstly the lack of sensitivity obtained using HPLC with ultra violet wavelength detection is overcome by using a sensitive immunoassay procedure and secondly the lack of specificity of certain antisera is overcome by using a chromatographic separation.

The present study was designed to characterize the urinary profiles of Δ^9-THC metabolites under controlled conditions. Although, other

workers [12,13] have reported on such profiles the urine samples were not collected under controlled conditions. In addition, the profiles described in this study were compared in frequent and infrequent marijuana users. For the purposes of this study an infrequent user was a person who smoked marijuana on average twice a month and a frequent user one who used marijuana daily.

MATERIALS

A Varian Model 5020 Liquid Chromatogarph equipped with a Varian UV-5 detector set at 215 nm (Varian Instrument Co., Walnut Creek, CA) was used for all HPLC assays. A Spherisorb-ODS (5 micron), 10 cm by 4.6 mm HPLC column was used (Supelco Inc., Bellefonte, PA). Eluent fractions were collected using a Buchler LC 100 fraction collector. All the solvents used were HPLC grade; all other chemicals were reagent grade. ß-glucuronidase (341,000 U/gr) was purchased from Sigma Chemical Co., St. Louis, MS. RIA analysis was performed using the Abuscreen® procedure for cannabinoids (Roche Diagnostics, Nutley, NJ).

Samples of Δ^9-THC, 11-hydroxy-Δ^9-THC (OH-THC), 8α, 11-dihydroxy-Δ^9-THC (8α, 11-OH-THC) and COOH-THC were supplied by the National Institute on Drug Abuse. A small sample of 8α-hydroxy-Δ^9-THC (8α-OH-THC) was generously supplied by Dr. Elsohly, University of Mississippi.

METHODS

Each subject smoked a single maijuana cigarette, containing 1.8% THC at 0800. They were asked not to smoke after midnight of the night before the test session. The infrequent user had not smoked in the previous 5 weeks. The frequent user had smoked at least one cigarette in the previous 24 hours, but none within 12 hours of testing. The frequent user was a daily user of marijuana. Both subjects were instructed to smoke in their usual manner except that the cigarette had to be smoked so that a 8mm butt remained. Urine samples were collected over a 14 day period.

The HPLC procedure used was that of Peat et al. [12] with one minor modification; the urine sample was adjusted to pH 3.0 with hydrochloric acid prior to injection. Briefly, two mls of urine are mixed with 2 mls of methanol and 2 mls of methanol:water (1:1) adjusted to pH 3.0 with 1N hydrochloric acid. After centrifuging, 5 mls of the supernatant are injected via a 5 mls. injection loop directly onto the HPLC column. The mobile phase is acetonitrile:water (adjusted to pH 3.3 with phosphoric acid); the following gradient is used: 0 to 10 min - 36% acetonitrile; 10 to 20 min - 36 to 70% acetonitrile and 20 to 25 min - 70% acetonitrile. The flow rate is 4 mls per min and fractions are collected every 30 secs.; the first four fractions are discarded. Each fraction collected is evaporated

to dryness at 40°C under vacuum using a Buchler vortex evaporator. Radioimmunoassay was carried out on each fraction, according to the manufacturer's instructions, after dissolving the residue in 100 µl of drug-free urine. For experiments involving hydrolysis, urine was incubated with ß-glucuronidase (5,000 units per ml of urine) at 37°C overnight after adjustment to pH 5.0 with 1.0 M sodium acetate.

Standards of COOH-THC ranging from 5.0 to 75 ng/ml were prepared in drug-free urine and analyzed by the above procedure to determine the concentration (ng/ml) of cross-reacting cannabinoids (CRC).

RESULTS

Figure 1 shows an HPLC-UV chromatogram of Δ^9-THC and metabolites, the retention volumes are as follows: 8α, 11-OH-THC: 22 mls, 8α -OH-THC: 44 mls, OH-THC: 54 mls, COOH-THC: 59 mls and THC: 79 mls.

Figure 2 illustrates HPLC-RIA chromatograms of urine samples collected from an infrequent user 2 and 4 hours after smoking a marijuana cigarette containing 1.8% THC. Only the sample collected at 4 hours contained detectable amounts of unconjugated COOH-THC, none of the other samples collected up to 8 hours after smoking contained unconjugated COOH-THC. Of note a peak (identified as I in Figure 2) was found in samples collected between 1 and 8 hours after smoking. The retention

volume of the peak was similar to 8α , 11-OH-THC. In each of these unhydrolyzed samples appreciable amounts of cross-reacting cannabinoids were found in an early eluting fraction (retention volume no greater than 12 mls).

Figure 3 shows HPLC-RIA chromatograms of the same urine samples after hydrolysis with ß-glucuronidase; significant differences were seen. In the samples collected for up to 8 hours after smoking, appreciable amounts of COOH-THC were detected (range of CRC was 25 to 73 ng/ml). In three of the samples, those collected between 1 and 4 hrs, a new peak appeared (identified as II) in the chromatograms. The concentration of this peak reached a maximum (15 ng/ml CRC) 2 hrs. after smoking. I, which was detected in the unhydrolyzed samples, was also detected in the hydrolyzed samples at lower concentrations. In all of the enzyme hydrozyled samples the amount of CRC detected in the early eluting fraction was dramatically decreased. Figure 4 shows the concentrations of CRC detected in the fractions corresponding to COOH-THC, I and II in the urine samples collected for up to 8 hrs after smoking in an infrequent user.

Figures 5, 6 and 7 show the corresponding data for samples from a frequent user. As can be seen there are significant differences from the infrequent user chromatograms. Firstly, between 17 and greater than 75 ng/ml of CRC were detected in the COOH-THC fraction of unhydrolyzed urine, with a peak being noted 1 hr. after smoking. I was also observed in

these urine samples but in this instance 42 ng/ml CRC of I was still detectable 24 hr after smoking. Of interest a new fraction (identified as III) was found in the urine samples from frequent marijuana smokers, this reached a peak of 15 ng/ml 2 hrs after smoking. The retention volume (38 mls) of this fraction was slightly lower than that of 8α -OH-THC. Similarly to the infrequent user significant amounts of CRC were detected in an early eluting fraction.

After enzyme hydrolysis a more complex HPLC-RIA chromatogram was observed (Figure 6). However, similar trends to those seen in the urine samples from an infrequent user were apparent. There was a significant reduction in the amount of CRC detected in the early eluting fraction and an increase in the CRC detected in the COOH-THC fraction, with a peak concentration of greater than 75 ng/ml being detected in the latter fraction 2 hrs after smoking. I was also detected in these hydrolyzed samples, although at lower concentrations than in the unhydrolyzed samples. II appeared after hydrolysis with a peak concentration of 55 ng/ml CRC after 2 hrs. III, which was not detected in the urine samples from an infrequent user, increased in concentration after hydrolysis of urine samples from a frequent user. Figure 7 summarises the time-course data for the samples from the frequent user.

DISCUSSION

The data reported here should be considered to be preliminary observations for three reasons. Firstly the study involved only one frequent and one infrequent marijuana user and the results should be confirmed by examining more urine samples. Secondly, although the differences seen appear to be qualitative they may only be quantitative because the sensitivity of the HPLC-RIA assay used was equivalent to 5 ng/ml of COOH-THC. Thirdly, the exact identification of I, II and III has not been determined. However, several of our findings may be of importance to forensic toxicologists.

Most importantly, it appears that in infrequent users virtually all the COOH-THC excreted in the first 8 hrs is in the conjugated form. This supports the suggestion of Law et al. (4) who reported that following the oral administration of THC the glucuronide conjugate of COOH-THC was the major metabolite detected in plasma and urine. However, this does not apply to frequent users as significant concentrations of unconjugated COOH-THC were detected in urine samples from this group. Our data do suggest that there may be a metabolite present in the urine of frequent marijuana users which is not found in that of infrequent users. In this report that metabolite is indicated as III and has a retention volume similar to 8α-OH-THC. It would also appear that it forms a glucuronide

conjugate because hydrolysis of the urine with ß-glucuronidase results in an increase in its concentration.

The dramatic difference observed in the excretion of unconjugated COOH-THC between the frequent and infrequent user may be due to several reasons. Firstly, heavy use of marijuana by the frequent user may have resulted in a saturation of the hepatic glucuronyl transferase system responsible for conjugation. This enzyme system may also have been altered by use of other drugs in this individual, although he admitted only to frequent use of marijuana and ethanol and occasional use of cocaine. Metabolites of THC are also excreted in the feces and it is possible that a switch of excretion pathways in the frequent user is responsible for the urinary excretion of unconjugated COOH-THC.

In both sets of urine samples significant concentrations of CRC were detected in an early eluting fraction; because of the reversed-phase HPLC system used this fraction is assumed to contain conjugates of either carboxylic acid or phenolic metabolites. After hydrolysis with ß-glucuronidase there was a significant increase in the COOH-THC fraction and a decrease in the early eluting fraction as would be expected if the early eluting fraction was primarily glucuronide conjugates. I and II were also found in urine samples from infrequent and frequent users. I, which has a retention volume similar to 8α, 11-OH-THC, decreased in concentration following ß-glucuronidase hydrolysis whereas II was only present after hydroysis. This suggest that II is an unconjugated form of a

conjugate detected in the early eluting fraction or in I; although the latter also contains other CRC because it is only reduced by approximately 25% following hydrolysis.

The time period over which unconjugated COOH-THC and I can be detected differs between the users. In fact, in an infrequent user unconjugated COOH-THC is detected at very low concentrations in only one of the urine specimens whereas in a frequent user it is detected for at least 24 hrs. Although I is detected in both sets of urine samples it is found for only 4 to 8 hrs in the infrequent user at appreciable concentrations, but for at least 24 hrs in a frequent user.

From our data it appears as if I contains at least two compounds, possibly a glucuronide conjugate of II and a dihydroxy metabolite of THC. In addition, III which was detected in frequent users also appears to form a glucuronide conjugate. This data would suggest therefore that II and III form glucuronide conjugates at either the C-11 carboxylic acid or the C-1 phenolic group. Haldin et al. [14] have reported that apart from COOH-THC two other carboxylic acid metabolites can be detected in urine samples from marijuana users at greater than trace amounts. The structures and abundances of these three metabolites are shown in Table 1. II and III may, therefore, be these metabolites; however, the study of Haldin et al. [14] was limited to identifying acidic metabolites and the possibility exists that II and III do not contain a carboxylic acid grouping and may simply form glucuronide conjugates on the phenolic position alone.

In summary this study has demonstrated two differentiating characteristics between frequent and infrequent users; one is that in the former the COOH-THC is excreted as glucuronide conjugates and that a metabolite III is present only in urine samples from a frequent user. With the difficulty in interpreting blood or plasma THC and metabolite concentrations these findings may assist in the identification of the type of user involved although it should be remembered that the users studied here represent extremes in their frequency of use. Further investigations are in progress to identify these metabolites and to elucidate the reasons for the differences.

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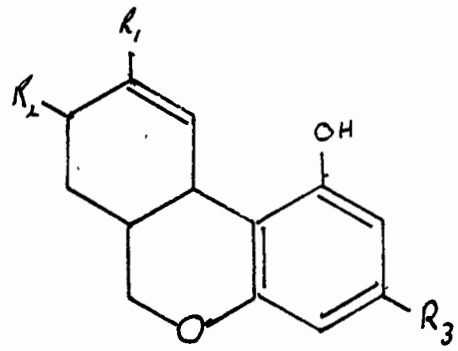
Table 1: Structures and Relative Abundances of the Three Major Carboxylic Metabolites in Urine.



Metabolite	R_1	R_2	R_3	% of Total Excreted*
11-nor-delta-9-carboxylic acid (COOH-THC)	COOH	H	C5H11	27
4',5'-bisno-delta-9-11,4'-dicarboxylic acid	COOH	H	C2H4COOH	8
4'-hydroxy-delta-9-11-carboxylic acid	COOH	H	C5H10OH	5

*Over 72 hr after last administered dose.

Ref: Haldin et al. (14).

FIGURE LEGENDS

Figure 1: An HPLC-UV chromatogram of 8,α,11-OH-THC, 8,α,OH-THC,11-OH-THC, COOH-THC and THC. For conditions see Methods.

Figure 2: HPLC-RIA chromatograms of urine samples collected from an infrequent user 2 and 4 hours after smoking a marijuana cigarette containing 1.8% THC.

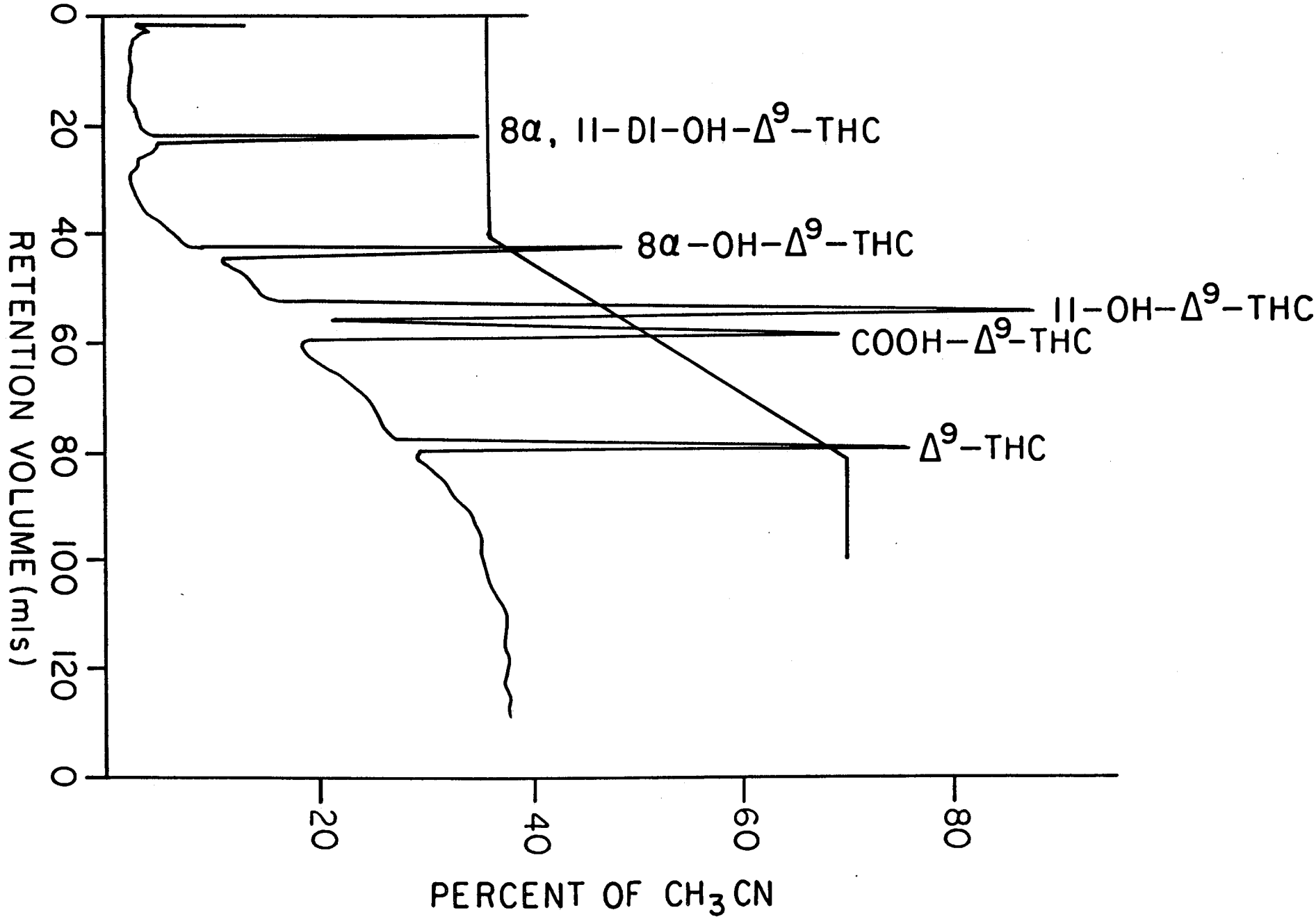
Figure 3: HPLC-RIA chromatograms of enzyme hydrolyzed urine samples collected from an infrequent user 2 and 4 hours after smoking a marijuana cigarette containing 1.8% THC.

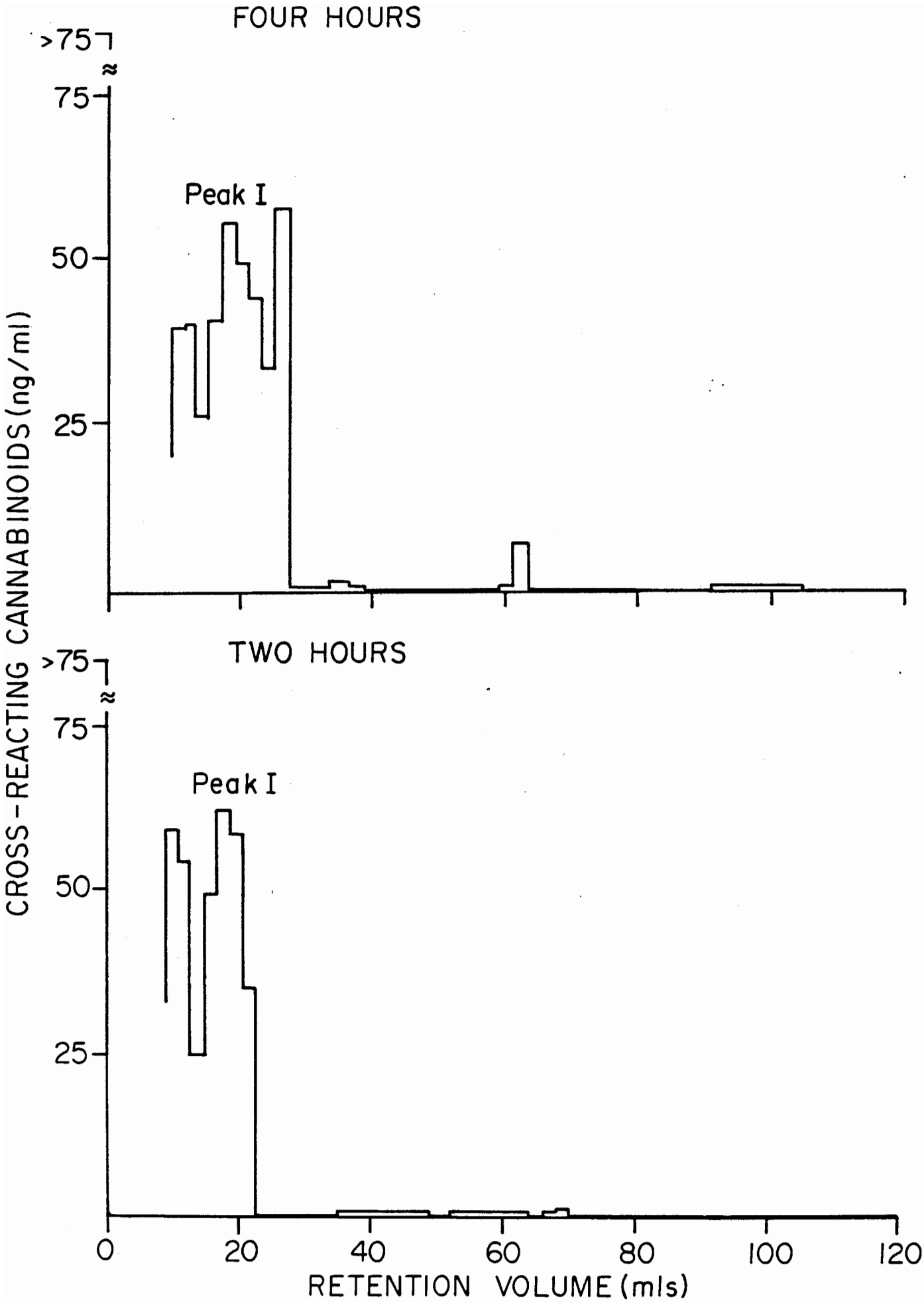
Figure 4: Concentrations of cross-reacting cannabinoids (CRC ng/mL) in the HPLC-RIA fractions corresponding to COOH-THC, I and II in an infrequent user.

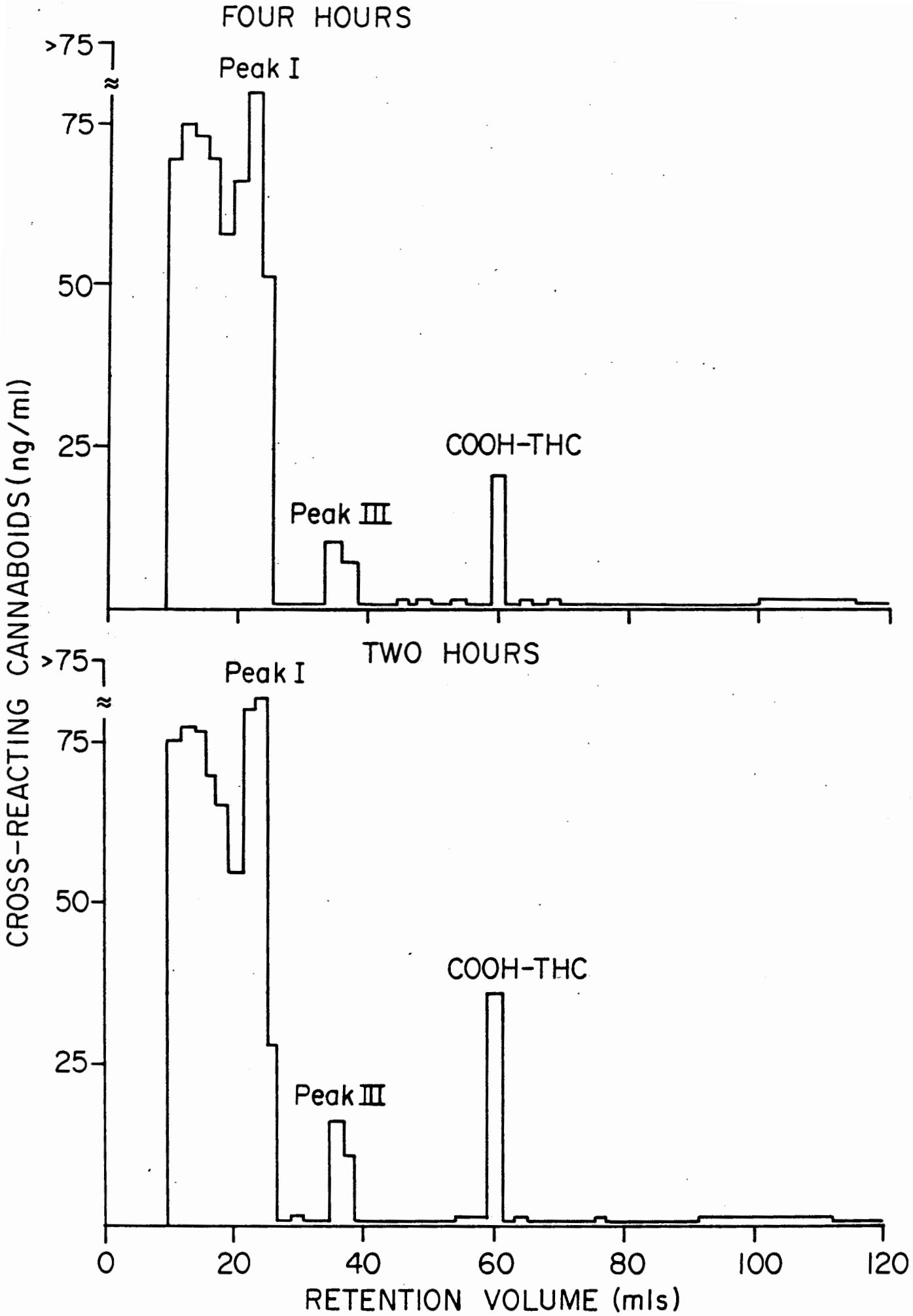
Figure 5: HPLC-RIA chromatograms of urine samples collected from a frequent user 2 and 4 hours after smoking a marijuana cigarette containing 1.8% THC.

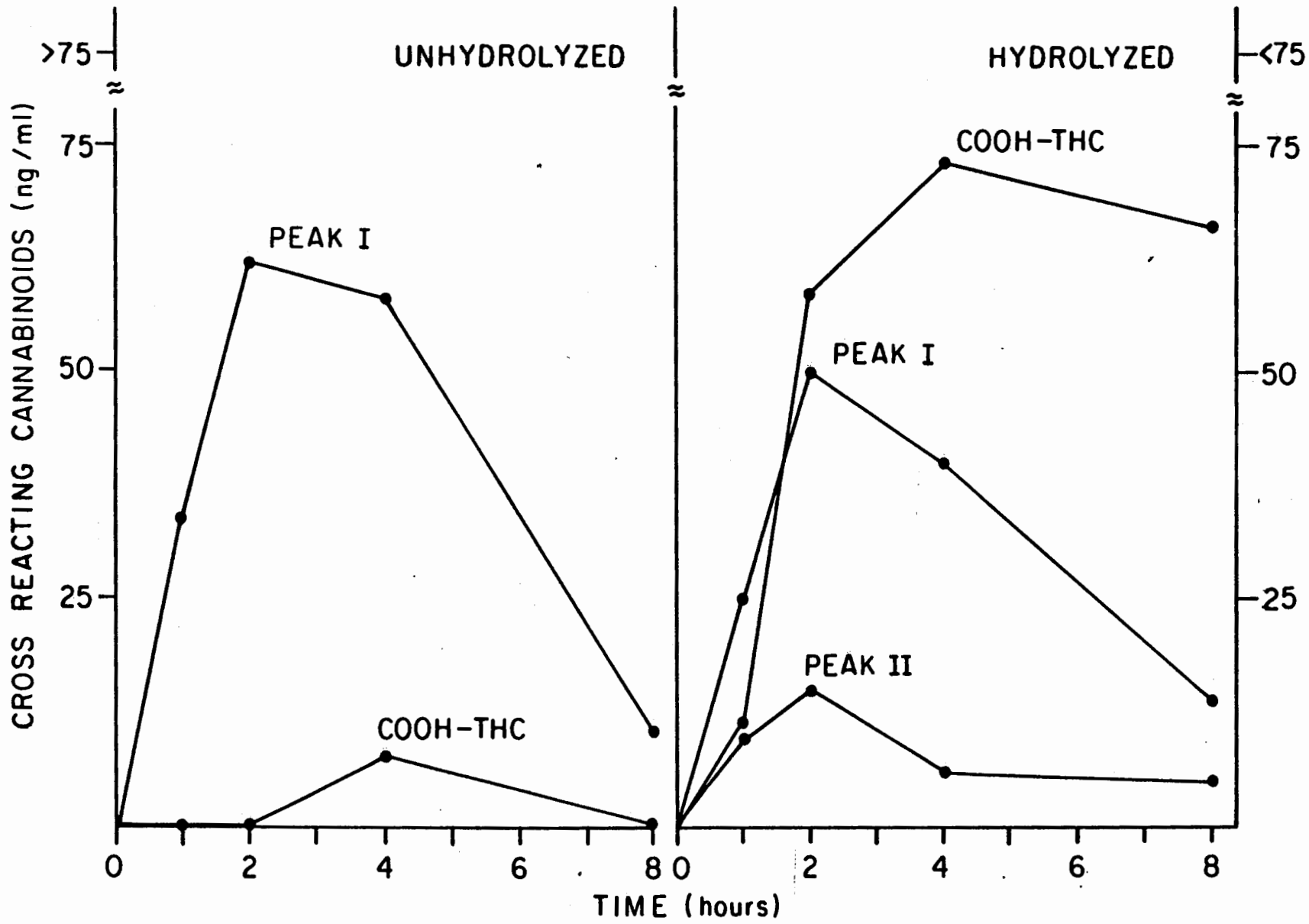
Figure 6: HPLC-RIA chromatograms of enzyme hydrolyzed urine samples collected from a frequent user 2 and 4 hours after smoking a marijuana cigarette containing 1.8% THC.

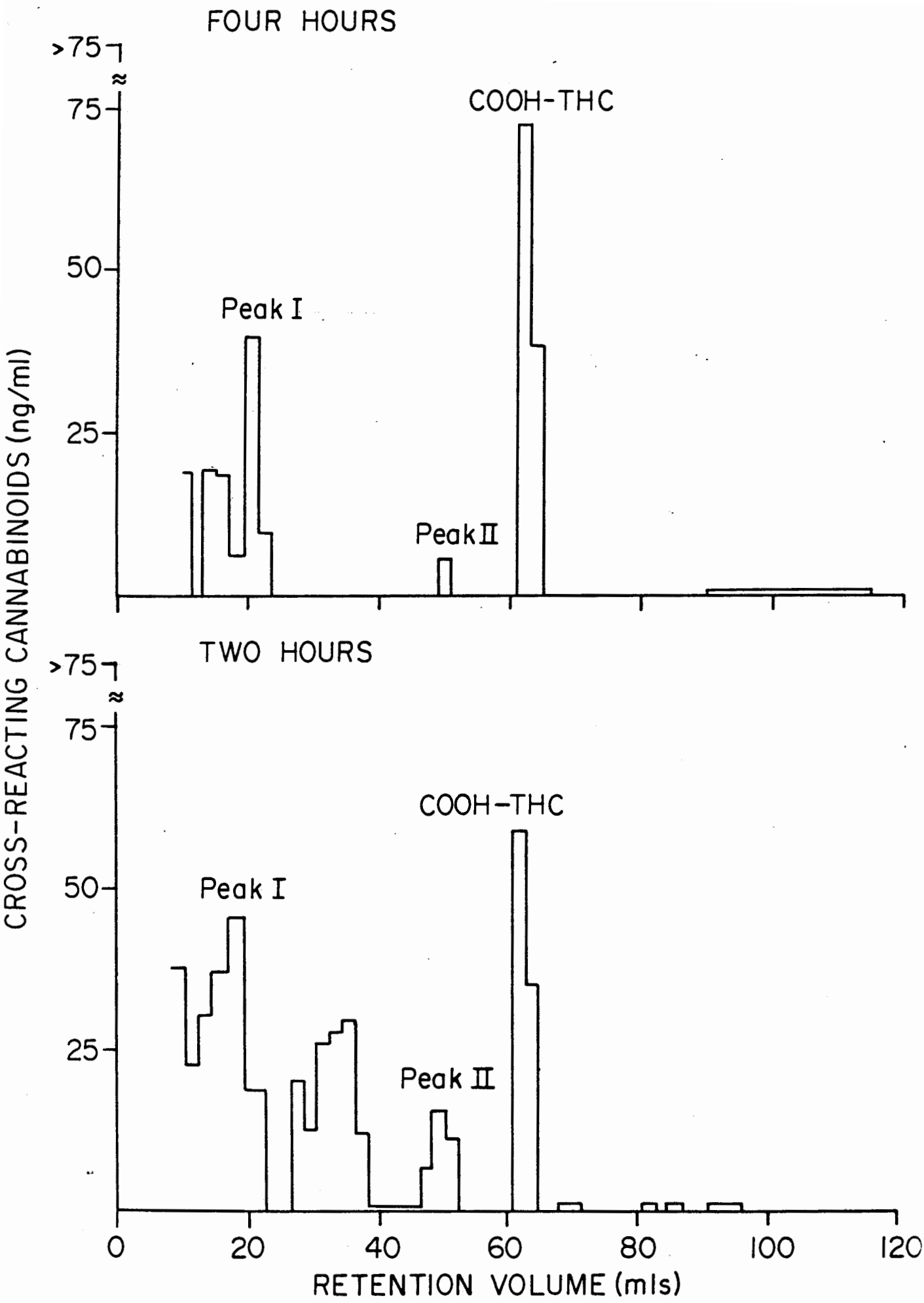
Figure 7: Concentrations of cross-reacting cannabinoids (CRC ng/mL) in the HPLC-RIA fractions corresponding to COOH-THC, I, II and III.

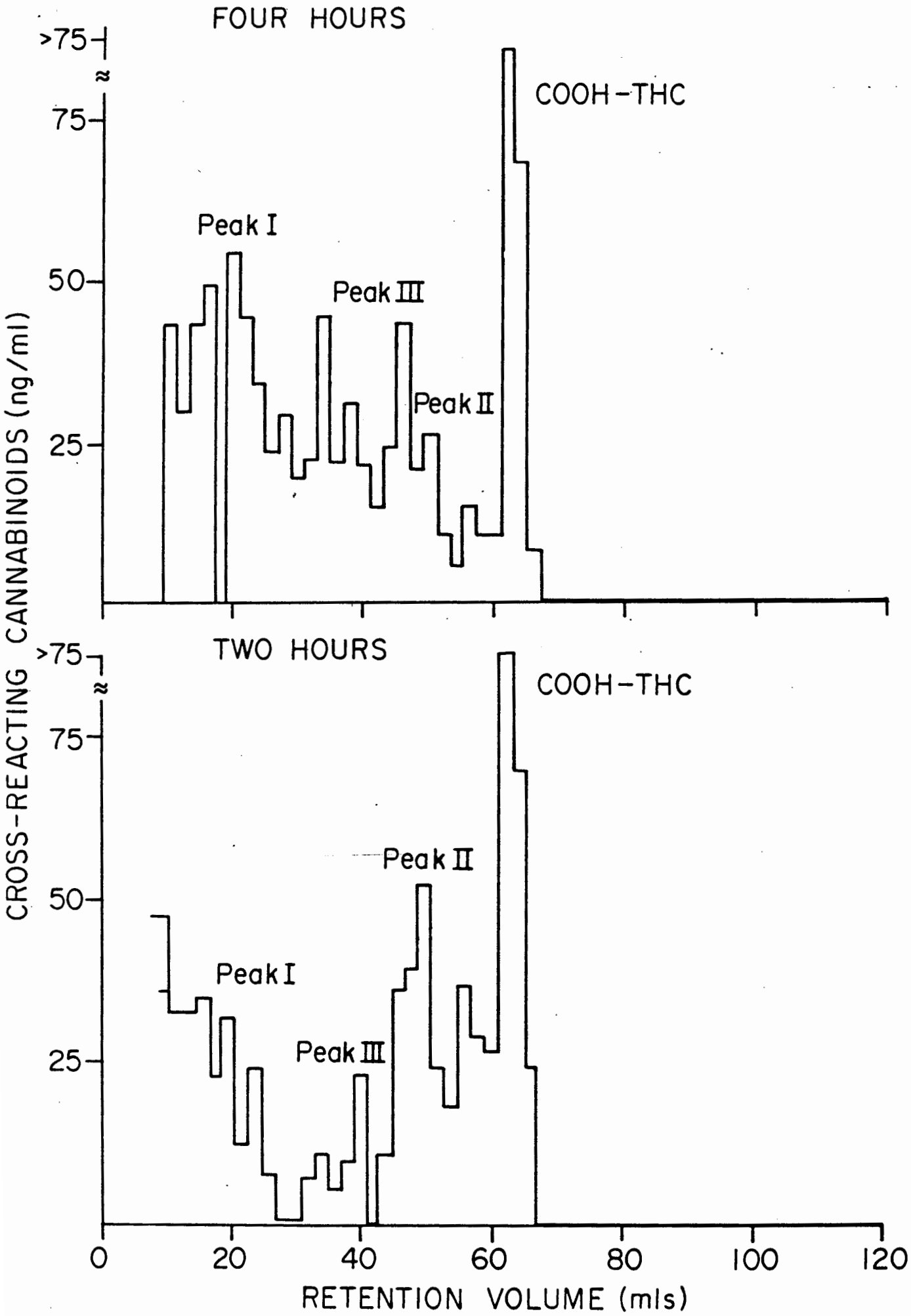

8α, 11-DI-OH-Δ9-THC
8α-OH-Δ9-THC
11-OH-Δ9-THC
COOH-Δ9-THC
Δ9-THC
RETENTION VOLUME (mls)
0
20
40
60
80
100
120
0
20
40
60
80
PERCENT OF CH3CN

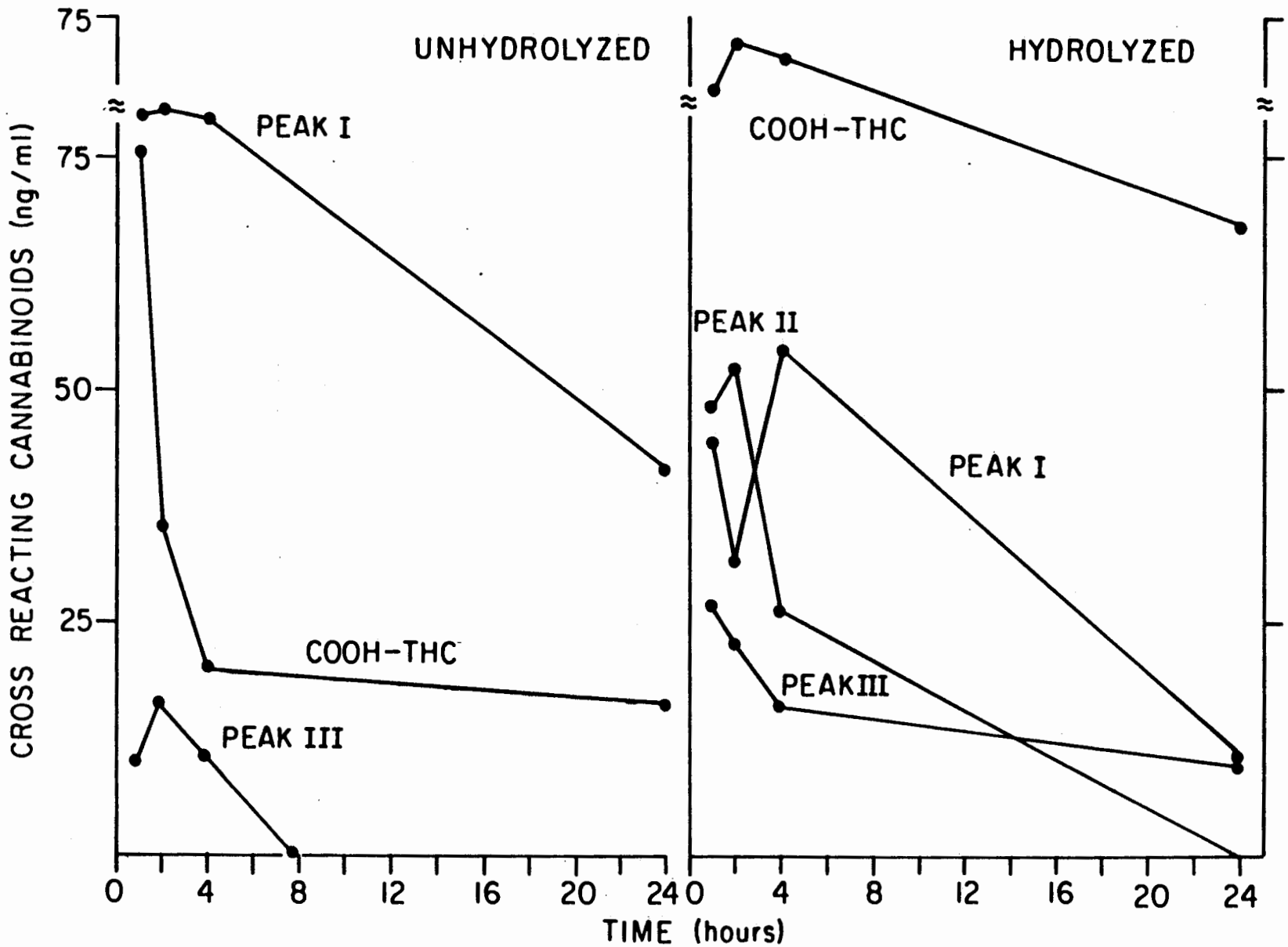

FOUR HOURS
>75
≈
75
50
25
Peak I
TWO HOURS
>75
≈
75
50
25
Peak I
CROSS-REACTING CANNABINOIDS (ng/ml)
0
20
40
60
80
100
120
RETENTION VOLUME (mls)


FOUR HOURS
>75
≈
75
50
25
Peak I
Peak III
COOH-THC
TWO HOURS
>75
≈
75
50
25
Peak I
Peak III
COOH-THC
CROSS-REACTING CANNABOIDS (ng/ml)
0
20
40
60
80
100
120
RETENTION VOLUME (mls)


UNHYDROLYZED
HYDROLYZED
>75
<75
75
50
25
CROSS REACTING CANNABINOIDS (ng/ml)
PEAK I
COOH-THC
PEAK I
PEAK II
COOH-THC
0
2
4
6
8
TIME (hours)


FOUR HOURS
>75
≈
75
50
25
COOH-THC
Peak I
Peak II
TWO HOURS
>75
≈
75
50
25
COOH-THC
Peak I
Peak II
CROSS-REACTING CANNABINOIDS (ng/ml)
0
20
40
60
80
100
120
RETENTION VOLUME (mls)


FOUR HOURS
>75
≈
75
50
25
COOH-THC
Peak I
Peak III
Peak II
TWO HOURS
>75
≈
75
50
25
COOH-THC
Peak II
Peak I
Peak III
CROSS-REACTING CANNABINOIDS (ng/ml)
0
20
40
60
80
100
120
RETENTION VOLUME (mls)


UNHYDROLYZED
HYDROLYZED
PEAK I
COOH-THC
PEAK III
PEAK II
PEAK I
PEAKIII
COOH-THC
CROSS REACTING CANNABINOIDS (ng/ml)
75
75
50
25
0
4
8
12
16
20
24
TIME (hours)