CHAPTER 4

Influence of Halogen Salts on the Production of the Ochratoxins by *Aspergillus Ochraceus* Wilh.

This chapter comprises a collaborative study between the School of Chemistry and Biochemistry, University of Potchefstroom; Foodtek (CSIR) and Department of Biochemistry, Imperial College of Science, Technology and Medicine, London. The development of some of the methodology used in this chapter is related in **Chapter 10**. This chapter was recently accepted for publication by the *Journal of Food and Agricultural Chemistry*. The purpose of the project was firstly the biopreparation of the halogen derivatives (bromo-ochratoxin B, fluoro-ochratoxin B and iodo-ochratoxin B) of OTA and secondly to study the influence of halides on OTA production.

Contribution made by the candidate

The candidate was responsible for the design, planning and conducting of the experiments done in South Africa. The candidate was assisted with the microbiological part of the work by Ms. Annelie Lübben and dr. Gert Marais (Foodtek). The candidate did the processing of the data and the results of the work done in South Africa and in London, and the writing of the publication. Prof. Peter Mantle (Imperial College) was responsible for writing the results and discussion of the work done in London. Prof. Peter Mantle and Prof. Pieter Steyn (University of Stellenbosch) made invaluable contributions in the proof-reading and compilation of the paper.

Influence of halogen salts on the production of the ochratoxins by *Aspergillus ochraceus* Wilh.

ABSTRACT

The first report of the biological production of bromo-ochratoxin B (Br-OTB) by *Aspergillus ochraceus* Wilh. is presented as well as a study of the influence of potassium bromide, potassium iodide, potassium fluoride and potassium chloride on the production of ochratoxin A and ochratoxin B. Potassium fluoride and potassium iodide inhibited the growth of the fungus, whereas potassium chloride substantially stimulated the production of ochratoxin A in shaken solid substrate fermentation on whole wheat or shredded wheat, generally giving high yield of ochratoxins. Increasing levels of potassium bromide led to a decline in ochratoxin A production and an increase in bromo-ochratoxin B, ochratoxin B and 4-hydroxyochratoxin B. Nevertheless, *A. ochraceus* was much less versatile in elaborating bromo-analogues than other fungi, which produce metabolites containing chlorine. Analysis included amino-propyl solid phase extraction column cleanup, followed by quantitative analysis on reversed phase HPLC using fluorescence detection and employing N-(5-chloro-2-hydroxybenzoyl)-phenylalanine as internal standard.

Keywords: Ochratoxin A, bromo-ochratoxin B, (4*R*)-hydroxyochratoxin B, *Aspergillus ochraceus* Wilh., Solid phase extraction.

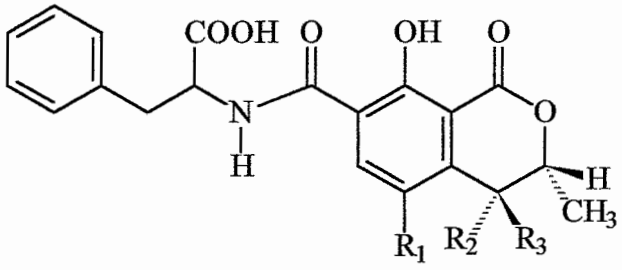
INTRODUCTION

Ochratoxin A (OTA) is a nephrotoxic, carcinogenic, teratogenic and immunogenic mycotoxin, produced mainly by isolates of *Aspergillus ochraceus,* Wilh. and *Penicillium verrucosum* (Van der Merwe *et al.*, 1965, Frisvad, 1989). OTA is a frequent contaminant in cereals, coffee (Pittet *et al.*, 1996), wine (Majerus and Ottender 1996), spices and beer (Speijers and Van Egmond, 1993). OTA is the cause of Danish porcine nephropathy (Krogh *et al.*, 1988) and is implicated as a cause of kidney diseases amongst humans, *viz* Balkan endemic nephropathy in the Balkans and Chronic interstitial nephropathy in North Africa (Creppy *et al.*, 1993). Ochratoxin B (OTB) the des-chloro analogue of OTA is approximately ten times less toxic than OTA (Xiao *et al.*, 1995), and is hydrolysed 200 times faster than OTA by carboxypeptidase A (Doster and Sinnhuber, 1972). The halogen-group is evidently important in the toxicity of the ochratoxins (see **Figure 1** for the structures). Preliminary tests

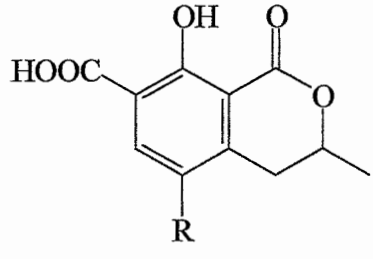
in kidney cells have indicated that bromo-ochratoxin B (Br-OTB) is more toxic than OTA (Creppy, 1999). The question is if the chlorine or bromine group plays a direct role in the toxicity or if it is the change in the compound's ability to chelate iron, to bind to DNA or the ability of enzymes to cleave the toxin that causes the variation in biological activity of the ochratoxins.

This conundrum has been investigated by our concerted efforts to produce the fluoro-, bromo- and iodo analogues of OTA which could provide invaluable information on structure-function relationships and the mode of action of the ochratoxins.

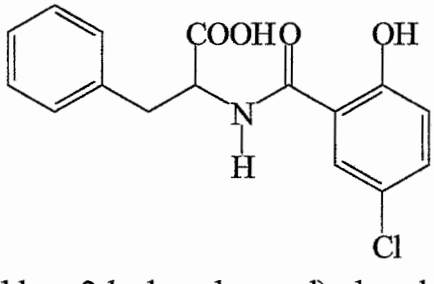
The precedent of halogenation enzymes of *Penicillium crustosum*, *Penicillium griseofulvum* and *Penicillium nigricans* readily accepting bromide ions to form bromo-analogues of penitrem A (Mantle *et al.*, 1983) or griseofulvin (MacMillan, 1954) allowed expectation that *A. ochraceus* might be similarly versatile.



Ochratoxin A: R_1 = Cl, R_2, R_3 = H
Ochratoxin B: R_1 = H, R_2, R_3 = H
(4*R*)-4-Hydroxyochratoxin A: R_1 = Cl, R_2 = OH, R_3 = H
(4*S*)-4-Hydroxyochratoxin A: R_1 = Cl, R_2 = H, R_3 = OH
(4*R*)-4-Hydroxyochratoxin B: R_1 = H, R_2 = OH, R_3 = H
Bromo-ochratoxin B: R_1 = Br, R_2 = H, R_3 = H



Ochratoxin α: R = Cl
Ochratoxin β: R = H



N-(5-chloro-2-hydroxybenzoyl)-phenylalanine

Figure 1: Structures of the ochratoxins

This paper relates the first report of the biological production of Br-OTB by South African and Australian isolates of *A. ochraceus* Wilh. and the effects of potassium bromide, potassium fluoride, potassium chloride and potassium iodide on the dynamics of production of ochratoxins. In addition, a number of minor metabolites of the South African isolate, e.g.

ochratoxin α (OTα), ochratoxin β (OTβ), (4*R*)-4-hydroxyochratoxin B [(4*R*)-OH-OTB], (4*R*)- and (4*S*)-4-hydroxyochratoxin A [(4*S*)-OH-OTA and (4*R*)-OH-OTA], and citrinin, were identified. These metabolites were reported previously by Xiao *et al.*, (1996). Methyl esters of OTA and OTB (esterified at the phenylalanine carboxylic acid), were recognised as significant minor metabolites of the Australian isolate.

MATERIAL AND METHODS

Experiments done in South Africa

Solid-Phase Extraction (SPE) columns

Aminopropyl SPE columns, 500 mg (SUPELCO, 5-7014) were used in conjunction with a vacuum manifold.

Thin layer (TLC) and column chromatography

A mobile phase of toluene/acetic acid (4:1) was used on Silica gel 60 F_{254} TLC plates (Merck). The ochratoxins display very strong fluorescence upon UV illumination. Preparative TLC was done on Silica gel 60 F_{254} 2 mm plates (Merck).
Column chromatography was done with either Silica gel 60 (230-400 mesh ASTM, supplied by Merck) or Sephadex LH20.

Instrumentation

A Hewlett Packard 1090, HPLC system, fitted with a diode array (HP 1090) and fluorescence detector (HP 1100), autosampler and ChemStation software was used. Separations were achieved using a 4,6 mm × 150 mm, 5 µm, C_{18} analytical column (Discovery C_{18}, SUPELCO) fitted with a C_{18} guard cartridge (Spherisorb ODS-2, SUPELCO) and a mobile phase of water/methanol/acetic acid (50:60:2). Injection volume was 5 µl and flow rates of 1 ml/min were used. The fluorescence detector was set at an excitation wavelength of 250 nm and an emission wavelength of 454 nm.

LC-ES-MS

A VG Quattro II - HPLC 1090 system was used with positive ionisation.

NMR

A Bruker 500 MHz spectrometer was used for NMR analysis.

Safety

The ochratoxins are potent nephrotoxic compounds and should be handled with care.

Analysis of halogen content of Durum wheat

Durum wheat was digested according to the method of Havlin and Soltanpour (1980), to determine its halogen content. The chloride content of the Durum wheat was determined by the method of Weiss (1986), fluoride by the method of McQuaker and Gurney (1977) and the bromide and iodide content of the wheat by ICP/MS analysis.

Preparation of the standards

OTA and OTB were obtained by cultivating *A. ochraceus* (MRC 10582) on wet sterilised Durum wheat (2.1 kg) at 25 °C for 14 days on a rotary shaker in 25 Erlenmeyer 500 ml flasks. The wheat was soaked with chloroform/methanol (1:1, 200 ml per flask containing 80 g wheat) and left at *ca.* 20 °C for 12 hrs and subsequently homogenised by blending at 3 000 rpm for 10 minutes, filtered, the residues thoroughly washed with chloroform/methanol (1:1, 20 ml) and the filtrate evaporated under vacuum to dryness. The crude extracts (121.5 g) were combined, resuspended in methanol/water (95:5, 3.5 l) and washed four times with hexane (4 x 1,5 l). The methanol layer was evaporated to dryness and partitioned between 1 M sodium bicarbonate (3.5 l) and chloroform (2.5 l). The aqueous layer containing the ochratoxins was acidified to pH 1 with 6 M hydrochloric acid and extracted three times with chloroform (1 l). The combined chloroform extracts were washed with water (2 x 0.5 l), dried over anhydrous sodium sulphate and evaporated to dryness. The ochratoxin-containing fraction (54.7 g) was separated on silica gel (1.5 kg, 70-230 mesh), on a column (1 m x 50 mm) with chloroform/acetic acid (97:3) as the mobile phase. The OTA and OTB containing fractions were combined and the solvent evaporated under reduced pressure, dissolved in chloroform, extracted twice with water and dried over anhydrous sodium sulphate. The chloroform was removed under reduced pressure and OTA (3.433 g, m.p. 91 °C, literature 90 °C, van der Merwe *et al.*, 1965) and OTB (1.466 g, m.p. 219 °C, literature 221 °C, van der Merwe *et al.*, 1965) were recrystallised from benzene and methanol, respectively.

Bromo-ochratoxin B and N-(5-chloro-2-hydroxybenzoyl)-phenylalanine were synthesised by Steyn and Payne (1999).

N-(5-chloro-2-hydroxybenzoyl)-phenylalanine was used as internal standard for HPLC analysis. It is a very stable compound with UV and fluorescence characteristics similar to those of the ochratoxins.

The four hydroxylated ochratoxins: (4*R*)-OH-OTB, (4*R*)-OH-OTA, (4*S*)-OH-OTA and 10-OH-OTA were supplied by Prof. R. Marquardt, Department of Animal Science, University of Manitoba, Canada.

Citrinin was kindly provided by Prof. F.C. Størmer, National Institute of Public Health, Oslo, Norway.

OTα and OTβ were produced by hydrolysing OTA and OTB respectively, under reflux in excess 6 M hydrochloric acid for 60 hours.

Cultivation of A. ochraceus at different levels of halogens

A lyophilized culture of *A. ochraceus* Wilh. (MRC 10582) kept at -70 °C, obtained from the CSIR Culture Collection, was plated onto potato dextrose agar plates. The petri dishes were incubated at 25 °C for three days in the dark. Twelve different concentrations, ranging from 0 to 2000 mg of potassium bromide, potassium iodide, potassium chloride and potassium fluoride per 40 g whole Durum wheat kernels, were prepared by adding the salt, wheat and 25 ml distilled water together in an Erlenmeyer flask (500 ml) for each concentration. A second set identical to the first was also prepared to give a total of 84 flasks. These were subsequently incubated for 16 h at 25 °C on a rotary shaker at 350 rpm, autoclaved for 30 min and cooled down to room temperature. The wheat was then inoculated with a 2 ml spore suspension of three day old cultures of *A. ochraceus*. The flasks were replaced on the rotary shaker for 14 days after which they were harvested for analysis. The content of the flasks was quantitatively transferred to beakers, methanol/chloroform (1:1, 200 ml) was added and milled for 10 min at 3000 rpm, sealed and left for 24 hrs. The wheat extracts were then vacuum filtered through a Buchner funnel containing Whatman No. 1 filter paper. The flask and filter pad were rinsed with 40 ml methanol/chloroform (1:1) followed by another filtering step using glass filter paper. The combined extracts from each separate experiment were then transferred to volumetric flasks (250 ml) and filled with methanol/chloroform (1:1). The recovery of the extraction step was 80%. This was determined by repeating the extraction

until no more OTA could be observed by HPLC. The total OTA as determined in all the extraction steps, was used as the total OTA in the wheat.

Purification of the ochratoxins by the SPE columns

The SPE columns were conditioned with 2.5 ml methanol/chloroform (1:1), 2 ml of the above extracts subsequently were placed on the columns and allowed to flow at ± 2 drops per second. The SPE columns were washed with 2.7 ml chloroform and allowed to run completely dry. The ochratoxins were eluted from the columns with 2.5 ml methanol/acetic acid (4:1) and collected into test tubes. A stock solution of internal standard was prepared by dissolving N-(5-chloro-2-hydroxybenzoyl)-phenylalanine (100 mg) in methanol (100 ml) and diluting it ten times. The eluant (1.5 ml) of the SPE extraction was transferred to autosampler vials and 100 µl of the internal standard was added to each vial for HPLC analysis.

Isolation of the different ochratoxins in the potassium bromide supplemented wheat

Three of the above Erlenmeyer flasks containing extracts of cultured wheat (3 x 40 g), each supplemented with 1500 mg and 2000 mg potassium bromide, were used to isolate the ochratoxins produced in wheat cultivated with *A. ochraceus* and supplemented with high levels of potassium bromide. The contents of the flasks were combined and evaporated to dryness under vacuum. The extract (15 g) was redissolved in methanol/water (95:5, 200 ml), hexane (250 ml) was added (to remove excess oils) and the two layers were separated. The methanol layer was concentrated under vacuum, redissolved in chloroform (350 ml) and the ochratoxins were extracted with 1 M sodium bicarbonate (2 x 200 ml). The aqueous layer was carefully acidified with 6 M hydrochloric acid and re-extracted three times with chloroform (3 x 150 ml). The chloroform extract was evaporated to dryness, and the residue (2.5 g) and transferred to a glass column (1 m x 50 mm) packed with silica (200 g) in chloroform. At first chloroform/acetic acid (98:2, 1 l) was used to elute most of the lipids still present in the extract. The first 180 fractions (10 ml each) containing ochratoxins were eluted with chloroform/acetic acid (92:8, 2 l) followed by 100 smaller fractions (5 ml) eluted with chloroform/acetic acid (90:10, 500 ml) and 80 fractions eluted with chloroform/acetic acid/methanol (85:12:3, 500 ml). These fractions were analysed by TLC and similar fractions were combined and evaporated to dryness to yield six ochratoxin-containing extracts. The

six extracts were compared with the reference standards by using HPLC and TLC (See **Table 1**).

Table 1: Fractions obtained after chromatography on silica gel of different ochratoxins present in cultivated wheat

Extract	Fractions	Amount (mg)	Ochratoxins
1	10-70	324	OTA, Br-OTB
2	81-135	427	OTB, OTβ
3	157-183	66	OTβ, OTα
4	229-251	23	(4*R*)-OH-OTA
5	253-318	42	(4*S*)-OH-OTA and/or 10-OH-OTA
6	329-349	208	(4-*R*)-OH-OTB

- The two ochratoxins (OTA and Br-OTB) present in extract 1 proved to be inseparable by using TLC or column chromatography, their identity was confirmed by comparison of their retention time on HPLC and the very distinctive molecular ion pattern of chlorine (M+1, *m/z* 404, 406, ratio 3:1) and bromine (M+ 1, *m/z* 448, 450, ratio1:1) as present in OTA and Br-OTB, respectively (see **Figure 2**).
- The ochratoxins (OTB and OTβ) present in extract 2 (427 mg) were separated on a glass column (1 m x 15 mm), containing Sephadex LH20 (10 g), using methanol as mobile phase. The substances were unambiguously identified as OTB and OTβ by comparisons of their TLC and HPLC retention and ES-MS characteristics.
- The two ochratoxins (OTβ and OTα) present in fraction 3 (66 mg) were separated by preparative TLC using 5 plates and chloroform/acetic acid (96:4) as mobile phase, and their identity was confirmed by ES-MS showing molecular ions at *m/z* 223, 257 (M+1) respectively.
- Extract 4 (23 mg) contained mostly one compound, a hydroxy-ochratoxin A, which was purified by preparative TLC using two preparative TLC plates and chloroform/acetic acid (98:2) as mobile phase. Its presence was confirmed as (4*R*)-OH-OTA by retention time comparison on HPLC and ES-MS (M+1, *m/z* 420).
- Extract 5 (42 mg) was at first cleaned up on a glass column (1 m x 15 mm, containing 5 g Sephadex LH 20) employing methanol as mobile phase, the ochratoxin containing fractions were evaporated to dryness, followed by preparative TLC using 8 analytical TLC plates and toluene/acetic acid (5:1) as mobile phase. The presence of (4*R*)-OH-OTA and

(4*S*)-OH-OTA was confirmed by ES-MS and retention time comparison on HPLC (See **Figure 3**).

- Extract 6 (208 mg) contained only one ochratoxin type compound as well as unidentified brown material. It was cleaned on a glass column (1 m x 15 mm, containing 8 g Sephadex LH 20) and methanol as mobile phase. It yielded crystals (150 mg, melting point 237-238°C) and was identified to be [(4*R*)-OH-OTB] by NMR (Xiao *et al.*, 1996) and ES-MS.

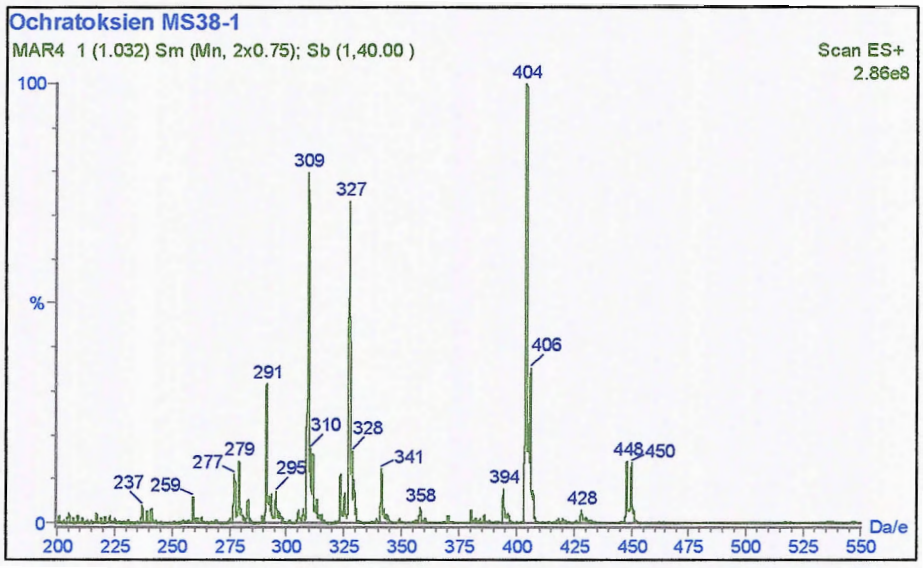


Figure 2: ES-MS spectrum of extract 1 from cultivated wheat supplemented with potassium bromide containing ochratoxin A (M+1, *m/z* 404,406) and bromo-ochratoxin B (M+1, *m/z* 448,450).

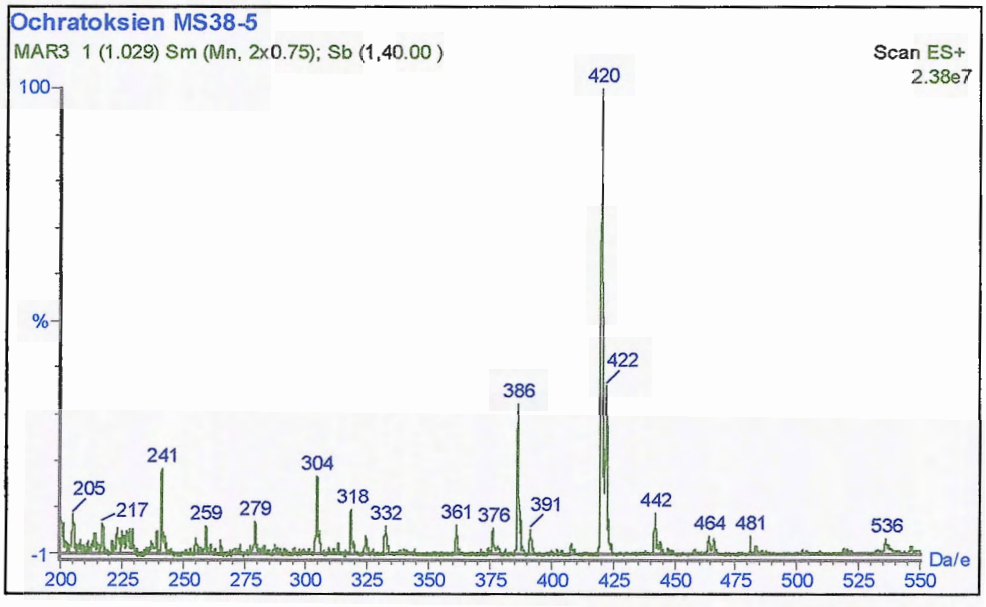


Figure 3: ES-MS spectrum of extract 5 from wheat cultivated with the South African isolate of *A. ochraceus*, supplemented with potassium bromide containing 4-hydroxyochratoxin A (M+1, *m/z* 420).

Table 2: The identification of ochratoxins produced on wheat inoculated with *A. ochraceus*

Number on Fig.2	Retention time	TLC R_f-value	Ochratoxin	Technique used
1	3.0	0.23	Ochratoxin α	a, b, c, d
2	4.7	0.19	Ochratoxin β	a, b, c
3	6.3	0.098	(4*R*)-4-Hydroxyochratoxin B	a, b, c, d
4	7.8	0.16(5)	(4*S*)-4-Hydroxyochratoxin A	a, b, c
5	9.1	0.19	(4*R*)-4-Hydroxyochratoxin A	a, b, c
6	9.4	0.46	Citrinin	a, c
7	10.0	0.35	Ochratoxin B	a, b, c, d
8	10.7	0.17(0)	10-Hydroxyochratoxin A	a, b, c
9	15.6		N-(5-chloro-2-hydroxybenzoyl)-phenylalanine	
10	21.0	0.50	Ochratoxin A	a, b, c
11	24.0	0.50	Bromo-ochratoxin B	a, b

a. HPLC retention time comparison with standard compound; b. ES-MS analysis; c. TLC retention time comparison; d. NMR

Experiments Mainly on the Australian Isolate

The principal differences from experimentation in South Africa concerned the fungus which was of Australian origin (Tapia and Seawright, 1984), the wheat substrates either in the form of whole UK wheat or as a processed food (shredded wheat; Cereal Partners UK), and the basic optimised solid substrate fermentation process for shredded wheat involving addition of aqueous spore suspension in dilute Tween 80 and sterile distilled water (total volume 16 ml) to sterile substrate (40 g) in a 500 ml Erlenmeyer flask. Incubation was at 29°C on a rotary shaker at 200 rpm and 10 cm eccentric throw (Harris, 1996). This system has been found to be particularly suitable for expression of the potential for production of OTA by *A. ochraceus* (Mantle and Chow, 2000). Analysis of ochratoxins involved optimised (according to Nesheim *et al.*, 1992) extraction with ethyl acetate/0.01 M H_3PO_4 (9:1), partition into 3 % $NaHCO_3$, acidification with 1 M HCl, partition into ethyl acetate and evaporation to dryness. A standard solution of the residue was made in methanol and analysed (20 µl) by reversed phase HPLC in acetonitrile/water/acetic acid (59:39:1) with diode array detection, facilitating monitoring of UV spectra of eluted compounds. Satisfactory validity of analytical methodology between London and South Africa was confirmed in representative samples.

RESULTS AND DISCUSSION

Studies on the South African Isolate of *A. Ochraceus* Wilh.

The use of the amino-propyl SPE columns proved to be very effective, with a percentage recovery of 98±4% (±RSD, n = 6). The SPE columns retain only compounds containing a free carboxylic acid group. The methyl esters of the ochratoxins were thus not retained (they were detected with TLC (toluene:acetic acid 5:1 R_f: 0.66 in the crude extract). To our knowledge; this is the first report of the use of this type of SPE columns in ochratoxin analysis, a more detailed report on their use and effectiveness will follow shortly. A typical HPLC chromatogram of the supplementation of the *A. ochraceus* growth medium with potassium bromide is shown in **Figure 4**. The presence of the Br-OTB was confirmed by using retention time studies and electrospray mass spectroscopy (M+1, *m/z* 448, 450) and comparison with synthetic Br-OTB (See **Table 2**).

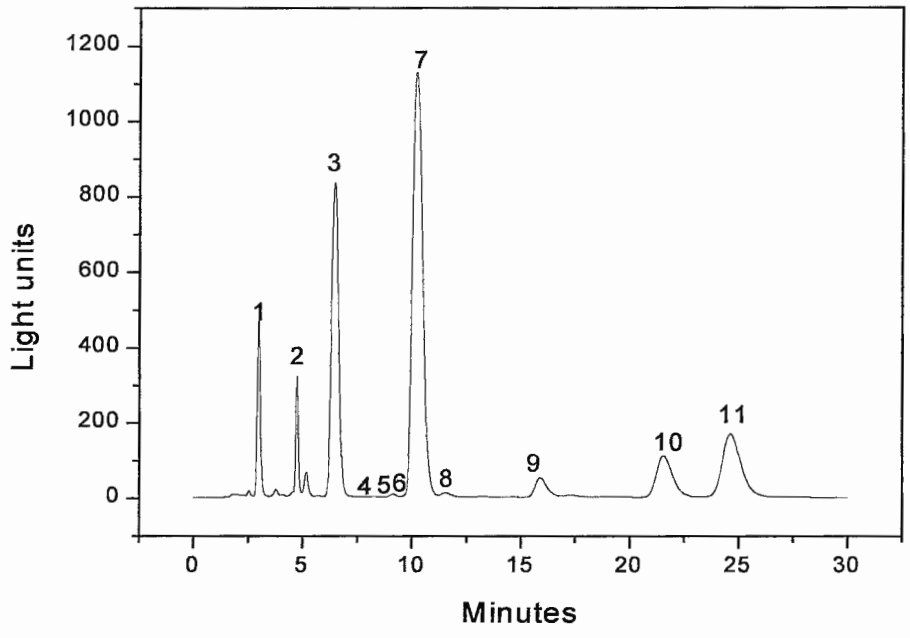


Figure 4: A HPLC chromatogram depicting the distribution of the ochratoxins produced by the South African isolate of *A. ochraceus* at a concentration of 1.5 g potassium bromide per 40 g Durum wheat.

The Durum wheat used in the South African experiments was found to contain much higher concentrations of chloride (827.5 mg/kg) than fluoride (34.3 mg/kg). Only trace amounts of bromide (<0.05 mg/kg) and iodide (<0.05mg/kg) were found.

The results of the potassium bromide experiment are summarised in **Figure 5**. The amount of OTA decreased markedly with the increase of potassium bromide concentration. However, the accumulated amounts of OTB and Br-OTB increased with an increase of potassium bromide concentration, but at high concentrations (e.g. 1.5 mg KBr / 40 g wheat) the yield of the total ochratoxins decreased due to the apparent poisoning of the microorganism. The results indicate that chloride is the preferred halogen for incorporation into the ochratoxin type molecules, and the organism only accepts bromide at relatively high bromide concentrations, there being a disproportionately weak influence of high concentration of bromide. OTB is the likely biosynthetic precursor to Br-OTB.

The results obtained upon addition of potassium iodide and potassium fluoride to cultures of *A. ochraceus* on wheat are summarised in **Figure 6**. The yield of both OTA and OTB decreased markedly as the amount of potassium iodide and potassium fluoride was increased, the fungus evidently being very sensitive to fluoride and iodide. No iodo-ochratoxin B and fluoro-ochratoxin B was detected using HPLC, ES-MS and FAB-MS (positive ionisation).

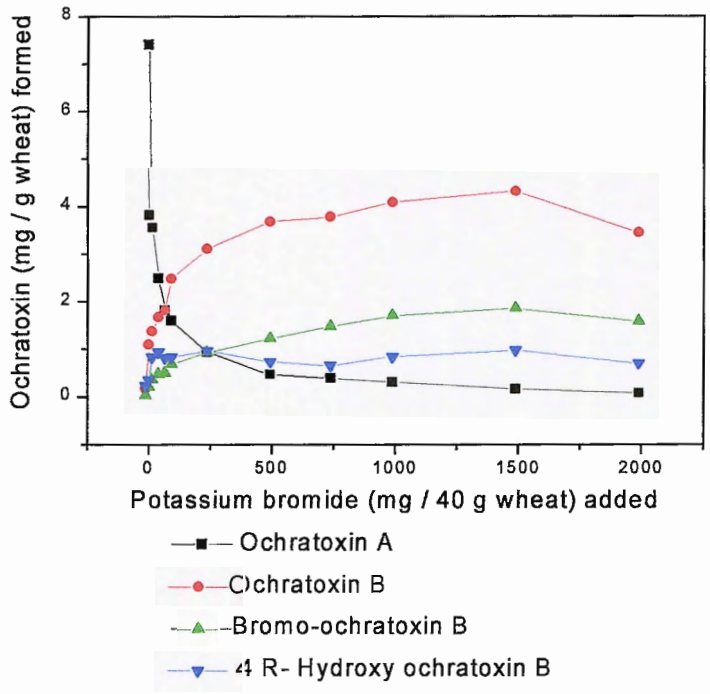


Figure 5: The production of OTA, OTB, Br-OTB and [(4*R*)-OH-OTB], at different concentrations of potassium bromide by the South African isolate of *A. ochraceus*.

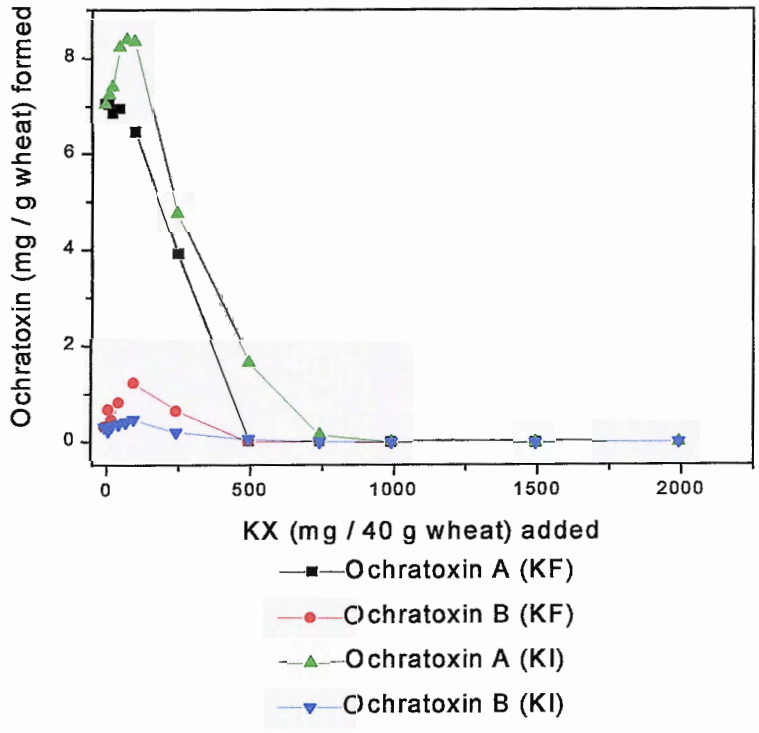


Figure 6: The influence of potassium fluoride and potassium iodide on the production of OTA and OTB in wheat: OTA and OTB produced by the South African isolate of *A. ochraceus* on wheat *versus* amount of potassium chloride added.

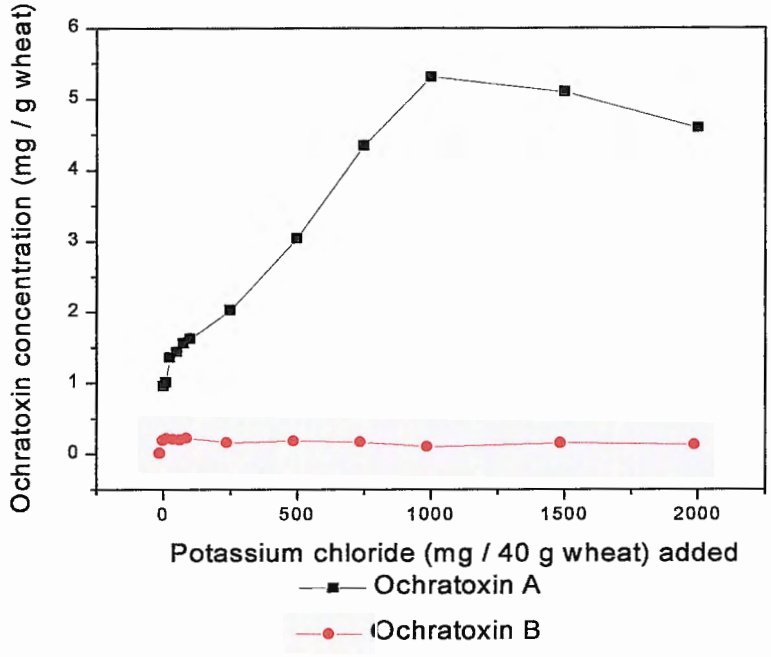


Figure 7: The influence of potassium chloride on the production of OTA and OTB in wheat by the South African isolate of *A. ochraceus*.

The chloride supplementation experiment was conducted two months after the initial supplementation experiments when the overall production of OTA was less than in previous experiments. However, a fivefold increase was observed (See **Figure 7)** in OTA production when 1 g of potassium chloride was added to 40 g wheat. Increased potassium chloride must have allowed corresponding increased OTB production and amounts of residual OTB remained roughly constant. This supposition is based on the premise that OTA is derived from OTB, though there is experimental evidence that OTA is also derived from OTα (Harris, 1996). However supplementary chloride was influential whether chlorination of the isocoumarin occurred before or after linkage of phenylalanine.

Studies Mainly on the Australian Isolate

Comparison between a culture of the South African isolate and the Australian isolate on whole wheat in shaken culture conditions in London showed that the latter yielded 3 to 4-fold more OTA than the former. On the shredded wheat substrate there was an even greater difference in OTA yield between the two fungi but they both showed the same profound adverse effect on total ochratoxin yield in response to increasing amounts of KBr (illustrated for the Australian isolate in **Figure 8**). In a further experiment with the Australian isolate only, which included analysis of ochratoxin methyl esters that have relatively long residence time in HPLC, the marked decrease in OTA yield with increasing KBr was matched by increased occurrence of OTB, which becomes the dominant ochratoxin at the highest concentrations of KBr (**Figure 9a**). Correspondingly, a methyl ester of OTB, and of OTA, identified by the mass spectral fragmentation pattern as esterified at the phenylalanine carboxyl rather than substitution of the isocoumarin hydroxyl, become notable metabolites with the addition of 50 mg KBr to the wheat (40 g). They were still evident with 100 mg KBr, but were absent with 250 mg KBr in 40 g wheat. Consequently, although total chlorinated ochratoxins again declined with increasing KBr (**Figure 9b**), des-halo-ochratoxins became the most abundant metabolites at 50 mg KBr in 40 g wheat, which also supported the highest total ochratoxins yield within which Br-OTB was identified as a significant though very minor component (**Figure 9a**). Thus both fungi were qualitatively similar in being shy to biosynthesize the bromo-analogue of OTA, and to have halogenation enzymes which were adversely sensitive to bromide by comparison with those of other fungi producing the chlorine-containing metabolites penitrem A and griseofulvin. In another experiment with the Australian isolate, using pure wheat substrate, either in the form of whole grain or as a

processed food product (shredded wheat), supported a high OTA:OTB ratio even at the higher overall yields obtained (illustrated for the shredded wheat substrate in **Figure 10**). However, a 50 mg potassium chloride supplement further enhanced chlorination and OTA yield reflected through a higher OTA:OTB ratio. In the same experiment, this contrasted with confirmed typical changes in ochratoxins in response to the same amount of KBr. Addition of 50 mg potassium chloride to 40 g shredded wheat approximately doubles the available chlorine (*ca.* 0.065%; Cereal Partners UK), and the highest yields of OTA in unsupplemented shredded wheat substrate in the present study apparently used all the available chlorine.

In conclusion:

- High levels of bromide in the wheat on which *A. ochraceus* was cultivated has a substantial influence on the yields and the formation of different ochratoxin metabolites. Br-OTB can be produced by *A. ochraceus* Wilh in the presence of high bromide levels, although not in high yield.
- An increase in the chloride concentration in wheat resulted in increased production of chloride-containing ochratoxins by *A. ochraceus*.
- Iodide and fluoride are too generally toxic to support formation of ochratoxin analogues.

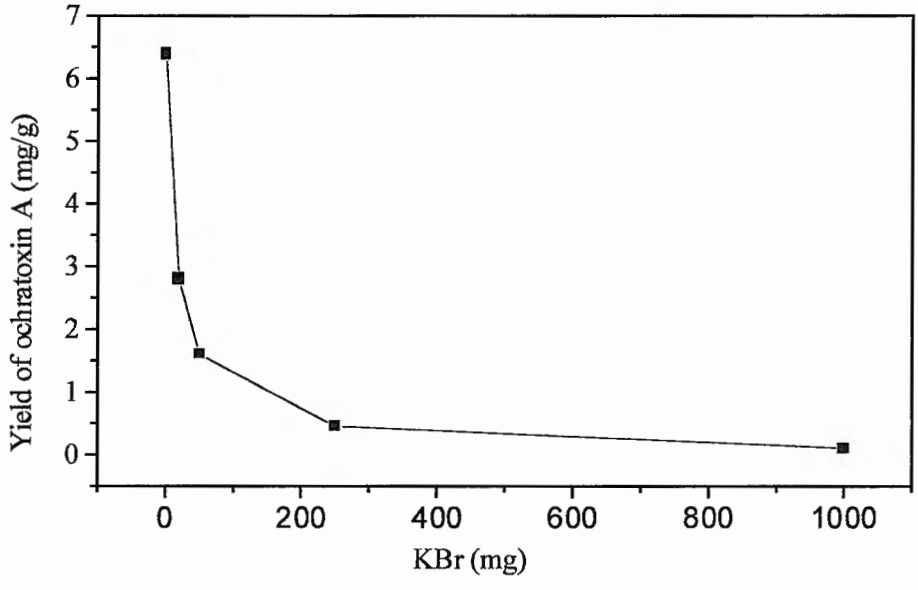
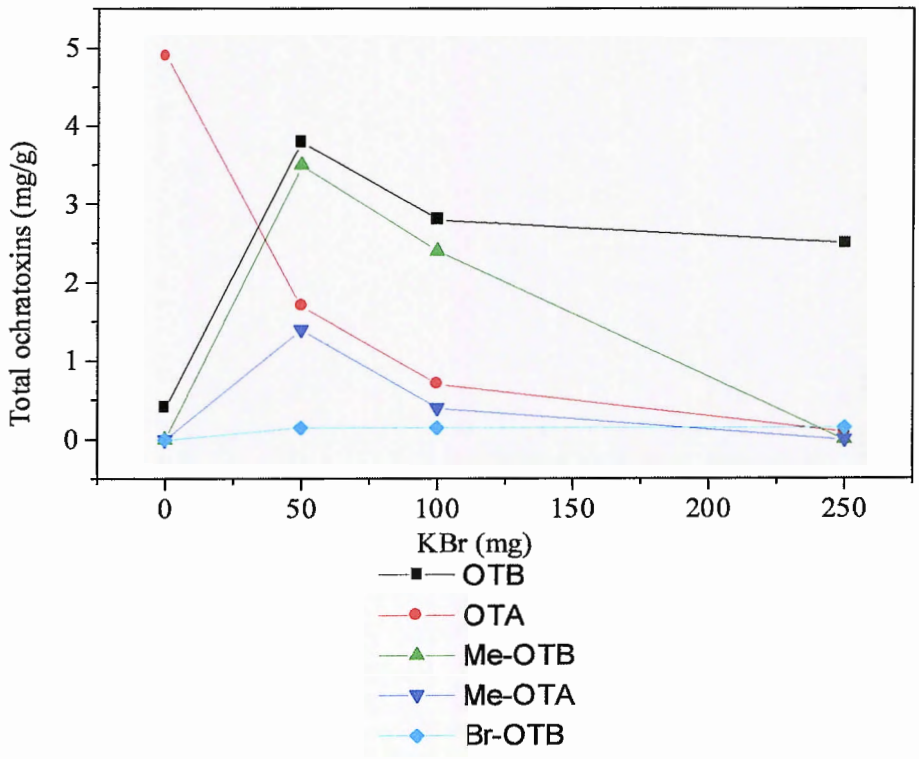
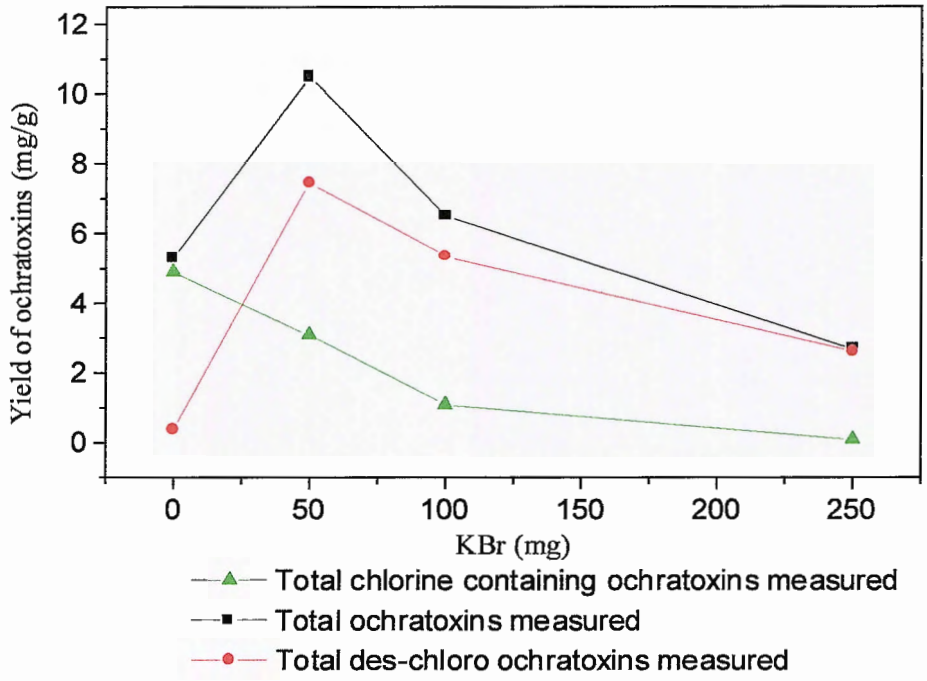


Figure 8: Effect of initial addition of batched potassium bromide on 17-day shaken shredded wheat fermentations (n = 4) of the Australian isolate of *A. ochraceus* concerning the mean yield of OTA.



a)



b)

Figure 9: Effect of batched potassium bromide on 14-day shaken shredded wheat fermentation of the Australian isolate of *A. ochraceus* concerning the yield a) of individual ochratoxins and b) of groups of chloro-, *des*-chloro-, and total ochratoxins.

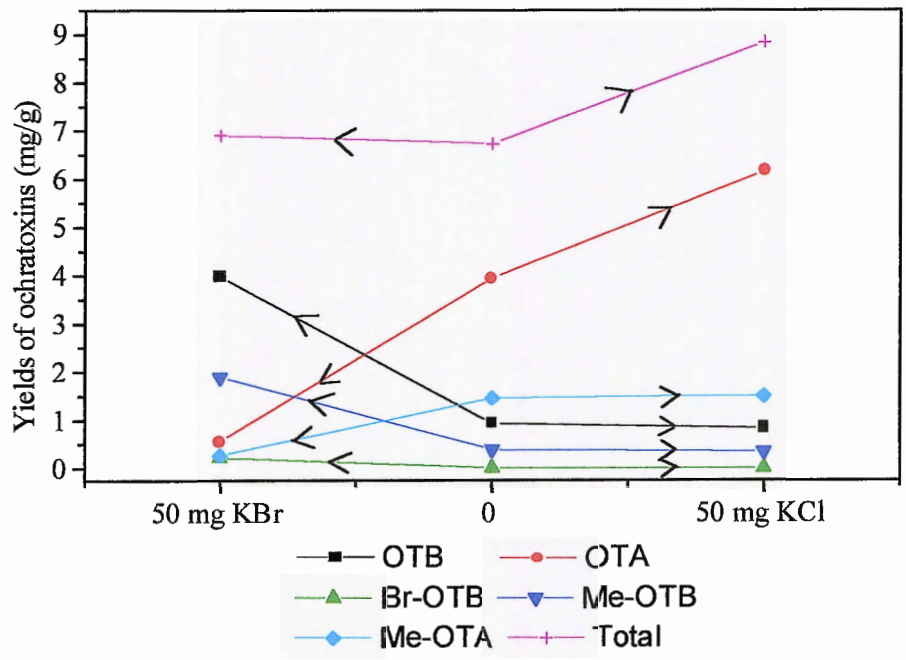


Figure 10: Direct comparison of the effects of addition of 50 mg of potassium bromide or potassium chloride to 17-day shaken shredded wheat fermentation of the Australian isolate *of A. ochraceus* on the mean yield (n = 3) of total and individual ochratoxins.

ACKNOWLEDGEMENTS

We thank Barry Payne for the Br-OTB and N-(5-chloro-2-hydroxybenzoyl)-phenylalanine standards, Lardus Erasmus for the ES-MS analysis (Potchefstroom University) and P.L. Wessels for NMR analysis (University of Pretoria). We are indebted to the National Research Foundation, Pretoria for financial assistance.

LITERATURE CITED

Creppy, E.E. Personal Communication, **1999**.

Creppy, E.E.; Castegnaro, M.; Dirheimer, G. (eds). Human ochratoxicosis and its pathologies, Proceedings of the International Symposium: Human ochratoxicosis and associated pathologies in Africa and developing countries, held in Bordeaux (France) on July 4-6, **1993**. Colloque INSERM Vol. 231, John Libbey Eurotext.

Doster, R.C.; Sinnhuber, R.O. Comparative rates of hydrolysis of ochratoxins A and B *in vitro., Food Cosmet. Toxicol.* **1972**, *10*, 389-394.

Frisvad, J.C. The connection between the *Penicillia* and *Aspergilli* and mycotoxins with special emphasis on misidentified isolates. *Arch. Environ. Contam. Toxicol.* **1989**, *18*, 452.

Harris, J.P. The biosynthesis of ochratoxin A and other structurally related polyketides by *Aspergillus ochraceus*. PhD Thesis. University of London. 1996.

Havlin, J.L.; Soltanpour, P.N. A nitric acid plant tissue digest method for use with inductively coupled plasma spectrometry. *Commun. in soil Science and Plant Analysis* **1980**, *11(10)*, 969-980.

Krogh, P.; Gyrd-Hansen, N.; Larsen, S.; Nielsen, J.P.; Smith, M.; Ivanoff, C.; Meisner, H. Renal enzyme activities in experimental ochratoxin A-induced porcine nephropathy: Diagnostic potential of phosphoenolpyruvate carboxykinase and gamma-glutamyl transpeptidase activity. *J. Toxicol. Environ. Health* **1988**, *23*, 1.

MacMillan, J. Griseofulvin. Part IX. Isolation of the bromo-analogue from *Penicillium griseofulvum* and *Penicillium nigricans*. *J. Chem. Soc.* **1954**, 2585-2587.

Majerus, P.; Ottender, H. Nachweiss und vorkommen von ochratoxin A in wein und traubensaft. Deutsche *Lebensmittel-Rundschau* **1996**, *92* (12), 388.

Mantle, P.G.; Chow, A.M. Ochratoxin formation in *Aspergillus ochraceus* with particular reference to spoilage of coffee. *Int. J. Food Microbiol.* In press.

Mantle, P.G.; Perera, K.P.W.C.; Maishman, N.J.; Mundy, G.R. Biosynthesis of penitrems and roquefortine by *Penicillium crustosum*. *App. Environ. Microbiol.* **1983**, *45*, 1486-1490.

McQuaker, N.R.; Gurney, M. Determination of total fluoride in soil and vegetation using an alkali fusion selective ion electrode technique. *Analytical Chemistry* **1977**, *49*(1), 53-56.

Nesheim, S.; Stack M.E.; Trucksess, M.W.; Eppley, R.M.; Krogh, P. Rapid solvent-efficient method for liquid-chromatographic determination of ochratoxin A in corn, barley, and kidney - collaborative study. *Journal of AOAC International* **1992**, *75*, 481-487.

Pittet, A.; Tornare, D.; Huggett, A.; Viani, R. Liquid chromatographic determination of ochratoxin in pure and adulterated soluble coffee using an immunoaffinity column cleanup procedure. *J. Agric. Food Chem.* **1996**, *44* (11), 3564.

Speijers, G.J.A.; Van Egmond, H.P. Worldwide ochratoxin A levels in food and feeds. In: Creppy, E.E.; Castegnaro, M.; Dirheimer, G. (eds.) Human ochratoxicosis and its pathologies. Colloque INSERM/John Libbey Eurotext, **1993**, *231*, 85-100.

Steyn, P.S.; Payne, B.E. The synthesis of bromo-ochratoxin B and iodo-ochratoxin B. *S.Afr.J.Chem.*, **1999**, 52(2/3), 69-70.

Tapia, M.O.; Seawright, A.A. Experimental ochratoxicosis A in pigs. *Aust. Vet. J.* **1984**, *61*, 219-222.

van der Merwe, K.J.; Steyn, P.S.; Fourie, L. Mycotoxins. Part II. The constitution of ochratoxins A, B and C, metabolites of *Aspergillus ochraceus* Wilh. *J. Chem. Soc.* **1965**, 7083-7088.

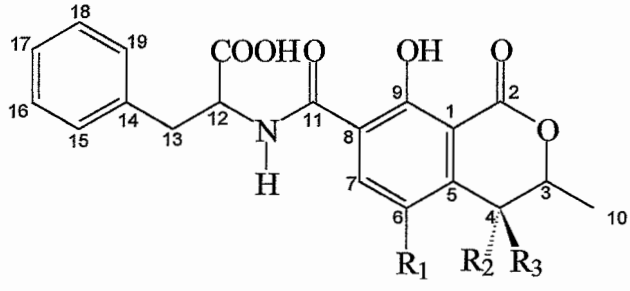
Weiss, J. In: Handbook of ion chromatography, M. Gurney (ed),. Dionex Corporation, Sunnyvale, California, **1986**.

Xiao, H.; Marquard, R.R.; Frohlich, A.A.; Ling, Y.Z. Synthesis and structure elucidation of analogues of ochratoxin A., *J. Agric. Food Chem.* **1995**, *43 (2),* 524-530.

Xiao, H.; Marquard, R.R.; Abramson, D.; Frohlich, A.A. Metabolites of ochratoxins in rat urine and in a culture of *Aspergillus ochraceus*. *Appl. Environ. Microbiol.* **1996**, *62 (2),* 648-655.

SUPPORTING INFORMATION AVAILABLE

^{1}H NMR (500 MHz) spectra of the ochratoxins



Ochratoxin B: R_{1-3} = H
Ochratoxin A: $R_{2,3}$ = H; R_1 = Cl
(4*R*)-4-Hydroxyochratoxin B: $R_{1,3}$ = H; R_2 = OH

Table 3: ^{1}H NMR (500 MHz) of ochratoxin B in $CDCl_3$

Proton	Shift [ppm]	J [Hz]	Multiplicity	Connections
OH	12.70		1	
NH	8.51	6.62	2	J(NH,H12)
H3	4.74	6.28	4	J(H3,H10)
		5.00	2	J(H3,H4a)
		10.25	2	J(H3,H4b)
H4a	2.99	5.00	2	J(H4a,H3)
		16.65	2	J(H4a,H4b)
H4b	2.95	16.65	2	J(H4b,H4a)
		10.25	2	J(H4b,H3)
H6	6.82	7.97	2	J(H6,H7)
H7	8.33	7.99	2	J(H7,H6)
3H10	1.53	6.28	2	J(H10,H3)
H12	4.97			
H13a	3.20	14.20	2	J(H13a,H13b)
		7.64	2	J(H13a, H12)
H13b	3.35	14.18	2	J(H13b,H13a)
		5.30	2	J(H13b, H12)
H15-19	7.26		M	

Table 4: ^{1}H NMR (500 MHz) of ochratoxin A in $CDCl_3$

Proton	Shift [ppm]	J [Hz]	Multiplicity	Connections
OH	12.74		1	
NH	8.50	7.07	2	J(NH, H12)
H3	4.76	11.75	2	J(H3,H4a)
		6.05	4	J(H3,H10)
		3.49	2	J(H3,H4b)
H4b	3.29	17.47	2	J(H4b,H4a)
		3.49	2	J(H4b,H3)
H4a	2.86	17.47	2	J(H4a,H4b)
		11.75	2	J(H4a,H3)
H7	8.43		1	
3H10	1.60	6.05	2	J(H10,H3)
H12	5.06	7.07	2	J(H12,H13a)
		7.07	2	J(H12,NH)
		5.35	2	J(H12,H13b)
H13a	3.23	14.08	2	J(H13a,H13b)
		7.07	2	J(H13a,H12)
H13b	3.36	14.08	2	J(H13b,H13a)
		5.35	2	J(H13b,H12)
H15-19	7.27		M	

Table 5: ^{1}H NMR (500 MHz) of ochratoxin α in $(CD_3)_2SO$

Proton	Shift [ppm]	J [Hz]	Multiplicity	Connections
H3	4.74	11.60	2	J(H3,H4a)
		6.22.	4	J(H3,H10)
		3.11	2	J(H3,H4b)
H4b	3.19	17.19	2	J(H4b,H4a)
		3.11	2	J(H4b,H3)
H4a	2.86	17.19	2	J(H4a,H4b)
		11.60	2	J(H4a,H3)
H7	7.99		1	
3H10	1.43	6.28	2	J(H10,H3)

Table 6: ^{1}H NMR (500 MHz) of (4*R*)-4-hydroxyochratoxin B in $(CD_3)_2SO$

Proton	Shift [ppm]	J [Hz]	Multiplicity	Connections
OH	12.60		1	
OH	5.86	6.40	2	J(R_2(OH), H4)
NH	8.51	7.47	2	J(NH, H12)
H3	4.79	6.59	4	J(H3,H10)
		2.17	2	J(H3,H4)
H4	4.56	6.40	2	J(H4,R_2(OH))
		2.17	2	J(H4,H3)
H6	7.08	7.90	2	J(H6,H7)
H7	8.13	7.90	2	J(H7,H6)
3H10	1.39	6.59	2	J(H10,H3)
H12	4.73	7.82	2	J(H12,H13A)
		7.47	2	J(H12,NH)
		4.95	2	J(H12,H13B)
H13a	3.08	13.80	2	J(H13A,H13B)
		7.82	2	J(H13A,H12)
H13b	3.20	13.80	2	J(H13B,H13A)
		4.95	2	J(H13B,H12)
H15-19	7.23			

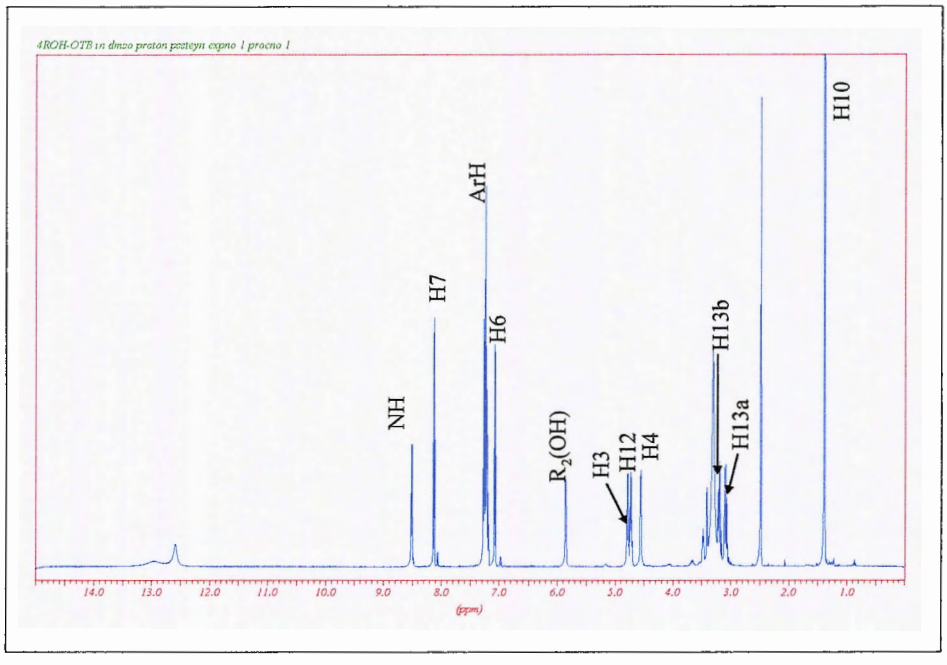


Figure 11: ^{1}H NMR(500 MHz) spectrum of (4*R*)-4-hydroxyochratoxin B in $(CD_3)_2SO$

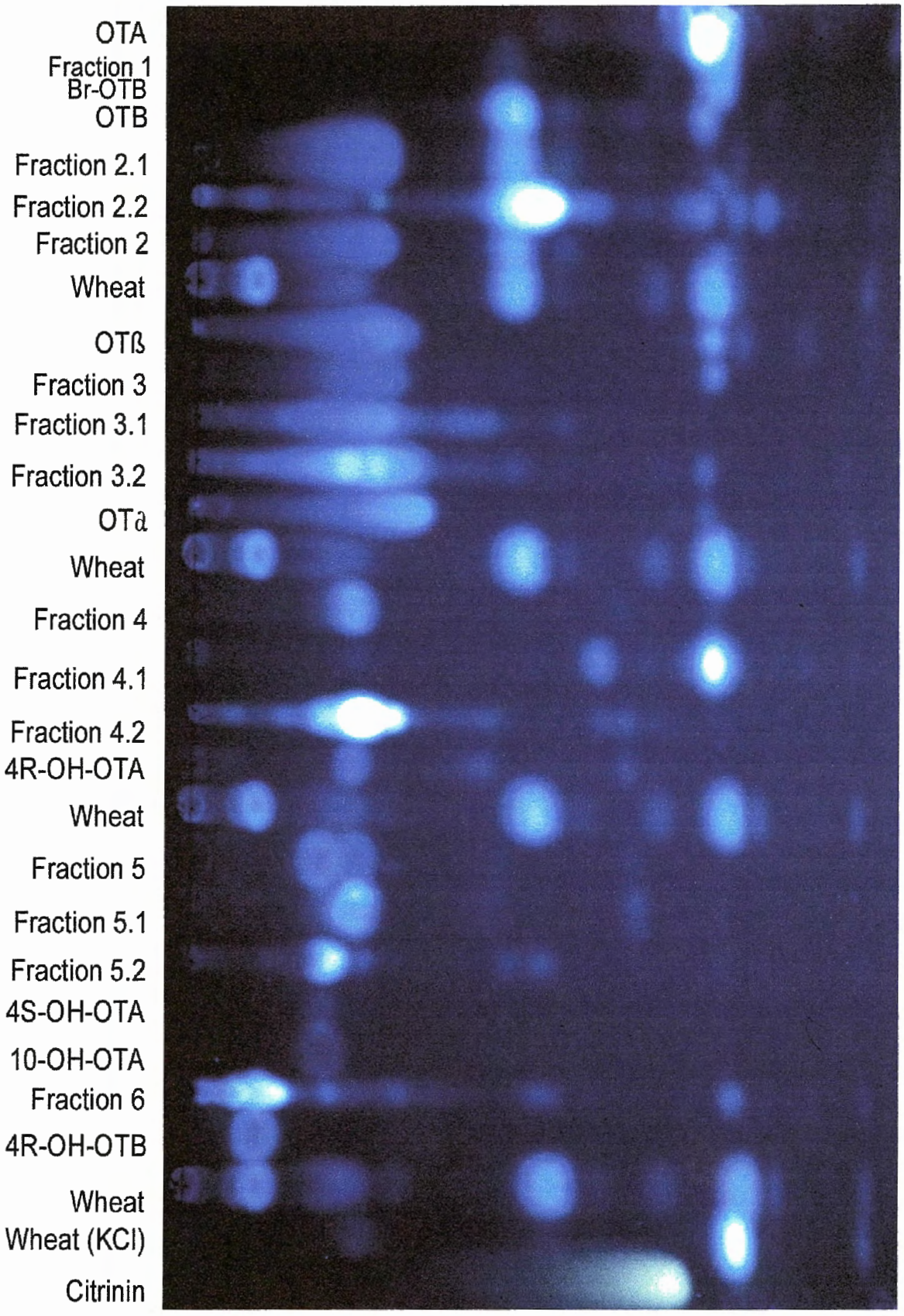


Figure 12: TLC plate of the different fractions of ochratoxins separated in the *A. ochraceus* cultivated heat supplemented with KBr and ochratoxin standards (Fractions 1-6 correspond to extracts 1-6 in the text).

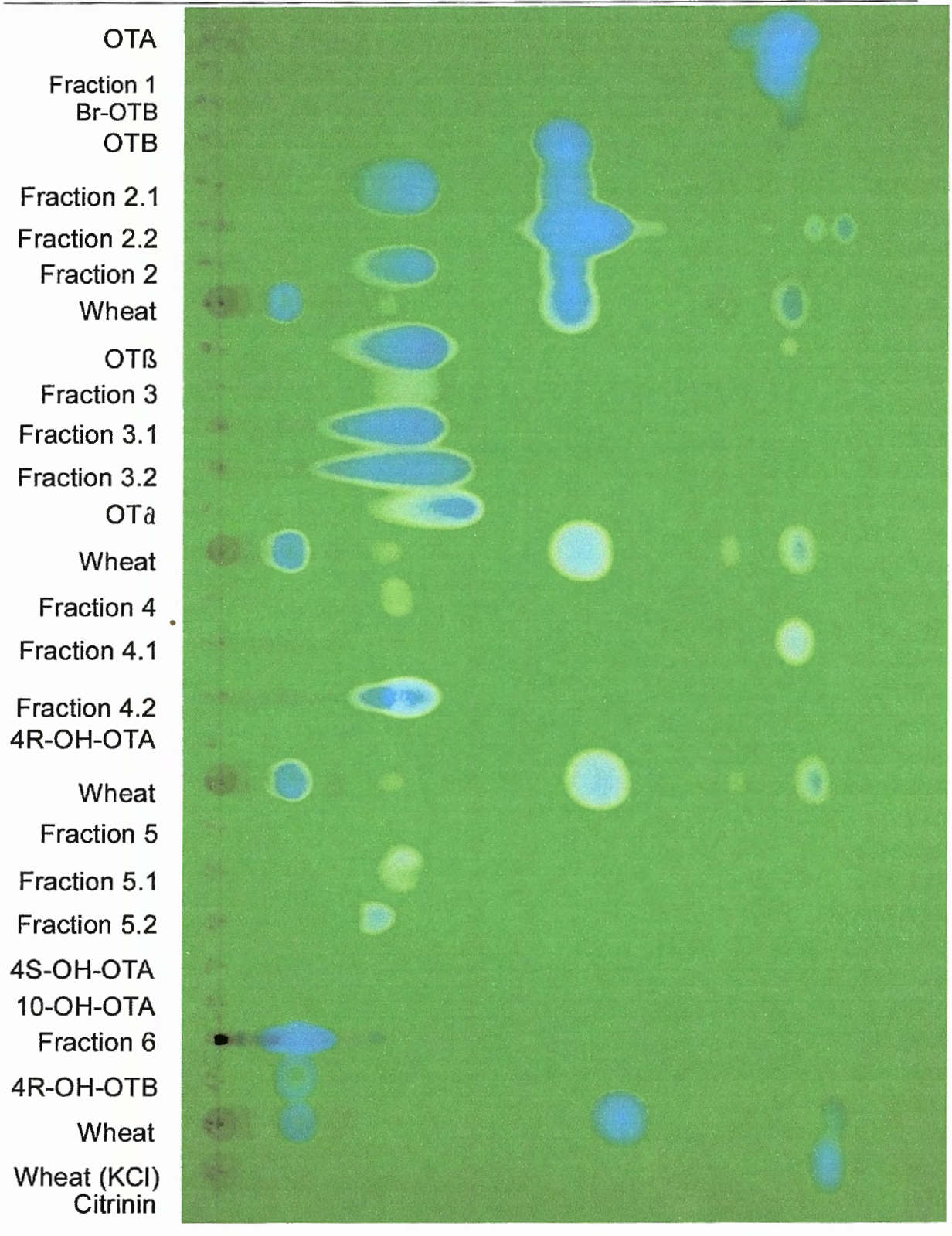
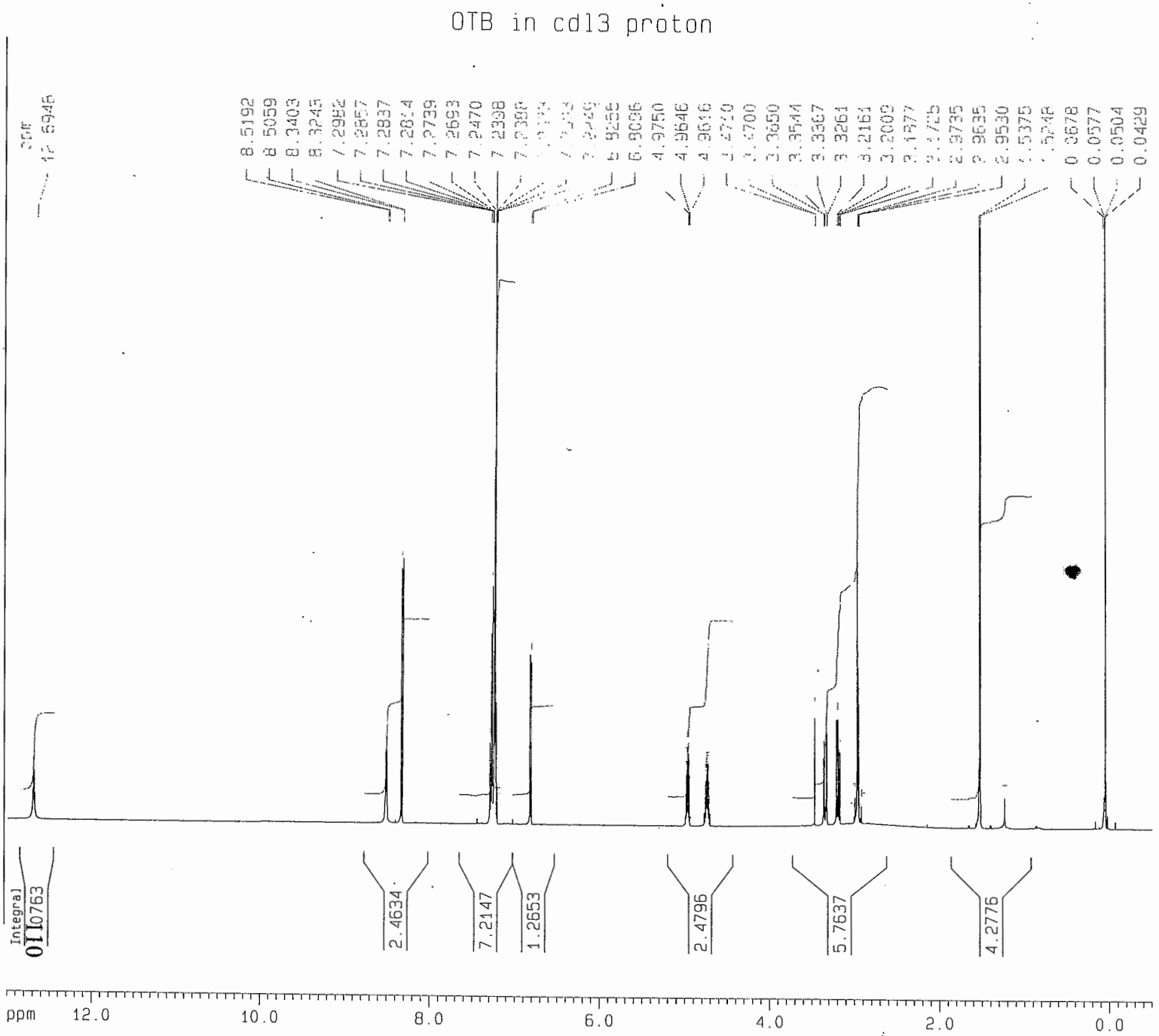


Figure 13: TLC plate of the different fractions of ochratoxins separated in the *A. ochraceus* cultivated wheat supplemented with KBr and ochratoxin standards (Fractions 1-6 correspond to extracts 1-6 in the text)



Current Data Parameters

NAME	pssteyn
EXPNO	5
PROCNO	1

F2 - Acquisition Parameters

Date_	990428
Time	11.53
INSTRUM	spect
PROBHD	5 mm TBI 13C
PULPROG	zg
TD	65536
SOLVENT	CDCl3
NS	512
DS	2
SWH	7507.507 Hz
FIDRES	0.114555 Hz
AQ	4.3647475 sec
RG	228.1
DW	66.600 usec
DE	6.00 usec
TE	300.0 K
D1	1.00000000 sec

========== CHANNEL f1 ==========

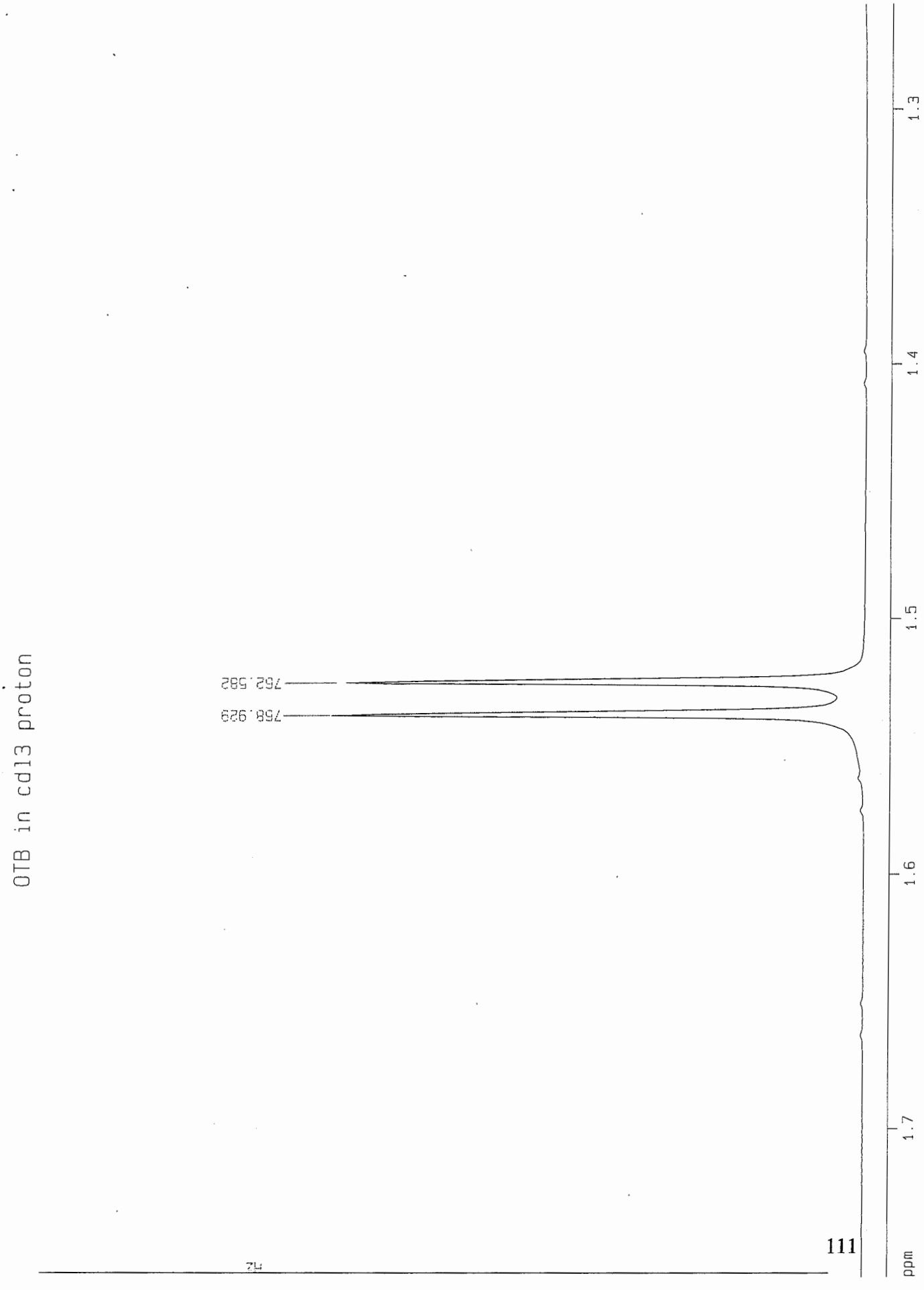
NUC1	1H
P1	9.00 usec
PL1	3.00 dB
SFO1	500.1332742 MHz

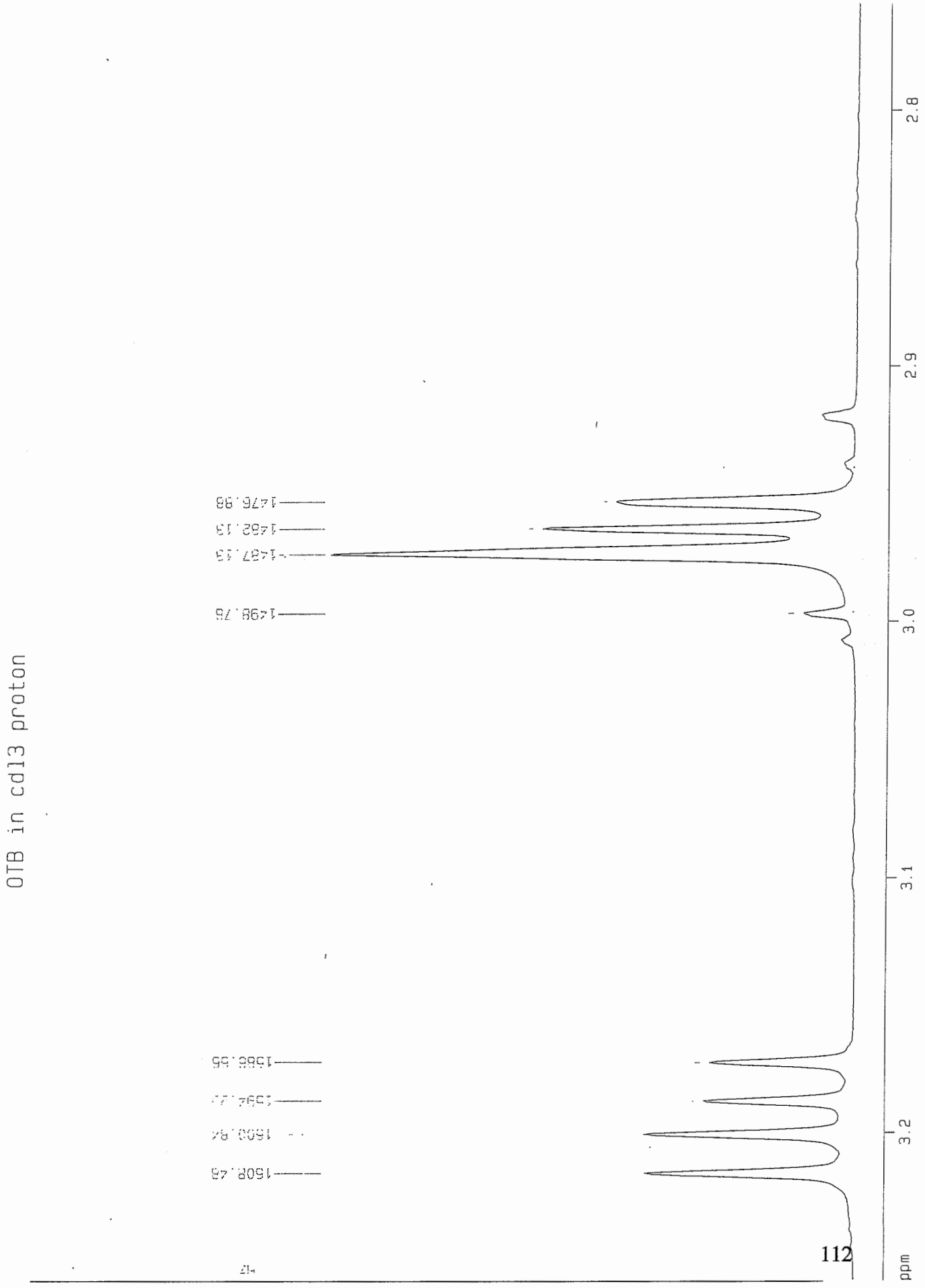
F2 - Processing parameters

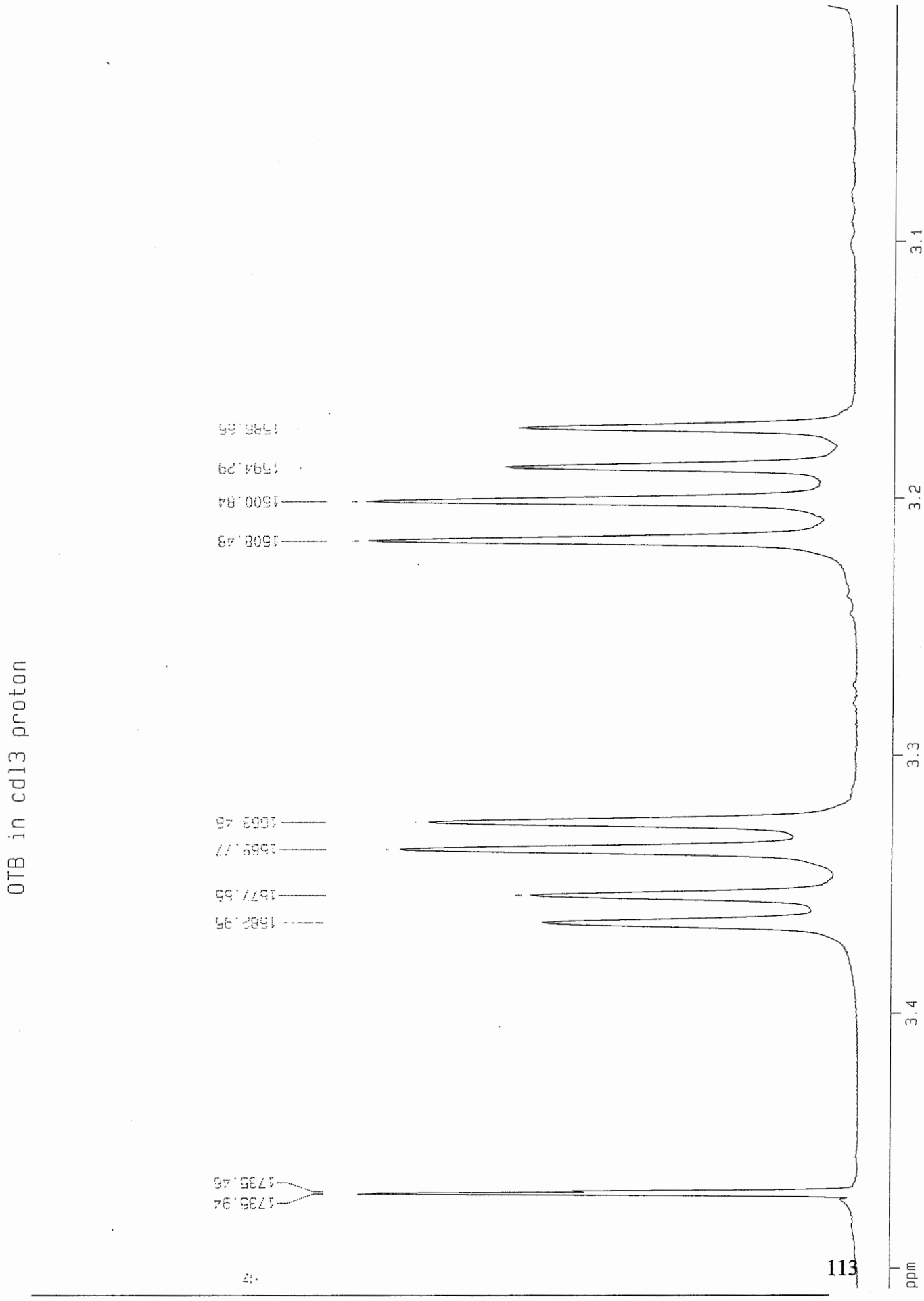
SI	32768
SF	500.1300235 MHz
WDW	GM
SSB	0
LB	-0.50 Hz
GB	0.5
PC	1.00

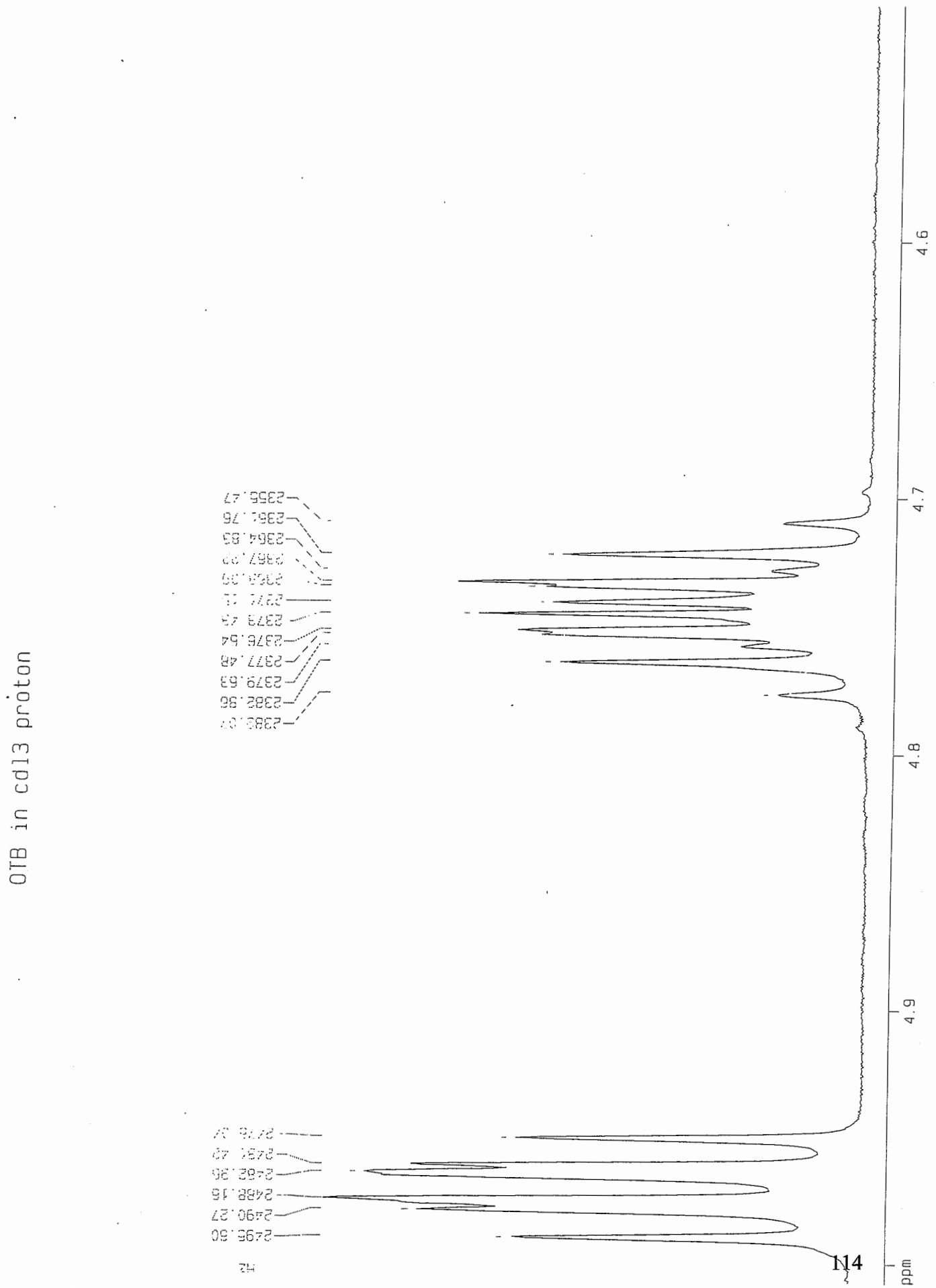
1D NMR plot parameters

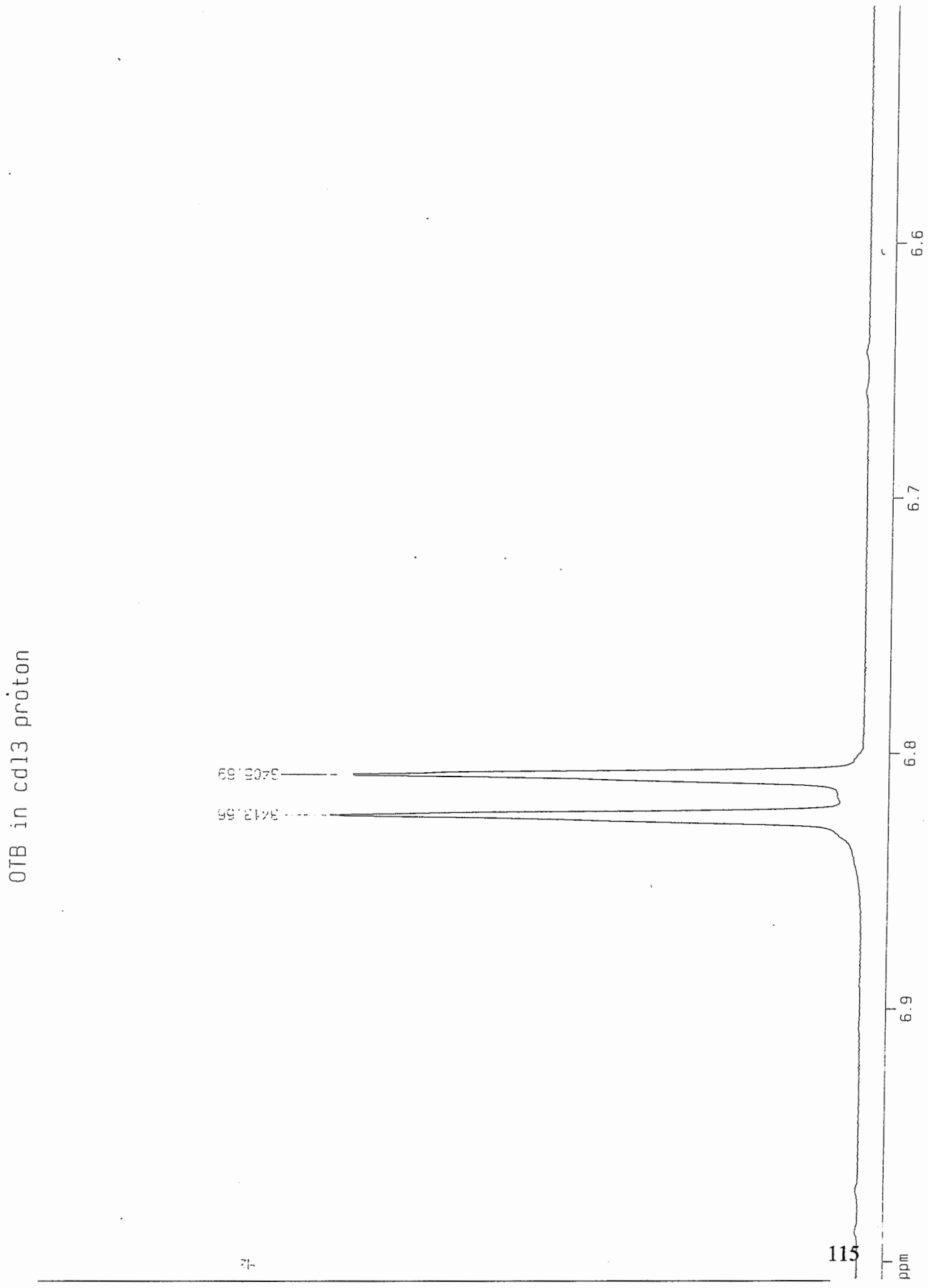
CX	20.00 cm
F1P	13.000 ppm
F1	6501.69 Hz
F2P	-0.500 ppm
F2	-250.07 Hz
PPMCM	0.67500 ppm/cm
HZCM	337.58780 Hz/cm

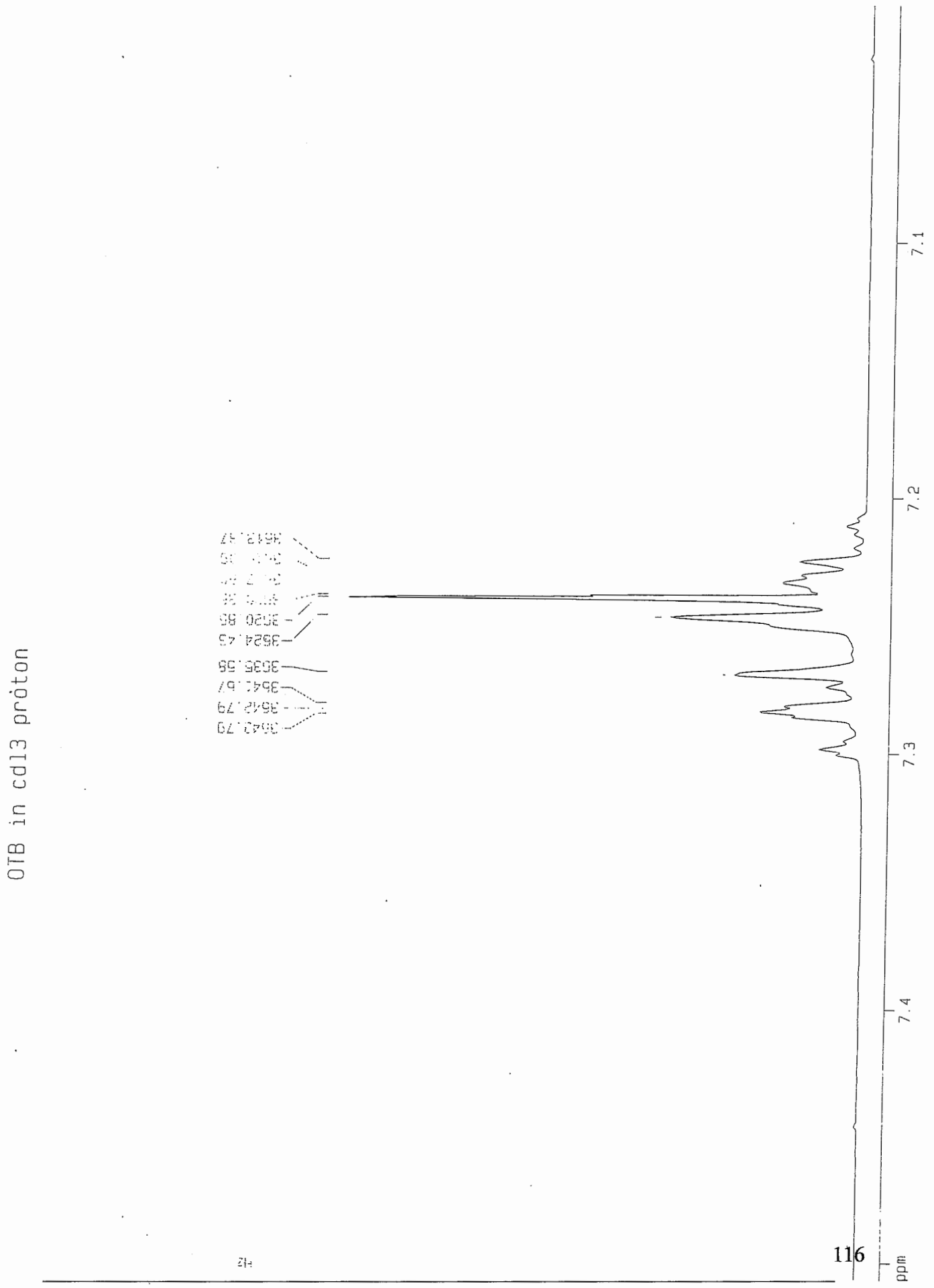

OTB in cdl3 proton
762.582
758.929
1.3
1.4
1.5
1.6
1.7
ppm

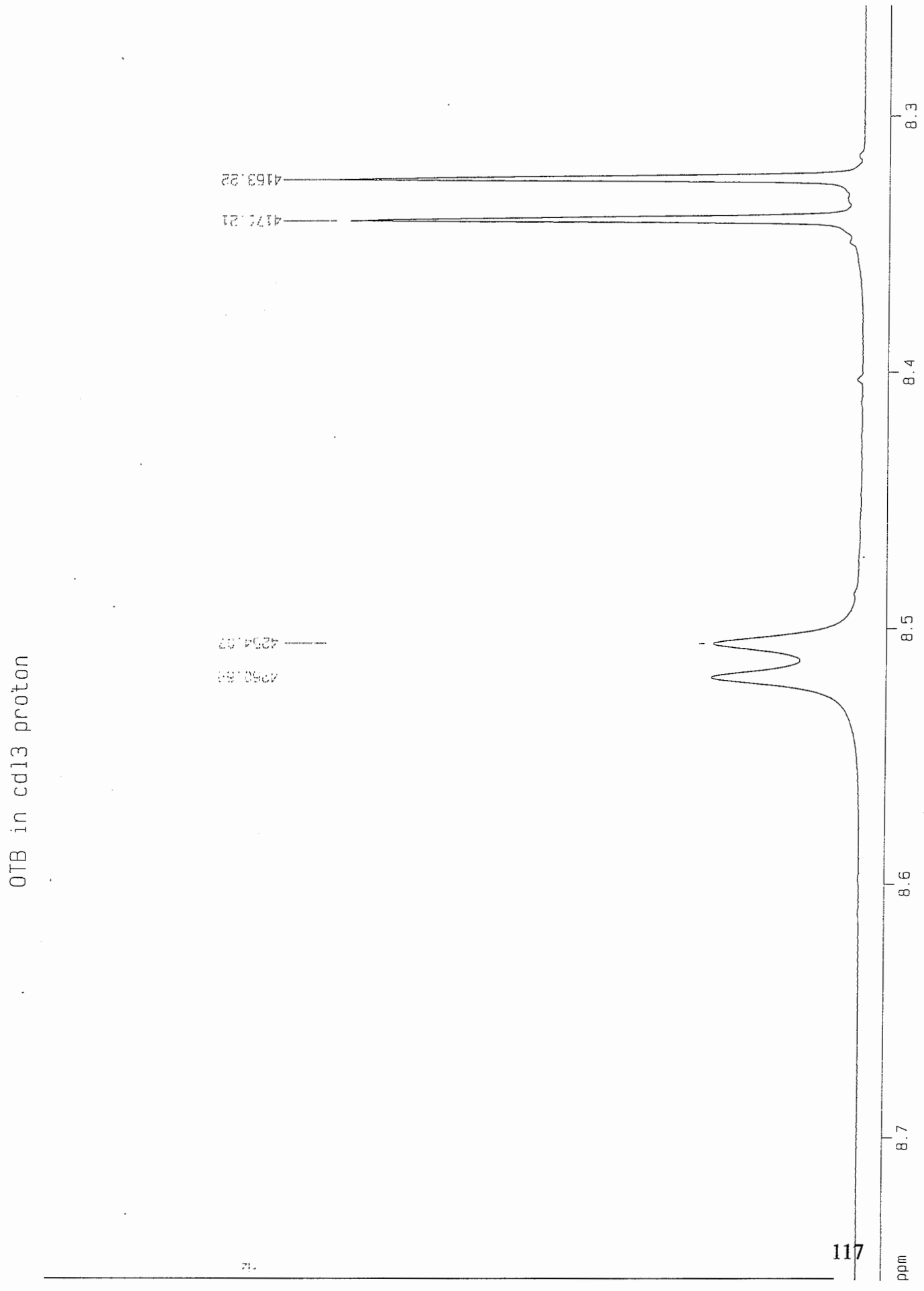

OTB in cd13 proton
1476.98
1462.13
1437.13
1398.78
1586.85
1508.46
2.8
2.9
3.0
3.1
3.2
ppm

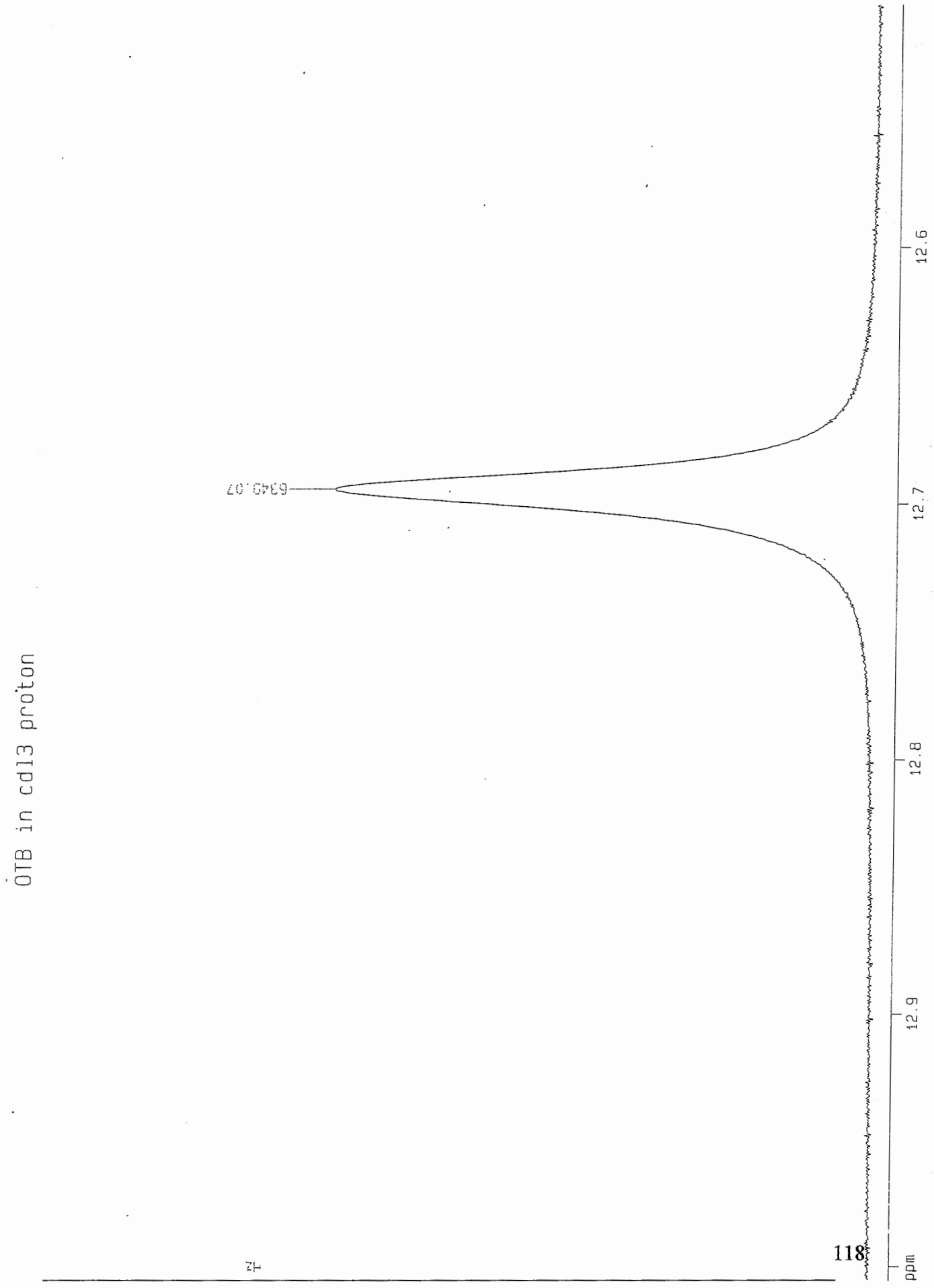

OTB in cdl3 proton
1736.46
1736.94
1582.95
1577.65
1569.77
1563.46
1508.48
1500.84
1594.29
1586.66
3.4
3.3
3.2
3.1
ppm


OTB in cdl3 proton
2355.47
2361.76
2364.85
2376.64
2377.48
2379.63
2382.86
2495.60
2490.27
2488.15
2482.36
ppm
4.6
4.7
4.8
4.9

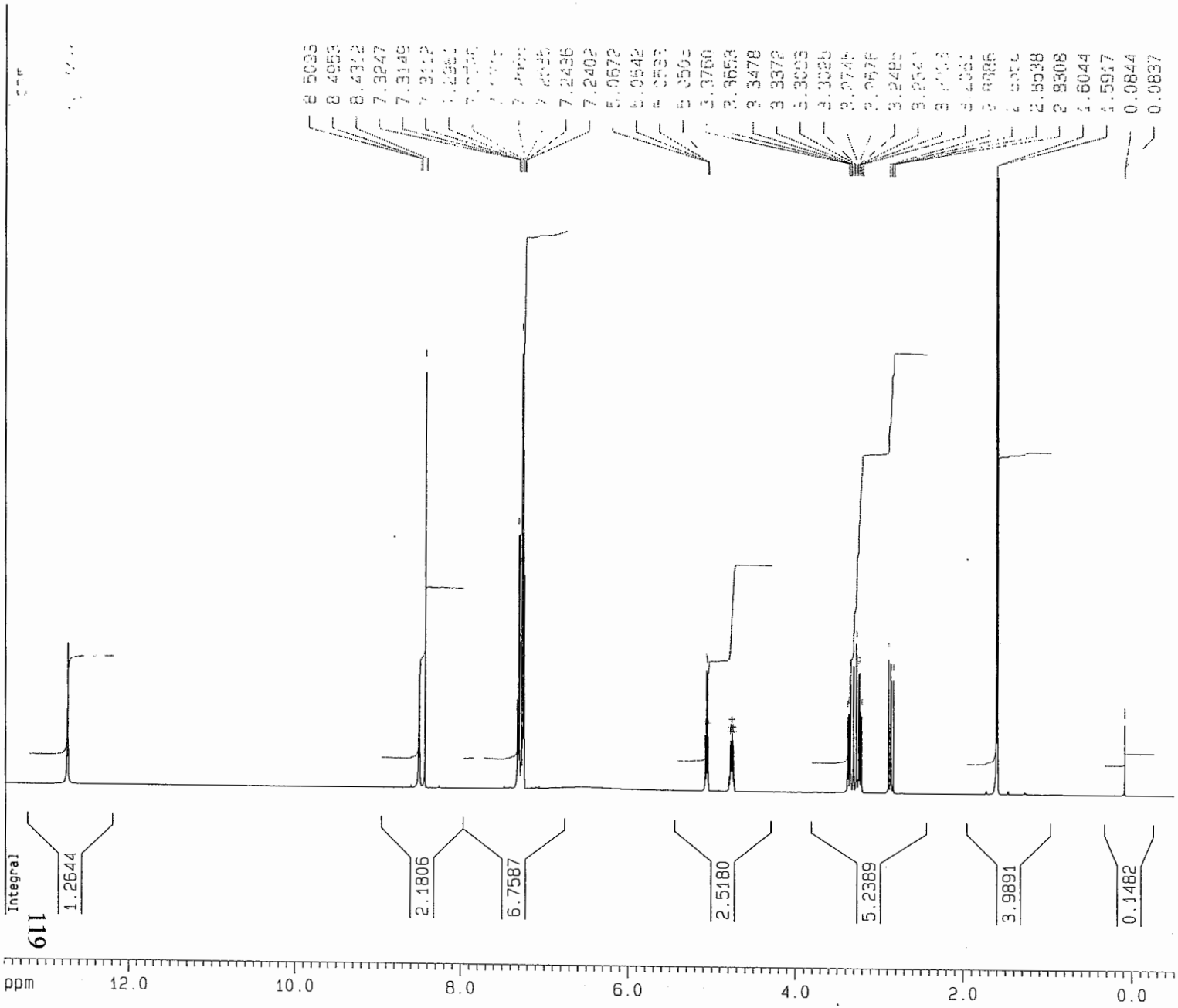

OTB in cdl3 proton
6.6
6.7
6.8
6.9
ppm


OTB in cdl3 proton
7.1
7.2
7.3
7.4
ppm


OTB in cd13 proton
4163.22
4172.21
4254.07
4266.64
8.3
8.4
8.5
8.6
8.7
ppm


OTB in cdl3 proton
6349.07
12.6
12.7
12.8
12.9
ppm

OTA in cdcl3 proton



Current Data Parameters

NAME	pssteyn
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	990422
Time	12.56
INSTRUM	spect
PROBHD	5 mm TBI 13C
PULPROG	zg
TD	65536
SOLVENT	CDCl3
NS	256
DS	2
SWH	7788.162 Hz
FIDRES	0.118838 Hz
AQ	4.2074614 sec
RG	71.8
DW	64.200 usec
DE	6.00 usec
TE	300.0 K
D1	1.00000000 sec

========== CHANNEL f1 ==========

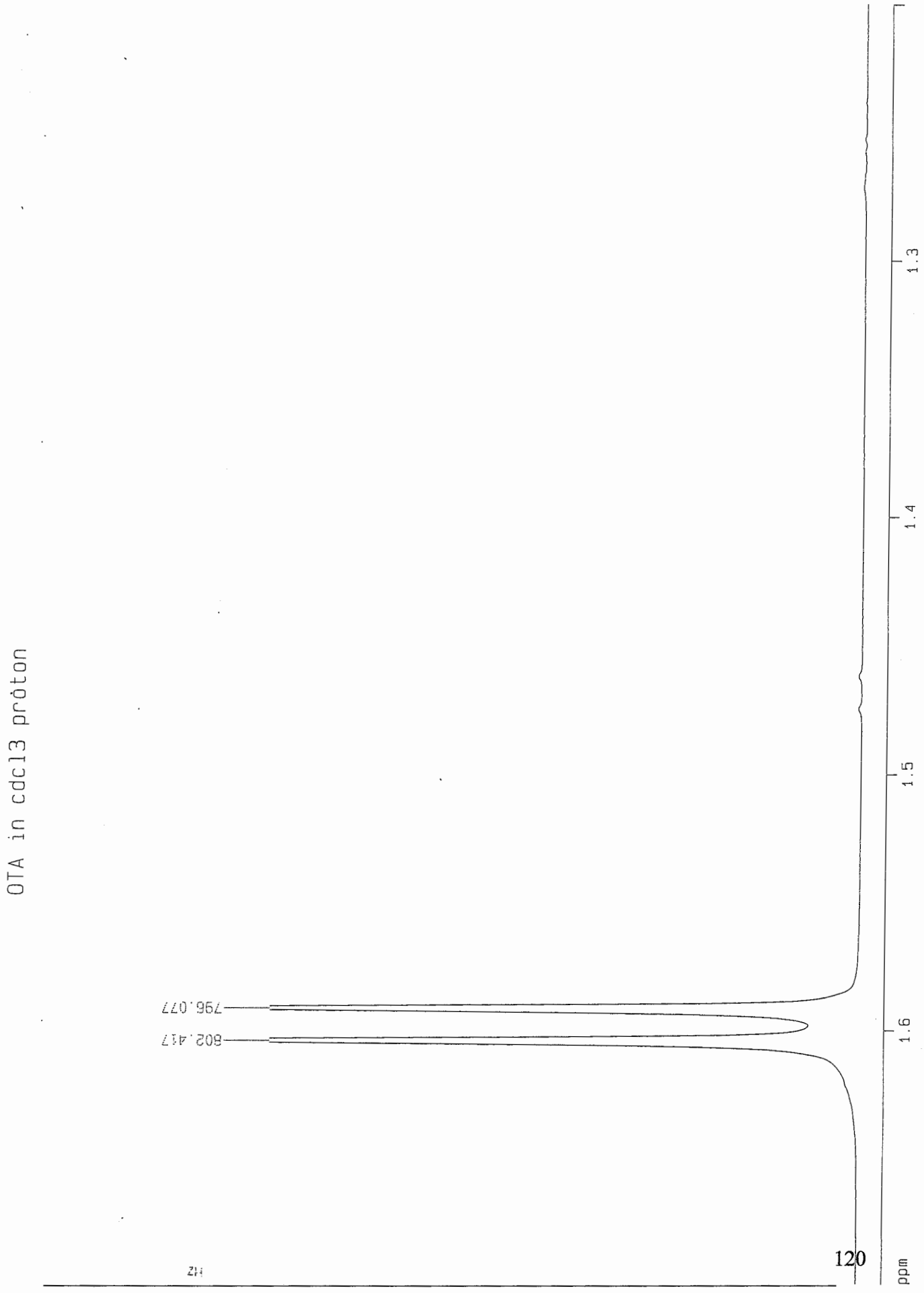
NUC1	1H
P1	9.00 usec
PL1	3.00 dB
SFO1	500.1331492 MHz

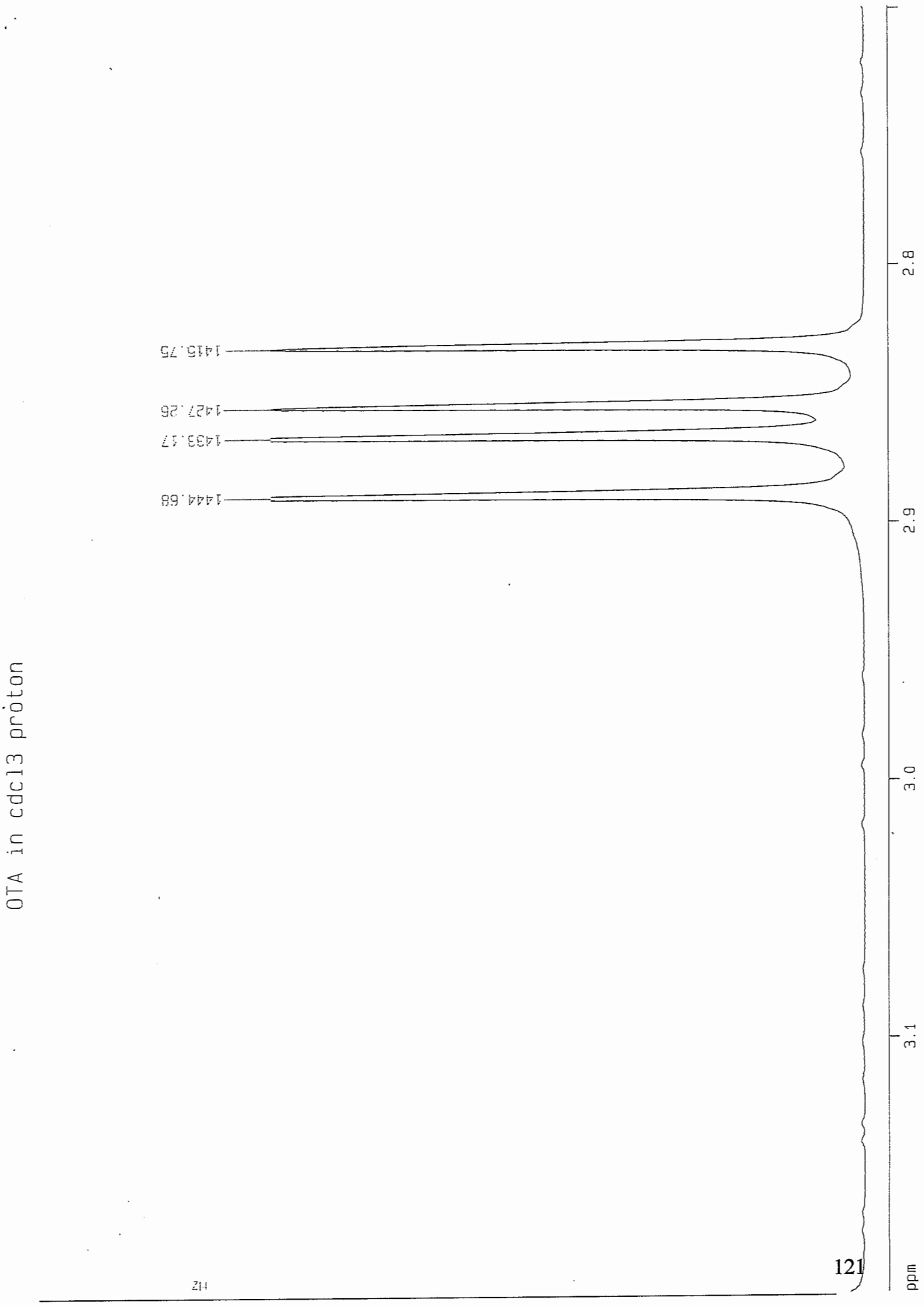
F2 - Processing parameters

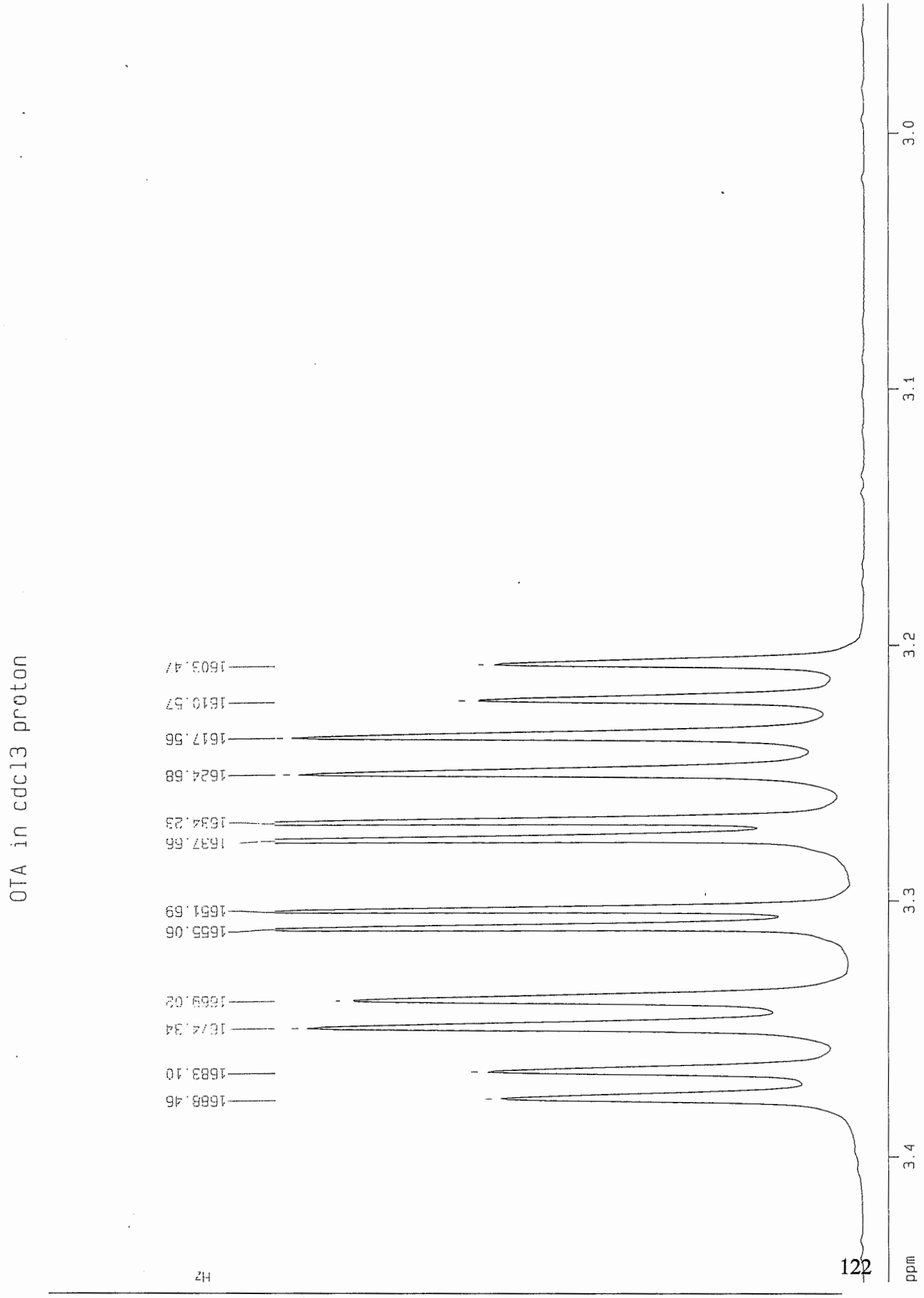
SI	32768
SF	500.1300072 MHz
WDW	GM
SSB	0
LB	-0.50 Hz
GB	0.5
PC	1.00

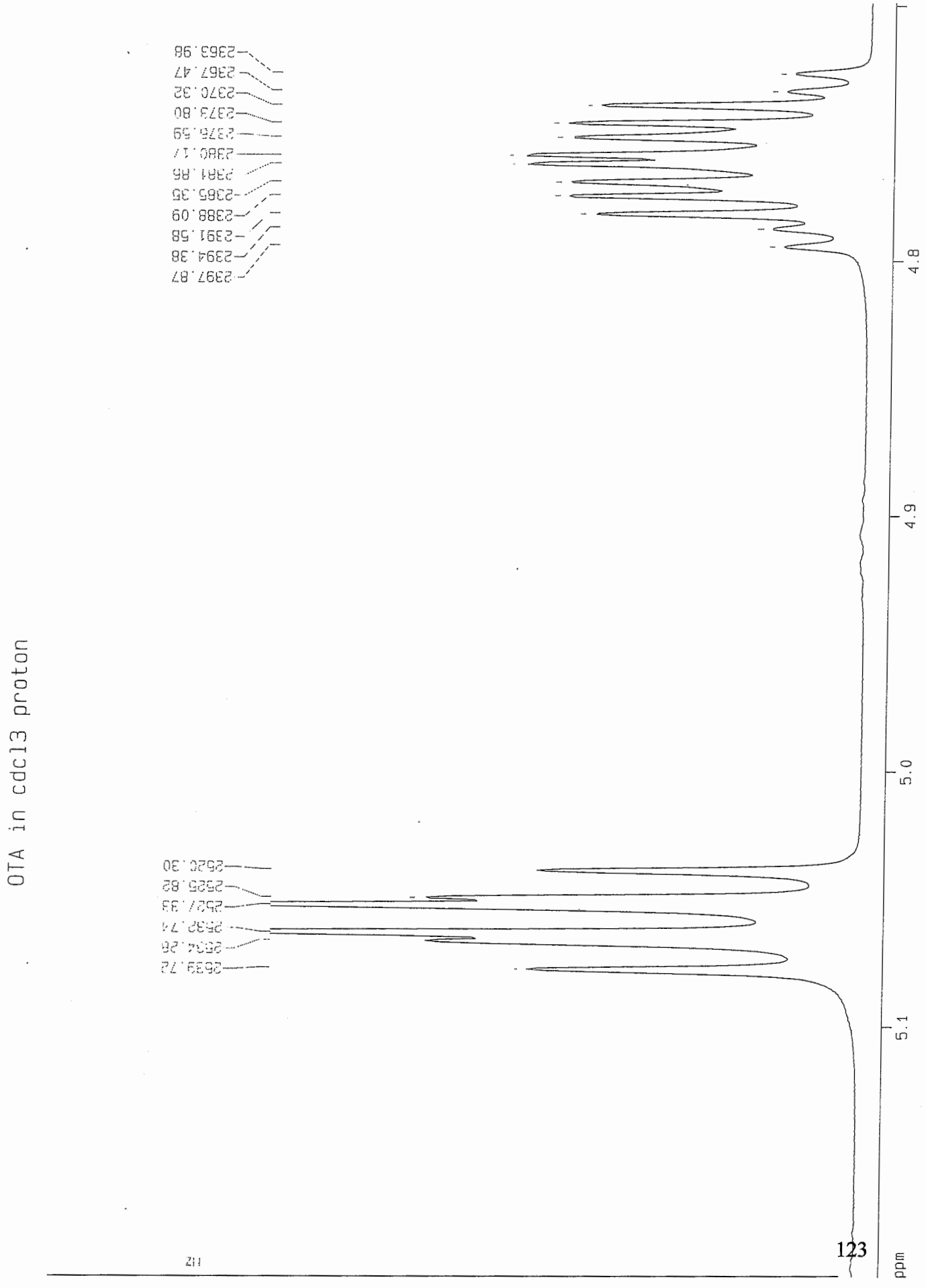
1D NMR plot parameters

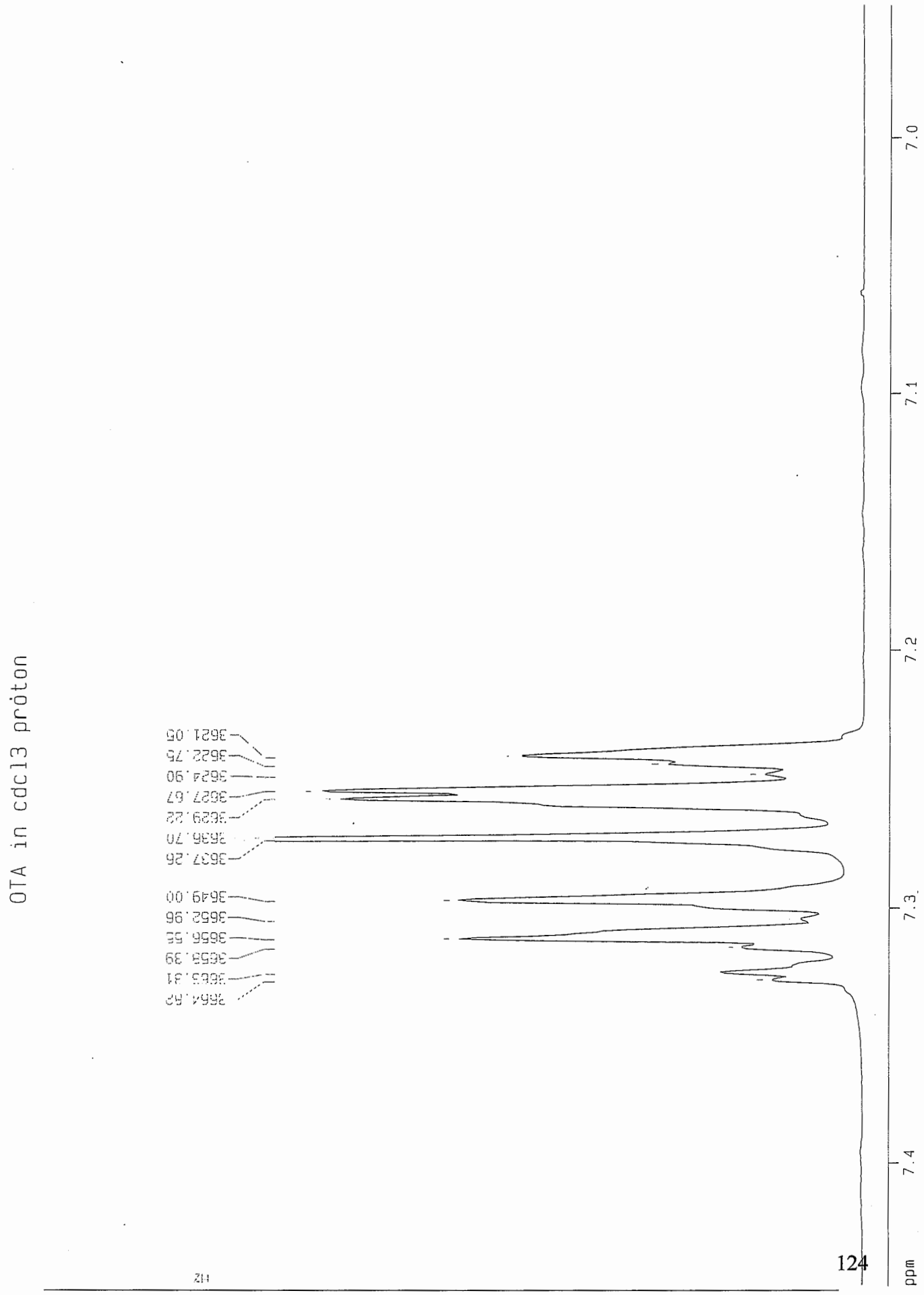
CX	20.00 cm
F1P	13.500 ppm
F1	6751.75 Hz
F2P	-0.500 ppm
F2	-250.07 Hz
PPMCM	0.70000 ppm/cm
HZCM	350.09103 Hz/cm

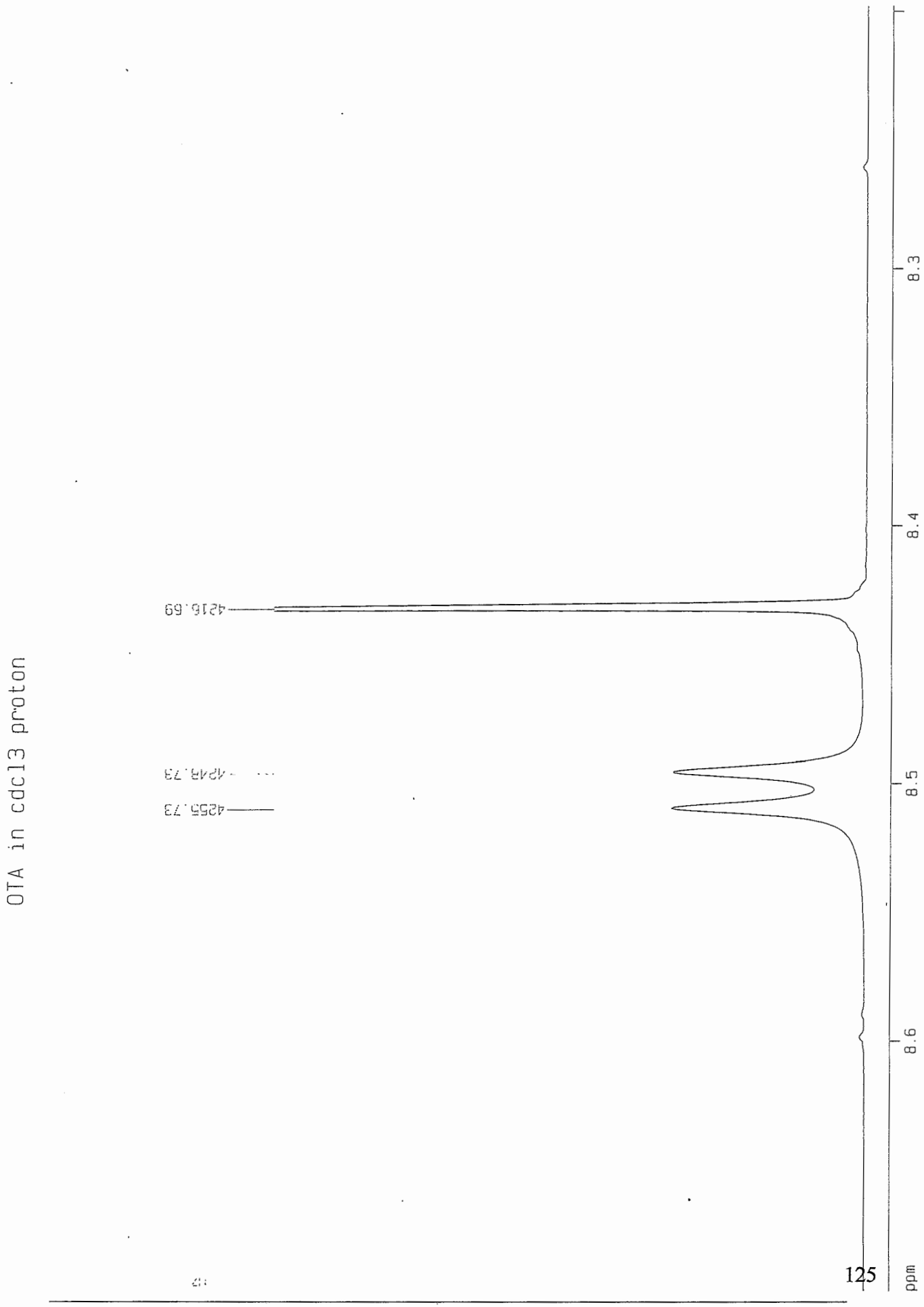

OTA in cdcl3 proton
796.077
802.417
1.3
1.4
1.5
1.6
ppm

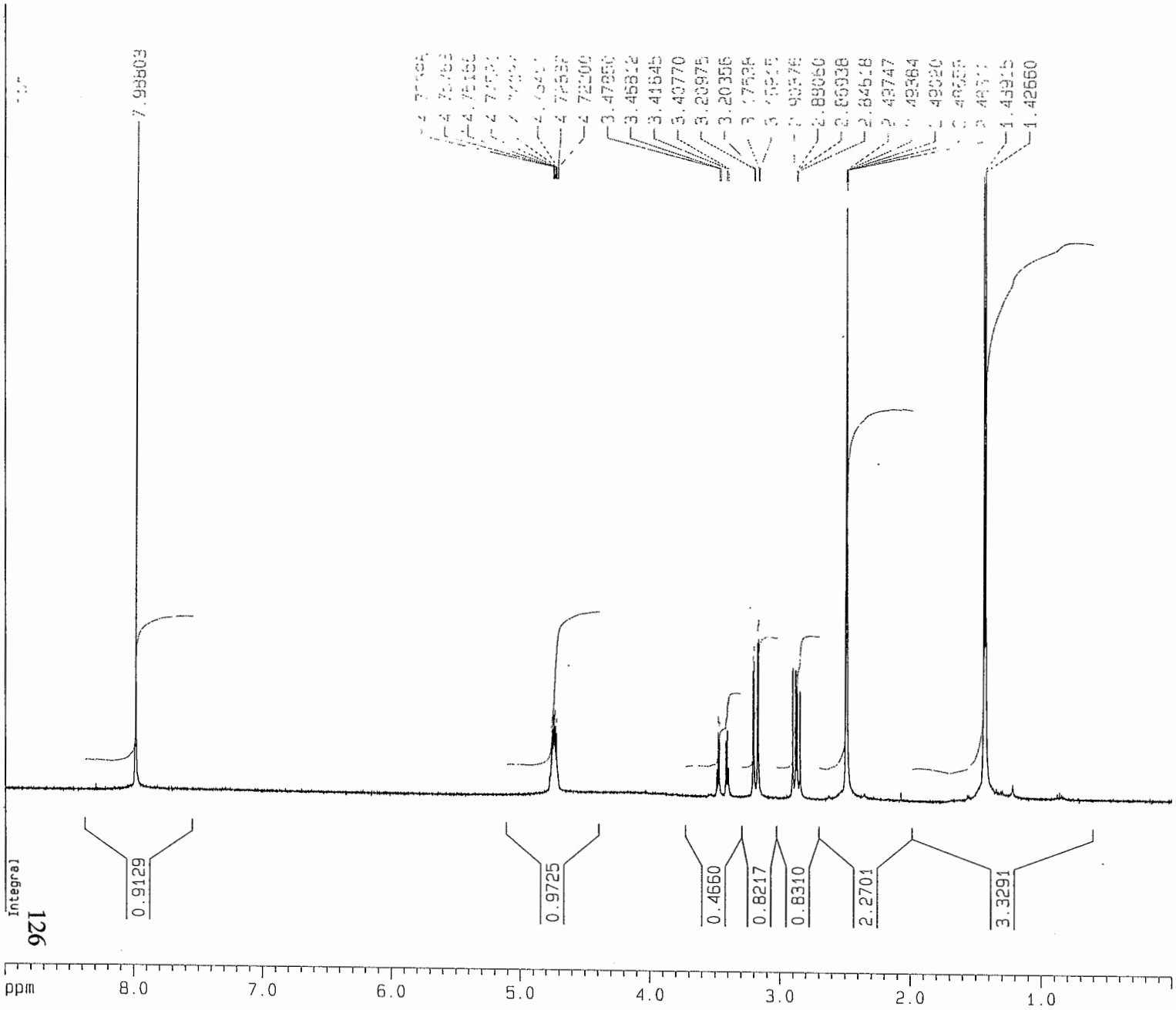

OTA in cdcl3 proton
1415.75
1427.26
1433.17
1444.68
2.8
2.9
3.0
3.1
ppm


OTA in cdcl3 proton
1603.47
1610.57
1617.56
1624.68
1634.23
1637.66
1651.69
1655.06
1669.02
1674.34
1683.10
1688.46
3.0
3.1
3.2
3.3
3.4
ppm
HZ


OTA in cdcl3 proton
2363.98
2367.47
2370.32
2373.80
2376.59
2380.17
2381.86
2385.35
2388.09
2391.58
2394.38
2397.87
2620.30
2625.82
2627.33
2632.74
2634.26
2639.72
4.8
4.9
5.0
5.1
ppm


OTA in cdcl3 proton
3621.05
3622.76
3624.90
3627.67
3629.22
7636.70
3637.26
3649.00
3652.96
3656.56
3658.39
3663.41
7664.62
7.0
7.1
7.2
7.3
7.4
ppm
HZ


OTA in cdcl3 proton
4216.69
4248.73
4255.73
8.3
8.4
8.5
8.6
ppm



Current Data Parameters

NAME	pssteyn
EXPNO	6
PROCNO	1

F2 - Acquisition Parameters

Date_	990428
Time	12.34
INSTRUM	spect
PROBHD	5 mm TBI 13C
PULPROG	zg
TD	65536
SOLVENT	DMSO
NS	16
DS	2
SWH	10330.578 Hz
FIDRES	0.157632 Hz
AQ	3.1719923 sec
RG	406.4
DW	48.400 usec
DE	6.00 usec
TE	300.0 K
D1	1.00000000 sec

========== CHANNEL f1 ==========

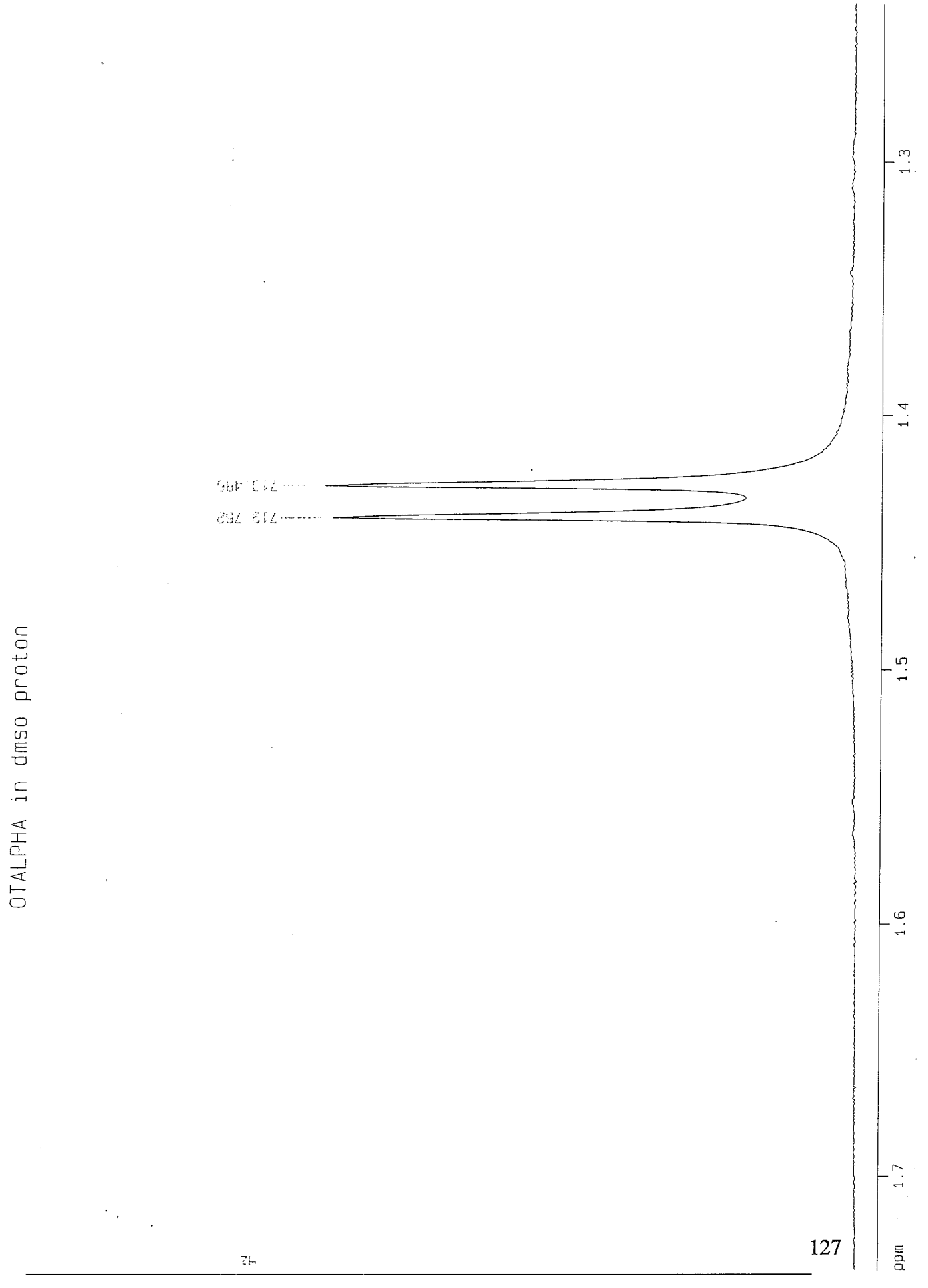
NUC1	1H
P1	9.00 usec
PL1	3.00 dB
SFO1	500.1330885 MHz

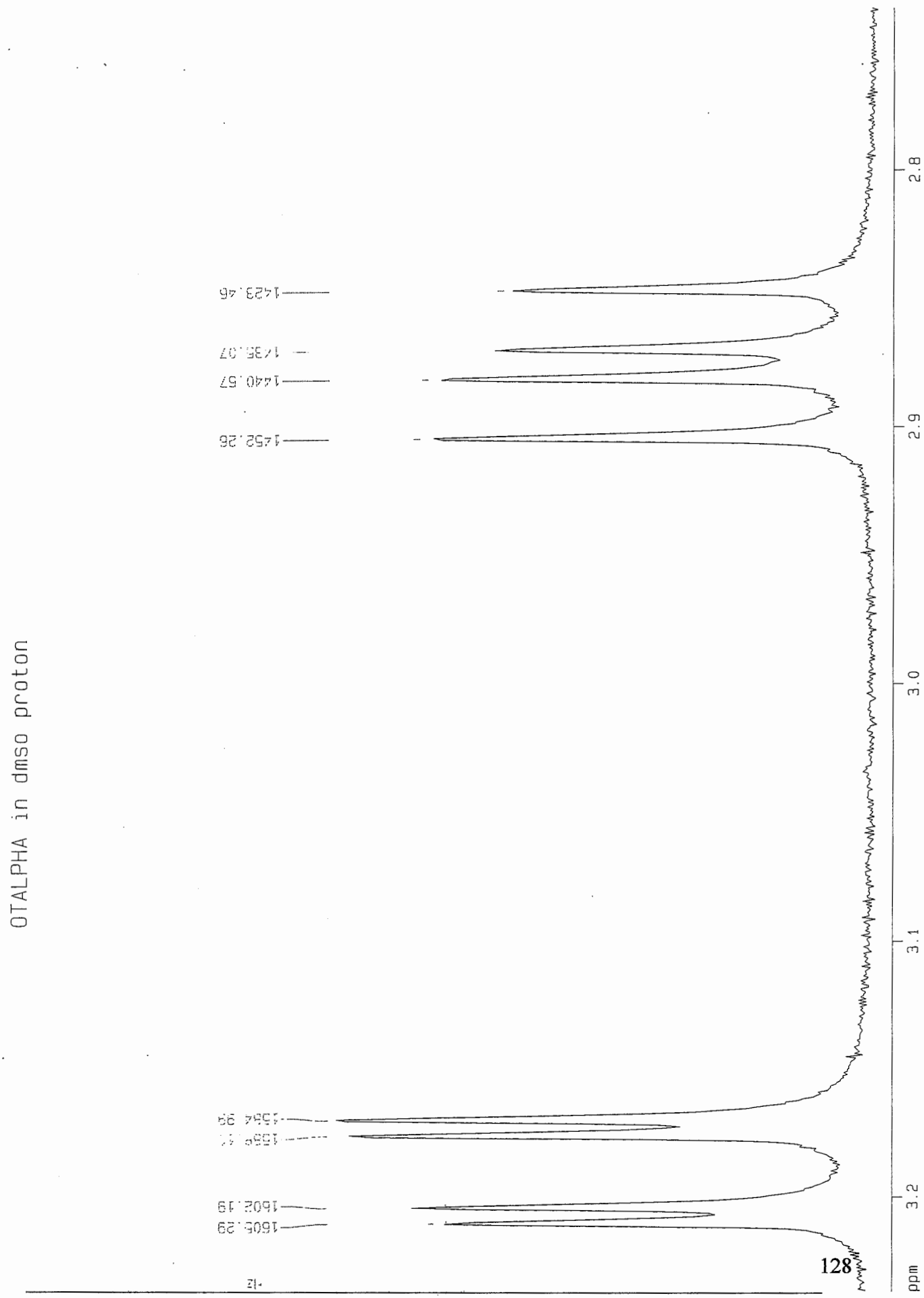
F2 - Processing parameters

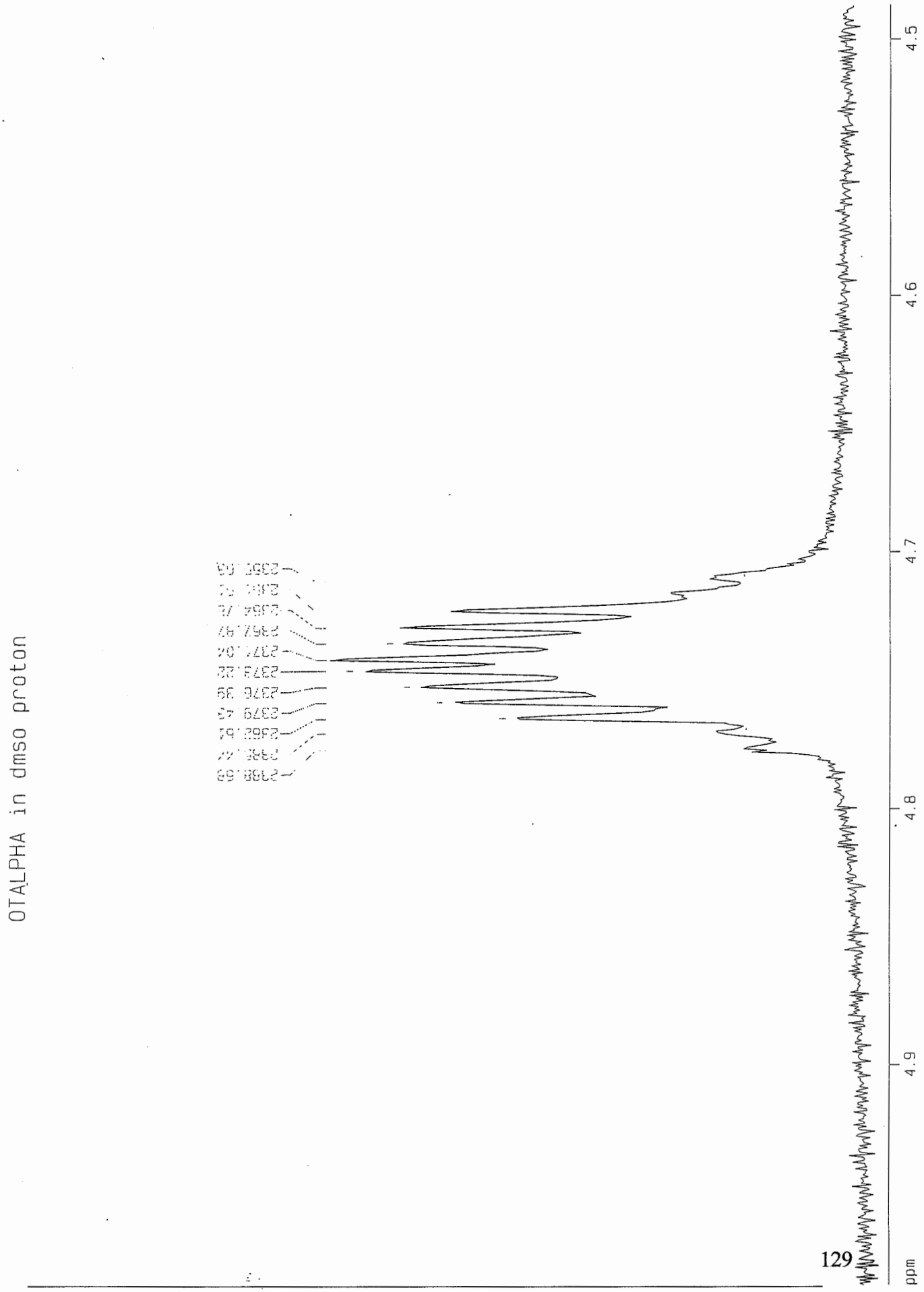
SI	32768
SF	500.1300099 MHz
WDW	GM
SSB	0
LB	-0.50 Hz
GB	0.5
PC	1.00

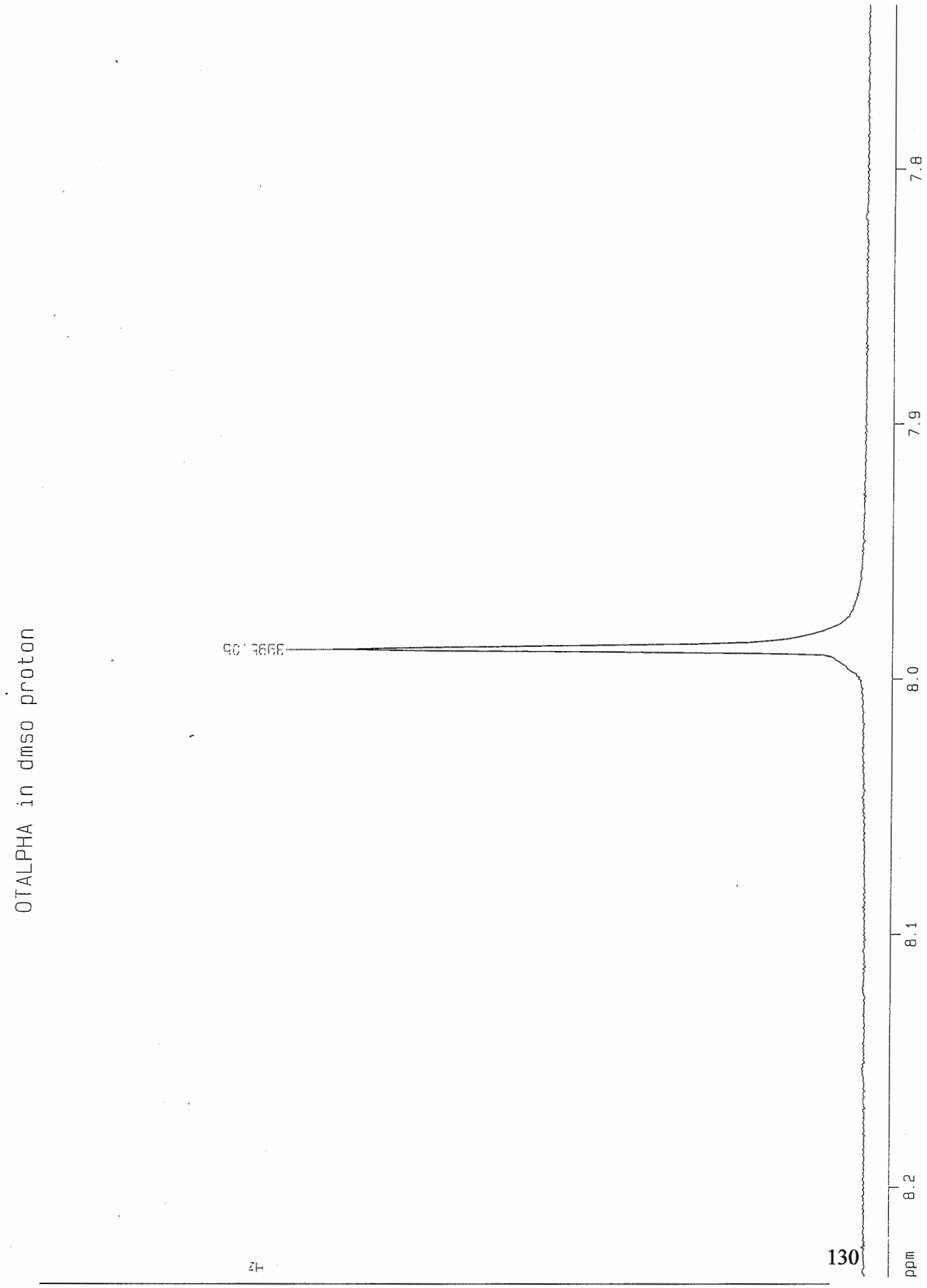
1D NMR plot parameters

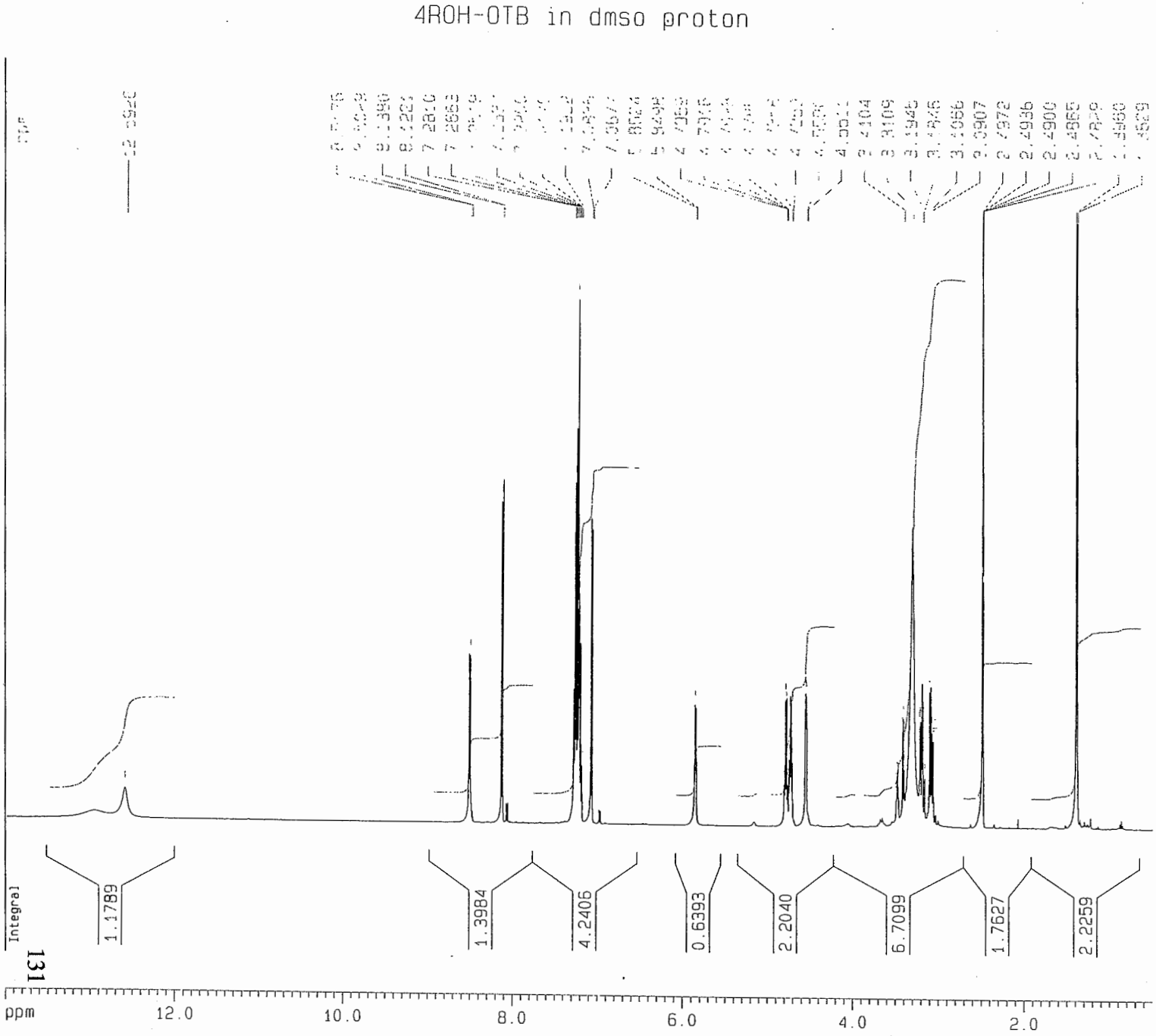
CX	20.00 cm
F1P	9.000 ppm
F1	4501.17 Hz
F2P	0.000 ppm
F2	0.00 Hz
PPMCM	0.45000 ppm/cm
HZCM	225.05849 Hz/cm


OTALPHA in dmso proton
719 768
713 496
1.3
1.4
1.5
1.6
1.7
ppm


OTALPHA in dmso proton
1606.29
1602.19
1559.47
1554.89
1452.26
1440.57
1435.07
1429.46
3.2
3.1
3.0
2.9
2.8
ppm


OTALPHA in dmso proton
4.5
4.6
4.7
4.8
4.9
ppm


OTALPHA in dmso proton
7.8
7.9
8.0
8.1
8.2
ppm



Current Data Parameters

NAME	pssteyn4
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	990422
Time	14.40
INSTRUM	spect
PROBHD	5 mm TBI 13C
PULPROG	zg
TD	65536
SOLVENT	DMSO
NS	256
DS	2
SWH	7507.507 Hz
FIDRES	0.114555 Hz
AQ	4.3647475 sec
RG	50.8
DW	66.600 usec
DE	6.00 usec
TE	300.0 K
D1	1.00000000 sec

========== CHANNEL f1 ==========

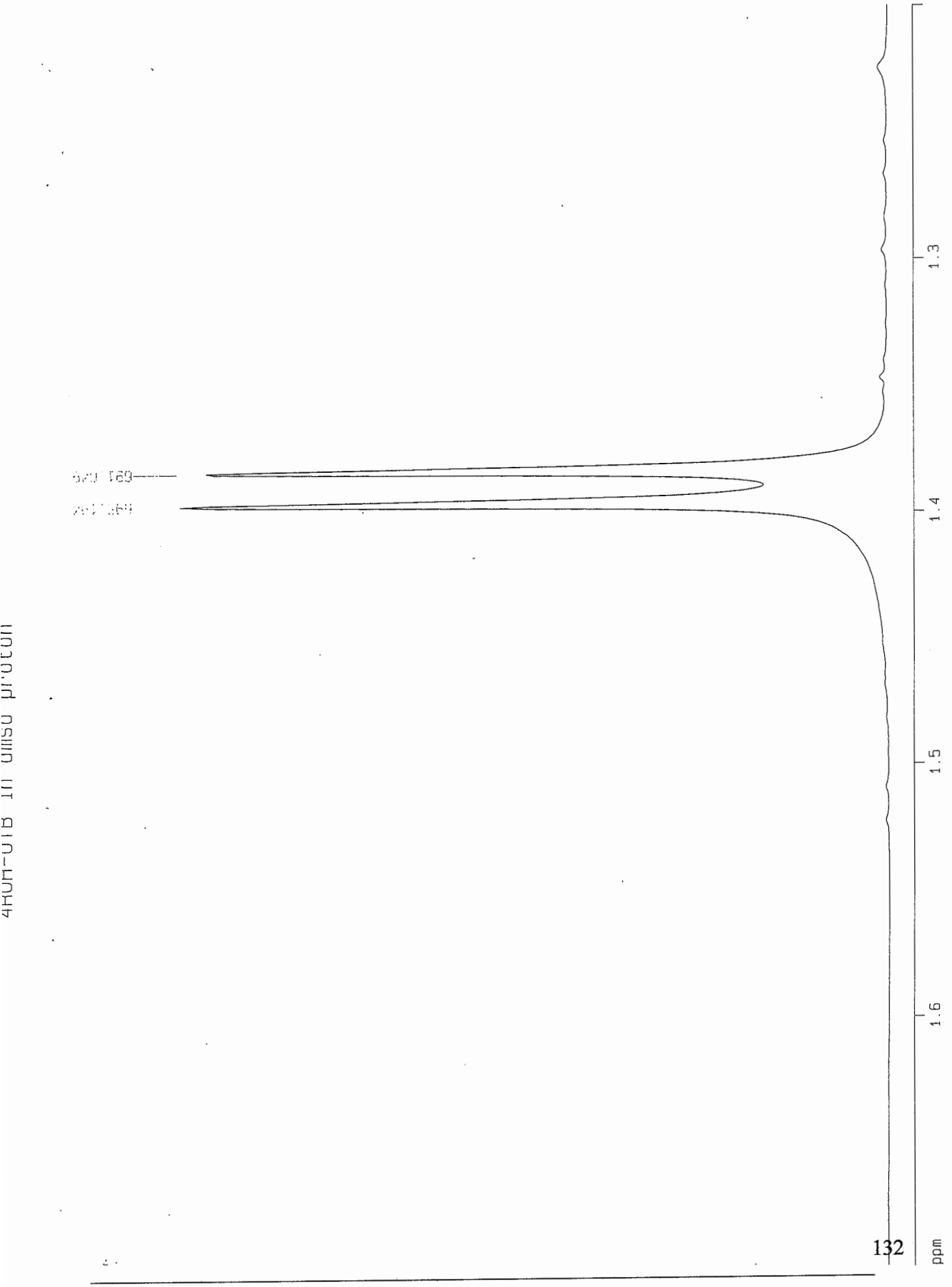
NUC1	1H
P1	9.00 usec
PL1	3.00 dB
SFO1	500.1337612 MHz

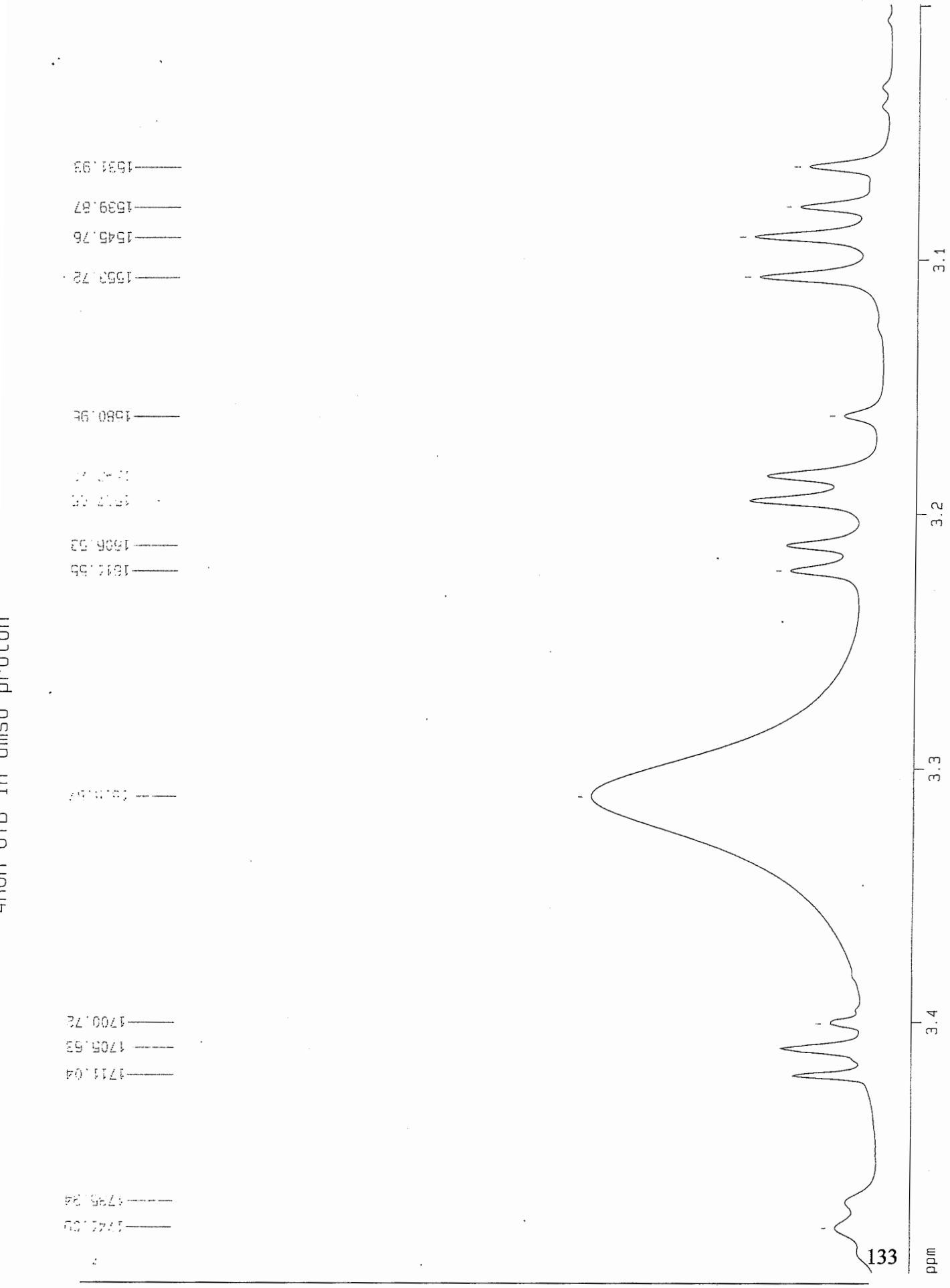
F2 - Processing parameters

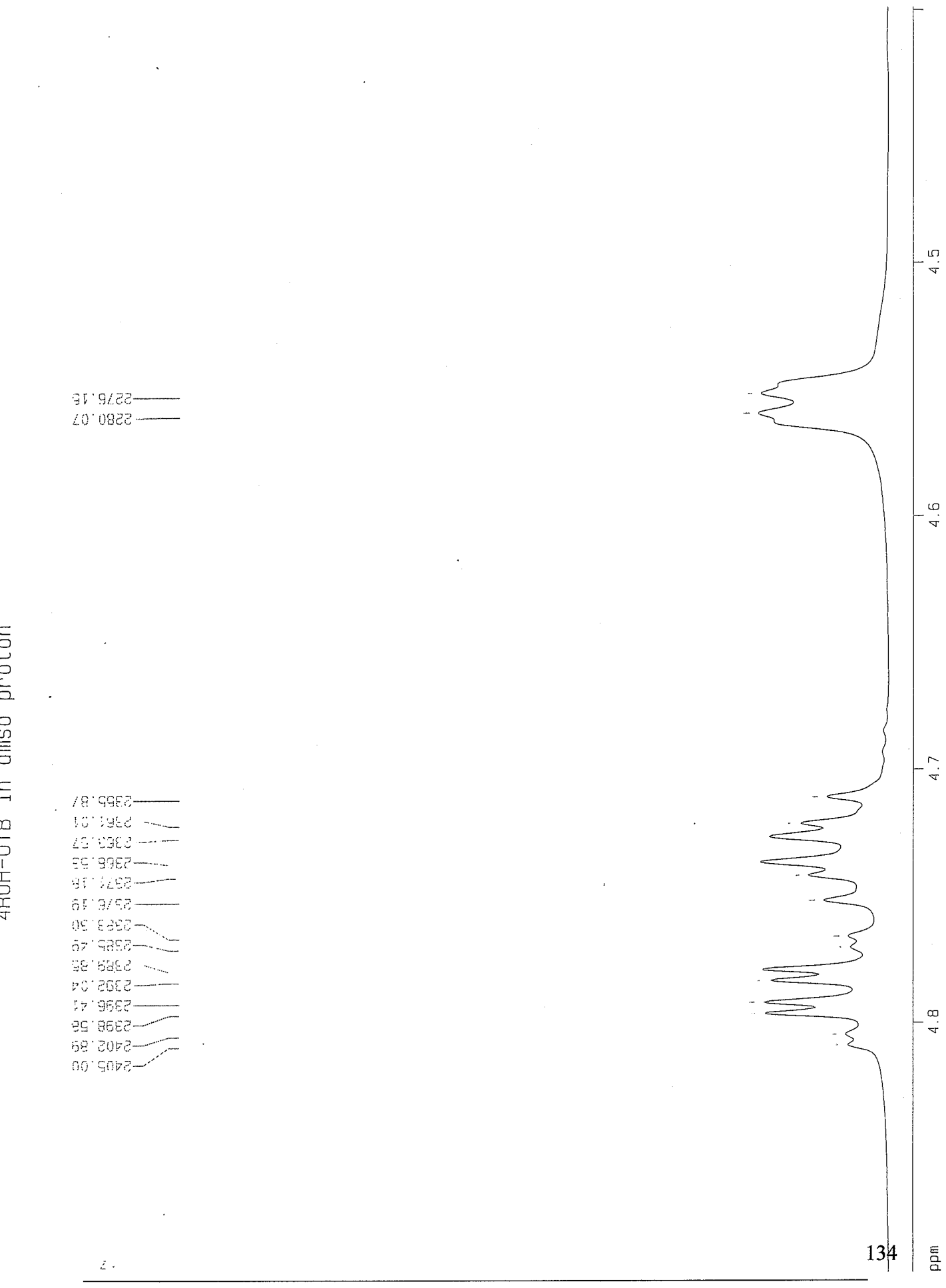
SI	32768
SF	500.1300099 MHz
WDW	GM
SSB	0
LB	-0.50 Hz
GB	0.5
PC	1.00

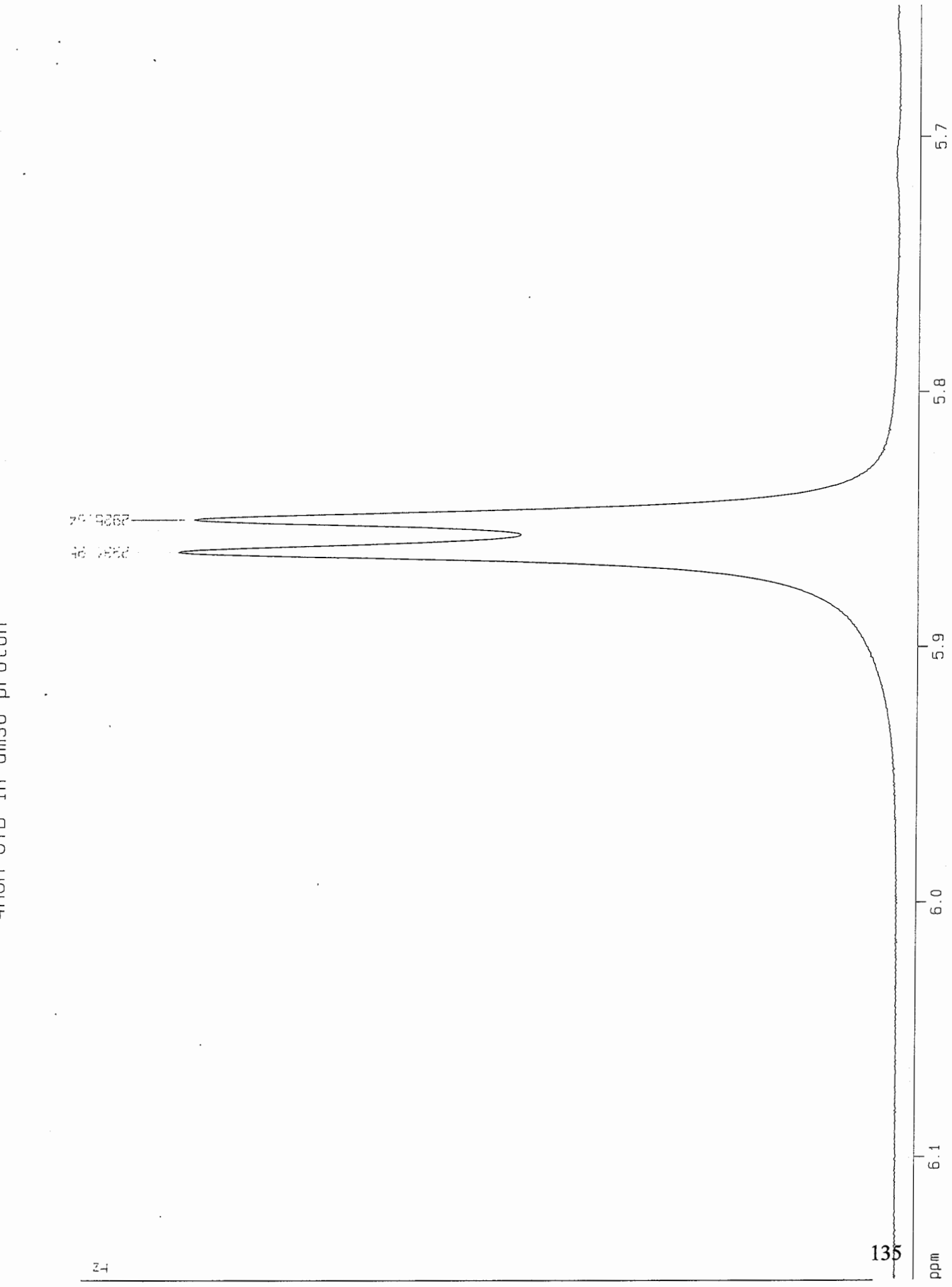
1D NMR plot parameters

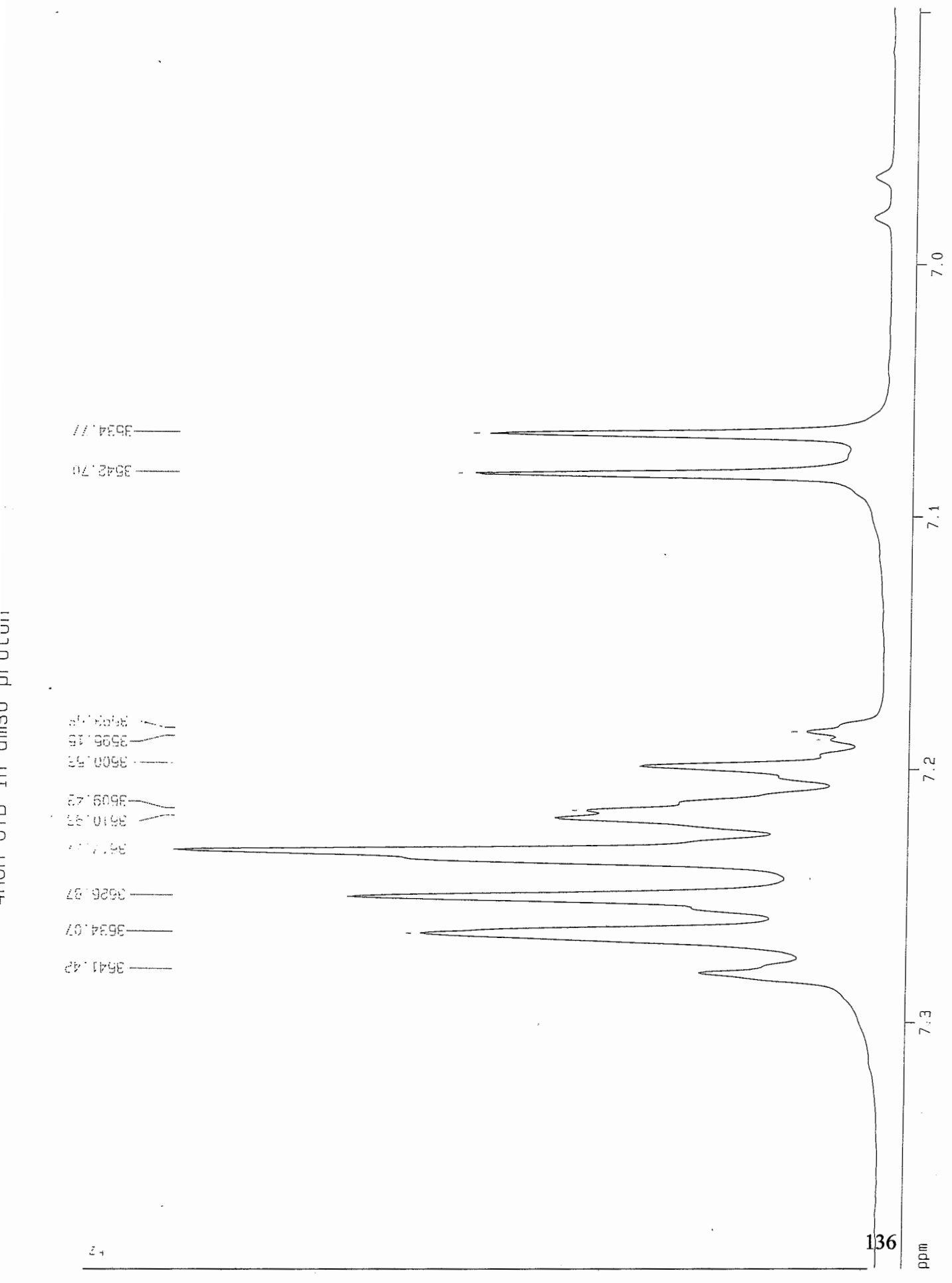
CX	20.00 cm
F1P	14.000 ppm
F1	7001.82 Hz
F2P	0.500 ppm
F2	250.07 Hz
PPMCM	0.67500 ppm/cm
HZCM	337.58777 Hz/cm

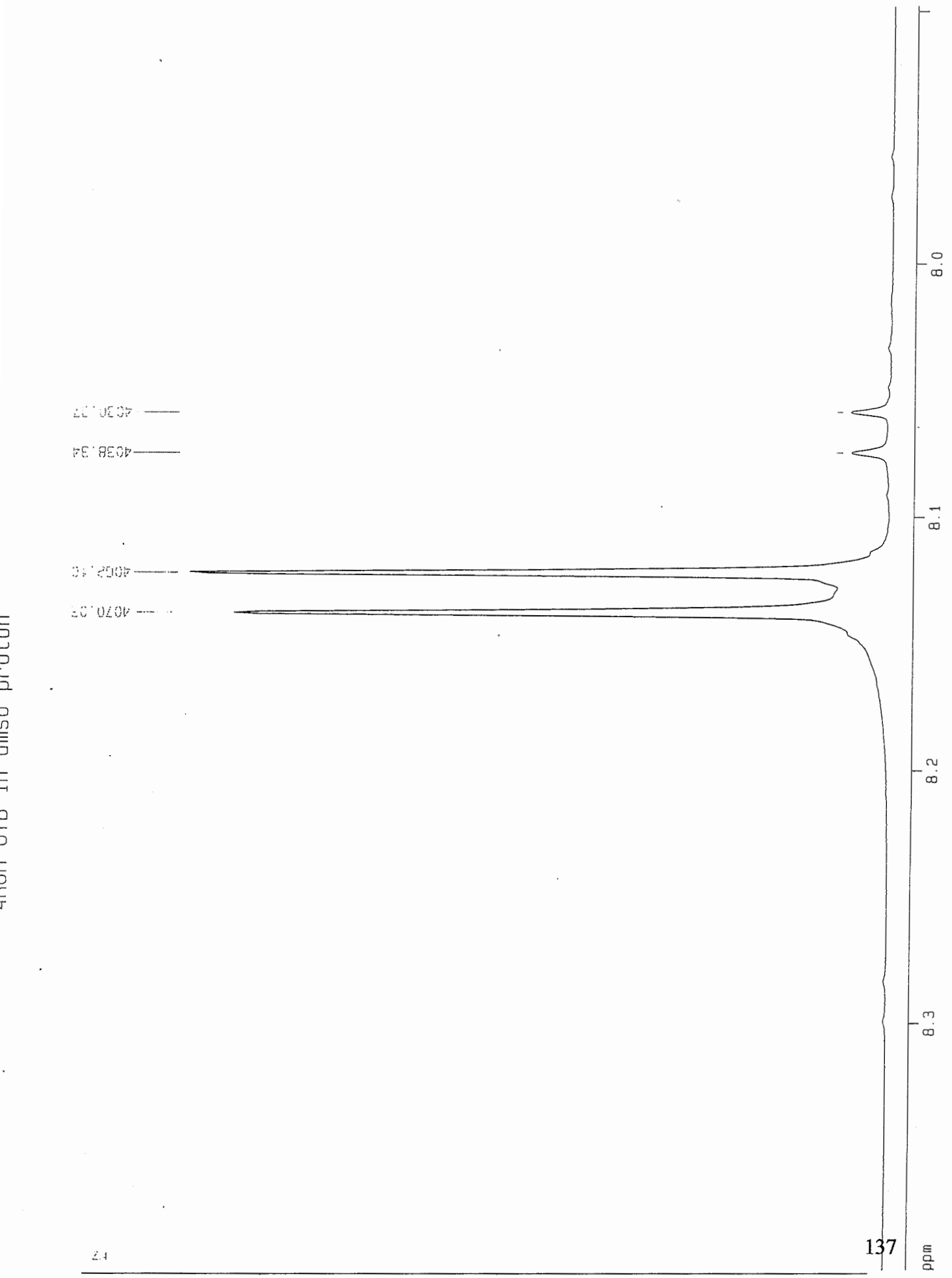

1.3
1.4
1.5
1.6
ppm

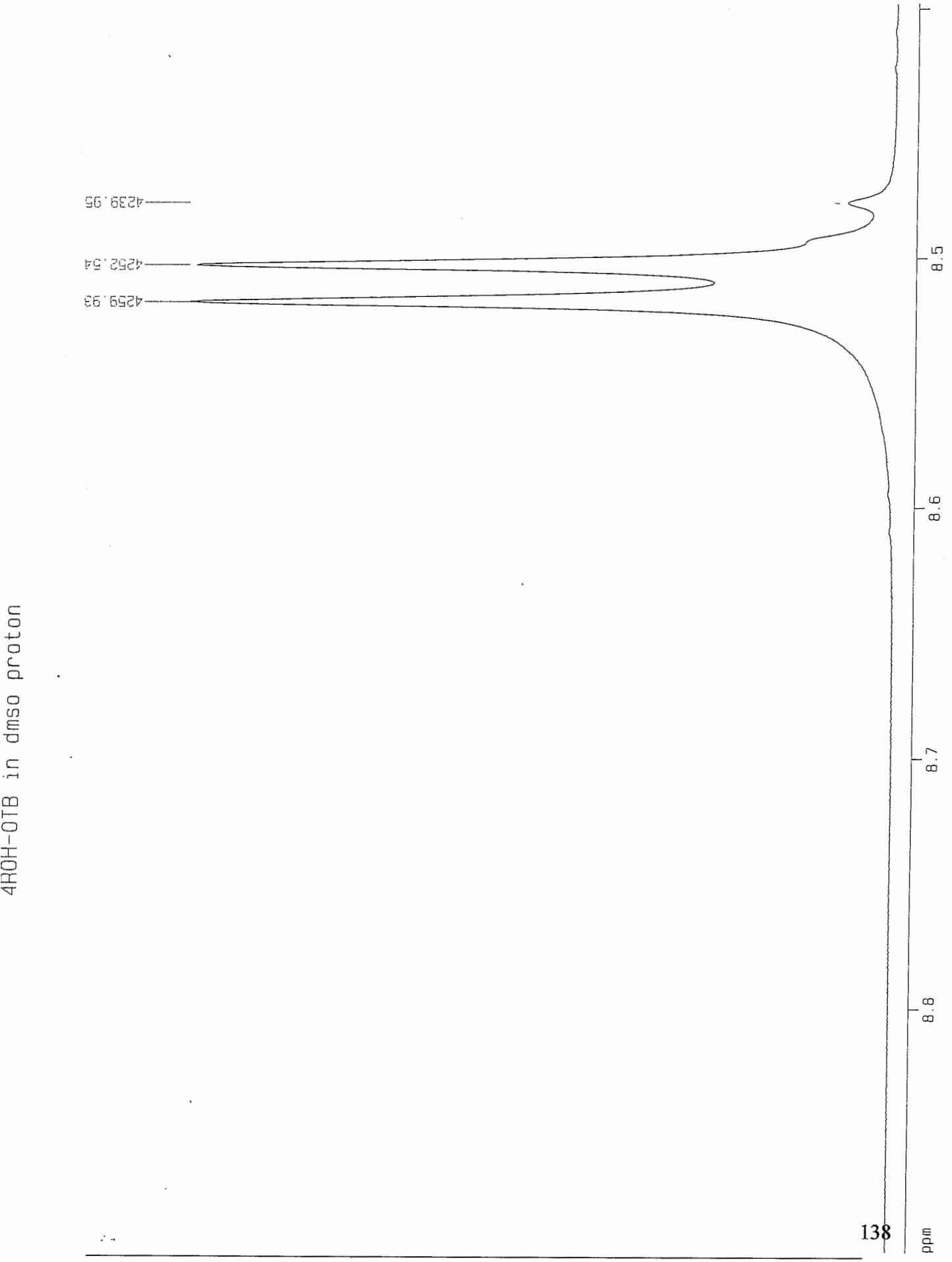

1531.93
1539.87
1545.76
1553.72
1580.96
1606.53
1612.66
1700.72
1708.63
1711.04
3.1
3.2
3.3
3.4
ppm

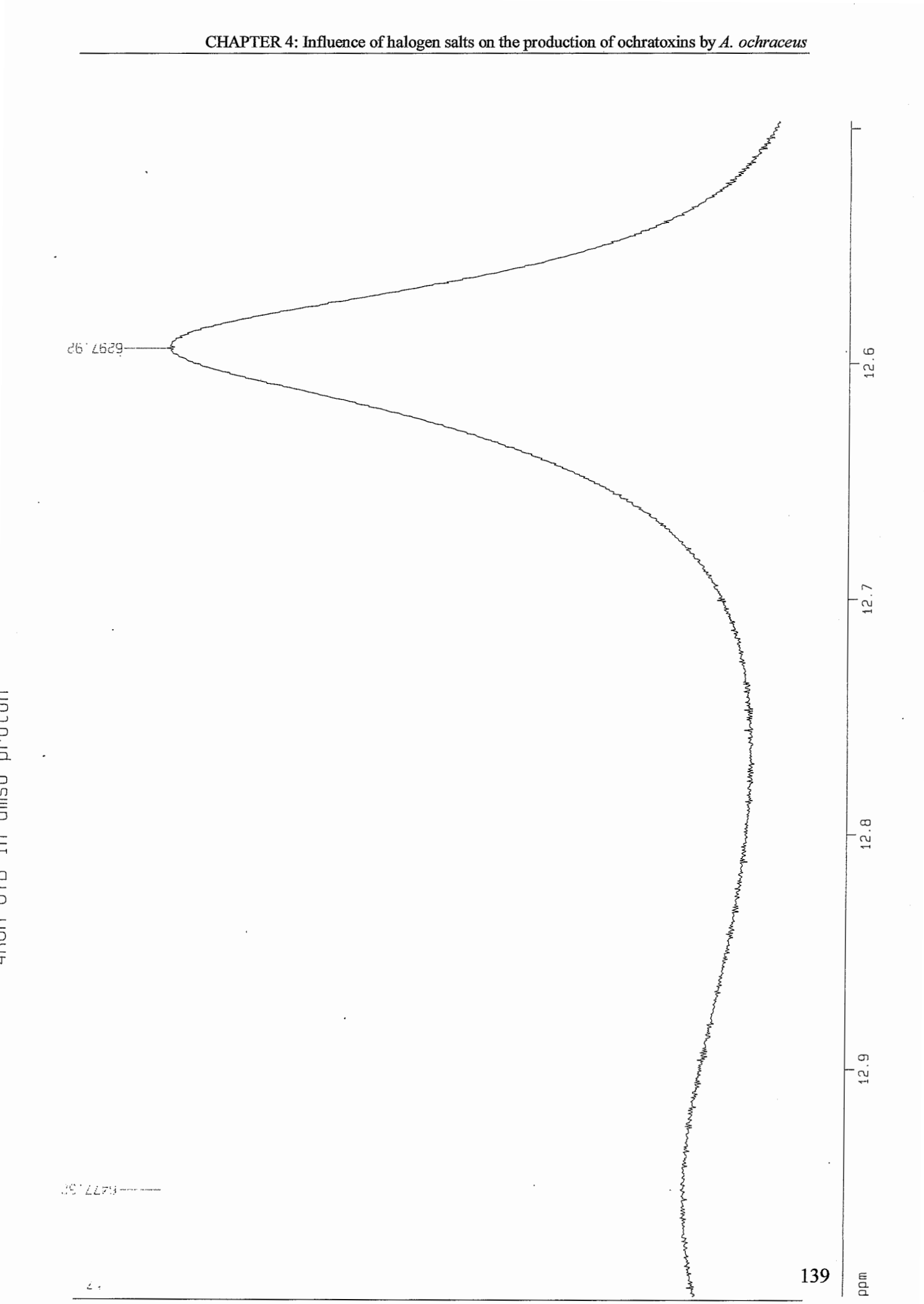

4ROH-OTB in dmso proton
2276.16
2280.07
2385.87
2961.01
2363.57
2366.56
2372.18
2376.49
2373.30
2385.49
2389.85
2302.04
2396.41
2398.58
2402.89
2405.00
4.5
4.6
4.7
4.8
ppm


5.7
5.8
5.9
6.0
6.1
ppm


3634.77
3542.70
3509.15
3500.53
3609.42
3610.93
3626.57
3534.07
3641.42
7.0
7.1
7.2
7.3
ppm


4070.03
4062.40
4038.34
4030.07
8.0
8.1
8.2
8.3
ppm


4239.95
4262.54
4259.93
8.5
8.6
8.7
8.8
ppm
4ROH-OTB in dmso proton


6297.92
12.6
12.7
12.8
12.9
ppm