Enhanced Oxidative Stability of Deuterated Squalene: The Impact of Labelling Extent on Chemical Resilience

Supporting Information

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General experimental

Reagents

D_2O (99.8%-*d*) and absolute ethanol were supplied by Merck. *n*-Hexane for flash column chromatography was obtained from Merck. Squalene-h_{50} (≥98%) was obtained from Merck. Triphenylphosphine (obtained from Merck) was subjected to flash column chromatography (*n*-hexane) then recrystallised from *n*-hexane and dried *in vacuo* then stored under nitrogen before use. Triphenyl phosphate as TraceCERT® ^{31}P-qNMR Standard (obtained from Merck) was used to quantify the concentration of triphenylphosphine solutions. NMR measurements were carried out in chloroform-d_1 ($CDCl_3$, 99.8%-*d*), obtained from Cambridge Isotope Laboratories. Chloroform-d_1 was stored under nitrogen, with silver foil, over $NaHCO_3$ and filtered then sparged with nitrogen prior to use.

Equipment and processes

A Gelman BH180 biohazard cabinet with UV emission between 100 – 280 nm was used to perform the kinetic fractionation and peroxidation studies at ambient temperature (20±5°C). The measured radiant intensity of UV was 1607 $mW \cdot m^{-2}$ at the position where samples were exposed (directly under the lamp on the floor of the biohazard cabinet). Throughout the experiment an air atmosphere was present.

The isotopic purity of the heavy water growth medium made by mixing D_2O and H_2O was confirmed with a Lantha D_2O analyser.

Column chromatography was performed using a Buchi Pure Chromatography System. Squalene was eluted with hexane, discarding the initial and final fractions. The remaining eluted solution of squalene in *n*-hexane was concentrated to *ca*. 10 mL then filtered twice through a 0.45 µm syringe filter, followed by drying *in vacuo*, with stirring, at room temperature (*ca*. 0.5 mBar, 16 h).

High-resolution mass spectrometry (HRMS) was recorded using Atmospheric Pressure Chemical Ionisation (APCI) on a LCMS-9050 Q-TOF mass spectrometer. The overall percentage deuteration of the molecules was calculated with the use of the DGet! software package.[1]

Nuclear magnetic resonance spectra were recorded at 300 K using a Bruker AVANCE DRX400 (400 MHz) spectrometer equipped with a 5 mm PABBO BB H/D z-gradient probe. ^{13}C resonances attached to deuterium appear as multiplets when only the proton nucleus is decoupled (*i.e.* $^{13}C\{^1H\}$) and resolve to singlets when both proton and deuterium nuclei are decoupled (*i.e.* $^{13}C\{^1H,^2H\}$). Integrals for residual hydrogen are normalised according to the overall deuteration level determined by mass spectrometry.

Gas chromatographic analysis of squalene for the kinetic fractionation study was performed on a Varian CP-3800 GC attached to a Varian 1200 Quadrupole MS, using electron ionisation (EI^+) as the detection method. Samples were injected onto a 30 m capillary column (Vf-5ms, Agilent) at 50°C for 2 min, followed by temperature ramp at $20°C \cdot min^{-1}$ to 280°C. Gas chromatographic analysis of squalene for purity analysis was performed on a Shimadzdu Nexis™ GC-2030 equipped with a Shimadzu triple-quad GCMS-TQ8040 NX. Samples were injected onto a 30 m capillary column (SH-I-5Sil MS, Shimadzu) at 100°C for 3 min,

followed by temperature ramp at 10°C·min^{-1} to 300°C then holding at 300°C for 10 minutes. Squalene retention times were inversely correlated with deuteration level using both methods. All samples of squalene prepared for this study were found to be pure by GC.

Preparation of the NMR standard solution

The standard solution of triphenylphosphine was produced by dissolving 1.60 g of triphenylphosphine purified as stated above in 50 mL of freshly nitrogen-sparged chloroform-d_1. The theoretical concentration was 0.122 M and the actual concentration was measured at 0.122, 0.121 and 0.122 M using triphenyl phosphate (99.97% purity) as standard prior to commencement of the study. The concentration of the standard was again measured at 0.121 M at the completion of the study, one week after preparation of the standard solution. The standard was kept under nitrogen, in a sealed Schlenk flask, at —20°C, in the dark, when not in use and given one hour to return to ambient temperature prior to dispensing by micropipette. All experiments took place in air-conditioned laboratories with temperatures of 20±5°C.

Standard solutions of squalene-h_{50} after flash column chromatography, [U-^{2}H]squalene (19%-*d*), [U-^{2}H]squalene (46%-*d*) and [U-^{2}H]squalene (74%-*d*) were prepared in *n*-hexane and the standard solution of squalene-h_{50} (commercial, used as received) was prepared in absolute ethanol. Squalene standard solutions were produced between 0.0147 and 0.0168 M such that between 765 and 877 µL was dispensed for experiments to give equimolar (1.29×10^{-5} mol) quantities for each repeat measurement.

Biosynthesis

Growth media were sterilised by vacuum filtration (0.22 µm, PES) prior to use. Optical density (OD) was measured at 600 nm using an Eppendorf D30 Biophotometer. Bead beating was performed with a BeadBeater, Biospec Products, USA. Biosynthesis and purification of deuterated squalene was conducted according to our reported protocol using a bioreactor with 2 L working volume (Minifors 2, Infors HT).[2] [U-^{2}H]Squalene at 19%-*d*, 46%-*d* and 74%-*d* was produced using a heavy water medium with, respectively, 33%, 63% and 90% deuterium enrichment.

References

1. T. E. Lockwood and A. Angeloski, *Journal of Cheminformatics*, 2024, **16**, 36.
2. C. Recsei, R. A. Russell, M. Cagnes and T. Darwish, *Organic & Biomolecular Chemistry*, 2023, **21**, 6537-6548.

DATA FOR THE PEROXIDATION STUDY

INTEGRAL OF TRIPHENYLPHOSPHINE OXIDE

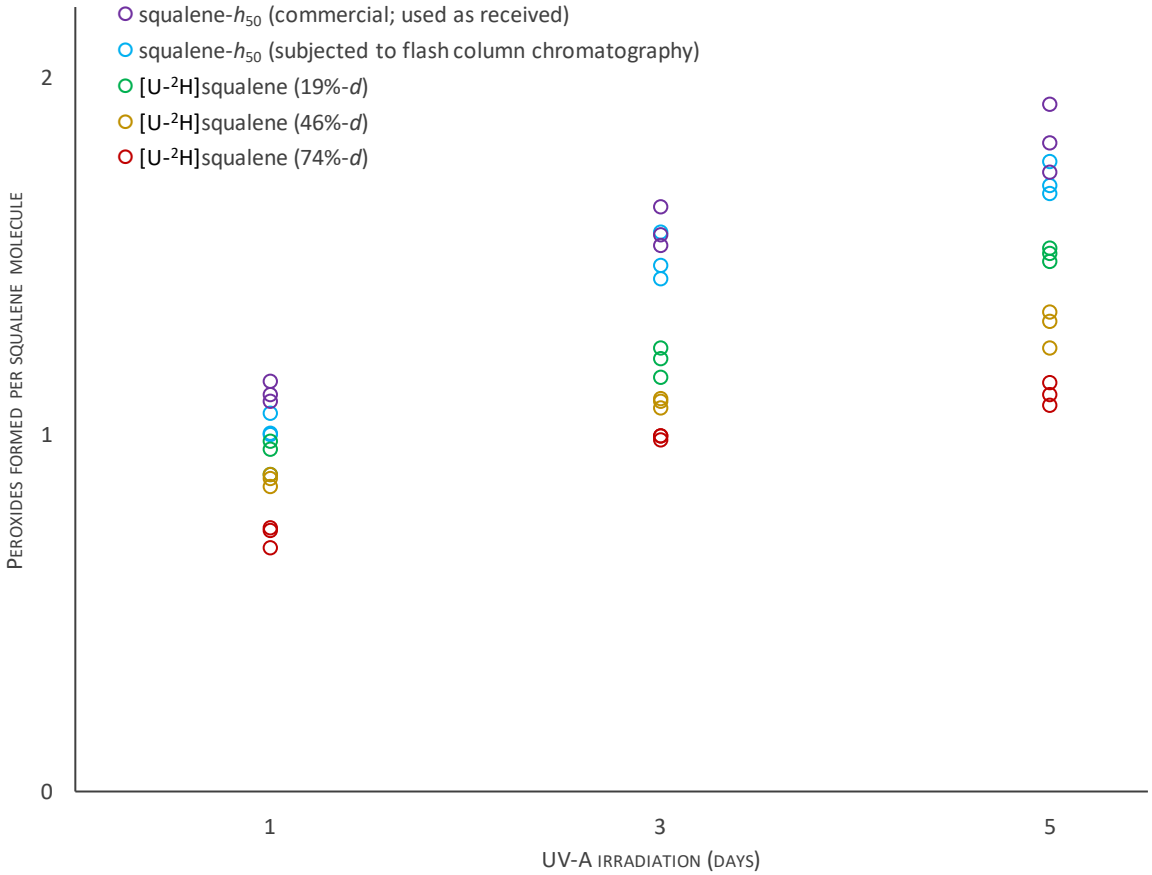
note: sum of PPh_3 + $P(O)Ph_3$ ^{31}P integrals normalised to 100

		Elapsed time (days)		
%-*d*		1	3	5
0%	repeat1	14.9523	20.7686	23.6303
(commercial)	r2	14.1501	22.1064	24.8878
	r3	14.0913	20.2395	23.9340
0%	r1	15.6632	21.9907	25.6117
(purified)	r2	16.2030	23.1197	24.4975
	r3	15.4207	21.5865	27.1770
19%	r1	13.8439	16.3766	21.2749
	r2	13.5465	17.0948	20.9595
	r3	12.5411	17.5111	21.4593
46%	r1	12.3706	15.1460	17.5122
	r2	12.5312	15.5047	18.5896
	r3	12.0617	15.4046	18.9524
74%	r1	10.3868	13.8929	15.2321
	r2	10.3158	14.0262	15.6838
	r3	9.6344	14.0237	16.1376

PEROXIDES PER SQUALENE

		Elapsed time (days)		
%-*d*		1	3	5
0%	repeat1	1.0622	1.4755	1.6788
(commercial)	r2	1.0053	1.5705	1.7681
	r3	1.0011	1.4379	1.7003
0%	r1	1.1122	1.5615	1.8186
(purified)	r2	1.1505	1.6416	1.7395
	r3	1.0950	1.5328	1.9297
19%	r1	0.9832	1.1631	1.5110
	r2	0.9621	1.2141	1.4886
	r3	0.8907	1.2437	1.5241
46%	r1	0.8784	1.0754	1.2435
	r2	0.8898	1.1009	1.3200
	r3	0.8564	1.0938	1.3457
74%	r1	0.7381	0.9873	1.0825
	r2	0.7331	0.9968	1.1146
	r3	0.6847	0.9966	1.1468
		0	0	0

GRAPH OF DATA FOR THE PEROXIDATION STUDY INCLUDING COMMERCIAL SQUALENE-h_{50} (USED AS RECEIVED)



Note: A small but detectable difference between the commercial squalene-h_{50} subjected to flash column chromatography and the commercial material used as received (violet and blue open circles, respectively) was observed. Although the peroxidation data overlap, one can note an apparent, small increased tendency toward oxidation after flash column chromatography. This small effect may be due to the presence of small traces of silica (although for all samples subjected to chromatography filtration was performed twice through a 0.45 µm PTFE syringe filter prior to removal of hexane *in vacuo*, and the silica particle size is nominally 40 – 63 µm).

NOTES ON THE STATISTICAL SIGNIFICANCE OF THE RESULTS OF THE PEROXIDATION STUDY

These studies assume that observed results on page 4 (see also Table **1** in the main article) are normally distributed around a measured mean. There is assumed to be a theoretical, true mean value for peroxidation as a function of deuteration level at each specific time point if all other variables are identical. We have used t-tests to interrogate the relationship between the measured means and the hypothetical, true means. For example, in the peroxidation study the measured mean number of peroxides formed per [U-^{2}H]squalene (19%-*d*) molecules is 1.21 after 3 days irradiation while the average for protiated, purified squalene is 1.58 ($\sigma_{19\%}$ = 0.041, $\sigma_{0\%}$ = 0.056). Assuming a null hypothesis that the true mean of squalene peroxidation events is invariant with deuteration level returns a P-value of P<0.001 (<0.1% chance) that the variance observed in the oxidation study between [U-^{2}H]squalene (19%-*d*) and squalene-h_{50} at t = 3 days would arise by chance, using a standard two-sample t-test. The same null hypothesis for [U-^{2}H]squalene (46%-*d* or 74%-*d*) versus squalene-h_{50} is even less likely. This calculation suggests that it is very likely that deuteration truly reduces the susceptibility of squalene to oxidation. It may be the case, however, that the true is effect is less pronounced than the data suggest. Assuming a null hypothesis that the mean number of peroxides per [U-^{2}H]squalene (19%-*d*) is at most two thirds of the measured value returns P<0.04 which is still considered statistically significant (<5% chance). For the more closely clustered data points of [U-^{2}H]squalene (46%-*d* and 74%-*d*), the likelihood of the true mean having less than ⅔ of the observed difference with purified, protiated squalene is extremely low (P<0.003). We therefore assert that the measured, reduced tendency toward peroxidation with increasing deuteration level represents a true phenomenon of a magnitude highly likely to be close to that observed in the current study.

DATA FOR THE KINETIC FRACTIONATION STUDY

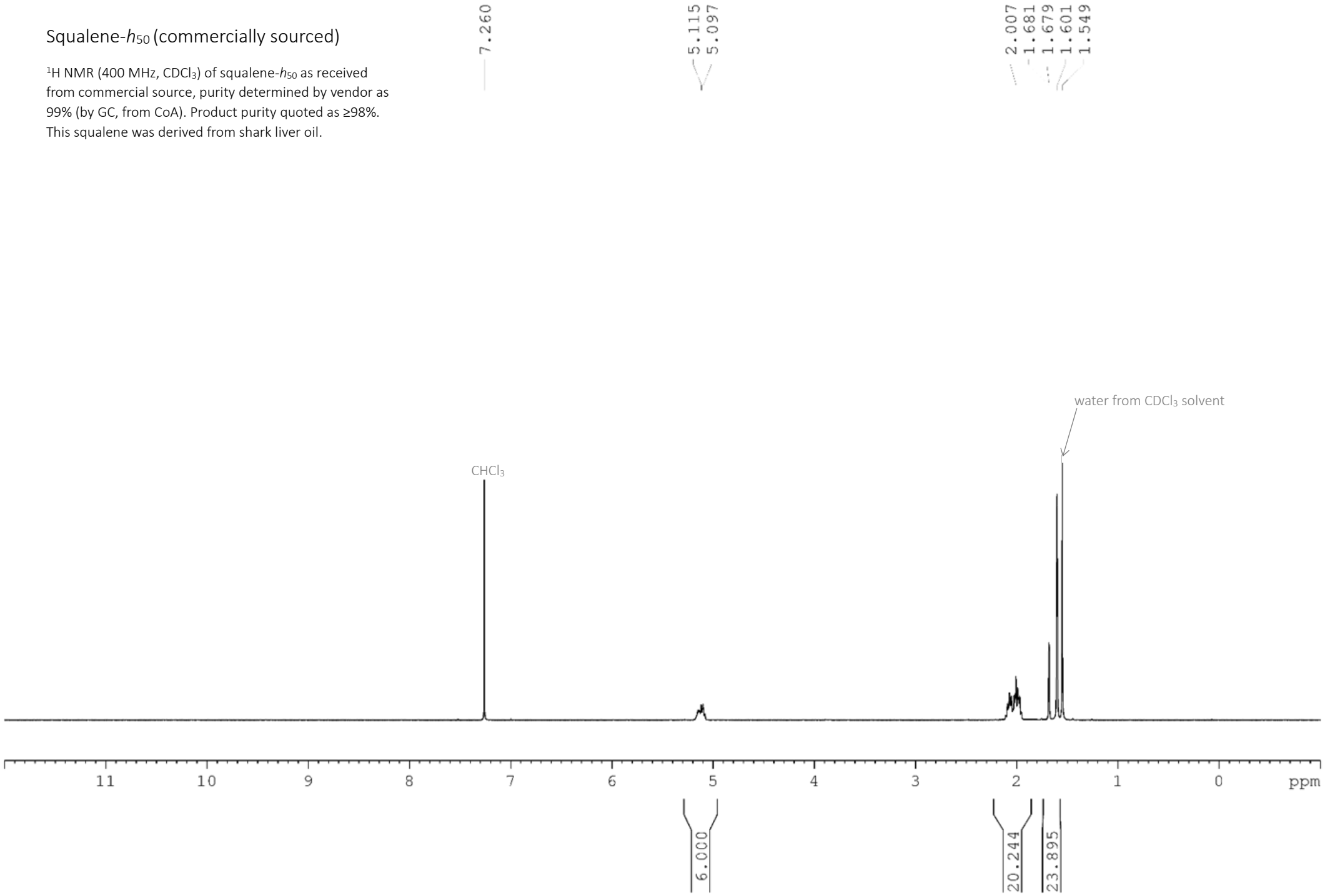
INTEGRAL RATIO

Ratio of persisting commercial squalene-h_{50} to persisting [U-^{2}H]squalene (79%-*d*) integrals (GC, EI^{+}) as a function of irradiation time. The two materials are present in equimolar quantities. The data is uncorrected for the difference in response factor associated with deuteration.

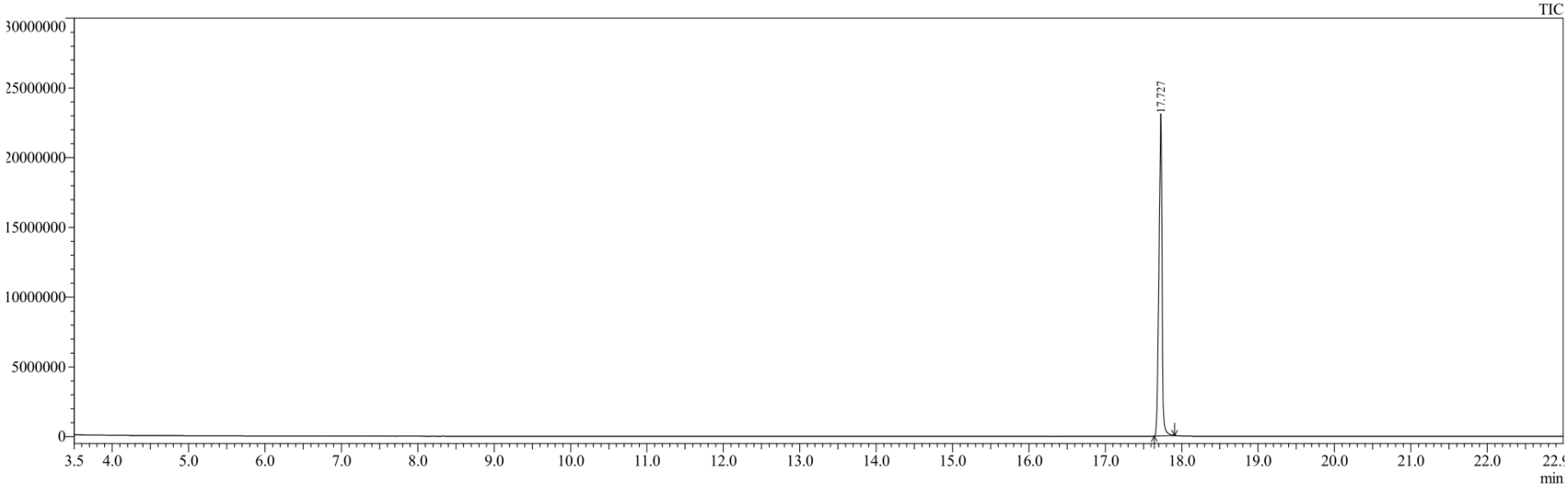
		UV-A irradiation (days)			
%-*d*		0	1	3	5
0% / 79%	repeat1	1.246	1.065	0.428	0.319
	r2	1.292	0.904	0.411	0.250
	r3	1.276	1.036	0.384	0.214

Squalene-h_{50} (commercially sourced)

^{1}H NMR (400 MHz, $CDCl_3$) of squalene-h_{50} as received from commercial source, purity determined by vendor as 99% (by GC, from CoA). Product purity quoted as ≥98%. This squalene was derived from shark liver oil.



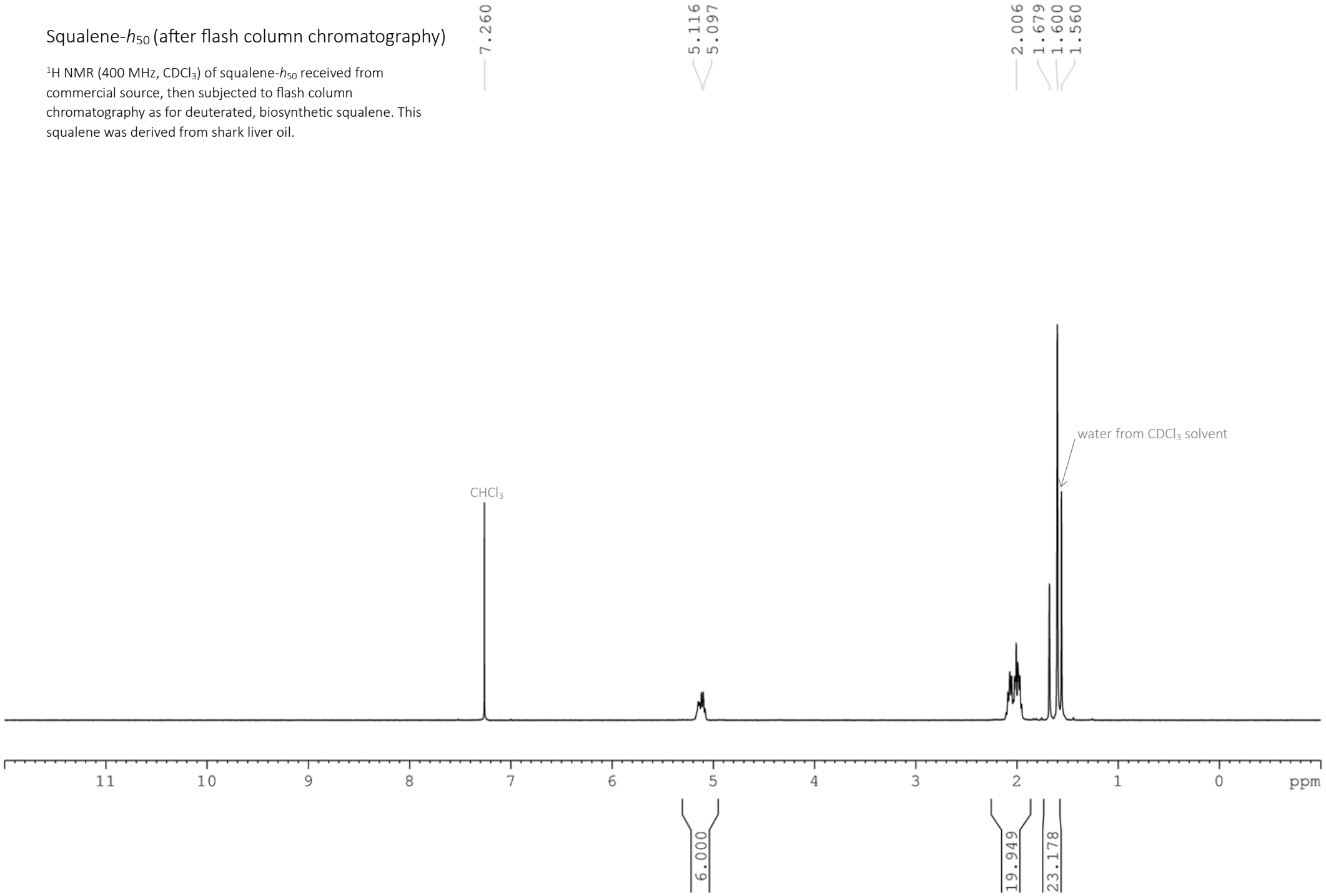
Squalene-h_{50} (commercially sourced)



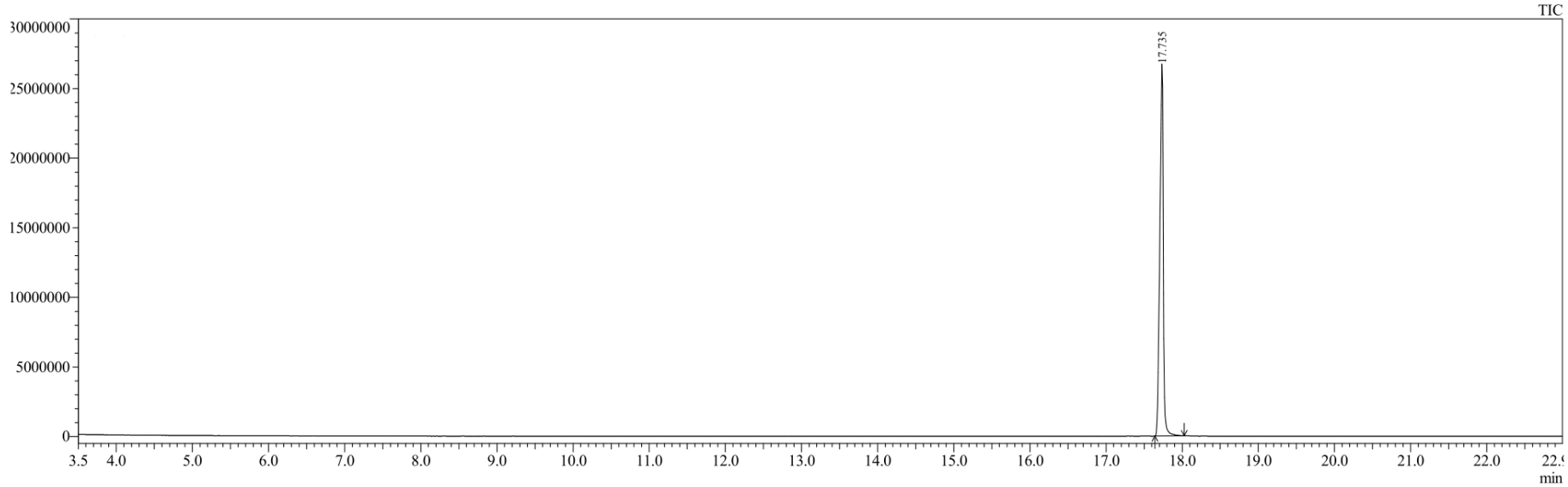
ABOVE: GC trace (TIC, EI^+) of squalene-h_{50} as received from commercial source. No other peaks were detected by automatic integration or manual inspection of the baseline. The peak was found to be a library match for squalene. The purity of this sample was determined by the vendor as 99% (by GC, from CoA). Product purity was quoted as ≥98%. This squalene was derived from shark liver oil.

Squalene-h_{50} (after flash column chromatography)

^{1}H NMR (400 MHz, $CDCl_3$) of squalene-h_{50} received from commercial source, then subjected to flash column chromatography as for deuterated, biosynthetic squalene. This squalene was derived from shark liver oil.



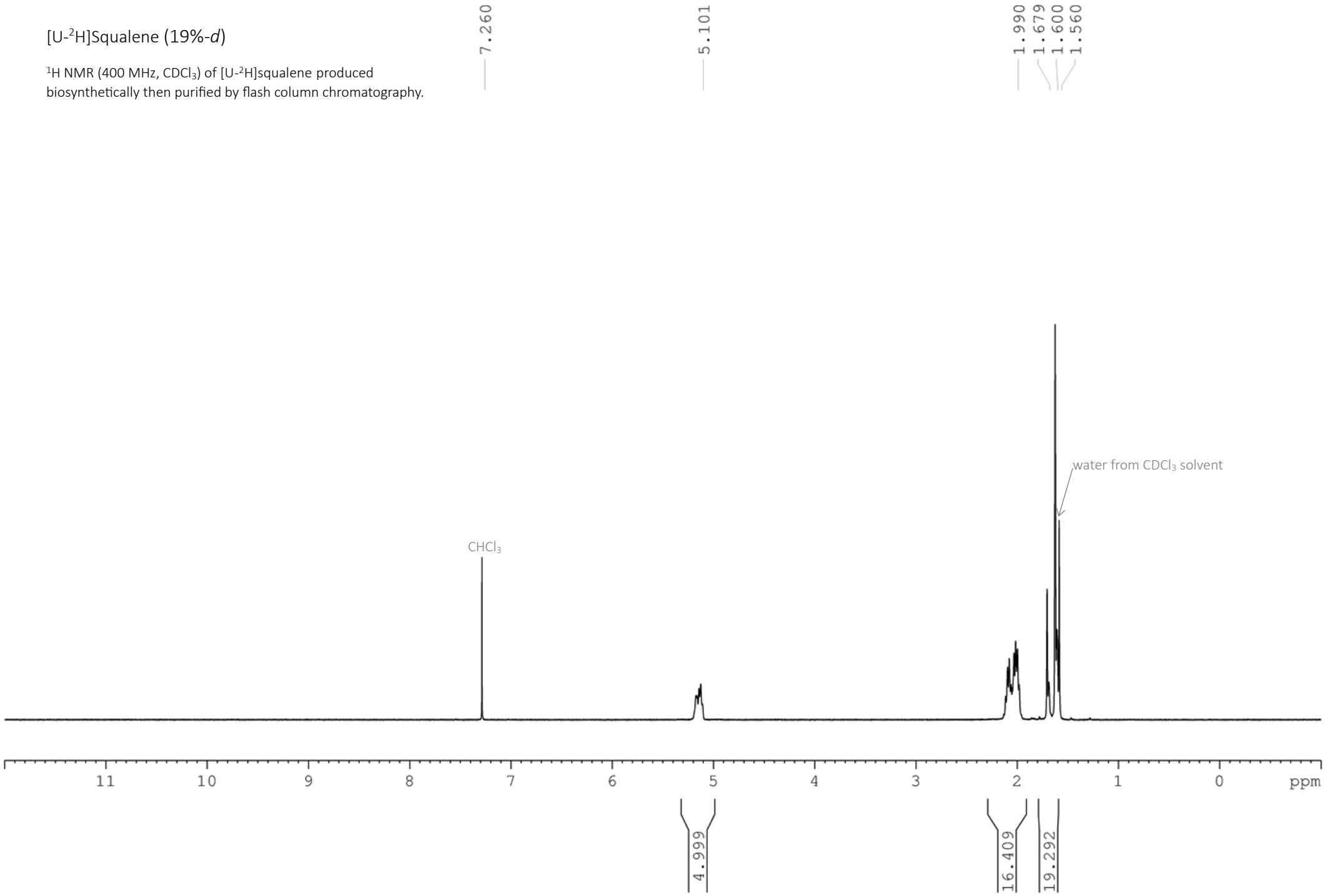
Squalene-h_{50} (after flash column chromatography)



ABOVE: GC trace (TIC, EI^+) of squalene-h_{50} as received from commercial source, then subjected to flash column chromatography as for deuterated, biosynthetic squalene. No other peaks were detected by automatic integration or manual inspection of the baseline. The peak was found to be a library match for squalene. This squalene was derived from shark liver oil.

[U-^{2}H]Squalene (19%-*d*)

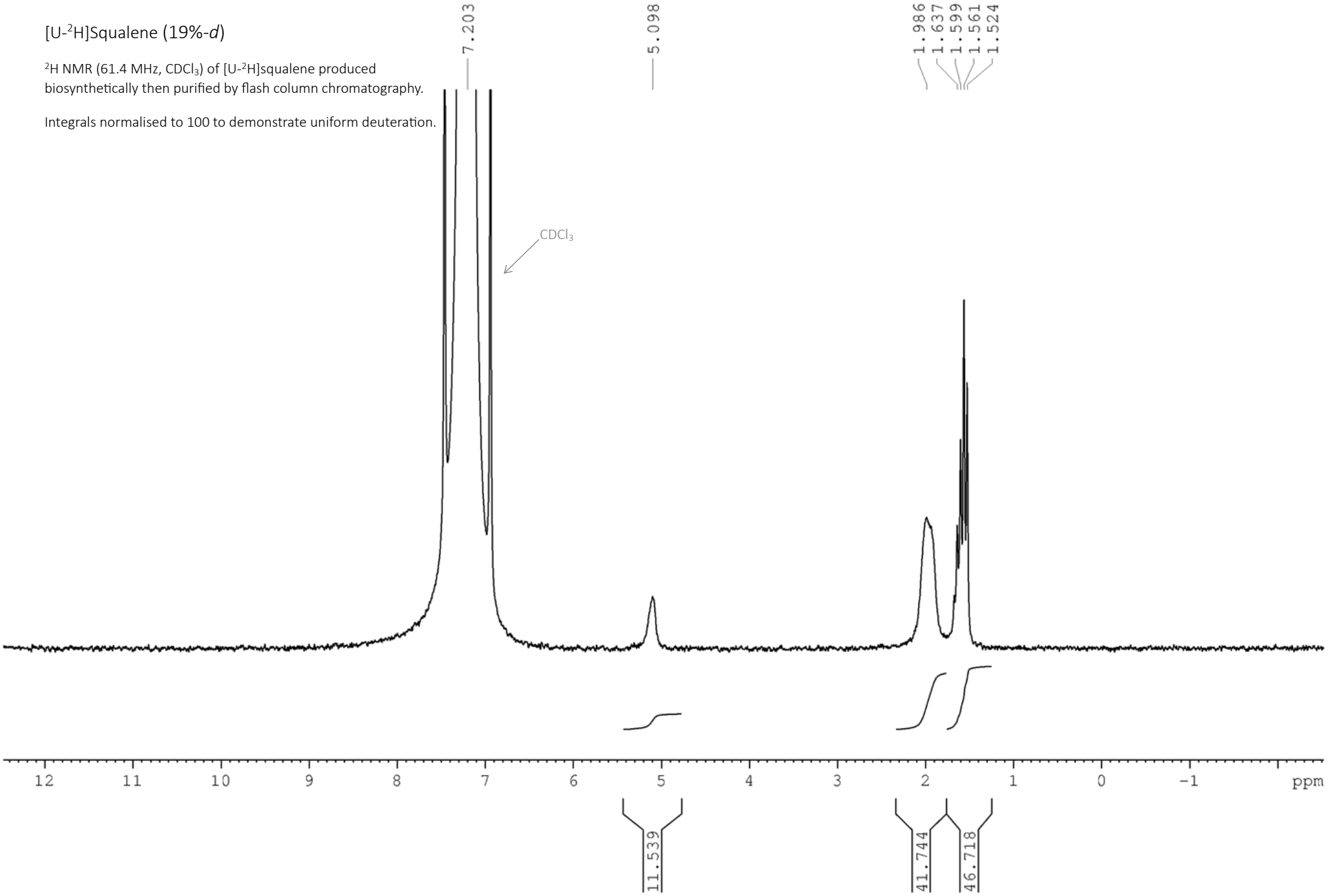
^{1}H NMR (400 MHz, $CDCl_3$) of [U-^{2}H]squalene produced biosynthetically then purified by flash column chromatography.



[U-^{2}H]Squalene (19%-*d*)

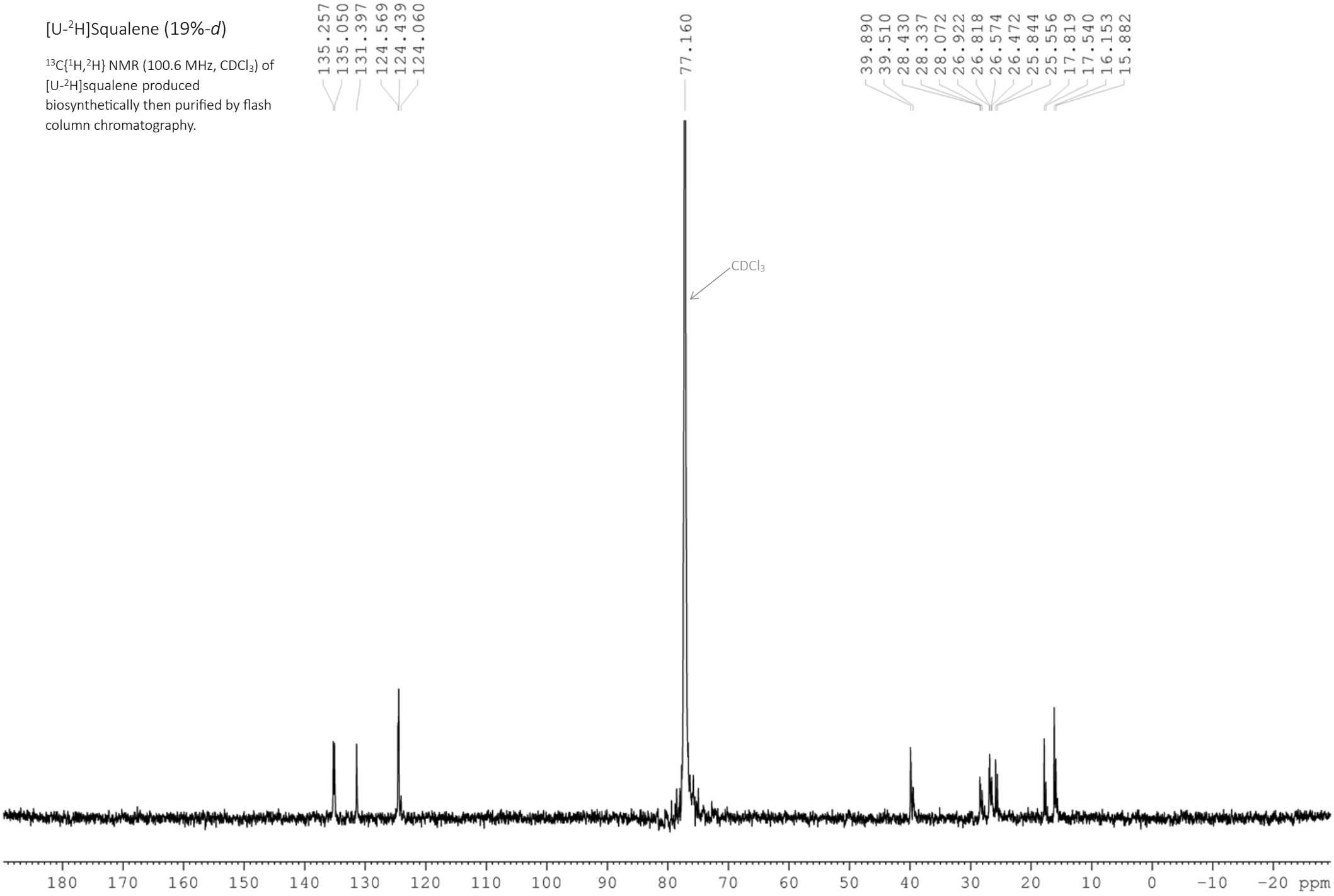
^{2}H NMR (61.4 MHz, $CDCl_3$) of [U-^{2}H]squalene produced biosynthetically then purified by flash column chromatography.

Integrals normalised to 100 to demonstrate uniform deuteration.



[U-^{2}H]Squalene (19%-*d*)

$^{13}C\{^{1}H,^{2}H\}$ NMR (100.6 MHz, $CDCl_3$) of [U-^{2}H]squalene produced biosynthetically then purified by flash column chromatography.



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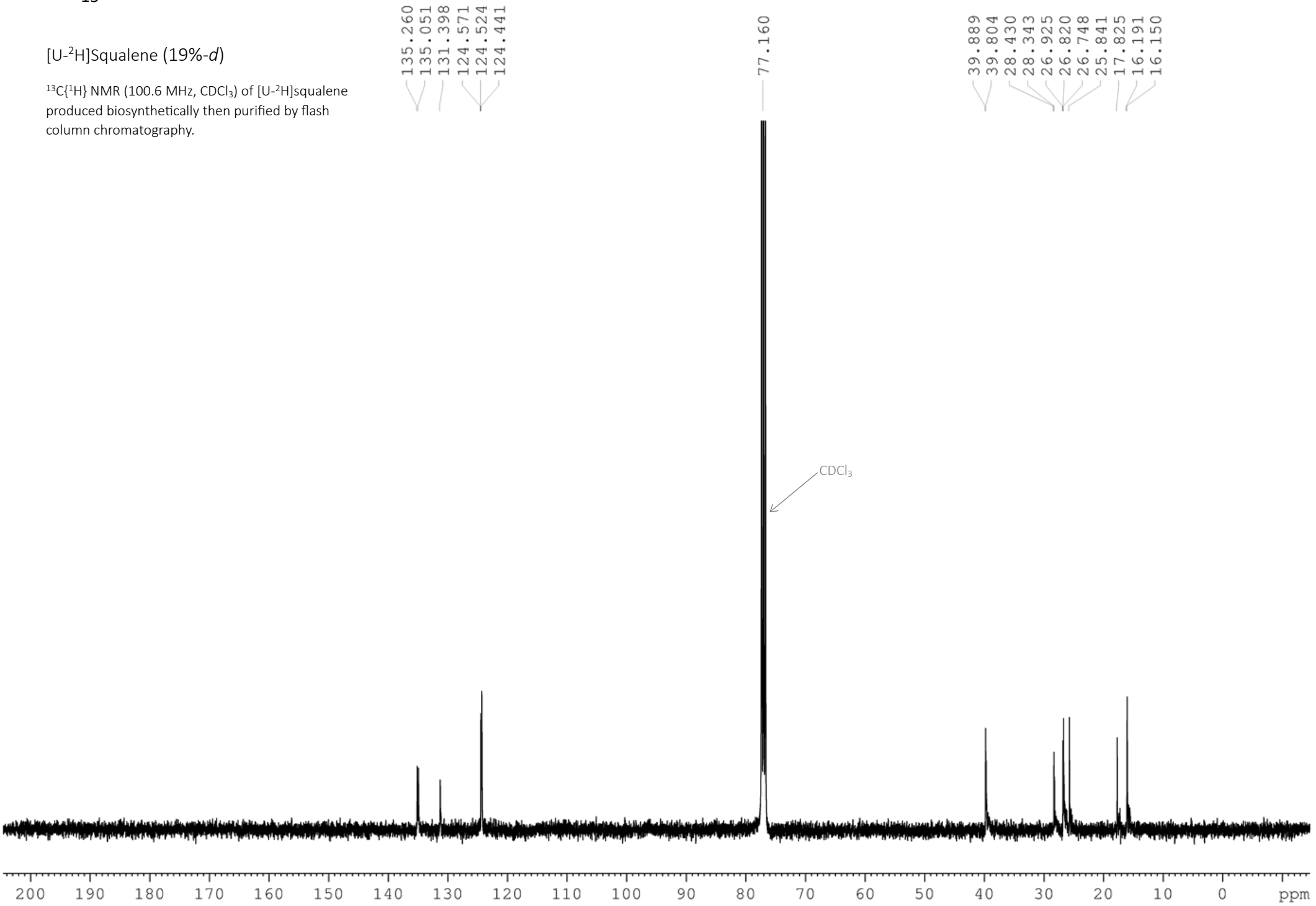
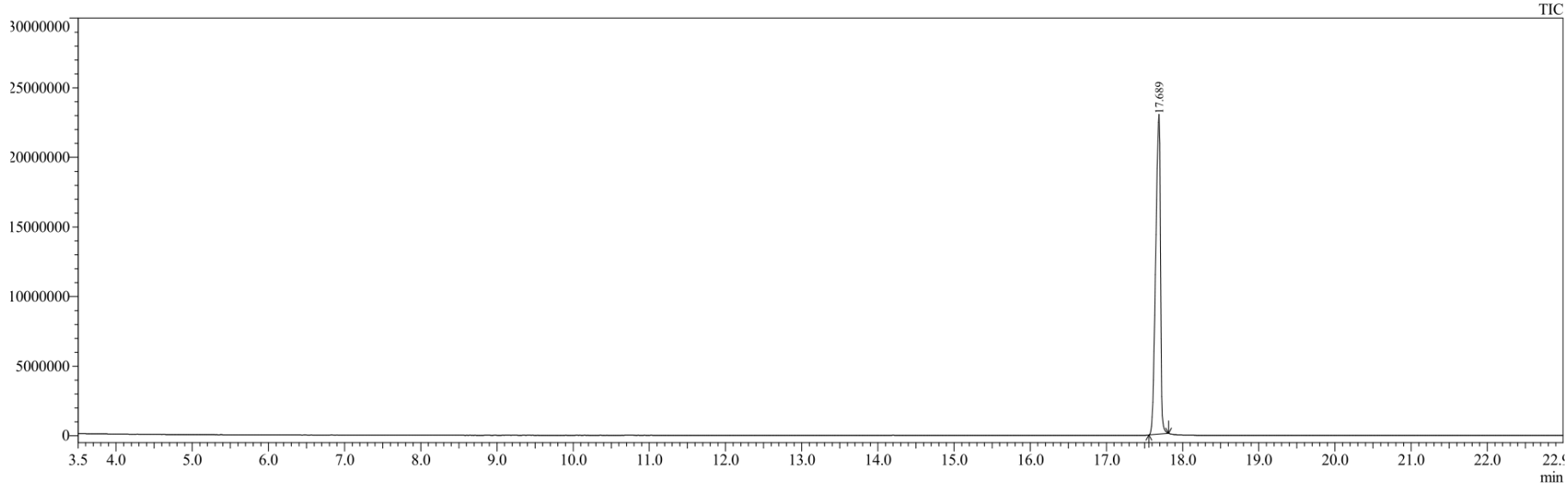


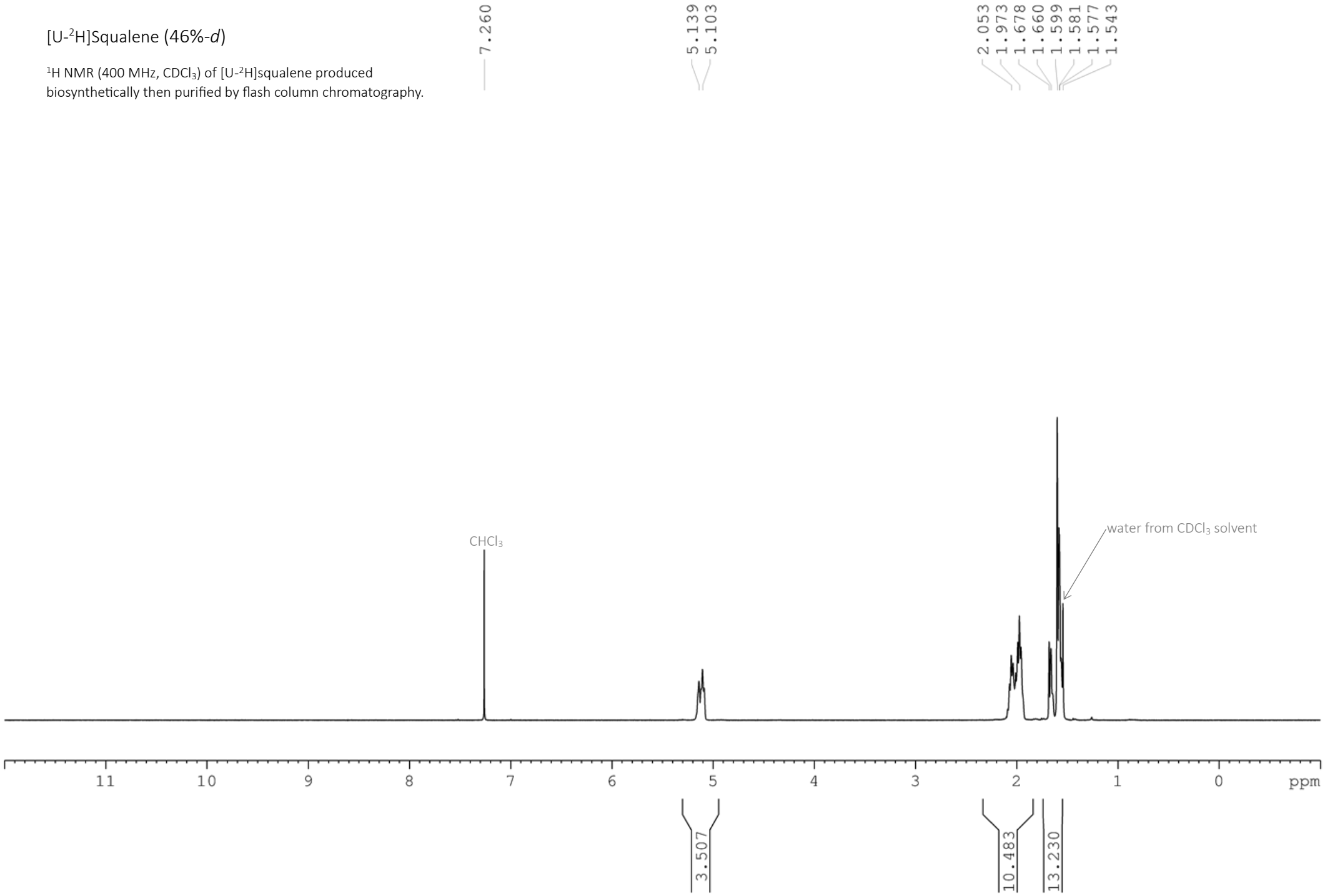
[U-^{2}H]squalene (19%-*d*)



ABOVE: GC trace (TIC, EI^{+}) of [U-^{2}H]squalene (19%-*d*) produced biosynthetically then purified by flash column chromatography. No other peaks were detected by automatic integration or manual inspection of the baseline.

[U-^{2}H]Squalene (46%-*d*)

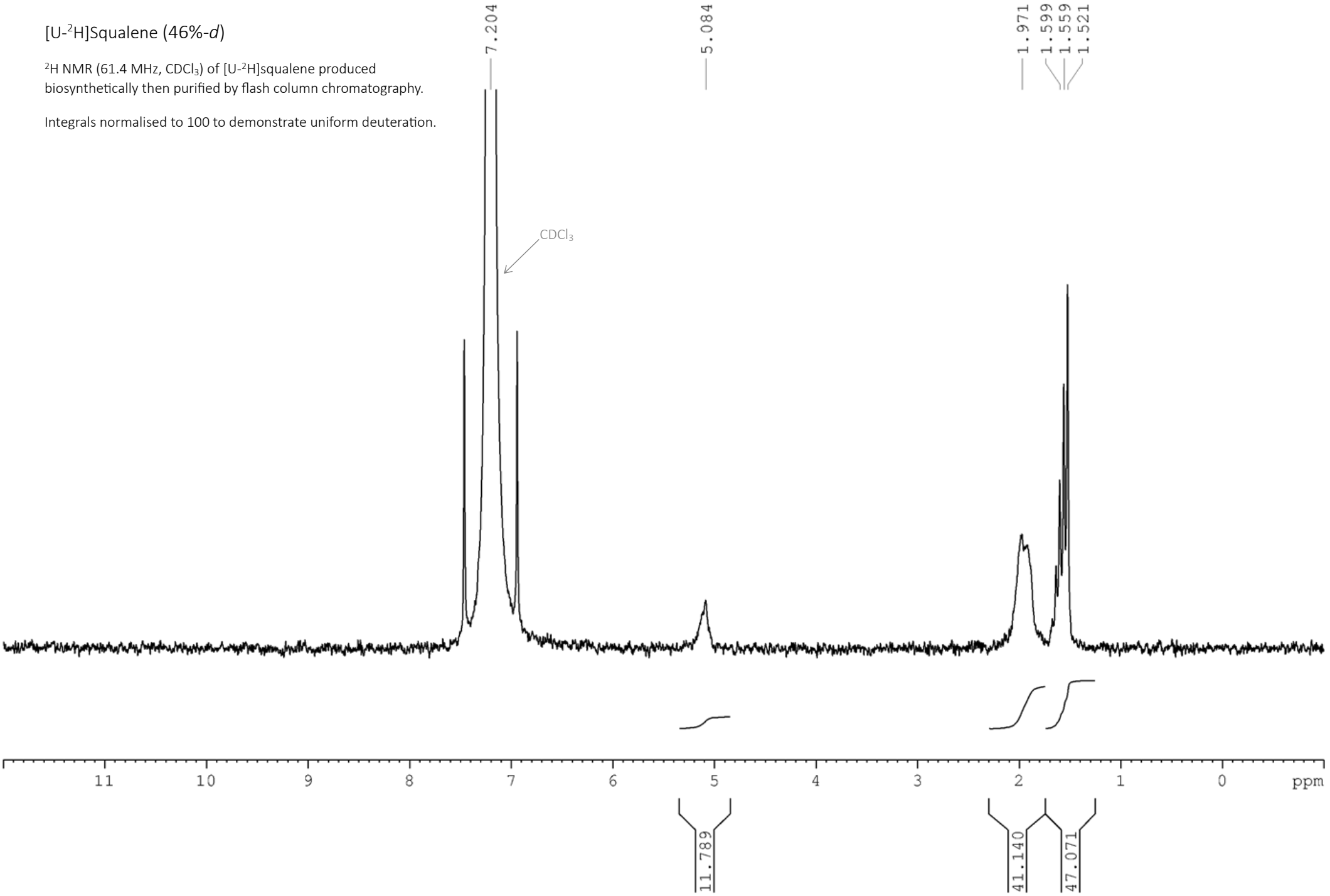
^{1}H NMR (400 MHz, $CDCl_3$) of [U-^{2}H]squalene produced biosynthetically then purified by flash column chromatography.



[U-^{2}H]Squalene (46%-*d*)

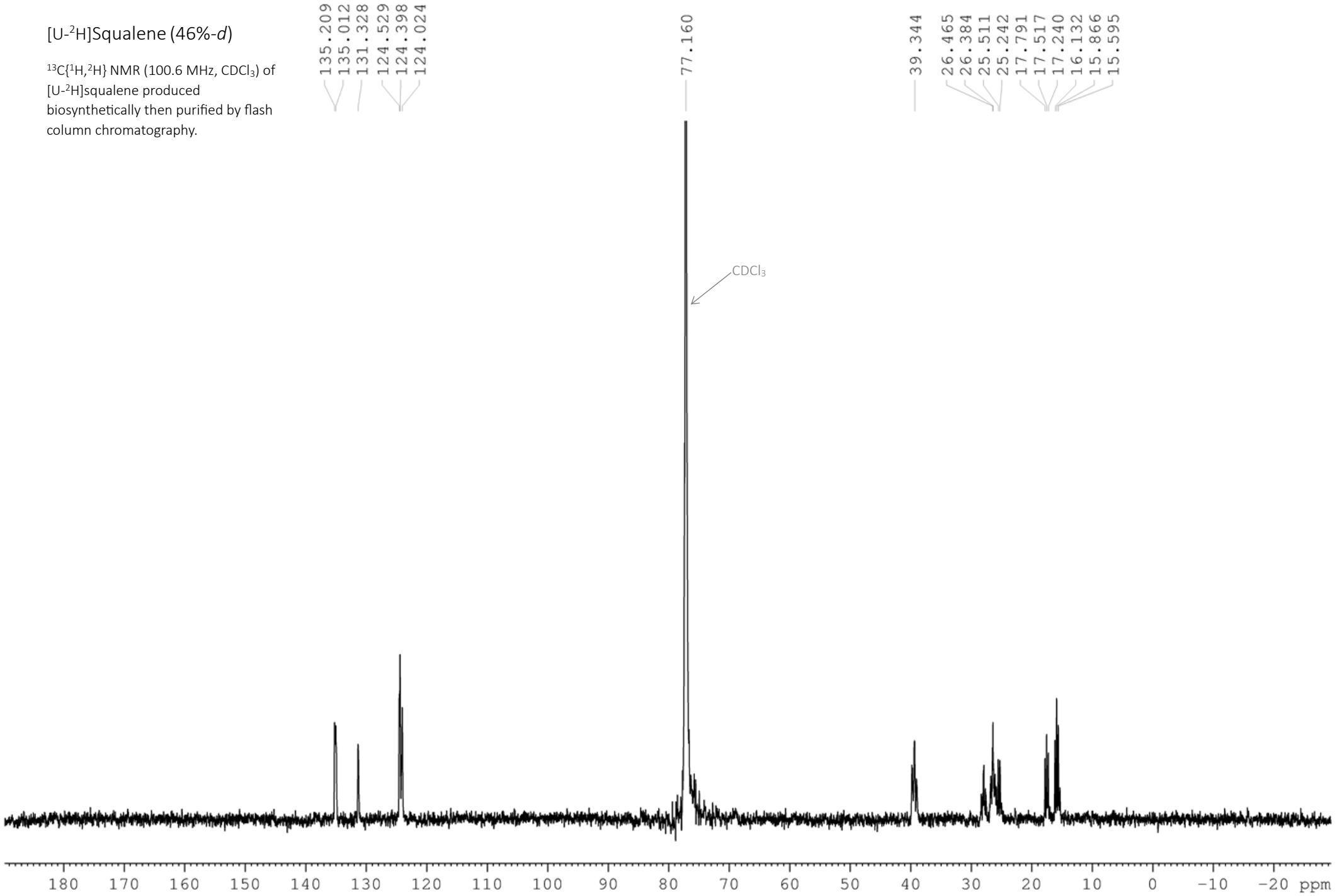
^{2}H NMR (61.4 MHz, $CDCl_3$) of [U-^{2}H]squalene produced biosynthetically then purified by flash column chromatography.

Integrals normalised to 100 to demonstrate uniform deuteration.



[U-^{2}H]Squalene (46%-*d*)

$^{13}C\{^{1}H,^{2}H\}$ NMR (100.6 MHz, $CDCl_3$) of [U-^{2}H]squalene produced biosynthetically then purified by flash column chromatography.



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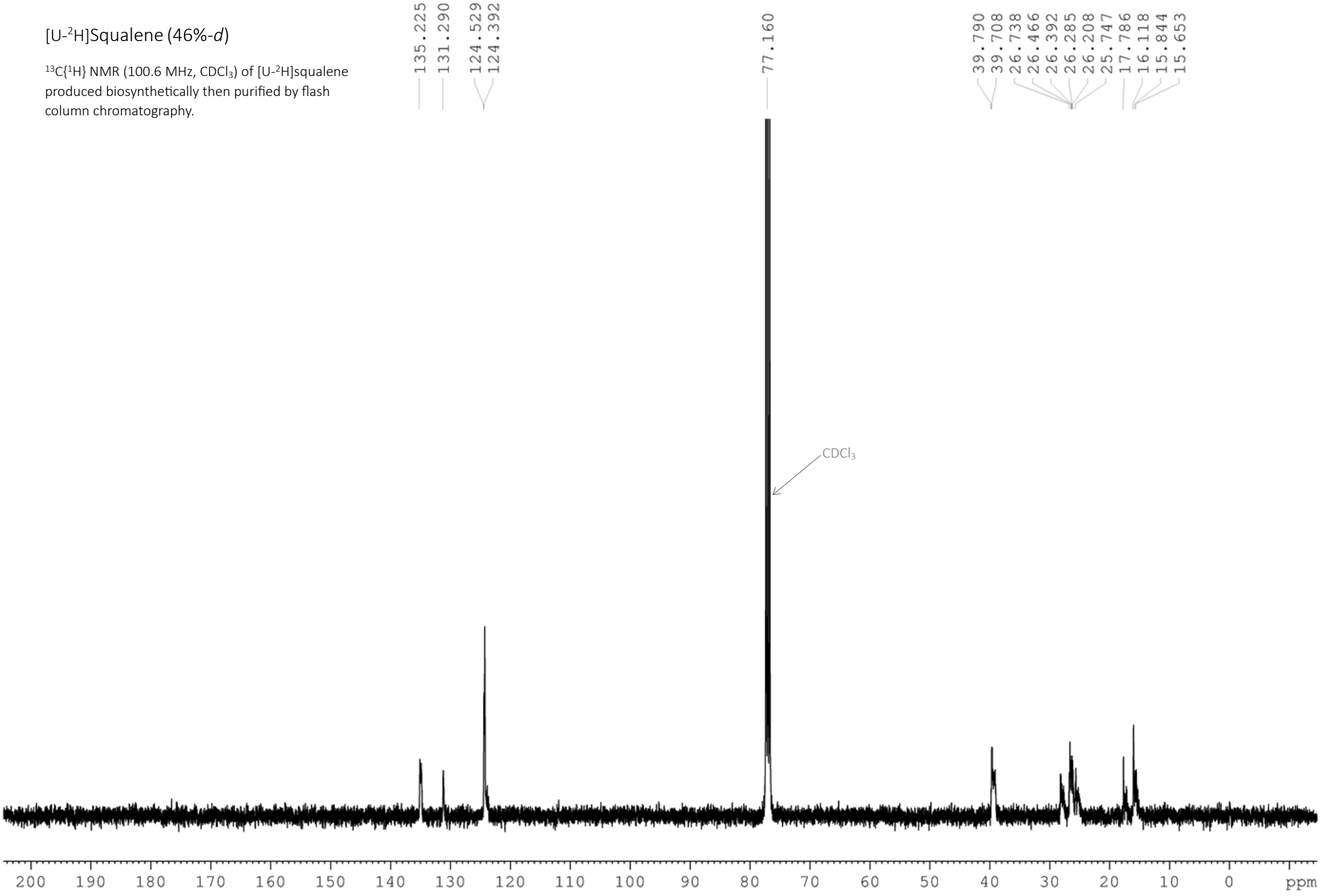
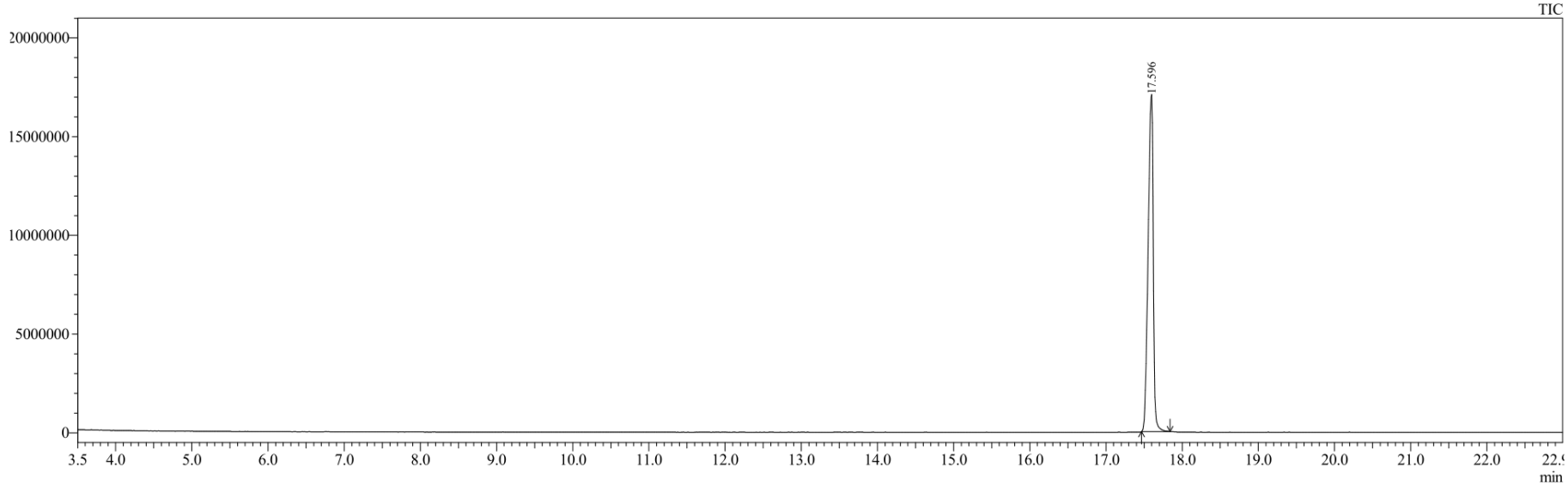


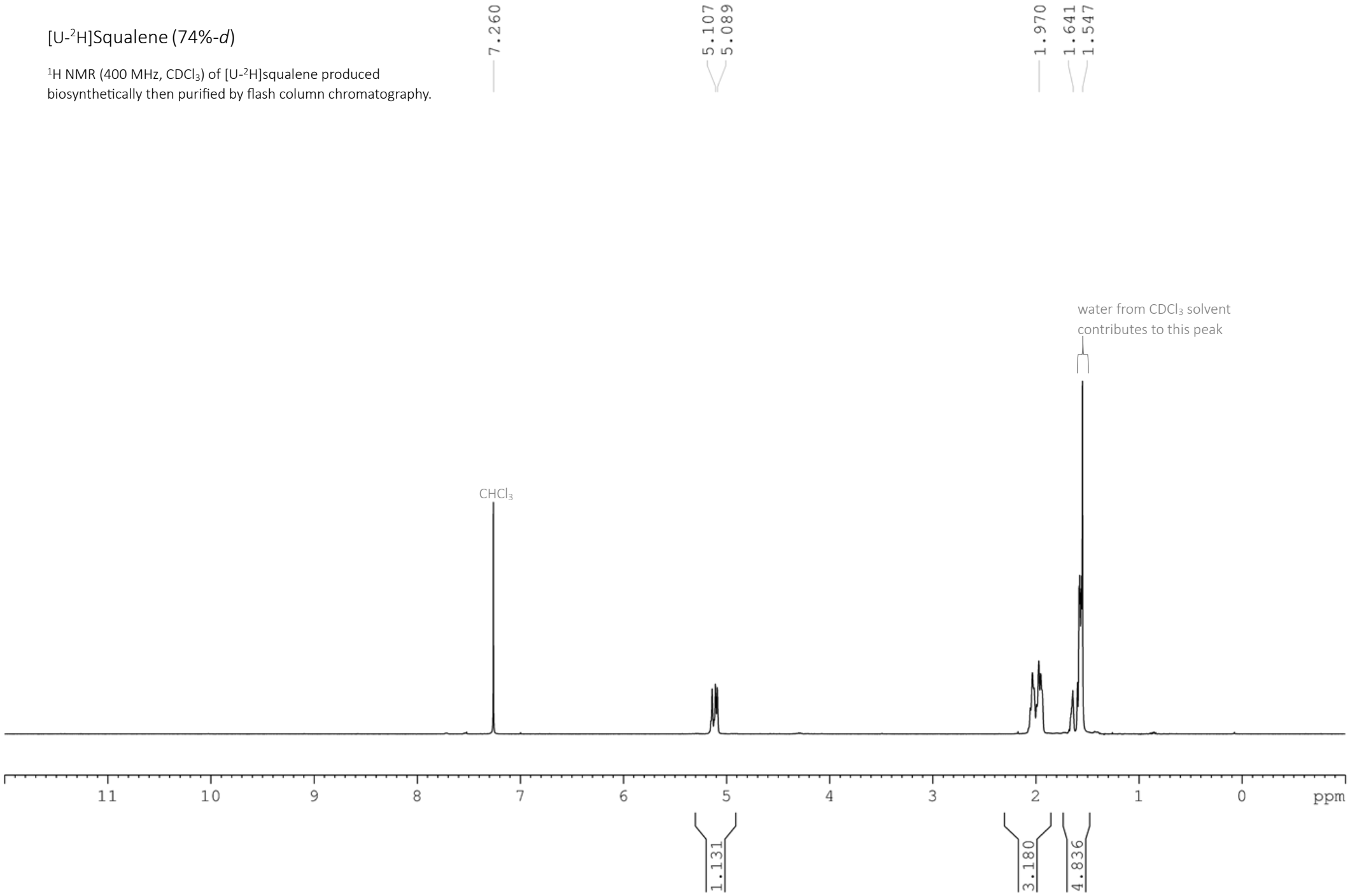
[U-^{2}H]squalene (46%-*d*)



ABOVE: GC trace (TIC, EI^{+}) of [U-^{2}H]squalene (46%-*d*) produced biosynthetically then purified by flash column chromatography. No other peaks were detected by automatic integration or manual inspection of the baseline.

[U-^{2}H]Squalene (74%-*d*)

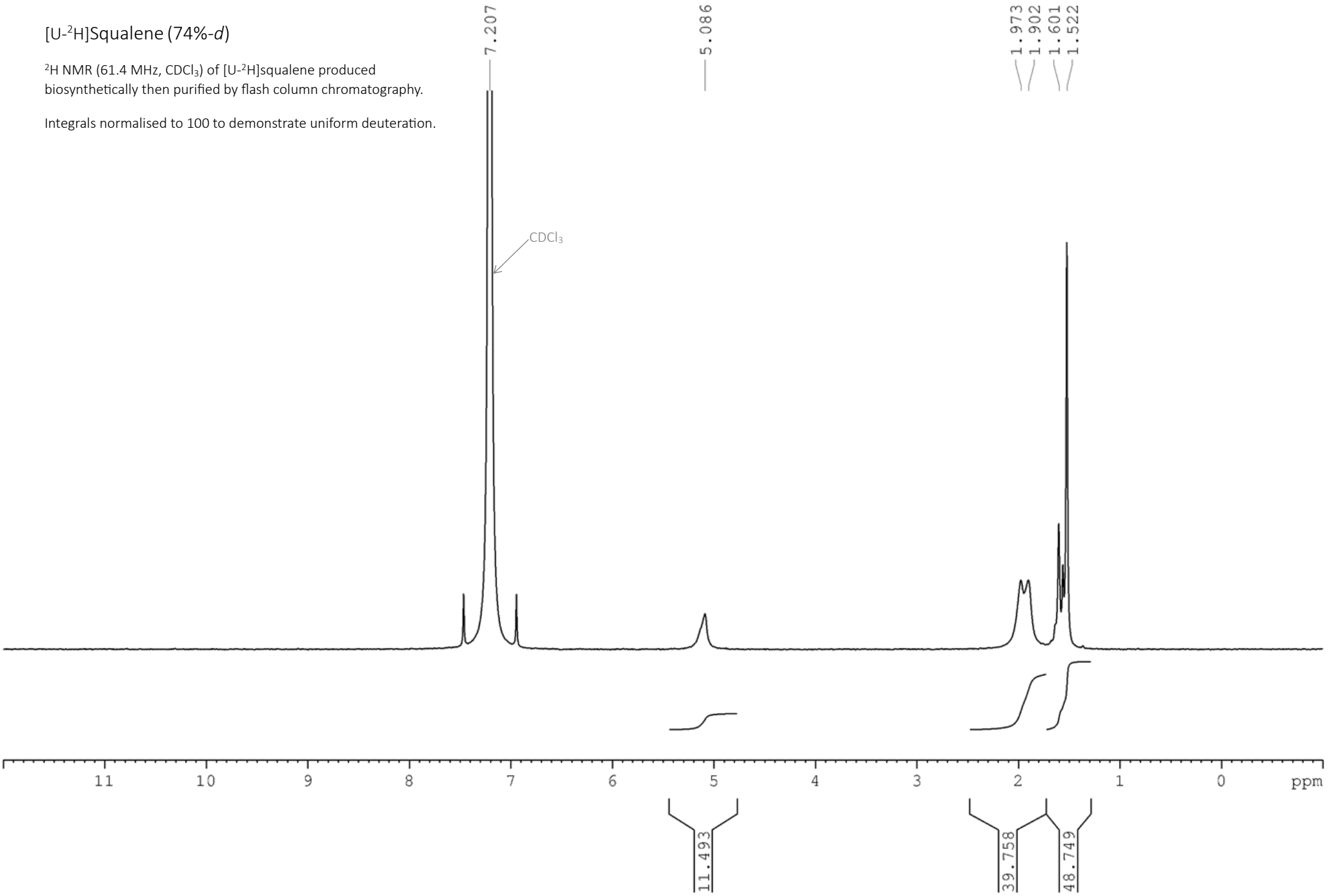
^{1}H NMR (400 MHz, $CDCl_3$) of [U-^{2}H]squalene produced biosynthetically then purified by flash column chromatography.



[U-^{2}H]Squalene (74%-*d*)

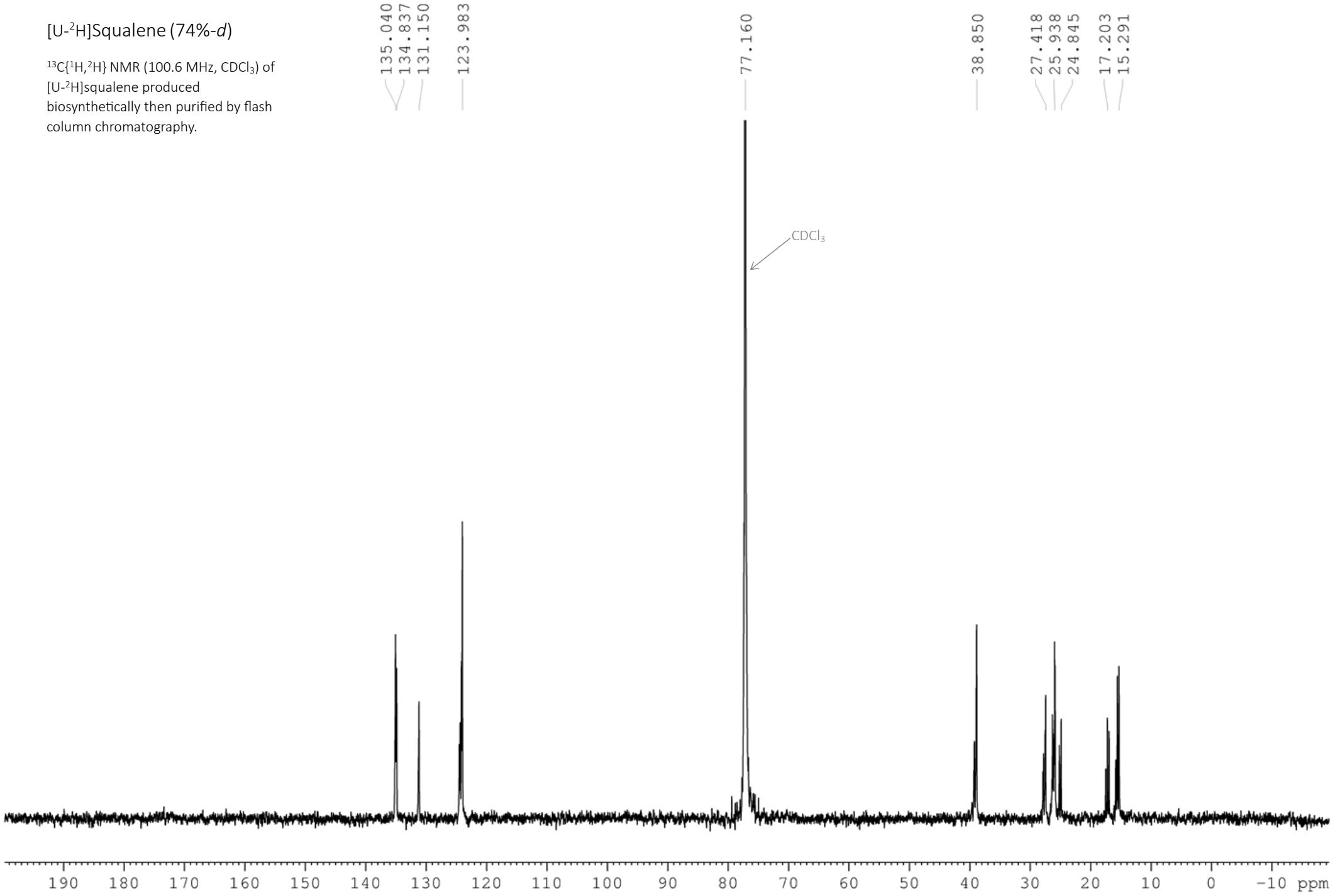
^{2}H NMR (61.4 MHz, $CDCl_3$) of [U-^{2}H]squalene produced biosynthetically then purified by flash column chromatography.

Integrals normalised to 100 to demonstrate uniform deuteration.



[U-^{2}H]Squalene (74%-*d*)

$^{13}C\{^{1}H,^{2}H\}$ NMR (100.6 MHz, $CDCl_3$) of [U-^{2}H]squalene produced biosynthetically then purified by flash column chromatography.



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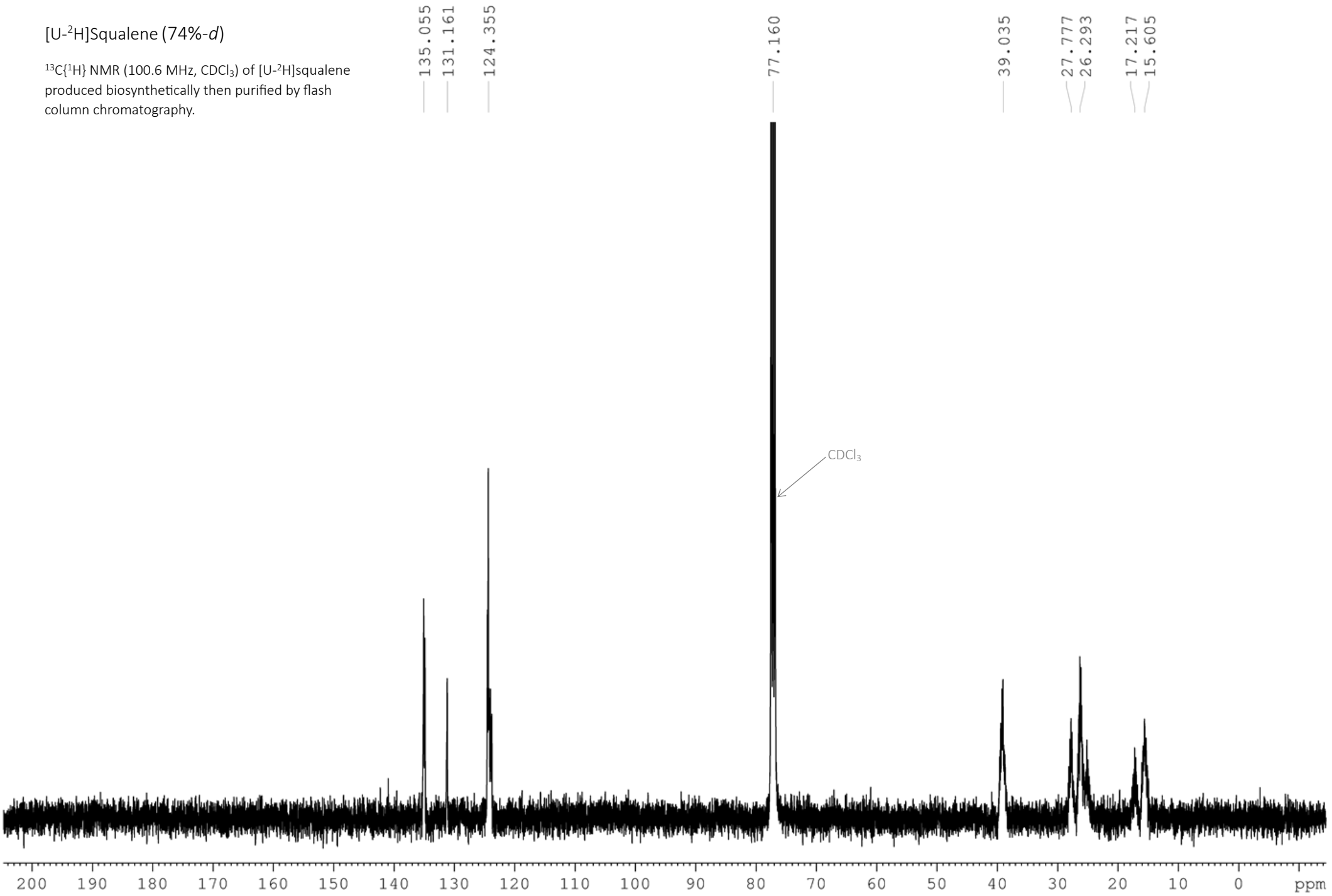
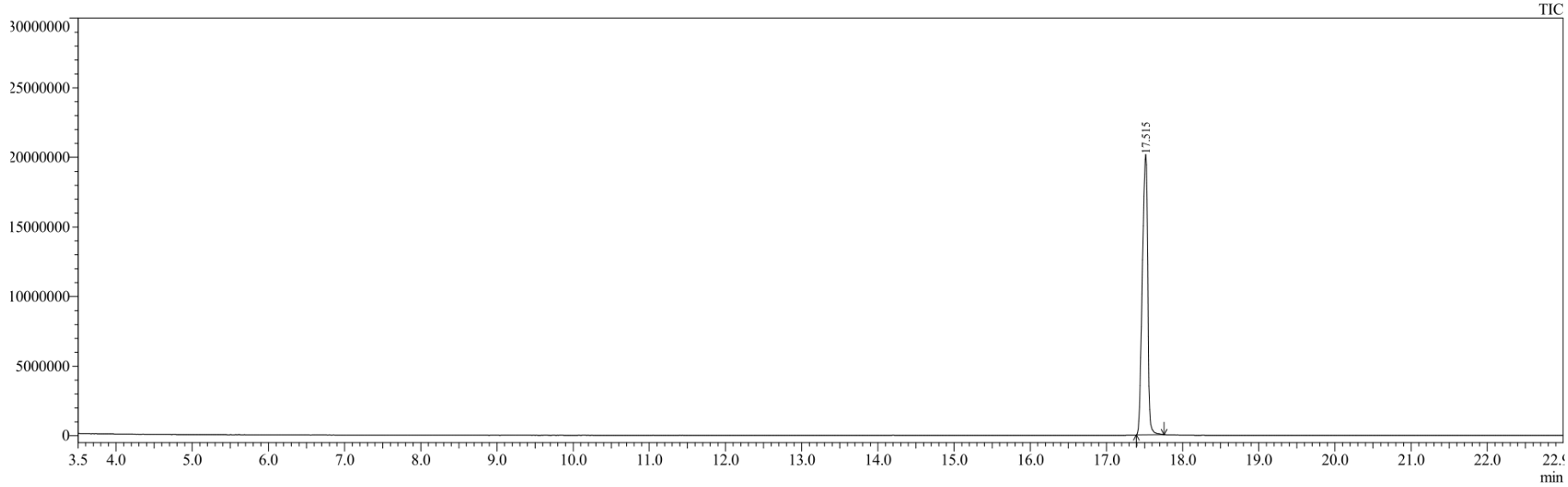
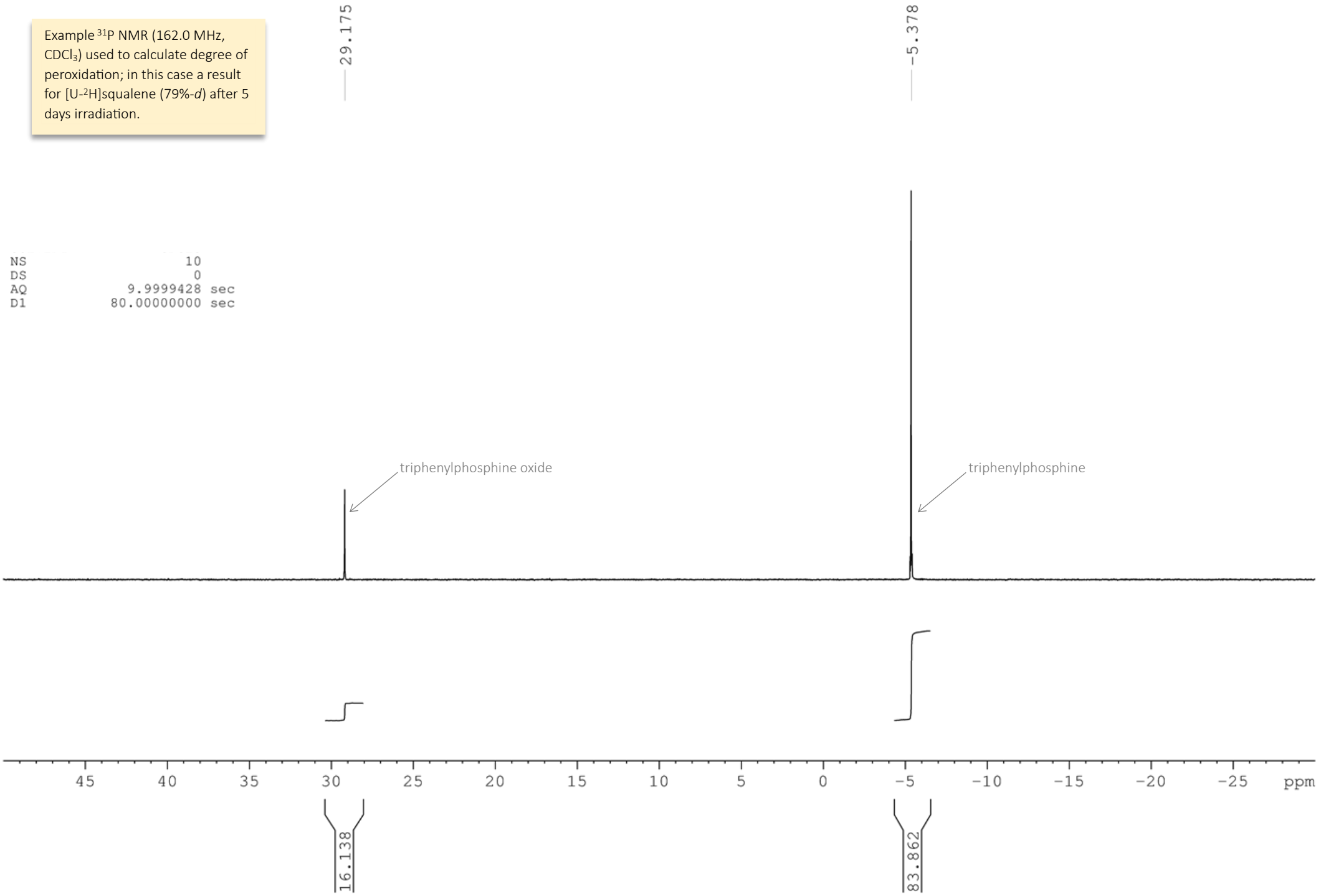


[U-^{2}H]squalene (74%-*d*)



ABOVE: GC trace (TIC, EI^{+}) of [U-^{2}H]squalene (74%-*d*) produced biosynthetically then purified by flash column chromatography. No other peaks were detected by automatic integration or manual inspection of the baseline.

Example ^{31}P NMR (162.0 MHz, $CDCl_3$) used to calculate degree of peroxidation; in this case a result for [U-^{2}H]squalene (79%-*d*) after 5 days irradiation.



Information

Date:	2024-05-10
Notes:	ex. 63%-d medium

Compound Information

Name / ID:	squalene-d50 (46%-d)
Formula:	C30D50
m/z:	460.7051
Adduct:	[M+Na+CH4O]+
Adduct m/z:	515.7205

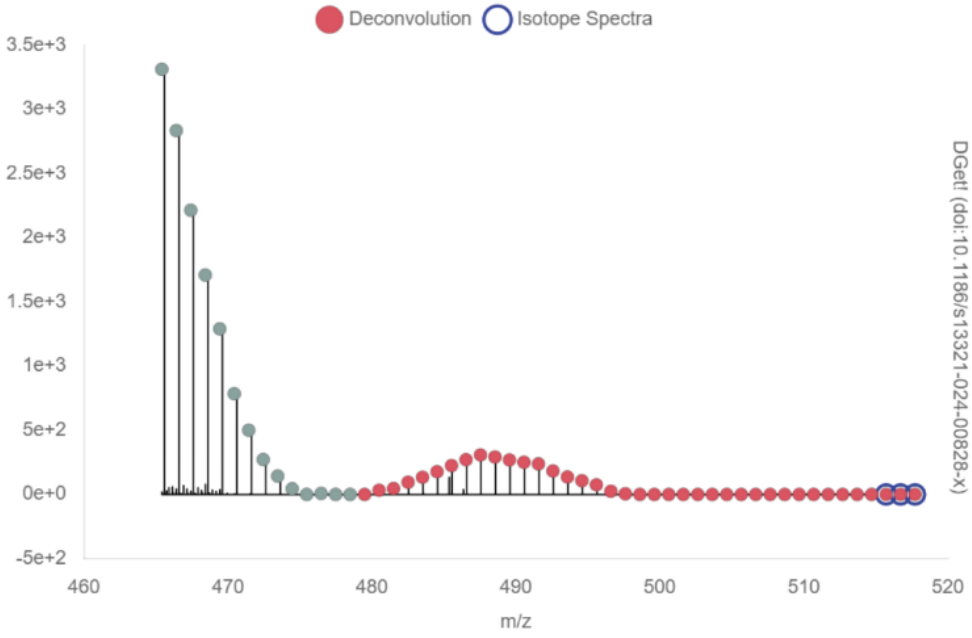
Results

Deuteration:	**45.54 %**
Deuteration Ratio Spectra	
D14:	0.0 %
D15:	1.6 %
D16:	1.69 %
D17:	3.95 %
D18:	5.08 %
D19:	6.66 %
D20:	8.39 %
D21:	9.95 %
D22:	11.12 %
D23:	9.87 %
D24:	9.11 %
D25:	8.56 %
D26:	8.15 %
D27:	5.65 %
D28:	4.24 %
D29:	3.45 %
D30:	2.24 %
D31:	0.27 %
D32:	0.03 %
D33:	0.0 %
D34:	0.01 %
D35:	0.0 %
D36:	0.0 %
D37:	0.0 %
D38:	0.0 %
D39:	0.0 %
D40:	0.0 %
D41:	0.0 %
D42:	0.0 %
D43:	0.0 %
D44:	0.0 %
D45:	0.0 %
D46:	0.0 %
D47:	0.0 %
D48:	0.0 %
D49:	0.0 %
D50:	0.0 %

Example calculation of the deuteration level of [U-^{2}H]squalene (46%-*d*) using the DGet! software platform.

Note that the most intense signal in APCI+ for squalene is generally $[M+H]^+$, however the contribution of the less intense $[M-H]^+$ to the distribution of peaks upon deuteration makes measurement of the deuteration level potentially unreliable using the more intense distribution of peaks centred around the molecular ion. Examination of the mass spectrum of squalene-h_{50} induced us to use the sodium + methanol adduct of squalene for calculation of the overall deuteration level as no other peaks were present in this part of the mass spectrum. In this particular case nearly the same result (45.59%) was calculated for $[M+H]^+$ as for $[M+CH_3OH+Na]^+$ This result was unsurprising as the intensity of [M-H]+ in the mass spectrum of squalene-h_{50} was very small compared to $[M+H]^+$.

Spectra



Report generated using the DGet (0.26) web application (0.27.3).

Please cite Lockwood and Angeloski (2024).

For more information see https://github.com/djdt/dget.