AFFIDAVIT OF JERRY L. ATWOOD, Ph.D.

I, the undersigned, Jerry L. Atwood, US Passport No. 028224440 with a business address of Department of Chemistry, 601 S. College Avenue, University of Missouri-Columbia, Columbia, MO 65211, after having been warned that I must state the truth and if I fail to do so I will be liable to penalties prescribed by law, hereby declare in writing as follows:

1. I reside at 5704 Short Line Dr., Columbia, Missouri 65203. I hold a B.S. degree in Chemistry and Mathematics from Southwest Missouri State University (1964) and a Ph.D. in Chemistry from the University of Illinois (1968).

2. Since 1994, I have been employed as Professor and Chairman of the Department of Chemistry at the University of Missouri-Columbia. From 1968 to 1994, I was employed by the University of Alabama, where I successively held the titles of Assistant Professor, Associate Professor, Professor, and University Research Professor. In 1999 I became Curators' Professor at the University of Missouri-Columbia.

3. From 1985 to 1998, I was Editor of the *Journal of Chemical Crystallography*. In 1999 I was named Consulting Editor for the *Journal of Chemical Crystallography*. I edited the *Journal of Supramolecular Chemistry* from 2000 to 2003, and I was Associate Editor of *Chemical Communications* from 1996 to 2005. From 1992 until 2000, I was Editor of *Supramolecular Chemistry*. From 1985 to 1993, I was Regional Editor for the *Journal of Coordination Chemistry*. I have been Co-Editor-in-Chief of the *New Journal of Chemistry* since 2005. I am Co-Editor of the *Inclusion Compounds* book series (five volumes), *Comprehensive Supramolecular Chemistry* (ten volumes), and the *Encyclopedia of Supramolecular Chemistry* (2 volumes). I currently serve on the Editorial Boards of *Crystal Growth & Design*, *Crystal Engineering*, the *Journal of Coordination Chemistry*, the *New Journal of Chemistry*, and *Supramolecular Chemistry*. I have published more than 650 articles in refereed journals. I have authored twelve patents. I have taught more than 10,000 students in undergraduate University chemistry courses and I have taught and supervised graduate students (at both the

Masters and Ph.D. level) with a primary emphasis on organic synthesis and crystallization. I am an expert in the fields of organic and inorganic chemistry and crystal growth and engineering. Over the years, I have consulted numerous innovative and generic pharmaceutical companies with regard to issues pertaining to synthesis, processes and pre-formulation including crystallization, salt formation and polymorphism. I have consulted widely for industry, particularly in the fields of pharmaceutical chemistry and polymer chemistry. A copy of my *curriculum vitae* is attached hereto as Appendix "A".

4. I was requested by Applicant to review Prof. Serajuddin's declaration and IL 172,563 and respond to Prof. Serajuddin's assertions relating to the formation of phosphoric acid salts of 4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine (sitagliptin).

5. Prof. Serajuddin essentially argues that a skilled person would have expected that the only possible stable phosphoric acid salt form of sitagliptin would be the dihydrogenphosphate salt and that other phosphoric acid salt forms of sitagliptin are not chemically feasible. Briefly, Prof. Serajuddin argues that the pKa difference between phosphoric acid and sitagliptin is not sufficient to allow a stable phosphoric acid salt other than the dihydrogenphosphate salt.

6. As detailed below, Prof. Serajuddin's assertions are without any scientific merit and contradict first principles of chemistry. Prof. Serajuddin's assertions are also inconsistent with his own publications. As I elaborate below, there is every expectation that sitagliptin can form various phosphoric acid salts. Moreover, had Prof. Serajuddin carried out some experiments, he would have immediately found out that his entire assertions fly in the face of reality.

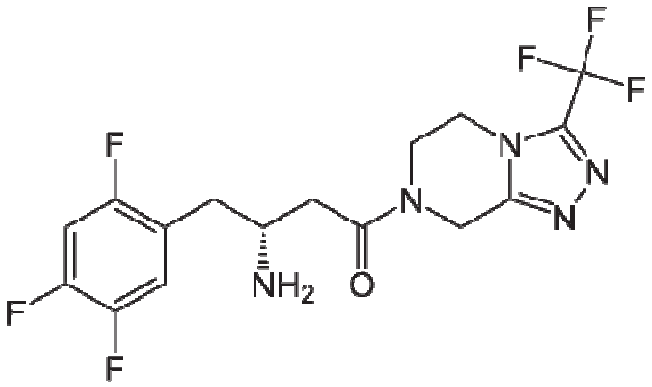
Phosphoric acids

7. Phosphoric acid (H_3PO_4) is a triprotic compound, i.e. it has three acidic protons which can be donated to an appropriate base to form salts. Therefore, phosphoric acid has the

potential for forming the dihydrogenphosphate salt ($H_2PO_4^-$), the monohydrogenphosphate salt (HPO_4^{-2}) and the phosphate salt (PO_4^{-3}). For instance, the known stable ammonium phosphates are $(NH_4)H_2PO_4$ (ammonium acid dihydrogenphosphate; CAS 7722-76-1), $(NH_4)_2HPO_4$ (diammonium acid phosphate; CAS 7783-28-0) and $(NH_4)_3PO_4$ (triammonium phosphate; CAS 10361-65-6). As phosphoric acid has three donatable protons, it is sometimes used with another cation such as potassium, sodium or ammonium. All these phosphoric acids are well known stable commercial products.

Sitagliptin base molecule and simple acid-base chemistry

8. The chemical structure of sitagliptin is as follows:



As can be readily observed, sitagliptin has several potential sites for protonation which can accept a proton from the acid.

9. The reaction of phosphoric acids with a base with multiple protonation sites such as sitagliptin is expected to result in various salts of different combinations, including differing stoichiometries. This is simple acid base chemistry.

pKa differences

10. As noted above, Prof. Serajuddin argues that the pKa difference between sitagliptin and phosphoric acid (H_3PO_4) does not allow the formation of any salt form other than the dihydrogenphosphate salt.

11. pKa is the negative log of the ionization constant for an acid or a base in aqueous solution. It is used as a measurement of the strength of the base or the acid in an aqueous medium (McMurry, pp. 52-53, Appendix "B").

12. The first pKa value of phosphoric acid is 2.12; the second is 7.21 and the third is 12.67. The pKa value of sitagliptin, as reported by Applicant during prosecution of IL 172,563, is 7.7. According to Prof. Serajuddin, in order to obtain a stable salt of a basic drug, "the pKa of the acid used should be at least 2-3 units below that of the drug". Therefore, Prof. Serajuddin concludes that phosphoric acid and sitagliptin can form a dihydrogenphosphate salt because the pKa difference between the first pKa of phosphoric acid (2.12) and sitagliptin (7.7) is above 3 units. However, the monohydrogenphosphate salt of sitagliptin cannot be formed because the pKa difference between the second pKa of phosphoric acid (7.21) and sitagliptin (7.7) is only 0.5.

13. As detailed below, Prof. Serajuddin's arguments are incorrect speculations.

pKa values in water are different from pKa values in other solvent systems

14. Prof. Serajuddin's starting point is totally flawed. He bases his entire argument on the pKa values of phosphoric acid and of sitagliptin in aqueous medium. These pKa values are an indication of ionization in water. However, salts are commonly formed in non-aqueous solutions. The pKa values in water are not a measure of the acid and base strength in a non-aqueous medium or in mixed organic–aqueous solvent systems and can vary significantly (F.G. Bordwell, "Equilibrium Acidities in Dimethyl Sulfoxide Solution", *Acc. Chem. Res.* 21 (1988), 456-463, Appendix "C"). The difference in pKa value of the same compound in aqueous medium vs. organic medium can be in many orders of magnitude.

15. For instance, A. Bhattacharyya et al., "Conductometric Studies on the Dissociation Constants of Phosphoric Acid in Methanol-Water Mixture", *Electrochimica Acta* 25 (1980), 559-561 (Appendix "D") teaches that in solutions of water and methanol in

varying concentrations the first pKa value of phosphoric acid can vary from 2.1 to 4.9. Similarly, H. Yüksek et al., "Synthesis and Determination of p*K*a Values of Some New 3,4-Disubstituted-4,5-Dihydro-1*H*-1,2,4-triazol-5-one Derivatives in Non-aqueous Solvents", *Molecules* 9 (2004), 232-240 (Appendix "E") also teaches that different medium can substantially impact the pKa value.

16. Therefore, even if Prof. Serajuddin's argument had any substance at all, it would have been relevant to the prediction of the making of sitagliptin phosphate salt **only** in water. It is not predictive for other commonly used solvents or solvent systems which are routinely used for salt formation. This is basic chemistry. It is also acknowledged by Prof. Serajuddin on page 159 of *Handbook of Pharmaceutical Salts* (Appendix 5 to Prof. Serajuddin's declaration). I am therefore surprised that Prof. Serajuddin did not mention this fact in his declaration.

pKa of sitagliptin was not known at the priority date of IL 172,563

17. It is my understanding that the pKa of sitagliptin was not known at the priority date of IL 172,563. Prof. Serajuddin also mentions that he relies on the pKa value of sitagliptin as reported by Applicant during prosecution of IL 172,563, well after the priority date of the application. Without the pKa value of sitagliptin, Prof. Serajuddin's arguments collapse as the skilled artisan had no reference value for sitagliptin.

18. Moreover, calculation or measurement of pKa value is an intricate exercise for a molecule of the chemical complexity of sitagliptin. Not only is the measurement itself difficult, but the stability of the molecule must also be ascertained throughout the entire measurement in which the molecule is exposed to highly acidic conditions. This is particularly so for a sensitive compound such as the free base of sitagliptin. The multiple ionization sites further add to the complexity of the exercise.

19. The pKa value of sitagliptin is the key data of Prof. Serajuddin's analysis. As indicated, the data were only known to Applicant and were not available at the priority date to the skilled person. Hence, in order to challenge the validity of the application, Prof.

Serajuddin relies on data that only became available much later than the priority date. This is pure hindsight.

Prof. Serajuddin ignores that sitagliptin has numerous protonation sites

20. Prof. Serajuddin discusses and dismisses the possibility of a phosphoric acid salt with more than one substrate base of sitagliptin. He however disregards the possibility that more than one site of the sitagliptin molecule will be protonated, thus forming a salt of sitagliptin cation with two or more dihydrogenphosphate anions (i.e. instead of a salt consisting of 1 phosphoric acid with 1, 2 or 3 molecules of base, a salt consisting of 1 molecule of base with 2 or more phosphoric acid molecules). According to Prof. Serajuddin's arguments, in order to predict whether or not such salts can be formed, one needs to know all the pKa values of sitagliptin and not only the first pKa. These pKa values were not known at the priority date of the application. I am not aware that they have been reported even today. Hence, the lack of pKa information for sitagliptin renders Prof. Serajuddin's analysis useless.

Salt formation is a trial and error endeavor

21. In any event, Prof. Serajuddin grossly exaggerates the significance of the ΔpKa between conjugate base and acid. This is not a scientific principle as Prof. Serajuddin seems to incorrectly imply. Even Prof. Serajuddin himself stated in his publications that, in any case, notwithstanding the pKa differences, the reality of salt formation remains "a trial and error endeavor" (C.G. Smith et al., *The Process of New Drug Discovery and Development* (editors), 2006, at p. 26, Appendix "F"). Moreover, in section 2.1 of *Handbook of Pharmaceutical Salts* on "feasibility assessment for salt formation" (Appendix 4 to Prof. Serajuddin's declaration), Prof. Serajuddin states that "no predictive procedure to determine whether a particular acidic or basic drug would form a salt with a particular counter-ion has been reported in the literature".

22. In addition, to the extent that the pKa difference can provide any guidance, it is that the pKa value of the acid should be equal to or lower than the pKa of the conjugate base in

order to create a salt (i.e. a 2-3 unit difference is not necessary). For instance, Gould states that "to form a salt the pKa of the conjugate acid has to be less than or equal to the pKa of a basic centre of the drug" (P.L. Gould, Salt Selection of Basic Drugs, *International Journal of Pharmaceutics*, 33 (1986) 201, at p. 202, Appendix "G"). As discussed below, salts may be formed even in cases where the pKa value of the acid is higher than the pKa value of the base. Prof. Serajuddin himself states that "although it is generally agreed that a successful salt formation requires the pKa of a conjugate acid to be less than the pKa of the conjugate base to ensure sufficient proton transfer from the acidic to the basic species, the salt formation still remains a trial and error endeavor" (Smith, page 26, Appendix "F").

23. In any event, if one were to rely on the pKa difference, any pKa difference would be considered sufficient. This is exactly the case for sitagliptin phosphate. The first pKa of sitagliptin is 7.7 and the second pKa of phosphoric acid is 7.21. Accordingly, contrary to Prof. Serajuddin's assertions, sufficient pKa differences exist for more than one ionization to occur. The pKa difference does not support any expectation that only the dihydrogenphosphate can be formed. Just the contrary.

Complete ionization in solution is not necessary to form a salt

24. Prof. Serajuddin further asserts that in order to form a stable salt, the acid must be completely ionized. According to Prof. Serajuddin's explanations, complete ionization of the second hydrogen atom of phosphoric acid occurs at a pH of 9.21 (section 49). Unless a pH of 9.21 is reached, it will be "extremely difficult" to obtain a stable monohydrogenphosphate salt. This is a total misconception.

25. The aim is to obtain a solid salt, not a salt in solution. A stable solid will be obtained by precipitation when the solution is super-saturated. When the solution is in equilibrium at pH 7.21, the concentration of dihydrogenphosphate salt equals the concentration of monohydrogenphosphate salt. If, under those conditions, the monohydrogen salt becomes super-saturated and precipitates, it will drive the equilibrium to form more

monohydrogenphosphate salt in solution which will further precipitate and so on. It is therefore not at all necessary to obtain complete ionization in solution and there is no need to reach a pH of 9.21.

Ammonium phosphate experiments are perfectly valid examples showing that the pKa of the conjugate acid does not have to be lower than the pKa of the base

26. As indicated above, ammonium phosphate experiments demonstrate that phosphoric acid has three acidic protons, which can be donated to an appropriate base to form dihydrogenphosphate, monohydrogenphosphate and phosphate salts. Prof. Serajuddin argues that this example is "inappropriate and irrelevant" and presents a long list of irrelevant properties of ammonia as a liquid and as a gas, all intended to demonstrate that ammonia is not useful in a pharmaceutical environment. Prof. Serajuddin totally misses the point. Ammonia is able to remove all three protons from phosphoric acid to make ammonium phosphate. The pKa of aqueous ammonia is 9.21 (see http://chemweb.unp.ac.za/chemistry/Physical_Data/pKa_compilation.pdf) whereas the third pKa of phosphoric acid is much higher (12.67). Therefore, the ammonium phosphate experiments are perfectly valid examples to demonstrate that in order to create a salt, the pKa of the conjugate acid does not have to be lower than the pKa of the base.

27. There are abundant additional examples in the literature to the same effect, including phosphate salts specifically. For instance, bis(4-amino-trans-azobenzene) hydrogen phosphate salt is reported in the literature (I. Halasz et al, "Hydrogen Phosphate and Dihydrogen Phosphate Salts of 4-aminoazobenzene", *Acta Cryst* C63 (2007), o61-o64, Appendix "H"). The pKa difference between the base and the first pKa of phosphoric acid (2.12) is 0.70. The pKa difference between the base and the second pKa of phosphoric acid (7.21) is -4.39 (the pKa value of the amino group of 4-aminoazobenzene is reported in K.N. Bascombe et al., "Acidity Functions of Some Aqueous Acids", J. Chem. Soc. (1959), 1096-1104, Appendix "I"). 4-aminoazobenzene also forms the dihydrogen phosphate phosphoric acid solvate salt (see, I. Halasz et al,

Appendix "H"). This salt is formed by the protonation of the azo group of the 4-aminoazobenzene molecule. The pKa value of the azo group of this molecule is less basic than the pKa of the amino group (see, G. Cilento, "Resolution of the Over-all Basicity of the Carcinogenic and Noncarcinogenic Derivatives of 4-Aminoazobenzene", *Cancer Res* 20 (1960), 120-124, Appendix "J"). Thus the pKa difference between the base and the first pKa of phosphoric acid (2.12) is even less than 0.70.

28. Other examples are guanine phosphate salts. Guaninium dihydrogenphosphate salt and bis(guaninium) hydrogen phosphate salt are reported in E. Bendeif et al., "Tautomerism and Hydrogen Bonding in Guaninium Phosphite and Guaninium Phosphate Salt", *Acta Cryst.* B63 (2007), 448-458 (Appendix "K") and J.N. Low et al., "Structure of Bis(guaninium) Hydrogenphosphate 2.5 hydrate", *Acta Cryst.* C42 (1986), 1045-1047 (Appendix "L"). The pKa differences are 1.18 and -3.91, respectively (the pKa value of guanine is reported in V. Verdolino et al., "Calculation of pKa Values of Nucleobases and the Guanine Oxidation Products Guanidinohydantoin and Spiroiminodihydantoin Using Density Functional Theory and a Polarizable Continuum Model", *J. Phys. Chem*, 112 (2008), 16860-16873, Appendix "M"). As can be seen, salts are formed not only when the pKa difference is below 2 or 3 units, but also when the pKa difference is negative.

29. To sum up, Prof. Serajuddin's arguments that sitagliptin and phosphoric acid can only form a dihydrogenphosphate salt and that this was the "expectation" based on the pKa values of the acid and the base are artificial and totally unfounded.

30. In any event, in order to completely rebut Prof. Serajuddin's assertions, I ran some experiments demonstrating that sitagliptin and phosphoric acids form additional salts. In all experiments, sitagliptin free base obtained from Merck was used (Batch no. 0000013867). Certificate of Analysis, XRPD and DSC of Batch no. 0000013867 of the free base of sitagliptin are attached hereto as Appendix "N". The Laboratory Notebook pages of the experiments are attached hereto as Appendix "O".

Bis(sitagliptin) phosphoric acid salt

31. Sitagliptin free base (1.50 g) (0.00368 moles) was combined with isopropanol (3.2 mL) and distilled water (1.4 mL). The mixture was stirred for 5-10 minutes to form a clear solution. Phosphoric acid (85% w/w) 0.215 g (0.00186 moles) was added with stirring. The solution was heated with stirring to 70ºC for 15 minutes, cooled to room temperature and left stirring overnight. The solution solidified. The solid was dried for ~ 6h at room temperature under vacuum and was analyzed by XRPD (file 0431_sample1_2.xrdml), TGA (file 0431_Sample_1_2.tai) and DSC (file 0431_Sample_1_2.002), Appendix "P". In addition, a sample was analyzed by elemental analysis (Appendix "Q").

	C	H	N	P
Observed	39.36%	3.83%	14.30%	3.02%
Calculated $(sitagliptin)_2(H_3PO_4)(H_2O)_3$	39.76%	4.07%	14.49%	3.20%

32. As can be determined from the analytical results, the compound produced is bis(sitagliptin)H_3PO_4 trihydrate, i.e., phosphoric acid can donate two protons to sitagliptin.

33. This conclusion is further supported by the agreement between the calculated and experimental values for the nitrogen and phosphorous content that corresponds to the stoichiometry of a bis(sitagliptin) phosphoric acid ($(sitagliptin)_2(H_3PO_4)$) salt.

34. To further demonstrate that phosphoric acid can donate two protons to sitagliptin, I also ran the following similar procedure, this time using methanol as a solvent:

Bis(sitagliptin) phosphoric acid salt – Procedure in Methanol

35. Sitagliptin free base (1.50 g) (0.00368 moles) was combined with methanol (4.6 mL). Phosphoric acid (85% w/w) (0.21 g) (0.0018 moles) was added to the solution. The

solution was heated with stirring to 70ºC for 15 minutes and cooled to room temperature. A white crystalline powder was formed. The solid was dried overnight at room temperature under vacuum and was analyzed by XRPD (file 0431_sample8.xrdml), TGA (file 0431_Sample_8.tai) and DSC (file 0431_SAMPLE8.008), Appendix "R". In addition, a sample was analyzed by elemental analysis (Appendix "S").

	C	H	N	P
Observed	40.81%	3.54%	14.82%	3.39%
Calculated $(sitagliptin)_2(H_3PO_4)(H_2O)$	41.30%	3.79%	15.05%	3.33%

36. As can be determined from the analytical results, the compound produced is bis(sitagliptin)H_3PO_4 monohydrate, again demonstrating that phosphoric acid can donate two protons to sitagliptin.

37. As elaborated above, this conclusion is further supported by the agreement between the calculated and experimental values for the nitrogen and phosphorous content that corresponds to the stoichiometry of a bis(sitagliptin) phosphoric acid ($(sitagliptin)_2(H_3PO_4)$) salt.

Sitagliptin ammonia phosphoric acid salt

38. As another demonstration that the second proton of phosphoric acid can be donated to sitagliptin, I reacted sitagliptin with ammonia phosphoric acid ($NH_4H_2PO_4$).

39. Sitagliptin free base (1.50 g) (0.00368 moles) was combined with isopropanol (3.2 mL) and distilled water (1.4 mL). The mixture was stirred for 5-10 minutes to form a solution. Ammonia phosphoric acid ($NH_4H_2PO_4$) (0.42 g) (0.00365 moles) was added with stirring. The mixture was heated to 70ºC with stirring for 15 minutes and then cooled to room temperature to yield a white crystalline powder. The solid was dried overnight at room temperature under vacuum and was analyzed by XRPD (file

0431_sample3.xrdml), TGA (file 0431_Sample_3.tai) and DSC (file 0431_SAMPLE3.004), Appendix "T". In addition, the sample was analyzed by elemental analysis (Appendix "U").

	C	H	N	P
Observed	34.36%	4.21%	14.67%	5.50%
Calculated (sitagliptin) ($NH_4H_2PO_4$)	33.87%	4.62%	14.81%	5.46%

40. As can be determined from the analytical results, the compound produced is (sitagliptin $NH_4H_2PO_4$)·2.5 H_2O, i.e., phosphoric acid can donate its second proton to sitagliptin. This conclusion is further supported by the agreement between the calculated and experimental values for the nitrogen and phosphorous content that corresponds to the stoichiometry of sitagliptin ammonia phosphoric acid salt.

Sitagliptin bis(phosphoric acid) salt

41. As a demonstration that sitagliptin can form salt with phosphoric acids by protonation of two sites, I carried out the following experiment.

42. Sitagliptin free base (0.75 g) (0.00184 moles) was combined with acetone (4.0 mL). The mixture was stirred for 5-10 minutes to form a solution. Anhydrous, crystalline H_3PO_4 (0.36 g) (0.00368 moles) was added with stirring. The mixture was heated to 50°C with stirring for 15 minutes and the clear solution was then cooled to room temperature. The acetone was removed under vacuum at room temperature. The white product was crushed with a spatula and then placed under vacuum overnight and was analyzed by XRPD, TGA and DSC (Appendix "V"). In addition, a sample was analyzed by elemental analysis (Appendix "W").

	C	H	N	P
Observed	31.80%	3.54%	10.71%	10.10%

Calculated (Sitagliptin) $(H_3PO_4)_2$ (with 2% acetone and 1% water residues)	32.14%	3.72%	11.26%	9.96%

43. As can be determined from the analytical results, the compound produced is amorphous (Sitagliptin)$(H_3PO_4)_2$, i.e., sitagliptin forms a salt with 2 molecules of phosphoric acid by protonation of two of its sites. This conclusion is further supported by the agreement between the calculated and experimental values for the nitrogen and phosphorous content.

44. The experiments reported above clearly demonstrate that contrary to Prof. Serajuddin's unfounded assumptions, the dihydrogenphosphate salt is by no means the inevitable result of reacting sitagliptin with phosphoric acids. As clearly exemplified in these experiments, reacting sitagliptin with phosphoric acids result in the formation of various salts. Therefore, Prof. Serajuddin's unfounded assumptions do not stand up to scientific reality.

45. In addition to the experiments described above, I ran additional experiments as described in the attached LNB's. These experiments would require additional work to isolate or obtain pure compounds. As the experiments reported above unequivocally rebut Prof. Serajuddin's assertions, I did not find it necessary to fine tune every experiment that I ran. However, there is no doubt in my mind that, in addition to the salt claimed in IL 172,563 and the salts reported above, additional phosphoric acid salts of sitagliptin can be made.

WO '498

46. In sections 44-47 and 54-55 of his declaration, Prof. Serajuddin comments on the WO '498 patent application. I reviewed WO '498 and, in particular, the sections on pages 9 line 27 – page 10, line 15, on which Prof. Serajuddin relies. It is my clear opinion that Prof. Serajuddin attempts to incorrectly interpret WO '498 based on hindsight and with the benefit of Applicant's subsequent research.

47. WO '498 describes a large class of beta-aminotetrahydrotriazolo[4,3-a]pyrazine compounds which are optionally in the form of pharmaceutically acceptable salts. Lists of potentially suitable salt-forming acids and bases are provided. WO '498 exemplifies 33 compounds belonging to this class, including sitagliptin. All 33 beta aminotetrahydrotriazolo[4,3-a]pyrazine compounds are exemplified as the hydrochloride salts. It is therefore incorrect to say that WO '498 directs the skilled person to sitagliptin, let alone to a phosphate salt of sitagliptin, and not to one of the many other salt formers listed in WO '498.

48. Even if the skilled person would have opted to make a phosphate salt, numerous phosphate salts are possible as I already explained above. A phosphate salt is not synonymous to a dihydrogenphosphate salt. Prof. Serajuddin's assertion that only one single species of phosphate salt of sitagliptin is possible or expected is erroneous as I demonstrated theoretically and empirically above. Therefore, any attempt to assert that WO '498 in any manner renders Applicant's invention "obvious" is totally misleading.

Declaration of Dr. Leonard Chyall

49. I am advised that on October 17, 2010, Opposer submitted an additional declaration of Dr. Leonard Chyall. At the request of Applicant, I also reviewed Dr. Chyall's declaration.

50. In his declaration, Dr. Chyall asserts that he ran salt formation experiments for the purpose of obtaining a phosphate salt other than a 1:1 adduct of sitagliptin and phosphoric acid. He further asserts that he varied common parameters used in the experiments in a "deliberate attempt" to obtain different possible salts and that his experiments "encompassed a wide range of experimental conditions in deliberate attempts to prepare a phosphate salt other than a 1:1 adduct of sitagliptin and phosphoric acid" (see, for instance, sections 23 and 72 of Dr. Chyall's declaration). Dr. Chyall nevertheless reports that he failed to obtain any salt other than the dihydrogenphosphate salt of sitagliptin. He concludes that his experiments "indicate"

that there is "only one possible molecular ratio, a 1:1 ratio", namely, dihydrogenphosphate salt of sitagliptin.

51. I was surprised to see that Dr. Chyall did not attach to his declaration the laboratory notebooks describing the experiments which he conducted. This is highly uncustomary and does not allow a thorough analysis of the experiments. I am advised that Applicant's counsel requested Opposer to provide the notebooks and that a disc containing the notebooks was thereafter received on October 28, 2010. A printout of the laboratory notebooks as received is attached hereto as Appendices "X1", "X2", "X3" and "X4". Upon review of the notebooks, it transpired that numerous analytical results or pages were missing, partial or unclear. I am advised that on January 4, 2011, January 11, 2011 and January 19, 2011, Applicant's counsel requested Opposer to provide the missing material and information. On February 24, 2011 Opposer provided another disc with some additional laboratory notebooks and analytical results. A printout of the additional material is attached as Appendix "Y". Also attached hereto is the correspondence between the parties' counsel with respect to the missing laboratory notebooks and data (Appendices "Z1" and "Z2", correspondence in Hebrew and translations to English).

Dr. Chyall's failed experiments

52. The experiments I ran unequivocally rebut Dr. Chyall's assertions and render Dr. Chyall's failed attempts to obtain other salts irrelevant. In any event, the procedures reported by Dr. Chyall do not represent attempts to deliberately obtain different possible molecular combinations of the sitagliptin and phosphoric acid. As detailed below, they rather appear as experiments that were designed to fail. Among others, Dr. Chyall conducted the experiments in temperatures expected to suppress the reaction, added one of the reagents (phosphoric acid) drop-wise, causing the reaction to start without the required excess molar amount, used excess solvent causing the salt to disproportionate, etc.

53. Moreover, contrary to Dr. Chyall's assertions, his experiments did not encompass a wide range of experimental conditions. Just the opposite. As detailed below, the experimental parameters used by Dr. Chyall were in fact extremely narrow. Among others, Dr. Chyall conducted his "salt screening" experiments in one single solvent (methanol), did not try any other solvent system and went to great lengths to avoid the presence of water in the reactions which he conducted.

54. I will now discuss in detail Dr. Chyall's experiments:

Two experiments are irrelevant

55. Overall, Dr. Chyall reported that he performed 12 experiments. In two of the experiments, Dr. Chyall used a molar ratio of 1:1.05 between the sitagliptin base and the acid (Nos. 4063-02-01 and 4063-34-01). In order to obtain salts of different molecular combinations, one needs to use an excess molar ratio of either the base or the acid, not a molar ratio which is essentially a 1:1 ratio. Thus, these experiments are irrelevant.

Five experiments with excess sitagliptin base

56. In five of the experiments (4063-18-01, 4063-04-01, 4063-35-01, 4063-51-01, 4063-57-01), Dr. Chyall used a molar excess of the sitagliptin base.

Temperature of the reaction

57. It is common to heat organic reactions including salt formation experiments. The heating is generally at temperatures just below the boiling point of the solvent (in the case of methanol, 65º C). Among others, the solubility of the compound greatly increases with the temperature. In addition, higher temperatures affect the kinetics of the reaction and may "favor" a reaction which is otherwise not energetically favored. Higher temperature also favors an increased yield.

58. Despite these well known realities, Dr. Chyall ran most of his experiments at low or ambient temperatures. In one of the experiments (4063-50-01), Dr. Chyall conducted the reaction in a water bath cooled to 0ºC. In three of the experiments (4063-03-01, 4063-19-01, 4063-32-01), he conducted the reaction at ambient temperature. Dr. Chyall heated the solution to reflux (65ºC) only in one experiment (4063-56-01). As elaborated below, although Dr. Chyall heated this particular reaction, he did not perform the experiment in a manner that allows the formation of other salt forms.

59. It is odd that out of the five experiments, Dr. Chyall conducted only a single experiment with heating and, instead, used ambient temperatures or even cooled the reaction to a temperature of 0ºC. These are not optimal conditions and may suppress a reaction creating a 2:1 sitagliptin to phosphoric acid salt from taking place.

Dr. Chyall ran the experiments only with a single solvent (methanol)

60. As noted, Dr. Chyall asserted that he used a wide range of experimental conditions and also varied the "composition of the solvent". Another striking feature in Dr. Chyall's failed experiments is that, contrary to his assertions, he in fact only used methanol as a solvent in all his experiments. If one fails to obtain the desired salt product using a particular solvent, it is expected to conduct the experiment in different solvent systems. As explained above, different solvent systems can affect the strength of the acid and the base and, therefore, affect the outcome of the salt forming reaction. In addition, different solvent systems can affect the crystallization of different solid state forms and this, in turn, could also affect the salt that is created in the experiment. This is an everyday reality in the lab. If Dr. Chyall endeavored to run experiments showing that salt adducts other than the 1:1 salt are possible, he should have tried different solvents and solvent systems. For some reason, Dr. Chyall did not do so and conducted his experiments only with methanol.

Dr. Chyall did not use a methanol – water solvent system

61. Dr. Chyall asserts that in one out of the five experiments (4063-35-01), he used as a reaction solvent "12.5% water in methanol" (see table 1 in section 24 of Dr. Chyall's declaration). This could lead one to assume that Dr. Chyall was actually using a solvent mixture of methanol (87.5%) and water (12.5%). This is not at all the case.

62. As transpires from Dr. Chyall's laboratory notebooks, this particular reaction was also conducted in methanol (see page 35 of LNB 4063). Dr. Chyall dissolved sitagliptin free base in methanol and then added drop-wise to the reaction solution phosphoric acid in water (85% phosphoric acid; 15% water; see page 17 of LNB 4036). Accordingly, the reaction started in a solvent system without water, continued in a solvent system containing minute amounts of water and contained 12.5% water only after Dr. Chyall completed the addition of the phosphoric acid drop-wise, long after the reaction started.

63. Generally, when one wants to conduct a reaction in a particular solvent system, one first mixes the solvents in the required concentrations and then conducts the reaction in the solvent mixture. Dr. Chyall's procedure (to add the second solvent drop-wise together with one of the reagents of the reaction) is uncustomary. It is not equivalent to a salt forming experiment conducted in a mixed aqueous – organic solvent system.

Dr. Chyall avoided water in his experiments

64. Furthermore, it appears that Dr. Chyall went to great lengths to conduct his salt forming experiments in a non-aqueous system. Phosphoric acid is sold and used as a stock solution of phosphoric acid in water (85%, 15% w/w). According to his laboratory notebooks, Dr. Chyall sourced an 85% aqueous solution of phosphoric acid from Sigma-Aldrich. Rather than using the phosphoric acid solution, Dr. Chyall diluted the solution with methanol so as to make the phosphoric acid solution substantially free of water and used it in his experiments. The use of a mixed solvent system containing an organic solvent and water is a common strategy in salt forming experiments. This was carefully avoided by Dr. Chyall.

The use of excessive amounts of methanol and the slurrying of the crystals

65. In his experiments, Dr. Chyall uses a ratio of 1 mL solvent (methanol) to 100 mg sitagliptin free base. This ratio is about three times more solvent than the ratio I used in my experiments (1 ml solvent to ~ 325 mg free base) and also used in IL 172,563 (p. 15). In Dr. Chyall's experiments, solids formed within minutes to several hours. Nevertheless, Dr. Chyall left the solids to slurry in methanol for an additional day. Thereafter, as transpires from the laboratory notebooks, he washed the solids three times with large amounts of methanol (3 x 2 mL).

66. Use of such excessive amounts of methanol is unnecessary and, in fact, can make the 2:1 salt disproportionate into a 1:1 salt. The poor yields obtained in Dr. Chyall's experiments are also indicative of excessive use of solvent.

67. In order to demonstrate this, I ran the following experiments:

68. First, I prepared another sample of bis(sitagliptin) phosphoric acid salt in a manner similar to the procedure described above for the sample prepared in isopropanol and water (the procedure is described in the attached laboratory notebook, Appendix "O"). It was analyzed by XRPD, TGA and DSC (Appendix "AA"). In addition, a sample was analyzed by elemental analysis (Appendix "BB").

	C	H	N	P	Solvent
Observed	39.80%	3.93%	14.51%	3.46%	5.9%
Calculated $(Sitagliptin)_2(H_3PO_4)(H_2O)_3$	39.76%	4.07%	14.49%	3.20%	5.6%

69. As can be determined from the agreement between the calculated and the experimental results, the compound produced is bis(sitagliptin) (H_3PO_4) trihydrate.

70. I then slurried a 500 mg sample of the material prepared above in 5 mL of methanol for 24 hours. I vacuum filtered the resulting white solids and solvent and washed the flask

and the collected precipitate three times with 2 mL methanol for each wash. The resulting collected precipitate was air dried to content weight yielding about 100 mg (see laboratory notebook attached as Appendix "O"). After slurrying, I analyzed the resultant solid by XRPD, DSC and TGA (attached as Appendix "CC"). These analytical data show that the sample converted from bis(sitagliptin)(H_3PO_4) trihydrate (2 Base: 1 Acid ratio) to sitagliptin dihydrogenphosphate salt (1 Base: 1 Acid ratio). In addition, the sample was sent to elemental analysis (attached hereto as Appendix "DD"). The results of the elemental analysis also demonstrate that the sample is sitagliptin dihydrogenphosphate salt (1 Base: 1 Acid ratio).

71. This experiment therefore shows that use of excess solvent prevents the formation of disitagliptin monohydrogenphosphate salt. It is also possible that disitagliptin monohydrogenphosphate salt was initially formed in Dr. Chyall's experiments. However, Dr. Chyall failed to isolate it because he had it slurried in methanol and then washed three times with significant volumes of methanol thus forcing conversion of the salt.

Five experiments with a molar excess of phosphoric acid

72. In the remaining five experiments (4063-03-01, 4063-19-01, 4063-32-01, 4063-50-01, 4063-56-01), Dr. Chyall used a molar excess of phosphoric acid. These experiments are flawed for the same reasons explained above. Other than one single experiment, Dr. Chyall ran the experiments at ambient and 0ºC. Dr. Chyall ran his experiments only with a single solvent (methanol). He did not use a methanol – water solvent system and tried to avoid water in his experiments. In addition, Dr. Chyall used excess methanol, slurried the crystals for an additional day and then washed them with excess amounts of methanol.

73. Moreover, in this series of five experiments, Dr. Chyall allegedly tried to create a 1 (sitagliptin): 2 (phosphoric acid) salt and, therefore, used an excess amount of phosphoric acid (1:2.1 in experiments 4063-03-01, 4063-32-01, 4063-50-01, 4063-56-

01 and 1:5.01 in experiment 4063-19-01). However, as previously indicated, Dr. Chyall adds the phosphoric acid drop-wise, i.e. the reaction starts without an excess phosphoric acid. Only after the reaction starts and its kinetics established, additional quantities of phosphoric acid are gradually added until there is an excess molar amount of phosphoric acid.

74. Reagents can be added drop-wise when it is necessary to prevent excessive heating of an exothermic reaction. There was no reason to add phosphoric acid drop-wise in Dr. Chyall's experiments. It is indeed odd that Dr. Chyall's experiments were conducted in this manner. In any event, this clearly affects both the stoichiometries and the kinetics of the reaction and could very well affect the results obtained by Dr. Chyall.

75. To sum up, contrary to his assertions, Dr. Chyall did not run a study intended to evaluate whether phosphate salts of sitagliptin other than the 1:1 salt can be formed using a broad range of common parameters. To the contrary. Dr. Chyall ran a small number of experiments using a very narrow set of parameters. Some of the parameters selected appear odd and may have prevented the forming of salts other than the 1:1 salt or caused the salts to disproportionate after they had been formed. Even after he failed to obtain salts other than the 1:1 salt, Dr. Chyall did not change the reaction parameters as he was expected to do. The fact that I successfully made other sitagliptin phosphate salts further demonstrates that Dr. Chyall's experiments were meaningless and poorly designed.

Dr. Chyall's pH solubility experiments

76. Dr. Chyall also conducted "pH solubility experiments" for sitagliptin. Dr. Chyall asserts that these experiments support his conclusion that "only a 1:1 salt of sitagliptin is isolable". As discussed above, Dr. Chyall's conclusion is clearly incorrect. Instead of obtaining other species of the salt by varying some common parameters in his experiments, Dr. Chyall opted to conduct a long series of solubility studies to justify his failed experiments.

77. In any event, these pH solubility studies are a truly futile exercise. pH solubility calculations require knowledge of the pKa of sitagliptin. In addition, experimental pH max studies require access to the API. It is my understanding that none of these were available at the priority date of the application. Accordingly, similarly to Prof. Serajuddin's analysis, Dr. Chyall's pH solubility studies are nothing but an artificial exercise in hindsight.

78. As indicated above, organic solvents and water-organic solvent systems are very frequently used to make salts. For instance, IL 172,563 exemplifies the making of the dihydrogenphosphate salt in isopropanol and water. Dr. Chyall himself purportedly attempts to make other species of phosphate salts in methanol. pH solubility studies are performed in water and therefore are totally irrelevant for organic solvent systems or for water – organic solvent systems as the organic solvent impacts the solubility of the compound and the pKa of the acid and the base. For some reason, there is not a word about this in Dr. Chyall's declaration.

79. pH max studies are conducted in a saturated solution, not in a supersaturated solution which is commonly used in salt formation experiments. The behavior of the base and the acid and the kinetics of the reaction are different in a supersaturated solution. By controlling supersaturation it should be possible to isolate additional salt forms. This is another reason why Dr. Chyall's experiments are practically meaningless.

80. Dr. Chyall's pH solubility experiments are also poorly designed and poorly executed. Dr. Chyall derived a theoretical solubility curve for sitagliptin based on the measurement of the intrinsic solubility of sitagliptin base and of the anhydrate dihydrogenphosphate salt. Dr. Chyall sought to validate the theoretical curve by measuring additional solubility points (Fig. 3). For some reason, he did not take any measurements in the critical area between pH 4.2 to 7. For practical purposes, pH max is considered as a range of pH over which a change in equilibrium occurs. The lack of measurements in the most critical areas below and above Dr. Chyall's calculated pH

max totally undermines the accuracy and credibility of the curves derived by Dr. Chyall.

81. Dr. Chyall's theoretical curve does not correlate with the experimental data points. Dr. Chyall attempts to justify this lack of correlation on the grounds that "the highly concentrated solutions with extremely high ionic strengths no longer behave as ideal solutions". This is merely an unsubstantiated speculation that cannot justify the lack of correlation up to ~ pH 4. In fact, review of the experimental data points confirms that sitagliptin has a second pH max. This is consistent with the fact that sitagliptin has more than one protonation site. However, Dr. Chyall's theoretical curve is based on the incorrect premises that sitagliptin is a mono-basic compound. These incorrect premises bias Dr. Chyall's solubility studies and, indeed, as can be clearly seen from Figure 3 of Dr. Chyall's declaration, the solubility curve on which Dr. Chyall relied is incorrect.

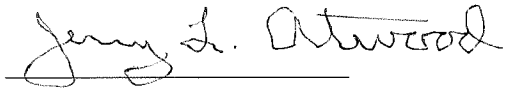
82. Moreover, Dr. Chyall only calculated the theoretical pH max and measured empirical solubility points for one salt form of sitagliptin (the anhydrous form of sitagliptin dihydrogenphosphate salt). Salt solubility can differ, sometimes substantially, between different salt forms. Therefore, in order to obtain the pH max range, one needs solubility information about different salts forms. Conducting pH solubility studies with solubility information of a single salt form, as done by Dr. Chyall, is a futile exercise which does not provide meaningful information on the range of pH max for sitagliptin and cannot predict which salt forms can be made.

83. In the concluding paragraphs of his declaration, it appears that Dr. Chyall qualifies his own conclusions and states that his experiments merely show that the 1:1 salt is the only "stable" salt, rather than the only possible phosphate salt of sitagliptin. Prof. Serajuddin also makes similar comments implying that he recognizes that other sitagliptin phosphate salt forms can be made but that they will not be stable. My understanding is that whether or not other phosphate salts of sitagliptin are stable is irrelevant. Opposer asserted that the only possible salt is the 1:1 salt and this has been unequivocally proven to be false. In addition, even if a compound is unstable it can be

protected by appropriate packaging and formulation and used as a pharmaceutically acceptable compound. This is well known.

84. In any event, I performed stability studies under accelerated conditions for bis(sitagliptin) phosphoric acid salt. The procedure is described in the attached laboratory notebooks (Appendix "O"). As can be seen from the attached XRPD, DSC and TGA (Appendix "EE") as well as elemental analysis (Appendix "FF") of the salt after two weeks under accelerated conditions (40C/75% RH), the salt is highly stable.

85. My name and signature follow and the contents of this affidavit are true.



Jerry L. Atwood

CURRICULUM VITAE

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Professional Experience

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Associate Professor, University of Alabama, 1972-1978
Professor, University of Alabama, 1978-1987
Visiting Professor, Imperial College, 1977
Visiting Professor, University of Sussex, 1985
University Research Professor, University of Alabama, 1987 - 1994
Professor and Chairman, University of Missouri-Columbia, 1994-
Curators' Professor, University of Missouri-Columbia, 1999-

Professional Activities

Co-Editor-in-Chief, *New Journal of Chemistry* (2005-)
Editor, *Journal of Supramolecular Chemistry* (2000-2004)
Editor, *Supramolecular Chemistry* (1992-2000)
Associate Editor, *Chemical Communications* (1996-2006)
Consulting Editor, *Journal of Chemical Crystallography* (1999-)
Editor, *Journal of Chemical Crystallography* (1985-1998)
Regional Editor, *Journal of Coordination Chemistry*, A & B (1985-1993)
Editor, *Journal of Inclusion Phenomena* (1983-1991)
Editorial Advisory Board, *Crystal Growth & Design* (2000-)
International Advisory Editorial Board, *New Journal of Chemistry* (2003-)
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Publications Summary

Publications in Refereed Journals	668
Patents	13

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Honors and Awards:

1986	Burnum Award for Teaching and Research, U of Alabama
1987	University Research Professor, U of Alabama
1989	von Humboldt Senior Scientist Award, Germany
1992	Japanese Society for the Promotion of Science Award
1996	Outstanding Alumni Award, SMSU
1999	Curators' Professor, University of Missouri (MU)
2000	President's Award for Research and Creative Activity, MU
2000	Izatt-Christensen International Macrocyclic Chemistry Award
2000	Polish Academy of Science, Elected Foreign Member
2002	Alumni-Faculty Award, MU
2005	Royal Society of Chemistry, Elected Fellow
2005	Honorary Medal of the Institute of Physical Chemistry, Polish Academy of Sciences
2005	Midwest Chemist Award, American Chemical Society
2010	Distinguished Faculty Alumni Award, MU

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1. "Liquid Clathrates"
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2. "Coal Liquefaction Using Liquid Clathrates"
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4. "Calixarene Chloride-Channel Blockers"
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   with L. R. MacGillivray, U. S. Patent 7,169,957 (2007).

7. "Formation of Nanometer-Scale Structures"
   with G. W. Orr and L. J. Barbour, U. S. Patent 6,495,669 (2002).

8. "Hexameric Complexes and Their Preparation"
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9. "Self-Assembled Calixarene Based Guest-Host Assemblies for Guest Storage by van der Waals Confinement"
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11. "Material for the Recovery of Hydrogen from Gas Mixtures"
with L. J. Barbour and A. Jerga
filed April 19, 2004.

12. “Processes for the Preparation of Calixarene Derivatives”
with C. L. Raston
filed June 13, 2008.

13. “New Strategy for Transforming Pharmaceutical Solids”
with J. Tian and S. J. Dalgarno
to be filed.

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proton to give the hydronium ion, H_3O^+. In its reaction with amide ion, $^-NH_2$, however, water is an acid that donates a proton to give ammonia, NH_3, and hydroxide ion, HO^-.

Problem 2.10 Nitric acid (HNO_3) reacts with ammonia (NH_3) to yield ammonium nitrate. Write the reaction, and identify the acid, the base, the conjugate acid product and the conjugate base product.

2.8 Acid and Base Strength

Acids differ in their ability to donate H^+. Stronger acids such as HCl react almost completely with water, whereas weaker acids such as acetic acid (CH_3COOH) react only slightly. The exact strength of an acid, HA, in water solution is described using the equilibrium constant K_{eq} for the acid-dissociation equilibrium. (Remember from general chemistry that brackets [ ] around a substance mean that the concentration of the enclosed species is given in moles per liter, M.)

$$HA + H_2O \rightleftharpoons A^- + H_3O^+$$

$$K_{eq} = \frac{[H_3O^+][A^-]}{[HA][H_2O]}$$

In the dilute aqueous solution normally used for measuring acidity, the concentration of water, $[H_2O]$, remains nearly constant at approximately 55.6 M. We can therefore rewrite the equilibrium expression using a new quantity called the **acidity constant, K_a**. The acidity constant for any generalized acid HA is simply the equilibrium constant for the acid dissociation multiplied by the molar concentration of pure water, 55.6 M:

$$HA + H_2O \rightleftharpoons A^- + H_3O^+$$

$$K_a = K_{eq}[H_2O] = \frac{[H_3O^+][A^-]}{[HA]}$$

Stronger acids have their equilibria toward the right and thus have larger acidity constants, whereas weaker acids have their equilibria toward the left and have smaller acidity constants. The range of K_a values for different acids is enormous, running from about 10^{15} for the strongest acids to about 10^{-60} for the weakest. The common inorganic acids such as H_2SO_4, HNO_3, and HCl have K_a's in the range 10^2–10^9, while organic acids generally have K_a's in the range 10^{-5}–10^{-15}. As you gain more experience in later chapters, you'll develop a rough feeling for which acids are "strong" and which are "weak" (remembering that the terms are always relative).

Acid strengths are normally expressed using pK_a values rather than K_a values, where the pK_a is the negative common logarithm of the K_a:

$$pK_a = -\log K_a$$

A *stronger* acid (larger K_a) has a *smaller* pK_a, and a *weaker* acid (smaller K_a) has a *larger* pK_a. Table 2.3 lists the pK_a's of some common acids in order of their strength. A more comprehensive table is given in Appendix B.

TABLE 2.3 Relative Strengths of Some Common Acids and Their Conjugate Bases

	Acid	Name	pK_a	Conjugate base	Name	
Weaker acid	CH_3CH_2OH	Ethanol	16.00	$CH_3CH_2O^-$	Ethoxide ion	Stronger base
	H_2O	Water	15.74	HO^-	Hydroxide ion	
	HCN	Hydrocyanic acid	9.31	CN^-	Cyanide ion	
	CH_3COOH	Acetic acid	4.76	CH_3COO^-	Acetate ion	
	HF	Hydrofluoric acid	3.45	F^-	Fluoride ion	
	HNO_3	Nitric acid	−1.3	NO_3^-	Nitrate ion	
Stronger acid	HCl	Hydrochloric acid	−7.0	Cl^-	Chloride ion	Weaker base

Notice that the pK_a value shown in Table 2.3 for water is 15.74, a value that results from the following calculation: The K_a for any acid in water is the equilibrium constant K_{eq} for the acid dissociation multiplied by the molar concentration of pure water. For the acid dissociation of water, we have

$$H_2O + H_2O \rightleftharpoons OH^- + H_3O^+$$

$$K_{eq} = \frac{[H_3O^+][OH^-]}{[H_2O]^2} \quad \text{and} \quad K_a = K_{eq} \times [H_2O] = \frac{[H_3O^+][OH^-]}{[H_2O]}$$

The numerator in this expression, $[H_3O^+][OH^-]$, is the so-called ion-product constant for water, $K_w = 1.0 \times 10^{-14}$, and the denominator is $[H_2O] = 55.6$ M. Thus, we have

$$K_a = \frac{1.0 \times 10^{-14}}{55.6} = 1.80 \times 10^{-16} \quad \text{and} \quad pK_a = 15.74$$

Notice also in Table 2.3 that there is an inverse relationship between the acid strength of an acid and the base strength of its conjugate base. To understand this relationship, think about what happens to the acidic

Equilibrium Acidities in Dimethyl Sulfoxide Solution

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Equilibrium acidities provide a fundamental data base for assessment of the electronic and steric effects brought about by structural variations in organic molecules. The Hammett equation,[1] based on the aqueous acidities of meta- and para-substituted benzoic acids, and the Taft equation, based partially on the aqueous acidities of substituted acetic acids, GCH_2CO_2H,[2] have served chemists in this regard for over 40 years. The Hammett H_0 acidity function and the like have allowed the aqueous acidity scale, which has a practical pK_a range of 0–12, to be extended downward into the negative pK_a region by about an equal amount.[3] The aqueous scale has also been extended upward by about 12 pK_a units by the use of H_- acidity functions that employ cosolvents and strong bases.[4] These models and functions have severe limitations, however.

The first acidity scale to be established in a pure solvent other than water was the result of the pioneering work of Conant, Wheland, and McEwen in ether or benzene.[5] During the past 20 years an ion-pair acidity scale covering an "effective pK_a range" from about 15 to 40 has been developed in cyclohexylamine (CHA),[6] and similar studies in other low-dielectric-constant solvents including 1,2-dimethoxyethane (DME)[7a] and tetrahydrofuran (THF)[7b,c] have been carried out. A more limited ion-pair acidity scale has been developed in liquid NH_3.[7d] Also, during this period, acidity scales have been established in the polar non-hydrogen-bond-donor (NHBD) solvents dimethyl sulfoxide (Me_2SO)[8] and *N*-methylpyrrolidin-2-one (NMP),[9] which have relatively high dielectric constants. The pK_a's measured in these solvents differ from ion-pair pK_a's in that they are absolute, in the sense that they are based on Me_2SO and NMP as the standard states, which allows direct comparisons to be made with H_2O and gas-phase pK_a's. A truly absolute acidity scale has been established in the gas phase, which, for the first time, provides intrinsic measures of structural effects free of solvent effects.[10] Our purpose in this Account is (a) to discuss briefly acidities in various solvent media, (b) to present a table of representative equilibrium acidity constants in Me_2SO solution, and (c) to illustrate ways in which these pK_a data can be used. In an accompanying Account we compare acidities in Me_2SO solution with intrinsic gas-phase acidities and discuss some of the insights into solvation effects provided thereby.

Acidities in H_2O and Me_2SO. It is important to recognize that pK_a values are solvent dependent. The dissociation constant of an acid, formally defined by eq 1, depends on the ability of the solvent to solvate the

$$H\text{-}A \rightleftarrows H^+ + A^- \qquad (1)$$

proton, the anion, and the undissociated acid. Since solvation of the proton is constant in a given solvent and solvation of most neutral acids is small compared to that of their conjugate bases, differences in acidities brought about by structural variations or solvent changes are usually caused by changes in the energies of the anions. The large acidity increases observed in changing from Me_2SO to H_2O for oxygen acids forming oxyanions that are strongly H-bonded to water provide examples (Table I).

In Table I we see that the acidities of very strong acids such as F_3CSO_3H, HBr, HCl, and CH_3SO_3H are

Frederick G. Bordwell is Professor Emeritus at Northwestern University. (For a biography summarizing his earlier research activities, see *Acc. Chem. Res.* **1972**, *5*, 374). In the period 1970–1980 the Bordwell research group established acidity scales in Me_2SO and *N*-methyl-2-pyrrolidone solvents using a method adapted from one developed by E. C. Steiner at Dow Chemical Co. Since 1980 the research focus has shifted to the application of the data in the Me_2SO scale to problems in physical organic chemistry, the results of which are summarized in this Account.

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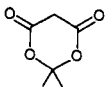
Table I
Equilibrium Acidities in Dimethyl Sulfoxide and in Water

acid	$pK_a(H_2O)$	$pK_a(Me_2SO)$[b]	acid	$pK_a(H_2O)$	$pK_a(Me_2SO)$[b]
F_3CSO_3H	−14[a]	0.3[c]	$F_3CSO_2NH_2$	6.3	9.7
HBr	−9[a]	0.9[c]	PhSH	6.5	10.31
HCl	−8[a]	1.8[c]	$(CH_3CO)_2CH_2$	8.9	13.3
CH_3SO_3H	−0.6[a]	1.6[c]	HCN	9.1	12.9[e]
2,4,6-$(NO_2)_3C_6H_2OH$	0	~0[d]	NH_4^+	9.2	10.5[d]
4-Cl-2,6-$(NO_2)_3C_6H_2OH$	3.0	3.6	CH_3NO_2	10.0	17.2
HF	3.2	15 ± 2	PhOH	10.0	18.0
$PhCO_2H$	4.25	11.1	$CH_2(CN)_2$	11.0	11.0[e]
CH_3CO_2H	4.75	12.3	F_3CCH_2OH	12.4	23.6
$PhNH_3^+$	4.6	3.6[d]	$(CH_3SO_2)_2CH_2$	12.7	15.0
HN_3	4.7	7.9[e]	CH_3CONH_2	15.1	25.5
	4.8	7.3[f]	CH_3OH	15.5	29.0
$PhSO_2H$	3.5	7.1	H_2O	15.75	32
$C_5H_5NH^+$	5.2	3.4[d]			

[a] Estimated by the H_0 method; in pure H_2O their acidities are leveled to that of H_3O^+ ($pK_a = -1.75$). [b] From measurements made in our laboratory, unless otherwise noted. [c] McCallum, C.; Pethybridge, A. D. *Electrochim. Acta* **1975**, *20*, 815–818. [d] Reference 8d. [e] Reference 8c. [f] Reference 34.

leveled in Me_2SO to that of Me_2SOH^+, just as they are leveled to that of H_3O^+ in H_2O. For strong oxygen acids such as picric acid and 4-chloro-2,6-dinitrophenol, which form highly delocalized anions on dissociation, acidities do not differ greatly in H_2O and Me_2SO. As the oxygen acids in Table I become weaker, charge delocalization in the anion decreases and the difference in acidity in H_2O vs Me_2SO (ΔpK_a) increases from near zero for picric acid to 15 pK_a units for the weakest acids, MeOH and H_2O. This change is due primarily to the strong H-bond donor properties of the water solvent, which achieve maximum effectiveness toward localized ions such as F^-, $F_3CCH_2O^-$, MeO^-, or HO^-. The strong H-bond acceptor properties of H_2O make $PhNH_3^+$ and pyridinium ions (but not the NH_4^+ ion) weaker acids in H_2O than in Me_2SO. (Me_2SO is also a good H-bond acceptor and solvates cations well.) Solvation of the $CH(CN)_2^-$ by H_2O and Me_2SO appears to be nearly equal.

Acidities in Solvents of Low Dielectric Constant. Ion-Pair pK_a's. Ion-pair acidity scales in cyclohexylamine (CHA),[6] DME, THF, or other solvents of low dielectric constant[7] complement that in Me_2SO in some respects but are more limited in scope. These scales were originally anchored arbitrarily on the H_ pK_a = 18.49 for 9-phenylfluorene (9-PhFlH) in H_2O/sulfolane, but more recently they have been anchored on the pK_a of fluorene in Me_2SO (22.3 on a per-hydrogen basis).[11]

The size of ion-pairing effects will depend somewhat on the nature of the cation. For example, with Li^+ counterion in CHA, PhC≡CH appears to be a stronger acid than in Me_2SO by 6.1 pK units, but with Cs^+ counterion in DME ΔpK is only 2.4 units.[12] Ion-pairing effects of anions with K^+ counterion are of little or no importance in dilute (millimolar) Me_2SO solution, except for strongly chelating anions such as that formed from $CH_3COCH_2COCH_3$. A method for detecting such ion-pairing effects and a spectroscopic method for measuring ion-pair association constants (K_{as}) have been devised.[13a] Ion pairing stabilizes the anion and leads to an apparent acidity increase. Small corrections of the pK_a values are therefore needed. For chelating anions the size of K_{as} increases along the series $K^+ < Na^+ < Li^+$.

Acidities in Other NHBD Solvents. An acidity scale in *N*-methylpyrrolidin-2-one (NMP) has also been established by using the overlapping indicator method.[9] Relative acidities in NMP and Me_2SO correlate beautifully (see Figure 5 in ref 9), and the absolute acidities do not differ greatly. Ion-pairing association constants with K^+ counterion for chelating anions and homohydrogen bonding constants for phenols[13b] are also similar in NMP and Me_2SO. Since differences in free energies of transfer from H_2O to solvents such as HMPA, Me_2SO, NMP, DMF, and MeCN do not differ greatly,[14] we can expect relative acidities in Me_2SO to provide a good model for those in these NHBD solvents. Differences in free energies of transfer of the proton in these solvents may be appreciable, however, and can lead to sizable differences in absolute acidities. For example, the pK_a's for PhOH in Me_2SO, NMP,[9] and MeCN[8d] are 18.0, 20.1, and 27.2, respectively.

Acids in the pK_a range 32–35 are difficult to measure in Me_2SO ($pK_a = 35$) because of the leveling effect of the solvent.[15] Very weak acids such as amines, alkyl sulfides or ethers, benzenes, alkylbenzenes, alkenes, and alkanes are not deprotonated by $MeSOCH_2K$ in Me_2SO, showing that their pK_a's are above 35. Conceivably, the pK_a's for some of these compounds could be measured in a more weakly acidic solvent such as HMPA, but problems with ion pairing can be expected to increase. It is possible to obtain at least a rough estimate of the pK_a's of some of these compounds by extrapolation, however.[16]

(11) Kaufman, M. J.; Streitwieser, A., Jr. *J. Am. Chem. Soc.* **1987**, *109*, 6092–6097.
(12) Bordwell, F. G.; Drucker, G. F.; Andersen, N. H.; Denniston, A. D. *J. Am. Chem. Soc.* **1986**, *108*, 7310–7313. See ref 11 and Kaufman et al. (Kaufman, M. J.; Gronert, S.; Burs, D. A.; Streitwieser, A., Jr. *J. Am. Chem. Soc.* **1987**, *109*, 602–603) for additional examples and discussion.
(13) (a) Olmstead, W. N.; Bordwell, F. G. *J. Org. Chem.* **1980**, *45*, 3299–3305. (b) Bordwell, F. G.; McCallum, R.; Olmstead, W. N. *J. Org. Chem.* **1984**, *49*, 1424–1427.
(14) Alexander, R.; Ko, E. C. F.; Parker, A. J.; Broxton, T. J. *J. Am. Chem. Soc.* **1968**, *90*, 5049–5069.
(15) Olmstead, W. N.; Margolin, Z.; Bordwell, F. G. *J. Org. Chem.* **1980**, *45*, 3295–3299.
(16) Bordwell, F. G.; Algrim, D. J. *J. Am. Chem. Soc.* **1988**, *110*, 2964–2968.

Table II
Equilibrium Acidities in Dimethyl Sulfoxide at 25 °C

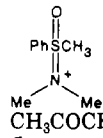
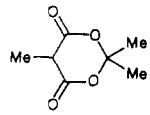
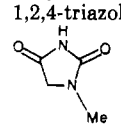
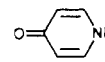
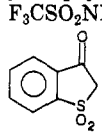
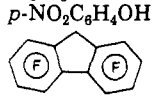
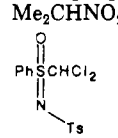
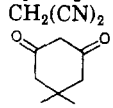
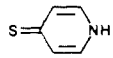
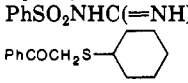
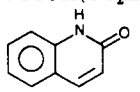
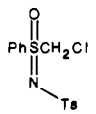
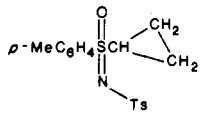
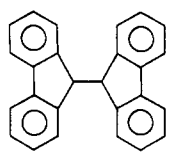
acid	pK_a[a]	acid	pK_a[a]
5-nitrobarbituric acid	0.8	PhCONHOH	13.65
$(F_3CSO_2)_2CH_2$	2.1	2,3-dihydroxynaphthalene	13.7
2,4-dinitronaphthol	2.1	*N*-acetyloxindol	13.8[g]
PhN^+HMe_2	2.45	1,2,3-triazole	13.9
F_3CCO_2H	3.45	uracil	14.1
saccharin	4.0	adenine	14.2
$PhCH(CN)_2$	4.2	$CH_3COCH_2CO_2Et$	14.2
2,6-dinitrophenol	4.9	$(MeSO_2)_2CHPh$	14.3
2,4-dinitrophenol	5.1	2,5-diphenylcyclopentadiene	14.3
$F_3CSO_2CH_2COPh$	5.1	9-cyano-9,10-dihydroanthracene	14.3
PhCOSH	5.2[b]		14.4
Cl_2CHCO_2H	6.4[c]		
$PhSCH(SO_2Ph)_2$	5.55		
$F_3CCH_2SO_2NHPh$	5.7		
$2,4,5\text{-}Cl_3C_6H_2SH$	6.0	$CH_3COCH_2CO_2Et$	14.4
$Ph_3P^+CH_2COPh$	6.1	fluorenone benzylimine	14.5
$Ph_3P^+CH_2CN$	7.05	$F_3CSO_2CH_2Ph$	14.55
$PhSO_2H$	7.1	succinimide	14.6
$PhSO_2CH_2NO_2$	7.1	$CH_3C(=S)NHPh$	14.7
PhSeH	7.1[b]	1,2,4-triazole	14.75
	7.4[d]		14.75
HONO	7.5		
$H_3N^+CH_2CO_2H$	7.5[e]		14.8
$CH_2{=}CHCH_2NO_2$	7.7		
$(C_6F_5)_2CHCN$	7.95	fluorenone phenylhydrazone	14.9
tetrazole	8.2	$MeCH(COCH_3)_2$	15.05
9-cyanofluorene	8.3	1,2,3-triphenylindene	15.2
barbituric acid	8.4	$PhCH_2SH$	15.4
$(CH_3CO)_3CH$	8.6	9-(phenylthio)fluorene	15.4
$H_3N^+CH_2CO_2Et$	8.7[e]	9-(benzylsulfinyl)fluorene	15.7
$p\text{-}NO_2C_6H_4CO_2H$	9.0	$PhSO_2NHNMe_2$	15.8
$F_3CSO_2NH_2$	9.7	nitrocycloheptane	15.8
	10.1	$C_6F_5CH_2CN$	15.8
		nitrocyclopentane	16.0
		$PhSO_2NH_2$	16.1
		$(PhSe)_2CHPh$	16.15
		fluorenone oxime	16.2
PhSH	10.3	$CH_2(CO_2Et)_2$	16.4
$PhCOCH_2CN$	10.2	benzimidazole	16.4
1,3-cyclohexanedione	10.3[d]	$CH_3CH(SO_2Et)_2$	16.7
9-(methoxycarbonyl)fluorene	10.35	$NO_2NH(=NH)NH_2$	16.7
fluoradene	10.5[f]	3-((phenylsulfonyl)methyl)pyridine	16.7
$(F_3C)_3COH$	10.7	isonicotinic hydrazide	16.8
$p\text{-}NO_2C_6H_4OH$	10.8	2,6-di-*tert*-butylphenol	16.85
	10.8	$PhC(=S)NH_2$	16.9
		H_2NCN	16.9
		$PhCH_2SO_2F$	16.9
$F_3CCH(CO_2Me)_2$	10.8	Me_2CHNO_2	16.9
$PhCO_2H$	11.0		16.95
$F_3CSO_2CH_2SPh$	11.0		
$CH_2(CN)_2$	11.0		
	11.2	2-indanone	16.95
		$CH_3CH_2CH_2CH_2SH$	17.0
$PhSO_2CH_2COPh$	11.4	2-pyridone	17.0
	11.8	$PhSO_2NHNH_2$	17.1
		2-naphthol	17.1
benzo-1,2,3-triazole	11.9	$PhCOCH_2SPh$	17.1
$PhSO_2CH_2CN$	12.0	F_3CCONH_2	17.15
$(PhSO_2)_2CH_2$	12.25	CH_3NO_2	17.2
$PhCH_2NO_2$	12.3	nicotinic hydrazide	17.5
CH_3CO_2H	12.3[c]	$CH_3SO_2NH_2$	17.5
9-(ethylsulfonyl)fluorene	12.3	$PhCOCH_2Ph$	17.65
9-isocyanofluorene	12.3	nitrocyclobutane	17.8
pentaphenylcyclopentadiene	12.5	nitrocyclohexane	17.9
5-fluorouracil	12.7	9-phenylfluorene	17.9
5,5-diethylbarbituric	13.0	$(CH_3CO)_2NH$	17.9
$(CH_3CO)_2CH_2$	13.3	cyclopentadiene	18.0
2-thiopyridone	13.3	PhOH	18.0
$(PhCO)_2CH_2$	13.35	$(PhSO)_2CH_2$	18.1
$(PhNH)_2C{=}S$	13.4	$(CH_3)_3S^+$	18.2

Table II (Continued)

acid	pK_a[a]
oxindole	18.2[g]
$(EtO)_2P(O)NHPh$	18.3
p-$CH_3C_6H_4SO_2CH_2N{=}C$	18.4
$CH_3C(=S)NH_2$	18.45
$PhCON(Me)OH$	18.5
N-methyloxindole	18.5[g]
imidazole	18.6
$PhCOCH_2SePh$	18.6
1,3-dimethylbenzoimidazolium ion	18.6
$PhCOCHPh_2$	18.7
$(PhCH_2)_2C{=}O$	18.7
$F_3CSO_2CH_3$	18.75
$PhCONHNH_2$	18.9
4-chloro-2-nitroaniline	18.9
$PhCOCH_2SCH_2Ph$	19.0
$Me_3SiCH(CO_2Et)_2$	19.0
$PhCH_2SO_2SCH_2Ph$	19.1
$Ph_2P(S)CH_2P(S)Ph_2$	19.3
2-phenylindene	19.4
$PhSO_2NHC(=NH)NH_2$	19.45
	19.45
$PhCH_2N{=}CHCO_2Et$	19.5
$(PhNH)_2C{=}O$	19.55
$NH_2C(=NH)NHCN$	19.6
pyrazole	19.8
carbazole	19.9
$PhCH_2SO_3Ph$	19.9
$PhCH_2COCH_3$	19.9
10-cyano-9-methylanthracene	20.0
indene	20.1
$Ph_2C{=}NOH$	20.1
$PhCH{=}CHCH_2SO_2Ph$	20.2
$PhCOCHF_2$	20.2
$PhSO_2CH_2PPh_2$	20.2
$PhCOCH_2NPh_2$	20.3
p-$NO_2C_6H_4CH_3$	20.4
$F_3CSO_2CH_2Me$	20.4
i-$PrCH(CO_2Et)_2$	20.5
	20.7
	20.7
2-benzylbenzothiazole	20.8
	20.8
	20.9[h]
	24.7[h] (pK_a^{II})
indole	20.95
$PhCH{=}NNHPh$	21.1
$PhCOCH_2OPh$	21.1
$(H_2N)_2C{=}S$	21.1
$PhCONHC(=NH)NH_2$	21.25
$PhSO_2CH_2SiPh_3$	21.3
$PhCH_2C(=S)NMe_2$	21.3
$PhSCH_2CO_2Me$	21.4
$CH_3CONHPh$	21.45
isonicotinamide	21.5
$PhC(Me){=}NNHPh$	21.5
phenoxazine	21.65
$PhCOCH_2F$	21.7
9-(trimethylsilyl)fluorene	21.7
4-acetylpyridine	21.8

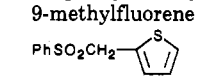
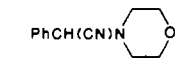
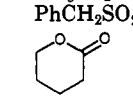
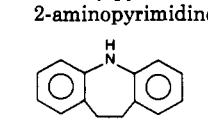
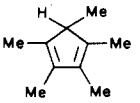
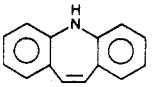
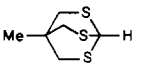
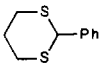
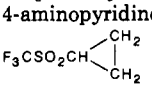
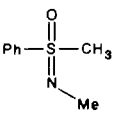
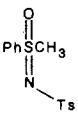
acid	pK_a[a]
$F_3CSO_2CHMe_2$	21.8
2-methylindene	21.8
$CH_3CONHNH_2$	21.8
$PhCH_2CN$	21.9
9-cyano-9,10-dihydrophenanthrene	21.9
nicotinamide	22.0
$CH_3COCH_2SO_2Ph$	22.1
$PhC{\equiv}CCH_2SO_2Ph$	22.1
4,5-methylenephenanthrene	22.2
2-thiophenecarboxamide	22.3
$PhSO_2CHPh_2$	22.3
2-((phenylsulfonyl)methyl)furan	22.3
9-methylfluorene	22.3
	22.35
	22.4
$CH_2{=}CHCH_2SO_2Ph$	22.5
3-methylindene	22.5
$Ph_3PCH_3^+$	22.5
2-furancarboxamide	22.55
fluorene	22.6
$PhCH_2CO_2Et$	22.6
phenothiazine	22.7
$(PhS)_3CH$	22.8
pyrrole	23.0
$PhCH(Me)CN$	23.0
$PhOCH_2CONH_2$	23.0
1-indanone	23.0[i]
	29.6[i] (pK_a^{II})
$PhSCH_2CONH_2$	23.0
$PhSeCH_2CONH_2$	23.1
2,3,4-trimethylimidazolium ion	23.2
4-aminopyrimidine	23.3[j]
$(H_2NNH)_2C{=}O$	23.3
$PhCONH_2$	23.35
$(Ph_2C{=}CH)_2CHPh$	23.4
$PhSO_2CH_2Ph$	23.4
$HCONH_2$	23.45
F_3CCH_2OH	23.45
$PhSO_2CH_2Cl$	23.8
$(PhCH_2)_2SO_2$	23.9
$MeOCH_2CONH_2$	23.9
t-$BuSCH_2CONH_2$	24.1
p-$F_3CSO_2C_6H_4CH_3$	24.1
2-pyrrolidone	24.2
3-((phenylsulfonyl)methyl)thiophene	24.2
cyclohexanone oxime	24.3
$Ph_2C{=}NCH_2Ph$	24.3
benzoxazole	24.4
$CH_3COCH_2CH_3$	24.4
H_2NCO_2Et	24.6
t-$BuCH(CO_2Et)_2$	24.7
$H_2NCH_2CONH_2$	24.7
$PhCH_2CONH_2$	24.7
$PhCOCH_3$	24.7
Ph_2NH	24.95
cyclobutanone	25.05
CH_3SO_2OPh	25.2
$PhCH_2SO_2NMe_2$	25.2
	25.2[d]
4-benzylpyridine N-oxide	25.2
2-aminopyrimidine	25.3[j]
	25.5
CH_3CONH_2	25.5
$Ph_2C{=}CHCH_2Ph$	25.6
$CH_3C(=S)NMe_2$	25.65
$Ph_2C{=}CHCHPh_2$	25.8
c-C_5H_9COPh	25.8
cyclopentanone	25.8

Table II (Continued)

acid	pK_a[a]	acid	pK_a[a]
	26.1	CH_3OH	29.0
		thiazole	29.4
		p-$PhSO_2C_6H_4CH_3$	29.85
		2-benzylthiophene	29.9
	26.1	xanthene	30.0
		3-benzylpyridine	30.15
		$(CH_3)_2CHOH$	30.25
		4-methylthiazole	30.3
c-C_4H_7COPh	26.15	camphor	30.4
$PhNHNHPh$	26.2		30.6
$Me_2CHCOPh$	26.25		
2-piperidone	26.4		
cyclohexanone	26.4	$PhNH_2$	30.6
CH_3COCH_3	26.5	Ph_3CH	30.6
4-aminopyridine	26.5		30.65
	26.6		
c-$C_6H_{11}COPh$	26.7	$PhSO_2CH_2OMe$	30.7
4-benzylpyridine	26.7	$PhSCH_2Ph$	30.8
cyclodecanone	26.8	9-methylanthracene	31.1
$PhSCHPh_2$	26.8	$CH_3SO_2CH_3$	31.1
$(H_2N)_2C{=}O$	26.95	$(n\text{-}PrS)_3CH$	31.3
benzothiazole	27.0	CH_3CN	31.3
$PhS(O)CH_2Ph$	27.2	H_2O	31.2
$PhCH_2N{\equiv}C$	27.4	Ph_2CH_2	32.2
heptamethylindene	27.4	$(CH_3)_3COH$	32.2
$(EtO)_2P(O)CH_2Ph$	27.55		(33)[k]
2-methylbenzothiazole	27.6		
2-aminopyridine	27.7		
	27.7	$CH_3S(O)CH_3$	35
		4-methylpyridine	(35)[k]
cycloheptanone	27.8		(39)[k]
9-phenylxanthene	27.9		
$PhSO_2CH_2OPh$	27.9	NH_3	(41)[k]
4-methyloxazole	28.0	2-methylnaphthalene	(42)[k]
$PhOCH_2CN$	28.1	$PhSCH_3$	(42)[k]
$[(CH_3)_2CH]_2C{=}O$	28.2	2-methylthiophene	(42)[k]
2-benzylpyridine	28.2	2-methylfuran	(43)[k]
c-C_3H_5COPh	28.25	$PhCH_3$	(43)[k]
$PhSO_2CH_2F$	28.5	$CH_2{=}CHCH_3$	(44)[k,l]
3-aminopyridine	28.5	CH_3SCH_3	(45)[k]
$PhC{\equiv}CH$	28.7	$PhOCH_3$	(49)[k]
$(EtO)_2P(O)CH_2SiMe_3$	28.7	CH_4	(56)[k]

[a] The pK_a's were selected from a list of about 1200 that have been measured in our laboratory. The pK_a's of oxygen acids have been corrected for homohydrogen bonding, and pK_a's of acids forming chelating anions have been corrected for ion pairing with K^+. The ylides formed from cations are often reactive, and these values should be regarded as tentative. Most pK_a's were measured by using two or more indicators or standard acids and are believed to be accurate to ±0.1 unit. [b] Courtot-Coupez, J.; Le Démézet, M. *Bull. Soc. Chim. Fr.* **1969**, 1033–1039. [c] Ritchie, C. D.; Lu, S., private communication. [d] Arnett, E. M.; Harrelson, J. A., Jr. *J. Am. Chem. Soc.* **1987**, *109*, 809–812. [e] Hughes, D. L.; Bergan, J. J.; Grabowski, E. J. *J. Org. Chem.* **1986**, *51*, 2579–2585. [f] Ritchie, C. D.; Uschold, R. E. *J. Am. Chem. Soc.* **1968**, *90*, 2821–2824. [g] Fried, H. E. Ph.D. Dissertation, Northwestern University, 1982. [h] Streitwieser, A., Jr *Acc. Chem. Res.* **1984**, *17*, 353–357. [i] Cornforth, F. W. Ph.D. Dissertation, Northwestern University, 1974. [j] Shkurko, O. P.; Terekhova, M. J.; Petrov, E. S.; Mamaev, V. P.; Shatenshtein, A. J. *J. Org. Chem. USSR* (*Engl. Transl.*) **1981**, *17*, 260–264. [k] Values in parentheses were extrapolated by methods such as those described in ref 16. [l] From ref 52, assuming a BDE of 81 for the C–H bond in Ph_3CH.[51]

The Me_2SO Acidity Scale. Structural Effects on Acidities. In Table II we present data for equilibrium acidities in Me_2SO for over 300 compounds. The effects of structural variations on acidities for many of these have been discussed in papers from our laboratory, including the effects of cyclopropyl rings,[20] α-electron-withdrawing groups,[21] α-heteroatoms,[22] phenyl groups,[23] phenylthio groups,[24] alkyl groups on C–H acids,[25] sp hybridization at carbon,[26] remote substituents (in

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(18) In CHA a Brønsted-type extrapolation gives an ion-pair pK_a for toluene of 41,[7b] and two extrapolations from azine acidities in DME have given a value of 42.

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(21) (a) Bordwell, F. G.; Van Der Puy, M.; Vanier, N. R. *J. Org. Chem.* **1976**, *41*, 1884–1885. (b) Bordwell, F. G.; Algrim, D. *J. Org. Chem.* **1976**, *41*, 2507–2508.

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fluorenes[27] and in acetophenones[28]), ion pairing,[13] steric inhibition of resonance,[29] alkyl groups in alcohols,[15] aromaticity,[30] thiol groups,[31] methyl effects on cyclopentadienes and indenes,[32] and homohydrogen bonding in phenols.[13b] Papers from other laboratories have discussed the first and second ionization constants of 9,9′-bifluorenyl,[33] the effects of cyclization on acidities of ketones and carboxylic esters containing β-dicarbonyl groups,[34] and the effects of aza groups on acidities.[35] In the accompanying paper we compare some of these structural effects on solution acidities with those on intrinsic gas-phase acidities and discuss insights into solvation effects derived therefrom.[36]

The Me_2SO acidity scale has proved useful in several ways. Jorgensen and his students have used the pK_a's as one of the parameters in an interactive computer program, CAMEO, that is being designed to predict products of organic reactions, given the starting materials and conditions.[37] By combining pK_a values in Me_2SO for 21 delocalized carbanions and 5 phenoxide ions with calculated π delocalization energies of 6 carbocations, Arnett has developed a "master equation" to correlate data for 30 reactions ($r = 0.9948$). Equations of this type are capable of providing a simple means of estimating heterolysis energies in solution for thousands of bonds that give resonance-stabilized anions and cations on cleavage.[38] In our laboratory we have found that rates (log k_{obsd}) of reactions between the conjugate bases of various families of acids and electrophiles can be correlated generally with pK_{HA} values to give linear Brønsted plots,[39] as will be brought out in the next section.

Acid–Base Families and the Brønsted Relationship. For acids in Table II containing an aromatic nucleus, Hammett-type families can be prepared by placing substituents in remote positions. Taft-type families such as GCH_2CONH_2 and GCH_2COPh can also be prepared, and other types of families can be constructed from various groups of acids, e.g., an azole family (pyrrole, pyrazole, imidazole, etc.). The ρ values in Table III, when combined with literature σ and σ_p^- values,[40] provide a means of estimating pK_a's for hundreds of additional acids.

Table III
Hammett ρ Values for Equilibrium Acidities in Me_2SO Solution at 25 °C

acid family	pK_a[a]	ρ[b]	n[f]	R^2	ref
$ArCH(CN)_2$	4.2	4.2 ± 0.1	5	0.997	*g*
$ArSO_2H$	7.1	2.4 ± 0.2	4	0.986	*h*
ArSH	10.2	4.8 ± 0.3	5	0.988	31
$ArCO_2H$	11.0	2.6	9		*i*
ArCONHOH	13.65	2.6	4	0.989	*j*
ArOH	18.0	5.3 ± 0.1	8	0.991	13b
$ArCH_2COCH_3$	19.9	4.7	4	0.999	*k*
$ArNHCOCH_3$	21.45	4.1	6		*k*
$ArCH_2CN$	21.9	5.9	8	0.939	*l*
$ArCH(NC_4H_4O)CN$	22.4	7.0 ± 0.1	7	0.996	*m*
fluorenes	22.6	7.5 ± 0.53[c]	14	0.939	27
fluorenes	22.6	5.7 ± 0.3[d]	7	0.989	*g*
phenothiazines	22.7	5.21	5	0.982	*n*
$ArCH_2SO_2Ph$	23.4	4.8	10	0.999	*g*
$ArCOCH_3$	24.7	3.55 ± 0.05	14	0.998	28
ArNHPh	24.95	5.4	3	0.997	*n*
GCH_2CONH_2	25.5	3.1 ± 0.3	13	0.976	22b
$ArNH_2$	30.6	5.7 ± 0.1	6	0.998	*o*
$ArCHPh_2$	30.6	5.7 ± 0.3[d]	7	0.989	*p*
9-methylanthracenes	36.1	>10[e]	9		*l*

[a] pK_a of the parent acid. [b] The Hammett plots are restricted for the most part to meta points; $\sigma_{m\text{-}OMe}$ is 0.02 in Me_2SO, however, rather than the value of 0.12 derived from benzoic acids in water.[28] The σ_p^- values for *p*-NO_2, *p*-RCO, and like substituents are made abnormally high, in part, by substituent solution-assisted resonance (SSAR) effects.[36] [c] For 2- and 2,7-substituents; ρ is abnormally high because PhCO, CN, etc. groups are included and the 2- and 2,7-positions have some para character. [d] For 3-substituents. [e] ρ is abnormally high; the correlation is poor since para substituents are used and steric effects in the 10-position are severe. [f] Number of substituents. [g] Branca, J. C. Ph.D. Dissertation, Northwestern University, 1979. [h] Hughes, D. L. Ph.D. Dissertation, Northwestern University, 1981. [i] Ritchie, C. D.; Uschold, R. E. *J. Am. Chem. Soc.* **1968**, *90*, 2821–2824. [j] Hughes, D. L.; Whang, Y., unpublished results. [k] Chehel-Amiran, M., unpublished results. [l] Bares, J. E. Ph.D. Dissertation, Northwestern University, 1976. [m] Mueller, M. E., unpublished results. [n] Cheng, J.-P. Ph.D. Dissertation, Northwestern University, 1987. [o] Algrim, D. J. Ph.D. Dissertation, Northwestern University, 1981. [p] Twyman, C. L. unpublished results.

Rates of reactions of electrophiles with the conjugate bases of acids within a family can be studied under conditions where steric, as well as solvent, effects are kept constant. Plots of log k_{obsd} vs pK_{HA} values give linear Brønsted plots, which are similar to Hammett plots but are much more precise since they do not depend on an arbitrary model (the pK_a's of benzoic acids in water). These Brønsted plots have been found to be linear for nearly all combinations of anions with electrophiles tried to date. The types of reactions include S_N2,[41] S_N2',[42] E2,[43] S_NAr,[44] H_T^+,[45] and e_T^-.[46] This means that for all of these reactions the nucleophilicities of the bases depend on only two factors, (a) their basicity, as measured by pK_{HA}, and (b) the sensitivity of the reac-

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(35) Terekhova, M. I.; Petrov, E. S.; Mikhaeleva, M. A.; Shkurko, O. P.; Mamaev, V. P.; Shatenshtein, A. I. *J. Org. Chem. USSR (Engl. Transl.)* **1982**, *18*, 6–10.
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(38) (a) Arnett, E. M.; Molter, K. E. *Acc. Chem. Res.* **1985**, *18*, 339–346. (b) Arnett, E. M.; Chawla, B.; Amarnath, K.; Whitsell, L. G., Jr. *Energy Fuels* **1987**, *1*, 17–23. Arnett, E. M.; Whitsell, L. G., Jr.; Amarnath, K.; Cheng, J.-P.; Marchot, E. *J. Macromol. Chem., Macromol. Symp.* **1988**, *13/14*, 21–31.
(39) For a review, see: Bordwell, F. G.; Hughes, D. L.; Cripe, T. A. in *Nucleophilicity*; Harris, M. J., McManus, S. P., Eds.; Advances in Chemistry 215; American Chemical Society: Washington, DC, 1987; Chapter 9.
(40) See Exner[1b] for an extensive list of σ vs σ^- constants. The σ^- values for the NO_2, RCO, CN, and RSO_2 groups are exalted in Me_2SO in part by substituted solvation-assisted resonance (SSAR) effects.[36]
(41) Bordwell, F. G.; Hughes, D. L. *J. Am. Chem. Soc*, **1986**, *108*, 7300–7309 and references cited therein.
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(46) Bordwell, F. G.; Bausch, M. J. *J. Am. Chem. Soc.* **1986**, *108*, 1985–1988.

tion to changes in basicity, as measured by the slope of the Brønsted plot, β_{Nu}. β_{Nu} values usually fall in the range 0.2–0.5 for S_N2, S_N2', and E2 reactions and in the range 0.5–1.0 for S_NAr, H_T^+, and e_T^- reactions.

Reactions of $PhCH_2Cl$ with families of delocalized anions bearing various types of donor atoms have been found to have similar β_{Nu} values. This has allowed the rate constant order for anions of the same basicity, but with different donor atoms, reacting with electrophiles such as $PhCH_2Cl$ to be approximated: S^- (10^3) > C^- (1.0) > O^- (0.3) > N^- (0.1).[47] For reactions having β_{Nu} values of 0.3 the total rate span is about 10^9 for delocalized anions derived from the acids in Table II, but the rate span increases exponentially as β_{Nu} increases. Thus, for some proton- or single-electron-transfer reactions, where β_{Nu} can approach unity, the rate span will be of the order of 10^{30}.

Acidities, Basicities, Reactivities, and Redox Potentials in Me_2SO. A plot of the oxidation potentials, $E_{ox}(A^-)$, of 2- and 2,7-substituted fluorenide ions vs the pK_{HA} values of their conjugate acids is linear with a slope near unity, indicating that substituents in the 2- and 2,7-positions do not stabilize (or destabilize) 9-fluorenyl radicals.[48] This explains the linearity of Brønsted plots, with slopes near unity, observed for 2-G- and 2,7-G_2FlH^- ions reacting by single-electron transfer (SET) with acceptors such as 1,1-dinitrocyclohexane.[46] On the other hand, 9-G substituents have strong stabilizing (or destabilizing) effects on 9-G-Fl$^\bullet$ radicals, the size of which can be measured, relative to 9-H-Fl$^\bullet$, by eq 2.[48]

$$\Delta E_s = 1.37\Delta pK_{HA} + 23.06\Delta E_{ox}(A^-) \qquad (2)$$

In eq 2, ΔE_s provides an estimate of the effect of the 9-G substituent on the energy of the 9-G-Fl$^\bullet$ radical, relative to that of the 9-H-Fl$^\bullet$ radical. The ΔE_s values range from a stabilizing effect of as much as −10 kcal/mol for G = R_2N to a destabilizing effect of +2 kcal/mol for G = RSO_2.[48] These ΔE_s values can be equated with the relative homolytic bond dissociation energies (ΔBDEs) of 9-C–H bonds in the corresponding fluorenes, 9-G-Fl-H.

Absolute BDEs for acidic C–H bonds in hydrocarbons or their derivatives can be estimated from eq 3, which

$$BDE = 1.37pK_{HA} + 23.06E_{ox}(A^-) + 55.9 \qquad (3)$$

is based on a thermodynamic cycle derived by Nicholas and Arnold.[49] (Equation 3 was derived earlier in a different way by Friedrich and used to estimate BDEs in water for hydroquinone and phenol.[50]) The BDEs in Me_2SO solution for the acidic C–H bonds in fluorene, indene, cyclopentadiene, 9-methylanthracene, diphenylmethane, triphenylmethane, xanthene, phenol, thiophenol, and aniline estimated in this way agree satisfactorily with gas-phase BDEs.[51]

The pK_a values for a few hydrocarbons, including cyclopentadiene (CpH_2), toluene, propene, and isobutane, relative to triphenylmethane have been estimated from the algebraic sum of the differences in their BDEs and the differences in the oxidation potentials of their conjugate bases.[52] For example, the $E_{ox}(A^-)$ value for the CpH^- ion was found to be less negative than that of the Ph_3C^- ion by 18 kcal/mol. When the 6 kcal/mol difference in the BDEs of CpH_2 (81 kcal/mol) and Ph_3CH (75 kcal/mol) was taken into account, an estimated difference in acidities of 8.8 pK_a units was arrived at. If we use the pK_a of 30.6 for Ph_3CH in Me_2SO as a reference, the estimated relative pK_a for CpH_2 is then 22. But, as Breslow points out, the pK_a of 22 rests in part on the BDE of 75 for Ph_3CH, which has not been checked by modern methods.[52] Indeed, if the BDE of 81 ± 3 estimated by eq 3 is used,[51] the pK_a calculated for CpH_2 becomes 17.6, which is in good agreement with the value of 18.0 determined in Me_2SO (Table II).

By combining pK_{HA} values with $E_{ox}(A^-)$ and $E_{ox}(HA)$ values, according to eq 4, it is possible to estimate

$$pK_{HA}^{\bullet+} = pK_{HA} + 23.06[E_{ox}(A^-) - E_{ox}(HA)]/1.37 \qquad (4)$$

acidities of radical cations of the type $HA^{\bullet+}$, where A may be S, O, N, C, and the like.[53] Direct experimental determination of $pK_{HA}^{\bullet+}$ values presents a formidable problem since establishment of the equilibrium $HA^{\bullet+} \rightleftarrows H^+ + A^\bullet$, which involves two radical species, is difficult, as is the measurement of the radical concentrations. The method is of particular value for estimating acidities of radical cation C–H acids, which generally have $pK_{HA}^{\bullet+}$ values of 0 to −30.[54] A similar method, which is also based on a thermodynamic cycle, has been used to estimate the acidities of the conjugate acids of radical anions.[55]

For single-electron-transfer (SET) reactions from fluorenide carbanions to an acceptor of the type 1,1-$(NO_2)_2$-c-C_6H_{10} or 1-NO_2-1-Ts-c-C_6H_{10}, Marcus-type plots of log k_{obsd} vs $E_{ox}(A^-)$ have been found to be linear in several instances.[46,56] Recently, a family of seven 9-R_2N-fluorenide ions having basicities that vary over a relatively small range (pK_{HA}'s = 20.4 ± 2.2) but have $E_{ox}(A^-)$ values varying over a substantial range (0.427 V; 9.8 kcal/mol) has proved useful for testing for the presence of an SET component in S_N2-type substitution reactions.[58] This family gave a linear Marcus-type plot for reactions with F_3CCH_2I, a known SET acceptor,[59] and the log k_{SET} values calculated with the Marcus equation, with λ and $\Delta G°$ values derived according to the method of Eberson,[60] were found to correspond well

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with the experimental log k_{obsd} values. Application of the test to reactions with Ph_2CHCl, which gives S_N2 kinetics and products with no evidence of radical-type products, gave linear Marcus-type plots and log k_{SET} values corresponding to the experimental log k_{obsd} values. It was concluded that the "S_N2 reactions" of 9-R_2N-Fl$^-$ ions with Ph_2CHCl are occurring by a radical pair mechanism. This approach promises to be of general use for elucidating the role of SET in reactions of families of anions with electrophiles.

Concluding Remarks. The Me_2SO acidity scale, for which about 300 representative values are given in Table II, furnishes (a) quantitative acidity data that can be related to intrinsic gas-phase data to provide information on solvation effects and (b) quantitative basicity data that can be related to reactivity data by means of Brønsted, Hammett, and Marcus equations. Combination of the pK_a data with electrochemical data can provide estimates of (a) relative radical stabilities, (b) homolytic bond dissociation energies of H–A acids, (c) radical cation acidities, and (d) the acidities of radicals.

The experimental results presented and referred to in this paper were obtained during the past 15 years by the students whose names appear in the references. Special thanks goes to W. S. Matthews, who modified the Steiner method, reducing the time necessary for each titration and at the same time making it more precise. The author wishes to express his sincere appreciation to these research associates and wishes to thank the National Science Foundation and Petroleum Research Fund for financial support during this period. Discussions with Prof. R. W. Taft were most helpful in preparing the manuscript. We are grateful to Crown Zellerbach Corp. and more recently to Gaylord Chemical Corp. for generous gifts of dimethyl sulfoxide.

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Structural and Solvent Effects Evaluated from Acidities Measured in Dimethyl Sulfoxide and in the Gas Phase[1]

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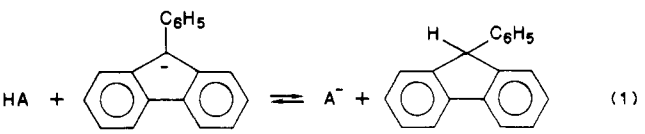
The preceding paper gives extensive data for equilibrium acidities in dimethyl sulfoxide (Me_2SO) and makes comparisons with corresponding results in other condensed-phase media.[2] In the present paper the results of 76 selected gas-phase acidities[3] are compared with corresponding results in Me_2SO as a means of separating inherent effects of molecular structure on acidities from solvent effects. Simplified concepts are presented on relationships between solvent effects and structure. Broad applicability of the results and concepts is shown.

Table I gives comparisons of gas-phase and Me_2SO acidities, expressed by $1.364\Delta pK_a = -\Delta G°$ values in kcal/mol (hereafter abbreviated as kcal) for the proton-transfer equilibria (eq 1) of 76 typical acids HA with



(1)

9-phenylfluorenide ion (9-PhFl$^-$). The acids have been selected to illustrate important kinds of structural and solvent effects. Positive values of $-\Delta G°$ indicate greater acidity (lower pK_a) for HA than for 9-phenylfluorene (9-PhFlH) and vice versa. The acidities from NH_4^+ to CH_4 cover a range of 211 kcal in the gas phase and 74 kcal in Me_2SO solution. The values in the table are arranged in order of increasing Me_2SO medium effects, as defined by $\Delta G°_{(g)} - \Delta G°_{(s)} = \delta_s \Delta G°$ (where s = Me_2SO), which cover a range of 160 kcal or 117 pK_a units.

Robert W. Taft is Professor of Chemistry at the University of California, Irvine. Born in Lawrence, KS, Taft received a B.S. in Chemistry from the University of Kansas and a Ph.D. from The Ohio State University where he worked with Melvin Newman. Following a postdoctoral year with Louis Hammett at Columbia University, Taft spent 15 years at The Pennsylvania State University. He has been at Irvine since it began in 1965. The present Account is taken from extensive studies of the effects of molecular structure on gas-phase proton-transfer equilibria, using ion cyclotron resonance spectroscopy. Current work also includes binding studies in the gas phase with a variety of univalent cations. Additional interests include studies of structural and solvent effects on hydrogen-bond acidities and basicities and their applications to treatments of solute partitioning between bilayers and biological activities.

Frederick G. Bordwell is Professor Emeritus at Northwestern University. (For a biography summarizing his earlier research activities, see *Acc. Chem. Res.* **1972**, *5*, 374). In the period 1970–1980 the Bordwell research group established acidity scales in Me_2SO and *N*-methyl-2-pyrrolidone solvents using a method adapted from one developed by E. C. Steiner at Dow Chemical Co. Since 1980 the research focus has shifted to the application of the data in the Me_2SO scale to problems in physical organic chemistry, the results of which are summarized in the preceding Account in this issue.

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(1) This work was supported by grants from the National Science Foundation (UCI and NU).
(2) Bordwell, F. G. *Acc. Chem. Res.*, preceding paper, in this issue.
(3) All neutral-acid $-\Delta G°_{(g)}$ values are from the gas-phase acidity scale of Prof. J. E. Bartmess (available by request in care of the Department of Chemistry, University of Tennessee, Knoxville, TN 37996-1600). The unpublished results of Drs. F. Anvia, A. D. Headley, J. F. Gal, I. Koppel, M. Mishima, R. W. Taft, and S. Ueji have been incorporated into this scale, which is anchored to the most reliable absolute thermodynamic acidities. The gas-phase acidities for the three positively charged acids in Table I are from ref 4 with correction to a proton affinity of NH_3 of 204.0 kcal/mol. All $-\Delta G°_{(s)}$ values are from pK_a's cited in ref 2.

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CONDUCTOMETRIC STUDIES ON THE DISSOCIATION CONSTANTS OF PHOSPHORIC ACID IN METHANOL–WATER MIXTURES

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Abstract – The dissociation constants of phosphoric acid have been determined in methanol–water mixtures (8.0–87% by weight of methanol) by the method suggested by Gelb. The results have been verified by Fuoss–Kraus method. Λ^0 values of $HClO_4$, $KClO_4$ and KH_2PO_4 (and hence of H_3PO_4) in mixed solvents have been determined in the usual way. The role of solvents on the dissociation constants has been discussed.

The dissociation constants of weak organic acids in different mixed solvents have been the subject of much study by different methods. However, relatively little is known regarding the dissociation constants of inorganic acids in mixed solvents. We present in this communication the determination of the dissociation constants of H_3PO_4, an acid readily available and widely used for the preparation of buffers involving a wide range of pHs. This will enable us to prepare buffers in different mixed solvents, the lack of which is one of the strong handicaps for the measurement of H^+ ion concentrations in mixed solvents by pH-metry.

We have applied conductometric methods as the methods involve no serious approximations[1, 2].

EXPERIMENTAL

Conductivity water was prepared by redistilling good distilled water with alkaline permanganate in an all-glass distilling set. Precautions were taken to prevent the water from contamination with CO_2. Methanol (E. Merck) was treated with sufficient quantity of preheated CaO, kept overnight and distilled in an all-glass distilling set. The middle fraction was collected and used without further treatment. The slight traces of water, if any, were neglected. Phosphoric acid (G.R.* E. Merck) was used. The purity of phosphoric acid was tested gravimetrically. $HClO_4$ (G.R. E. Merck) used were estimated by acid-base titration with standardized caustic soda solution. Succinic acid (G.R. E.M.) was used as a primary standard. KH_2PO_4 (A.R.†–B.D.H.) was recrystallised from water, dried and estimated gravimetrically. $KClO_4$ was prepared by adding fairly concentrated KOH (G.R. E.M.) to solutions of $HClO_4$ acid. The precipitated $KClO_4$ was recrystallized twice from water and used. The weight percentages of methanol in the solvent-mixtures were obtained in the same way as described previously[3].

Conductance measurements were made with the aid of a Leeds and Northrup model 4959 conductance bridge with a sensitivity of ±0.1%. A 1000 Hz signal was employed. A dip-type Philips condutance cell with cell constant, $\theta_1 = 0.82\,cm^{-1}$ was utilized. Cell constant was determined in the usual way. The measurements were done at 25° ± 0.02°C.

In the "titration" method[4], the conductance of a known amount of the acid solution of known strength was measured. At regular intervals definite amounts of $HClO_4$ of higher strength in appropriate solvent were added. The conductance at each stage was recorded after allowing the mixture to attain equilibrium. The blank "titrations" were performed in the same way, the experimental solution being replaced by the same amount of the appropriate solvent mixtures. The conductances of the solvent mixtures were also taken to apply corrections where necessary. The experiment was repeated to obtain concurrent results.

Similarly, experiments were performed both with different concentrations of the acid and salts in different percentages of methanol. The ionic strength were kept as low as practicable ($\approx 10^{-3} - 10^{-4}$ M).

RESULTS

For a mixture of completely dissociated $HClO_4$ (an assumption which is regarded to be valid in mixed solvents containing high percentages of organic solvents and at low concentrations of $HClO_4$) and partly dissociated HA, the dissociation constant for the reaction (only the first dissociation constant of H_3PO_4 is considered).

$$HA \rightleftharpoons H^+ + A^- \qquad (1)$$

can be written as

$$K = \frac{\alpha(C_{HA}\alpha + C_{HClO_4}) \times f_{\pm}^2}{(1-\alpha)} \qquad (2)$$

The terms have their usual significance.

However, when the conductances of the mixture, $1/R$ (containing HA and $HClO_4$) and blank solutions ($1/R^*$) (containing $HClO_4$ only) are equal, we obtain

* G. R. – Guaranted Reagent.
† A.R. – Analar Reagent.

following Gelb[4],

$$C_{HClO_4}\Lambda'_{HClO_4} + \alpha C_{HA}\Lambda'_{HA} = C_{*HClO_4}\Lambda'_{*HClO_4} \quad (3)$$

or

$$C_{HClO_4} + \alpha C_{HA} = C_{*HClO_4} \quad (4)$$

(* indicates the quantities in blank solution)

assuming $\Lambda'_{HClO_4} = \Lambda'_{HA}$ and $\Lambda'_{HClO_4} = \Lambda'_{*HClO_4}$ in aqueous and mixed solvents from which α can be calculated. Here, Λ' = molar conductance of the completely dissociated electrolyte at any ionic strength and Λ = the molar conductance of the electrolyte at that ionic strength *ie* $\Lambda = \alpha\Lambda'$

$\alpha = 1$, in the case of $HClO_4$.

However, refinement of α values are possible taking

$$\alpha = (C_{*HClO_4} - C_{HClO_4})/\beta C_{HA}$$

where

$$\beta = \frac{\Lambda'_{HA}}{\Lambda'_{HClO_4}} \approx \frac{\Lambda^0_{HA}}{\Lambda^0_{HClO_4}}$$

as we have used dilute solutions and kept ionic strengths sufficiently low.

Λ^0 values of perchloric acids in different mixed-solvents have been determined in the same way as described before. The values of Λ^0_{HA} and K were obtained from the plot of ΛC against $1/\Lambda$ of a number of dilute solutions of HA utilizing the equation

$$\Lambda C = -K\Lambda^0 + K\Lambda^{0^2}\,1/\Lambda.$$

The method, however, is an approximate one particularly for weak electrolytes and in solvents of low dielectric constants. The values of K and Λ^0 can be considerably improved by a method of computation used by Fuoss and Kraus[5,6] utilising the equation

$$\frac{F(z)}{\Lambda} = \frac{1}{K\Lambda^{0^2}} \cdot \frac{\Lambda C f_\pm^2}{F(z)} + \frac{1}{\Lambda^0}$$

The value of the Onsager constants

$$\left[\sigma = \frac{82.4}{(\varepsilon T)^{1/2}\eta}\right],$$

and

$$\theta = \frac{8.20 \times 10^5}{(\varepsilon T)^{3/2}}$$

used to calculate

$$z = \frac{(\theta\Lambda^0 + \sigma)\sqrt{\Lambda C)}}{\Lambda^{0^{3/2}}}$$

were calculated from the interpolated values of viscosities and relative permittivities of methanol–water mixtures obtained from the literature[7]. Previously determined Λ^0 values in different percentages of mixed solvents and θ and σ values were utilized to calculate z and hence $F(z)$. The values of $\log f_\pm$ were calculated from

$$-\log f_\pm = A\sqrt{(\alpha C)}$$

using appropriate values of A in different mixed solvents. Generally, two iterations are sufficient to have consistent results.

However, the improvement in the values of K and Λ^0 is very small in the present case, since the solutions under study are of low ionic strengths. The accuracy of extrapolated Λ^0 values are limited. Improved values of $\Lambda^0_{H_3PO_4}$ were obtained from the relation

$$\Lambda^0_{H_3PO_4} = \Lambda^0_{HClO_4} + \Lambda^0_{KH_2PO_4} - \Lambda^0_{KClO_4}$$

where $\Lambda^0_{HClO_4}$, $\Lambda^0_{KH_2PO_4}$ and $\Lambda^0_{KClO_4}$ are obtained in the usual way.

DISCUSSIONS

One of the aims of the present work is to find a suitable method of determining the dissociation constants of weak acids in mixed solvents. The results definitely indicate that the method suggested by Gelbe[4] can be well-adopted in mixed solvents. The method is rapid and ensures high accuracy. The accuracy could be considerably improved with sensitive bridges and better facilities. The method due to Fuoss and Kraus[5,6] along with measured Λ^0 values

Table 1. Dissociation constants of phosphoric in methanol–water mixtures (temp. 25°C)

Mole fraction of MeOH	$1/\varepsilon \times 100$	$\Lambda^0/\Omega^{-1}\,cm^2 \cdot mol^{-1}$				pK_a of H_3PO_4			
		$HClO_4$	$KClO_4$	KH_2PO_4	H_3PO_4	*	†	‡	§
0.0000	1.27	415.0	142.0	111.5	384.5	—	2.11	—	2.14
0.0466	1.33	381.3	118.5	96.0	358.5	2.34	2.38	2.42	2.37
0.0968	1.40	331.3	99.2	84.6	316.7	2.56	2.61	2.64	2.61
0.1593	1.48	280.5	88.0	72.1	264.6	2.76	2.80	2.72	2.70
0.2279	1.57	228.0	80.0	64.4	212.4	2.88	2.99	2.96	2.90
0.3126	1.69	195.1	74.2	60.0	180.9	3.12	3.18	3.16	3.10
0.3997	1.84	171.7	71.0	58.2	158.9	3.46	3.50	3.51	3.45
0.5000	2.01	151.5	68.2	55.0	138.3	3.77	3.82	3.84	3.81
0.6392	2.25	131.0	75.6	60.0	115.4	4.20	4.23	4.20	4.30
0.8034	2.57	117.5	92.0	67.0	92.5	4.89	4.88	5.00	4.87

* pK_a from a plot of ΛC *vs* $1/\Lambda$.
† pK_a from Fuoss–Kraus's method.
‡ pK_a from titration method without β correction.
§ pK_a from titration method with correction.

(as given before) appears to be one of the best methods of determining pK values of weak acids free from assumptions.

The accuracy of the results may be taken to be ± 0.04 pK in water to ± 0.10 pK in high percentages of organic solvents.

Λ^0 values of KH_2PO_4, $KClO_4$, $HClO_4$ and H_3PO_4 in methanol and water mixtures are given in Table 1. It has been found that $\Lambda^0_{H_3PO_4}$ values obtained by indirect method have some disagreement as expected. Comparison of the results in mixed solvents are not possible due to lack of data. The reliability of the results can be had from the following considerations.

The $\Lambda^0_{HClO_4}$ in water has been found to be $415.0\,\Omega^{-1}$ cm^2 mol^{-1} in good agreement with the values obtained in the literature ($\lambda^0_{H^+} = 349.8\,\Omega^{-1}$ cm^2 mol^{-1} and $\lambda^0_{ClO_4^-} = 65.4^8$ or $67.32^9\,\Omega^{-1}$ cm^2 mol^{-1} in water at 25°C). The $\Lambda^0_{KH_2PO_4}$ and $\Lambda^0_{KClO_4}$ values in water at 25°C have been found 111.50 and $142.00\,\Omega^{-1}$ cm^2 mol^{-1} respectively ($\lambda^0_{K^+} = 73.50^{10}$ and $\lambda^0_{H_2PO_4^-} = 36\,\Omega^{-1}$ cm^2 mol^{-1} at 25°C). The values, therefore, agree fairly well with the values reported in the literature. The Λ^0 values decrease as the percentage of organic solvent increases. This cannot be simply related with the change in dielectric constant. The decrease is generally found to be very small in case of KH_2PO_4 and $KClO_4$. Λ^0 values decrease up to 60 wt% but increase from 70 wt% of methanol and onwards in case of KH_2PO_4 and $KClO_4$ whereas in case of $HClO_4$ and H_3PO_4, Λ^0 values show a decreasing trend. Shedlovsky *et al*[11] observed an increase in the equivalent conductance values at infinite dilutions at about 90 wt% CH_3OH and beyond.

The pK values of H_3PO_4 in methanol and water mixtures are presented in Table 1. In view of absence of pK values of inorganic acids in mixed solvents, it is difficult to compare the results. In spite of the limited accuracy of the conductivity bridge, the results, obtained by two distinctly different methods compare favourably well. The expected trend of increasing pK-values (about 2 in pK from 0 to 87 wt% org. solv.) with increasing alcohol concentration is observed.

The results show a good linearity when the pK values are plotted against mole fraction of organic solvent and $1/\varepsilon$ up to about 64 wt% of organic solvents. Deviations are apparent at higher percentages. Marginal improvements occur when the activities of water are taken into consideration as suggested by Yasuda[12]. The deviations must be due to ion–solvent interactions.

The $\Delta pK = pK_s - pK_w$ can be divided into

$$\Delta pK = \Delta pK_{el} + \Delta pK_{nonel}.$$

The "electrostatic part" can be written as[13]

$$\Delta pK_{el} = 121.6\left(\frac{1}{\varepsilon} - 0.0128\right)\left(\frac{1}{r_{H_2PO_4^-}} + \frac{1}{r_{H^+}}\right)$$

But in view of lack of accurate values of the radii of ions and the approximate nature of the equation, the equation had not been applied. It is clear that precise values of the radii of ions are prerequisite to calculate "electrostatic part" using Born[13] equation or modified equations[14–16]. Moreover, the dielectric constant is known to be function of the distance.

It is imperative, however, that proper knowledge of the "non-electrostatic" contributions particularly basicity and ion-solvent interactions are necessary to understand the behaviour of the solvents. But we are unable to throw much light on these aspects from our present study.

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Synthesis and Determination of pK_a Values of Some New 3,4-Disubstituted-4,5-Dihydro-1*H*-1,2,4-triazol-5-one Derivatives in Non-aqueous Solvents

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Abstract: 3-Alkyl(Aryl)-4-amino-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**2**) reacted with 2-furoyl chloride and thiophene-2-carbonyl chloride to afford the corresponding 3-alkyl(aryl)-4-(2-furoylamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**3**) and 3-alkyl(aryl)-4-(2-thienylcarbonylamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**4**), respectively. The new compounds synthesized were characterized by using IR, ^{1}H-NMR, ^{13}C-NMR and UV spectral data together with elemental analysis. In addition, to investigate the effects of solvents and molecular structure upon acidity, compounds **3** and **4** were titrated potentiometrically with tetrabutylammonium hydroxide in four non-aqueous solvents (isopropyl alcohol, *tert*-butyl alcohol, *N*,*N*-dimethylformamide and acetonitrile). The half-neutralization potential values and the corresponding pK_a values were determined for all cases.

Keywords: 4,5-Dihydro-1*H*-1,2,4-triazol-5-ones; acylation; acidity; potentiometric titration; syntheses.

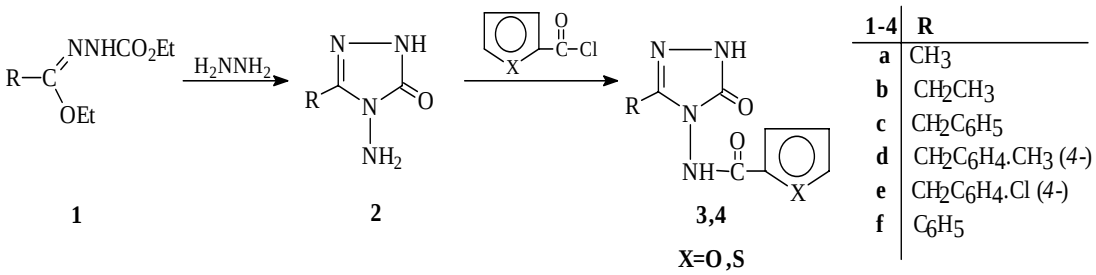
Introduction

1,2,4-Triazole and 4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives are reported to show a broad spectrum of biological activities such as antifungal, antimicrobial, hypoglycemic, antihypertensive, analgesic, antiparasitic, hypocholesteremic, antiviral, anti-inflammatory, antitumor and anti-HIV properties [1-14]. These observations prompted us to synthesize some new 4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives with potential biological activity. In addition, several articles, involving the acylation of 4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives, have also been published up to date [11, 12,15,16].

On the other hand, it is known that 1,2,4-triazole and 4,5-dihydro-1*H*-1,2,4-triazol-5-one rings have weak acidic properties, so some 1,2,4-triazole and 4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives were titrated potentiometrically with tetrabutylammonium hydroxide in non-aqueous solvents, and the pK_a values of the compounds were determined [11, 17-20]. We have previously described the synthesis and potentiometric titrations of some new 4,5-dihydro-1H-1,2,4-triazol-5-one derivatives in different non-aqueous medium [21, 22], where we determined the pKa values of these compounds for each non-aqueous solvent.

The aim of this work is to synthesize a series of 3-alkyl(aryl)-4-(2-furoylamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**3**) and 3-alkyl(aryl)-4-(2-thienylcarbonylamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**4**) from the reactions of 3-alkyl(aryl)-4-amino-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**2**) with 2-furoyl chloride and thiophene-2-carbonyl chloride, respectively (Scheme 1). Moreover, the synthesized compounds **3** and **4** were titrated potentiometrically with tetrabutylammonium hydroxide (TBAH) in four non-aqueous solvents, including isopropyl alcohol, *tert*-butyl alcohol, *N*,*N*-dimethylformamide and acetonitrile to determine their pK_a values. For each new compound synthesized, the half-neutralization potential (HNP) and the corresponding pK_a value were determined in the four mentioned non-aqueous solvents. The data obtained from the potentiometric titrations were interpreted, and the effect of the C-3 substituent and solvent effects were studied [17-22]. Determination of pK_a values of active constituents of certain pharmaceutical preparations is important, because their distribution, transport behavior, bonding to receptors, and contributions to metabolic behavior depend on the ionization constant [23].

Scheme 1



1-4	R
a	CH_3
b	CH_2CH_3
c	$CH_2C_6H_5$
d	$CH_2C_6H_4.CH_3$ (*4*-)
e	$CH_2C_6H_4.Cl$ (*4*-)
f	C_6H_5

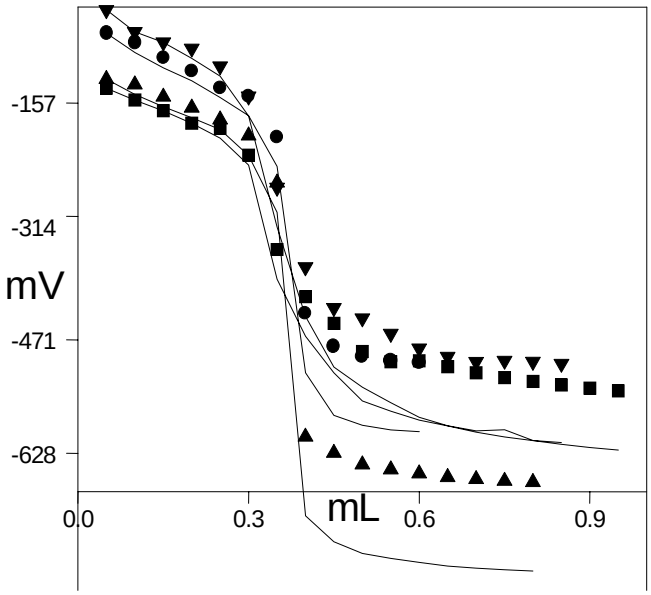
Results and Discussion

In this study, the structures of the newly synthesized 3-alkyl(aryl)-4-(2-furoylamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**3**) and 3-alkyl(aryl)-4-(2-thienyl-carbonylamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**4**) were identified using elemental analysis and IR, ^{1}H-NMR, ^{13}C-NMR and UV spectral data, and these obtained spectral values were seen to be compatible with literature reports [24, 25]. In addition, these newly synthesized compounds **3** and **4** were titrated potentiometrically with tetrabutyl-ammonium hydroxide (TBAH) in non-aqueous solvents such as isopropyl alcohol (ε=19.4), *tert*-butyl alcohol (ε=12), *N*,*N*-dimethylformamide (ε=37) and acetonitrile (ε=36).

The mV values were plotted versus TBAH volumes (mL) added, and thus potentiometric titration curve was formed for all the cases. From these curves, the HNP values were measured, and the corresponding pK_a values were calculated.

As an example, the potentiometric titration curves for 0.001 M 3-Benzyl-4-(2-furoylamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-one (**3c**) solutions titrated with 0.05 M TBAH in isopropyl alcohol, *tert*-butyl alcohol, *N*,*N*-dimethylformamide and acetonitrile are given in Figure **1**. As it is clearly seen in Figure **1**, a typical S-shaped titration curve was obtained.

Figure 1. Potentiometric titration curves of 10^{-3} M 3-Benzyl-4-(2-furoylamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-one (**3c**) solutions titrated with 0.05 M TBAH in isopropyl alcohol (●), *tert*-butyl alcohol (▲), *N*,*N*-dimethylformamide (■) and acetonitrile (▼) at 25 °C.



The half-neutralization potentials (HNP) and the corresponding pK_a values for compounds **3** and **4**, obtained from the potentiometric titrations with 0.05 M TBAH in isopropyl alcohol, *tert*-butyl alcohol, *N*,*N*-dimethylformamide and acetonitrile, are given in Table **1**.

Table 1. The half-neutralization potentials (HNP) and the corresponding pK_a values of compounds **3** and **4** in isopropyl alcohol, *tert*-butyl alcohol, *N*,*N*-dimethylformamide and acetonitrile.

Compd. no	Isopropyl alcohol		*Tert*-butyl alcohol		*N*,*N*-Dimethyl formamide		Acetonitrile	
	HNP (mV)	pK_a	HNP (mV)	pK_a	HNP (mV)	pK_a	HNP (mV)	pK_a
3a	-292	11.63	-206	9.84	-447	14.56	-430	14.57
3b	-298	11.69	-260	11.01	-459	15.69	-388	13.69
3c	-111	8.79	-162	9.49	-172	10.38	-96	8.48
3e	-290	11.52	-248	10.76	-443	15.30	-409	14.10
3f	-312	11.97	-186	9.52	-491	15.26	-329	12.48
4a	-275	11.07	-273	11.10	-450	14.37	-374	13.38
4b	-297	11.87	-254	10.86	-324	12.39	-333	12.54
4c	-291	11.42	-175	11.63	-347	12.42	-352	12.91
4d	-214	10.66	-285	11.84	-320	12.82	-227	11.52
4e	-286	11.48	-277	11.26	-436	14.23	-284	11.60
4f	-293	11.47	-291	11.40	-388	13.17	-333	12.51

As it is well known, the acidity of a compound depends on some factors. The two most important factors are the solvent effect and molecular structure [18-22, 26-28]. Table **1** shows that the HNP values and the corresponding pK_a values obtained from potentiometric titrations depend on the non-aqueous solvents used. The results obtained illustrate that *tert*-butyl alcohol is the best solvent. As can be observed in Figure **1**, for example, the potential jump of compound **3c** in the end-point is very large for *tert*-butyl alcohol ranging from –266 mV to –599 mV. In addition, Table **1** shows that the molecular structure of titrated compounds affects the HNP and corresponding pK_a values depending on the substituents at C-3 in the same solvent.

Experimental

General

Melting points were taken on a Electrothermal digital melting point apparatus and are uncorrected. IR spectra were registered using KBr disks on a Perkin-Elmer 1600 FTIR spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded in DMSO-d_6 with TMS as internal standard on a Varian Mercury spectrometer at 200 MHz and 50 MHz, respectively. UV absorption spectra were measured for ethanol solutions in 10 mm quartz cells between 200 and 400 nm using a Shimadzu UV-1201 spectrophotometer. For potentiometric titrations, a Jenway 3040 ion analyser pH meter (calibrated according to the instructions of the manufacturer) equipped with an Ingold pH electrode were used. During the titrations, the titrant was added in increments of 0.05 mL after each stable reading, and the corresponding mV values were recorded. Chemicals were supplied from Fluka and Merck. After purification, isopropyl alcohol was used to prepare 0.05 M tetrabutylammonium hydroxide (TBAH). For all potentiometric titrations, 0.05 M TBAH in isopropyl alcohol was used. The starting compounds **2a-e** were prepared from the reactions of the corresponding ester ethoxycarbonylhydrazones (**1a-e**) with hydrazine hydrate according to literature [16,29].

General Method for the Preparation of 3-alkyl(aryl)-4-[2-furoylamino]-4,5-dihydro-1H-1,2,4-triazol-5-ones (**3**) or *3-alkyl(aryl)-4-[2-thienylcarbonyamino]-4,5-dihydro-1H-1,2,4-triazol-5-ones* (**4**):

3-Alkyl(aryl)-4-amino-4,5-dihydro-1*H*-1,2,4-triazol-5-one (**2**) (0.01 mol) was refluxed with a solution of the appropriate heteroaroyl chloride (2-furoyl chloride or 2-thiophenecarbonyl chloride) (0.01 mol) in n-butyl acetate (40 mL) for 6 hours and then allowed to cool. The product was recrystallized from an appropriate solvent to give **3** or **4**. The following compounds were prepared applying this procedure:

3-Methyl-4-(2-furoylamino)-4,5-dihydro-1H-1,2,4-triazol-5-one (**3a**). Yield 90%; m.p. 83 °C (H_2O); Calculated for $C_8H_8N_4O_3$ (208.18): 46.16% C, 3.87% H, 26.91% N; found: 46.70% C, 4.18% H, 26.79% N. ^{1}H-NMR: δ 2.06 (s, 3H, CH_3), 6.76 (s, 1H, Ar-H), 7.40 (s, 1H, Ar-H), 8.02 (s, 1H, Ar-H), 11.38 (s, 1H, NH); 11.73 (s, 1H, NH); ^{13}C-NMR: δ 10.72 (aliphatic carbon), 112.65, 116.87, 145.05, 147.07 (aromatic carbons), 145.36 (triazole C_3), 152.89 (triazole C_5), 157.24 (C=O); IR: 3500, 3170 (NH), 1715, 1680 (C=O), 1596 (C=N) cm^{-1}; UV λ_{max} nm, (ε, $L \cdot mol^{-1} \cdot cm^{-1}$): 253 (26350), 212 (16510) nm.

3-Ethyl-4-(2-furoylamino)-4,5-dihydro-1H-1,2,4-triazol-5-one (**3b**). Yield 87%; m.p. 111-112 °C (EtOH-toluene, 1:3); Calculated for $C_9H_{10}N_4O_3$ (222.20): 48.65% C, 4.54% H, 25.21% N; found: 48.35% C, 4.74% H, 24.93% N. ^{1}H-NMR: δ 1.11 (t, 3H, CH_3), 2.33 (q, 2H, CH_2), 6.70 (s, 1H, Ar-H), 7.33 (s, 1H, Ar-H), 7.95 (s, 1H, Ar-H), 11.33 (s, 1H, NH); 11.70 (s, 1H, NH); ^{13}C-NMR: δ 9.88, 18.17

(aliphatic carbons), 112.57, 116.74, 145.00, 149.10 (aromatic carbons), 147.00 (triazole C_3), 152.96 (triazole C_5), 157.14 (C=O); IR: 3450, 3260 (NH), 1715, 1695 (C=O), 1595 (C=N) cm^{-1}; UV λ_{max} nm, (ε, $L \cdot mol^{-1} \cdot cm^{-1}$): 211 (11390) nm.

3-Benzyl-4-(2-furoylamino)-4,5-dihydro-1H-1,2,4-triazol-5-one (**3c**). Yield 82%; m.p. 160-162 °C (EtOH); Calculated for $C_{14}H_{12}N_4O_3$ (284.27): 59.15% C, 4.25% H, 19.71% N; found: 59.48% C, 4.18% H, 18.79% N. ^{1}H-NMR: δ 3.72 (s, 2H, CH_2), 6.74 (s, 1H, Ar-H), 7.20-7.36 (m, 7H, Ar-H), 11.71 (s, 1H, NH); 11.90 (s, 1H, NH); ^{13}C NMR: δ 30.40 (aliphatic carbon), 112.80, 117.10, 126.89, 128.47 (3C), 128.63 (3C), 134.60 (aromatic carbons), 146.20 (triazole C_3), 152.00 (triazole C_5), 163.30 (C=O); IR: 3450, 3230 (NH), 1746, 1715 (C=O), 1590 (C=N), 770, 705 (monosubstituted benzenoid ring) cm^{-1}; UV λ_{max} nm, (ε, $L \cdot mol^{-1} \cdot cm^{-1}$): 246 (28720), 221 (23950) nm.

3-(4-Chlorobenzyl)-4-(2-furoylamino)-4,5-dihydro-1H-1,2,4-triazol-5-one (**3e**). Yield 90%; m.p. 177-178 °C (EtOH-H_2O, 1:3); Calculated for $C_{14}H_{11}N_4O_3Cl$ (318.72): 52.76% C, 3.48% H, 17.58% N; found: 52.45% C, 3.87% H, 17.56% N. ^{1}H-NMR: δ 3.82 (s, 2H, CH_2), 6.75 (s, 1H, Ar-H), 7.25-7.38 (m, 5H, Ar-H), 8.02 (s, 1H, Ar-H), 11.36 (s, 1H, NH); 11.92 (s, 1H, NH); ^{13}C-NMR: δ 30.30 (aliphatic carbon), 112.40, 117.10, 128.58 (3C), 130.96 (2C), 131.90, 134.10, 147.03 (aromatic carbons), 145.00 (triazole C_3), 153.00 (triazole C_5), 157.30 (C=O); IR: 3400, 3210 (NH), 1725, 1688 (C=O), 1595 (C=N), 810 (1,4-disubstituted benzenoid ring) cm^{-1}; UV λ_{max} nm, (ε, $L \cdot mol^{-1} \cdot cm^{-1}$): 256 (14340), 221 (16830) nm.

3-Phenyl-4-(2-furoylamino)-4,5-dihydro-1H-1,2,4-triazol-5-one (**3f**). Yield 79%; m.p. 278-239 °C (H_2O); Calculated for $C_{13}H_{16}N_4O_3$ (270.25): 57.78% C, 3.73% H, 20.73% N; found: 58.02% C, 3.50% H, 20.43% N. ^{1}H-NMR: δ 6.75 (s, 1H, Ar-H), 7.47-7.52 (m, 4H, Ar-H), 7.85-7.86 (m, 2H, Ar-H), 8.03 (s, 1H, Ar-H), 11.82 (s, 1H, NH); 12.49 (s, 1H, NH); ^{13}C-NMR: δ 112.80, 117.20, 126.25, 127.14 (2C), 129.30 (2C), 130.91, 146.53, 147.17 (aromatic carbons), 145.21 (triazole C_3), 153.62 (triazole C_5), 157.45 (C=O); IR: 3450, 3150 (NH), 1713, 1670 (C=O), 1590 (C=N), 760, 695 (monosubstituted benzenoid ring) cm^{-1}; UV λ_{max} nm, (ε, $L \cdot mol^{-1} \cdot cm^{-1}$): 246 (29600), 216 (22900) nm.

3-Methyl-4-(2-thienylcarbonylamino)-4,5-dihydro-1H-1,2,4-triazol-5-one (**4a**). Yield 83%; m.p. 255-256 °C (EtOH); Calculated for $C_8H_8N_4O_2S$ (224.24): 42.85% C, 3.60% H, 24.99% N; found: 43.26% C, 3.45% H, 24.98% N. ^{1}H-NMR: δ 1.99 (s, 3H, CH_3), 7.06-7.29 (m, 1H, Ar-H), 7.45-8.22 (m, 2H, Ar-H), 11.46 (s, 1H, NH); 11.70 (s, 1H, NH); ^{13}C-NMR: δ 11.87 (aliphatic carbon), 129.30, 131.56, 134.00, 135.84 (aromatic carbons), 146.21 (triazole C_3), 153.82 (triazole C_5), 161.84 (C=O); IR: 3300, 3150 (NH), 1720, 1660 (C=O), 1610 (C=N) cm^{-1}; UV λ_{max} nm, (ε, $L \cdot mol^{-1} \cdot cm^{-1}$): 248 (29140), 212 (19790) nm.

3-Ethyl-4-(2-thienylcarbonylamino)-4,5-dihydro-1H-1,2,4-triazol-5-one (**4b**). Yield 82%; m.p. 202-203 °C (EtOH-H_2O, 1:3); Calculated for $C_9H_{10}N_4O_2S$ (238.26): 45.37% C, 4.23% H, 23.51% N;

found: 45.03% C, 4.18% H, 23.57% N. ^{1}H-NMR: δ 1.14 (t, 3H, CH_3), 2.41 (q, 2H, CH_2), 7.27-7.29 (m, 1H, Ar-H), 7.97-7.99 (m, 2H, Ar-H), 11.47 (s, 1H, NH); 11.79 (s, 1H, NH); ^{13}C-NMR: δ 10.11, 18.34 (aliphatic carbons), 128.79, 130.76, 133.47, 135.40 (aromatic carbons), 149.26 (triazole C_3), 153.19 (triazole C_5), 161.05 (C=O); IR: 3500, 3175 (NH), 1720, 1675 (C=O), 1600 (C=N) cm^{-1}; UV λ_{max} nm, (ε, $L \cdot mol^{-1} \cdot cm^{-1}$): 250 (22220), 207 (16500) nm.

3-Benzyl-4-(2-thienylcarbonylamino)-4,5-dihydro-1H-1,2,4-triazol-5-one (**4c**). Yield 86%; m.p. 220-222 °C (EtOH-H_2O, 1:3); Calculated for $C_{14}H_{12}N_4O_2S$ (300.33): 55.99% C, 4.03% H, 18.65% N; found: 55.73% C, 3.73% H, 18.38% N. ^{1}H-NMR: δ 3.82 (s, 2H, CH_2), 7.26 (s, 6H, Ar-H), 7.95 (s, 2H, Ar-H), 11.46 (s, 1H, NH); 11.94 (s, 1H, NH); ^{13}C-NMR: δ 31.10 (aliphatic carbon), 127.18, 128.76 (3C), 129.08 (2C), 131.10, 133.90, 135.40, 135.90 (aromatic carbons), 147.90 (triazole C_3), 153.40 (triazole C_5), 161.20 (C=O); IR: 3250, 3100 (NH), 1725, 1660 (C=O), 1600 (C=N), 770, 705 (monosubstituted benzenoid ring) cm^{-1}; UV λ_{max} nm, (ε, $L \cdot mol^{-1} \cdot cm^{-1}$): 251 (22220), 210 (30440) nm.

3-(4-Methylbenzyl)-4-(2-thienylcarbonylamino)-4,5-dihydro-1H-1,2,4-triazol-5-one (**4d**). Yield 77%; m.p. 167-168 °C (EtOH-H_2O, 1:3); Calculated for $C_{15}H_{14}N_4O_2S$ (314.36): 57.31% C, 4.48% H, 17.82% N; found: 57.10% C, 4.27% H, 17.93% N. ^{1}H-NMR: δ 2.27 (s, 3H, CH_3), 3.95 (s, 2H, CH_2), 7.11-7.27 (m, 5H, Ar-H), 7.95-8.00 (m, 2H, Ar-H), 11.43 (s, 1H, NH); 11.89 (s, 1H, NH); IR: 3250, 3120 (NH), 1760, 1670 (C=O), 1615 (C=N), 790 (1,4-disubstituted benzenoid ring) cm^{-1}; UV λ_{max} nm, (ε, $L \cdot mol^{-1} \cdot cm^{-1}$): 269 (23580), 211 (24660) nm.

3-(4-Chlorobenzyl)-4-(2-thienylcarbonylamino)-4,5-dihydro-1H-1,2,4-triazol-5-one (**4e**). Yield 90%; m.p. 190-191 °C (EtOH-H_2O, 1:3); Calculated for $C_{14}H_{11}N_4O_2SCl$ (334.78): 50.23% C, 3.31% H, 16.74% N; found: 50.55% C, 2.99% H, 16.50% N. ^{1}H-NMR: δ 3.82 (s, 2H, CH_2), 7.24-7.37 (m, 5H, Ar-H), 7.93-7.97 (m, 2H, Ar-H), 11.41 (s, 1H, NH); 11.93 (s, 1H, NH); ^{13}C-NMR: δ 29.00 (aliphatic carbon), 127.25 (3C), 129.39, 129.57 (2C), 130.90, 131.99, 132.63, 134.30 (aromatic carbons), 145.90 (triazole C_3), 152.90 (triazole C_5), 159.80 (C=O); IR: 3220, 3100 (NH), 1730, 1665 (C=O), 1595 (C=N), 805 (1,4-disubstituted benzenoid ring) cm^{-1}; UV λ_{max} nm, (ε, $L \cdot mol^{-1} \cdot cm^{-1}$): 251 (15100), 222 (24400) nm.

3-Phenyl-4-(2-thienylcarbonylamino)-4,5-dihydro-1H-1,2,4-triazol-5-one (**4f**). Yield 85%; m.p. 160-161 °C (EtOH-H_2O, 1:3); Calculated for $C_{13}H_{10}N_4O_2S$ (286.31): 55.54% C, 3.52% H, 19.57% N; found: 55.16% C, 3.52% H, 19.43% N. ^{1}H-NMR: δ 7.29-8.05 (m, 8H, Ar-H), 11.85 (s, 1H, NH); 12.46 (s, 1H, NH); ^{13}C-NMR: δ 126.10, 126.98 (2C), 127.10, 129.25 (2C), 130.90 (2C), 133.80, 135.50 (aromatic carbons), 146.20 (triazole C_3), 153.70 (triazole C_5), 161.10 (C=O); IR: 3250 (NH), 1730, 1675 (C=O), 1610 (C=N), 770, 700 (monosubstituted benzenoid ring) cm^{-1}; UV λ_{max} nm, (ε, $L \cdot mol^{-1} \cdot cm^{-1}$): 253 (25710), 210 (16990) nm.

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Sample availability: Contact the authors.

The Process of New Drug Discovery and Development

Second Edition

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successful solid oral dosage forms of drug that are highly unstable at GI pH (didonasine, esomaprazole magnesium[47]), excessive degradation in the GI tract limits oral absorption and may require heroic efforts to address the problem via formulation. Although no hard and fast rule exists on acceptable GI stability, Balbach and Korn[37] considered <2 to 5% degradation under simulated *in vivo* conditions (37°C, pH 1.2 to 8, fed and fasted conditions) as acceptable. Higher degradation may require additional investigation. The effect of the GI enzymes on drug stability should also be evaluated.

3.4.2 Preformulation Activities: Dependent on Solid Form

A new drug substance may exist in a multitude of crystalline and salt forms with different physical properties such as shape, melting point, and solubility that can profoundly impact the manufacturing and performance of its dosage form.

3.4.2.1 Solubility

Solubility is highly influenced by the solid-state form (e.g., crystalline or amorphous) of the drug. Rigorous solubility studies using the final solid form (i.e., salt form or crystal form) as a function of temperature (i.e., 25 and 37°C) and pH (range 1 to 7.5) are conducted during preformulation. Solubility in nonaqueous solvents is also screened. Solubility in simulated gastrointestinal fluids is also important.

For accurate determination of solubility:

- Attainment of equilibrium must be ensured by analyzing solution concentration at multiple time points until the concentration does not change considerably (i.e., <5% change in concentration).
- The pH of the saturated solution must be measured.
- The solid phase in equilibrium with the saturated solution must be analyzed by techniques such as hot stage microscopy, differential scanning calorimetry, or powder x-ray diffraction, to verify if the starting material has undergone a phase transformation.

3.4.2.2 Salt-Form Selection

The selection of an optimal chemical and physical form is an integral part of the development of an NCE. If an NCE is neutral or if its pK_a value(s) is not conducive to salt formation, it has to be developed in the neutral form (unless a prodrug is synthesized) and the only form selection involves the selection of its physical (crystal) form. However, if it exists as a free acid or a free base, then the "form" selection involves the selection of both chemical and physical forms. A decision must be made whether a salt or its free acid or base form should be developed. As will be described in Section 3.5, a salt form may lead to a higher dissolution rate and higher bioavailability for a poorly water-soluble drug. For a drug with adequate aqueous solubility, a salt form may not be necessary, unless, of course, a salt provides an advantage with respect to its physical form. In the pharmaceutical industry, salt selection is usually performed by a multidisciplinary team comprising representatives from the drug discovery, chemical development, pharmaceutical development, ADME, and drug safety departments. Serajuddin and Pudipeddi[27] reported that the following questions need to be satisfactorily addressed by the team in the selection of an optimal salt form for a compound: "Is the acid or base form preferred because of biopharmaceutical considerations? Is the salt form more suitable? Is the preparation of stable salt forms feasible? Among

various potential salt forms of a particular drug candidate, which has the most desirable physicochemical and biopharmaceutical properties?" With respect to physical properties, questions involve whether the compound exists in crystalline or amorphous form, and, if crystalline, whether it exhibits polymorphism.

At the outset of any salt selection program, it is important to determine whether the salt formation is feasible for the particular compound and, if yes, what counterions are to be used? Although it is generally agreed that a successful salt formation requires that the pK_a of a conjugate acid be less than the pK_a of the conjugate base to ensure sufficient proton transfer from the acidic to the basic species, the salt formation still remains a "trial and error" endeavor. Hundreds of individual experiments for salt formation of a particular compound are not uncommon. Because of the availability of HTS screening techniques in recent years there is no pressure to limit the number of such experiments. Serajuddin and Pudipeddi[27] reported that the number of feasibility experiments can be greatly reduced by studying the solubility vs. pH relationship of the drug and identifying the pH_{max} (the pH of maximum solubility). The nature of the pH–solubility profile and the position of pH_{max} depends on pK_a, intrinsic solubility (solubility of unionized species), and the solubility of any salt (K_{sp}) formed. For a basic drug, the pH must be decreased below the pH_{max} by using the counterion for a salt to be formed, and, for an acidic drug, the pH must be higher than the pH_{max}. Any counterion that is not capable of changing the pH in this manner may be removed from consideration. While salts may be formed from organic solvents by counterions that are not capable of changing the aqueous pH in this manner, such salts may readily dissociate in an aqueous environment. When the synthesis of multiple salts for a compound is feasible, the number may be narrowed down and the optimal salt may ultimately be selected by characterizing physicochemical properties of solids according to a multitier approach proposed by Morris et al.[48]

3.4.2.3 *Polymorphism*

Polymorphism is defined as the ability of a substance to exist as two or more crystalline phases that have different arrangements or conformations of the molecules in the crystal lattice. Many drug substances exhibit polymorphism. The definition of polymorphism according to the International Conference on Harmonization (ICH) guideline Q6A[49] includes polymorphs, solvates, and amorphous forms. Amorphous solids lack long-range order and do not possess a distinguishable crystal lattice. Solvates are crystal forms containing stoichiometric or nonstoichiometric amounts of solvent in the crystal. When the solvent is water they are termed hydrates. A thorough screening of possible crystal forms is conducted during candidate lead selection or shortly thereafter.

Typical methods for generation of polymorphs include sublimation, crystallization from different solvents, vapor diffusion, thermal treatment, melt crystallization, and rapid precipitation. High-throughput screening methods have been reported for polymorph screening.[50]

Methods for characterization of polymorphs include crystallographic techniques (single crystal and powder x-ray diffraction), microscopic characterization of morphology, thermal characterization (DSC/TGA), solution calorimetry, solid-state spectroscopic methods (IR, Raman, NMR), and solubility and intrinsic dissolution rate methods. Of these, the relative solubility or intrinsic dissolution rate is directly related to the free energy difference, and, hence the relative stability of polymorphs. Thermal data can also be used to assess relative stability of polymorphs. The form with the lowest solubility and, hence, free energy is the most stable form at a given temperature. Other forms would eventually transform to the stable form. The kinetics of crystal nucleation and growth determines the crystal form obtained during crystallization. Sometimes metastable forms are more readily crystallized than the most stable (and often desired) form. The kinetics of transformation of a metastable form to the stable form may be very slow and unpredictable. The

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Hydrogen phosphate and dihydrogen phosphate salts of 4-aminoazobenzene

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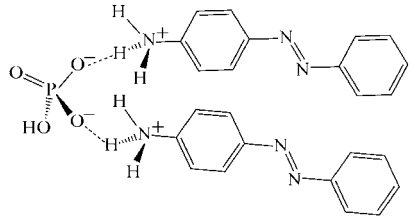
4-Amino-*trans*-azobenzene {or 4-[(*E*)-phenyldiazenyl]aniline} can form isomeric salts depending on the site of protonation. Both orange bis{4-[(*E*)-phenyldiazenyl]anilinium} hydrogen phosphate, $2C_{12}H_{12}N_3^+\cdot HPO_4^{2-}$, and purple 4-[(*E*)-phenyldiazenyl]anilinium dihydrogen phosphate phosphoric acid solvate, $C_{12}H_{12}N_3^+\cdot H_2PO_4^-\cdot H_3PO_4$, (II), have layered structures formed through O—H···O and N—H···O hydrogen bonds. Additionally, azobenzene fragments in (I) are assembled through C—H···π interactions and in (II) through π–π interactions. Arguments for the colour difference are tentatively proposed.

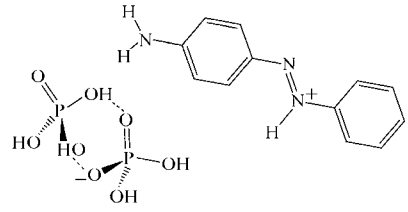
Comment

4-Aminoazobenzene has three N atoms, each possessing an unshared electron pair. In addition to the *cis–trans* isomerism, each of the N atoms can be protonated and isomeric cations can thus be formed. This property is potentially applicable in the design of piezochromic materials because two groups of salts, according to colour, can be distinguished. So far, purple and orange salts have been isolated. In our preliminary studies, we have found that the orange hydrogen phosphate salt of 4-amino-*trans*-azobenzene {4-[(*E*)-phenyldiazenyl]-aniline}, when pressed into a KBr pellet, turns purple over a period of a few minutes to a few days (Lukić *et al.*, 2007). We have encountered difficulties in determining the exact reaction taking place in the KBr pellet that causes the colour change. To the best of our knowledge, no work has reported results on colour changes in the solid state of salts of 4-amino-azobenzene. Even though the number of salts of 4-amino-azobenzene characterized in the solid state is still relatively small, an assumption can be made about the cause of the colour change. Tentatively, we propose that the colour of 4-aminoazobenzene salts depends on the site of protonation. In the Cambridge Structural Database (Version 5.27; August 2006; Allen, 2002), three orange salts are reported, all having the amino group protonated, and one purple salt with only the azo group protonated. Undoubtedly, finding an unequivocal answer about the origin of this effect requires a broader study.

In this paper, as a first step in this investigation, we report the crystal structures of the hydrogen phosphate, (I), and dihydrogen phosphate, (II), salts of 4-aminoazobenzene.



(I) (orange)



(II) (purple)

The formula unit of the orange salt (I) consists of two 4-aminoazobenzene molecules, both in the *trans* configuration and protonated on the amino N atom, along with a hydrogen phosphate anion (Fig. 1). The purple compound (II) consists of one 4-aminoazobenzene molecule, also in the *trans* configuration, protonated on an azo group N atom, a dihydrogenphosphate anion and one solvent molecule of phosphoric acid (Fig. 2). In compound (II), alternatively, the hydrogen-bonded phosphoric acid and dihydrogen phosphate units could be considered as jointly forming the anion. Different sites of protonation of 4-aminoazobenzene result in quite different geometries for the cations. This is probably the cause of the different colours of these salts. In both compounds, the geometry of the cation deviates significantly from planarity, but the deviation is more pronounced in the purple salt (II). The relative twist of the phenyl ring is 18.0 (1)° in compound (II), and 2.2 (3) and 6.8 (2)° in compound (I). This larger value in (II) can be explained by repulsions between H atoms of the phenyl ring and a hydrogen-bond acceptor which approaches the protonated azo group.

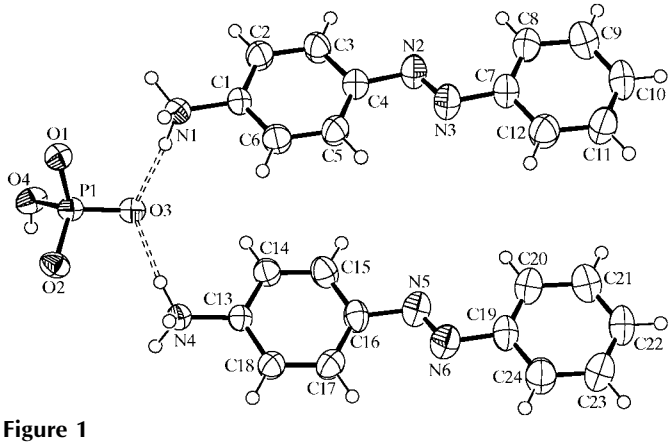


Figure 1
A view of (I), showing the atom-labelling scheme. Displacement ellipsoids are drawn at the 50% probability level.

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Both compounds have many possible hydrogen-bond donor groups of the type NH or OH and they are all, in accord with Etter's first rule (Etter *et al.,* 1990), involved in hydrogen bonds. In (I), the anions are linked through O—H···O hydrogen bonds (Table 1) and form a chain running in the [001] direction (Fig. 3). There are adjacent chains of anions in the (100) plane, which are related by inversion. In this way, layers of anions are formed even though there is no hydrogen bonding between the chains. A layer of anions is surrounded by cations which, through N—H···O hydrogen bonds, connect the chains. This forms a complex hydrogen-bonding network and a sheet structure parallel to the (100) plane (Fig. 4). There are no strong interactions between the sheets. The morphology of the orange crystals also reveals this. Crystals, obtained by evaporation from ethanol, are plate-like with {100} as the two most developed planes. Crystals also show pronounced cleavage parallel to the same planes. The hydroxyl group of the hydrogen phosphate ion is involved in hydrogen bonding only as a donor group so that, with seven remaining hydrogen-bond donor groups in (I), two O atoms are acceptors in two interactions and one in three (Table 1). The relative orientation of the non-polar azobenzene fragments is such that a C—H···π interaction is formed. Atoms H2 and H8 are at distances of 3.304 and 3.090 Å, respectively, from the mean planes of the benzene rings of the azobenzene fragment at (x, $\frac{3}{2} - y$, $-\frac{1}{2} + z$), and at distances of 3.322 and 3.140 Å, respectively, from the centroids of these rings. The second independent azobenzene fragment forms C—H···π interactions with two adjacent molecules. Firstly, atoms H14 and H20 lie 2.885 and 3.061 Å, respectively, from the mean planes (2.999 and 3.101 Å, respectively, from the centroids) of the benzene rings of the azobenzene fragment at (x, $\frac{1}{2} - y$, $-\frac{1}{2} + z$). The second interaction is towards one benzene ring of the azobenzene fragment at (x, $\frac{1}{2} - y$, $\frac{1}{2} + z$). The distance of H23 from the mean plane of the benzene ring is 3.47 Å (2.96 Å from its centroid). This interaction could also account for the deviation from planarity of the azobenzene fragment.

Compound (II) is also in accord with Etter's rule as it has eight independent possible hydrogen-bond donor groups and all of them are involved in hydrogen bonds. A network is formed through five O—H···O and three N—H···O hydrogen bonds (Table 2). Dihydrogen phosphate anions and molecules of phosphoric acid are connected through O—H···O hydrogen bonds and form a chain running in the [100] direction. This chain is surrounded by cations, each forming three hydrogen bonds of the N—H···O type and linking anionic chains. A two-dimensional network is thus formed parallel to the (001) plane (Fig. 5). Azobenzene fragments within this layer are related by translation in the [100] direction and are in contact through π–π stacking interactions.

In order to elucidate the chemical reaction taking place in the KBr pellet, we shall try to obtain further structural evidence with other salts of 4-amino-*trans*-azobenzene and to record UV–vis spectra of these salts in KBr pellets.

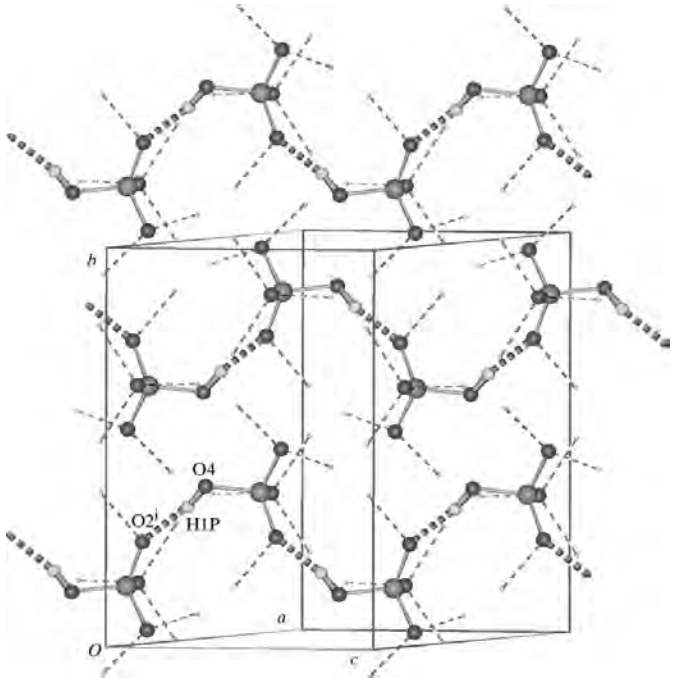

Figure 3
A layer of anions in (I) in the (100) plane. Chains of anions are formed in the [001] direction. Hydrogen bonds connecting hydrogen phosphate ions are shown with thicker dashed lines. [Symmetry code: (i) $x, -y + \frac{1}{2}, z - \frac{1}{2}$.]

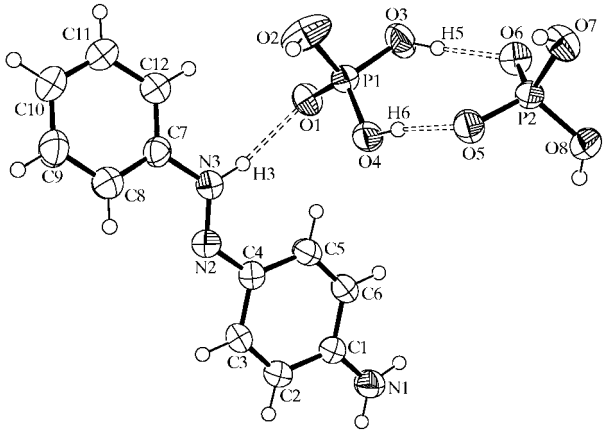

Figure 2
A view of (II), showing the atom-labelling scheme. Displacement ellipsoids are drawn at the 50% probability level.

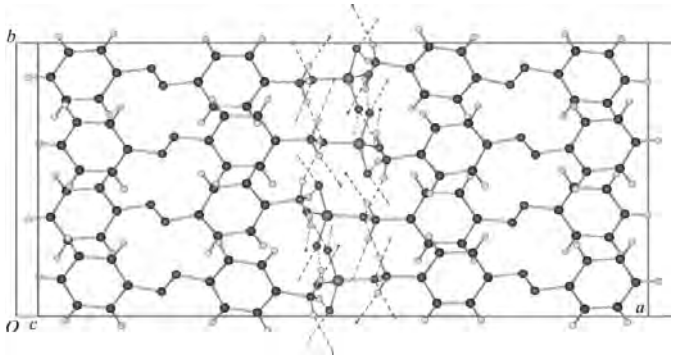

Figure 4
The two-dimensional network of hydrogen bonds in (I), extending in the (100) plane, perpendicular to the plane of the drawing.

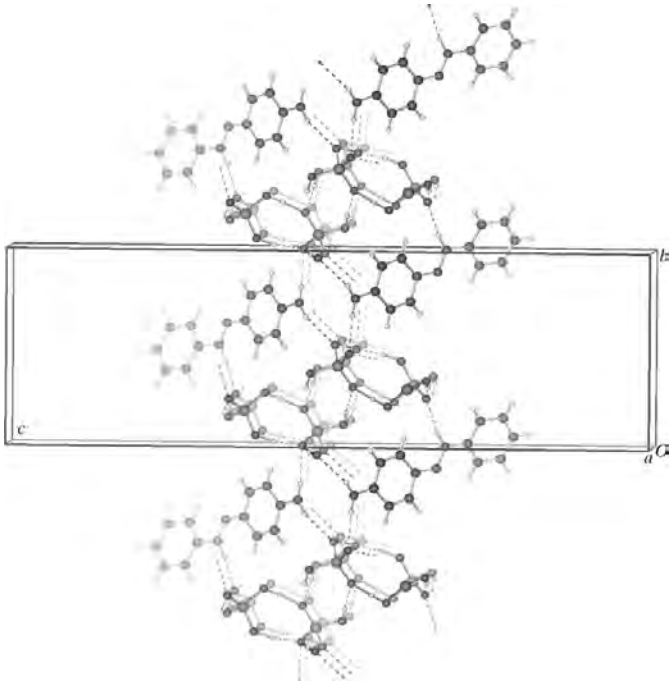

Figure 5
The two-dimensional network of hydrogen bonds in (II), extending in the (001) plane.

Experimental

For the preparation of (I), 4-aminoazobenzene (5 mmol, 0.986 g) and H_3PO_4 (5.5 mmol, 5.5 ml of 1.0 mol dm^{-3} aqueous solution) were dissolved in 96% EtOH (10 ml), with mild heating and stirring over a period of 3 h. This resulted in a dark-purple solution. Orange crystals precipitated after cooling. The crystals were rinsed three times with 96% EtOH and dried in air (yield 1.08 g, 88%). Crystals of (I) suitable for single-crystal X-ray diffraction were obtained after one week by slow evaporation of an EtOH solution [50 mg of (I) in 5 ml of 96% EtOH] at room temperature. For the preparation of (II), a further 10 ml of H_3PO_4 (aqueous, c = 1.0 mol dm^{-3}) was added to the mother liquor left from the preparation of (I) and the resulting purple solution was left to evaporate at room temperature. After approximately three weeks, purple plate-shaped crystals of (II) were isolated and used as obtained in the diffraction experiment.

Salt (I)

Crystal data

$2C_{12}H_{12}N_3^+\cdot HPO_4^{2-}$
M_r = 492.47
Monoclinic, $P2_1/c$
a = 26.757 (4) Å
b = 11.2998 (15) Å
c = 7.9943 (12) Å
β = 96.709 (12)°
V = 2400.5 (6) Å^3
Z = 4
D_x = 1.363 Mg m^{-3}
Mo $K\alpha$ radiation
μ = 0.16 mm^{-1}
T = 293 (2) K
Plate, orange
0.45 × 0.40 × 0.04 mm

Data collection

Oxford Xcalibur-3 CCD area-detector diffractometer
ω scans
17388 measured reflections
4676 independent reflections
3382 reflections with $I > 2\sigma(I)$
R_{int} = 0.058
θ_{max} = 26.0°

Refinement

Refinement on F^2
$R[F^2 > 2\sigma(F^2)]$ = 0.075
$wR(F^2)$ = 0.229
S = 1.05
4676 reflections
338 parameters
H atoms treated by a mixture of independent and constrained refinement
$w = 1/[\sigma^2(F_o^2) + (0.140P)^2 + 1.0658P]$ where $P = (F_o^2 + 2F_c^2)/3$
$(\Delta/\sigma)_{max}$ = 0.003
$\Delta\rho_{max}$ = 0.95 e Å^{-3}
$\Delta\rho_{min}$ = −0.55 e Å^{-3}

Table 1
Hydrogen-bond geometry (Å, °) for (I).

D—H···A	D—H	H···A	D···A	D—H···A
N1—H3A···O2^{ii}	0.88 (2)	1.84 (3)	2.721 (3)	173 (3)
N4—H4A···O3	0.88 (2)	2.02 (3)	2.901 (3)	176 (3)
N1—H2A···O1^{iii}	0.89 (2)	1.86 (3)	2.737 (3)	166 (3)
N1—H1A···O3	0.87 (2)	1.97 (3)	2.811 (3)	161 (3)
N4—H6A···O1^{iv}	0.88 (2)	1.78 (3)	2.663 (3)	173 (3)
N4—H5A···O3^{v}	0.88 (2)	1.96 (3)	2.816 (3)	161 (3)
O4—H1P···O2^{i}	0.87 (2)	1.68 (3)	2.523 (3)	164 (3)

Symmetry codes: (i) $x, -y+\frac{1}{2}, z-\frac{1}{2}$ (ii) $-x+1, y+\frac{1}{2}, -z+\frac{1}{2}$; (iii) $-x+1, -y+1, -z+1$; (iv) $-x+1, y-\frac{1}{2}, -z+\frac{1}{2}$; (v) $x, -y+\frac{1}{2}, z+\frac{1}{2}$.

Salt (II)

Crystal data

$C_{12}H_{12}N_3^+\cdot H_2PO_4^-\cdot H_3PO_4$
M_r = 393.23
Orthorhombic, $P2_12_12_1$
a = 4.5515 (4) Å
b = 10.5973 (9) Å
c = 34.705 (3) Å
V = 1673.9 (3) Å^3
Z = 4
D_x = 1.560 Mg m^{-3}
Mo $K\alpha$ radiation
μ = 0.31 mm^{-1}
T = 293 (2) K
Plate, purple
0.55 × 0.15 × 0.02 mm

Data collection

Oxford Xcalibur-3 CCD area-detector diffractometer
ω scans
Absorption correction: analytical (Alcock, 1970)
T_{min} = 0.911, T_{max} = 0.993
22198 measured reflections
4015 independent reflections
3323 reflections with $I > 2\sigma(I)$
R_{int} = 0.041
θ_{max} = 28.1°

Refinement

Refinement on F^2
$R[F^2 > 2\sigma(F^2)]$ = 0.040
$wR(F^2)$ = 0.101
S = 1.07
4015 reflections
251 parameters
H atoms treated by a mixture of independent and constrained refinement
$w = 1/[\sigma^2(F_o^2) + (0.0585P)^2]$ where $P = (F_o^2 + 2F_c^2)/3$
$(\Delta/\sigma)_{max}$ < 0.001
$\Delta\rho_{max}$ = 0.23 e Å^{-3}
$\Delta\rho_{min}$ = −0.24 e Å^{-3}
Absolute structure: Flack (1983), 1631 Friedel pairs
Flack parameter: −0.11 (10)

Table 2
Hydrogen-bond geometry (Å, °) for (II).

D—H···A	D—H	H···A	D···A	D—H···A
O3—H5···O6	0.86 (3)	1.78 (3)	2.647 (3)	176 (3)
O4—H6···O5	0.84 (3)	1.72 (3)	2.554 (3)	169 (3)
N3—H3···O1	0.91 (3)	2.03 (3)	2.899 (3)	161 (3)
O2—H4···O1^{i}	0.87 (4)	1.71 (4)	2.568 (3)	168 (4)
O7—H7···O6^{i}	0.82 (3)	1.72 (3)	2.537 (2)	171 (3)
N1—H2···O7^{ii}	0.90 (4)	2.17 (3)	2.994 (3)	149 (3)
O8—H8···O5^{iii}	0.85 (4)	1.75 (4)	2.579 (3)	167 (4)
N1—H1···O6^{iv}	0.91 (4)	2.12 (4)	3.011 (3)	168 (3)

Symmetry codes: (i) $x+1, y, z$; (ii) $x-\frac{3}{2}, -y+\frac{3}{2}, -z$; (iii) $x-\frac{1}{2}, -y+\frac{3}{2}, -z$; (iv) $x-1, y-1, z$.

In both structures, the H atoms bonded to N and O atoms were located from a difference Fourier map and then refined isotropically with a common U_{iso} value, and with N—H bond distances restrained to 0.86 Å and O—H bond distances restrained to 0.90 Å. H atoms bonded to C atoms were placed at geometrically calculated positions, with C—H bond distances fixed at 0.93 Å and U_{iso}(H) values of $1.2U_{eq}$(C). Refinement of the Flack parameter (Flack, 1983; Flack & Bernardinelli, 2000) was attempted for structure (II) using the TWIN and BASF commands in *SHELXL97* (Sheldrick, 1997), but it did not converge (shift/s.u. = 1.06 consecutively in an indefinite number of refinement cycles). Attempted refinement of the inverted structure led to instabilities in *SHELXL97*, but we observed that the value of x had settled at approximately 1.08 (9). If the Flack parameter was refined without refining the other parameters, the value $x = -0.1$ (1) was found. For the inverted structure, also without refinement of the atomic parameters, the result was $x = 1.1$ (1). From these results (high s.u. and lack of convergence) we cannot make a definite decision about the absolute structure of (II). The reported absolute structure was chosen as the more probable one.

For both compounds, data collection: *CrysAlis CCD* (Oxford Diffraction, 2003); cell refinement: *CrysAlis RED* (Oxford Diffraction, 2003); data reduction: *CrysAlis RED*; program(s) used to solve structure: *SHELXS97* (Sheldrick, 1997); program(s) used to refine structure: *SHELXL97* (Sheldrick, 1997); molecular graphics: *ORTEP-3* (Farrugia, 1997) and *SCHAKAL99* (Keller, 1999); software used to prepare material for publication: *PARST* (Nardelli, 1995) and *SHELXL97* (Sheldrick, 1997).

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Supplementary data for this paper are available from the IUCr electronic archives (Reference: FA3051). Services for accessing these data are described at the back of the journal.

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223. *Acidity Functions of Some Aqueous Acids.*

By K. N. Bascombe and R. P. Bell.

Measurements have been made of the indicator acidities at 25° of aqueous solutions of sulphuric, phosphorous, methanesulphonic, formic, acetic, monochloroacetic, dichloroacetic, and trichloroacetic acids, several of which were studied over the whole range of compositions. The dissociation constants of several of the indicators were re-determined, and measurements over a range of wavelengths were used to detect effects on the absorption spectra of the indicators due to the medium. Such effects were prominent in the most concentrated solutions of the carboxylic acids, and it is concluded that acidity functions cannot be accurately defined in these solutions. The theoretical treatment previously developed for solutions of strong acids is used to derive an approximate value of 4 for the dissociation constant of methanesulphonic acid.

The Hammett acidity function has been widely used in assessing the mechanistic significance of kinetic measurements in concentrated acid solutions, and recent reviews [1,2] have dealt thoroughly with this subject. There is also a close connection between indicator acidities and the general problem of interpreting the equilibrium properties of concentrated electrolyte solutions.[3,4] Nevertheless, a large proportion of the available experimental data is derived from Hammett's pioneer work, which employed a visual colorimeter without temperature control. The present paper reports measurements at 25°, in which a spectrophotometer was used, and special attention was paid to limitations upon the accuracy with which the acidity function can be defined or measured. In particular, measurements were carried out over a range of wavelengths, so as to detect any effect of changes in the medium on the absorption spectra of the species concerned. Many of the measurements relate to solutions of weak acids which have not previously been fully investigated.

Experimental

Of the acids used, perchloric, hydrochloric, sulphuric, acetic, formic, monochloroacetic trichloroacetic, and iodic were " AnalaR " products. Phosphorous, dichloroacetic, and toluene-*p*-sulphonic acids were of " Laboratory Reagent " grade, the last being recrystallised from alcohol. Methanesulphonic acid was manufactured by Boots Pure Drug Co., Ltd., and its purity checked by titration.

The following indicators were B.D.H. Laboratory Reagents, recrystallised from alcohol, and having m. p. within 2° of the accepted value: *p*-aminoazobenzene, *m*-nitroaniline, phenylazodiphenylamine, *p*-nitroaniline, *o*-nitroaniline, *p*-nitrodiphenylamine, 2 : 4-dinitroaniline, benzylideneacetophenone, and *NN*-dimethyl-*p*-nitroaniline. 4-Chloro-2-nitroaniline was a laboratory specimen recrystallised from alcohol. 4-Methyl-2 : 6-dinitroaniline was prepared as described by Brady, Day, and Rolt [5] (m. p. 165° from alcohol). 6-Bromo-2 : 4-dinitroaniline was prepared by brominating 2 : 4-dinitroaniline in glacial acetic acid at 100° (cf. Elion [6]). The product separated on cooling and was filtered off, washed with water, dried, and twice recrystallised from alcohol (m. p. 151°). *NN*-Dimethyl-2 : 4-dinitroaniline was prepared by adding solid *NN*-dimethyl-*p*-nitroaniline to excess of 1 : 1 aqueous nitric acid. The amine dissolved, but after a few minutes yellow crystals of the nitrate of the required product began to separate. After half an hour the mixture was neutralised with an excess of aqueous sodium hydroxide and the solid product filtered off, washed with water, dried, and recrystallised from alcohol (m. p. 86°).

The acid solutions were standardised by volume either against borax, or (through sodium hydroxide solution) against constant-boiling hydrochloric acid. The weight concentrations

[1] Paul and Long, *Chem. Rev.*, 1957, **57**, 1.
[2] Long and Paul, *Chem. Rev.*, 1957, **57**, 935.
[3] Bascombe and Bell, *Discuss. Faraday Soc.*, 1957, **24**, 158.
[4] Wyatt, *Discuss. Faraday Soc.*, 1957, **24**, 162.
[5] Brady, Day, and Rolt, *J.*, 1922, 527.
[6] Elion, *Rec. Trav. chim.*, 1923, **42**, 171.

given in the Tables were calculated from published densities, supplemented by our measurements on solutions of phosphorous and methanesulphonic acids. Stock indicator solutions were made up by weight in water or in the acid solution being investigated and the solutions for measurement were made by adding a known weight of indicator solution to a much larger volume of acid solution, giving indicator concentrations of about 10^{-5}M. The same indicator stock solution was used for measuring the absorption of the acidic and the basic form of the indicator and of the solution being investigated, so that it was not necessary to know its concentration accurately.

Optical densities were measured with a Unicam S.P. 500 spectrophotometer with a cell compartment regulated at 25° ± 0·02°. The latter consisted of a metal block resembling that described by Evans, Herington, and Kynaston:[7] the temperature was controlled by a platinum resistance thermometer embedded in the block, which formed one arm of a Wheatstone bridge and actuated a relay. The optical-density scale was tested by making measurements with solutions of potassium chromate which have been shown[8] to obey Beer's law with high accuracy. The apparent extinction coefficient at a given wavelength varied with concentration, decreasing by 2% for each unit increase in optical density. This shows that the scale of the instrument is slightly in error, and the true optical densities (D) were calculated from those read from the scale (D^*) by the relation $D = 50D^*/(50 - D^*)$. Similar difficulties in the accurate use of the Unicam S.P. 500 instrument have been reported by Davies and Prue.[9]

All measurements employed stoppered 10 mm. silica cells which were optically matched. Water was used in the comparison cell, and measurements were made over a range of 1000—1500 Å at intervals of 100 or 200 Å. In any set of measurements the observed optical densities (after application of the correction described above) were converted to a standard indicator concentration, usually that used in measuring the spectrum of the basic form of the indicator: these converted optical densities are represented by D'.

The acidity function H_0, as measured by an uncharged basic indicator B, is defined by

$$H_0 = pK + \log [B]/[BH^+] = pK + \log (D' - D'_{BH^+})/(D'_B - D') \quad . \quad . \quad (1)$$

where pK refers to BH^+, and D'_{BH^+} and D'_B are the converted optical densities for solutions in which the indicator is present entirely in the acidic and in the basic form respectively. D'_{BH^+} was usually measured in sulphuric acid of about 50% concentration. It was much smaller than D'_B for all the indicators used, with the exception of benzylideneacetophenone, which was used only for a few measurements with methanesulphonic acid: it was therefore not necessary to know D'_{BH^+} accurately. Indicators with p$K > -1$ are appreciably protonated even in dilute acid solution, and for these the value of D'_B was measured in 0·1M-aqueous sodium hydroxide. Since these indicators were not used in very concentrated acid solutions it is reasonable to use this value throughout in calculating H_0 from eqn. (1). The validity of this assumption can be tested to some extent by making measurements at different wavelengths, since it is unlikely that a change of medium would change the value of D'_B at a given wavelength without also changing the shape of the absorption curve. This test was carried out in most of the measurements, and examples are given under the individual acids. The most weakly basic indicators (pK −3 to −7) were used in very concentrated acid solutions, sometimes up to 100% acid, and it is certainly not appropriate to use the value of D'_B measured in water or dilute alkali. In most instances D'_B was measured in a solution of the acid being studied just below the concentration range in which protonation begins to become appreciable: this procedure was used by Hammett and Deyrup.[10] However, other procedures are possible and their use does not lead to identical results in concentrated solutions, as illustrated by our measurements with *p*-nitrodiphenylamine in sulphuric acid. The same uncertainty is revealed by the apparent variation of indicator ratio when the same solution is investigated at different wavelengths.

The indicators used, together with the values assumed for pK_a, are listed in Table 1. The pK values for the first three indicators were obtained from measurements in dilute solutions of hydrochloric, perchloric, and toluene-*p*-sulphonic acid, which were assumed to be completely

[7] Evans, Herington, and Kynaston, *Trans. Faraday Soc.*, 1953, **49**, 1284.
[8] Kortüm, *Z. phys. Chem.*, 1936, *B*, **33**, 243.
[9] Davies and Prue, *Trans. Faraday Soc.*, 1955, **51**, 1045.
[10] Hammett and Deyrup, *J. Amer. Chem. Soc.*, 1932, **54**, 4239.

dissociated. As would be expected in very dilute solution, the observed values of log {[BH$^+$]/[B][H$^+$]} showed no trend with acid concentration, and the values given for pK are averages for all measurements. As an example the results for *p*-aminoazobenzene are given in Table 2. The limits of error given for the indicator ratio I and the value of log {[BH$^+$]/[B][H$^+$]} calculated from it are based upon the precision and reproducibility of the instrumental readings.

TABLE 1. pK *values of indicators.*

	Indicator	pK (i)	Method	pK (ii)
I.	*p*-Aminoazobenzene	2·82	(a)	2·76
II.	*m*-Nitroaniline	2·50	(a)	2·50
III.	*p*-Nitroaniline	1·02	(a)	0·99
IV.	*o*-Nitroaniline	−0·29	(c)	−0·29
V.	*NN*-Dimethyl-2 : 4-dinitroaniline	−1·00	(c)	—
VI.	4-Chloro-2-nitroaniline	−1·02	(c)	−1·03
VII.	*p*-Nitrodiphenylamine	−2·4 to −2·9	(c)	−2·50
VIII.	4 : 6-Dichloro-2-nitroaniline	−3·61	(b)	−3·32
IX.	4-Methyl-2 : 6-dinitroaniline	−3·96	(b)	−4·44
X.	6-Bromo-2 : 4-dinitroaniline	−6·64	(b)	−6·71

(i) Present paper.
(ii) " Best value " given by Paul and Long.[1]
(a) Measured directly in dilute solutions of strong acids. (b) From overlap in solutions of methanesulphonic acid. (c) From overlap in solutions of sulphuric acid.

Table 2 shows that the observed indicator ratio is independent of the wavelength, and the same is true for *m*-nitroaniline and *p*-nitroaniline. The values obtained for the pK of these indicators agree fairly well with the " best values " of Paul and Long.[1] The values obtained for the remaining seven indicators are discussed in connection with the measurements on solutions of sulphuric and methanesulphonic acids.

Results.—In the Tables the indicators are numbered as in Table 1. Unless otherwise stated, measurements were made at the wavelengths of maximum absorption of the basic form, which were as follows: I 3800 Å, II 3600 Å, III 3800 Å, IV 4100 Å, V 3900 Å, VI 4200 Å, VII 4100 Å, VIII 4200 Å, IX 4500 Å, X 3550 Å. The acid concentration (concn.) is in moles/l. Details follow for the individual acids.

TABLE 2. *Measurement of* pK *for* p-*aminoazobenzene.*

[HCl]	I	log {[BH$^+$]/[B][H$^+$]}	[*p*-Me·C$_6$H$_4$·SO$_3$H]	I	log {[BH$^+$]/[B][H$^+$]}
0·00358	2·25 ± 0·12	2·80 ± 0·03	0·00171	1·17 ± 0·03	2·83 ± 0·01
	2·65 ± 0·09	2·86 ± 0·02		2·75 ± 0·09	2·80 ± 0·02
0·00366	2·60 ± 0·09 *	2·84 ± 0·02	0·00435	2·79 ± 0·08 *	2·82 ± 0·02
	2·73 ± 0·09 †	2·89 ± 0·02		2·68 ± 0·08 †	2·77 ± 0·02
0·00540	3·66 ± 0·12	2·83 ± 0·02	0·00870	5·73 ± 0·26	2·82 ± 0·02
0·00658	3·87 ± 0·15	2·77 ± 0·03			
0·00715	4·96 ± 0·17	2·84 ± 0·02			
0·00880	5·94 ± 0·18	2·83 ± 0·02			
[HClO$_4$]					
0·00087	0·53 ± 0·02	2·78 ± 0·02			
0·00266	1·72 ± 0·04	2·81 ± 0·01			
	2·94 ± 0·06	2·81 ± 0·01			
0·0455	2·98 ± 0·06 *	2·83 ± 0·01			
	2·95 ± 0·06 †	2·81 ± 0·01			
0·00957	7·10 ± 0·27	2·87 ± 0·02			

I = [BH$^+$]/[B].
Measurements at 3800 Å unless otherwise stated.
* Measurements at 3600 Å.
† Measurements at 4000 Å.

Sulphuric acid. This was studied in order to check the pK values for some of the indicators, and also to provide more accurate data for the intermediate range of concentrations. Most of the results are given in Table 3; they are independent of the wavelength within experimental error (2—5% in I, or 0·01 to 0·02 in H_0, depending upon the value of I and the part of the instrumental scale being used). The values of H_0 derived from indicators I, II, and III are in good mutual agreement, by use of the pK values in Table 1, and up to a concentration of c = 0·01 they agree within 0·02 unit with $H_0 = -\log [H^+]$, where [H$^+$] is calculated from $f_2\,[H^+]([H^+] - c)/(2c - [H^+]) = 0{\cdot}0103$, where f_2 is given in terms of the ionic strength μ by $-\log f_2 = 2\mu^{\frac{1}{2}}/(1 + \mu^{\frac{1}{2}})$. This calculation assumes that $f_{H^+} = f_{HSO_4^-}$, and the ionic strength is

obtained by successive approximations, while $K = 0{\cdot}0103$ is the value given by Davies, Jones, and Monk [11] for the second dissociation constant of sulphuric acid.

The pK values for indicators IV, V, and VI have been chosen to give concordant values of H_0 in the region of overlap, as shown in Table 3. pK(IV) and pK(VI) are in good agreement with the best values of Paul and Long [1] (cf. Table 1). Indicator V has not previously been investigated, and is of interest in being a tertiary amine: nevertheless, it gives results closely parallel with those of the primary amine VI of similar pK. The indicator *NN*-dimethyl-2 : 4 : 6-

TABLE 3. *Sulphuric acid.*

Key to wavelengths (λ): *m* maximum absorption, *a* 3400 Å, *b* 3600 Å, *c* 3700 Å, *d* 3800 Å, *e* 3900 Å, *f* 4000 Å, *g* 4400 Å, *h* 4600 Å.

Concn.	Wt. %	Indr.	λ	I	H_0	Concn.	Wt. %	Indr.	λ	I	H_0
0·00050	0·0049	I	*m*	0·62	3·03			III	*b*	28·6	−0·44
0·00058	0·0057	I	*m*	0·76	2·94			III	*c*	28·2	−0·43
		I	*b*	0·80	2·92	1·20	10·9	III	*m*	28·7	−0·44
0·00059	0·0058	I	*m*	0·76	2·94			III	*e*	29·0	−0·44
		I	*f*	0·78	2·93			III	*f*	29·0	−0·44
		I	*b*	1·02	2·81			IV	*d*	1·45	−0·44
0·00067	0·0066	I	*m*	0·98	2·83	1·22	11·1	IV	*m*	1·49	−0·46
		I	*f*	1·02	2·81			IV	*g*	1·47	−0·45
0·00098	0·0096	I	*m*	1·13	2·77	1·49	13·4	IV	*m*	2·13	−0·62
0·00118	0·0116	I	*m*	1·43	2·67	1·54	13·8	VI	*m*	0·415	−0·64
0·00134	0·0131	I	*m*	2·04	2·51	1·56	14·1	IV	*m*	2·43	−0·68
0·00239	0·0234	I	*m*	2·99	2·35	1·57	14·2	III	*m*	48·3	−0·66
0·00253	0·0248	I	*m*	3·09	2·33	1·66	14·75	III	*m*	53·3	−0·71
0·00409	0·0401	I	*m*	4·84	2·14	1·85	16·3	IV	*m*	3·51	−0·83
0·00434	0·0425	I	*m*	5·07	2·12	1·86	16·4	VI	*m*	0·65	−0·83
0·00480	0·0470	I	*m*	5·48	2·08	1·91	16·8	VI	*m*	0·72	−0·88
0·00603	0·0590	I	*m*	6·8	1·99	1·94	17·1	IV	*m*	3·74	−0·86
0·00615	0·0602	II	*m*	3·34	1·98			III	*b*	81	−0·89
0·00750	0·0734	I	*m*	8·3	1·90	2·04	17·8	III	*m*	83	−0·90
0·00908	0·0890	I	*m*	9·8	1·83			III	*f*	82	−0·90
		II	*a*	7·6	1·62	2·20	19·1	V	*m*	0·84	−0·93
0·0139	0·136	II	*m*	7·1	1·65			VI	*f*	1·57	−1·22
		II	*d*	7·0	1·66	2·61	22·2	VI	*m*	1·55	−1·21
		II	*a*	12·4	1·41			VI	*g*	1·53	−1·20
0·0254	0·248	II	*m*	12·1	1·42	2·68	22·6	IV	*m*	9·1	−1·25
		II	*d*	11·3	1·45	2·95	24·6	V	*m*	2·38	−1·38
0·0333	0·325	II	*m*	15·6	1·32	2·95	24·6	VI	*m*	2·30	−1·38
0·0410	0·400	III	*m*	0·63	1·22			IV	*d*	13·2	−1·40
0·0625	0·612	III	*m*	0·91	1·06	3·02	25·1	IV	*m*	13·6	−1·42
0·235	2·26	III	*m*	3·44	0·48			IV	*g*	13·5	−1·42
0·278	2·67	III	*m*	4·11	0·41	3·55	28·7	V	*m*	5·21	−1·72
0·280	2·69	III	*m*	4·14	0·40	3·55	28·7	VI	*m*	4·38	−1·66
0·355	3·39	III	*m*	5·47	0·28	4·29	33·6	V	*m*	10·8	−2·03
0·540	5·10	III	*m*	8·5	0·09	4·33	33·8	IV	*m*	75	−2·16
0·90	8·3	III	m	17·3	−0·22	4·54	35·2	V	*m*	14·1	−2·15
0·90	8·3	IV	*m*	0·89	−0·24			VI	*f*	15·9	−2·22
0·98	9·1	III	*m*	19·5	−0·27	4·61	35·6	VI	*m*	15·9	−2·22
1·18	10·75	III	*m*	27·7	−0·42			VI	*g*	15·5	−2·21
						4·84	37·1	V	*m*	23·7	−2·37
						5·08	38·6	VI	*m*	26·7	−2·45

trinitroaniline gives ionisation curves in 65—75% aqueous sulphuric acid and in acetic acid solutions of sulphuric acid which deviate considerably from those given by primary amines,[10,12] but individual deviations are more prominent in these highly acid solutions.

Attempts were made to study sulphuric acid solutions more concentrated than 5M by using indicator VII (*p*-nitrodiphenylamine). Since this will not be appreciably protonated at concentrations less than about 2M, it is possible to investigate the effect of acid concentration upon D'_B, the absorption of the basic form. Table 4 shows the results for three wavelengths in the neighbourhood of the maximum (4100 Å). It is clear that there is a considerable medium effect, which varies with wavelength. Three different assumptions have been made in calculating the indicator ratios in the range 4M—7M-sulphuric acid: (i) D'_B has the same value as in

[11] Davies, Jones, and Monk, *Trans. Faraday Soc.*, 1952, **48**, 921.
[12] Hall and Spengemann, *J. Amer. Chem. Soc.*, 1940, **62**, 2487.

neutral aqueous solution. (ii) D'_B has the same value as in 1·8M-sulphuric acid (*i.e.*, just below the protonation range). (iii) D'_B is calculated from Table 4 by linear extrapolation to the relevant acid concentration. Table 5 shows the results of these three assumptions. The values of I differ considerably according to the assumption made about D'_B: with assumptions (i) and (ii) there is also a considerable variation with wavelength, but with assumption (iii)

TABLE 4. *Medium effect on the absorption of* p-*nitrodiphenylamine.*

	[H_2SO_4]	0·575	0·98	1·38	1·83
D'_B	3800 Å	2·82	2·87	2·85	2·89
	4100 Å	3·80	3·90	3·94	4·05
	4400 Å	2·50	2·61	2·72	2·76

this variation is no greater than the uncertainty in the extrapolation for obtaining D'_B. However, this may be coincidental, since the values of I on assumption (iii) are not consistent with the results of Table 3 obtained with indicators IV, V, and VI. The choice of pK = −2·4 for *p*-nitrodiphenylamine gives coincident values of H_0 at 4·4M, but the plots of H_0 against Concn. diverge sharply at higher concentrations. On the other hand, either assumption (i) with pK = −2·93 or assumption (ii) with pK = −2·60 leads to values of H_0 which are consistent with Table 3. The values of H_0 from measurements at 4100 Å are given in Table 6: as can be seen from Table 5, somewhat different values would be obtained by using other wavelengths.

TABLE 5. *Apparent indicator ratios for* p-*nitrodiphenylamine in sulphuric acid.*

		I					*I*		
Concn.	λ (Å)	(i)	(ii)	(iii)	Concn.	λ (Å)	(i)	(ii)	(iii)
4·38	3800	0·199	0·384	0·465	6·36	3800	1·80	2·23	2·54
	4100	0·156	0·323	0·495		4100	1·64	2·03	2·72
	4400	0·130	1·290	1·457		4400	1·33	1·54	2·55
4·87	3800	0·328	0·532	0·665	7·04	3800	4·39	5·21	6·3
	4100	0·268	0·457	0·684		4100	4·31	5·09	6·7
	4400	0·226	0·305	0·644		4400	3·80	4·19	6·8
5·68	3800	0·673	1·08	1·25					
	4100	0·644	0·89	1·26					
	4400	1·516	0·63	1·28					

Tables 4—6 illustrate the difficulties which can arise in more concentrated solutions and the consequent uncertainties in H_0. These uncertainties arise partly from effects of the medium upon absorption spectra, and partly from a more fundamental breakdown in the concept of acidity functions, in that H_0 may no longer be independent of the indicator used for its measurement. It is probable that these medium effects are particularly large for *p*-nitrodiphenylamine, since most of the other indicators showed a much smaller dependence of apparent indicator ratio upon wavelength even when no allowance was made for the variation of D'_B with concentration.

TABLE 6. H_0 *from* p-*nitrodiphenylamine in sulphuric acid.*

(All measurements at 4100 Å, maximum.)
pK for indicator: (i) −2·93, (ii) −2·60, (iii) −2·40.

Concn.	Wt. %	Assumption	D'_B	I	H_0	Concn.	Wt. %	Assumption	D'_B	I	H_0
4·38	34·2	i	3·53	0·156	−2·11	6·36	45·9	i	3·53	1·64	−3·14
		ii	4·05	0·323	−2·11			ii	4·05	2·03	−2·91
		iii	4·57	0·495	−2·10			iii	4·97	2·72	−2·84
4·87	37·2	i	3·53	0·268	−2·36	7·04	49·6	i	3·53	4·31	−3·57
		ii	4·05	0·457	−2·26			ii	4·05	5·09	−3·31
		iii	4·67	0·68	−2·24			iii	5·10	6·70	−3·33
5·68	42·2	i	3·53	0·64	−2·74						
		ii	4·05	0·89	−2·55						
		iii	4·83	1·26	−2·50						

The values of H_0 in Tables 3 and 6 are in general agreement with Hammett and Deyrup's measurements [10] as recalculated by Paul and Long,[1] but show much less scatter.

Methanesulphonic acid. This acid was studied over the whole concentration range 0—100%,

partly in order to provide pK-values for indicators VIII, IX, and X, and partly because of the interest of an acid slightly weaker than the common mineral acids. Of the eight indicators used two were unsatisfactory. *p*-Nitrodiphenylamine gave inconsistent results, and appeared to react slowly with the acid. With benzylideneacetophenone the basic form has no absorption in the range usually examined, while the protonated form has a peak at 4300 Å in concentrated sulphuric acid. This appears at 4100 Å in 100% methanesulphonic acid; on addition of water the extinction coefficient increases by 30% in 95% acid, and then falls in the range 80—90%, simultaneously shifting to 3900 Å. This behaviour suggests either that several species are present or that the absorption spectra are subject to large medium effects: in either event the indicator is unsuitable for measuring acidities. The results for the remaining indicators are given in Table 7.

TABLE 7. *Methanesulphonic acid.*

Key to wavelengths: *m* maximum absorption, *a* 3300 Å, *b* 3400 Å, *c* 3800 Å, *d* 4200 Å, *e* 4400 Å, *f* 4600 Å, *g* 4800 Å.

Concn.	Wt. %	Indr.	λ	I	H_0	Concn.	Wt. %	Indr.	λ	I	H_0
0·302	3·0	III	*m*	2·72	0·43			VIII	*c*	0·165	−2·82
		III	*b*	11·0	−0·02	8·32	61·5	VIII	*m*	0·151	−2·79
0·749	6·8	III	*m*	11·3	−0·03			VIII	*f*	0·170	−2·84
		III	*c*	11·5	−0·04	9·38	67·4	VIII	*m*	0·538	−3·34
1·557	13·9	III	*m*	24·7	−0·37	10·46	73·3	VIII	*m*	1·99	−3·91
		IV	*c*	1·78	−0·54			IX	*d*	1·23	−4·05
1·925	17·0	IV	*m*	1·81	−0·55	10·78	74·9	IX	*m*	1·21	−4·04
		IV	*e*	1·77	−0·54			IX	*g*	1·20	−4·04
2·51	21·9	IV	*m*	2·28	−0·65	10·85	75·3	VIII	*m*	2·91	−4·07
2·98	25·5	VI	*m*	0·59	−0·79	11·85	80·6	VIII	*m*	9·6	−4·59
3·30	28·0	VI	*m*	0·72	−0·88	12·12	82·1	IX	*m*	7·6	−4·84
		VI	*c*	2·18	−1·36	12·74	85·2	IX	*m*	26·7	−5·39
4·82	39·5	VI	*m*	2·27	−1·38	13·45	88·9	X	*m*	0·309	−6·12
		VI	*f*	2·18	−1·36	13·95	91·4	X	*m*	0·94	−6·61
6·16	48·3	VI	*m*	7·4	−1·89			X	*a*	6·4	−7·44
6·44	50·2	IV	*m*	56·9	−2·05	14·77	95·7	X	*m*	6·1	−7·42
7·15	54·5	VI	*m*	19·1	−2·30			X	*c*	6·3	−7·43
						15·07	100·0	X	*m*	16·7	−7·86

There is no detectable variation of apparent indicator ratio with wavelength for any of the indicators studied, even close to 100% acid. The pK values taken for indicators III, IV, and VI are those previously obtained, while those for VIII, IX, and X are chosen so as to give good overlap or alignment in the present set of measurements (cf. Table 1). The values for VIII and IX differ somewhat from Paul and Long's best values, but the latter are based mainly on Hammett's early measurements with sulphuric acid and the differences probably reflect the spread of his experimental data in this region of concentration.

Trichloroacetic acid. An " AnalaR " specimen showed a small absorption in the range 3500—4500 Å, which was increased by vacuum distillation. The unpurified acid was therefor used, and a small correction applied. The results are given in Table 8.

TABLE 8. *Trichloroacetic acid.*

Measurements at peak wavelength, except those marked *a* (3400 Å), *b* (3800 Å), *c* (4200 Å), *d* (4400 Å).

Concn.	Wt. %	Indr.	I	H_0	Concn.	Wt. %	Indr.	I	H_0
0·221	4·0	IV	0·111	0·67	3·89	49·5	III	9·5	+0·04
0·574	9·1	IV	0·306	0·22			III	11·4 (*a*)	−0·03
		IV	0·435 (*b*)	0·07	4·99	59·9	III	10·8	−0·01
0·786	12·5	IV	0·406	1·10			III	10·9 (*c*)	−0·01
		IV	0·429 (*d*)	0·08			IV	1·12 (*b*)	−0·34
1·07	16·6	IV	0·509	0·00	6·68	74·0	IV	1·07	−0·32
1·72	25·5	III	10·7	−0·01			IV	1·12 (*d*)	−0·34
		III	10·8 (*a*)	0·00	7·26	78·6	III	34·3	−0·53
2·70	37·0	III	10·4	+0·01					
		III	10·0 (*c*)	+0·02					

The results are satisfactorily independent of wavelength, and the two indicators give concordant values. If allowance is made for the different pK values taken for *p*-nitroaniline, our

results agree fairly well with those of Randles and Tedder,[13] who allowed for the absorption of the acid solution, but they are much lower than the three measurements reported by Hammett and Paul,[14] who made no such allowance.

Dichloroacetic acid. Measurements were made over the whole range of composition 0—100% with three indicators, but only the measurements with *p*-nitroaniline are recorded in Table 9. *o*-Nitroaniline and 4-chloro-2-nitroaniline were used in the range 80—100% acid, but the apparent indicator ratios varied greatly with wavelength in the neighbourhood of maximum absorption, and no reliance can be placed on the results.

The values in Table 9 agree fairly well with the less accurate measurements of Bell and Brown [15] when allowance is made for the different pK values assumed for the indicator.

Monochloroacetic acid. Measurements were extended up to the saturated solution (82%) and are given in Table 10.

TABLE 9. *Dichloroacetic acid.*

Indicator *p*-nitroaniline. Measurements at peak wavelength, except those marked *a* (3400 Å) or *b* (4200 Å)

Concn.	Wt. %	I	H_0	Concn.	Wt. %	I	H_0
0·281	3·1	1·18	0·95	5·49	55·5	2·51	0·62
0·558	6·8	1·76	0·77	6·46	63·0	3·03	0·54
1·098	13·1	2·32	0·65	7·28	68·4	3·65	0·46
2·35	26·9	2·34	0·65			5·37 (*a*)	0·29
		2·34 (*a*)	0·66	8·35	76·4	5·33	0·29
2·93	33·0	2·37	0·65			5·68 (*b*)	0·26
		2·15 (*b*)	0·61			11·5 (*a*)	−0·05
3·44	37·8	2·28	0·66	9·76	85·5	15·7	−0·19
		2·43 (*a*)	0·64			15·9 (*b*)	−0·19
5·07	52·0	2·36	0·65				
		2·29 (*b*)	0·66				

TABLE 10. *Monochloroacetic acid.*

All measurements with *p*-nitroaniline, except those marked *, which are with *p*-aminoazobenzene. Measurements at peak wavelength, except those marked *a* (3400 Å) or *b* (4200 Å).

Concn.	Wt. %	I	H_0	Concn.	Wt. %	I	H_0
		0·217 (*a*)	1·68			0·420 (*a*)	1·39
0·363	3·38	0·234	1·65	4·90	40·0	0·396	1·42
		0·226 (*b*)	1·66			0·432 (*b*)	1·38
0·684	6·30	0·276	1·58			0·499 (*a*)	1·32
0·728	6·70	0·277	1·58	6·67	52·2	0·535	1·29
0·748	6·89	17·7 *	1·57			0·625 (*b*)	1·23
1·357	12·24	0·322	1·51	7·40	56·7	0·624	1·23
1·462	13·12	0·352	1·47			0·780 (*a*)	1·13
2·10	18·4	0·361	1·46	8·06	60·9	0·762	1·14
2·13	18·8	0·360	1·46			1·18 (*b*)	0·94
2·17	19·1	0·366	1·46			1·45 (*a*)	0·86
2·19	19·3	0·363	1·46	9·36	68·8	1·58	0·82
2·40	21·0	22·0 *	1·48			2·11 (*b*)	0·68
2·75	23·8	0·354	1·47			2·03 (*a*)	0·72
2·87	24·7	0·364	1·46	10·86	77·4	2·07	0·70
3·29	28·0	0·362	1·46			2·65 (*b*)	0·60
		0·346 (*a*)	1·48			1·77 (*a*)	0·76
3·33	28·3	0·358	1·47	11·21	79·3	2·06	0·70
		0·347 (*b*)	1·48			2·86 (*b*)	0·57
3·43	29·1	0·362	1·46			0·99 (*a*)	1·02
3·56	30·1	0·363	1·46	11·65	81·6	1·11	0·98
4·59	37·8	22·5 *	1·47			1·40 (*b*)	0·88

The results of measurements with indicators I and III are in good agreement, but for solutions more concentrated than about 50% there is a considerable dependence on wavelength, and in this region the H_0 scale is certainly not defined to better than about $\pm 0{\cdot}1$.

Formic acid. With this acid the observed optical densities changed with time, possibly

[13] Randles and Tedder, *J.*, 1955, 1218.
[14] Hammett and Paul, *J. Amer. Chem. Soc.*, 1934, **56**, 827.
[15] Bell and Brown, *J.*, 1954, 774.

because of slow reaction between the acid and the amines (cf. Davis [16]). It was possible to extrapolate back to zero time with fair accuracy, but the results were still unsatisfactory. Measurements with indicator II suggested only small variations of H_0 in the range 1·6—2·0 for 7—80% formic acid solutions. By contrast, the use of indicators III and IV produced H_0 values which decrease continuously by more than 3 units over the same range of concentrations: however, with the pK values of Table 1 there is a discrepancy of about 0·25 unit between the values of H_0 derived from these two indicators. It is therefore not possible to define an acidity scale on the basis of these measurements. Because of the variation with time, measurements were carried out at the peak wavelength only.

Acetic acid. Measurements were carried out over the whole range of compositions: there was no change with time during the time necessary for measurement, but it was not found possible to define an acidity scale. Indicator II at its peak wavelength showed very small changes in indicator ratio in the range 5—85% acetic acid, corresponding to H_0 values in the range 2·25—2·45, but the use of different wavelengths introduced discrepancies of up to 0·3 in H_0. In confirmation of this, both nitrobenzene and 2 : 4-dinitroaniline (which are not appreciably protonated in the solutions used) showed considerable changes in absorption spectra when the acetic acid concentration was varied from 5% to 85%. Measurements with indicator I suggested a decrease of H_0 from 2·3 to 1·9 in the range 7—60% acetic acid, but there is no reason to believe that this result is reliable. The most that can be said is that there are no very large changes in indicator acidity in the range 5—85%.

Phosphorous acid. This was investigated up to the limit of solubility (about 50%) with indicators III, IV, and VI, and the results are given in Table 11. There is satisfactory agreement between the different indicators, but the effect of varying the wavelength shows that there are increasing uncertainties in the acidity scale above about 20% acid.

TABLE 11. *Phosphorous acid.*

Key to wavelengths: *m* maximum absorption, *a* 3400 Å, *b* 3800 Å, *c* 4200 Å, *d* 4400 Å, *e* 4600 Å.

Concn.	Wt. %	Indr.	λ	*I*	H_0	Concn.	Wt. %	Indr.	λ	*I*	H_0
0·096	0·436	III	*m*	0·57	1·26	6·04	22·7	IV	*m*	1·22	−0·38
0·235	1·06	III	*m*	1·16	0·96			IV	*b*	1·36	−0·43
		III	*a*	2·31	0·65	7·46	26·9	IV	*m*	1·76	−0·54
0·596	2·66	III	*m*	2·23	0·67			IV	*d*	1·55	−0·48
		III	*c*	2·17	0·68	8·32	29·6	VI	*m*	0·408	−0·63
		IV	*b*	0·188	0·43			VI	*b*	0·181	−0·28
0·99	4·36	IV	*m*	0·208	0·39	9·38	32·6	VI	*m*	0·449	−0·67
		IV	*d*	0·181	0·45			VI	*e*	0·373	−0·59
		III	*a*	4·77	0·34	10·24	35·8	IV	*m*	3·21	−0·80
1·18	5·15	III	*m*	5·12	0·31	10·86	37·2	VI	*m*	0·78	−0·91
		III	*c*	4·46	0·36	12·05	40·8	IV	*m*	4·75	−0·97
		IV	*b*	0·307	0·22	12·50	42·2	VI	*m*	1·08	−1·05
2·01	8·55	IV	*m*	0·322	0·20			VI	*b*	0·68	−0·86
		IV	*d*	0·288	0·25	13·8	46·1	VI	*m*	2·07	−1·34
		IV	*b*	0·585	−0·05			VI	*e*	1·71	−1·25
4·01	15·6	IV	*m*	0·619	−0·08						
		IV	*d*	0·627	−0·09						

Iodic acid. No satisfactory measurements were obtained with this acid. The " AnalaR " material gave a cloudy solution in water. Clear solutions 0·2—1·6M were obtained after recrystallisation, but their acidity towards *p*-nitroaniline decreased slowly on keeping, without reaching any steady value. This phenomenon may be due to a slow polymerisation or depolymerisation of iodic acid species in solution. The existence of polymerised species has been suggested several times, but the constitution of these solutions is obscure.[17]

DISCUSSION

The above measurements provide revised pK values for a number of indicators (including one new one, *NN*-dimethyl-2 : 4-dinitroaniline) and for the acidity functions of solutions of sulphuric, trichloroacetic, and dichloroacetic acids. Solutions of methanesulphonic, monochloroacetic, and phosphorous acid were investigated for the first time, and measurements were also made with solutions of acetic, formic, and iodic acids, though it

[16] Davis, *Z. phys. Chem.*, 1912, **78**, 353.
[17] Morgan, *Quart. Rev.*, 1954, **8**, 123.

was not possible to define any reliable acidity functions for the last three. Our measurements show that under favourable conditions it is possible to define H_0 with an accuracy of about 0·02 unit, and it is noteworthy that this is the case for methanesulphonic acid over the whole range of compositions, the pure acid having $H_0 = -7{\cdot}86$. Trichloroacetic acid also gives a satisfactory acidity scale up to the solubility limit of 79% acid, and dichloroacetic acid up to about 80%. However, measurements at different wavelengths show that the spectra of indicators often show considerable changes in concentrated solutions of weak acids, and this was specially marked for acetic, formic, and monochloroacetic acids. Under these conditions the acidity function cannot be accurately defined, and it is dangerous to draw conclusions from measurements at a single wavelength. The indicator *p*-nitrodiphenylamine shows large medium effects even with sulphuric acid solutions, and it is unfortunate that no other indicator has been investigated between pK −1 and pK −3.

Solutions of phosphorous acid show a steady increase of acidity over the concentration range investigated, thus resembling all other inorganic acids which have been studied. In the intermediate range of concentrations the values of H_0 resemble those for orthophosphoric acid and potassium hydrogen sulphate, which have similar dissociation constants.* The three chloroacetic acids (and also trifluoroacetic acid [13]) show quite a different type of behaviour, in that the acidity is almost independent of concentration over the range 1—5M, and is much less than for an inorganic acid of similar dissociation constant. The cause of this behaviour is obscure, but it is possible that, as suggested by Paul and Long,[1] it depends upon the " salting in " effect of the large organic molecules or ions upon the basic form of the indicator.

Methanesulphonic acid behaves as a much stronger acid than any of the others investigated here, though its solutions are less acidic than those of hydrochloric, perchloric, sulphuric, or nitric acids. It has been recently shown [3] that the acidity functions of the strong acids can be accounted for quantitatively up to about 8M by a simple treatment involving the assumption that the hydrogen ion in aqueous solution is firmly associated with four water molecules. If we assume that the same treatment can be applied to methanesulphonic acid, then by inserting the observed values of H_0 in eqn. (5) of ref. 3, it is possible to compute the true hydrogen-ion concentration and hence the degree of dissociation. This gives 89%, 86%, 64%, and 53% dissociation at 0·5, 1·0, 2·0, and 3·0M, corresponding to a concentration dissociation constant of about 4: *i.e.*, methane sulphonic acid is intermediate in strength between trifluoroacetic acid ($K = 0{\cdot}18$) and nitric acid ($K = 23$).[19]

The validity of this procedure can be tested by using it to calculate the acidity functions of aqueous nitric acid, for which the true degrees of dissociation are known with some certainty from measurements of Raman spectra and proton magnetic resonance.[19] The results of such calculations are shown in Table 12: the agreement is as good as could be expected.

TABLE 12. *Observed and calculated acidity functions for aqueous nitric acid.*

Concn.	2·00	3·54	4·55	5·61	6·74
a	0·95	0·89	0·84	0·76	0·71
H_0 calc.	−0·66	−1·21	−1·51	−1·79	−2·10
H_0 obs.	−0·67	−1·18	−1·47	−1·71	−1·94

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* The values given by Bell and Brown [15] for potassium hydrogen sulphate indicate a levelling off of the acidity at a relatively low value in the range 1—3M, but recent and more accurate measurements by Satchell [18] show that it does in fact behave like the other inorganic acids.

[18] Satchell, *J.*, 1958, 3904.

[19] Young, Wu, and Krawetz, *Discuss. Faraday Soc.*, 1957, **24**, 37 and personal communication from Professor Young.

Cancer Research

Resolution of the Over-all Basicity of Carcinogenic and Noncarcinogenic Derivatives of 4-Aminoazobenzene

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Resolution of the Over-all Basicity of Carcinogenic and Noncarcinogenic Derivatives of 4-Aminoazobenzene

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SUMMARY

The pK_a of the over-all basicity of 4-aminoazobenzene and derivatives has been resolved in pK_{am} and pK_{az}, which represent the pK_a of the two basic centers, the amino nitrogen and the β-azo nitrogen.

This resolution has made possible for the first time a comparison of the carcinogenic activity of these dyes with a property related to electron density at these centers.

It appears that excessive electron density around the amino nitrogen brings about loss of activity.

The recent hypothesis of the Pullmans that both basic centers are concerned in the activity of aminoazo dyes is consistent with the results of the present work.

There is a fairly good correspondence between our pK_{am} and pK_{az} data and the electric charges on the amino nitrogen and β-azo nitrogen atoms as theoretically evaluated by the Pullmans.

In contrast to the polycyclic aromatic hydrocarbons, little is known about the relationship between electronic structure and carcinogenic activity for derivatives of 4-aminoazobenzene. Pullman (9) suggested that an optimum electron density at the azo group could be a crucial factor. Badger and Lewis (2) attempted to test this hypothesis by studying the rate of peracid oxidation of azo dyes. However, since the amino group reacted under these conditions (1), the study had to be limited to noncarcinogenic azobenzenes without this substituent, and the extrapolated results led to no convincing conclusion.

The addition of protons to aminoazo compounds has also been studied with the aim of correlating basicity with carcinogenic activity (1, 3, 4, 16, 17). However, this approach also appeared to be unsatisfactory for the evaluation of the relative electron densities, since more than one conjugate acid is formed (1, 3, 6, 13–15), and the pK_a merely measures the over-all basicity.

We have now calculated from pK_a data reported by Sawicki (15) the pK_a of the amino group and the pK_a of the azo group for a number of derivatives of 4-aminoazobenzene. We have thus compared for the first time the carcinogenic activity of aminoazo dyes with a property related to the electron density of the two basic centers.

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THEORETICAL

The very fact that a mixture of cations is formed on protonation of derivatives of 4-aminoazobenzene contributes to the over-all basicity by an entropy of mixing effect. This entropy is given by

$$-R\,(N_{am} \ln N_{am} + N_{az} \ln N_{az}) \quad ,$$

where N_{am} and N_{az} are the mole fractions of the ammonium cation and of the azonium cation, respectively. To calculate the effect of this entropy of mixing on the pK_a, one uses the equation

$$\Delta F = \Delta H - T\Delta S = -2.3RT\,p K_a \ ,$$

where ΔS is the total change on entropy on protonation. A part of ΔS, such as the entropy of mixing $\Delta\Delta S$, will contribute to pK_a by an amount ΔpK_a. One has

$$T\Delta\Delta S = 2.3RT\Delta pK_a \ .$$

Hence,

$$-2.3RT\,(N_{am} \log N_{am} + N_{az} \log N_{az}) = 2.3RT\Delta pK_a$$

Therefore,

$$\Delta pK_a = -\,(N_{am} \log N_{am} + N_{az} \log N_{az}) \ .$$

This contribution, ΔpK_a, attains the largest value in those cases in which both cations are formed in equal amounts. Then:

$$\Delta pK_a = -\,(0.5 \log 0.5 + 0.5 \log 0.5) = 0.3$$

The N_{am} and N_{az} values can be calculated roughly from the intensities $E_{\sim 500}$ and $E_{\sim 320}$, that is, of the longer wave-length transition (azonium cation) around 500 mμ and of the ultraviolet band around 320 mμ (ammonium cation) in the "acid spectrum" of the dye, the acidity being such that all the dye is protonated and none is biprotonated. These data are available (15). On the other hand, the molar absorbances ϵ necessary for the calculations are also available (3): ϵ is close to 6.1×10^4 for the azonium cation and to 2.2×10^4 for the ammonium cation. For all compounds here studied the sum of the mole fraction of these cations is near unity. When this was not the case (substituent[s] markedly affecting the absorption bands of the two parent conjugate acids), the compounds were not included in this study.

From the experimental value of pK_a, subtracting ΔpK_a, one obtains a corrected pK_a (pK_a corr). On the other hand, the equations for the basicity of both centers are:

$$K_{am} = \frac{[\text{Dye}]_{free}\,[H^+]}{[\text{Am}^+]} \qquad (1)$$

$$K_{az} = \frac{[\text{Dye}]_{free}\,[H^+]}{[\text{Az}^+]}\,, \qquad (2)$$

where $[\text{Dye}]_{free}$, $[\text{Am}^+]$ and $[\text{Az}^+]$ represent the concentrations of the free base, of the ammonium cation and of the azonium cation, respectively. Inverting and summing up equations (1) and (2), one gets

$$\frac{1}{K_{am}} + \frac{1}{K_{az}} = \frac{[\text{Am}^+]}{[\text{Dye}]_{free}\,[H^+]} + \frac{[\text{Az}^+]}{[\text{Dye}]_{free}\,[H^+]}\,.$$

Hence,

$$\frac{K_{am} + K_{az}}{K_{am}K_{az}} = \frac{1}{[H^+]}\,\frac{[\text{Am}^+] + [\text{Az}^+]}{[\text{Dye}]_{free}}\,,$$

from which,

$$\log \frac{K_{am} + K_{az}}{K_{am}K_{az}} = \text{pH} + \log \frac{[\text{Am}^+] + [\text{Az}^+]}{[\text{Dye}]_{free}}\,.$$

The right side of this equation is analogous to the Henderson-Hasselbach equation. Hence,

$$\log \frac{K_{am} + K_{az}}{K_{am}K_{az}} = pK_{a_{corr}}\,,$$

from which

$$K_{a_{corr}} = \frac{K_{am}K_{az}}{K_{am} + K_{az}}\,. \qquad (3)$$

To calculate K_{am} and K_{az} another equation is obviously necessary. Let us divide equation (1) by equation (2):

$$\frac{K_{am}}{K_{az}} = \frac{[\text{Az}^+]}{[\text{Am}^+]} = \frac{\dfrac{E_{\sim 500}}{6.1 \times 10^4}}{\dfrac{E_{\sim 320}}{2.2 \times 10^4}} = \frac{Q}{3}\,. \qquad (4)$$

Q represents the ratio of the intensities of the two absorption bands $E \sim 500/E \sim 320$; it is the C_e/A_e ratio of Sawicki (15).

From equation (4) one has:

$$K_{az} = \frac{3K_{am}}{Q}\,.$$

Inserting this value in the inverted form of equation (3) and simplifying, one arrives at

$$\frac{1}{K_{a_{corr}}} = \frac{3+Q}{3} \times \frac{1}{K_{am}}\,.$$

Separating $1/K_{am}$ and passing to the logarithms:

$$\log \frac{1}{K_{am}} = pK_{am} = pK_{a_{corr}} + \log 3 - \log (Q+3)\,. \qquad (5)$$

Similarly one arrives at

$$pK_{az} = pK_{a_{corr}} + \log Q - \log (Q+3)\,. \qquad (6)$$

Clearly, only approximate values can be expected from these equations, since Q is only an approximate ratio of the concentration of the two cations. Since Q depends upon the acidity (15) all values used were taken at about the same normality (1.0–1.2 N HCl). Obviously, not too much significance should be attached to the second decimal in the pK values presented in Tables 2 and 3, this being especially true for 2′-substituted compounds, because for these 2′-derivatives there is a reduced molar absorption of the azonium form (3).

RESULTS AND DISCUSSION

The aminoazo dyes which have been tested for the induction of liver tumors in the rat are arranged in order of increasing pK_a values in Table 1, together with the corresponding carcinogenic activities. The same comparison with the pK_{am} and pK_{az} values as calculated from equations (5) and (6) is presented in Tables 2 and 3, respectively. It can be seen that somewhat less scattering occurs with the data represented in Table 2. If one realizes that many factors probably determine the relative carcinogenic potencies, it seems reasonable to suspect that the activity disappears when the electron density at the amino nitrogen is above a certain value. Clearly this is only a possibility, another good one being the necessity of a N-CH_3 group for carcinogenic activity (7). These two possibilities are not necessarily independent of each other.

TABLE 1

THE OVER-ALL BASICITIES AND CARCINOGENICITIES OF VARIOUS DERIVATIVES OF 4-AMINOAZOBENZENE

Compound*	pK_a†	Carcinogenic activity‡
(N-CH_3, N-$CH_2C_6H_5$)-AB	1.6	0
3′-NO_2-DAB	1.67	5
2′-Cl-DAB	1.74	2
3′-CF_3-DAB	1.84	0
2′, 5′-diCH_3-DAB	2.0	0
4′-F-DAB	2.00	10–12
3′-Cl-DAB	2.01	5–6
2′-CH_3-DAB	2.04	2–3
4′-CH_3CONH-DAB	2.25	0
AB	2.28	0
DAB	2.28	6
2′, 3-diCH_3-AB	2.29	<1
4′-C_2H_5-DAB	2.30	10
3′-CH_3-DAB	2.33	10–12
4′-CH_3-DAB	2.36	<1
MAB	2.37	6
3,4′-diCH_3-AB	2.39	0
3′-CH_3-MAB	2.43	10–12
4′, 5-diCH_3-2-aminoazobenzene	2.5	0
(N-CH_3, N-CH_2CH_3)-AB	2.58	6
(N-C_2H_5)-AB	2.58	0
2,3′-diCH_3-AB	2.92	0
2,4′-diCH_3-AB	2.92	0
(N,N-diC_2H_5)-AB	3.08	0
2-CH_3-DAB	3.08	0
3-CH_3-DAB	3.48	0

* The following abbreviations are used: AB, 4-aminoazobenzene; MAB, N-methyl-4-aminoazobenzene; DAB, N,N-dimethyl-4-aminoazobenzene.

† In 50 per cent aqueous ethanol. All the data taken from reference (15).

‡ Carcinogenic activities taken from reference (7) and from J. A. Miller, E. C. Miller, and G. C. Finger, *Cancer Research*, 17:387, 1957.

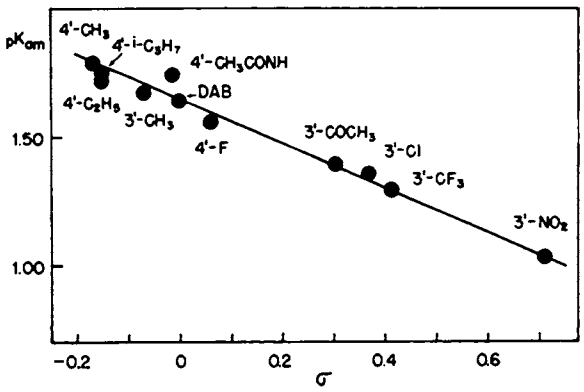

CHART 1.—pK_{am} values for various 3′ or 4′ monosubstituted 4-dimethylaminoazobenzenes vs. Hammett's substituent constant σ for the group. The 4′-i-C_3H_7 and 3′-$COCH_3$ derivatives are not included in Tables 1, 2, and 3 because they have not been tested for carcinogenic activity.

TABLE 2

THE CALCULATED BASICITIES OF THE AMINO NITROGEN IN VARIOUS DERIVATIVES OF 4-AMINOAZOBENZENE

Compound	pK_{am}	Carcinogenic activity
(N-CH_3, N-$CH_2C_6H_5$)-AB	0.9	0
3′-NO_2-DAB	1.08	5
3′-CF_3-DAB	1.30	0
3′-Cl-DAB	1.37	5–6
2′-Cl-DAB	1.47	2
4′-F-DAB	1.55	10–12
MAB	1.64	6
DAB	1.64	6
3′-CH_3-DAB	1.67	10–12
3′-CH_3-MAB	1.68	10–12
4′-C_2H_5-DAB	1.73	10
4′-CH_3CONH-DAB	1.75	0
2′-CH_3-DAB	1.78	2–3
4′-CH_3-DAB	1.79	<1
2′, 5′-diCH_3-DAB	1.8	0
(N-C_2H_5)-AB	1.95	0
AB	1.97	0
3,4′-diCH_3-AB	2.01	0
(N-CH_3, N-C_2H_5)-AB	2.14	6
2′,3-diCH_3-AB	2.08	<1
2-CH_3-DAB	2.20	0
2,3′-diCH_3-AB	2.32	0
2,4′-diCH_3-AB	2.39	0
4′,5-diCH_3-2-aminoazobenzene	2.5	0
(N,N-diC_2H_5)-AB	2.88	0
3-CH_3-DAB	3.4	0

TABLE 3

THE BASICITIES OF THE β-AZO NITROGEN IN VARIOUS DERIVATIVES OF 4-AMINOAZOBENZENE

Compound*	pK_{as}	Carcinogenic activity
2′-Cl-DAB	0.50	2
2′,3-diCH_3-AB	0.72	<1
2′-CH_3-DAB	0.76	2–3
2′,5′-diCH_3-DAB	0.8	0
(N-CH_3, N-$CH_2C_6H_5$)-AB	1.1	0
3′-NO_2-DAB	1.10	5
4′-F-DAB	1.24	10–12
3′-CF_3-DAB	1.33	0
AB	1.35	0
3′-Cl-DAB	1.44	5–6
3,4′-diCH_3-AB	1.54	0
4′-CH_3CONH-DAB	1.55	0
4′-C_2H_5-DAB	1.66	10
DAB	1.71	6
4′-CH_3-DAB	1.72	<1
3′-CH_3-DAB	1.80	10–12
(N-CH_3, N-C_2H_5)-AB	1.81	6
MAB	1.90	6
3′-CH_3-MAB	1.97	10–12
(N-C_2H_5)-AB	2.02	0
N,N-diC_2H_5-AB	2.04	0
2,4′-diCH_3-AB	2.23	0
2,3′-diCH_3-AB	2.33	0
2-CH_3-DAB	2.72	0

* Compounds 3-CH_3-DAB and 4′,5-diCH_3-2-aminoazobenzene are missing from this table because they undergo only very little, if any, protonation at the azo group.

There are only two apparently serious exceptions to this threshold concept: 4′-CH_3CONH-DAB and (N-CH_3, N-C_2H_5)-AB, both readily explained by conversion in the inactive 4′-NH_2-DAB (8)[1] and the active N-CH_3-AB (7) (that is, MAB).

The necessity of an optimum electron density at the amino nitrogen suggests that the carcinogen *in vivo* is subjected to another reaction which would take place at the amino group and which would divert it into another path, distinct from that which leads to malignancy. It is interesting to note that if this observation of a threshold is correct, then, as a consequence, the data in Table 3 tend to confirm the original Pullman hypothesis. Hence, the results will be consistent with—although not proof of—the more recent views of the Pullmans (11), namely, that both basic centers might determine the carcinogenic activity.

In their theoretical studies of chemical carcinogenesis the Pullmans (10, 11) have calculated the electric charges on the nitrogen atoms in 4-

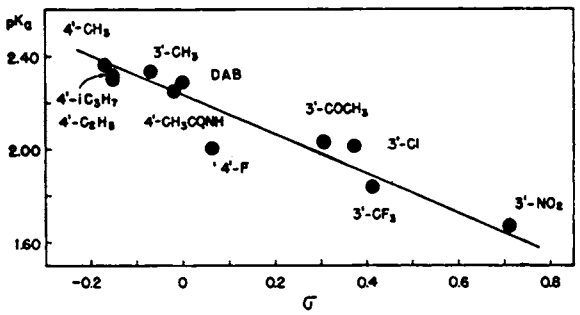

CHART 2.—pK_a values for various 3′ or 4′ monosubstituted 4-dimethylaminoazobenzenes vs. Hammett's substituent constant σ for the group. To draw the straight line the method of averages was employed.

aminoazobenzene ("AB") and in its ring monomethyl derivatives. Their theoretical results found strong experimental support in the work of Cilento, Miller, and Miller (3). Thus, the effect of methyl substitution on the relative proportion of azo protonation and amino protonation observed by the latter authors was found—as pointed out by the Pullmans (12)—in perfect agreement with the theoretical prediction.

Clearly, it would be important to compare the pK_{am} and pK_{as} values with the theoretically evaluated charges on the amino nitrogen and on the β-azo nitrogen. In view of the approximations involved and also because our data are for DAB compounds, whereas that of the Pullmans are for AB derivatives, we can only say that, except for two points, a general correspondence is found. The two points are: (*a*) the increase in basicity of the amino group by a 2-methyl group is higher than expected; and (*b*) although the 4′-CH_3 substituent does not increase the basicity of the azo nitrogen, it nevertheless does not reduce this basicity as predicted.

Finally, it is interesting to examine the relationship of pK_{am} and pK_{as} with the Hammett (5) "sigma" constants of 3′ and 4′ substituents.

Chart 1 shows that there is a close relationship between pK_{am} and σ values.[2] This is a most gratifying result, because the relationship with pK_a values is not striking (Chart 2); moreover, it adds further support for the calculations pre-

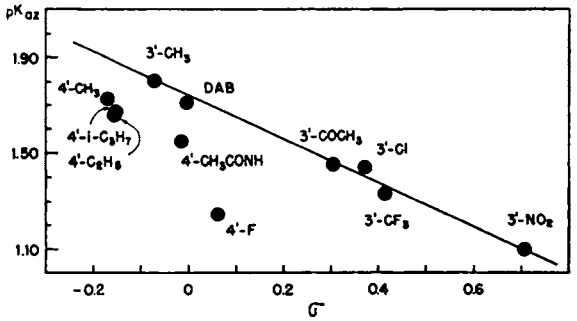

CHART 3.—pK_{as} values for various 3′ or 4′ monosubstituted 4-dimethylaminoazobenzenes vs. Hammett's substituent constant σ for the group.

sented in this paper. On the other hand, the relationship with pK_{as} values is poor (Chart 3). This is, of course, as expected. For instance, Pullmans' calculations (11) show an increasing deactivation of the protonable azo nitrogen with increasing electron donor ability of a 2′ or 4′-substituent. Now, if the 4′-derivatives are excluded, a close linear relationship is observed (Chart 3).

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[2] Values of σ were taken from H. H. Jaffé, *Chem. Rev.*, 53:191, 1953.

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Tautomerism and hydrogen bonding in guaninium phosphite and guaninium phosphate salts

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The crystal structures of three similar guaninium salts, guaninium monohydrogenphosphite monohydrate, $C_5H_6N_5O^+\cdot H_2O_3P^-\cdot H_2O$, guaninium monohydrogenphosphite dihydrate, $C_5H_6N_5O^+\cdot H_2O_3P^-\cdot 2H_2O$, and guaninium dihydrogenmonophosphate monohydrate, $C_5H_6N_5O^+\cdot H_2O_4P^-\cdot H_2O$, are described and compared. The crystal structures have been determined from accurate single-crystal X-ray data sets collected at 100 (2) K. The two phosphite salts are monoclinic, space group $P2_1/c$, with different packing and the monophosphate salt is also monoclinic, space group $P2_1/n$. An investigation of the hydrogen-bond network in these guaninium salts reveals the existence of two ketoamine tautomers, the N9H form and an N7H form.

1. Introduction

Under physiological conditions the purine base guanine exists predominantly in the neutral, keto tautomeric form. It has long been postulated that the presence of unpreferred or rare tautomeric forms might be involved in base mispair formation during polymerase-mediated DNA replication, resulting in genetic mutations (Yun *et al.*, 2003). However, it has also been estimated that these unpreferred tautomeric forms might be present, under physiological conditions, at a very low frequency of 10^{-6} to 10^{-5} (Topal & Fresco, 1976). The complex network of hydrogen-bond interactions that modulate DNA base recognition is based on the assumption of specific tautomeric and ionic states for the nucleic acid bases. The importance of tautomeric equilibria has been widely recognized since the early work of Watson & Crick (1953). Several models of spontaneous mutation in DNA are based on the existence of minor tautomeric forms (Kwiatkowski & Pullman, 1975; Topal & Fresco, 1976; Cohen *et al.*, 2003; Slósarek *et al.*, 2006; Guille & Clegg, 2006). In the Watson–Crick base-pairing scheme of nucleic acids, the nucleic acid bases are assumed to have the amino or the lactam structure (see Fig. 1). Although it has been suggested that the purine and pyrimidine bases can also exist in their minor tautomeric imino and lactim forms (Wong, 1973), the fraction of the minor tautomers, as determined by IR, UV and thermodynamic measurements, is very small, typically less than 1% (Kenner *et al.*, 1955; Katritzky & Waring, 1963; Brown & Hewlins, 1968; Wolfenden, 1969; Schweizer & Hollis, 1969; Kokko *et al.*, 1962; Miles *et al.*, 1963; Becker *et al.*, 1965). However, ^{1}H NMR results have indicated that the minor tautomers of cytosine and guanine are present to 15% at room temperatures in neutral aqueous solution (Lee *et al.*, 1971, 1972; Lee & Chan, 1972; Chan & Lee, 1972).

This explains the great experimental and theoretical effort focused on the study of tautomerism of nucleic acid bases. Recently some theoretical studies have been conduced on the tautomerism of neutral guanine (Colominas *et al.*, 1996; Barsky & Colvin, 2000; Choi & Miller, 2006). It was found that neutral guanine exists in the aqueous phase as a mixture of two major ketoamine tautomers, the N9H form (*A*, population 85%) and a N7H form (*B*, population 15%; Fig. 2). Among these two tautomers, *B* has been shown by theoretical studies to be more stable than *A* for isolated guanine (Lin *et al.*, 1980). However, *A* was known to be the only tautomeric form found in polar solvents (Miles *et al.*, 1963; Shapiro, 1968) or in the crystalline state (Thewalt *et al.*, 1971).

Using structural data retrieved from the Cambridge Structural Database, Taylor & Kennard (1982) determined that the N7 position (*B* form) is the most favourable protonation site of the guanine molecule. They found significant changes in the geometry of the purine skeleton owing to protonation, especially in the C5—N7—C8 angle, 104.2 (3)°, in the neutral guanine molecule and 108.0 (2)° in the protonated case. Del Bene (1983) optimized the geometry of both neutral and protonated guanine molecules and calculated the protonation energies for four different protonation sites (N1, N3, N7 and N9). She came to the same conclusions as Taylor & Kennard (1982), *i.e.* the most favourable site is N7 with C5—N7—C8 angles of 104.0 and 109.1° in the neutral and the protonated guanine, respectively.

We describe the crystal structures of guaninium monohydrogenphosphite monohydrate, $C_5H_6N_5O^+\cdot H_2O_3P^-\cdot H_2O$ (I), guaninium monohydrogenphosphite dihydrate, $C_5H_6N_5O^+\cdot H_2O_3P^-\cdot 2H_2O$ (II), and guaninium dihydrogenmonophosphate monohydrate, $C_5H_6N_5O^+\cdot H_2O_4P^-\cdot H_2O$ (III). Crystals of these salts are also of interest because they serve as convenient model systems to compare the structural properties of the two tautomeric forms in the crystalline state.

2. Experimental

2.1. Syntheses

The synthesis of (I) was carried out by dissolving the guanine base (Aldrich, 98%) in a concentrated acidic aqueous solution of H_3PO_3 (Merck, 30%). The solution was gently heated and then set aside for evaporation. Colorless single crystals of a prismatic form grew from the solution, by slow evaporation at room temperature, over a period of a few days, from which one small specimen was selected and used for X-ray analysis. Crystals of (II) were obtained by slow evaporation at room temperature of a dilute aqueous solution containing the guanine base and phosphorous acid in stoichiometric ratios. A few days later, crystals grew as white needles. Crystals of (III) were prepared by mixing two dilute aqueous solutions of guanine and orthophosphoric acid, H_3PO_4 (Carlo ERBA, 85%), so as to obtain an equimolar ratio in the resulting solution. This solution was then kept at room temperature and colorless needles appeared after a very long 9 month period.

2.2. Single-crystal X-ray diffraction

The crystal structures of the three guanine hybrid materials, *i.e.* guaninium monohydrogenphosphite monohydrate (I), guaninium monohydrogenphosphite dihydrate (II) and guaninium dihydrogenmonophosphate monohydrate (III), have been determined by single-crystal X-ray diffraction analysis. Diffraction data were collected at 100 (2) K using an Oxford–Xcalibur–Sapphire2 CCD-based diffractometer on crystals of 0.40 × 0.15 × 0.10 mm for (I), 0.40 × 0.15 × 0.10 mm for (II) and 0.42 × 0.10 × 0.07 mm for (III) with graphite-monochromated Mo $K\alpha$ radiation (λ = 0.71073 Å) equipped with a liquid-nitrogen Oxford Cryostream cooling device. The temperature control was calibrated using a K-type Chromel–Alumel thermocouple positioned at the same place on the crystal. The crystal temperature was stable to within 2 K. The cell parameters were determined from an analysis of the Bragg peak positions collected on the same sets of 15 images. X-ray diffraction data were collected at a fixed detector position using ω step scans repeated at eight different values of the angle. Each frame covered a 1° omega rotation

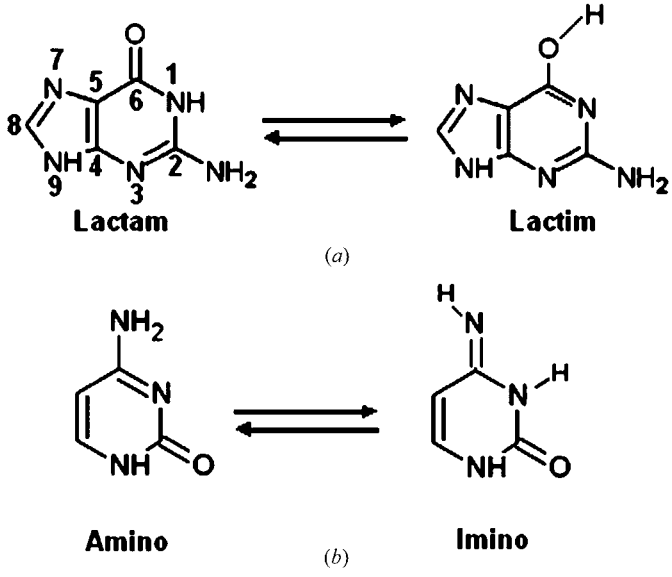


Figure 1
The tautomers of (*a*) guanine and (*b*) cytosine.

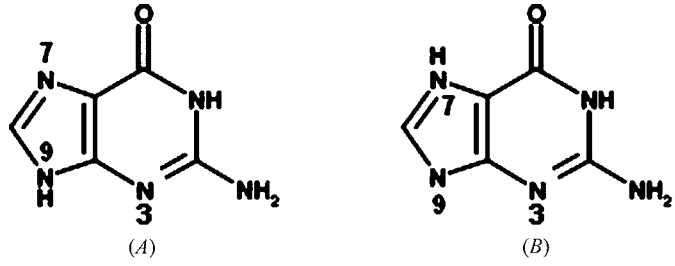


Figure 2
The two neutral guanine tautomeric forms N9H (*A*) and N7H (*B*).

Table 1
Experimental details.

	(I)	(II)	(III)
Crystal data			
Chemical formula	$C_5H_6N_5O^+\cdot H_2PO_3^-\cdot H_2O$	$C_5H_6N_5O^+\cdot H_2PO_3^-\cdot 2H_2O$	$C_5H_6N_5O^+\cdot H_2PO_4^-\cdot H_2O$
M_r	251.15	269.17	267.15
Cell setting, space group	Monoclinic, $P2_1/c$	Monoclinic, $P2_1/c$	Monoclinic, $P2_1/n$
Temperature (K)	100 (2)	100 (2)	100 (2)
a, b, c (Å)	4.9700 (2), 12.7506 (7), 15.0499 (8)	4.6812 (4), 24.0561 (15), 9.5186 (7)	4.5414 (3), 12.5774 (6), 18.1485 (9)
β (°)	92.293 (4)	99.773 (7)	93.689 (5)
V (Å^3)	952.96 (8)	1056.35 (14)	1034.48 (10)
Z	4	4	4
D_x (Mg m^{-3})	1.751	1.692	1.715
Radiation type	Mo $K\alpha$	Mo $K\alpha$	Mo $K\alpha$
μ (mm^{-1})	0.308	0.291	0.296
Crystal form, color	Needle, white	Needle, white	Needle, white
Crystal size (mm)^3	0.40 × 0.15 × 0.10	0.60 × 0.15 × 0.10	0.42 × 0.10 × 0.07
Data collection			
Diffractometer	Xcalibur-Saphire2	Xcalibur-Saphire2	Xcalibur-Saphire2
Data collection method	φ	φ	φ
Absorption correction	Integration	Integration	Integration
T_{min}	0.93	0.845	0.87
T_{max}	0.98	0.972	0.98
No. of measured, independent and observed reflections	13 648, 2749, 2727	31 247, 3086, 2911	42 941, 3013, 2971
Criterion for observed reflections	$I>2\sigma(I)$	$I>2\sigma(I)$	$I>2\sigma(I)$
R_{int}	0.0410	0.0402	0.0628
θ_{max} (°)	30.0	30.0	30.0
Intensity decay	None	None	None
Refinement			
Refinement on	F^2	F^2	F^2
$R[F^2>2\sigma(F^2)]$, $wR(F^2)$, S	0.043, 0.105, 1.15	0.040, 0.098, 1.17	0.042, 0.107, 1.11
No. of reflections	2749	3086	3013
No. of parameters	185	202	194
H-atom treatment	Mixture of independent and constrained refinement	Mixture of independent and constrained refinement	Mixture of independent and constrained refinement
Weighting scheme	$w = 1/[\sigma^2(F_o^2) + (0.0523P)^2 + 0.7658P]$, where $P = (F_o^2 + 2F_c^2)/3$	$w = 1/[\sigma^2(F_o^2) + (0.0432P)^2 + 0.8027P]$, where $P = (F_o^2 + 2F_c^2)/3$	$w = 1/[\sigma^2(F_o^2) + (0.064P)^2 + 0.5194P]$, where $P = (F_o^2 + 2F_c^2)/3$
$(\Delta/\sigma)_{max}$	0.001	< 0.0001	< 0.0001
$\Delta\rho_{max}$, $\Delta\rho_{min}$ (e Å^{-3})	0.62, −0.28	0.54, −0.27	0.54, −0.38

Computer programs used: *CrysAlis CCD* and *CrysAlis RED* (Oxford Diffraction, 2004), *SHELXS*97 and *SHELXL*97 (Sheldrick, 1997), *ORTEP*III (Farrugia, 1997), *WinGX* (Farrugia, 1999).

step. The intensity decay was monitored by repeating the initial frames at the end of the data collections and analysing the duplicate reflections. Coverage of reciprocal space was more than 99% complete to $\sin\theta/\lambda$ of 0.7 Å^{-1}. Data processing was performed using the *CrysAlis RED* program (Oxford Diffraction, 2004). Absorption effects were corrected by numerical methods based on crystal face indexing (using the program *ABSORB*; DeTitta, 1985). Equivalent reflections were scaled and averaged using *SORTAV* (Blessing, 1995). The structures were solved by direct methods (Sheldrick, 1997) and successive Fourier synthesis, and then refined by full-matrix least-squares refinements on F^2. All calculations were carried out using the *WinGX* software package (Farrugia, 1999). The electron density of the H atoms was clearly identified in the Fourier difference maps, and their atomic coordinates and isotropic displacements parameters were refined. Other details of the crystallographic and refinement data are summarized in Table 1.[1]

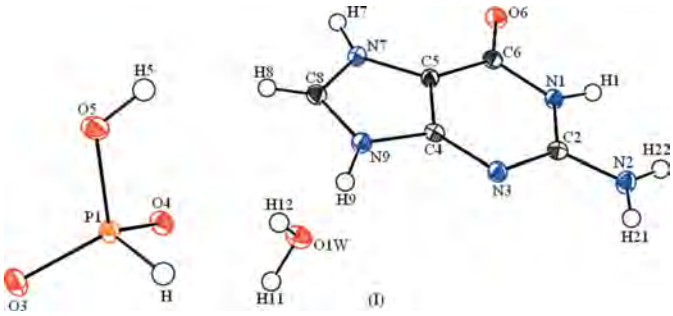


Figure 3
A perspective view of the guaninium monohydrate monohydrogenphosphite with atom-labeling scheme. Displacement ellipsoids are drawn at the 50% probability level. H atoms are represented by spheres.

[1] Supplementary data for this paper are available from the IUCr electronic archives (Reference: LB5008). Services for accessing these data are described at the back of the journal.

Table 2
Bond lengths (Å) and angles (°) for monohydrogenphosphite anions in (I) and (II) and for dihydrogenmonophosphate in (III).

(I)				
P1	**O3**	**O4**	**O5**	**H12**
O3	*1.490 (1)*	2.552 (1)	2.510 (2)	2.260 (2)
O4	115.81 (6)	*1.523 (1)*	2.523 (1)	2.252 (2)
O5	109.73 (4)	108.87 (6)	*1.579 (1)*	2.247 (2)
H	109.2 (10)	108.2 (10)	104.4 (10)	*1.26 (2)*
(II)				
P1	**O3**	**O4**	**O5**	**H12**
O3	*1.504 (1)*	2.570 (2)	2.546 (2)	2.248 (2)
O4	116.72 (6)	*1.514 (1)*	2.483 (2)	2.255 (2)
O5	111.68 (6)	107.14 (6)	*1.572 (1)*	2.272 (2)
H	107.6 (9)	108.7 (9)	104.2 (9)	*1.28 (2)*
(III)				
P1	**O1**	**O2**	**O3**	**O4**
O1	*1.5090 (9)*	2.533 (1)	2.531 (1)	2.466 (1)
O2	114.01 (6)	*1.510 (1)*	2.523 (1)	2.546 (1)
O3	110.49 (6)	109.84 (6)	*1.572 (1)*	2.485 (2)
O4	106.30 (6)	111.32 (6)	104.42 (6)	*1.573 (1)*

The P—O and P—H distances in (I) and (II), and the P—O distances in (III) run diagonally across the table (in italics). The three O—P—O angles and the three O—P—H angles in (I) and (II), and the six O—P—O angles in (III) are below the diagonal. The five internal O···O distances as well as the O···H distances in (I) and (II), and the six internal O···O distances in (III) are above the diagonal.

Table 3
Bond lengths (Å) and angles (°) for the guaninium cations.

Compound	(I)	(II)	(III)
Pyrimidine ring			
C6—N1	1.391 (2)	1.395 (2)	1.383 (2)
N1—C2	1.376 (2)	1.376 (2)	1.380 (2)
C2—N3	1.334 (2)	1.336 (2)	1.330 (2)
N3—C4	1.349 (2)	1.352 (2)	1.349 (2)
C4—C5	1.384 (2)	1.382 (2)	1.387 (2)
C5—C6	1.426 (2)	1.426 (2)	1.417 (2)
C6—O6	1.235 (2)	1.234 (2)	1.247 (2)
C2—N2	1.336 (2)	1.341 (2)	1.333 (2)
C6—N1—C2	126.0 (1)	125.7 (1)	125.2 (1)
N1—C2—N3	123.4 (1)	123.5 (1)	123.2 (1)
C2—N3—C4	112.2 (1)	112.4 (1)	113.0 (1)
N3—C4—C5	127.7 (1)	127.5 (1)	127.2 (1)
C4—C5—C6	120.0 (1)	120.3 (1)	119.5 (1)
C5—C6—N1	110.4 (1)	110.5 (1)	111.8 (1)
Imidazolyle ring			
C5—N7	1.386 (2)	1.385 (2)	1.389 (2)
N7—C8	1.327 (2)	1.326 (2)	1.321 (2)
C8—N9	1.345 (2)	1.350 (2)	1.347 (2)
N9—C4	1.376 (2)	1.382 (2)	1.376 (2)
C5—N7—C8	107.9 (1)	107.4 (1)	107.3 (1)
N7—C8—N9	110.1 (1)	110.5 (1)	111.0 (1)
C8—N9—C4	108.1 (1)	107.6 (1)	107.5 (1)
N9—C4—C5	106.8 (1)	106.6 (1)	106.7 (1)
N7—C5—C4	107.1 (1)	107.7 (1)	107.4 (1)

3. Results

3.1. Structures and crystal packing

3.1.1. Guaninium monohydrogenphosphite monohydrate, $C_5H_6N_5O^+\cdot H_2O\cdot P^-\cdot H_2O$ (I). A perspective view of (I) is given in Fig. 3. The guaninium cations, phosphite anions and water molecule build almost perpendicular layers (Fig. 4). The main feature of this stacking is the presence of centrosymmetric $(H_4P_2O_6)^{2-}$ dimers holding two layers together through strong hydrogen bonds. The guaninium entities are bonded together by two N—H···N bonds, and by four N—H···O and one O—H···O hydrogen bonds to the $H_2O_3P^-$ phosphite groups and the water molecule. Their intermolecular packing appears to be controlled by a three-dimensional network of hydrogen bonds.

The monohydrogenphosphite anion shows, as expected, a distorted tetrahedral configuration (Table 2), with a long protonated P—OH [1.5790 (1) Å] bond in agreement with that already described by Harrison (2003*a*) and Bendeif *et al.* (2005). As observed in the crystal structures of guanine picrate monohydrate and thioguanine picrate monohydrate (Bugg & Thewalt, 1975), and bisguaninium hydrogenphosphate hydrate (Low *et al.*, 1986), the guanine base is monoprotonated at the imino group of the imidazolyl portion N7 owing to the reaction with phosphorous acid, while the pyrimidine imino N3 group is not protonated. The geometrical features of the guaninium cations, $C_5H_6N_5O^+$ (Table 3), are in accordance with those previously observed in similar guaninium complexes (Bugg & Thewalt, 1975; Low *et al.*, 1986; Maixner & Zachová, 1991).

The anion behaves as both hydrogen-bond donor (through the O5 atom) and acceptor (through the O3, O4 and O5 atoms). Both O3 and O4 atoms are bifurcated hydrogen-bond acceptors *via* H1 and H22 atoms, and *via* H5 and H7 atoms, respectively, while the O5 atom acts as both a hydrogen-bond donor *via* the H5 atom and an acceptor *via* the H11 atom (Table 4). These different roles explain the significant differences between the P—O distances in the $H_2PO_3^-$ tetrahedron.

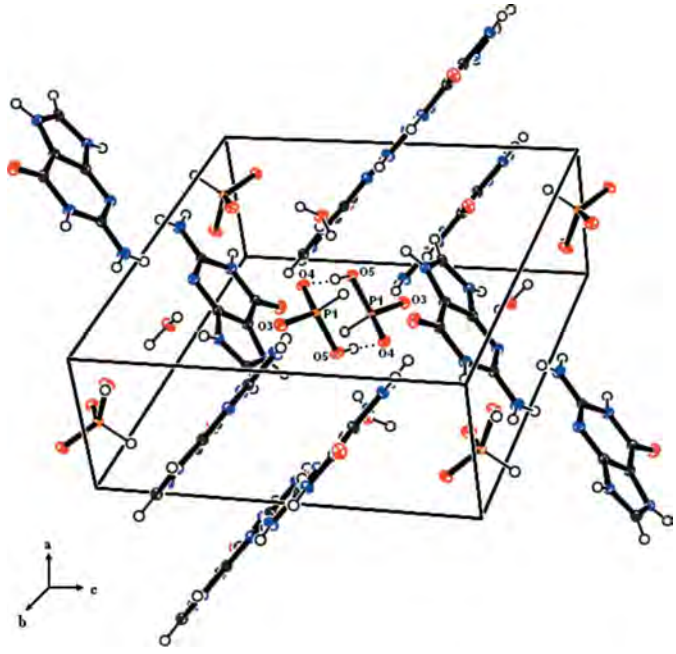


Figure 4
A perspective view of the packing of (I), showing the alternating $C_5H_6N_5O^+\cdot H_2O_3P^-$ and H_2O moieties.

These entities generate centrosymmetric $(H_4P_2O_6)_2^-$ dimers through two strong hydrogen bonds between the O4 and O5 atoms (Fig. 4).

Guaninium cations are hydrogen bonded to the anionic layers by three means: two strong hydrogen bonds (N7—H7···O4 and N1—H1···O3) and a weaker one (N2—H22···O3; Fig. 5*a*). The organic cations are laced together by only one hydrogen bond from the N2 amino group to the pyrimidine N3 imino group (N2—H21···N3) to form infinite perpendicular layers. The water molecule is involved in three strong hydrogen bonds connecting two guaninium cations *via* N9—H9···O1*W* and O1*W*—H11···O6, and one monohydrogenphosphite anion *via* O1*W*—H12···O5, so that it plays an important role in the stability of such an arrangement.

3.1.2. Guaninium monohydrogenphosphite dihydrate, $C_5H_6N_5O^+\cdot H_2O_3P^-\cdot 2H_2O$ (II). The crystal structure of (II) (Figs. 6 and 7) can be described by layers of guaninium cations, monohydrogenphosphite anions and water molecules. The asymmetric unit contains one guaninium cation, one monohydrogenphosphite anion and two water molecules. The monohydrogenphosphite chains and guaninium layers are parallel to the (001) plane and alternate at approximately $y =$

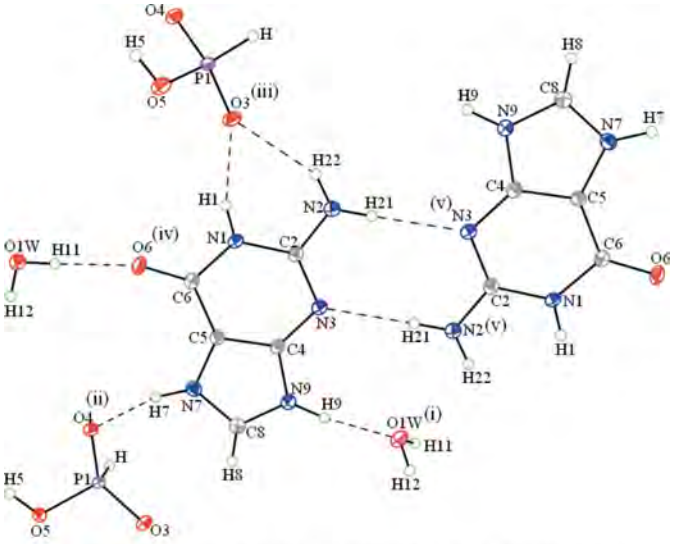


Figure 5
Hydrogen bonding involving guaninium cations (*a*) in (I) and (*b*) in (II).

Table 4
Hydrogen-bond geometry (Å, °).

D—H···A	D—H	H···A	D···A	D—H···A
(I)				
O5—H5···O4^{i}	0.88 (3)	1.66 (3)	2.535 (2)	177 (3)
N7—H7···O4^{ii}	0.92 (2)	1.80 (3)	2.698 (2)	165 (2)
N9—H9···O1*W*	0.91 (3)	1.80 (3)	2.702 (2)	170 (2)
N1—H1···O3^{iii}	0.84 (2)	1.91 (2)	2.714 (2)	160 (2)
O1*W*—H12···O5^{i}	0.88 (3)	1.88 (3)	2.755 (2)	176 (2)
O1*W*—H11···O6^{iv}	0.90 (3)	1.92 (3)	2.817 (2)	172 (3)
N2—H22···O3^{iii}	0.84 (3)	2.24 (3)	2.967 (2)	145 (2)
N2—H21···N3^{v}	0.88 (2)	2.14 (2)	3.022 (2)	178 (2)
(II)				
O5—H5···O4^{i}	0.83 (3)	1.74 (3)	2.559 (2)	173 (4)
N7—H7···O1*W*^{ii}	0.91 (3)	1.75 (3)	2.658 (2)	177 (3)
N9—H9···O3^{iii}	0.87 (2)	1.85 (2)	2.721 (2)	172 (2)
O1*W*—H11···O6^{iv}	0.79 (2)	1.95 (2)	2.730 (2)	169 (3)
N1—H1···O2*W*	0.84 (3)	1.96 (3)	2.774 (2)	164 (3)
O2*W*—H24···O3^{v}	0.82 (3)	2.00 (3)	2.814 (2)	170 (3)
O2*W*—H23···O4^{i}	0.83 (3)	2.07 (3)	2.874 (2)	164 (3)
O1*W*—H12···O4^{vi}	0.81 (3)	2.18 (3)	2.907 (2)	151 (3)
N2—H21···N3^{iii}	0.88 (2)	2.09 (2)	2.971 (2)	179 (1)
N2—H22···O3^{ii}	0.89 (2)	2.59 (2)	3.127 (2)	120 (2)
N2—H21···O2*W*^{iii}	0.89 (2)	2.38 (2)	3.130 (2)	142 (2)
(III)				
O3—H3···O1^{i}	0.90 (3)	1.67 (3)	2.567 (1)	174 (2)
O4—H4···O6^{ii}	0.85 (3)	1.75 (3)	2.592 (1)	169 (3)
N7—H7···O2^{iii}	0.92 (3)	1.74 (3)	2.651 (2)	173 (3)
N9—H9···O1*W*^{iv}	0.94 (2)	1.73 (2)	2.665 (2)	170 (2)
O1*W*—H12···O1^{v}	0.85 (3)	1.90 (3)	2.737 (1)	168 (3)
N1—H1···O2	0.90 (2)	1.85 (2)	2.751 (2)	172 (2)
O1*W*—H11···O3^{vi}	0.78 (4)	2.06 (4)	2.824 (1)	165 (3)
N2—H22···O1	0.88 (2)	1.96 (2)	2.838 (2)	175 (2)
N2—H21···N3^{vii}	0.82 (2)	2.20 (2)	3.016 (2)	178 (3)

Symmetry codes: for (I): (i) $1-x, 1-y, 1-z$; (ii) $1+x, \frac{3}{2}-y, z-\frac{1}{2}$; (iii) $x, y, z-1$; (iv) $x-1, \frac{3}{2}-y, \frac{1}{2}+z$; (v) $-x, 1-y, -z$. For (II): (i) $x, \frac{1}{2}-y, \frac{1}{2}+z$; (ii) $x, y, 1+z$; (iii) $1-x, -y, 1-z$; (iv) $-1-x, -y, 1-z$; (v) $x-1, y, z$; (vi) $-x, y-\frac{1}{2}, \frac{1}{2}-z$. For (III): (i) $1+x, y, z$; (ii) $\frac{3}{2}-x, y-\frac{1}{2}, \frac{1}{2}-z$; (iii) $\frac{3}{2}-x, \frac{1}{2}+y, \frac{1}{2}-z$; (iv) $x-1, y, z$; (v) $1-x, 1-y, 1-z$; (vi) $x, 1+y, z$; (vii) $-x, 1-y, 1-z$.

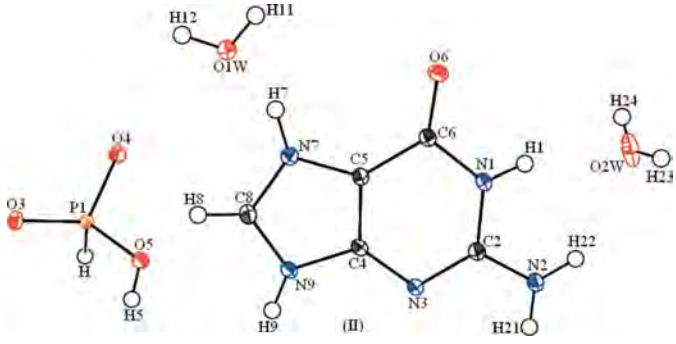


Figure 6
A perspective view of the guaninium dihydrate monohydrogenphosphite (*ORTEP*3; Farrugia, 1999) with atom-labeling scheme. Displacement ellipsoids are drawn at the 50% probability level. H atoms are represented by spheres.

$\frac{1}{4}$, $y = \frac{3}{4}$ and at $y = 0$, $y = \frac{1}{2}$, respectively (Fig. 7). The stability of such an arrangement results from a hydrogen-bond network which maintains the cohesion of the monohydrogenphosphite chains, guaninium layers and water molecules in the crystal.

Contrary to its behavior in (I), the inorganic $H_2O_3P^-$ moiety builds a zigzag polymeric network of tetrahedra, linked together by strong P—O···H—O—P hydrogen bonds along the **c** direction. Inside these infinite chains each $H_2O_3P^-$ group is connected to two adjacent neighbors by strong hydrogen bonds, O5—H5··· O4 = 2.559 (2) Å.

As in (I), the guanine base is monoproronated at N7. The geometrical features of the monohydrogenphosphite anion and of the pyrimidine and imidazolyl rings (Tables 2 and 3) are also similar to those observed in (I). Infinite layers of guaninium cations are linked to the anionic layers through two hydrogen bonds: firstly *via* a strong N9—H9···O3 hydrogen bond and secondly *via* a weaker N2—H22···O3 hydrogen bond (Fig. 5*b*). The functional groups N3 and N2 of the pyrimidine ring are a hydrogen-bond acceptor and donor, respectively, that hold together the guaninium cations through a long N3···H21—N2 hydrogen bond (Table 4). The first water molecule's O1*W* atom acts as a donor of two hydrogen bonds *via* the H11 and H12 atoms towards the O6 atom of the guaninium and O4 atom of the phosphite group, respectively, and as a hydrogen-bond acceptor *via* the H7 atom. However, the second water molecule's O2*W* atom is a donor of two hydrogen bonds to two phosphite anions *via* H24 and H23 towards O4 and O3 atoms, respectively, and a two hydrogen-bond acceptor from guaninium cations *via* H1 and H22 atoms. Therefore, the water molecules play an important role in the three-dimensional network of hydrogen bonding.

3.1.3. Guaninium dihydrogenmonophosphate monohydrate, $C_5H_6N_5O^+\cdot H_2O_4P^-\cdot H_2O$ (III). The crystal structure of (III) (Fig. 8) can be described as being composed of chains of $H_2PO_4^-$ groups extending along the **a** direction and alternating with $C_5H_6N_5O^+$ guaninium-stacked layers and water molecules. The $H_2PO_4^-$ chains are interconnected to the guaninium layers through several O···H—O and O···H—N hydrogen bonds (Fig. 8).

As expected, the distorted tetrahedral geometry of the $H_2PO_4^-$ anions (Table 2) clearly shows two main types of P—O distances: two long P—OH bonds [P—O3 1.572 (1) and P—O4 1.573 (1) Å] owing to the presence of the acidic H atoms on the PO_4 tetrahedron, and two short P—O(T) bonds [P—O1 1.5090 (9) and P—O2 1.510 (1) Å] corresponding to the

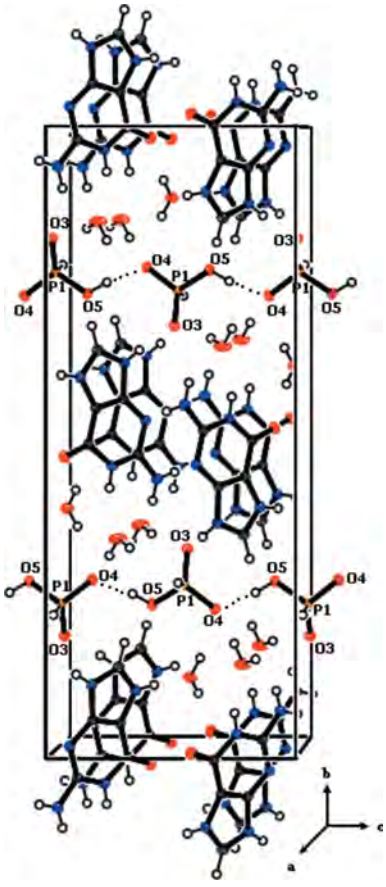


Figure 7
Unit-cell projection on the (100) plane of the packing of (II) showing the alternating $C_5H_6N_5O^+\cdot H_2O_3P^-$ and H_2O moieties.

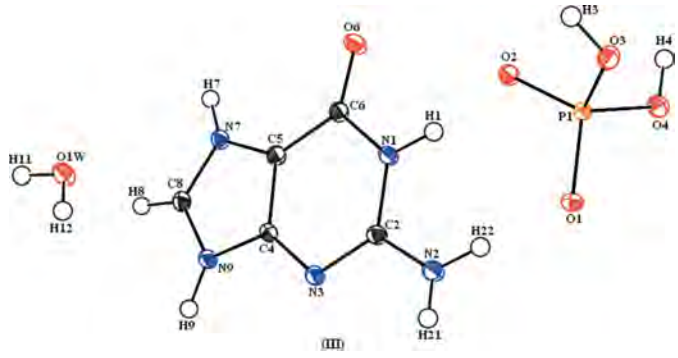
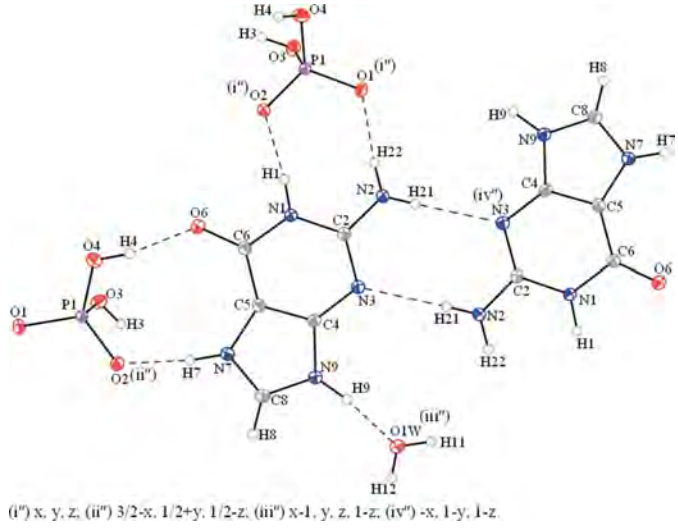


Figure 8
A perspective view of the guaninium monohydrate dihydrogen monophosphate with the atom-labeling scheme. Displacement ellipsoids are drawn at the 50% probability level. H atoms are represented by spheres.



(i'') x, y, z, (ii'') 3/2-x, 1/2+y, 1/2-z, (iii'') x-1, y, z, 1-z; (iv'') -x, 1-y, 1-z

Figure 9
Hydrogen bonding involving guaninium cations in (III).

terminal O atoms commonly observed in dihydrogen monophosphate groups (Masse & Durif, 1990; Boukhris *et al.*, 1994).

As previously observed in (I) and (II), the guanine base is also monoprotonated at the N7 imino group. The angle between the mean plane of the imidazolyl and pyrimidine rings (*i.e.* the dihedral angle) is 3.68 (2)° and this puckering along the C4—C5 bond is commonly found in purine structures (Bugg, 1972). The strong hydrogen bonds between the neighboring imino N1 atom and the amino N2 atom of the pyrimidine ring, and the $H_2PO_4^-$ anion as well as the contacts between N9 and O1*W* and N7 and $H_2PO_4^-$ are all above the average guaninium plane (Fig. 9); therefore, they prevent the two rings from being completely coplanar. The exocyclic carbonyl (O6) and amino (N2) groups deviate only slightly by 0.014 (1) and −0.008 (1) Å, respectively, from the mean least-squares plane of the purine base.

Within the inorganic chains, the $H_2PO_4^-$ tetrahedra are interconnected through a strong hydrogen bond [O3···O1 2.567 (1) Å] and form infinite chains extending along the **a** direction, with a P···P distance of 4.541 (5) Å (Fig. 10) as observed in (II) [P···P 4.814 (6) Å]. In contrast, in (I) the $H_2PO_3^-$ anions are held together in pairs yielding $H_4P_2O_6^{2-}$ dimers.

Guaninium cations are anchored to two $H_2PO_4^-$ groups belonging to two different chains by four hydrogen bonds: one $H_2PO_4^-$ anion interacts with the guaninium cation *via* strong hydrogen bonds [O4···O6 = 2.592 (1) and N7···O2 2.651 (1) Å], whereas the other $H_2PO_4^-$ anion is weakly bonded to the guaninium cation [N1···O2 2.751 (1) and N2···O1 2.838 (2) Å]. Finally, the guaninium cations are hydrogen-bonded together through a centrosymetric N2···N3 $R_2^2(8)$ ring (Fig. 9), as defined by Bernstein *et al.* (1995). These $R_2^2(8)$ rings give rise to inclined layers with an interplanar separation of 3.6105 Å; because of this long interplanar distance no layer–layer interaction was observed.

The water molecule is located in the same planes as the guanine base pairs and provides protons to form a strong hydrogen bond with $H_2PO_4^-$ groups [O1*W*···O1 2.737 (2) Å], and a relatively weaker one with another $H_2PO_4^-$ group belonging to another phosphoric chain [O1*W*···O3 2.824 (1) Å], so the water molecules ensure the connection between the phosphoric chains (Fig. 10). On the other hand, the water molecule acts as a hydrogen-bond acceptor *via* the H9 atom of the imino group containing N9 [N9···O1*W* 2.665 (1) Å].

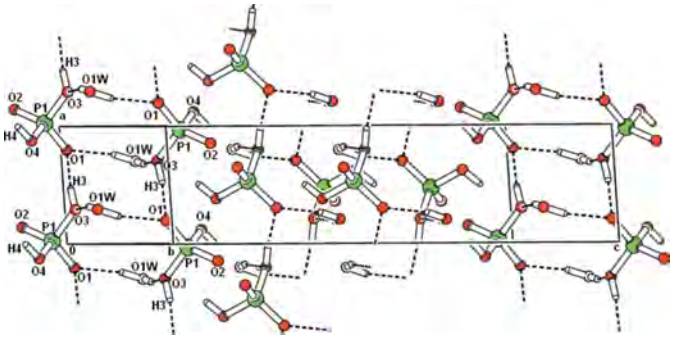


Figure 10
A perspective view of the arrangement of the monophosphate anions in (III). *PLATON* (Spek, 2003).

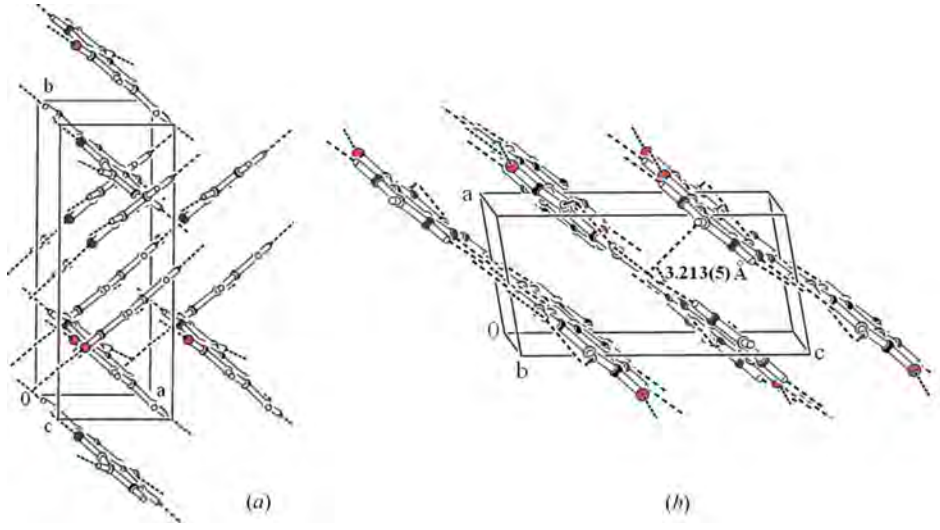


Figure 11
Guaninium arrangement (*a*) in (I) and (*b*) in (II). *PLATON* (Spek, 2003).

4. Discussion

4.1. Guaninium monohydrogenphosphite salts

4.1.1. Monohydrogenphosphite anions. The geometry around the P atom is tetrahedraly distorted in each structure: inside the $H_2PO_3^-$ tetrahedron of (I), the P—O4 bond [1.523 (1) Å] is relatively longer than the P—O3 bond [1.490 (1) Å]. This significant difference in P—O bond distances in (I) is due to the fact that O4 is involved in strong hydrogen bonding with O5 and N7 [O5···O4 2.535 (2) and N7··· O4 2.698 (2) Å] compared with O3 which is bonded *via* a weaker hydrogen bond to the N1 atom of the pyrimidine ring [N1···O3 2.714 (2) Å] and compared with the amino group N2 atom [N2···O3 2.967 (2) Å]. In (I) each $H_2PO_3^-$ tetrahedron is linked to an equivalent one by inversion symmetry through two strong hydrogen bonds between O4 and O5 atoms. This type of aggregation gives rise to strongly bonded dimers of $(H_4P_2O_6)^{2-}$ characterized by the short intermolecular O5···O4 distances [2.535 (1) Å] between the $H_2PO_3^-$ units, which are of the same order of magnitude as the O··· O distances in the tetra-

hedral unit. The internal P1···P1 distance is 4.139 (5) Å. Similar inter-anion linkages have been seen in other related organic phosphite structures, such as $2A5NP^{+}\cdot H_2PO_3^{-}$ (Pecaut & Bagieu-Beucher, 1993), $C_6H_8N^{+}\cdot H_2PO_3^{-}$ (Paixão *et al.*, 2000) and $C_7H_8NO_2^{+}\cdot H_2PO_3^{-}$ (Bendeif *et al.*, 2005).

By contrast, in (II) $H_2PO_3^{-}$ units are linked into a polymeric chain by P—O—H···O—P hydrogen bonds in the [001] direction, resulting in a P1···P1 separation of 4.814 (6) Å. The presence of such an arrangement has already been noticed in related ionic compounds, such as $CH_6N_3^{+}\cdot H_2PO_3^{-}$ (Harrison, 2003*b*), $C_2H_6NO_2^{+}\cdot H_2PO_3^{-}$ and $C_4H_9N_2O_3^{+}\cdot H_2PO_3^{-}$ (Averbuch-Pouchot, 1993).

4.1.2. Guaninium cations. The dihedral angle between the imidazolyl and the pyrimidine rings in (I) is 2.2 (2)°, while in (II) the two rings are nearly coplanar and their deviation from the mean plane is only 0.35 (2)°. This can be explained by the strong interconnection of the guaninium cation with two $(H_4P_2O_6)^{2-}$ dimers in the *cis* conformation in (I). The deviation of the amino and carbonyl groups from the least-squares plane of pyrimidine rings is −0.068 (1), +0.018 (1) Å for (I) and of 0.052 (1), −0.025 (1) Å for (II), respectively. The delocalization of the electron density appears to be weaker in the N7—C8—N9 fragment compared with the N3—C2—N2 fragment, as shown from the interatomic distances C4—N3, N3—C2 and C2—N2 (Table 3). The shortening of the C2—N2 bond is also influenced by inter cations N2···N3 and cation–anion N2···O3 hydrogen bonds involving both H atoms of the N2 amino group. The C2—N2 bond distance is slightly longer in (II) compared with (I): the exocyclic amino group N2 atom is involved in three hydrogen bonds in (II), while in (I) only two hydrogen bonds occur (Table 4). The interplanar separation between guaninium layers in (II) is only 3.213 (5) Å (Fig. 11*b*), leading to a $\pi\cdots\pi$ stacking interaction between the base pairs, in contrast to the structure of (I) in which guaninium cations are linked together to form perpendicular layers (Fig. 11*a*). No interlayer contact is observed in such an arrangement. In conclusion, the strength of the intermolecular hydrogen-bond interactions is in agreement with the subtle intramolecular bond-length changes.

4.1.3. Hydrogen bonding. In (I) the guaninium cations are linked to the anionic layers *via* three N—H···O hydrogen bonds, N7—H7···O4, N1—H1···O3 and N2—H22···O3, while in (II) only two N—H···O hydrogen bonds occur between them, N9—H9···O3 and N2—H22···O3. The strong N7···O4 2.698 (2) Å hydrogen bond, which directly links the monohydrogenphosphite anion $H_2PO_3^{-}$ and the imino group N7 atom in (I) (Fig. 5*a*), leads to the suggestion that the guanine base exists in a N9H tautomeric form compared with compound (II), where the monohydrogenphosphite anion is hydrogen-bonded directly to the imino group N9 atom of the imidazolyl moiety *via* a strong hydrogen bond [N9···O3 2.721 (2) Å; Fig. 5*b*].

In the three guanine salts the guaninium cations are related together through a centrosymmetric N2···N3 $R_2^2(8)$ ring, as already observed in similar guanine compounds: guanine hydrochloride dihydrate (Iball & Wilson, 1963) and bisguaninium hydrogenphosphate hydrate (Low *et al.*, 1986), and also to that in guanine hydrochloride monohydrate (Broomhead, 1951) in which the base pairs are hydrogen-bonded together *via* centrosymmetric N7—H7···O6 bonds.

Water molecules play an important role in the three-dimensional network. They maintain the cohesion between the organic and inorganic layers in the crystal structure stacking. O1*W* plays the same role in (I) and (II), acting as a hydrogen-bond acceptor and as a double hydrogen-bond donor, while the water molecule O2*W* in (II) is a bifurcated hydrogen-bond donor and acceptor.

The hydrogen-bonding schemes for (I) and (III), although similar, exhibit slight differences in hydrogen-bond lengths (Table 4). The major differences occur in the hydrogen bonds involving the water molecule. Indeed, in (I) the water molecule is connected to one $H_2PO_3^{-}$ group and to the carbonyl group (O6) of the guanine base, whereas in (III) it is connected to two different $H_2PO_4^{-}$ groups belonging to two parallel phosphoric chains.

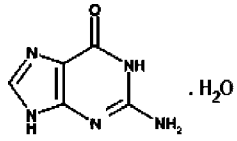
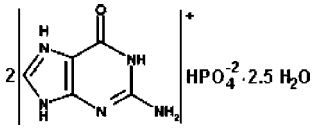
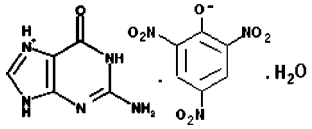
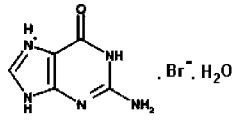
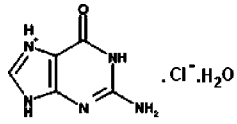
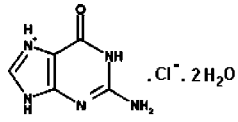
As also observed previously (Doran *et al.*, 2001; Harrison, 2001) the phosphite H atom is not involved in the hydrogen-bonding scheme since it is slightly negatively charged (Bendeif, 2007).

4.2. Cambridge Structural Database

A Cambridge Structural Database (CSD Version 5.27, November 2005; Allen, 2002) search revealed only nine organic–inorganic guaninium salts, which are listed in Tables 5 and 6. All the crystals are centrosymetric, most are monoclinic (seven entries), with one compound triclinic ($P\bar{1}$) and one orthorhombic (*Pnma*). Guanine cations are divided into three categories, monoprotonated at N7 (entries 2–6), diprotonated at N7 and N3 (entries 7–9) and not protonated (entry 1). Tables 5 and 6 show that monoprotonated guaninium cations connect together in two ways: either *via* only one relatively weak centrosymetric $R_2^2(8)$ N2···N3 hydrogen bond (entries 3 and 6) or *via* two strong hydrogen bonds, centrosymetric $R_2^2(8)$ N2···N3 and $R_2^2(10)$ N7···O6 (entries 2, 4 and 5). It is interesting to note that in guanine hydrobromide and hydrochloride monohydrate (entries 4 and 5, respectively) guaninium cations connect together in a similar fashion giving rise to the formation of perpendicular layers. In guanine hydrochloride dihydrate (entry 6) guaninium cations show a different type of interconnection, but they always form perpendicular layers. However, in the diprotonated cases guanine cations are not held together as seen in entries 8 and 9; in one case they are connected *via* only one strong hydrogen bond, N9···O6 (entry 7). In non-protonated guanine (entry 1), pairs of guanine bases are linked through three weak hydrogen bonds first *via* centrosymetric $R_2^2(8)$ N9···N3, secondly through N7···N1 and thirdly *via* N2···O6.

The monoprotonation of the guanine base at N7 shortens the C4—C5, C5—N7 and C8—N9 bonds by 0.0296, 0.0217 and 0.0238 Å, respectively (Fig. 12 – see supplementary material) and enlarges the C5—N7—C8 angle by 3.94°, while reducing the N7—C8—N9 angle by 3.66° (Fig. 12 – see supplementary

Table 5
CSD search on hybrid guanine salts.

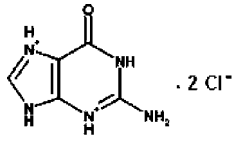
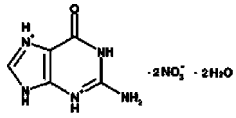
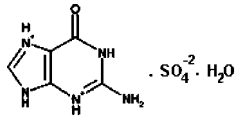
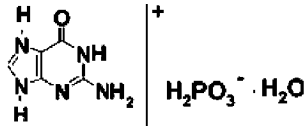
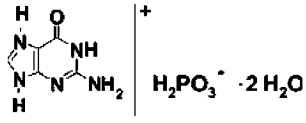
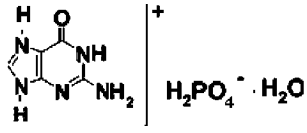
Entry	Compound name and refcode as given in CSD	Chemical structure as given in CSD	Overall supramolecular network	Anion position†	Comment
1	Guanine monohydrate (GUANMH10)		Three-dimensional	–	Water molecule makes a strong hydrogen bond with O6 and a weaker one with N2. Guaninium cations are related together in two ways: (i) *via* centrosymmetric N3···N9 hydrogen bonds and (ii) *via* O6···N2 and N7···N1.
2	Bisguaninium hydrogenphosphate hydrate (DUKKOJ)		Three-dimensional	N9	Water molecule O*W*1 makes a hydrogen bond with N1 and the water molecule O*W*2 atom is not involved in hydrogen bonding. Guaninium cations are related in two ways: (i) *via* centrosymmetric $R_2^2(8)$ N3···N2 hydrogen bonds and (ii) *via* centrosymmetric $R_2^2(10)$ O6···N7. They also form layers parallel to the diagonal of the *ab* plane.
3	Guanine picrate monohydrate (GUNPIC10)		Three-dimensional	N9	Water molecule makes a hydrogen bond with N7 and O6. Guaninium cations are related by centrosymmetric $R_2^2(8)$ N3···N2 hydrogen bonds.
4	Guanine hydrobromide monohydrate (GUANBM)		Three-dimensional	N9	Water molecule makes a hydrogen bond with N1. Guaninium cations are related in two ways: (i) *via* a centrosymmetric $R_2^2(8)$ N3···N2 hydrogen bond and (ii) *via* centrosymetric $R_2^2(10)$ O6···N7 and form perpendicular layers.
5	Guanine hydrochloride monohydrate (GUANCH01)		Three-dimensional	N9	Water molecule makes a hydrogen bond with N1. Guaninium cations are related in two ways: (i) *via* a centrosymmetric $R_2^2(8)$ N3···N2 hydrogen bond and secondly *via* centrosymmetric $R_2^2(10)$ O6···N7 and form perpendicular layers.
6	Guanine hydrochloride dihydrate (GUANCD)		Three-dimensional	N1	Water molecule O1*W* atom makes a hydrogen bond with N7 and O2*W* makes a hydrogen bond with N9. Guaninium cations are related by a centrosymmetric $R_2^2(8)$ N3···N2 hydrogen bond and also form perpendicular layers.

† For atom numbering see Fig. 1.

material). The diprotonation of the guanine base at N7 and N3 increases the N3—C2 bond by 0.037 Å and reduces the N1—C2 bond by 0.0213 Å (Fig. 13 – see supplementary material). The C2—N3—C4 angle increases by 5.4°, while the N1—C2—N3 and N3—C4—C5 angles decrease by 4.99 and 4.06°, respectively (Fig. 13 – see supplementary material).

As seen from Tables 5 and 6, anions, *i.e.* proton donors interacting with guanine bases, are in most cases connected directly to the imino group N9 atom in the guanine monoprotonated salts and are connected to both the N9 and N3 atom imino groups in the diprotonated cases, whereas water molecules are observed to form strong hydrogen bonds with the imino group N7 atom. However, in compounds (I) and (III) studied here, the $H_2PO_3^-$ and $H_2PO_4^-$ anions are hydrogen bonded directly to the N7 imino group, whereas water molecules form strong hydrogen bonds with the imino group N9. This is the first case in which phosphate or phosphite anions are linked directly to the imino group N7 *via* a strong hydrogen bond in guaninium salts. This raises the question: Does protonation at N7 indicate that before protonation the N9H tautomer was the favored form? In fact, inspection of hydrogen bonding in all the compounds listed in Tables 5 and 6 revealed that the hydrogen bonds between the imino group N7 atom and the anions or water molecules appear to be shorter than those observed between the imino group N9 atom and the anions or water molecules. To confirm such a hypothesis, deuterated phosphorous acid should be used and a neutron diffraction experiment performed to follow the protonation process in these salts and to show definitively where the protonation occurs.

Table 6
CSD search on hybrid guanine salts and compounds studied here (continuation of Table 5).

Entry	Compound name and refcode as given in CSD	Chemical structure as given in CSD	Overall supramolecular network	Anion position	Comment
7	Guanine dihydrochloride (GODYUT)		Two-dimensional	N3 and N9	Guaninium cations are related by only one strong O6···N9 hydrogen bond.
8	Guaninium dinitrate dihydrate (HUMNEI)		Two-dimensional	N3 and N9	Water molecule O1*W* atom makes a hydrogen bond with N7 and O2*W* makes a hydrogen bond with NO_2 groups. The two water molecules are connected by a strong hydrogen bond. Guaninium cations are not held together.
9	Guaninium sulfate monohydrate (HUSBEC)		Three-dimensional	N3 and N9	Water molecule makes a hydrogen bond with N7 and O6. Guaninium cations are not held together.
(I)	Guaninium hydrogenphosphite monohydrate (this work)		Three-dimensional	N7	Water molecule makes a strong hydrogen bond with N9 and guaninium cations are related by a centrosymetric $R_2^2(8)$ N3···N2 and form perpendicular layers.
(II)	Guaninium hydrogenphosphite dihydrate (this work)		Three-dimensional	N9	Water molecule O*W*1 atom makes a strong hydrogen bond with N7 and O*W*2 makes a hydrogen bond with N1. Guaninium cations are related by centrosymetric $R_2^2(8)$ N3···N2.
(III)	Guaninium dihydrogenmonophosphate monohydrate (this work)		Three-dimensional	N7	Water molecule makes a strong hydrogen bond with N9 and guaninium cations are related by centrosymetric $R_2^2(8)$ N3···N2.

5. Conclusion

The crystal structures of the guaninium salts have been determinated and show different anion packing. Inspection of hydrogen bonding between the phosphite (phosphate) anion and the guanine cation shows, for the first time, a direct hydrogen-bond interaction between the guanine N7—H residue and OPO_2H (OPO_3H).

This is in favor of the N9H tautomeric form. A neutron diffraction experiment using deuterated phosphorous or phosphoric acid might help to confirm such a hypothesis. Further work in this direction is planned.

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Structure of Bis(guaninium) Hydrogenphosphate 2·5-Hydrate

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Abstract. $2C_5H_6N_5O^+.HPO_4^{2-}.2{\cdot}5H_2O$, $M_r=445{\cdot}3$, triclinic, $P\bar{1}$, $a=9{\cdot}607$ (4), $b=10{\cdot}221$ (4), $c=10{\cdot}603$ (9) Å, $\alpha=84{\cdot}5$ (1), $\beta=108{\cdot}2$ (1), $\gamma=119{\cdot}7$ (1)°, $U=856{\cdot}9$ Å^3, $Z=2$, $D_x=1{\cdot}73$ Mg m^{-3}, Cu $K\alpha$, $\lambda=1{\cdot}5418$ Å, $\mu=1{\cdot}978$ mm^{-1}, $F(000)=462$, $T=293$ K, $R=0{\cdot}07$ for 1085 unique reflections. The title compound consists of two protonated guanine bases which form a network of hydrogen-bonded ribbons which run parallel to each other. Between these ribbons lie channels containing the hydrogenphosphate anion and the water molecules. P–O bond lengths are 1·44 (1)–1·47 (1) Å.

Introduction. The crystals of the title compound were obtained during an attempt to crystallize 2′-deoxyguanosine monophosphate. This compound apparently dissociated giving the title compound. A similar dissociation occurred during an attempt to crystallize ApT, adenine–thymine dinucleoside monophosphate, when an adeninium compound similar to the present compound was formed (Walker, Tollin & Low, 1982).

Experimental. Crystals grown from aqueous solution. Crystal dimensions approx. 0·5 × 0·5 × 0·5 mm. Cell dimensions and intensity data from Weissenberg photographs processed by the SERC Microdensitometer Service. Data collected in range $h=0$–5 from crystal mounted about **a**, $k=0$–6 from *b*-axis crystal and $l=0$ from *c*-axis crystal. Range of indices: $-10<h<9$, $-11<k<12$, $0<l<12$. No absorption corrections applied. 2649 reflections measured of which 1088 unique, $R_{int}=0{\cdot}085$. 1085 reflections used in refinement: three very strong reflections, 210, $2\bar{1}1$ and 003, omitted on grounds of extinction. This number of reflections constitutes about one quarter of those possible in the unique hemisphere of reciprocal space. Atoms of the bases located using *MULTAN*78 (Main, Hull, Lessinger, Germain, Declercq & Woolfson, 1978); other atoms obtained after Fourier recycling. Refinement (on *F*) by blocked-matrix least squares with *SHELX*76 (Sheldrick, 1976). Non-H atoms except water O atoms refined anisotropically; H atoms included at calculated positions with isotropic temperature factors fixed at 0·05 Å^2. Difference map did not reveal any peaks which could be assigned unambiguously to the H atoms; H atom attached to N(7) of each base on the basis of an N(7)···O(6) intermolecular short contact. This short contact, by implying the presence of H on N(7), also implies that the bases exist in a protonated form, H atom of HPO_4^{2-} anion not included; 257 parameters refined, $R=0{\cdot}070$, unit weights; $(\Delta/\sigma)_{max}=0{\cdot}012$; max. difference-map peak 0·47 e Å^{-3}, min. −0·54 e Å^{-3}. Site occupancies of atoms O(2*W*) and O(3*W*) refined initially and, on the basis of this refinement, then fixed at 1·0 and 0·5, respectively. An examination of the Fourier peaks for these two atoms indicated that they were somewhat elongated, indicating a certain amount of positional disorder. This is reflected in their higher temperature parameters. Other programs used, *XANADU* (Roberts & Sheldrick, 1975) and *PLUTO* (Motherwell & Clegg, 1978). Scattering factors from *International Tables for X-ray Crystallography* (1974). No correction for secondary extinction.

Discussion. Atomic coordinates are given in Table 1,* with bond lengths and angles in Table 2. The atomic numbering is given in Fig. 1. The two independent bases have almost identical bond lengths and angles. The bases form hydrogen-bonded ribbons as shown in Fig. 2: N(2*A*)···N(3*A*)(−*x*, 1 − *y*, 2 − *z*) 3·03 (2), N(2*B*)···N(3*B*)(1 − *x*, 1 − *y*, 2 − *z*) 3·01 (2), N(7*A*)···O(6*B*)(1 − *x*, −*y*, 2 − *z*) 2·69 (2), N(7*B*)···O(6*A*)(1 − *x*, −*y*, 2 − *z*) 2·71 (2) Å. There are several other short contacts involving the bases, the phosphate O atoms and O(1*W*). N(1*A*)···O(1*P*)(*x*, *y*, *z*) 2·75 (2), N(9*A*)···O(2*P*)(*x*, *y*, 1 + *z*) 2·66 (2), N(9*B*)···O(4*P*)(1 − *x*,

* Lists of structure amplitudes, anisotropic thermal parameters and calculated H-atom coordinates have been deposited with the British Library Lending Division as Supplementary Publication No. SUP 42863 (10 pp.). Copies may be obtained through The Executive Secretary, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England.

0108-2701/86/081045-03$01.50

$1-y$, $2-z$) 2·65 (2) and N(1*B*)···O(1*W*)(x, y, z) 2·74 (2) Å. These contacts can, on the basis of the lengths and angles involving the H atoms in their calculated positions, be safely assumed to be H bonds. There are several other short contacts of less than 3 Å between the phosphate anion and the water molecules which lie in a channel between the base ribbons. The guanine bases exist in a protonated form. Taylor & Kennard (1982) suggest that guanine bases can be classified according to the rule: protonated if C(5)–N(7)–C(8) > 106·1°, neutral if C(5)–N(7)–C(8) < 106·1°. The present structure confirms this conclusion, C(5)–N(7)–C(8) is 109 (1)° for both bases.

Table 1. *Coordinates* ($\times 10^4$) *for non-*H *atoms with e.s.d.'s in parentheses and equivalent isotropic temperature factors* (Å^2 × 10^3); *for water* O *atoms, temperature factors are refined isotropic values*

$$U_{eq} = \tfrac{1}{3}\sum_i\sum_j U_{ij} a_i^* a_j^* \mathbf{a}_i.\mathbf{a}_j.$$

	x	y	z	U_{eq}
Molecule *A*				
N(1)	840 (11)	2643 (10)	8453 (8)	22 (3)
C(2)	458 (14)	3509 (13)	9056 (10)	19 (3)
N(2)	−155 (12)	4323 (10)	8258 (9)	30 (3)
N(3)	652 (12)	3566 (10)	10335 (9)	23 (3)
C(4)	1301 (14)	2701 (12)	11003 (10)	21 (3)
C(5)	1690 (14)	1820 (13)	10473 (11)	23 (4)
C(6)	1470 (14)	1715 (12)	9129 (10)	21 (3)
O(6)	1764 (10)	977 (8)	8491 (7)	30 (2)
N(7)	2311 (11)	1133 (10)	11540 (9)	24 (3)
C(8)	2269 (15)	1574 (13)	12648 (11)	28 (4)
N(9)	1633 (12)	2530 (10)	12331 (8)	24 (3)
Molecule *B*				
N(1)	5848 (11)	2604 (10)	8565 (9)	21 (3)
C(2)	5560 (14)	3530 (13)	9169 (11)	23 (3)
N(2)	4981 (12)	4355 (11)	8345 (9)	33 (3)
N(3)	5853 (12)	3670 (11)	10458 (9)	25 (3)
C(4)	6461 (15)	2767 (13)	11134 (11)	24 (4)
C(5)	6751 (15)	1810 (13)	10609 (11)	23 (4)
C(6)	6440 (14)	1653 (13)	9261 (12)	26 (4)
O(6)	6675 (10)	861 (8)	8604 (7)	32 (2)
N(7)	7379 (11)	1143 (10)	11680 (9)	25 (3)
C(8)	7453 (16)	1684 (13)	12791 (12)	34 (4)
N(9)	6870 (12)	2686 (11)	12475 (9)	30 (3)
Phosphate and oxygen (water)				
P(1)	1739 (4)	3999 (3)	5286 (3)	21 (1)
O(1P)	397 (11)	3518 (10)	5888 (8)	41 (3)
O(2P)	1104 (10)	4038 (9)	3864 (7)	33 (3)
O(3P)	2397 (11)	2947 (10)	5551 (9)	47 (3)
O(4P)	3107 (11)	5522 (9)	5858 (8)	45 (3)
O(1*W*)	5149 (12)	2682 (11)	5860 (9)	55 (3)
O(2*W*)	6894 (20)	815 (17)	5733 (16)	138 (6)
O(3*W*)*	870 (30)	9868 (28)	5216 (24)	96 (8)

* Site occupancy 0·5.

Table 2. *Interatomic distances* (Å) *and angles* (°)

	A	*B*
C(2)–N(1)	1·38 (2)	1·37 (2)
C(6)–N(1)	1·40 (2)	1·40 (2)
N(2)–C(2)	1·34 (2)	1·34 (2)
N(3)–C(2)	1·31 (1)	1·31 (2)
C(4)–N(3)	1·35 (2)	1·36 (2)
C(5)–C(4)	1·35 (2)	1·35 (2)
N(9)–C(4)	1·36 (1)	1·36 (1)
C(6)–C(5)	1·38 (2)	1·37 (2)
N(7)–C(5)	1·40 (2)	1·40 (2)
O(6)–C(6)	1·24 (2)	1·26 (2)
C(8)–N(7)	1·32 (2)	1·32 (2)
N(9)–C(8)	1·36 (2)	1·36 (2)
O(1P)–P(1)	1·46 (1)	
O(2P)–P(1)	1·44 (1)	
O(3P)–P(1)	1·46 (1)	
O(4P)–P(1)	1·47 (1)	
C(6)–N(1)–C(2)	124 (1)	123 (1)
N(2)–C(2)–N(1)	116 (1)	115 (1)
N(3)–C(2)–N(1)	124 (1)	124 (1)
N(3)–C(2)–N(2)	120 (1)	120 (1)
C(4)–N(3)–C(2)	112 (1)	112 (1)
C(5)–C(4)–N(3)	126 (1)	127 (1)
N(9)–C(4)–N(3)	126 (1)	125 (1)
N(9)–C(4)–C(5)	108 (1)	109 (1)
C(6)–C(5)–C(4)	122 (1)	121 (1)
N(7)–C(5)–C(4)	106 (1)	106 (1)
N(7)–C(5)–C(6)	131 (1)	132 (1)
C(5)–C(6)–N(1)	111 (1)	112 (1)
O(6)–C(6)–N(1)	119 (1)	118 (1)
O(6)–C(6)–C(5)	131 (1)	130 (1)
C(8)–N(7)–C(5)	109 (1)	109 (1)
N(9)–C(8)–N(7)	108 (1)	108 (1)
C(8)–N(9)–C(4)	109 (1)	108 (1)
O(2P)–P(1)–O(1P)	110 (1)	
O(3P)–P(1)–O(1P)	110 (1)	
O(3P)–P(1)–O(2P)	109 (1)	
O(4P)–P(1)–O(1P)	109 (1)	
O(4P)–P(1)–O(2P)	109 (1)	
O(4P)–P(1)–O(3P)	109 (1)	

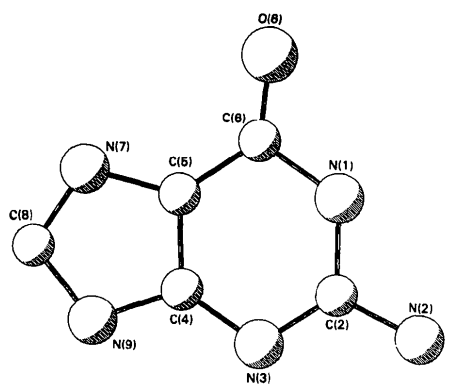

Fig. 1. Atomic numbering of the guaninium base.

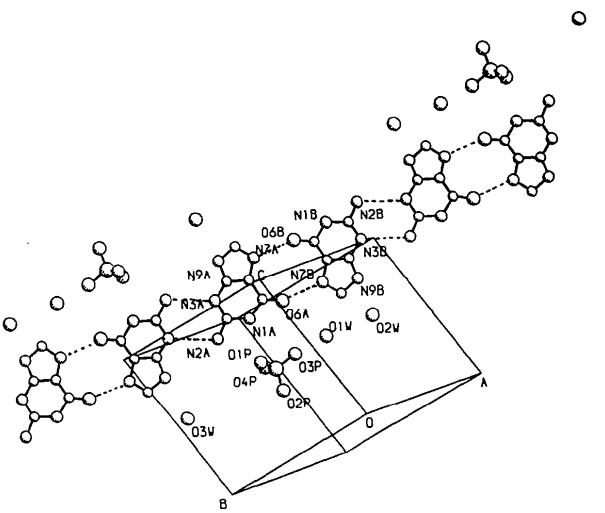

Fig. 2. View perpendicular to base *A* showing the hydrogen-bonded ribbon formed by bases.

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Structure of 9,10-Dihydro-9,10-ethenoanthracene-11,12-dicarbonitrile

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Abstract. $C_{18}H_{10}N_2$, $M_r = 254{\cdot}2$, monoclinic, $P2_1/c$, $a = 9{\cdot}885$ (5), $b = 14{\cdot}210$ (7), $c = 9{\cdot}6066$ (5) Å, $\beta = 103{\cdot}08$ (10)°, $U = 1314$ Å^3, $Z = 4$, $D_m = 1{\cdot}28$ (1), $D_x = 1{\cdot}28$ g cm^{-3}, Cu $K\alpha$, $\lambda = 1{\cdot}5418$ Å, $\mu = 5{\cdot}21$ cm^{-1}, $F(000) = 528$, room temperature, $R = 0{\cdot}046$ for 1052 observed reflections. The X-ray crystallographic structure is similar to that of 1-bromotriptycene [Palmer & Templeton (1968). *Acta Cryst.* B**24**, 1048–1052]. The short cyanide–ethane bond lengths [1·439 (5), 1·429 (5) Å] may furnish some evidence of electron delocalization, though the cyanide bond lengths [1·141 (4), 1·139 (4) Å] are of the expected magnitude. The other bond lengths and angles do not reveal any peculiarities.

Introduction. The Diels–Alder addition reaction of dicyanoacetylene with anthracene has been used for the preparation of the title compound (Weis, 1963). No doubt prompted by the similarity of the dicyano grouping in this compound to that of phthalonitrile, a commonly used precursor in the synthesis of phthalocyanines, the use of the former compound has been described (Kopranenkov & Rumyantseva, 1975). In order to gain some idea of the volume available in the axial positions of the metallobarrelenoporphoryrazine as well as pave the way to the interpretation of their X-ray crystallographic data the molecular structure of the title compound has been determined. In addition, such a study provides the opportunity of studying the structural effect of a single ethene group, which is not part of a peripheral benzene group, on the central ring system.

Experimental. A more convenient method for the preparation of the dicyano compound starts with the Diels–Alder addition of the dimethyl ester of dicarboxyacetylene to anthracene (Diels & Thiele, 1931; Holmes, 1949), followed by conversion of the diester product to the diamide by treatment with ammonia and final conversion to the dicyano form by reaction of the diamide with thionyl chloride in dimethylformamide. The final product after recrystallization from acetonitrile was characterized as follows: m.p. 540–541 K; composition: calculated: C 85·02, H 3·96, N 11·02%, found: C 85·38, H 4·20, N 10·72%; MS m/e (rel. int. %) 254 (M^+, 100%), 227 (38), 203 (12), 178 (38). ^{1}H NMR 7·54 (4H), 7·13 (4H), 6·03 (2H) p.p.m.

Crystal dimensions 0·2 × 0·1 × 0·1 mm. D_m by flotation. Cell dimensions determined from 24 reflections. 1948 reflections measured, $\theta = 3$–60°, Philips PW 1100 diffractometer, 1052 [$I \geq 3\sigma(I)$] used, index range h −11/10, k 0/15, l 0/10; Lorentz–polarization and absorption corrections (transmission coefficients max. 0·955, min. 0·924) applied; standard reflections measured every 4 h showed no reduction in intensity over the data-collection period; structure solved by direct methods; refined by full-matrix least squares using *SHELX76* (Sheldrick, 1976), F values, anisotropic temperature factors for non-hydrogen atoms and H atoms in geometrically calculated positions (riding model, C—H 1·08 Å) with a common isotropic temperature factor [U 0·071 (4) Å^2], $R = 0{\cdot}046$, $wR = 0{\cdot}047$, where $w = \sigma^{-2}(F)$; Δ/σ_{max} 0·001 in final cycle; no correction for extinction; scattering factors taken from *International Tables for X-ray Crystallography*

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Calculation of pK_a Values of Nucleobases and the Guanine Oxidation Products Guanidinohydantoin and Spiroiminodihydantoin using Density Functional Theory and a Polarizable Continuum Model

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An efficient computational method has been identified that uses B3LYP density functional theory, IEF-PCM solvation modeling with a modified UFF cavity, and Boltzmann weighting of tautomers to predict the site-specific and global pK_a of DNA nucleobases and their oxidation products. The method has been used to evaluate the acidity of guanidinohydantoin (Gh) and spiroiminodihydantoin (Sp), two highly mutagenic guanine oxidation products. The trend observed for the pK_a values of Gh (9.64 and 8.15) is consistent with the experimentally observed values for guanidine cation (13.7) and hydantoin (9.16). The pK_{a1}(calc) value for deprotonation of Sp cation ($Sp^+ \rightarrow Sp$) is very close to the experimentally observed pK_{a1} for 8-oxoG and is consistent with the similarity in their structures. The data suggest that the imide (N7) proton in Sp is considerably more acidic than that in Gh, possibly due to the presence of the through-space electronic effects of the carbonyl group located at C6. This difference in the acidity of Gh and Sp may be an indication of their potential toxicity and mutagenicity in vivo and remains a fertile area for experimental study.

1. Introduction

Damage to DNA via oxidation is thought to be related to a variety of cancers and neurological disorders as well as cell aging and death.[1−5] Typical damage involves chemical oxidation of the nucleobases or the pentose sugars, base cleavage, or the formation of DNA/protein cross-links. Oxidation of the guanine base leads to a variety of products including 8-oxo-7,8-dihydro-2′-deoxyguanine (8-oxoG), guanidinohydantoin (Gh), and spiroiminodihydantoin (Sp).[6−8] Gh and Sp are two of the major products observed in guanine oxidation and are thought to be formed via a common intermediate, 5-hydroxy-8-oxo-7,8-dihydroguanosine, with the product branching ratio dependent on the pH of the reaction environment.[9−13] Computational studies conducted by our group[14] confirmed that protonation or deprotonation of various sites within the intermediate species involved in the mechanism of formation of Gh and Sp had a significant effect on the predicted kinetics and thermodynamics of the various pathways. For future studies of acid- or base-catalyzed chemical reactions, it would be useful to have an efficient computational method to predict the site-specific protonation state or pK_a of intermediate species at experimental reaction pHs.

In addition to understanding the likely mechanisms for pH-sensitive reactions, a theoretical method for computing relative pK_as might prove useful for predicting the relative toxicity or mutagenicity of these adducts. The structural and functional changes observed in DNA following oxidation of its nucleobases may be due, in part, to changes in the hydrogen-bonding characteristics of the oxidized adducts. Hydrogen bonding between the nucleobases in DNA and RNA duplexes is known to be very important to their structure and function in vivo.[15] The strength of these hydrogen bonds is correlated to the relative pK_a values of the donor and acceptor nucleobases.[16] For several decades, research groups have used a variety of experimental techniques to measure the pK_a of the isolated DNA and RNA nucleobases as well as those attached to the ribose, deoxyribose, and the phosphate backbone.[8,17−22] The pK_a of nucleobases has been demonstrated to fluctuate depending on its location in the base sequence in the backbone of DNA and RNA,[18] its proximity to metal ions,[21] and its oxidation state.[8,19,20] Research groups have used computational methods to predict either the pK_a or protonation state of both modified[23,24] and unmodified nucleobases,[25−31] nucleotides,[32] and the guanine oxidation product, 8-oxoguanine.[33] The latter compound can be further oxidized in vivo to form Gh and Sp, two highly mutagenic DNA lesions.[34−37] In UV melting studies, both Gh and Sp were shown to decrease the thermodynamic stability of duplex DNA relative to guanine and 8-oxoguanine, with the effect of the Sp being more severe.[34,38] Molecular dynamics simulations indicate that Sp lesions alter the base stacking and Watson−Crick hydrogen-bonding interactions of the duplexes.[39,40] Although steric differences between the planar guanine and 8-oxoguanine and the nonplanar Gh and Sp compounds could certainly contribute to these conformational changes, it is also possible that the duplex destabilization is due, at least in part, to changes in the site specific pK_a values of these lesions relative to their parent compounds guanine and 8-oxoguanine.

The prediction of accurate pK_a values using computational techniques has been the subject of study for many years.[16,41−63] It is particularly challenging due to the fact that an error of 1.36 kcal/mol in the free energy calculation results in a error of 1 pK_a unit. A variety of computational methods have been used to study the relative acidity and basicity of the nucleobases and nucleosides.

Theoretical studies of the gas phase acidity of the isolated nucleobases were conducted by several groups. Giese and

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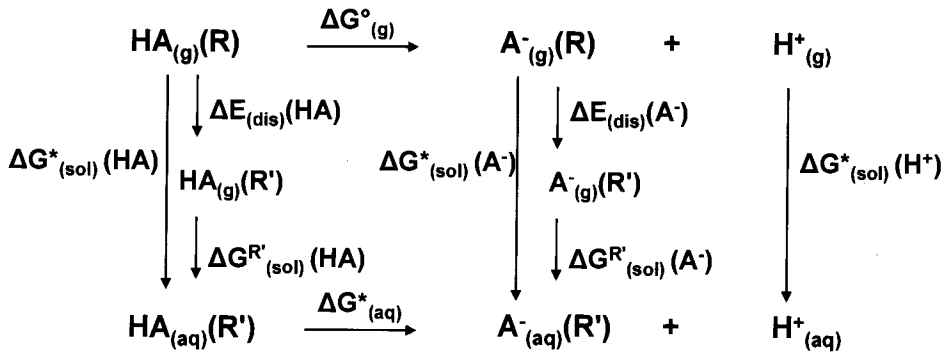
SCHEME 1: Thermodynamic cycle used in the calculation of the pK_a[a]



[a] The standard state reference for the gas phase free energy, $\Delta G^{\circ}_{(g)}$, is one atmosphere of pressure and 298.15 K, and for the free energy in water, $\Delta G^{*}_{(aq)}$, and free energy of solvation, $\Delta G^{*}_{(sol)}$, it is 1 M and 298.15 K. R is the structure optimized in the gas phase at B3LYP/6-31+G(d,p) and R′ is the structure optimized in solution at IEF-PCM/B3LYP/6-31+G(d,p). $\Delta E_{(dis)}$ is the electronic distortion energy between the gas and solution phase optimized structures calculated at the same level of theory with the aug-cc-pVTZ basis set. $\Delta G^{R'}_{(sol)}$ is the calculated free energy of solvation of the R′ structure.

McNaughton studied the site-specific proton affinities of guanine and its heptahydrate at the B3LYP/6−31++G(d,p) level of theory. The proton affinity was calculated to be 229.7 kcal/mol and found to be consistent with the experimental value of 227.3 ± 11.5 kcal/mol.[31] Leszczynski calculated the proton affinities of the five nucleobases and the rare tautomers of guanine, adenine, and cytosine at the MP4(SDTQ)/6−31+G(d,p)//MP2/6−31+G(d,p) and MP2/6−311++G(d,p)//MP2/6−31+G(d,p) levels of theory. The calculated values were reported to be within 2.1% of the experimental values, and their data suggested that the rare tautomers of the nucleobases were a significant portion of the gas-phase equilibrium composition.[30] Chandra and Zeegers-Huyskens used B3LYP density functional theory and the 6−31++G(d,p) and 6−311++G(d,p) basis sets[26−29] to calculate the relative acidity of various tautomers of cytosine, thymine, and uracil, both alone and complexed with one molecule of water. They reported the relative acidities of the five nucleobases as uracil > thymine > guanine > adenine > cytosine.

The solution-phase pK_a values of nucleotides in RNA have been calculated to within 1−2 pK_a units using a modified version of the multiconformational continuum electostatics (MCCE) program that employs a variation of the Poisson−Boltzmann equation coupled with Monte Carlo treatment of the multiple ionization states.[32] This group predicted that significant shifts in the pK_a of the adenine and cytosine nucleotides could be brought about by changes in the 3D structure of the RNA backbone and may be responsible for its function. Chatterjee et al.[22] estimated the effect of modification of the pentose-sugar on the site-specific pK_a values for adenosine, guanosine, cytidine, thymidine, and uridine nucleosides using the closed-shell Hartree−Fock (HF) level of theory with a 6−31G(d,p) basis set. The conductor-like polarizable continuum model (CPCM) was used to calculate the free energy of solvation for each of the modified nucleosides, and the results were compared to experimental data. For these calculations, the free energy of solvation for the proton was assumed to be −263.47 kcal/mol. The authors reported a good linear correlation [R = 0.98, pK_a(exp) = 0.4690(±0.0170) × pK_a(calc) − 2.1087(±0.3270)] between the predicted pK_a values of the nucleosides and the experimental values obtained with their corresponding bis-ethylphosphate nucleotides.

Using the B3LYP density functional theory and the Poisson−Boltzmann continuum-solvation model with modified atomic radii, Goddard's group has calculated both the site specific and overall pK_a values of guanine, cytosine, isoguanine, 9-methylisoguanine, xanthine, and 8-oxoguanine.[23,25,33] By treating the solvation free energy of the proton as a variable ($\Delta G_{sol}(H^+)$ = −263.47 kcal/mol), they were able to predict the pK_a of guanine within 0.2 units of experiment and the values for cytosine, isoguanine, xanthine, and 9-methylisoguanine within 1 unit of experiment. Goddard also estimated the relative abundance of deprotonated guanine at physiologic pH and discussed the role of the deprotonated species in base-pair mismatching during DNA replication. Using the same method, Hwang and Jang[24,64] predicted the pK_a values of 9-methylguanine, 9-methyladenine, and 9-methylhypoxanthine to within 0.6−1.5 units of the experimental data. In this paper, we propose an efficient computational method for calculating the pK_a of DNA and RNA nucleobases and the guanine oxidation products, Gh and Sp, employing the thermodynamic cycle outlined in Scheme 1, Boltzmann weighting of relevant tautomers, and modification of the UFF solvation cavity. The thermodynamic cycle is a modification of the one proposed by Nasimento et al.[59] for the calculation of absolute pK_a values for carboxylic acids.

2. Computational Methods

General Formulas for Calculation of Global and Site-specific pK_a Values. As noted in work previously published by Goddard's group,[25,33] the calculation of pK_a values is complicated by the presence of multiple tautomers having various site-specific pK_a values. We have followed the method outlined by Goddard for calculating a global pK_a value from a Boltzmann weighting of the site-specific values of the various tautomers investigated in our study (eq 1). Where pK_a^{ij} is the site-specific value for deprotonation of tautomer i resulting in the formation of the deprotonated tautomer j, and f_i and f_j are the Boltzmann weighted fractions of tautomers i and j, respectively.

$$pK_a = pK_a^{ij} - \log f_i + \log f'_j \quad (1)$$

The site-specific pK_a of an acid HA is given by eq 2,

$$\mathrm{p}K_a = \frac{1}{2.303RT}\Delta G^{*}_{(aq)} \quad (2)$$

where R is the gas constant, T is the temperature, and $\Delta G^{*}_{(aq)}$ is the free energy of the deprotonation reaction, HA → H^+ + A^-,

for a standard state of 1 mol/L and room temperature. Using the thermocycle defined in Scheme 1, $\Delta G^*_{(aq)}$ is defined as the difference in the free energies in solution between the proton (H^+) and unprotonated tautomer (A^-) and the protonated tautomer (HA) (eq 3).

$$\Delta G^*_{(aq)} = G^*_{(aq)}(A^-_{R'}) + G^*_{(aq)}(H^+) - G^*_{(aq)}(HA_{R'}) \quad (3)$$

For each species, $G^*_{(aq)}$ is the sum of the gas phase standard free energy, $G^*_{(g)}$ and the free energy of solvation in water, $\Delta G^*_{(sol)}$, where the "*" indicates that all terms are in the standard state of 1 mol/L. To convert the calculated gas phase standard free energy, $G^\circ_{(g)}$, from its standard state of 1 atm gas phase/1 M solution to $G^*_{(g)}$ with a standard state of 1 M gas/1 M solution phase, it is necessary to add 1.89 kcal/mol ($RT \ln(1/R_gT)$,[65] (eq 4)

$$G^*_{(aq)} = G^*_{(g)} + \Delta G^*_{(sol)} = (G^\circ_{(g)} + 1.89) + \Delta G^*_{(sol)} \quad (4)$$

where R is 1.987 cal/mol·K and R_g is 8.206×10^{-2} liter·atm/mol·K. Combining equations 2, 3, and 4 yields a general expression for the calculation of the site-specific pK_a of a given tautomer (eqs 5.1−5.3).

$$pK_a = \frac{1}{2.303RT}(G^\circ_{(g)}(A^-) + 1.89 + \Delta G^*_{(sol)}(A^-) + G^\circ_{(g)}(H^+) + 1.89 + \Delta G^*_{(sol)}(H^+) - G^\circ_{(g)}(HA) - 1.89 - \Delta G^*_{(sol)}(HA)) \quad (5.1)$$

$$= \frac{1}{2.303RT}(G^\circ_{(g)}(A^-) + \Delta G^*_{(sol)}(A^-) + G^\circ_{(g)}(H^+) + 1.89 + \Delta G^*_{(sol)}(H^+) - G^\circ_{(g)}(HA) - \Delta G^*_{(sol)}(HA)) \quad (5.2)$$

$$= \frac{1}{2.303RT}(G^\circ_{(g)}(A^-) + \Delta G^*_{(sol)}(A^-) + G^\circ_{(g)}(HA) - \Delta G^*_{(sol)}(HA) - 270.29) \quad (5.3)$$

The gas and solution phase free energies of the proton were taken from the literature to be $G^\circ_{(g)} = -6.28$ kcal/mol[41] and $\Delta G^*_{(sol)} = -265.9$ kcal/mol,[65] respectively.

Calculation of Gas and Solution Phase Free Energies. Molecular orbital calculations were carried out using the E05 development version of the GAUSSIAN series of programs (Note: the PCM parameters change significantly between versions E05 and F01).[66] Optimized geometries and energies in the gas phase and in aqueous solution were computed with the B3LYP density functional method[67−69] using the 6−31+G-

SCHEME 2: Major tautomers and experimental pK_a values of guanine, 8-oxoguanine, adenine, thymine, and cytosine, evaluated for this study

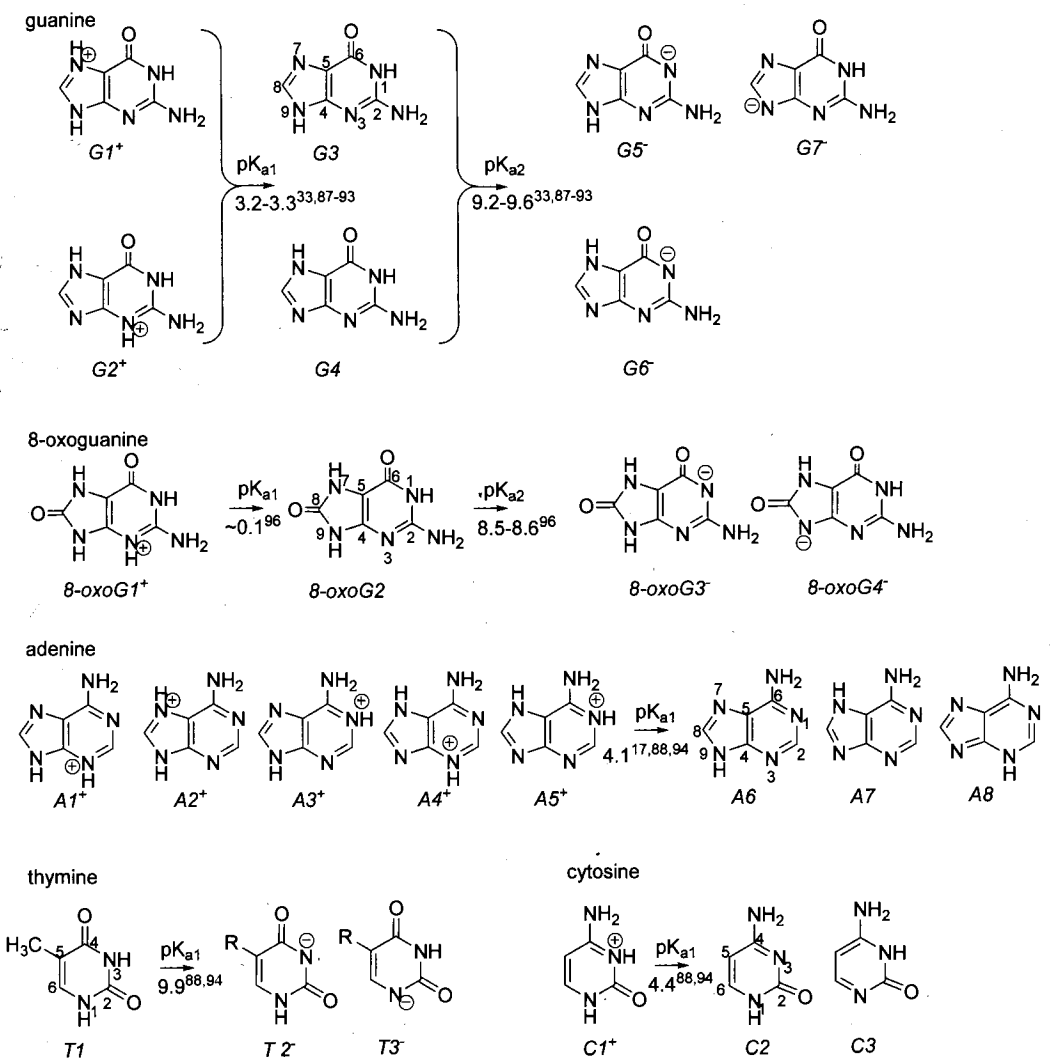


TABLE 1: Relative Free Energies (kcal/mol), Calculated and Experimental pK_a Values for Major Tautomers of Guanine, Adenine, Cytosine, Thymine, 8-oxoguanine, Hydantoin, Phenytoin, Guanidine, N-formylguanidine, and N-acetylguanidine

system	$\Delta G_{(g)}{}^{a}$ (kcal/mol)	$\Delta G^{*}_{(aq)}{}^{a}$ (kcal/mol)	$\Delta G^{R'}{}_{(sol)}{}^{a}$ (kcal/mol)	pK_a (calc)	pK_a (exp)
Guanine					
G1$^+$	0.0	0.0	−64.8		
G2$^+$	4.3	0.6	−68.9	3.4	3.2−3.3[b]
G3	0.3	1.5	−18.5		
G4	0.0	0.0	−19.0		
G5$^-$	2.8	0.0	−75.4	9.6	9.2−9.6[b]
G6$^-$	0.0	0.3	−72.0		
G7$^-$	0.8	0.7	−73.1		
Adenine					
A1$^+$	1.7	1.5	−60.4		
A2$^+$	8.0	2.8	−65.6		
A3$^+$	0.5	0.0	−60.7		
A4$^+$	0.0	0.3	−59.9	4.2	4.1[c]
A5$^+$	10.1	1.0	−69.5		
A6	0.0	0.0	−9.9		
A7	8.5	2.0	−16.7		
A8	8.5	4.5	−14		
Thymine					
T1	0.0	0.0	−11.1		
T2$^-$	0.0	0.6	−60.3	10.5	9.9[c]
T3$^-$	10.8	0.0	−72.3		
Cytosine					
C1$^+$	0.1	0.0	−65.8		
C2	0.0	0.0	−17.8	4.2	4.4[c]
C3	6.8	3.9	−21.0		
8-oxoG					
8-oxoG1$^+$	7.0	0.0	−82.6	−0.4	−0.1[d]
8-oxoG2	0.0	0.0	−21.0		
8-oxoG3$^-$	0.0	2.0	−69.6	8.0	8.5−8.6[d]
8-oxoG4$^-$	6.8	0.0	−79.1		
Hydantoin					
N1 anion	4.6	6.1	−69.9	11.0	9.16[e]
N3 anion	0.0	0.0	−67.9		
Phenytoin					
N1 anion	4.2	6.3	−54.1	8.7	8.31[e]
N3 anion	0.0	0.0	−55.8		
Guanidine	0.0	0.0	−62.5	14.5	13.7[f]
N-formyl guanidine					
FG1$^+$	0.0	0.0	−67.0	6.7	not available
FG2$^+$	7.6	3.3	−71.7		
FG3	0.0	0.0	−12.2		
FG8	8.1	0.8	−21.3		
N-acetyl guanidine					
AG1$^+$	0.0	0.0	−61.6	8.5	8.32[g]
AG2$^+$	11	7.5	−67.0		
AG3a	0.0	0.0	−9.78		
AG3b	0.1	0.1	−9.78		
AG5	2.0	2.8	−8.54		

[a] Energies were calculated using B3LYP density functional theory and the 6−31+G(d,p) and aug-cc-pVTZ basis sets. See eqs 4, 6, 13, 14, and 15. [b] References 25, 87−93. [c] Reference 88. [d] Reference 96. [e] Reference 103. [f] Reference 102. [g] Reference 108.

(d,p) basis set[70−75] (BS-I) with the latter calculations also employing integral equation formalism of the polarizable continuum model (IEF-PCM).[76−78] Tight convergence criteria and the "nosymm" options were used for all optimizations. The solution phase optimizations employed a solvent-excluding surface cavity model,[79] UFF radii,[80] and tesserae with an average area of 0.200 Å^2. For this study, all Gh and Sp chiral tautomers were the *R* stereoisomer. Given the symmetric nature of the PCM solvation model, it is anticipated that the calculated pK_a values would be the same for the *S* isomer. Single-point calculations (in the gas phase) were also conducted with the gas phase and solution phase optimized geometries with the aug-cc-pVTZ[81] basis set (BS-II) and tight convergence criteria. Cartesian coordinates for the optimized geometries and electronic energies for all compounds are provided in the Supporting Information. Vibrational frequencies were computed in the gas phase at the B3LYP level with the 6−31+G(d,p) basis set (BS-I) and were used without scaling since the B3LYP frequencies agree quite well with experimental values for a wide range of second and third period compounds.[82] Thermal corrections and free energies were calculated by standard statistical thermodynamic methods[83] using the unscaled B3LYP frequencies and the ideal gas/rigid rotor/harmonic oscillator approximations. To improve the accuracy of the pK_a calculated values versus experimental data, the solution phase single point calculation of the free energy of solvation employed a modified UFF cavity using an α value of 0.91 for the cationic and neutral species and 0.83 for the anionic species. Selection of the optimal α values was made by the empirical fitting process described below.

Calculation of pK_a. Following optimization of each of the species in the gas phase, the standard gas phase free energy, $G^{\circ}_{(g)}$, for each species was calculated as the sum of the electronic energy at 0 K, the unscaled zero point energy, and the change in the free energy from 0 to 298 K (eq 6). Here R refers to the gas phase optimized structure.

$$G^{\circ}_{(g)}(\mathrm{R}) = E^{\mathrm{B3LYP/aug-cc-pVTZ}}_{(0\ \mathrm{K})}(\mathrm{R}) + \mathrm{ZPE}^{\mathrm{B3LYP/6-31+G(d,p)}}(\mathrm{R}) + \Delta G^{\mathrm{B3LYP/6-31+G(d,p)}}_{(0\rightarrow 298\ \mathrm{K})}(\mathrm{R}) \quad (6)$$

The standard free energy of solvation in water $\Delta G^{*}_{(sol)}$, as described by Ben-Naim et al.,[84] is defined as the difference between the gas phase free energy at the gas phase optimized geometry, R, and the solution phase free energy at the solution phase optimized geometry, R′ (eqs 7 and 8).

$$\Delta G^{*}_{(sol)}(\mathrm{A}^-) = G^{*}_{(aq)}(\mathrm{A}^-_{R'}) - G^{*}_{(g)}(\mathrm{A}^-_{R}) \quad (7)$$

$$\Delta G^{*}_{(sol)}(\mathrm{HA}) = G^{*}_{(aq)}(\mathrm{HA}_{R'}) - G^{*}_{(g)}(\mathrm{HA}_{R}) \quad (8)$$

In the thermodynamic cycle outlined in Scheme 1, the standard free energy of solvation, $\Delta G^{*}_{(sol)}$, is partitioned into two physically meaningful parts (eqs 9 and 10): a deformation term that captures the electronic distortion energy resulting from the change in the geometry of the solute as the species moves from the gas phase (R) to the solution phase (R′), $\Delta E_{(dis)}$ (eqs 11 and 12), and a free energy of solvation term for the molecule at its geometry optimized in solution, $\Delta G^{R'}_{(sol)}$ (eqs 13 and 14). $\Delta G^{R'}_{(sol)}$ was obtained from a single-point calculation at the IEF-PCM/B3LYP/6−31+G(d,p) level of theory using the modified UFF cavity. The distortion energy ($\Delta E_{(dis)}$) for each species was computed from single-point calculations conducted at the B3LYP/aug-cc-pVTZ level of theory on the gas phase (R) and solution phase optimized (R′) structures optimized at B3LYP/6−31+G(d,p) and IEF-PCM/B3LYP/6−31+G(d,p) respectively.

$$\Delta G^{*}_{(sol)}(\mathrm{A}^-) = \Delta G^{R'}_{(sol)}(\mathrm{A}^-) + \Delta E_{(dis)}(\mathrm{A}^-) \quad (9)$$

TABLE 2: Relative Free Energies (kcal/mol) and Population of Neutral Tautomers of Gh

	Gh1	Gh2	Gh3	Gh4	Gh5	Gh6	Gh7	Gh8	Gh9
gas phase									
$\Delta G^{\circ}_{(g)rel}$[a]	6.4	0.0	27.6	24.2	20.9	21.7	23.4	17.5	25.9
population	1.9×10^{-5}	1.0	5.4×10^{-21}	1.9×10^{-18}	4.3×10^{-16}	1.2×10^{-16}	6.0×10^{-18}	1.4×10^{-13}	1.2×10^{-19}
aqueous phase									
$\Delta G^{*}_{(aq)rel}$[b]	2.3	0.0	23.0	18.6	16.3	17.3	na[c]	16.3	0.7
population	0.02	0.76	9.8×10^{-18}	1.9×10^{-14}	8.8×10^{-13}	1.7×10^{-13}	na[c]	7.9×10^{-13}	0.22

[a] Relative energies with respect to $\Delta G^{\circ}_{(g)}$ for **Gh2**. [b] Relative energies with respect to $\Delta G^{*}_{(aq)}$ for **Gh2**. [c] Data not available.

$$\Delta G^{*}_{(sol)}(HA) = \Delta G^{R'}_{(sol)}(HA) + \Delta E_{(dis)}(HA) \quad (10)$$

$$\Delta E_{(dis)}(A^{-}) = E^{\circ}_{(g)}(A^{-}_{R'}) - E^{\circ}_{(g)}(A^{-}_{R}) \quad (11)$$

$$\Delta E_{(dis)}(HA) = E^{\circ}_{(g)}(HA_{R'}) - E^{\circ}_{(g)}(HA_{R}) \quad (12)$$

$$\Delta G^{R'}_{(sol)}(A^{-}) = G^{*}_{(aq)}(A^{-}_{R'}) - G^{*}_{(g)}(A^{-}_{R'}) \quad (13)$$

$$\Delta G^{R'}_{(sol)}(HA) = G^{*}_{(aq)}(HA_{R'}) - G^{*}_{(g)}(HA_{R'}) \quad (14)$$

This approximation has been employed for computational reasons, as it avoids the calculation, often problematic, of the normal modes in solution. As a check of the validity of this approximation, the partition functions for the gas phase and solution phase optimized geometries of two cationic, two neutral, and two anionic species were evaluated. The mean absolute deviation in the calculated relative free energy, $\Delta\Delta G$, of the species was found to be 0.1−0.7 kcal/mol. Semiempirical modification of the cavity scaling factor to more accurately predict the experimental pK_a values should reduce the impact of the differences in the partition functions.

When the standard free energy of solvation is expressed in terms of the distortion energy and the solvation energy of the distorted geometry, $\Delta G_{(sol)} = \Delta E_{(dis)} + \Delta G^{R'}_{(sol)}$, eq 5.3 becomes

$$pK_a = \frac{1}{2.303RT}(G^{\circ}_{(g)}(A^{-}_{R}) + \Delta G^{R'}_{(sol)}(A^{-}) + \Delta E^{\circ}_{(dis)}(A^{-}) - G^{\circ}_{(g)}(HA_{R}) - \Delta E^{\circ}_{(dis)}(HA) - \Delta G^{R'}_{(sol)}(HA) - 270.29) \quad (15)$$

This equation is used to calculate the site-specific pK_a for each tautomer.

3. Results and Discussion:

Selection of the Model Cavity. As anticipated in the previous section, the critical issue for exploring a biological system characterized by several tautomers and equilibrium in solution is to create a model capable of reproducing the experimental pK_a values. In particular, as the pK_a values are logarithmic functions of the free energy, a very high accuracy (within 2 kcal/mol) is needed. As has been demonstrated by Goddard's group,[25,33] the computational level adopted in this work is satisfactory for reproducing the gas phase proton affinity of nucleobases. However, there is not currently a standard procedure using the polarizable continuum model (PCM) to predict the solution free energies of the nucleobases with the same accuracy.

The PCM parameter optimized for the test set of DNA nucleobases considered in this study (Scheme 2) is the electrostatic scaling factor (α), which is a real number that is used to increase or decrease the radii of each sphere centered on the atoms.[79] The UFF set of atomic radii (see Table 1 in ref 80), with default value of the scaling factor α = 1.0 (Gaussian

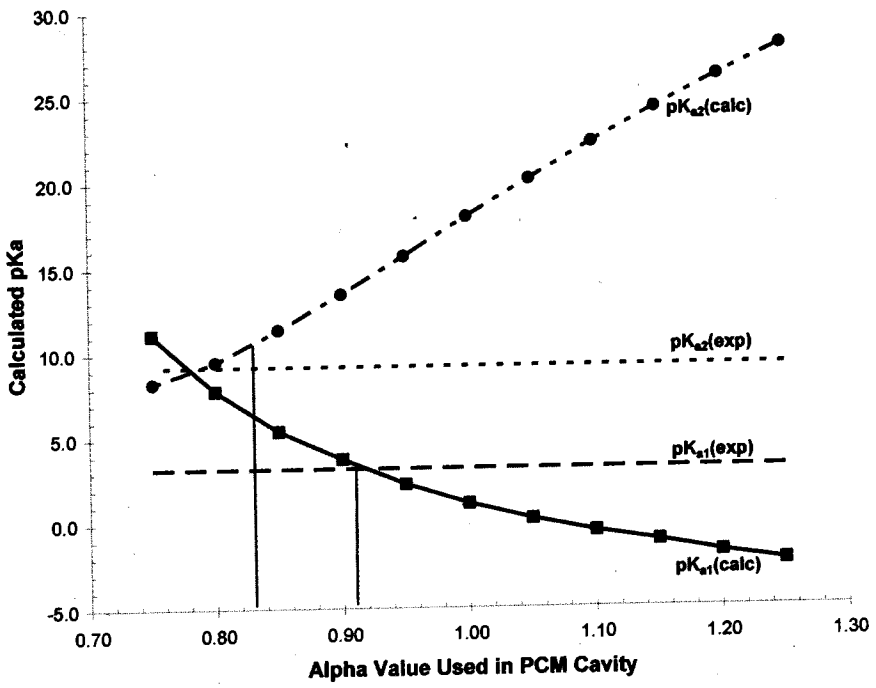
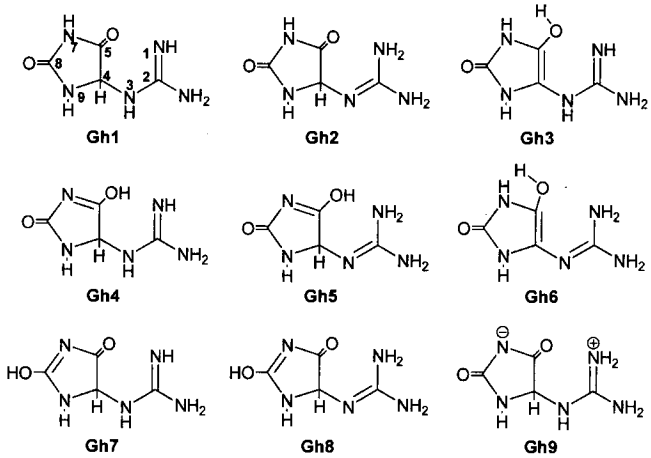


Figure 1. Effect of the UFF alpha value on the calculated pK_{a1} and pK_{a2} values of guanine.

SCHEME 3: Neutral Tautomers of Guanidinohydantoin Evaluated During This Study



Development Version E05), ensures a good balance between the computational stability and applicability and the reasonable accuracy of the free energy of solution for the neutral solutes.[79] For charged solutes, the scaling factor must be reconsidered. Due to the strong electrostatic interactions, solvent molecules are, in general, closest to the atoms of charged solutes rather than those of the neutral one.[85] In a recent study conducted by Camaioni's group, improved quantitative estimates of solvation effects in solution were obtained by decreasing the size of the cavity for cations and anions.[86] The representative nucleobase studied to set up the cavities used in this work was guanine, and various values of α were evaluated by comparing the calculated pK_a value with guanine's experimental first and second pK_a. Two different values of the scaling factor were adopted: to surround cationic and neutral intermediates a value of α = 0.91 showed the best results whereas, for anions, a smaller cavity with α = 0.83 has been used to provide good accuracy (Figure 1, Supporting Information Tables S12−S16).

Table 1 contains a summary of the relative free energy in the gas and solution phases, the free energy of solution, and the calculated and experimental values for the tautomers of guanine and the other nucleobases evaluated during this study. The calculated pK_a values and associated energy data for each deprotonation of the various guanine tautomers are provided in Supporting Information Scheme S1 and Table S1. On the basis of previous studies,[25] two cationic, two neutral, and three anionic tautomers were considered for the first and second guanine deprotonation (Scheme 2). In Table 1, the gas and solution phase energy for each tautomer is expressed relative to the most stable species of each class (e.g., guanine cations relative to the most stable guanine cation). The solvation free energy is also reported and clearly shows the effects of this contribution on the relative stability of the three anionic tautomers as the stability of **G5**$^-$ and **G6**$^-$ are reversed between the gas and solution phases. The two pK_a values computed are 3.4 and 9.6 for the first and the second deprotonation respectively, which are in good agreement with the experimental values of 3.2−3.3[33,87−93] and 9.2−9.6.[33,87−93]

Test of the Model Cavity on Other Nucleobases. To be sure that these new cavities could be extended to all the other nucleobases under study (Scheme 2), we have performed several test calculations on adenine, cytosine, thymine, and 8-oxoG and compared the predicted pK_a to the available experimental data. The data for the most abundant gas and solution phase tautomers are provided in Table 1. A complete summary of the data for all tautomers tested for adenine, cytosine, thymine, and 8-oxoG is provided in the Supporting Information Schemes S2−S5 and Tables S2−S5. In the adenine system, the two most stable cationic tautomers are **A3**$^+$ (protonated at N1) and **A4**$^+$ (protonated at N3). In aqueous solution, the two most stable neutral tautomers are predicted to be **A6** and **A7**, which are similar in structure to the lowest energy guanine tautomers, **G3** and **G4**.

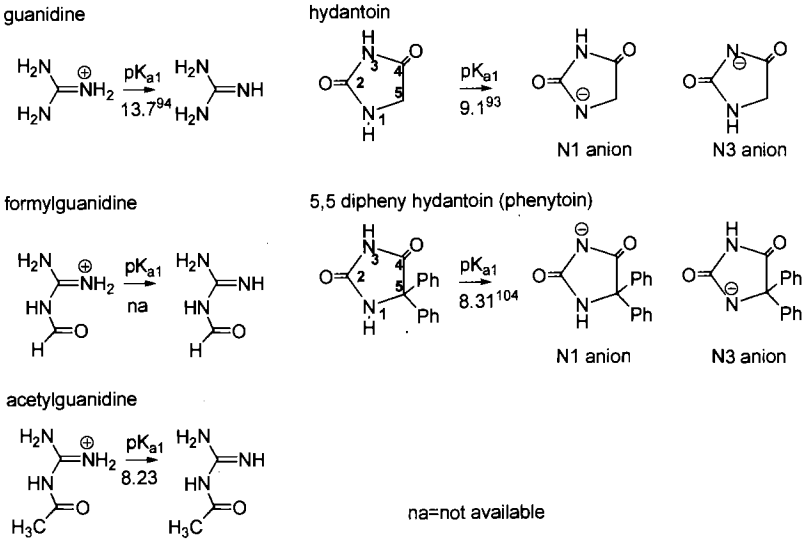
The global pK_a for adenine deprotonation has been estimated to be 4.2, in very good agreement with the experimental value of 4.1.[88,94] The calculations carried out on thymine show essentially only one neutral tautomer, the diketo **T1**, (Scheme 2) and two anions, **T2-** (deprotonation at N1) and **T3-** (deprotonation at N3), which are close in energy (Supporting Information Scheme S4 and Table S4) and predicted to be present at equilibrium in aqueous solution. All the other enol tautomers are predicted to be either completely absent or at such a low level as to not be relevant to the global pK_a calculation. In this case, the calculated pK_a was 10.5, which is in good agreement with the experimental value of 9.9.[88,94] Cytosine is a similar system to the pyrimidine base, thymine, but the presence of an amine functional group rather than a carbonyl group at C4 implies the potential existence of several enol imine tautomers (Supporting Information, Scheme S3 and Table S3). For cytosine, just one cationic and one neutral tautomer are predicted to be relevant in aqueous solution (i.e., **C1**$^+$ and **C2**, Table 1). Again a good agreement between calculated (4.2) and experimental pK_a (4.4) values has been found. Finally, in the 8-oxoG system, the data indicate that the presence of the carbonyl group at C8, instead of −CH, provides a drastic increase of acidity in N3, moving from 2.9 (local pK_a in guanine, see Supporting Information Scheme S1) to −0.4 (local and global first pK_a in 8-oxoG). This is in good agreement with the value found experimentally (~0.1). Two different energies contribute to this value: the first one is a large difference between the gas phase basicity, which is calculated to be 218.3 kcal/mol for **G2**$^+$ → **G4** and 202.8 kcal/mol for **8-oxoG1**$^+$ → **8-oxoG2**. These calculations compare favorably with those of Goddard's group,[25,33] which predicted values of 216.18 and 200.8 kcal/mol for the same reactions, respectively, at B3LYP/6−31++G(d,p)//B3LYP/6−31G(d,p). The experimental value for the gas phase basicity of guanine is 222 ± 2 kcal/mol.[95] This energy gap is in part reduced by the contribution of polarization, and this is very clear if we consider the solvation free energies of the cationic and neutral intermediates of these two systems. As reported in Table 1 for guanine and 8-oxoG, the solvation energies of the neutral tautomers are comparable (−18.5 to −19.0 kcal/mol for guanine versus −21.0 kcal/mol for 8-oxoG) despite the presence of a more polar group in C8 of the 8-oxoG. In contrast, when the molecule is charged (i.e., cationic tautomers), the free energy of solvation for 8-oxoG is approximately 14−18 kcal/mol greater than for guanine, the energy gap between the 8-oxoG neutral and cationic species decreases, leading to a pK_a value near to −0.4. The second deprotonation of 8-oxoG involves two anionic tautomers, and also in this case, the calculated pK_a (8.00 units) is close to the experimental value (8.5−8.6[96]).

Summary of the Computational Method. Upon the basis of the good agreement between the predicted and experimental pKa values for the five nucleobases, the following steps were taken to calculate the pKa values for Gh and Sp:

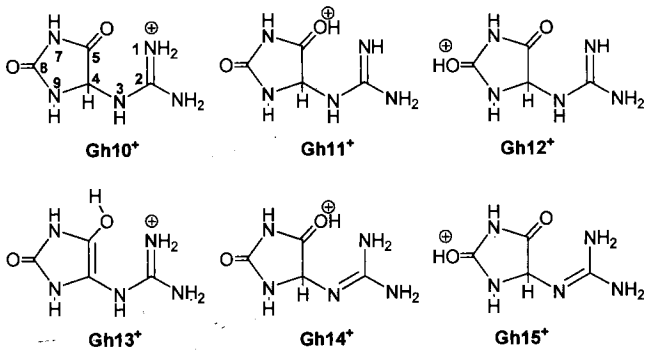
(1) The geometry of each tautomer was optimized at B3LYP/6−31+G(d,p) and the frequencies were calculated.

(2) A gas phase single-point calculation was conducted on the gas phase optimized geometry (from Step 1) at B3LYP/aug-cc-pVTZ.

SCHEME 4: Structure and pK_a of Guanidine, Formylguanidine, Acetylguanidine, Hydantoin, and Phenytoin



SCHEME 5: Cationic Tautomers of Gh Evaluated During This Study



(3) The geometry of each tautomer was optimized in aqueous solution at IEF-PCM/B3LYP/6−31+G(d,p) using the gas phase optimized geometry as a starting point. The alpha value for this optimization was the default value of 1.00.

(4) A gas phase single point calculation was conducted on the aqueous phase optimized geometry (from Step 3) at B3LYP/aug-cc-pVTZ.

(5) The free energy of solvation for the solution phase optimized geometry (from Step 3) was obtained via a single-point calculation at IEF-PCM/B3LYP/6−31+G(d,p) using alpha values of 0.91 for the neutral and cationic tautomers and 0.83 for the anions.

(6) The free energy in solution of each tautomer and the site-specific or local pKa values are calculated using eqs 14 and 15, respectively.

Guanidinohydantoin Results. Several tautomers are possible for Gh for each ionization state and each is expected to contribute to an experimentally observable pK_a in proportion to its population in solution at 298 K. The free energies in the gas and aqueous phases of each of tautomer were calculated using the method described previously and are provided in Supporting Information Tables S8 and S9. The relative population of each species at 298 K was estimated assuming that the tautomers follow the Boltzmann distribution.

Tautomers of Neutral Guanidinohydantoin. The nine tautomers of neutral Gh considered in this study are shown in Scheme 3, and their relative free energies and populations in the gas and aqueous phases are given in Table 2. The gas phase electronic energies, sum of the zero point energy and thermal corrections, and free energies of solvation for each species are provided in the Supporting Information Table S7. In the gas phase, the free energy of the neutral tautomers increases in the following order: **Gh2** < **Gh1** ≪ **Gh8** < **Gh5** < **Gh6** < **Gh7** < **Gh4** < **Gh9** < **Gh3**. The diketo forms of Gh (**Gh1**, **Gh2**, and **Gh9**) are 15−26 kcal/mol more stable than the enol tautomers (**Gh3**−**Gh8**). This result for the diketo forms of Gh are consistent with NMR, IR, UV, and dipole moment experimental data for hydantoin (Scheme 4), which demonstrated that the diketo species is predominant.[97−101] Earlier semiempirical calculations on hydantoin performed by Kleinpeter et al.[97] estimated that the diketo tautomer was 13−21.7 kcal/mol (PM3) and 15.6−25.2 kcal/mol (AM1) more stable the various imine enol species. **Gh2**, the N3−C2 imine tautomer, is the most stable species and is 6.4 kcal/mol lower in energy than **Gh1**, the C2−N1 imine species, due at least in part to the formation of a hydrogen bond between the terminal amino group and the oxygen of the C5 carbonyl group. Upon the basis of the calculated free energies, the gas phase equilibrium of Gh would consist almost exclusively of the **Gh2** tautomer.

In aqueous solution, the free energy of tautomers of neutral Gh increase in a slightly different order from that observed in the gas phase: **Gh2** ≈ **Gh9** < **Gh1** ≪ **Gh8** < **Gh5** < **Gh6** < **Gh7** < **Gh4** < **Gh3**. These data also indicate that the diketo tautomers—**Gh1**, **Gh2**, and **Gh9**—are more stable then the enol tautomers by 14−23 kcal/mol. Of the diketo tautomers, **Gh2** is predicted to be the most stable species followed by **Gh9** (+0.7 kcal/mol), a zwitterion formed by movement of a proton from N7 to N1, and **Gh1** (+2.3 kcal/mol). The stability of the zwitterion tautomer containing a protonated guanidine subunit (−N(H)C(NH$_2$)$_2$) is consistent with the experimentally observed basicity of guanidine (pK_a = 13.7)[102] and acidity of hydantoin (pK_a = 9.16) (Scheme 4).[103] The equilibrium in aqueous solution is predicted to consist of 76% **Gh2**, 22% **Gh9**, and 2% **Gh1**.

Tautomers of Cationic Guanidinohydantoin. Tautomers of cationic Gh considered in this study are shown in Scheme 5, and their relative free energies and populations in the gas and aqueous phases are given in Table 3. The N1 site appears to be the major site for protonation in both the gas and solution phases as the data suggest that the equilibrium concentration will consist almost entirely of the **Gh10**$^+$ tautomer, the diketo hydantoin protonated at N1 of the guanidinyl subunit. This result is consistent with the known acidity and basicity of hydantoin and

TABLE 3: Relative Free Energies (kcal/mol) and Population of Cationic Tautomers of Gh

	Gh10$^+$	Gh11$^+$	Gh12$^+$	Gh13$^+$	Gh14$^+$	Gh15$^+$
Gas Phase						
$\Delta G^{\circ}_{(g)rel}$	0.0	47.8	41.4	23.0	32.1	25.0
population	1.0	3.7×10^{-29}	1.9×10^{-24}	5.5×10^{-11}	5.5×10^{-11}	2.0×10^{-12}
Aqueous Phase						
$\Delta G^{*}_{(aq)rel}$	0.0	36.2	32.2	20.2	35.5	27.7
population	1.0	2.8×10^{-27}	2.3×10^{-24}	1.5×10^{-15}	9.3×10^{-27}	4.9×10^{-21}

[a] Relative energies with respect to $\Delta G^{\circ}_{(g)}$ for **Gh10**$^+$. [b] Relative energies with respect to $\Delta G^{*}_{(aq)}$ for **Gh10**$^+$.

SCHEME 6: Anionic Tautomers of Gh Evaluated During This Study

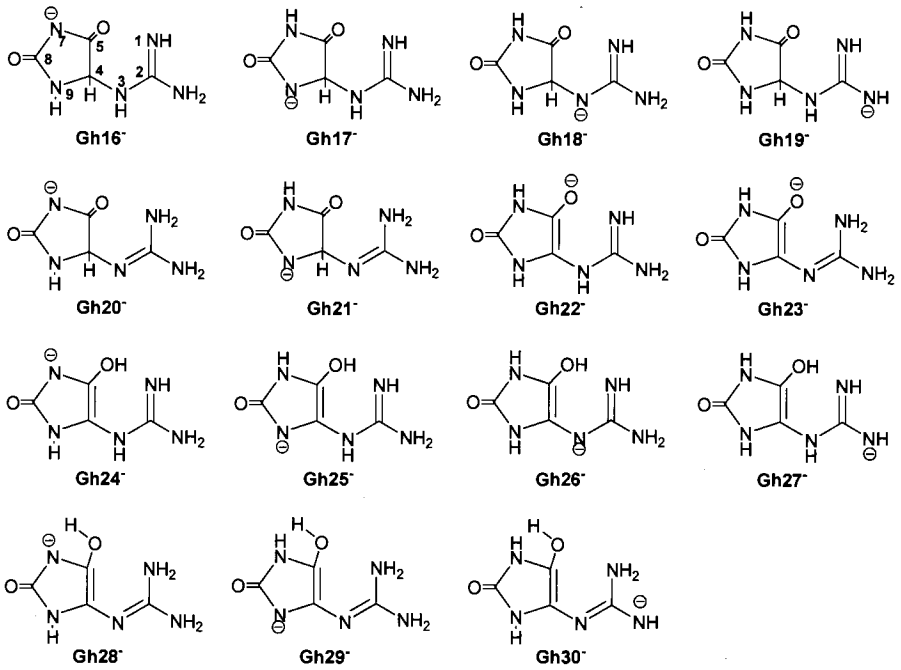


TABLE 4: Relative Free Energies (kcal/mol) and Population of Anion Tautomers of Gh

	Gh16$^-$	Gh17$^-$	Gh18$^-$	Gh19$^-$	Gh20$^-$	Gh21$^-$	Gh22$^-$	Gh23$^-$
Gas Phase[a]								
$\Delta G^{\circ}_{(g)rel}$	0.0	6.4	10.8	26.0	2.2	9.8	19.3	11.3
population	0.98	1.9×10^{-5}	1.1×10^{-8}	8.9×10^{-20}	0.02	6.3×10^{-8}	7.3×10^{-15}	5.4×10^{-9}
Aqueous Phase[b]								
$\Delta G^{*}_{(aq)rel}$	0.9	6.6	14.1	17.7	0.0	8.4	16.0	13.0
population	0.18	1.2×10^{-5}	3.8×10^{-11}	8.1×10^{-14}	0.82	5.6×10^{-7}	1.5×10^{-12}	2.3×10^{-10}

	Gh24$^-$	Gh25$^-$	Gh26$^-$	Gh27$^-$	Gh28$^-$	Gh29$^-$	Gh30$^-$
Gas Phase[a]							
$\Delta G^{\circ}_{(g)rel}$	33.1	30.5	9.7	37.6	27.0	28.3	25.5[c]
population	5.0×10^{-25}	4.6×10^{-23}	8.2×10^{-8}	2.8×10^{-28}	1.6×10^{-20}	1.6×10^{-21}	2.1×10^{-19}
Aqueous Phase[b]							
$\Delta G^{*}_{(aq)rel}$	24.8	21.5	12.8	39.0	23.3	25.0	33.0
population	5.2×10^{-19}	1.4×10^{-16}	3.2×10^{-10}	2.1×10^{-29}	6.3×10^{-18}	3.7×10^{-19}	5.5×10^{-25}

[a] Relative energies with respect to $\Delta G^{\circ}_{(g)}$ for **Gh16**$^-$. [b] Relative energies with respect to $\Delta G^{*}_{(aq)}$ for **Gh20**$^-$. [c] The data shown are for the lowest energy rotamer of **Gh30**$^-$. The relative free energy ranged from 25.5 to 36.0 kcal/mol and from 33.0 to 34.3 kcal/mol in the gas and aqueous phases, respectively.

guanidine. Similar to the pattern observed with the neutral tautomers, the **Gh10**$^+$ diketo form is significantly more stable than the enol tautomers **Gh13**$^+$, **Gh14**$^+$, and **Gh15**$^+$ in both the gas and solution phases. Protonation of the diketo form of Gh at either the C5 (**Gh11**$^+$) or C8 (**Gh12**$^+$) carbonyl oxygen results in a 23.0−47.8 kcal/mol increase in free energy in the gas phase and 20.2−36.2 kcal/mol increase in aqueous solution.

Tautomers of Anionic Guanidinohydantoin. Tautomers of anionic Gh considered in this study are shown in Scheme 6, and their relative free energies and populations in the gas and aqueous phases are given in Table 4. The data indicate that deprotonation at the N7H imide position of the Gh diketone tautomers is energetically favored over all other sites as these tautomers, **Gh16**$^-$ and **Gh20**$^-$, are at least 4 kcal/mol lower in energy than the next most stable species, **Gh17**$^-$, a diketo N9 anion. These results are consistent with NMR data published by Kleinpeter[97] for a series of 5,5-disubstituted hydantoins demonstrating that the N3H imide proton (Scheme 4) located

SCHEME 7: Local and Global pK$_a$ of Major Tautomers of Gh Evaluated During This Study

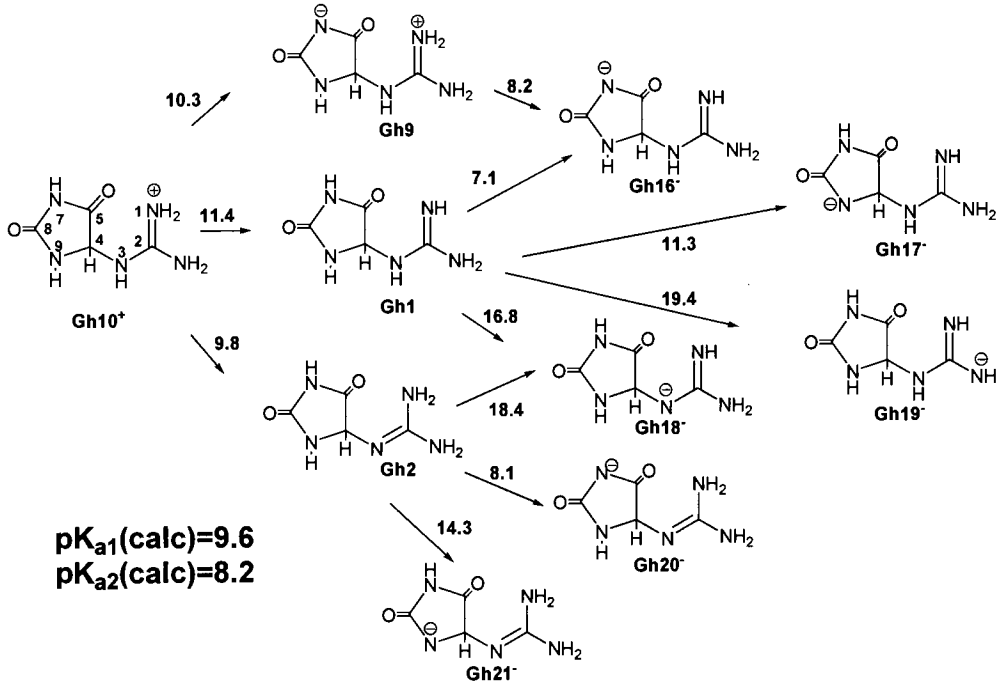


TABLE 5: Relative Free Energies (kcal/mol) and Population of Neutral Tautomers of Sp

	Sp1	Sp2	Sp3	Sp4	Sp5	Sp6	Sp7	Sp8
Gas Phase[a]								
$\Delta G^{\circ}_{(g)rel}$	0.0	1.4	1.3	2.0	19.5	19.7	19.7	12.2
population	0.81	0.08	0.08	0.03	4.1×10^{-15}	3.0×10^{-15}	2.8×10^{-15}	8.6×10^{-10}
Aqueous Phase[b]								
$\Delta G^{*}_{(aq)rel}$	0.0	3.7	6.8	7.0	19.9	19.7	22.1	19.1
population	1.0	1.8×10^{-3}	1.1×10^{-5}	7.0×10^{-6}	2.6×10^{-15}	3.6×10^{-15}	6.8×10^{-17}	1.1×10^{-14}

	Sp9	Sp10	Sp11	Sp12	Sp13	Sp14	Sp15
Gas Phase[a]							
$\Delta G^{\circ}_{(g)rel}$	19.9	20.3	18.2	20.0	19.5	19.3	15.6
population	2.1×10^{-15}	1.1×10^{-15}	3.7×10^{-14}	1.8×10^{-15}	3.9×10^{-15}	6.0×10^{-15}	3.2×10^{-12}
Aqueous Phase[b]							
$\Delta G^{*}_{(aq)rel}$	16.2	15.9	17.5	23.1	22.9	24.2	22.1
population	1.3×10^{-12}	2.1×10^{-12}	1.4×10^{-13}	1.2×10^{-18}	1.7×10^{-17}	1.8×10^{-18}	6.3×10^{-17}

[a] Relative energies with respect to $\Delta G^{\circ}_{(g)}$ for **Sp1**. [b] Relative energies with respect to $\Delta G^{*}_{(aq)}$ for **Sp1**.

between the two carbonyl groups was more acidic than the N1H amide proton. Similar to the data for the other Gh ionization states, the enol anions are much higher in energy than the diketo tautomers. As a consequence, in the gas phase, the population of anionic Gh is predicted to be 98% **Gh16**$^-$ and 2% **Gh20**$^-$. In aqueous solution, the N3−C2 imine species, **Gh20**$^-$, is 0.9 kcal/mol more stable than **Gh16**$^-$ and the population ratio is predicted to shift to 18% **Gh16**$^-$ and 82% **Gh20**$^-$.

pK$_a$ of Guanidinohydantoin. Site-specific pK_a values (pK_a^{ij}) for deprotonation of the various cationic and neutral tautomers of Gh were calculated from the change in free energy for each of the identified reactions using eqs 13–15 and are provided in Supporting Information Table S9. The global pK_a of Gh was calculated using the Boltzmann weighting method outlined by Goddard[25,33] (eq 1) and assumes rapid equilibrium between the various tautomers in aqueous solution. The calculated values for site-specific and global deprotonation of the major tautomers of Gh are summarized in Scheme 7. The predicted pK_{a1} value suggests that deprotonation of the Gh cation will likely occur at a pH of 9.6 at either the N3 position to form **Gh2** or at the imide (N7) position to form the **Gh9** zwitterionic tautomer. Once

SCHEME 8: Neutral Tautomers of Sp Evaluated During This Study

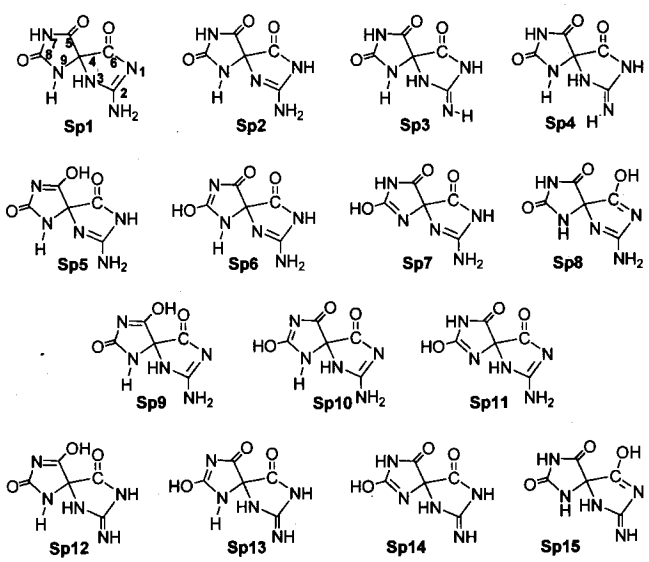
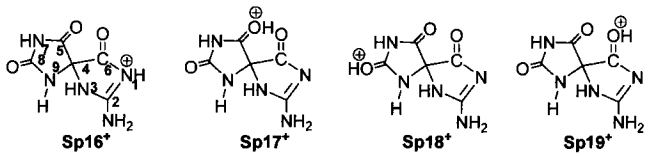


TABLE 6: Relative Free Energies (kcal/mol) and Population of Cationic Tautomers of Sp

	$Sp16^+$	$Sp17^+$	$Sp18^+$	$Sp19^+$
Gas Phase[a]				
$\Delta G^{\circ}_{(g)rel}$	0.0	21.1	15.7	26.6
population	1.0	3.7×10^{-16}	3.0×10^{-12}	3.0×10^{-20}
Aqueous Phase[b]				
$\Delta G^{*}_{(aq)rel}$	0.0	na[c]	22.3	26.0
population	1.0	na[c]	4.3×10^{-17}	8.2×10^{-20}

na = not available. [a] Relative energies with respect to $\Delta G^{\circ}_{(g)}$ for **Sp16**$^+$. [b] Relative energies with respect to $\Delta G^{*}_{(aq)}$ for **Sp16**$^+$. [c] Data not available.

SCHEME 9: Cationic Tautomers of Sp Evaluated During This Study



formed, the calculated pK_{a2} of 8.2 indicates that these neutral species should almost immediately undergo a second deprotonation to form the two N7 anion species, **Gh16**$^-$ and **Gh20**$^-$. The data suggests that Gh will behave in a similar manner to its two subunits: guanidine ($pK_a = 13.7$) and hydantoin ($pK_a = 9.16$) but is slightly more acidic than either moiety alone. Using the same computational method discussed previously, the calculated pK_a values for guanidine and hydantoin are predicted to be 14.5 and 11.0, respectively. The increase in acidity of Gh is not unexpected given that some substituted guanidines and hydantoins demonstrate similar behavior. The experimental pK_a values for acetylguanidine and 5,5 diphenylhydantoin (phenytoin) are 8.32 and 8.31, respectively. The method described in this study predicts values of 8.5 for acetyl guanidine and 8.7 for phenytoin (Table 1, Supporting Information Scheme S7 and Tables S6 and S7). Experimental values for Gh have not been published to date; however, our results are consistent with SciFinder Scholar's online database for Gh, which lists empirically predicted pK_{a1} and pK_{a2} values of 15.83 ± 0.40 and 12.17 ± 0.70 (calculated using Advanced Chemistry Development software V8.14.).[104] Using the same software, the predicted values for guanidine and hydantoin are 13.27 ± 0.70 and 8.7 ± 0.50, respectively.

Neutral Tautomers of Spiroiminodihydantoin. The relative free energies and populations in the gas and aqueous phases of the various neutral tautomers of Sp considered in this study are given in Table 5 and in Tables S10 and S11 of the Supporting Information; their structures are provided in Scheme 8.

In the gas phase, the free energy of each neutral species increases in the following order: **Sp1** < **Sp3** ≈ **Sp2** < **Sp4** ≪ **Sp8** < **Sp15** < **Sp11** < **Sp14** < **Sp5**, **Sp13** < **Sp6**, **Sp7** < **Sp9** < **Sp12** < **Sp14**. Of the 15 tautomers, the 4 triketo species — **Sp1**−**Sp4** — are estimated to be the most stable species with a free energy difference between them of 2 kcal/mol. The remaining 11 tautomers are imine enol species and are at least 10−18 kcal/mol higher in energy than **Sp1**−**Sp4**, making their population negligible. In the gas phase, the equilibrium concentration is predicted to be 81% **Sp1** (the C2−N1 imine), 8% **Sp2** (the N3−C2 imine), 8% **Sp3** (a C2−N10 imine), and 3% **Sp4** (a rotamer of **Sp3**).

In aqueous solution, the free energy of the tautomers increases in a slightly different order: **Sp1** < **Sp2** < **Sp3** ≈ **Sp4** ≪ **Sp10** ≈ **Sp9** < **Sp11** < **Sp8** < **Sp6** ≈ **Sp5** < **Sp7**, **Sp15** < **Sp13** < **Sp12** < **Sp14**. The imine enol species—**Sp5**−**Sp15**—are again predicted to be significantly less stable than the four triketo tautomers, **Sp1**−**Sp4**. These results are also consistent with the experimental and theoretical data for hydantoin, which indicates that the keto species is thermodynamically preferred over the enol tautomers.[97−101] In aqueous solution, the difference in energy between **Sp1**, the lowest energy species, and **Sp2**, **Sp3**, and **Sp4** is 3.7, 6.8, and 7.0 kcal/mol, respectively. Therefore, at equilibrium, an aqueous solution of Sp will almost exclusively consist of the **Sp1** C2−N1 imine tautomer.

Cationic Tautomers of Spiroiminodihydantoin. Protonation of Sp can occur at the N1 position and on the oxygen of the carbonyl groups at C5, C6, or C8. The relative free energies and populations in the gas and aqueous phases of the four cationic tautomers of Sp considered in this study are given in Table 6; their structures are provided in Scheme 9. Both the gas and solution phase data indicate that **Sp16**$^+$ is approximately 15−26 kcal/mol lower in energy than the other three cations, suggesting that Sp is almost exclusively protonated at a guanidinyl nitrogen rather than at one of the carbonyl oxygens.

Anionic Tautomers of Spiroiminodihydantoin. Depending on the neutral triketo tautomer, deprotonation of Sp can occur at the N1, N3, N7, or N9 positions of the hydantoin rings or from the terminal amino group at C2. For the imine enol tautomers, deprotonation can also occur at the C5, C6, or C8 hydroxyl groups. The relative free energies and populations in the gas and aqueous phases of the 12 anion tautomers of Sp considered in this study are given in Table 7; their structures are provided in Scheme 10. In the gas phase, the most stable tautomers are **Sp23**$^-$ (0.0 kcal/mol), **Sp22**$^-$ (0.86 kcal/mol), **Sp26**$^-$ (1.7 kcal/mol), and **Sp29**$^-$ (3.3 kcal/mol). The free energy of the remaining anionic tautomers increases in the following order: **Sp20**$^-$ < **Sp31**$^-$ ≈ **Sp27**$^-$ < **Sp30**$^-$ < **Sp28**$^-$ ≈ **Sp21**$^-$ ≈ **Sp24**$^-$ < **Sp25**$^-$. In equilibrium in the gas phase, the composition is predicted to comprise 77% **Sp23**$^-$, 18% **Sp22**$^-$, and 4% **Sp26**$^-$.

In aqueous solution, the data suggest that the equilibrium composition will consist almost exclusively of the **Sp20**$^-$ N7 anion triketo tautomer. All other tautomers are at least 4.5 kcal/mol higher in energy and will therefore represent less than 0.05% of the equilibrium population.

pK_a of Spiroiminodihydantoin. Site specific pK_a values (pK_a^{ij}) for deprotonation of the various cationic and neutral tautomers of Sp were calculated from the change in free energy for each of the identified reactions using eqs 13–15 and are provided in Supporting Information Table S10. The global pK_a of Sp was calculated using the Boltzmann weighting method discussed previously (eq 1). The calculated values for site-specific and global deprotonation of the major tautomers of Sp are summarized in Scheme 11. The predicted pK_{a1} value suggests that deprotonation of the Sp cation will likely occur at a pH of 0.5 and will most likely result from a loss of a proton at the N1 position. This calculated value is in line with both the experimentally observed (~0.1) and calculated (−0.3) values for deprotonation of 8-oxoguanine.[23,25,33] Experimental data measuring the pK_a of Sp are not available; however, SciFinder Scholar's online database for Sp lists empirically predicted values of $pK_{a1} = -0.19$ and $pK_{a2} = 7.79$ (calculated using Advanced Chemistry Development software V8.14.).[104]

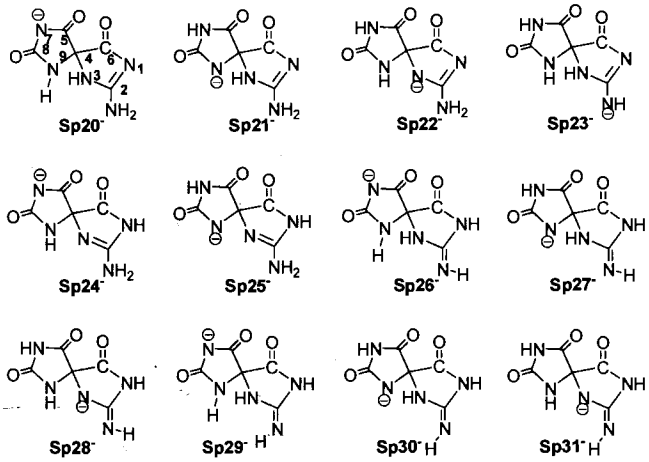
Once formed, the calculated pK_{a2} of 4.8 for deprotonation of neutral Sp indicates that this molecule is significantly more acidic than Gh ($pK_{a2} = 8.2$). A close review of the free energy data for the anion tautomers shows that the values for the global pK_{a2} of Sp and Gh are predominately driven by deprotonation

TABLE 7: Relative Free Energies (kcal/mol) and Population of Anionic Tautomers of Sp

	Sp20$^-$	Sp21$^-$	Sp22$^-$	Sp23$^-$	Sp24$^-$	Sp25$^-$
Gas Phase[a]						
$\Delta G^{\circ}_{(g)rel}$	5.15	11.8	0.86	0.0	12.0	18.2
population	1.3×10^{-4}	1.6×10^{-9}	0.18	0.77	1.2×10^{-9}	3.3×10^{-14}
Aqueous Phase[b]						
$\Delta G^{*}_{(aq)rel}$	0.0	5.3	4.6	7.4	4.5	10.5
population	1.0	1.4×10^{-4}	4.0×10^{-4}	3.8×10^{-6}	4.7×10^{-4}	2.1×10^{-8}
	Sp26$^-$	**Sp27$^-$**	**Sp28$^-$**	**Sp29$^-$**	**Sp30$^-$**	**Sp31$^-$**
Gas Phase[a]						
$\Delta G^{\circ}_{(g)rel}$	1.7	6.9	11.6	3.3	8.5	6.4
population	0.04	7.2×10^{-6}	2.4×10^{-9}	2.9×10^{-3}	4.7×10^{-7}	1.5×10^{-5}
Aqueous Phase[b]						
$\Delta G^{*}_{(aq)rel}$	5.9	11.4	14.5	6.0	11.3	14.4
population	4.6×10^{-5}	4.5×10^{-9}	2.2×10^{-11}	3.9×10^{-5}	5.5×10^{-9}	2.8×10^{-11}

[a] Relative energies with respect to $\Delta G^{\circ}_{(g)}$ for **Sp23$^-$**. [b] Relative energies with respect to $\Delta G^{*}_{(aq)}$ for **Sp20$^-$**.

SCHEME 10: Anionic Tautomers of Sp Evaluated During This Study



at the N7 position of each molecule. These results are consistent with NMR data indicating that the imide proton in both unmodified and 5,5-disubstituted hydantoins (i.e., the N3H proton, Scheme 4) is also the most acidic ring proton.[97] For Sp, based upon the Boltzmann weighting, the global pK_{a2} is almost exclusively represented by the deprotonation of **Sp1** at N7 leading to formation of **Sp20$^-$**. For Gh, three reactions contribute to the global pK_{a2}: **Gh1** → **Gh16$^-$** (pK_a = 7.1), **Gh2** → **Gh20$^-$** (pK_a = 8.1); and **Gh9** → **Gh16$^-$** (pK_a = 8.2). The first two reactions are deprotonations at the N7 position; the third is a deprotonation from N1 of the guanidinyl subunit of the zwitterion.

The predicted pK_a value of 4.8 for Sp seems surprisingly low for a substituted hydantoin; however, experimental data indicates that the pK_a of hydantoins is very much affected by substituents at N1 and C5 of the ring.[97] The experimentally measured pK_a of 5,5-diphenyl hydantoin, sold commercially as phenytoin, is 8.31, almost one full unit lower than the unmodified moiety.[105,106] The predicted value for phenytoin using the method described in this study, is 8.7 (Table 1). Modification of phenytoin at the N1 position to yield 1-phenylsulfonyl-5,5-diphenyl hydantoin, reduces the pK_a of the resulting substituted hydantoin to 4.89.[104,105]

In an effort to examine in detail, the factors contributing to the predicted difference in acidity between Sp and Gh, the contribution of each energy component of the calculation to the site specific pK_a was evaluated and is presented in Table 8. Through-space substituent effects were also evaluated and are presented in the same table. Comparing the deprotonation of **Sp1** to **Gh2** (Gh N3 imine), the major neutral Gh tautomer, the difference in acidity is being driven by the gas phase free energy of the N7 anion relative to its neutral moiety. In the case of **Gh1** (C2N1 imine), and the zwitterion, **Gh9,** the difference in acidity is a consequence of the greater free energy of solvation associated with the Sp anion, **Sp20$^-$**.

The hydantoin ring containing the N7 proton is the same structure for both Gh and Sp. One key difference between the two molecules is the presence of a carbonyl group located at the C6 position of Sp. The optimized geometry of Sp orients this carbonyl group directly over the hydantoin ring, and it is possible that the lone pair electrons of the oxygen are influencing the acidity of the imine proton (Figure 2). Through-space electronic effects[107] on the acidity of the imine (N7) and amine (N9) protons were evaluated by performing calculations on Sp by replacing the carbonyl at the C6 position with an ethylene group (C=CH$_2$) and a CH$_2$ group (Table 8). Deprotonation of four neutral tautomers of SpC6CH$_2$ at the N7 position was evaluated (Supporting Information Table S17 and S18). The predicted pK_a of Sp increases significantly as the electron-withdrawing nature of the substituent decreases with calculated values of 6.6 for **Sp(C6=CH$_2$)** and 11.9 for **Sp(C6H$_2$)**. Visual inspection of the predicted highest occupied molecular orbitals (HOMOs) for **Sp1**, **Sp(C6=CH$_2$)**, **Sp(C6H$_2$)** and **Gh1** (Figure 2) indicate that there is significant orbital overlap between the hydantoin ring of **Sp1**and the electrons of the C6 carbonyl group. This overlap is not observed for the other species. Through-space electronic effects of the lone-pair may therefore be partly responsible for the predicted increase in acidity of Sp vs Gh.

As indicated by the data in Table 7, the predicted relative population of Sp anions in the gas phase and in aqueous solution are significantly different. The difference is, in large part, the result of the approximately 10 kcal/mol larger free energy of solvation of the Sp N7 and N9 anions — **Sp20$^-$**, **Sp21$^-$**, **Sp24$^-$**, and **Sp25$^-$** — relative to the other anionic tautomers of Sp (Supporting Information Table S9). Careful review of the solvation data for these four N7 and N9 anionic tautomers indicates that they also have larger solution phase dipole moments (15.2−20.9 debye) relative to the other tautomers (4.6−13.7) due to the location of the anion and the lone pairs of the three carbonyl groups. It is possible that this large polarization of the solute cavity may result in an overestimation

SCHEME 11: Site-specific and global pK_a of major tautomers of Sp evaluated during this study

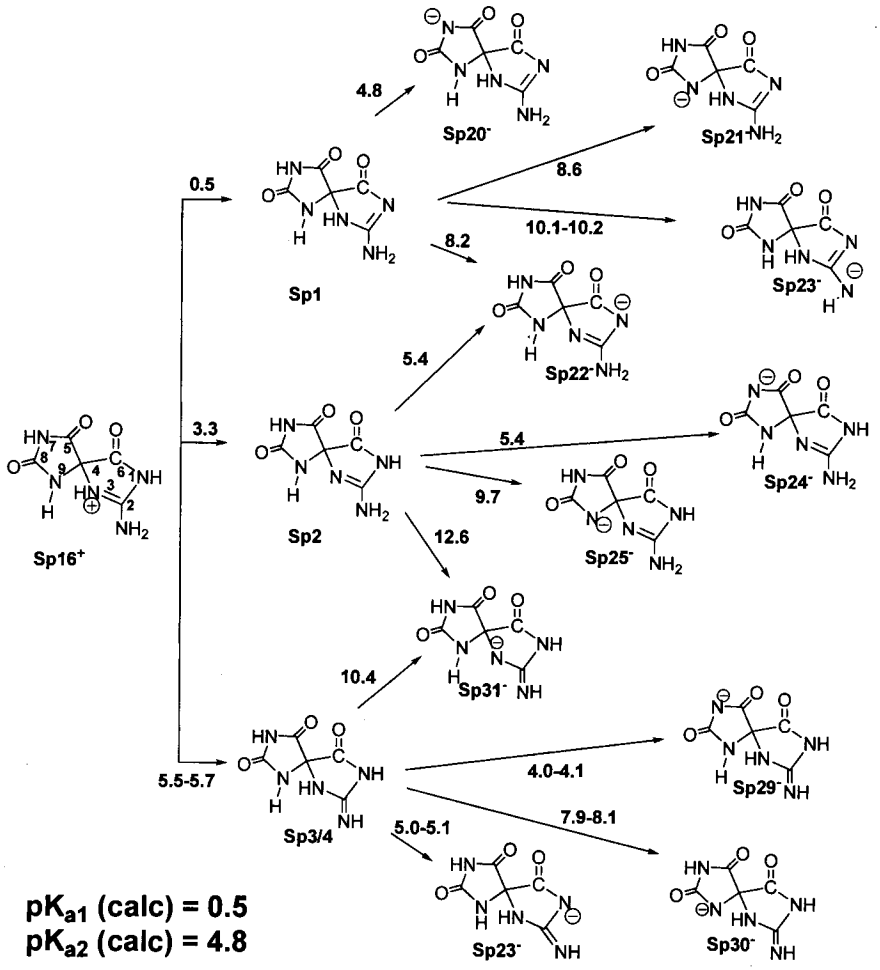
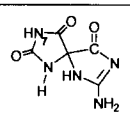
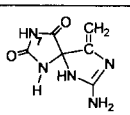
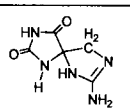
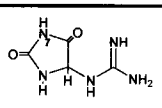
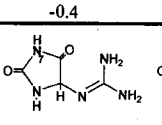
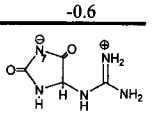


TABLE 8: Acidity of N7 Proton: Comparison of Sp1 with Sp1 (C6=CH_2), Sp (C6CH_2), Gh1, Gh2, and Gh9

Site-Specific H^+ loss at N7			
Energy Components[a]	**Sp1 (C6=O) (1.0)[b]**	**Sp1 (C6=CH_2)**	**Sp1 (C6H_2)**
$\Delta G_{(g)}$	325.26	328.26	330.27
$\Delta\Delta G^{R'}_{(sol)}$	-56.90	-56.84	-51.37
$\Delta E_{(dis)}$	2.11	1.58	1.28
Site-Specific pK_a	**4.8**	**6.6**	**11.9**
Δ pK_a vs Sp5		*+1.8*	*+7.1*
diff $G_{(g)}$		+2.2	+3.7
diff $G_{(sol)}$		+0.0	+4.0
diff $\Delta E_{(dis)}$		-0.4	-0.6
Site-Specific H^+ loss at N7			
Energy Components[a]	**Gh1 (0.02)[b]**	**Gh2 (0.76)[b]**	**Gh9 (0.22)[b]**
$\Delta G_{(g)}$	325.81	334.47	306.36
$\Delta\Delta G^{R'}_{(sol)}$	-54.30	-60.63	-29.7
$\Delta E_{(dis)}$	2.17	1.22	-1.43
Site-Specific pK_a	**7.1**	**8.1**	**8.2**
Δ pK_a vs Sp5	*+2.3*	*+3.3*	*+3.4*
diff $G_{(g)}$	+0.4	+6.7	-13.9
diff $G_{(sol)}$	+1.9	-2.7	+19.9
diff $\Delta E_{(dis)}$	+0.0	-0.7	-2.6

[a] All energies are expressed in kcal/mol and represent the change in energy resulting from deprotonation of the neutral molecule at N7. [b] Boltzmann weighting of neutral molecule.

of the free energy of solvation by the PCM model and a correspondingly low predicted pK_a value for this molecule. A similar effect was noted in the calculation of the pK_a of formylguanidine where a value of 6.7 was obtained. One of

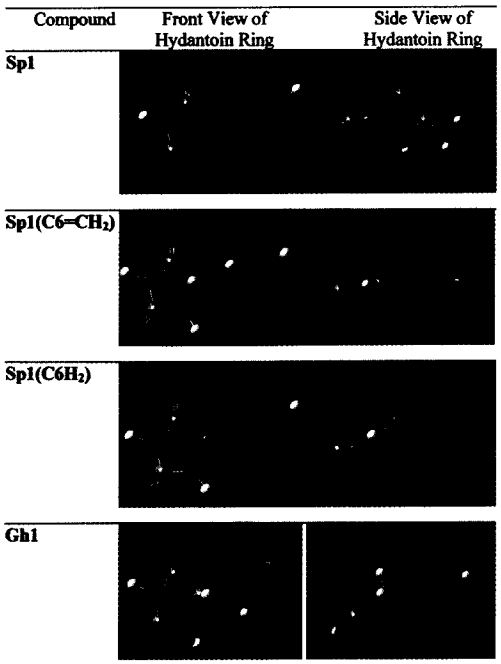


Figure 2. Comparison of HOMO molecular orbitals for **Sp1**, **Gh1**, **Sp1(C6=CH$_2$)** and **Sp1(C6H$_2$)**.

the tautomers, **FG8** (Supporting Information Scheme S6), had a calculated free energy of solvation nearly twice that of the other species and a local pK_a value of 5.0. No experimental value for N-formylguanidine was found in the literature; however, SciFinder Scholar's online database lists an empirically predicted value of pK_{a1} = 7.75 for this molecule (calculated using Advanced Chemistry Development software V8.14).[104]

Upon the basis of the analysis provided above, we believe that the observed pK_{a2} value for Sp is likely to be lower than that of Gh but higher than the 4.8 value predicted by the computational model described in this study. Calculations conducted with a methylene subtituent at the C6 carbon instead of a carbonyl group suggest that the pK_{a2} value for Sp may be in the range of 6.6−7.7 (Supporting Information Table S-18).

4. Conclusions

An efficient computational method has been identified that uses B3LYP density functional theory, IEF-PCM solvation modeling with a modified UFF cavity, and Boltzmann weighting of tautomers to predict the site-specific and global pK_a of DNA nucleobases and their oxidation products. The method is shown to be capable of predicting the global pK_a of the DNA nucleobases guanine, 8-oxoG, adenine, cytosine, and thymine to within 0.6 pK_a units of their experimental value. Predictions of the experimental values of phenytoin and N-acetylguanidine were within 0.4 pK_a units. The method works less-well for smaller molecules such as guanidine and hydantoin, where the calculated values are higher by 0.8 and 1.9 pK_a units, respectively.

The method has been used to evaluate the acidity of Gh and Sp, two highly mutagenic guanine oxidation products. The trend observed for the pK_a values of Gh (9.6 and 8.2) is consistent with the experimentally observed values for guanidine cation (13.7) and hydantoin (9.16). Molecular orbital predictions of the HOMO indicate that there is very little interaction between the hydantoin and guanidine subunits of Gh.

The pK_{a1}(calc) value for deprotonation of Sp cation ($Sp^+ \rightarrow$ Sp) is very close to the experimentally observed pK_{a1} for 8-oxoG and is consistent with the similarity in their structures. The data suggest that the imide (N7) proton in Sp may be more acidic than that in Gh, possibly due to the presence of the through-space electronic effects of the carbonyl group located at C6. This difference in the acidity of Gh and Sp may be an indication of their potential toxicity and mutagenicity in vivo and remains a fertile area for experimental study.

Acknowledgment. This work was supported by a grant from the National Science Foundation (CHE 0512144) and by financial support from the Italian Ministero dell' Istruzione, Universita' e Ricerca (PRIN2005), and Gaussian, Inc. The authors wish to thank Dr. Jason Sonnenberg for many helpful discussions and the C&IT and Chemistry Departments at Wayne State University for computer time.

Supporting Information Available: Data used in the calculation of site-specific and global pK_as for the various species discussed in this manuscript are provided in the Supporting Information. The molecular geometries in Cartesian coordinates for the tautomers optimized in the gas phase are also provided. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

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JP8068877

USER SAMPLE ID	40000009202.0000013867
MATERIAL	2-56291
MATERIAL TYPE	SITAGLIPTIN FREEBASE

Submission level Approval

SUBMISSION APPROVED

Blanca M. Guzman 03-JUN-2009 02:52 PM

Electronic Signature

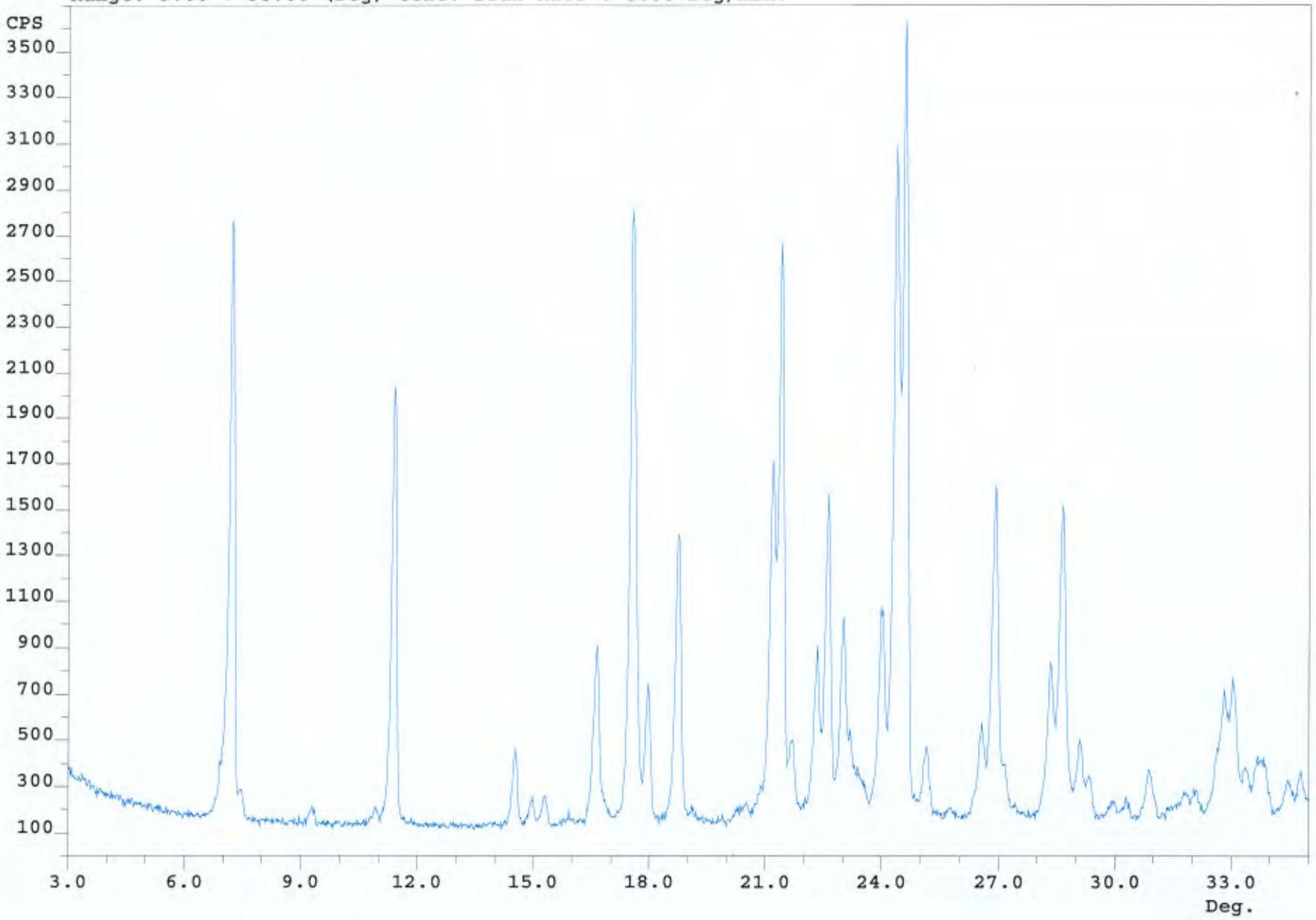
SAMPLE ID	200135006	**LOGIN DATE**	22-MAY-2009 06:56 AM
SAMPLE PLAN	3001652.04	**STATUS**	COMPLETE
DATAGROUP	API_BULK_PRODUCTS		
BATCH NUMBER	0000013867	**LEGEND**	* Regulatory Requirements
LEGEND	** Internal Requirements	**LOT NUMBER**	N/A
QS EDITION DATE	09-Dec-2007	**REVISION NUMBER**	2
SAMPLES RECEIVED	1		

Test	Specification	Result	Pass/Fail	Analyst
CHIRAL PURITY (HPLC)	Max. 0.5% *	0.3 %	PASS	BESALELI
TRIAZOLE	Max. 0.1% *	<= 0.05 %	PASS	BESALELI
2,5-DIFLUORO	Max. 0.2% *	<= 0.05 %	PASS	BESALELI
2,4-DIFLUORO	Max. 0.2% *	<= 0.05 %	PASS	BESALELI
KETO AMIDE	Max. 1.6% *	<= 0.05 %	PASS	BESALELI
METHYL ESTER	Max. 0.1% *	<= 0.05 %	PASS	BESALELI
ENAMINE AMIDE	Max. 7.5% *	0.2 %	PASS	BESALELI
OLEFIN 1	Max. 0.4% *	0.1 %	PASS	BESALELI
OLEFIN 2	Max. 2.3% *	0.6 %	PASS	BESALELI
ELIMINATION	Max. 1.9% *	0.4 %	PASS	BESALELI
RRT 2.85	Max. 0.4% *	0.2 %	PASS	BESALELI
DIMER	Max. 4.8% *	0.4 %	PASS	BESALELI
ANY UNSPECIFIED IMPURITY	Max. 0.1% *	CONFORMS	PASS	BESALELI
2,5-DIFLUORO	Max. 0.15% * (Australia / New Zealand)	<= 0.05 %	PASS	BESALELI
2,4-DIFLUORO	Max. 0.15% * (Australia / New Zealand)	<= 0.05 %	PASS	BESALELI
RHODIUM	Max. 20 ppm *	3 ppm	PASS	BESALELI
IRON	Max. 20 ppm *	<3 ppm	PASS	BESALELI
IDENTITY (CHIRAL PURITY)	Conforms to the Chiral Purity specifications	CONFORMS	PASS	BESALELI

Sitagliptin Free Base

J Atwood

File: sitagliptin free base, ID: sitagliptin free base, lot 0000013867
Date: 01/28/11 17:25 Step : 0.020° Cnt Time: 2.000 Sec.
Range: 3.00 - 35.00 (Deg) Cont. Scan Rate : 0.60 Deg/min.



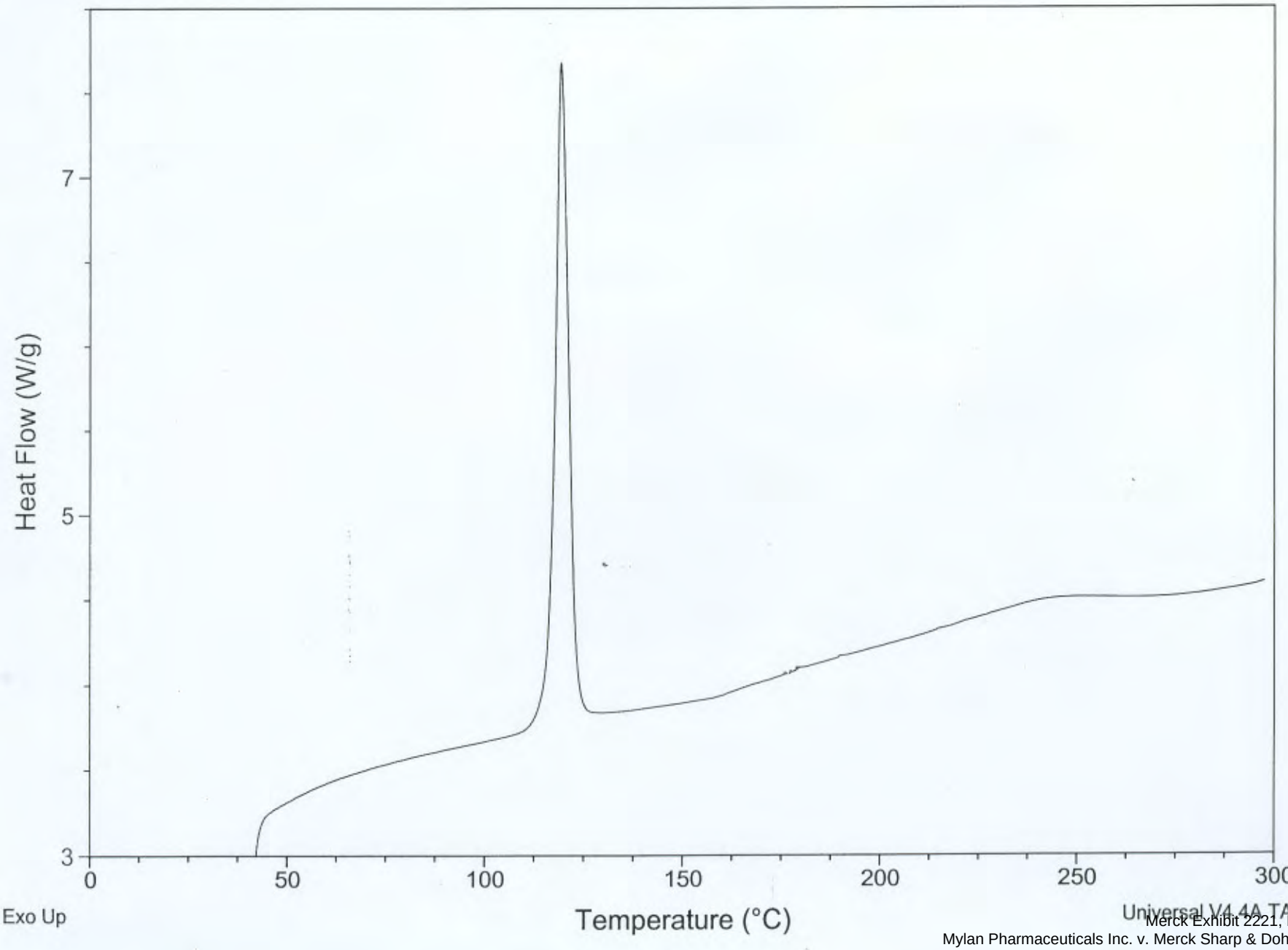
Sitagliptin Free Base

J Atwood

Sample: sitagliptin free base
Size: 3.7000 mg
Method: Ramp
Comment: 13867

DSC

File: C:\TA\Data\Atwood\sitafb.001
Operator: Atwood
Run Date: 20-Apr-2011 19:04
Instrument: DSC Q100 V9.8 Build 296



9/13/10 Preparation of (sitagliptin)$_2$ H_3PO_4

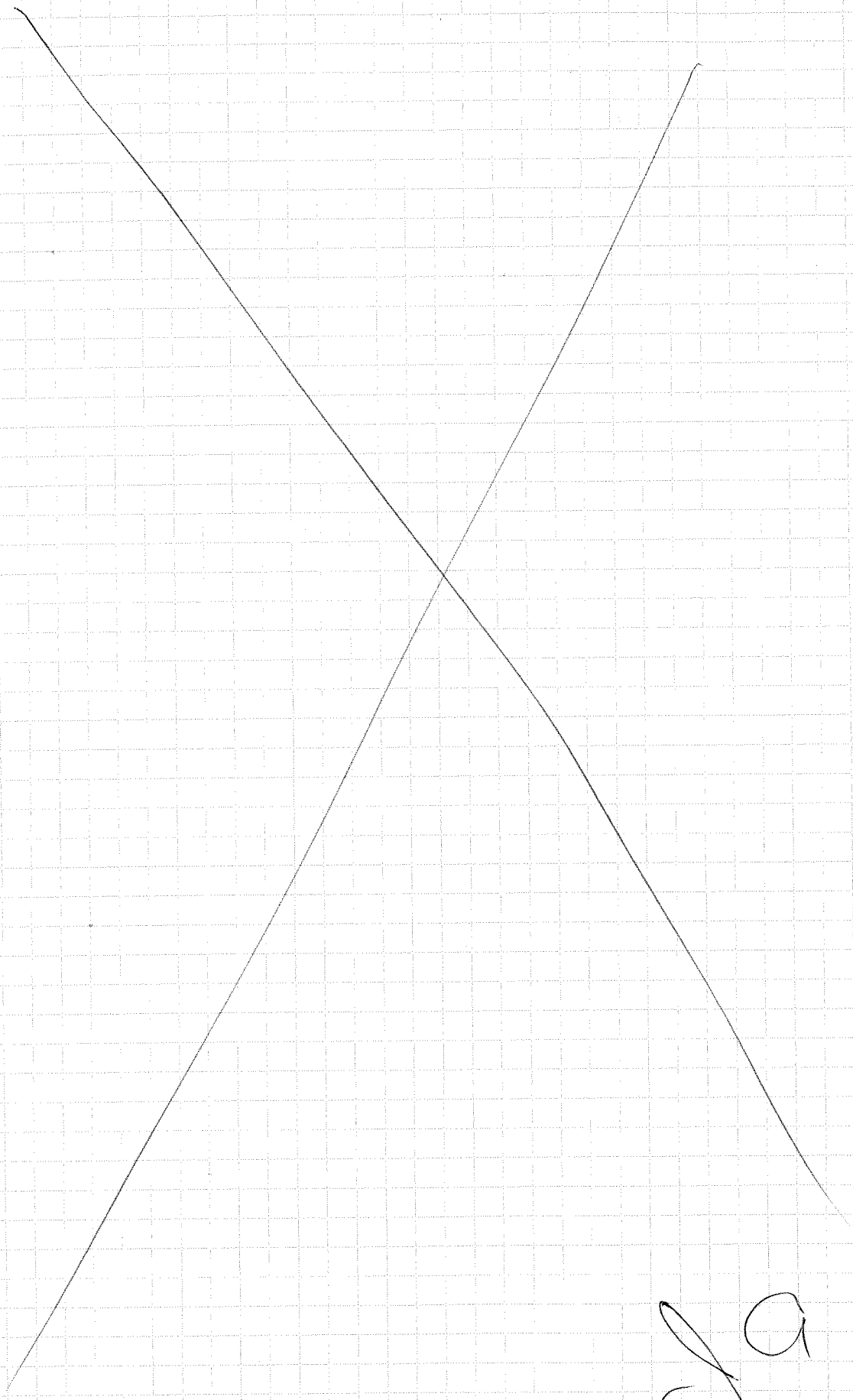
1.50 g sitagliptin base (0.00368 moles, lot No. 000001 3867) was combined with isopropanol (3.2 mL) (Sigma Aldrich) and distilled water (1.4 mL). The mixture was stirred 5-10 min to form a clear solution. To this solution was added 0.215 g H_3PO_4 (85% w/w, Sigma Aldrich, 0.00186 moles) with stirring. The mixture was heated with stirring at 70°C for 15 min, then cooled to rt and left stirring overnight.

Sample 1-1

Preparation of (sitagliptin)$_2$ H_3PO_4

1.50 g sitagliptin base (0.00368 moles, lot No. 000001 3867) was combined with isopropanol (3.2 mL) (Sigma Aldrich) and distilled water (1.4 mL). The mixture was stirred 5-10 min to form a clear solution. To this solution was added 0.215 g H_3PO_4 (85% w/w, Sigma Aldrich, fresh bottle, 0.00186 moles) with stirring. The mixture was heated with stirring at 70°C for 15 min, then cooled to rt and left stirring overnight.

Sample 1-2



Preparation of (sitagliptin)$(H_3PO_4)_2$

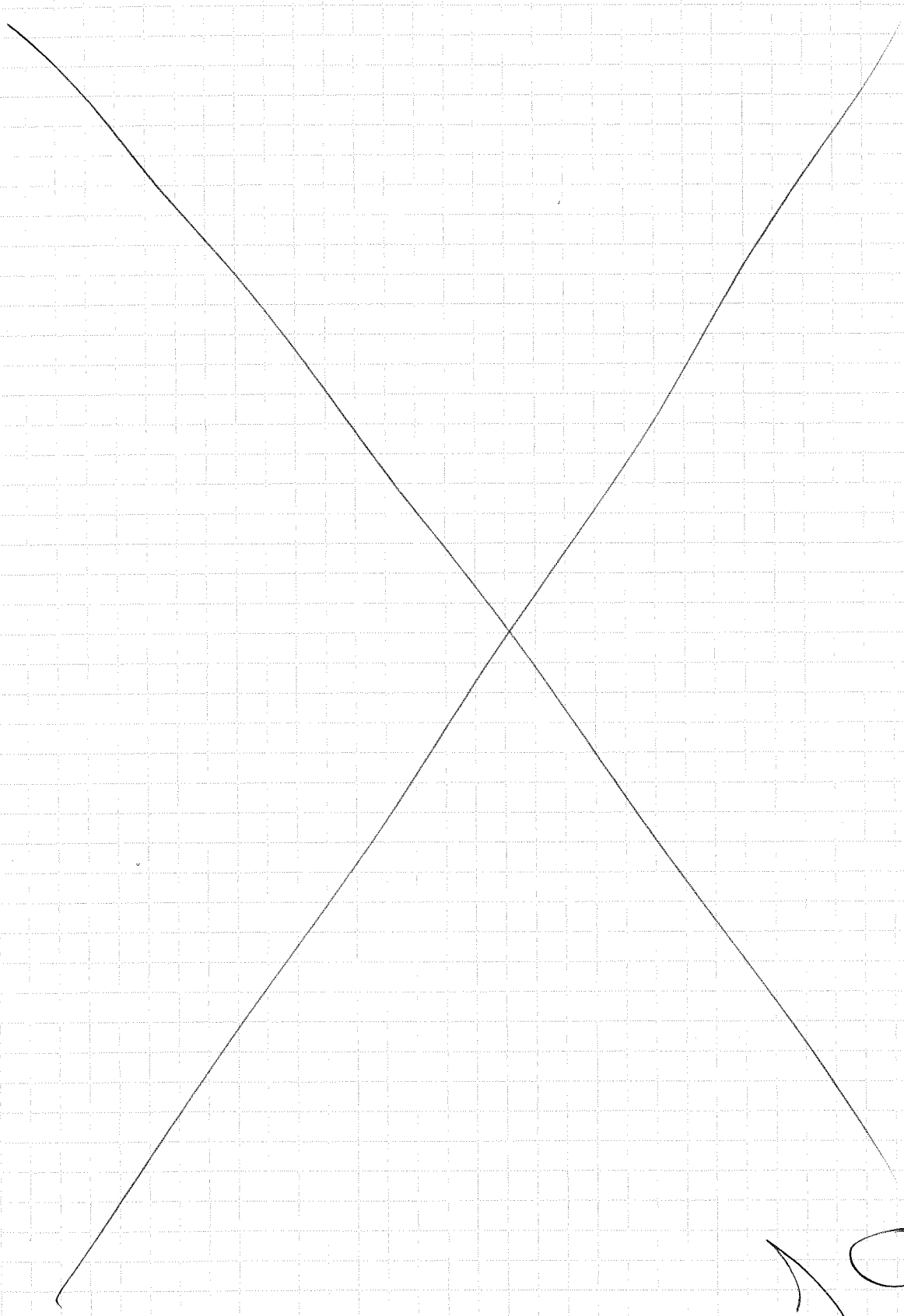
1.50 g sitagliptin base (0.00368 moles, lot No. 000001387) was combined with isopropanol (3.2 mL) and distilled water (1.4 mL). The mixture was stirred for 5-10 min to form a solution. H_3PO_4, 0.85 g (85% w/w, Sigma Aldrich) was added with stirring. The mixture was heated to 70°C with stirring for 15 min, then cooled to rt. The pale yellow solution which formed was left stirring overnight.

Sample 2

Preparation of (sitagliptin)$(NH_4H_2PO_4)$

1.50 g sitagliptin base (0.00368 moles, lot No. 000001387) was combined with isopropanol (3.2 mL) and distilled water (1.4 mL). The mixture was was stirred for 5-10 min to form a solution. $NH_4H_2PO_4$, 0.42 g, Fisher Scientific, was added with stirring. The mixture was heated to 70°C with stirring for 15 min, then cooled to rt to yield a white crystalline powder. The solid was dried overnight at rt under vacuum.

Sample 3



Preparation of (sitagliptin)(NaH_2PO_4)

1.50 g sitagliptin base (0.00368 moles, lot No. 000001 3867) was combined with isopropanol (3.2 mL) and distilled water (1.4 mL). The mixture was stirred 5-10 min to form a solution. NaH_2PO_4, 0.44 g, was added with stirring. The solution was heated to 70°C for 15 min, then cooled to rt. A white crystalline powder formed. The solid was dried overnight at rt under vacuum.

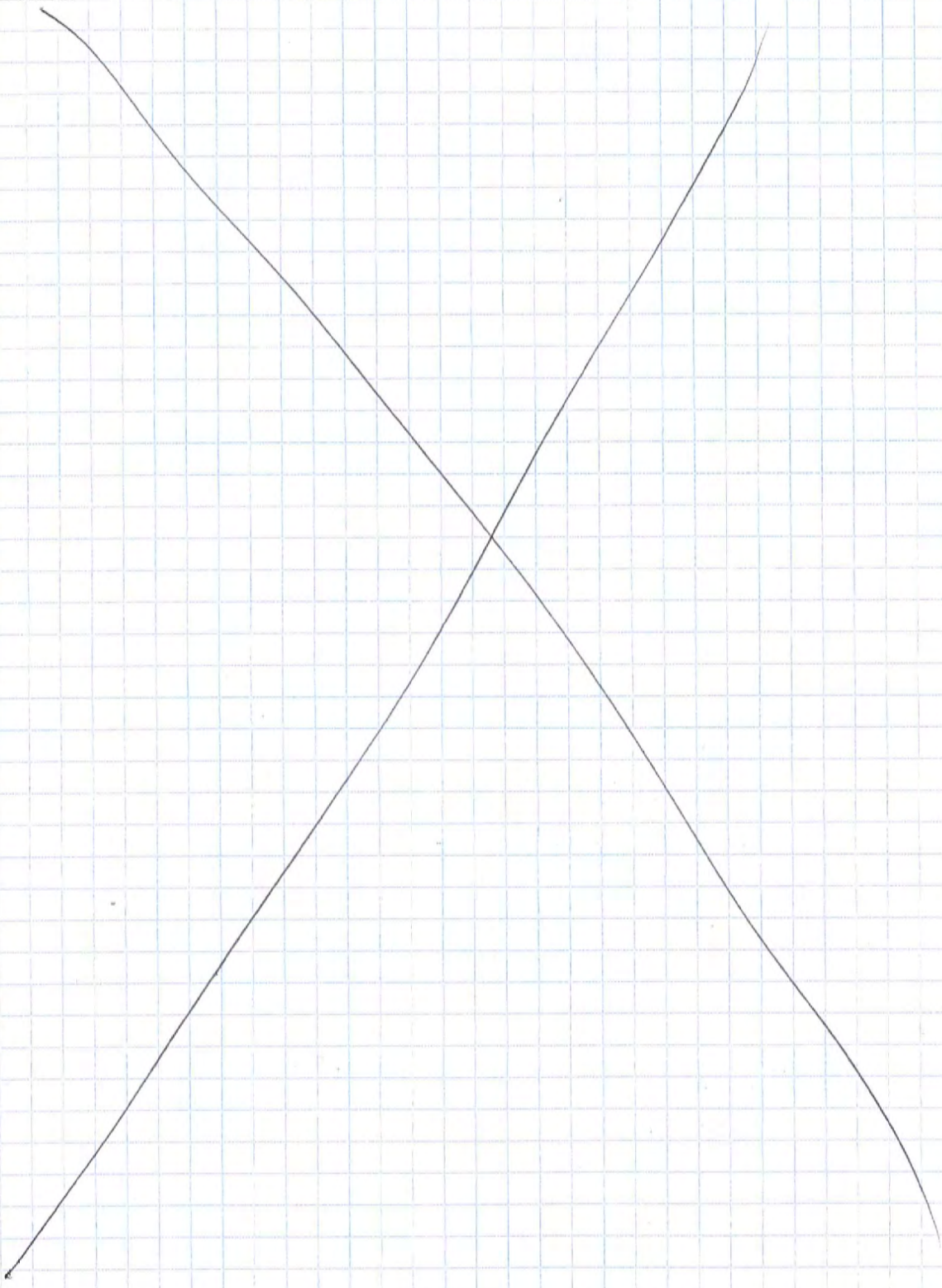
Sample 4

9/14/10 Sample 1-1: The solution had solidified. The white crystalline powder was placed in a vacuum chamber and dried at rt for ca. 6 h. Samples labelled 1-1 were taken for XRPD, DSC, TGA, and elemental analysis.

Sample 1-2 was found to have solidified, the white crystalline powder was treated as for Sample 1-1.

Preparation of (sitagliptin)$_2(NH_4H_2PO_4)$, Sample 5

1.50 g sitagliptin base (0.00368 moles, lot No. 000001 3867) was combined with isopropanol (3.2 mL) and distilled water (1.4 mL).

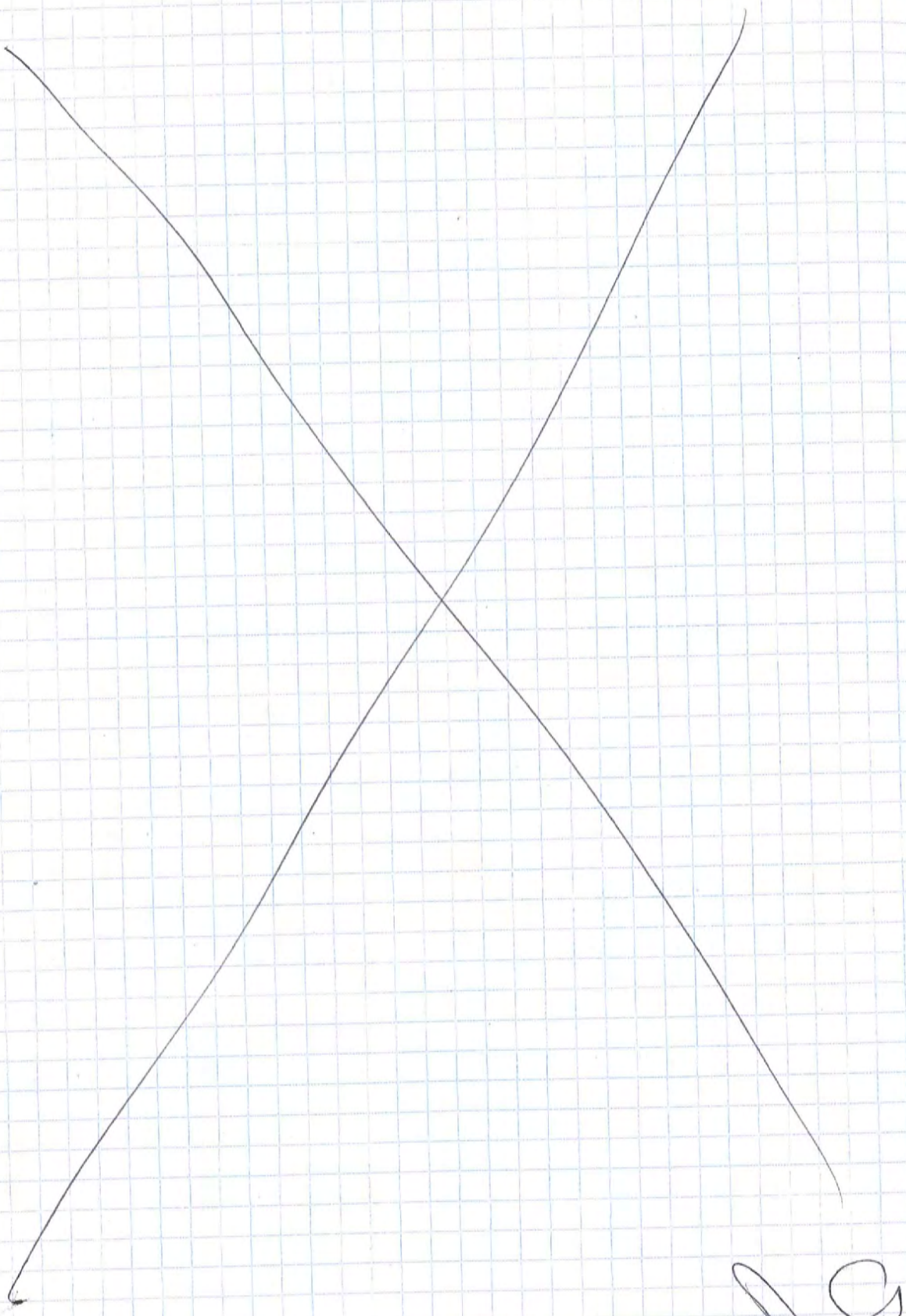


The mixture was stirred for 5-10 min to form a solution. $NH_4H_2PO_4$, 0.21g, was added with stirring. The solution was heated to 70°C for 15 min, then cooled to rt. A white crystal powder formed. The solid was left to dry overnight at rt under vacuum.

Sample 3 - samples labelled Sample 3 were taken for XRPD, DSC, TGA, and elemental analysis.

Sample 4 - samples labelled Sample 4 were taken for XRPD, DSC, TGA, and elemental analysis.

Preparation of (sitagliptin)$_3$(H_3PO_4), Sample 6
1.50 g sitagliptin base (0.00365 mole, lot No. 000001386 7) was combined with isopropanol (3.2 mL) and distilled water (1.4 mL). The mixture was stirred 5-10 min to form a solution. H_3PO_4, 0.07g (85% w/w) was added. The solution was heated to 70°C for 15 min, then cooled to rt. The soln was left at rt under vacuum overnight.

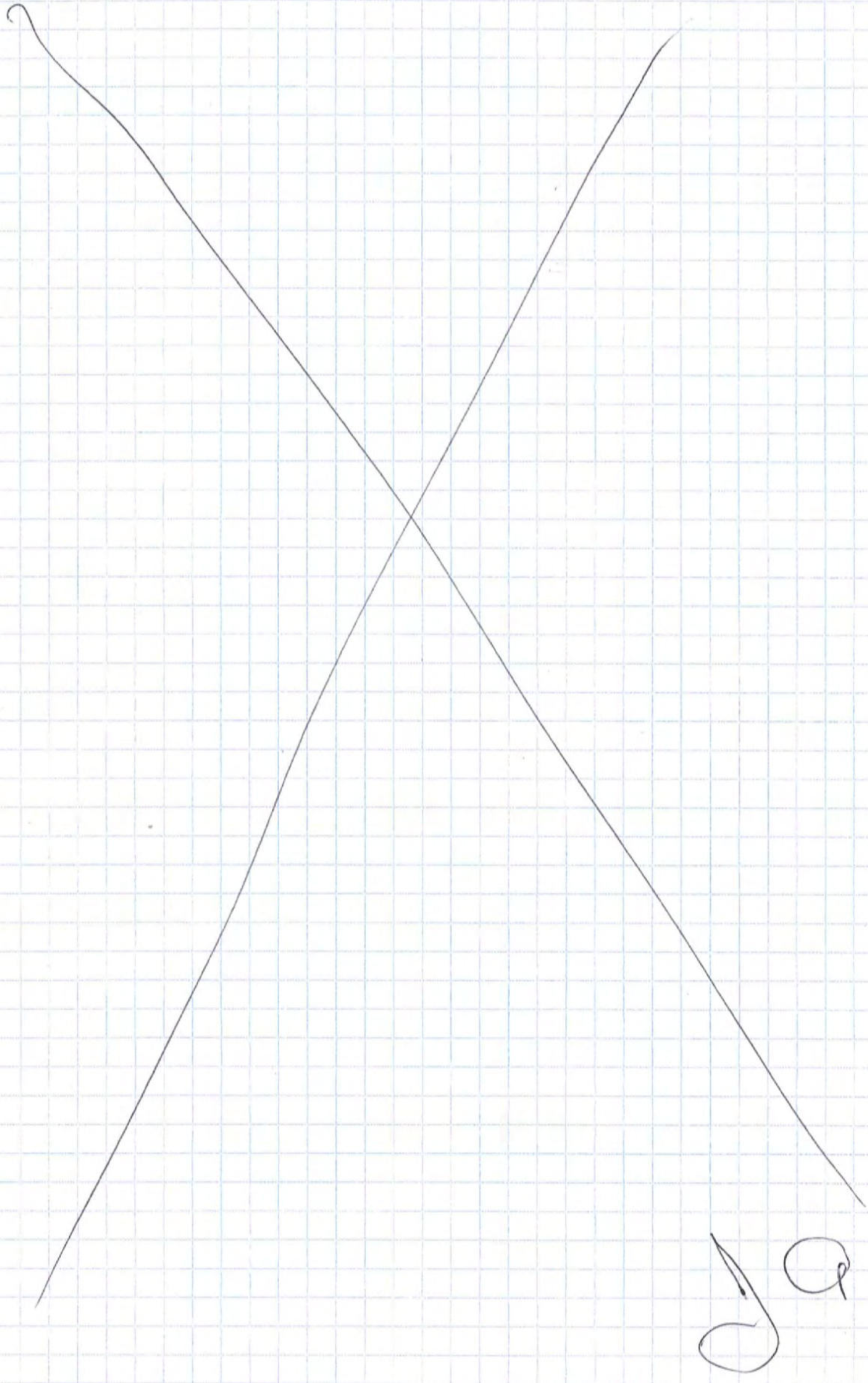


Preparation of (sitagliptin)$_3$(H_3PO_4) ~~Sample~~ 7

1.50 g sitagliptin base (0.00368 moles, lot No. 000001386 7) was combined with isopropanol (3.2 mL) and distilled water (1.4 mL). To this solution with stirring was added 0.36 mL conc. HCl (37%), 0.13 g, to produce sitagliptin hydrochloride, 0.20 g Na_3PO_4 was added with continued stirring. The solution was left overnight at r.t under vacuum.

Preparation of (sitagliptin)$_2$(H_3PO_4)

1.50 g sitagliptin base (0.00368 mole), (lot No. 000001386 7) was combined with methanol (4.6 mL). To the resulting soln was added 0.21 g H_3PO_4 (85% w/w). The solution was heated with stirring to 70°C for 15 min, then cooled to r.t. A white crystalline powder formed which was left for drying overnight under vacuum.



9/15/10 Sample 2 - samples labelled Sample 2 were taken for XRPD, DSC, TGA, and elemental analysis.

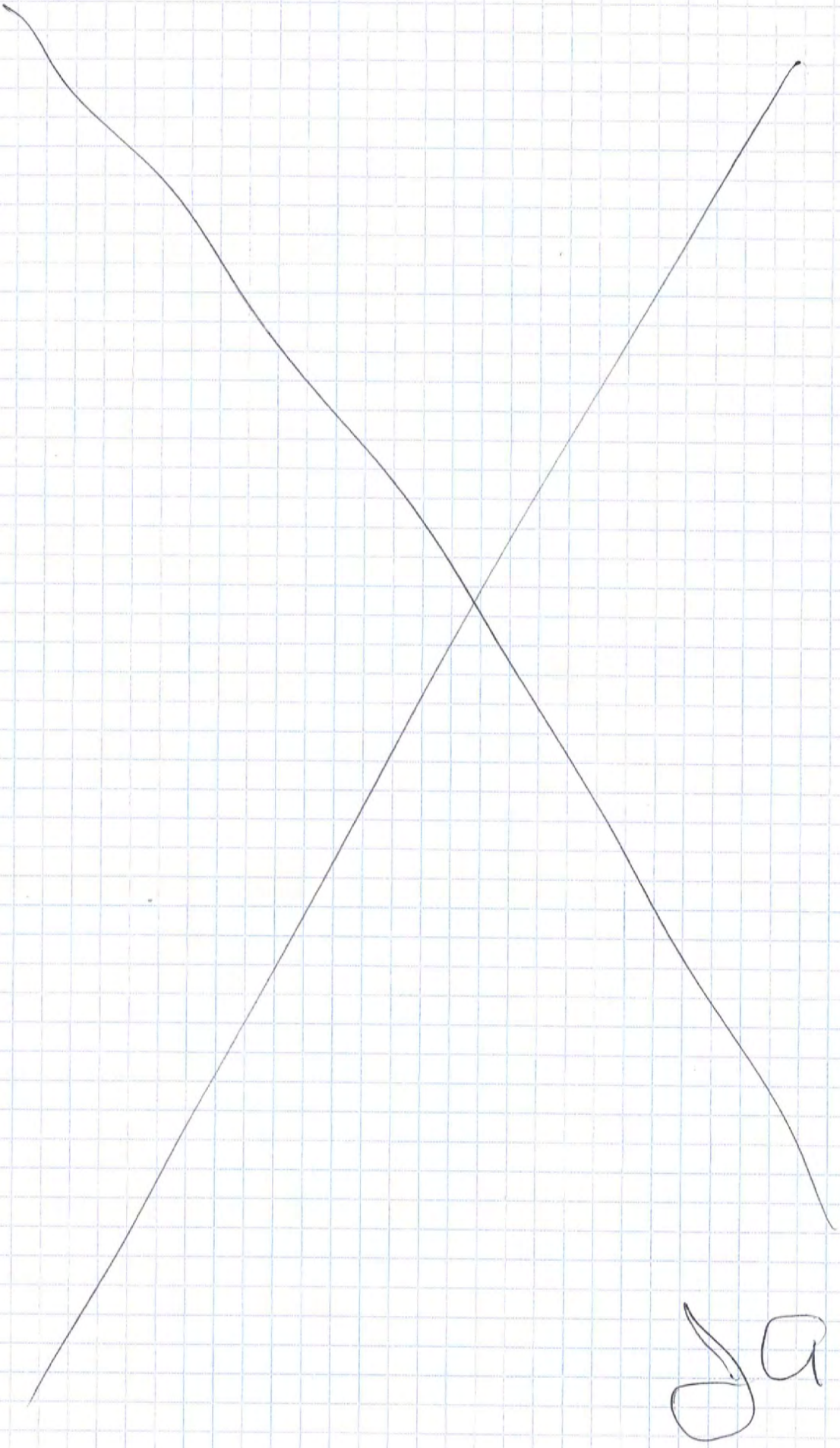
Sample 5 - samples labelled Sample 5 were taken for XRPD, DSC, TGA, and elemental analysis.

Sample 6 - crystalline material from Sample 4 were labelled as Sample 6 and taken for XRPD, DSC, TGA, and elemental analysis.

Sample 7 - produced a gel from the overnight drying. Further drying failed to produce a solid substance. No analysis was performed.

Sample 8 - samples labelled Sample 8 were taken for XRPD, DSC, TGA, and elemental analysis.

J. Atwood
9/15/10



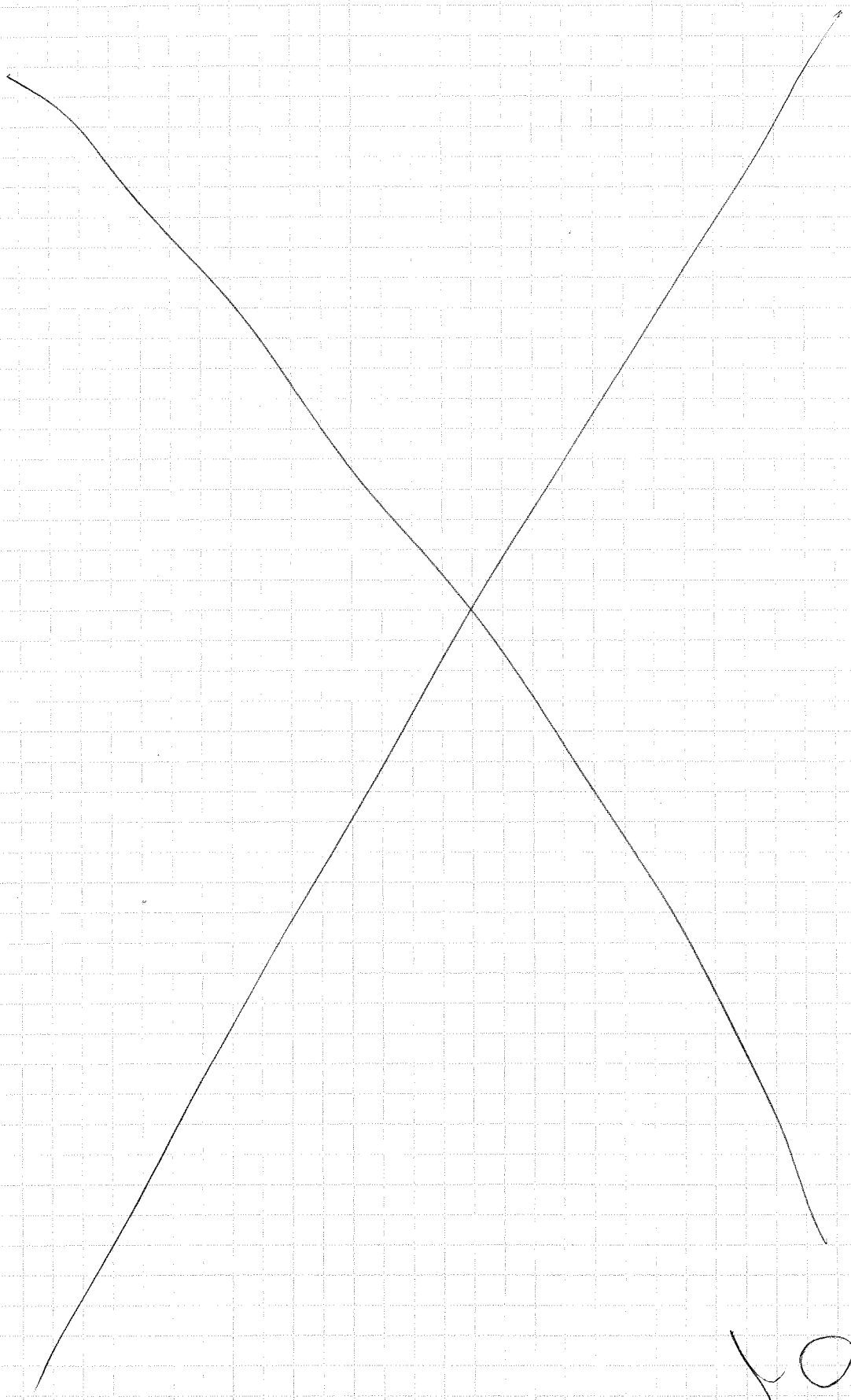
1/28/11 Preparation of (sitagliptin)$_2$(H_3PO_4)$\cdot 3H_2O$ Sample 9
1.50 g sitagliptin base (0.00368 moles, lot No. 0000013867) was combined with isopropanol (3.2 mL) and distilled water (1.4 mL). The mixture was stirred for 5-10 min to form a clear solution. To this solution was added 0.215 g H_3PO_4 (85% w/w, Sigma Aldrich), with stirring. The mixture was heated with stirring at 70°C for 15 min, then cooled to rt and left stirring overnight.

1/29/11 Sample 9 had solidified overnight. The sample was placed in the rt vacuum chamber for 24 h.

1/30/11 A portion of Sample 9 was taken for XRPD, DSC, TGA, and elemental analysis.

2/4/11 An accelerated stability chamber (40°C, 75% RH) was set up. A portion of Sample was placed in the chamber.

2/9/11 I performed a treatment of 500 mg (sitagliptin)$_2$(H_3PO_4)$\cdot 3H_2O$ according to the conditions in the Chygall Declaration, para 36 (for the sitagliptin base: H_3PO_4 (2.04 : 1.00) in methanol). That is to say, I slurried the 500 mg (sitagliptin)$_2$(H_3PO_4)$\cdot 3H_2O$ in 5 mL methanol for 24 h.

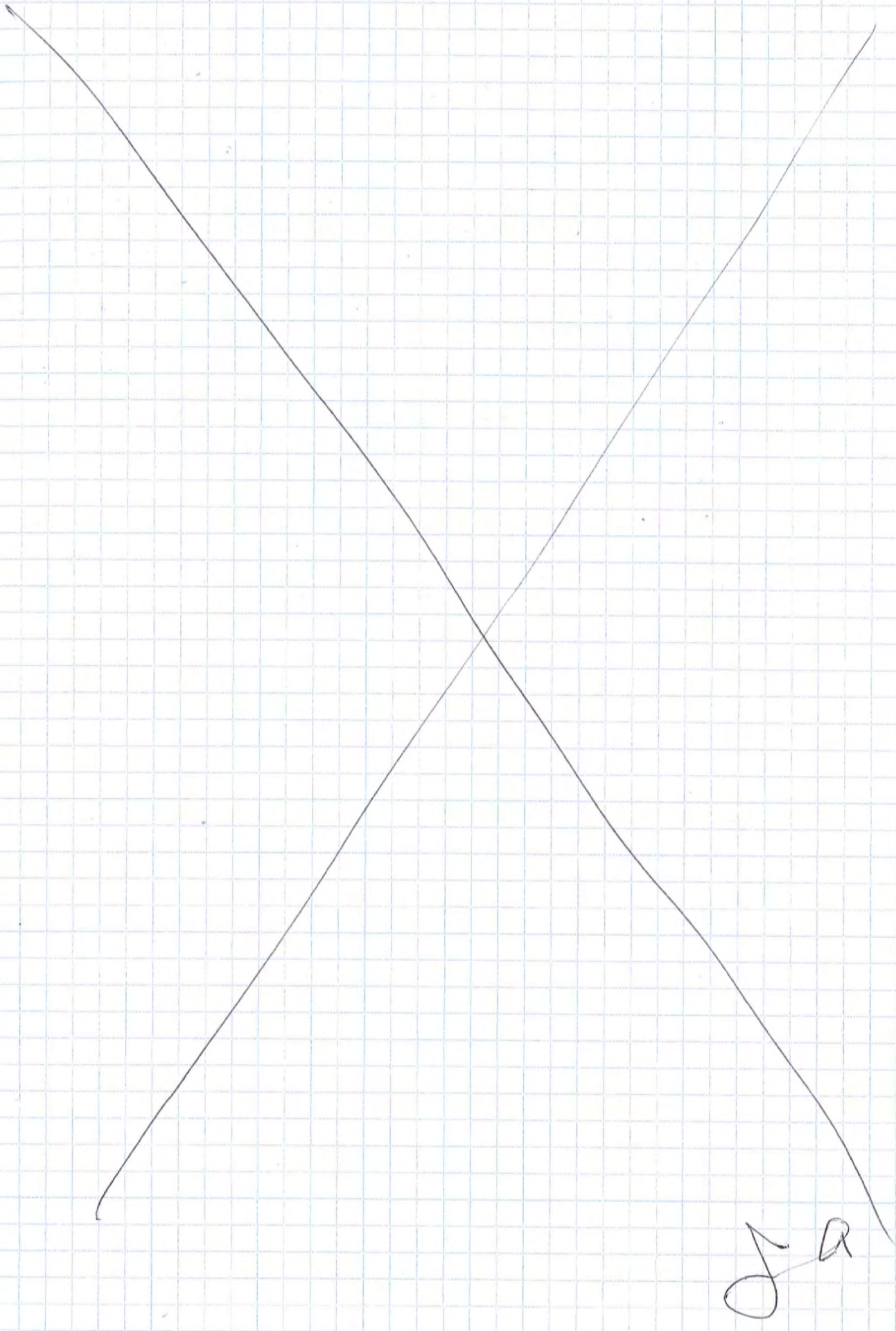


2/10/11 I vacuum filtered the resulting white solids + solvent, and I washed the flask and the collected ppt 3 x 2mL methanol. (This step according to Chygall lab notebook 4063-04-01.) The resulting collected ppt was air dried to constant weight, yielding ca. 100mg, labelled as Sample 11. Sample 11 was taken for XRPD, DSC, TGA, and elemental analysis.

2/11/11 The accelerated stability sample from 2/4, Sample 9, was sampled for XRPD, DSC, TGA,

2/18/11 The accelerated stability sample from 2/4 was removed from the chamber after 2wks, labelled Sample 10, and taken for XRPD, DSC, TGA, and elemental analysis.

J Atwood
2/18/11



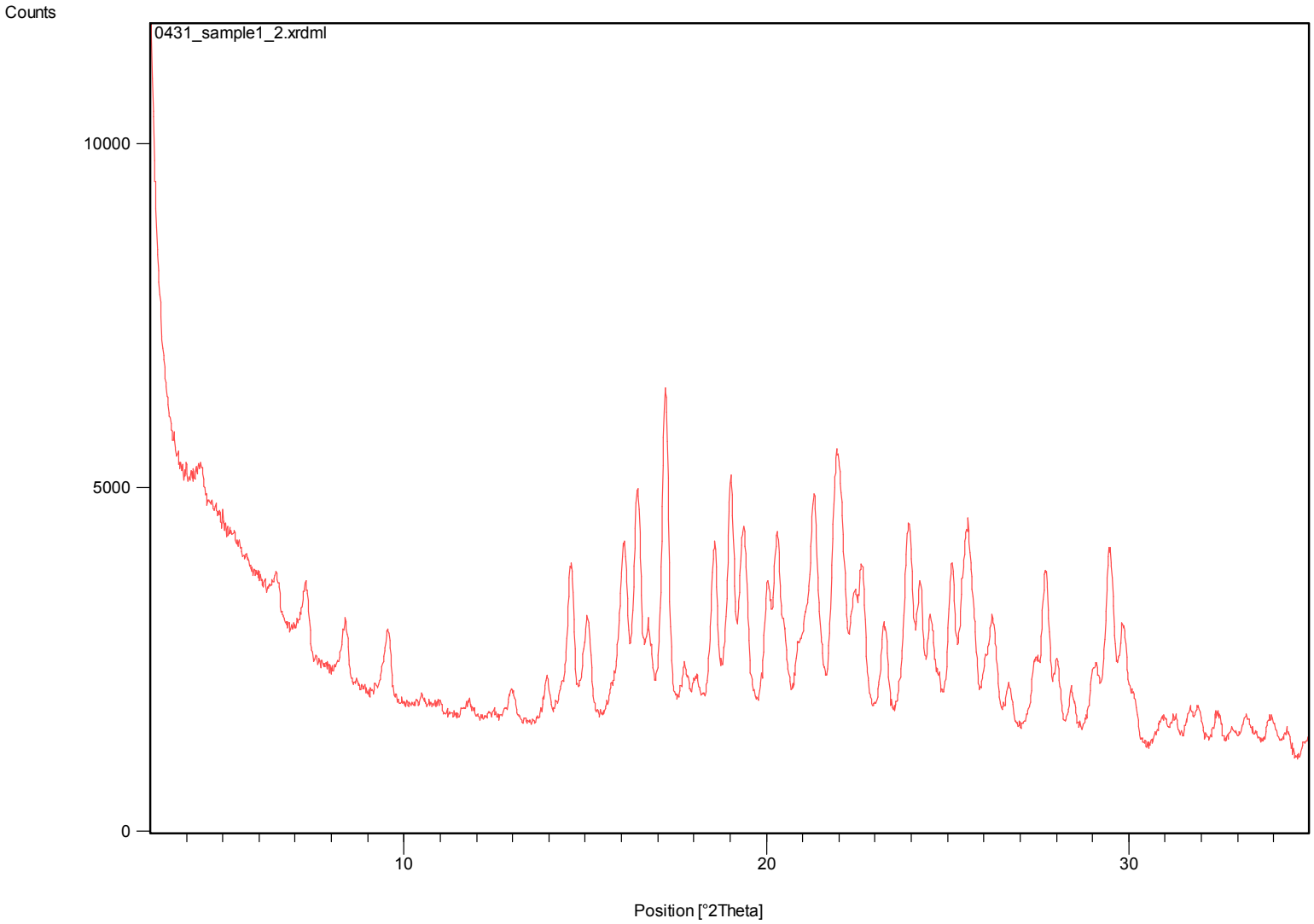
5/2/11 Preparation of (sitagliptin)$(H_3PO_4)_2$

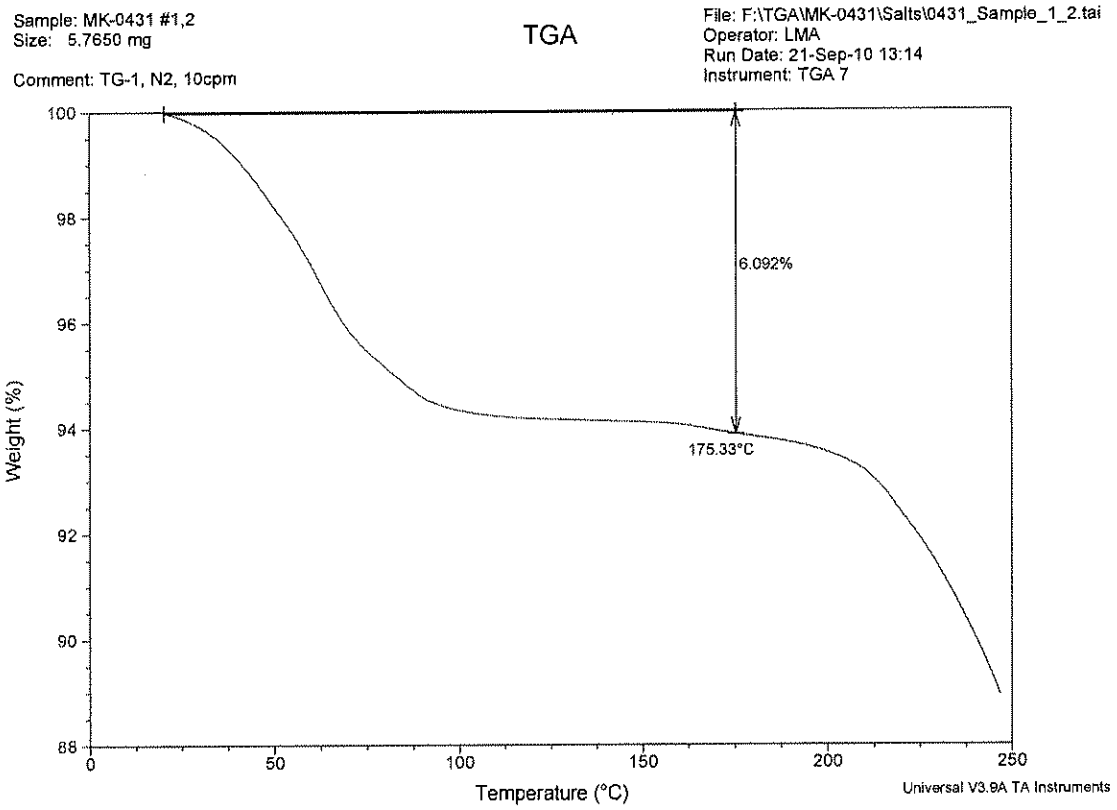
sitagliptin base, 0.75g (0.00184 moles, lot 000001386 7) was combined with acetone (4 mL). The mixture was stirred 5-10 min to form a solution. Anhydrous, crystalline H_3PO_4, 0.36 ~~mL~~ g, 0.00368 moles (Aldrich) was added with stirring. The mixture was heated to 50°C with stirring for 15 min, and the clear soln was then cooled to r.t. The acetone was removed under vacuum at r.t. The white, colorless product was crushed with a spatula and then placed under vacuum at r.t overnight.

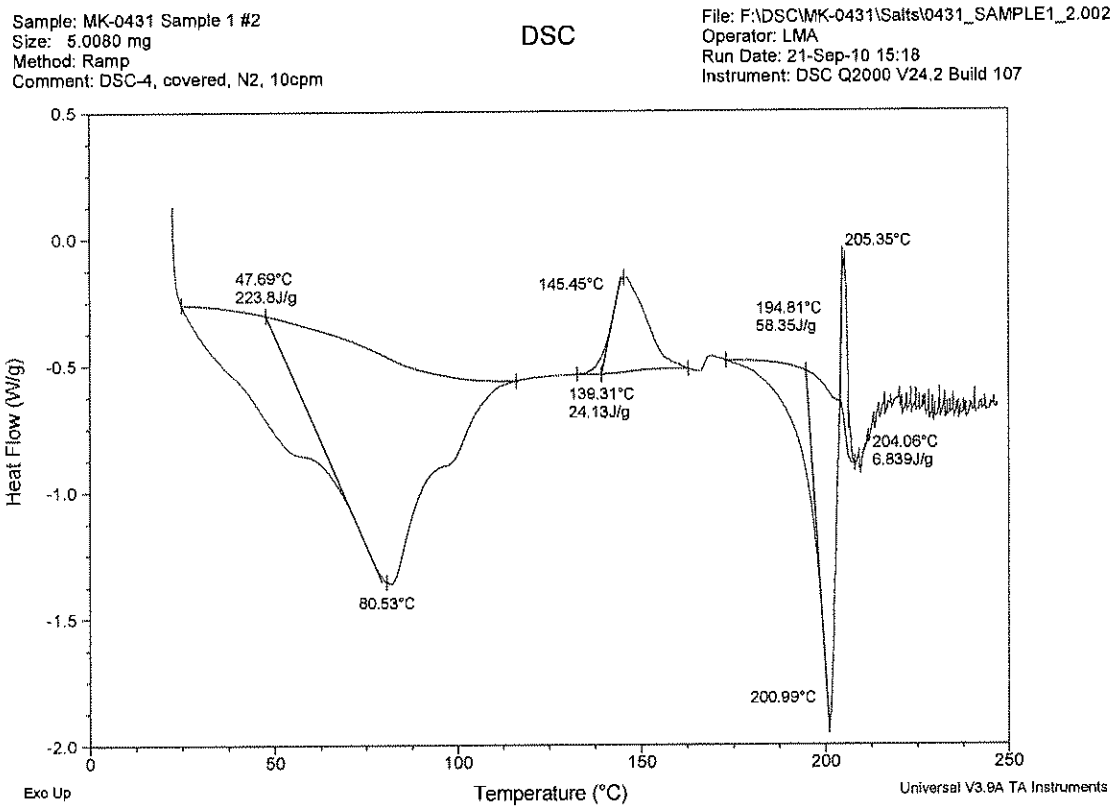
5/3/11 Samples of the white, free flowing powder were labelled Sample 12 (S12), and taken for XRPD, DSC, TGA, and elemental analysis.

J Atwood

5/10/2011


Counts
0431_sample1_2.xrdml
10000
5000
0
10
20
30
Position [°2Theta]


Sample: MK-0431 #1,2
Size: 5.7650 mg
Comment: TG-1, N2, 10cpm
TGA
File: F:\TGA\MK-0431\Salts\0431_Sample_1_2.tai
Operator: LMA
Run Date: 21-Sep-10 13:14
Instrument: TGA 7
6.092%
175.33°C
Weight (%)
Temperature (°C)
Universal V3.9A TA Instruments


Sample: MK-0431 Sample 1 #2
Size: 5.0080 mg
Method: Ramp
Comment: DSC-4, covered, N2, 10cpm
DSC
File: F:\DSC\MK-0431\Salts\0431_SAMPLE1_2.002
Operator: LMA
Run Date: 21-Sep-10 15:18
Instrument: DSC Q2000 V24.2 Build 107
47.69°C
223.8J/g
145.45°C
205.35°C
194.81°C
58.35J/g
139.31°C
24.13J/g
204.06°C
6.839J/g
80.53°C
200.99°C
Heat Flow (W/g)
Temperature (°C)
Exo Up
Universal V3.9A TA Instruments

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Certificate of Analysis

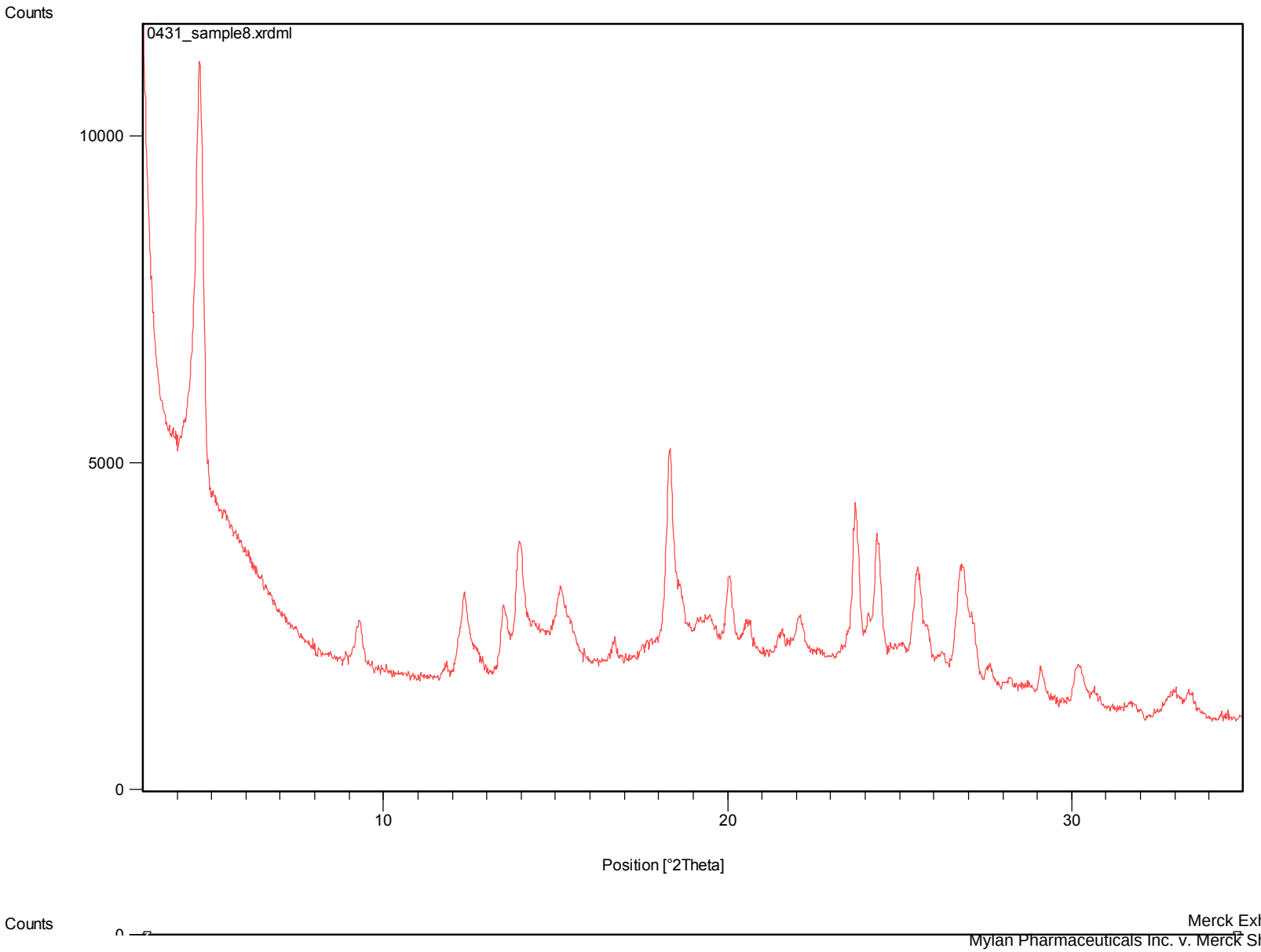
Company: Merck & Company

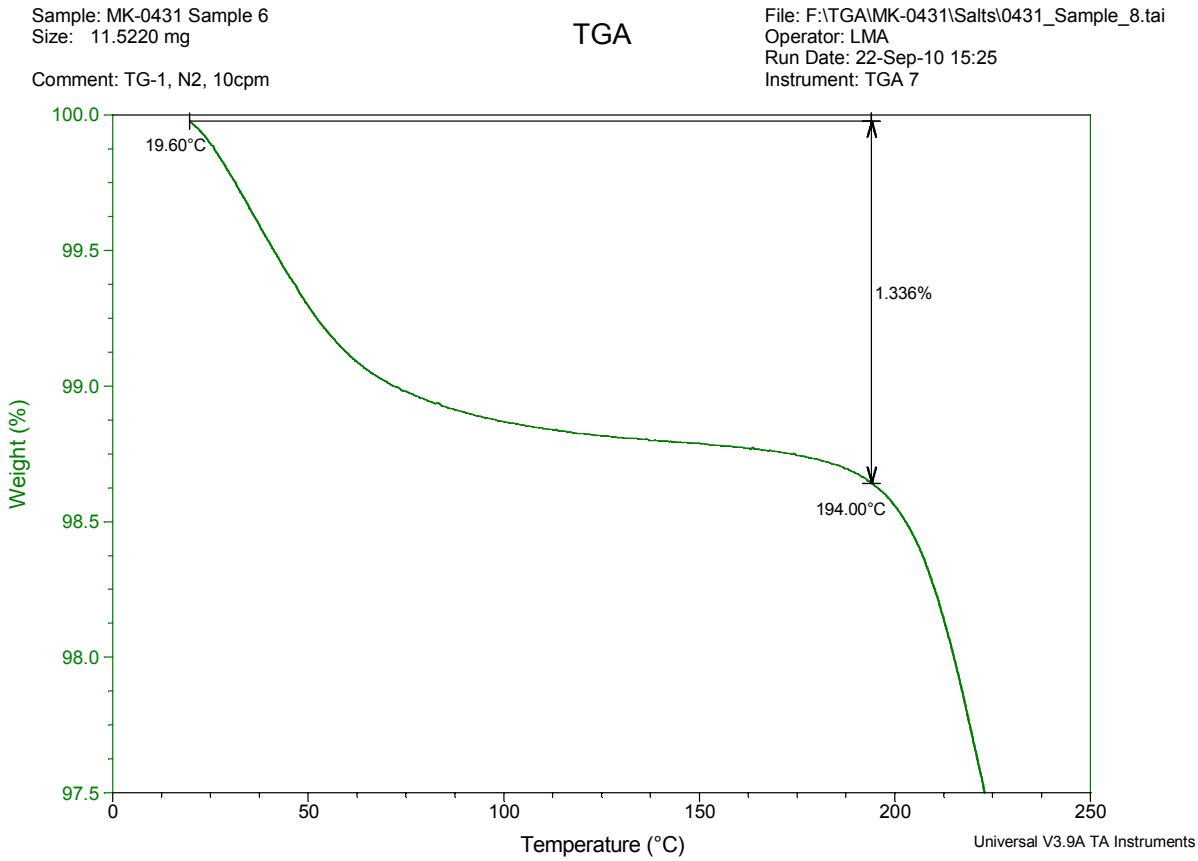
Submitter: Wang, Tiebang Sample ID.: 1-2 Date of Analysis: 10/4/2010

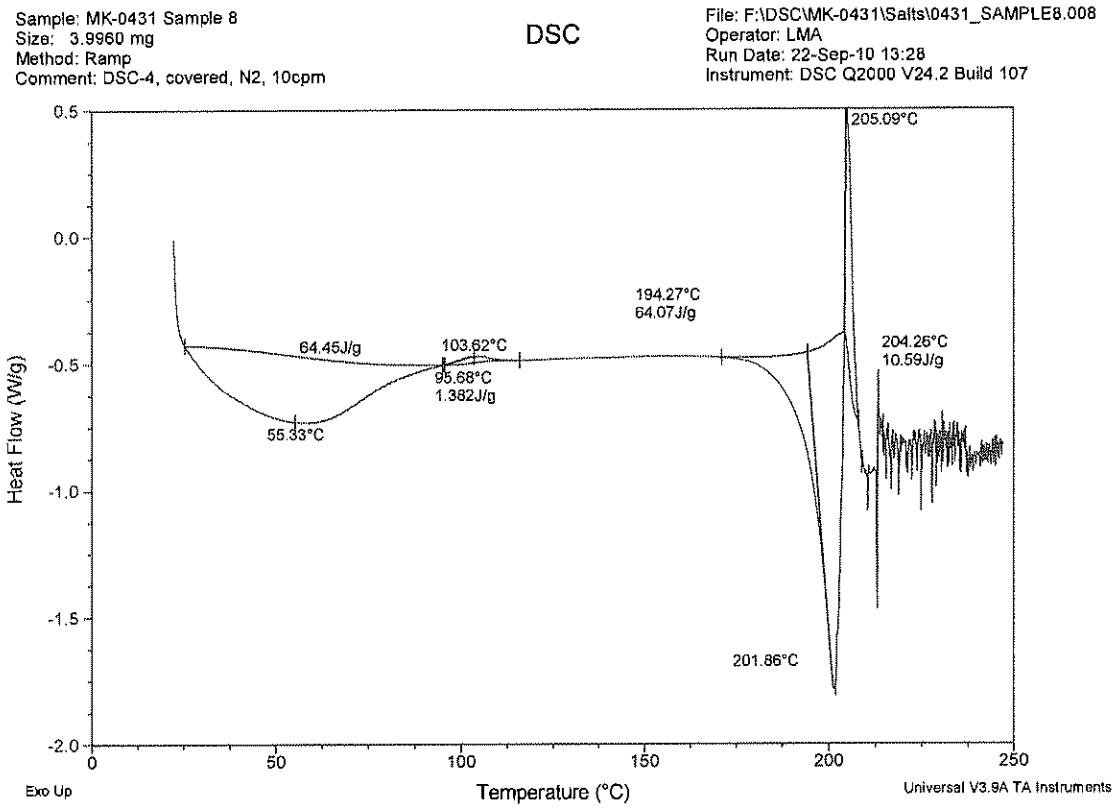
Analysis	Result
Test #: 1	
C	39.36 %
H	3.83 %
N	14.30 %
Na	196 ppm
P	3.02 %

Comments: Date Received: 09/27/2010

Reference: MERC01	Authorized Signature: Mike Hatoleki
	Date: 4/15/2011


Counts
0431_sample8.xrdml
10000
5000
0
10
20
30
Position [°2Theta]
Counts


Sample: MK-0431 Sample 6
Size: 11.5220 mg
Comment: TG-1, N2, 10cpm
TGA
File: F:\TGA\MK-0431\Salts\0431_Sample_8.tai
Operator: LMA
Run Date: 22-Sep-10 15:25
Instrument: TGA 7
100.0
99.5
99.0
98.5
98.0
97.5
Weight (%)
19.60°C
1.336%
194.00°C
0
50
100
150
200
250
Temperature (°C)
Universal V3.9A TA Instruments


Sample: MK-0431 Sample 8
Size: 3.9960 mg
Method: Ramp
Comment: DSC-4, covered, N2, 10cpm
DSC
File: F:\DSC\MK-0431\Salts\0431_SAMPLE8.008
Operator: LMA
Run Date: 22-Sep-10 13:28
Instrument: DSC Q2000 V24.2 Build 107
205.09°C
194.27°C
64.07J/g
64.45J/g
103.62°C
95.68°C
1.382J/g
204.26°C
10.59J/g
55.33°C
201.86°C
Heat Flow (W/g)
Temperature (°C)
Exo Up
Universal V3.9A TA Instruments

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Certificate of Analysis

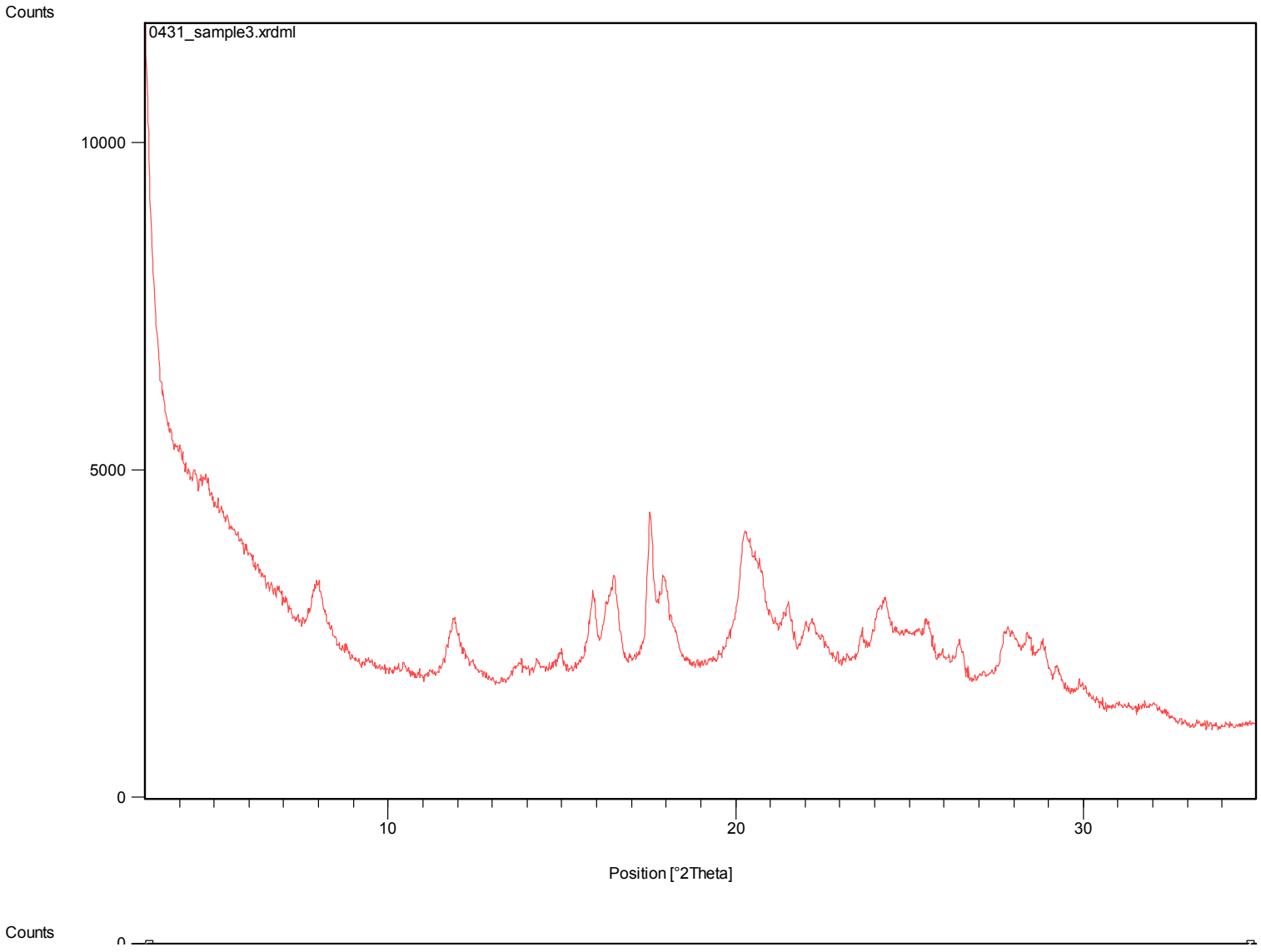
Company: Merck & Company

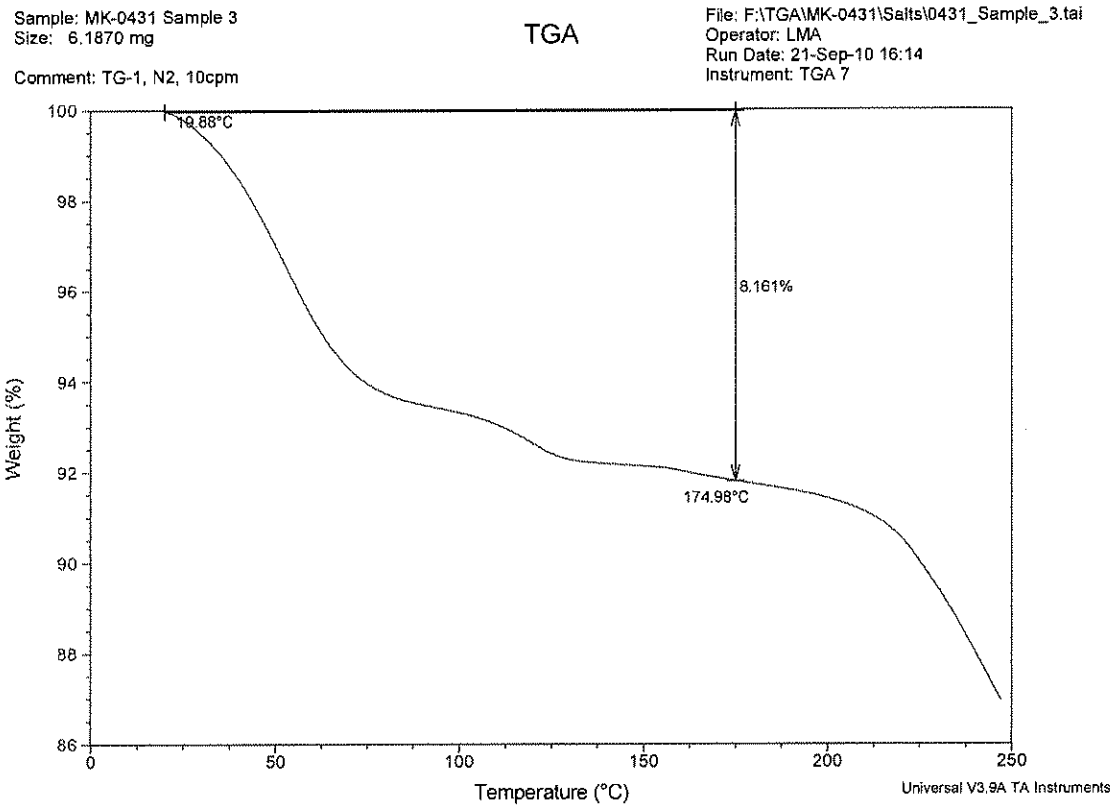
Submitter: Wang, Tiebang Sample ID.: 1-8 Date of Analysis: 10/4/2010

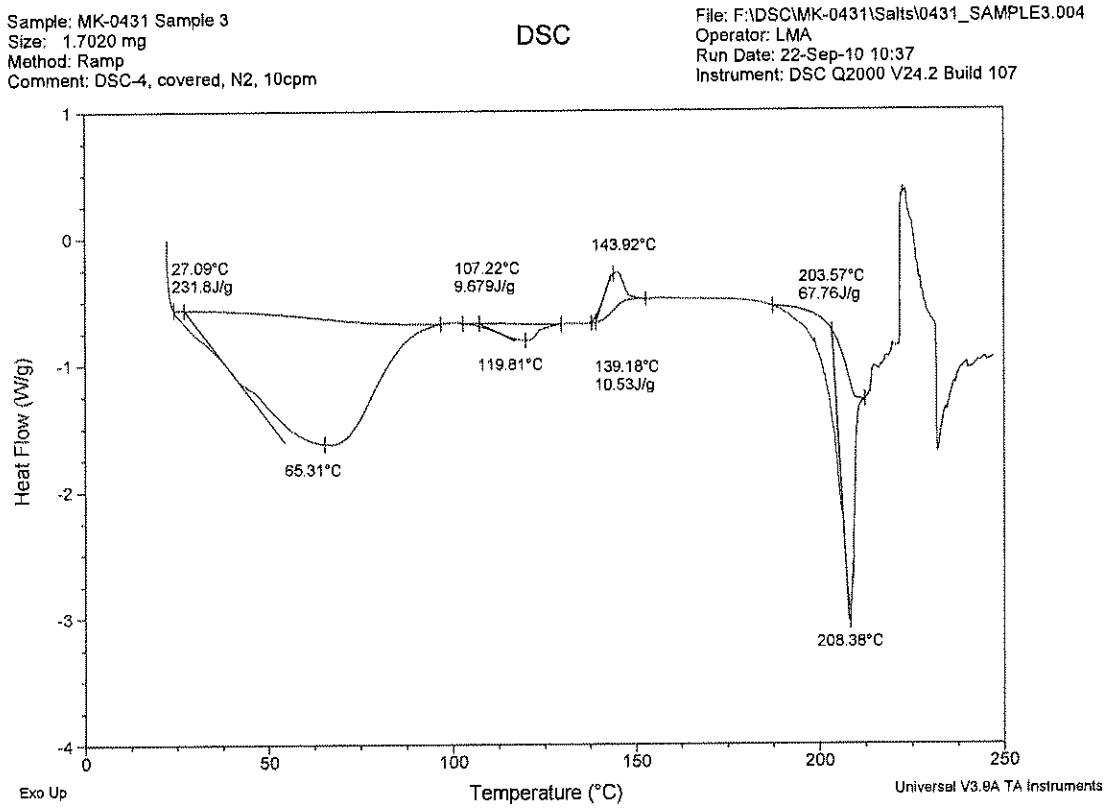
Analysis	Result
Test #: 1	
C	40.81 %
H	3.54 %
N	14.82 %
Na	116 ppm
P	3.39 %

Comments: Date Received: 09/27/2010

Reference: MERC001	Authorized Signature: Mike Hatolski
	Date: 4/15/2011


Counts
0431_sample3.xrdml
10000
5000
0
10
20
30
Position [°2Theta]
Counts
0


Sample: MK-0431 Sample 3
Size: 6.1870 mg
Comment: TG-1, N2, 10cpm
TGA
File: F:\TGA\MK-0431\Salts\0431_Sample_3.tai
Operator: LMA
Run Date: 21-Sep-10 16:14
Instrument: TGA 7
100
98
96
94
92
90
88
86
Weight (%)
19.68°C
8.161%
174.98°C
0
50
100
150
200
250
Temperature (°C)
Universal V3.9A TA Instruments


Sample: MK-0431 Sample 3
Size: 1.7020 mg
Method: Ramp
Comment: DSC-4, covered, N2, 10cpm
DSC
File: F:\DSC\MK-0431\Salts\0431_SAMPLE3.004
Operator: LMA
Run Date: 22-Sep-10 10:37
Instrument: DSC Q2000 V24.2 Build 107
27.09°C
231.8J/g
65.31°C
107.22°C
9.679J/g
119.81°C
143.92°C
139.18°C
10.53J/g
203.57°C
67.76J/g
208.38°C
Heat Flow (W/g)
Temperature (°C)
Exo Up
Universal V3.9A TA Instruments

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Certificate of Analysis

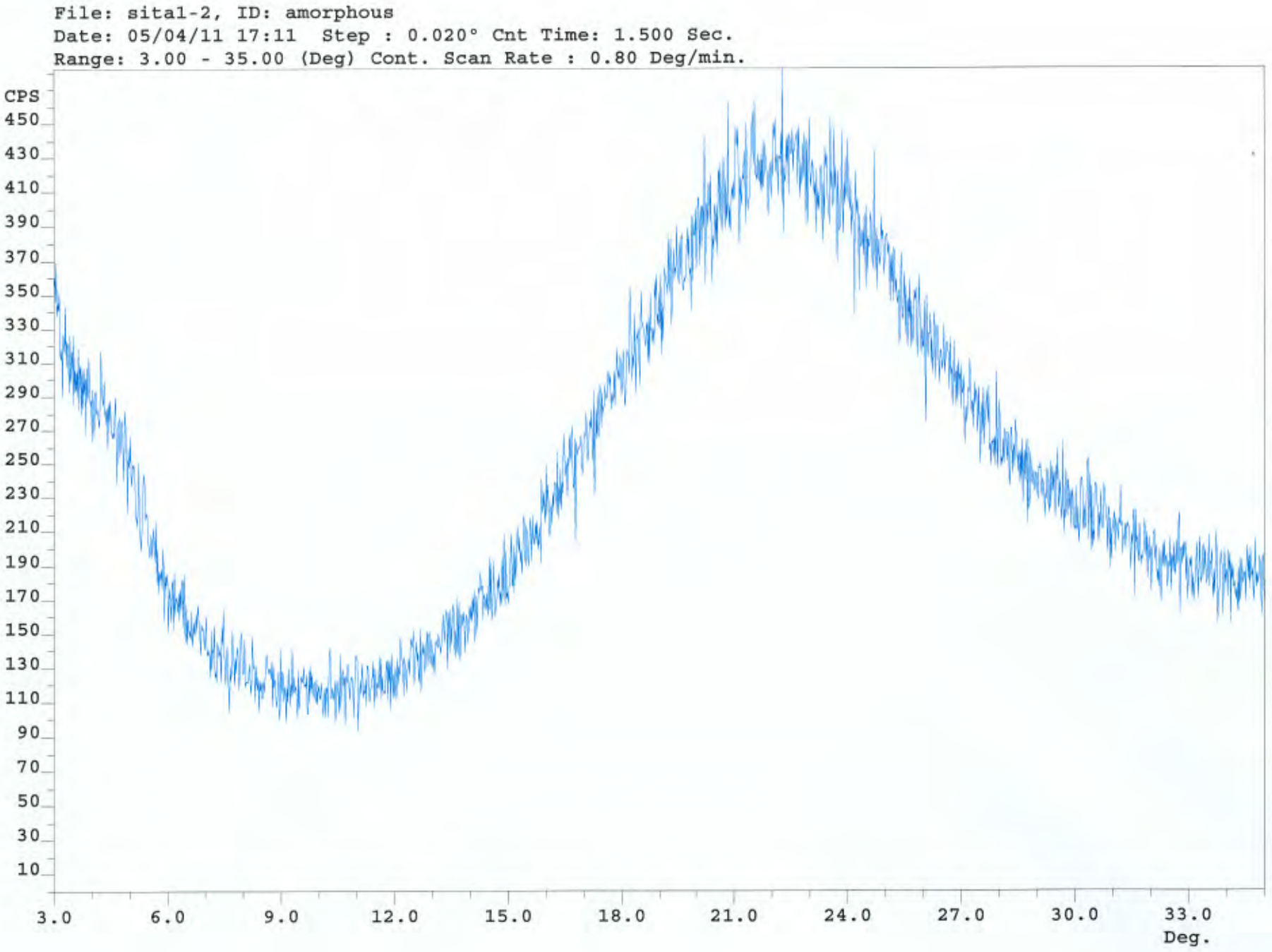
Company: Merck & Company

Submitter: Wang, Tiebang Sample ID.: 1-3 Date of Analysis: 10/4/2010

Analysis	Result
Test #: 1	
C	34.36 %
H	4.21 %
N	14.67 %
Na	80 ppm
P	5.50 %

Comments: Date Received: 09/27/2010

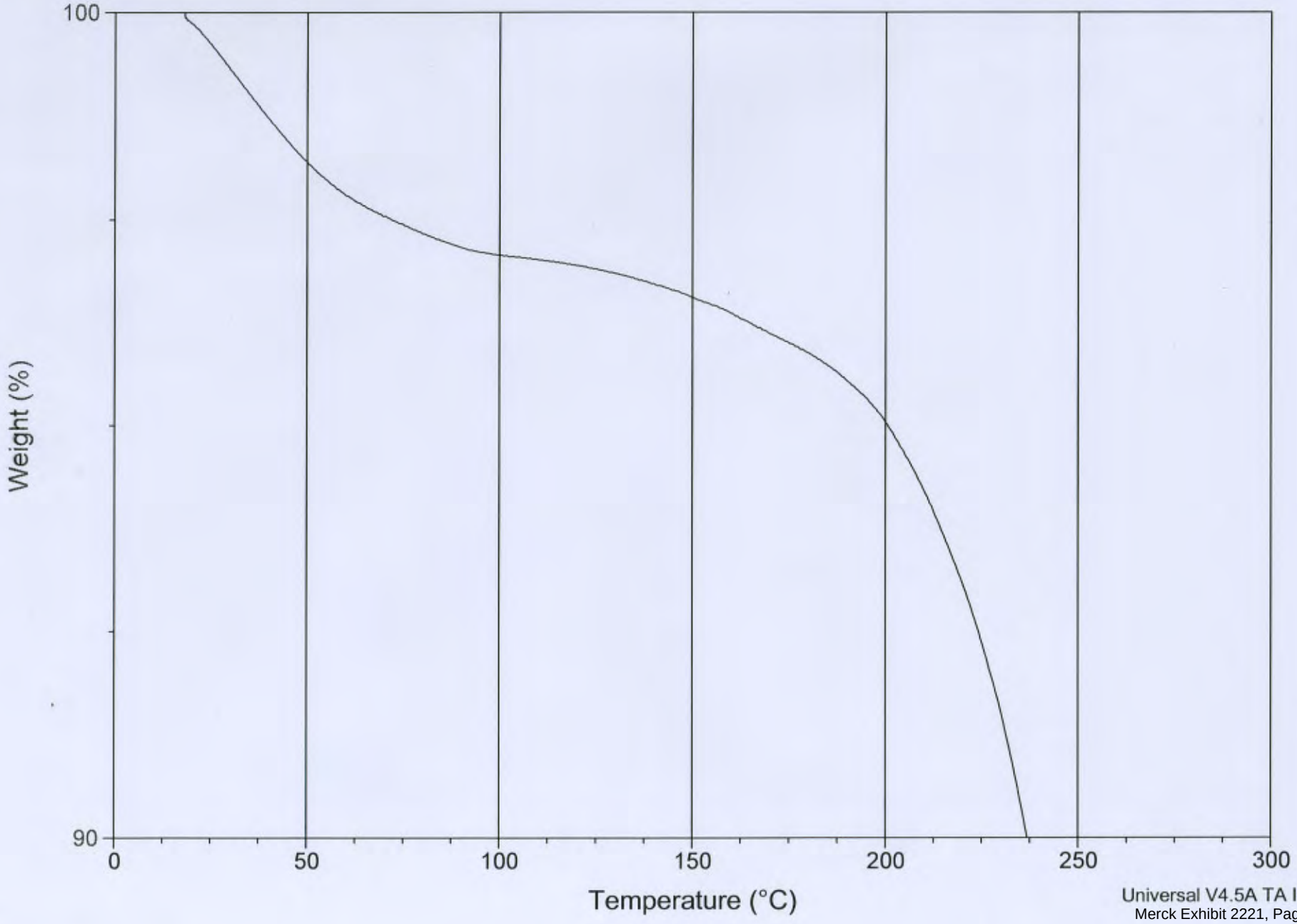
Reference: MER001	Authorized Signature: Mike Hatoleh
	Date: 4/15/2011


File: sita1-2, ID: amorphous
Date: 05/04/11 17:11 Step : 0.020° Cnt Time: 1.500 Sec.
Range: 3.00 - 35.00 (Deg) Cont. Scan Rate : 0.80 Deg/min.
CPS
450
430
410
390
370
350
330
310
290
270
250
230
210
190
170
150
130
110
90
70
50
30
10
3.0
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0
30.0
33.0
Deg.

Sample: sita, H3PO4, 1:2
Size: 4.2820 mg
Method: Ramp
Comment: as sent for analysis

TGA

File: C:\TA\Data\TGA\Atwood\sita.010
Operator: Atwood
Run Date: 10-May-2011 14:59
Instrument: TGA Q50 V20.10 Build 36

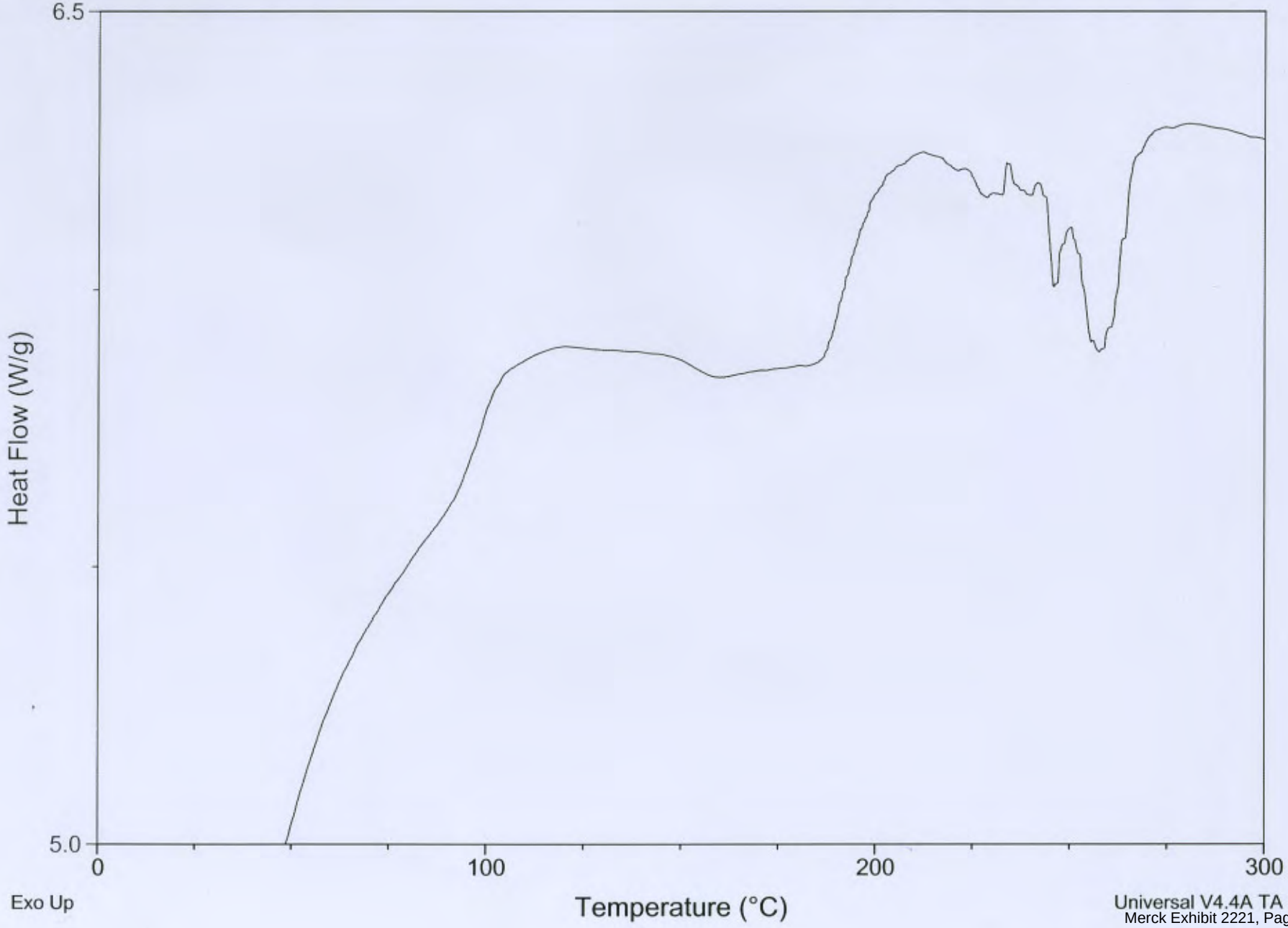


Universal V4.5A TA Instruments

Sample: sita , H3PO4, 1:2
Size: 2.1000 mg
Method: Ramp
Comment: as sent for analysis

DSC

File: C:\TA\Data\Atwood\sita.069
Operator: Atwood
Run Date: 10-May-2011 14:55
Instrument: DSC Q100 V9.8 Build 296

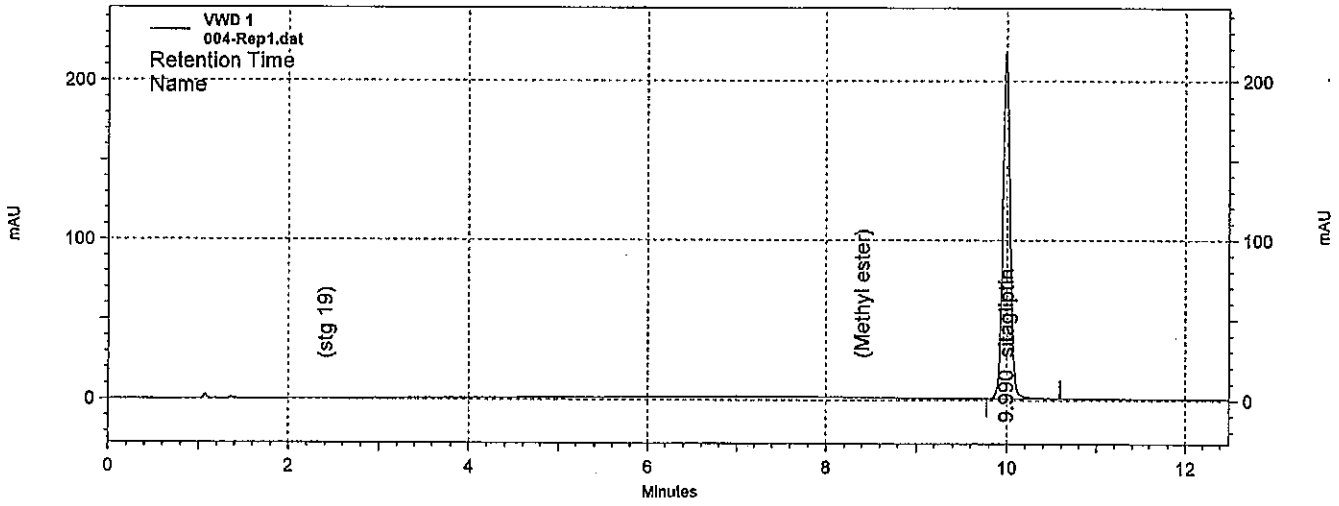


Universal V4.4A TA Instruments

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: stg std1 21.13/20*5/20 {Data Description}
Acquired: 24/03/2010 17:26:38 (GMT +02:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201043 STG Assay hp36.met
Method rev.: 24/02/2010 15:22:27 (GMT +02:00)
Instrument: HPLC_36 **Vial:** 12 **Inj. Vol.:** 10 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg assay_24.03.2010.seq001.ol.ssizip | stg assay_24.03.2010.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg assay_24.03.2010.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (25/03/2010 08:01:54 (GMT +02:00)) (Reprocessed)



VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width	Resolution (USP)
stg 19							
Methyl ester							
sitagliptin	1	9.990	1265858	100.000	217071	0.81	0.00
stg OH							
stg 57							
stg 58							
Eliminate 1							
Eliminate 2							
Des Amino							
Dimer							
Totals			1265858	100.000	217071		

STD 1895ST01-T-39342109
see original prints
in work from 22.03.2010

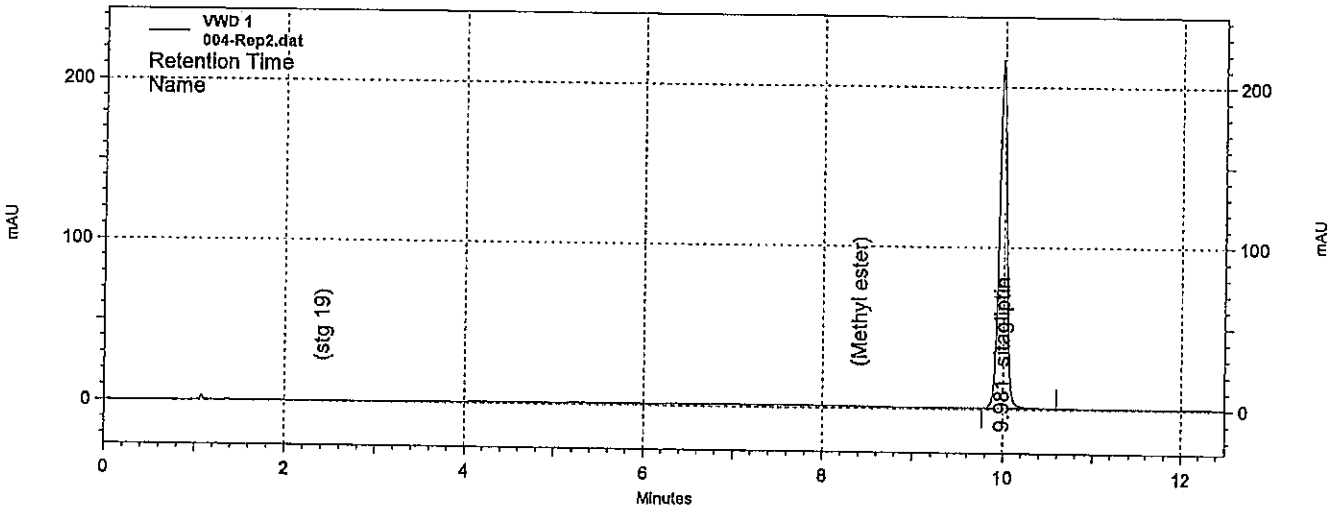
Printed: 25/03/2010 08:01:55 (GMT +02:00) by Dahlia Weinberg (TEVAIL\DWEINBERG)

stg std1 21.13/20*5/20

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: stg std1 21.13/20*5/20 {Data Description}
Acquired: 24/03/2010 17:46:41 (GMT +02:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201043 STG Assay hp36.met
Method rev.: 24/02/2010 15:22:27 (GMT +02:00)
Instrument: HPLC_36 **Vial:** 12 **Inj. Vol.:** 10 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg assay_24.03.2010.seq001.ol.ssizip | stg assay_24.03.2010.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg assay_24.03.2010.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (25/03/2010 08:01:58 (GMT +02:00)) (Reprocessed)



VWD 1 Results

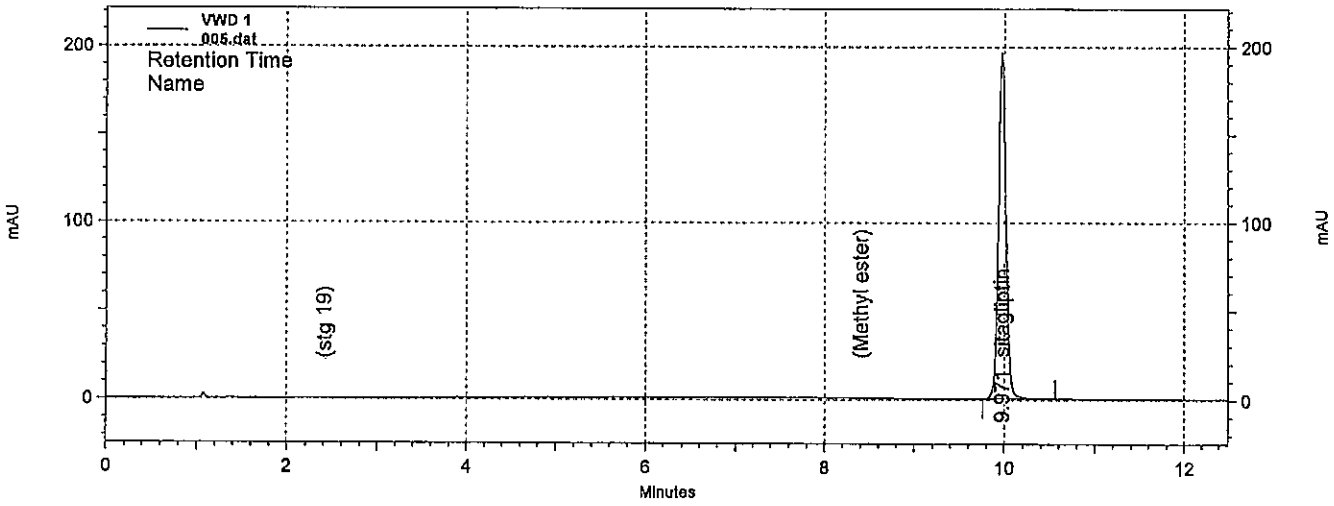
Name	Pk #	Retention Time	Area	Area Percent	Height	Width	Resolution (USP)
stg 19							
Methyl ester							
sitagliptin	1	9.981	1263116	100.000	215840	0.83	0.00
stg OH							
stg 57							
stg 58							
Eliminate 1							
Eliminate 2							
Des Amino							
Dimer							
Totals			1263116	100.000	215840		

Printed: 25/03/2010 08:01:59 (GMT +02:00) by Dahlia Weinberg (TEVAIL\DWEINBERG)

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: stg std2 19.28/20*5/20 {Data Description}
Acquired: 24/03/2010 18:06:45 (GMT +02:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201043 STG Assay hp36.met
Method rev.: 24/02/2010 15:22:27 (GMT +02:00)
Instrument: HPLC_36 **Vial:** 13 **Inj. Vol.:** 10 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg assay_24.03.2010.seq001.ol.ssizip | stg assay_24.03.2010.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg assay_24.03.2010.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (25/03/2010 08:02:02 (GMT +02:00)) (Reprocessed)



VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width	Resolution (USP)
stg 19							
Methyl ester							
sitagliptin	1	9.971	1143945	100.000	196333	0.80	0.00
stg OH							
stg 57							
stg 58							
Eliminate 1							
Eliminate 2							
Des Amino							
Dimer							
Totals			1143945	100.000	196333		

STD 1895ST01-T-39342 109
see original prints
in work from 22.03.2010

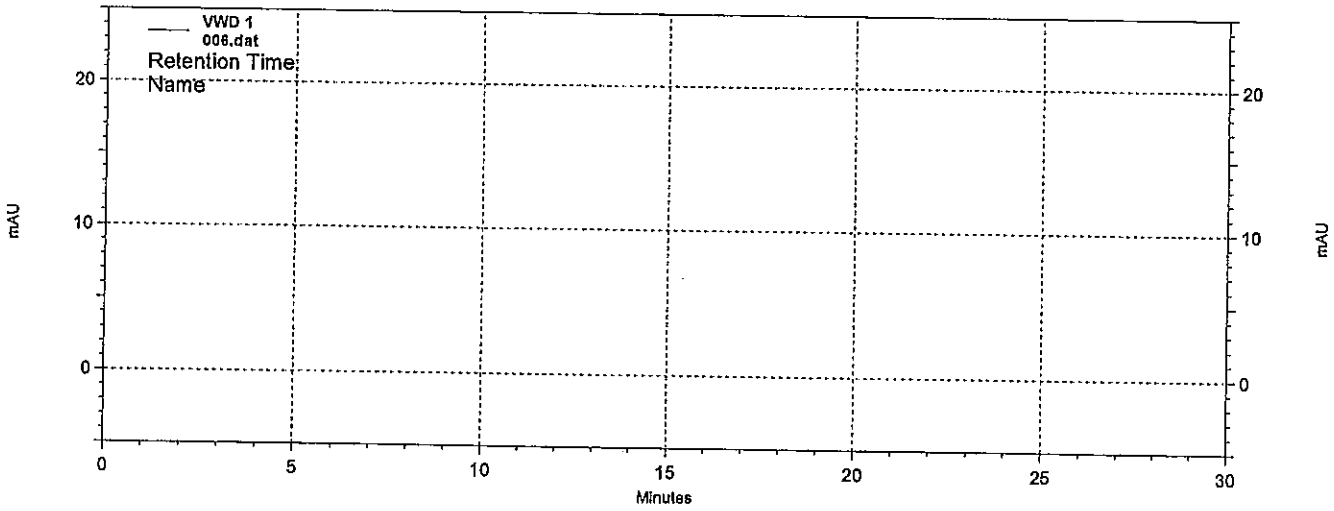
Printed: 25/03/2010 08:02:03 (GMT +02:00) by Dahlia Weinberg (TEVAIL\DWEINBERG)

dil

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: dil {Data Description}
Acquired: 24/03/2010 18:25:26 (GMT +02:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201041 STG Imp-long hp36.met
Method rev.: 25/03/2010 07:49:27 (GMT +02:00)
Instrument: HPLC_36 **Vial:** 7 **Inj. Vol.:** 10 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg assay_24.03.2010.seq001.ol.ssizip | stg assay_24.03.2010.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg assay_24.03.2010.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (25/03/2010 08:02:06 (GMT +02:00)) (Reprocessed) (Aborted Run)



VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width	RRT
stg 19							
BAA							
sitagliptin							
stg OH							
Totals							

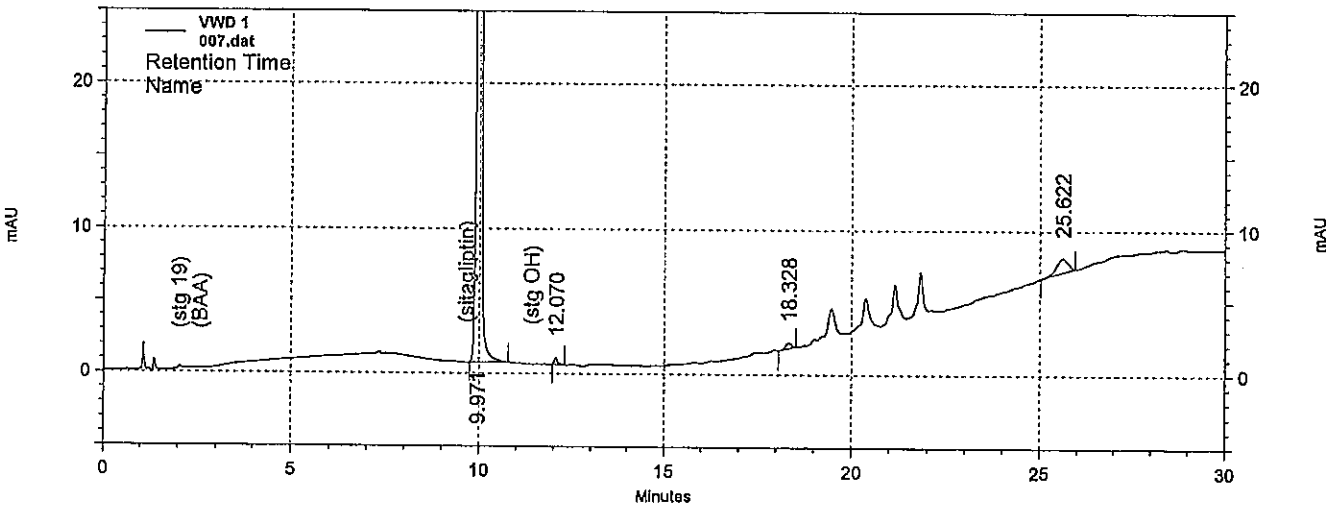
Printed: 25/03/2010 08:02:07 (GMT +02:00) by Dahlia Weinberg (TEVAIL\DWEINBERG)

stg base sal 069 21.01/5*1/20

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: stg base sal 069 21.01/5*1/20 {Data Description}
Acquired: 24/03/2010 18:27:30 (GMT +02:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201041 STG Imp-long hp36.met
Method rev.: 25/03/2010 07:49:27 (GMT +02:00)
Instrument: HPLC_36 **Vial:** 15 **Inj. Vol.:** 10 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg assay_24.03.2010.seq001.ol.ssizip | stg assay_24.03.2010.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg assay_24.03.2010.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (25/03/2010 08:02:09 (GMT +02:00)) (Reprocessed)



VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width	RRT
stg 19 BAA sitagliptin	1	9.971	1267073	97.840	216712	1.03	0.00
stg OH	2	12.070	2874	0.222	470	0.34	0.00
	3	18.328	4082	0.315	394	0.47	0.00
	4	25.622	21014	1.623	978	0.91	0.00
Totals			1295043	100.000	218554		

24.Mar 2010 15:16
User 123
Type AX205
SNR 1126311588
Balance No.14
SAMPLE STG B
ID SAL 069

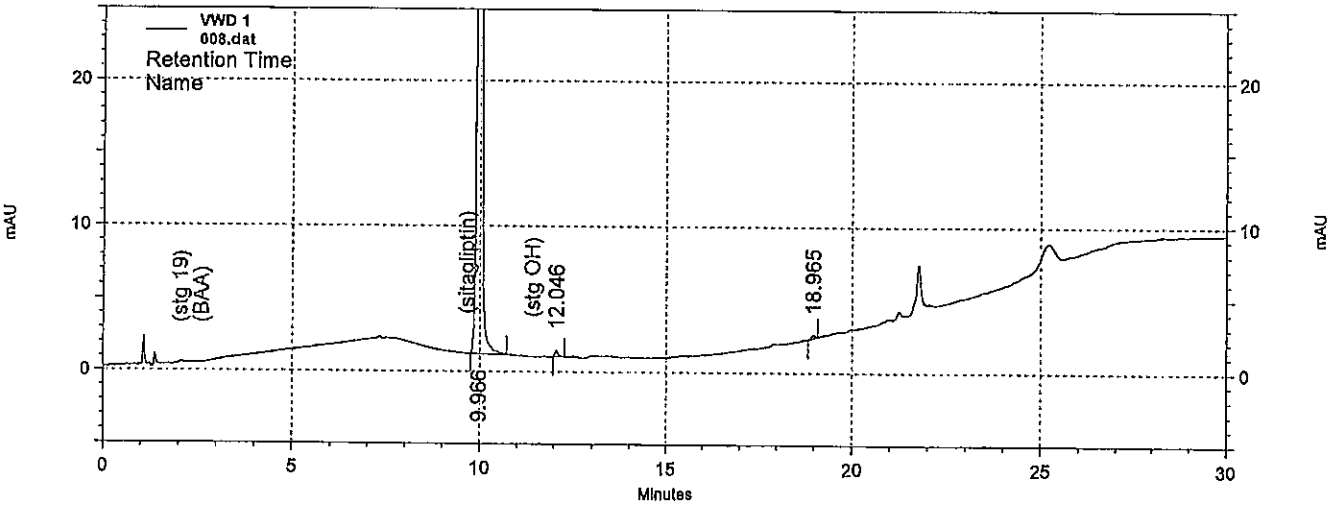
0.00 mg
21.01 mg

Printed: 25/03/2010 08:02:10 (GMT +02:00) by Dahlia Weinberg (TEVAIL\DWEINBERG)

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: stg base sal 069 19.51/5*1/20 {Data Description}
Acquired: 24/03/2010 19:06:04 (GMT +02:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201041 STG Imp-long hp36.met
Method rev.: 25/03/2010 07:49:27 (GMT +02:00)
Instrument: HPLC_36 **Vial:** 16 **Inj. Vol.:** 10 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg assay_24.03.2010.seq001.ol.ssizip | stg assay_24.03.2010.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg assay_24.03.2010.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (25/03/2010 08:02:13 (GMT +02:00)) (Reprocessed)



VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width	RRT
stg 19							
BAA							
sitagliptin							
	1	9.966	1176005	99.670	202285	0.97	0.00
stg OH							
	2	12.046	2562	0.217	434	0.30	0.00
	3	18.965	1328	0.113	208	0.25	0.00
Totals			1179895	100.000	202927		

24.Mar 2010 15:15
User 123
Type AX205
SNR 1126311588
Balance No.14
SAMPLE STG B
ID SAL 069

0.00 mg
19.51 mg

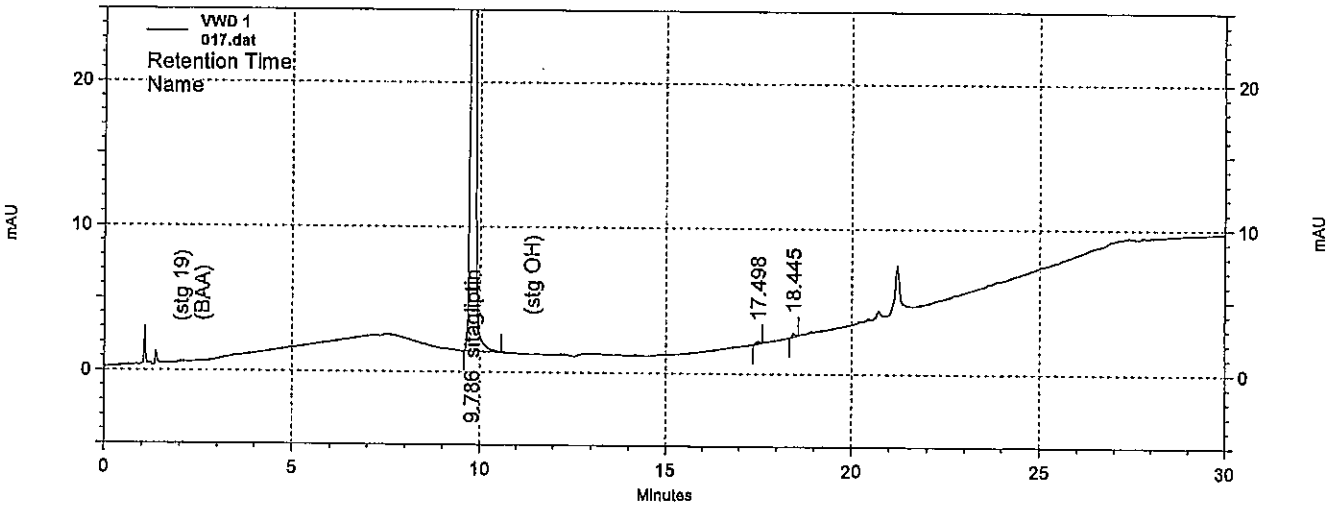
Printed: 25/03/2010 08:02:14 (GMT +02:00) by Dahlia Weinberg (TEVAIL\DWEINBERG)

stg std1 21.13/20*5/20

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: stg std1 21.13/20*5/20 {Data Description}
Acquired: 25/03/2010 00:53:39 (GMT +02:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201041 STG Imp-long hp36.met
Method rev.: 25/03/2010 07:49:27 (GMT +02:00)
Instrument: HPLC_36 **Vial:** 12 **Inj. Vol.:** 10 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg assay_24.03.2010.seq001.ol.ssizip | stg assay_24.03.2010.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg assay_24.03.2010.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (25/03/2010 08:02:47 (GMT +02:00)) (Reprocessed)



VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width	RRT
stg 19							
BAA							
sitagliptin	1	9.786	1265023	99.801	221322	1.01	1.00
stg OH							
	2	17.498	1145	0.090	147	0.24	1.79
	3	18.445	1380	0.109	225	0.25	1.89
Totals			1267548	100.000	221694		

Printed: 25/03/2010 08:02:48 (GMT +02:00) by Dahlia Weinberg (TEVAIL\DWEINBERG)

ASSAY DETERMINATION

S:\PROJECTS\SITAGLIPTIN\Analytical\assay\Assay STG base 24.03.11

Project: stg
Instrument: hp 36

NOT ORIGINAL

ANALYTICAL STANDARDS

Name: stg
C.N / Batch:
% Potency: 99.9
Exp/Retest date:

Weight 1 (mg): 21.13
Init.Volume 1 (ml): 20
Total Dilution 1 : 4

Weight 2 (mg): 19.28
Init.Volume 2 (ml): 20
Total Dilution 2 : 4

Multiplic. Factor : 0.61

Standard 1 Nomin Conc. (mg/ml): 2.6386E-01				Standard 2 Nomin Conc. (mg/ml): 2.4076E-01			
#	RT (min)	Std. Area	Resp.Factor	#	RT (min)	Std. Area	Resp. Factor
1	9.99	1255856	5922771.44	1	9.97	1143945	5865939.71
2	9.98	1263116	5909942.01	2			
3				3			
Average area of 3 inj :		1264487.00		Average area of 3 inj :		1143945.00	
SD:				SD:			
RSD,% :				RSD,% :			

Average factor of 6 injections : 5899551.05
SD : 29806.72
RSD,% : 0.5

Control Std: As control std used Standard No 1
Nomin Conc. (mg/ml): 2.6386E-01

#	RT (min)	Std. Area	Resp. Factor	Accept. value, %
1	9.98	1255194	5919664.68	100.3

A S S A Y (weight/weight, %)

Initial Volume (ml): 5 Total Dilution: 20

	#	LOD(%) / Water (%)	Weight (mg)	Conc. (mg/ml)	RT (min)	Area	Resp. Factor	Assay (%)
Name: stg base C.N / Batch: sal 069	1		21.01	2.1010E-01	9.97	1267073	6030809.14	102.22
	2		19.51	1.9510E-01	9.97	1176005	6027703.74	102.17
							Average :	102.2
							Differ.,% :	0.1

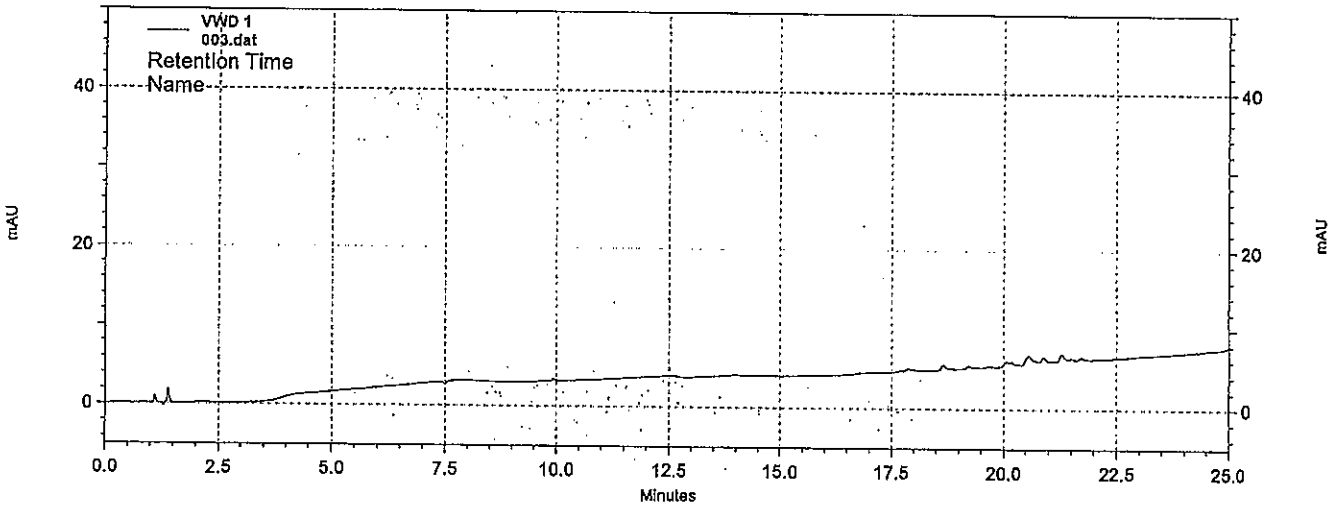
	#	LOD(%) / Water (%)	Weight (mg)	Conc. (mg/ml)	RT (min)	Area	Resp. Factor	Assay (%)
Name: [redacted] C.N / Batch: [redacted]	1		[redacted]					
	2							
							Average :	
							Differ.,% :	

diluent

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: diluent {Data Description}
Acquired: 12/05/2010 19:24:33 (GMT +03:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201041 STG Imp hp25.met
Method rev.: 12/05/2010 10:11:36 (GMT +03:00)
Instrument: HPLC_25 **Vial:** 1 **Inj. Vol.:** 10 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg assay_12.05.2010_1.seq001.ol.ssizip | stg assay_12.05.2010_1.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg assay_12.05.2010_1.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (09/06/2010 14:26:26 (GMT +03:00)) (Reprocessed)



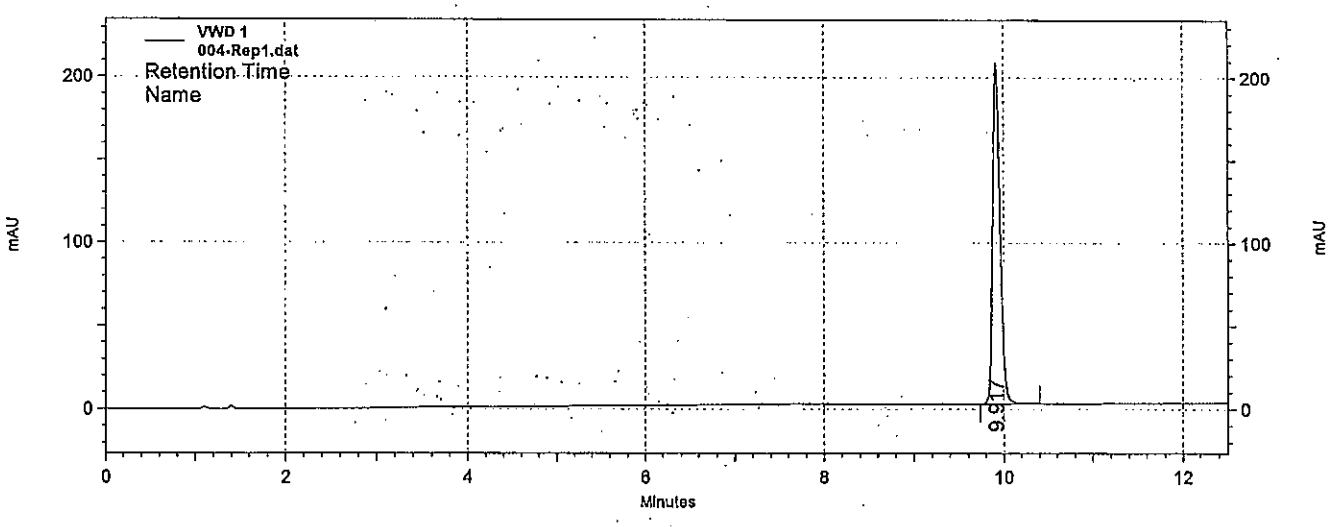
VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width	Resolution (USP)

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: stg std1 19.53/20*5/20 {Data Description}
Acquired: 12/05/2010 19:58:05 (GMT +03:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201043 STG Assay hp25.met
Method rev.: 04/05/2010 14:20:23 (GMT +03:00)
Instrument: HPLC_25 **Vial:** 4 **Inj. Vol.:** 10 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg assay_12.05.2010_1.seq001.ol.ssizip | stg assay_12.05.2010_1.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg assay_12.05.2010_1.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (09/06/2010 14:26:29 (GMT +03:00)) (Reprocessed)



VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width	Resolution (USP)
	1	9.917	1146273	100.000	205593	0.66	0.00
Totals			1146273	100.000	205593		

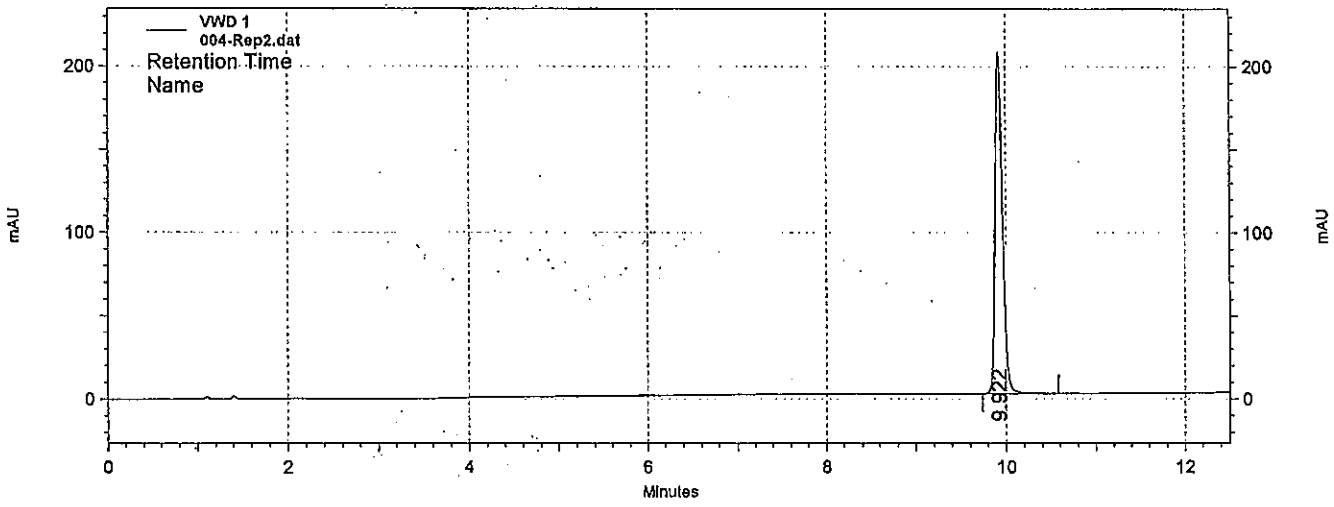
std 1895ST01-T-39342109
see original prints
in work from 09.05.2010

Printed: 09/06/2010 14:26:30 (GMT +03:00) by Dahlia Weinberg (TEVAIL\DWEINBERG)

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: stg std1 19.53/20*5/20 {Data Description}
Acquired: 12/05/2010 20:18:09 (GMT +03:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201043 STG Assay hp25.met
Method rev.: 04/05/2010 14:20:23 (GMT +03:00)
Instrument: HPLC_25 **Vial:** 4 **Inj. Vol.:** 10 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg assay_12.05.2010_1.seq001.ol.ssizip | stg assay_12.05.2010_1.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg assay_12.05.2010_1.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (09/06/2010 14:26:32 (GMT +03:00)) (Reprocessed)



VWD 1 Results

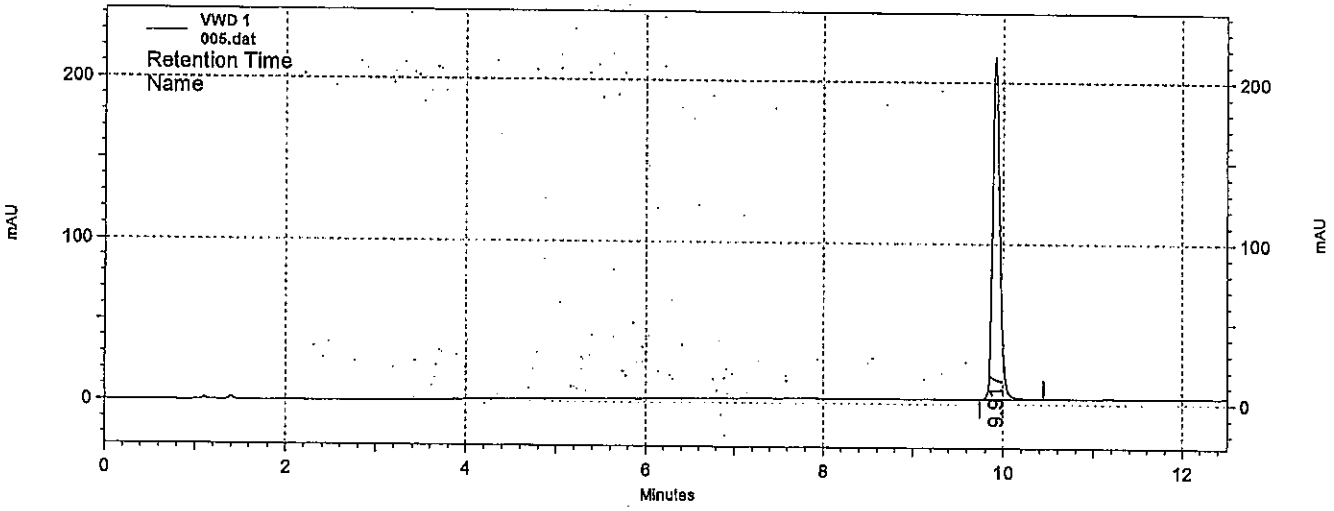
Name	Pk #	Retention Time	Area	Area Percent	Height	Width	Resolution (USP)
	1	9.922	1149596	100.000	205474	0.85	0.00
Totals			1149596	100.000	205474		

stg std2 20.46/20*5/20

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: stg std2 20.46/20*5/20 (Data Description)
Acquired: 12/05/2010 20:38:17 (GMT +03:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201043 STG Assay hp25.met
Method rev.: 04/05/2010 14:20:23 (GMT +03:00)
Instrument: HPLC_25 **Vial:** 5 **Inj. Vol.:** 10 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg assay_12.05.2010_1.seq001.ol.ssizip | stg assay_12.05.2010_1.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg assay_12.05.2010_1.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (09/06/2010 14:26:36 (GMT +03:00)) (Reprocessed)



VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width	Resolution (USP)
	1	9.917	1186982	100.000	212479	0.71	0.00
Totals			1186982	100.000	212479		

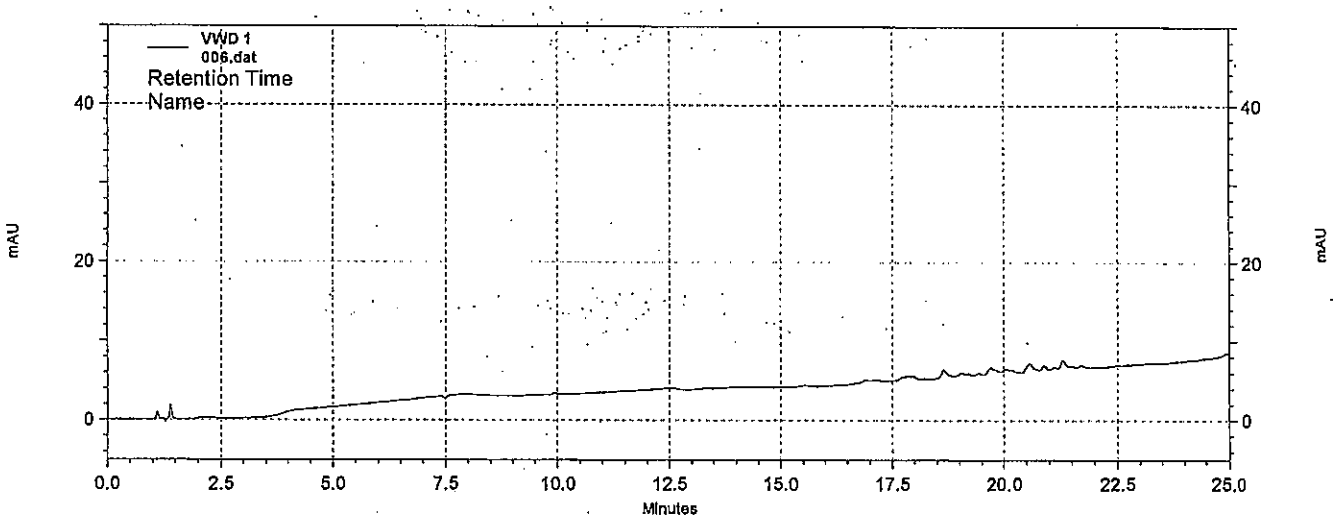
STD 1895 STO1-T-39342109
see original prints
in work from 09.05.2010

Printed: 09/06/2010 14:26:37 (GMT +03:00) by Dahlia Weinberg (TEVAIL\DWEINBERG)

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: dil {Data Description}
Acquired: 12/05/2010 20:58:20 (GMT +03:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201041 STG Imp hp25.met
Method rev.: 12/05/2010 10:11:36 (GMT +03:00)
Instrument: HPLC_25 **Vial:** 1 **Inj. Vol.:** 10 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg assay_12.05.2010_1.seq001.ol.ssizip | stg assay_12.05.2010_1.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg assay_12.05.2010_1.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (09/06/2010 14:26:40 (GMT +03:00)) (Reprocessed)



VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width	Resolution (USP)

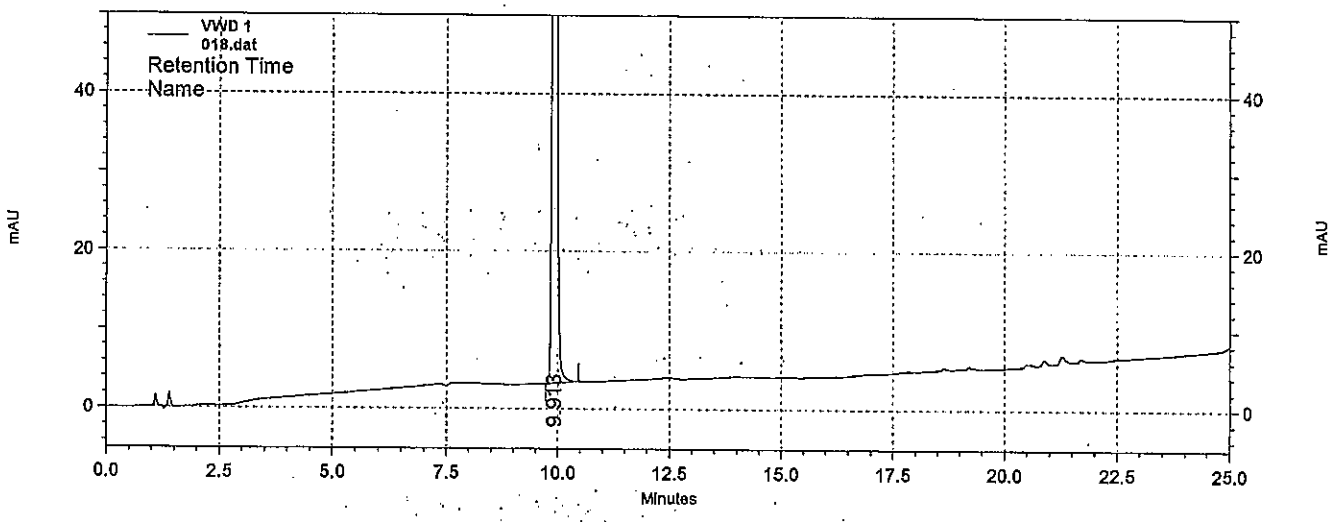
Printed: 09/06/2010 14:26:42 (GMT +03:00) by Dahlia Weinberg (TEVAIL\DWEINBERG)

stg base sal 087-088 40.38/10*1/20

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: stg base sal 087-088 40.38/10*1/20 {Data Description}
Acquired: 12/05/2010 21:31:56 (GMT +03:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201041 STG Imp hp25.met
Method rev.: 12/05/2010 10:11:36 (GMT +03:00)
Instrument: HPLC_25 **Vial:** 12 **Inj. Vol.:** 10 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg assay_12.05.2010_2.seq001.ol.ssizip | stg assay_12.05.2010_2.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg assay_12.05.2010_2.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (09/06/2010 14:26:52 (GMT +03:00)) (Reprocessed)



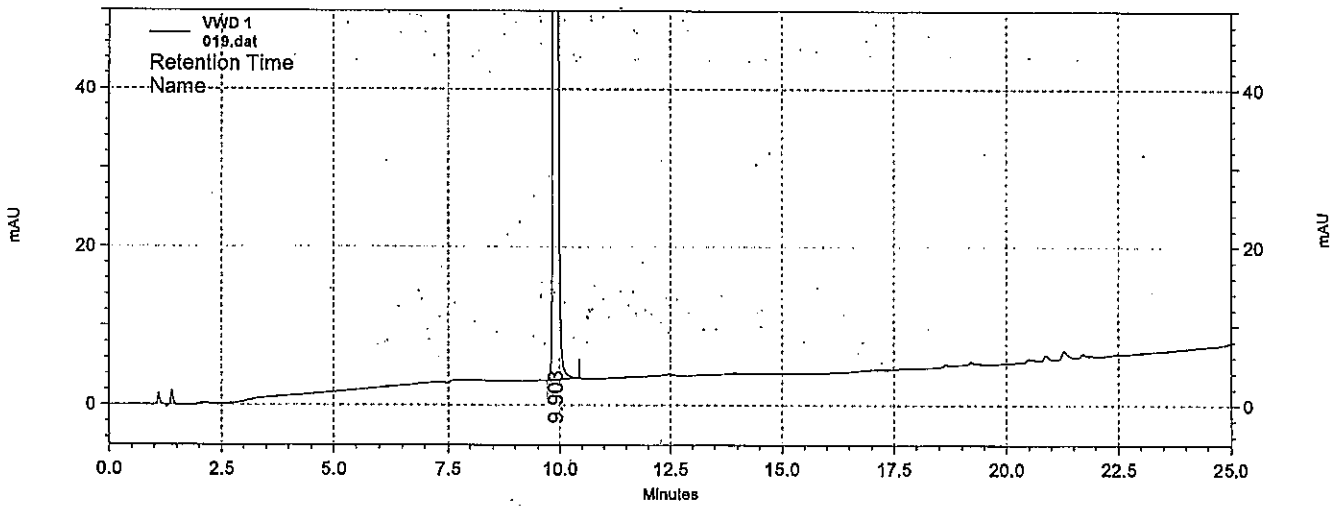
VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width	Resolution (USP)
	1	9.913	1175758	100.000	210590	0.75	0.00
Totals			1175758	100.000	210590		

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: stg base sal 087-088 40.42/10*1/20 {Data Description}
Acquired: 12/05/2010 22:05:30 (GMT +03:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201041 STG Imp hp25.met
Method rev.: 12/05/2010 10:11:36 (GMT +03:00)
Instrument: HPLC_25 **Vial:** 13 **Inj. Vol.:** 10 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg assay_12.05.2010_2.seq001.ol.ssizip | stg assay_12.05.2010_2.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg assay_12.05.2010_2.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (09/06/2010 14:26:58 (GMT +03:00)) (Reprocessed)



VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width	Resolution (USP)
	1	9.903	1186280	100.000	211783	0.73	0.00
Totals			1186280	100.000	211783		

12.May 2010 14:54
User ANNA
Type AX205
SNR 1121102961
Balance 8
SAMPLE STG BASE
ID SAL 87-88

4.98 mg
40.42 mg

ASSAY DETERMINATION

&\PROJECTS\TAGLIPTIN\Analytical\Assay\[Assay STG base 12.05.11

NOT ORIGINAL

Project: stg
Instrument: hp25

ANALYTICAL STANDARDS

Name: STG Ph
C.N / Batch: 1895STG1-T-39342109
% Potency: 99.8
Exp/Retest date: 10.2010

Weight 1 (mg): 19.53
Init.Volume 1 (ml): 20
Total Dilution 1 : 4

Weight 2 (mg): 20.46
Init.Volume 2 (ml): 20
Total Dilution 2 : 4

Multipla. Factor : 0.81

Standard 1				Standard 2			
Nomin Conc. (mg/ml): 2.4364E-01				Nomin Conc. (mg/ml): 2.5524E-01			
#	RT (min)	Std. Area	Resp.Factor	#	RT (min)	Std. Area	Resp. Factor
1	9.92	1146273	5808449.88	1	9.92	1166982	5741335.56
2	9.92	1149596	5825288.35	2			
3				3			
Average area of 3 inj :		1147934.60		Average area of 3 inj :		1166982.00	
SD:				SD:			
RSD ,% :				RSD ,% :			

Average factor of 6 injections : 5791891.28
SD : 44414.59
RSD ,% : 0.8

Control Std: As control std used Standard No 1
Nomin Conc. (mg/ml): 2.4364E-01

#	RT (min)	Std. Area	Resp. Factor	Accept. value, %

ASSAY (weight/weight, %)

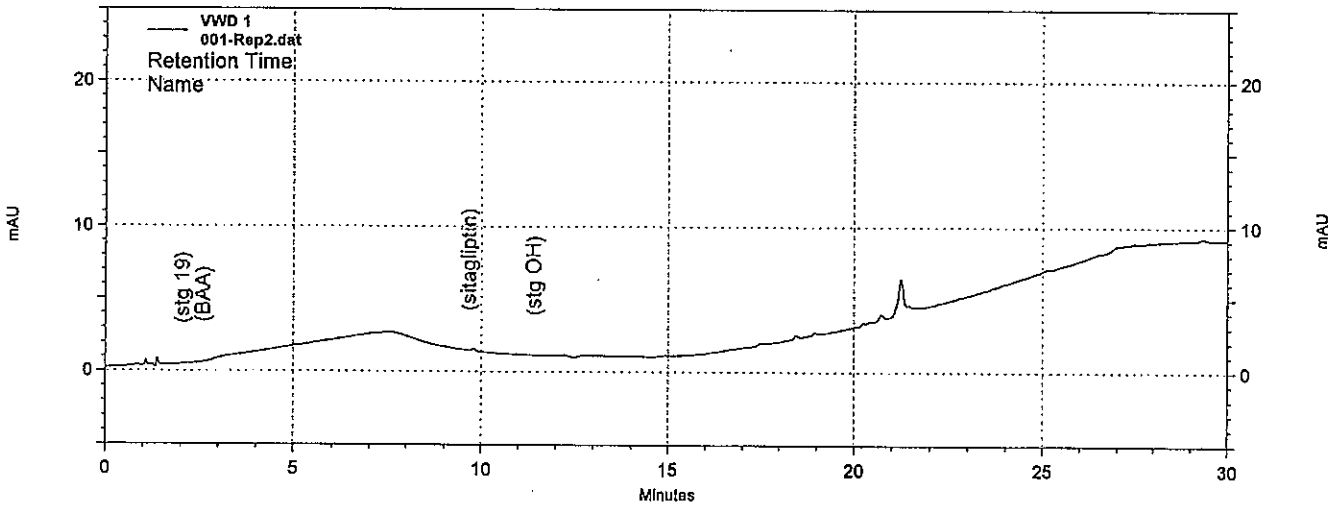
Initial Volume (ml): 10
Total Dilution: 20

Name: stg base	#	LOD(%) / Water (%)	Weight (mg)	Conc. (mg/ml)	RT (min)	Area	Resp. Factor	Assay (%)
C.N / Batch: sg0087-099	1		40.39	2.0190E-01	9.91	1175758	5823467.06	100.65
	2		40.42	2.0210E-01	9.9	1166280	5869767.44	101.35
							Average :	100.9
							Differ.,% :	0.8

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: dil {Data Description}
Acquired: 25/03/2010 16:20:16 (GMT +03:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201041 STG Imp-long hp36.met
Method rev.: 11/03/2010 16:36:12 (GMT +03:00)
Instrument: HPLC_36 **Vial:** 1 **Inj. Vol.:** 5 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg impurity_25.03.2010_2.seq001.ol.ssizip | stg impurity_25.03.2010_2.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg impurity_25.03.2010_2.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (28/03/2010 10:10:39 (GMT +03:00)) (Reprocessed)



VWD 1 Results

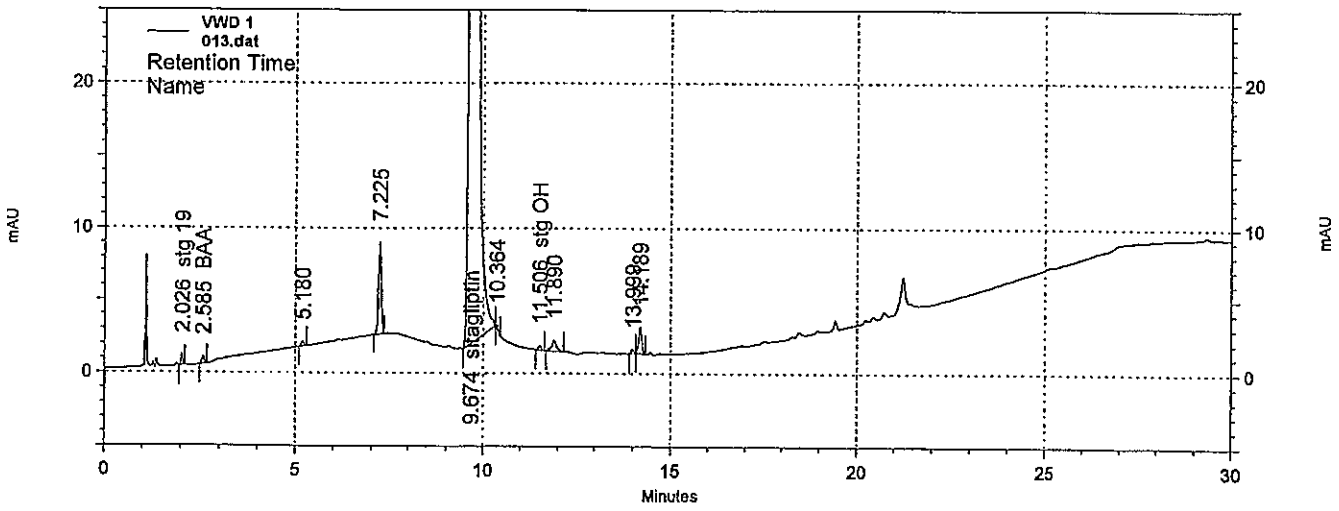
Name	Pk #	Retention Time	Area	Area Percent	Height	Width	RRT
stg 19							
BAA							
sitagliptin							
stg OH							
Totals							

Printed: 28/03/2010 10:10:41 (GMT +03:00) by Dahlia Weinberg (TEVAIL\DWEINBERG)

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: stg-b sal 069 19.51/5 {Data Description}
Acquired: 26/03/2010 00:46:18 (GMT +03:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201041 STG Imp-long hp36.met
Method rev.: 11/03/2010 16:36:12 (GMT +03:00)
Instrument: HPLC_36 **Vial:** 36 **Inj. Vol.:** 5 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg impurity_25.03.2010_2.seq001.ol.ssizip | stg impurity_25.03.2010_2.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg impurity_25.03.2010_2.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (28/03/2010 10:11:36 (GMT +03:00)) (Reprocessed)



VWD 1 Results

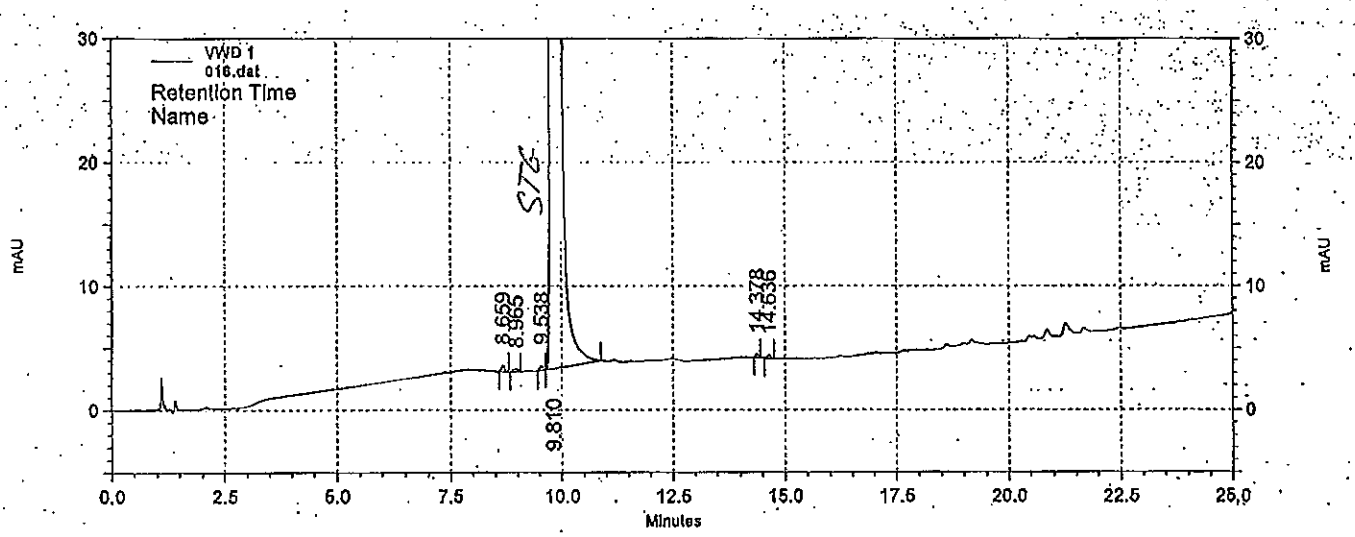
Name	Pk #	Retention Time	Area	Area Percent	Height	Width	RRT
stg 19	1	2.026	2813	0.028	748	0.17	0.21
BAA	2	2.585	2212	0.022	513	0.19	0.27
	3	5.180	1446	0.015	311	0.18	0.53
	4	7.225	33528	0.339	6361	0.29	0.75
sitagliptin	5	9.674	9834776	99.387	1202458	0.86	1.00
	6	10.364	1159	0.012	271	0.13	1.07
stg OH	7	11.506	1737	0.018	316	0.24	1.19
	8	11.890	6554	0.066	736	0.48	1.23
	9	13.999	1297	0.013	261	0.17	1.45
	10	14.189	9875	0.100	1780	0.24	1.47
Totals			9895397	100.000	1213755		

Printed: 28/03/2010 10:11:37 (GMT +03:00) by Dahlia Weinberg (TEVAIL\DWEINBERG)

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: stg base sal 087-088 40.38/10 {Data Description}
Acquired: 12/05/2010 17:23:53 (GMT +03:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201041 STG Imp hp25.met
Method rev.: 13/05/2010 09:41:05 (GMT +03:00)
Instrument: HPLC_25 **Vial:** 11 **Inj. Vol.:** 5 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg impurity_11.05.2010_3.seq001.ol.ssizip | stg impurity_11.05.2010_3.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg impurity_11.05.2010_3.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (13/05/2010 09:48:09 (GMT +03:00)) (Reprocessed)



VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width	Resolution (USP)
	1	8.659	2327	0.024	471	0.21	0.00
	2	8.965	1641	0.017	215	0.23	1.94
	3	9.538	1784	0.019	384	0.17	3.66
	4	9.810	9630820	99.913	1227709	1.25	1.61
	5	14.378	1239	0.013	269	0.16	27.23
	6	14.636	1367	0.014	257	0.20	1.95
Totals			9639178	100.000	1229305		

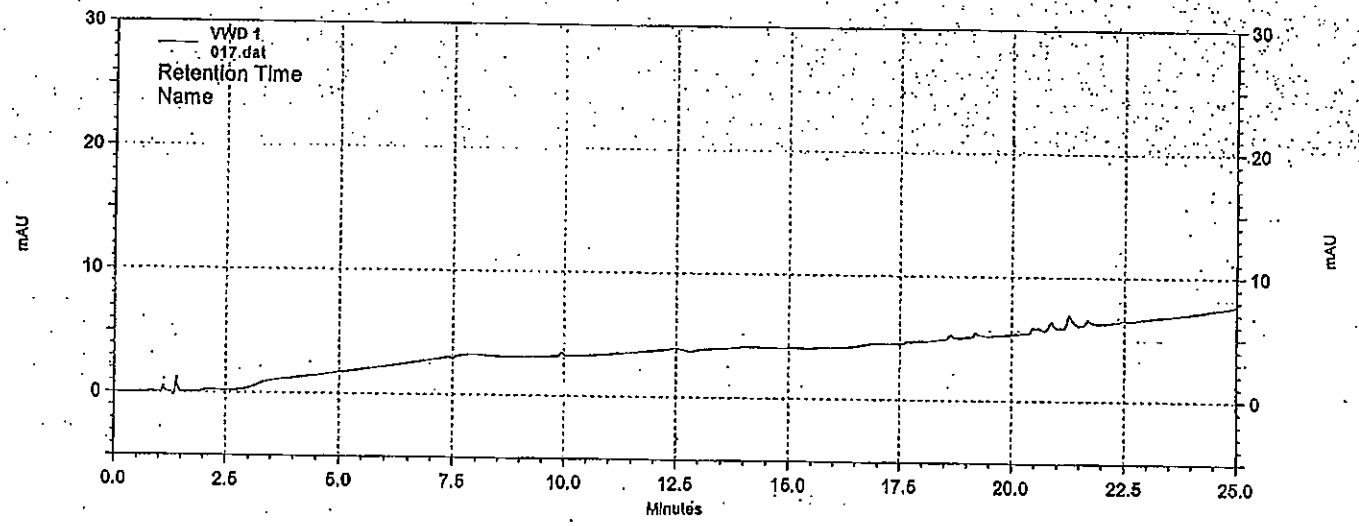
12.May 2010 14:52
User ANNA
Type AX205
SNR 1121102961
Balance 8
SAMPLE STG BASE
ID SAL 87-88

8.00 mg
48.38 mg
48.38 mg

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: diluent {Data Description}
Acquired: 12/05/2010 17:57:23 (GMT +03:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201041 STG Imp hp25.met
Method rev.: 13/05/2010 09:41:05 (GMT +03:00)
Instrument: HPLC_25 Vial: 1 Inj. Vol.: 5 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg impurity_11.05.2010_3.seq001.ol.ssizip | stg impurity_11.05.2010_3.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg impurity_11.05.2010_3.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (13/05/2010 09:48:13 (GMT +03:00)) (Reprocessed)



VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width	Resolution (USP)

056 TITLE

PROJECT NO.

BOOK NO.

24.03.10 Sitagliptin

STG-Base: Assay

HPLC 36. All conditions are the same as on page 1.

SST in Notebook: 43371001/63 14.3.10 Resolution = 11.00

Project: stg
Instrument: hp 36

S:\PROJECTS\SITAGLIPTIN\Analytical\assay\Assay STG base 24.03.1

NOT ORIGINAL

Samples: Sal 069, [redacted]

ANALYTICAL STANDARDS

Name: stg
C.N / Batch:
% Potency: 99.9
Exp/Retest date:

Weight 1 (mg): 21.13
Init.Volume 1 (ml): 20
Total Dilution 1 : 4

Weight 2 (mg): 19.28
Init.Volume 2 (ml): 20
Total Dilution 2 : 4

Multiplic. Factor : 0.81

Standard 1
Nomin Conc. (mg/ml): 2.5386E-01

#	RT (min)	Std. Area	Resp.Factor
1	9.99	1265858	5922771.44
2	9.98	1263116	5909942.01
3			
Average area of 3 inj :		1264487.00	
SD:			
RSD,% :			

Standard 2
Nomin Conc. (mg/ml): 2.4076E-01

#	RT (min)	Std. Area	Resp. Factor
1	9.97	1143945	5865939.71
2			
3			
Average area of 3 inj :		1143945.00	
SD:			
RSD,% :			

Average factor of 6 injections : 5899551.05
SD : 29306.72
RSD,% : 0.5

Control Std: As control std used Standard No 1
Nomin Conc. (mg/ml): 2.6386E-01

#	RT (min)	Std. Area	Resp. Factor	Accept. value, %
1	9.96	1265104	5919664.68	100.3

ASSAY (weight/weight, %)

Initial Volume (ml): 5
Total Dilution: 20

Name: stg base
C.N / Batch: sal 069

#	LOD(%) / Water (%)	Weight (mg)	Conc. (mg/ml)	RT (min)	Area	Resp. Factor	Assay (%)
1		21.01	2.1010E-01	9.97	1267073	6030609.14	102.22
2		19.51	1.9510E-01	9.97	1176005	6027703.74	102.17
						Average :	102.2
						Differ.,% :	0.1

Name:
C.N / Batch:

#	LOD(%) / Water (%)	Weight (mg)	Conc. (mg/ml)	RT (min)	Area	Resp. Factor	Assay (%)
1		[redacted]					
2							
						Average :	
						Differ.,% :	

Project: stg
Instrument: hp 36

W:\save.corp\pta\pladata\DATA\Data\MGPPROJECTS\SITAGLIPTIN\An

NOT ORIGINAL

ANALYTICAL STANDARDS

Name: stg

Weight 1 (mg): 21.13
Init.Volume 1 (ml): 20

Weight 2 (mg): 19.28
Init.Volume 2 (ml): 20

24.03.10
43111001/56

SIGNATURE

DISCLOSED TO AND UNDERSTOOD BY

DATE 6.4.10

WITNESS

DATE 28.3.10

DATE

060 TITLE PROJECT NO.

BOOK NO.

25.03.10 Sitagliptin

STG: %Area SST: Res. 8.4, S/N = 7

Samples: [redacted] S. 069

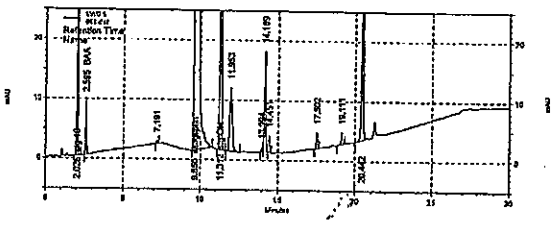
25.03.10											STG %AREA											
RT	9.80	2.03	2.60	7.25	8.47	8.74	MTAB	9.33	10.24	10.92	11.48	11.84	14.04	14.28	14.52	14.78	14.85	15.57	15.68	16.46	16.85	17.28
RRT	1.00	0.21	0.27	0.76	0.88	0.91	0.94	0.97	1.07	1.14	1.20	1.23	1.46	1.49	1.52	1.54	1.56	1.62	1.65	1.71	1.76	1.80
Name	STG	STG-19 (RRF 0.45)	BAA (RRF 0.4)					di-flouro	STG-i-Bu		stg OH				eliminate	STG-plv					STG bac	
Sample																						
[redacted]																						
stg base sal 069	99.39	0.07	0.05	0.34			LT 0.01		0.01		0.02	0.07	0.01	0.10								

See Marker prepration in n/b 43371001, p001

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.1 OEM ID 1

Sample Name: marker (Data Description)
Acquired: 25/03/2010 7:16:16 PM (GMT +02:00)
Method: Analytical R&DSTG\User 6 (DishiliW)\Method\51-201041 STG Imp-long hp35.mth
Method rev.: 11/03/2010 3:06:12 PM (GMT +02:00)
Instrument: HPLC_36 Vials: 4 Inj. Vol: 5 µL
Sequence: Analytical R&DSTG\User 6 (DishiliW)\Data\hplc_3\stg impurity_25.03.2010_2.seq001.sl.sisisip\stg impurity_25.03.2010_2.sl
Data File: Analytical R&DSTG\User 6 (DishiliW)\Data\hplc_3\stg impurity_25.03.2010_2.seq001.sl.sisisip
Results Source: TEVA\LVARUDYAK (06/04/2010 10:17:10 AM (GMT +02:00)) (Reprocessed)



SWD 1 results

Name	Pk#	Retention Time	Area	Area Percent	Width	RRT	Theoretical plates (USP)	Resolution (USP)
stg 19	1	2.026	195703	1.438	0.43	0.21	8472	0.00
BAA	2	2.595	34139	0.251	0.25	0.27	10781	6.05
	3	7.191	2167	0.016	0.23	0.74	52244	40.71
sitagliptin	4	9.650	12437520	91.416	1.37	1.00	24835	13.26
stg OH	5	11.312	425314	3.126	0.52	1.17	90433	8.41
	6	11.553	117907	0.867	0.88	1.24	26753	2.90
	7	13.994	2334	0.017	0.16	1.45	220126	9.92
	8	14.189	94122	0.692	0.27	1.47	140128	1.43
	9	14.451	14568	0.107	0.24	1.50	155617	1.76
	10	17.502	16053	0.118	0.34	1.81	190348	19.89
	11	19.111	12883	0.095	0.53	1.98	236372	10.13
	12	20.442	252647	1.857	0.63	2.12	202753	8.64
Totals			13605357	100.000				

SIGNATURE RDS DATE 29.03.10

DISCLOSED TO AND UNDERSTOOD BY Rey DATE 06.4.10 WITNESS DATE

Rey 19.10.10

SIGNATURE

DISCLOSED TO

088 TITLE

PROJECT NO.

BOOK NO.

12.5.10 Sitagliptin

STG: % Area + Assay (For sal 087/88 only)

Res = 11.3 c

SST: 43371001/63 14.3.10

HPLC 25. All HPLC conditions are the same as on page 1.

Samples: [redacted]

STG Base - sal 087-088

12.05.10

Project: stg

Instrument: hp25

S:\PROJECTS\STAGLIPTIN\Analytical\Assay\Assay STG base 12.05.10

ANALYTICAL STANDARDS

Name: STG Ph
C.N / Batch: 18955101-T-39342109
% Potency: 99.8
Exp/Retest date: 10.2010

Weight 1 (mg): 19.53
Init.Volume 1 (ml): 20
Total Dilution 1: 4

Weight 2 (mg): 20.46
Init.Volume 2 (ml): 20
Total Dilution 2: 4

Multiple Factor: 0.81

Standard 1
Nomin Conc. (mg/ml): 2.4364E-01

#	RT (min)	Std. Area	Resp.Factor
1	9.92	1146273	5808449.88
2	9.92	1149596	5825268.95
3			
Average area of 3 inj:		1147934.50	
SD:			
RSD,%:			

Standard 2
Nomin Conc. (mg/ml): 2.5524E-01

#	RT (min)	Std. Area	Resp. Factor
1	9.92	1156982	5741335.56
2			
3			
Average area of 3 inj:		1156982.00	
SD:			
RSD,%:			

Average factor of 6 injections: 5791691.26
SD: 44414.59
RSD,%: 0.8

Control Std: As control std used Standard No 1
Nomin Conc. (mg/ml): 2.4364E-01

#	RT (min)	Std. Area	Resp. Factor	Accept. value, %

ADT ORIGINAL

43111001/88

DMS 43111001/88

12.5.10

12.05.10

Sar

stg base s

ASSAY (weight/weight, %)

Initial Volume (ml): 10

Total Dilution: 20

Name: stg base C.N / Batch: sal087-088	#	LOD(%) / Water (%)	Weight (mg)	Conc. (mg/ml)	RT (min)	Area	Resp. Factor	Assay (%)
	1		40.38	2.0190E-01	9.91	1175758	5823467.06	100.55
	2		40.42	2.0210E-01	9.9	1166290	5769787.44	101.35
							Average:	100.9
							Differ.,%:	0.8

See Marker preparation in n/b 43371001 P 94

12.05.10

%Area of impurities

[redacted]

*samples were stored at room temperature and not in refrigeration

12.05.10

SAMPLE	NOTES	PURITY OF STG	%Area of impurities							
			RT	STG-19	2.64	2.5-dif	2.4-dif	9.54	Elim.1	Elim.2
			RRT	0.22	0.27	0.88	0.91	0.97	1.47	1.49
stg base sal 087-088		99.910				0.024	0.017	0.019	0.013	0.014

SIGNATURE DMS

DISCLOSED TO AND UNDERSTOOD BY

DATE 16.05.10

WITNESS

DATE 13.05.10

SIGNATURE DMS

DISCLOSED TO AND UNDE

DATE

231202, Compound 184, Lot D-1895NN-13067/3, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

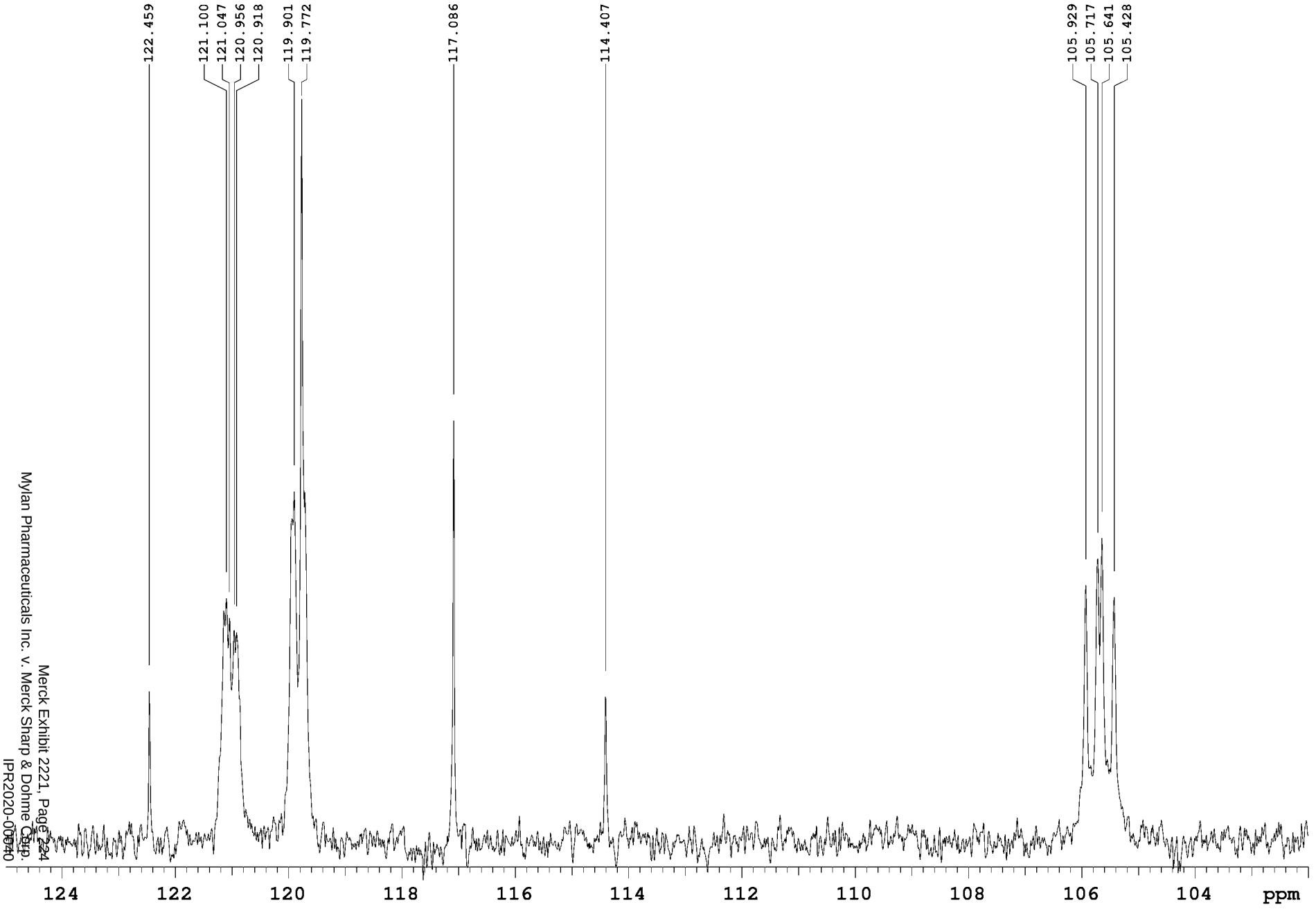
File: 409909

INDEX	FREQUENCY	PPM	HEIGHT
1	17012.545	169.232	65.5
2	17000.338	169.110	141.8
3	15823.885	157.408	24.2
4	15814.730	157.317	25.7
5	15580.508	154.987	25.5
6	15571.353	154.896	31.0
7	15168.520	150.888	127.2
8	15157.839	150.782	60.5
9	15037.295	149.583	14.9
10	15023.562	149.446	31.3
11	15009.066	149.302	15.0
12	14789.340	147.117	30.7
13	14775.607	146.980	50.9
14	14762.637	146.851	20.8
15	14544.436	144.680	25.1
16	14532.229	144.559	23.2
17	14384.219	143.087	12.6
18	14346.072	142.707	43.6
19	14330.050	142.548	19.2
20	14307.162	142.320	43.7
21	14291.140	142.161	18.8
22	14268.252	141.933	15.0

Plot file: 409909-2_peaks

231202, Compound 184, Lot D-1895NN-13067/3, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

File: 409909



Plot file: 409909-3

231202, Compound 184, Lot D-1895NN-13067/3, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

File: 409909

INDEX	FREQUENCY	PPM	HEIGHT
1	12310.549	122.459	28.6
2	12173.983	121.100	46.4
3	12168.643	121.047	42.6
4	12159.487	120.956	40.2
5	12155.673	120.918	39.8
6	12053.439	119.901	66.7
7	12040.469	119.772	141.8
8	11770.388	117.086	80.3
9	11501.071	114.407	27.5
10	10648.867	105.929	48.8
11	10627.505	105.717	53.9
12	10619.875	105.641	57.9
13	10598.513	105.428	46.5

Plot file: 409909-3_peaks

231202, Compound 184, Lot D-1895NN-13067/3, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

File: 409909

INDEX	FREQUENCY	PPM	HEIGHT
1	4782.626	47.575	15.2
2	4382.845	43.598	13.7
3	4322.573	42.999	6.5
4	4186.007	41.640	7.7
5	4121.157	40.995	14.6
6	4033.419	40.122	19.9
7	4012.820	39.917	60.1
8	3991.458	39.705	120.5
9	3970.858	39.500	141.7
10	3949.496	39.287	122.4
11	3928.896	39.083	61.8
12	3907.534	38.870	21.1
13	3860.995	38.407	16.6
14	3761.813	37.421	7.4
15	3574.893	35.561	7.1
16	3536.746	35.182	14.1
17	3200.289	31.835	7.1
18	3183.505	31.668	12.9

Plot file: 409909-4_peaks

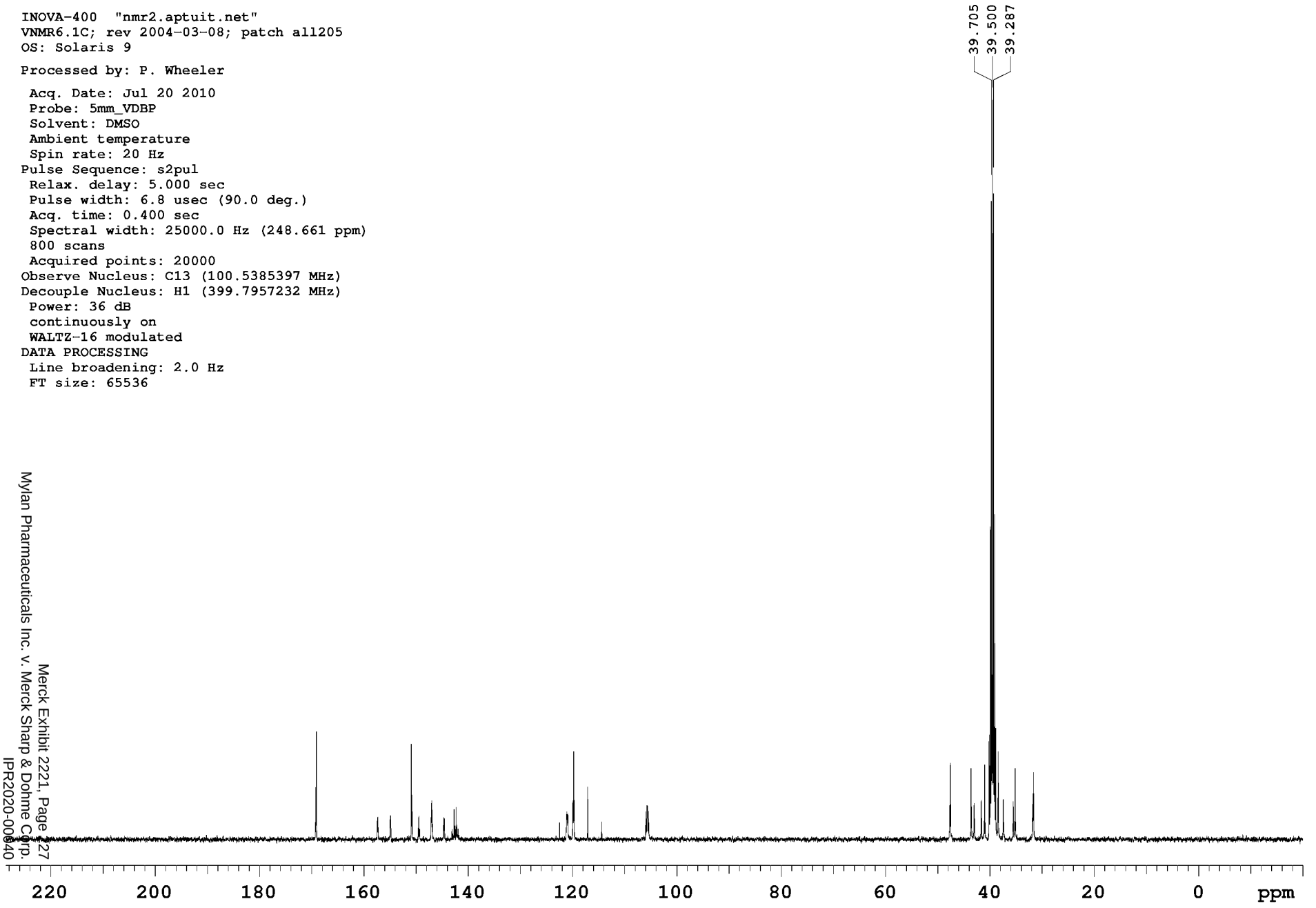
233141, 4063-03-01, Compound 184, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

File: 409910

INOVA-400 "nmr2.aptuit.net"
VNMR6.1C; rev 2004-03-08; patch all205
OS: Solaris 9

Processed by: P. Wheeler

Acq. Date: Jul 20 2010
Probe: 5mm_VDBP
Solvent: DMSO
Ambient temperature
Spin rate: 20 Hz
Pulse Sequence: s2pul
Relax. delay: 5.000 sec
Pulse width: 6.8 usec (90.0 deg.)
Acq. time: 0.400 sec
Spectral width: 25000.0 Hz (248.661 ppm)
800 scans
Acquired points: 20000
Observe Nucleus: C13 (100.5385397 MHz)
Decouple Nucleus: H1 (399.7957232 MHz)
Power: 36 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening: 2.0 Hz
FT size: 65536



Plot file: 409910-1

233141, 4063-03-01, Compound 184, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

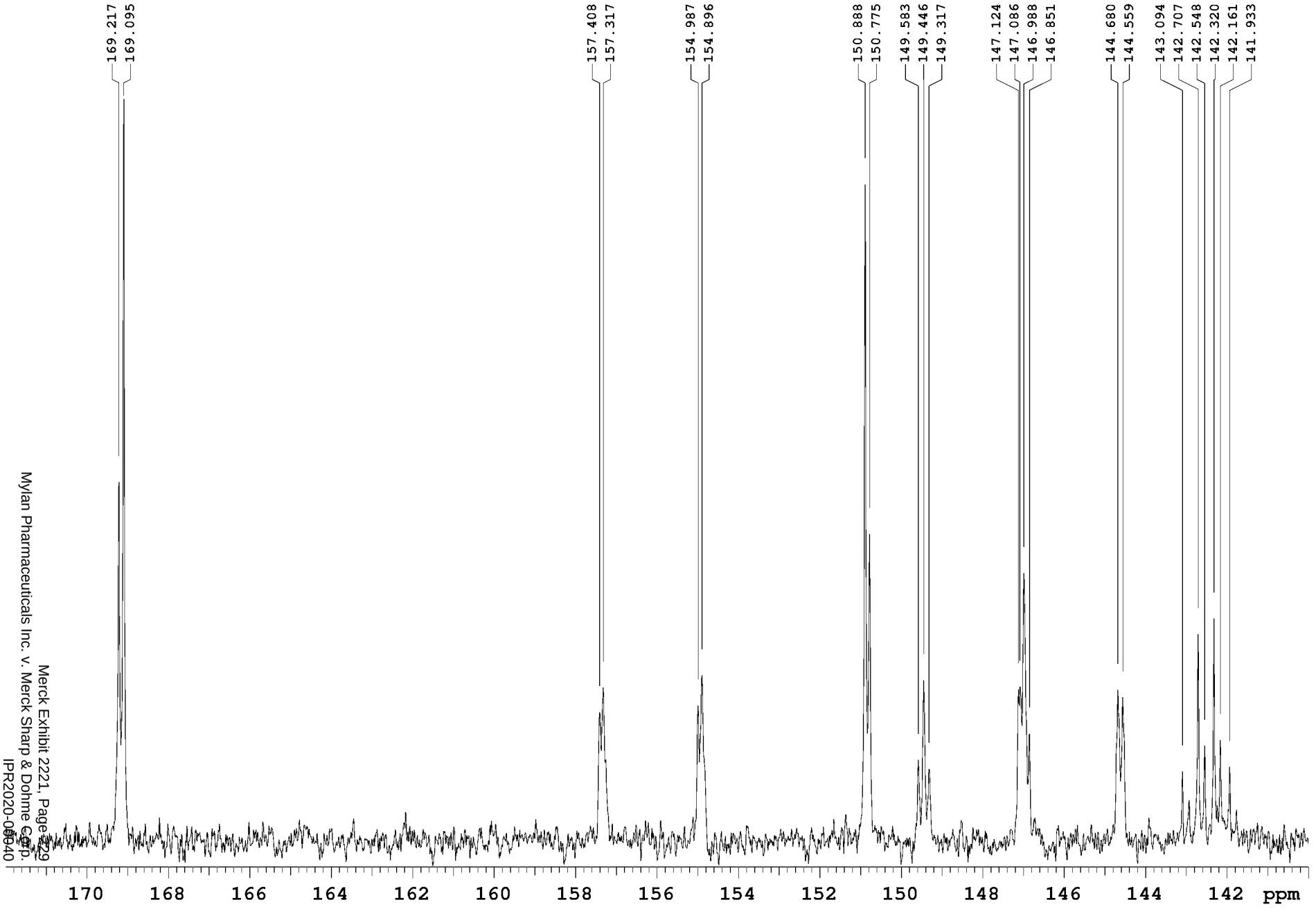
File: 409910

INDEX	FREQUENCY	PPM	HEIGHT
1	3991.458	39.705	122.4
2	3970.858	39.500	141.8
3	3949.496	39.287	123.9

Plot file: 409910-1_peaks

233141, 4063-03-01, Compound 184, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

File: 409910



Plot file: 409910-2

233141, 4063-03-01, Compound 184, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

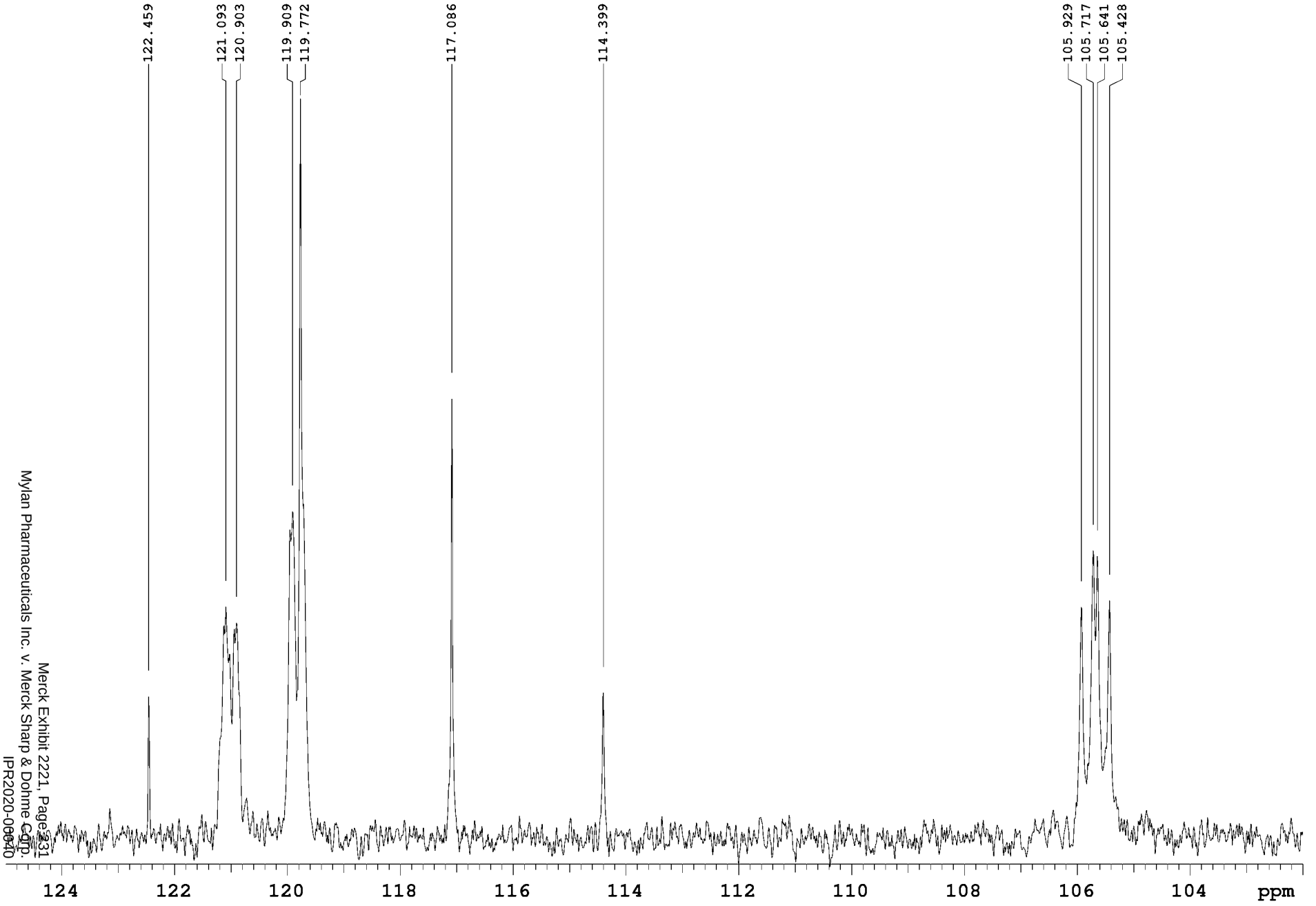
File: 409910

INDEX	FREQUENCY	PPM	HEIGHT
1	17011.019	169.217	68.6
2	16998.812	169.095	141.8
3	15823.885	157.408	24.6
4	15814.730	157.317	29.3
5	15580.508	154.987	25.9
6	15571.353	154.896	31.6
7	15168.520	150.888	125.4
8	15157.076	150.775	58.6
9	15037.295	149.583	15.5
10	15023.562	149.446	30.7
11	15010.592	149.317	13.8
12	14790.103	147.124	28.9
13	14786.288	147.086	29.5
14	14776.370	146.988	51.1
15	14762.637	146.851	20.5
16	14544.436	144.680	28.9
17	14532.229	144.559	27.5
18	14384.982	143.094	13.3
19	14346.072	142.707	39.5
20	14330.050	142.548	18.2
21	14307.162	142.320	42.5
22	14291.140	142.161	19.3
23	14268.252	141.933	14.2

Plot file: 409910-2_peaks

233141, 4063-03-01, Compound 184, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

File: 409910



Plot file: 409910-3

233141, 4063-03-01, Compound 184, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

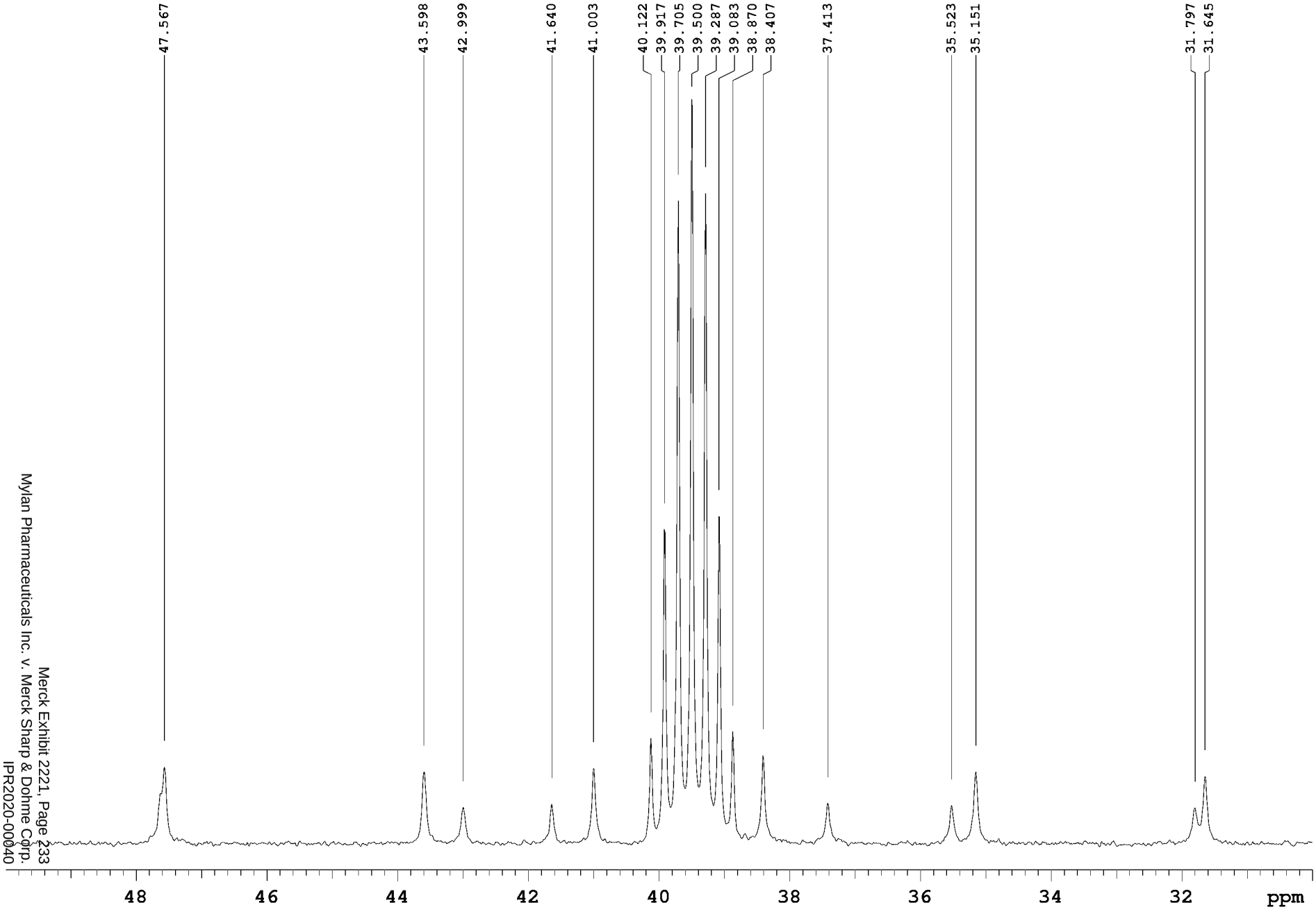
File: 409910

INDEX	FREQUENCY	PPM	HEIGHT
1	12310.549	122.459	27.1
2	12173.220	121.093	44.3
3	12154.147	120.903	41.3
4	12054.202	119.909	62.6
5	12040.469	119.772	141.8
6	11770.388	117.086	84.2
7	11500.308	114.399	27.9
8	10648.867	105.929	44.3
9	10627.505	105.717	55.1
10	10619.875	105.641	54.1
11	10598.513	105.428	45.5

Plot file: 409910-3_peaks

233141, 4063-03-01, Compound 184, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

File: 409910



Plot file: 409910-4

233141, 4063-03-01, Compound 184, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

File: 409910

INDEX	FREQUENCY	PPM	HEIGHT
1	4781.863	47.567	14.6
2	4382.845	43.598	13.7
3	4322.573	42.999	6.9
4	4186.007	41.640	7.5
5	4121.920	41.003	14.3
6	4033.419	40.122	20.1
7	4012.820	39.917	59.9
8	3991.458	39.705	122.4
9	3970.858	39.500	141.8
10	3949.496	39.287	123.9
11	3928.896	39.083	62.3
12	3907.534	38.870	21.4
13	3860.995	38.407	16.8
14	3761.050	37.413	7.7
15	3571.078	35.523	7.3
16	3533.694	35.151	13.7
17	3196.475	31.797	6.8
18	3181.216	31.645	12.8

Plot file: 409910-4_peaks

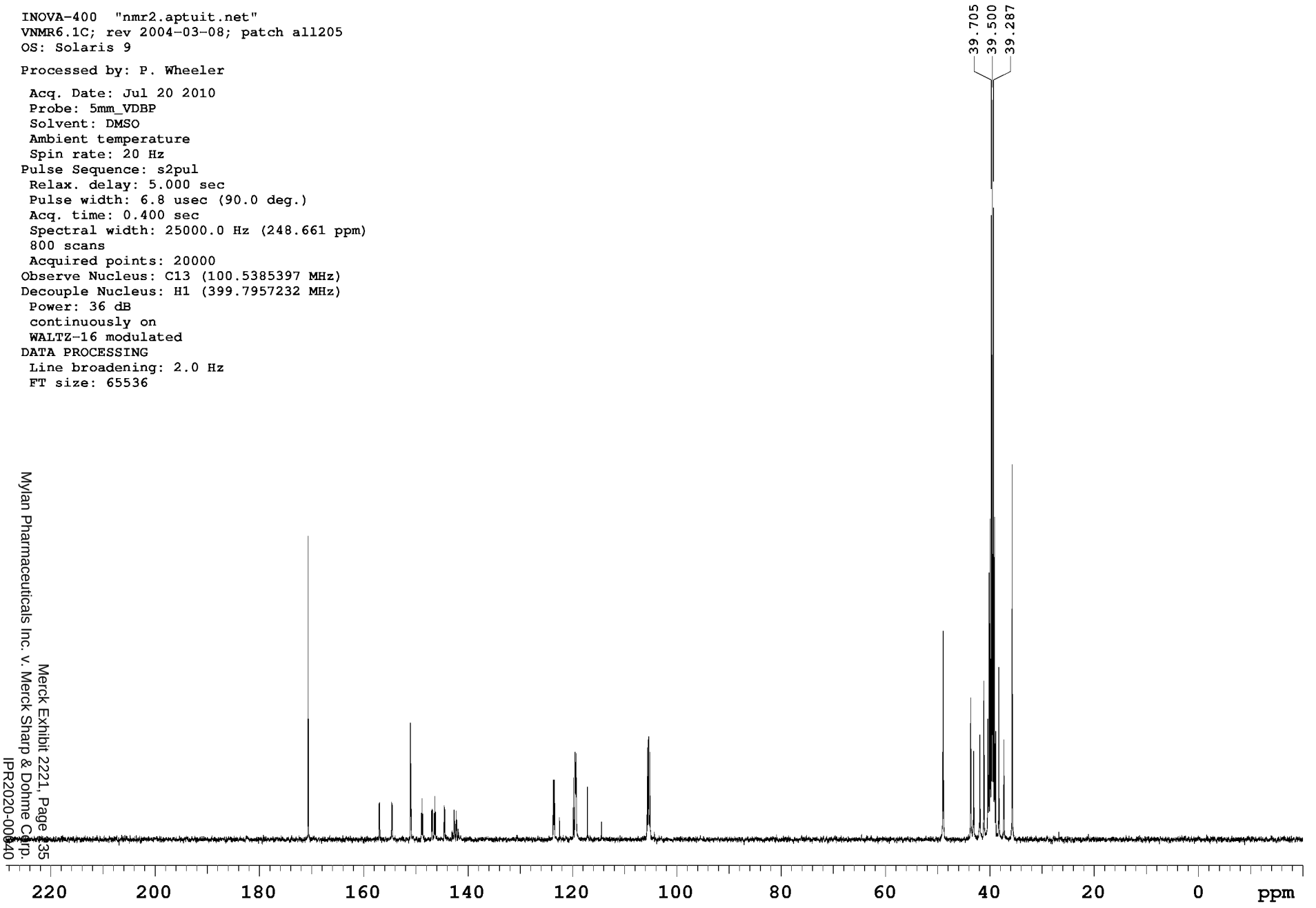
233285, Compound 184, Lot SAL-088,087, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

File: 410032

INOVA-400 "nmr2.aptuit.net"
VNMR6.1C; rev 2004-03-08; patch all205
OS: Solaris 9

Processed by: P. Wheeler

Acq. Date: Jul 20 2010
Probe: 5mm_VDBP
Solvent: DMSO
Ambient temperature
Spin rate: 20 Hz
Pulse Sequence: s2pul
Relax. delay: 5.000 sec
Pulse width: 6.8 usec (90.0 deg.)
Acq. time: 0.400 sec
Spectral width: 25000.0 Hz (248.661 ppm)
800 scans
Acquired points: 20000
Observe Nucleus: C13 (100.5385397 MHz)
Decouple Nucleus: H1 (399.7957232 MHz)
Power: 36 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening: 2.0 Hz
FT size: 65536



Plot file: 410032-1

233285, Compound 184, Lot SAL-088,087, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

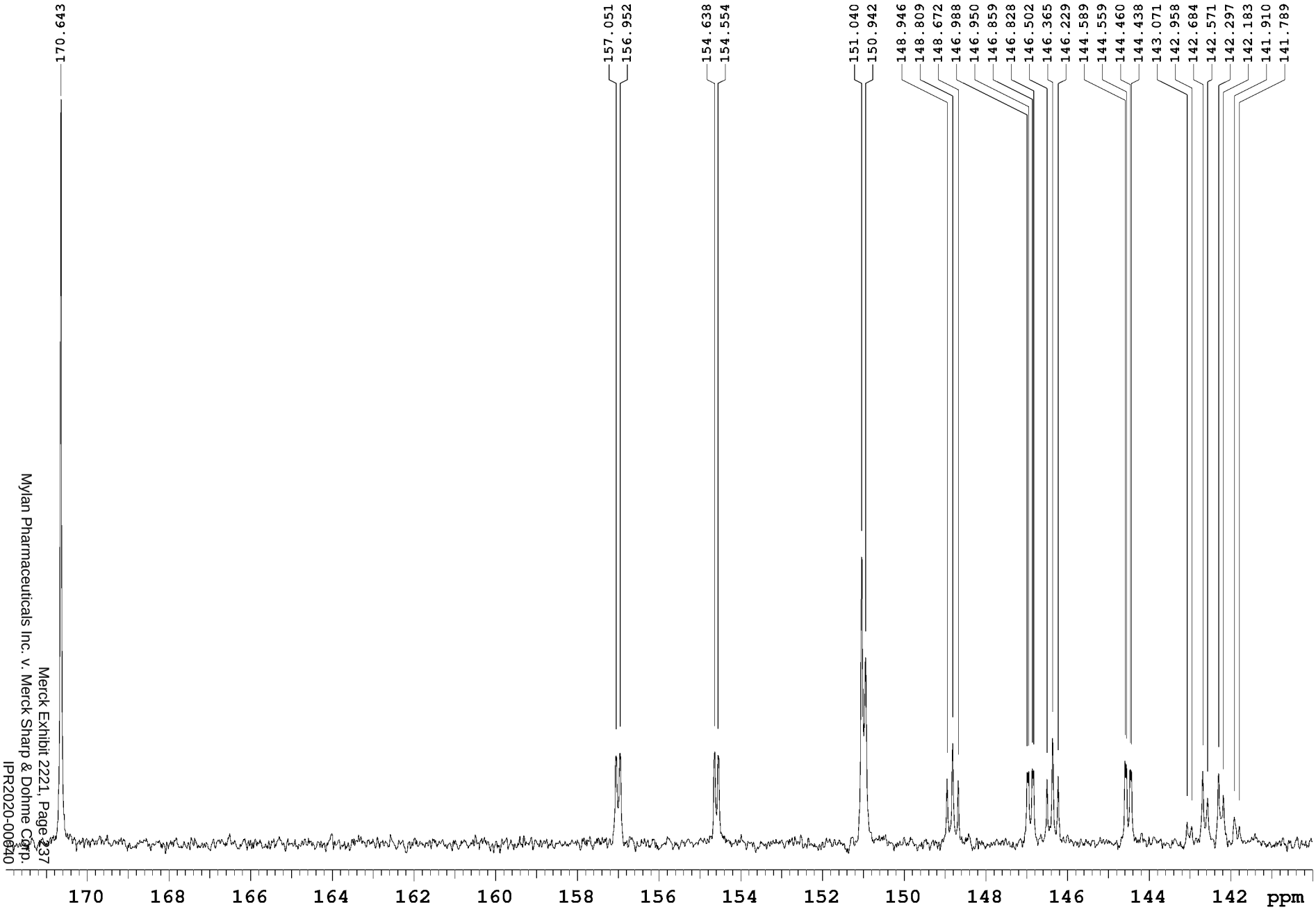
File: 410032

INDEX	FREQUENCY	PPM	HEIGHT
1	3991.458	39.705	119.6
2	3970.858	39.500	141.8
3	3949.496	39.287	121.1

Plot file: 410032-1_peaks

233285, Compound 184, Lot SAL-088,087, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

File: 410032



Plot file: 410032-2

233285, Compound 184, Lot SAL-088,087, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

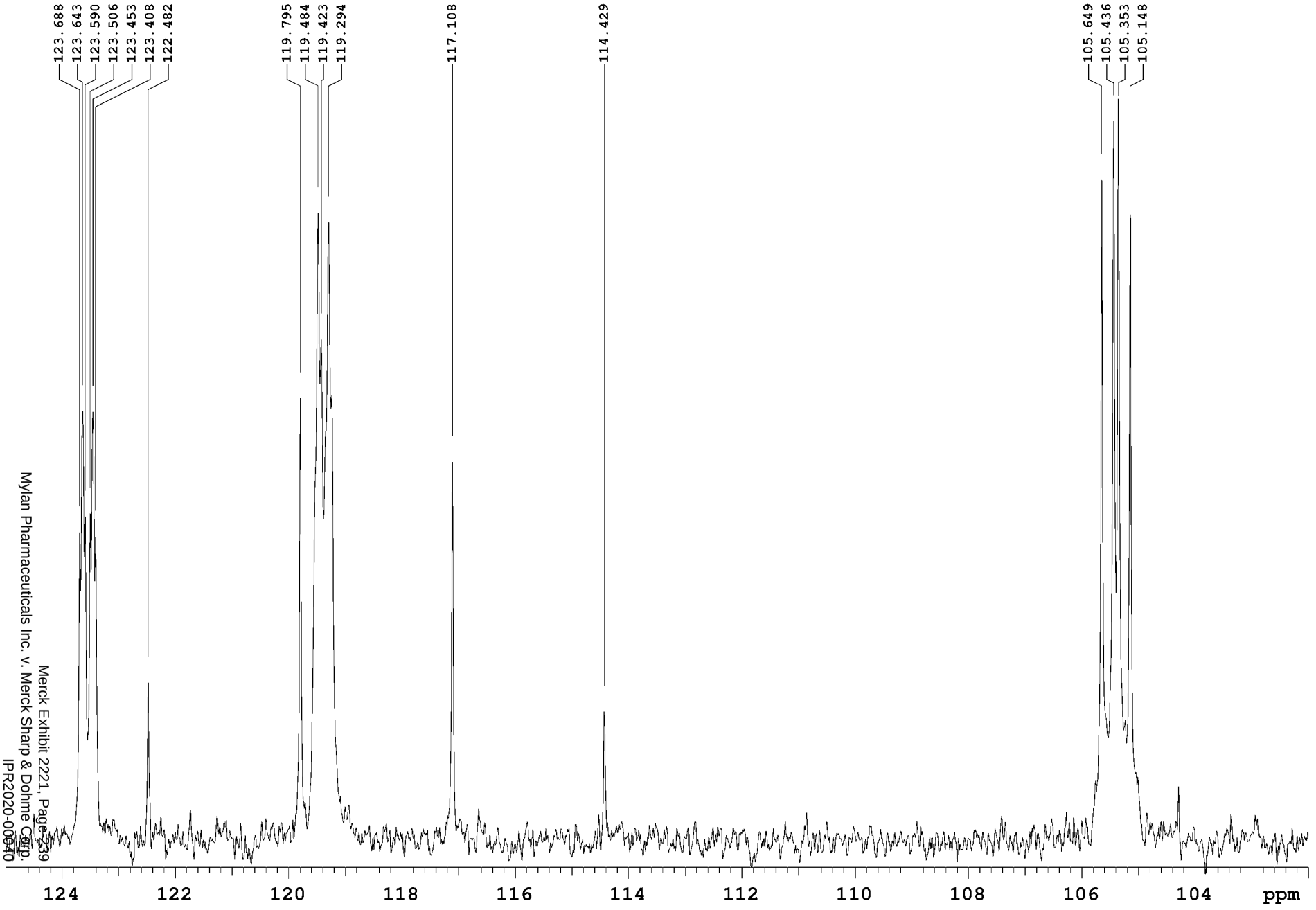
File: 410032

INDEX	FREQUENCY	PPM	HEIGHT
1	17154.452	170.643	141.8
2	15788.027	157.051	16.8
3	15778.109	156.952	17.3
4	15545.413	154.638	17.5
5	15537.020	154.554	16.9
6	15183.779	151.040	54.6
7	15173.861	150.942	35.4
8	14973.208	148.946	12.4
9	14959.475	148.809	19.1
10	14945.742	148.672	12.0
11	14776.370	146.988	13.6
12	14772.555	146.950	13.7
13	14763.400	146.859	14.3
14	14760.348	146.828	13.9
15	14727.542	146.502	12.3
16	14713.809	146.365	20.1
17	14700.076	146.229	12.9
18	14535.281	144.589	15.8
19	14532.229	144.559	15.1
20	14522.311	144.460	14.2
21	14520.022	144.438	13.9
22	14382.693	143.071	4.1
23	14371.249	142.958	3.3
24	14343.783	142.684	13.8
25	14332.339	142.571	8.7
26	14304.873	142.297	13.4
27	14293.429	142.183	9.2
28	14265.963	141.910	5.1
29	14253.756	141.789	3.3

Plot file: 410032-2_peaks

233285, Compound 184, Lot SAL-088,087, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

File: 410032



Plot file: 410032-3

233285, Compound 184, Lot SAL-088,087, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

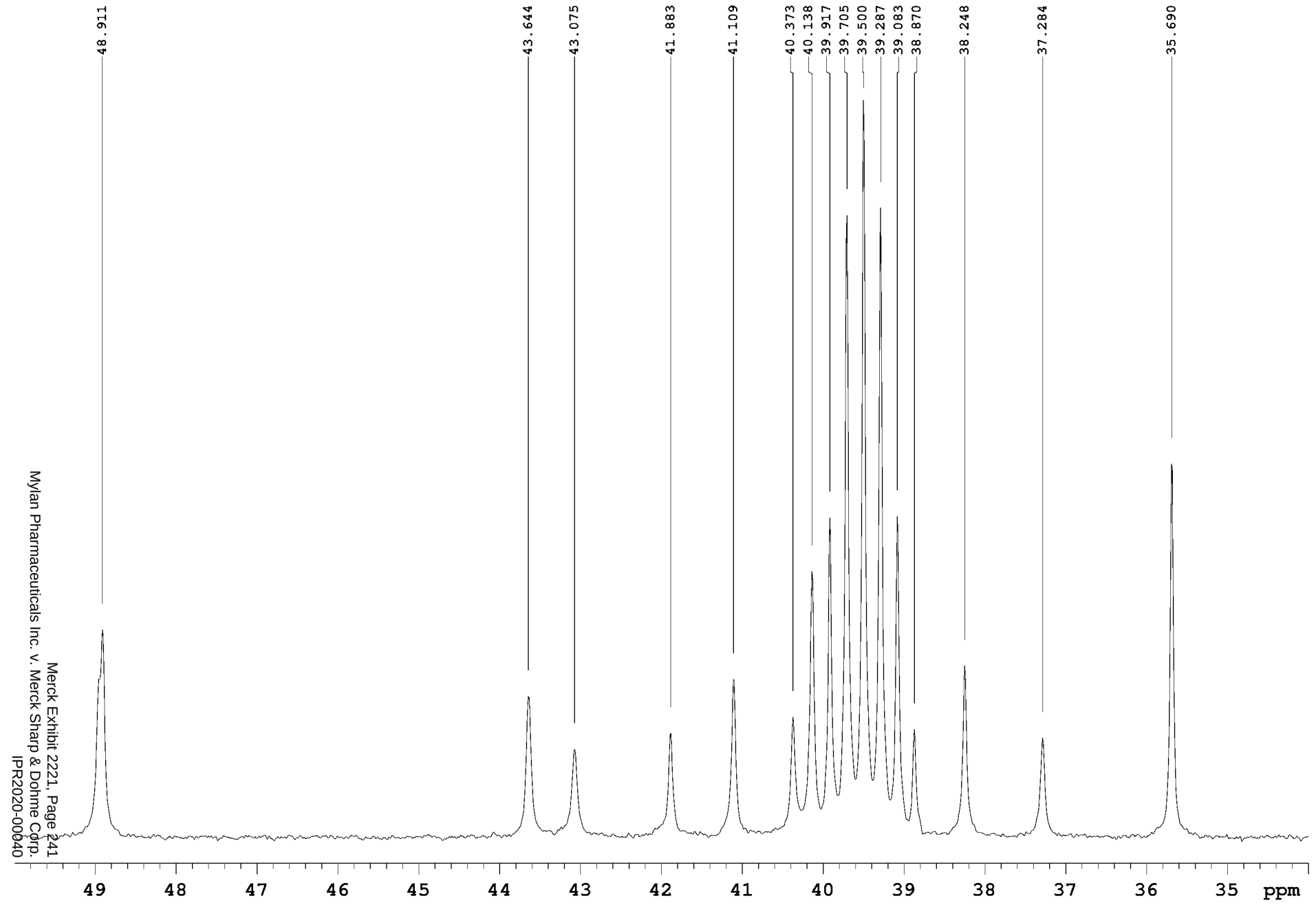
File: 410032

INDEX	FREQUENCY	PPM	HEIGHT
1	12434.146	123.688	59.0
2	12429.568	123.643	82.1
3	12424.227	123.590	62.0
4	12415.835	123.506	62.6
5	12410.494	123.453	82.0
6	12405.917	123.408	58.1
7	12312.838	122.482	30.3
8	12042.758	119.795	84.6
9	12011.477	119.484	120.0
10	12005.374	119.423	95.8
11	11992.404	119.294	118.2
12	11772.677	117.108	72.5
13	11503.359	114.429	24.7
14	10620.639	105.649	126.3
15	10599.276	105.436	137.6
16	10590.884	105.353	141.8
17	10570.284	105.148	119.7

Plot file: 410032-3_peaks

233285, Compound 184, Lot SAL-088,087, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

File: 410032



Plot file: 410032-4

233285, Compound 184, Lot SAL-088,087, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

File: 410032

INDEX	FREQUENCY	PPM	HEIGHT
1	4916.903	48.911	40.0
2	4387.423	43.644	27.1
3	4330.203	43.075	17.0
4	4210.421	41.883	20.1
5	4132.601	41.109	30.4
6	4058.596	40.373	23.1
7	4034.945	40.138	51.2
8	4012.820	39.917	61.5
9	3991.458	39.705	119.6
10	3970.858	39.500	141.8
11	3949.496	39.287	121.1
12	3928.897	39.083	61.8
13	3907.534	38.870	20.7
14	3844.973	38.248	33.0
15	3748.080	37.284	19.1
16	3587.863	35.690	71.9

Plot file: 410032-4_peaks

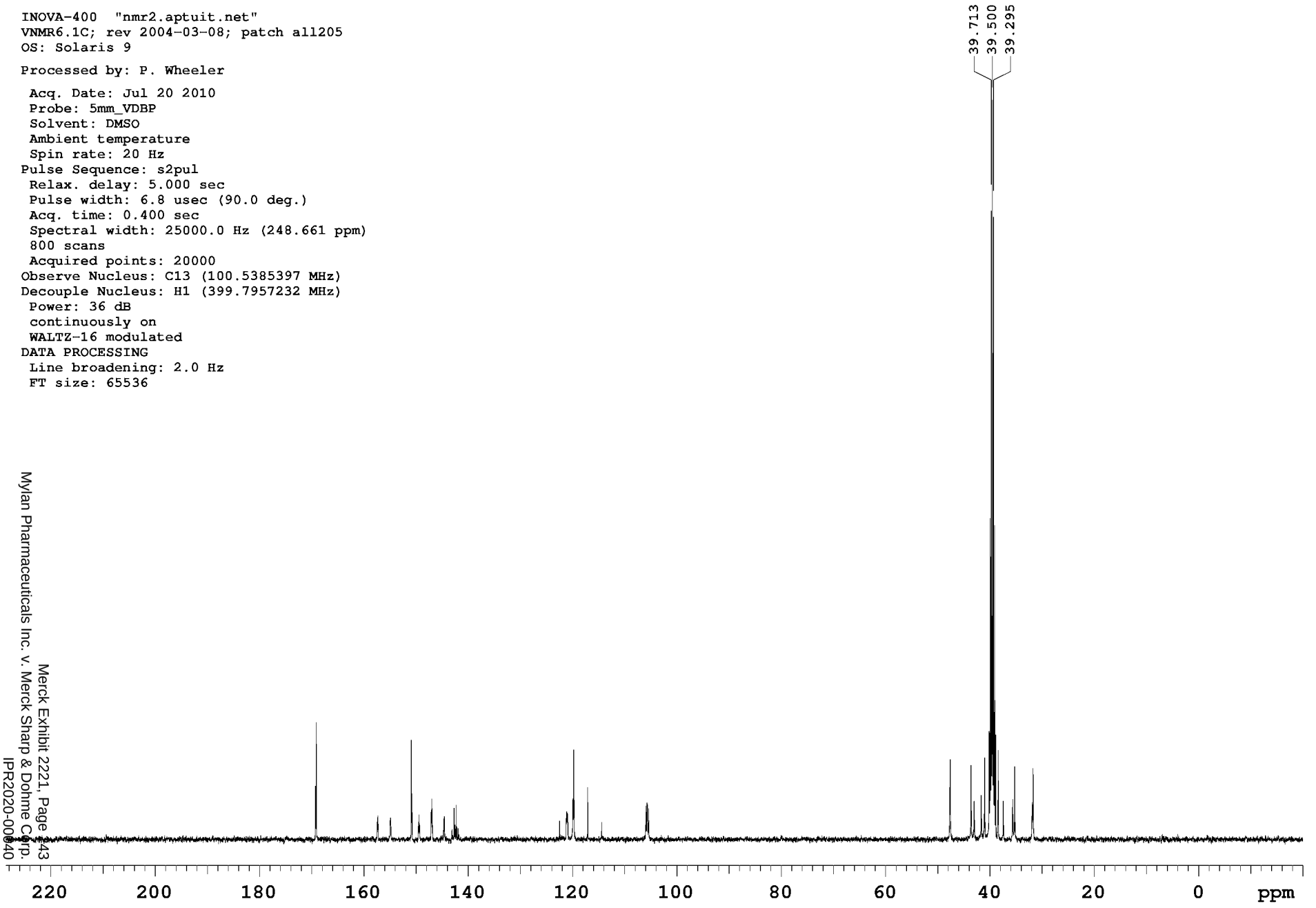
235849, 4063-57-01, Compound 184, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

File: 410114

INOVA-400 "nmr2.aptuit.net"
VNMR6.1C; rev 2004-03-08; patch all205
OS: Solaris 9

Processed by: P. Wheeler

Acq. Date: Jul 20 2010
Probe: 5mm_VDBP
Solvent: DMSO
Ambient temperature
Spin rate: 20 Hz
Pulse Sequence: s2pul
Relax. delay: 5.000 sec
Pulse width: 6.8 usec (90.0 deg.)
Acq. time: 0.400 sec
Spectral width: 25000.0 Hz (248.661 ppm)
800 scans
Acquired points: 20000
Observe Nucleus: C13 (100.5385397 MHz)
Decouple Nucleus: H1 (399.7957232 MHz)
Power: 36 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening: 2.0 Hz
FT size: 65536



Plot file: 410114-1

235849, 4063-57-01, Compound 184, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

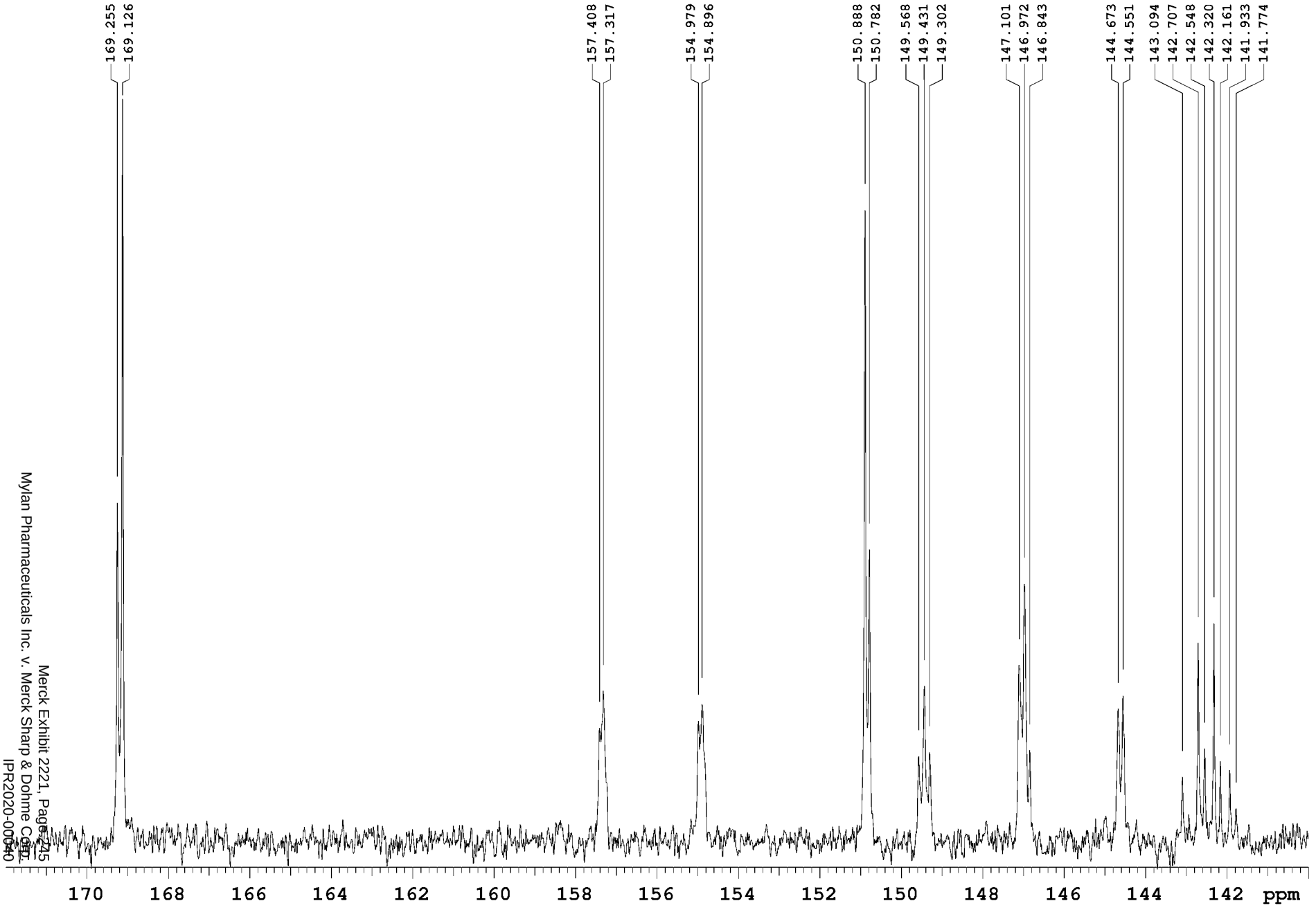
File: 410114

INDEX	FREQUENCY	PPM	HEIGHT
1	3992.220	39.713	120.5
2	3970.858	39.500	141.8
3	3950.259	39.295	119.4

Plot file: 410114-1_peaks

235849, 4063-57-01, Compound 184, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

File: 410114



Plot file: 410114-2

235849, 4063-57-01, Compound 184, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

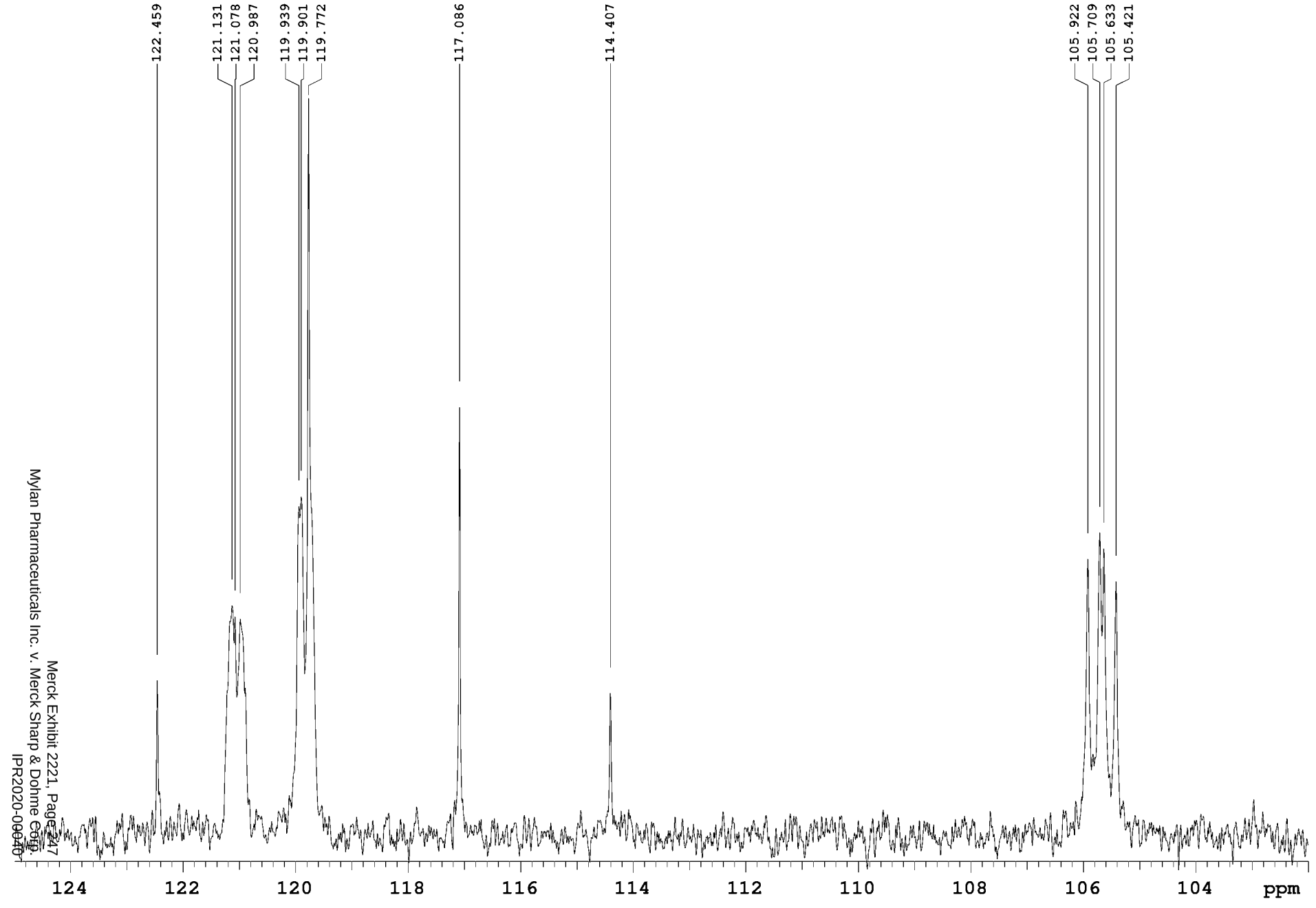
File: 410114

INDEX	FREQUENCY	PPM	HEIGHT
1	17014.834	169.255	64.6
2	17001.864	169.126	141.8
3	15823.885	157.408	21.6
4	15814.730	157.317	28.7
5	15579.745	154.979	23.0
6	15571.353	154.896	26.2
7	15168.520	150.888	120.5
8	15157.839	150.782	55.7
9	15035.769	149.568	16.2
10	15022.036	149.431	29.7
11	15009.066	149.302	17.0
12	14787.814	147.101	33.6
13	14774.844	146.972	49.2
14	14761.874	146.843	17.4
15	14543.673	144.673	25.3
16	14531.466	144.551	27.8
17	14384.982	143.094	12.2
18	14346.072	142.707	37.9
19	14330.050	142.548	17.7
20	14307.162	142.320	41.6
21	14291.140	142.161	15.2
22	14268.252	141.933	13.6
23	14252.230	141.774	6.2

Plot file: 410114-2_peaks

235849, 4063-57-01, Compound 184, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

File: 410114



Plot file: 410114-3

235849, 4063-57-01, Compound 184, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

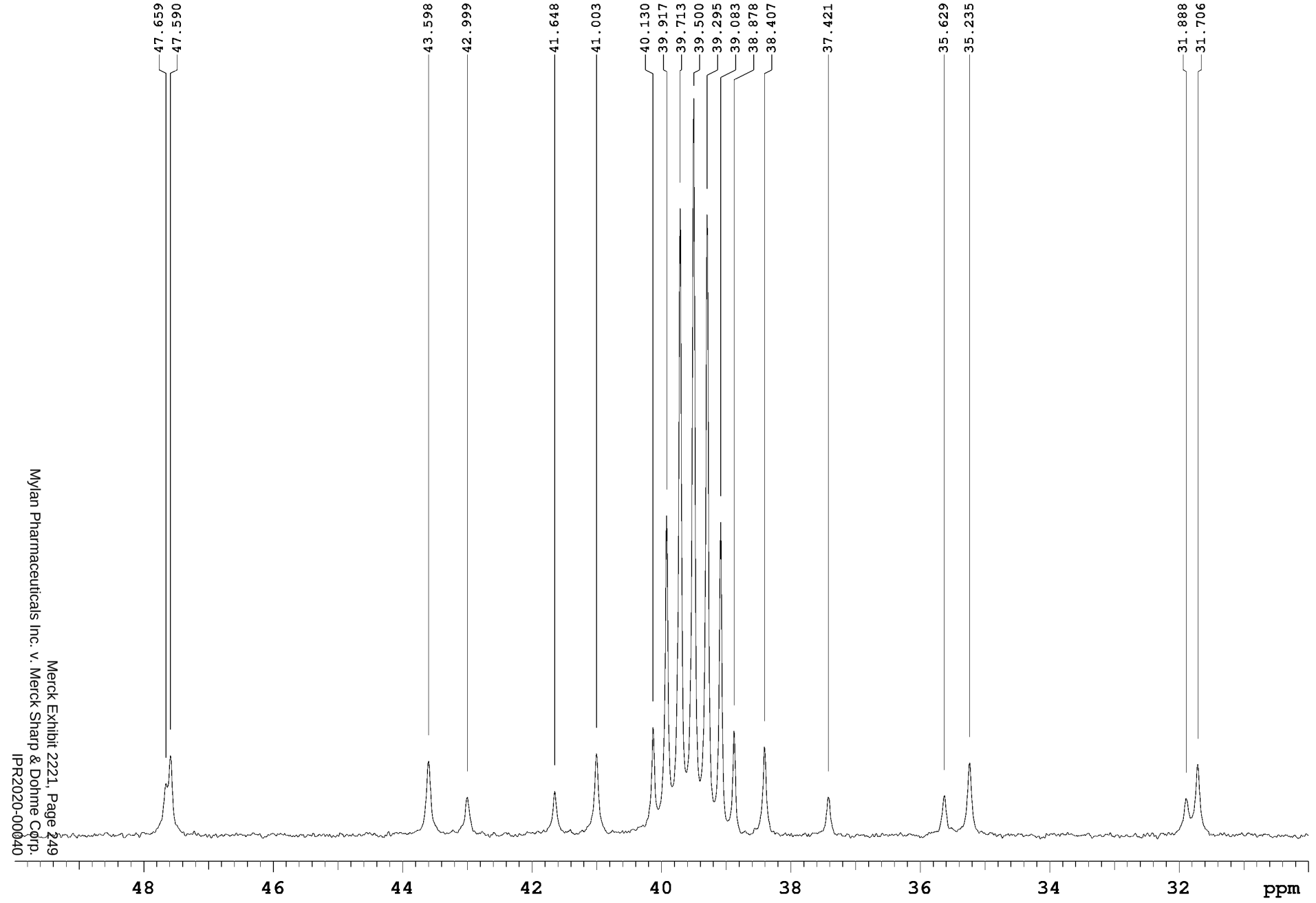
File: 410114

INDEX	FREQUENCY	PPM	HEIGHT
1	12310.549	122.459	29.8
2	12177.035	121.131	44.3
3	12171.694	121.078	42.1
4	12162.539	120.987	41.7
5	12057.253	119.939	63.3
6	12053.439	119.901	65.1
7	12040.469	119.772	141.8
8	11770.388	117.086	82.4
9	11501.071	114.407	27.4
10	10648.104	105.922	53.2
11	10626.742	105.709	58.2
12	10619.113	105.633	55.2
13	10597.750	105.421	48.8

Plot file: 410114-3_peaks

235849, 4063-57-01, Compound 184, in DMSO-d6, 13C NMR, referenced to solvent at 39.5 ppm

File: 410114



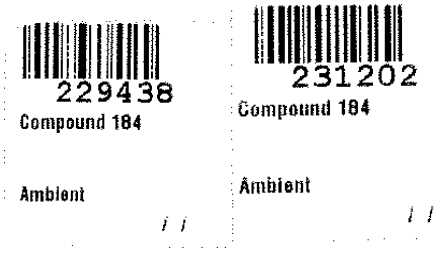
Plot file: 410114-4

235849, 4063-57-01, Compound 184, in DMSO-d6, 13C NMR, referenced to so1vent at 39.5 ppm

File: 410114

INDEX	FREQUENCY	PPM	HEIGHT
1	4791.018	47.659	10.0
2	4784.152	47.590	15.3
3	4382.845	43.598	14.3
4	4322.573	42.999	7.4
5	4186.770	41.648	8.5
6	4121.920	41.003	15.7
7	4034.182	40.130	20.8
8	4012.820	39.917	61.5
9	3992.220	39.713	120.5
10	3970.858	39.500	141.7
11	3950.259	39.295	119.4
12	3928.896	39.083	60.3
13	3908.297	38.878	20.1
14	3860.995	38.407	17.1
15	3761.813	37.421	7.4
16	3581.759	35.629	7.7
17	3542.086	35.235	14.0
18	3205.630	31.888	7.1
19	3187.319	31.706	13.6

Plot file: 410114-4_peaks



LIMS 229438, 231202 – submitted for X-ray
- submitted for SM images

229438 – observed on SM#3, 5x objective, pictures saved to D: drive

231202 – observed on SM#3, 5x objective, pictures saved to D: drive

4031-09-01 – in agate mortar and pestle ground small amount 229438
For ~30 seconds
- submitted for X-ray

4031-09-02 in agate mortar and pestle ground small amt 231202
for ~30 seconds
- submitted for X-ray

Read and Understood By

Skylar Wolfe	5/3/10	Len Chyall	7/17/10
Signed	Date	Signed	Date

Water bath 2062 set to 25 °C pipette
H_2O
pH meter 0607 calibrated 4,7,10

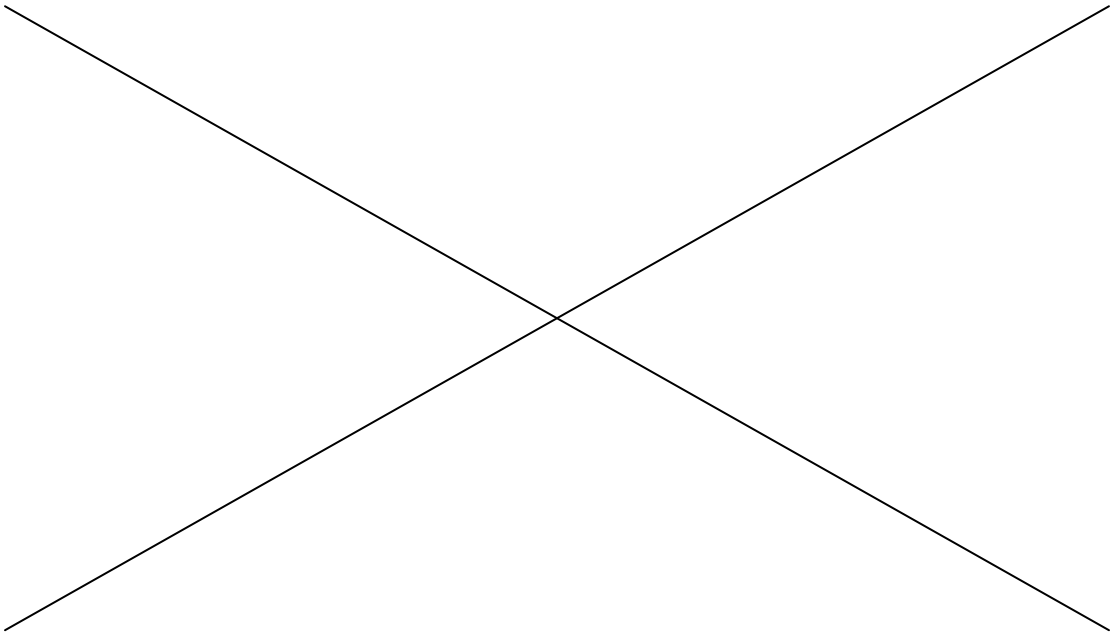
4031-13-01 – ground 229438 (FB) using agate mortar, pestle for ~30 seconds, transferred to 2 dram vial, added cross stir bar, 5 mL H_2O

4031-13-02 – ground 229438 (FB) using agate mortar, pestle for ~30 seconds, transferred to 2 dram vial, added cross stir bar, 5 mL H_2O

4031-13-03 – ground 231202 (Salt) using agate mortar, pestle for ~15 seconds, transferred to 2 dram vial, added cross stir bar, 5 mL H_2O

4031-13-04 – ground 231202 (Salt) using agate mortar, pestle for ~15 seconds, transferred to 2 dram vial, added cross stir bar, 5 mL H_2O

At t=0 (timer#20283072 exp 7/22/10)
Samples placed in water bath to stir

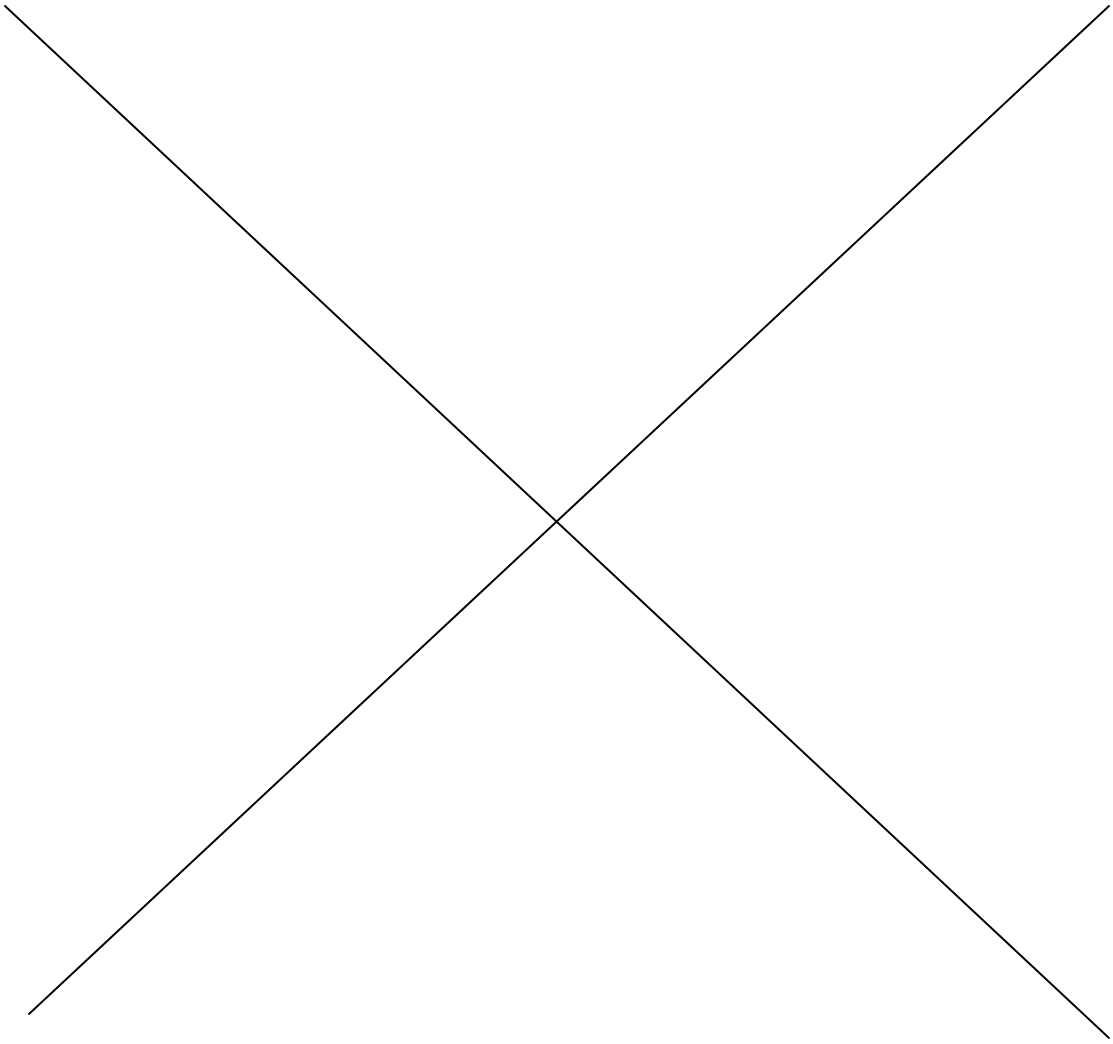


Read and Understood By

Skylar Wolfe	5/6/10	Len Chyall	7/17/10
Signed	Date	Signed	Date

4031-21-01 – ground 229438 in mortar pestle for ~30 seconds
transferred to vial

4031-21-02 - ground 231202 in mortar/pestle for ~ 15 seconds
transferred to vial



Continued 22

Read and Understood By

Skylar Wolfe	5/12/10	Len Chyall	7/19/10
Signed	Date	Signed	Date

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Injection Date  : 5/4/2010 8:53:56 PM          Seq. Line :   7
Sample Name     : 233004                      Location : Vial 3
Acq. Operator   : DC                             Inj :   1
Acq. Instrument : Instrument 1                Inj Volume : 10 µl
Sequence File   : D:\LIMSDATA\CONSULT\397811.S
Method          : C:\HPCHEM\1\METHODS\40601302.M
Last changed    : 5/4/2010 6:34:37 PM by DC
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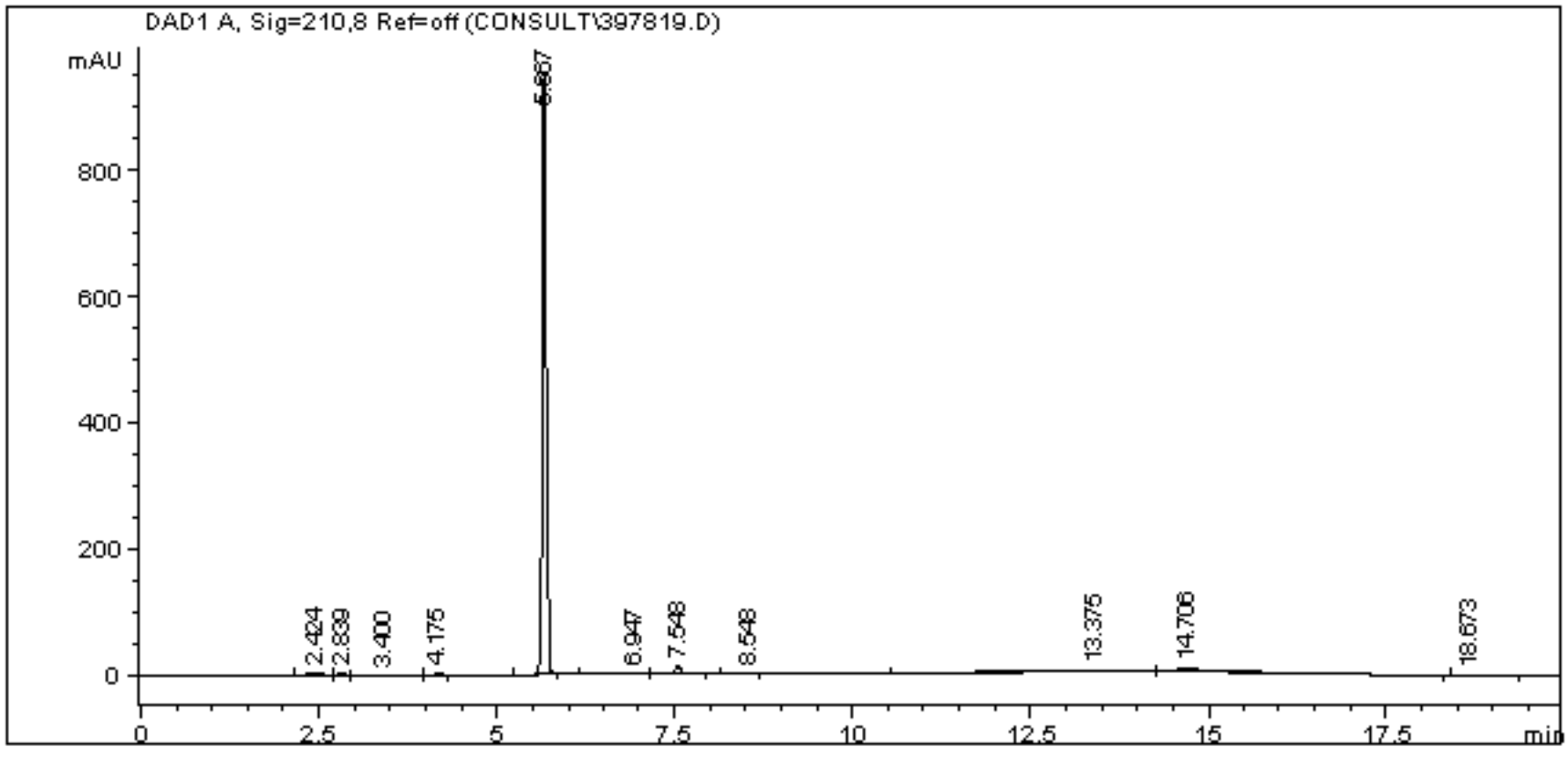


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==========================================================================
                          Area Percent Report
==========================================================================

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


Signal 1: DAD1 A, Sig=210,8 Ref=off

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   2.424 BB    0.1287   17.86463    1.84910   0.4109
   2   2.839 BB    0.1039   21.09403    3.24132   0.4851
   3   3.400 BB    0.6020   67.42688    1.38012   1.5507
   4   4.175 BB    0.0821   12.56670    2.36154   0.2890
   5   5.667 BB    0.0685 3923.11230  943.95648  90.2247
   6   6.947 BB    0.4334    9.17026 2.69229e-1   0.2109
   7   7.548 BB    0.0866   62.54363   10.96022   1.4384
   8   8.548 BB    0.0743    5.17250    1.11037   0.1190
   9  13.375 BB    0.6918  121.28802    2.13161   2.7894
  10  14.706 BBA   0.3259  103.89158    4.77313   2.3893
  11  18.673 BBA   0.2886    4.02870 1.69897e-1   0.0927

Totals :                  4348.15923  972.20302

 Results obtained with enhanced integrator!
==========================================================================
                          *** End of Report ***
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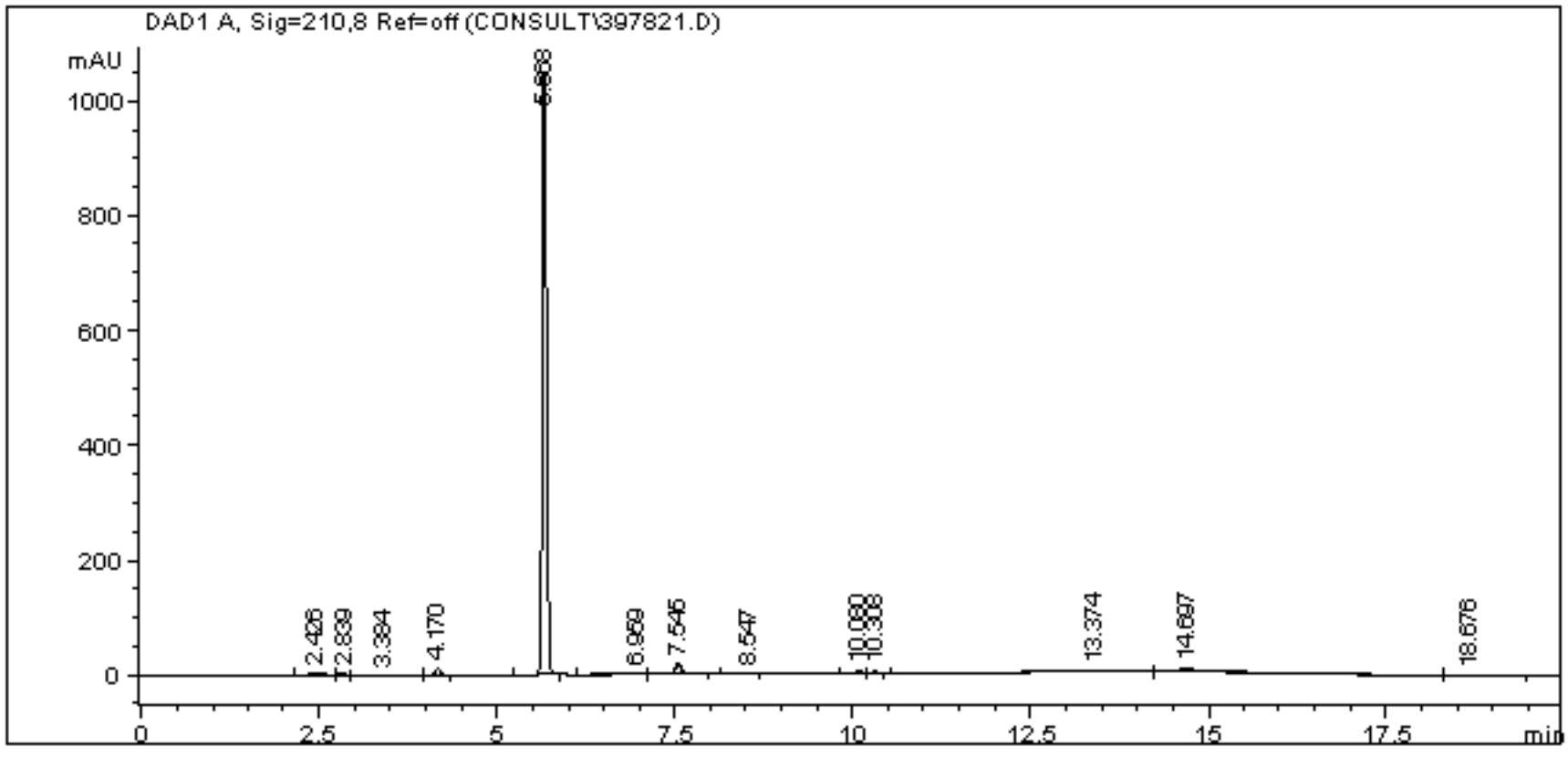
Injection Date	: 5/4/2010 9:37:10 PM	Seq. Line	: 9
Sample Name	: 233006	Location	: Vial 5
Acq. Operator	: DC	Inj	: 1
Acq. Instrument	: Instrument 1	Inj Volume	: 10 µl
Sequence File	: D:\LIMSDATA\CONSULT\397811.S		
Method	: C:\HPCHEM\1\METHODS\40601302.M		
Last changed	: 5/4/2010 6:34:37 PM by DC		



Area Percent Report

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

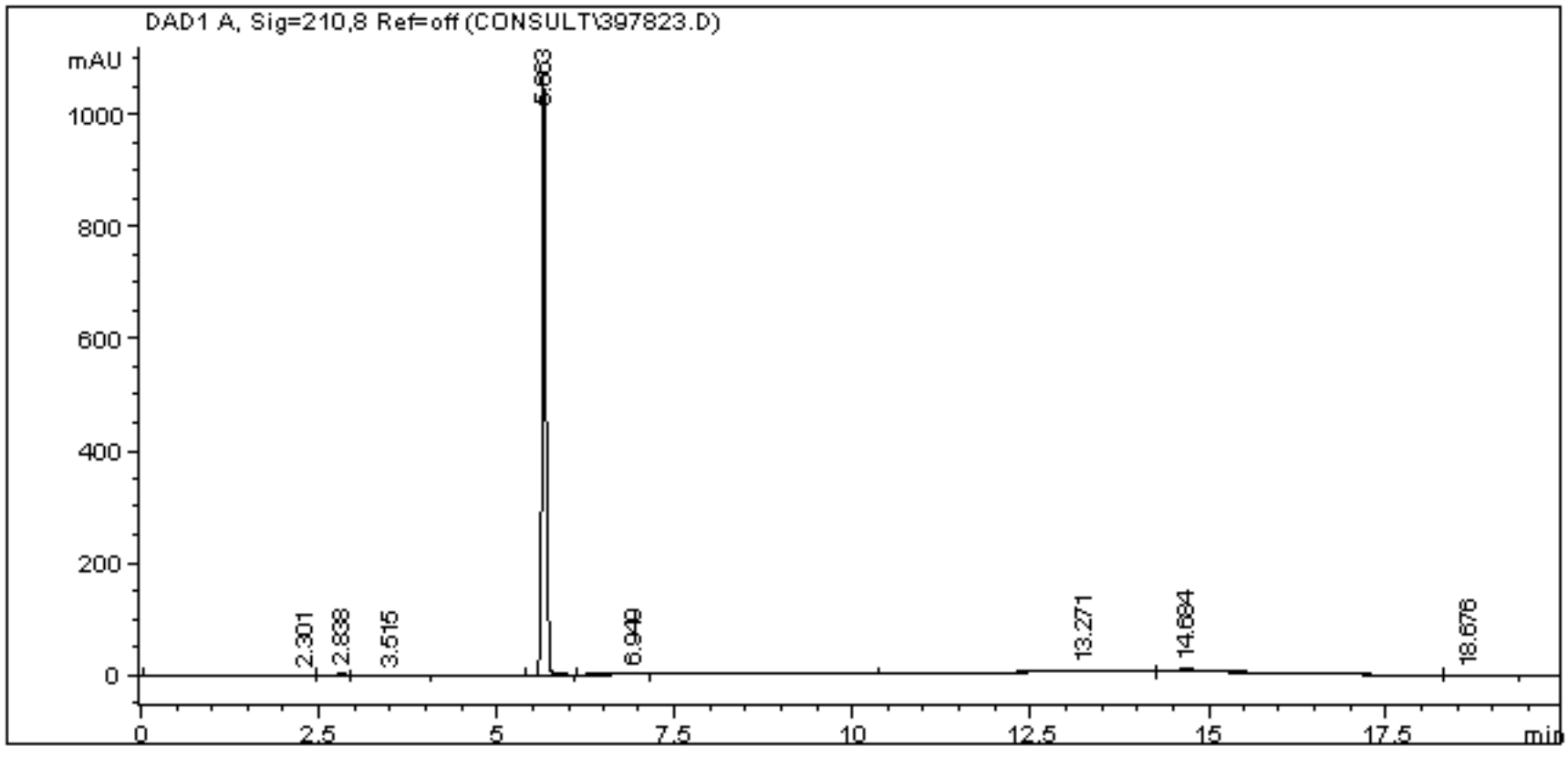
Signal 1: DAD1 A, Sig=210,8 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.426	BB	0.1034	33.07724	4.41490	0.6764
2	2.839	BB	0.1019	20.54387	3.24474	0.4201
3	3.384	BB	0.5778	68.52557	1.45399	1.4013
4	4.170	BB	0.0818	54.99649	10.37575	1.1247
5	5.668	BB	0.0687	4342.47998	1040.12170	88.8021
6	6.959	BB	0.3976	7.72264	2.36117e-1	0.1579
7	7.545	BB	0.0858	93.88944	16.63844	1.9200
8	8.547	BB	0.0729	7.25609	1.59908	0.1484
9	10.080	BB	0.0740	14.13773	3.05397	0.2891
10	10.308	BB	0.0757	9.06458	1.89838	0.1854
11	13.374	BB	0.7055	126.14094	2.18516	2.5795
12	14.697	BB	0.3406	108.18127	4.76996	2.2123
13	18.676	BBA	0.2547	4.04650	1.97773e-1	0.0827
Totals :				4890.06233	1090.18996	

Results obtained with enhanced integrator!

*** End of Report ***

```
==========================================================================
Injection Date  : 5/4/2010 10:41:58 PM          Seq. Line :  12
Sample Name     : 233008                        Location : Vial 7
Acq. Operator   : DC                               Inj :   1
Acq. Instrument : Instrument 1                  Inj Volume : 10 µl
Sequence File   : D:\LIMSDATA\CONSULT\397811.S
Method          : C:\HPCHEM\1\METHODS\40601302.M
Last changed    : 5/4/2010 6:34:37 PM by DC
==========================================================================
```




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

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

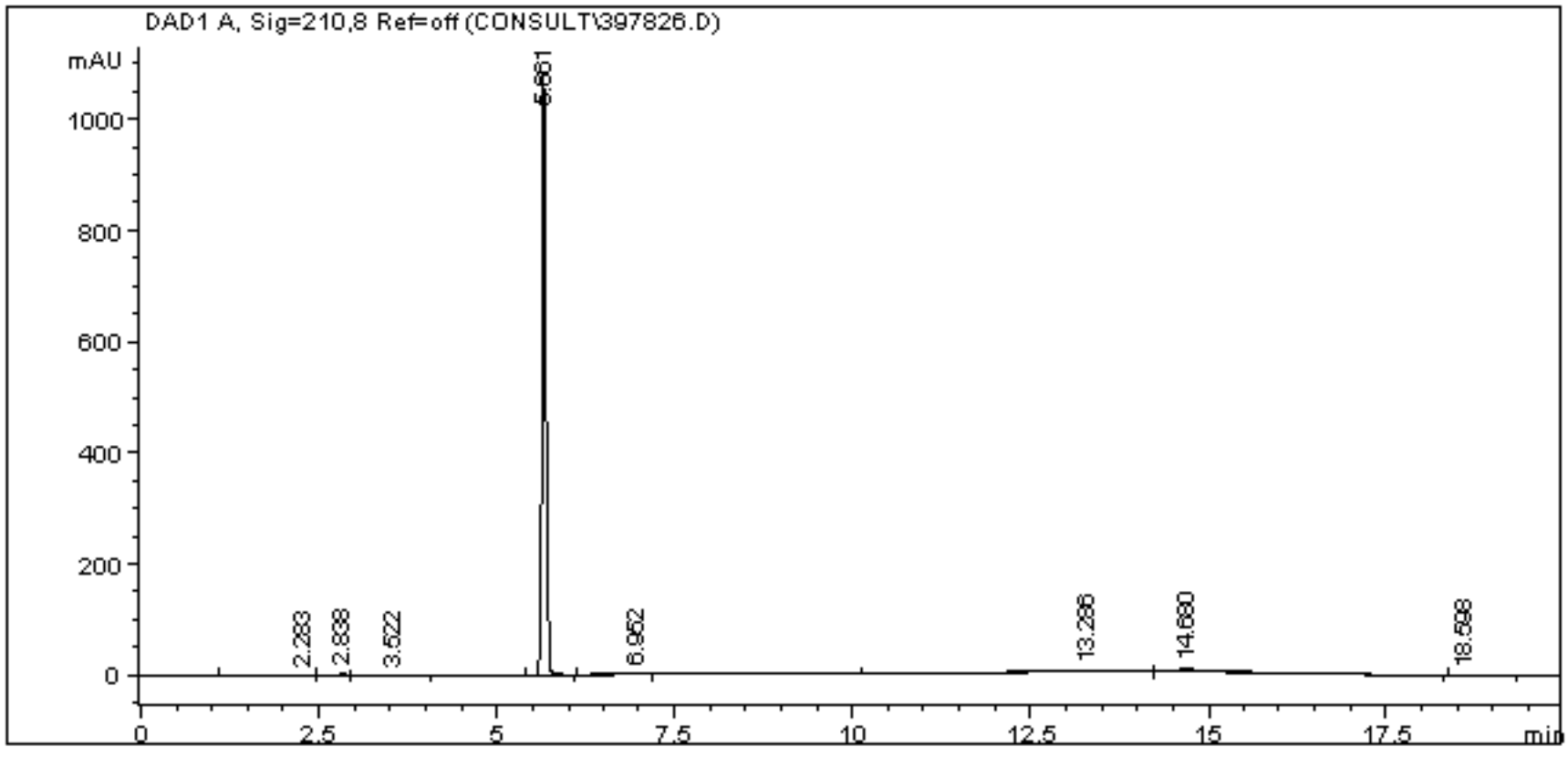

Signal 1: DAD1 A, Sig=210,8 Ref=off

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   2.301 BB    0.3144    7.43116 2.90514e-1   0.1556
   2   2.838 BB    0.1432   36.29916    3.67764   0.7600
   3   3.515 BB    0.7596   77.87273    1.23508   1.6305
   4   5.663 BBA   0.0650 4427.44336 1054.46252  92.7009
   5   6.949 BB    0.3888    7.64413 2.47193e-1   0.1601
   6  13.271 BB    0.6933  126.29224    2.21470   2.6443
   7  14.684 BB    0.2917   87.12482    4.62566   1.8242
   8  18.676 BB    0.3479    5.94610 2.14163e-1   0.1245

Totals :                  4776.05371 1066.96747

 Results obtained with enhanced integrator!
==========================================================================
                          *** End of Report ***
```

Injection Date	: 5/4/2010 11:25:10 PM	Seq. Line	: 14
Sample Name	: 233010	Location	: Vial 9
Acq. Operator	: DC	Inj	: 1
Acq. Instrument	: Instrument 1	Inj Volume	: 10 µl
Sequence File	: D:\LIMSDATA\CONSULT\397811.S		
Method	: C:\HPCHEM\1\METHODS\40601302.M		
Last changed	: 5/4/2010 6:34:37 PM by DC		



Area Percent Report

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

Signal 1: DAD1 A, Sig=210,8 Ref=off

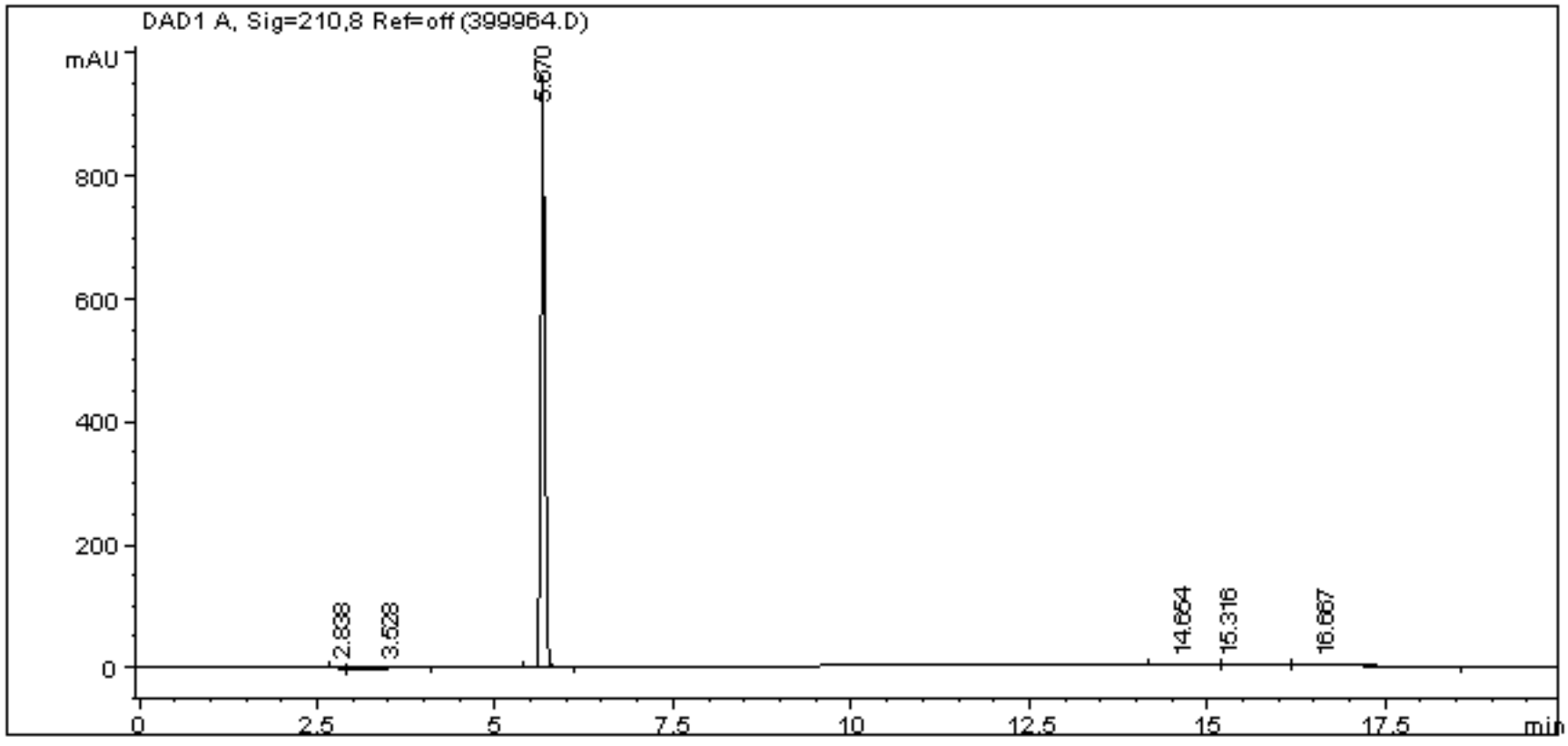
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.283	BB	0.3040	7.08928	3.13338e-1	0.1455
2	2.838	BB	0.1437	36.11237	3.64220	0.7410
3	3.522	BB	0.7568	76.87045	1.20403	1.5773
4	5.661	BBA	0.0653	4497.85645	1064.19238	92.2915
5	6.952	BB	0.3957	7.81252	2.53426e-1	0.1603
6	13.286	BB	0.7123	120.97987	2.08708	2.4824
7	14.680	BBA	0.3661	121.91664	4.77065	2.5016
8	18.598	BBA	0.3908	4.89559	1.52378e-1	0.1005
Totals :				4873.53317	1076.61549	

Results obtained with enhanced integrator!

*** End of Report ***

Data File D:\LIMSDATA\399964.D Sample Name: 234037

Injection Date	: 5/13/2010 6:31:33 PM	Seq. Line	: 5
Sample Name	: 234037	Location	: Vial 3
Acq. Operator	: MHC	Inj	: 1
Acq. Instrument	: Instrument 1	Inj Volume	: 10 µl
Sequence File	: D:\LIMSDATA\399961.S		
Method	: C:\HPCHEM\1\METHODS\40602701.M		
Last changed	: 5/13/2010 2:12:18 PM by LL		



Area Percent Report

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

Signal 1: DAD1 A, Sig=210,8 Ref=off

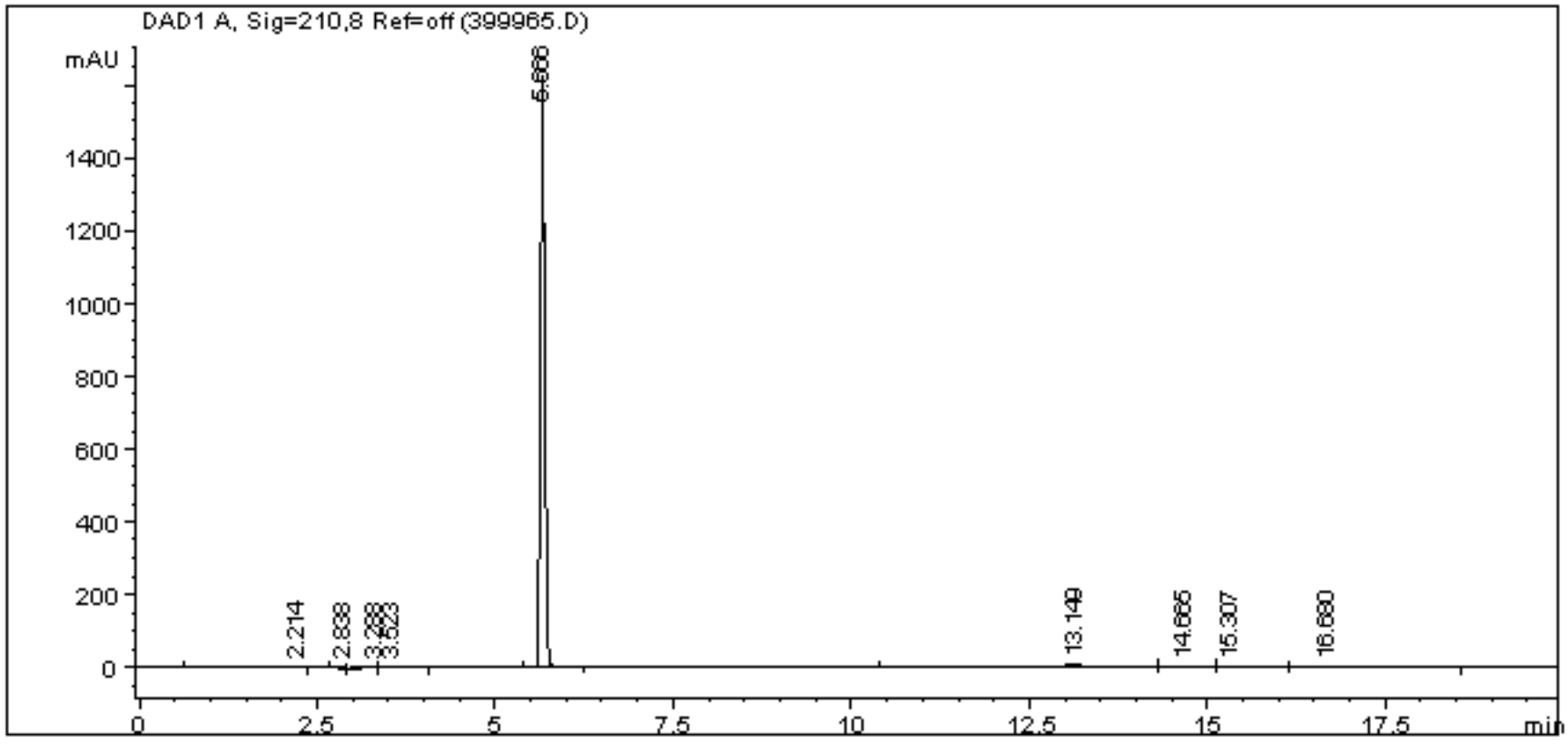
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.838	BB	0.1097	23.81708	3.39898	0.5704
2	3.528	BB	0.7752	82.18034	1.27624	1.9683
3	5.670	BBA	0.0691	4036.35596	959.86176	96.6755
4	14.654	BB	0.2437	10.24884	5.64800e-1	0.2455
5	15.316	BB	0.2834	5.60212	2.73015e-1	0.1342
6	16.667	BBA	0.2467	16.95432	1.21594	0.4061
Totals :				4175.15866	966.59073	

Results obtained with enhanced integrator!

*** End of Report ***

Data File D:\LIMSDATA\399965.D Sample Name: 234038

```
==========================================================================
Injection Date  : 5/13/2010 6:53:03 PM          Seq. Line :   6
Sample Name     : 234038                        Location : Vial 4
Acq. Operator   : MHC                              Inj :   1
Acq. Instrument : Instrument 1                 Inj Volume : 10 µl
Sequence File   : D:\LIMSDATA\399961.S
Method          : C:\HPCHEM\1\METHODS\40602701.M
Last changed    : 5/13/2010 2:12:18 PM by LL
==========================================================================
```




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

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


Signal 1: DAD1 A, Sig=210,8 Ref=off

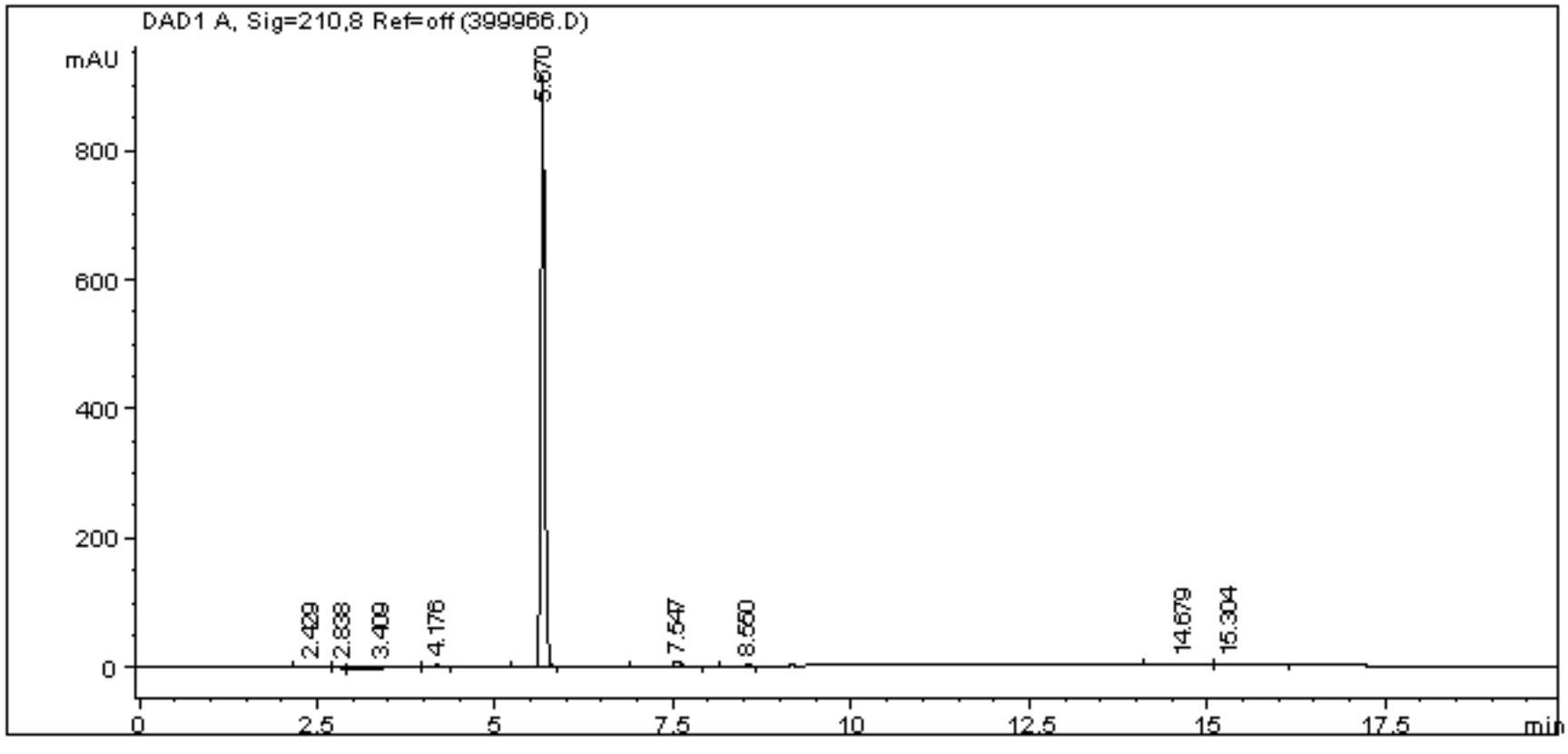
Peak RetTime Type  Width     Area      Height     Area
  #   [min]       [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   2.214 PB    0.1899    4.11751 3.20462e-1   0.0561
   2   2.838 BB    0.1060   22.03981    3.29791   0.3005
   3   3.288 BB    0.9262   22.50424 2.92316e-1   0.3068
   4   3.523 BB    0.3825    3.36934 1.07261e-1   0.0459
   5   5.666 BBA   0.0719 7165.20410 1609.88123  97.6812
   6  13.149 BB    0.7473   80.82142    1.33310   1.1018
   7  14.665 BB    0.2664   11.03487 5.49181e-1   0.1504
   8  15.307 BB    0.2988    7.28221 3.13682e-1   0.0993
   9  16.680 BBA   0.2761   18.91888    1.25315   0.2579

Totals :                  7335.29238 1617.34829

 Results obtained with enhanced integrator!
==========================================================================
                        *** End of Report ***
```

Data File D:\LIMSDATA\399966.D Sample Name: 234039

```
==========================================================================
Injection Date  : 5/13/2010 7:14:32 PM          Seq. Line :   7
Sample Name     : 234039                        Location : Vial 5
Acq. Operator   : MHC                              Inj :   1
Acq. Instrument : Instrument 1                 Inj Volume : 10 µl
Sequence File   : D:\LIMSDATA\399961.S
Method          : C:\HPCHEM\1\METHODS\40602701.M
Last changed    : 5/13/2010 2:12:18 PM by LL
==========================================================================
```




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

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


Signal 1: DAD1 A, Sig=210,8 Ref=off

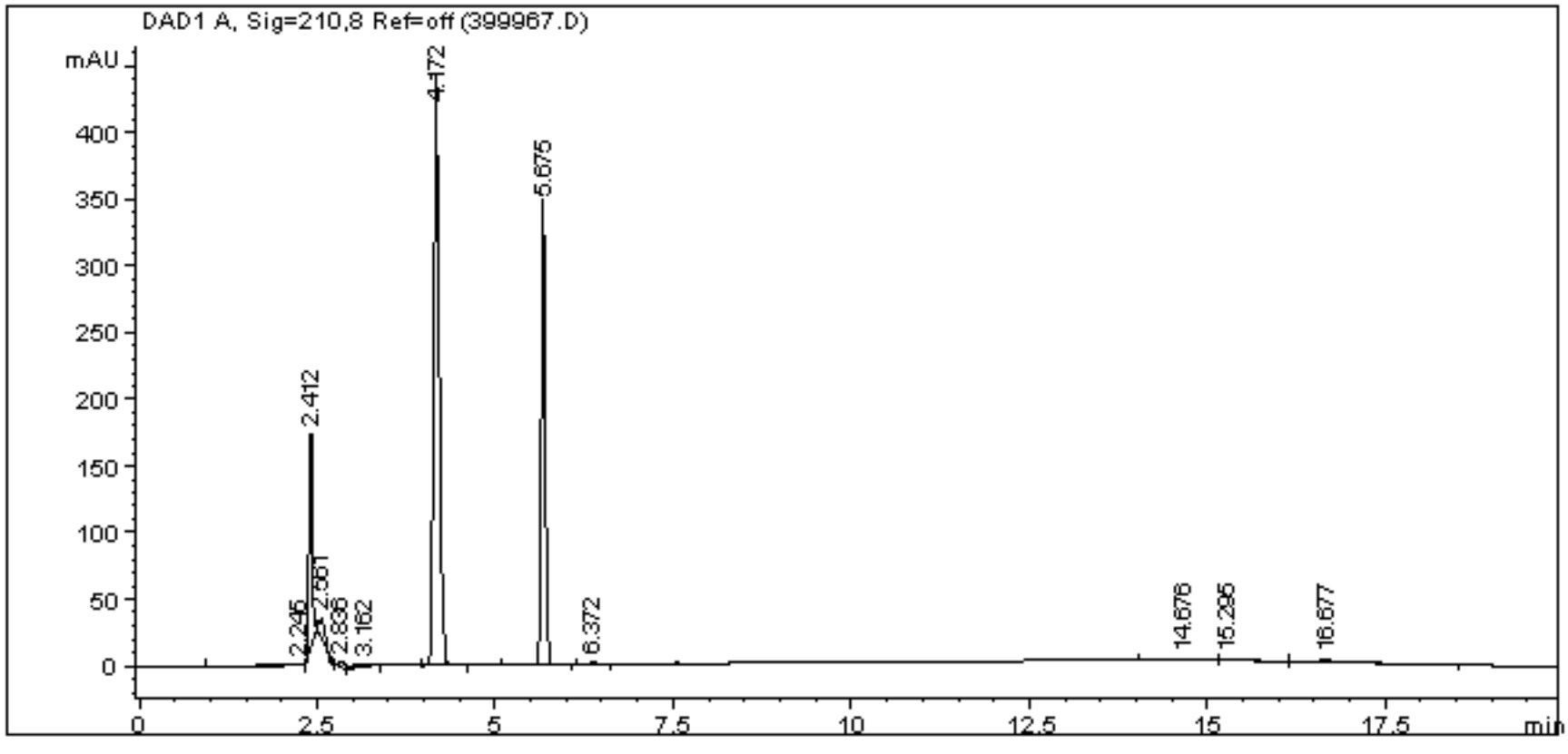
Peak RetTime Type  Width     Area      Height     Area
  #   [min]       [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   2.429 BB    0.1069   12.13023    1.55735   0.3048
   2   2.838 BB    0.1050   21.58258    3.27137   0.5423
   3   3.409 BB    0.6078   69.50925    1.40835   1.7465
   4   4.176 BB    0.0825   16.30547    3.04465   0.4097
   5   5.670 BB    0.0648 3790.39355  906.53589  95.2398
   6   7.547 BB    0.0854   46.65509    8.32658   1.1723
   7   8.550 BB    0.0720    3.81933 8.56296e-1   0.0960
   8  14.679 BB    0.2955    9.67989 4.96432e-1   0.2432
   9  15.304 BB    0.3558    9.76531 3.51809e-1   0.2454

Totals :                  3979.84070  925.84872

 Results obtained with enhanced integrator!
==========================================================================
                        *** End of Report ***
```

Data File D:\LIMSDATA\399967.D Sample Name: 234040

Injection Date	: 5/13/2010 7:36:01 PM	Seq. Line :	8
Sample Name	: 234040	Location :	Vial 6
Acq. Operator	: MHC	Inj :	1
Acq. Instrument	: Instrument 1	Inj Volume :	10 µl
Sequence File	: D:\LIMSDATA\399961.S		
Method	: C:\HPCHEM\1\METHODS\40602701.M		
Last changed	: 5/13/2010 2:12:18 PM by LL		



Area Percent Report

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

Signal 1: DAD1 A, Sig=210,8 Ref=off

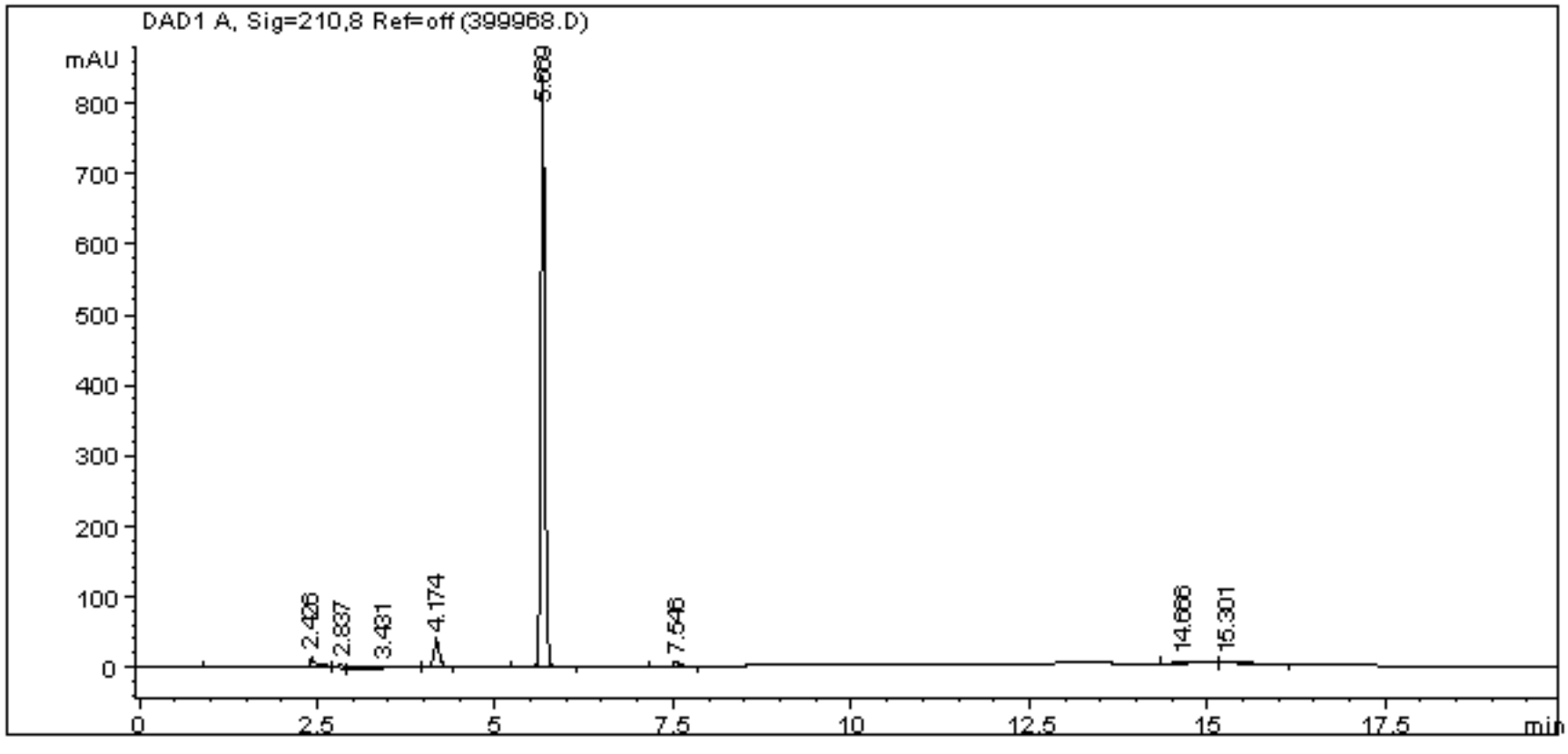
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.245	PB	0.1076	3.96995	5.5409le-1	0.0883
2	2.412	BB	0.0496	510.55173	158.34679	11.3524
3	2.561	BB	0.0732	51.91171	13.39030	1.1543
4	2.836	BB	0.0940	15.29675	2.70216	0.3401
5	3.162	BB	0.2837	23.29053	1.01516	0.5179
6	4.172	BBA	0.0839	2417.73071	441.76682	53.7594
7	5.675	BBA	0.0640	1419.47827	344.91879	31.5628
8	6.372	PB	0.0869	15.36246	2.67993	0.3416
9	14.676	BB	0.2579	9.64672	4.98254e-1	0.2145
10	15.295	BB	0.3289	6.40589	2.55186e-1	0.1424
11	16.677	BBA	0.3066	23.67000	1.28660	0.5263
Totals :				4497.31473	967.41407	

Results obtained with enhanced integrator!

*** End of Report ***

Data File D:\LIMSDATA\399968.D Sample Name: 234041

```
==========================================================================
Injection Date  : 5/13/2010 7:57:34 PM          Seq. Line :   9
Sample Name     : 234041                        Location : Vial 7
Acq. Operator   : MHC                              Inj :   1
Acq. Instrument : Instrument 1                 Inj Volume : 10 µl
Sequence File   : D:\LIMSDATA\399961.S
Method          : C:\HPCHEM\1\METHODS\40602701.M
Last changed    : 5/13/2010 2:12:18 PM by LL
==========================================================================
```




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

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


Signal 1: DAD1 A, Sig=210,8 Ref=off

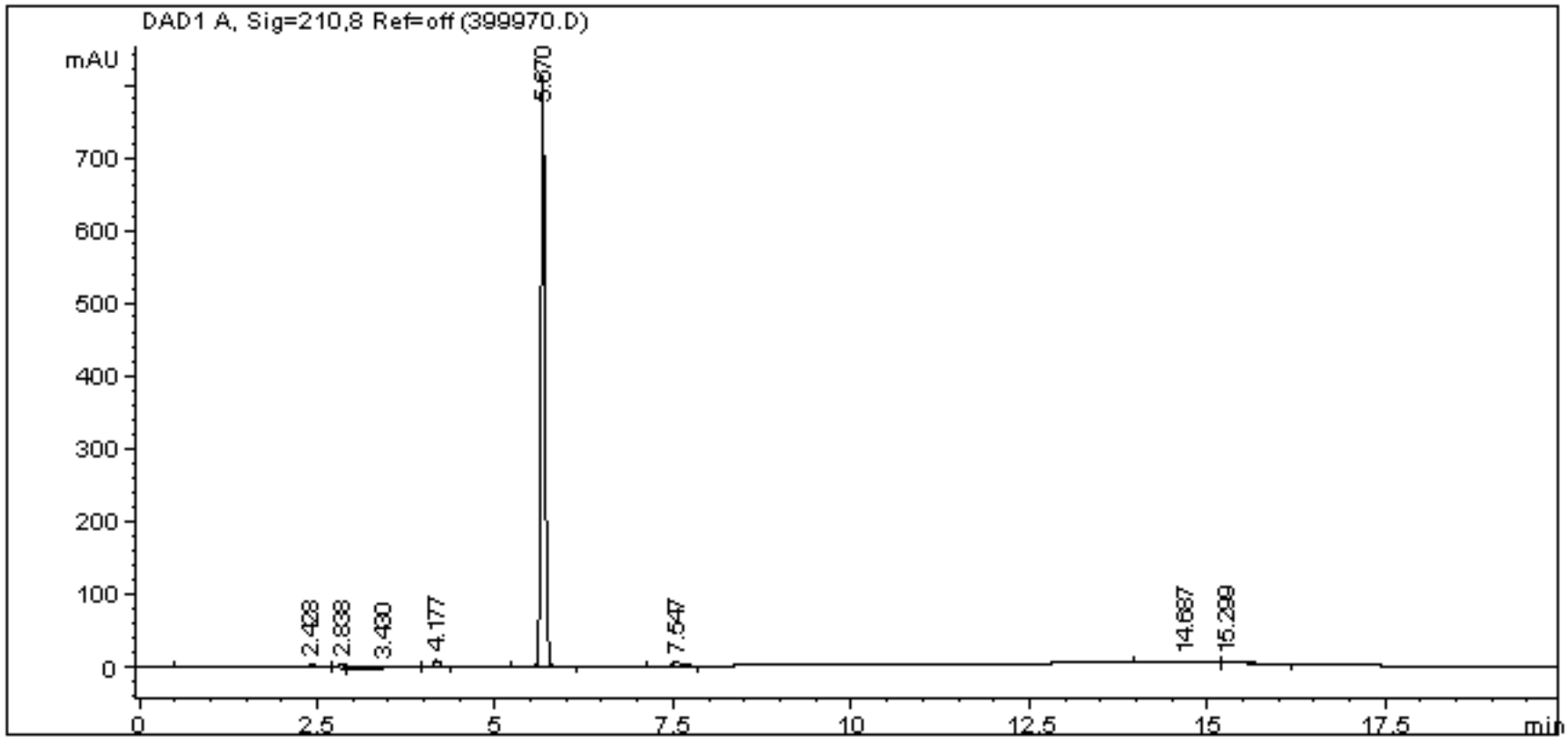
Peak RetTime Type  Width     Area      Height     Area
  #   [min]       [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   2.426 PB    0.0798   75.41846   12.97574   1.9345
   2   2.837 BB    0.1007   19.41878    3.11612   0.4981
   3   3.431 BB    0.6534   68.22099    1.24866   1.7499
   4   4.174 BBA   0.0821  208.14311   39.11160   5.3390
   5   5.669 BBA   0.0649 3481.73779  830.93817  89.3089
   6   7.546 BB    0.0884   29.18499    4.98109   0.7486
   7  14.666 BB    0.2675   10.32344 5.11434e-1   0.2648
   8  15.301 BB    0.3056    6.08526 2.45152e-1   0.1561

Totals :                  3898.53283  893.12797

 Results obtained with enhanced integrator!
==========================================================================
                          *** End of Report ***
```

Data File D:\LIMSDATA\399970.D Sample Name: 234042

```
==========================================================================
Injection Date  : 5/13/2010 8:40:33 PM          Seq. Line :  11
Sample Name     : 234042                        Location : Vial 8
Acq. Operator   : MHC                           Inj :  1
Acq. Instrument : Instrument 1                  Inj Volume : 10 µl
Sequence File   : D:\LIMSDATA\399961.S
Method          : C:\HPCHEM\1\METHODS\40602701.M
Last changed    : 5/13/2010 2:12:18 PM by LL
==========================================================================
```




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

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


Signal 1: DAD1 A, Sig=210,8 Ref=off

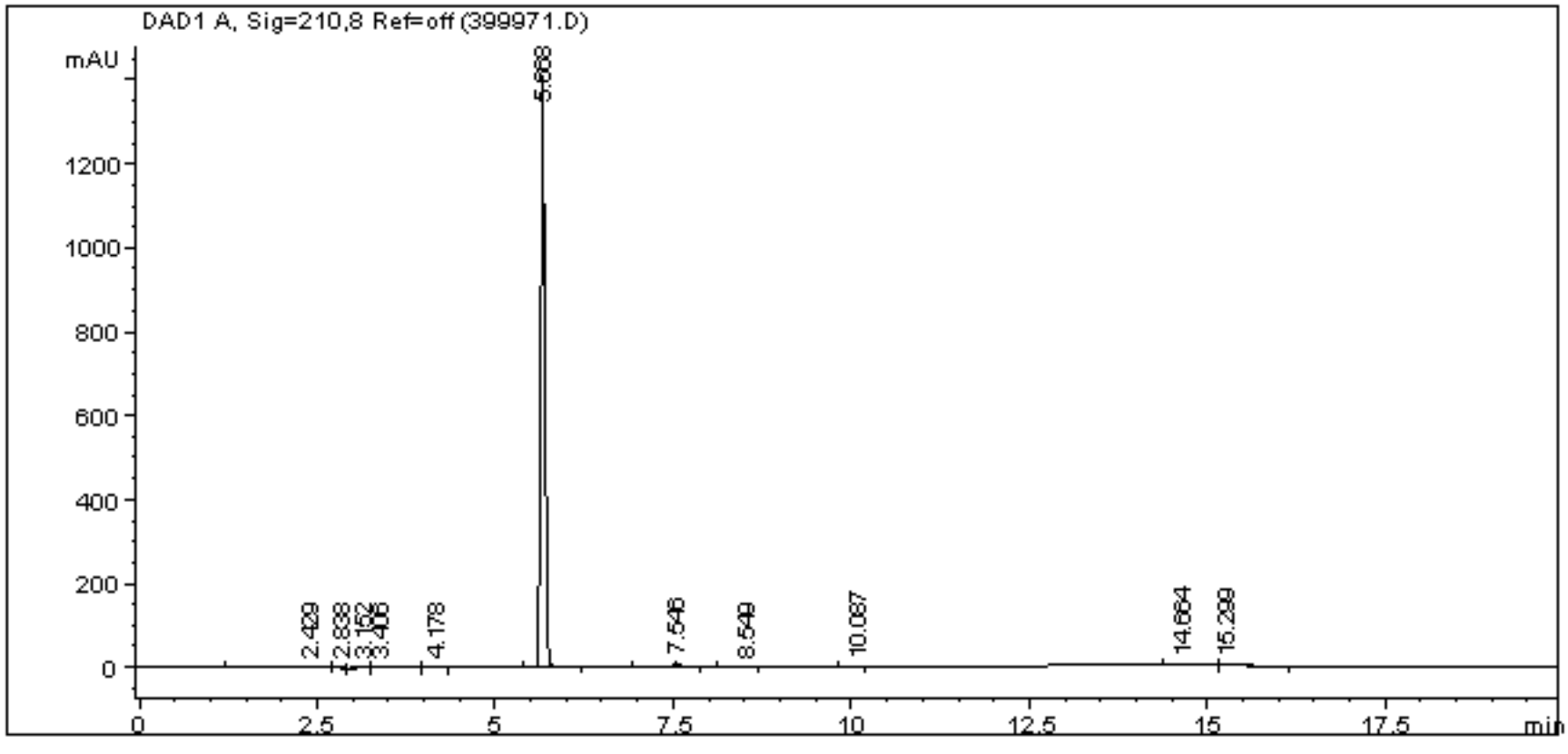
Peak RetTime Type  Width     Area      Height     Area
  #   [min]       [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   2.428 PB    0.0612   14.96918    3.55209   0.4167
   2   2.838 BB    0.1027   20.43370    3.19105   0.5688
   3   3.430 BB    0.6281   69.45502    1.34139   1.9335
   4   4.177 BB    0.0860   52.48671    9.87596   1.4611
   5   5.670 BB    0.0650 3384.05713  804.98627  94.2053
   6   7.547 BB    0.0890   36.83683    6.22523   1.0255
   7  14.687 BB    0.3016    9.91926 4.95308e-1   0.2761
   8  15.299 BB    0.2786    4.05802 2.01805e-1   0.1130

Totals :                  3592.21586  829.86909

 Results obtained with enhanced integrator!
==========================================================================
                          *** End of Report ***
```

Data File D:\LIMSDATA\399971.D Sample Name: 234043

```
==========================================================================
Injection Date  : 5/13/2010 9:02:04 PM          Seq. Line :  12
Sample Name     : 234043                        Location : Vial 9
Acq. Operator   : MHC                              Inj :   1
Acq. Instrument : Instrument 1                 Inj Volume : 10 µl
Sequence File   : D:\LIMSDATA\399961.S
Method          : C:\HPCHEM\1\METHODS\40602701.M
Last changed    : 5/13/2010 2:12:18 PM by LL
==========================================================================
```




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

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


Signal 1: DAD1 A, Sig=210,8 Ref=off

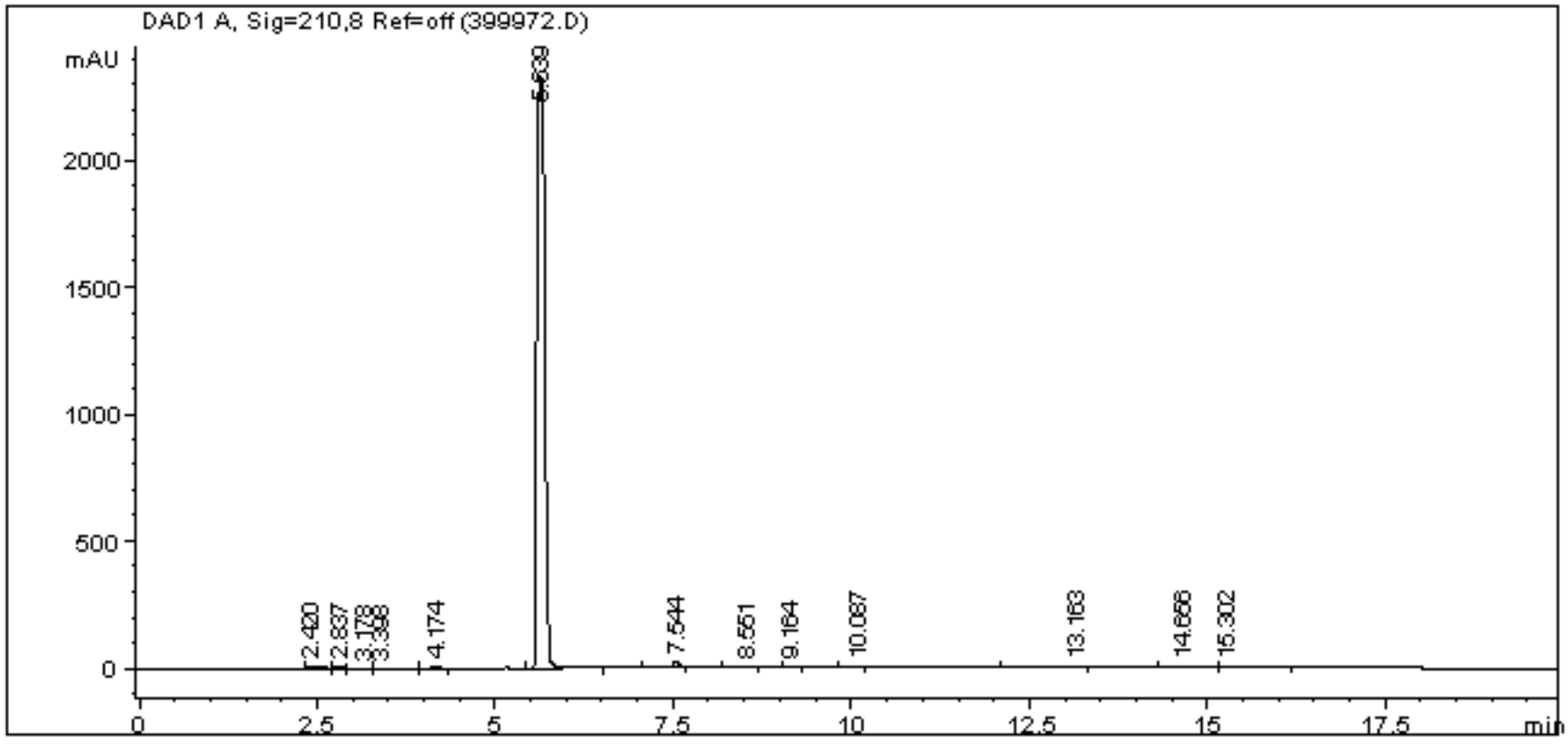
Peak RetTime Type  Width     Area      Height     Area
  #   [min]       [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   2.429 PB    0.0691    7.38437    1.62298   0.1187
   2   2.838 BB    0.1050   21.70236    3.28723   0.3489
   3   3.152 BB    0.3571   15.71806 6.43308e-1   0.2527
   4   3.406 BB    0.2816    3.61543 1.69010e-1   0.0581
   5   4.178 BB    0.0817   19.33064    3.65709   0.3108
   6   5.668 BB    0.0704 6080.69482 1407.72571  97.7511
   7   7.546 BBA   0.0853   47.36104    8.45998   0.7614
   8   8.549 BB    0.0777    4.68787 9.47116e-1   0.0754
   9  10.087 BB    0.0747    3.32501 7.09474e-1   0.0535
  10  14.664 PB    0.2561    9.65572 4.93950e-1   0.1552
  11  15.299 BB    0.3515    7.11145 2.50252e-1   0.1143

Totals :                  6220.58678 1427.96610

 Results obtained with enhanced integrator!
==========================================================================
                          *** End of Report ***
```

Data File D:\LIMSDATA\399972.D Sample Name: 234044

Injection Date	: 5/13/2010 9:23:40 PM	Seq. Line	: 13
Sample Name	: 234044	Location	: Vial 10
Acq. Operator	: MHC	Inj	: 1
Acq. Instrument	: Instrument 1	Inj Volume	: 10 µl
Sequence File	: D:\LIMSDATA\399961.S		
Method	: C:\HPCHEM\1\METHODS\40602701.M		
Last changed	: 5/13/2010 2:12:18 PM by LL		



Area Percent Report

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

Signal 1: DAD1 A, Sig=210,8 Ref=off

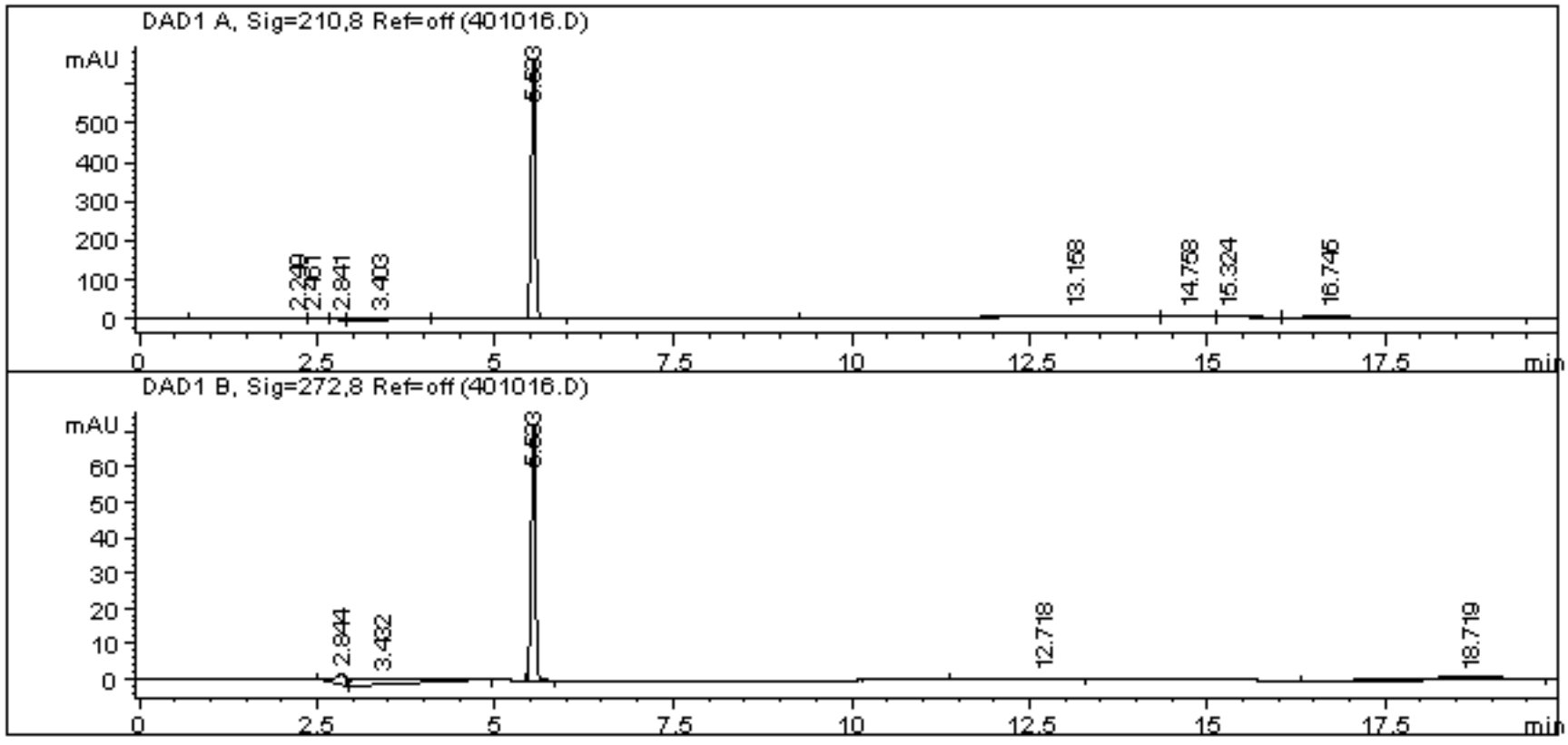
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.420	BB	0.0956	19.66948	2.74531	0.1102
2	2.837	BB	0.1047	21.45417	3.26194	0.1202
3	3.178	BB	0.3257	17.97447	6.77136e-1	0.1007
4	3.398	BB	0.1841	4.02475	3.08620e-1	0.0225
5	4.174	BB	0.0825	24.90322	4.64791	0.1395
6	5.639	BB	0.1284	1.76376e4	2328.98804	98.7984
7	7.544	BB	0.0684	77.71806	18.74678	0.4353
8	8.551	BB	0.0806	12.59490	2.42636	0.0706
9	9.164	BB	0.0771	10.87706	2.22170	0.0609
10	10.087	BB	0.0777	5.32535	1.07721	0.0298
11	13.163	BB	0.2103	5.80841	4.28055e-1	0.0325
12	14.656	BB	0.2585	9.99444	5.05777e-1	0.0560
13	15.302	BB	0.2700	4.16496	2.18922e-1	0.0233
Totals :				1.78521e4	2366.25377	

Results obtained with enhanced integrator!

*** End of Report ***

Data File D:\LIMSDATA\401016.D Sample Name: 234572

```
==========================================================================
Injection Date  : 5/19/2010 5:05:19 PM          Seq. Line :   2
Sample Name     : 234572                        Location : Vial 3
Acq. Operator   : MHC                              Inj :   1
Acq. Instrument : Instrument 1                 Inj Volume : 10 µl
Sequence File   : D:\LIMSDATA\401014.S
Method          : C:\HPCHEM\1\METHODS\40603102.M
Last changed    : 5/19/2010 2:44:21 PM by MHC
==========================================================================
```




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

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


Signal 1: DAD1 A, Sig=210,8 Ref=off

Peak RetTime Type  Width     Area      Height     Area
  #   [min]       [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   2.249 BB    0.4846   16.75420 4.43664e-1   0.5554
   2   2.461 BB    0.1053    4.90451 6.70638e-1   0.1626
   3   2.841 BB    0.1083   22.22395    3.22814   0.7368
   4   3.403 BB    0.6622   79.69419    1.44780   2.6420
   5   5.533 BBA   0.0680 2712.99512  659.92786  89.9407
   6  13.158 BB    0.9967  123.21251    1.50892   4.0847
   7  14.758 BB    0.2170    6.40590 4.03957e-1   0.2124
   8  15.324 BB    0.3348    8.06854 3.15006e-1   0.2675
   9  16.745 BBA   0.3203   42.16923    2.11810   1.3980

Totals :                  3016.42815  670.06408

 Results obtained with enhanced integrator!
```

```
==========================================================================
Injection Date  : 5/19/2010 5:05:19 PM          Seq. Line :   2
Sample Name     : 234572                        Location : Vial 3
Acq. Operator   : MHC                              Inj :   1
Acq. Instrument : Instrument 1                 Inj Volume : 10 µl
Sequence File   : D:\LIMSDATA\401014.S
Method          : C:\HPCHEM\1\METHODS\40603102.M
Last changed    : 5/19/2010 2:44:21 PM by MHC
==========================================================================


Signal 2: DAD1 B, Sig=272,8 Ref=off

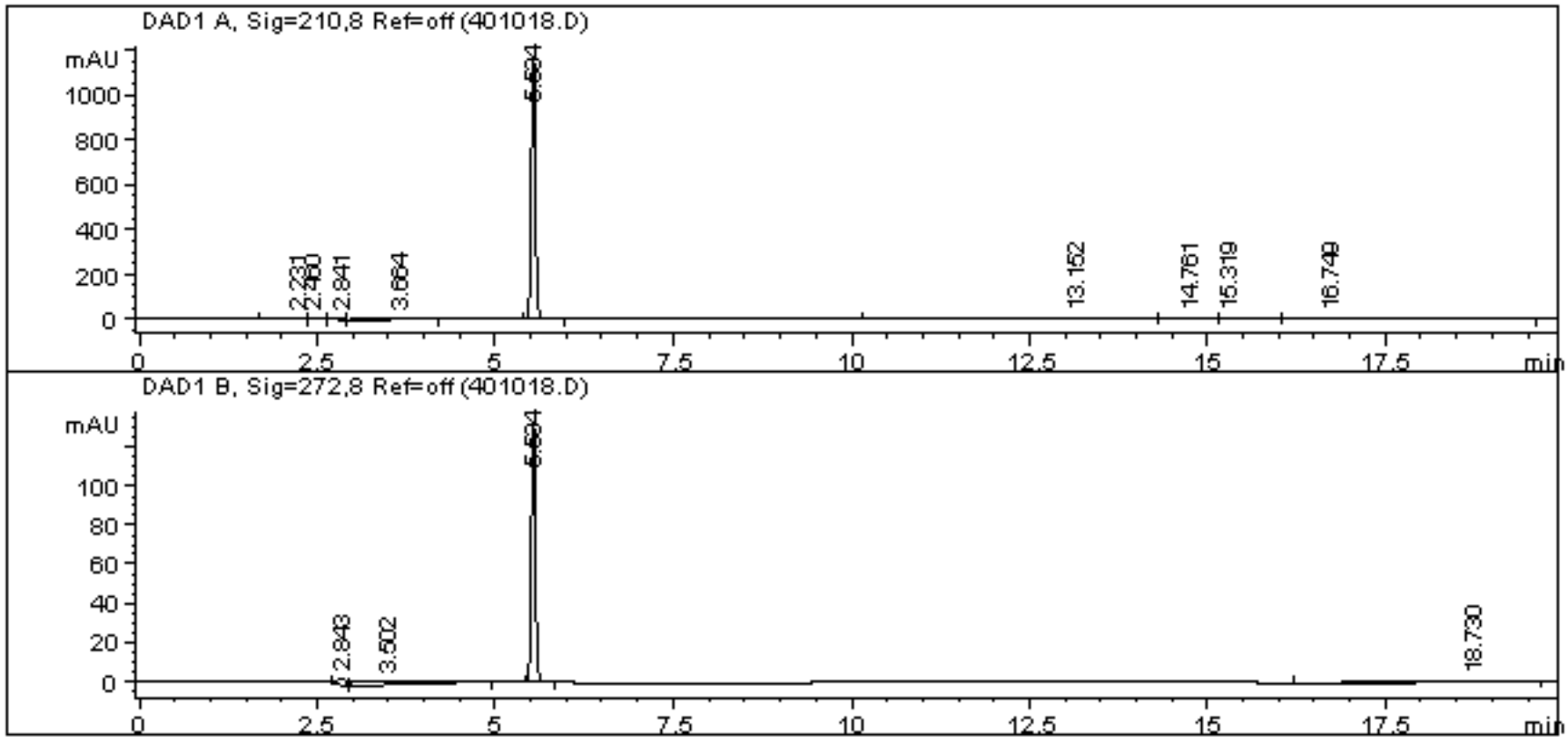
Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|-------|
   1   2.844 BB    0.1487   27.86911    2.78584   5.8163
   2   3.432 BBA   0.8958  102.42530    1.39637  21.3764
   3   5.533 BBA   0.0678  296.16168   72.28495  61.8096
   4  12.718 PB    0.3035    5.91520 2.70097e-1   1.2345
   5  18.719 PBA   0.8806   46.78012 6.34189e-1   9.7631

Totals :                   479.15140   77.37145

 Results obtained with enhanced integrator!
==========================================================================
                          *** End of Report ***
```

Data File D:\LIMSDATA\401018.D Sample Name: 234573

```
==========================================================================
Injection Date  : 5/19/2010 5:26:48 PM          Seq. Line :   3
Sample Name     : 234573                        Location : Vial 4
Acq. Operator   : MHC                           Inj :   1
Acq. Instrument : Instrument 1                  Inj Volume : 10 µl
Sequence File   : D:\LIMSDATA\401014.S
Method          : C:\HPCHEM\1\METHODS\40603102.M
Last changed    : 5/19/2010 2:44:21 PM by MHC
==========================================================================
```




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

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


Signal 1: DAD1 A, Sig=210,8 Ref=off

Peak RetTime Type  Width     Area      Height     Area
  #   [min]       [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   2.231 BB    0.2951    9.75991 4.53400e-1   0.1905
   2   2.460 BB    0.0910    3.62911 5.96336e-1   0.0708
   3   2.841 BB    0.1166   25.67869    3.37963   0.5013
   4   3.664 BB    0.9382   86.29074    1.11088   1.6844
   5   5.534 BBA   0.0691 4841.80371 1151.54822  94.5137
   6  13.152 BB    0.8484   99.31306    1.45493   1.9386
   7  14.761 BB    0.2440   16.72730 9.95559e-1   0.3265
   8  15.319 BB    0.2889    5.77607 2.66411e-1   0.1128
   9  16.749 BBA   0.2646   33.87984    2.14086   0.6613

Totals :                  5122.85843 1161.94622

 Results obtained with enhanced integrator!
```

```
==========================================================================
Injection Date  : 5/19/2010 5:26:48 PM          Seq. Line :   3
Sample Name     : 234573                        Location : Vial 4
Acq. Operator   : MHC                              Inj :   1
Acq. Instrument : Instrument 1                 Inj Volume : 10 µl
Sequence File   : D:\LIMSDATA\401014.S
Method          : C:\HPCHEM\1\METHODS\40603102.M
Last changed    : 5/19/2010 2:44:21 PM by MHC
==========================================================================

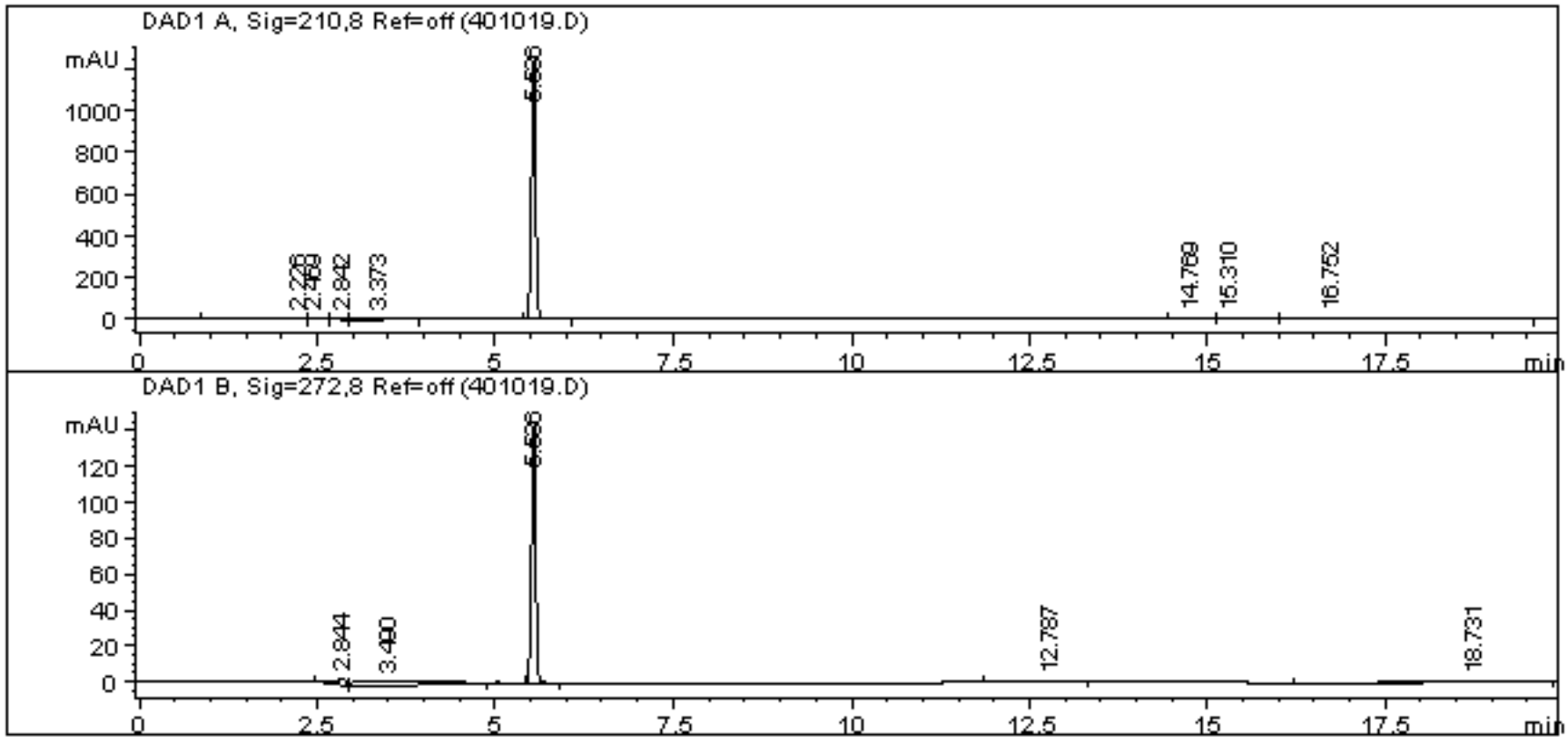

Signal 2: DAD1 B, Sig=272,8 Ref=off

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|-------|
   1   2.843 BB    0.1198   21.68844    2.75631   3.0349
   2   3.502 BB    0.8914  103.65039    1.37501  14.5038
   3   5.534 BBA   0.0683  542.95044  131.25127  75.9748
   4  18.730 PBA   0.9232   46.35599 6.29460e-1   6.4866

Totals :                   714.64527  136.01204

 Results obtained with enhanced integrator!
==========================================================================
                          *** End of Report ***
```

```
==========================================================================
Injection Date  : 5/19/2010 5:48:17 PM          Seq. Line :   4
Sample Name     : 234574                        Location : Vial 5
Acq. Operator   : MHC                              Inj :   1
Acq. Instrument : Instrument 1                  Inj Volume : 10 µl
Sequence File   : D:\LIMSDATA\401014.S
Method          : C:\HPCHEM\1\METHODS\40603102.M
Last changed    : 5/19/2010 2:44:21 PM by MHC
==========================================================================
```




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

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


Signal 1: DAD1 A, Sig=210,8 Ref=off

Peak RetTime Type  Width     Area      Height     Area
  #   [min]       [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   2.226 BB    0.3809   13.78271 4.83068e-1   0.2557
   2   2.459 BB    0.0974    4.36745 6.58308e-1   0.0810
   3   2.842 BB    0.1094   22.34771    3.20461   0.4146
   4   3.373 BB    0.5881   63.37391    1.28338   1.1758
   5   5.536 BBA   0.0693 5229.49463 1238.00464  97.0287
   6  14.769 BB    0.2029    5.69447 3.88673e-1   0.1057
   7  15.310 BB    0.3151    7.26366 2.95191e-1   0.1348
   8  16.752 BBA   0.3221   43.31462    2.19614   0.8037

Totals :                  5389.63916 1246.51401

 Results obtained with enhanced integrator!

Signal 2: DAD1 B, Sig=272,8 Ref=off
```

Injection Date : 5/19/2010 5:48:17 PM Seq. Line : 4
Sample Name : 234574 Location : Vial 5
Acq. Operator : MHC Inj : 1
Acq. Instrument : Instrument 1 Inj Volume : 10 µl
Sequence File : D:\LIMSDATA\401014.S
Method : C:\HPCHEM\1\METHODS\40603102.M
Last changed : 5/19/2010 2:44:21 PM by MHC

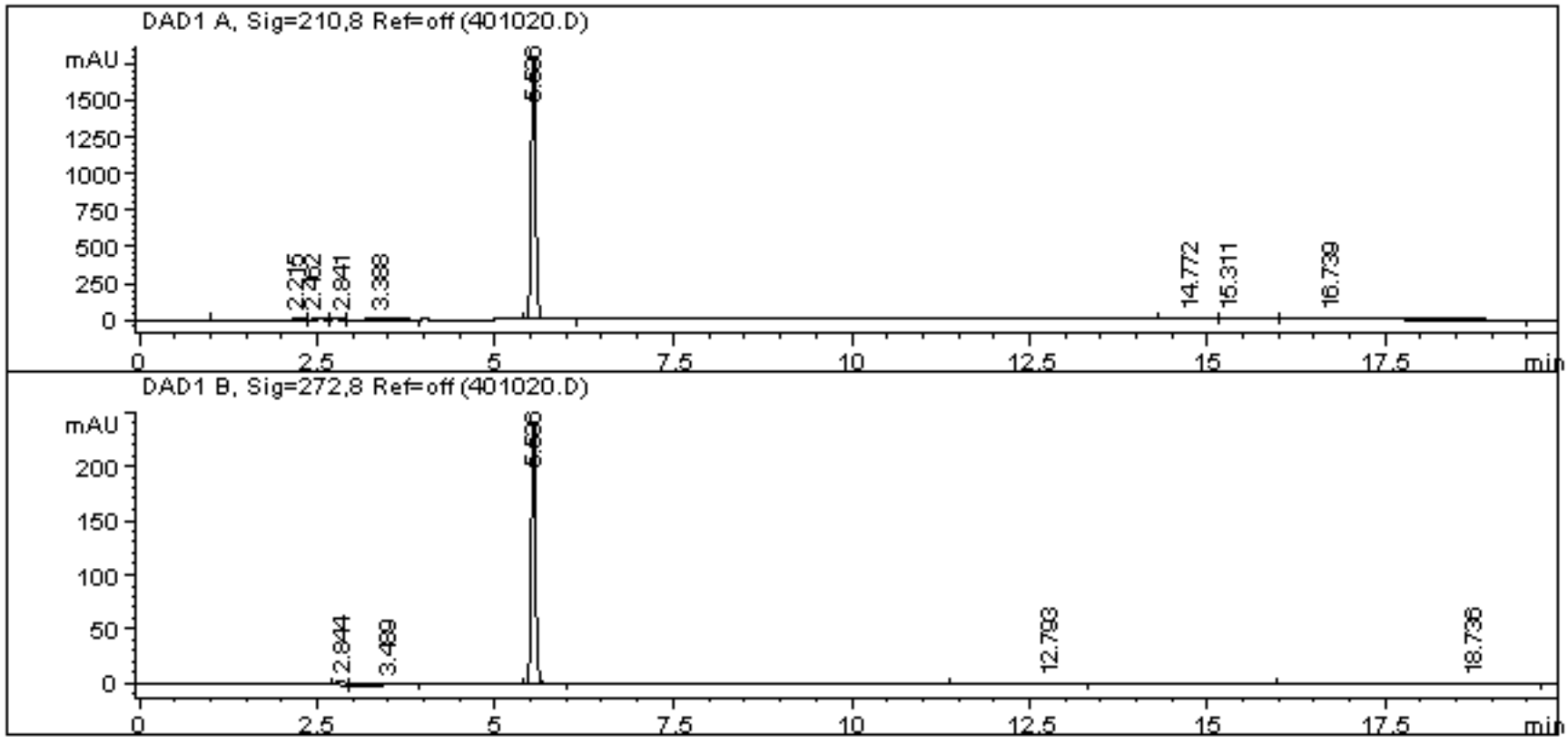
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.844	BB	0.1500	28.29282	2.79719	3.6542
2	3.490	BB	0.8833	102.14489	1.37404	13.1927
3	5.536	BBA	0.0643	591.26190	142.71515	76.3652
4	12.787	PB	0.4157	4.70649	1.47709e-1	0.6079
5	18.731	PBA	0.9266	47.84933	6.38304e-1	6.1800
Totals :				774.25544	147.67240	

Results obtained with enhanced integrator!

*** End of Report ***

Data File D:\LIMSDATA\401020.D Sample Name: 234575

```
==========================================================================
Injection Date  : 5/19/2010 6:09:47 PM          Seq. Line :   5
Sample Name     : 234575                        Location : Vial 6
Acq. Operator   : MHC                              Inj :   1
Acq. Instrument : Instrument 1                  Inj Volume : 10 µl
Sequence File   : D:\LIMSDATA\401014.S
Method          : C:\HPCHEM\1\METHODS\40603102.M
Last changed    : 5/19/2010 2:44:21 PM by MHC
==========================================================================
```




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

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


Signal 1: DAD1 A, Sig=210,8 Ref=off

Peak RetTime Type  Width     Area      Height     Area
  #   [min]       [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   2.215 BB    0.2853   11.03969 5.42775e-1   0.1344
   2   2.462 BB    0.0978    3.70831 5.56086e-1   0.0451
   3   2.841 BB    0.1113   23.46390    3.28781   0.2856
   4   3.388 BB    0.5944   64.44897    1.29990   0.7846
   5   5.536 BBA   0.0728 8037.33447 1775.99963  97.8403
   6  14.772 BB    0.2029    5.73484 3.91498e-1   0.0698
   7  15.311 BB    0.3393    6.85356 2.63605e-1   0.0834
   8  16.739 BBA   0.4163   62.16166    2.26390   0.7567

Totals :                  8214.74540 1784.60521

 Results obtained with enhanced integrator!

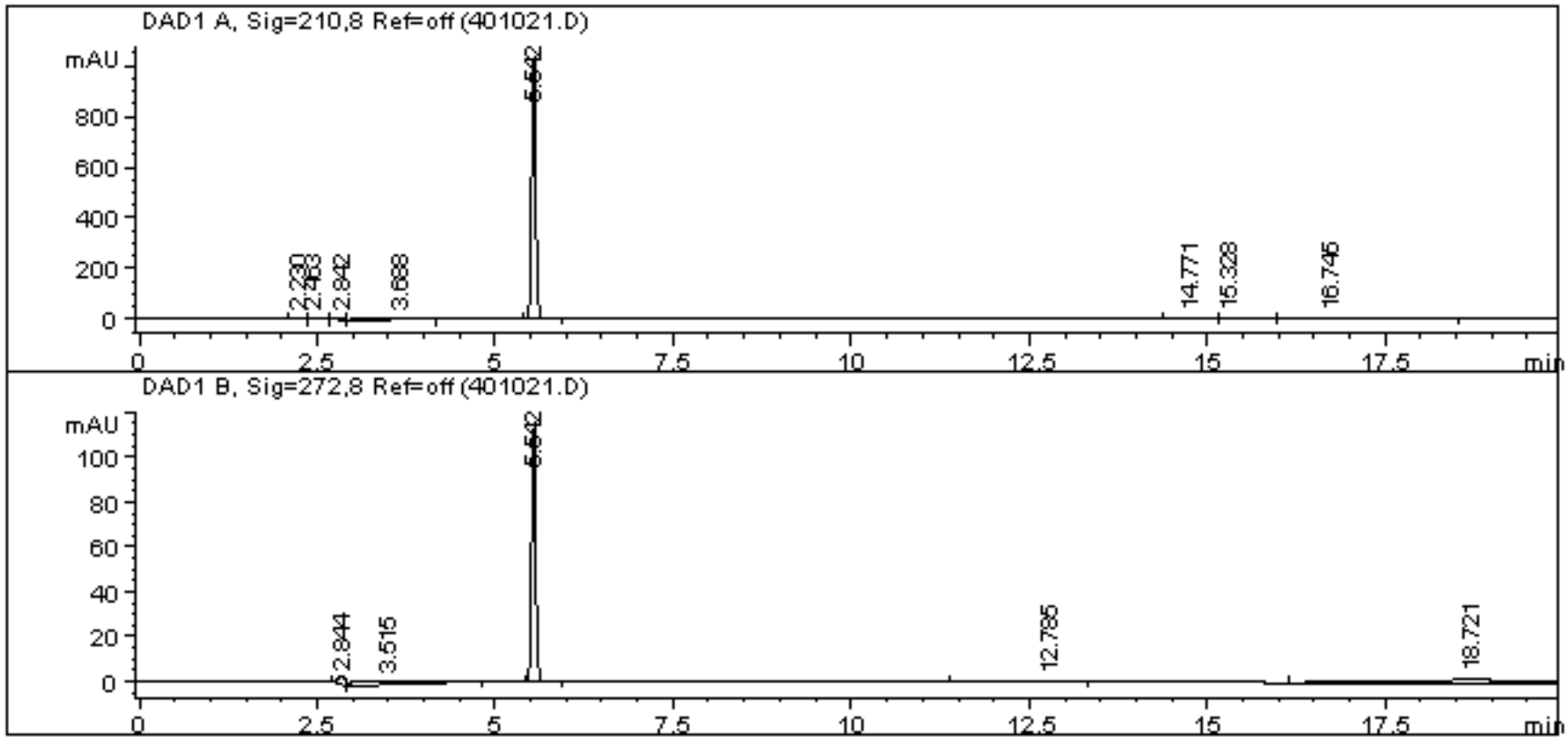
Signal 2: DAD1 B, Sig=272,8 Ref=off
```

```
==========================================================================
Injection Date  : 5/19/2010 6:09:47 PM          Seq. Line :   5
Sample Name     : 234575                        Location : Vial 6
Acq. Operator   : MHC                               Inj :   1
Acq. Instrument : Instrument 1                 Inj Volume : 10 µl
Sequence File   : D:\LIMSDATA\401014.S
Method          : C:\HPCHEM\1\METHODS\40603102.M
Last changed    : 5/19/2010 2:44:21 PM by MHC
==========================================================================
```

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.844	BB	0.1178	20.79928	2.70346	1.8387
2	3.489	BB	0.6715	51.94810	9.36073e-1	4.5923
3	5.536	BBA	0.0693	1007.98926	238.70695	89.1082
4	12.793	PB	0.5043	5.07309	1.33198e-1	0.4485
5	18.736	BBA	0.8774	45.38725	6.29324e-1	4.0123
Totals :				1131.19698	243.10901	

```
 Results obtained with enhanced integrator!
==========================================================================
                          *** End of Report ***
```

```
==========================================================================
Injection Date  : 5/19/2010 6:31:18 PM          Seq. Line :   6
Sample Name     : 234576                        Location : Vial 7
Acq. Operator   : MHC                              Inj :   1
Acq. Instrument : Instrument 1                  Inj Volume : 10 µl
Sequence File   : D:\LIMSDATA\401014.S
Method          : C:\HPCHEM\1\METHODS\40603102.M
Last changed    : 5/19/2010 2:44:21 PM by MHC
==========================================================================
```




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

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


Signal 1: DAD1 A, Sig=210,8 Ref=off

Peak RetTime Type  Width     Area      Height     Area
  #   [min]       [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   2.230 BB    0.1566    3.01976 3.24333e-1   0.0675
   2   2.463 BB    0.1103    4.38110 5.92239e-1   0.0980
   3   2.842 BB    0.1129   22.26269    3.21058   0.4978
   4   3.688 BB    0.9421   81.87652    1.03141   1.8309
   5   5.542 BBA   0.0649 4257.39063 1015.49957  95.2033
   6  14.771 BB    0.2136    6.18153 3.97195e-1   0.1382
   7  15.328 BB    0.2912    6.55643 2.77942e-1   0.1466
   8  16.745 BBA   0.5596   90.22715    2.35172   2.0176

Totals :                  4471.89581 1023.68498

 Results obtained with enhanced integrator!

Signal 2: DAD1 B, Sig=272,8 Ref=off
```

Injection Date : 5/19/2010 6:31:18 PM Seq. Line : 6
Sample Name : 234576 Location : Vial 7
Acq. Operator : MHC Inj : 1
Acq. Instrument : Instrument 1 Inj Volume : 10 µl
Sequence File : D:\LIMSDATA\401014.S
Method : C:\HPCHEM\1\METHODS\40603102.M
Last changed : 5/19/2010 2:44:21 PM by MHC

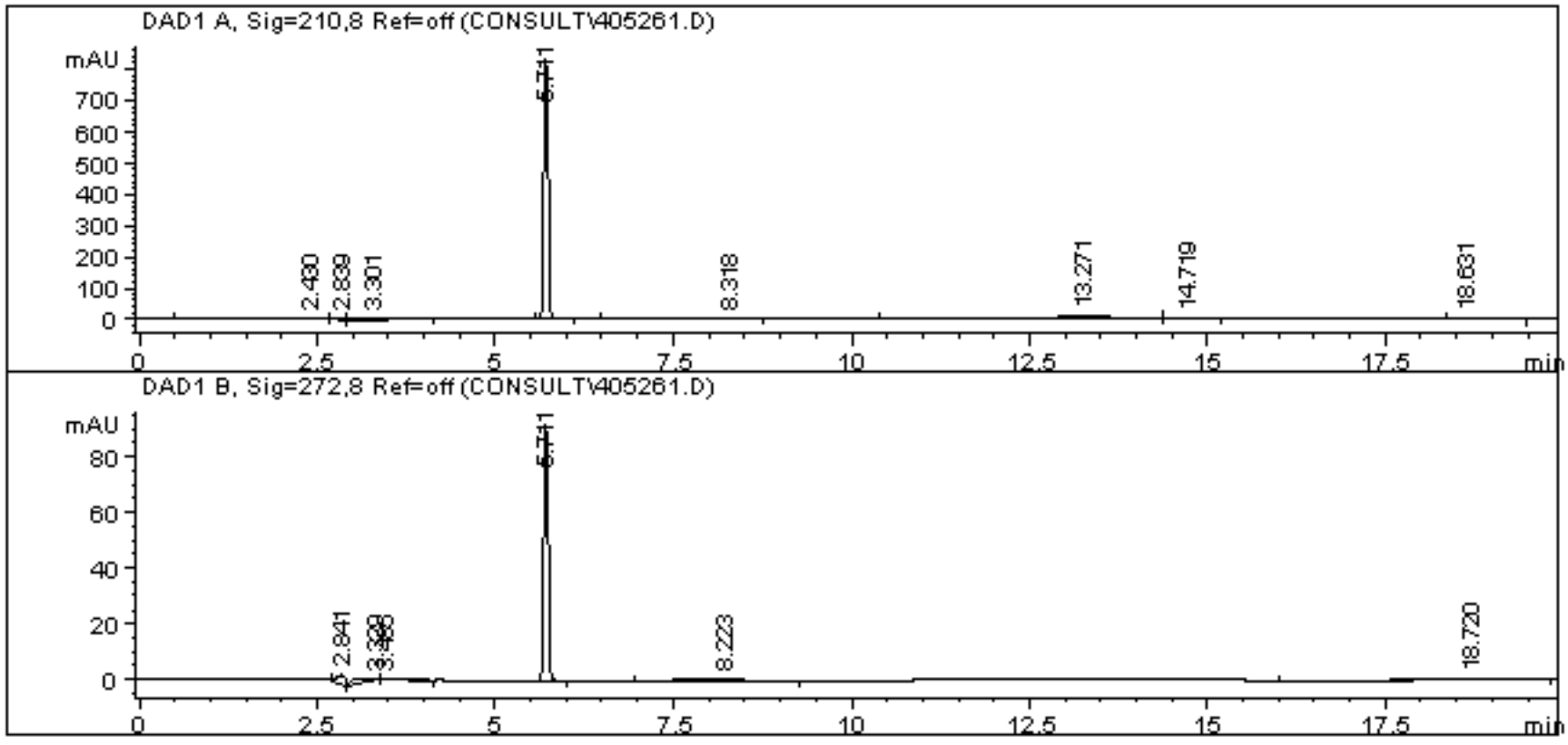
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.844	BB	0.1200	20.34879	2.69658	2.7691
2	3.515	BB	0.8974	100.28277	1.35196	13.6465
3	5.542	BBA	0.0644	472.94495	114.10406	64.3583
4	12.785	PB	0.4992	5.71267	1.43874e-1	0.7774
5	18.721	BBA	1.4127	135.57281	1.15898	18.4487
Totals :				734.86199	119.45546	

Results obtained with enhanced integrator!

*** End of Report ***

Data File D:\LIMSDATA\CONSULT\405261.D Sample Name: 236763

Injection Date	: 6/17/2010 11:49:00 AM	Seq. Line	: 3
Sample Name	: 236763	Location	: Vial 3
Acq. Operator	: MHC	Inj	: 1
Acq. Instrument	: Instrument 1	Inj Volume	: 10 µl
Sequence File	: D:\LIMSDATA\CONSULT\405256.S		
Method	: C:\HPCHEM\1\METHODS\40604201.M		
Last changed	: 6/17/2010 11:04:09 AM by MHC		



Area Percent Report

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

Signal 1: DAD1 A, Sig=210,8 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.430	PB	0.0952	3.77798	5.56273e-1	0.1007
2	2.839	BB	0.1068	23.63398	3.49929	0.6298
3	3.301	BB	0.5962	88.85009	1.78659	2.3679
4	5.711	BBA	0.0683	3423.11133	826.03040	91.2263
5	8.318	BB	0.7547	45.99421	7.42468e-1	1.2258
6	13.271	BB	0.7415	154.35214	2.53804	4.1135
7	14.719	BB	0.2244	9.41421	5.59249e-1	0.2509
8	18.631	BBA	0.2354	3.19467	1.86948e-1	0.0851

Totals : 3752.32861 835.89926

Results obtained with enhanced integrator!

Signal 2: DAD1 B, Sig=272,8 Ref=off

Injection Date : 6/17/2010 11:49:00 AM Seq. Line : 3
Sample Name : 236763 Location : Vial 3
Acq. Operator : MHC Inj : 1
Acq. Instrument : Instrument 1 Inj Volume : 10 µl
Sequence File : D:\LIMSDATA\CONSULT\405256.S
Method : C:\HPCHEM\1\METHODS\40604201.M
Last changed : 6/17/2010 11:04:09 AM by MHC

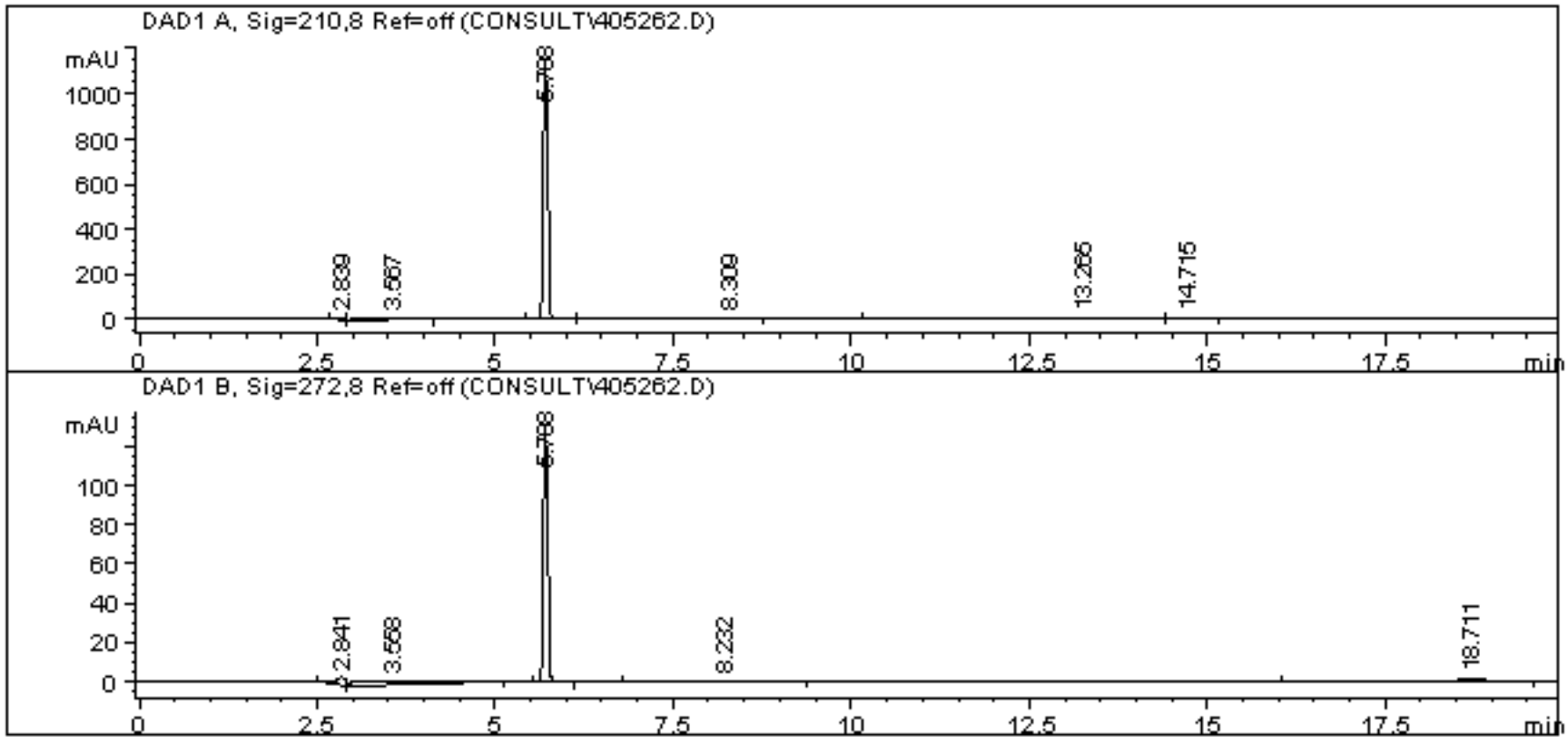
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.841	BB	0.1136	21.97510	2.99482	4.4691
2	3.329	BB	1.1352	21.68601	2.25260e-1	4.4103
3	3.485	BB	0.1914	4.62286	3.22163e-1	0.9402
4	5.711	BBA	0.0680	377.17407	91.73744	76.7070
5	8.223	PB	0.7436	17.83737	2.86023e-1	3.6276
6	18.720	BBA	0.9171	48.41209	6.71494e-1	9.8457
Totals :				491.70749	96.23720	

Results obtained with enhanced integrator!

*** End of Report ***

Data File D:\LIMSDATA\CONSULT\405262.D Sample Name: 236764

Injection Date	: 6/17/2010 12:10:30 PM	Seq. Line	: 4
Sample Name	: 236764	Location	: Vial 4
Acq. Operator	: MHC	Inj	: 1
Acq. Instrument	: Instrument 1	Inj Volume	: 10 µl
Sequence File	: D:\LIMSDATA\CONSULT\405256.S		
Method	: C:\HPCHEM\1\METHODS\40604201.M		
Last changed	: 6/17/2010 11:04:09 AM by MHC		



Area Percent Report

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

Signal 1: DAD1 A, Sig=210,8 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.839	BB	0.1090	24.39016	3.51247	0.4735
2	3.567	BB	0.8004	89.66148	1.34035	1.7405
3	5.708	BBA	0.0693	4837.36084	1144.88110	93.9016
4	8.309	BB	0.7060	39.02866	7.13746e-1	0.7576
5	13.265	BB	0.7527	152.95956	2.48990	2.9692
6	14.715	BB	0.2184	8.11815	5.19213e-1	0.1576

Totals : 5151.51886 1153.45678

Results obtained with enhanced integrator!

Signal 2: DAD1 B, Sig=272,8 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.841	BB	0.1388	29.03085	3.05567	3.8112
2	3.558	BB	0.9545	121.63163	1.51197	15.9681
3	5.708	BBA	0.0685	545.47534	131.25829	71.6114

Injection Date	: 6/17/2010 12:10:30 PM	Seq. Line	: 4
Sample Name	: 236764	Location	: Vial 4
Acq. Operator	: MHC	Inj	: 1
Acq. Instrument	: Instrument 1	Inj Volume	: 10 µl
Sequence File	: D:\LIMSDATA\CONSULT\405256.S		
Method	: C:\HPCHEM\1\METHODS\40604201.M		
Last changed	: 6/17/2010 11:04:09 AM by MHC		

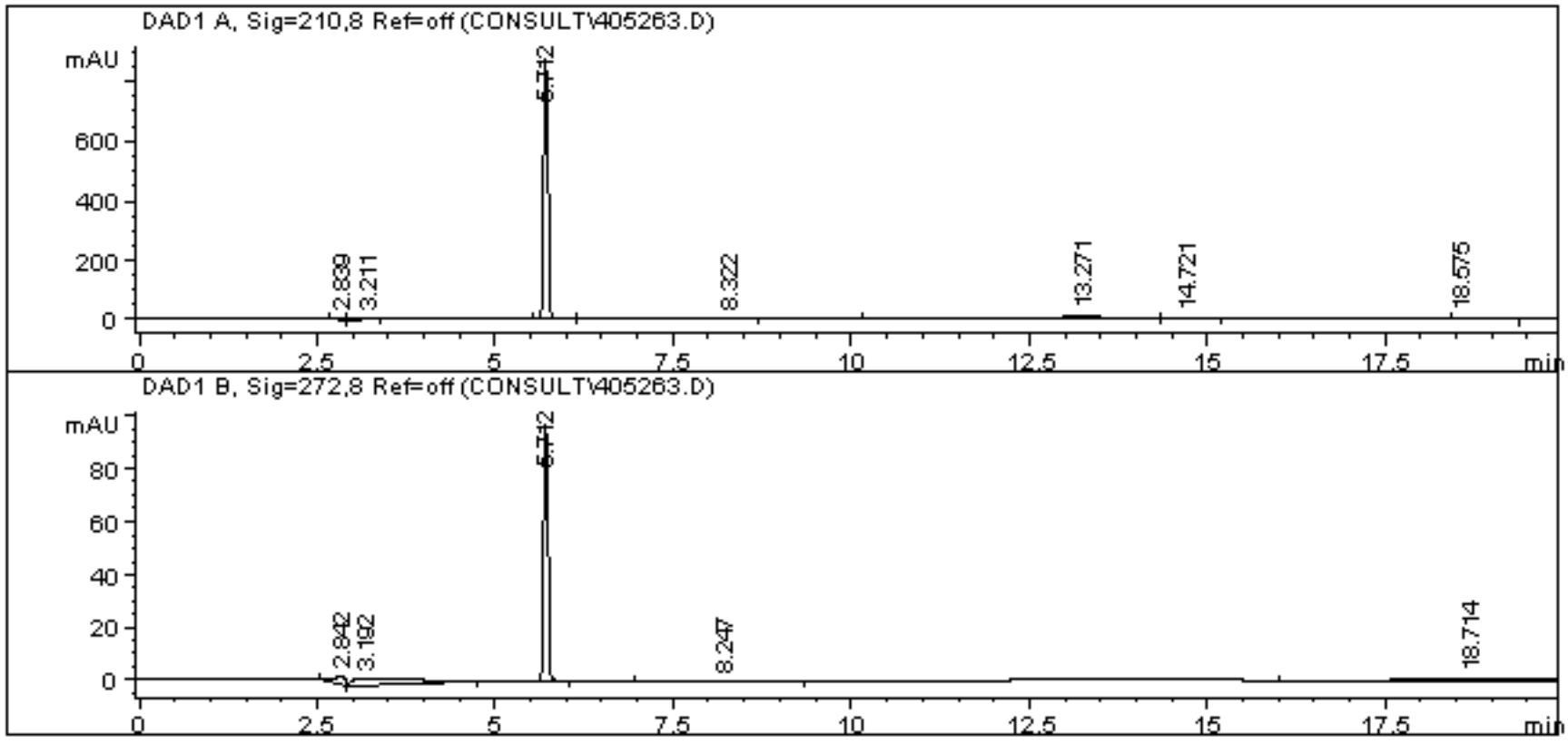
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
4	8.232	PBA	0.7395	16.35445	2.66661e-1	2.1471
5	18.711	BBA	0.9182	49.22304	6.75420e-1	6.4621
Totals :				761.71531	136.76801	

Results obtained with enhanced integrator!

*** End of Report ***

Data File D:\LIMSDATA\CONSULT\405263.D Sample Name: 236765

Injection Date	: 6/17/2010 12:32:01 PM	Seq. Line	: 5
Sample Name	: 236765	Location	: Vial 5
Acq. Operator	: MHC	Inj	: 1
Acq. Instrument	: Instrument 1	Inj Volume	: 10 µl
Sequence File	: D:\LIMSDATA\CONSULT\405256.S		
Method	: C:\HPCHEM\1\METHODS\40604201.M		
Last changed	: 6/17/2010 11:04:09 AM by MHC		



Area Percent Report

Sorted By	: Signal
Multiplier	: 1.0000
Dilution	: 1.0000

Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 A, Sig=210,8 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.839	BB	0.1082	24.02176	3.49619	0.6182
2	3.211	BB	0.3785	26.59621	8.75381e-1	0.6845
3	5.712	BBA	0.0649	3644.21606	869.25842	93.7863
4	8.322	BB	0.6244	33.54130	6.65070e-1	0.8632
5	13.271	BB	0.7233	146.77354	2.49130	3.7773
6	14.721	BB	0.2219	7.40105	4.54613e-1	0.1905
7	18.575	BBA	0.2980	3.10789	1.26757e-1	0.0800
Totals :				3885.65782	877.36773	

Results obtained with enhanced integrator!

Signal 2: DAD1 B, Sig=272,8 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.842	BB	0.1376	28.57011	3.04245	4.3516
2	3.192	BBA	0.7467	103.13490	1.68347	15.7089

```
==========================================================================
Injection Date  : 6/17/2010 12:32:01 PM          Seq. Line :   5
Sample Name     : 236765                        Location : Vial 5
Acq. Operator   : MHC                               Inj :   1
Acq. Instrument : Instrument 1                 Inj Volume : 10 µl
Sequence File   : D:\LIMSDATA\CONSULT\405256.S
Method          : C:\HPCHEM\1\METHODS\40604201.M
Last changed    : 6/17/2010 11:04:09 AM by MHC
==========================================================================
```

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
3	5.712	BBA	0.0645	402.04245	96.80908	61.2367
4	8.247	PBA	0.7577	16.77390	2.68190e-1	2.5549
5	18.714	PBA	1.2736	106.01717	9.96175e-1	16.1479
Totals :				656.53852	102.79937	

```
 Results obtained with enhanced integrator!
==========================================================================
                          *** End of Report ***
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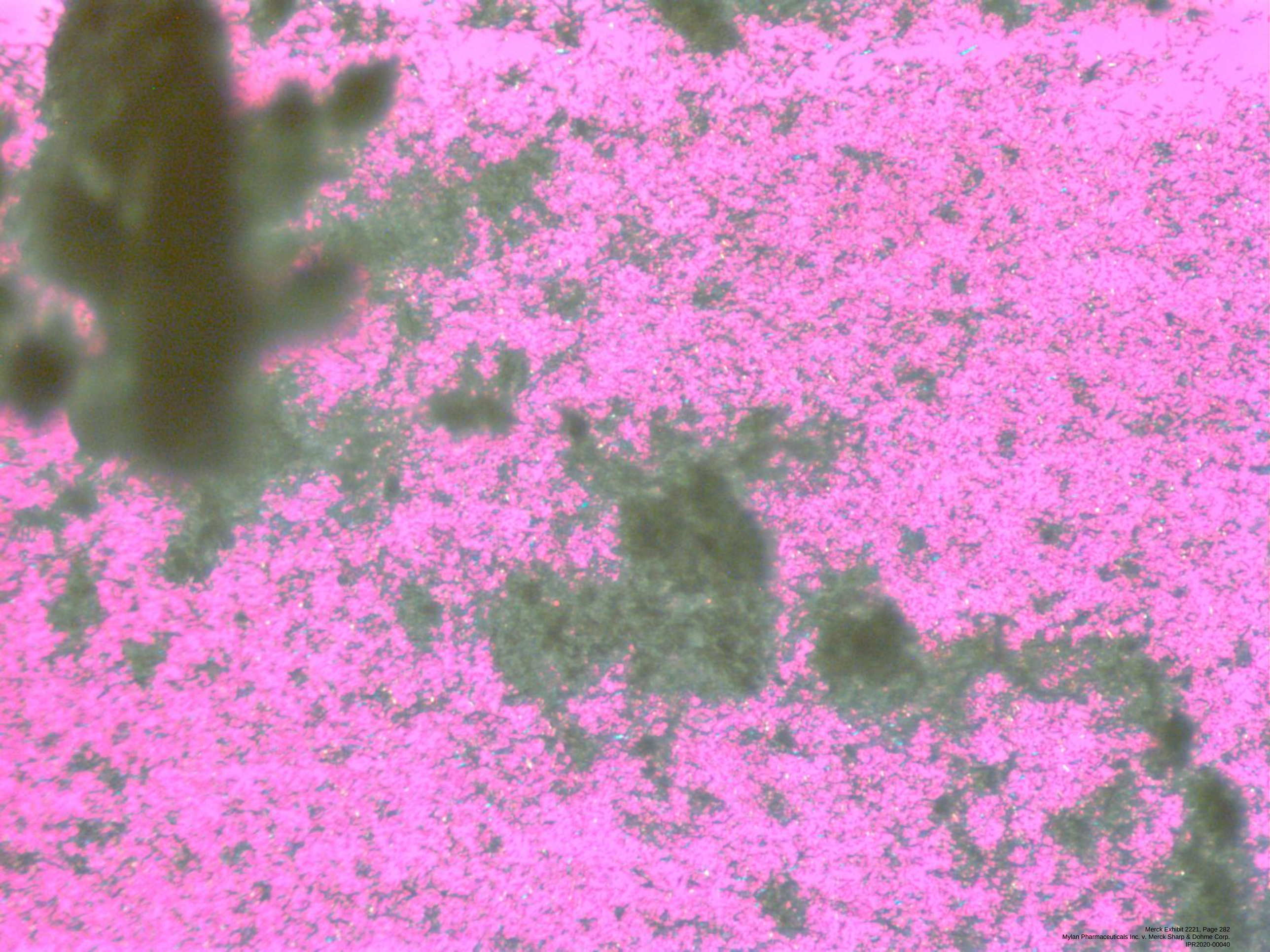
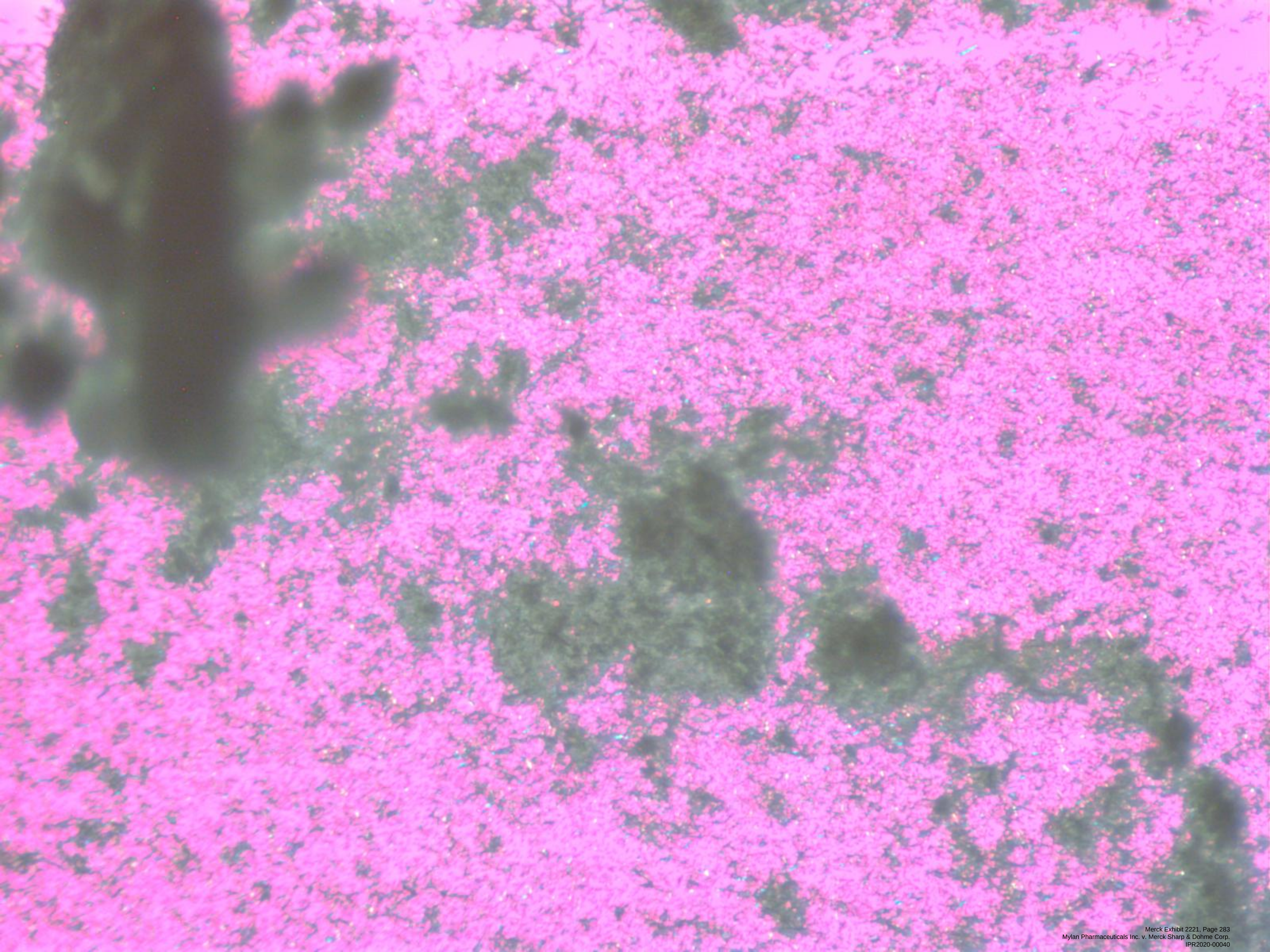
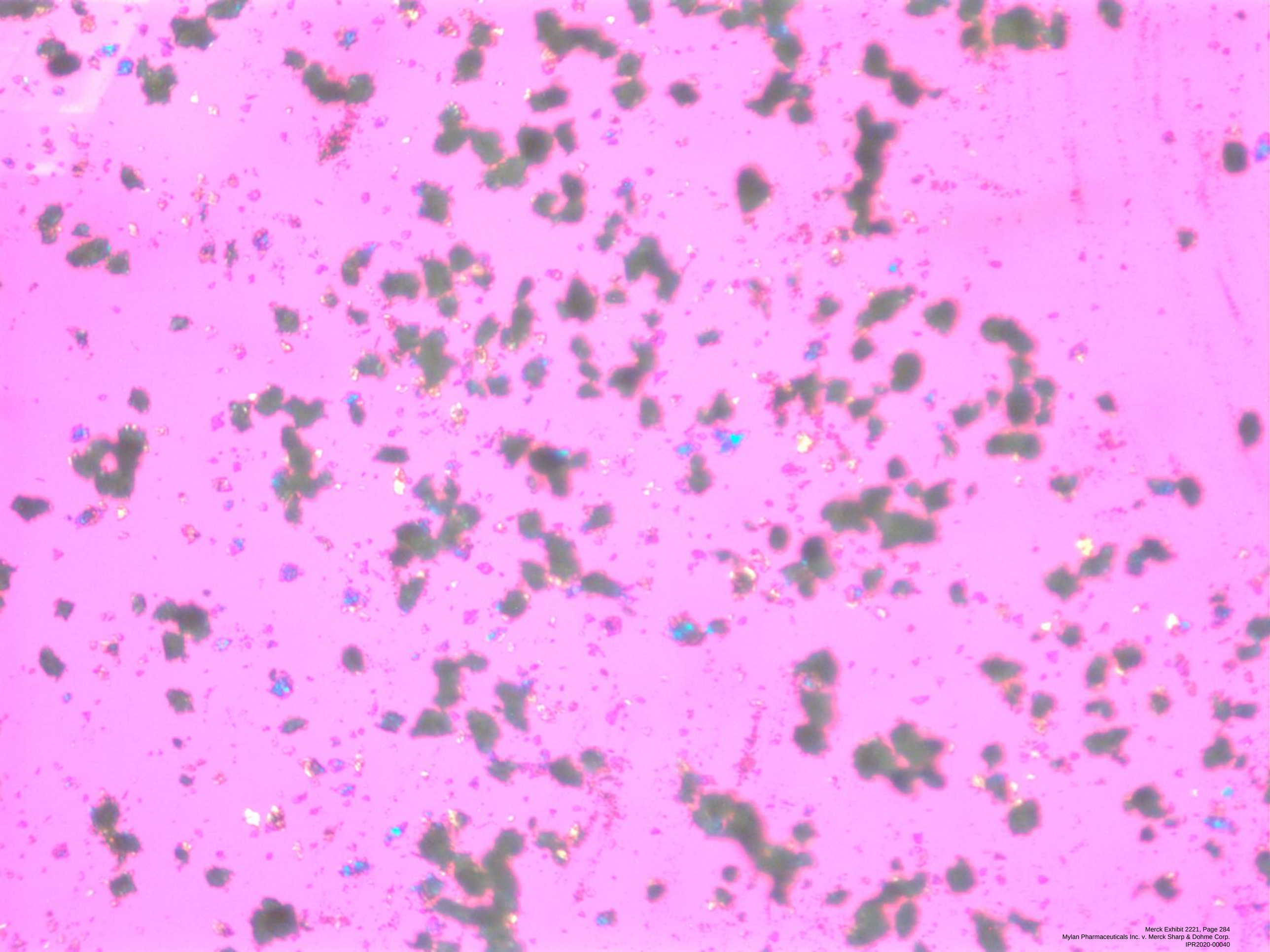
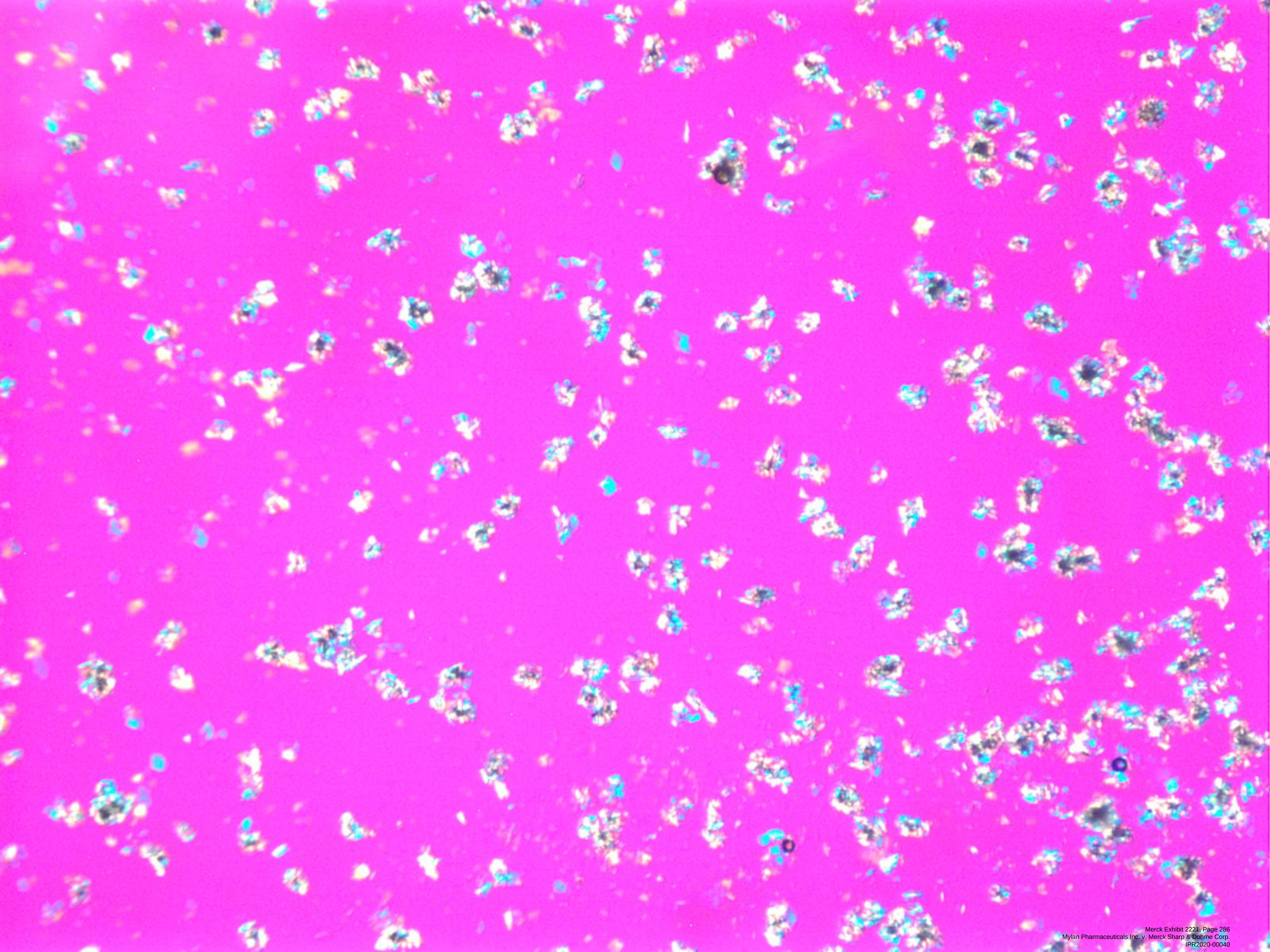
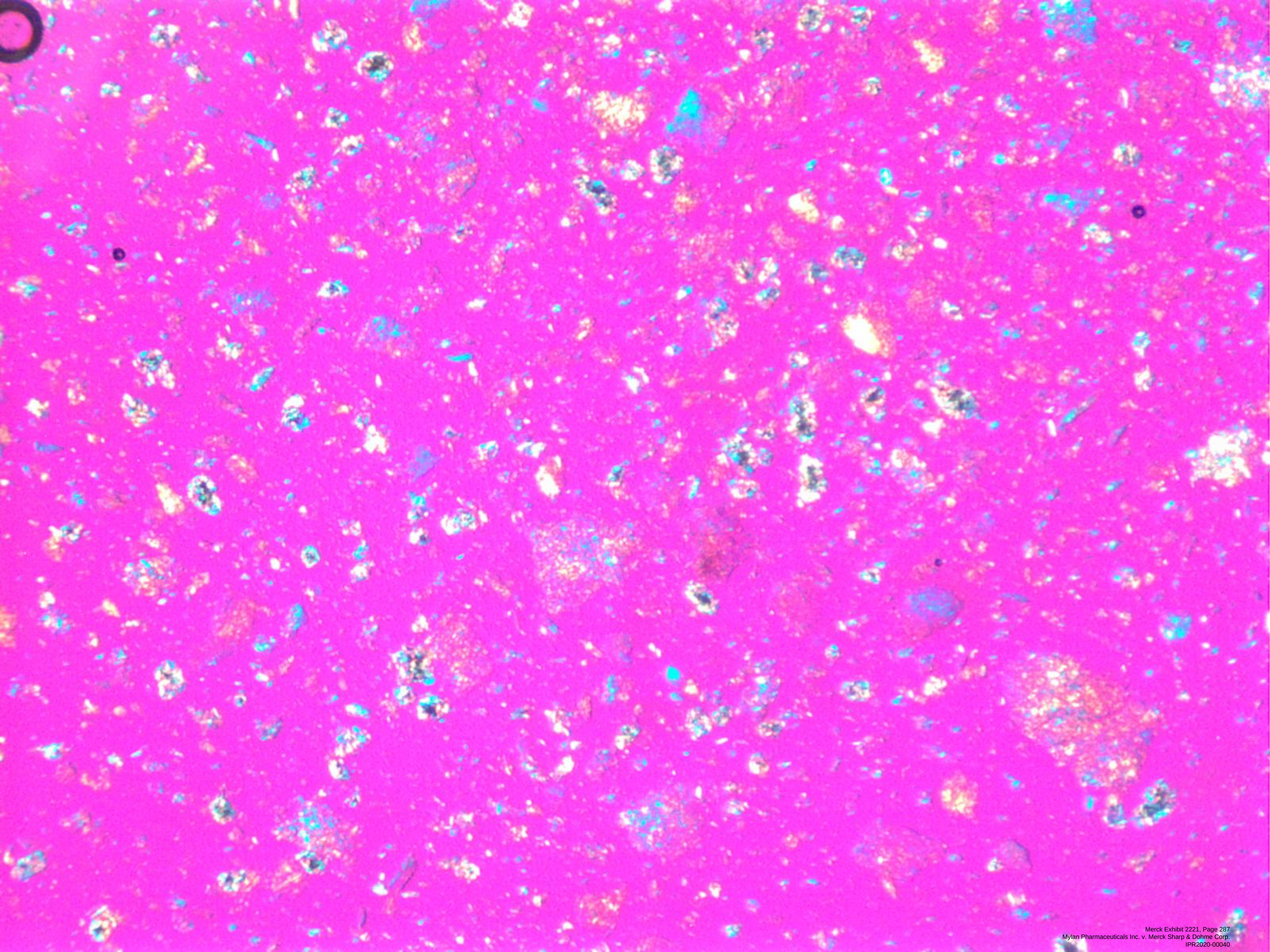










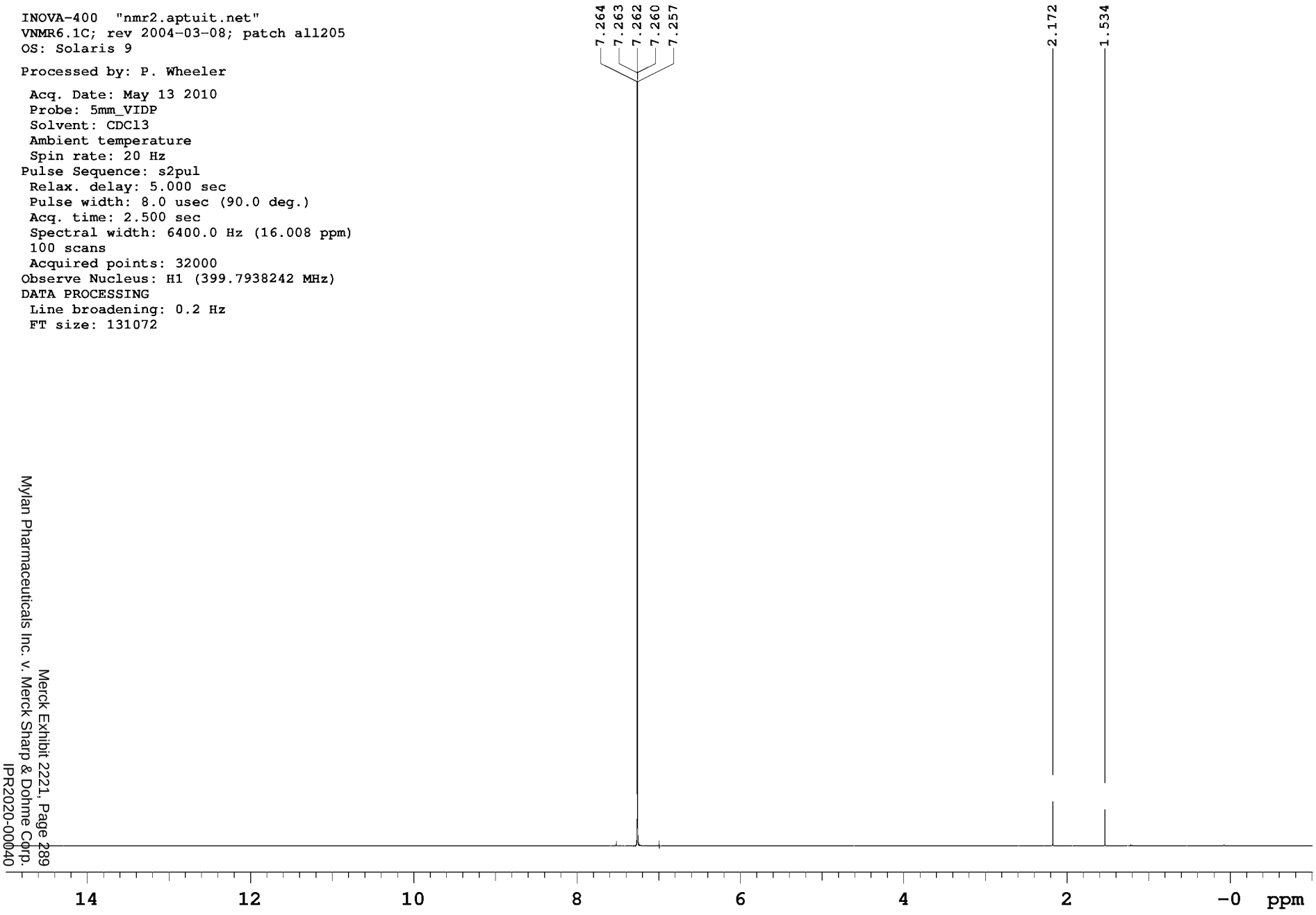
231202, Compound 184, Lot D-1895NN-13067/3, in CDCl3, 1H NMR, referenced to solvent at 7.26 ppm

File: 399496

INOVA-400 "nmr2.aptuit.net"
VNMR6.1C; rev 2004-03-08; patch all205
OS: Solaris 9

Processed by: P. Wheeler

Acq. Date: May 13 2010
Probe: 5mm_VIDP
Solvent: CDCl3
Ambient temperature
Spin rate: 20 Hz
Pulse Sequence: s2pul
Relax. delay: 5.000 sec
Pulse width: 8.0 usec (90.0 deg.)
Acq. time: 2.500 sec
Spectral width: 6400.0 Hz (16.008 ppm)
100 scans
Acquired points: 32000
Observe Nucleus: H1 (399.7938242 MHz)
DATA PROCESSING
Line broadening: 0.2 Hz
FT size: 131072



Plot file: 399496-1

231202, Compound 184, Lot D-1895NN-13067/3, in CDC13, 1H NMR, referenced to solvent at 7.26 ppm

File: 399496

INDEX	FREQUENCY	PPM	HEIGHT
1	2904.143	7.264	4.8
2	2903.752	7.263	6.2
3	2903.362	7.262	9.3
4	2902.483	7.260	141.8
5	2901.213	7.257	4.7
6	868.498	2.172	8.5
7	613.420	1.534	7.0

Plot file: 399496-1_peaks

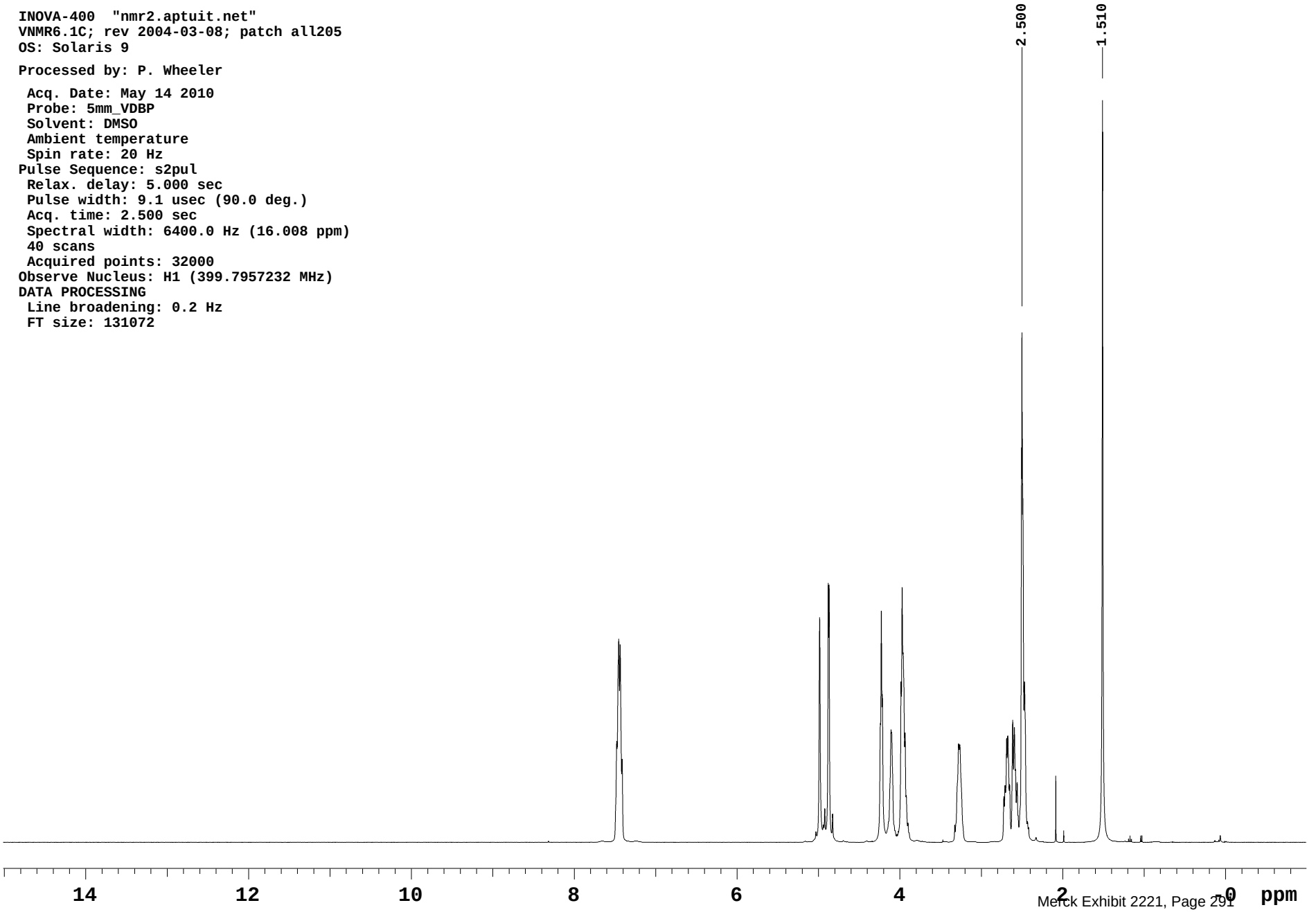
229438, Compound 184, Lot sal-069, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

File: 399497

INOVA-400 "nmr2.aptuit.net"
VNMR6.1C; rev 2004-03-08; patch all205
OS: Solaris 9

Processed by: P. Wheeler

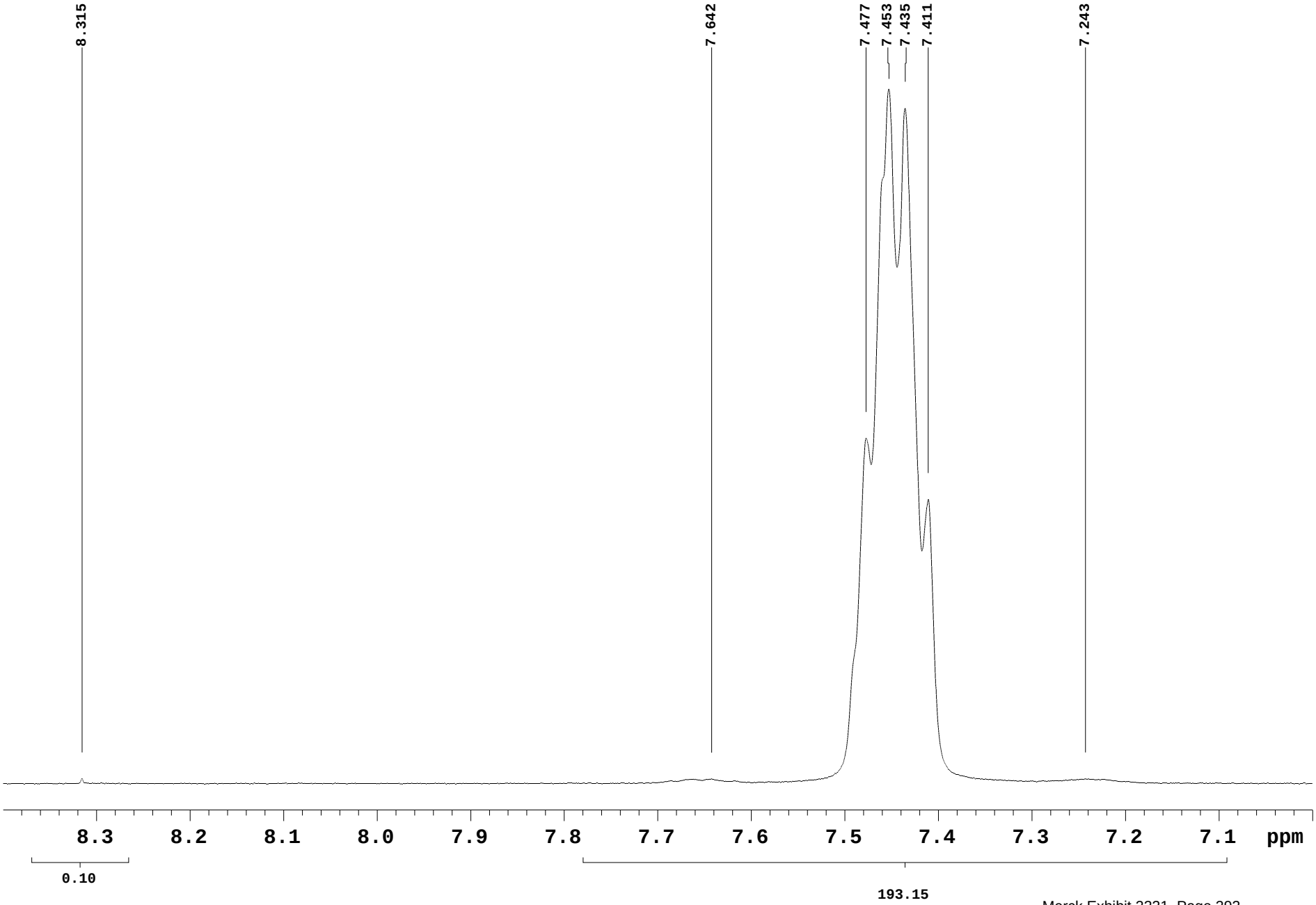
Acq. Date: May 14 2010
Probe: 5mm_VDBP
Solvent: DMSO
Ambient temperature
Spin rate: 20 Hz
Pulse Sequence: s2pul
Relax. delay: 5.000 sec
Pulse width: 9.1 usec (90.0 deg.)
Acq. time: 2.500 sec
Spectral width: 6400.0 Hz (16.008 ppm)
40 scans
Acquired points: 32000
Observe Nucleus: H1 (399.7957232 MHz)
DATA PROCESSING
Line broadening: 0.2 Hz
FT size: 131072



Plot file: 399497-1

229438, Compound 184, Lot sal-069, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

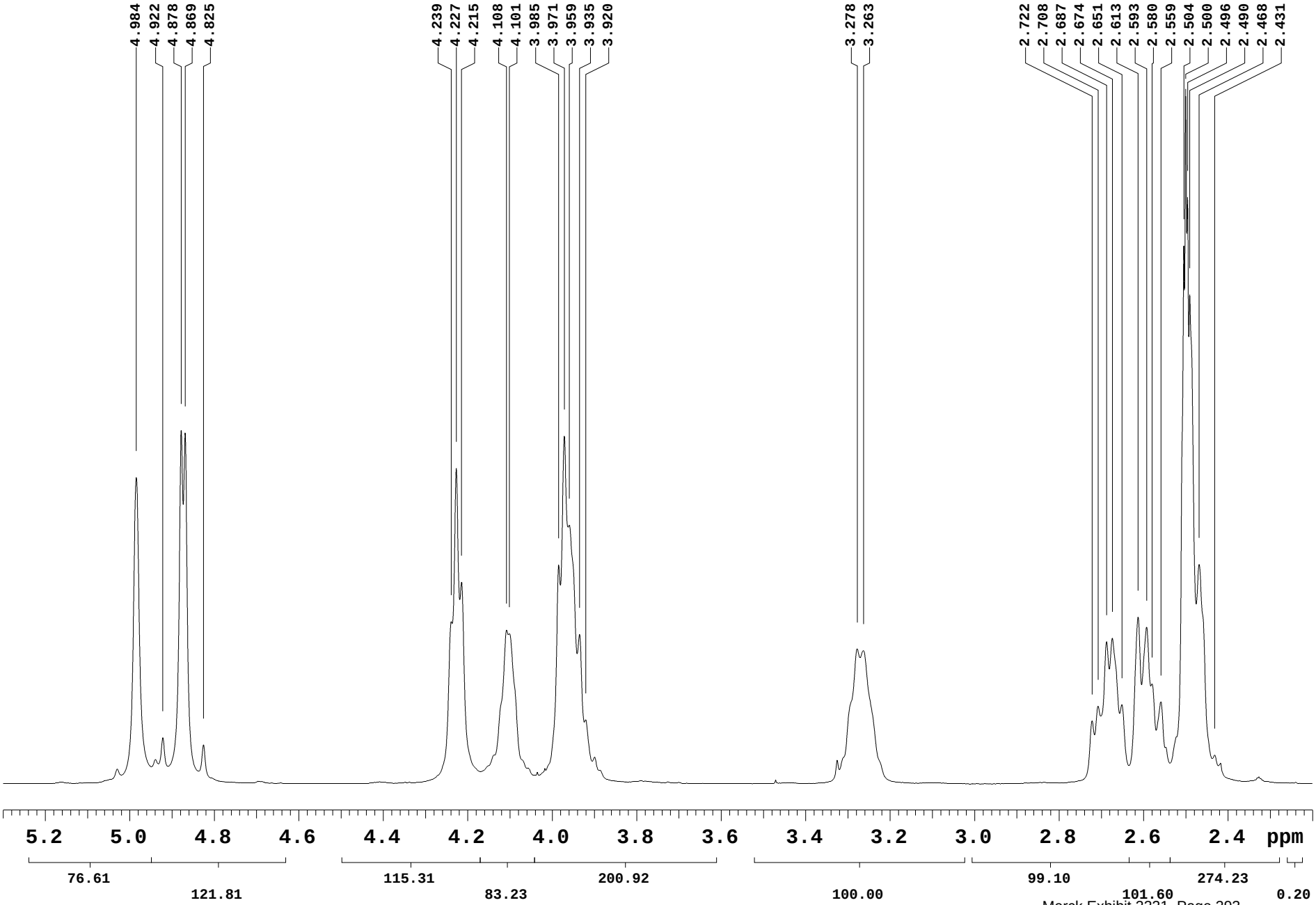
File: 399497



Plot file: 399497-2

229438, Compound 184, Lot sal-069, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

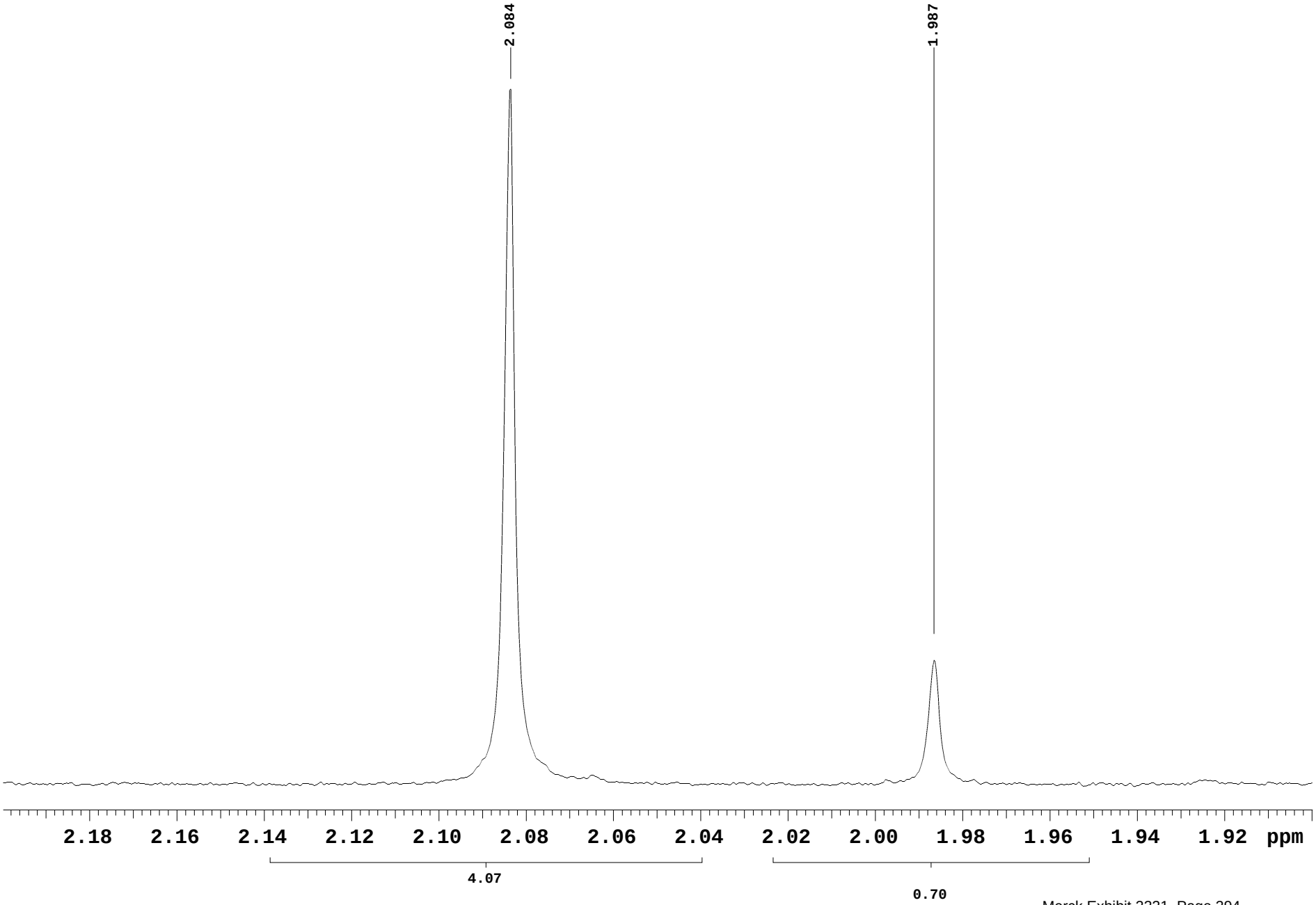
File: 399497



Plot file: 399497-3

229438, Compound 184, Lot sal-069, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

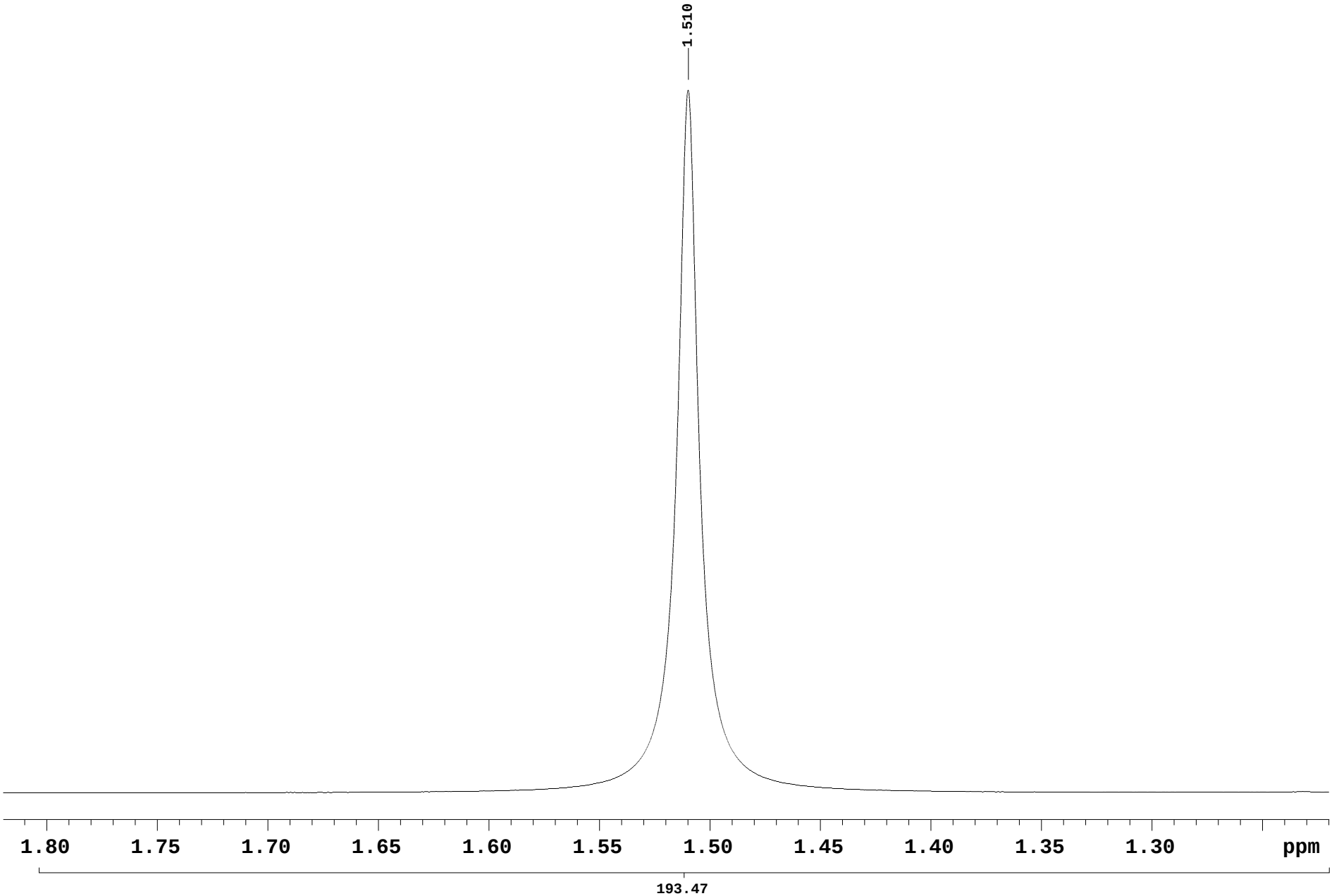
File: 399497



Plot file: 399497-4

229438, Compound 184, Lot sal-069, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

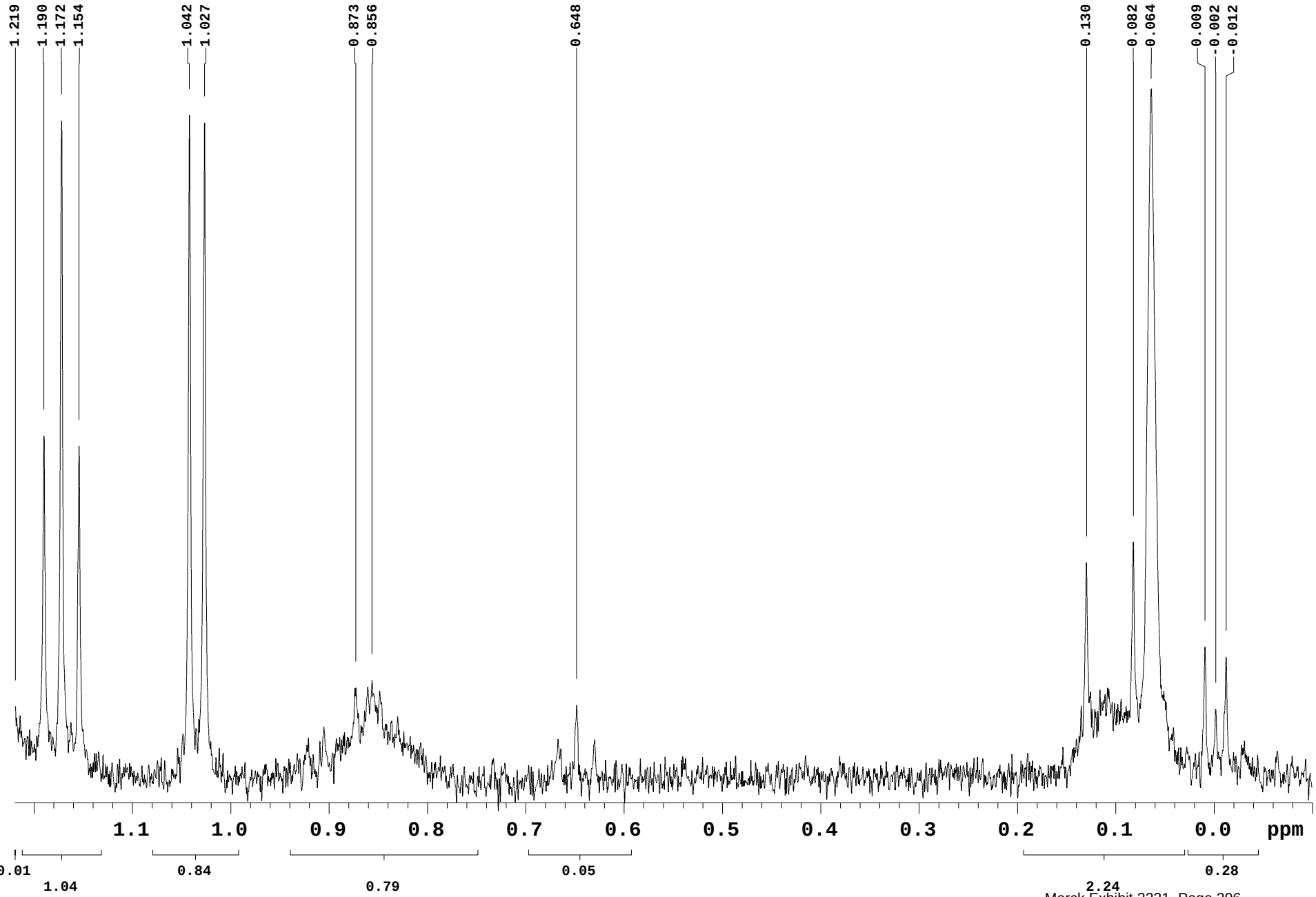
File: 399497



Plot file: 399497-5

229438, Compound 184, Lot sal-069, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

File: 399497



Plot file: 399497-6

229438, Compound 184, Lot sal-069, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

File: 399497

INDEX	FREQUENCY	PPM	HEIGHT
1	999.482	2.500	97.4
2	603.584	1.510	141.8

Plot file: 399497-1_peaks

229438, Compound 184, Lot sal-069, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

File: 399497

INDEX	FREQUENCY	PPM	HEIGHT
1	3324.385	8.315	0.9
2	3055.342	7.642	0.9
3	2989.326	7.477	65.6
4	2979.560	7.453	132.0
5	2972.627	7.435	128.4
6	2962.764	7.411	54.0
7	2895.576	7.243	0.9

Plot file: 399497-2_peaks

229438, Compound 184, Lot sal-069, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

File: 399497

INDEX	FREQUENCY	PPM	HEIGHT
1	1992.744	4.984	58.2
2	1967.646	4.922	8.8
3	1950.264	4.878	67.1
4	1946.553	4.869	66.7
5	1929.170	4.825	7.4
6	1694.599	4.239	30.8
7	1689.912	4.227	59.9
8	1684.932	4.215	38.3
9	1642.353	4.108	29.2
10	1639.717	4.101	28.5
11	1593.037	3.985	41.6
12	1587.666	3.971	66.1
13	1582.978	3.959	49.1
14	1573.213	3.935	28.4
15	1567.353	3.920	12.1
16	1310.420	3.278	25.6
17	1304.463	3.263	25.3
18	1088.057	2.722	11.9
19	1082.490	2.708	14.7
20	1074.385	2.687	27.0
21	1068.916	2.674	27.7
22	1059.932	2.651	15.0
23	1044.599	2.613	31.7
24	1036.494	2.593	29.8
25	1031.318	2.580	18.9
26	1022.920	2.559	15.5
27	1001.240	2.504	102.2
28	999.482	2.500	132.0
29	997.724	2.496	111.4
30	995.674	2.490	92.9
31	986.787	2.468	41.7
32	972.041	2.431	5.5

Plot file: 399497-3_peaks

229438, Compound 184, Lot sal-069, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

File: 399497

INDEX	FREQUENCY	PPM	HEIGHT
1	832.978	2.084	132.0
2	794.209	1.987	23.5

Plot file: 399497-4_peaks

229438, Compound 184, Lot sal-069, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

File: 399497

INDEX	FREQUENCY	PPM	HEIGHT
1	603.584	1.510	132.0

Plot file: 399497-5_peaks

229438, Compound 184, Lot sal-069, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

File: 399497

INDEX	FREQUENCY	PPM	HEIGHT
1	487.373	1.219	13.5
2	475.752	1.190	65.4
3	468.623	1.172	125.9
4	461.494	1.154	63.5
5	416.572	1.042	127.0
6	410.420	1.027	125.5
7	348.896	0.873	17.1
8	342.353	0.856	18.5
9	259.150	0.648	13.8
10	51.826	0.130	41.1
11	32.783	0.082	45.1
12	25.557	0.064	132.0
13	3.682	0.009	24.9
14	-0.713	-0.002	13.0
15	-4.912	-0.012	23.0

Plot file: 399497-6_peaks

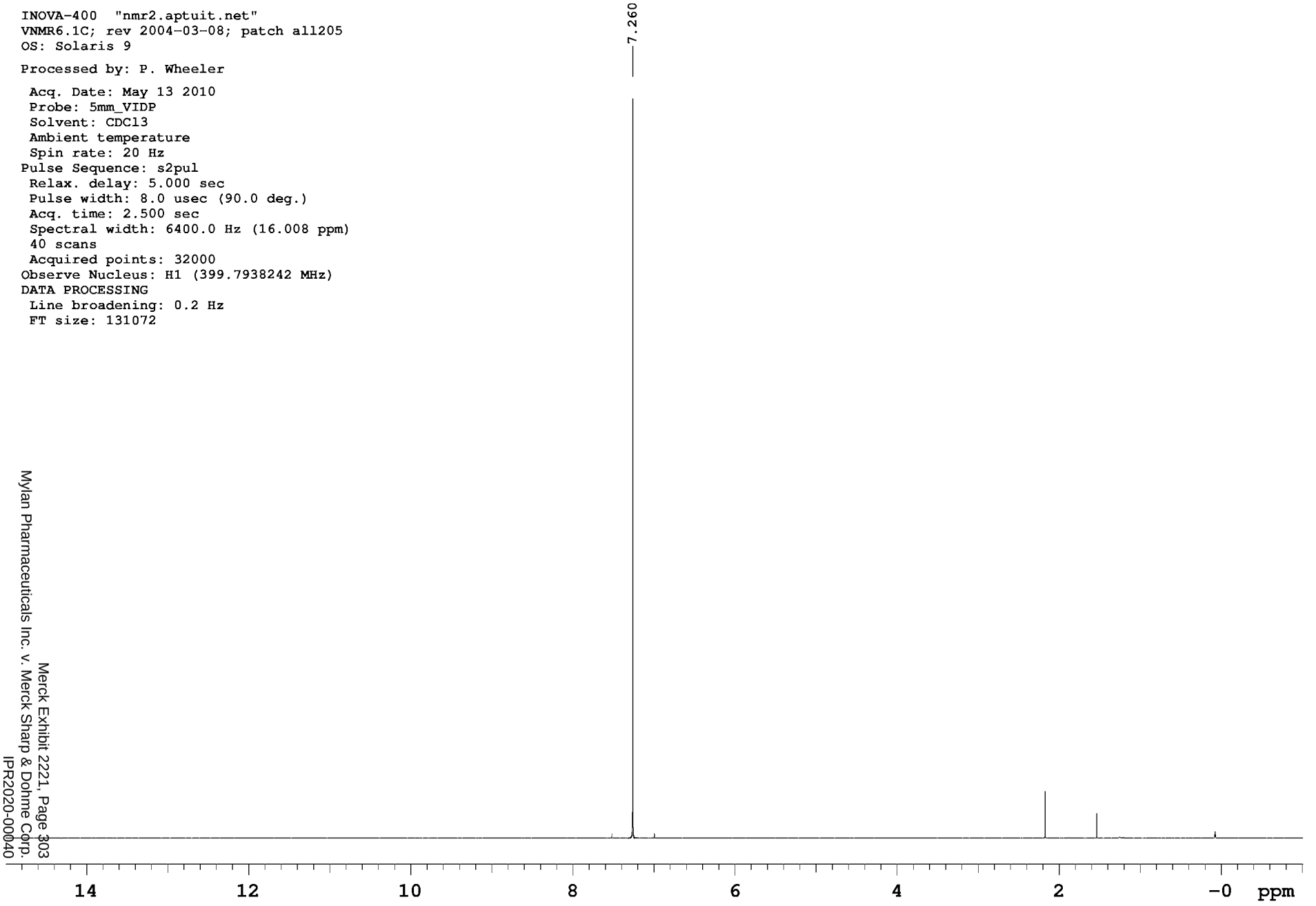
233959, 4063-14-03, Chloroform-D, in CDCl3, 1H NMR, referenced to solvent at 7.26 ppm

File: 399793

INOVA-400 "nmr2.aptuit.net"
VNMR6.1C; rev 2004-03-08; patch all205
OS: Solaris 9

Processed by: P. Wheeler

Acq. Date: May 13 2010
Probe: 5mm_VIDP
Solvent: CDCl3
Ambient temperature
Spin rate: 20 Hz
Pulse Sequence: s2pul
Relax. delay: 5.000 sec
Pulse width: 8.0 usec (90.0 deg.)
Acq. time: 2.500 sec
Spectral width: 6400.0 Hz (16.008 ppm)
40 scans
Acquired points: 32000
Observe Nucleus: H1 (399.7938242 MHz)
DATA PROCESSING
Line broadening: 0.2 Hz
FT size: 131072



Plot file: 399793-1

233959, 4063-14-03, Chloroform-D, in CDCl3, 1H NMR, referenced to solvent at 7.26 ppm

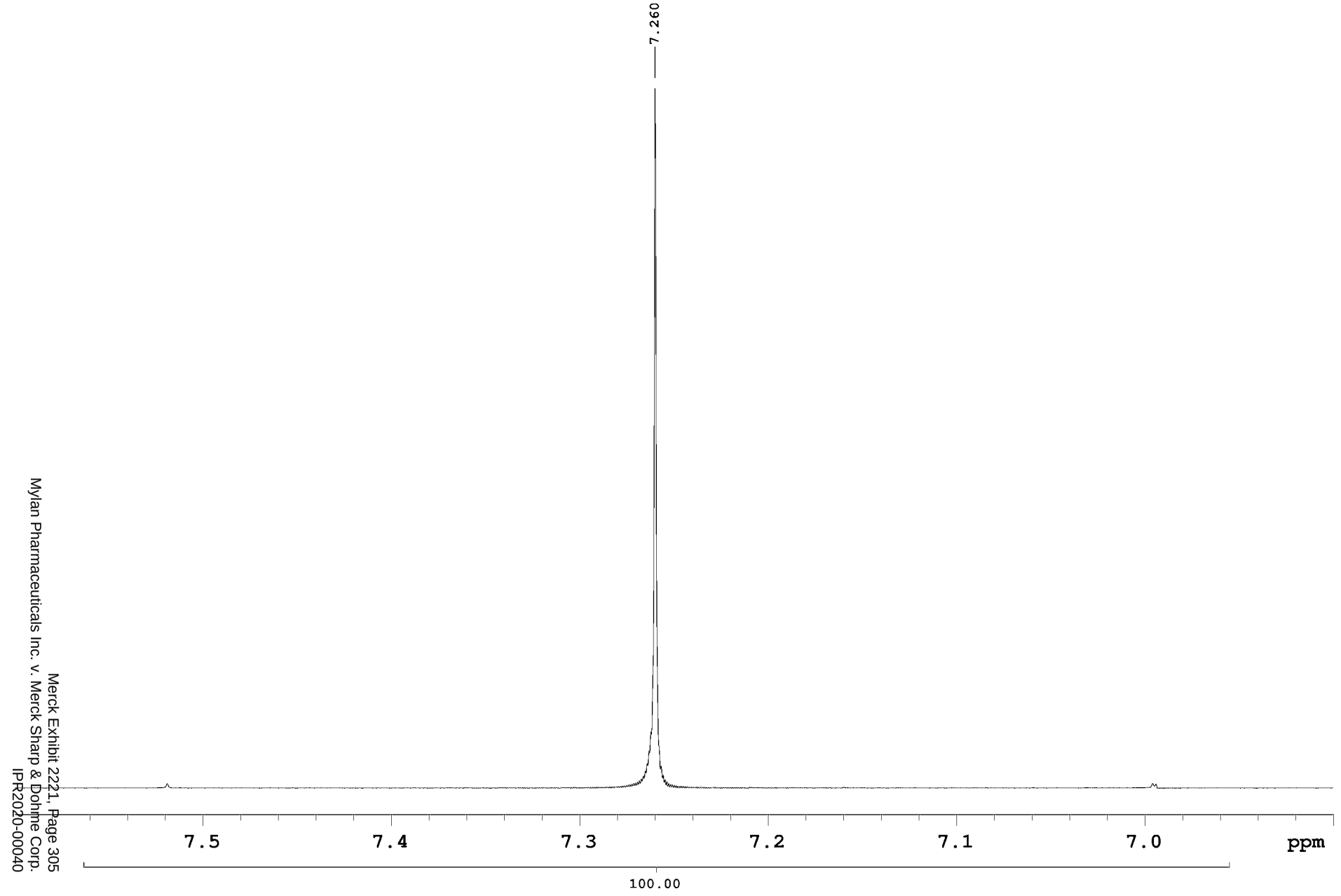
File: 399793

INDEX	FREQUENCY	PPM	HEIGHT
1	2902.483	7.260	141.8

Plot file: 399793-1_peaks

233959, 4063-14-03, Chloroform-D, in CDCl3, 1H NMR, referenced to solvent at 7.26 ppm

File: 399793



Plot file: 399793-2

233959, 4063-14-03, Chloroform-D, in CDCl3, 1H NMR, referenced to solvent at 7.26 ppm

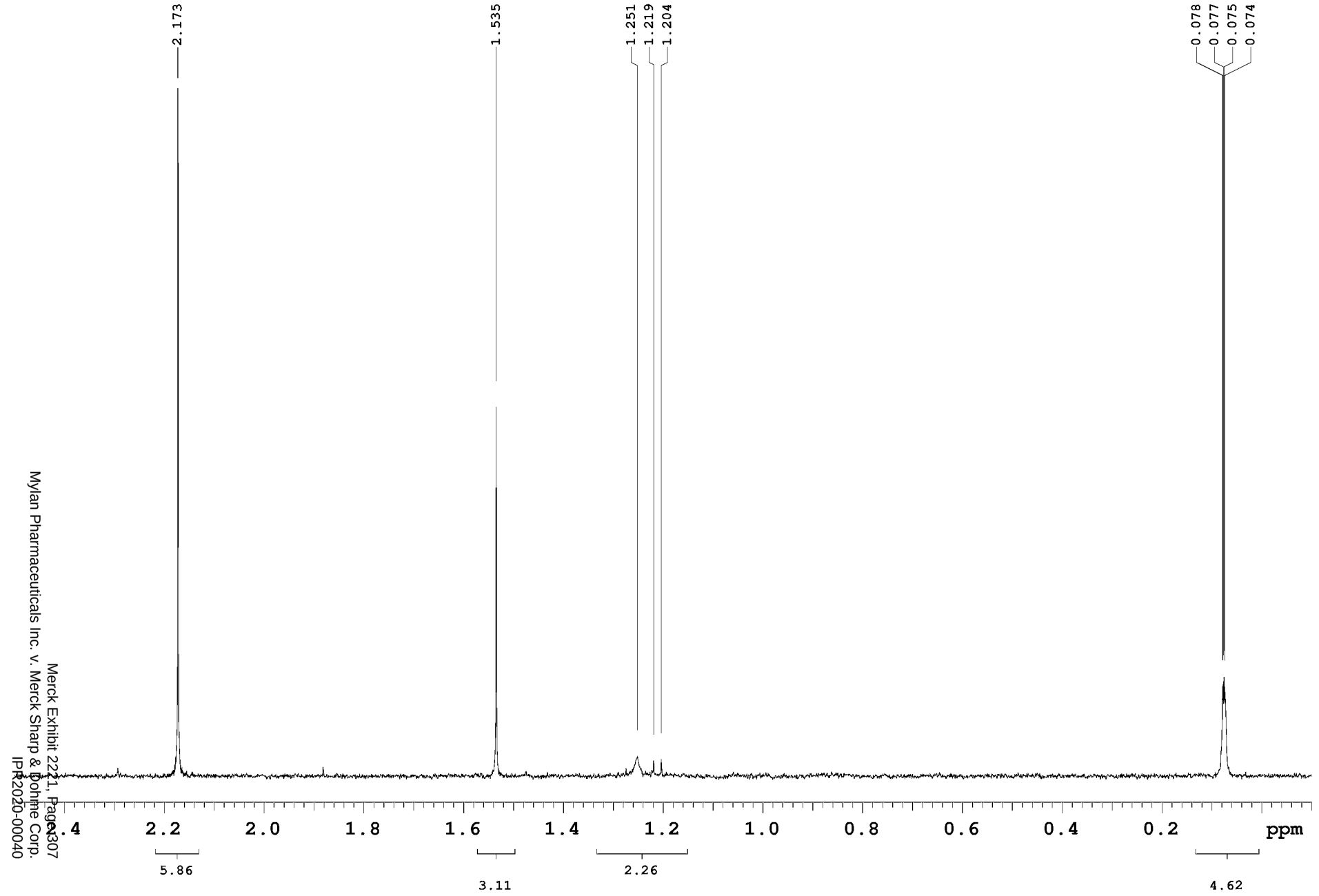
File: 399793

INDEX	FREQUENCY	PPM	HEIGHT
1	2902.483	7.260	132.0

Plot file: 399793-2_peaks

233959, 4063-14-03, Chloroform-D, in CDCl3, 1H NMR, referenced to solvent at 7.26 ppm

File: 399793



Plot file: 399793-3

233959, 4063-14-03, Chloroform-D, in CDCl3, 1H NMR, referenced to solvent at 7.26 ppm

File: 399793

INDEX	FREQUENCY	PPM	HEIGHT
1	868.596	2.173	132.0
2	613.518	1.535	70.8
3	500.334	1.251	3.8
4	487.248	1.219	3.0
5	481.194	1.204	3.2
6	31.194	0.078	17.3
7	30.608	0.077	18.2
8	30.022	0.075	19.1
9	29.534	0.074	17.2

Plot file: 399793-3_peaks

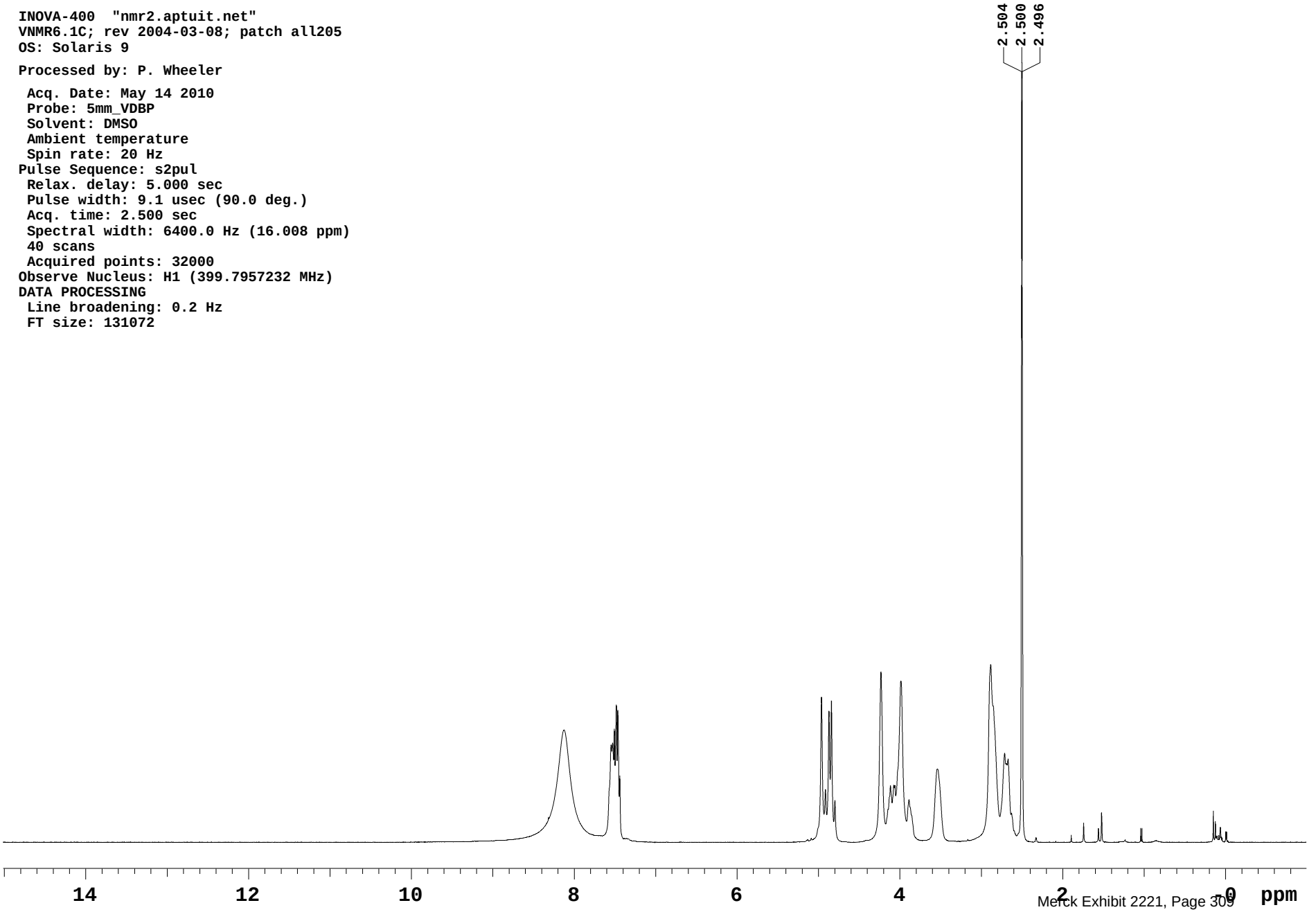
231202, Compound 184, Lot D-1895NN-13067/3, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

File: 399864

INOVA-400 "nmr2.aptuit.net"
VNMR6.1C; rev 2004-03-08; patch all205
OS: Solaris 9

Processed by: P. Wheeler

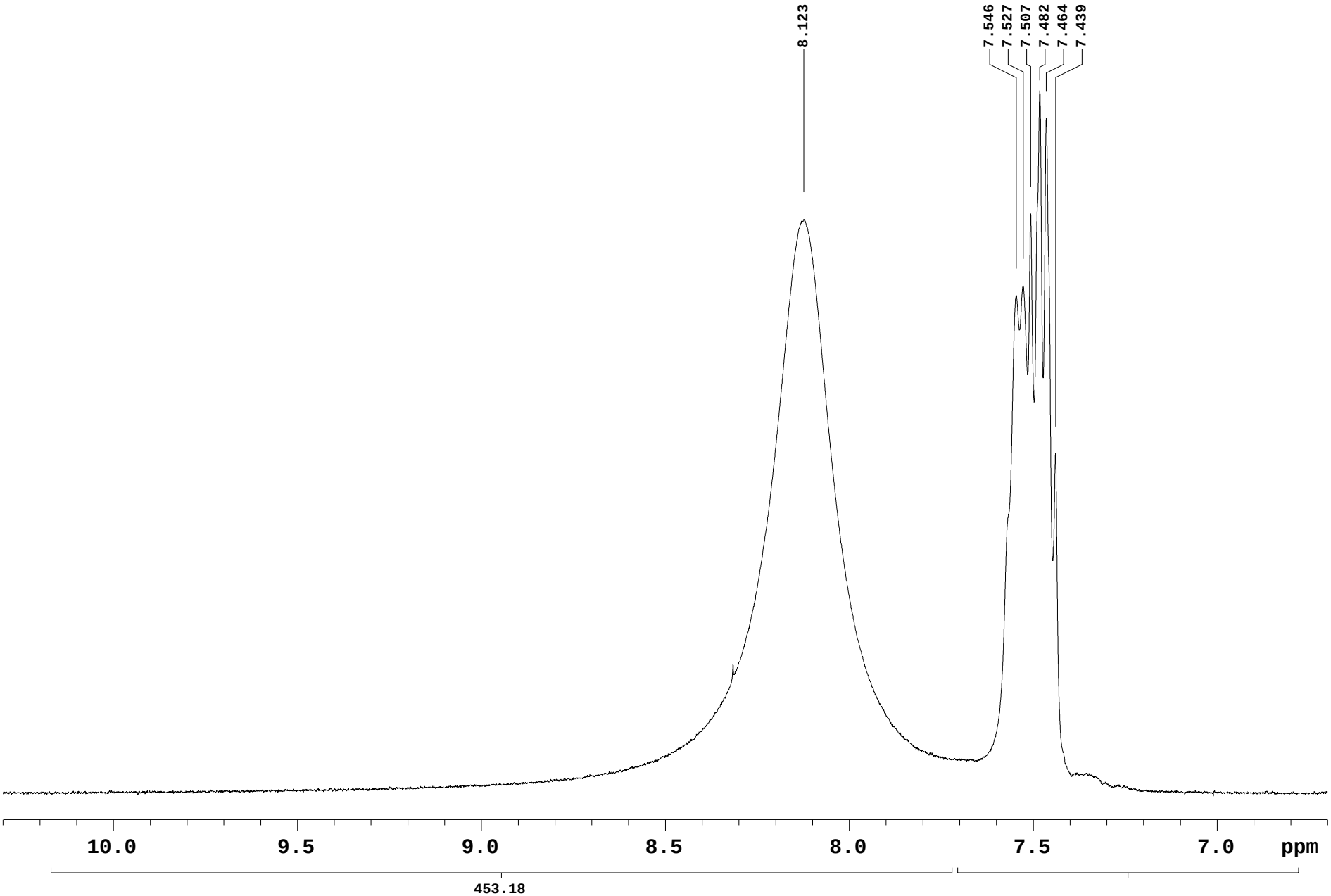
Acq. Date: May 14 2010
Probe: 5mm_VDBP
Solvent: DMSO
Ambient temperature
Spin rate: 20 Hz
Pulse Sequence: s2pul
Relax. delay: 5.000 sec
Pulse width: 9.1 usec (90.0 deg.)
Acq. time: 2.500 sec
Spectral width: 6400.0 Hz (16.008 ppm)
40 scans
Acquired points: 32000
Observe Nucleus: H1 (399.7957232 MHz)
DATA PROCESSING
Line broadening: 0.2 Hz
FT size: 131072



Plot file: 399864-1

231202, Compound 184, Lot D-1895NN-13067/3, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

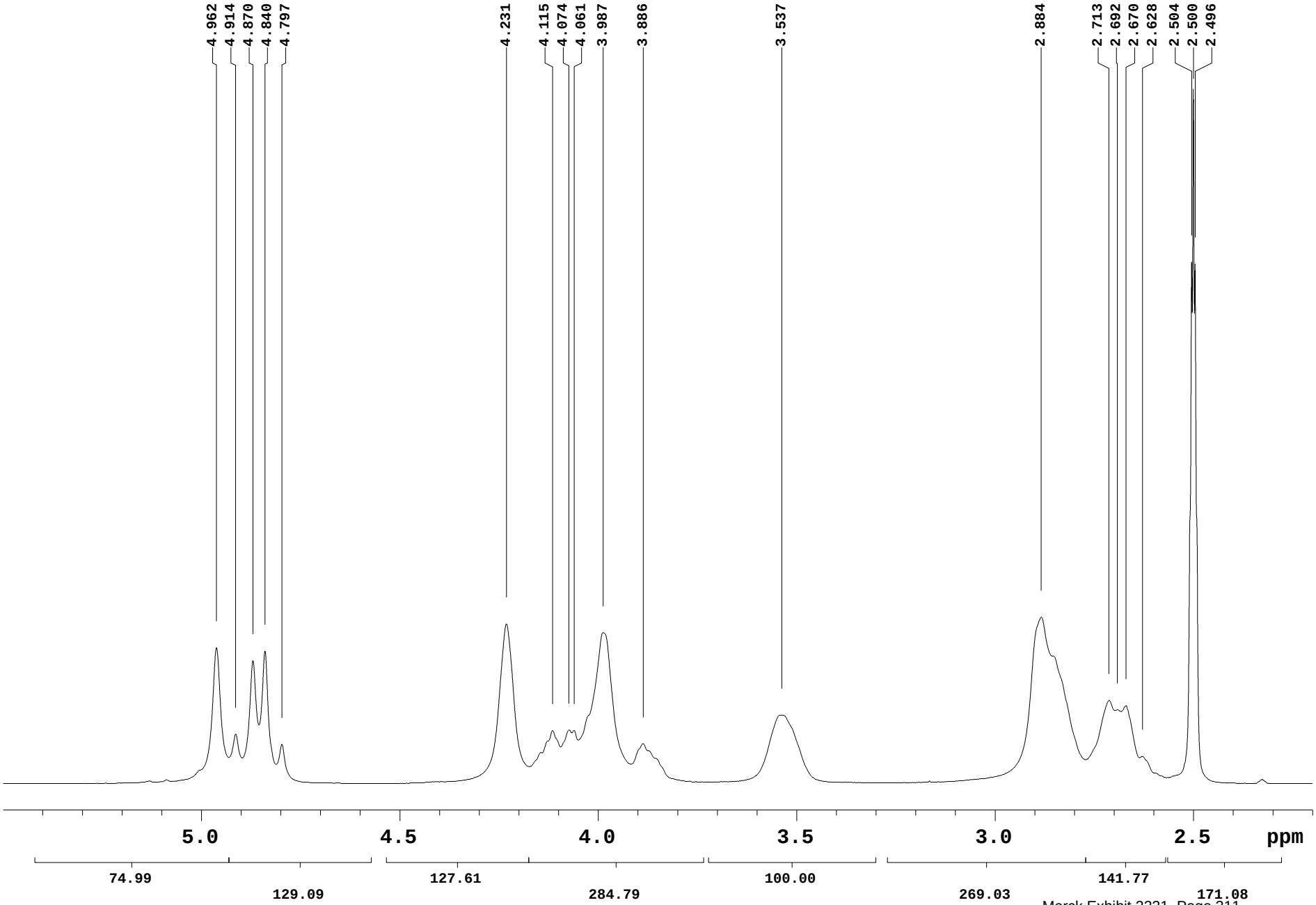
File: 399864



Plot file: 399864-2

231202, Compound 184, Lot D-1895NN-13067/3, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

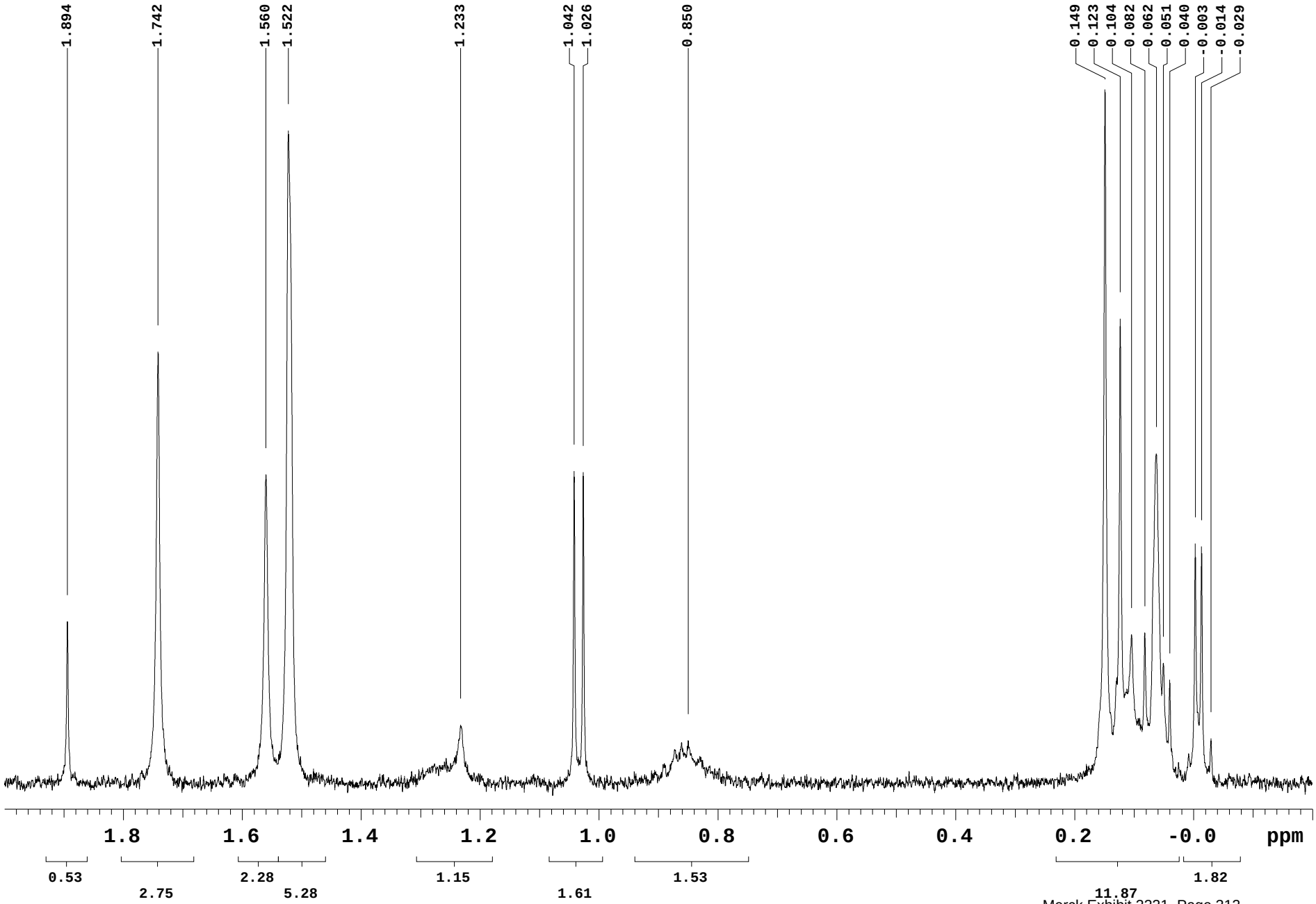
File: 399864



Plot file: 399864-3

231202, Compound 184, Lot D-1895NN-13067/3, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

File: 399864



Plot file: 399864-4

231202, Compound 184, Lot D-1895NN-13067/3, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

File: 399864

INDEX	FREQUENCY	PPM	HEIGHT
1	1001.240	2.504	106.4
2	999.482	2.500	141.8
3	997.724	2.496	106.1

Plot file: 399864-1_peaks

231202, Compound 184, Lot D-1895NN-13067/3, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

File: 399864

INDEX	FREQUENCY	PPM	HEIGHT
1	3247.627	8.123	107.9
2	3016.865	7.546	93.6
3	3009.346	7.527	95.4
4	3001.142	7.507	108.9
5	2991.279	7.482	132.0
6	2984.150	7.464	126.9
7	2974.092	7.439	63.9

Plot file: 399864-2_peaks

231202, Compound 184, Lot D-1895NN-13067/3, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

File: 399864

INDEX	FREQUENCY	PPM	HEIGHT
1	1983.857	4.962	25.8
2	1964.521	4.914	9.4
3	1947.041	4.870	23.4
4	1934.932	4.840	25.2
5	1917.842	4.797	7.5
6	1691.670	4.231	30.4
7	1645.088	4.115	10.1
8	1628.682	4.074	10.2
9	1623.408	4.061	10.1
10	1594.111	3.987	28.7
11	1553.682	3.886	7.6
12	1414.228	3.537	13.1
13	1152.998	2.884	31.6
14	1084.736	2.713	15.8
15	1076.142	2.692	14.1
16	1067.353	2.670	14.9
17	1050.752	2.628	5.2
18	1001.240	2.504	99.1
19	999.482	2.500	132.0
20	997.724	2.496	98.8

Plot file: 399864-3_peaks

231202, Compound 184, Lot D-1895NN-13067/3, in DMSO-d6 w/ TMS, 1H NMR, referenced to solvent at 2.5 ppm

File: 399864

INDEX	FREQUENCY	PPM	HEIGHT
1	757.197	1.894	30.7
2	696.260	1.742	82.1
3	623.701	1.560	58.7
4	608.564	1.522	124.2
5	492.842	1.233	11.1
6	416.474	1.042	59.4
7	410.322	1.026	59.1
8	339.814	0.850	8.1
9	59.541	0.149	132.0
10	49.287	0.123	88.4
11	41.670	0.104	28.3
12	32.783	0.082	28.6
13	24.971	0.062	62.7
14	20.283	0.051	22.8
15	15.986	0.040	19.7
16	-1.201	-0.003	45.5
17	-5.401	-0.014	45.0
18	-11.748	-0.029	8.5

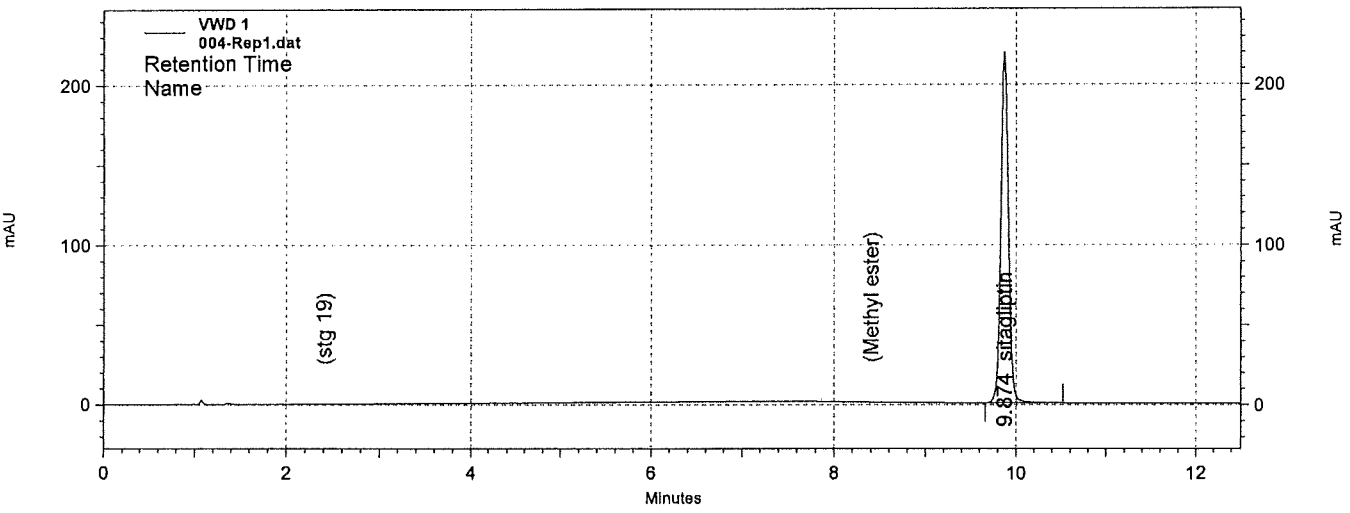
Plot file: 399864-4_peaks

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: **stg std1 21.13/20*5/20** {Data Description}
Acquired: 22/03/2010 13:23:58 (GMT +02:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201043 STG Assay hp36.met
Method rev.: 24/02/2010 15:22:27 (GMT +02:00)
Instrument: **HPLC_36** **Vial:** 12 **Inj. Vol.:** 10 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg assay_22.03.2010_2.seq001.ol.ssizip | stg assay_22.03.2010_2.rst

Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg assay_22.03.2010_2.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (23/03/2010 07:44:27 (GMT +02:00)) (Reprocessed)



VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width	Resolution (USP)
stg 19							
Methyl ester							
sitagliptin	1	9.874	1264257	100.000	218921	0.86	0.00
stg OH							
stg 57							
stg 58							
Eliminate 1							
Eliminate 2							
Des Amino							
Dimer							
Totals			1264257	100.000	218921		

STD 1895 ST01-T-393412109

22.Mar 2010 13:06
User 123
Type AX205
SNR 1126311588
Balance No.14
SAMPLE STG
ID STD1

0.00 mg
21.13 mg

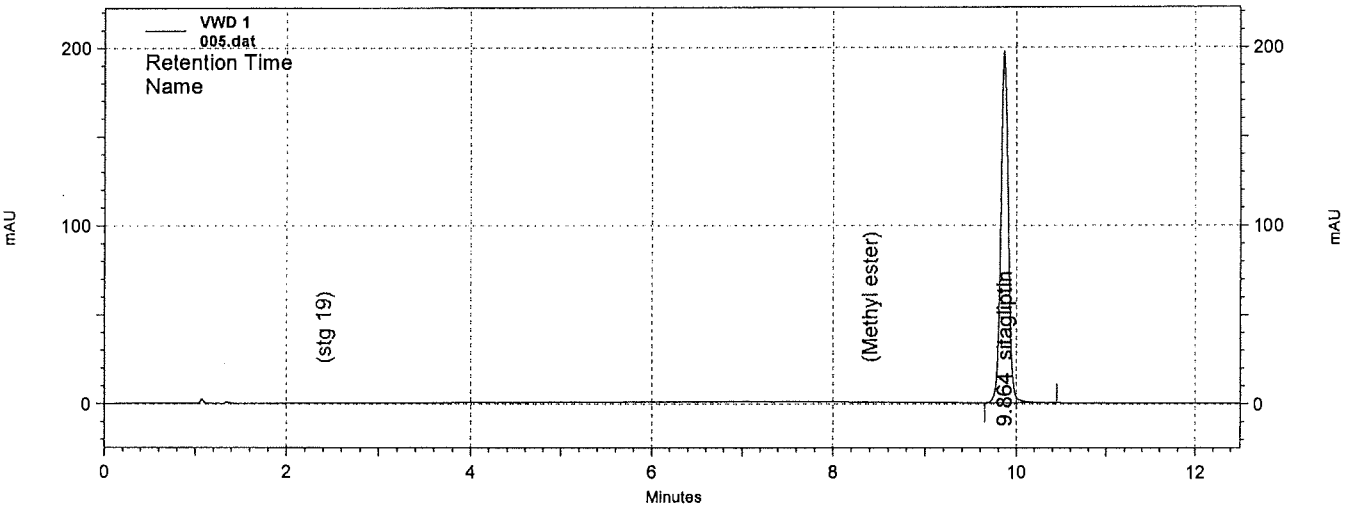
Printed: 23/03/2010 07:44:28 (GMT +02:00) by Dahlia Weinberg (TEVAIL\DWEINBERG)

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: **stg std2 19.28/20*5/20** {Data Description}
Acquired: 22/03/2010 14:04:06 (GMT +02:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201043 STG Assay hp36.met
Method rev.: 24/02/2010 15:22:27 (GMT +02:00)
Instrument: **HPLC_36** **Vial:** 13 **Inj. Vol.:** 10 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg assay_22.03.2010_2.seq001.ol.ssizip | stg assay_22.03.2010_2.rst

Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_36stg assay_22.03.2010_2.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (23/03/2010 07:44:34 (GMT +02:00)) (Reprocessed)



VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width	Resolution (USP)
stg 19							
Methyl ester							
sitagliptin	1	9.864	1143514	**100.000**	197127	0.80	**0.00**
stg OH							
stg 57							
stg 58							
Eliminate 1							
Eliminate 2							
Des Amino							
Dimer							
Totals			1143514	**100.000**	197127		

STD 1895 STO1-T-393H2109

22.Mar 2010 13:08
User 123
Type AX205
SNR 1126311588
Balance No.14
SAMPLE STG
ID STD2

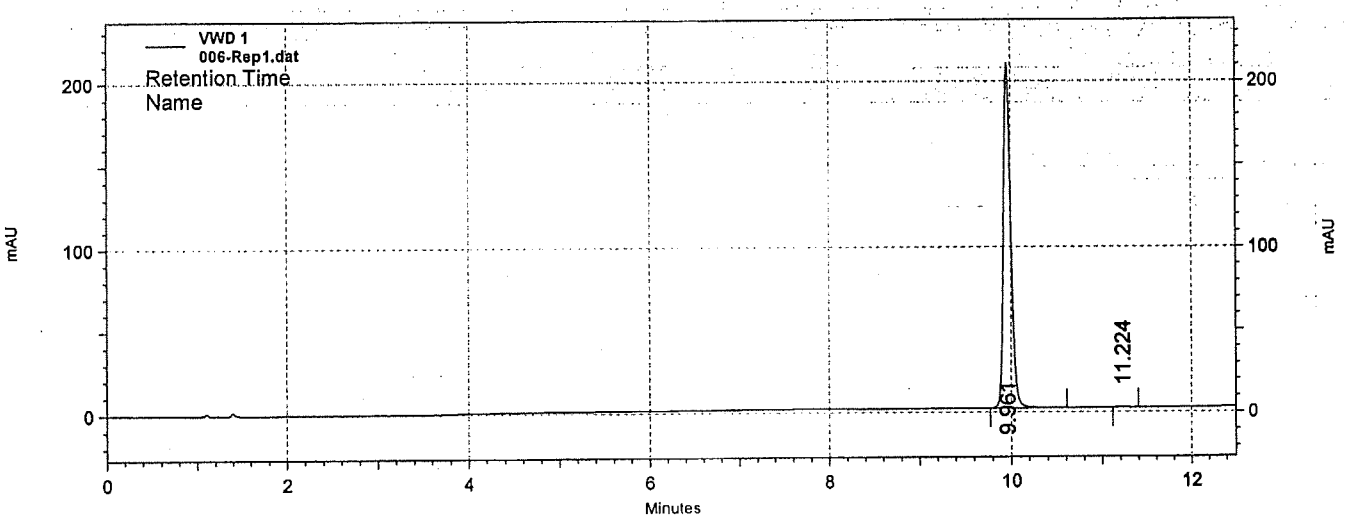
0.00 mg
19.28 mg

Printed: 23/03/2010 07:44:36 (GMT +02:00) by Dahlia Weinberg (TEVAIL\DWEINBERG)

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID 1

Sample Name: stg std1 19.53/20*5/20 {Data Description}
Acquired: 09/05/2010 14:24:15 (GMT +03:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\SI-201043 STG Assay hp25.met
Method rev.: 04/05/2010 14:20:23 (GMT +03:00)
Instrument: HPLC_25 **Vial:** 4 **Inj. Vol.:** 10 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg assay_09.05.2010_1.seq001.ol.ssizip | stg assay_09.05.2010_1.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg assay_09.05.2010_1.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (16/05/2010 14:23:57 (GMT +03:00)) (Reprocessed)



VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width	Resolution (USP)
	1	9.961	1153901	[illegible]	208175	0.84	0.00
	2	11.224	3115	0.269	487	0.28	8.06
Totals			1157016	100.000	208662		

STD 1895 ST 01-T-393421 09

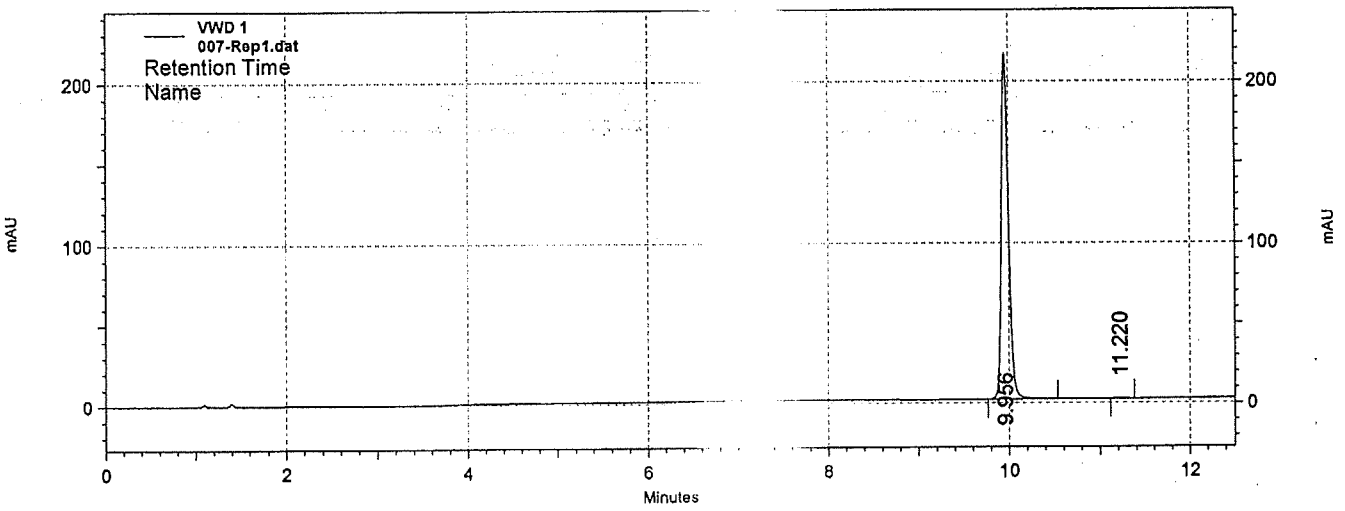
9.May 2010 14:00
User ANNA
Type AX205
SNR 1121102961
Balance 8
SAMPLE STG P
ID, STD1

0.00 mg
19.53 mg

Standard Report

Agilent OpenLAB ICM Build 3.3.2.14 Version 3.3.2 OEM ID

Sample Name: stg std2 20.46/20*5/20 {Sample Description}
Acquired: 09/05/2010 15:24:37 (GMT +03:00)
Method: Analytical R&D\STG\User 6 (DahliaW)\Method\ 13 STG Assay.hp25.met
Method rev.: 04/05/2010 14:20:23 (GMT +03:00)
Instrument: HPLC_25 **Vial:** 5 **Inj. Vol.:** 10 µL
Sequence: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg assay_09 1.seq001.ol.ssizip | stg assay_09.05.2010_1.rst
Data File: Analytical R&D\STG\User 6 (DahliaW)\Data\hplc_25stg 5.2010_1.seq001.ol.ssizip
Results Source: TEVAIL\DWEINBERG (16/05/2010 14:24:09 (GM) (Reprocessed)



VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width	Resolution (USP)
	1	9.956	1189375		214821	0.77	**0.00**
	2	11.220	2916		468	0.26	**8.16**
Totals			1192291		215289		

STD 1895 ST01-T-393422109

9.May 2010 14:05
User ANNA
Type AX205
SNR 1121102961
Balance 8
SAMPLE STG P
ID STD2

0.00 mg
20.46 mg

Printed: 16/05/2010 14:24:11 (GMT +03:00) by Dahlia Weinberg (TEVAIL\DWEINBERG)

TITLE PROJECT NO. BOOK NO. H31111001 **001**

26.01.10 Sitagliptin

STG - phosphate : Assay

HPLC 34

column : Waters XBridge Phenyl, 3.5µm 150 * 4.6 ; Col temp = 43°C ± 1°C

Buffer : 20mM $NH_4H_2PO_4$ pH = 7.0 w/ NH_4OH (concentration ~ 25%)

Eluent A : 78% Buffer : 16% MeOH : 06% Acetonitrile

Eluent B : 30% Buffer : 16% MeOH : 54% Acetonitrile

Gradient :

Time	A	B
0	100	0
25	0	100
25.1	100	0

Runtime = 25 min

equilibration time = 7 min

Sample volume = 10 µl (for assay

5 µl (for impuri

Flow = 1.5mL/min

Detector = 215nm

Diluent = Buffer (adjusted to pH 9) : Acetonitrile (50:50)

Autosampler : 10°C

* STD run time is 12.5 minutes. At this time the gradient is 50% A : 50% B ~~28.10.03.10~~

Sample prep: for Assay: STDs - 20mg/20mL * 5ml/20 ml (diluted w/diluent)

samples - 50mg/10mL * 1/20mL (diluted w/diluent)

for Impurity : samples - 50mg/10mL

System Suitability test : (see NOTEBOOK H337090/151) 04.11.09

Resolution - 12.22

Sample : MH1856

H31111001/1

26.01.2010

SIGNATURE [signature] DATE 27.01.10

10.3.10 [signature]

DISCLOSED TO AND UNDERSTOOD BY [signature] DATE 14.03.10 WITNESS DATE

STG

Impurity validation

PROJECT NO.

001

TITLE

BOOK NO. 43371001

30.12.09 Impurity Profile

SI-201041/03 of Sitagliptin Phosphate by HPLC

instrument PDA 40

Column Packing: Waters X Bridge Phenyl, 3.5 µm, 150 x 4.6 mm,

p.n. 176003335

Buffer: 20mM Ammonium Dihydrogen Phosphate ($NH_4H_2PO_4$)

adjusted to pH = 7.0 ± 0.05 with NH_4OH conc. (a 25%)

Eluent A: 78 : 16 : 6, Buffer : Methanol : Acetonitrile

Eluent B: 30 : 16 : 54, Buffer : Methanol : Acetonitrile

Gradient:

Time	Eluent A	Eluent B
0	100	0
25	0	100
25.1	100	0

Equilibrium time: 7 min

Sample volume: 5 µL

Flow rate: 1.5 mL/min

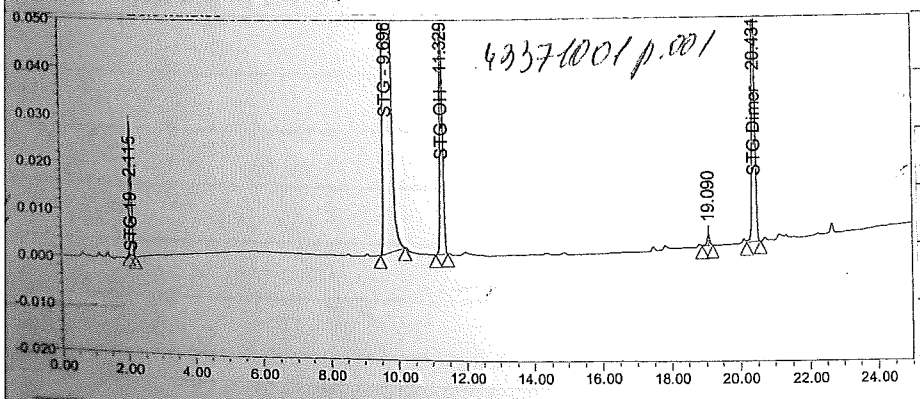
Detector: 215 nm

Column temperature: 43 °C ± 1 °C

Autosampler temperature: 19 °C

Diluent: Buffer adjusted to pH 9.0 : ACN 50 : 50

SST



STG-Dimer 1995TO5-GP-5257

10.69 mg/10mL 1mL/10mL

STG

1295TO1-T-39342109

51.61 mg/10mL

STG-19

1895TO6-239-03

11.74 mg/10mL 1mL/10mL

STG-OH

1895T10-MK-625

10.32 mg/10mL 1mL/10mL

SIGNATURE		DATE 30.12.09
DISCLOSED TO AND UNDERSTOOD BY	DATE 03.01.2010	WITNESS / DATE

STG

TITLE Assay

PROJECT NO.

BOOK NO. 43371001

063

14.03.2010

SI-201043 Assay Determination of Sitagliptin Phosphate

Instrument PDA46

Column&Packing: Waters XBridge Phenyl 3.5 µm, 150x4.6 186003335

Buffer: 20mM Ammonium Phosphate to pH 7.0±0.05 with NH4OH conc.

Eluent A: 78:16:6 Buffer: Methanol: Acetonitrile

Eluent B: 30:16:54 Buffer: Methanol: Acetonitrile

Gradient:	Time	Eluent A	Eluent B
	0	100	0
	12.5	50	50
	12.6	100	0

Equilibration time: 6 min

Sample volume: 10 µl

Flow rate: 1.5 ml/min

Detector: 215 nm

Column temperature: 43°C

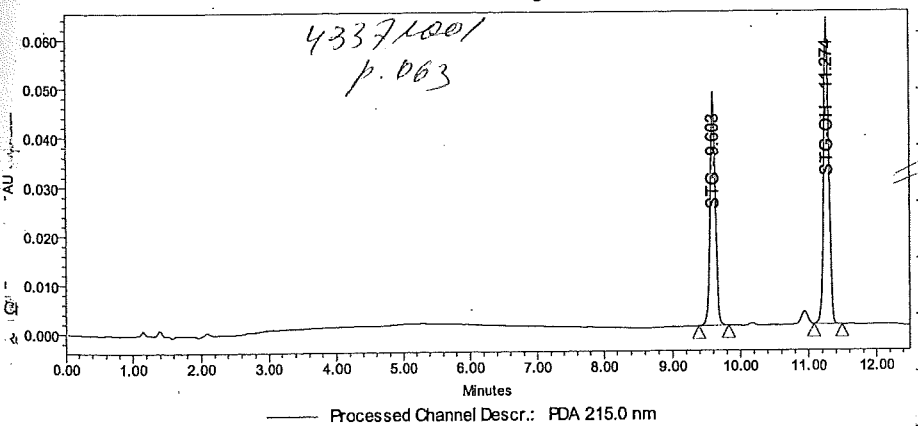
Autosampler: 10°C

Diluent: Buffer adjusted to pH 8 : ACN - 50:50

SST

Auto-Scaled Chromatogram

43371001
p. 063



SST solution preparation see on p. 151 = notebook number 43370901

Resolution between STG and STG-OH is 11.4 > 8.0

Peak Results

	Name	RT	Area	% Area	USP Resolution	Height (µV)	Width @ 50%	Purity1 Angle	Purity1 Threshold	USP Plate Count	USP Tailing
1	STG	9.603	253044	43.167		45910	0.08	0.273	0.504	71288	0.96
2	STG-OH	11.274	333148	56.833	11.40	60806	0.08	0.222	0.436	98203	0.93

SIGNATURE [signature] DATE 14.03.2010

DISCLOSED TO AND UNDERSTOOD BY [signature] DATE 18.03.2010 WITNESS DATE

STG
impurity profile validation

094 TITLE PROJECT NO.

BOOK NO. 43371001

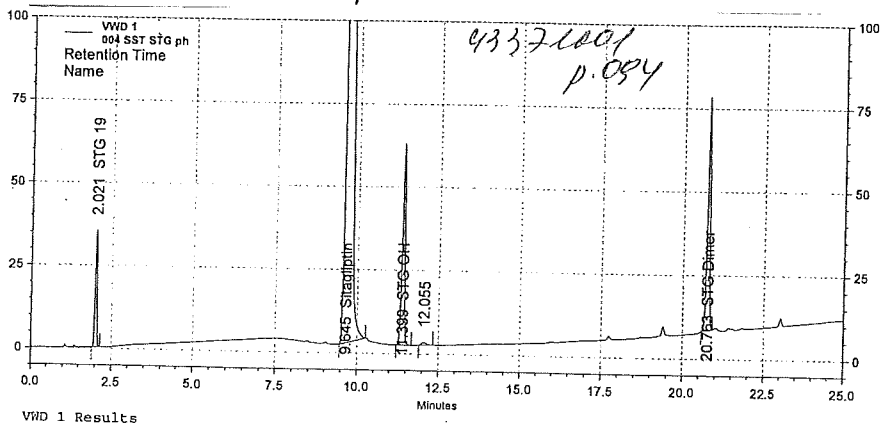
25.04.2010 Sample solution Stability time 0

SI-20104 1/03 Impurity profile of Sitagliptin phosphate by HPLC

Instrument ~~PDA40~~ HP36 Analyst 25.04.10

Column, operating conditions see on p. 001.

SST



43371001 p. 084

VWD 1 Results

Name	Pk #	Retention Time	Area	Area Percent	Height	Width at 50% height	Resolution (USP)
STG 19	1	2.021	133747	1.272	35178	0.06	0.00
Sitagliptin	2	9.645	9675325	92.051	1191231	0.13	47.12
STG OH	3	11.399	322672	3.070	60540	0.08	9.69
	4	12.055	7664	0.073	754	0.16	3.20
STG Dimer	5	20.763	371430	3.534	69860	0.08	42.48
Totals			10510838	100.000	1357563		

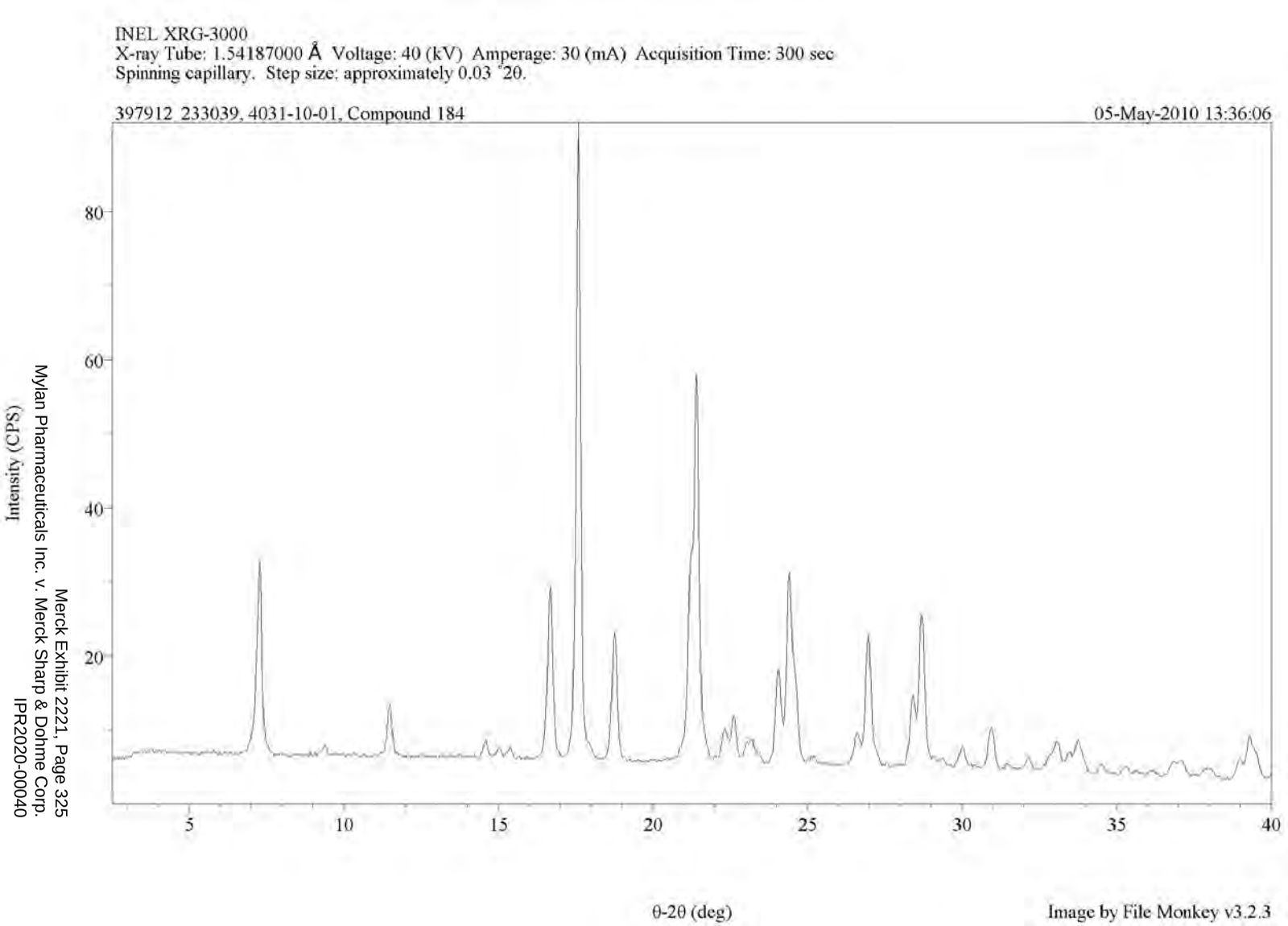
STG-19
1895STO6-239-03
Potency 89.7 % /to base
Retest 02.2011 Analyst
10.95 mg/10 ml 1 ml/10 ml
STG 1895 STO1-T-39342107
Potency 99.8 %. Retest 10.2010
STG OH, 1895T10-MK-625
Potency 98.1 % Retest 03.2011
9.99 mg/10 ml 1 ml/10 ml
STG 46.66 mg/10 ml
STG-Dimer

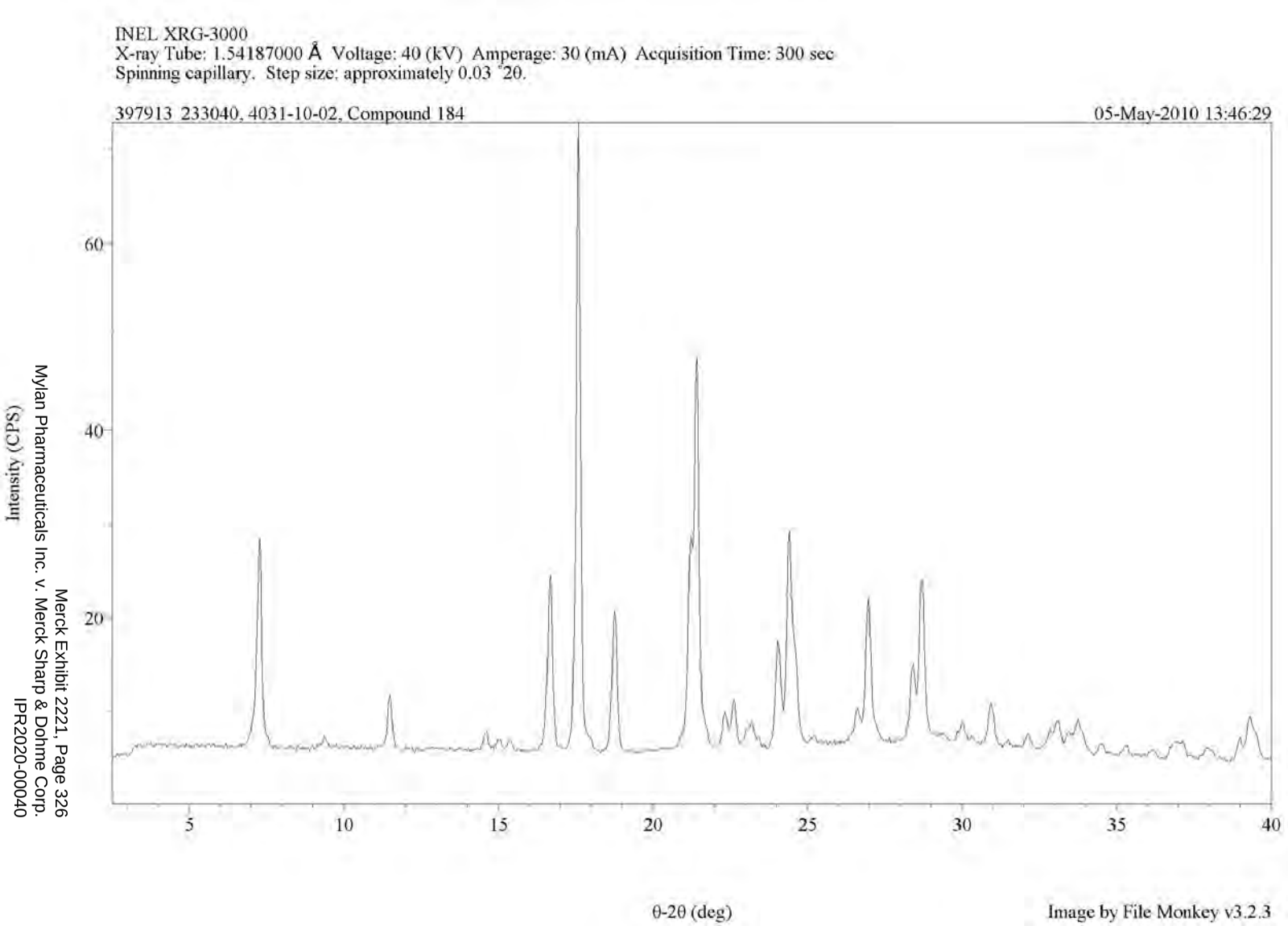
1895T10-MK-625 Potency 89.7 %. Retest 02.2011
Resolution between STG-OH and STG is 9.69 > 8.0 STG Theor plates 1029886
QL STG 19.41 mg/20 ml 5 ml/20 ml 1 ml/50 ml 3 ml/10 ml S/N = 11.7
Std 1 STG 19.41 mg/20 ml
5 ml/20 ml 1 ml/50 ml
Std 2 STG 20.57 mg/20 ml
5 ml/20 ml 1 ml/50 ml

43371001 p. 084

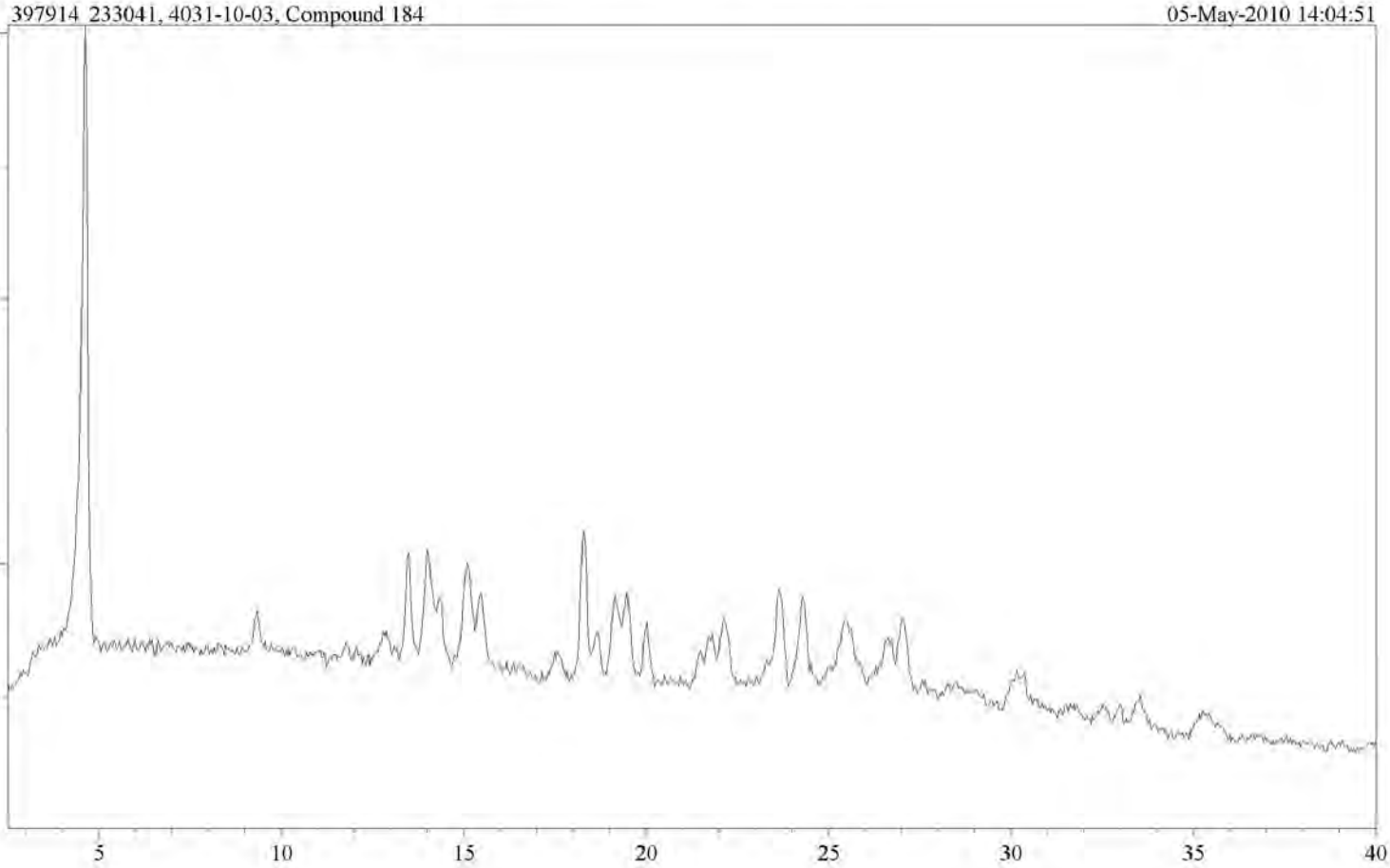
SIGNATURE Analyst DATE 26.04.10 25.04.10

DISCLOSED TO AND UNDERSTOOD BY Reg DATE 26.04.10 WITNESS DATE



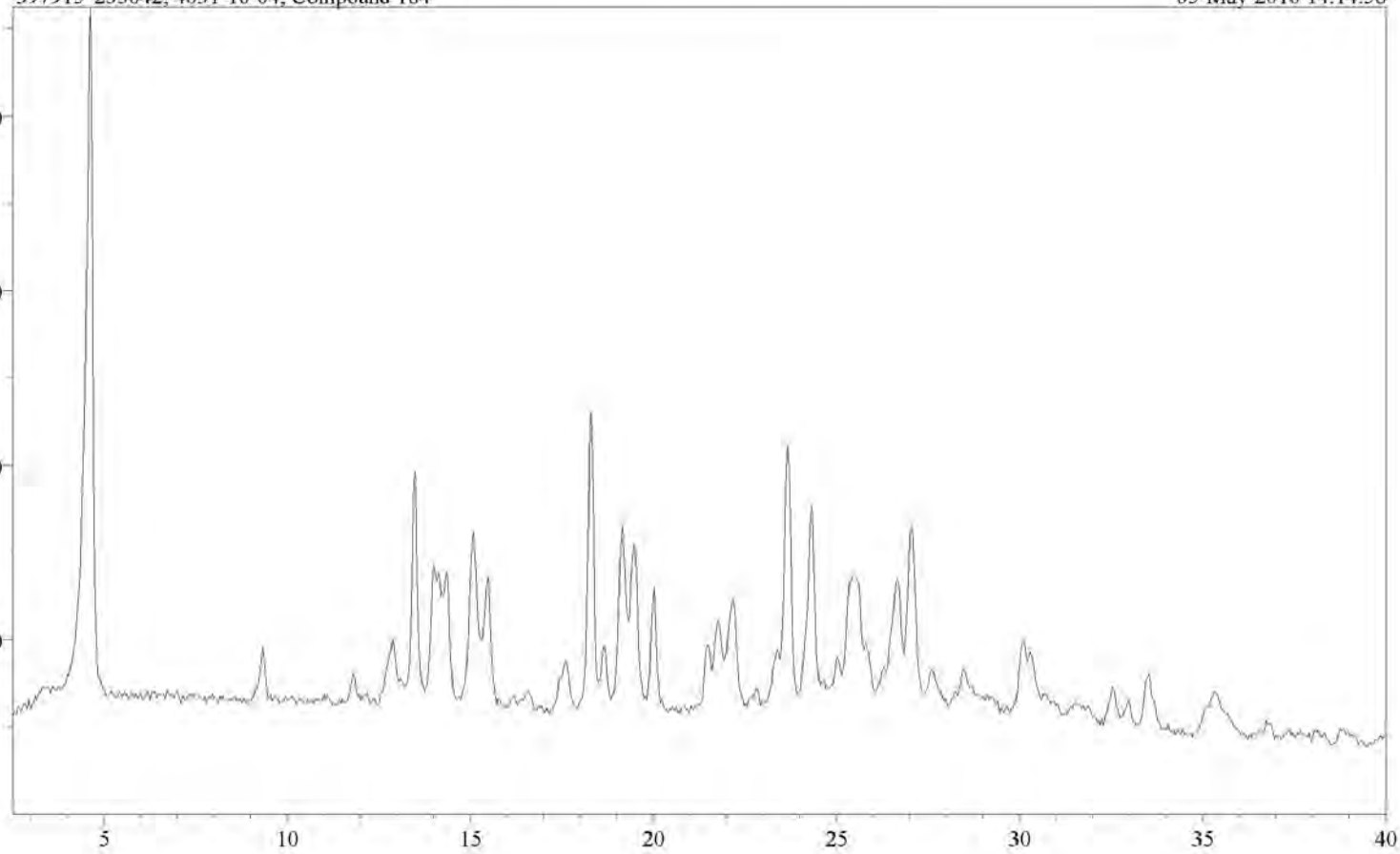


INEL XRG-3000
X-ray Tube: 1.54187000 Å Voltage: 40 (kV) Amperage: 30 (mA) Acquisition Time: 300 sec
Spinning capillary. Step size: approximately 0.03 °2θ.



INEL XRG-3000
X-ray Tube: 1.54187000 Å Voltage: 40 (kV) Amperage: 30 (mA) Acquisition Time: 300 sec
Spinning capillary. Step size: approximately 0.03 °2θ.

397915 233042, 4031-10-04, Compound 184 05-May-2010 14:14:58



INEL XRG-3000
X-ray Tube: 1.54187000 Å Voltage: 40 (kV) Amperage: 30 (mA) Acquisition Time: 300 sec
Spinning capillary. Step size: approximately 0.03 °2θ.

397961 233074, 4031-09-01, Compound 184 05-May-2010 12:45:29

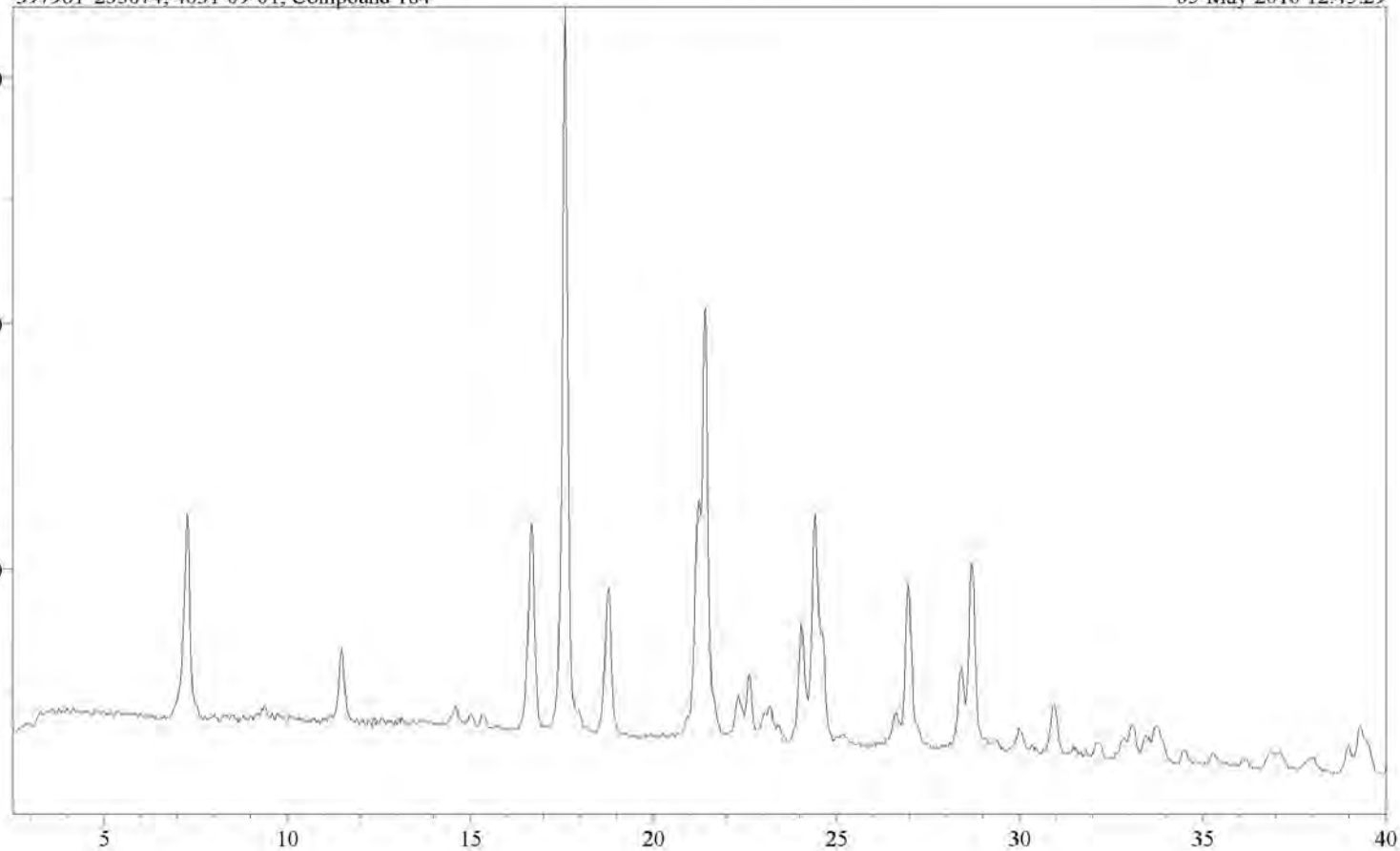


Image by File Monkey v3.2.3

INEL XRG-3000
X-ray Tube: 1.54187000 Å Voltage: 40 (kV) Amperage: 30 (mA) Acquisition Time: 300 sec
Spinning capillary. Step size: approximately 0.03 °2θ.

397962 233075, 4031-09-02, Compound 184 05-May-2010 12:58:13

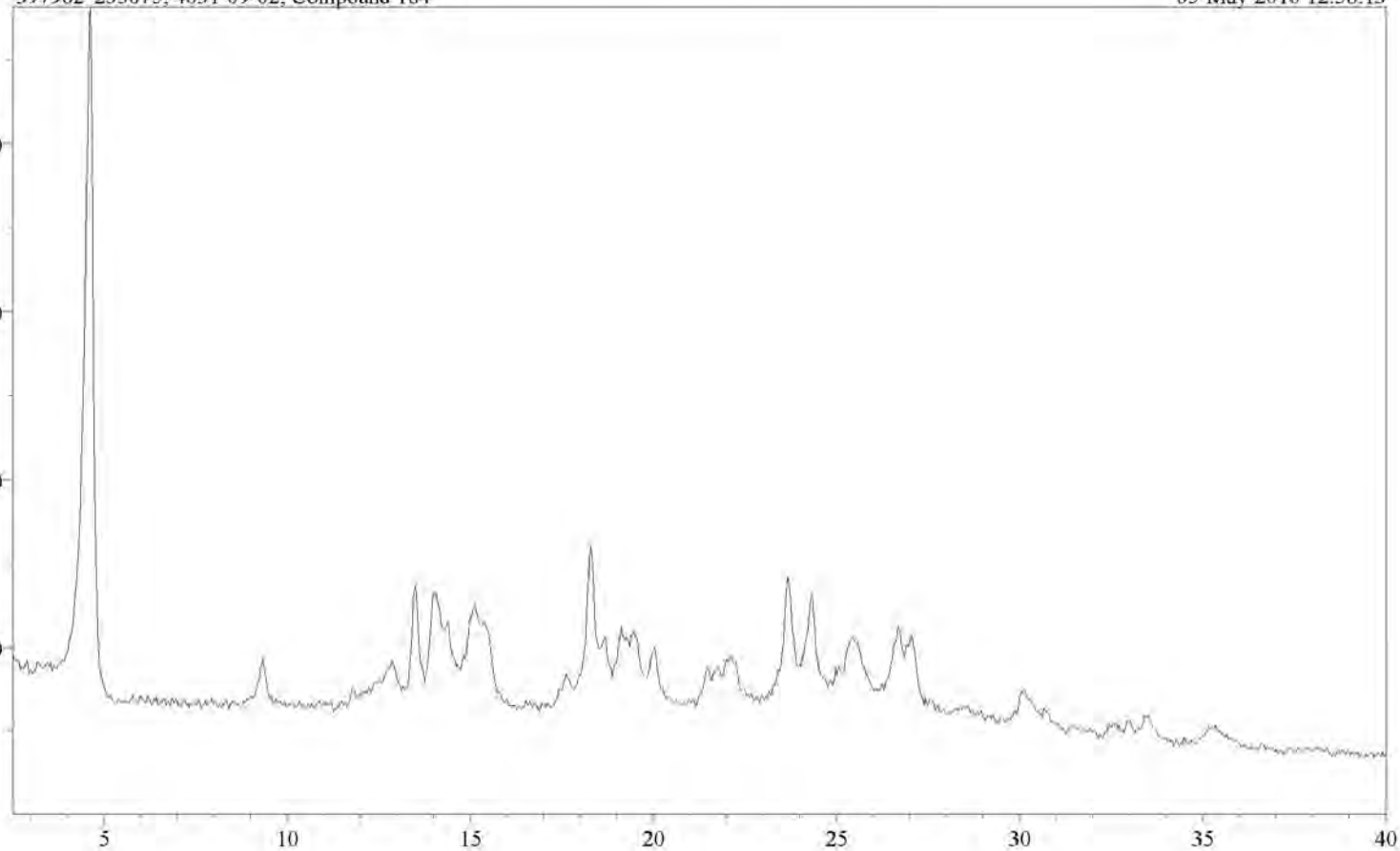
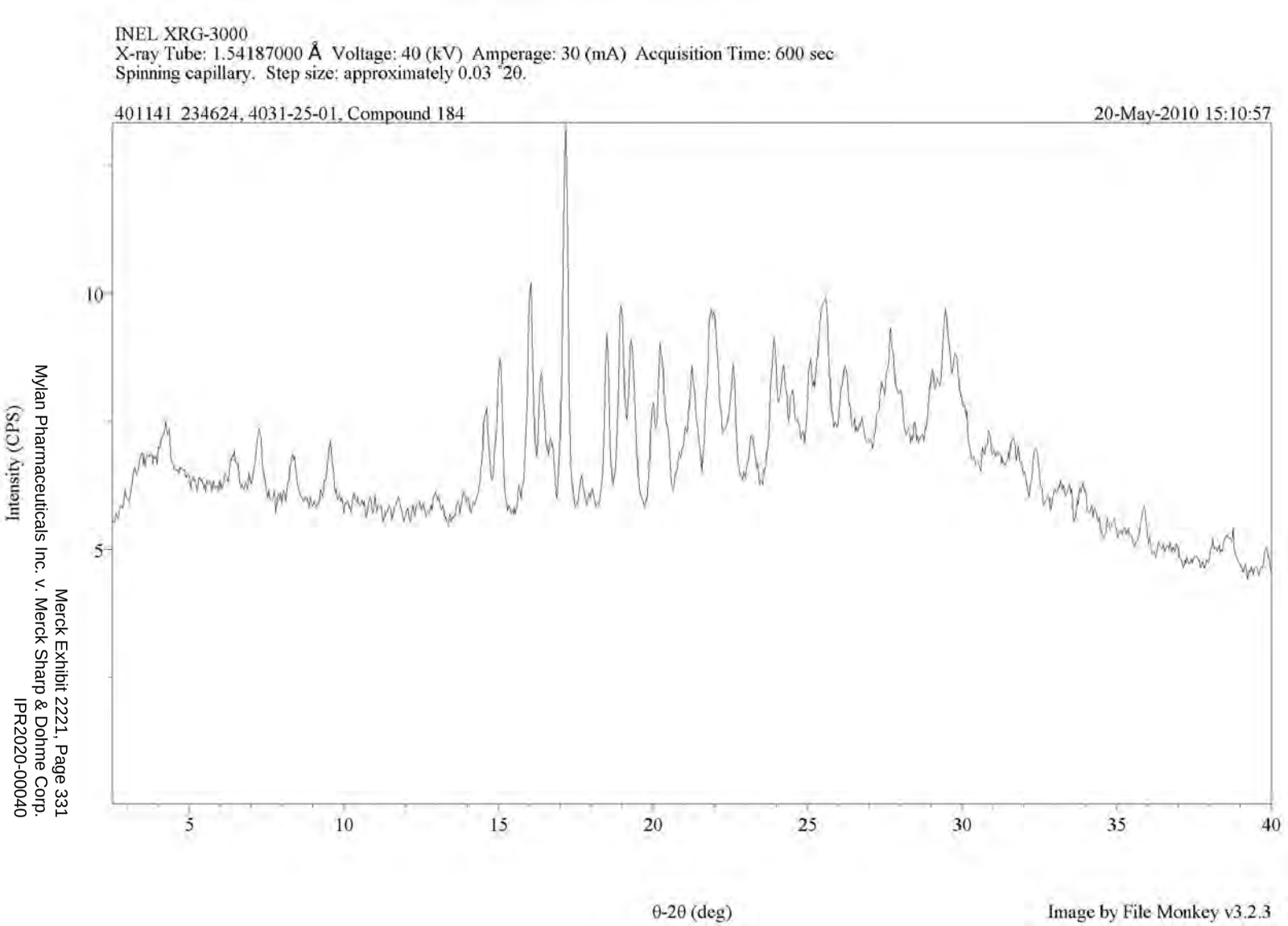
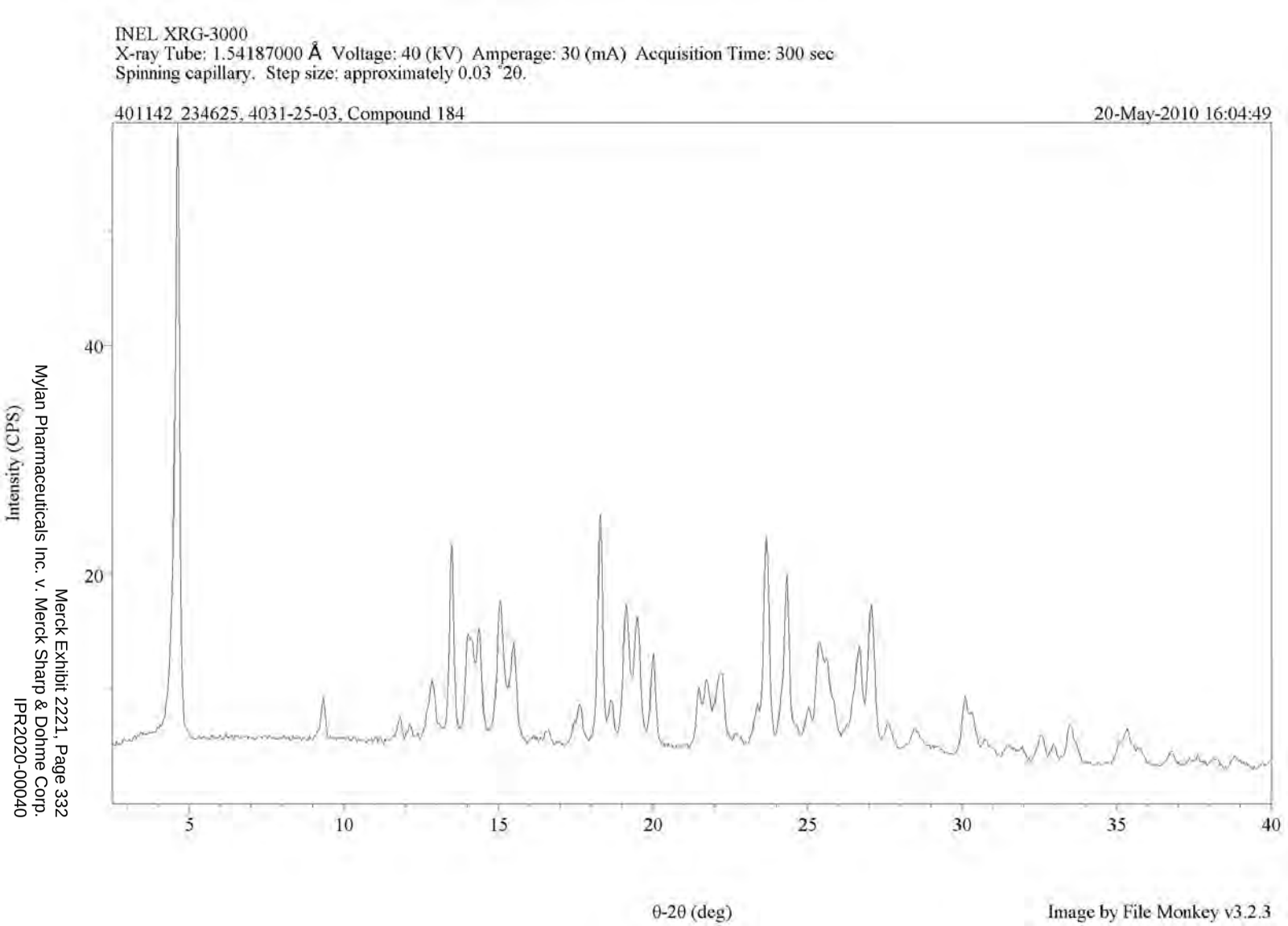


Image by File Monkey v3.2.3





INEL XRG-3000
X-ray Tube: 1.54187000 Å Voltage: 40 (kV) Amperage: 30 (mA) Acquisition Time: 300 sec
Spinning capillary. Step size: approximately 0.03 °2θ.

401143 234626, 4031-25-04, Compound 184 20-May-2010 16:19:56

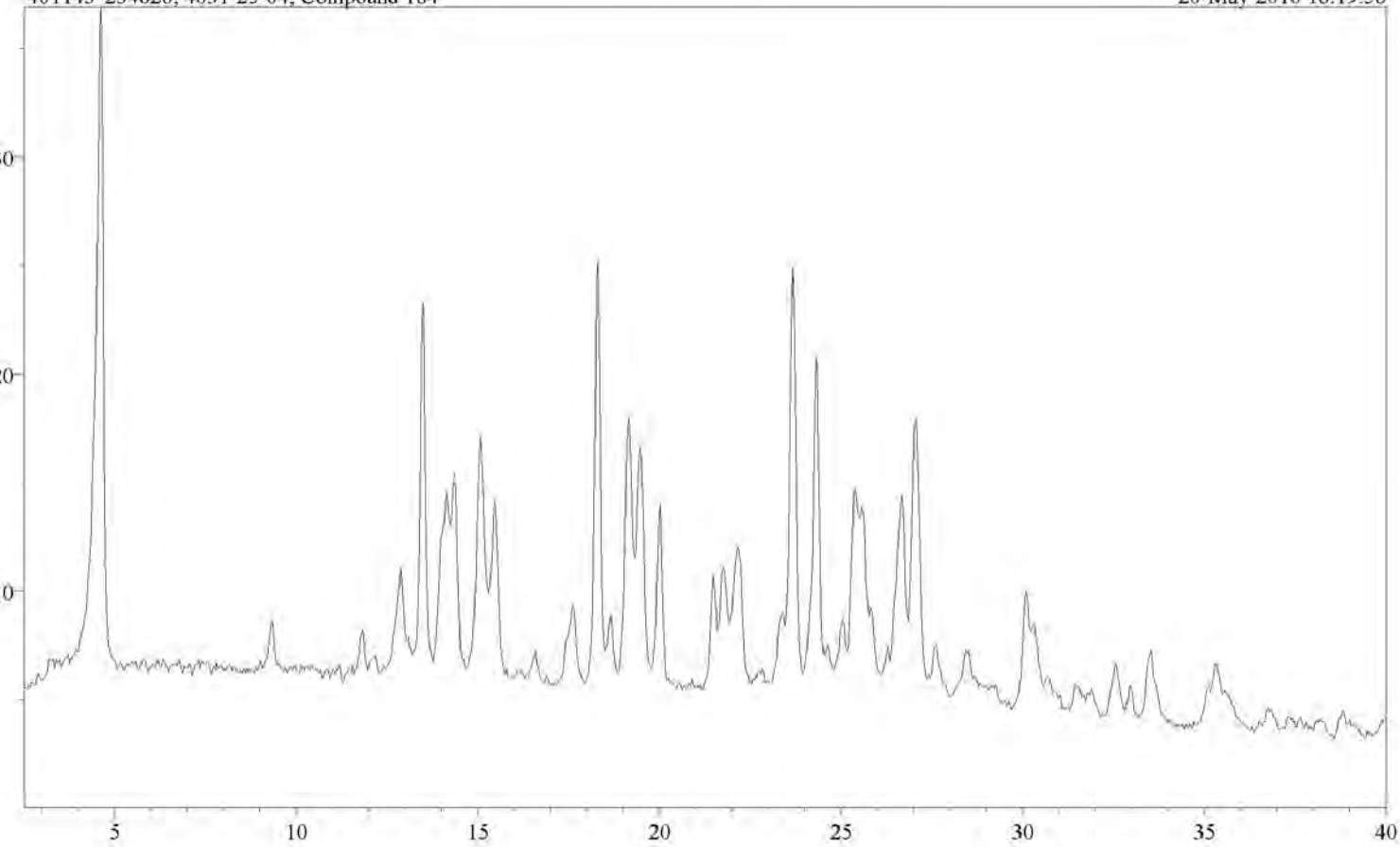
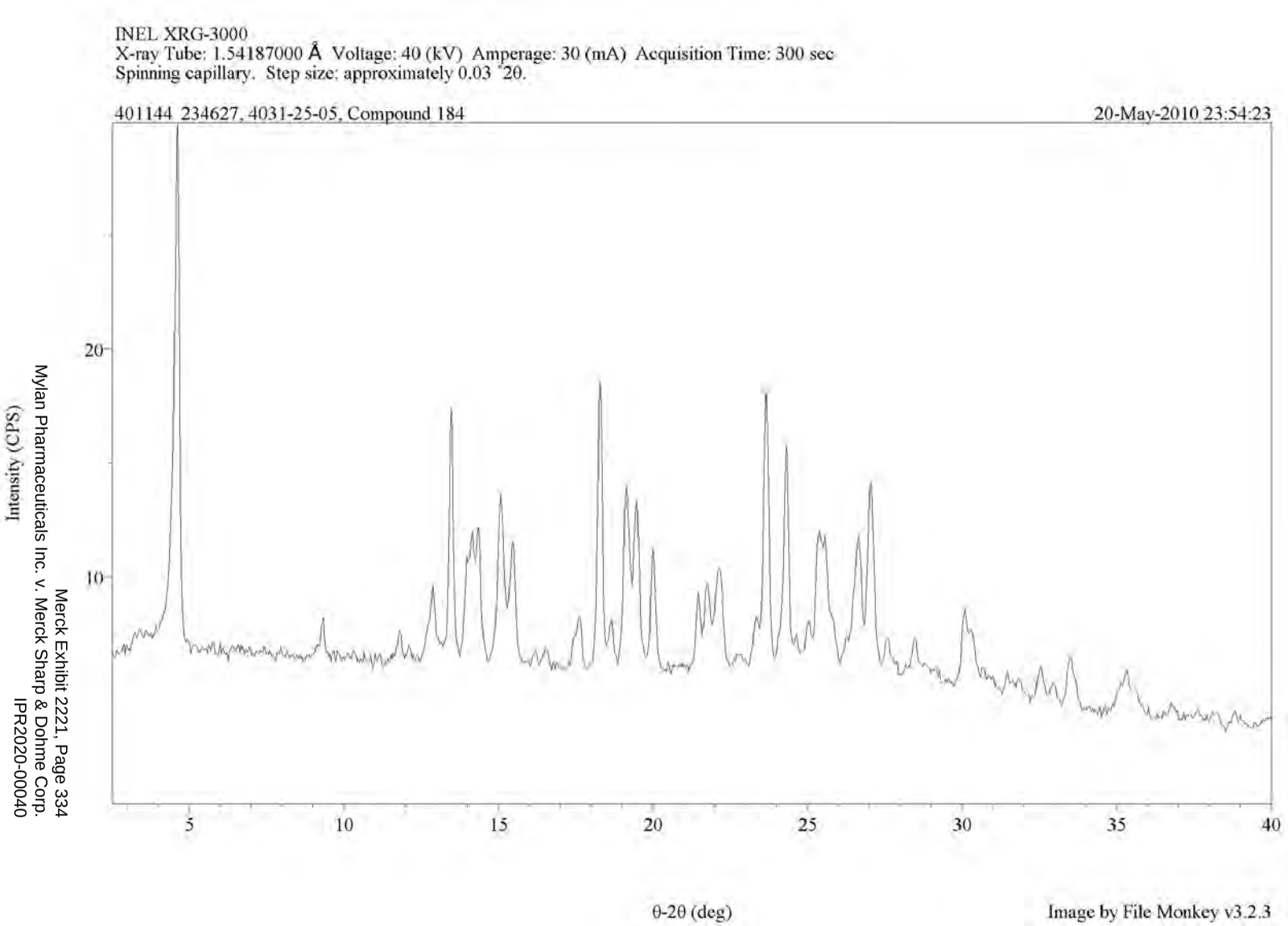
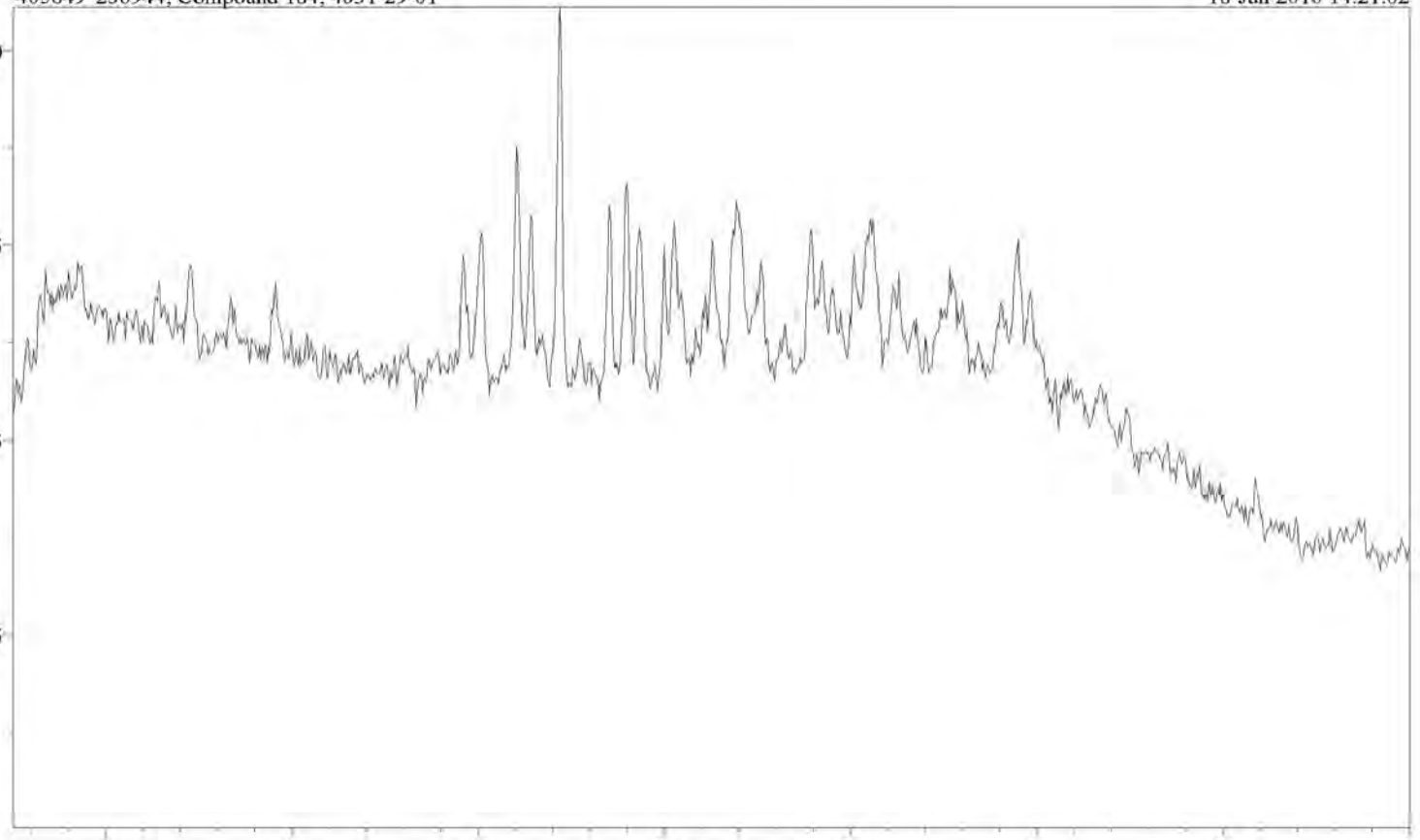


Image by File Monkey v3.2.3



INEL XRG-3000
X-ray Tube: 1.54187000 Å Voltage: 40 (kV) Amperage: 30 (mA) Acquisition Time: 300 sec
Spinning capillary. Step size: approximately 0.03 °2θ.

405649 236944, Compound 184, 4031-29-01 18-Jun-2010 14:21:02



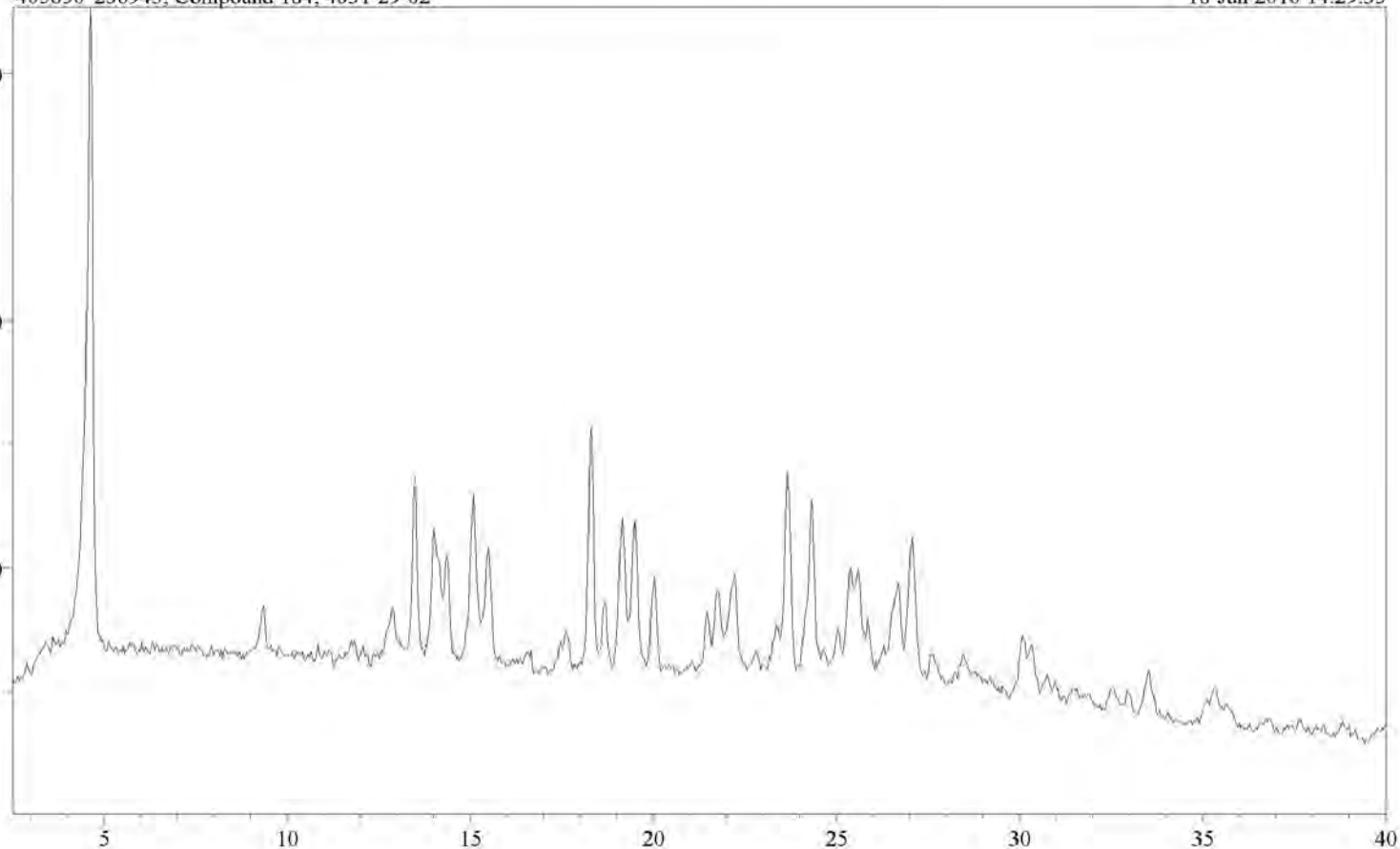
Intensity (CPS)

θ-2θ (deg)

Image by File Monkey v3.2.3

INEL XRG-3000
X-ray Tube: 1.54187000 Å Voltage: 40 (kV) Amperage: 30 (mA) Acquisition Time: 300 sec
Spinning capillary. Step size: approximately 0.03 °2θ.

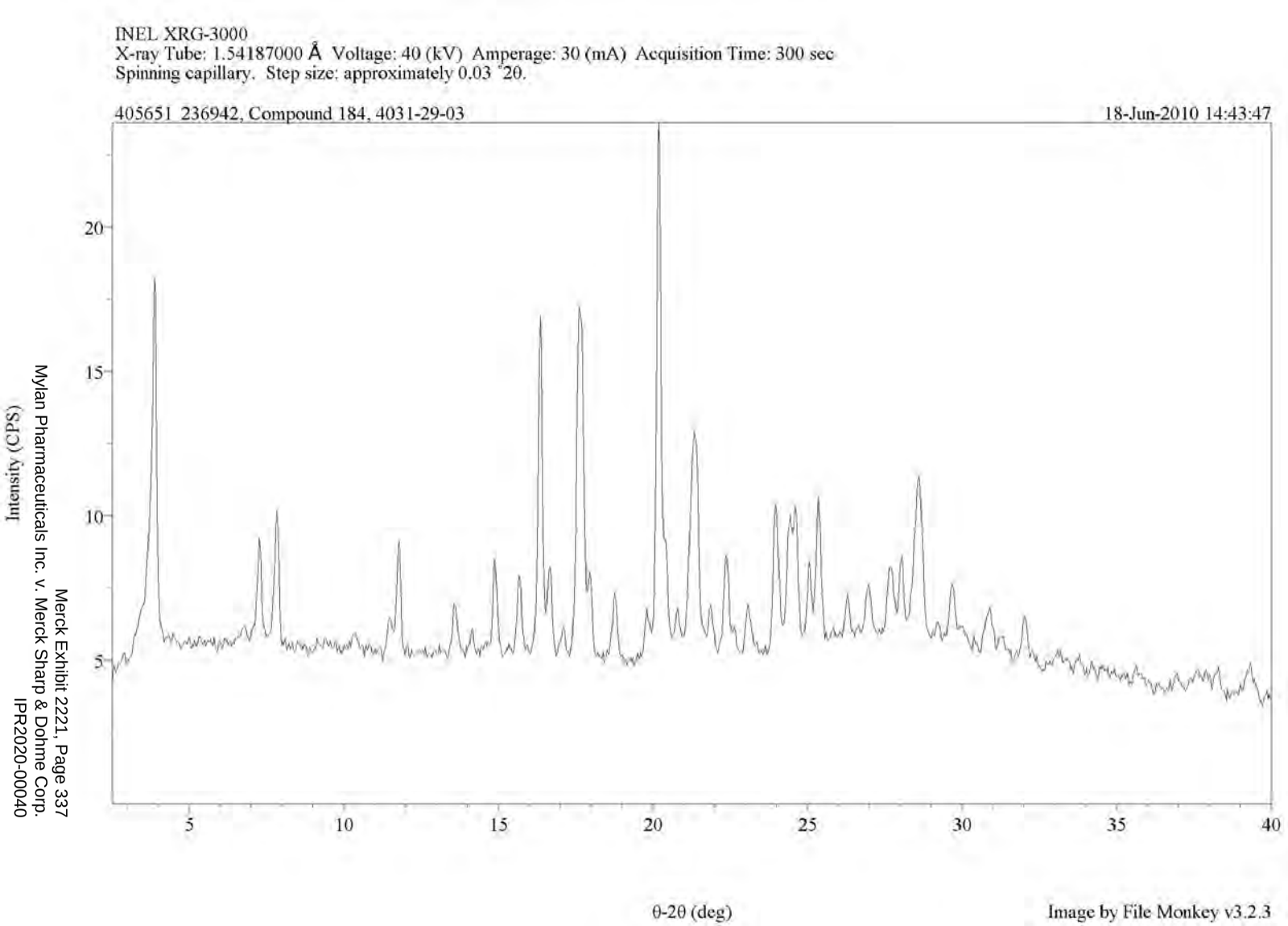
405650 236943, Compound 184, 4031-29-02 18-Jun-2010 14:29:35



Intensity (CPS)

θ-2θ (deg)

Image by File Monkey v3.2.3



Date: 01/22/2011
Time: 3:13:51PM

Project Sample Report

Project ID: EL20100011

Sample Information

Lims No	Notebook #	Compound	Lot #	Storage	Retain Location	Hazard Code	Priority	Status	Sample Comments
229438		Compound 184	sal-069	Ambient	Legal sample roo		NC_Urgent	Done	03/29/2010 14:24:11 AMARCOV: Entry No: Sitagliptin Base Received from: Teva Pharmaceutical Industries 16 Bazel Street Petach Tikva IL 49131

Lims No	Notebook #	Compound	Lot #	Storage	Retain Location	Hazard Code	Priority	Status	Sample Comments
231202		Compound 184	D-1895NN-13067/3	Ambient	Legal sample roo		NC_Urgent	Done	04/16/2010 14:29:22 CGILMAN: Received from Teva Pharmaceuticals Received on 4/16/10 Sitagliptin Phosphate 654671-77-9 CAS#: 790712-60-6
232384		Compound 184	D1895MM14084/2	Ambient	Trans. to another p		NC_Urgent	Logged	04/28/2010 11:30:27 CGILMAN: Received from Teva Pharaceuticals Received on 4/28/10 Sitagliptin Phosphate Date: 15/04/10 10/08/2010 09:21:36 CGILMAN: Transferred to EL20101477, LIMS 246222

Lims No	Notebook #	Compound	Lot #	Storage	Retain Location	Hazard Code	Priority	Status	Sample Comments
233285		Compound 184	SAL-088.087	Ambient	Legal sample roo		NC_Urgent	Done	05/06/2010 13:20:33 CGILMAN: Received from Teva Received on 5/6/10 STG-Base

EL20100011
Cmpd 134
Lims 229438

ORIG. ID:
FROM: Sarah Levene Goffer 4048538806
Teva Pharmaceutical Industries, Ltd
16 BAZEL ST.

PETACH TIKVA
49131 (IL)

TO: LEONARD J. CHYALL, PH.D (111) 111-1111
SSCI - A DIVISION OF APTUIT
3065 KENT AVE

WEST LAFAYETTE
IN 47906 USA (US)

FedEx.
1 OF 1

Cad#: 8582185
SHIP DATE: 25MAR10
WEIGHT: 0.5 KGS

DESCRIPTION
PHARMACEUTICAL SAMPLES FOR R&D ONLY

CTRY MFG
IL

CUSTOM VALUE: 7.00 USD
CARRIAGE VALUE: .00 USD

BILL T/C: S 216607740
BILL D/T: S 216607740

REF: MNG 240894
SIGNATURE: Sarah Levene Goffer

TRK # 7921 7929 3466 FORM 0430

INTL PRIORITY

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EL20100011
LIMS 231202
cmpd 184
4/16/10
CG

Courtney Gilman

From: Len Chyall
Sent: Friday, April 16, 2010 11:16 AM
To: Courtney Gilman
Subject: RE: Legal Sample

EL20100011 Compound 184

From: Courtney Gilman
Sent: Friday, April 16, 2010 10:39 AM
To: Len Chyall
Subject: Legal Sample

Hi,

You received a sample from Teva yesterday. What project would you like this logged into?

Thanks!

Courtney Gilman
Administrative Assistant
SSCI, A Division of Aptuit
3065 Kent Ave.
West Lafayette, IN 47906
800.375.2179 X3621
765.463.0112 X3621
Fax: 765.463.4722

ORIG. ID:
FROM: Naama Nevo 97239267278
Teva Pharmaceutical Industries, Ltd
16 BAZEL ST.

PETACH TIKVA
49131 (IL)

TO: LEONARD J. CHYALL, PH.D (765) 463-0112
SSCI - A DIVISION OF APTUIT
3065 KENT AVE

WEST LAFAYETTE
IN 47906 USA (US)

DESCRIPTION
PHARMACEUTICAL SAMPLES FOR R&D ONLY

CTRY MFG
IL

FedEx.
1 OF 1

Cad#: 8582185
SHIP DATE: 12APR10
WEIGHT: 1.0 KGS

CUSTOM VALUE: 11.00 USD
CARRIAGE VALUE: .00 USD

BILL T/C: S 216607740
BILL D/T: S 216607740

REF: MNG 243214
SIGNATURE: Naama Nevo

TRK # 7994 4812 4412

FORM
0430

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Invoice Date:	12/4/2010
Invoice Number:	243214
Purchase Order Number:	N/A

INCOTERMS:	DDP
Terms of Payment:	FREE-OF-CHARGE
Type of Currency:	USD

Name & Address of Shipper:

Naama Nevo Teva
Pharmaceutical Industries 16
Basel street Petach- Tikva ,
Israel.

Name & Address of Importer of Record &Ultimate Consignee:

Leonard J. Chyall, Ph.D.
SSCI, A Division of Aptuit
3065 Kent Ave
West Lafayette, IN 47906
USA
Phone: 1-765-463-0112 X 3361
Fax: 1-765-463-4772
E-mail: len.chyall@aptuit.com

Purpose of Shipment:

Detailed Product Description	Quantity	CAS#	Batch #	Country of Origin	Unit Price	Total Price (USD)	Packed in
Sitagliptin Phosphate: 654671-77-9	11 GR	790712-60-6	D-1895NN-13067/3	Assia Chemical Industries LTD Teva Tech Site Ramat Hovav P.O Box 2049, Emek Sara, Be'er Sheva 84874, Israel Tel: (972)-8-6509555; Fax: (972)-8-6509500 For Emergency (24 hours/day): Tel: (972)-8-6509555	$1/1 GR	11	Plastic Container
Totals:	**11 GR**					**11 USD**	

Temperature Requirements: 25c
Total Cartons:
Total Pallets:
Pallet Measurements:
Total Gross Weight KG:

Responsible Individual with Knowledge of the Transaction:

Name:	Naama Nevo
Title:	Corporate Patent Dept - Administrator
E-mail:	Naama.Nevo@teva.co.il
Phone:	972 3 9267278

To the best of my knowledge, this invoice is true and correct.

Signature:

SAMPLES FOR RESEARCH ONLY

EL20100011
LIMS 232384
CMPd 184
4/28/10 CG

Courtney Gilman

From: Len Chyall
Sent: Wednesday, April 28, 2010 10:56 AM
To: Courtney Gilman
Subject: RE: Legal Sample

Is it sitagliptin? If so, it goes into EL20100011.

From: Courtney Gilman
Sent: Wednesday, April 28, 2010 10:55 AM
To: Len Chyall
Subject: Legal Sample

You received a sample from Teva today. What project would you like this logged into?

Thanks!

Courtney Gilman
Administrative Assistant
SSCI, A Division of Aptuit
3065 Kent Ave.
West Lafayette, IN 47906
800.375.2179 X3621
765.463.0112 X3621
Fax: 765.463.4722

XH/CAFA

ORIG. ID:
FROM: Naama Nevo 97239267278
Teva Pharmaceutical Industries, Ltd
16 BAZEL ST.

PETACH TIKVA
49131 (IL)

TO: LEONARD J. CHYALL, PH.D (765) 463-0112
SSCI - A DIVISION OF APTUIT
3065 KENT AVE

WEST LAFAYETTE
IN 47906 USA (US)

FedEx.
1 OF 1

Cad#: 8582185
SHIP DATE: 25APR10
WEIGHT: 1.0 KGS

DESCRIPTION
PHARMACEUTICAL SAMPLES FOR R&D ONLY

CTRY MFG
IL

CUSTOM VALUE: 10.00 USD
CARRIAGE VALUE: .00 USD

BILL T/C: S 216607740
BILL D/T: S 216607740

REF: PTNT 245167
SIGNATURE: Naama Nevo

TRK # 7907 0284 3390 FORM 0430

INTL PRIORITY

47906-IN-US

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IE CG 5/6/10
EL20010011
LIMS 233285
5/6/10 CG
20100011
cmpd 184

ORIG. ID:
FROM: Naama Nevo 97239267278
Teva Pharmaceutical Industries, Ltd
16 BAZEL ST.

PETACH TIKVA
49131 (IL)

TO: LEONARD J. CHYALL, PH.D (765) 463-0112
SSCI - A DIVISION OF APTUIT
3065 KENT AVE

WEST LAFAYETTE
IN 47906 USA (US)

FedEx.
1 OF 1

Cad#: 8582185
SHIP DATE: 03MAY10
WEIGHT: 1.0 KGS

DESCRIPTION
PHARMACEUTICAL SAMPLES FOR R&D ONLY

CTRY MFG
IL

CUSTOM VALUE: 20.00 USD
CARRIAGE VALUE: .00 USD

BILL T/C: S 216607740
BILL D/T: S 216607740

REF: PTNT 246802
SIGNATURE: Naama Nevo

TRK # 7994 4980 3887

FORM
0430

INTL PRIORITY

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ד"ר שלמה כהן ושות'

עורכי-דין

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אבן גבירול 124
תל-אביב 62038
טלפון 03 5271919
פקס 03 5272666
cohens@shlomocohen.co.il
www.shlomocohen.co.il

לכבוד
מר טל בנד, עו"ד
ש. הורוביץ ושות'
רח' אחד העם 31
תל אביב 65202

4 בינואר 2011
9615/71
בפקס
מבלי לפגוע בזכויות

טל שלום,

הנדון: **התנגדות לרישום פטנט מס' 172563 – ראיות נוספות מטעם המתנגדת**

מעיון במחברות המעבדה בקשר לחומרים שקיבל ד"ר Chyall והמתארות את הניסויים שערך, עולה כי חומרים מסויימים חסרים או בלתי ברורים כדלקמן:

1. בס' 16 לתצהירו, מציין ד"ר Chyall כי קיבל מטבע דוגמאות של בסיס חופשי של sitagliptin מאצווה sal-069 ומאצווה sal-008,087. בס' 18 לתצהירו, מציין ד"ר Chyall כי קיבל מטבע דוגמאות של מלח פוספאט של sitagliptin (אצוות D-1895NN-13067/3 ו-D1895MM14084/2). במחברות המעבדה שקיבלנו לא מופיע תיעוד של קבלת החומר, שקילתו, מספרי ה-LIMS שהוקצו לו וכו'. אבקשך להעביר את הדפים החסרים.

2. ב-assays של אצוות הבסיס החופשי יש הפניות למחברות מעבדה שלא נמסרו לנו (כגון, 43371001/63) וכן חלקים מחוקים (redacted). נא להעביר לעיוננו עותקים לא מחוקים של מחברות המעבדה וכן את העמודים החסרים.

3. על פי התיעוד במחברת 4031, הדוגמאות 4031-02-01 עד 4031-02-05, 4031-03-02, 4031-04-01, 4031-05-01, 4031-06-01, 4031-07-01 נשלחו לבדיקת UV. נא להעביר לעיוננו את תוצאות בדיקות ה-UV.

4. לא ניתן לקרוא את הפרוצדורה להכנת דוגמאות 4031-09-01, 4031-09-02 (מחברת 4031, בע' 9). נא למסור את הפרוצדורה באופן ברור. כמו כן, נא להעביר לעיוננו את תוצאות בדיקות ה-X-Ray. תוצאות אלו אינן מדווחות במחברות המעבדה ואינן מצורפות.

5. נא להעביר לעיוננו את תוצאות בדיקות ה-X-Ray של דוגמאות 4031-10-01 עד 4031-10-04, דוגמאות 4031-26-01 עד 4031-26-05 ודוגמאות 4031-29-01 עד 4031-29-03 (מחברת 4031, בע' 9, 26 ו-29 בהתאמה). תוצאות אלו אינן מדווחות במחברות המעבדה ואינן מצורפות.

6. לא ניתן לקרוא את הפרוצדורה להכנת דוגמאות 4031-13-01 עד 4031-13-04 (מחברת 4031, בע' 13). נא למסור את הפרוצדורה באופן ברור.

7. נא לזהות את דוגמאות LIMS 233075, 233074 (מחברת 4031, בע' 17). כמו כן, נא להעביר לעיוננו את תוצאות ה-microscopy images של דוגמאות אלו ודוגמאות אלה ודוגמאות LIMS 231202, 229438.

8. לא ניתן לקרוא את הפרוצדורה להכנת דוגמאות 4031-21-01, 4031-21-02 (מחברת 4031, בע' 21). נא למסור את הפרוצדורה באופן ברור.

9. נא להסביר כיצד הותאם ה-pH בדוגמא 4031-25-05 (מחברת 4031, בע' 25).

10. דוגמאות 4031-24-01 עד 4031-24-08 נשלחו לבדיקת HPLC. פרוצדורה הכנת הדוגמאות לבדיקת ה-HPLC מתוארת במחברת 4060, בע' 28 (מספור חדש כדוגמאות 4060-28-01 עד 4060-28-08). אולם, תוצאות הבדיקות אינן מדווחות במחברות המעבדה ואינן מצורפות. נא לצרף את תוצאות הבדיקות.

11. דוגמאות 4031-27-01 עד 4031-27-05 נשלחו לבדיקות HPLC (מחברת 4031, בע' 27). הפרוצדורה להכנת הדוגמאות לבדיקת ה-HPLC מתוארת במחברת 4060 (בע' 32, דוגמאות 4060-32-01 עד 4060-32-05). אולם, תוצאות הבדיקות אינן מדווחות במחברות המעבדה ואינן מצורפות. נא לצרף את תוצאות הבדיקות.

12. דוגמאות 4031-30-04 עד 4031-30-06 נשלחו לבדיקות HPLC. הפרוצדורה להכנת הדוגמאות לבדיקת ה-HPLC מתוארת במחברת 4060 (בע' 42-43, דוגמאות 4060-42-01 עד 4060-42-03). אולם, תוצאות הבדיקות אינן מדווחות במחברות המעבדה ואינן מצורפות. נא לצרף את תוצאות הבדיקות.

13. ד"ר Cyall מתייחס לדוגמאות מס' 4031-11-01, 4031-11-03, 4031-11-05, 4031-11-07 בטבלה 4 לתצהירו. נא להעביר לעיוננו את תוצאות בדיקות ה-X-Ray ו-HPLC שבוצעו בקשר לדוגמאות אלו. במחברת המעבדה מצוין כי הדוגמאות נשלחו לבדיקות X-Ray אך תוצאות אלו אינן מדווחות במחברות המעבדה ואינן מצורפות.

14. דוגמא 4063-09-01 (מחברת 4063, עמ' 9-10) הוכנה מחומר LIMS 232802. נא לזהות את החומר האמור. כמו כן, נא לציין לצורך איזה שימוש הוכנה דוגמא זו.

15. נא להסביר לשם מה הוכנו הדוגמאות הבאות ואיזה שימוש נעשה בהן:

- 4063-14-01 עד 4063-14-03 (מחברת 4063, עמ' 14-15)
- דוגמאות 4063-16-01 ו-4063-16-02 (מחברת 4063, עמ' 16)
- דוגמא 4063-36-01 (מחברת 4063, עמ' 36)
- דוגמאות 4063-47-01, 4063-48-01 (מחברת 4063, עמ' 47-48)

על מנת שנוכל להתקדם בהכנת התשובה לתצהירו של ד"ר Chyall ללא עיכובים מיותרים, נודה לכם על תשובתכם המהירה.

ב ב ר כ ה,

ליעד רוטשטיין, עו"ד

ד"ר שלמה כהן ושות'

עורכי-דין

שלמה כהן
ליעד וטשטיין
שי ישראל
עינת אלירז
אמירה מנגלוס
אורן תג
אריה בנימין
עמיר אלגר
קרן בן שחר
אורי פרוכטמן
סריה בדיר
מוחמד כרים
ליאב שפירא
ליעד אלופר

מגדל המאה
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תל-אביב 62038
טלפון 03-5271919
פקס 03-5272666
cohens@shlomocohen.co.il
www.shlomocohen.co.il

11 בינואר 2011

9615/71

בפקס

מבלי לפגוע בזכויות

לכבוד
מר טל בנד, עו"ד
ש. הורוביץ ושות'
רח' אחד העם 31
תל אביב 65202

טל שלום,

הנדון: **התנגדות לרישום פטנט מס' 172563 – ראיות נוספות מטעם המתנגדת**

בהמשך לפנייתנו מיום 4 בינואר 2011, נבקשכם להעביר לעיוננו את החומרים החסרים הנוספים שתועדו במחברות המעבדה המתארות את הניסויים המדווחים בתצהירו של ד"ר Chyall, כדלקמן:

1. תוצאות בדיקות C^{13} NMR של דוגמאות D1895NN-13067/3, 4063-03-01, SAL-087,088 (מחברת 4063, בע' 62).

2. תוצאות בדיקת C^{13} NMR של דוגמא המזוהה כ-LIMS 235849 (מחברת 4063, בע' 63-64). נא לזהות את החומר האמור ולהעביר לעיוננו את תוצאת הבדיקה.

על מנת שנוכל להתקדם בהכנת התשובה לתצהירו של ד"ר Chyall ללא עיכובים מיותרים, נודה לכם על תשובתכם המהירה למכתבנו זה ולמכתב מיום 4.1.11.

בברכה,

ליעד וטשטיין, עו"ד

ד"ר שלמה כהן ושות'

עורכי-דין

שלמה כהן
ליעד וטשטיין
שי ישראל
עינת אלירז
אמירה מנגלוס
אורן חג
אריה בנימין
עמיר אלגר
קרן בן שחר
אורי פרוכטמן
סריה בדיר
מוחמד כרים
ליאב שפירא
ליעד אלופר

מגדל המאה
אבן גבירול 124
תל-אביב 62038
טלפון 03-5271919
פקס 03-5272666
cohens@shlomocohen.co.il
www.shlomocohen.co.il

19 בינואר 2011

9615/71

בפקס

מבלי לפגוע בזכויות

לכבוד
מר טל בנד, עו"ד
ש. הורוביץ ושות'
רח' אחד העם 31
תל אביב 65202

טל שלום,

הנדון: **התנגדות לרישום פטנט מס' 172563 – ראיות נוספות מטעם המתנגדת**

טרם קיבלנו את החומר המפורט במכתבנו מהימים 4.1.11 ו-11.1.11.

מחברות המעבדה המתארות את הניסויים המדווחים בתצהירו של ד"ר Chyall, לרבות החומר והמידע שהתבקשה המתנגדת להשלים, אמורים היו להיות מצורפים מלכתחילה לראיותיה הנוספות של המתנגדת. משום מה, המתנגדת מסרה את מחברות המעבדה רק לאור פנייתה של המבקשת. גם לאחר שעשתה כן, העבירה רק חומר חלקי וחסר ללא מסמכים ותוצאות אנליטיות רבות הנזכרים במחברות המעבדה, המהווים חלק בלתי נפרד מהן ודרושים לבדיקת הניסויים שהגישה המתנגדת.

כמו כן, בנוסף לחומרים שהתבקשה המתנגדת להשלים, מתבקשת המתנגדת לפרט כיצד חישב ד"ר Chyall את עקומת המסיסות המתוארת בס' 67-68 לתצהירו וב-Figure 2 וכיצד חישב את הערך של ה-pHmax בעקומה זו.

לאור לוחות הזמנים הקבועים בתיק ועל מנת לקדם את ההליך ללא עיכובים מיותרים, נדרשת המתנגדת למסור את כל החומר והמידע החסרים לא יאוחר מיום 25.1.11. לאור העובדה שמכתבנו הראשון נשלח כבר ביום 4.1.11, ומכיוון שיש להניח שחומרים אלו ממילא נבדקו בטרם הגישה המתנגדת את ראיותיה הנוספות, אין הצדקה לכל עיכוב נוסף במסירת החומר שהיה אמור להיות מצורף מלכתחילה לתצהירו של ד"ר Chayll.

בברכה,

ליעד וטשטיין, עו"ד

g:\petros2011\9615\71\faxes\let3-19.1.11.doc

ש. הורוביץ ושות'

עורכי דין, נוטריונים, עורכי פטנטים

19 בינואר 2011
T/44/548

לכבוד
מר ליעד וטשטיין, עו"ד
שלמה כהן ושות, משרד עורכי דין
מגדל המאה
אבן גבירול 124
<u>תל-אביב 62038</u>

בפקסימיליה

ליעד שלום,

הנדון: <u>התנגדות לבקשת פטנט 172563</u>

1. אני מאשר קבלת מכתביך מהימים 4.1.2011, 11.1.2011 ו-19.1.2011 בעניין הנדון.

2. מחברות המעבדה של ד"ר צ"ל, וכן תיעוד אנליזות ה- HPLC שנערכו לאצוות הבסיס החופשי, נמסרו למרשתך עוד ביום 28.10.2010, בסמוך להגשת הראיות הנוספות. תמוהה העובדה כי מרשתך נזכרה, רק בחלוף כמעט שלושה חודשים (ובמקביל להגשת בקשת ארכה להגשת ראיותיה), לפנות למרשתי ברשימה ארוכה של בקשות הנוגעות לחומר שנמסר, וממשיכה "לטפטף" בקשות חדשות נוספות. קשה להשתחרר מן הרושם, כי מרשתך לא טרחה כלל לעבור על החומר שנמסר לה, עד לאחרונה ממש. בנסיבות אלה, אין למרשתך להלין אלא על עצמה.

3. יתירה מכך: מבלי להתייחס בשלב זה לבקשות מרשתך לגופן, ומבלי לגרוע מכל זכות או טענה העומדות לטבע, יובהר כי ממילא לא חלה על מרשתי כל חובה שבדין להשיב לבקשות שהופנו אליה. כמו כן, ובניגוד לרושם שמתקבל מחלק מהבקשות המופיעות במכתביך האמורים, ד"ר צ"ל בוודאי אינו נתון כעת לחקירה נגדית על ידי מרשתך. ככל שלמרשתך שאלות לגופם של הניסויים, הרי שהדרך והשלב לבררן אינם במסווה של "בקשות להשלמת מידע", כביכול.

4. על אף האמור, ולפנים משורת הדין בלבד, מרשתי מבררת את רשימת הבקשות הארוכה של מרשתך ותשיב למכתביך הנזכרים לעיל בהקדם.

L/80044/5480/1786769/1

ש. הורוביץ ושות'

אחד העם 31, תל-אביב 65202, תא דואר 2499, תל אביב 61024. (ט): 03-5670700 (פ): 03-5660974 (דוא"ל): info@s-horowitz.co.il

ש. הורוביץ ושות'
עורכי דין, נוטריונים, עורכי פטנטים

5. מובן, כי העובדה שמרשתך בחרה לפצל את בקשותיה על פני שלושה מכתבים שונים, גורמת להתארכות בירורן של הבקשות. בנסיבות אלה, ולרקע האמור לעיל, מרשתי דוחה מכל וכל את הנסיון העולה ממכתבך "להכין את הקרקע" לטענה כאילו מרשתי גורמת לעיכוב כלשהו בהכנת ראיותיה של מרשתך.

6. מובן, כי כי אין במכתבי זה כדי להוות הסכמה ו/או הודאה בטענה כלשהי של מרשתך, או כדי לפגוע בכל זכות ו/או טענה של טבע בכל הנוגע לעניין הנדון.

בכבוד רב ובברכה,

טל בנד, עו"ד
ש. הורוביץ ושות'

ד"ר שלמה כהן ושות'

עורכי-דין

שלמה כהן
ליעד וטשטיין
שי ישראל
עינת אלירז
אמירה מנגלוס
אורן חג
אריה בנימין
עמיר אלגר
קרן בן שחר
אורי פרוכטמן
סריה בדיר
מוחמד כרים
ליאב שפירא
ליעד אלופר

מגדל המאה
אבן גבירול 124
תל-אביב 62038
טלפון 03-5271919
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www.shlomocohen.co.il

19 בינואר 2011

9615/71

<u>בפקס</u>

<u>מבלי לפגוע בזכויות</u>

לכבוד
מר טל בנד, עו"ד
ש. הורוביץ ושות'
רח' אחד העם 31
<u>תל אביב 65202</u>

טל שלום,

הנדון: **<u>התנגדות לרישום פטנט מס' 172563 – ראיות נוספות מטעם המתנגדת</u>**

קיבלתי את מכתבך מהיום.

הנסיון של מרשתך לטשטש את מחדליה לא יעלה יפה.

מרשתך החסירה והשמיטה מסמכים ומידע רבים וחיוניים שהינם חלק בלתי נפרד ממחברות המעבדה ושהיה עליה לצרף מלכתחילה.

רשימת הבקשות אכן ארוכה אך היא תוצאה של ההשמטות המסיביות ממחברות המעבדה.

על מנת לגלות את החוסרים וההשמטות נאלצה מרשתי להשקיע עבודה בהיקף עצום בין השאר בפענוח כתב היד הבלתי קריא של מחברות המעבדה. מרשתך תישא בכל ההוצאות הגדולות שנגרמו למרשתי בשל כך.

כל החומר מצוי בהישג ידה של מרשתך. לא היתה כל הצדקה לכך שלא נמסר מלכתחילה. אין כל הצדקה לעיכוב הנוסף במסירתו.

אם לא ימסר החומר עד למועד הנקוב במכתבי מיום 19.1.11, לא יהיה מנוס אלא לפנות לרשם הפטנטים על מנת שיורה למרשתך למסור את כל החומר החסר ללא עיכובים נוספים.

בברכה

ליעד וטשטיין, עו"ד

ש. הורוביץ ושות'
עורכי דין, נוטריונים, עורכי פטנטים

רות אוך
היו קוברסקי
עזגד שטרן
יהושע חורש
אלקס הרסמן
סל בבד
שושנה גביש
אהוד אתצ
אנטוני בלוך
אבי אורלי
אביגיל קסטיאל
אתיר כספרי
ק'פורד דיוס
אליה צונץ קולר
מיכל ליברמן
חגי דורון
רן פוגל
בנימין שפר
מרדכי מלכה
ליאור מימון
אורית יולס-דבי
אייל דהרן
ד"ר אסף רתצר

פיליפ וולדקס
עמית שטיימן
פנינה שפר-עמנואל
רתן ברומר
גיא פירר
דתבב אפל
אופיר פחנר
ערן בצלאל
יהודית שיגו
מיכל זלצמן
צהלה הורמן
אופיר קפלן
אחדלית דמן-שלום
מורן כ"ץ
נועם בלי
יפעת שקדי-שץ
קיהל שאו
אוהד בן-יהודה
שירלי קציר
ענת גרימלנד
ליאור מימון
אוריאל פרינץ
שי גימלשטיין

אריתר אזולאי
ליאל גולני ברנס
גלעד זבידה
עינת מיטל
רן פלדמן
גל וינגולד
פרח רוסלר
ליאור מלר
אמיר ויצנהליס
גיא ורטהים
נועה גלזר-בכר
שלומי דלג'
גלעד כ"ץ
שי שטרן
זארון אבניאלי
איילת בן-גיגי
שי אבניאלי
סרוזי דוינש
משה כהן
שמר כרמל
רן קמיל
לילך דנביץ
איל מילר

ניר סרדן
גילי דגב
יאיר זק
נועם זמיר
גיתית לוין גרינברג
לטה ערמון
יעל חזנברג
עמיאל רז
לי בכר כספי
מיכל ברנד-גולד
הילה ביסון
ערי זונדר
ורד גיפמן
צוני רוזנוביץ
מיטל מלכיאל
ניר פרלשטין
צוז רויטנברג
עדי שהם
תמר שוירמן
סעה שמידע
אבנר יצחקי
עמיקם אשל
עדן גוסליב

ליאור סער
סיון אורגד
ענת שנור
דניאל לבנשטיין
נועה מלצר
הילה דילר
יאיר אנגל
ניקולס גיפס
עבבל מילס
מוני סבן
גילת בכד
סיון וולקן
רעות כהן
נועם קומי
איתי פרקש

לילך גולדמן, עו"פ
חיה רחמנקוף, ע"פ

ז"ר אמנון גולדנברג (1935 - 2005)
אברהם לין (1901 - 1988)
נחמיה סלומון (1903 – 1990)

24 בינואר 2011
T/44/548

לכבוד
מר ליעד וטשטיין, עו"ד
שלמה כהן ושות, משרד עורכי דין
מגדל המאה
אבן גבירול 124
תל-אביב 62038

בפקסימיליה

ליעד שלום,

הנדון: <u>התנגדות לבקשת פטנט 172563</u>

1. אני מאשר קבלת מכתבך מיום 19.1.2011.

2. טבע דוחה מכל וכל את טענת מרשתך בדבר "החסרות" ו"השמטות" של מסמכים ומידע, כביכול. האמת שנתשפה בניסויים בוודאי אינה נוחה למרשתך. יחד עם זאת, מוטב כי זו תמקד את מאמציה בהרמת הנטל המוטל על כתפיה, ולא בנסיונות חסרי יסוד להשחיר את פניה של טבע.

3. כאמור במכתבי הקודם אליך, על טבע לא חלה כל חובה שבדין להשיב ל"בקשות להשלמת מידע" של מרשתך. למרות זאת, ולפנים משורת הדין בלבד, אנו מבררים את רשימת הבקשות שנכללו בשלושת מכתביך הקודמים. בשל ריבוי הבקשות, והעובדה כי מרשתך בחרה להעבירן באופן מפוצל, הבירור הנדרש אורך זמן.

4. למען הסר ספק, טבע אינה כפופה לתכתיבים כלשהם של מרשתך, ולמועד שהיא מתיימרת לקצוב לה להשלמת הבירור. כפי שצויין במכתבי הקודם, אנו נשיב למכתביך בהקדם האפשרי. מרשתך בחרה להעביר את בקשותיה רק שלושה חודשים לאחר שקיבלה את מחברות המעבדה, "בדקה התשעים" לפני המועד להגשת ראיותיה. בנסיבות אלה, אין לה להלין אלא על עצמה.

5. במידה ומרשתך תבחר לחטריח את הרשם בבקשות סרק בעניין זה, מובן כי כל ההוצאות שייגרמו לטבע יחולו על מרשתך, ועליה בלבד.

אחד העם 31, תל-אביב 65202, תא דואר 2499, תל אביב 61024. (ס): 03-5670700 (פ): 03-5660974 (דוא"ל): info@s-horowitz.co.il

ש. הורוביץ ושות'
עורכי דין, נוטריונים, עורכי פטנטים

6. מובן, כי כי אין במכתבי זה כדי להוות הסכמה ו/או הודאה של טבע בטענה כלשהי של מרשתך, או כדי לפגוע בכל זכות ו/או טענה של טבע בכל הנוגע לעניין הנדון.

בכבוד רב ובברכה,

טל בנד, עו"ד
ש. הורוביץ ושות'

ד"ר שלמה כהן ושות'

עורכי-דין

שלמה כהן
ליעד וטשטיין
שי ישראל
עינת אלירז
אמירה מנגלוס
אורן חג
אריה בנימין
עמיר אלגר
קרן בן שחר
אורי פרוכטמן
סריה בדיר
מוחמד כרים
ליאב שפירא
ליעד אלופר

מגדל המאה
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www.shlomocohen.co.il

24 בינואר 2011

9615/71

<u>בפקס</u>

<u>מבלי לפגוע בזכויות</u>

לכבוד
מר טל בנד, עו"ד
ש. הורוביץ ושות'
רח' אחד העם 31
<u>תל אביב 65202</u>

טל שלום,

הנדון: **<u>התנגדות לרישום פטנט מס' 172563 – ראיות נוספות מטעם המתנגדת</u>**

קיבלתי את מכתבך הנוסף מהיום.

מכבסת המילים של מרשתך איננה יכולה לשנות את המציאות: מחברות המעבדה חלקיות וחסרות ו"ריבוי הבקשות" כהגדרתכם נובע מריבוי ההשמטות.

מרשתך עושה כל שלאל ידה על מנת לגרור את ההליך. תחילה נזכרה להגיש ראיות נוספות חודשים ארוכים לאחר שחלף המועד להגשת ראיותיה. לאחר מכן מסרה את הראיות ללא מחברות המעבדה. כשהואילה סוף סוף למסור את מחברות המעבדה, היו אלה כאמור חלקיות וחסרות. ועתה, למרות שכל החומר החסר ממילא אמור להיות בידיה, נדרשת מרשתך לפרק זמן ממושך מאד ולארכות חוזרות ונשנות על מנת להשלים את החסר.

תלונתה של מרשתך כאילו מרשתי התעכבה פרק זמן של שלושה חודשים עד שפנתה בדרישה להשלמת החוסרים היא עיוות מוחלט של המציאות. מרשתי נאבקה במשך זמן רב בנסיון לפענח את מחברות המעבדה הבלתי קריאות ופנתה אליכם כחודשיים לאחר שקיבלה את מחברות המעבדה, לאחר שהחלה להתברר מסכת ההחסרות וההשמטות.

בניגוד לרושם העשוי להתקבל ממכתבה של מרשתך, אין היא עושה למרשתי "טובה" כלשהי בכך שהיא משיבה לדרישות מרשתי. ללא החומר החסר, משקלם הראייתי של הניסויים אותם הגישה מרשתך הינו משקל נוצה. מעבר לכך, מרשתי כמובן תתייחס בהרחבה רבה בראיותיה לניסויים המפוקפקים שהגישה מרשתך.

העובדה שמרשתך מגישה את ראיותיה טיפין טיפין ובעיכובים גדולים גורמת למרשתי טרחה רבה והוצאות גדולות. מרשתי תעמוד על כך שמרשתך תשפה את מרשתי בגין כל ההוצאות המיותרות הנגרמות לה ומעניקה למרשתך, לפנים משורת הדין, ארכה אחרונה עד ליום 31.1.11 למסירת החומר והמידע החסרים. לאחר מכן, תגיש מרשתי בקשות מתאימות ללא כל התראה נוספת.

בברכה,

ליעד וטשטיין, עו"ד

9615/71

אמירה, קרן, פרי
+
ג'וסף

ש. הורוביץ ושות'

עורכי דין, נוטריונים, עורכי פטנטים

24 בפברואר 2011
T/44/548

לכבוד
מר ליעד וטשטיין, עו"ד
שלמה כהן ושות', משרד עורכי דין
מגדל המאה
אבן גבירול 124
<u>תל-אביב 62038</u>

<u>באמצעות שליח</u>

Shlomo Cohen & Co
Law offices
24.02.2011
RECEIVED

ליעד שלום,

הנדון: <u>התנגדות לבקשת פטנט 172563</u>

אנו משיבים בזאת על מכתביך מהימים 4.1.2011, 11.1.2011 ו-19.1.2011 בעניין שבנדון.

המענה לשאלות שהועלו על ידי מרשתך מופיע להלן על פי סדר הופעתן של השאלות בכל אחד מהמכתבים.

<u>תשובות לבקשות מרשתך במכתב מיום 4.1.2011</u>:

1. מעבדת Aptuit מתעדת קבלת דוגמאות ממקורות חיצוניים באופן אלקטרוני, באמצעות מערכת LIMS (Laboratory Information Management System). בתקליטור המצורף למכתבי זה, בקובץ CMPD184ExternalSamples.pdf, תמצא תדפיס ממערכת LIMS של הדוגמאות שמעבדת Aptuit קיבלה מטבע, וכן מסמכי משלוח של הדוגמאות האמורות.

כפי שצוין במכתבנו אליכם מיום 28.10.2010, בסעיף 16 לחוות דעתו של ד"ר צ'ייל נפלה טעות סופר במספרה של אחת מדוגמאות ה- sitagliptin base, אשר צוין כ- sal-0<u>0</u>8/087. מספרה הנכון של דוגמה זו הוא sal-0<u>8</u>8,087.

מעבדת Aptuit אינה מבצעת שקילה של דוגמאות המתקבלות ממקורות חיצוניים עם קבלתן, אלא אם התומר נשוא הדוגמה מפוקח על ידי הסוכנות הפדרלית למלחמה בסמים בארה"ב (ה- DEA).

אחד העם 31, תל-אביב 65202, תא דואר 2499, תל אביב 61024. (ט): 03-5670700 (פ): 03-5660974 (דוא"ל): info@s-horowitz.co.il

2. עמודי המחברת אליהם מופיעה הפניה בתיעוד אנליזות ה- HPLC של אצוות ה- sitagliptin base, שנמסר למרשתך ביום 28.10.2010, מצויים בתקליטור המצורף למכתבי זה בספריה "Teva Notebooks". כפי שציינו במכתבנו מיום 28.10.2010, החלקים המושחרים בתיעוד שנמסר למרשתך מתייחסים לדוגמאות שאינן נוגעות לניסויים שנערכו בקשר להליך ההתנגדות. לפיכך, אין כל מקום למסור למרשתך עמודים אלה ללא ההשחרות.

3. להלן טבלה המציגה את מספרי הדוגמאות נשוא השאלה, מספרי ה- LIMS שלהן ומספרי הקבצים המתייחסים לאנליזות ה- UV שנערכו להן. הקבצים מצויים בתקליטור המצורף למכתבי זה, בספריה "Microplate".

Notebook No.	LIMS No.	UV Microplate filename
4031-02-01	230669	393629, 393633, 393634
4031-02-02	230681	393663
4031-02-03	230682	393664
4031-02-04	230683	393665
4031-02-05	230684	393666
4031-03-02	230804	393882
4031-04-01	230832	393958
4031-05-01	231012	394231
4031-06-01	231183	394473
4031-07-01	231351	394782, 394848

4. בספריה "Aptuit Notebooks" שבתקליטור המצורף למכתבי זה מצוי עותק מודפס של עמ' 9 במחברת מעבדה מס' 4031. כמו כן, בספריה "XRPD" מצויים קבצי אנליזות ה- XRPD של דוגמאות 4031-09-01 ו- 4031-09-02, כדלקמן:

Sample	LIMS	XRPD Filename
4031-09-01	223074	397961
4031-09-02	223075	397962

5. להלן טבלה המציגה את מספרי הדוגמאות נשוא השאלה, מספרי ה- LIMS שלהן ומספרי הקבצים המתייחסים לאנליזות ה- XRPD שנערכו להן. הקבצים מצויים בתקליטור המצורף למכתבי זה, בספריה "XRPD". דוגמאות 4031-26-01 עד 4031-26-05 מתייחסות לתמיסות מסוננות שהתקבלו בניסויים עם דוגמאות אלה. המוצקים שהתקבלו בניסויים האמורים נאספו ונבדקו ב- XRPD כדוגמאות 4031-25-01, 4031-25-03, 4031-25-04 ו- 4031-25-05, כאמור בעמ' 26 של מחברת מעבדה מס' 4031.

יצויין כי, בשל טעות שנפלה בקבצים 405649 ו- 405651, ביחס למספרי ה- LIMS ומספרי הדוגמאות של LIMS 236932 ו- LIMS 236944 (מסומנים בטבלה שלהלן ב- *), לא ניתן היה לשייך את אנליזות ה- XRPD בקבצים אלה לדוגמאות שנבדקו בפועל. מכל מקום, ד"ר צ'ייל לא הסתמך על אנליזות אלה בחוות דעתו.

Sample	LIMS	XRPD Filename
4031-10-01	233039	397912
4031-10-02	233040	397913
4031-10-03	233041	397914
4031-10-04	233042	397915
4031-25-01	234624	401141
4031-25-03	234625	401142
4031-25-04	234626	401143
4031-25-05	234627	401144
4031-29-01[a]	236932	405649
4031-29-02	236943	405650
4031-29-03[a]	236944	405651

6. בספריה "Aptuit Notebooks" שבתקליטור המצורף למכתבי זה מצוי עותק מודפס של עמ' 13 במחברת מעבדה מס' 4031.

7. דוגמה מס' LIMS 233074 הינה חדוגמה המופיעה במחברת המעבדה במספר 4031-09-01, שהינה דוגמה מתוך LIMS 229438 שנכתשה בעלי ומכתש, כמופיע בעמ' 9 של מחברת מעבדה מס' 4031.

דוגמה מס' LIMS 233075 הינה הדוגמה המופיעה במחברת המעבדה במספר 4031-09-02, שהינה דוגמה מתוך LIMS 231202 שנכתשה בעלי ומכתש, כמופיע בעמ' 9 של מחברת מעבדה מס' 4031.

קבצי אנליזות ה- microscopy של הדוגמאות האמורות מצויים בתקליטור המצורף למכתבי זה בספריה "microscopy", כדלקמן:

Notebook No.	LIMS No.	Microscopy File
4031-09-01	233074	398703-1.jpg
4031-09-02	233075	398649-1.jpg
n/a	231202	397467-1.jpg (analyzed without oil) 398647-1.jpg (analyzed under oil)
n/a	229438	397466-011.jpg (analyzed without oil) 397466-012.jpg (analyzed without oil) 398646-1.jpg (analyzed under oil)

8. בספריה "Aptuit Notebooks" שבתקליטור המצורף למכתבי זה מצוי עותק מודפס של עמ' 21 במחברת מעבדה מס' 4031.

9. בקשתך של מרשתך להסביר כיצד חותאם ה- pH בדוגמא 4031-25-05 חורגת מגדר בקשה לקבלת תיעוד של הניסויים, ואין מקומה בשלב זה של ההליך. מבלי לגרוע מכל זכות או טענה של טבע, שעתן של החקירות הנגדיות בתיק טרם הגיעה.

10. לדוגמאות 4031-24-01 עד 4031-24-08 ניתנו מספרי מחברת מעבדה שונים לצורך עריכת אנליזות ה-HPLC, כמפורט בטבלה שלהלן:

Sample No.	HPLC Sample No.	LIMS No. of HPLC Sample	HPLC Filename
4031-24-01	4060-28-01	234037	399964
4031-24-02	4060-28-02	234038	399965
4031-24-03	4060-28-03	234039	399966
4031-24-04	4060-28-04	234040	399967
4031-24-05	4060-28-05	234041	399968
4031-24-06	4060-28-06	234042	399970
4031-24-07	4060-28-07	234043	399971
4031-24-08	4060-28-08	234044	399972

קבצי אנליזות ה- HPLC של הדוגמאות האמורות מצויים בתקליטור המצורף למכתבי זה, בספריה "HPLC".

11. לדוגמאות 4031-27-01 עד 4031-27-05 ניתנו מספרי מחברת מעבדה שונים לצורך עריכת אנליזות ה-HPLC, כמפורט בטבלה שלהלן:

Sample No.	HPLC Sample No.	LIMS No. of HPLC Sample	HPLC Filename
4031-27-01	4060-32-01	234572	401016
4031-27-02	4060-32-02	234573	401018
4031-27-03	4060-32-03	234574	401019
4031-27-04	4060-32-04	234575	401020
4031-27-05	4060-32-05	234576	401021

קבצי אנליזות ה- HPLC של הדוגמאות האמורות מצויים בתקליטור המצורף למכתבי זה, בספריה "HPLC".

12. לדוגמאות 4031-30-04 עד 4031-30-06 ניתנו מספרי מחברת מעבדה שונים לצורך עריכת אנליזות ה-HPLC, כמפורט בטבלה שלהלן:

Sample No.	HPLC Sample No.	LIMS No. of HPLC Sample	HPLC Filename
4031-30-04	4060-42-01	236763	405261
4031-30-05	4060-42-02	236764	405262
4031-30-06	4060-42-03	236765	405263

קבצי אנליזות ה- HPLC של הדוגמאות האמורות מצויים בתקליטור המצורף למכתבי זה, בספריה "HPLC".

13. לדוגמאות 4031-11-01, 4031-11-03, 4031-11-05 ו- 4031-11-07 ניתנו מספרי מחברת מעבדה שונים לצורך עריכת אנליזות XRPD ו- HPLC, כמפורט בטבלאות שלהלן:

<u>אנליזות HPLC</u>:

Sample No.	HPLC Sample No.	LIMS No. of HPLC Sample	HPLC Filename
4031-11-01	4060-12-02	233004	397819
4031-11-03	4060-12-04	233006	397821
4031-11-05	4060-12-10	233008	397823
4031-11-07	4060-12-08	233010	397826

קבצי אנליזות ה- HPLC של הדוגמאות האמורות מצויים בתקליטור המצורף למכתבי זה, בספריה "HPLC".

<u>אנליזות XRPD</u>:

Sample No.	XRPD Sample No.	LIMS No. of XRPD Sample	XRPD Filename
4031-11-01	4060-10-01	233039	397912
4031-11-03	4031-10-02	233040	397913
4031-11-05	4031-10-03	233041	397914
4031-11-07	4031-10-04	233042	397915

קבצי אנליזות ה- XRPD של הדוגמאות האמורות מצויים בתקליטור המצורף למכתבי זה, בספריה "XRPD".

14. דוגמה מס' 4063-09-01 תינה חלק מדוגמה מס' LIMS 232802 (Molecular Sieves, 3A, 1.6mm Sigma-Aldrich Batch #MKAA0920), אשר הוכנס לתנור ואקום (vaccum oven) וחומם ב- 200°C~ למשך הלילה, כאמור בעמ' 9 של מחברת מעבדה מס' 4063.

בקשת מרשתך לקבל הסבר באשר לשימוש שעבורו הוכנה הדוגמה נשוא השאלה תורגת מגדר בקשה לקבלת תיעוד של הניסויים, ואין מקומה בשלב זה של ההליך. מבלי לגרוע מכל זכות או טענה של טבע, שעתן של התקירות הנגדיות בתיק טרם הגיעה.

15. בקשת מרשתך לקבל הסבר לשם מה הוכנו הדוגמאות נשוא השאלה, ואיזה שימוש נעשה בהן, תורגת מגדר בקשה לקבלת תיעוד של הניסויים, ואין מקומה בשלב זה של ההליך. מבלי לגרוע מכל זכות או טענה של טבע, שעתן של התקירות הנגדיות בתיק טרם הגיעה.

מבלי לגרוע מהאמור לעיל, ומכל זכות או טענה של טבע, קבצי אנליזות proton NMR שנערכו בקשר לדוגמאות 4063-14-02, 4063-14-03, 4063-16-01 ו- 4063-16-02 מצויות בתקליטור המצורף למכתבי זה, בספריה "PNMR", כדלקמן:

Sample No.	PNMR Filename
4063-14-02	399496
4063-14-03	399793
4063-16-01	399497
4063-16-02	399864

<u>תשובות לבקשות מרשתך במכתב מיום 11.1.2011</u>:

16. קבצי אנליזות ה- C^{13} NMR של דוגמאות D1895NN-13067/3, 4063-03-01 ו- sal-087,088 מצויים בתקליטור המצורף למכתבי זה בספריה "C13 NMR", כדלקמן:

Sample	LIMS	^{13}C NMR filename
D1895NN-13067/3	231202	409909
4063-03-01	233141	409910
SAL-087,088	233285	410032

17. דוגמה LIMS 235849 הינה של מלח הזרחן הדו-מימני של סיטגליפטין. הכנת דוגמה זו מתוארת בסעיף 44 לחוות דעתו של ד"ר צייל, והיא מופיעה בטבלה מס' 1 של חוות הדעת. קובץ אנליזת ה- C^{13} NMR של דוגמה LIMS 235849 (4063-57-01) מצוי בתקליטור המצורף למכתבי זה בספריה "C13 NMR", תחת שם הקובץ 410114.

<u>תשובה לבקשת מרשתך במכתב מיום 19.1.2011</u>:

18. בקשת מרשתך לקבל הסבר על האופן שבו חישב ד"ר צייל את עקומת המסיסות המתוארת בסעיפים 67-68 וב- Figure 2 לחוות דעתו, וכיצד חישב את ערך ה- pH max בעקומת האמורה, חורגת מגדר בקשה לקבלת תיעוד של הניסויים, ואין מקומה בשלב זה של ההליך. מבלי לגרוע מכל זכות או טענה של טבע, מבלי לגרוע מכל זכות או טענה של טבע, שעתן של החקירות הנגדיות בתיק טרם הגיעה.

בשולי הדברים יובהר, כי ההתכתבות בכל הנוגע לבקשות המידע של מרשתך מיצתה את עצמה. התומר שנמסר למרשתך, במענה לבקשות האמורות, נמסר לפנים משורת הדין. ככל שדעתה של מרשתך עדיין לא נחה מהתומר שנמסר לה, ממילא פתוחה בפניה הדרך לפנות בעניין זה לכבי' חרשם. מובן, כי טבע תעמוד על כך שתיפסקנה נגד מרשתך הוצאות בגין בקשה חסרת יסוד כזו.

קשה לחמוק מן הרושם, כי כל מטרת ההטרדה המתמשכת מצד מרשתך אינה אלא להניח תשתית לבקשה להארכת מועד להגשת ראיות, בקשה שאין לה כל הצדקה. מרשתי דוחה נסיונות אלו ליצור תשתית מלאכותית לבקשה כזו, ומודיעה כבר עתה כי לא תסכים לארכה (אלא מטעמים קולגיאליים בלבד).

מובן כי אין באמור במכתבי זה, או במה שלא נאמר בו, כדי לתוות הודאה או הסכמה של טבע לטענה כלשהי של מרשתך, כשם שאין בו כדי לגרוע מכל זכות או טענה של טבע, ואלו שמורות לה במלואן.

בכבוד רב ובברכה,

טל בנד, עו"ד
ש. הורוביץ ושות'

ד"ר שלמה כהן ושות'

עורכי-דין

שלמה כהן
ליעד וטשטיין
שי ישראל
עינת אלירז
אמירה מנגלוס
אורן חג
אריה בנימין
עמיר אלגר
קרן בן שחר
אורי פרוכטמן
סריה בדיר
מוחמד כרים
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28 בפברואר 2011

לכבוד
מר טל בנד, עו"ד
ש. הורוביץ ושות'
רח' אחד העם 31
תל אביב 65202

9615/71

בפקס

מבלי לפגוע בזכויות

טל שלום,

הנדון: **התנגדות לרישום פטנט מס' 172563 – ראיות נוספות מטעם המתנגדת**

קיבלתי את מכתבך מיום 24.2.2011.

מרשתך מקדישה את עיקר מאמציה לגרירת ההליך באופן חסר תקדים ממש.

תחילה פיצלה את ראיותיה והגישה ראיות נוספות חודשים ארוכים לאחר שחלף המועד להגשת ראיותיה.

לאחר מכן, מסרה את ראיותיה הנוספות ללא מתברות מעבדה בתמיכה לניסויים שערכה.

כאשר הואילה מרשתך למסור סוף סוף את מחברות המעבדה לאחר פניית מרשתי, היו אלה חלקיות וחסרות.

בהמשך, נאלצה מרשתי לנהל מסכת ארוכה של התכתבויות עם מרשתך בקשר לבקשתה להשלמת החומר החסר.

רק לאחר שהשתהתה במשך כחודשיים נוספים ממועד מכתבי הראשון, ממש סמוך למועד הגשת הראיות בתשובה מטעם מרשתי, הואילה מרשתך למסור סוף סוף את החומר החסר.

וגם הפעם גילתה טפח אך כיסתה טפחיים...

מרשתי מצוייה עתה בעיצומה של בדיקת החומר. ככל שתזדקק לארכה, היא תפנה בבקשה מתאימה ללשכה. בנסיבות הענין, בהן מרשתכם התעכבה במשך פרק זמן ממושך במסירת החומר שאמור היה להיות מצורף לראיותיה מלכתחילה, האחריות לכל ארכה שתידרש, אם תידרש, רובצת באופן בלעדי על מרשתך ואיננה כפופה להסכמה של מרשתך.

אין באמור לעיל כדי לגרוע מכל טענה של המבקשת, לרבות בדבר התנהלותה הבלתי תקינה של מרשתך והשלכותיה על משקלם הראייתי של הניסויים שהגישה.

בברכה,

ליעד וטשטיין, עו"ד

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DR. SHLOMO COHEN & CO
LAW OFFICE

January 4, 2011
9615/71
by fax
Without prejudice

Mr. Tal Band, Adv.
S. Horowitz & Co.
31 Ahad Haam Street
Tel Aviv 65202

Dear Tal,

re: **Opposition to Registration of Patent No. 172563 – Additional Evidence by the Opponent**

Based on a perusal of the laboratory notebooks in connection with the materials Dr. Chyall received and which describe the experiments he carried out, it turns out that certain materials are missing or unclear, as follows:

1. In Paragraph 16 of his affidavit, Dr. Chyall mentions that he received from Teva samples of sitagliptin free base from batch sal-069 and batch sal-008,087. In Paragraph 18 of his affidavit, Dr. Chyall mentions that he received from Teva samples of a phosphate salt of sitagliptin (batches D-1895NN-13067/3 and D1895MM14084/2). In the laboratory notebooks which we received no documentation appears about receipt of the material, the weighing thereof, the LIMS numbers that were allocated to it, etc. Please send the missing pages.

2. In the assays of the free base batches there are references to laboratory notebooks that were not delivered to us (such as 43371001/63) and deleted portions (redacted). Please send un-erased copies of the laboratory notebooks for our perusal as well as the missing pages.

3. According to the documentation in notebook 4031, the samples 4031-02-01 to 4031-02-05, 4031-03-02, 4031-04-01, 4031-05-01, 4031-06-01, 4031-07-01 were sent for UV examination. Please send the results of the UV examinations for our perusal.

4. It is not possible to read the preparation procedure of samples 4031-09-01, 4031-09-02 (notebook 4031, p. 9). Please report the procedure in a clear manner. Likewise, please send the results of the X-ray examinations for our perusal. These results are not reported in the laboratory notebooks and are not attached.

5. Please send for our inspection results of the X-ray examinations of samples 4031-10-01 to 4031-10-04, samples 4031-26-01 to 4031-26-05 and samples 4031-29-01 to 4031-29-

03 (notebook 4031, pages 9, 26 and 29 respectively). These results are not reported in the laboratory notebooks and are not attached.

6. It is not possible to read the preparation procedure of samples 4031-13-01 to 4031-13-04 (notebook 4031, at p. 13). Please deliver the procedure clearly.

7. Please identify samples LIMS 233075, 233074 (notebook 4031, p. 17). In addition, please send for our perusal the results of the microscopy images of these samples and samples LIMS 231202, 229438.

8. It is not possible to read the preparation procedure of samples 4031-21-01, 4031-21-02 (notebook 4031, p. 21). Please deliver the procedure clearly.

9. Please explain how the pH of sample 4031-25-05 (notebook 4031, p. 25) was adapted.

10. Samples 4031-24-01 to 4031-24-08 were sent for HPLC analysis. The procedure for preparation of the samples for the HPLC analysis is described in notebook 4060, p. 28 (re-numbered as samples 4060-28-01 to 4060-28-08). However, the analysis results are not reported in the laboratory notebooks and are not attached. Please attach the analysis results.

11. Samples 4031-27-01 to 4031-27-05 were sent for HPLC analysis (notebook 4031, p. 27). The procedure for preparation of the samples for the HPLC analysis is described in notebook 4060 (p. 32, samples 4060-32-01 to 4060-32-05). However, the analysis results are not reported in the laboratory notebooks and are not attached. Please attach the analysis results.

12. Samples 4030-30-04 to 4030-30-06 were sent for HPLC analysis. The procedure for preparation of the samples for the HPLC analysis is described in book 4060 (p. 42-43, samples 4060-42-01 to 4060-42-03). However, the analysis results are not reported in the laboratory notebooks and are not attached. Please attach the analysis results.

13. Dr. Chyall relates to samples no. 4031-11-01, 4031-11-03, 4031-11-05, 4031-11-07 in Table 4 of his affidavit. Please send for our perusal the results of the X-ray and the HPLC analysis performed in connection with these samples. In the laboratory notebook it is mentioned that the samples were sent for X-ray examinations, but these results are not reported in the laboratory notebooks and are not attached.

14. Sample 4063-09-01 (notebook 4063, p. 9-10) was prepared from the substance LIMS 232802. Please identify the aforesaid substance. In addition, please indicate for what use this sample was prepared.

15. Please explain for what purpose the following samples were prepared and what use was made of them:

- 4063-14-01 to 4063-14-03 (notebook 4063, p. 14-15).

- Samples 4063-16-01 and 4063-16-02 (notebook 4063, p. 16)
- Sample 4063-36-01 (notebook 4063, p. 36).
- Samples 4063-47-01, 4063-48-01 (notebook 4063, p. 47-48).

In order that we can make progress in preparing the reply to Dr. Chyall's affidavit without unnecessary delays, we would appreciate your prompt reply.

Sincerely

(-)
Liad Whatstein, Adv.

DR. SHLOMO COHEN & CO
LAW OFFICE

January 11, 2011

Mr. Tal Band, Adv.
S. Horowitz & Co.
31 Ahad Haam Street
Tel Aviv 65202

9615/71
by fax
Without prejudice

Dear Tal,

re: **Opposition to Registration of Patent No. 172563 – Additional Evidence by the Opponent**

Further to our letter of January 4, 2011, we would request you to send for our perusal the additional missing material which was documented in the laboratory notebooks that describe the experiments reported in Dr. Chyall's affidavit, as follows:

1. Results of the C^{13} NMR tests of samples D1895NN-13067/3, 4063-03-01, SAL-087,088 (notebook 4063, p. 62).

1. Results of the C^{13} NMR tests of the sample identified as LIMS 235849 (notebook 4063, p. 63-64). Please identify the aforesaid substance and send for our perusal the test result.

In order for us to be able to make progress in preparing the reply to Dr. Chyall's affidavit without unnecessary delays, we would appreciate your prompt reply to this letter of ours and to the letter of January 4, 2011.

Sincerely,

(-)
for/ Liad Whatstein, Adv.

(174)

DR. SHLOMO COHEN & CO
LAW OFFICE

January 19, 2011
9615/71
by fax
Without prejudice

Mr. Tal Band, Adv.
S. Horowitz & Co.
31 Ahad Haam Street
Tel Aviv 65202

Dear Tal,

re: **Opposition to Registration of Patent No. 172563 – Additional Evidence by the Opponent**

We have not yet received the material listed in our letters of January 4, 2011 and January 11, 2011.

The laboratory notebooks which describe the experiments reported in Dr. Chyall's affidavit, including the material and information which the Opponent was requested to supplement, ought to have been attached to the Opponent's additional evidence from the outset. For some reason the Opponent delivered the laboratory notebooks only in light of the Applicant's request. Even after it did so, it sent only partial and deficient material, without documents and results of many analyses which are mentioned in the laboratory notebooks, which form an integral part thereof and are required for examining the experiments which the Opponent submitted.

Likewise, in addition to the material the Opponent was requested to supplement, the Opponent is requested to give details as to how Dr. Chyall calculated the solubility graph described in Paragraphs 67-68 of his affidavit and in Figure 2 and how he calculated the value of the pHmax in that graph.

In view of the timetable that has been set for this case and in order to move forward with the proceeding without unnecessary delays, the Opponent is called upon to deliver all the missing material and information by not later than January 25, 2011. In light of the fact that our first letter was sent already on January 4, 2011, and because it is to be assumed that such material was in any event studied and examined before the Opponent submitted its additional evidence, there is no justification for any additional delay in the delivery of the material which ought to have been attached to Dr. Chyall's affidavit in the first place.

Sincerely,

(-)
Liad Whatstein, Adv.

S. HOROWITZ & CO.

Attorneys, Notaries, Patent Attorneys

January 19, 2011
T/44/548

Mr. Liad Whatstein, Adv.
Shlomo Cohen & Co., Law Office
Century Tower
124 Ibn Gvirol Street
Tel Aviv 62038

By facsimile

Dear Liad,

re: Opposition to Patent Application 172563

1. I acknowledge receipt of your letters of January 4, 2011, January 11, 2011 and January 19, 2011 in the above connection.

2. Dr. Chyall's laboratory notebooks, and the documentation of the HPLC analyses which were carried out on batches of the free base were delivered to your client as far back as October 28, 2010, shortly after the filing of the additional evidence. The fact that your client has remembered, only after the elapse of nearly three months (and concurrent with the filing of an application for an extension of time for the submission of its evidence) to refer to my client with a long list of requests pertaining to the material that was delivered, is most surprising, and it continues "to push in slowly" additional new requests. It is difficult to get away from the impression that your client did not take the trouble at all to go over the material that was delivered to it until very recently. In these circumstances your client has no one but itself to blame.

3. Furthermore: without relating at this stage to your client's requests on their merits, and without derogating from any right or argument available to Teva, it must be made clear that, in any event, there is no obligation on my client according to law to respond to the requests that were made to it. Likewise, and contrary to the impression to be gained from some of the requests appearing in your abovementioned letters, Dr. Chyall is certainly not available now for cross-examination by your client. If your client has questions on the merit and substance of the experiments, then the way and the stage for clarifying them is not under the guise of so-called "requests for supplementing of information".

4. Notwithstanding the foregoing, and on an *ex gratia* basis only, my client is clarifying your client's long list of requests and will reply to your above-referenced letters shortly.

5. Obviously, the fact that your client has chosen to split its requests into three different letters, causes a lengthening of the process for clarifying the requests. In these circumstances, and in light of the afore said, my client strenuously rejects the attempt,

which is to be inferred from your letter, "at preparing the ground" for an allegation that my client is supposedly causing any sort of delay in the preparation of your client's evidence.

6. Clearly, nothing in this letter of mine constitutes acquiescence to and/or admission of any of your client's arguments or allegations, or has the effect of prejudicing any right and/or argument Teva has with regard to the abovementioned matter.

Respectfully yours,

(-)
for/Tal Band, Adv.
S. Horowitz & Co.

DR. SHLOMO COHEN & CO
LAW OFFICE

January 19, 2011
9615/71
by fax
Without prejudice

Mr. Tal Band, Adv.
S. Horowitz & Co.
31 Ahad Haam Street
Tel Aviv 65202

Dear Tal,

re: **Opposition to Registration of Patent No. 172563 – Additional Evidence by the Opponent**

I am in receipt of your letter of today's date.

Your client's attempt to blur and cover up its own omissions will be of no avail.

Your client left out and omitted many documents and a great deal of vital information which form an integral part of the laboratory notebooks and which it was obliged to attach from the inception.

The list of requests is indeed a long one, but that is the consequence of the massive omissions from the laboratory notebooks.

In order to discover the missing items and the omissions, my client was compelled to devote an immense amount of work, *inter alia*, in deciphering the illegible handwriting in the laboratory notebooks. Your client will bear all the immense expenses that my client has incurred by virtue of this.

All the material is readily available to your client. There was no justification for the fact that it was not delivered from the outset. There is no justification for an additional delay in the delivery thereof.

If the material is not delivered up to the time specified in my letter of January 19, 2011, there will be no other option than to apply to the Registrar of Patents in order for him to direct your client to deliver all the missing material without additional delays.

Sincerely,

(-)
for/Liad Whatstein, Adv.

S. HOROWITZ & CO.

Attorneys, Notaries, Patent Attorneys

January 24, 2011
T/44/548

Mr. Liad Whatstein, Adv.
Shlomo Cohen & Co., Law Office
Century Tower
124 Ibn Gvirol Street
Tel Aviv 62038

By facsimile

Dear Liad,

re: Opposition to Patent Application 172563

1. I acknowledge receipt of your letter dated January 19, 2011.
2. Teva vigorously rejects your client's allegation in regard to so-called "missing items" and "omissions" of documents and information. The truth that was revealed in the experiments is obviously not to your client's liking. However, it would be best if your client were to focus its efforts on discharging the onus imposed upon it, and not in unfounded attempts to blacken Teva's name.
3. As stated in my previous letter, there is no obligation in law on Teva to respond to the "requests for the supplementing of information" sent by your client. In spite of this, and on *ex gratia* basis only, we are investigating the list of requests that were included in your last three letters. By virtue of the multitude of requests, and the fact that your client chose to send them in a split form, means that the required investigation will take a longer time.
4. For the avoidance of doubt, Teva is not subject to any of your client's dictates, and to the time which it purports to allocate for completion of the investigation. As mentioned in my previous letter, we will reply to your letters as soon as possible. Your client chose to send its requests only three months after it had received the laboratory notebooks, "in the 90th minute" before the time for s of its evidence. In these circumstances, it has no one but itself to blame.
5. If your client chooses to bother and harass the Registrar with groundless applications in this regard, it is obvious that all the expenses which Teva will incur shall be borne by your client, and by it alone.
6. Clearly, nothing in this letter of mine constitutes any form of acquiescence and/or admission by Teva of any of your client's allegations, or has the effect of prejudicing any right and/or argument Teva has in connection with the abovementioned matter.

Respectfully yours,

(-)
Tal Band, Adv.
S. Horowitz & Co.

DR. SHLOMO COHEN & CO
LAW OFFICE

January 24, 2011
9615/71
by fax
Without prejudice

Mr. Tal Band, Adv.
S. Horowitz & Co.
31 Ahad Haam Street
Tel Aviv 65202

Dear Tal,

re: **Opposition to Registration of Patent No. 172563 – Additional Evidence by the Opponent**

I received your additional letter of today's date.

Your client's flurry of words cannot change the realities: partial and defective laboratory notebooks and "a multitude of requests" as you defined them, are the outcome of multiple omissions.

Your client is doing everything it can in order to drag out the proceeding. To start with it suddenly remembered to submit additional evidence many months after the time for the submission of its evidence had passed. Subsequently it delivered evidence without the laboratory notebooks. When it eventually condescended to deliver up the laboratory notebooks, these were, as already mentioned, only partial and incomplete. Now, even though all the missing material ought in any event to be in its possession, your client requires a very protracted period of time and repeated extensions in order to supplement what is missing.

Your client's complaint that my client delayed for a period of three months until it referred with a request for the supplementing of the missing items is a complete distortion of the true realities. My client struggled for a lengthy period in an attempt to decipher the illegible laboratory notebooks and referred to you about two months after it had received the laboratory notebooks, once the scope of missing items and omission began to become clear.

Contrary to the impression likely to be obtained from your client's letter, it is not doing my client any "favor" by responding to my client's demands. Without the missing material, the evidentiary weight of the experiments which your client submitted is virtually feather-weight. Over and above that, my client will naturally relate extensively in its evidence to the dubious experiments which your client submitted.

The fact that your client is submitting its evidence "little by little" and with huge delays, is something that causes my client immense bother and harassment and large expenses. My client will insist that your client compensate my client for all the unnecessary expenses being incurred by it and, on an *ex gratia* basis, is granting your client a last extension until January 31, 2011 to deliver the missing material and information. Thereafter, my client will file appropriate applications without any further warning.

Sincerely,

(-)
Liad Whatstein, Adv.

S. HOROWITZ & CO.

Attorneys, Notaries, Patent Attorneys

February 24, 2011
T/44/548

Mr. Liad Whatstein, Adv.
Shlomo Cohen & Co., Law Office
Century Tower
124 Ibn Gvirol Street
Tel Aviv 62038

By facsimile

Dear Liad,

re: Opposition to Patent Application 172563

We are replying herewith to your letters of January 4, 2011, January 11, 2011 and January 19, 2011 in the above connection.

The answer to the questions that were raised by your client appear below in the same sequence in which the questions appeared in each of the letters.

Answers to your client's requests in the letter of January 4, 2011:

1. Aptuit Laboratory documents the receipt of samples from outside sources electronically, by means of the LIMS (Laboratory Information Management Services) system. On the compact disk which is attached to this letter, in the file CMPD184ExternalSamples.pdf, you will find a printout from the LIMS system of the samples which Aptuit Laboratory received from Teva, as well as delivery documents in respect of the aforesaid samples.

   As mentioned in our letter to you of October 28, 2010, a clerical error was made in Paragraph 16 of Dr. Chyall's opinion in the number of one of the samples of the sitagliptin base, which was marked as sal-0**0**8/087. The correct number of this sample is sal-0**8**8,087.

   The Aptuit Laboratory does not perform a weighing of samples received from external sources at the time of receipt thereof, unless the substance that is the subject of the sample is controlled by the Federal Agency for the War on Drugs in the USA (DEA).

2. The pages of the notebook on which a reference appears to the documentation of the HPLC analyses of the sitagliptin base batches, which were delivered to your client on October 28, 2010, are to be found on the compact disk which is attached to this letter in the library "Teva Notebooks". As we stated in our letter of October 28, 2010, the blacked-out portions in the documentation delivered to your client relate to samples which have nothing to do with the experiments that were performed in connection with the opposition proceeding. Accordingly, there is no basis for delivering these pages to your client without the blackings out.

3. A table appears below which presents the numbers of the samples that are the subject matter of the question, their LIMS numbers and the file numbers which relate to the UV analyses that were conducted on them. The files are contained in the compact disk which is attached to this letter, under the library "Microplate".

Notebook	LIMS No.	UV Microplate filename
4031-02-01	230669	393629, 393633, 393634
4031-02-02	230681	393663
4031-02-03	230682	393664
4031-02-04	230683	393665
4031-02-05	230684	393666
4031-03-02	230804	393882
4031-04-01	230832	393958
4031-05-01	231012	394231
4031-06-01	231183	394473
4031-07-01	231351	394782, 394848

4. In the library "Aptuit Notebooks" on the compact disk which is attached to this letter, a printed copy is to be found on p. 9 of laboratory notebook no. 4031. Likewise, in the library "XRPD" are the XRPD are the analysis files of samples 4031-09-01 and 4031-09-02, as follows:

Sample	LIMS	XRPD Filename
4031-09-01	223074	397961
4031-09-02	223075	397962

5. Set forth below is a table which presents the numbers of the samples that are the subject matter of the question, their LIMS numbers and the numbers of the files which relate to the XRPD analyses that were performed on them. The files are contained in the compact disk which is attached to this letter in the "XRPD" library. Samples 4031-26-01 to 4031-26-05 relate to filtered solutions that were obtained in experiments with these samples. The solids that were obtained in the aforesaid experiments were collected and were examined under XRPD in samples 4031-25-01, 4031-25-03, 4031-25-04 and 4031-25-05, as mentioned on p. 26 of laboratory notebook no. 4031.

It must be mentioned that due to an error which occurred in files 405649 and 405651, in relation to the LIMS numbers and the numbers of the samples of LIMS 236932 and LIMS 236944 (marked in the table below with an [a]), it was not possible to attribute the XRPD analyses in these files to the samples that were actually examined. In any event, Dr. Chyall did not rely on these analyses in his opinion.

Sample	LIMS	XRPD Filename
4031-10-01	233039	397912
4031-10-02	233040	397913
4031-10-03	233041	397914
4031-10-04	233042	397915
4031-25-01	234624	401141
4031-25-03	234625	401142
4031-25-04	234626	401143
4031-25-05	234627	401144
4031-29-01[a]	236932	405649
4031-29-02	236943	405650
4031-29-03[a]	236944	405651

6. In the "Aptuit Notebooks" library on the compact disk, which is attached to this letter, there is a printed copy of p. 13 of laboratory notebook no. 4031.

7. Sample no. LIMS 233074 is the sample that appears in the laboratory notebook under number 4031-09-01, which is a sample from LIMS 229438 that was crushed with a pestle and mortar, as appears on p. 9 of laboratory notebook no. 4031.

   Sample no. LIMS 233075 is the sample appearing in the laboratory notebook under number 4031-09-02, which is a sample from LIMS 231202 that was crushed with a pestle and mortar, as appears on p. 9 of laboratory notebook no. 4031.

   The microscopy analyses files of the aforesaid samples are contained in the compact disk which is attached to this letter in the "microscopy" library, as follows:

Notebook	LIMS No.	Microscopy File
4031-09-01	233074	398703-1.jpg
4031-09-02	233074	398649-1.jpg
n/a	231202	397467-1.jpg (analyzed without oil) 398647-1.jpg (analyzed under oil)
n/a	229438	397466-011.jpg (analyzed without oil) 397466-012.jpg (analyzed without oil) 398646-1.jpg (analyzed under oil)

8. In the "Aptuit Notebooks" library on the compact disk, which is attached to this letter, there is a printed copy of p. 21 of laboratory notebook 4031.

9. Your client's request for an explanation as to how the pH in sample 4031-25-05 was adapted goes beyond the scope of a request to receive documentation of the experiments, and this stage of the proceedings is not the correct place for it. Without derogating from any right or argument which Teva may have, the time for cross-examinations in this case has not yet arrived.

10. Samples 4031-24-01 to 4031-24-08 were given additional laboratory notebook numbers for purposes of performing the HPLC analysis, as set forth in the table below:

Sample No.	HPLC Sample No.	LIMS No. of HPLC Sample	HPLC Filename
4031-24-01	4060-28-01	234037	399964
4031-24-02	4060-28-02	234038	399965
4031-24-03	4060-28-03	234039	399966
4031-24-04	4060-28-04	234040	399967
4031-24-05	4060-28-05	234041	399968
4031-24-06	4060-28-06	234042	399970
4031-24-07	4060-28-07	234043	399971
4031-24-08	4060-28-08	234044	399972

The HPLC analyses files of the aforesaid samples are on the compact disk, which is attached to this letter, in the "HPLC" library.

11. Samples 4031-27-01 to 4031-27-05 were given additional laboratory notebook numbers for purposes of performing the HPLC analysis, as set forth in the table below:

Sample No.	HPLC Sample No.	LIMS No. of HPLC Sample	HPLC Filename
4031-27-01	4060-32-01	234572	401016
4031-27-02	4060-32-02	234573	401018
4031-27-03	4060-32-03	234574	401019
4031-27-04	4060-32-04	234575	401020
4031-27-05	4060-32-05	234576	401021

The HPLC analyses files of the aforesaid samples are on the compact disk which is attached to this letter, in the "HPLC" library.

12. Samples 4031-30-04 to 4031-30-06 were given additional laboratory notebook numbers for purposes of performing the HPLC analysis, as set forth in the table below:

Sample No.	HPLC Sample No.	LIMS No. of HPLC Sample	HPLC Filename
4031-30-04	4060-42-01	236763	405261
4031-30-05	4060-42-02	236764	405262
4031-30-06	4060-42-03	236765	405263

The HPLC analyses files of the aforesaid samples are on the compact disk, which is attached to this letter, in the "HPLC" library.

13. Samples 4031-11-01, 4031-11-03, 4031-11-05 and 4031-11-07 were given different laboratory notebook numbers for purposes of performing XRPD and HPLC analyses, as described in the following tables:

HPLC analyses:

Sample No.	HPLC Sample No.	LIMS No. of HPLC Sample	HPLC Filename
4031-11-01	4060-12-02	233004	397819
4031-11-03	4060-12-04	233006	397821
4031-11-05	4060-12-10	233008	397823
4031-11-07	4060-12-08	233010	397826

The HPLC analyses files of the aforesaid samples are on the compact disk attached to this letter, in the "HPLC" library.

XRPD analyses:

Sample No.	XRPD Sample No.	LIMS No. of XRPD Sample	XRPD Filename
4031-11-01	4060-10-01	233039	397912
4031-11-03	4060-10-02	233040	397913
4031-11-05	4060-10-03	233041	397914
4031-11-07	4060-10-04	233042	397915

The XRPD analyses files of the aforesaid samples are on the compact disk, which is attached to this letter, in the "XRPD" library.

14. Sample no. 4063-09-01 is part of sample no. LIMS 232802 (Molecular Sieves, 3A, 1.6 mm Sigma-Aldrich Batch #MKAA0920), which was inserted into a vacuum oven and heated at ~200ºC overnight, as stated on p. 9 of laboratory notebook no. 4063.

    Your client's request to receive an explanation regarding the use for which the sample that is the subject of the question was prepared goes beyond the scope of a request to receive documentation of the experiments, and this stage of the proceedings is not the right place for it. Without derogating from any right or argument Teva has, the time for cross-examinations in this case has not yet arrived.

15. Your client's request to receive an explanation as to why the samples that are the subject of the question were prepared, and what use was made of them, is a clear departure from the scope of a request to receive documentation of the experiments, and this stage of the proceedings is not the place for it. Without derogating from any right or argument Teva has, the time for cross-examinations in this file has not yet arrived.

    Without derogating from foregoing, and from any right or argument of Teva, files of the proton NMR analyses that were carried out in connection with samples 4063-14-02, 4063-14-03, 4063-16-01 and 4063-16-02 can be found on the compact disk attached to this letter, in the library "PNMR", as follows:

Sample No.	PNMR Filename
4063-14-02	399496
4063-14-03	399793
4063-16-01	399497
4063-16-02	399864

Replies to your client's request in the letter dated January 11, 2011:

16. The C^{13} NMR analyses files of samples D1895NN-13067/3, 4063-03-01 and sal-087,088 are to be found on the compact disk, which is attached to this letter, in the "C13 NMR" library, as follows:

Sample	LIMS	C^{13} NMR Filename
D1895NN-13067/3	231202	409909
4063-03-01	233141	409910
SAL-087,088	233285	410032

17. Sample LIMS 235849 is the dihydrate phosphate salt of sitagliptin. Preparation of this sample is described in Paragraph 44 of Dr. Chyall's opinion, and it appears in table no. 1 to his opinion. The C^{13} NMR analysis of sample LIMS 235849 (4063-57-01) is contained in the compact disk which is attached to this letter in the "C13 NMR" library under the file name 410114.

Answer to your client's request in the letter dated January 19, 2011:

18. Your client's request to receive an explanation about the manner in which Dr. Chyall calculated the solubility graph described in Paragraphs 67-68 and Figure 2 of his opinion, and how he calculated the pH max value of the aforesaid graph, constitutes a departure from the scope of a request to receive documentation of the experiments, and this stage of the proceedings is not the proper place for it. Without derogating from any of Teva's rights or arguments, the time for cross-examinations in this matter as yet to arrive.

As an aside, it should be clarified that the correspondence regarding your client's request for information has fully exhausted itself. The material that has been delivered to your client, in answer to the aforesaid requests, has been furnished on an *ex gratia* basis. If your client is still not satisfied with the material that has been delivered to it, it obviously has the possibility of applying in this connection the honorable Registrar. Clearly Teva will insist on costs being awarded against your client in respect of such an unfounded application.

It is difficult to escape the impression that the sole purpose of the prolonged harassment by your client is nothing more than laying a foundation for an application for an extension of time in which to file its evidence, which is an application that is totally unjustified. My client rejects these attempts to create an artificial basis for such an application, and gives notice already at this point that it will not agree to an extension of time (other than on collegial grounds only).

Obviously nothing contained in this letter, or anything that is not stated herein, constitutes an admission or acquiescence on the part of Teva to any allegation or argument advanced by your client, in the same way that nothing herein derogates from any right or argument which Teva has, and these are fully reserved to it.

Respectfully yours,

(-)
Tal Band, Adv.
S. Horowitz & Co.

DR. SHLOMO COHEN & CO
LAW OFFICE

February 28, 2011
9615/71
by fax
Without prejudice

Mr. Tal Band, Adv.
S. Horowitz & Co.
31 Ahad Haam Street
Tel Aviv 65202

Dear Tal,

re: **Opposition to Registration of Patent No. 172563 – Additional Evidence by the Opponent**

I have received your letter of February 24, 2011.

Your client devotes most of its efforts to dragging the proceeding out in really unprecedented fashion.

To begin with it split up its evidence and submitted additional evidence many months after the date for submission of its evidence had passed.

Thereafter, it gave its additional evidence without laboratory notebooks in support of the experiments it had conducted.

When your client eventually condescended to delivering the laboratory notebooks subsequent to my client's request, these were partial and deficient.

Later on, my client was compelled to conduct a lengthy correspondence with your client in connection with its request for the supplementing of the missing material.

Only after it had procrastinated for about two additional months from the time of my first letter, precisely before the time for filing of the evidence in reply on behalf of my client, did your client eventually see fit to deliver up the missing material.

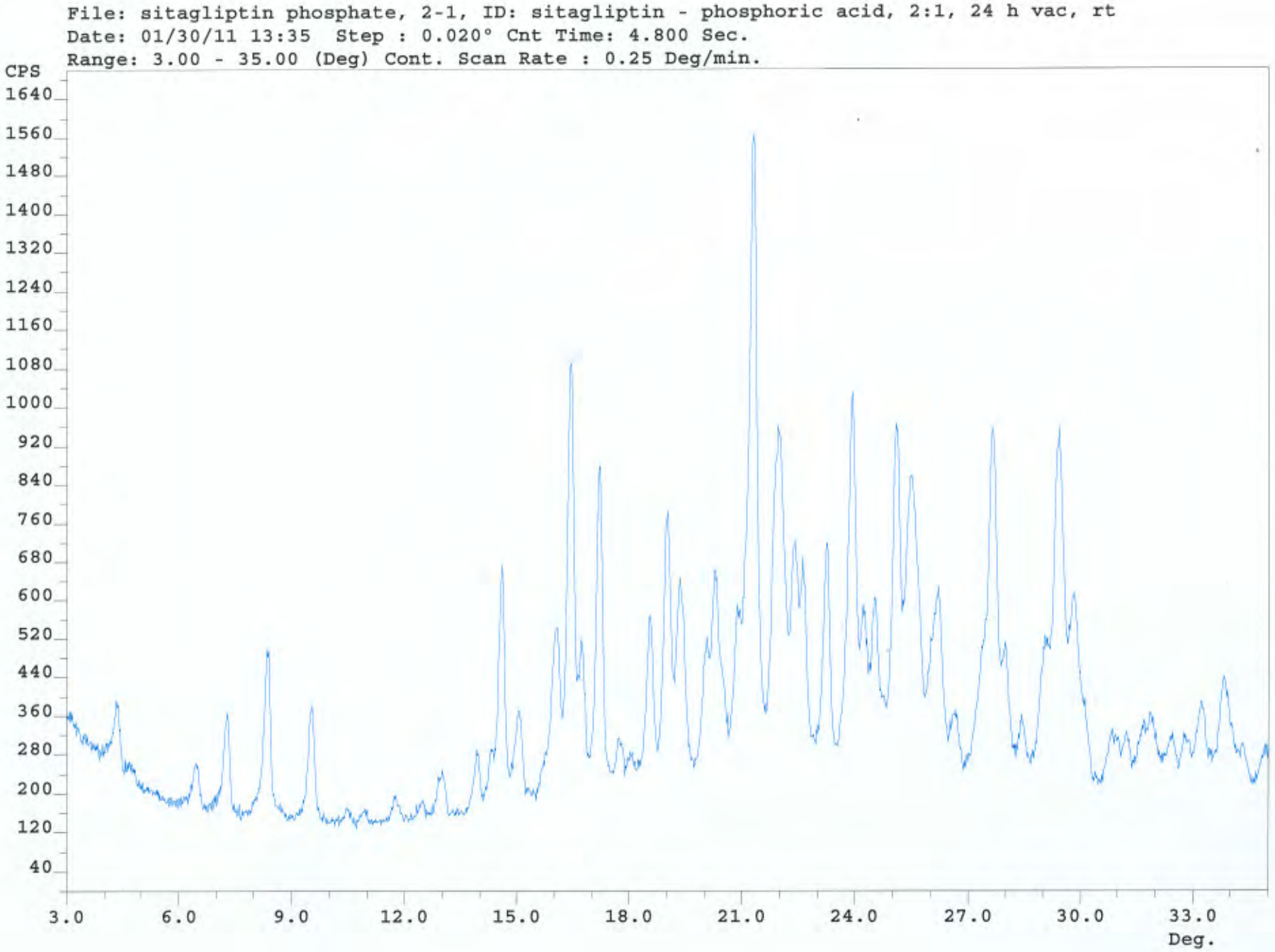
And this time, too, it revealed a little and covered up twice as much...

My client is now in the midst of examining the material. If an extension of time is required, my client will make an appropriate application to the Office. In the circumstances of the case, in which your client delayed for a protracted period of time in delivering the material that ought to have been attached to its evidence in the first place, the responsibility for any extension that may be needed, if needed, is placed squarely and solely on your client's shoulders and is not subject to your client's consent.

Nothing in the foregoing derogates from any of the Applicant's arguments, including with regard to your client's improper conduct and behavior and the impact thereof on the evidentiary weight of the experiments which it submitted.

Sincerely
(-)
Liad Whatstein, Adv.

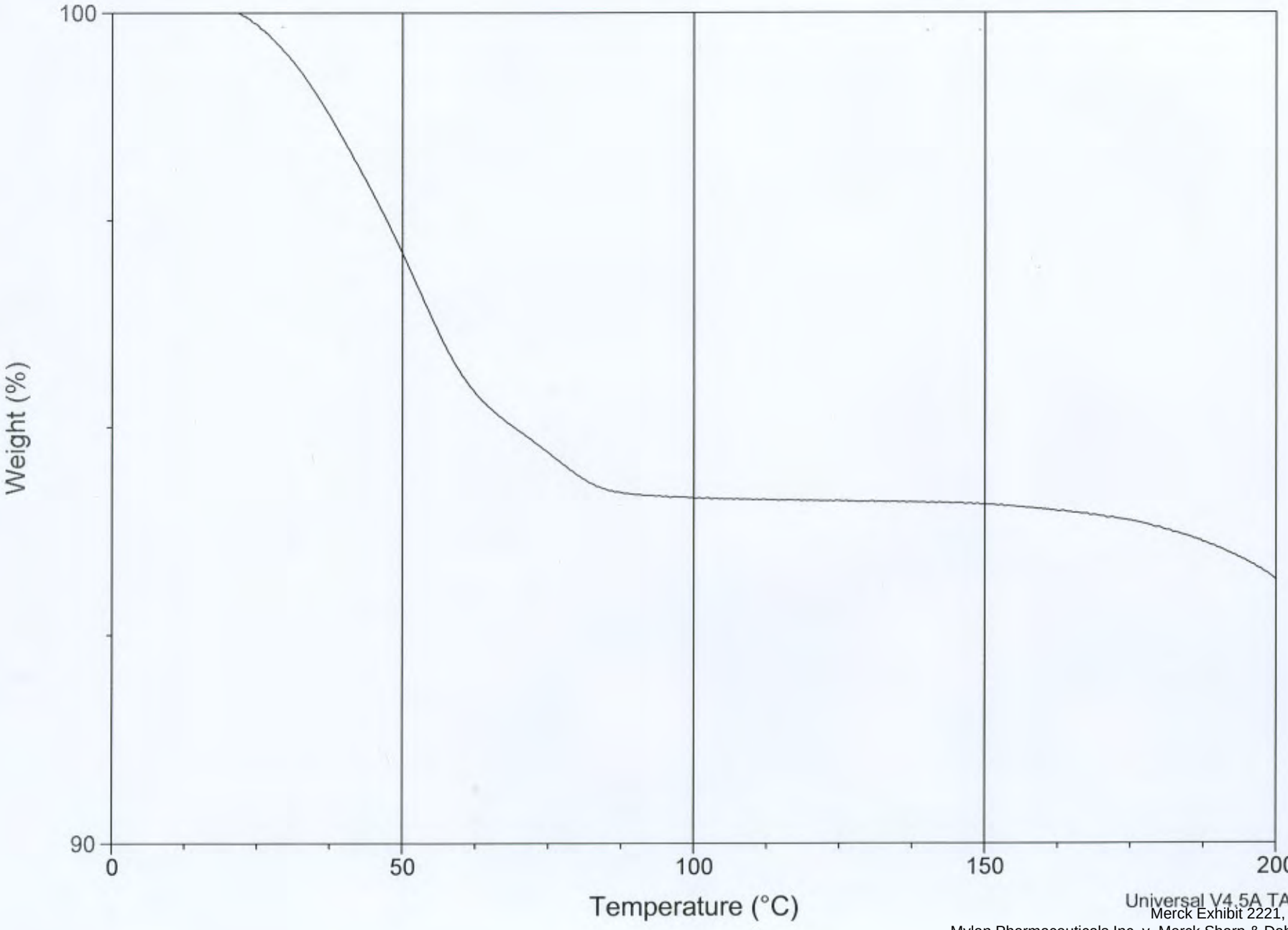
(5151)


File: sitagliptin phosphate, 2-1, ID: sitagliptin - phosphoric acid, 2:1, 24 h vac, rt
Date: 01/30/11 13:35 Step : 0.020° Cnt Time: 4.800 Sec.
Range: 3.00 - 35.00 (Deg) Cont. Scan Rate : 0.25 Deg/min.
CPS
1640
1560
1480
1400
1320
1240
1160
1080
1000
920
840
760
680
600
520
440
360
280
200
120
40
3.0
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0
30.0
33.0
Deg.

Sample: sita2(H3PO4, off xrpd
Size: 3.5970 mg
Method: Ramp

TGA

File: C:\TA\Data\TGA\Atwood\(sita)2H3PO4.001
Operator: ~~Drew~~
Run Date: 30-Jan-2011 19:18
Instrument: TGA Q50 V20.10 Build 36

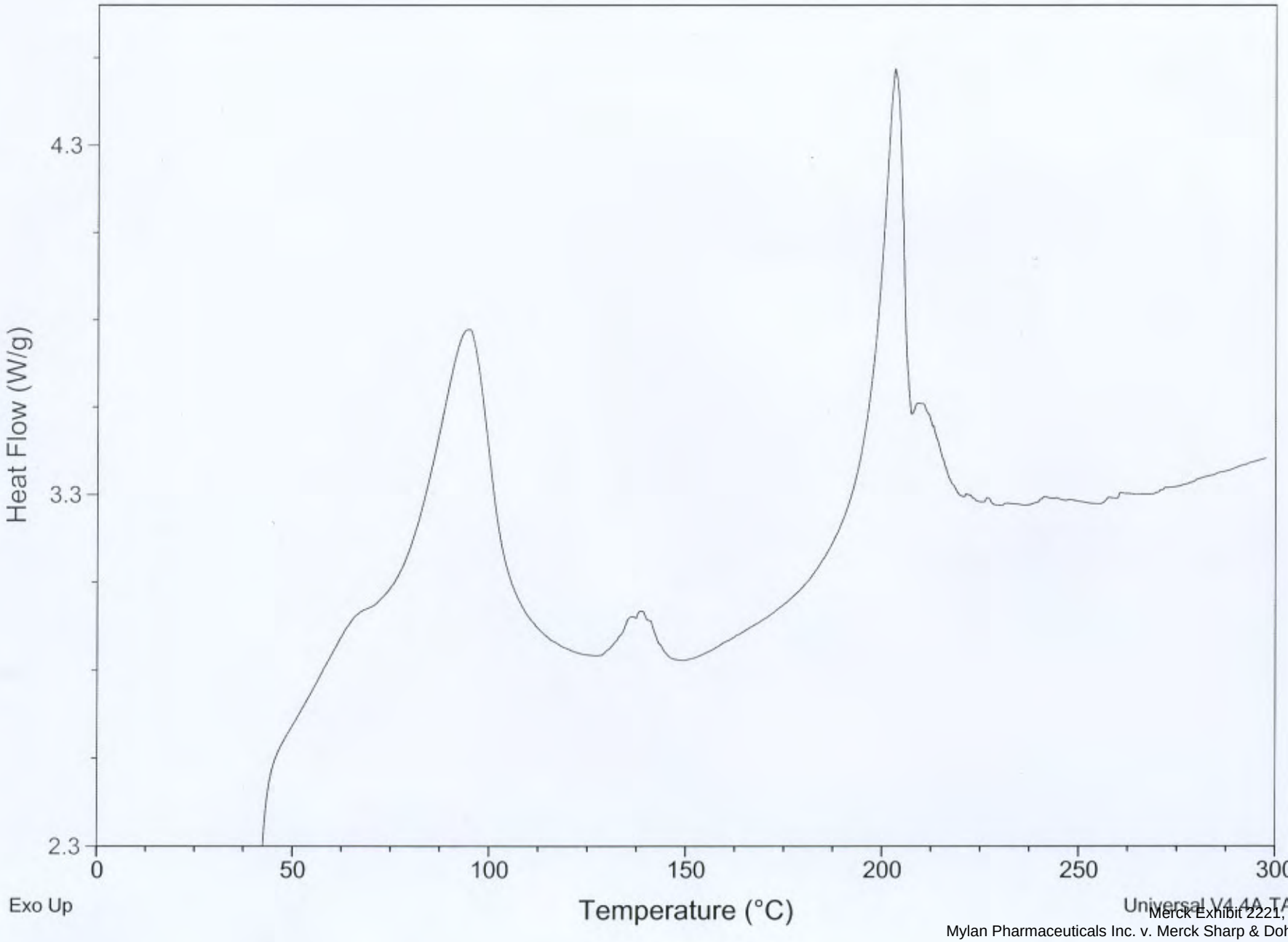


Universal V4.5A TA Instruments

Sample: sita2+H3PO4, off xrpd
Size: 4.5000 mg
Method: Ramp

DSC

File: C:\TA\Data\Atwood\sita.063
Operator: Atwood
Run Date: 30-Jan-2011 19:15
Instrument: DSC Q100 V9.8 Build 296



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1705 U.S. Highway 46 / Suite 1D/ Ledgewood, NJ 07852 / (973) 966-6668 / Fax: (973) 966-0136
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Certificate of Analysis

Company: Merck & Company

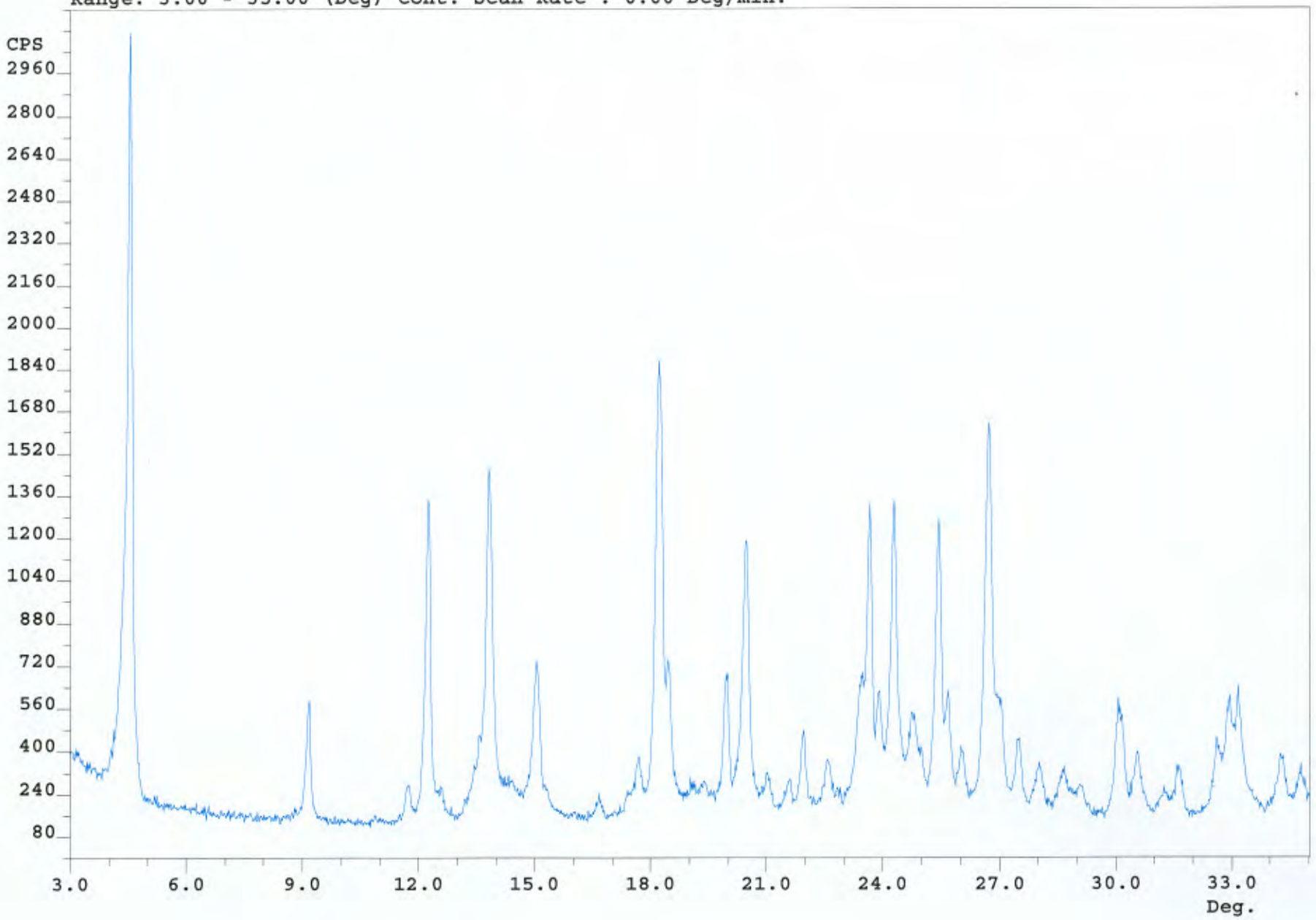
Submitter: Wang, Tiebang Sample ID.: M601 S9 Date of Analysis: 3/4/2011

Analysis	Result
Test #: 1	
C	39.80 %
H	3.93 %
N	14.51 %
Na	364 ppm
P	3.46 %

Comments: Date Received 02/25/2011

Reference: MERC01	Authorized Signature: Mike Hatoleski
	Date: 4/15/2011

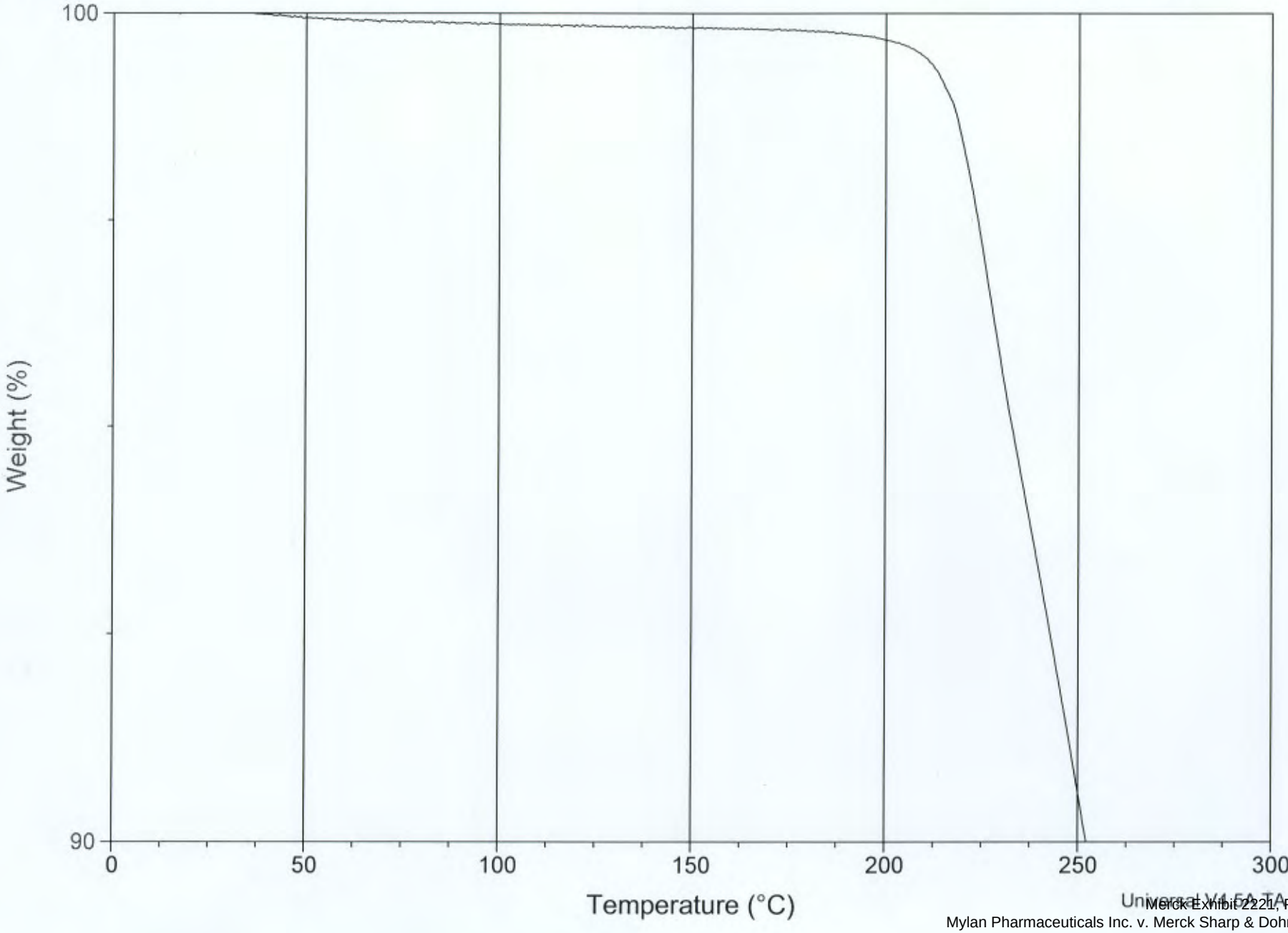
File: chyall test, ID: sita2(HPO4), slurried with MeOH as per Chyall
Date: 02/10/11 13:07 Step : 0.020° Cnt Time: 2.000 Sec.
Range: 3.00 - 35.00 (Deg) Cont. Scan Rate : 0.60 Deg/min.



Sample: chyall test
Size: 3.7510 mg
Method: Ramp
Comment: off xrpd

TGA

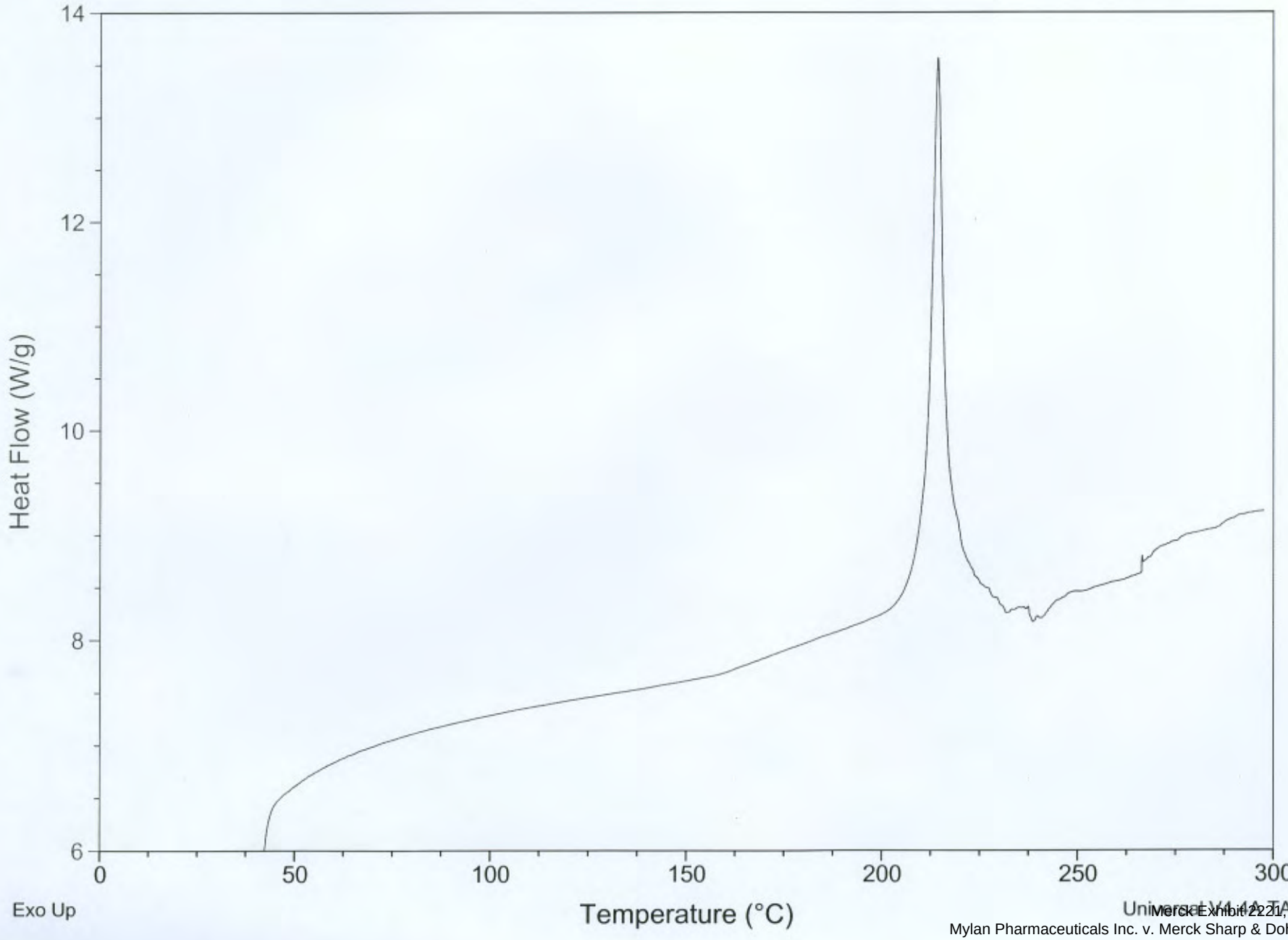
File: C:\TA\Data\TGA\Atwood\chyall.001
Operator: Drew
Run Date: 10-Feb-2011 15:51
Instrument: TGA Q50 V20.10 Build 36



Sample: chyall test, off xrpd
Size: 1.7000 mg
Method: Ramp

DSC

File: C:\TA\Data\Atwood\chyall.001
Operator: Atwood
Run Date: 10-Feb-2011 15:44
Instrument: DSC Q100 V9.8 Build 296



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Certificate of Analysis

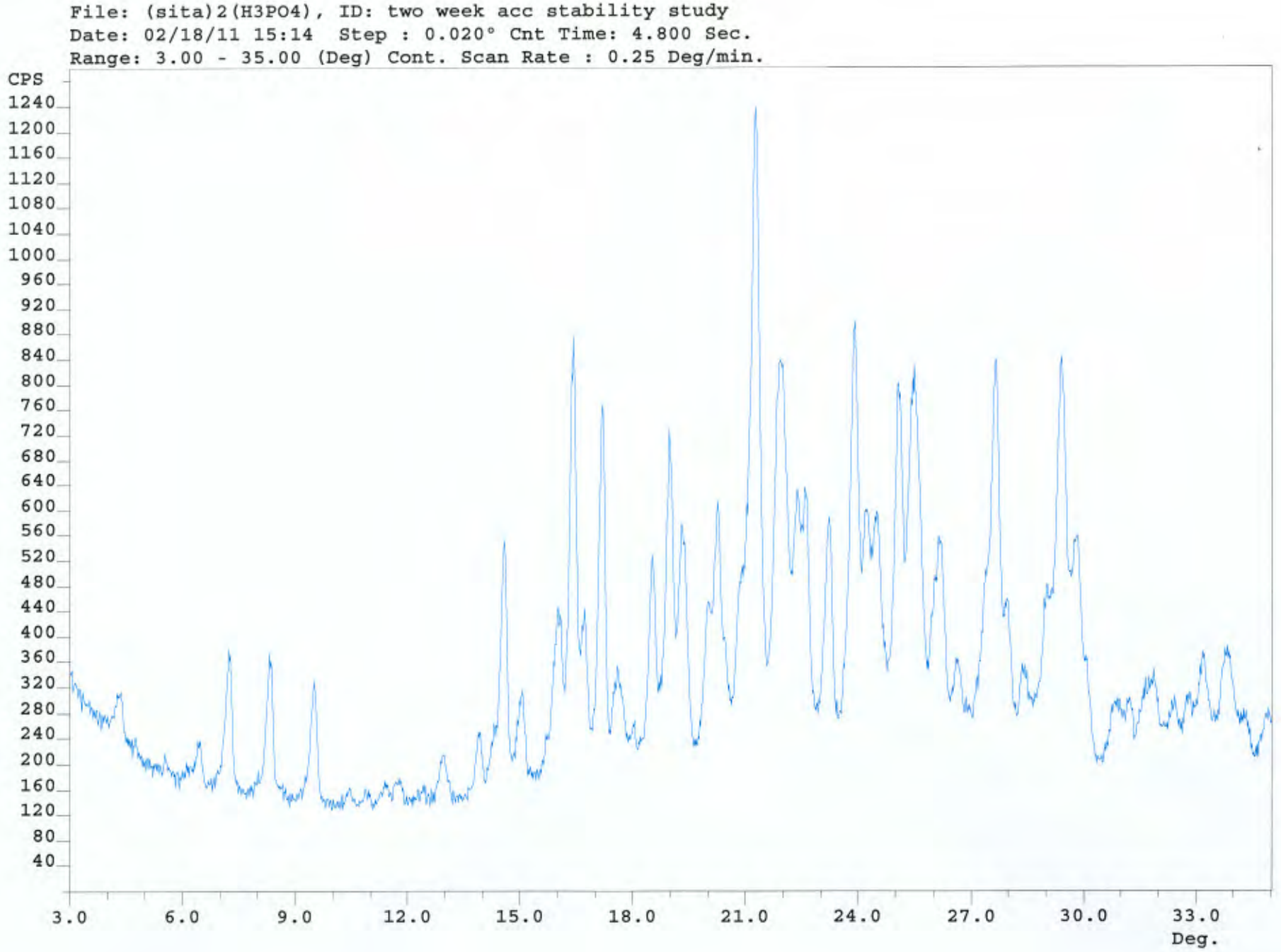
Company: Merck & Company

Submitter: Wang, Tiebang Sample ID.: M603 S11 Date of Analysis: 3/4/2011

Analysis	Result
Test #: 1	
C	38.42 %
H	3.57 %
N	14.04 %
Na	19 ppm
P	5.67 %

Comments: Date Received 02/25/2011

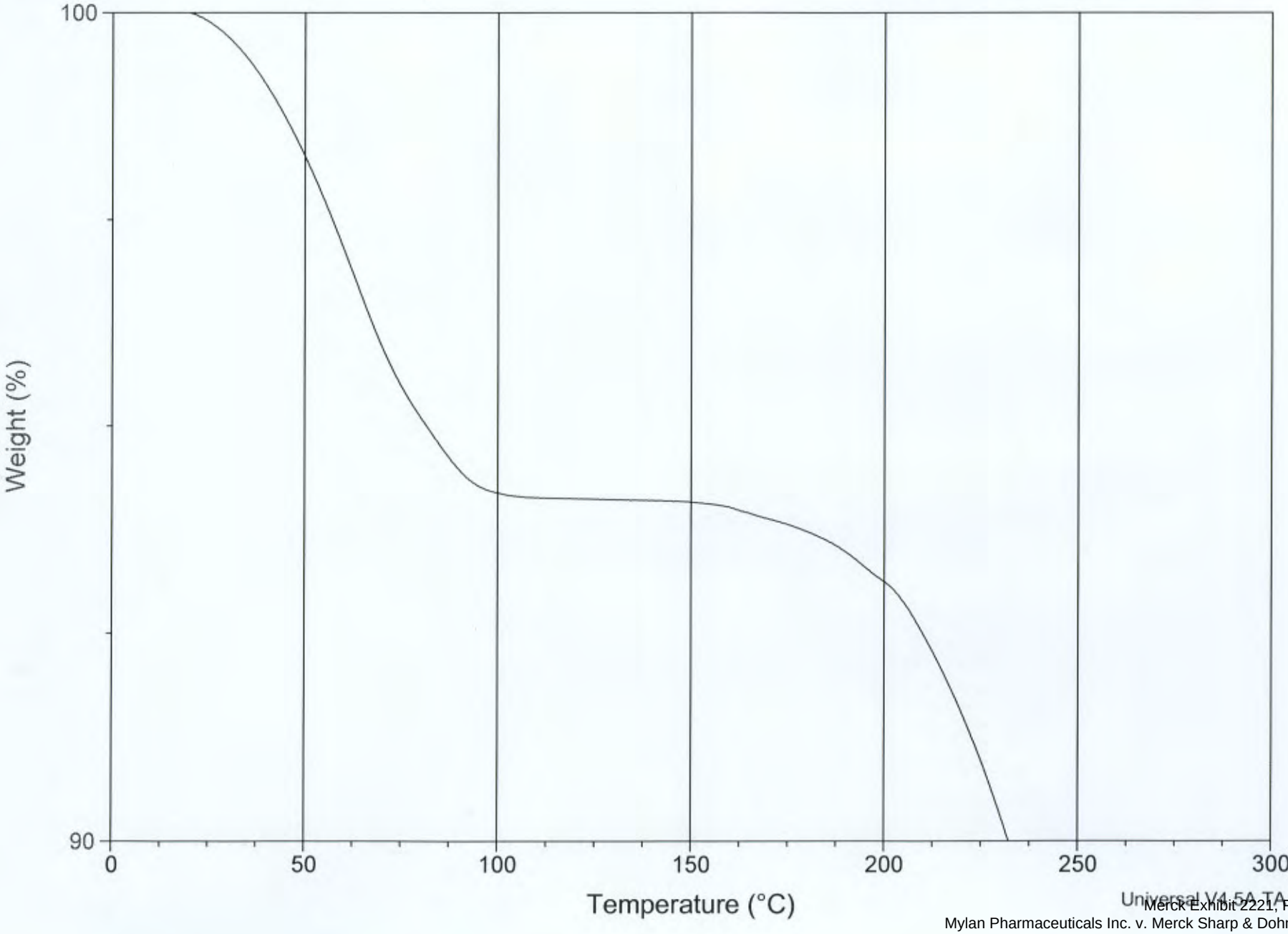
Reference: MEROOI	Authorized Signature: Mike Hatolek
	Date: 4/15/2011


File: (sita)2(H3PO4), ID: two week acc stability study
Date: 02/18/11 15:14 Step : 0.020° Cnt Time: 4.800 Sec.
Range: 3.00 - 35.00 (Deg) Cont. Scan Rate : 0.25 Deg/min.
CPS
1240
1200
1160
1120
1080
1040
1000
960
920
880
840
800
760
720
680
640
600
560
520
480
440
400
360
320
280
240
200
160
120
80
40
3.0
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0
30.0
33.0
Deg.

Sample: sita2(H3PO4, 2 wk acc study
Size: 7.1740 mg
Method: Ramp

TGA

File: C:\TA\Data\TGA\Atwood\sita.004
Operator: Drew
Run Date: 18-Feb-2011 15:26
Instrument: TGA Q50 V20.10 Build 36

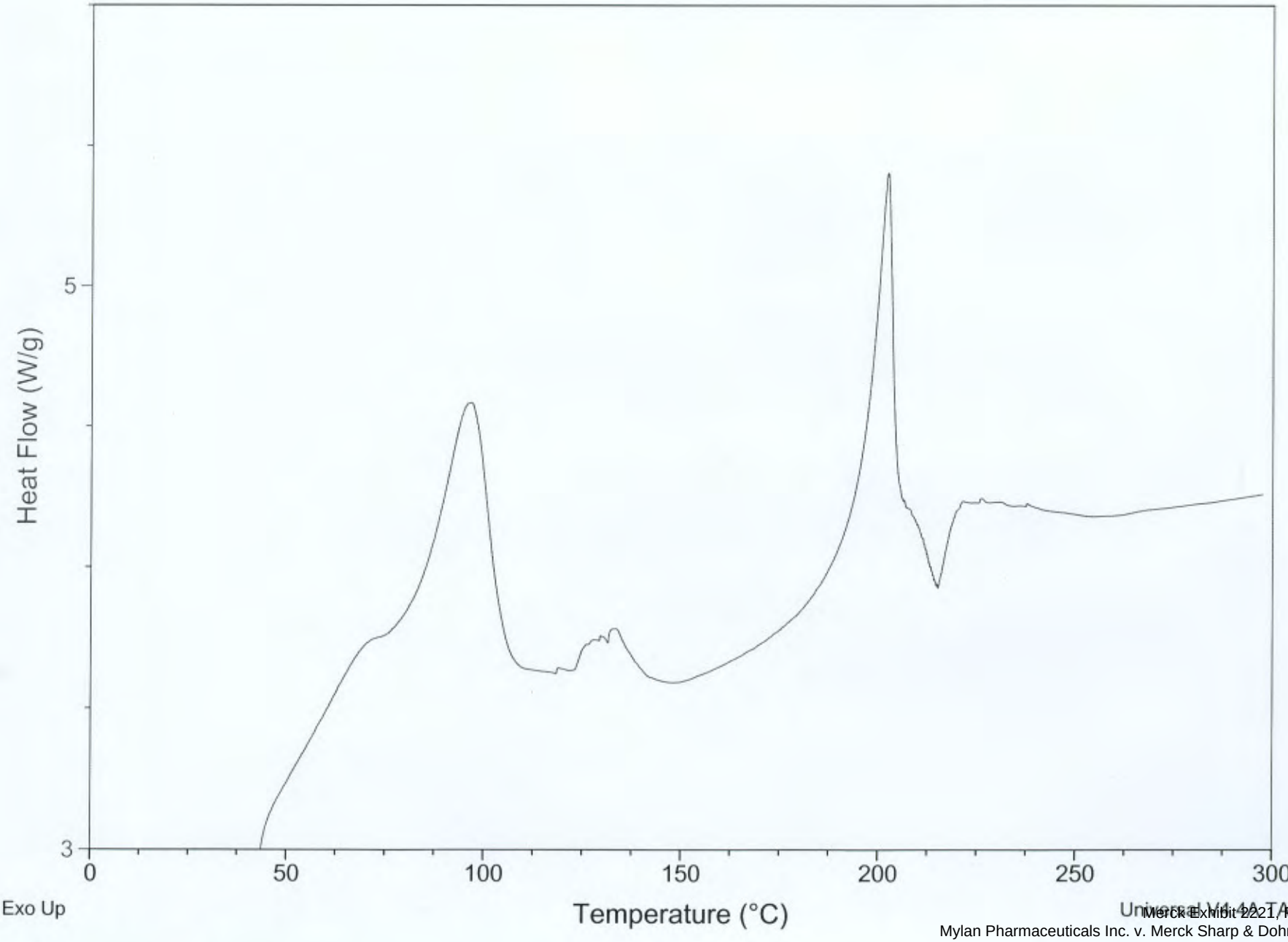


Universal V4.5A TA Instruments

Sample: sita2(H3PO4), 2 WK acc
Size: 3.5000 mg
Method: Ramp

DSC

File: C:\TA\Data\Atwood\sita.002
Operator: Atwood
Run Date: 18-Feb-2011 15:23
Instrument: DSC Q100 V9.8 Build 296



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Certificate of Analysis

Company: Merck & Company

Submitter: Wang, Tiebang Sample ID.: M602 S10 Date of Analysis: 3/4/2011

Analysis	Result
Test #: 1	
C	39.34 %
H	4.20 %
N	14.29 %
Na	344 ppm
P	3.31 %

Comments: Date Received 02/25/2011

Reference: MERC051	Authorized Signature: Mike Hatolski
	Date: 4/15/2011