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Synthesis, identification and *in vitro* biological evaluation some novel 5-imidazopyrazole incorporated pyrazoline and isoxazole derivatives

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Supporting information

1. Biological evaluation

1.1. *In vitro* antimicrobial assay

The *in vitro* antimicrobial activity of newly synthesized compounds was carried out by broth microdilution method. Mueller – Hinton broth was used as nutrient medium to grow and dilute the compound suspension for the test bacteria. 2% DMSO in water was used as the diluent to get desired concentration of compounds to test upon standard bacterial strains. Sabouraud Dextrose broth was used for fungal nutrition. Inoculum size for test strain was adjusted to 10^8 CFU mL^{-1} by comparing the turbidity. Serial dilutions were prepared in primary and secondary screening. Each synthesized compound and the standard drugs were diluted obtaining 2000 µg/mL concentration as a stock solution. The drugs which were found to be active in primary screening (i.e. 500, 250 and 200 µg/mL concentrations) were further screened in their second set of dilution at 100, 50, 25 and 12.5 µg/mL concentration against all microorganisms. 10 µL suspensions were further inoculated on appropriate media and growth was noted after 24 and 48 hrs. The control tube containing no antibiotic was instantaneously subcultured by spreading a consistently over an area of plate of medium fitting for the growth of the test organism. The tubes were then put for incubation at 37°C overnight. The maximum dilution preventing appearance of turbidity after spot subculture was considered as minimal inhibitory concentration (MIC, µ/L). All the tubes showing no visible growth (same as control tube) were subcultured and incubated overnight at 37 °C. The amount of growth from the control tube before incubation was compared. The outcomes are summarized in **Table 1**. In this study Ampicillin, Chloramphenicol and Norfloxacin were used as the standard antibacterial drugs while Nystatin and Griseofulvin were used as standard antifungal drugs.

1.2. *In vitro* antituberculosis assay

The antitubercular activity of pyrazoline **6a-l** / isoxazole **7a-l** derivatives against *Mycobacterium tuberculosis* H37Rv was performed by Lowensteine-Jensen method [1] with minor modification where 250 µg/mL and 100 µg/mL dilution of each compound was added to Lowensteine-Jensen medium and then media was uncontaminated by inspissations method. A culture of *Mycobacterium tuberculosis* H37Rv growing on Lowensteine-Jensen medium was harvested in 0.85% saline in bijou bottle. The stock solutions of the title compounds were prepared in DMSO i.e. 250 µg/mL and 100 µg/mL. These tubes were then incubated at 37 °C for 1 day followed by

streaking of *Mycobacterium tuberculosis* H37Rv (5×10^{-4} bacilli per tube). The growth of bacilli was seen after two weeks, three weeks and finally after four weeks of incubation. The tubes having the compounds were compared with control tubes where medium alone was incubated with *Mycobacterium tuberculosis* H37Rv. The concentration at which complete inhibition of colonies occurred was taken as active concentration of the tested compound. The standard drugs isoniazid and rifampicin were used for comparison purpose. The results are summarized in **Table 2**.

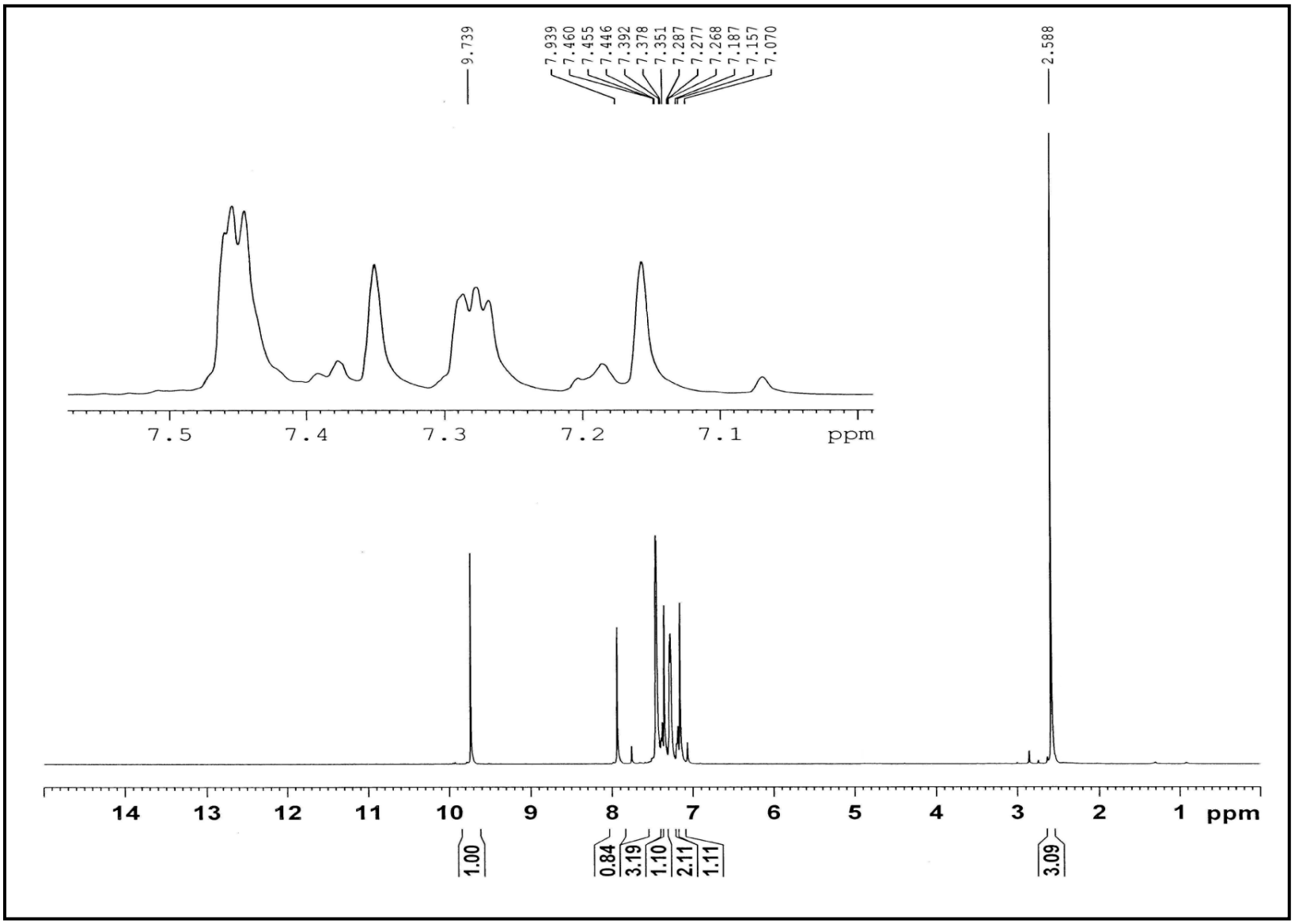
1.3. *In vitro* antimalarial assay

All the synthesized compounds were screened for their antimalarial activity against the *P. falciparum* strain. The *P. falciparum* strain was acquired from Shree R. B Shah Mahavir Super-speciality hospital, Surat, Gujarat, India. The *P. falciparum* strains were cultivated by a modified method described by Trager and Jensen [2]. Compounds were dissolved in DMSO. The final concentration of DMSO used was not toxic and did not interfere with the assay. The antiparasitic effect of the compounds was measured by growth inhibition percentage as described by Carvalho and Krettli [3]. For experimental purposes, the cultures were synchronized with 5% D-sorbitol when the parasites were in the ring stage [4]. The parasite suspension, consisting of predominately the ring stage, was adjusted to a 1-2 % parasitaemia and 2.5 % haematocrit in hypoxanthine-free RPMI-1640 culture medium with 10% human plasma and was exposed to 7 concentrations of each compound for a single cycle of parasite growth of 48 h at 37 °C. A positive control with reference to antimalarial drugs in standard concentrations was used in each experiment. The stock solutions were additionally diluted in whole medium (RPMI 1640 plus 10% human serum) to each of the used concentrations. The concentration that inhibited 50% of parasite growth (IC_{50} value) was determined by interpolation using Microcal Origin software. The standard drugs chloroquine and quinine were used as the reference antimalarial agents, blood smears were read blind and each duplicate experiment was repeated thrice. The results are summarized in **Table 3**.

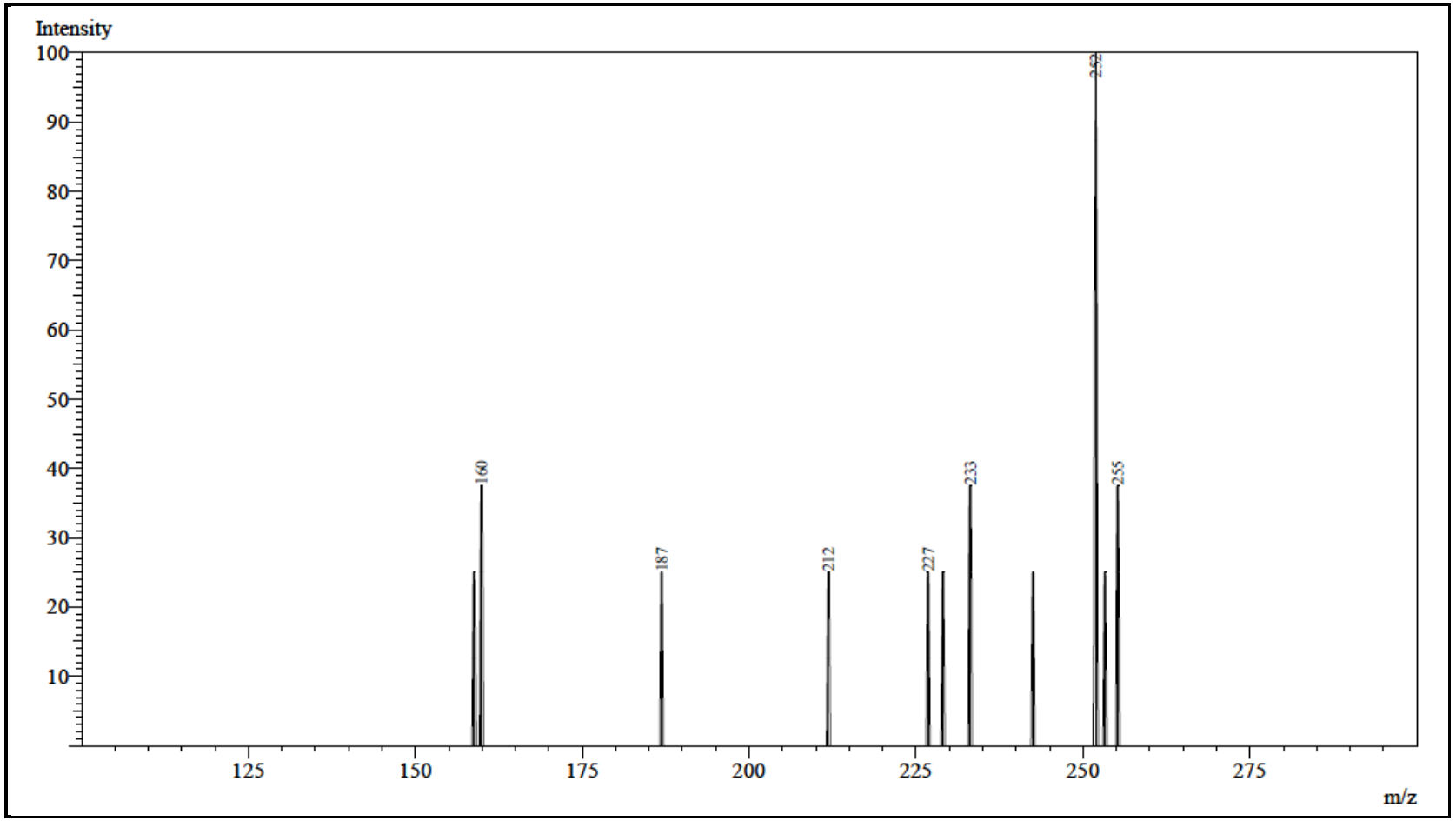
1.4. *In vitro* antioxidant assay

FRAP assay measure a reduction power of the compounds, converting ferric tripyridyl triazine (Fe (III)-TPTZ) complex into a blue colour ferrous tripyridyl triazine (Fe (II)-TPTZ) complex at 593 nm [5]. Fe(II)-TPTZ(2,4,6-tripyridyl-s-triazine) reagent was prepared by mixing a 10.0 mL TPTZ solution (0.155 gm TPTZ was dissolved in 100 mL 40 mM HCl), 10 mL $FeCl_3 \cdot 6H_2O$ solution (0.324 gm FeCl3 was dissolved in 100 mL distilled water) and 100 mL acetate buffer (0.187 gm sodium acetate and 1.6 mL acetic acid dissolved in double distilled water to make 100 mL) at pH 3.6. A mixture of 200.0 mL sample solution and 3 mL of Fe(II)TPTZ reagent was incubated at 37 ºC for 25 min. The absorbance of colour complex Fe(II)TPTZwas measured at 593 nm using ascorbic acid as the standard. The results were expressed as ascorbic equivalent (mmol/100 gm compound).

^{1}H-NMR Spectra of Compound 3a



Mass Spectra of Compound 3a



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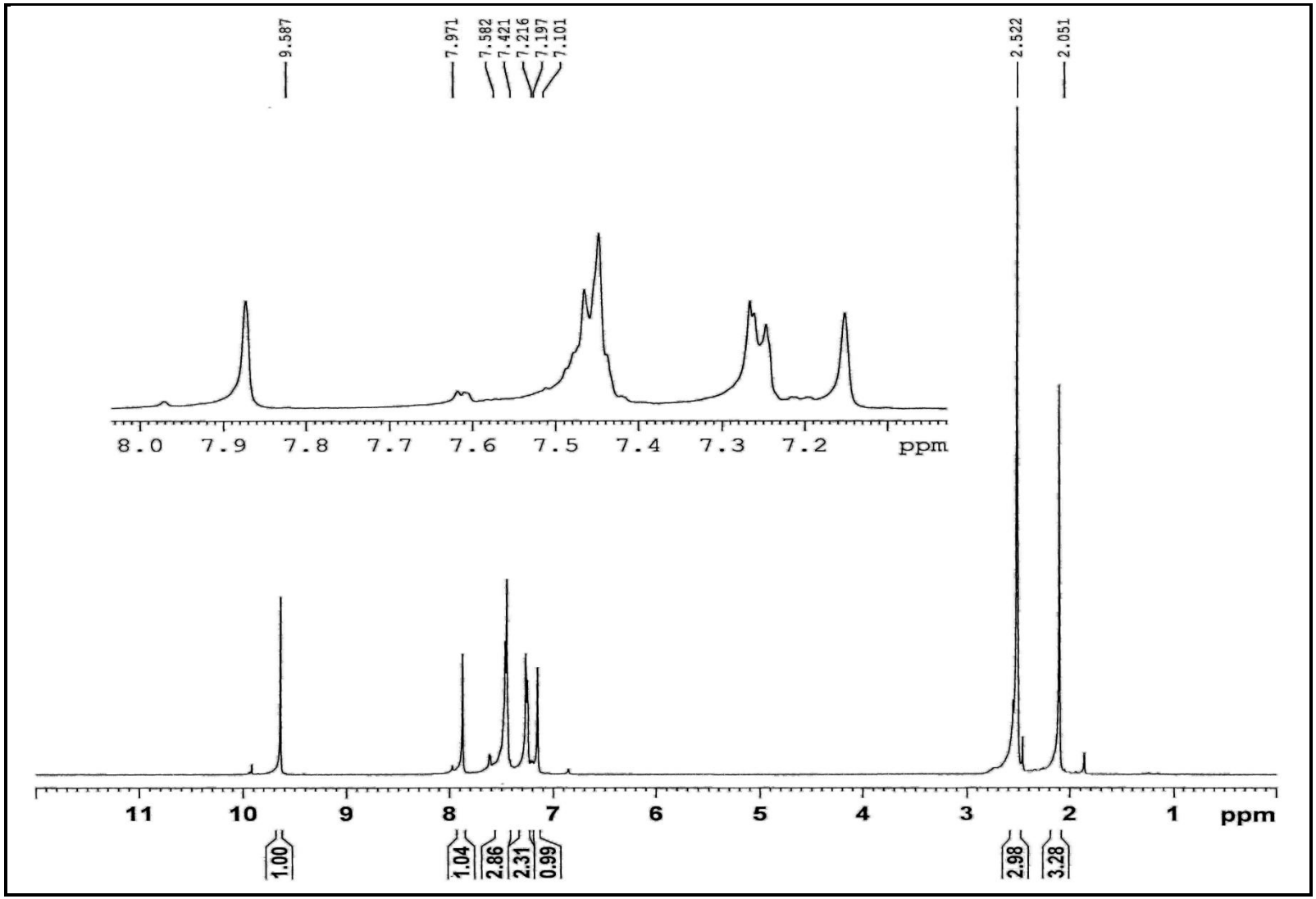

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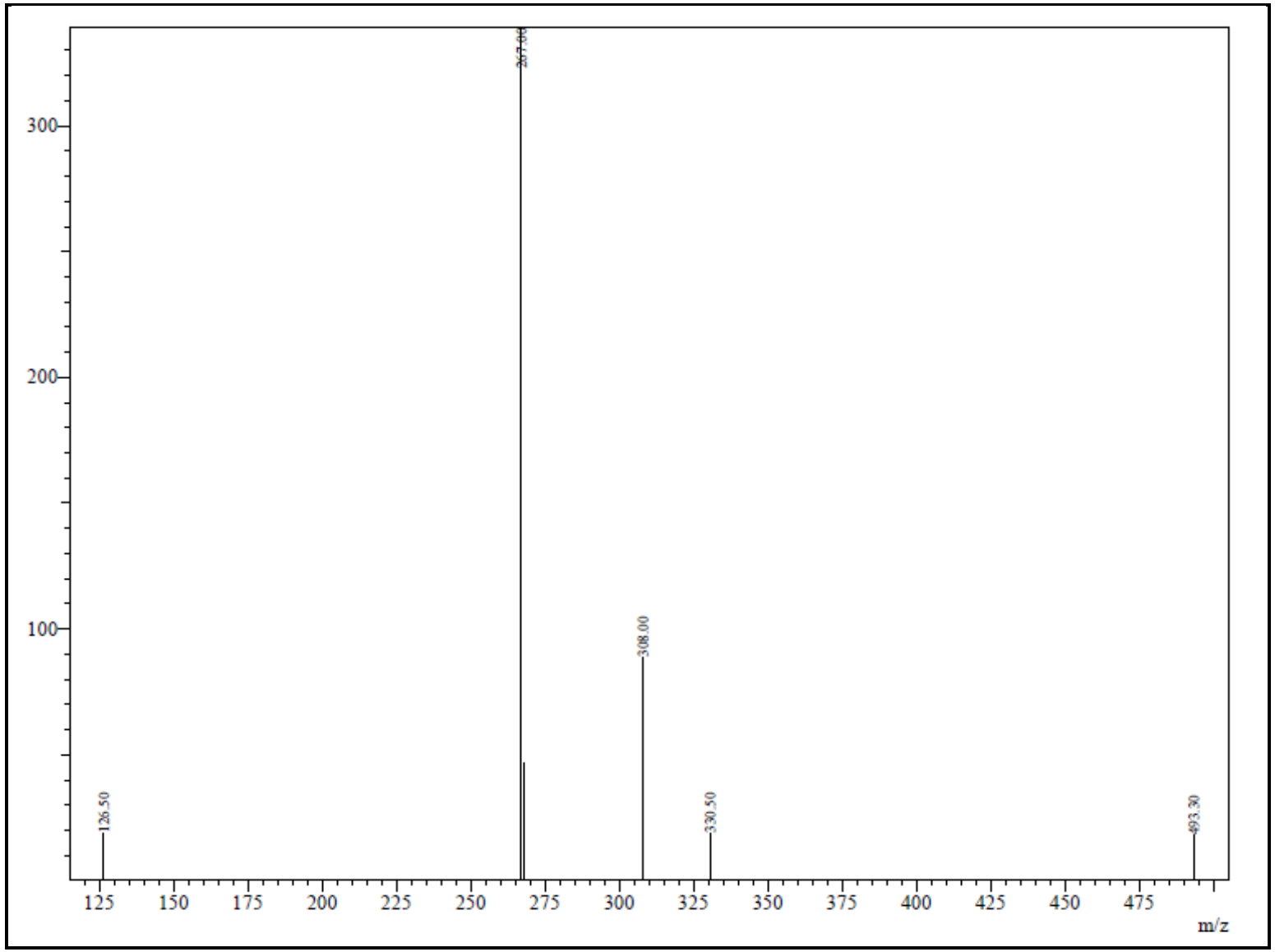

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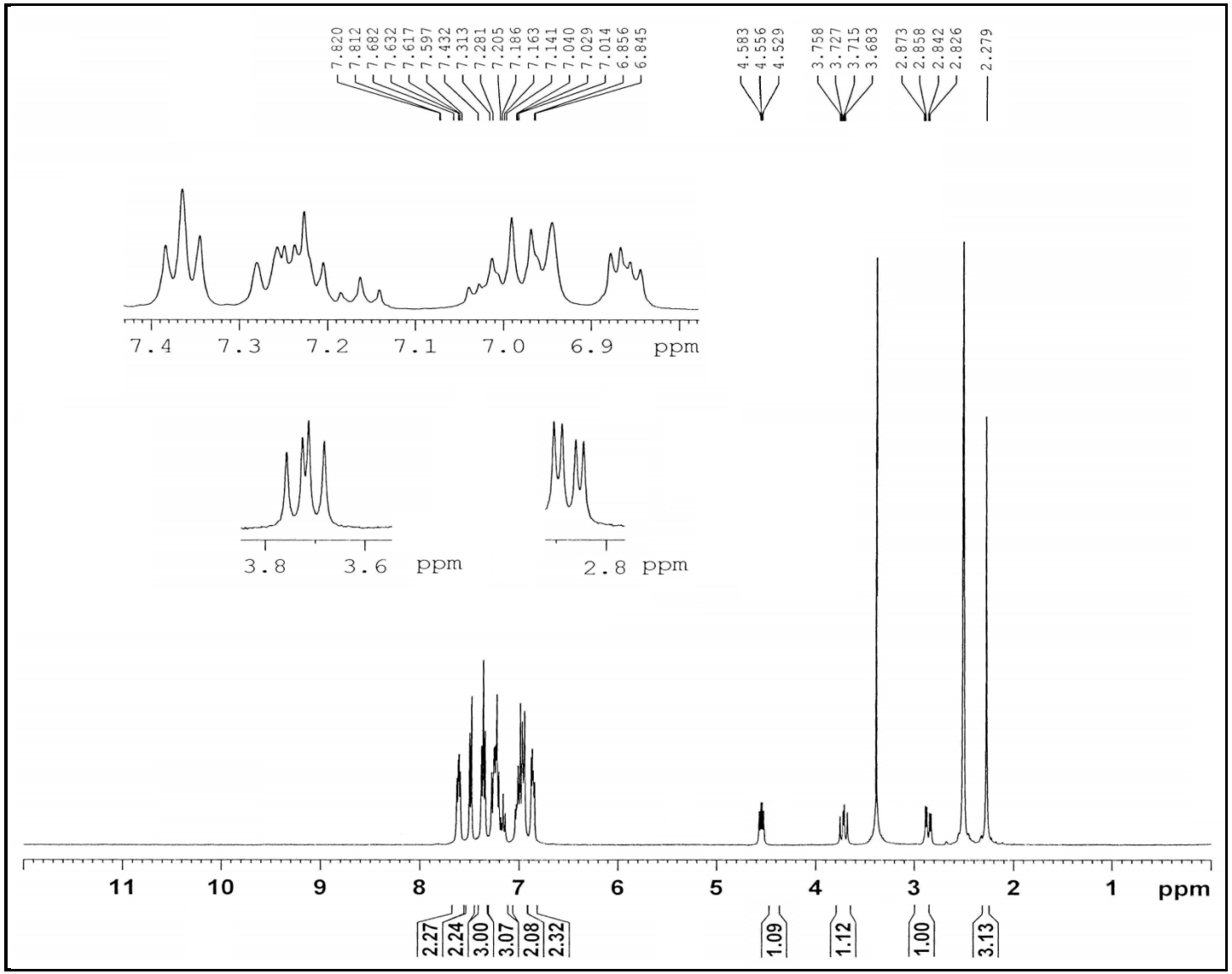

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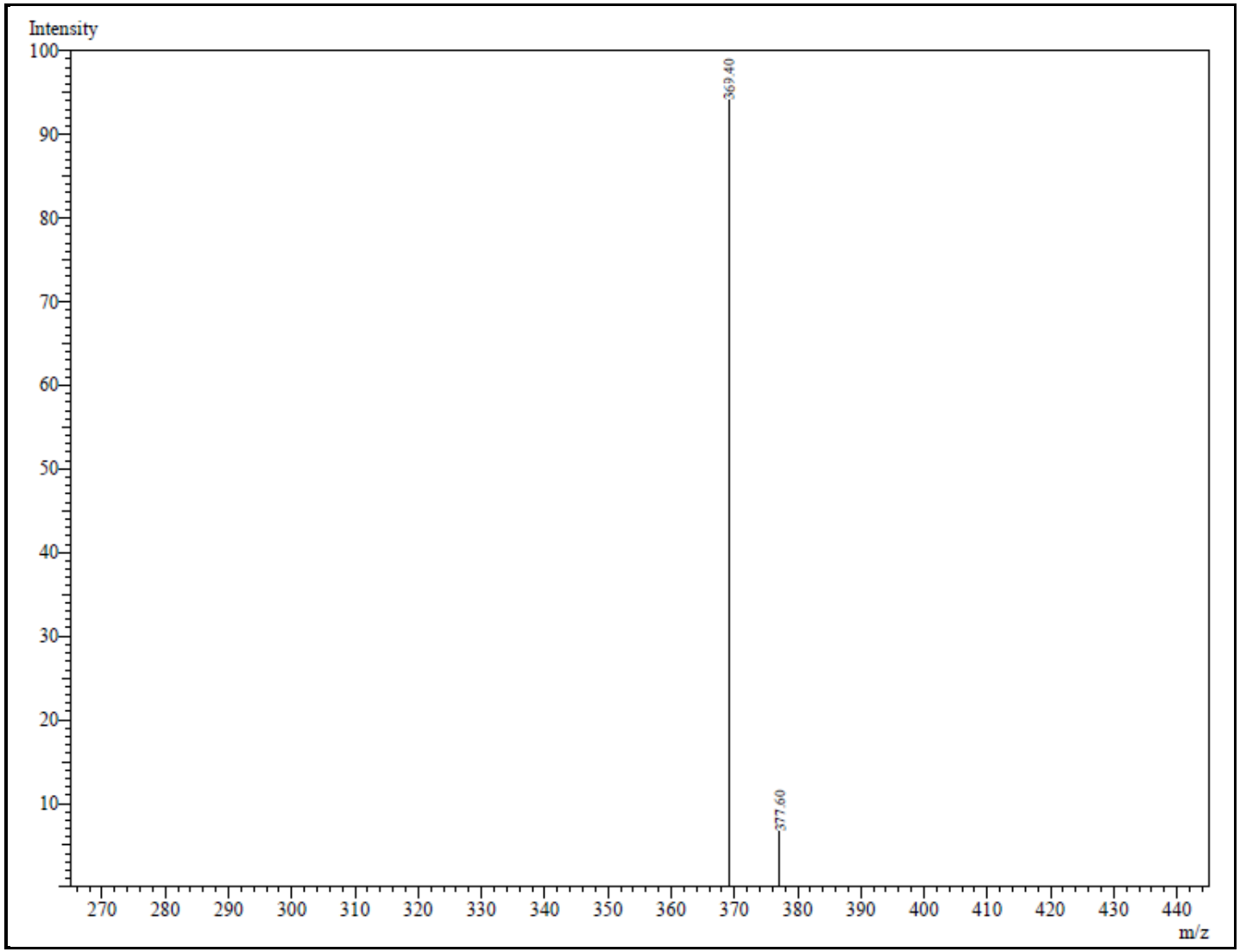

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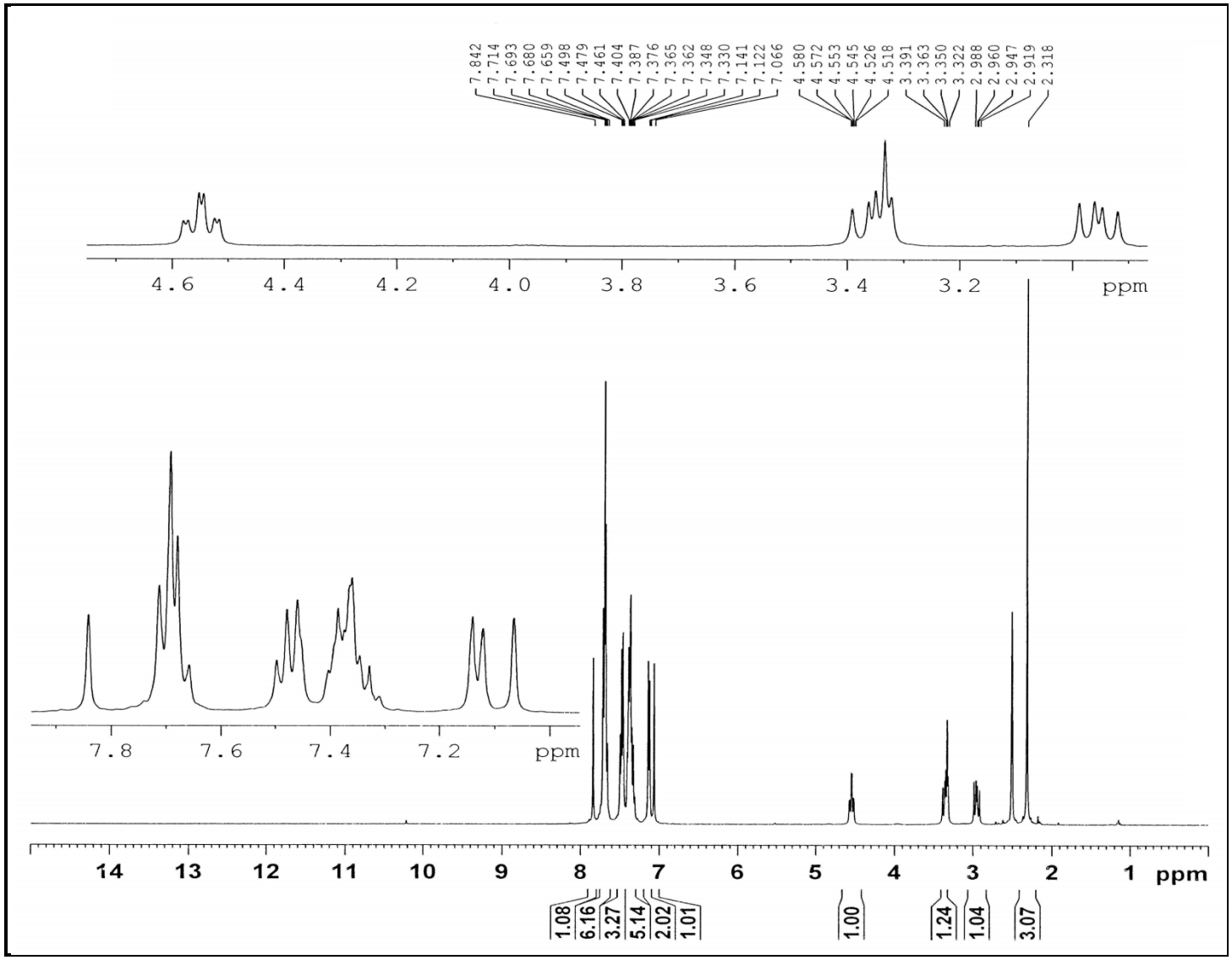

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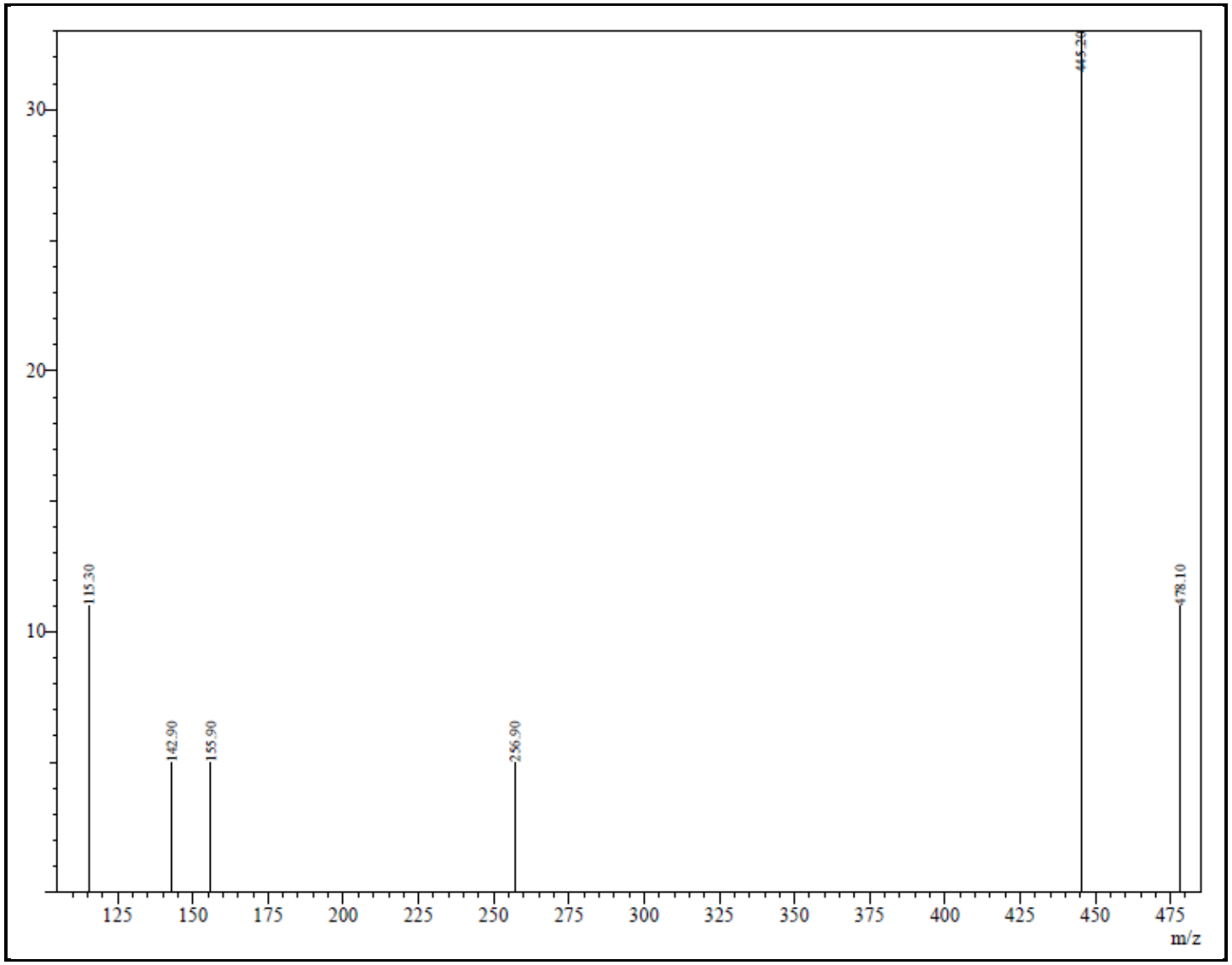

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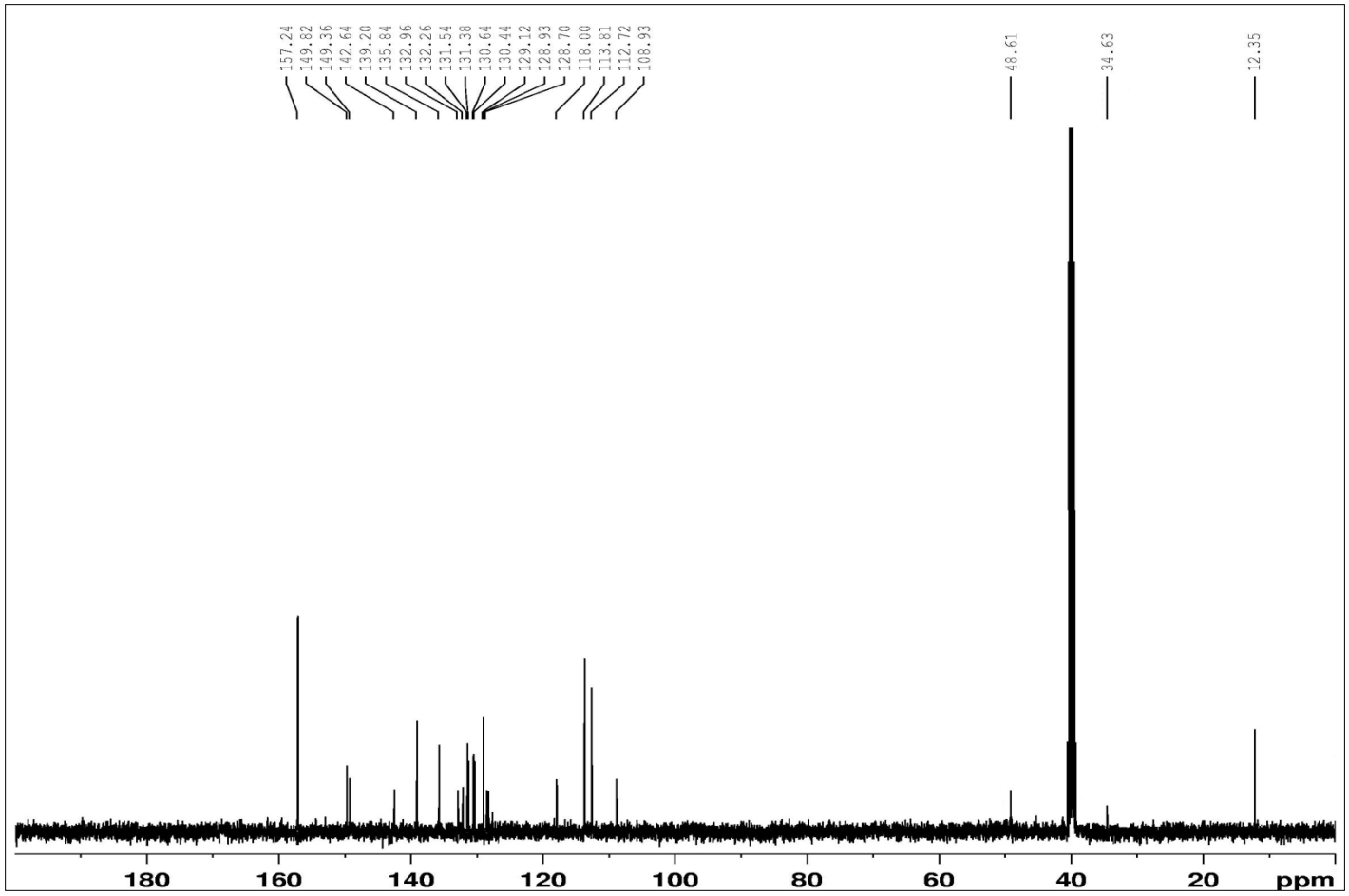

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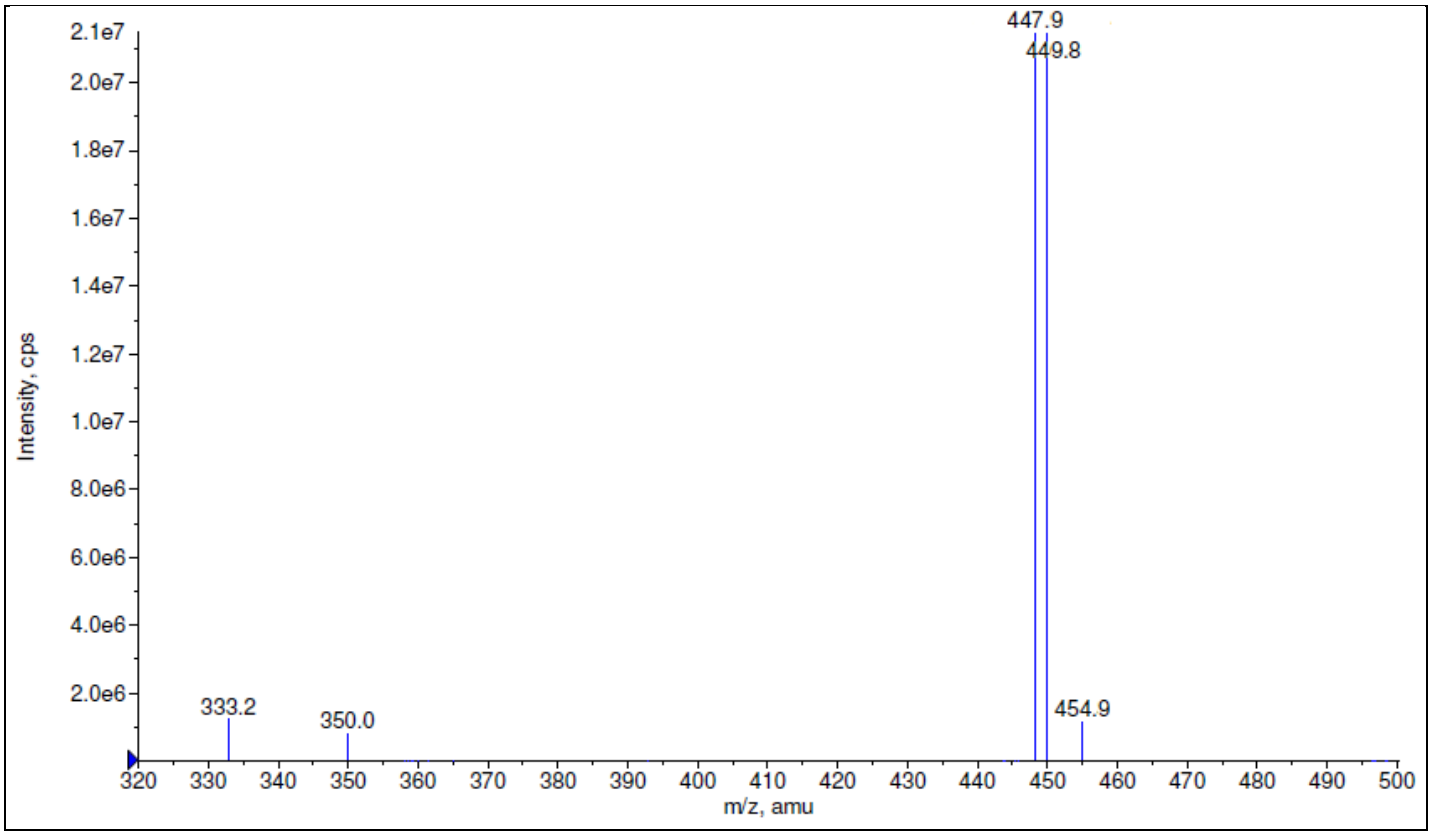

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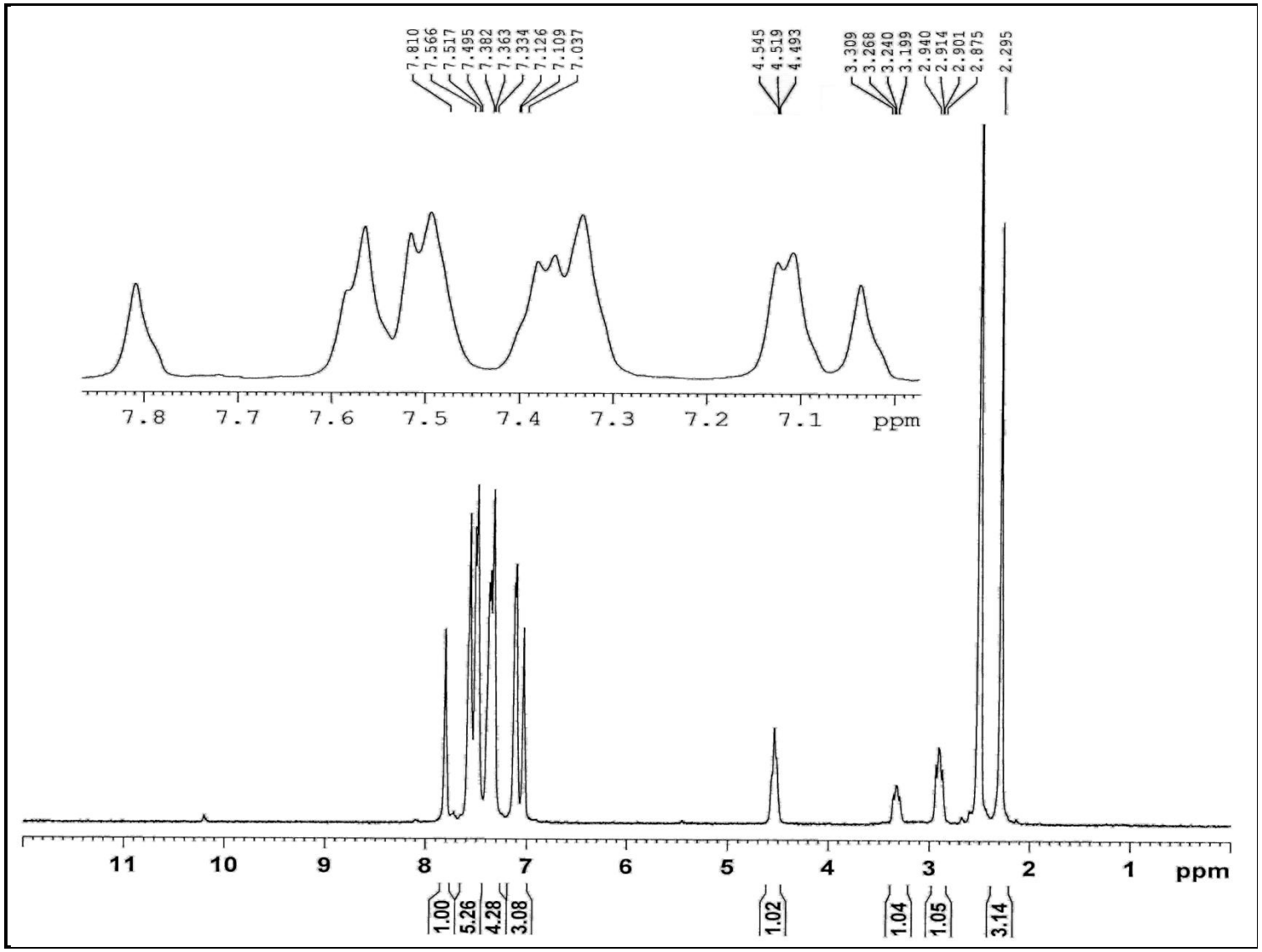

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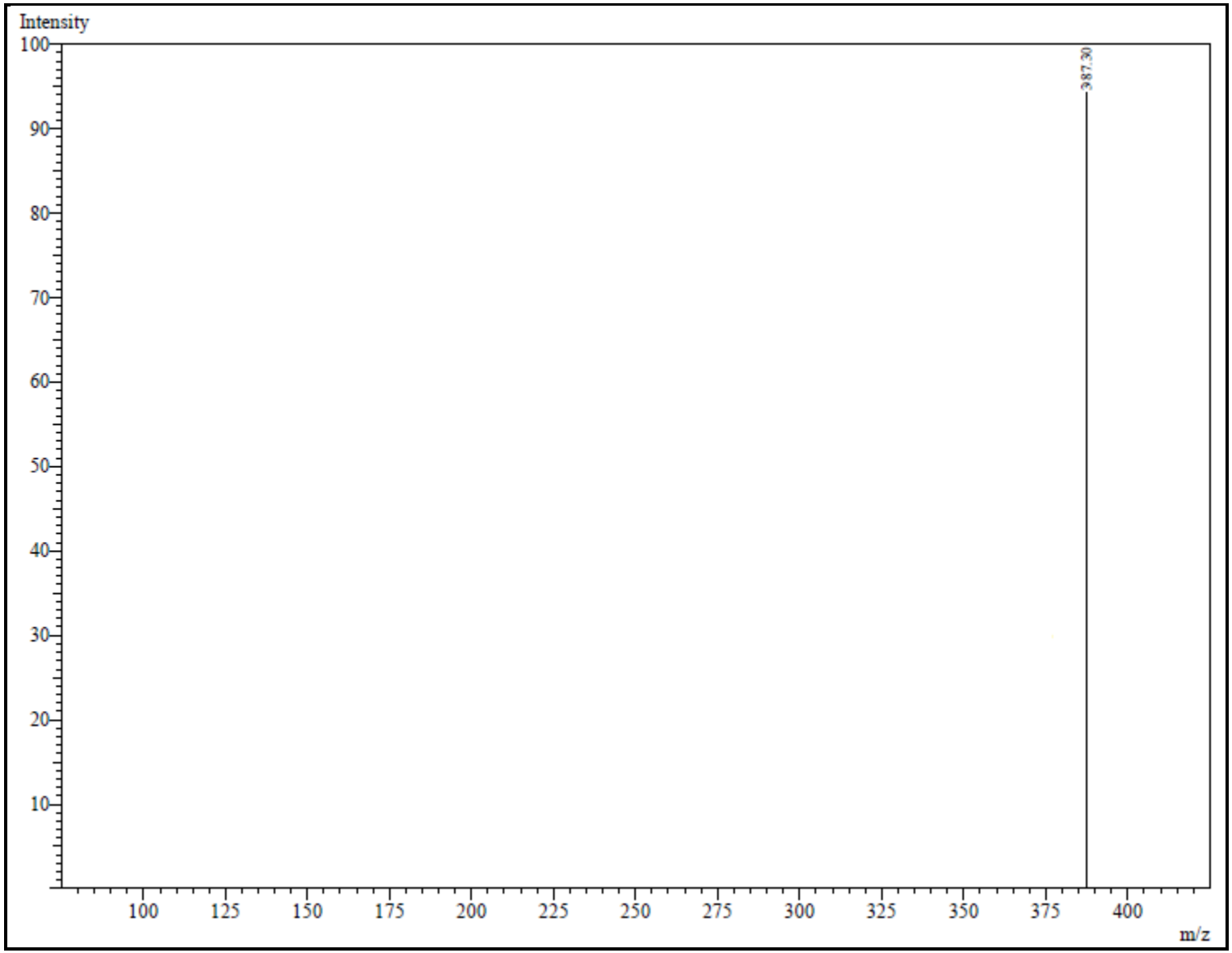

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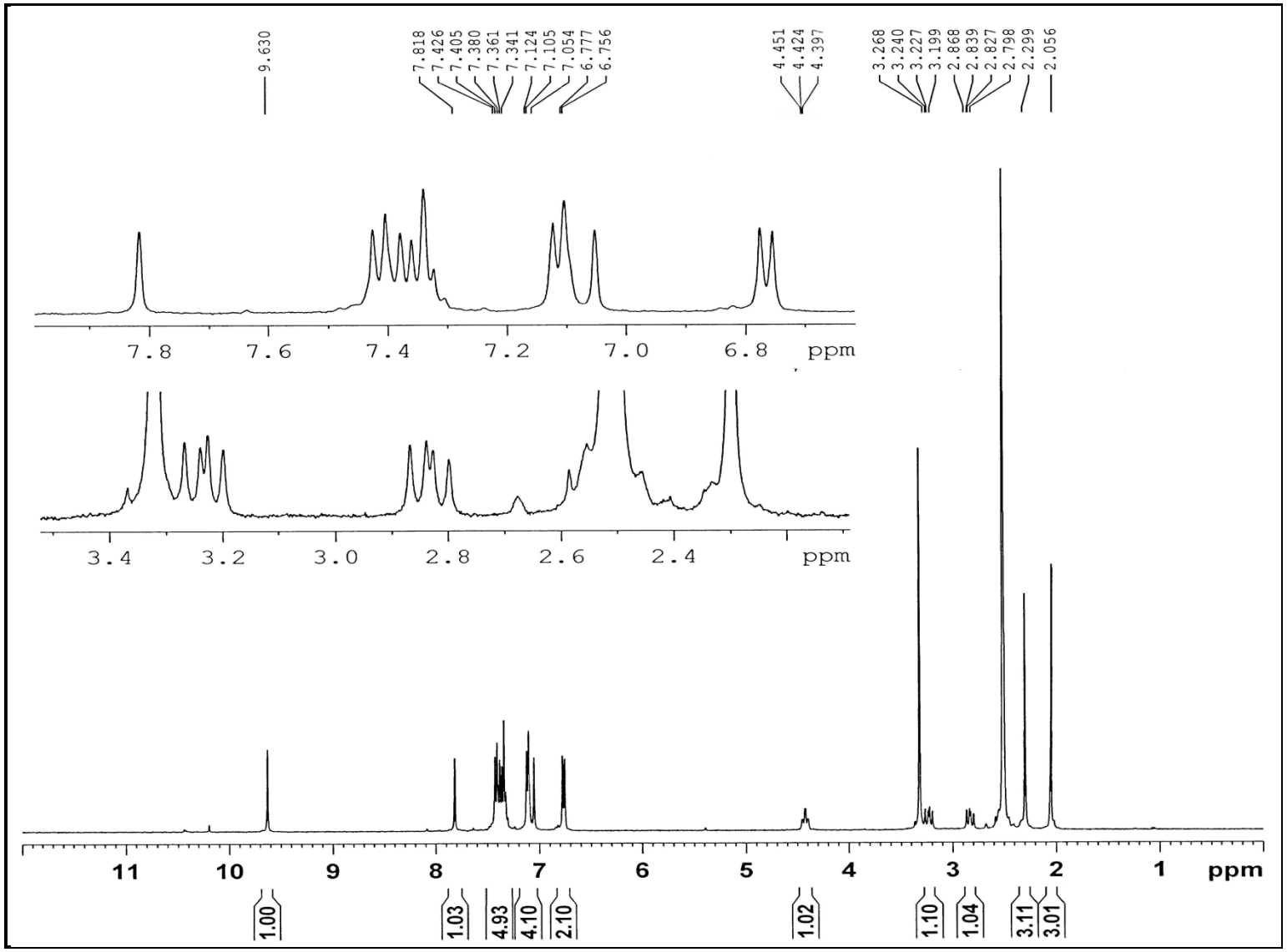

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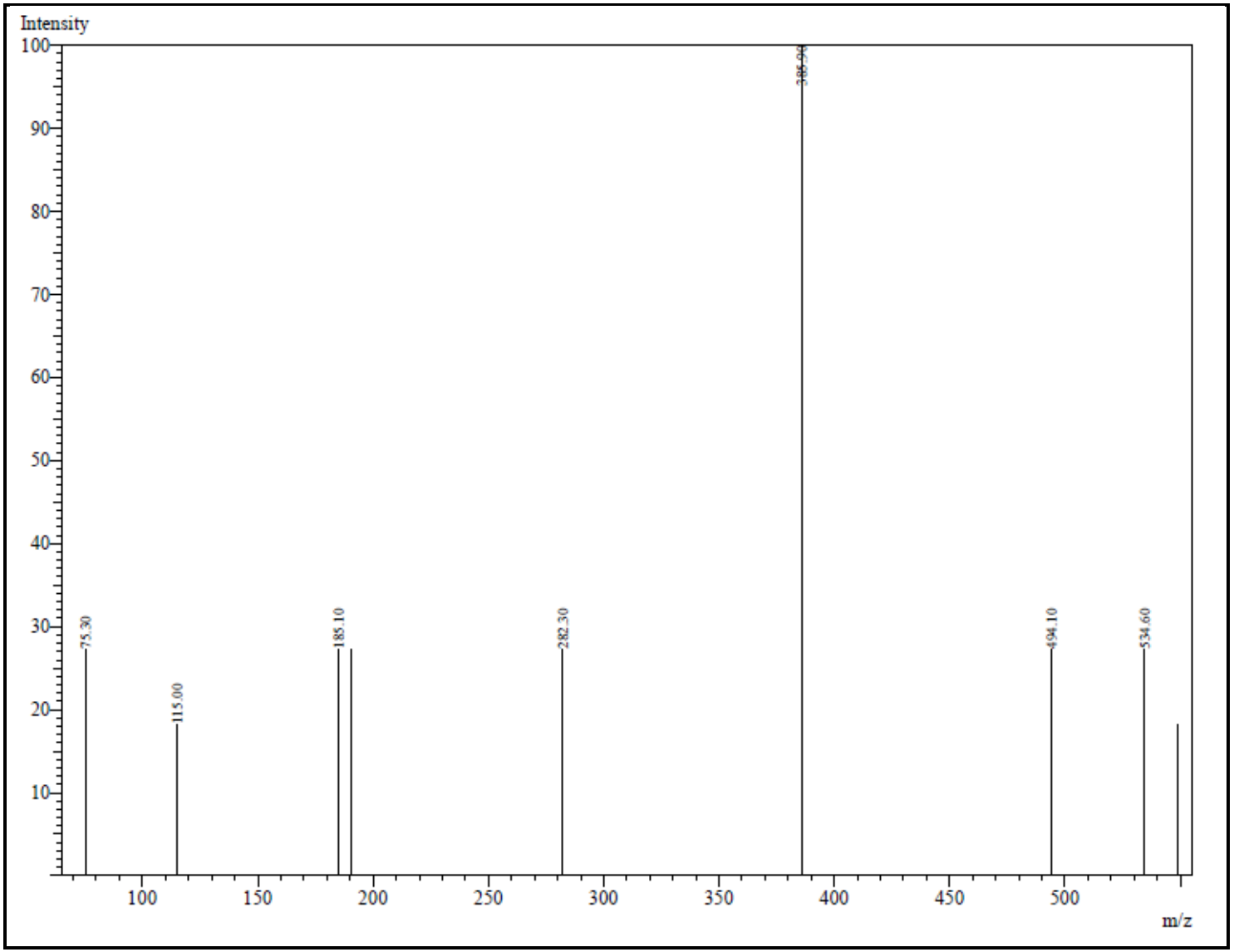

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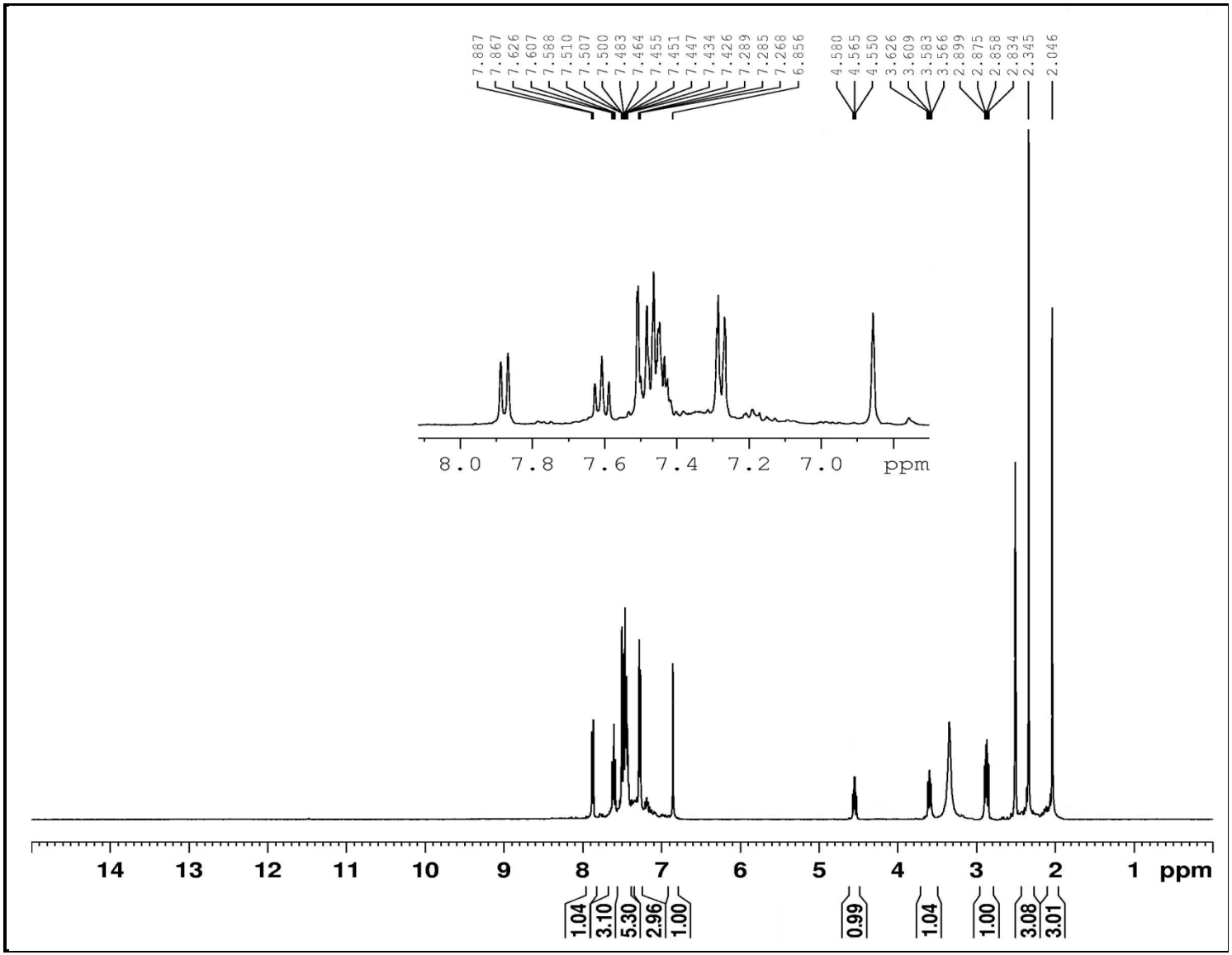

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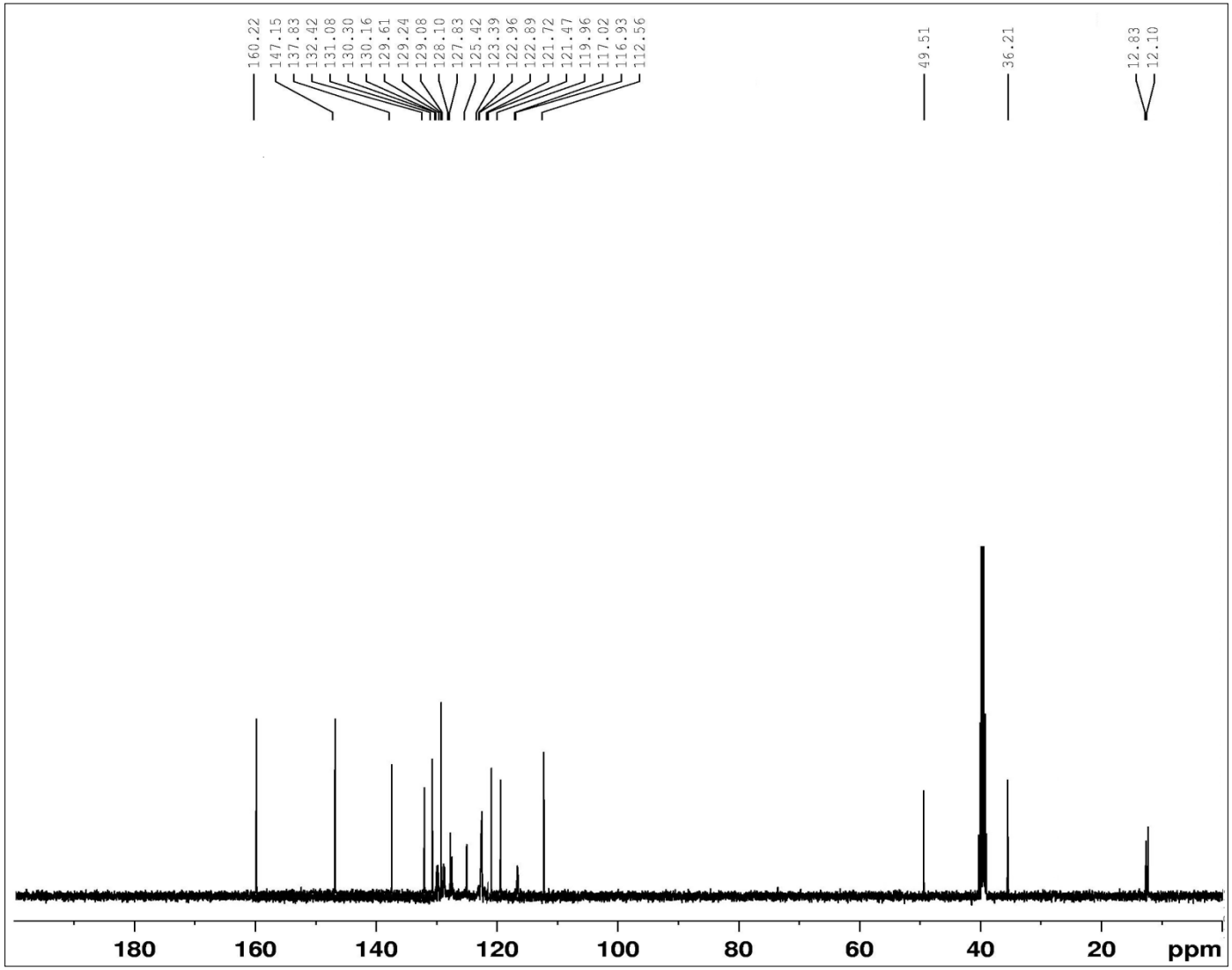

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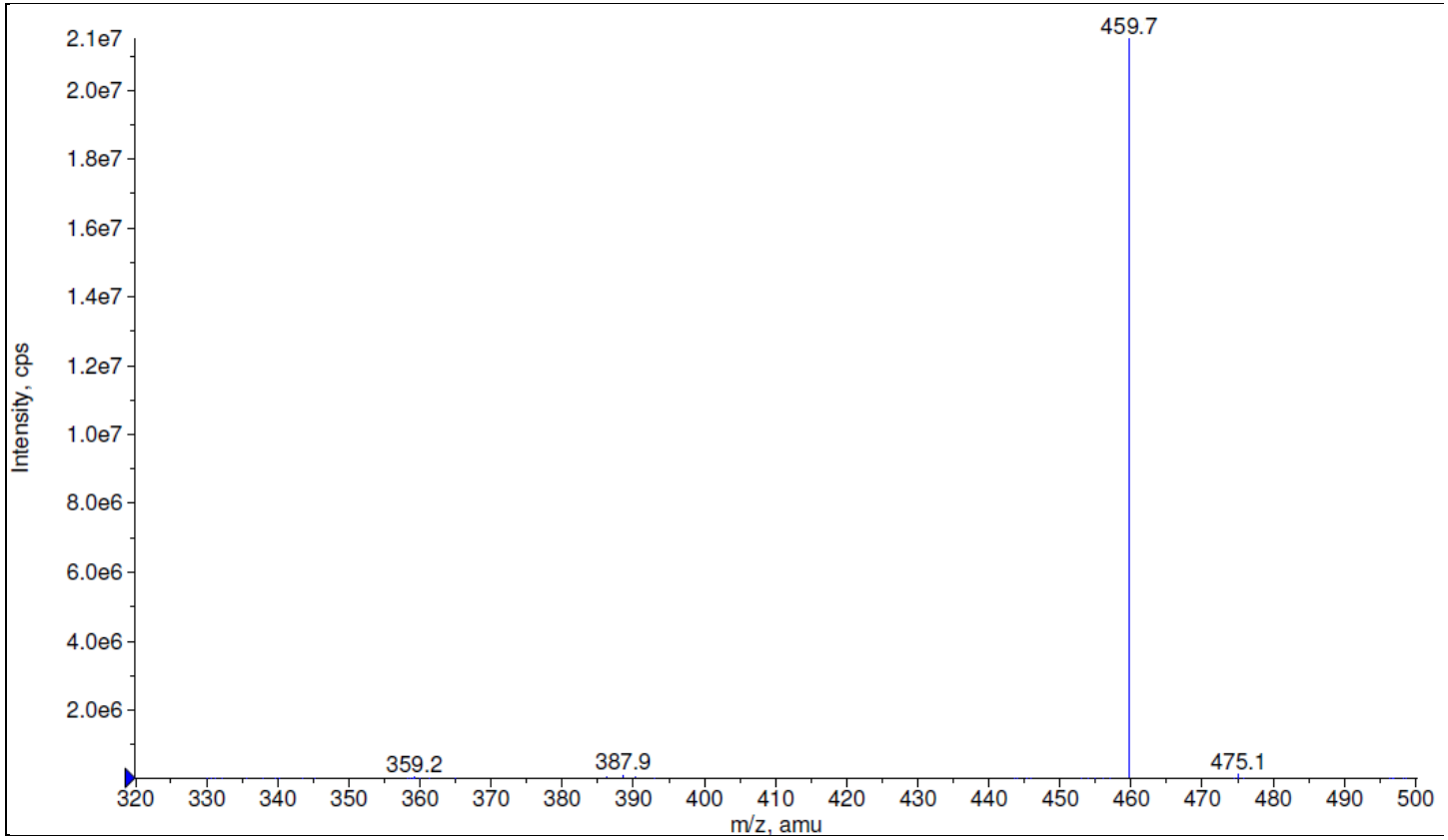

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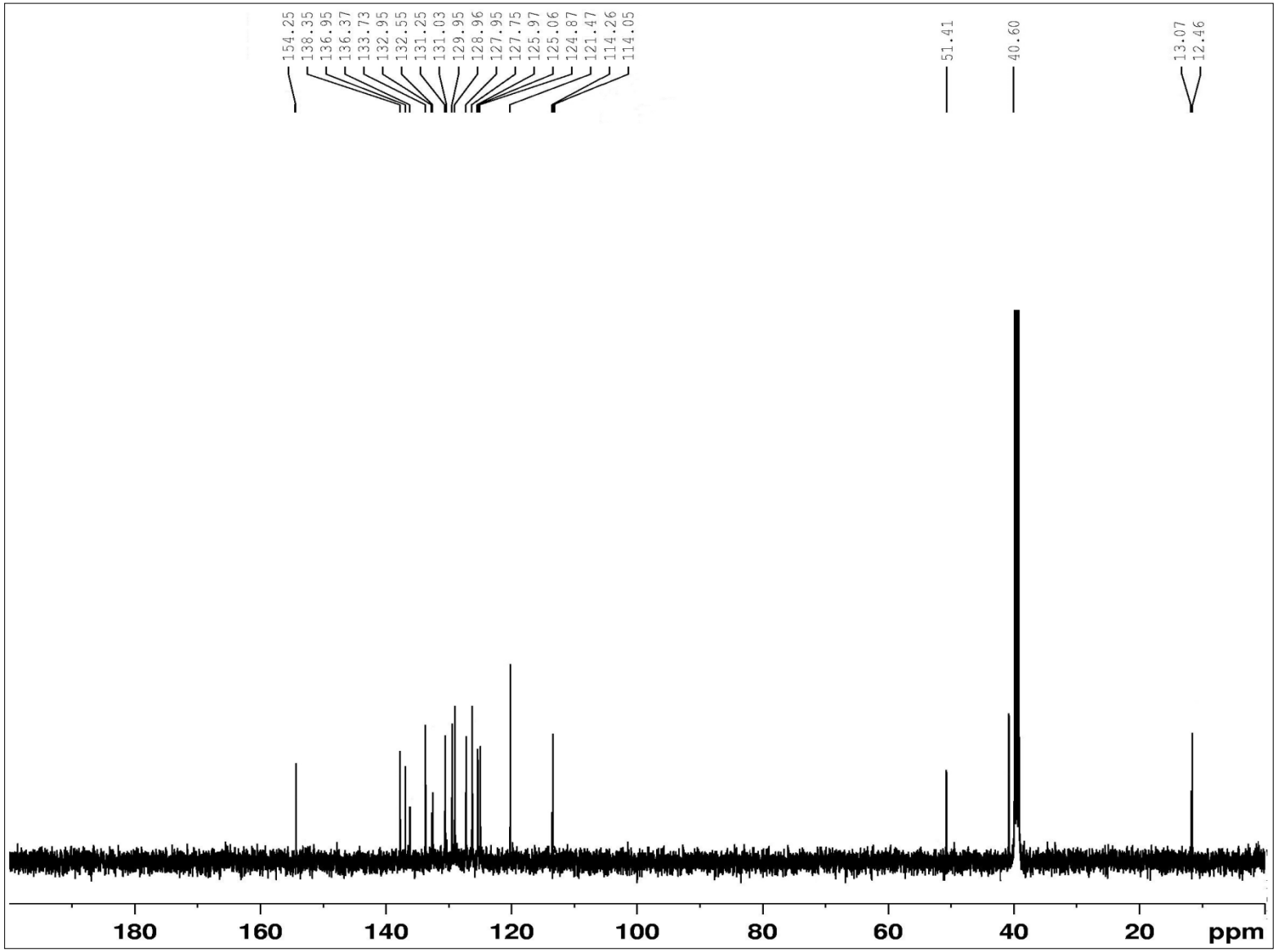

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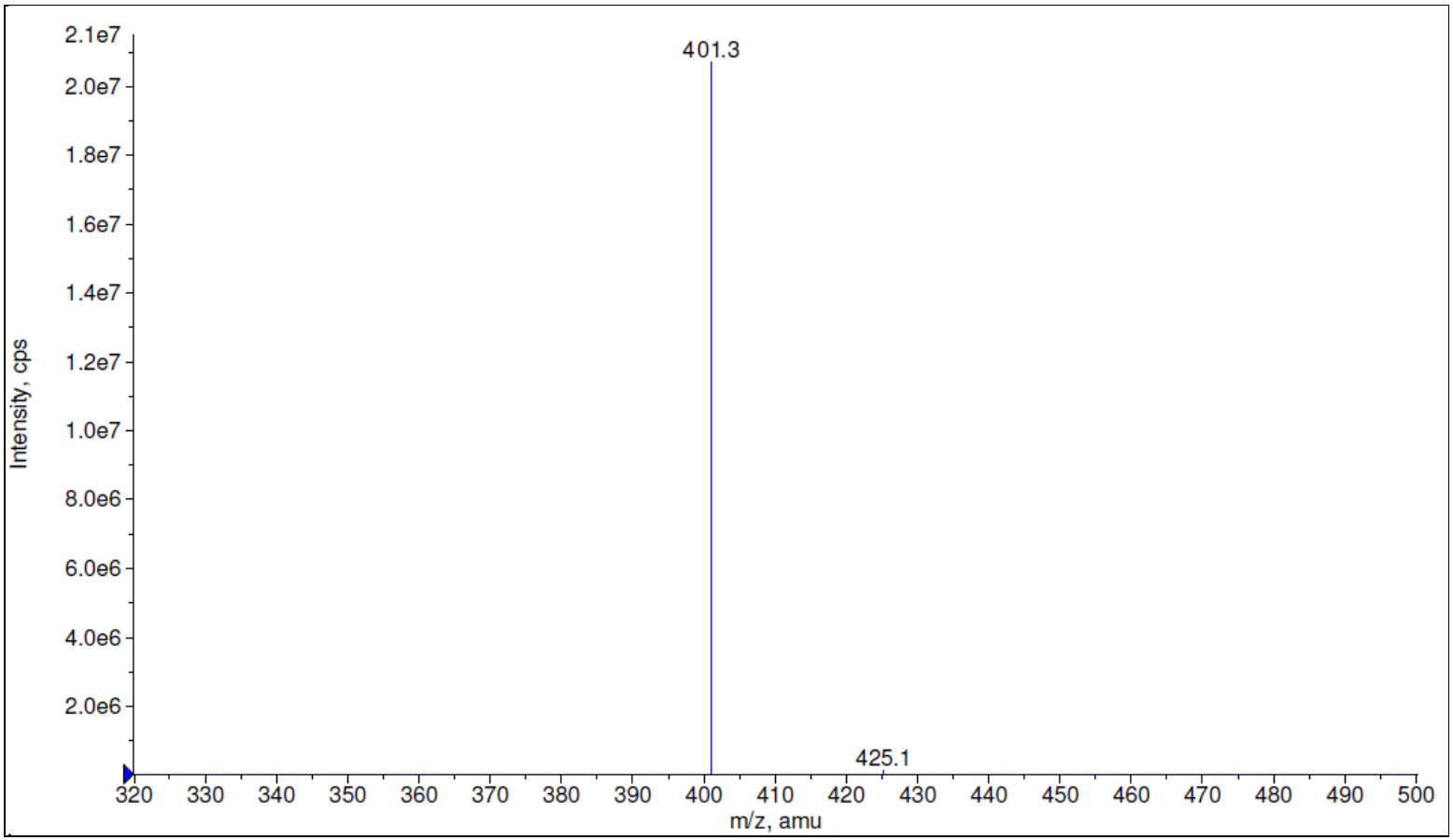

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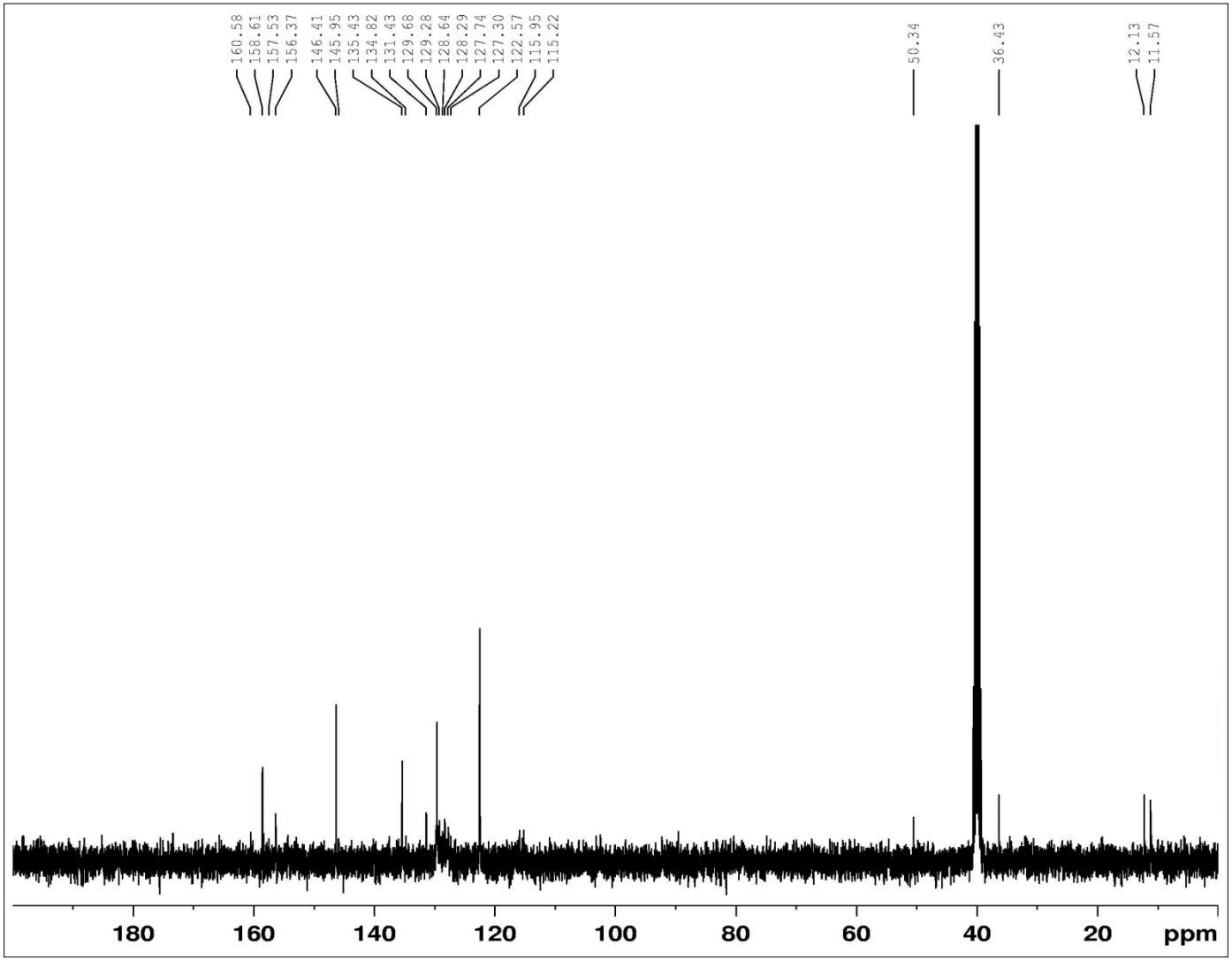
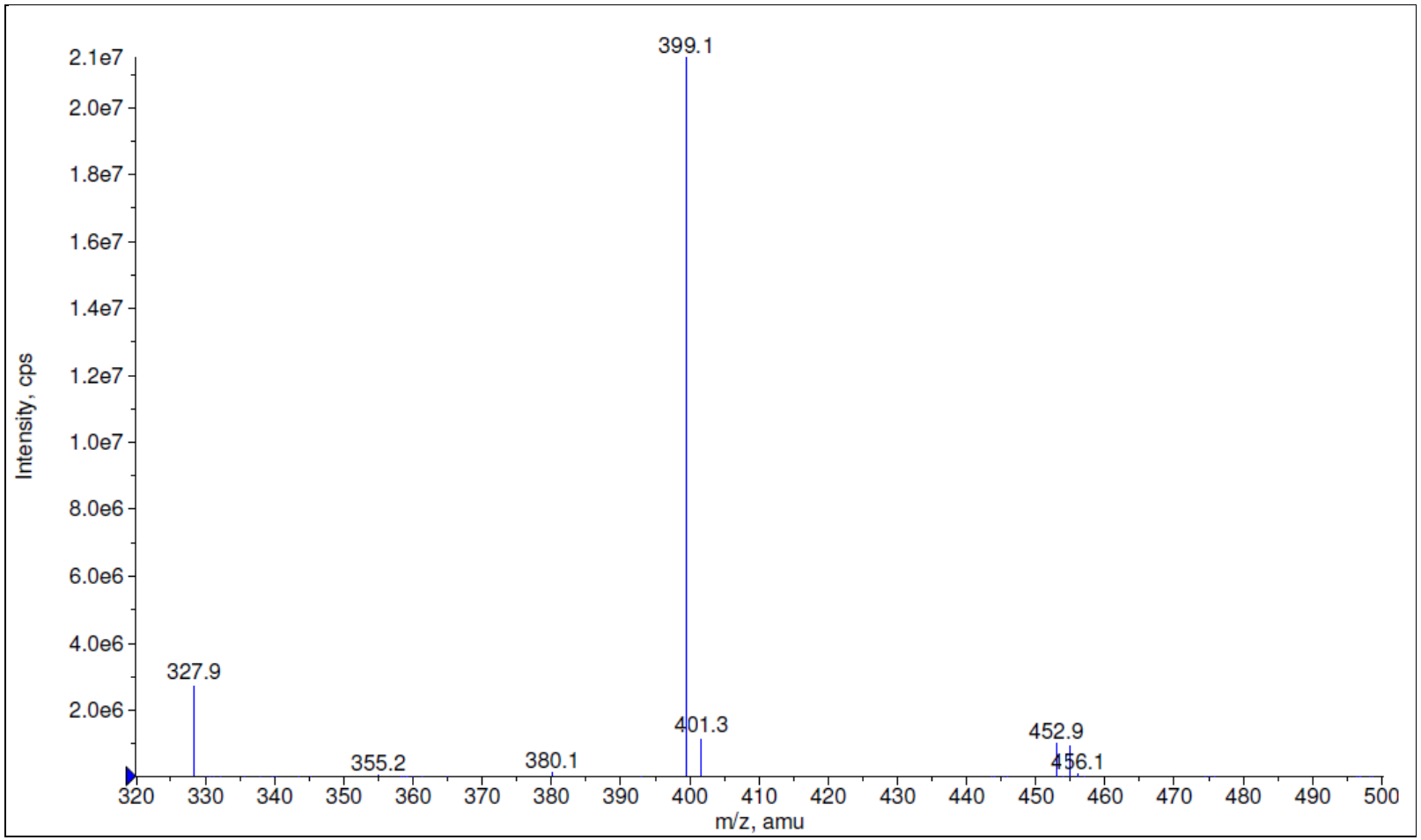


Mass Spectra of Compound 6l



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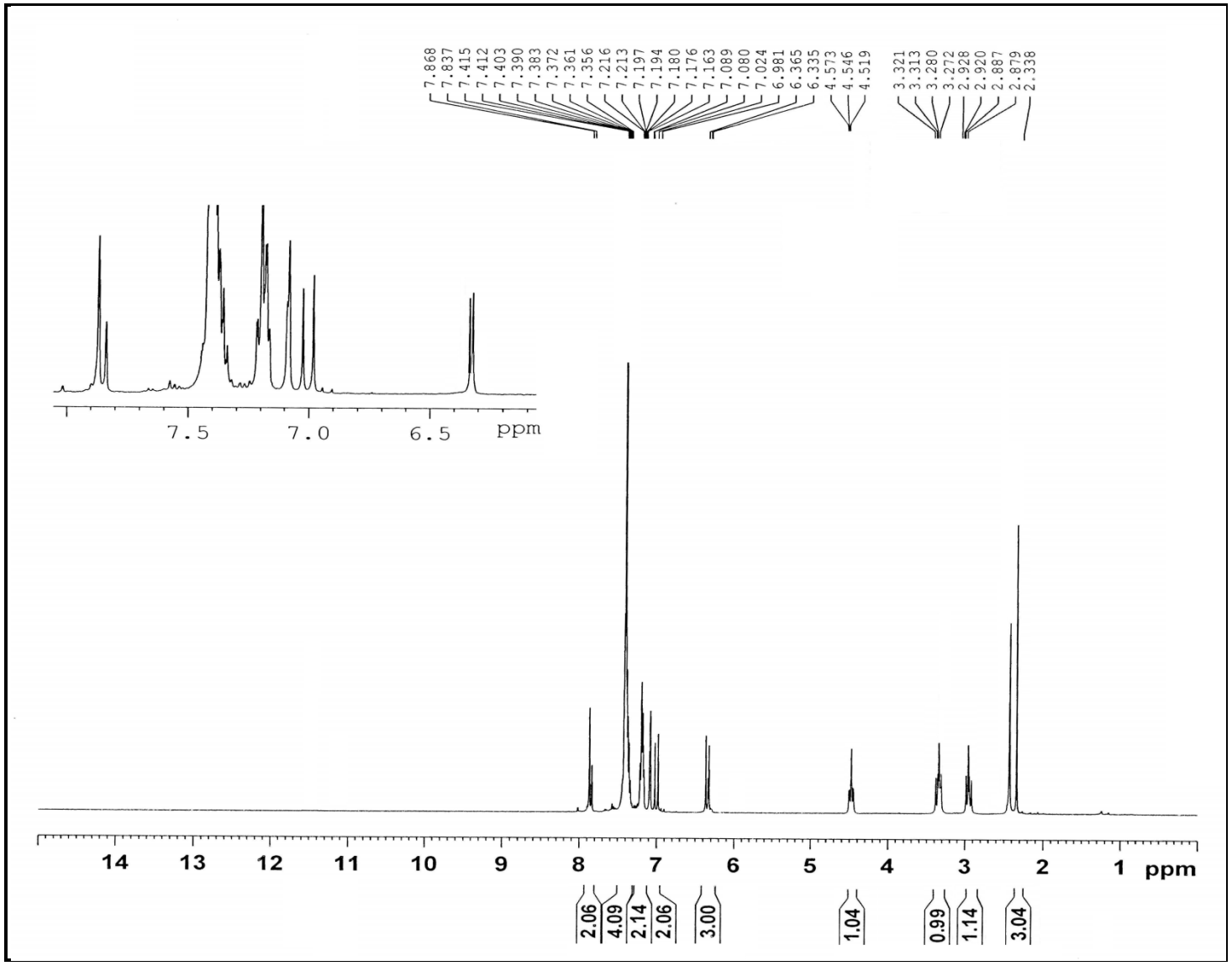
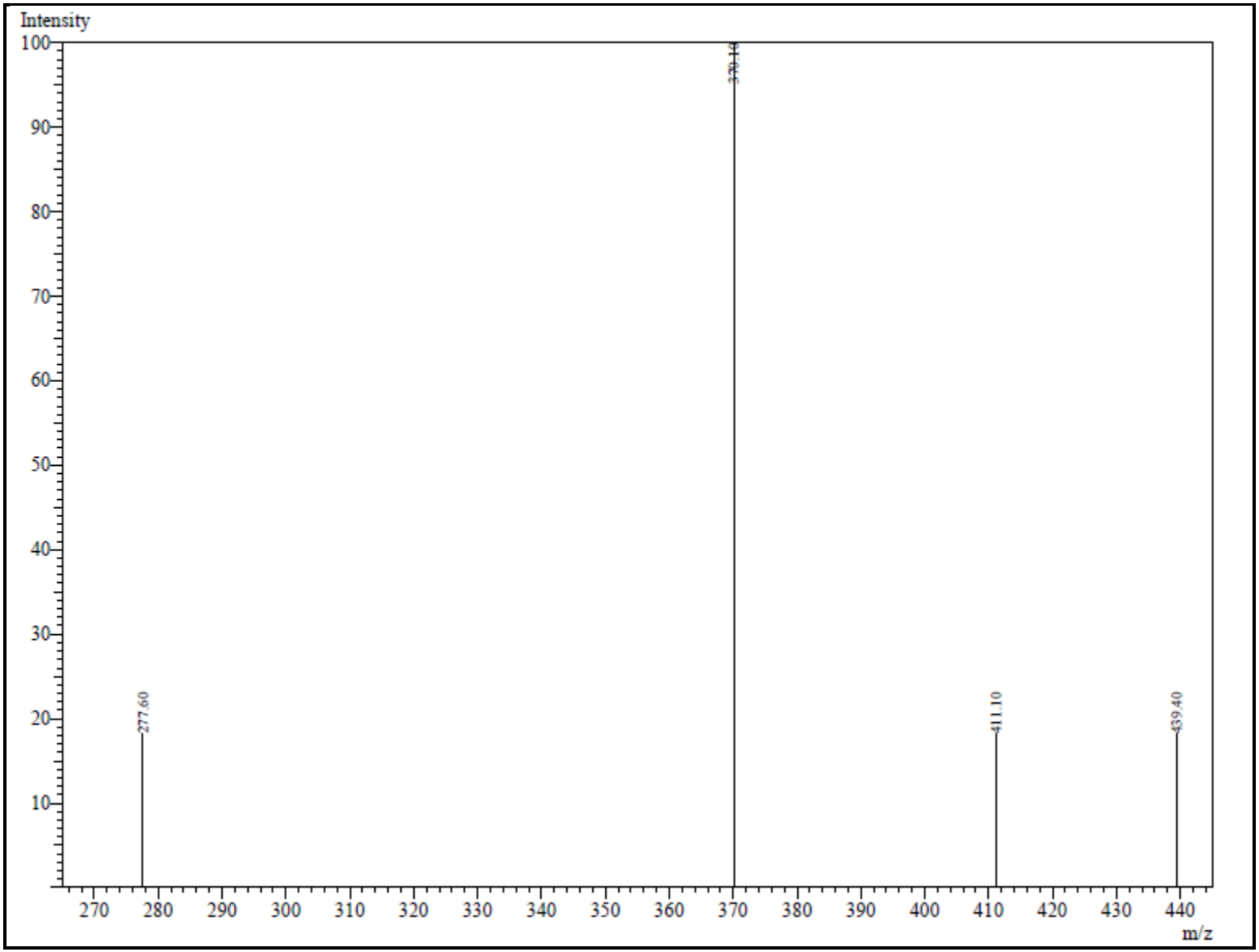


Mass Spectra of Compound 7a



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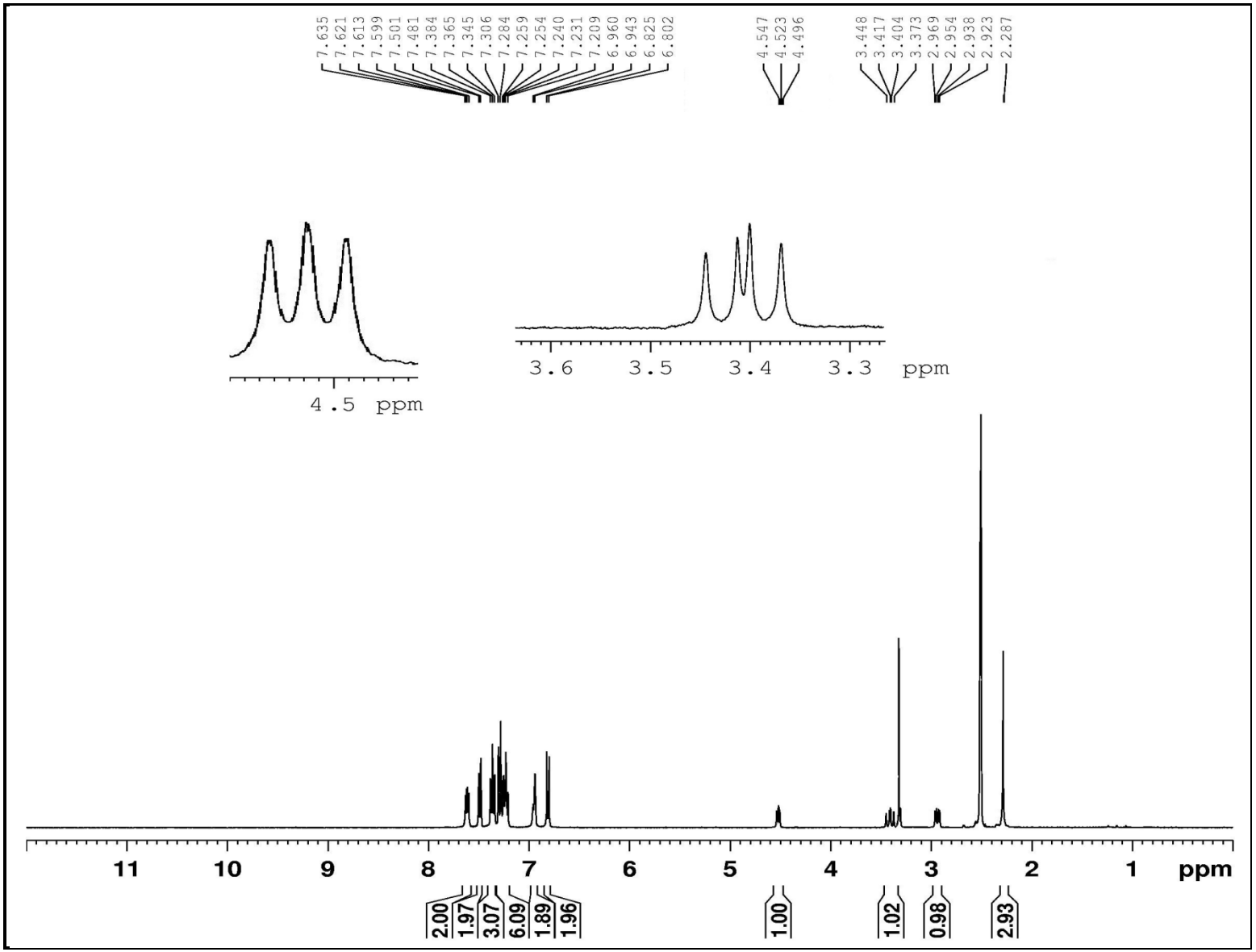

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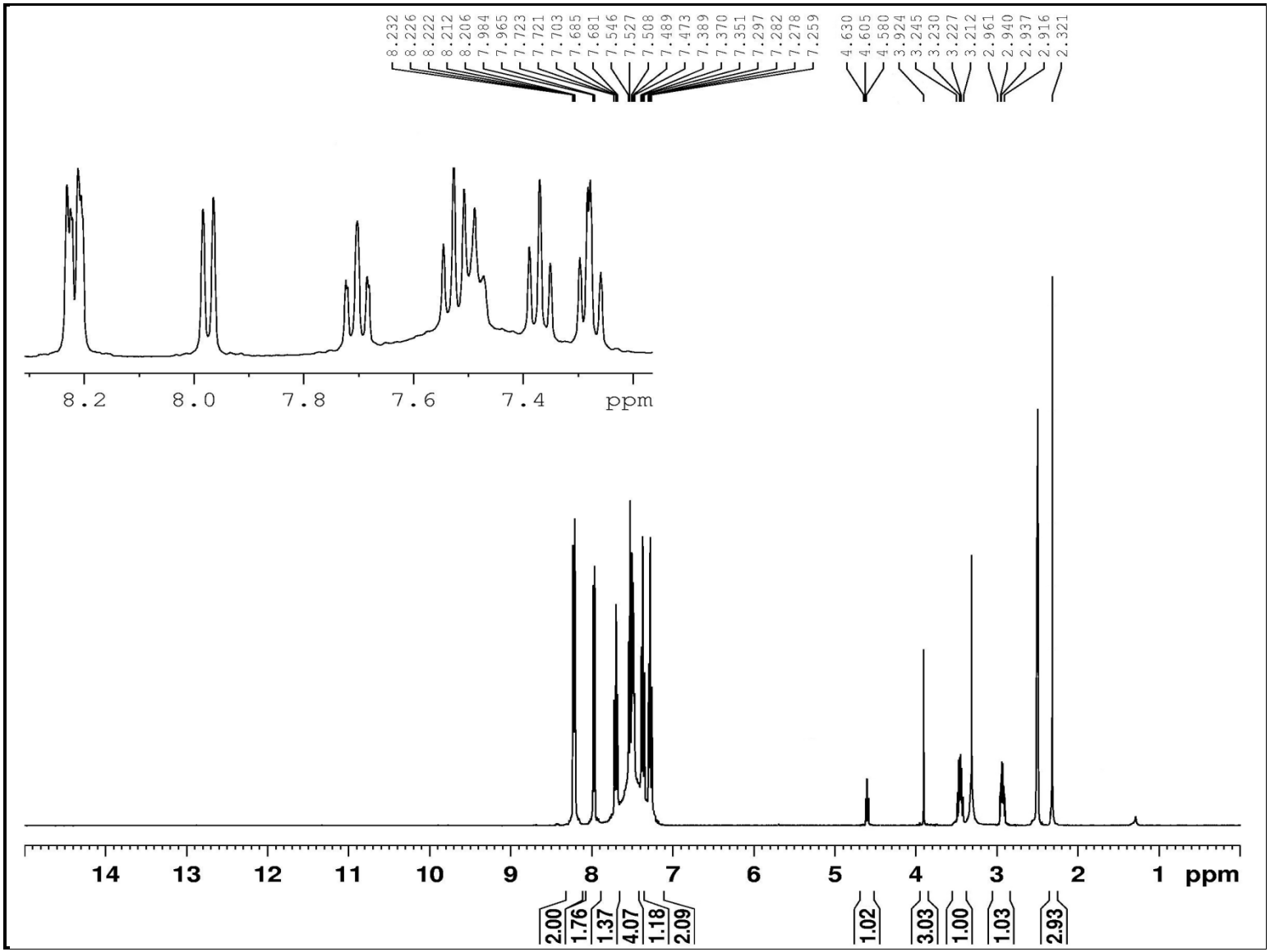

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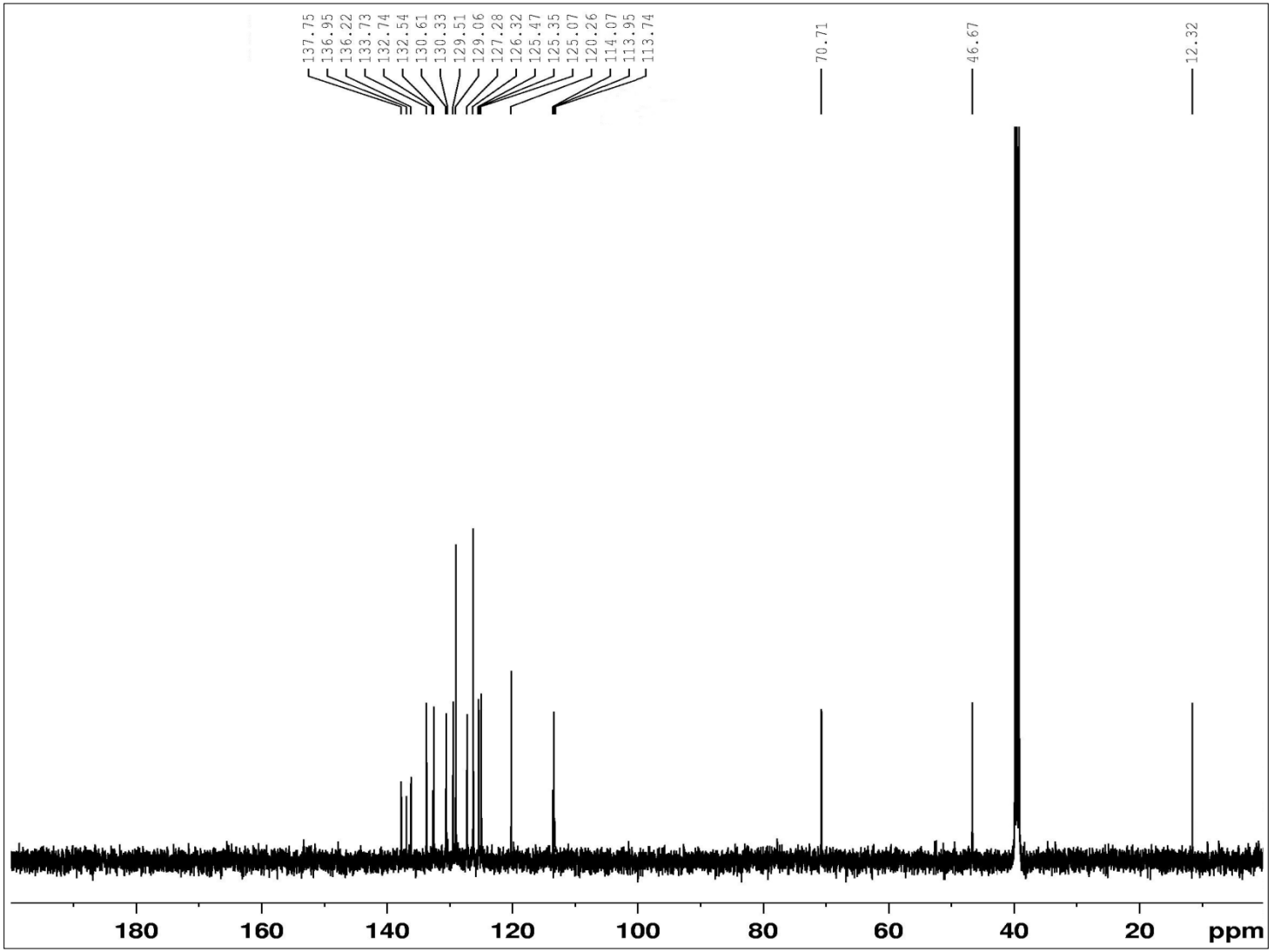

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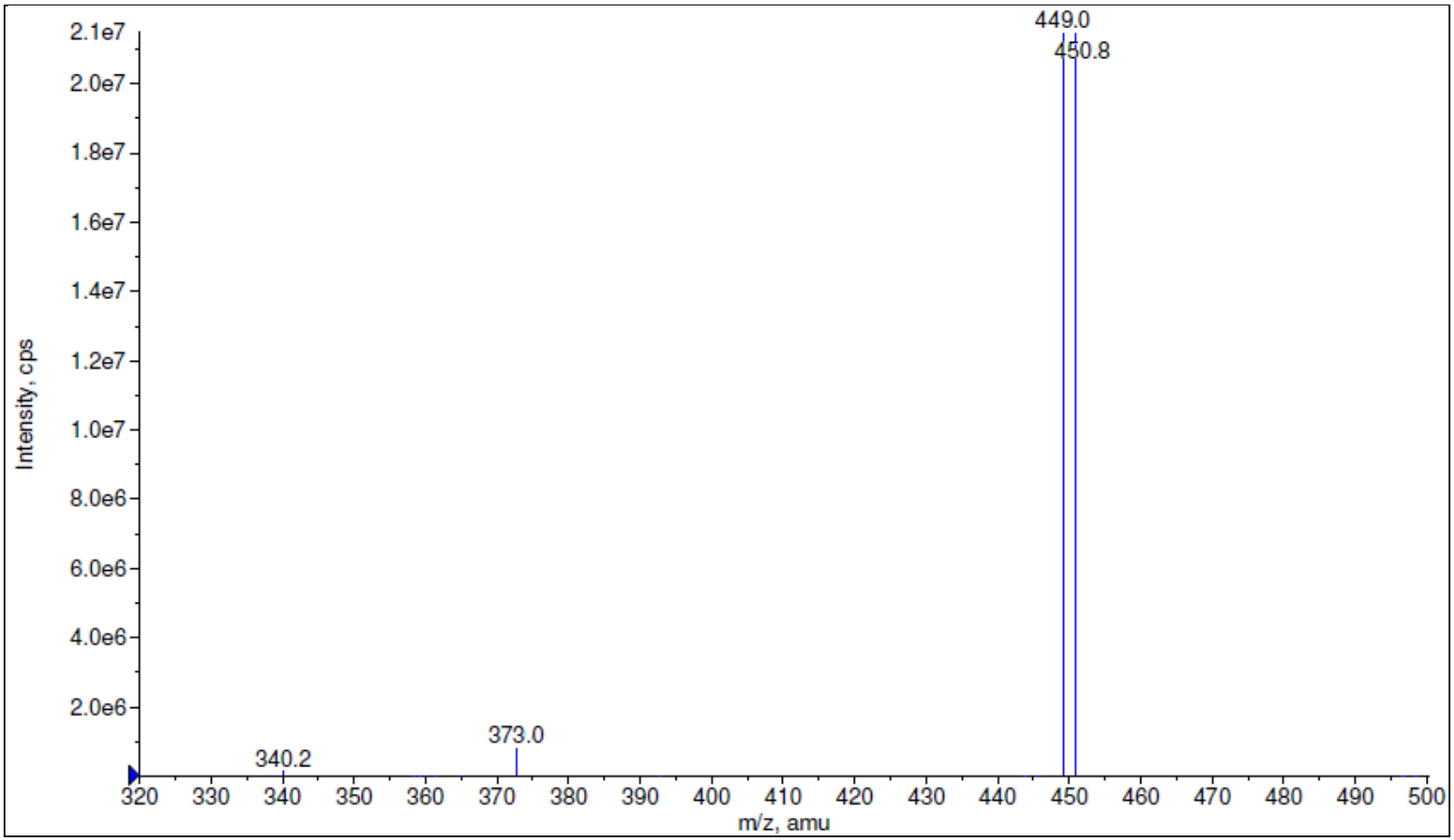

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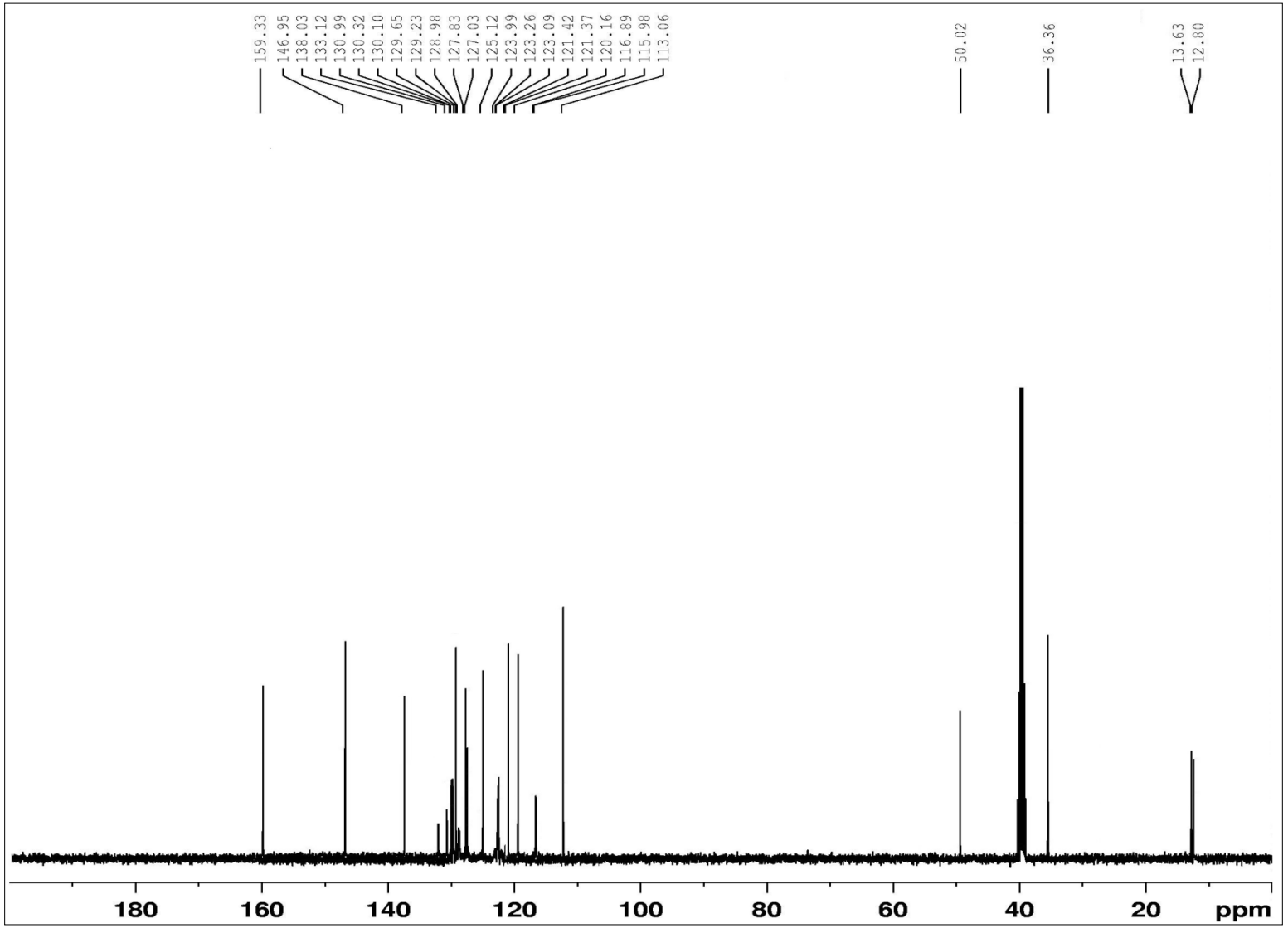

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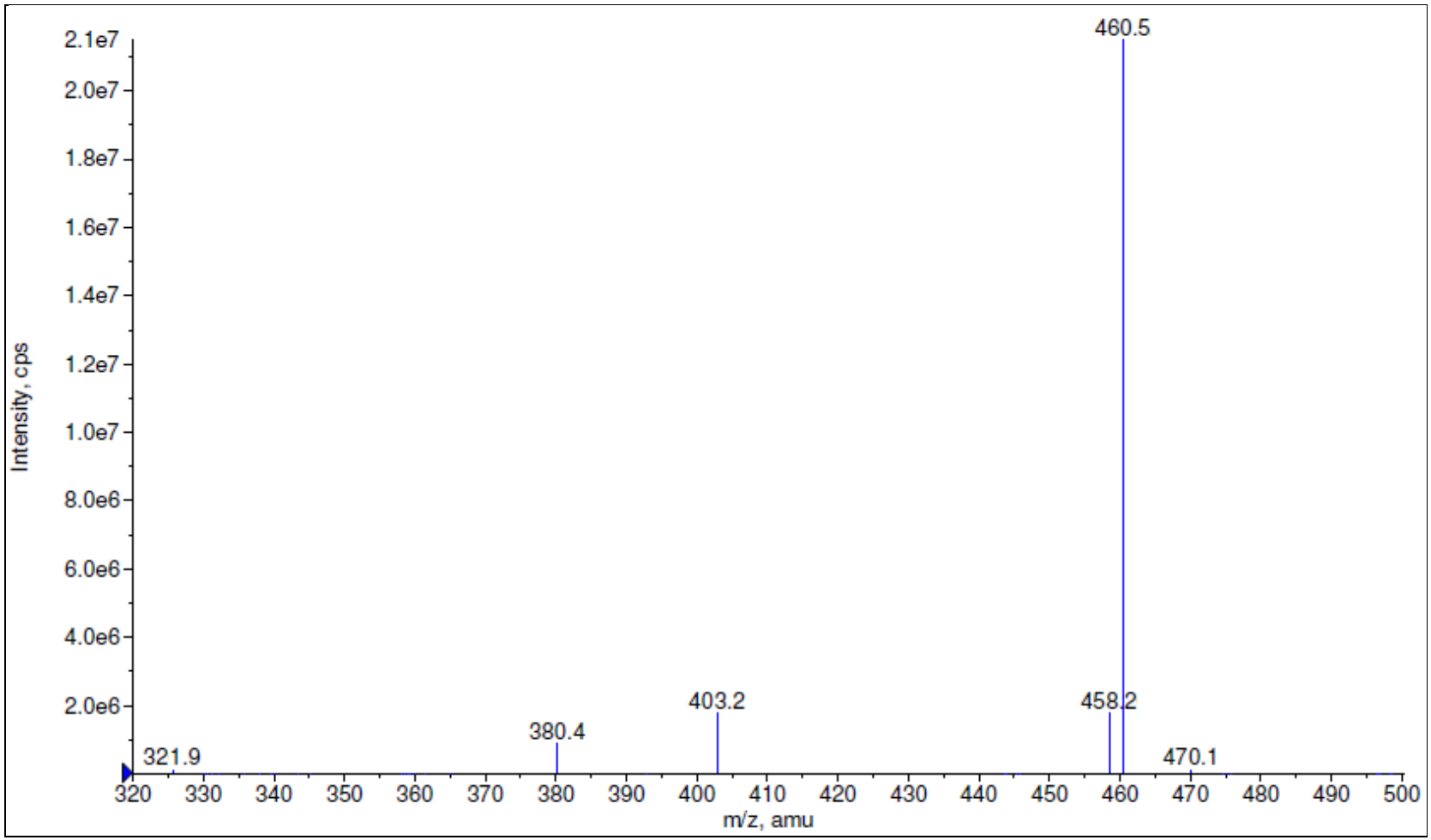

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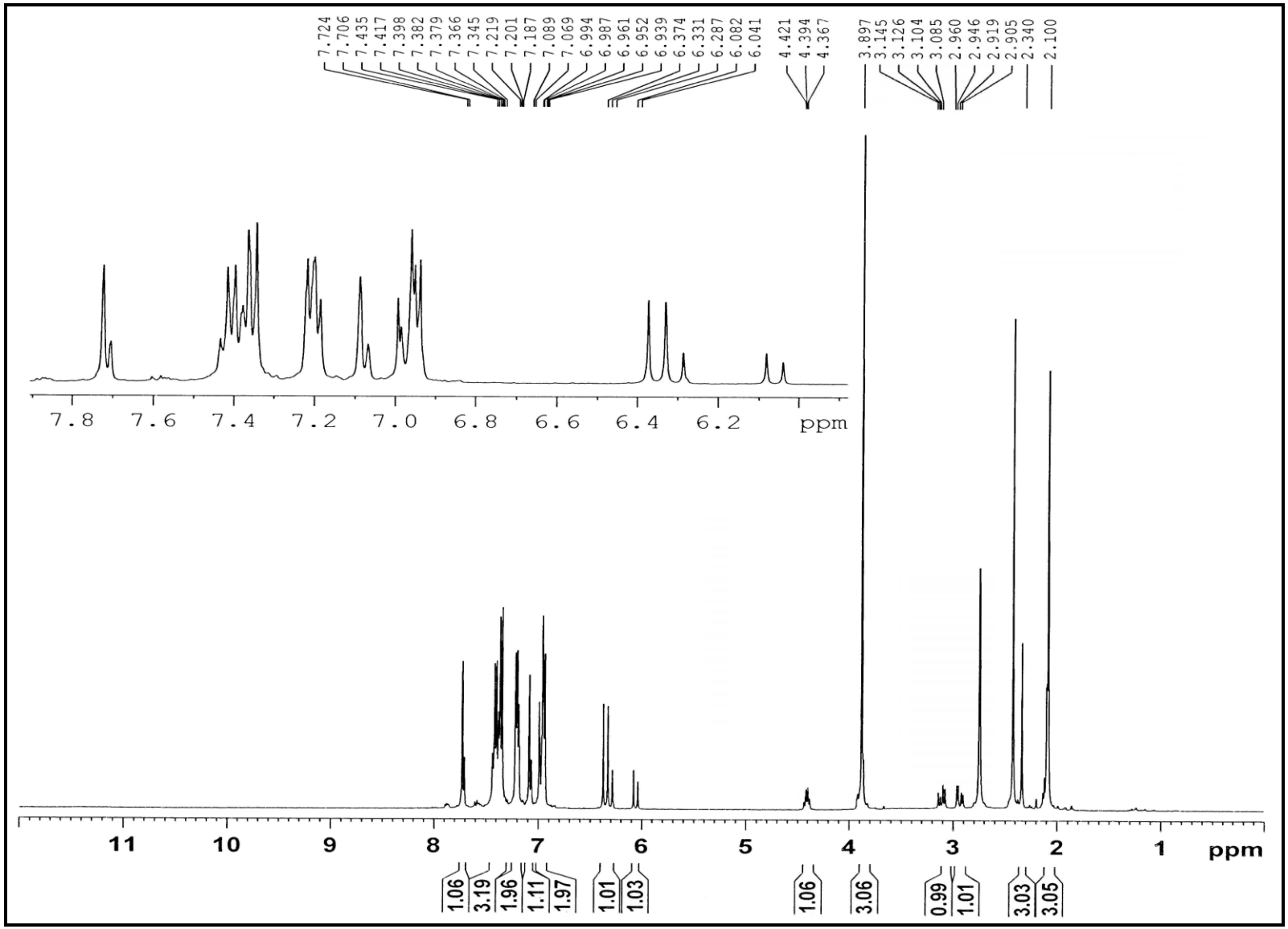
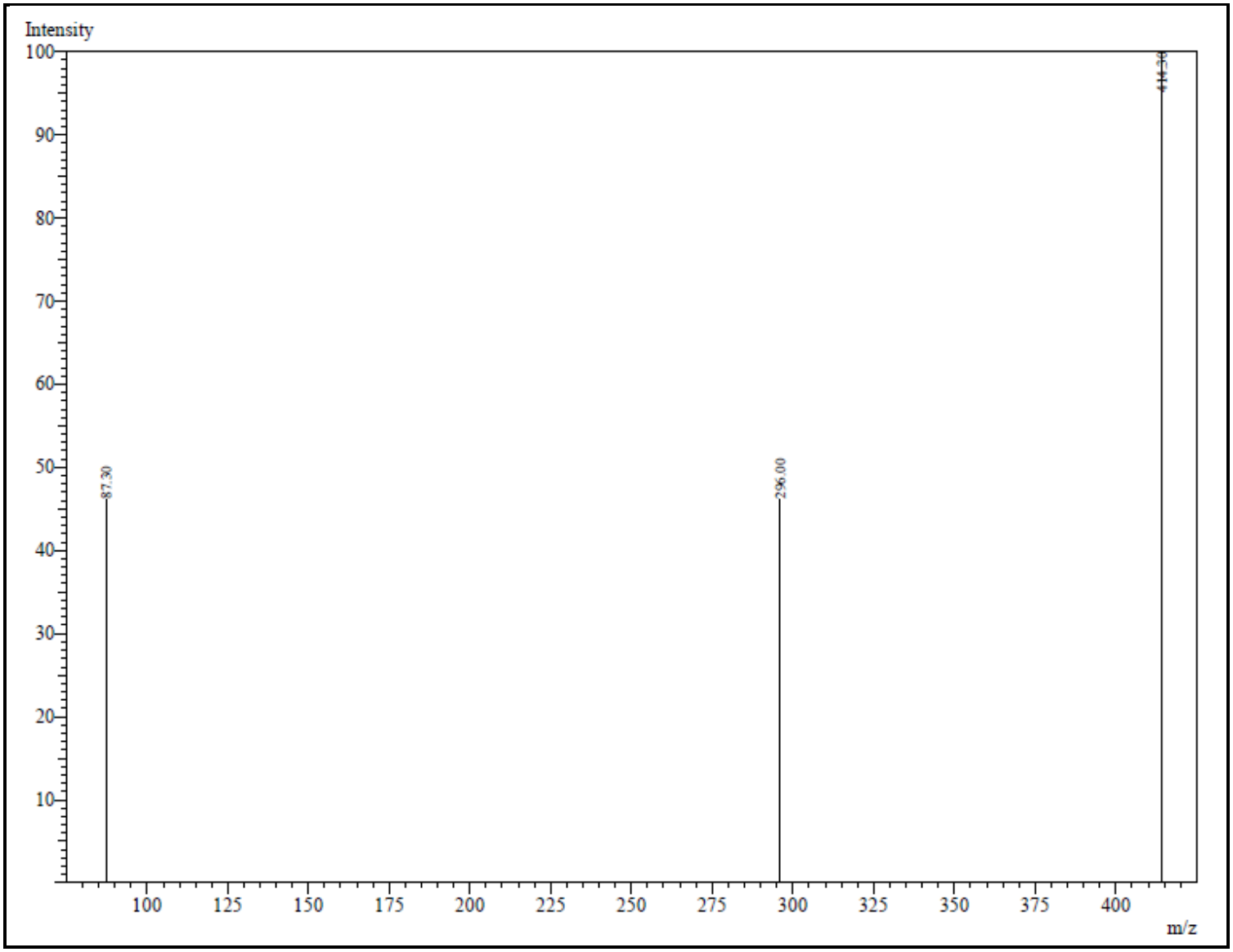


Mass Spectra of Compound 7i



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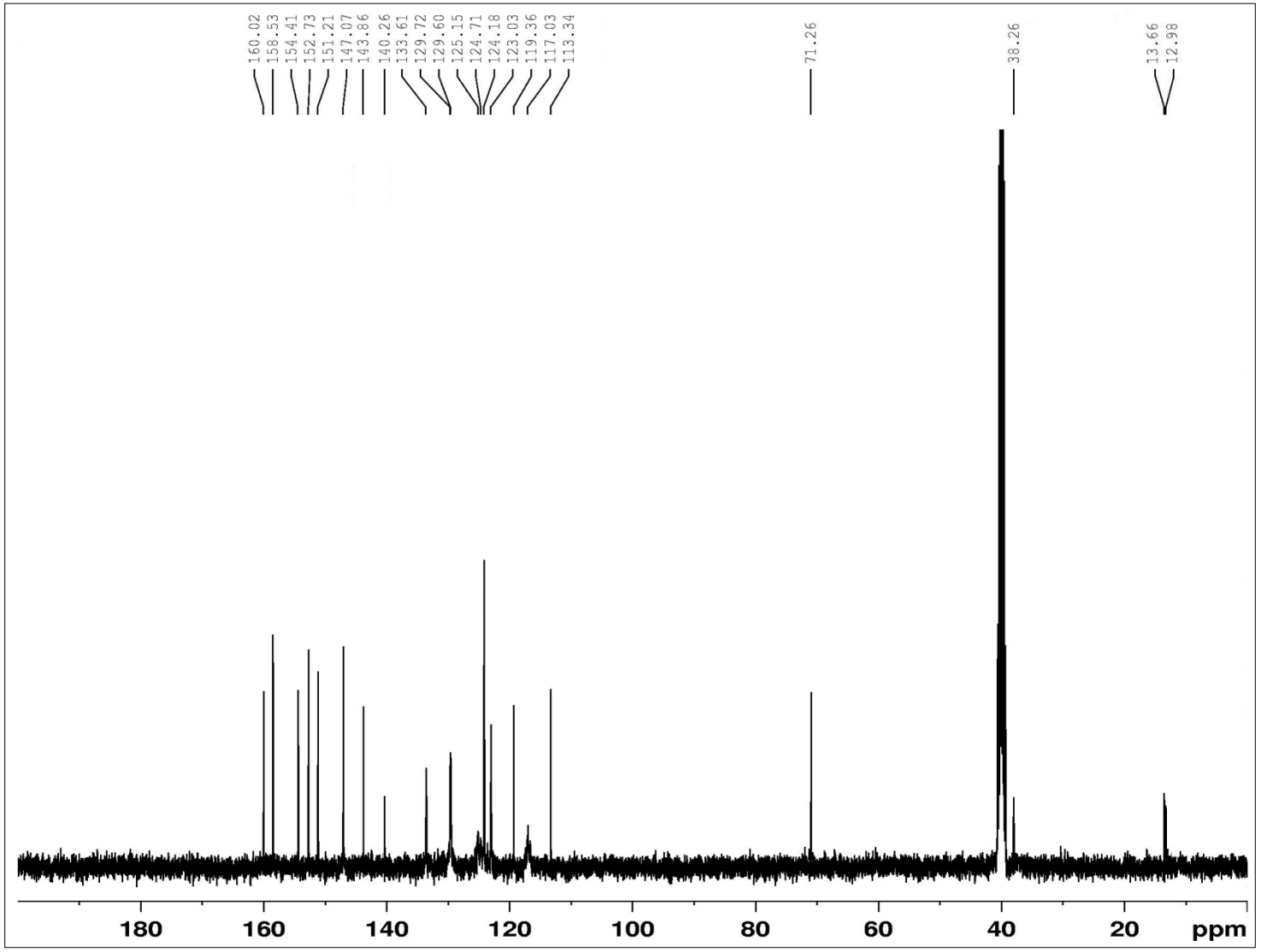

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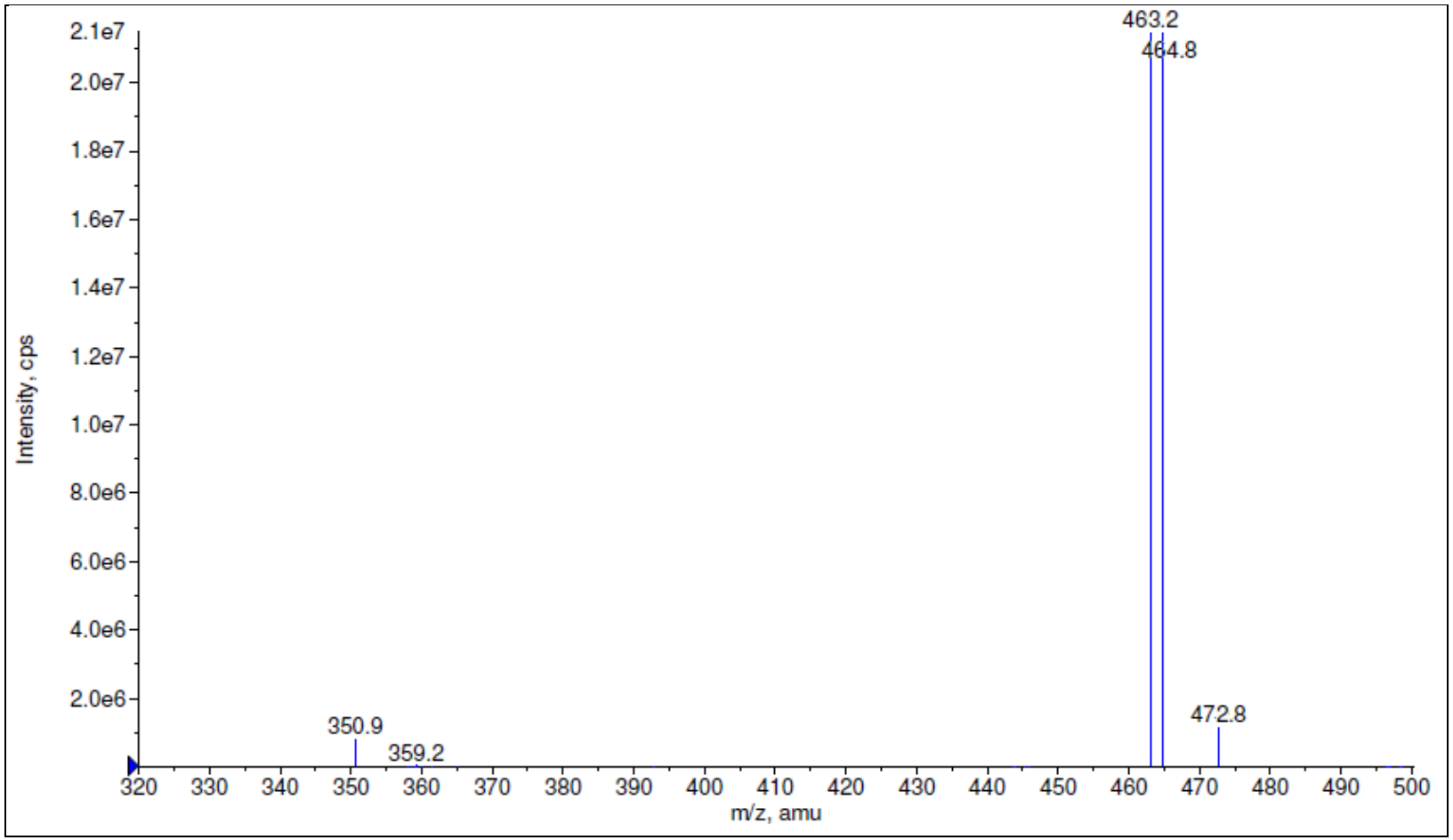

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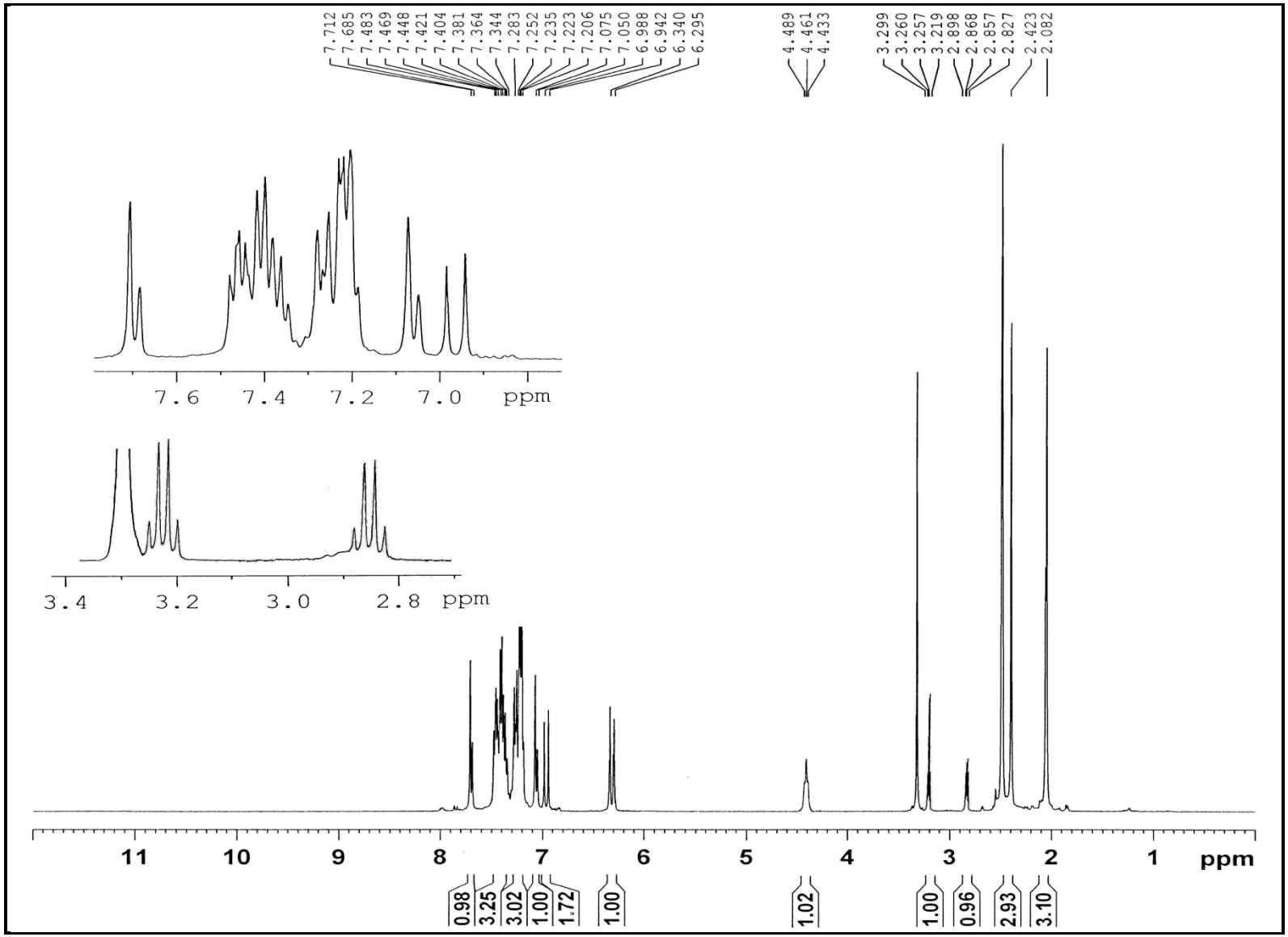

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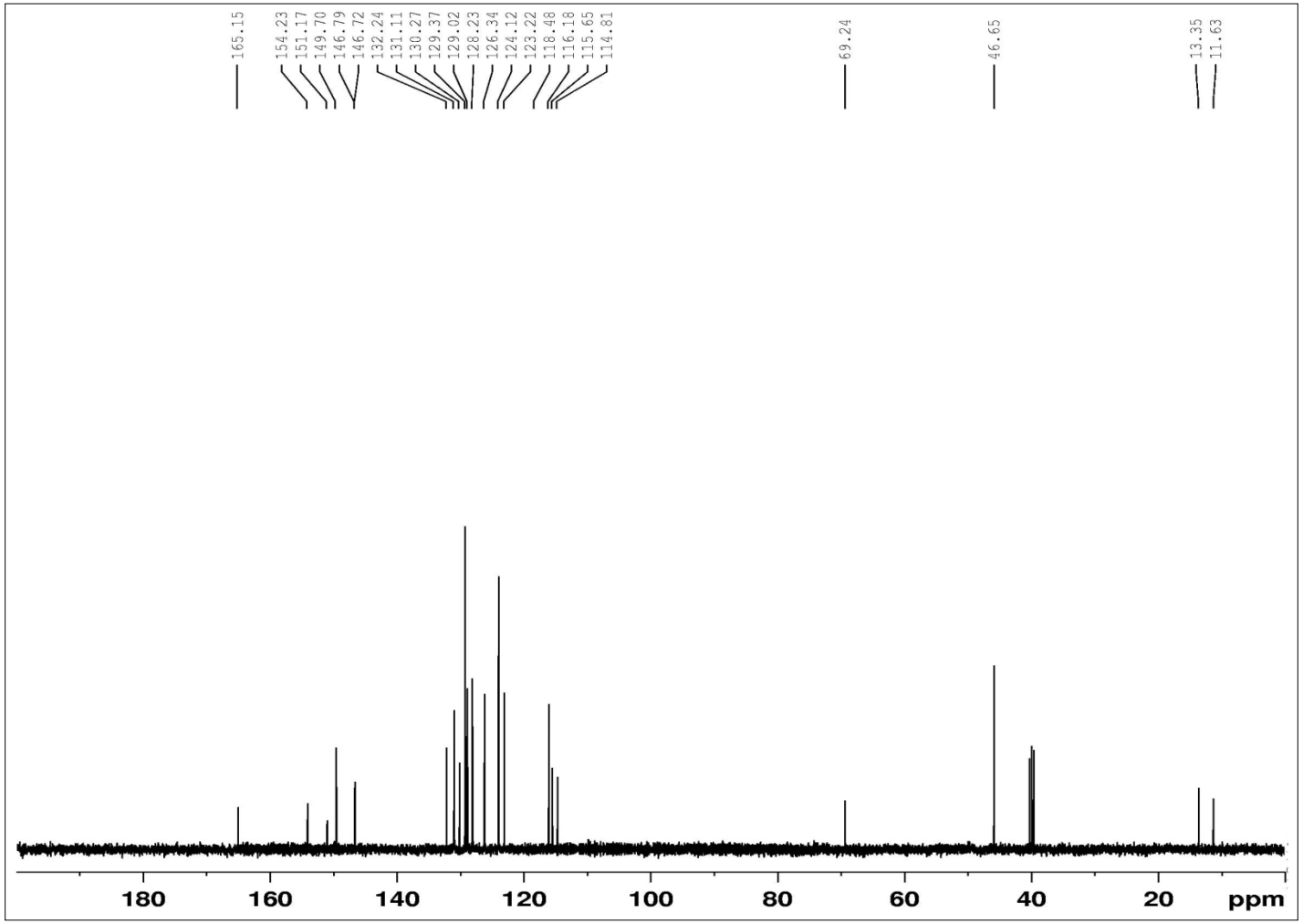

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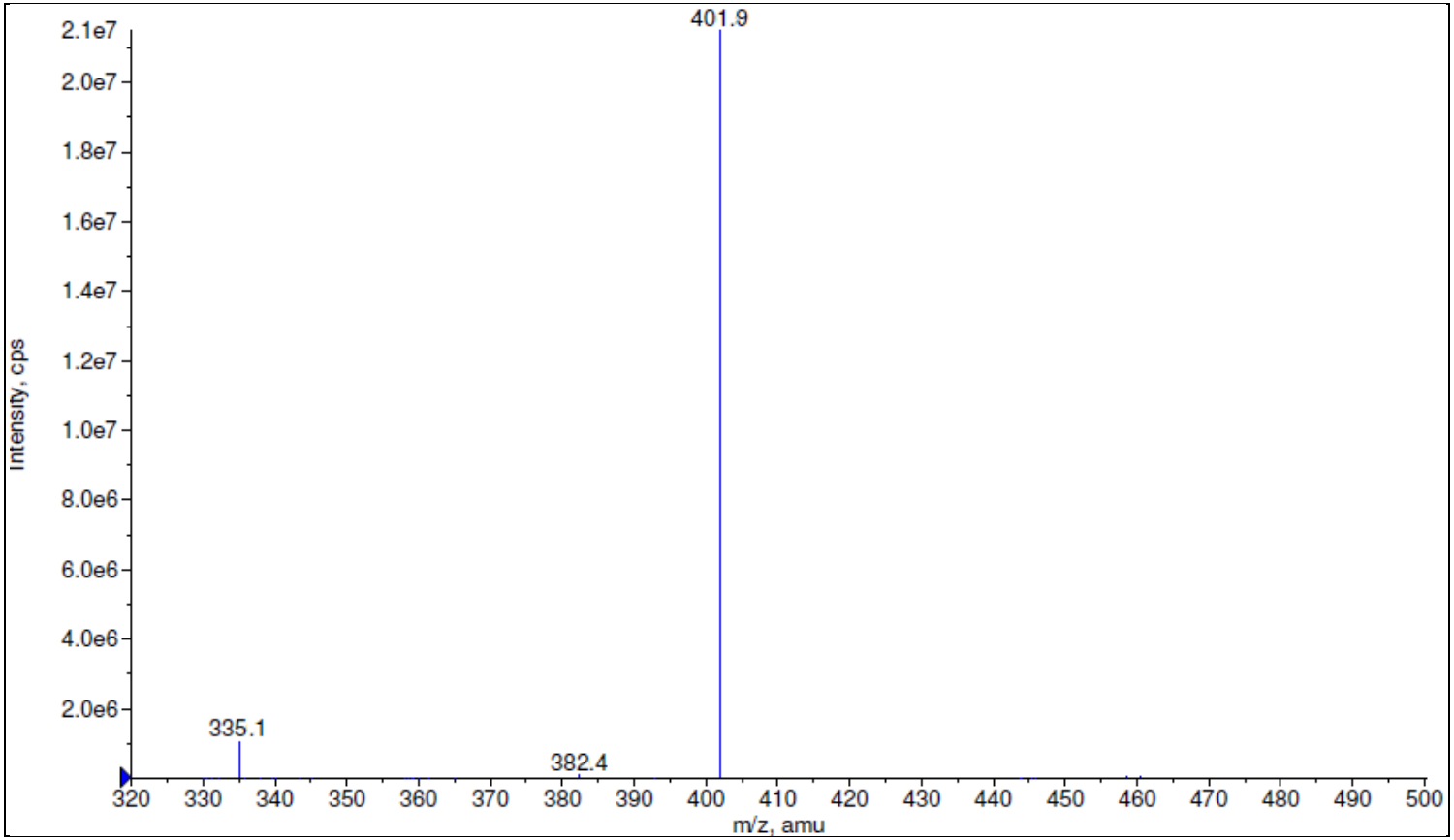

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4. J. P. v. d. B. C. Lambros, J., *Parasitol.* , (1979), **65** 418.
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