The Structural Basis of *Cryptosporidium*-Specific IMP Dehydrogenase Inhibitor Selectivity

Iain S. MacPherson[†], Sivapriya Kirubakaran[†], Suresh Kumar Gorla[†], Thomas V. Riera[¥], J. Alejandro D'Aquino[¥], Minjia Zhang[†], Gregory D. Cuny[§] and Lizbeth Hedstrom[† ¶*]

Departments of [†]Biology, [¥]Biochemistry and [¶]Chemistry, Brandeis University, MS009, 415 South St. Waltham, MA 02454; [§]Laboratory for Drug Discovery in Neurodegeneration, Brigham & Women's Hospital, Harvard Medical School, 65 Landsdowne Street, Cambridge, MA 02139

Supporting Information

Complete References

(4) Abrahamsen, M. S.; Templeton, T. J.; Enomoto, S.; Abrahante, J. E.; Zhu, G.; Lancto, C. A.; Deng, M.; Liu, C.; Widmer, G.; Tzipori, S.; Buck, G. A.; Xu, P.; Bankier, A. T.; Dear, P. H.; Konfortov, B. A.; Spriggs, H. F.; Iyer, L.; Anantharaman, V.; Aravind, L.; Kapur, V. *Science* **2004**, *304*, 441-445.

(5) Xu, P.; Widmer, G.; Wang, Y.; Ozaki, L. S.; Alves, J. M.; Serrano, M. G.; Puiu, D.; Manque, P.; Akiyoshi, D.; Mackey, A. J.; Pearson, W. R.; Dear, P. H.; Bankier, A. T.; Peterson, D. L.; Abrahamsen, M. S.; Kapur, V.; Tzipori, S.; Buck, G. A. *Nature* **2004**, *431*, 1107-1112.

Construction of CpIMPDH-pET28a

A ligase-free cloning method [1] was used with modifications to insert the *Cp*IMPDH coding sequence into pET28a, yielding an N-terminal His-tagged construct with thrombin cleavage signal. First, using pTactac-CpIMPDH as template [2], coding sequence was amplified with primers CpIMPDH-pETfor and CpIMPDH-pETrev where the 5' ends of each primer in bold lettering correspond to the pET28a vector and 3' ends correspond to the *Cp*IMPDH coding sequence (Table S1). The PCR product was purified using a Qiagen PCR-cleanup kit and used as template with pET28a forward extender and pET28a reverse extender, where the 3' ends of each primer overlap with the 5' ends of the previous primer pair, resulting in an increase in the amount of sequence overlap with pET28a on the flanking regions of the construct. In both PCR steps, reactions contained 1X Phusion HF buffer, 200μM dNTPs, 200 nM each primer, 4% DMSO, and 1 U Phusion Hotstart polymerase (New England Biolabs). Cycling was performed with a 68° annealing temperature, according to manufacturer's instructions. The resulting product was purified using a Qiagen PCR-cleanup kit, and used as a megaprimer for whole plasmid synthesis, using EcoRI-digested pET28a as the template. Reactions contained 1XPhusion HF buffer, 200 μM dNTPs, 300 ng PCR product megaprimer, 50 ng pET28a-EcoRI and 1 U Phusion Hotstart in a 50 μl reaction. Cycling was performed at 98° for 50 sec, 98° for 10 sec, 60° for 30 sec, 72° for 2 min, and cycles 2-4 repeated 23 more times. The resulting product was transformed into XL10-gold cells (Stratagene) and resulting clones sequenced to ensure that no mutations were introduced.

Generation of CpIMPDH-Δ90-134 deletion mutant.

Using the CpIMPDH-pET28a clone obtained above as template, codons for residues 90-134 were replaced with codons for SerGlyGly by a modification of the megaprimer cloning method [1]. First, the Δ90-134 deletion primer and T7 primer were used to amplify a region of CpIMPDH-pET28a ranging from the T7 promoter to Val143, while replacing codons for residues 90-134 with codons for SerGlyGly. The resulting product was purified using a Qiagen PCR-cleanup kit and used as megaprimer in the whole plasmid synthesis reaction, with NcoI-digested CpIMPDH-pET28a as template. Reactions contained 1X Phusion HF buffer, 200 μM dNTPs, 300 ng PCR product megaprimer, 50 ng CpIMPDH-pET28a-NcoI, and 1 U Phusion Hotstart in a 50 μl reaction. Cycling was performed at 98° for 50 sec, 98° for 10 sec, 72° for 2.5 min, and cycles 2-4 repeated 23 more times. The resulting product was transformed into XL10-gold cells (Stratagene) and resulting clones sequenced.

Generation of ΔguaB strain of BL21(DE3).

To eliminate the possibility of contamination from endogenous *E. coli* IMPDH, the *guaB* gene was deleted using the methods and plasmids developed in [3]. First, primers IMPDH-KO-for and IMPDH-KO-rev were used in a PCR using PKD3 as a template, resulting in the chloramphenicol resistance gene flanked by FRT sites and chromosomal sequence adjacent to the *guaB* (IMPDH) gene of *E. coli*. The product was electroporated into BL21(DE3) containing PKD46, which codes for arabinose-dependent expression of FLP recombinase. Chloramphenicol-resistant colonies were confirmed by PCR with IMPDH-KO-for and -rev primers, and were cured of PKD46 by growth at 37° overnight. The resulting strain was able to grow on minimal media only when supplemented with guanosine.

Table S1. Primer Sequence.

CpIMPDH-pETfor	5'**TGGTGCCTCGTGGTAGCCAT**ATGGGTACAAAAAACATAGGAAAAGGCT
CpIMPDH-pETrev	5'**CTCAGCTTCCTTTCGGGCTTTGTTA**TTTACTATAATTCATTACTTCTTTTACGATTTCAA
pET28a forward extender	5'ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCTCGTGGTAGCCAT
pET28a reverse extender	5'TATGCTAGTTATTGCTCAGCGGTGGCAGCAGCCAACTCAGCTTCCTTTCGGGCTTTGTTA
Δ90-134 deletion primer	5'TACACCTATTGCTGCTCCAACTCTTAAACCACCACTATTCTTGACTTTCAATACTTCATT
T7 primer	5'TAATACGACTCACTATAGGGG
IMPDH-KO-for	5'CGGCAATATTTATTAACCACTCTGGTCGAGATATTGCCCTGTAGGCTGAGCTGCTTCG
IMPDH-KO-rev	5'GTCCAGAATGAGGATGCGATGCTTATGAATGTTTTCCGTCATATGAATATCCTCCTTAG

Expression of CpIMPDH-Δ90-134

CpIMPDH-Δ90-134-pET28a plasmid was transformed into BL21(DE3Δ*guaB*), spread onto LB agar plates supplemented with 25 μg/ml kanamycin and grown overnight at 37°. An isolated colony was inoculated into 5 ml LB containing 25 μg/ml kanamycin cultures and shaken overnight at 37°. The cultures were used to inoculate 1 L of LB containing 25 μg/ml kanamycin, and the resulting culture was shaken at 30° until reaching an OD_{600} = 1, after which the culture was moved to 25° and shaken for an

additional 30 minutes, before induction with 0.5 mM IPTG and shaking overnight at 25°. Cells were harvested by centrifugation, lysed by sonication in 1X binding buffer (0.2 M sodium phosphate, 0.5 M NaCl) supplemented with 20 mM imidazole pH 8. The lysate was cleared by centrifugation and applied to Ni-NTA HisTrap beads (GE Healthcare). The beads were washed with binding buffer supplemented with 20, 50 and 100 mM imidazole and *Cp*IMPDH-Δ90-134 was eluted with binding buffer supplemented with 500 mM imidazole. The eluted fractions were dialyzed against 20 mM Tris pH 7.5, 50 mM NaCl, after which thrombin (Haematologic Technologies) was added at a ratio of 1:500 (thrombin:IMPDH, w:w) and incubated overnight at 4°. Thrombin was subsequently removed by incubation with 50 µl benzamidine-Sepharose beads (GE Healthcare). The protein was concentrated to 5 mg/ml and used for crystallization trials.

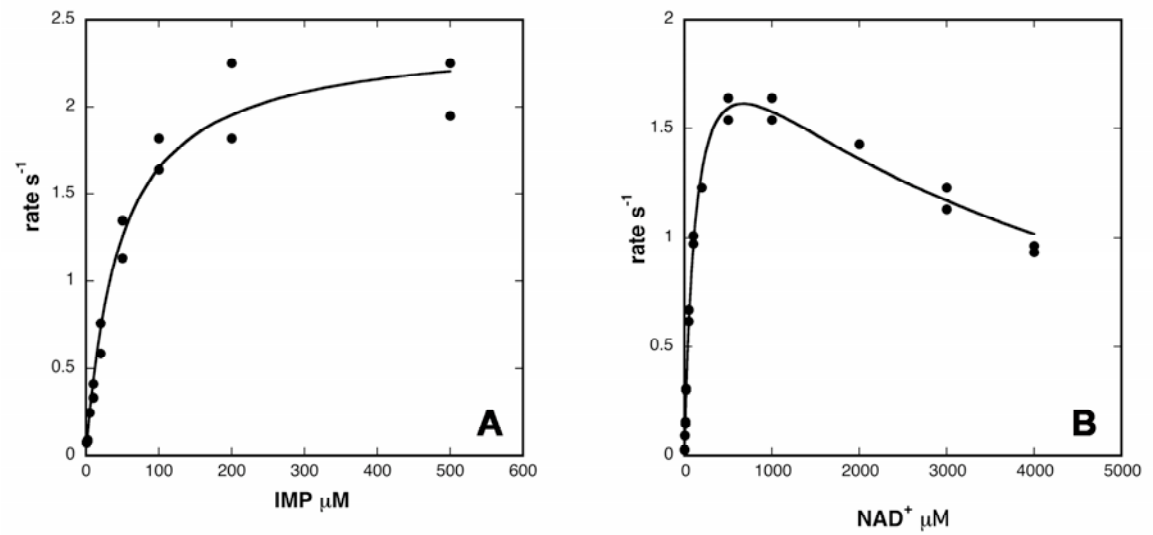


Figure S1. Kinetics of *Cp*IMPDH-Δ90-134. Conditions: 50 mM TrisHCl, pH 8.0, 100 mM KCl, 1 mM dithiothreitol. A. IMP varied, [NAD^+] = 500 µM. k_{cat} = 2.4 ± 0.1 s^{-1}, K_m(IMP) = 46 ± 5 µM. B. NAD^+ varied, [IMP] = 1 mM. k_{cat} = 2.3 ± 0.1, K_m(NAD^+) = 130 ± 10 µM, K_{ii}(NAD^+) = 3.4 ± 0.4 mM. These values are very similar to those of the wild-type enzyme: k_{cat} = 3.3 s^{-1}, K_m(IMP) = 29 µM, K_m(NAD^+) = 150 µM, K_{ii}(NAD^+) = 2.9 ± 0.7 mM [8]

Crystallography.

The structure of CpIMPDH-Δ90-134-C64 was solved using the CCP4 molecular replacement program MOLREP [4] using a *Cp*IMPDH monomer as the search model. The structure was refined using REFMAC [5] and modeling was performed with COOT [6]. Refinement statistics are shown in Table S2.

Table S2: Statistics for Data Collection and Refinement

	Data Collection	
	WtCpIMPDH	CpIMPDH(*Δ90-134*)-C64
x-ray wavelength (Å)	0.97946	0.9194
temperature	100K	100K
space group	$P2_12_12$	$P2_1$
unit cell (Å)		
a	119.1	83.481
b	153.3	166.141
c	98.2	101.289
Cell angles (degrees)		
α	90	90
β	90	105.14
γ	90	90
resolution (Å)	3.2	2.8
total no. of reflections	207,910	3,394,714
no. of unique reflections	30,575	65,589
$\langle I/\sigma(I)\rangle$	12.1 (3.1)	19.4 (5.2)
completeness of data (%)	99 (99)	100 (100)
R-merge[a] (%)	18 (63)	9.3 (38.3)
	Refinement	
resolution range (Å)	50 - 3.2	100-2.8
reflections used (working/free)	28,947/1,536	61,921/3,303
R-factor[b]/R-free (%)	26.9/32.8	22.4/26.6
total number of non-hydrogen atoms in asymmetric unit	8,563	18,779
rms deviations from ideal geometry		
bond length (Å)	0.01	0.006
bond angles (deg)	1.45	1.00
mean B value	54	53
PDB accession code	3FFS	3KHJ

[a] R-merge = $\Sigma|I_{obs} - I_{avg}|/\Sigma I_{avg}$, over all symmetry-related observations. [b] R-factor = $\Sigma|F_{obs} - F_{avg}|/\Sigma F_{avg}$, over all reflections.

Synthetic materials and methods.

Unless otherwise noted, all reagents and solvents were purchased from commercial sources and used without further purification. All reactions were performed under nitrogen atmosphere unless otherwise noted. The NMR spectra were obtained using a 400 MHz spectrometer. All ^{1}HNMR spectra are reported in δ units ppm and are referenced to tetramethylsilane (TMS) if conducted in $CDCl_3$ or to the central line of the quintet at 2.49 ppm for samples in DMSO-d6. All chemical shift values are also reported with multiplicity, coupling constants, and proton count. All ^{13}C NMR spectra are reported in δ units ppm and are referenced to the central line of the triplet at 77.23 ppm if conducted in $CDCl_3$ or to the central line of the septet at 39.5 ppm for samples in DMSO-d6. Coupling constants (J values) are reported in hertz. Column chromatography was carried out on SILICYCLE SiliaFlash silica gel F60 (40-63 μm, mesh 230-400). All test compounds had purity 95% as determined by high performance liquid chromatography (HPLC) analysis, unless otherwise noted. The elemental composition of compounds agreed to within (0.4% of the calculated values. Chemical and enantiomeric purities were determined using high performance liquid chromatography (HPLC) analysis on a Hewlett-Packard 1100 series instrument equipped with a quaternary pump and a Daicel Chiralpak AD column (250 x 4.6 mm). UV absorption was monitored at λ=254 nm. The injection volume was 1 μL. HPLC gradient was 50% *n*-hexane and 50% *i*-propanol with a flow rate of 1.0 mL/min. In some cases, chemical purity was determined using a Agilent 1100 HPLC instrument equipped with a quaternary pump and a Zorbax SB-C8 column (30 mm x 4.6 mm, 3.5 μm). UV absorption was monitored at λ=254 nm. The injection volume was 5 μL. HPLC gradient went from 5% acetonitrile and 95% water to 95% acetonitrile and 5% water (both solvents contain 0.1% trifluoroacetic acid) over 1.9 min with a total run time of 2.5 min and a flow rate of 3.0 mL/min.

Synthesis of bromoacetylamides 3.

To a dried round-bottom flask fitted with a nitrogen inlet was charged with anhydrous DCM (4 mL), 4-bromoaniline (200 mg, 1.16 mmol), and a catalytic amount of DMAP. The reaction mixture was cooled to 0 °C and bromoacetylchloride (274 mg, 1.74 mmol) was added dropwise over the period of 10 min. The resulting solution was stirred at 0 °C for 30 min and then for another 2-3 h at room temperature. After 2h the reaction mixture was quenched with water. The reaction mixture was further diluted with water (50 mL) and extracted with DCM (3 x 50 mL). The organic extracts were combined, dried over anhydrous $MgSO_4$, filtered, concentrated *in vacuo*, and the product obtained was used for the next step without any further purification.

Synthetic materials and methods.

Unless otherwise noted, all reagents and solvents were purchased from commercial sources and used without further purification. All reactions were performed under nitrogen atmosphere unless otherwise noted. The NMR spectra were obtained using a 400 MHz spectrometer. All ^{1}HNMR spectra are reported in δ units ppm and are referenced to tetramethylsilane (TMS) if conducted in $CDCl_3$ or to the central line of the quintet at 2.49 ppm for samples in DMSO-d_6. All chemical shift values are also reported with multiplicity, coupling constants, and proton count. All ^{13}C NMR spectra are reported in δ units ppm and are referenced to the central line of the triplet at 77.23 ppm if

conducted in $CDCl_3$ or to the central line of the septet at 39.5 ppm for samples in DMSO-d6. Coupling constants (J values) are reported in hertz. Column chromatography was carried out on SILICYCLE SiliaFlash silica gel F60 (40-63 μm, mesh 230-400). All test compounds had purity 95% as determined by high performance liquid chromatography (HPLC) analysis, unless otherwise noted. The elemental composition of compounds agreed to within (0.4% of the calculated values. Chemical and enantiomeric purities were determined using high performance liquid chromatography (HPLC) analysis on a Hewlett-Packard 1100 series instrument equipped with a quaternary pump and a Daicel Chiralpak AD column (250 x 4.6 mm). UV absorption was monitored at λ=254 nm. The injection volume was 1 μL. HPLC gradient was 50% *n*-hexane and 50% *i*-propanol with a flow rate of 1.0 mL/min. In some cases, chemical purity was determined using a Agilent 1100 HPLC instrument equipped with a quaternary pump and a Zorbax SB-C8 column (30 mm x 4.6 mm, 3.5 μm). UV absorption was monitored at λ=254 nm. The injection volume was 5 μL. HPLC gradient went from 5% acetonitrile and 95% water to 95% acetonitrile and 5% water (both solvents contain 0.1% trifluoroacetic acid) over 1.9 min with a total run time of 2.5 min and a flow rate of 3.0 mL/min.

Synthesis of 2-substituted benzimidazoles.

To a dried round-bottom flask fitted with a nitrogen inlet was added anhydrous DMF (4 mL), *o*-phenylenediamine (200 mg, 1.85 mmol) and the respective aryl aldehyde (2.2 mmol) and the mixture was stirred for 5 min before sodium metabisulfite (420 mg, 2.2 mmol) was added. The reaction mixture was stirred at room temperature for 7-8 h. Once the starting materials were consumed (followed by TLC) the solvent was removed by in vacuo. The solid obtained was dissolved in ethyl acetate (50 mL) and washed with water (50 mL). The aqueous layer was extracted with ethyl acetate (3 x 50 mL). The organic extracts were combined, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by flash column chromatography eluting with methanol/chloroform (1:9) to furnish 2-substituted benzimidazole in 65-70% yield.

Synthesis of C-derivatives.

To a dried round-bottom flask fitted with a nitrogen inlet was added anhydrous DMF (4 mL), a 2-aryl benzimidazole (0.50 mmol) a bromoacetylamide (0.52 mmol) and anhydrous K_2CO_3 (540 mg, 1.56 mmol). The reaction mixture was stirred at room temperature for 7-8 h. Once the starting materials were consumed (followed by TLC) the solvent was removed by in vacuo. The solid obtained was dissolved in ethyl acetate (20 mL) and washed with water (50 mL). The aqueous layer was extracted with ethyl acetate (3 x 50 mL). The organic extracts were combined, dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by flash column chromatography eluting with methanol/chloroform (1:9) to furnish **C** derivatives in 60-80% yield.

C10

Yield 87 %; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 5.66 (s, 2H), 7.24 (bs, 2H), 7.33 (d, J = 8 Hz, 2H), 7.54 (d, J = 7.6 Hz, 2H), 7.62 (bs,1H), 7.66 (bs,1H), 8.52 (s, 1H), 9.25 (s, 1H), 10.56 (s, 1H); ^{13}C NMR (DMSO-d6, 100 MHz) δ 48.82, 111.36, 119.59, 121.22, 122.87, 123.0, 123.44, 127.53, 129.40, 137.16, 138.44, 142.93, 147.71, 155.99, 166.74

C14

Yield 80 %; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.21 (s, 2H), 7.24 (bs,1H), 7.36-7.46 (m, 3H), 7.70 (d, *J* = 7.2 Hz, 1H), 7.79 (d, *J* = 8 Hz, 1H), 8.38 (s, 1H), 9.09 (s, 1H), 10.07 (s, 1H) ; ^{13}C NMR ($CDCl_3$ & DMSO-d_6, 100 MHz) δ 48.20, 109.92, 102.35, 114.73, 119.46, 120.99, 122.97, 123.39, 128.70, 137.14, 139.92, 142.68, 144.47, 151.65, 165.57

C61

Yield 85 %; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 5.72 (s, 2H), 7.27-7.33 (m, 4H), 7.54 (d, *J* = 8.4 Hz, 2H), 7.70 (dd, J_1 = 18.8 Hz, J_2 = 8.0 Hz, 2H), 7.92 (d, *J* = 2.8 Hz, 1H), 7.99 (d, *J* = 3.2 Hz, 1H), 10.62 (s, 1H) ; ^{13}C NMR (DMSO-d_6, 100 MHz) δ 48.45, 111.68, 120.11, 121.30, 123.66, 123.76, 124.69, 129.43, 137.62, 138.31, 138.34, 142.56, 144.89, 166.40

C64

Yield 75 %; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.33 (s, 2H), 7.23 (s, 2H), 7.35-7.42 (m, 4H), 7.64 (d, *J* = 2.8 Hz, 1H), 7.74 (d, *J* = 8 Hz, 1H), 7.82 (d, *J* = 7.6 Hz, 1H), 8.08 (d, *J* = 3.2 Hz, 1H), 10.07 (s, 1H) ; ^{13}C NMR (DMSO-d_6, 100 MHz) δ 48.62, 111.37, 119.60, 121.23, 122.88, 123.00, 123.04, 123.45, 123.45, 123.48, 129.41, 138.44, 142.94, 147.71, 156.00, 166.74

C90

Yield 82 %; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.24 (s, 2H), 7.19-7.36 (m, 5H), 7.65-7.75 (m, 5H), 8.16 (s, 1H), 8.34 (s, 1H), 9.07 (s, 1H), 10.07 (s, 1H) ; ^{13}C NMR (DMSO-d6, 100 MHz) δ 48.67, 111.37, 115.78, 119.59, 120.37, 122.88, 122.98, 123.43, 125.30, 127.12, 127.89, 128.10, 129.15, 130.38, 134.02, 137.02, 137.19, 142.97, 147.57, 147.77, 155.98, 166.80

C97

Yield 79 %; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.44 (s, 2H), 7.33-7.47 (m, 5H), 7.67 (d, *J* = 3.2 Hz, 1H), 7.72-7.76 (m, 3H), 7.82 (d, *J* = 8 Hz, 1H), 7.85 (d, *J* = 8.8 Hz, 1H), 8.16 (d, *J* = 3.2 Hz, 1H), 8.23 (bs, 1H), 10.29 (s, 1H) ; ^{13}C NMR (DMSO-d6, 100 MHz) ^{13}C NMR (DMSO-d6, 100 MHz) δ 48.5, 111.77, 115.81, 120.12. 120.36, 123.62 (2C), 124.60, 125.34, 127.15, 127.92, 128.11, 129.19, 130.41, 134.03, 136.99, 137.68, 142.66, 144.89, 145.85, 159.95, 166.49.

C84

Yield 78 %; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.34 (s, 2H), 7.28 (t, *J* = 8.4 Hz, 1H), 7.31 (t, *J* = 8.8 Hz, 1H), 7.38 (t, *J* = 7.2 Hz, 1H), 7.45 (t, *J* = 7.6 Hz, 1H), 7.69 (d, *J* = 3.2 Hz, 1H), 7.74 (s, 1H), 7.76 (d, *J* = 8.0 Hz, 1H), 7.84 (d, *J* = 8.0 Hz, 1H), 8.12 (d, *J* = 3.2 Hz, 1H), 10.45 (s, 1H) ; ^{13}C NMR (DMSO-d6, 100 MHz) δ 48.60, 111.74, 119.74, 120.12, 120.86, 123.64 (2C), 124.62, 125.50, 131.50, 131.74, 137.60, 139.49, 142.60, 144.88, 145.71, 159.82, 166.90.

C86

Yield 75 %; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 5.70 (s, 2H), 7.26 (t, *J* = 2 Hz, 1H), 7.28 (t, *J* = 2 Hz, 1H), 7.46 (dd, J_1 = 8.8 Hz, J_2 = 2.4 Hz 1H), 7.57 (d, *J* = 8.8 Hz, 1H), 7.64-7.66 (m, 1H), 7.69-7.71 (m, 1H), 7.91 (d, *J* = 2.4 Hz, 1H), 8.5 (d, *J* = 1.6 Hz, 1H), 9.28

(d, J = 2.4 Hz, 1H), 10.77 (s, 1H) ; ^{13}C NMR (DMSO-d6, 100 MHz) δ 48.71, 111.37, 119.60, 119.75, 120.86, 122.90, 123.03, 123.47, 125.45, 131.48, 131.72, 137.15, 139.55, 142.92, 147.43, 147.66, 156.03, 167.23

REFERENCES

(1) Geiser, M.; Cebe, R.; Drewello, D.; Schmitz, R. *Biotechniques* **2001**, *31*, 88-90, 92.

(2) Umejiego, N. N.; Li, C.; Riera, T.; Hedstrom, L.; Striepen, B. *J Biol Chem* **2004**, *279*, 40320-40327.

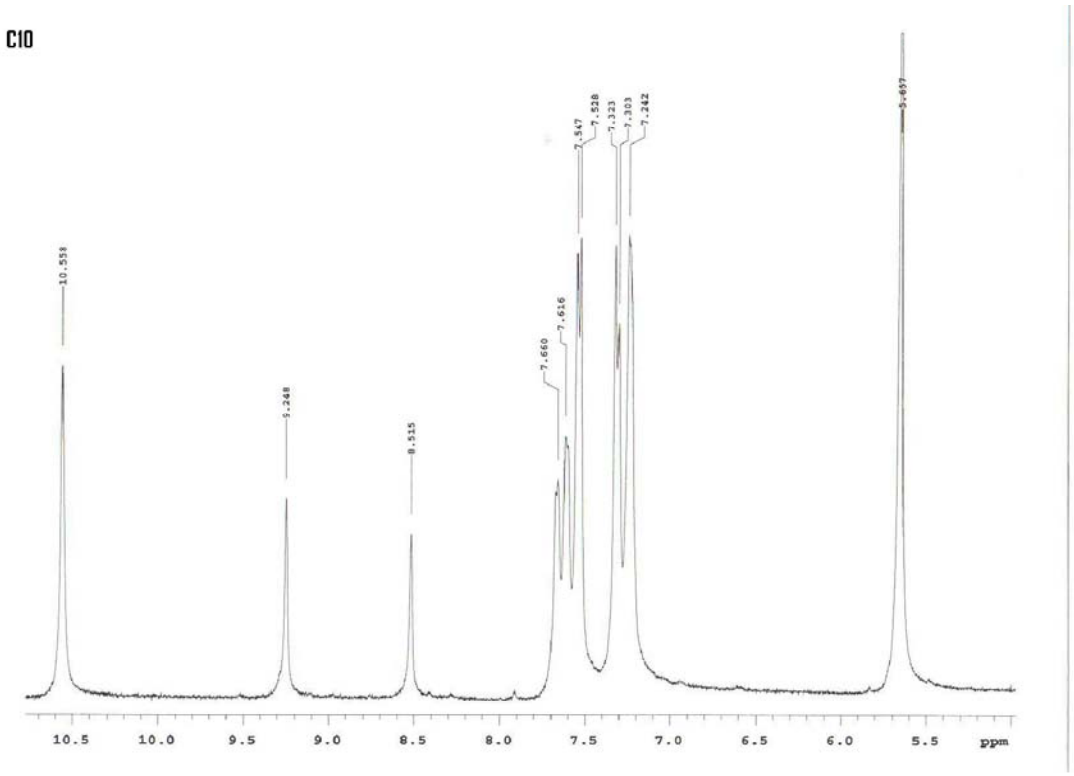
(3) Datsenko, K. A.; Wanner, B. L. *Proc Natl Acad Sci U S A* **2000**, *97*, 6640-5.

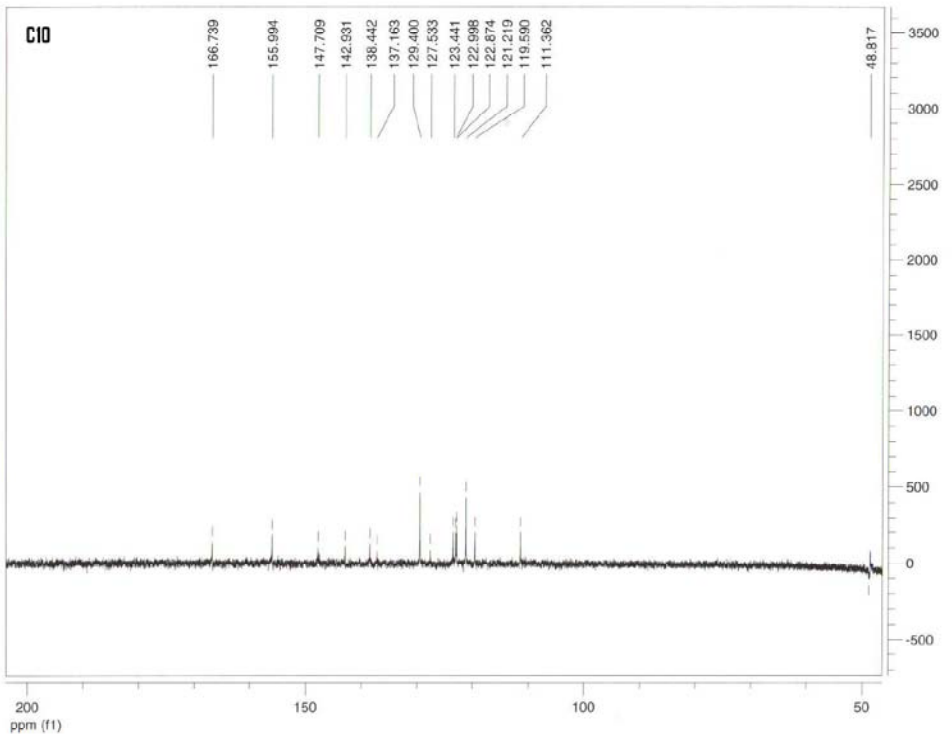
(4) Vagin, A.; Teplyakov, A. *J. Appl. Cryst.* **1997**, *30*, 1022-1025.

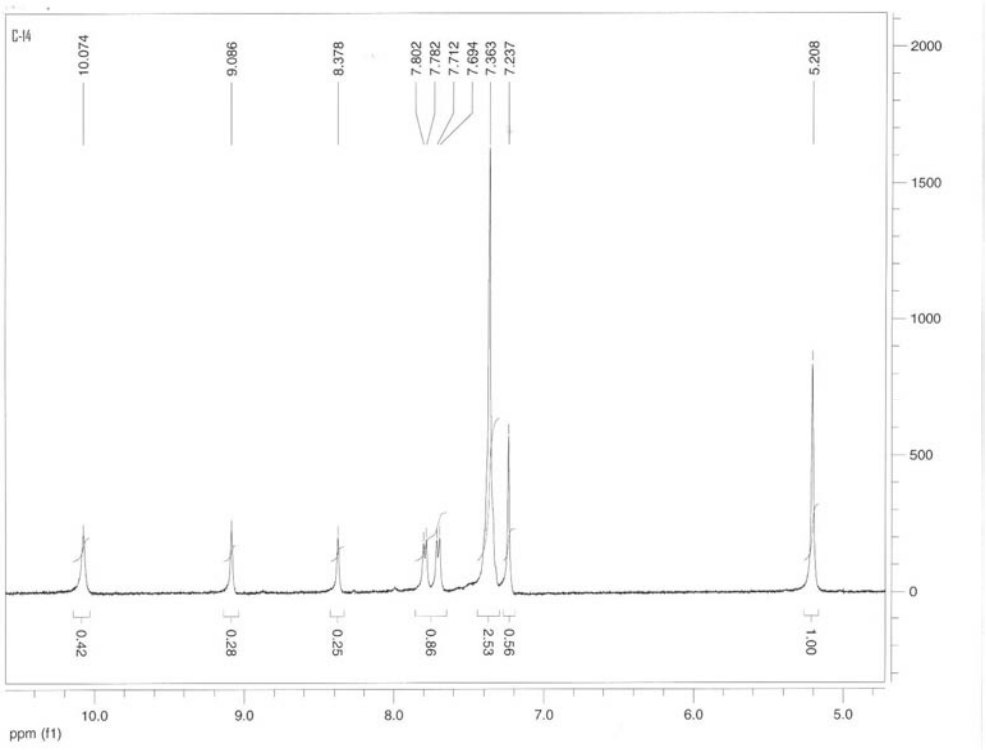
(5) Murshudov, G. N.; Vagin, A. A.; Dodson, E. J. *Acta crystallographica* **1997**, *53*, 240-255.

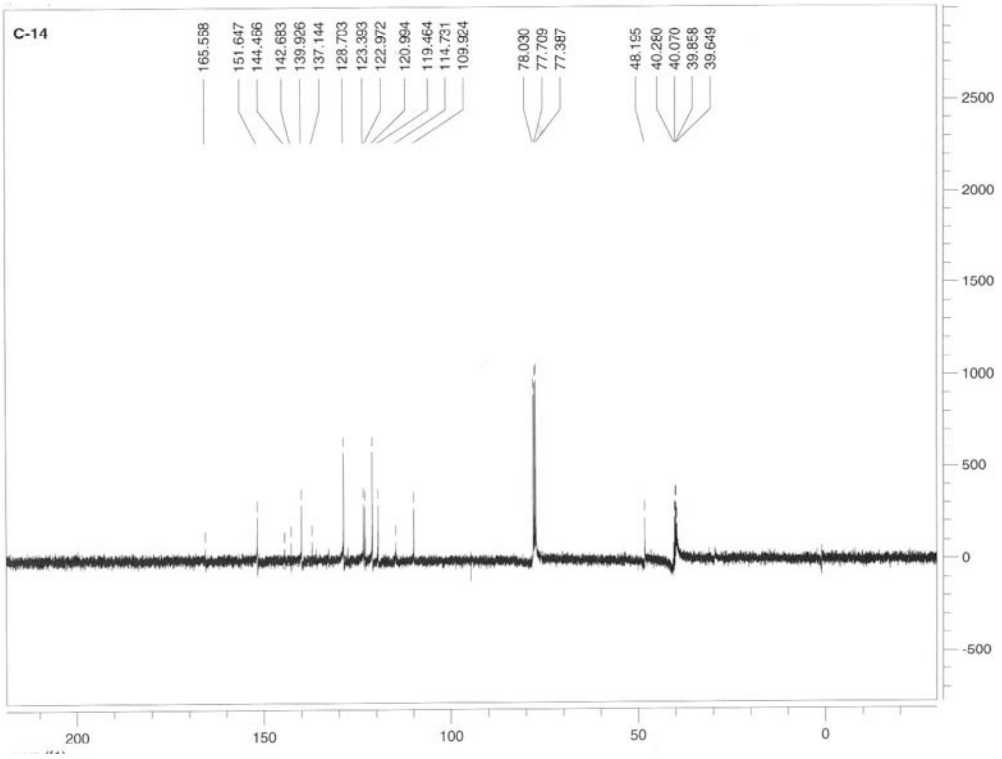
(6) Emsley, P.; Cowtan, K. *Acta crystallographica* **2004**, *60*, 2126-2132.

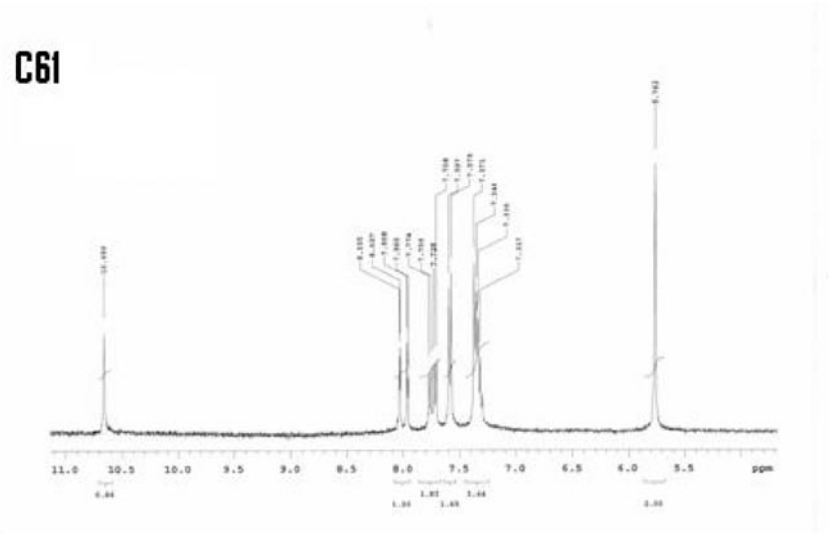
NMR Spectra

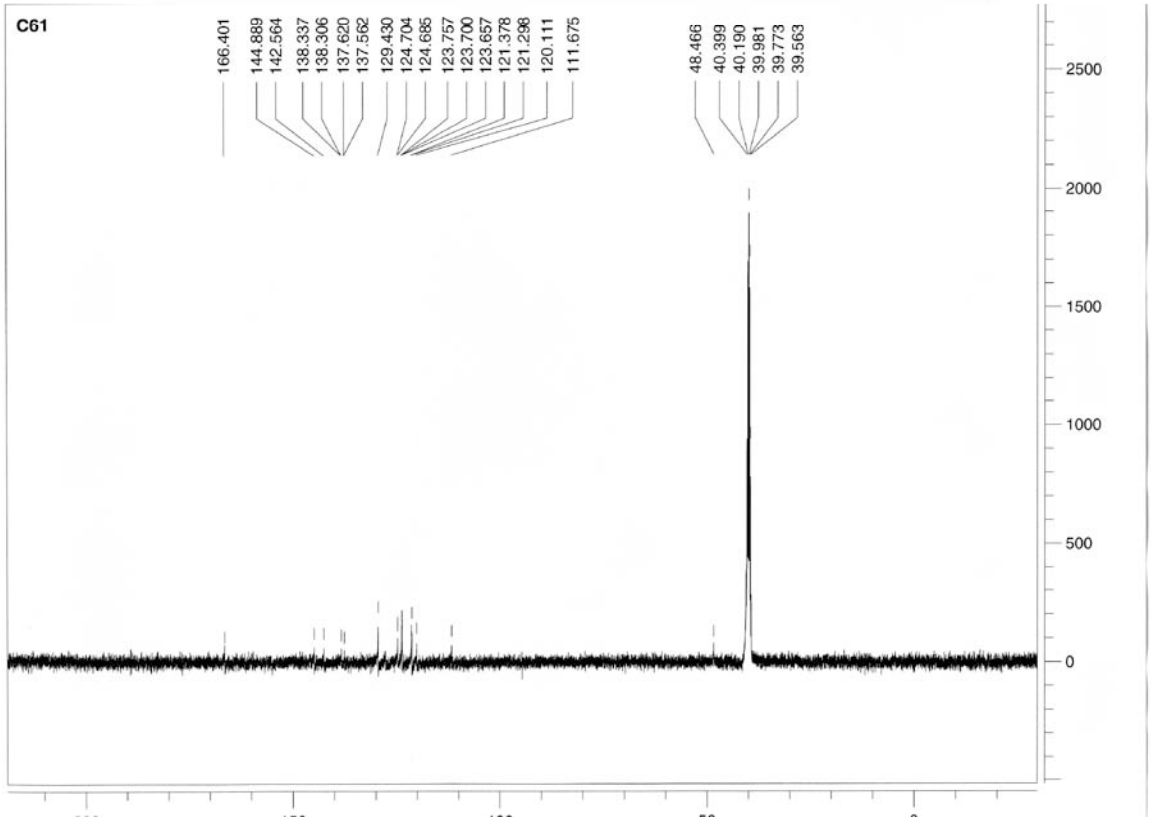

C10
10.553
9.248
8.535
7.660
7.616
7.547
7.528
7.323
7.303
7.242
5.457
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
ppm

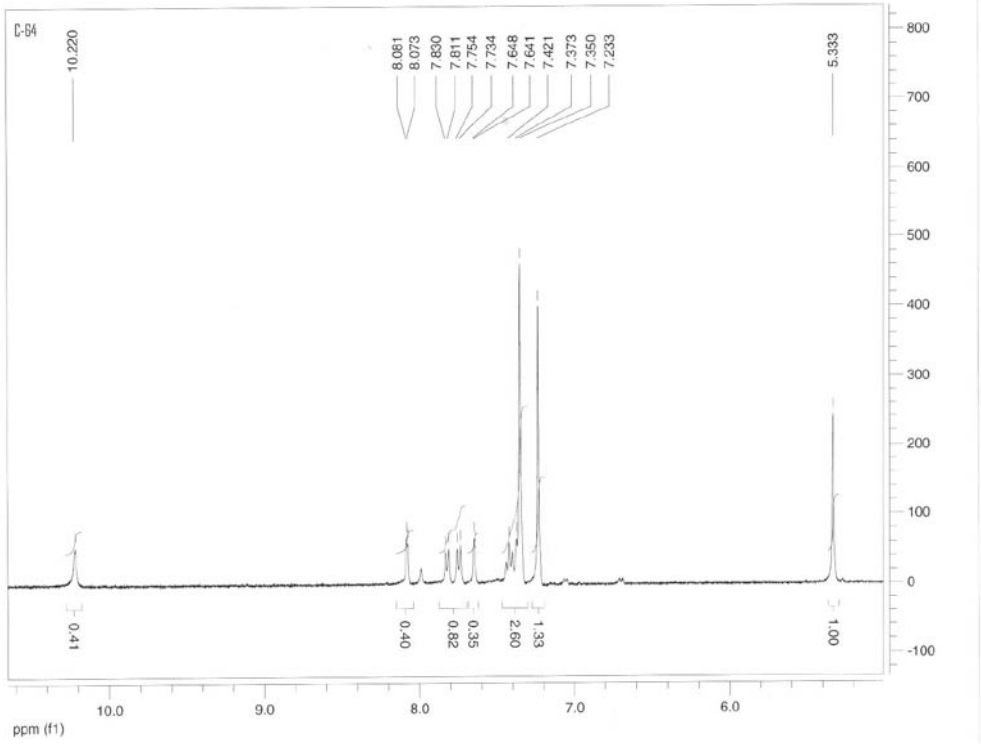

C10
166.739
155.994
147.709
142.931
138.442
137.163
129.400
127.533
123.441
122.998
122.874
121.219
119.590
111.362
48.817
3500
3000
2500
2000
1500
1000
500
0
-500
200
150
100
50
ppm (f1)

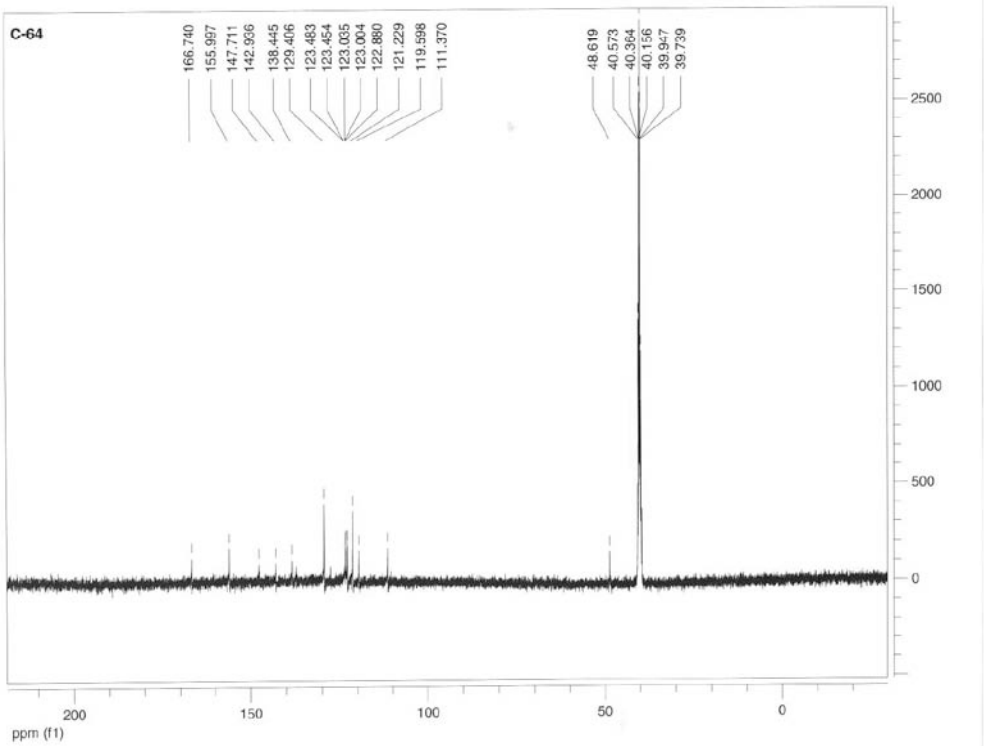

C-14
10.074
9.086
8.378
7.802
7.782
7.712
7.694
7.363
7.237
5.208
0.42
0.28
0.25
0.86
2.53
0.56
1.00
ppm (f1)

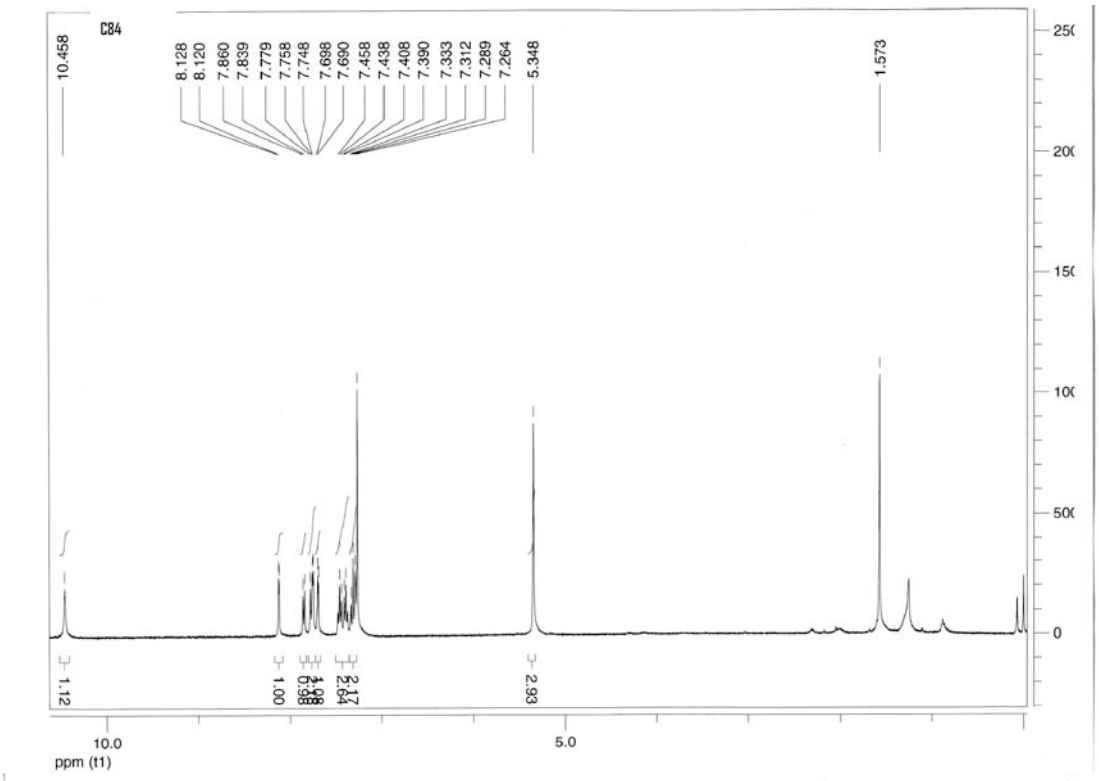

C-14
165.558
151.647
144.466
142.683
139.926
137.144
128.703
123.393
122.972
120.994
119.464
114.731
109.924
78.030
77.709
77.387
48.155
40.260
40.070
39.858
39.649

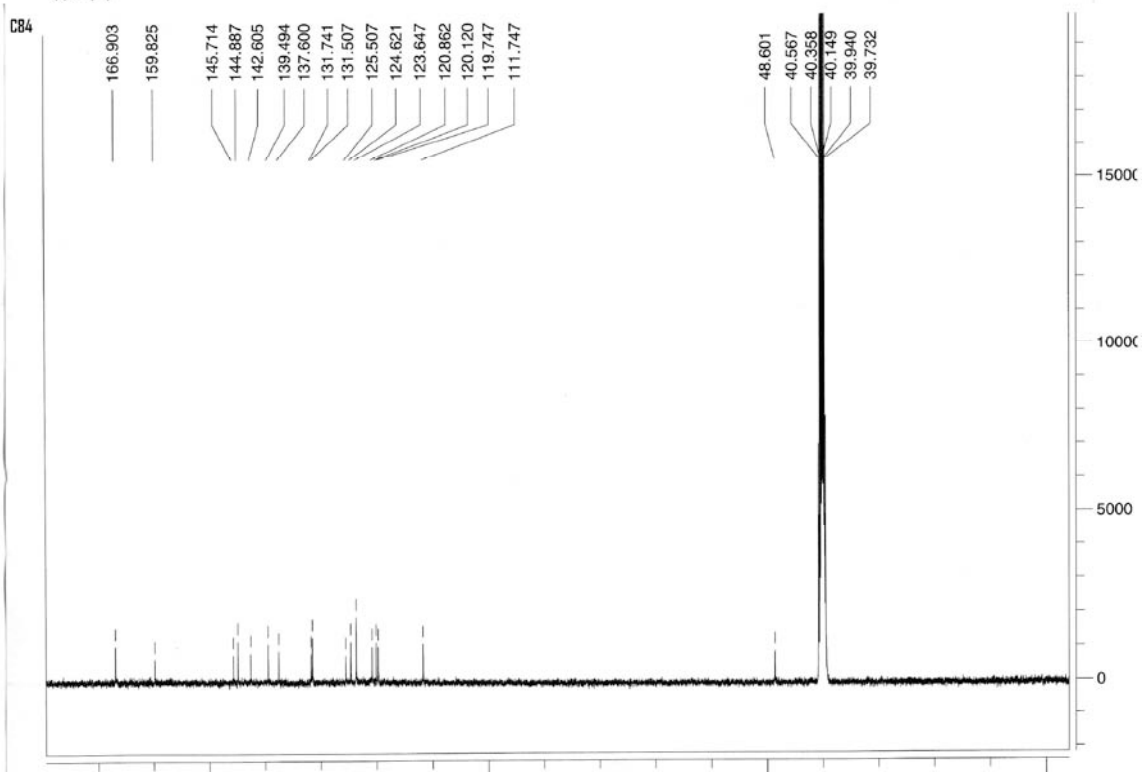

C61
11.0
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
ppm

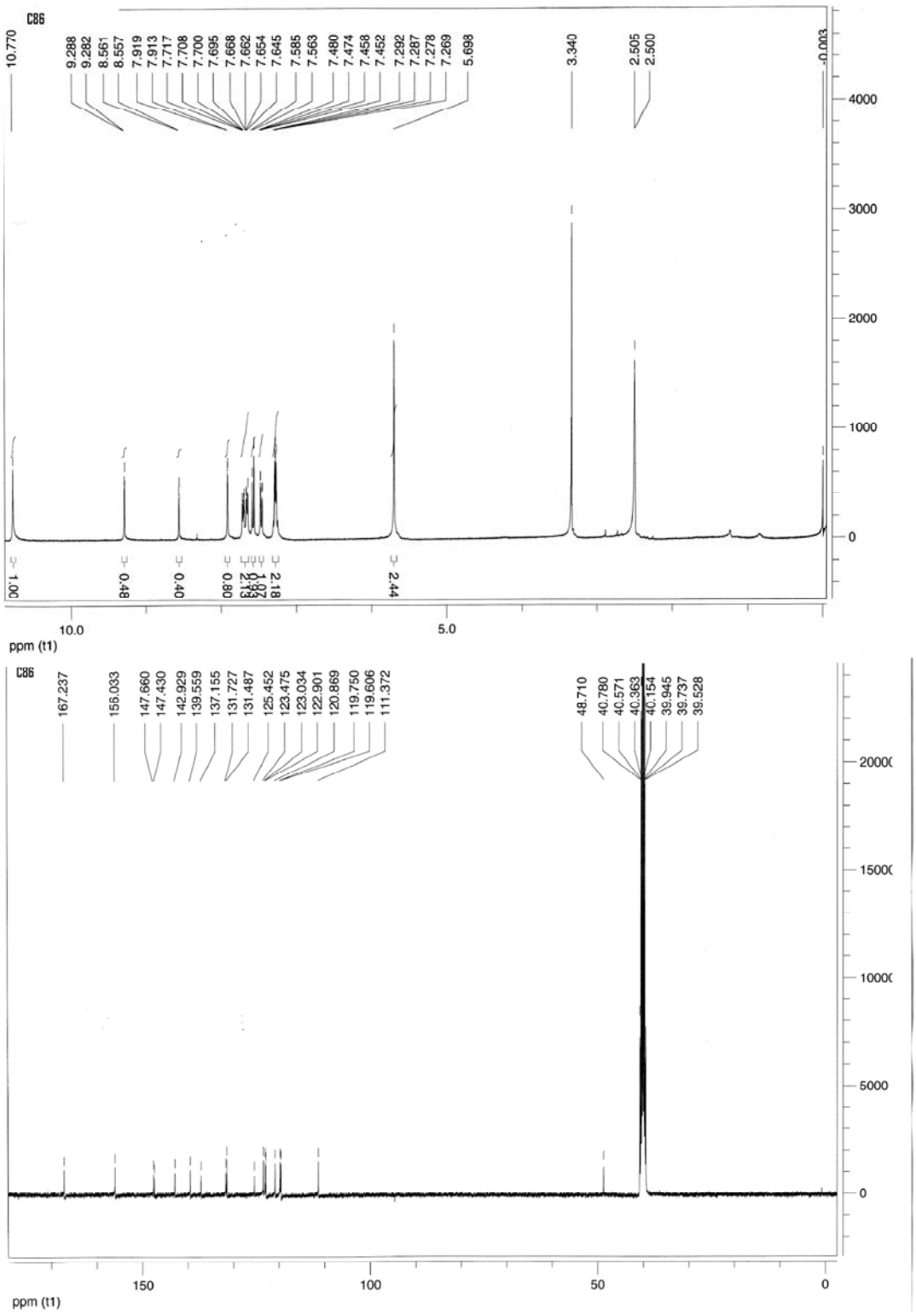

C61
166.401
144.889
142.564
138.337
138.306
137.620
137.562
129.430
124.704
124.685
123.757
123.700
123.657
121.378
121.296
120.111
111.675
48.466
40.399
40.190
39.981
39.773
39.563
2500
2000
1500
1000
500
0

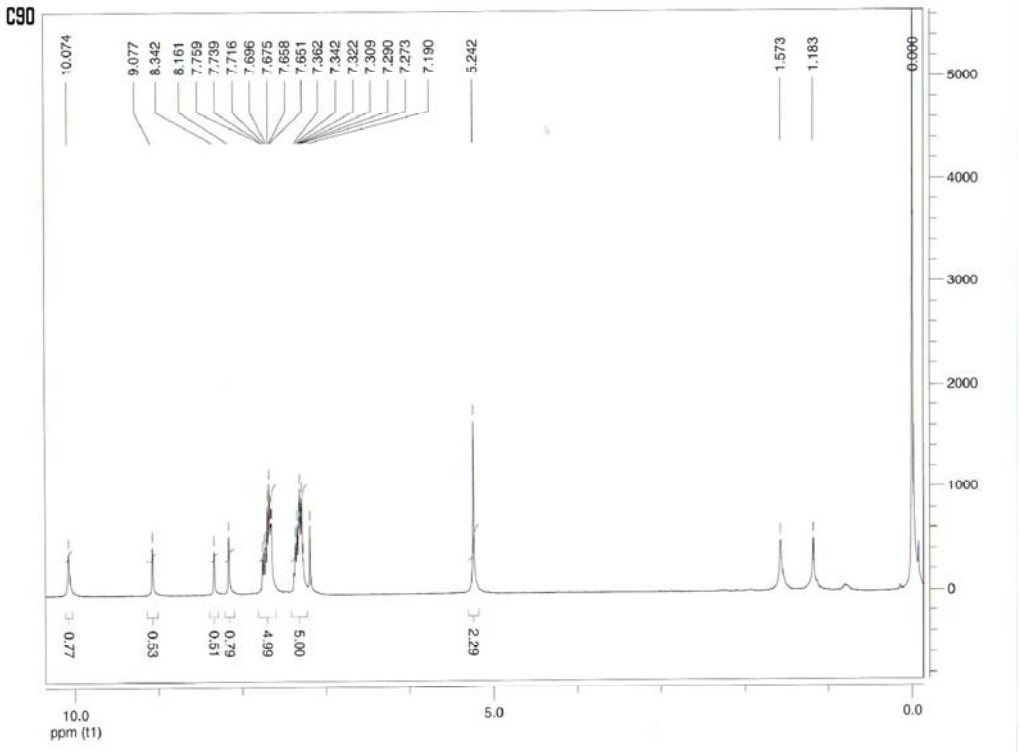

C-64
10.220
8.061
8.073
7.830
7.811
7.754
7.734
7.648
7.641
7.421
7.373
7.350
7.233
5.333
0.41
0.40
0.82
0.35
2.69
1.33
1.00
ppm (f1)

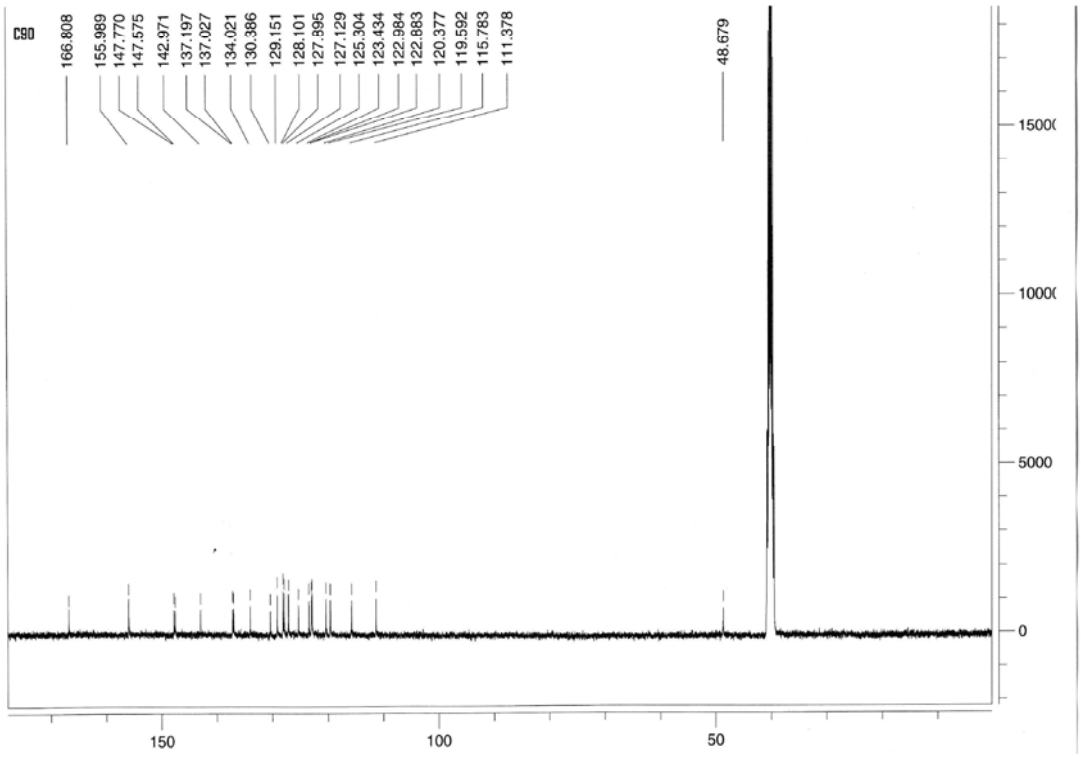

C-64
166.740
155.997
147.711
142.936
138.445
129.406
123.483
123.454
123.035
123.004
122.880
121.229
119.598
111.370
48.619
40.573
40.364
40.156
39.947
39.739
ppm (f1)

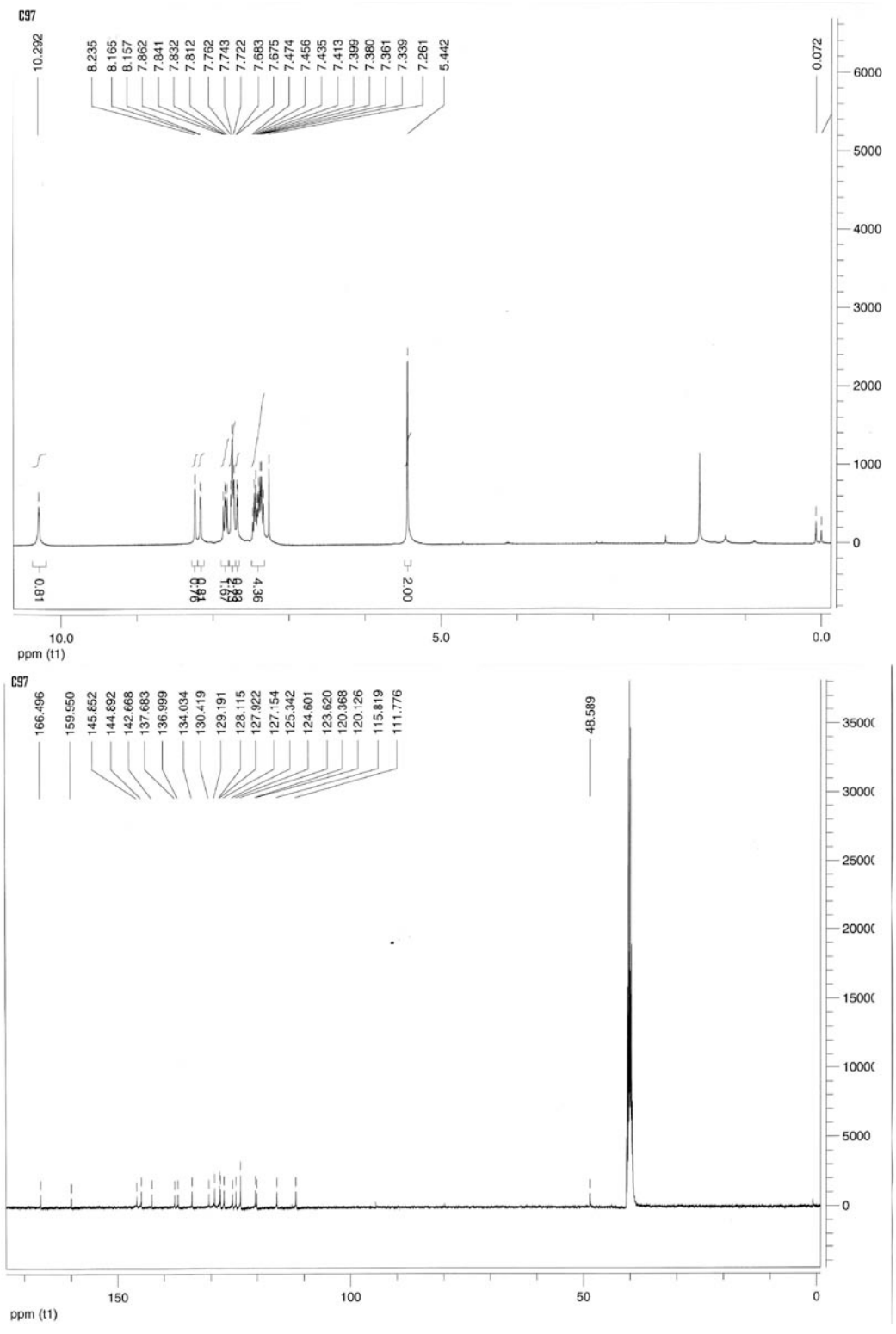

C84
10.458
8.128
8.120
7.860
7.839
7.779
7.758
7.748
7.698
7.690
7.458
7.438
7.408
7.390
7.333
7.312
7.289
7.264
5.348
1.573
1.12
1.00
2.17
2.93
10.0
5.0
ppm (t1)


C84
166.903
159.825
145.714
144.887
142.605
139.494
137.600
131.741
131.507
125.507
124.621
123.647
120.862
120.120
119.747
111.747
48.601
40.567
40.358
40.149
39.940
39.732


C86
10.770
9.288
9.282
8.561
8.557
7.919
7.913
7.717
7.708
7.700
7.695
7.668
7.662
7.654
7.645
7.585
7.563
7.480
7.474
7.458
7.452
7.292
7.287
7.278
7.269
5.698
3.340
2.505
2.500
-0.003
1.00
0.48
0.40
0.80
2.18
2.44
10.0
5.0
ppm (t1)
C86
167.237
156.033
147.660
147.430
142.929
139.559
137.155
131.727
131.487
125.452
123.475
123.034
122.901
120.869
119.750
119.606
111.372
48.710
40.780
40.571
40.363
40.154
39.945
39.737
39.528
150
100
50
0
ppm (t1)


C90
10.074
9.077
8.342
8.161
7.759
7.739
7.716
7.696
7.675
7.658
7.651
7.362
7.342
7.322
7.309
7.290
7.273
7.190
5.242
1.573
1.183
0.000
0.77
0.53
0.51
0.79
4.99
5.00
2.29
10.0
ppm (t1)
5.0
0.0

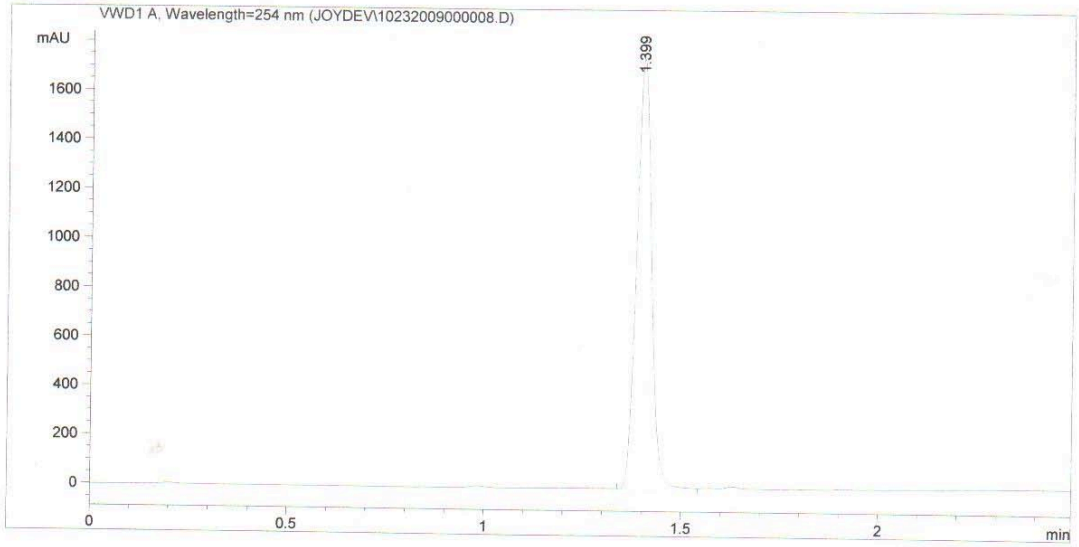

C90
166.808
155.989
147.770
147.575
142.971
137.197
137.027
134.021
130.386
129.151
128.101
127.995
127.129
125.304
123.434
122.984
122.883
120.377
119.592
115.783
111.378
48.679
150
100
50


C97
10.292
8.235
8.165
8.157
7.862
7.841
7.832
7.812
7.762
7.743
7.722
7.683
7.675
7.474
7.456
7.435
7.413
7.399
7.380
7.361
7.339
7.261
5.442
0.072
0.81
4.36
2.00
10.0
5.0
0.0
ppm (t1)
C97
166.496
159.550
145.852
144.892
142.668
137.683
136.999
134.034
130.419
129.191
128.115
127.922
127.154
125.342
124.601
123.620
120.368
120.126
115.819
111.776
48.589
150
100
50
0
ppm (t1)

HPLC Chromatograms. Method: Acetonitrile/ water (1:9)

C10

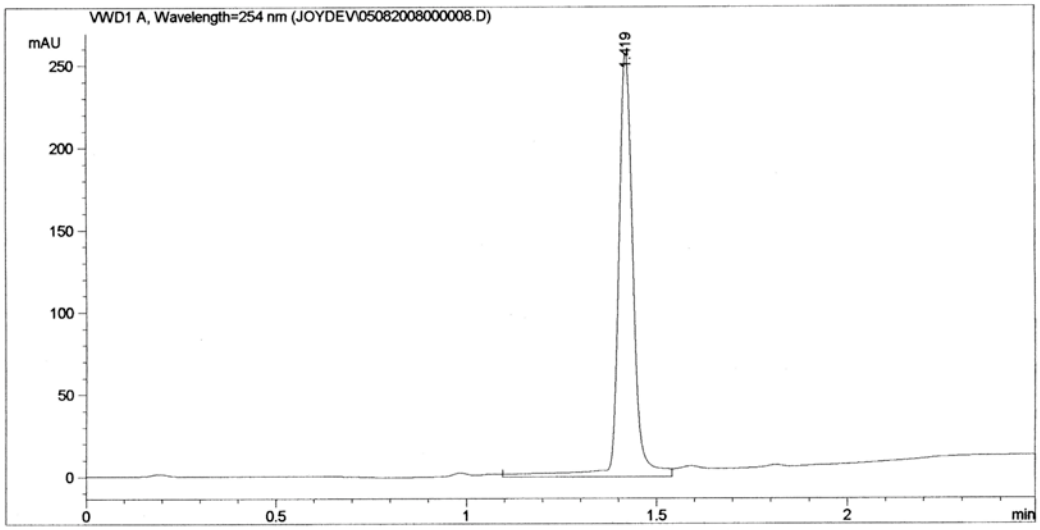
```
================================================================================
Acq. Operator   : Joydev-shiva                    Seq. Line :   1
Acq. Instrument : Instrument 1                     Location : Vial 41
Injection Date  : 10/23/2009 4:49:40 PM               Inj :   1
                                                  Inj Volume : Inj prog
Sequence File   : C:\CHEM32\1\SEQUENCE\DEF_LC1.S
Method          : C:\CHEM32\1\METHODS\SB-C8-254NM.M
Last changed    : 4/4/2009 7:35:01 PM by Joydev
Method Info     : injector program
```




```
================================================================================
                          Area Percent Report
================================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=254 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]       [min]  mAU   *s    [mAU   ]     %
----|-------|----|-------|----------|----------|--------|
   1   1.399 VV    0.0409 4627.63037 1757.30823 100.0000

Totals :                  4627.63037 1757.30823

================================================================================
                          *** End of Report ***
```

C14

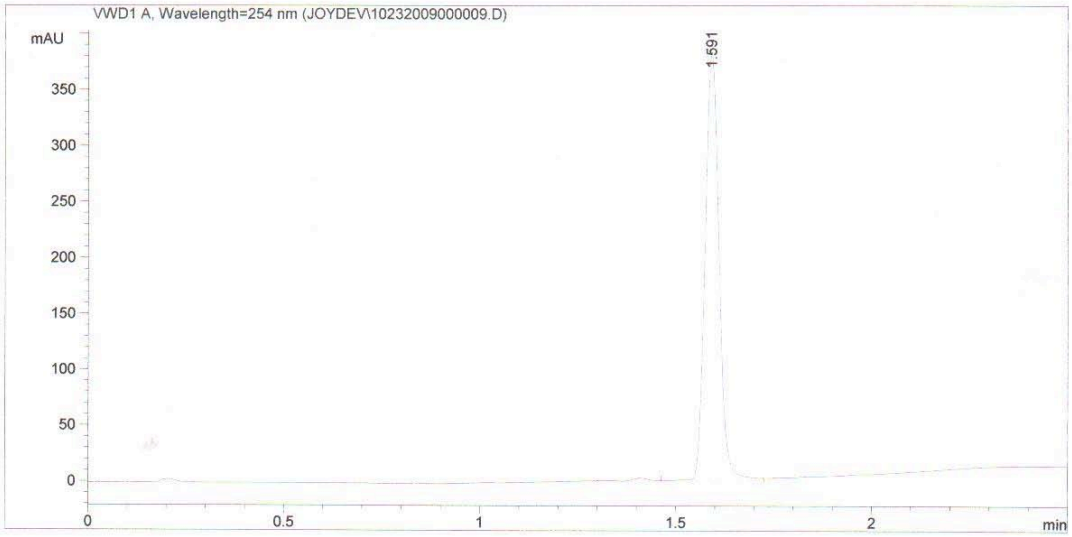
```
==========================================================================
Acq. Operator   : Joydev                          Seq. Line :   1
Acq. Instrument : Instrument 1                     Location : Vial 21
Injection Date  : 5/8/2008 3:06:04 PM                 Inj :   1
                                                  Inj Volume : Inj prog
Sequence File   : C:\CHEM32\1\SEQUENCE\DEF_LC1.S
Method          : C:\Chem32\1\METHODS\SB-C8-254NM.M
Last changed    : 1/28/2008 2:54:39 PM by lqiao
Method Info     : injector program
```




```
==========================================================================
                          Area Percent Report
==========================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=254 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]  mAU   *s    [mAU   ]      %
----|-------|----|-------|----------|----------|--------|
   1   1.419 VV    0.0403  702.65326  259.75424 100.0000

Totals :                   702.65326  259.75424

==========================================================================
                          *** End of Report ***
```

C6I

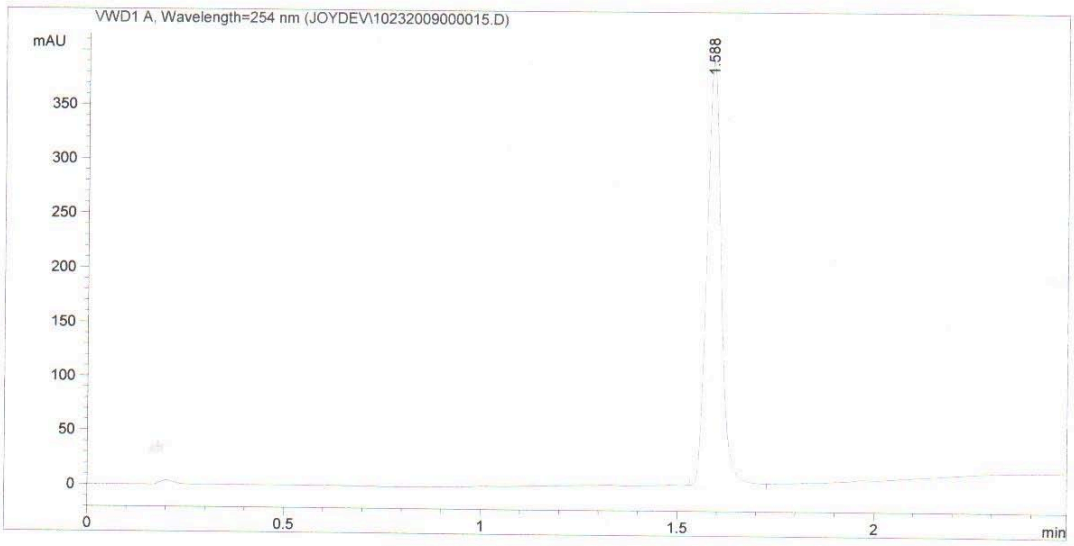
```
Acq. Operator   : Joydev-shiva                    Seq. Line :   2
Acq. Instrument : Instrument 1                    Location : Vial 42
Injection Date  : 10/23/2009 4:54:12 PM           Inj :   1
                                                  Inj Volume : Inj prog
Sequence File   : C:\CHEM32\1\SEQUENCE\DEF_LC1.S
Method          : C:\CHEM32\1\METHODS\SB-C8-254NM.M
Last changed    : 4/4/2009 7:35:01 PM by Joydev
Method Info     : injector program
```




```
                        Area Percent Report

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=254 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]  mAU   *s   [mAU   ]      %
----|-------|----|-------|----------|----------|--------|
   1   1.591 VV    0.0403  998.38293  386.41644 100.0000

Totals :                   998.38293  386.41644

                        *** End of Report ***
```

C64

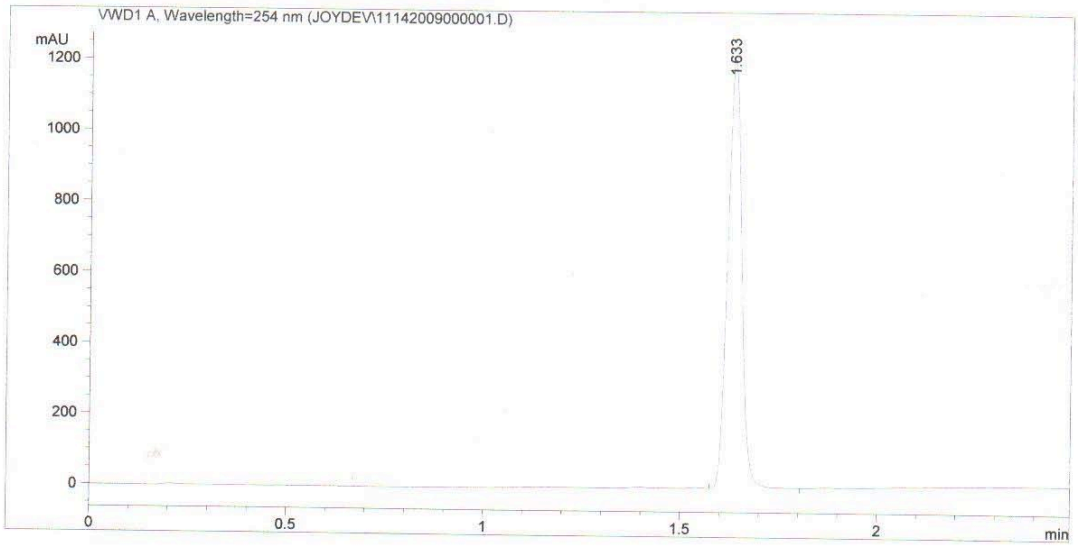
```
================================================================================
Acq. Operator    : Joydev-shiva                      Seq. Line :   1
Acq. Instrument  : Instrument 1                       Location : Vial 41
Injection Date   : 10/23/2009 5:33:46 PM                  Inj :   1
                                                   Inj Volume : Inj prog
Sequence File    : C:\CHEM32\1\SEQUENCE\DEF_LC1.S
Method           : C:\CHEM32\1\METHODS\SB-C8-254NM.M
Last changed     : 4/4/2009 7:35:01 PM by Joydev
Method Info      : injector program
```




```
================================================================================
                          Area Percent Report
================================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=254 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]  mAU   *s   [mAU   ]      %
----|-------|----|-------|----------|----------|--------|
   1   1.588 VV    0.0406 1032.09961  395.72729 100.0000

Totals :                  1032.09961  395.72729

================================================================================
                          *** End of Report ***
```

C84

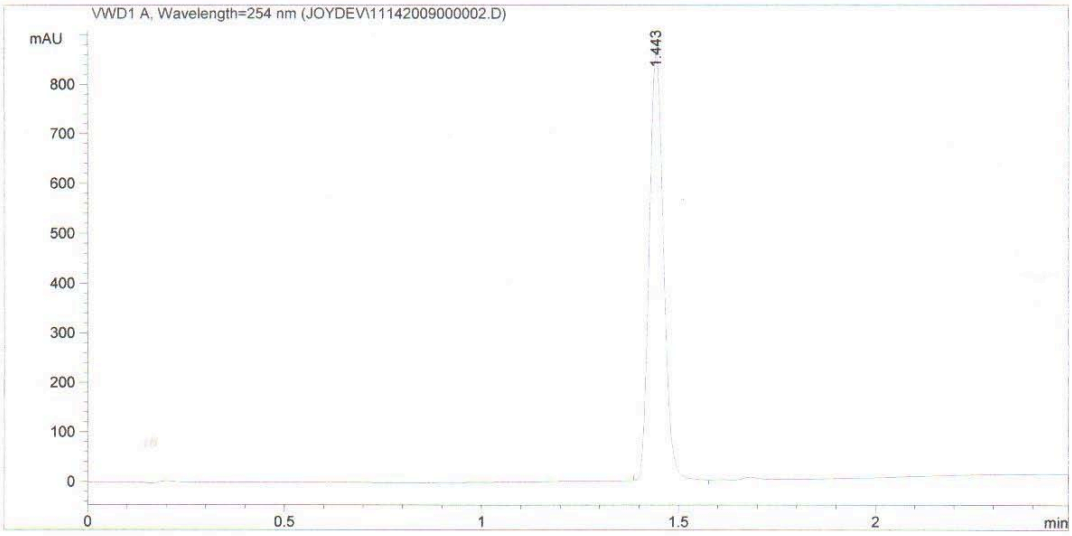
```
Acq. Operator    : Joydev-shiva                    Seq. Line :   1
Acq. Instrument  : Instrument 1                     Location : Vial 41
Injection Date   : 11/14/2009 4:14:22 PM               Inj :   1
                                                  Inj Volume : Inj prog
Sequence File    : C:\CHEM32\1\SEQUENCE\DEF_LC1.S
Method           : C:\CHEM32\1\METHODS\SB-C8-254NM.M
Last changed     : 4/4/2009 7:35:01 PM by Joydev
Method Info      : injector program
```




```
                        Area Percent Report

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=254 nm

Peak RetTime Type  Width     Area       Height      Area
  #   [min]        [min]  mAU   *s    [mAU   ]       %
----|-------|----|-------|----------|----------|--------|
   1   1.633 VV    0.0380 3077.17993 1225.28430 100.0000

Totals :                  3077.17993 1225.28430

                        *** End of Report ***
```

C90

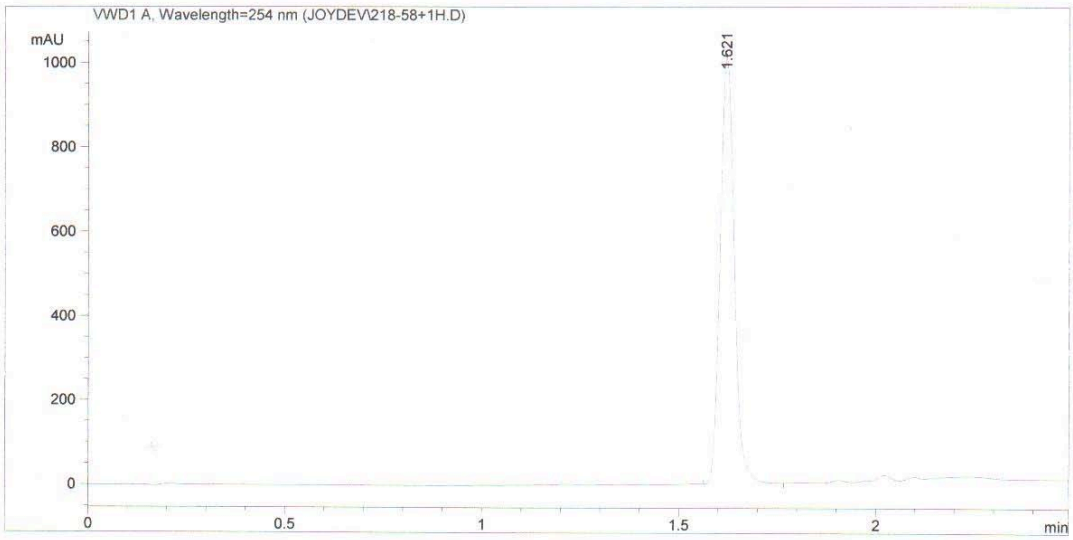
```
==========================================================================
Acq. Operator   : Joydev-shiva                    Seq. Line :   2
Acq. Instrument : Instrument 1                     Location : Vial 42
Injection Date  : 11/14/2009 4:18:53 PM               Inj :   1
                                                  Inj Volume : Inj prog
Sequence File   : C:\CHEM32\1\SEQUENCE\DEF_LC1.S
Method          : C:\CHEM32\1\METHODS\SB-C8-254NM.M
Last changed    : 4/4/2009 7:35:01 PM by Joydev
Method Info     : injector program
```




```
==========================================================================
                        Area Percent Report
==========================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=254 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]  mAU   *s    [mAU   ]     %
----|-------|----|-------|----------|----------|--------|
   1   1.443 VV    0.0400 2224.03418  869.30493 100.0000

Totals :                  2224.03418  869.30493

--------------------------------------------------------------------------
```

C97

```
Acq. Operator   : Joydev-Suresh                    Seq. Line :   3
Acq. Instrument : Instrument 1                     Location : Vial 43
Injection Date  : 11/6/2009 10:42:28 AM                Inj :   1
                                                  Inj Volume : Inj prog
Sequence File   : C:\CHEM32\1\SEQUENCE\DEF_LC1.S
Method          : C:\CHEM32\1\METHODS\SB-C8-254NM.M
Last changed    : 4/4/2009 7:35:01 PM by Joydev
Method Info     : injector program
```

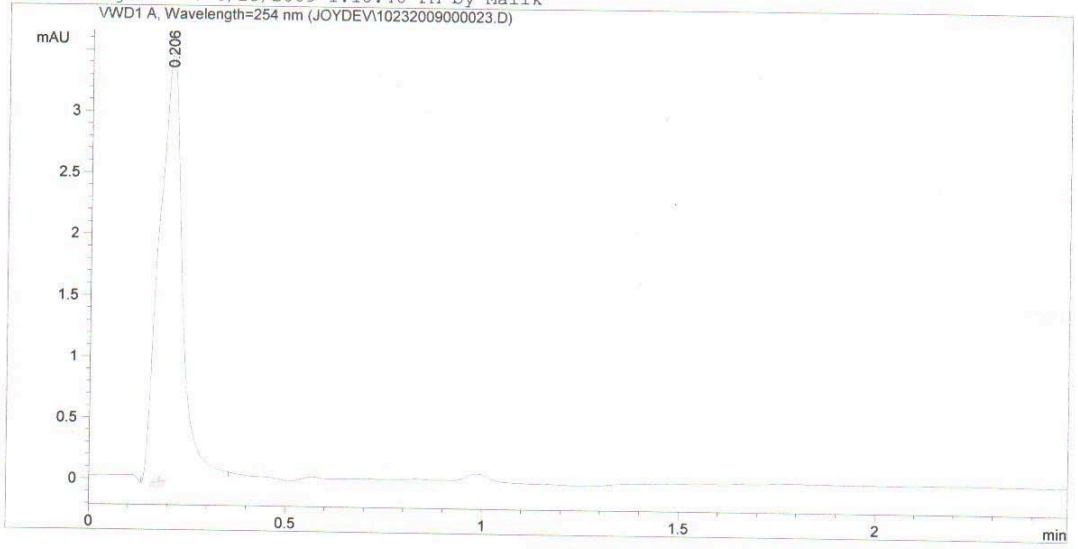



```
                        Area Percent Report

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=254 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]  mAU   *s   [mAU   ]      %
----|-------|----|-------|----------|----------|--------|
   1   1.621 VV    0.0392 2527.57642 1018.20734 100.0000

Totals :                  2527.57642 1018.20734

                        *** End of Report ***
```

HPLC Chromatograms. Method: methanol/ water (1:9)

C10

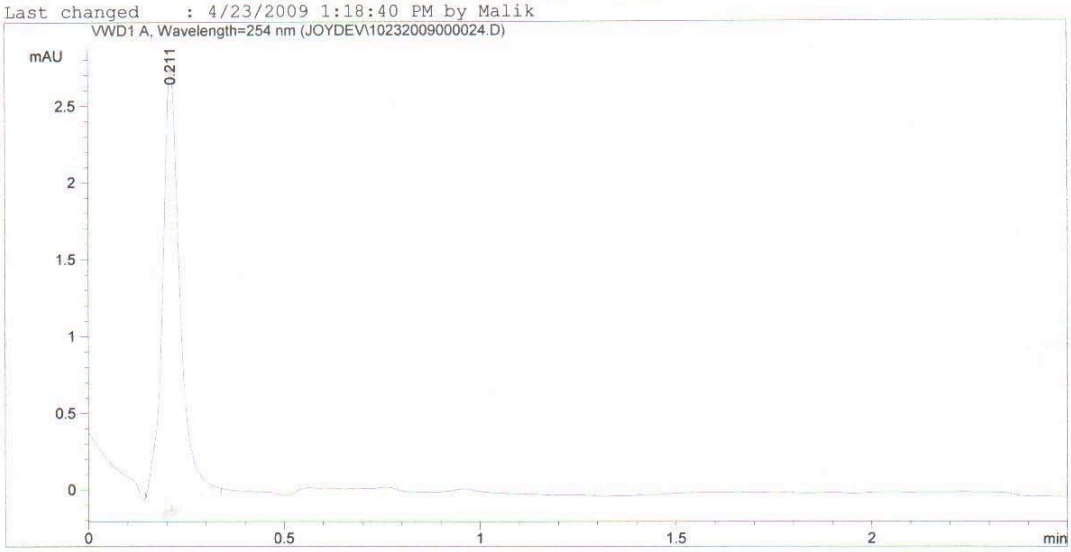
```
Acq. Operator    : Joydev-shiva                    Seq. Line :   1
Acq. Instrument  : Instrument 1                     Location : Vial 41
Injection Date   : 10/23/2009 6:02:11 PM               Inj :   1
                                                  Inj Volume : 5 µl
Sequence File    : C:\CHEM32\1\SEQUENCE\DEF_LC1.S
Method           : C:\CHEM32\1\METHODS\MALIK90WATER10MEOH.M
Last changed     : 4/23/2009 1:18:40 PM by Malik
```




```
                          Area Percent Report

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=254 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]       [min]  mAU   *s    [mAU   ]     %
----|-------|----|-------|----------|----------|--------|
   1   0.206 BB    0.0574   14.19989    3.51214 100.0000

Totals :                    14.19989    3.51214

                        *** End of Report ***
```

C6I

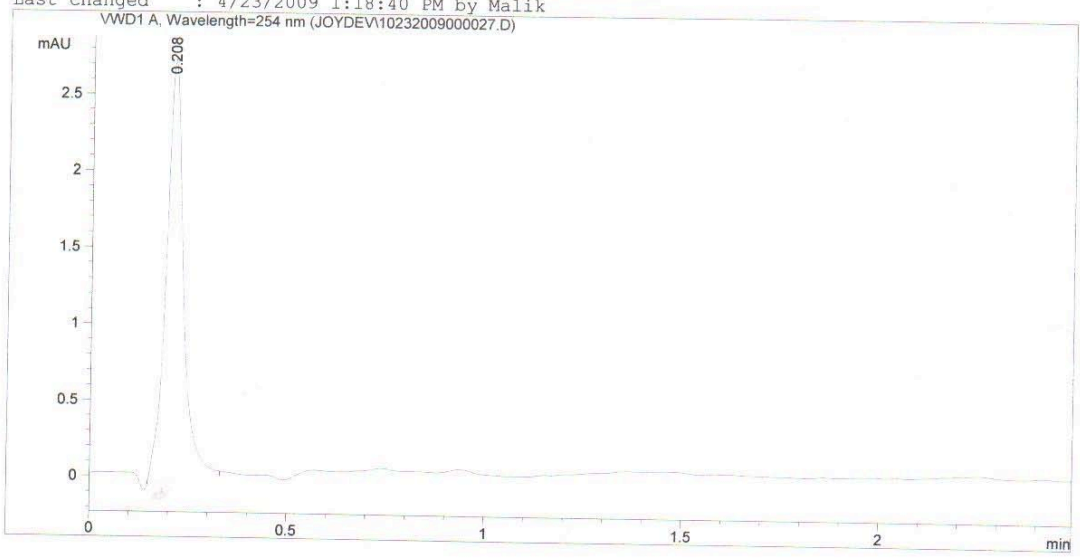
```
Acq. Operator    : Joydev-shiva                    Seq. Line :   2
Acq. Instrument  : Instrument 1                     Location : Vial 42
Injection Date   : 10/23/2009 6:05:46 PM                Inj :   1
                                                  Inj Volume : 5 µl
Sequence File    : C:\CHEM32\1\SEQUENCE\DEF_LC1.S
Method           : C:\CHEM32\1\METHODS\MALIK90WATER10MEOH.M
Last changed     : 4/23/2009 1:18:40 PM by Malik
```




```
                          Area Percent Report

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=254 nm

Peak RetTime Type  Width     Area      Height      Area
  #   [min]        [min]  mAU   *s   [mAU   ]       %
----|-------|----|-------|----------|----------|--------|
   1   0.211 BB    0.0448    8.70308    2.81324 100.0000

Totals :                     8.70308    2.81324

                          *** End of Report ***
```

C64

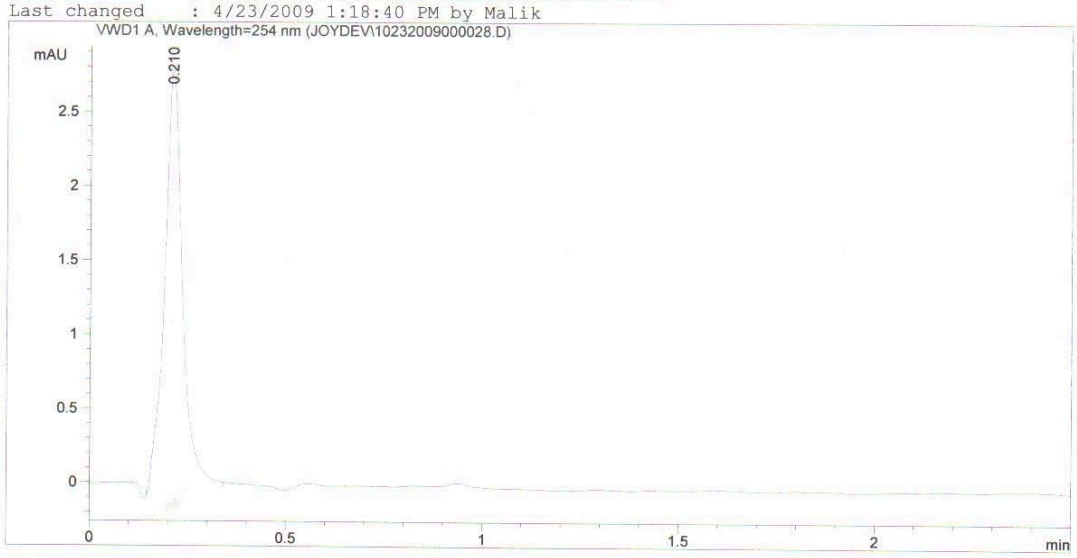
```
Acq. Operator   : Joydev-shiva                    Seq. Line :   1
Acq. Instrument : Instrument 1                    Location : Vial 41
Injection Date  : 10/23/2009 6:27:46 PM              Inj :   1
                                                  Inj Volume : 5 µl
Sequence File   : C:\CHEM32\1\SEQUENCE\DEF_LC1.S
Method          : C:\CHEM32\1\METHODS\MALIK90WATER10MEOH.M
Last changed    : 4/23/2009 1:18:40 PM by Malik
```




```
                        Area Percent Report

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=254 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]  mAU   *s   [mAU   ]      %
----|-------|----|-------|----------|----------|--------|
   1   0.208 BB    0.0461    8.54374    2.77505 100.0000

Totals :                     8.54374    2.77505

                      *** End of Report ***
```

C86

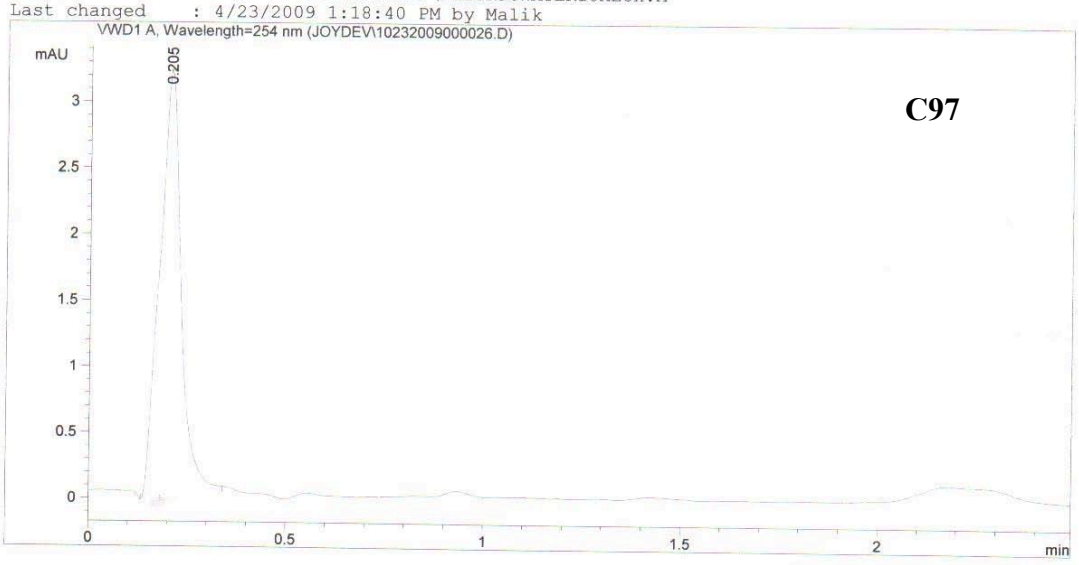
```
==================================================================================
Acq. Operator   : Joydev-shiva                      Seq. Line :   2
Acq. Instrument : Instrument 1                       Location : Vial 42
Injection Date  : 10/23/2009 6:31:23 PM                  Inj :   1
                                                   Inj Volume : 5 µl
Sequence File   : C:\CHEM32\1\SEQUENCE\DEF_LC1.S
Method          : C:\CHEM32\1\METHODS\MALIK90WATER10MEOH.M
Last changed    : 4/23/2009 1:18:40 PM by Malik
```




```
==================================================================================
                          Area Percent Report
==================================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=254 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]  mAU   *s   [mAU   ]      %
----|-------|----|-------|----------|----------|--------|
   1   0.210 BB    0.0464    9.28219    2.87325 100.0000

Totals :                     9.28219    2.87325

==================================================================================
                          *** End of Report ***
```

C97

```
Acq. Operator    : Joydev-shiva                    Seq. Line :   4
Acq. Instrument  : Instrument 1                     Location : Vial 44
Injection Date   : 10/23/2009 6:12:58 PM                Inj :   1
                                                  Inj Volume : 5 µl
Sequence File    : C:\CHEM32\1\SEQUENCE\DEF_LC1.S
Method           : C:\CHEM32\1\METHODS\MALIK90WATER10MEOH.M
Last changed     : 4/23/2009 1:18:40 PM by Malik
```



C97

```
                        Area Percent Report

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=254 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]       [min]  mAU   *s   [mAU   ]      %
----|-------|----|-------|----------|----------|--------|
   1   0.205 BB    0.0531   12.34572    3.24812 100.0000

Totals :                    12.34572    3.24812

                        *** End of Report ***
```