

## Supplementary Material

### Identification, synthesis and characterization of novel Ensifentrine impurities

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Email: [r.dandela@iocb.ictmumbai.edu.in](mailto:r.dandela@iocb.ictmumbai.edu.in)

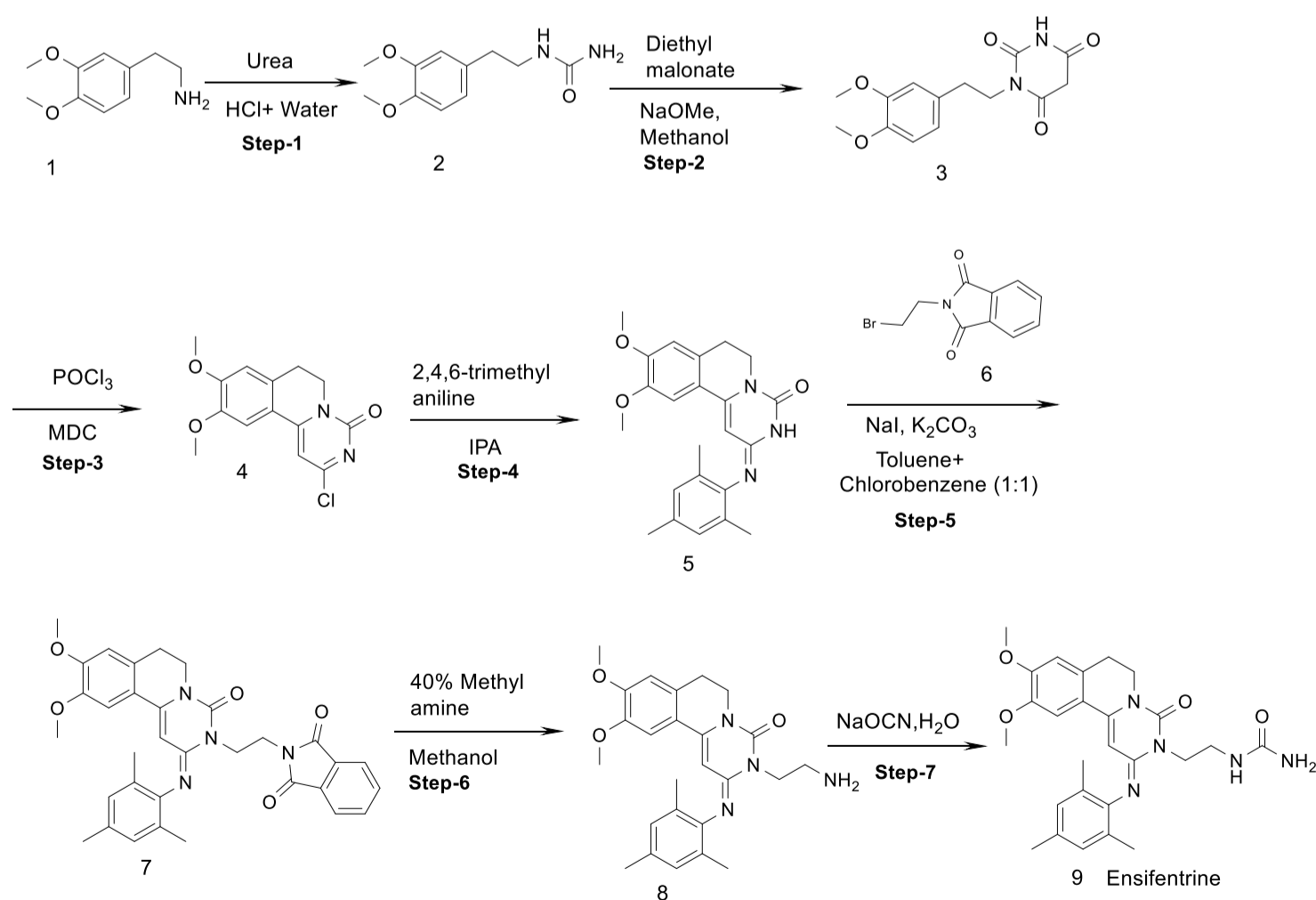
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### General Information

The samples used in this study were synthesized in Chemsar Laboratory. ENF N-alkyl Ensifentrine impurity, ENF N-methyl amide impurity and ENF Carboxylic acid impurity were synthesized and then purified using preparative HPLC if required. All the HPLC- grade solvents, NMR solvents, and reagents were procured from Merck Life Sciences, India. A Waters (LC2695) HPLC system was used for the present study using a PDA-2996 detector set at 260 nm. Empower 2 software was used to process the HPLC data. The Epic C-18 (PerkinElmer, 250 mm × 4.6 mm, 5 μm) analytical column was used to perform the analysis. Mobile phase A was 10 mM ammonium acetate, and mobile phase B was methanol/acetonitrile (50:50, v/v). The linear gradient program was set as follows: T<sub>min</sub>/B(mL/min); T 0/10; T15/ 90; T25/90; T26/10; T30/10. The flow rate was set at 1.0 mL/min, and the injection volume was 10 μL. The homogeneous mixture of HPLC-grade water and HPLC- grade methanol in the ratio 1:1 was used as a diluent for sample preparation. LC–MS analysis of the degraded sample of Ensifentrine was done on a Waters 2695 (Water Corporation) ACQUITY HPLC-MS system. The EPIC C18 column (4.6 × 150 mm, 3 μm) was used as an analytical column for chromatographic separation. The wavelength of the UV detector was set at 260 nm. Mobile phase A consisted of 0.1% formic acid. Mobile phase B consisted of HPLC-grade acetonitrile. The flow rate was set at 0.8 mL per min, and the column oven temperature was set at 25 °C. The cone voltage was 30 V, and the capillary voltage was 3.5 kV. The source temperature was maintained at 120 °C. Nitrogen gas was used as both the desolation and cone gases with flow rates of 350 L/h and 50 L/h, respectively. The linear gradient program was set as follows: T<sub>min</sub>/B (%) T 0/10; T15/90; T25/90; T26/10; T30/10. Purification of ENF carboxylic acid impurity was done from the enriched samples obtained from the reaction of deprotection of ENF phthalimide compound (7) using the described procedure mentioned in Scheme 1. The required impurity peak was isolated using the Waters 2545 preparative HPLC system equipped with the Phenomenex C18 column (250 × 20 mm, 10 μm), and the PDA2996 detector was set at 260 nm. Mass Lynx software was used to process the data. Ammonium and the run time was 40 min. The linear gradient program was set as follows: T<sub>min</sub>/B(mL/min): T0/20; T5/30; T15/40; T20/50; T25/80; T35/90; T40/100. The ENF carboxylic acid impurity obtained from the preparative HPLC fraction was purified heat-cool, heat-cool method in acetonitrile to get pure desired impurity as a pale-yellow solid. The isolated sample was further used for its complete characterization. NMR spectra were recorded on a Bruker Avance 400 spectrometer (<sup>1</sup>H at 400 MHz and <sup>13</sup>C at 100 MHz). Chemical shifts for <sup>1</sup>H NMR spectra are reported in parts per million (ppm) relative to tetramethylsilane, with the solvent resonance as the internal standard (CDCl<sub>3</sub>: δ 7.26 ppm). Similarly, <sup>13</sup>C NMR spectra are reported in ppm relative to tetramethylsilane, using the solvent as the internal standard (CDCl<sub>3</sub>: δ 77.0 ppm).

### 1. Chemical Synthesis of Ensifentrine



## 2. Characterization Data

**2.1. ENF N-methyl amide impurity, (  $N^1$ -{2-[(*E*)-9,10-dimethoxy-4-oxo-2-[(2,4,6-trimethylphenyl)imino]-6,7-dihydro-2*H*-pyrimido[6,1- $\alpha$ ]isoquinolin-3(4*H*)-yl]ethyl}-*N*<sup>2</sup>-methylbenzene-1,2-dicarboxamide):** 10 g Phthalimide compound (7) was taken in 70 ml of methanol. To that 40% aqueous methyl amine solution was added at room temperature. Reaction mass was stirred for 4 hours at 45 °C. Reaction mass was then quenched by 40 ml Conc. HCl. Formed precipitate was filtered and washed with 10 ml methanol. Filtrate was distilled and residue was purified by flash column chromatography to obtain 0.2 g impurity with good purity.

**<sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>)**  $\delta$  8.42 (s, 1H), 8.07 (s, 1H), 7.46 (dd, *J* = 8.2Hz, 2.1 Hz, 4H), 6.96 (s, 1H), 6.85 (s, 2H), 6.67 (d, 1H), 5.34 (s, 1H), 4.34 (t, *J* = 6.4Hz, 2H), 3.93 (t, *J* = 6.8Hz, 2H), 3.80 (s, 3H), 3.61 (broad hump, 5H), 2.90 (t, *J* = 6.8Hz, 2H), 2.70 (d, 3H), 2.22 (s, 3H), 1.98 (s, 6H).

**<sup>13</sup>C NMR (500 MHz, DMSO-*d*<sub>6</sub>)**  $\delta$  168.9, 168.6, 152.1, 151.7, 148.6, 144.5, 142.7, 136.7, 136.7, 131.0, 130.7, 129.7, 129.7, 129.6, 128.8, 128.8, 128.2, 128.2, 128.0, 119.4, 111.8, 109.0, 88.3, 56.5, 41.3, 40.0, 37.1, 27.5, 26.5, 20.9, 18.5. **IR interpretation:** 3288 (N-H Stretching), 2937, 2911, 1636, 1511, 1474, 1436, 1289, 1268, 1033, 986. *m/z* (M + H)<sup>+</sup> calc for C<sub>34</sub>H<sub>38</sub>N<sub>5</sub>O<sub>5</sub>, 595.7002; found 596.2761.

HSQC: Formation of N-Methyl is confirmed by HSQC. Carbon no. 44 shows 3 protons at  $\delta$  2.69 and the same carbon seen at  $\delta$  26.5.

COSY: Neighboring protons of NH (43) at  $\delta$  8.07 and CH<sub>3</sub> (44) at  $\delta$  2.69 correlated and confirmed by COSY.

HMBC: NH (43) at  $\delta$  8.07 and CH<sub>3</sub> (44)  $\delta$  2.69 at are having two bond heteronuclear multiple bond correlation with C (41) 168.9  $\delta$ .

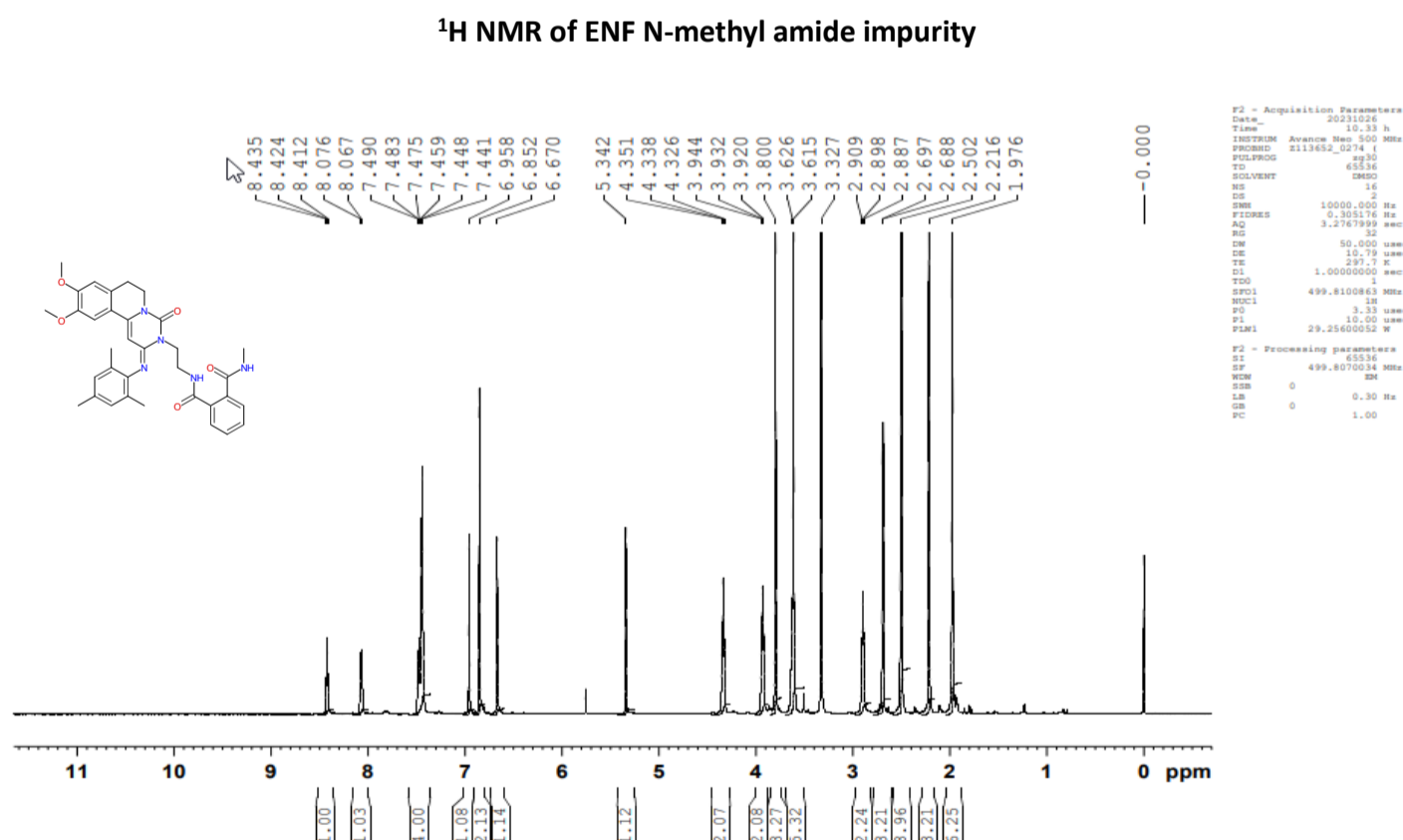
Complete correlation data ( $\delta$ H ppm,  $\delta$ C ppm): (6.58, 108.6), (6.97, 110.7), (6.93, 128.9), (7.07, 119.6), (5.24, 84.9), (3.94, 39.1), (2.89, 26.7), (3.55, 49.2), (3.25, 37.8), (3.59, 55.6), (3.81, 56.3), (2.26, 20.4), (2.03, 17.6).

Quaternary and non-protonated carbons observed at  $\delta$  151.4, 147.7, 118.9, 129.8, 149.7, 155.5, 162.6, 137.2, 134.9, 139.7, 135.8, and 158.7.

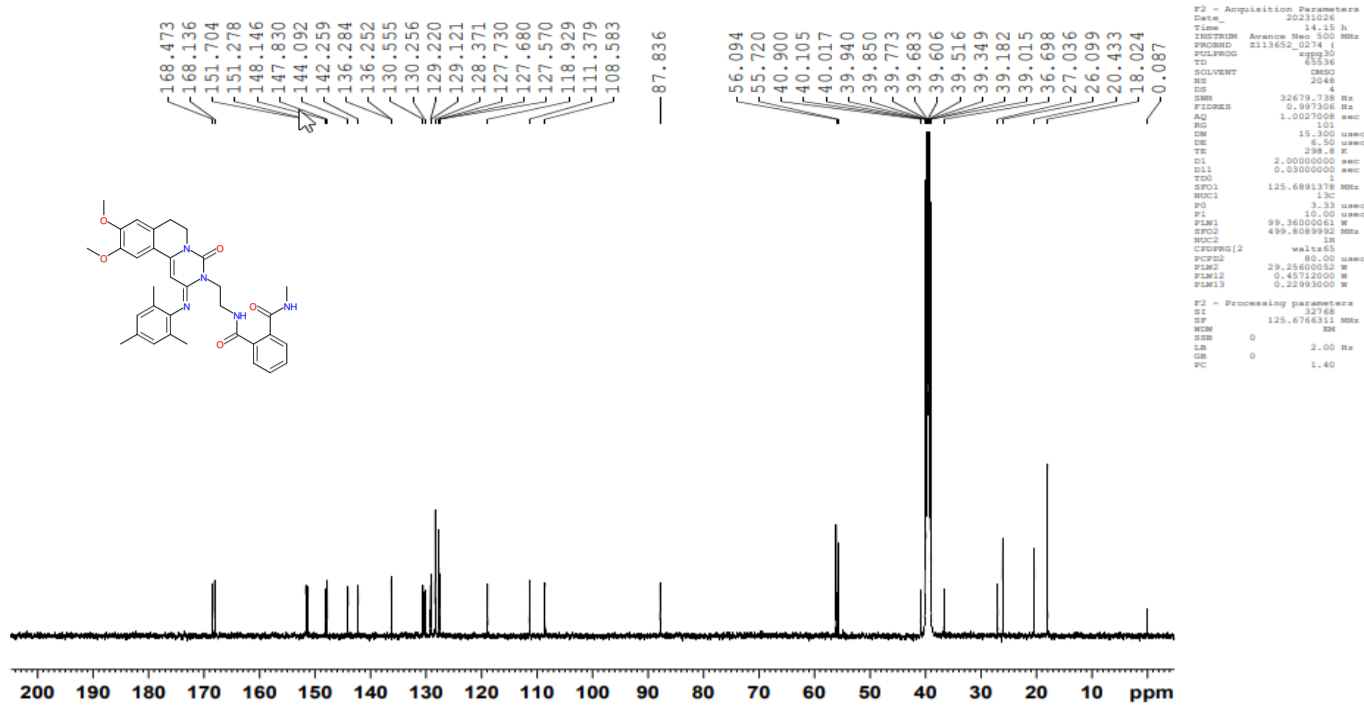
DSC: Melting point peak observed at 134.58°C

HPLC: ENFN-METHYL AMIDE detected at retention time 38.866 min

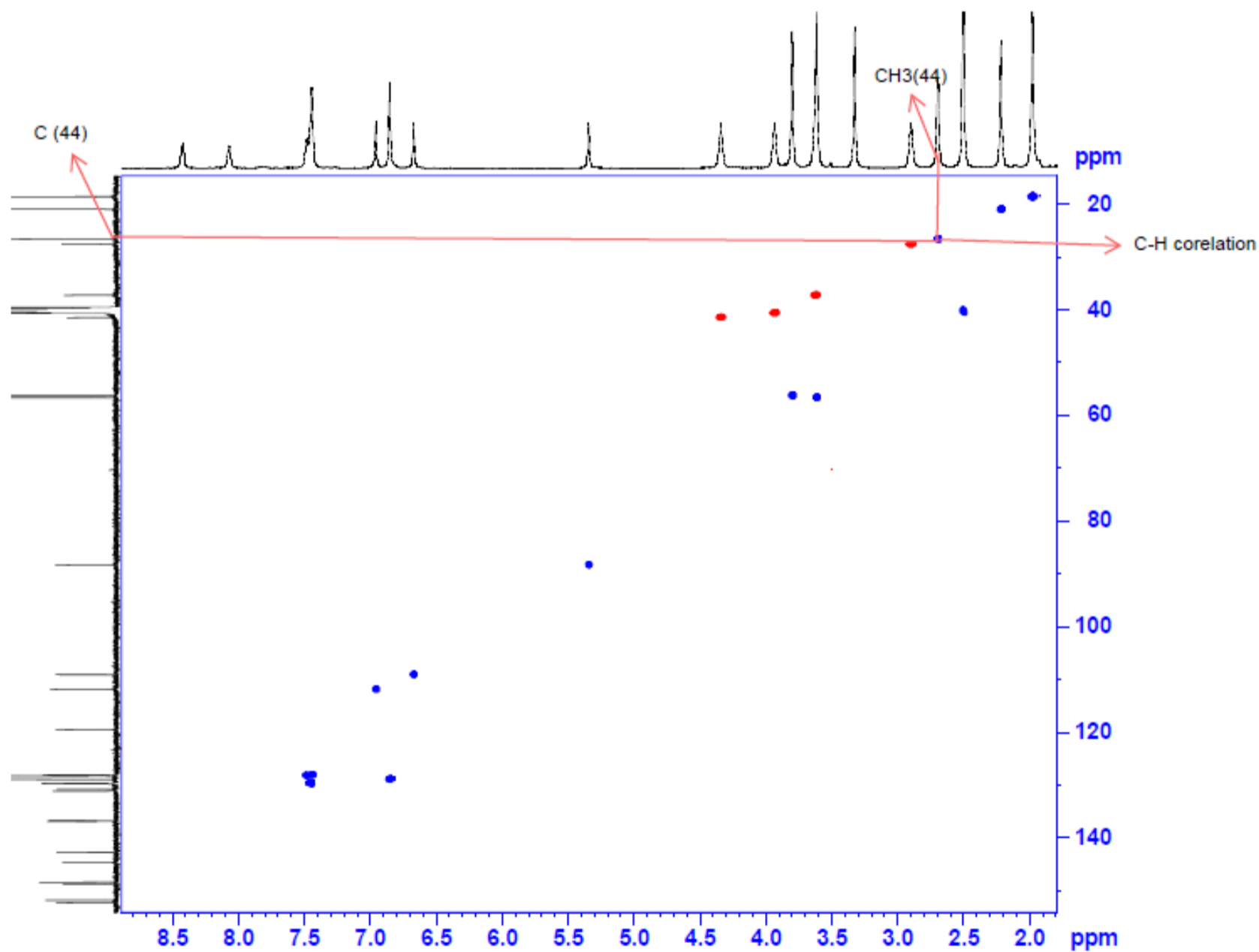
IR and UV spectra confirms the functional group in the impurity.



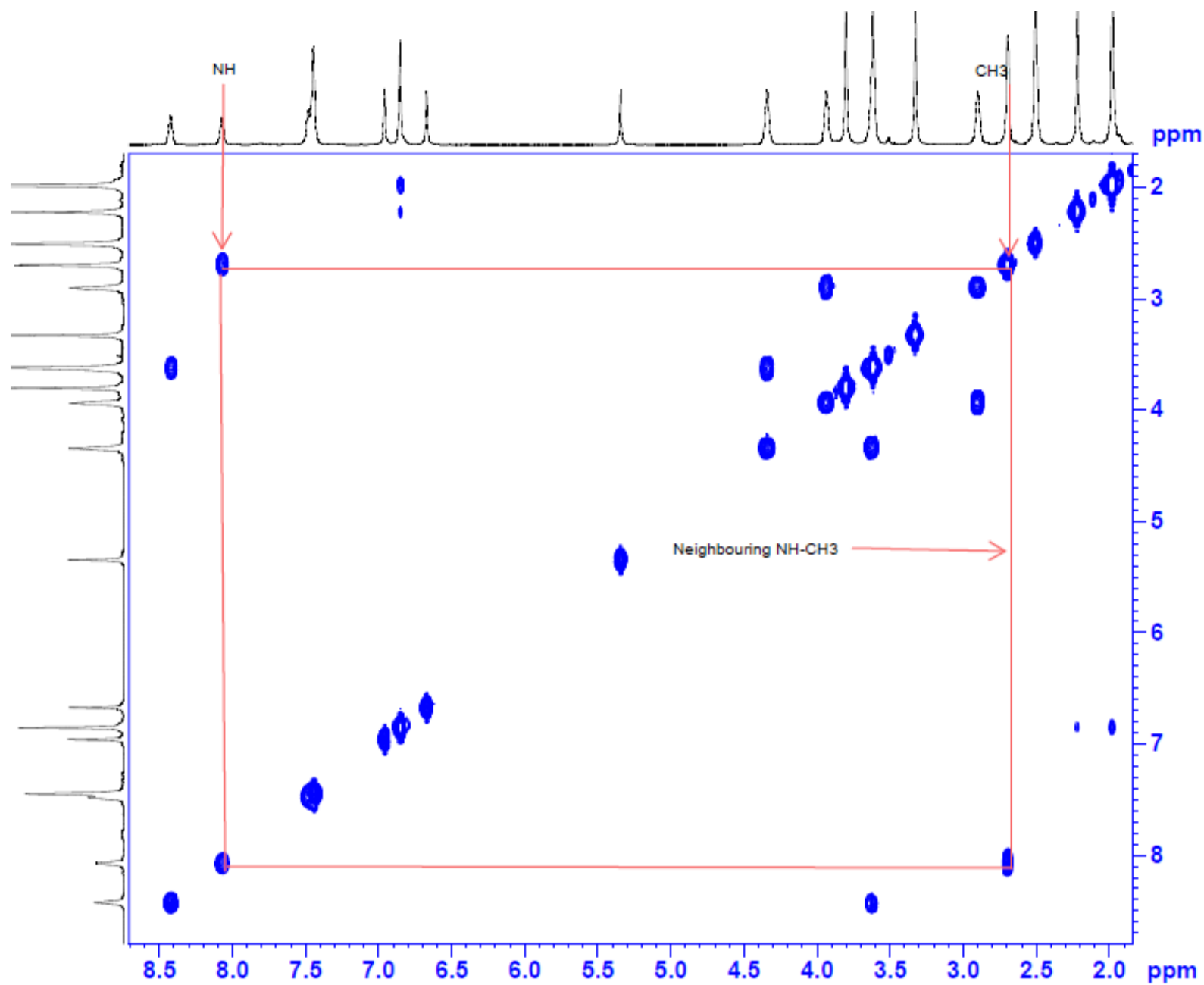
<sup>13</sup>C NMR of ENF N-methyl amide impurity



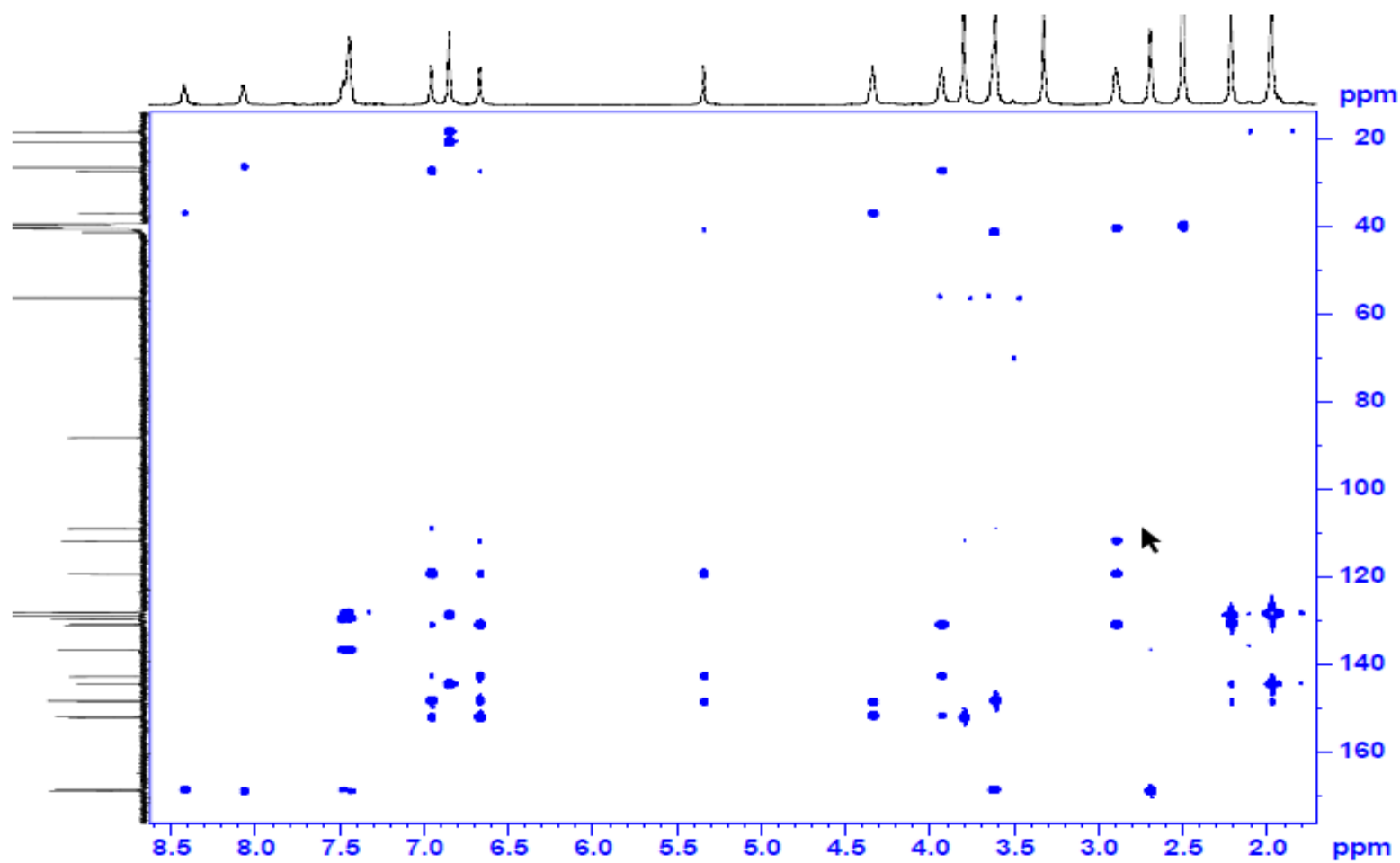
HSQC of ENF N-methyl amide impurity



COSY of ENF N-methyl amide impurity



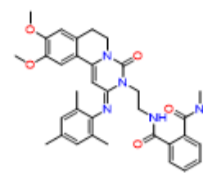
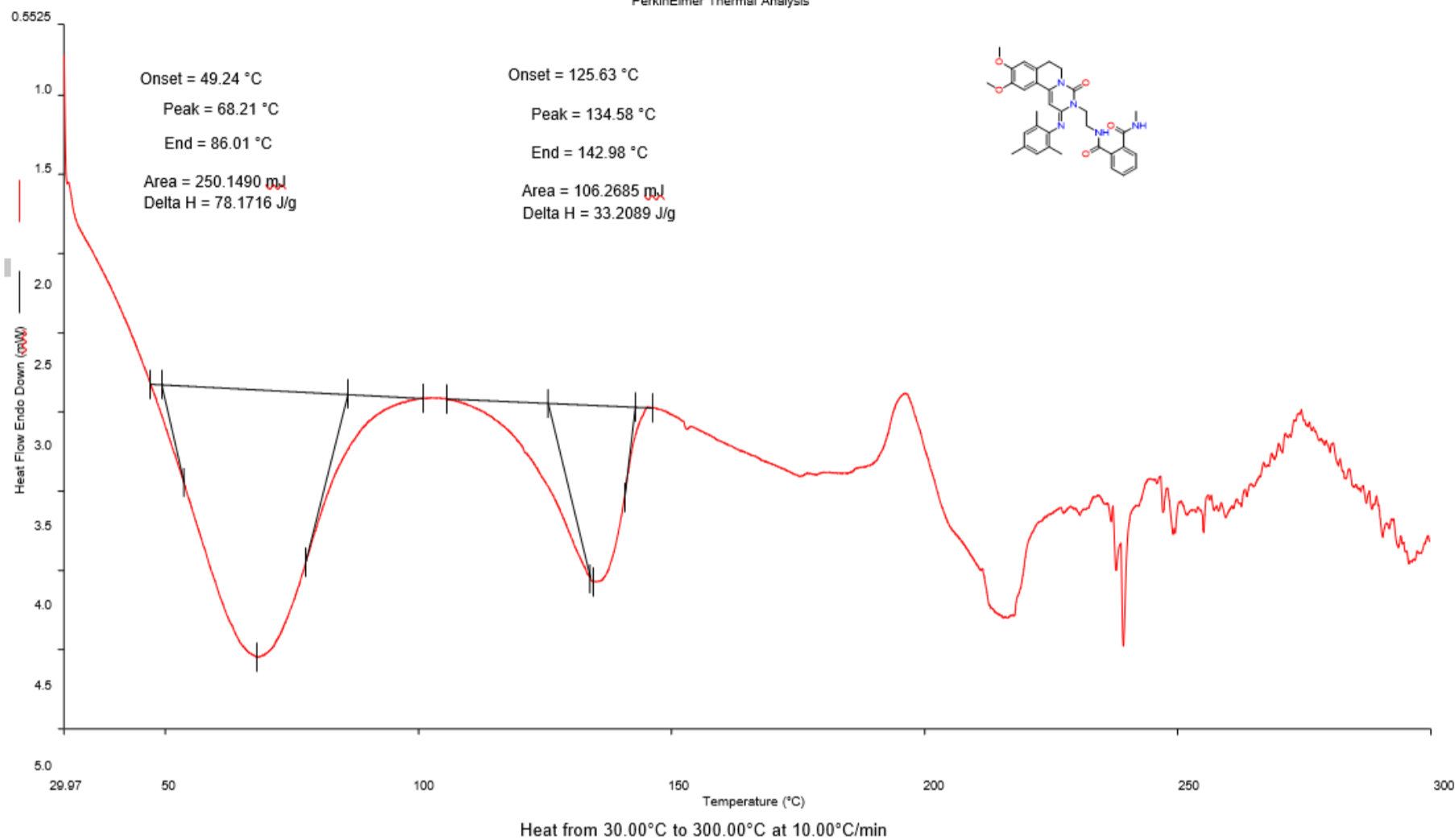
HMBC of ENF N-methyl amide impurity



### DSC Analysis of ENF N-methyl amide impurity

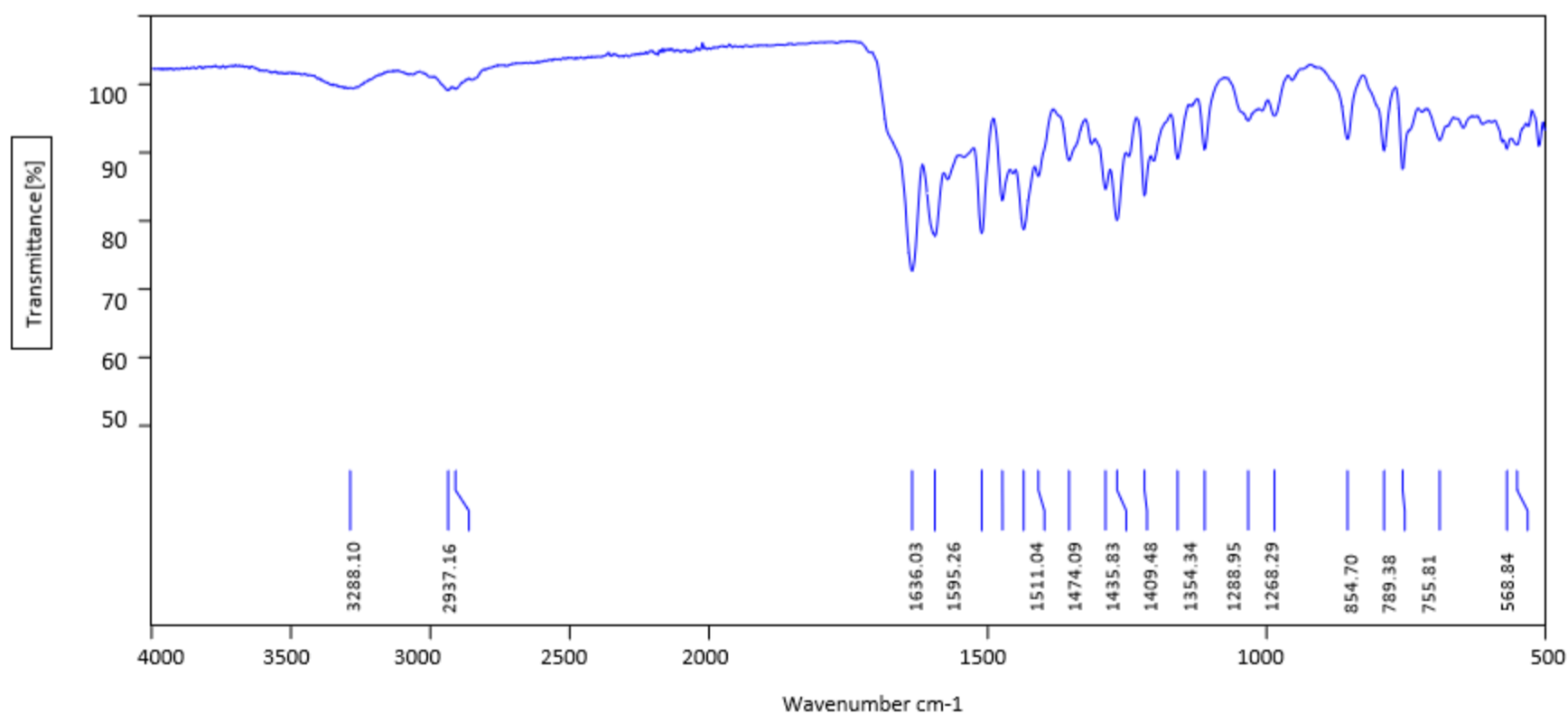
ENF N-methyl amide Impurity  
 Operator ID: Dattatray G  
 Sample Weight: 3.200 mg

PerkinElmer Thermal Analysis



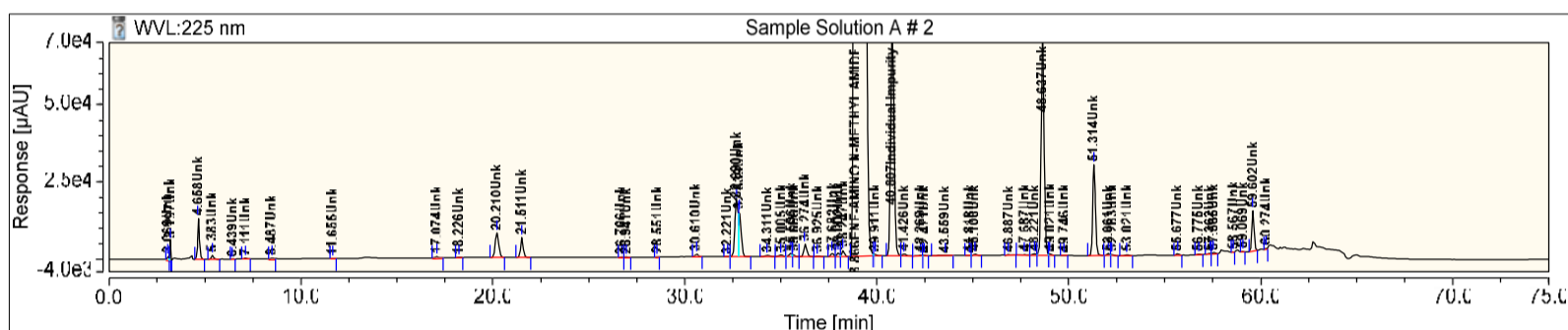
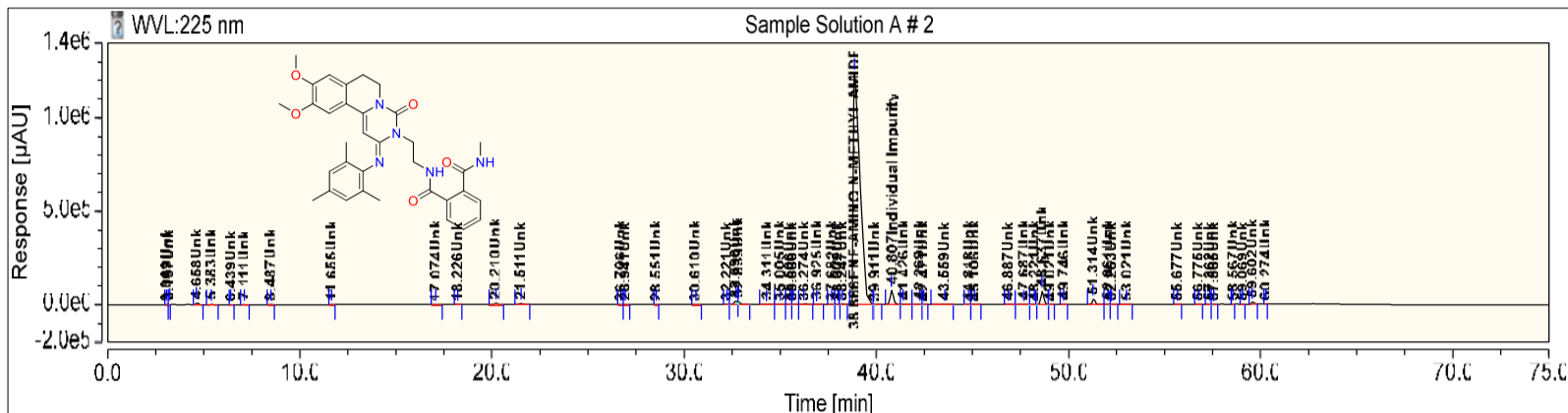
### IR Spectra of ENF N-methyl amide impurity

ENF N-methyl amide impurity



HPLC Analysis of ENF N-methyl amide impurity

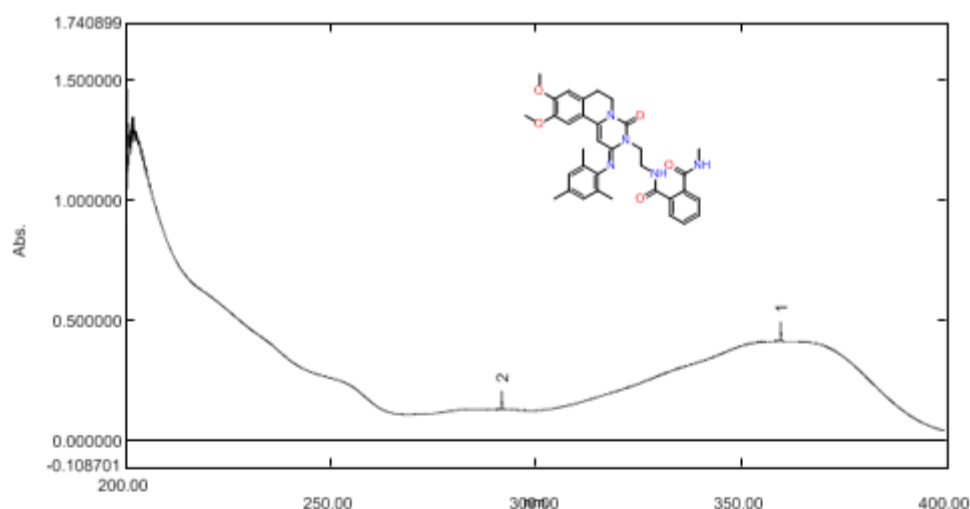
ENF N-methyl amide Impurity  
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 Detector: PDA\_1  
 Injection volume: 10.0 µL  
 Wavelength: 225 nm



UV Spectra of ENF N-methyl amide impurity

Spectrum Measurement Report

Data set: 1068638\_Identification\_SPL\_18 - RawData



Software Information  
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 Version: 2.62  
 Mode: LabSolutions

Data Information  
 Data is:  
 Method: I-003 - Identification By UV\_200\_

Instrument Information  
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 Model (S/N): UV Vis Spectrophotometer (A11454907900)

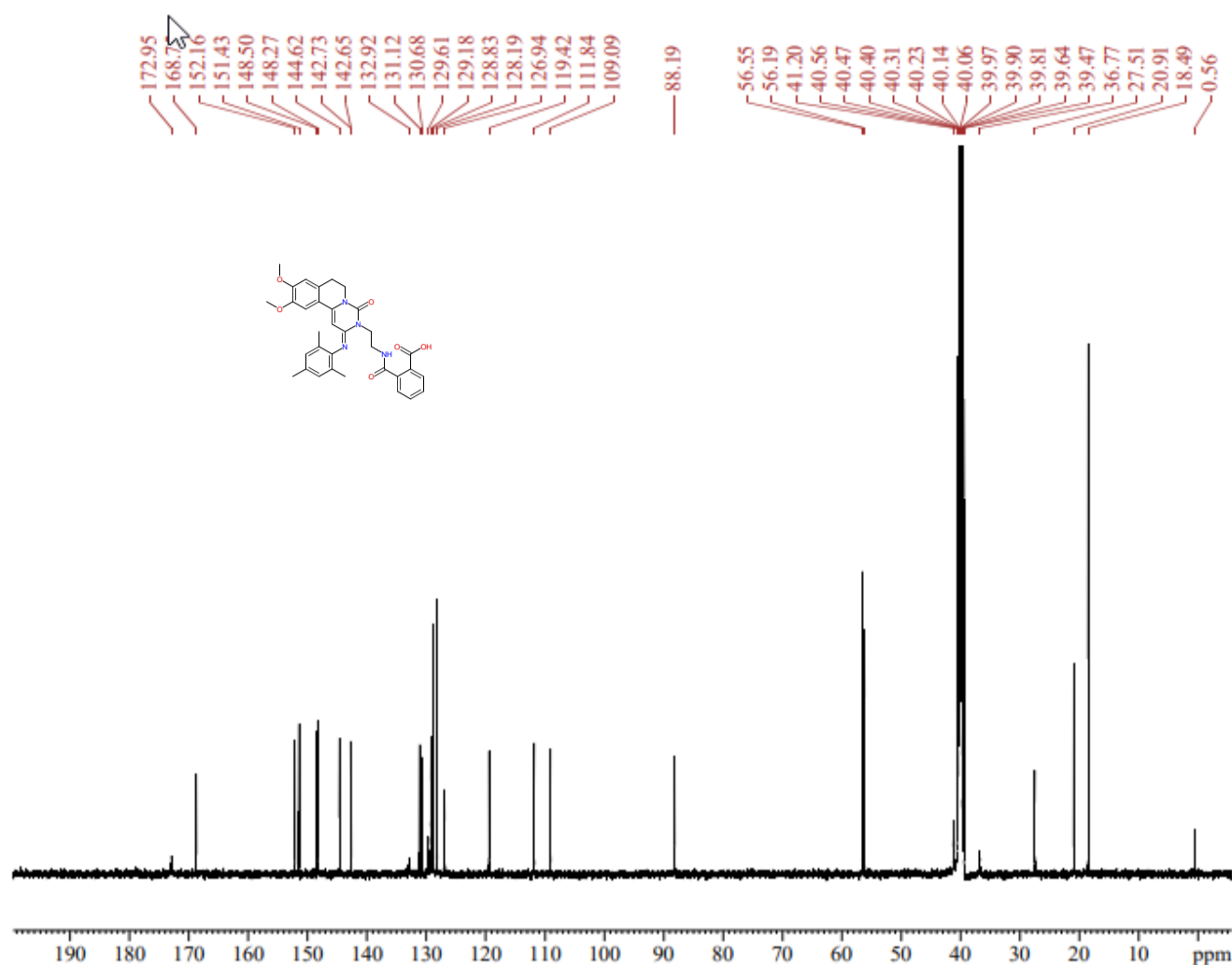
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 Scan Mode: Single

[Instrument Properties]  
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 Measuring Mode: Absorbance  
 Slit Width: 1.0 nm  
 Light Source Change Wavelength: 360.0 nm  
 S/R Exchange: Normal

No.	P/V	Wavelength	Abs.	Description
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<sup>13</sup>C NMR ENF Carboxylic acid Impurity



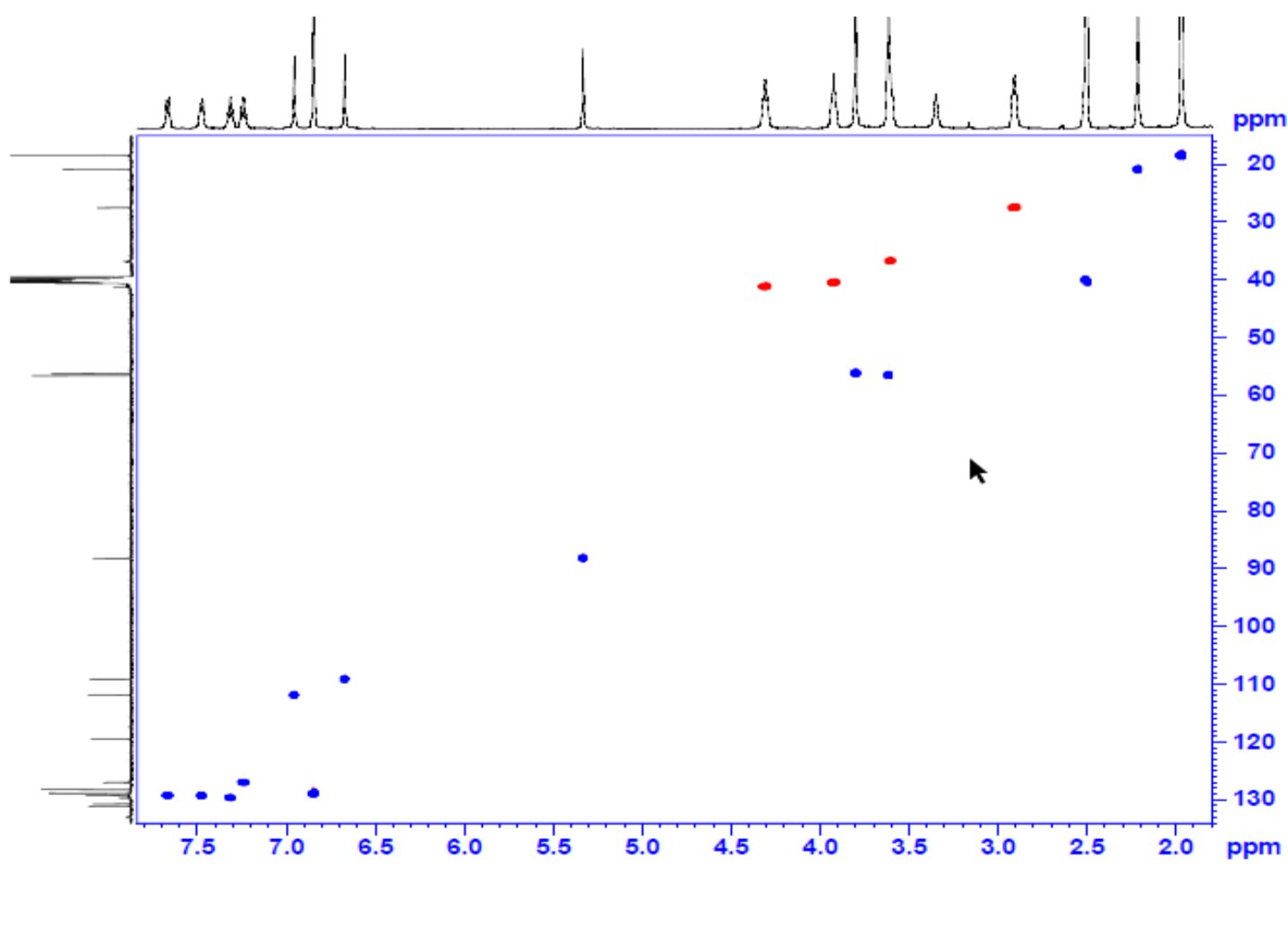
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FIDRES    0.919204 Hz
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DE         6.50 usec
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D11       0.03000000 sec
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HSQC of ENF Carboxylic acid Impurity



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kishor.jadhav1
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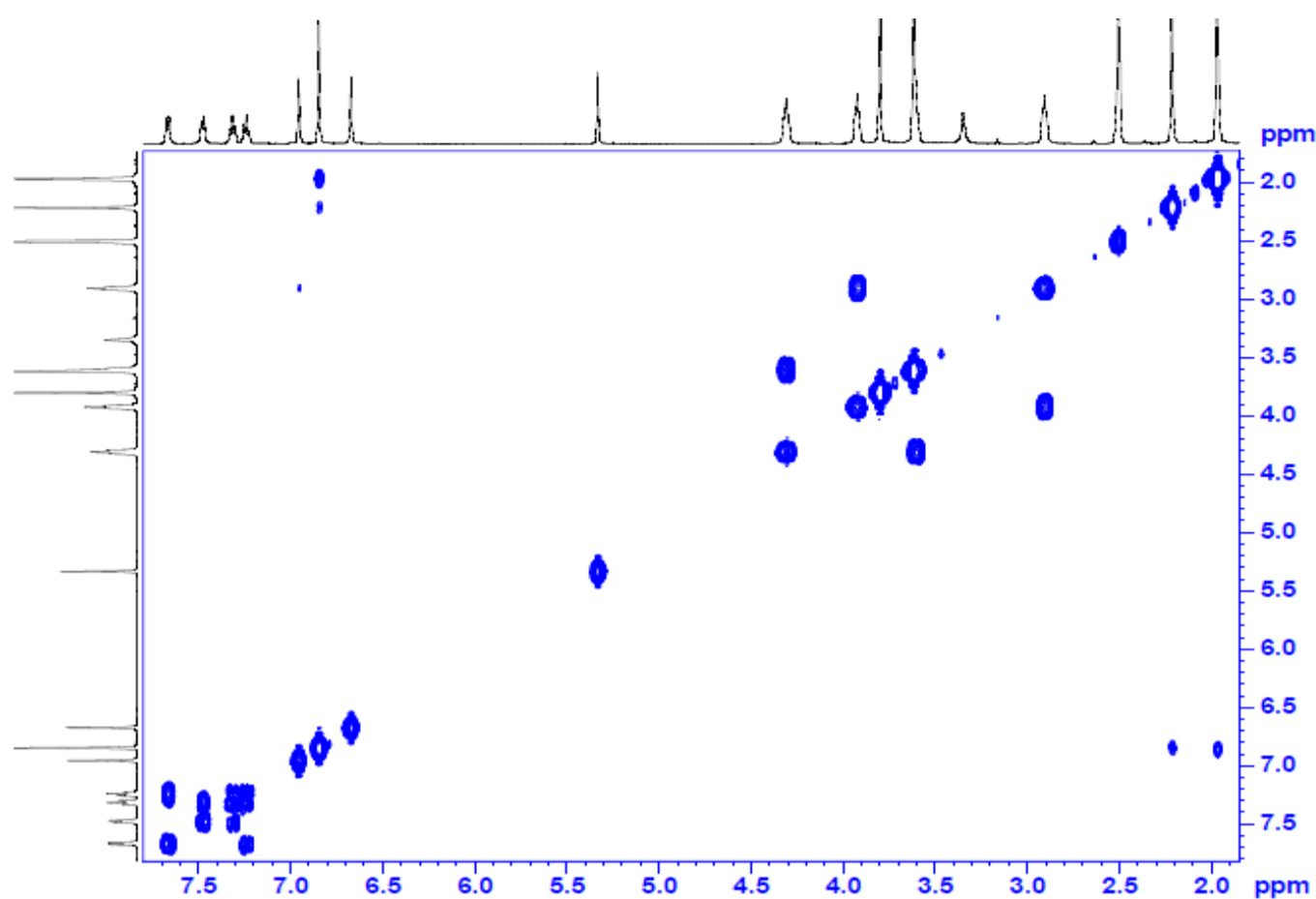
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RG         101
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DE         6.50 usec
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NUC1      13C
P0         3.00 usec
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NUC2      1H
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F1 - Processing parameters
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COSY of ENF Carboxylic acid Impurity



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kshor_jedhar\
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EXPNO    34
PROCNO   1

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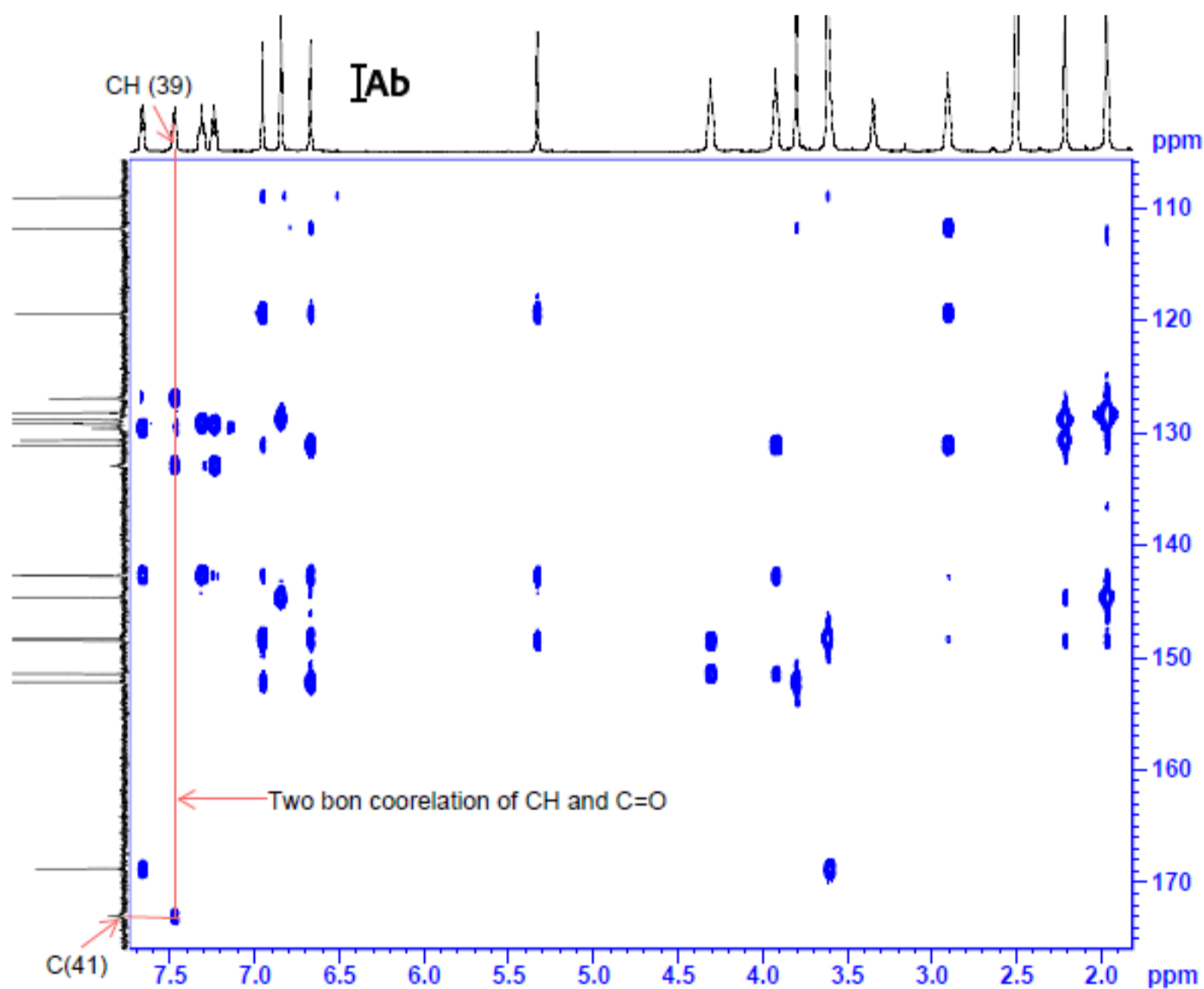
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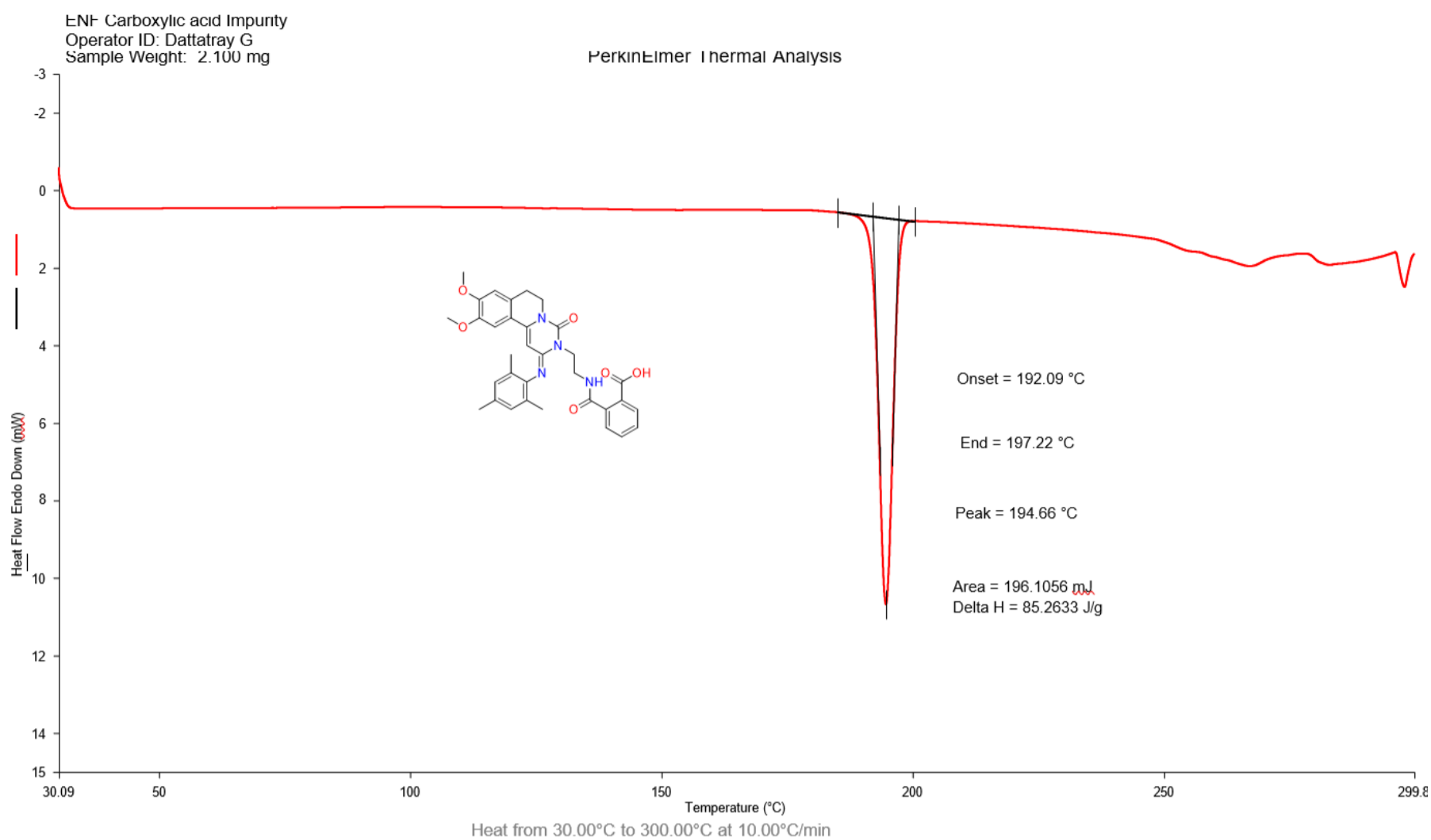
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HMBC of ENF Carboxylic acid Impurity

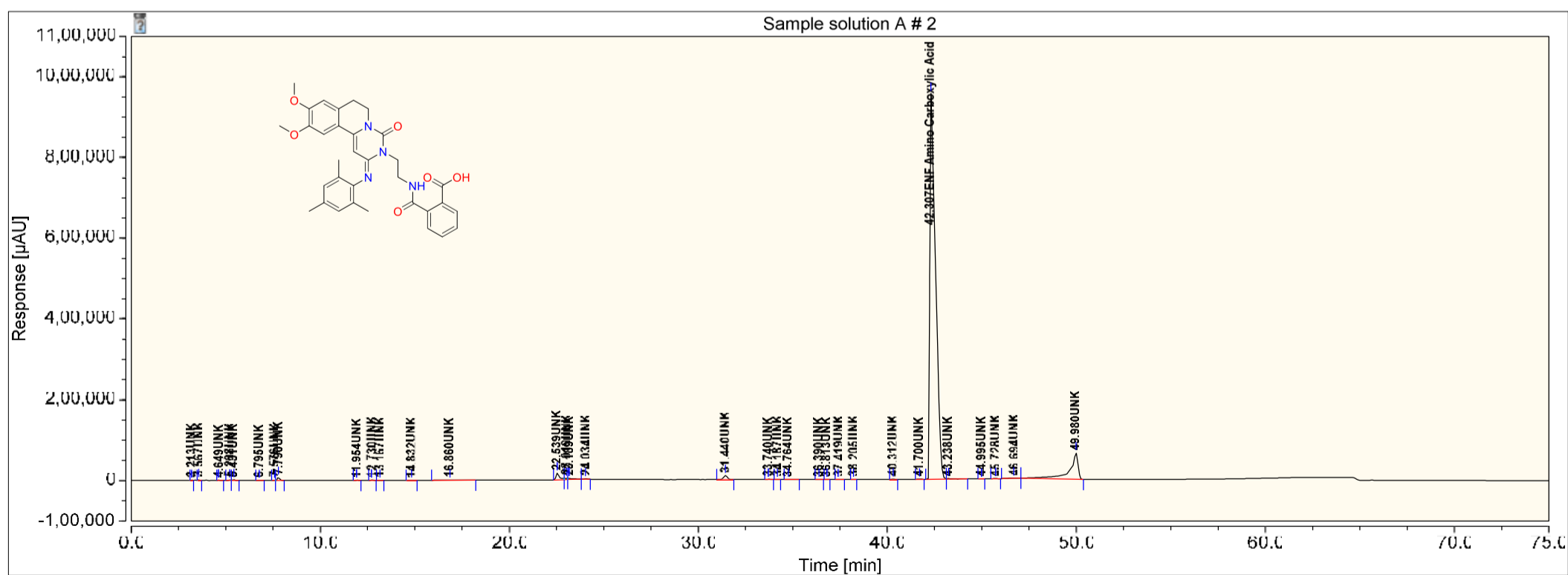


DSC Analysis of ENF Carboxylic acid Impurity

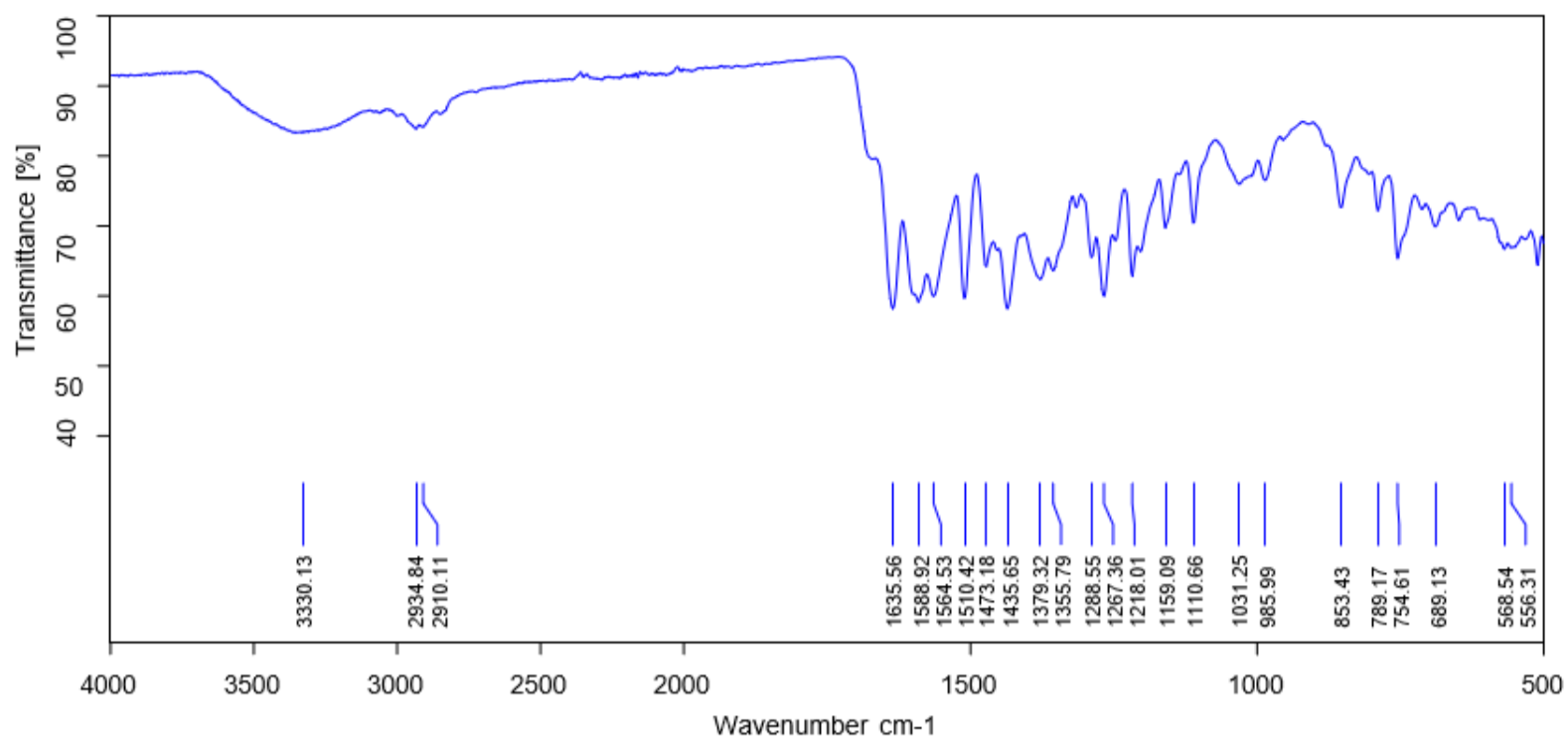


HPLC Analysis of ENF Carboxylic acid Impurity

ENF Carboxylic acid Impurity  
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Detector: VWD\_Signal A  
Injection volume: 10.0 µL.  
Wavelength: 225 nm



### IR Spectra of ENF Carboxylic acid Impurity

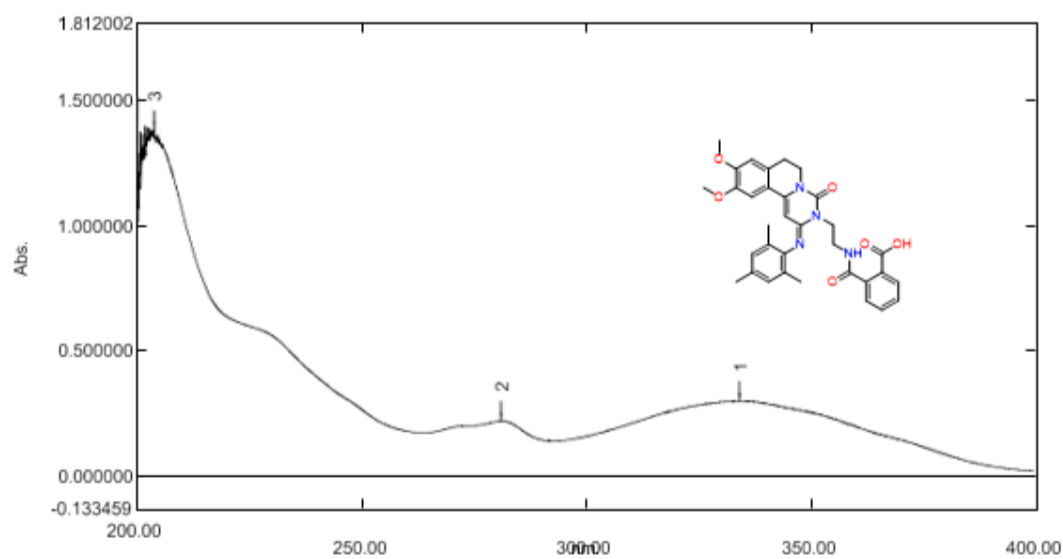


\\nptg01i5w-006\c\$\FTIR\5\_I-025\2024\Ensifentrine\CA 0922845\_SPL\_24 Solid as such

### UV Analysis of ENF Carboxylic acid Impurity

#### Spectrum Measurement Report

Data set: 1068647\_Identification\_SPL\_18 - RawData



<b>Software Information</b> Software Name: UVProbe Version: 2.62 Mode: LabSolutions	<b>[Measurement Properties]</b> Wavelength Range (nm.): 200.00 to 400.00 Scan Speed: Fast Sampling Interval: 0.05 Auto Sampling Interval: Disabled Scan Mode: Single
<b>Data Information</b> Data is: Method: I-003 - Identification By UV_200_	<b>[Instrument Properties]</b> Instrument Type: UV-1800 Series Measuring Mode: Absorbance Slit Width: 1.0 nm Light Source Change Wavelength: 360.0 nm S/R Exchange: Normal
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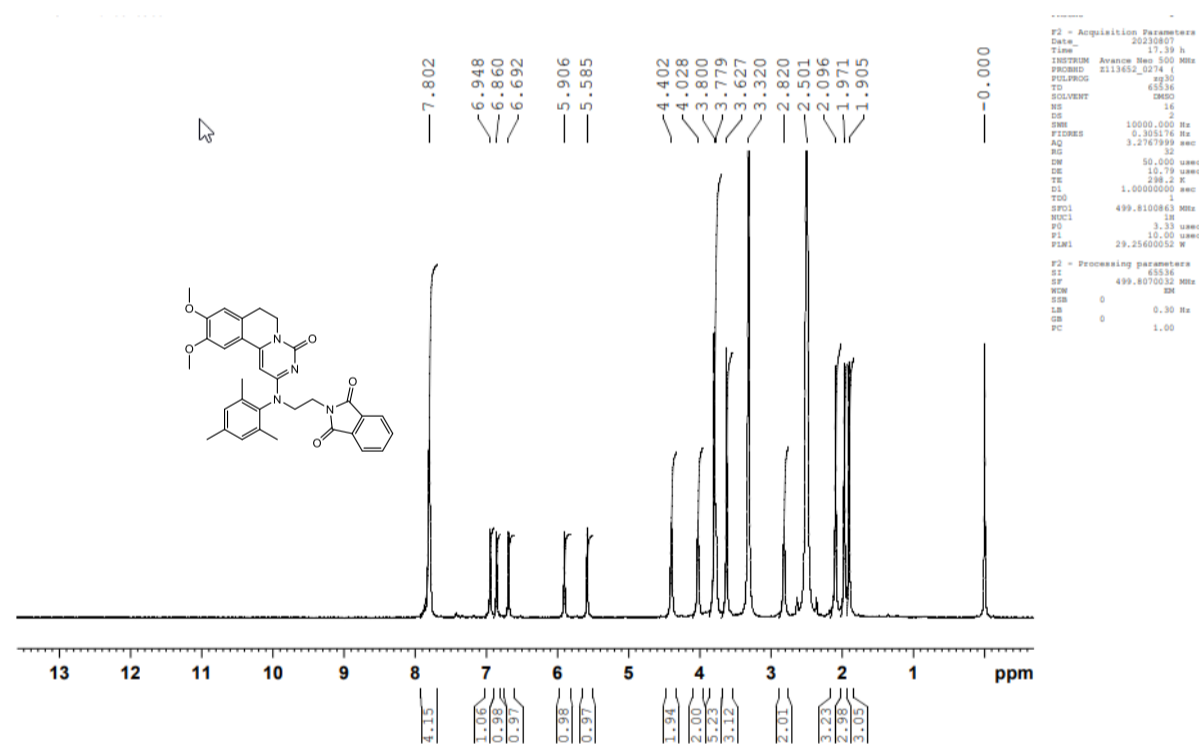
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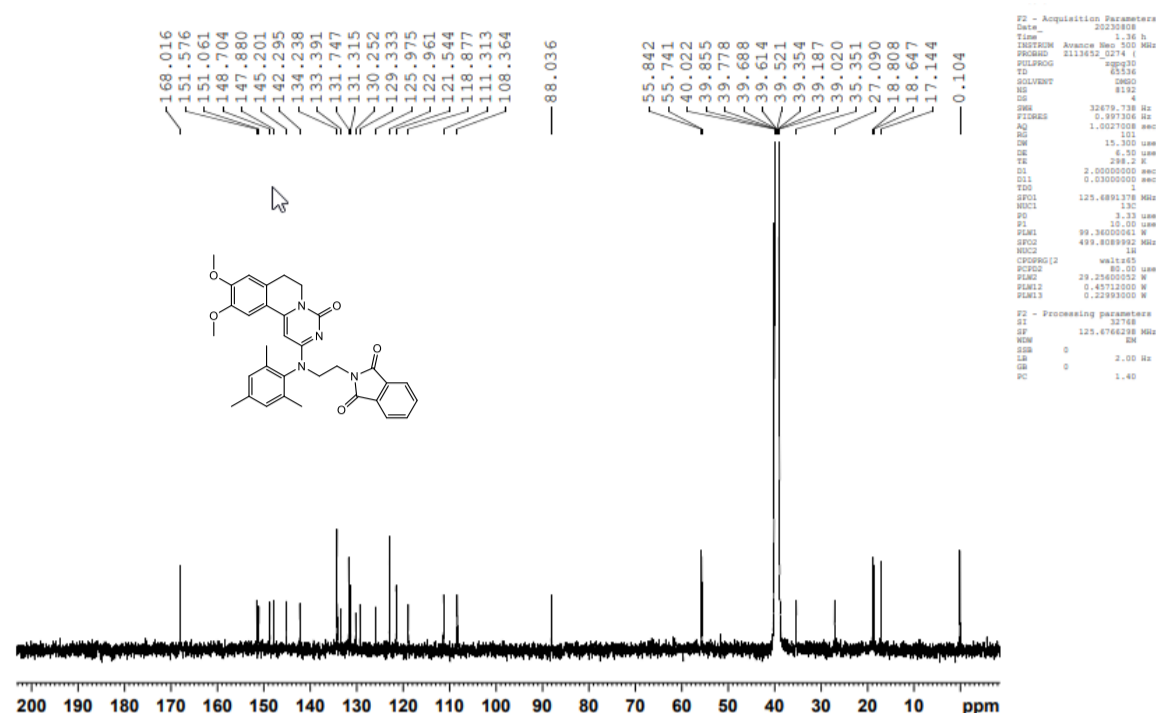
### 2.3. ENF N-Alkyl substitution impurity, (*N*-{2-[(9,10-dimethoxy-4-oxo-6,7-dihydro-4*H*-pyrimido[6,1-*a*]isoquinolin-2-yl)(2,4,6-trimethylphenyl)amino]ethyl}urea)<sup>1</sup>

#### Stage-1: Synthesis of 2-{2-[(9,10-dimethoxy-4-oxo-6,7-dihydro-4*H*-pyrimido[6,1-*a*]isoquinolin-2-yl)(2,4,6-trimethylphenyl)amino]ethyl}-1*H*-isoindole-1,3(2*H*)-dione (Synthesis of *N*-alkyl phthalimide) (7a)

(2*E*)-9,10-dimethoxy-2-[(2,4,5-trimethylphenyl)imino]-2,3,6,7-tetrahydro-4*H*-pyrimido[6,1-*a*]isoquinolin-4-one (10 g, 25.54 mmol) (5), was taken into 50 ml Chlorobenzene. To that *N*-(2-bromoethyl)phthalimide (20 g, 3.08 equiv), Sodium iodide (8 g, 2.08 equiv) and Potassium carbonate powder 40 mesh (20 g, 5.7 equiv) were added and reaction mass was heated for 5 hours at 115-120 °C. Then the reaction mass 50 ml toluene was added and the content was heated at 105-110 °C for 20 hours. Reaction mass was then cooled to 55-60 °C and to that 2 g hyflo bed was added. Reaction mass was stirred for 1 hr at 25-30 °C and then filtered. Given material was adsorbed on hyflo bed. Hyflo bed material was taken into 500 ml RBF. 200 ml MDC: methanol (8:2) was added into RBF. The RM was stirred at 35-40 °C for 1 hr. RM was then cooled to 25-30 °C and stirred for 1 hr. RM was then filtered and washed with 20 ml MDC. Filtrate was distilled under vacuum to get off white material. Isolated material was further purified in 50 ml toluene by heat cool- heat cool method at 55-60 °C. Obtained material was taken in 50 ml methanol and stirred for 1 hour at 40-45 °C and then cool, filtered to afford 4 g pure ENF *N*-Alkyl phthalimide compound (7a). <sup>1</sup>H NMR and <sup>13</sup>C NMR data of ENF *N*-Alkyl phthalimide compound (7a) are presented in Table 1. <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ 7.84 (dd, *J* = 8.2Hz, 2.1 Hz, 2H), 7.72 (dd, *J* = 8.2Hz, 2.1 Hz, 2H), 6.99 (s, 2H), 6.72 (s, 1H), 6.67 (s, 1H), 5.39 (s, 1H), 4.24 (t, *J* = 6.4Hz, 2H), 4.18 (t, *J* = 6.8Hz, 2H), 3.99 (t, *J* = 6.4Hz, 2H), 3.93 (s, 3H), 3.75 (s, 3H), 2.91 (t, *J* = 6.8Hz, 2H), 2.32 (s, 3H), 2.22 (s, 6H). <sup>13</sup>C NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ 167.9, 163.4, 157.1, 152.0, 150.0, 148.1, 138.1, 136.7, 136.5, 133.8, 132.0, 130.5, 129.8, 123.1, 119.9, 110.4, 108.8, 86.3, 56.3, 56.0, 46.3, 40.0, 36.1, 27.6, 20.9, 18.2. **IR interpretation:** 3080, 3002, 2939, 2918, 1769, 1703, 1654, 1510, 1462, 1154, 1130, 1066, 1036 (C-N Stretching). *m/z* (M + H)<sup>+</sup> calc for C<sub>33</sub>H<sub>33</sub>N<sub>4</sub>O<sub>5</sub>, 564.6424 u; found 565.5532 u.

#### <sup>1</sup>H NMR of *N*-alkyl phthalimide (7a)

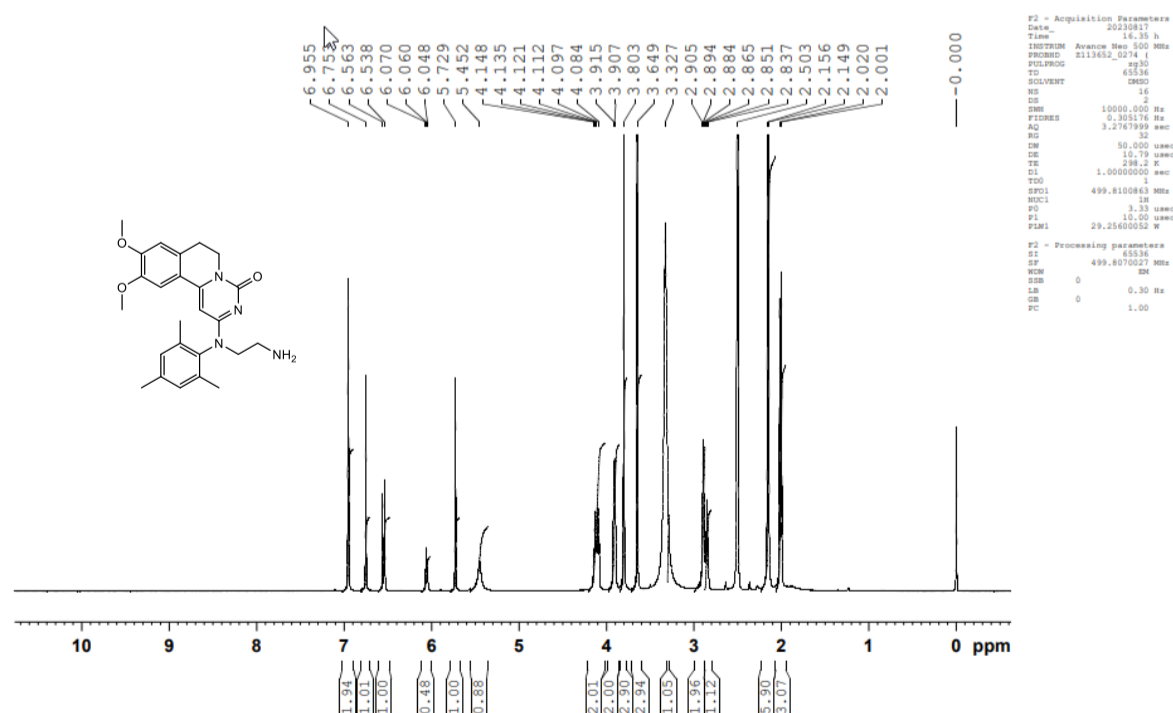


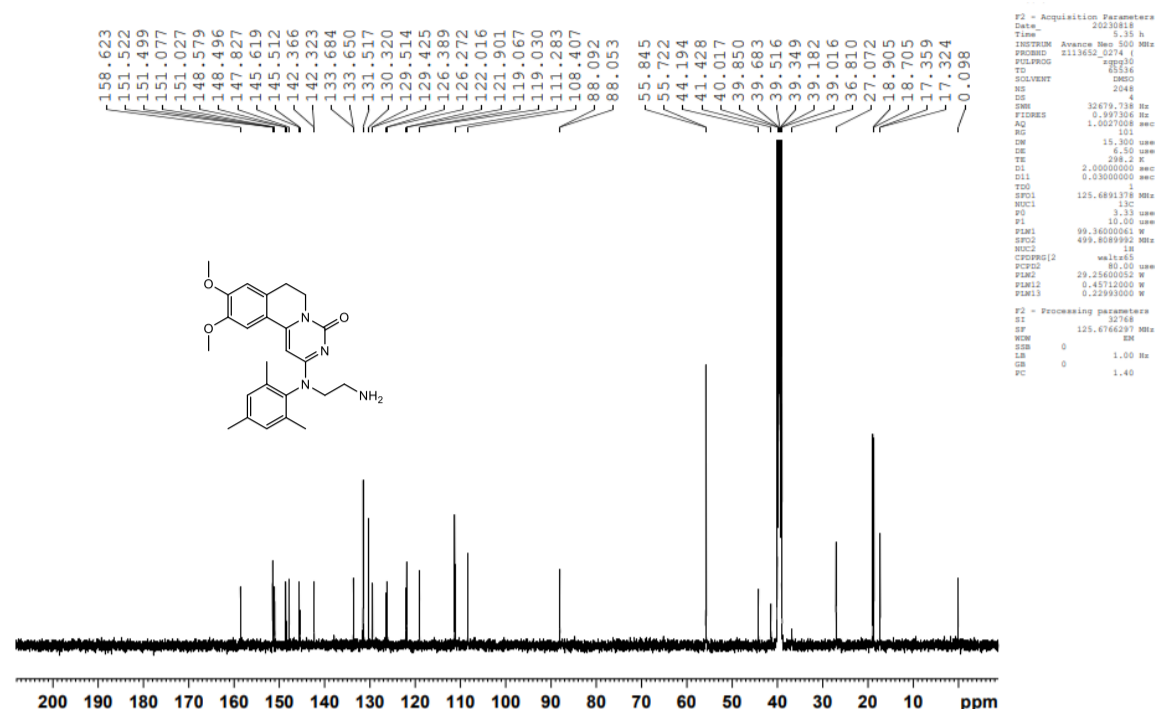
<sup>13</sup>C NMR of N-alkyl phthalimide (7a)

## Stage-2

## Synthesis of 2-[(2-aminoethyl)(2,4,6-trimethylphenyl)amino]-9,10-dimethoxy-6,7-dihydro-4H-pyrimido[6,1-a]isoquinolin-4-one. (Synthesis of N-alkyl amine) (8a)

2-[2-((2E)-9,10-dimethoxy-2-[(2,4,6-trimethylphenyl)imino]-6,7-dihydro-2H-pyrimido[6,1-a]isoquinolin-4-yl)oxy]ethyl]-1H-indole-1,3(2H)-dione (4 g, 7.092 mmol) (7a) was diluted in 7 vol methanol. To that 14 ml 40% methyl amine was added and reaction mass was stirred at 40-45 °C. After 4 hours reaction mass was cooled to 0-5°C and then pH of reaction mass was adjusted to 1-2 by using Conc HCl. After 1 hour stirring at cool temperature reaction mass was filtered and washed with 10 ml methanol. Obtained precipitate was purified in ACN: Water (3 vol :0.3 vol) at 50-55°C and then filtered at RT affording white compound with good yield. <sup>1</sup>H NMR (500 MHz, DMSO-d<sub>6</sub>) δ 7.07 (s, 2H), 6.98 (s, 1H), 6.58 (s, 1H), 5.23 (s, 1H), 3.93 (m, 2H), 3.81 (s, 3H), 3.73 (t, J = 6.4Hz, 2H), 3.59 (s, 3H), 2.88 (m, 2H), 2.77 (m, J = 6.4Hz, 2H), 2.30 (s, 3H), 2.11 (s, 6H). <sup>13</sup>C NMR (500 MHz, DMSO-d<sub>6</sub>) δ 162.6, 155.7, 151.9, 149.5, 147.7, 137.3, 137.2, 135.8, 130.7, 129.5, 119.0, 111.3, 108.5, 84.8, 55.8, 55.7, 51.7, 40.0, 39.0, 26.7, 20.5, 20.4, 17.6. **IR interpretation:** 3366, 3288 (N-H Stretching), 2997, 2919, 1734, 1636, 1503, 1459, 1178, 1145, 1079, 1049, 1031 (C-N Stretching). m/z (M + H)<sup>+</sup> calc for C<sub>25</sub>H<sub>31</sub>N<sub>4</sub>O<sub>3</sub>, 434.1235 u; found 435.1235 u.

<sup>1</sup>H NMR of N-alkyl amine) (8a)

**<sup>13</sup>C NMR of N-alkyl amine) (8a)****Stage-3****Synthesis of N-{2-[(9,10-dimethoxy-4-oxo-6,7-dihydro-4H-pyrimido[6,1-a]isoquinolin-2-yl)(2,4,6-trimethylphenyl)amino]ethyl}urea (Synthesis of ENF N-alkyl Ensifentrine) (9a)**

2-((2*E*)-9,10-dimethoxy-2-[(2,4,6-trimethylphenyl)imino]-6,7-dihydro-2*H*-pyrimido[6,1-*a*]isoquinolin-4-yl)oxyethan-1-amine (1 g, 2.30 mmol) (8a) was taken in 30 v methanol. Reaction mass was heated to 65-70 °C. Sodium cyanate solution (0.25 g, 1.8 equiv in 5 ml water) was added slowly into the reaction mass. After 1 hour reaction mass was cooled to 25-30 °C and to that 10 ml water was added. Reaction mass was then stirred for 1 hour and filtered to get white solid material. Purification was done in MDC: Methanol: EA (10: 1.5: 15) at room temperature to afford white solid material. **<sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>)** δ 7.78 (s, 1H), 7.27 (s, 1H), 7.06 (s, 1H), 6.98 (s, 1H), 6.93 (s, 1H), 6.30-6.21 (broad hump, 1H), 5.70 (s, 1H), 5.58 (broad hump, 1H), 3.97 (t, *J* = 6.4Hz, 2H), 3.94 (t, *J* = 6.8Hz, 2H), 3.84 (s, 3H), 3.70 (m, 1H), 3.58 (s, 1H), 3.53 (m, 1H), 3.25 (t, *J* = 6.8Hz, 2H), 2.89 (t, *J* = 6.4Hz, 2H), 2.29 (s, 3H), 2.10 (s, 6H). **<sup>13</sup>C NMR (500 MHz, DMSO-*d*<sub>6</sub>)** δ 163.1, 162.6, 159.4, 155.5, 152.0, 151.4, 150.5, 148.1, 147.7, 139.7, 137.4, 137.2, 135.8, 135.7, 134.9, 130.7, 129.8, 129.6, 128.9, 119.6, 118.9, 111.3, 110.7, 109.3, 108.6, 85.2, 84.9, 56.3, 55.8, 55.7, 55.6, 50.9, 49.2, 37.8, 26.8, 26.7, 20.5, 20.4, 17.9, 17.6. **IR interpretation:** 3469 (N-H Stretching), 2996, 2953, 1637, 1596, 1563, 1269, 1218, 1157, 1115 (C-N Stretching). *m/z* (M + H)<sup>+</sup> calc for C<sub>26</sub>H<sub>32</sub>N<sub>5</sub>O<sub>4</sub>, 477.5651u ; found 478.2369 u.

HSQC: NH<sub>2</sub> not showing any correlation in HSQC with carbon, it indicates that they are exchangeable proton of NH<sub>2</sub> (32)

HMBC: C (16) having two bond correlation with C (25), which confirm that N-alkylation formed at Nitrogen which is outside the ring.

Complete correlation data: (6.67, 108.6), (6.96, 111.3), (6.65, 128.3), (5.53, 87.6), (3.92, 40.1), (2.90, 27.0), (4.18, 41.5), (3.36, 36.8), (3.62, 56.0), (3.80, 55.7), (2.22, 20.4), (1.97, 18.0).

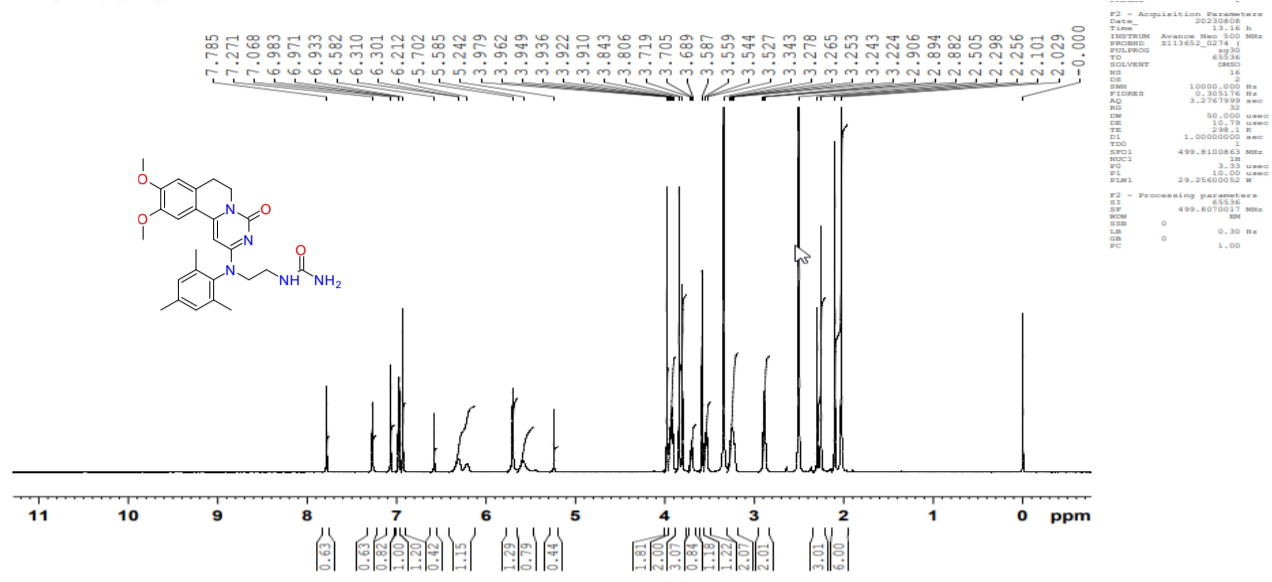
Quaternary and non-protonated carbons appeared at δ 151.7, 147.8, 118.6, 130.5, 142.3, 148.1, 150.9, 144.0, 127.7, and 158.6.

DSC: Melting point peak at 161.69°C

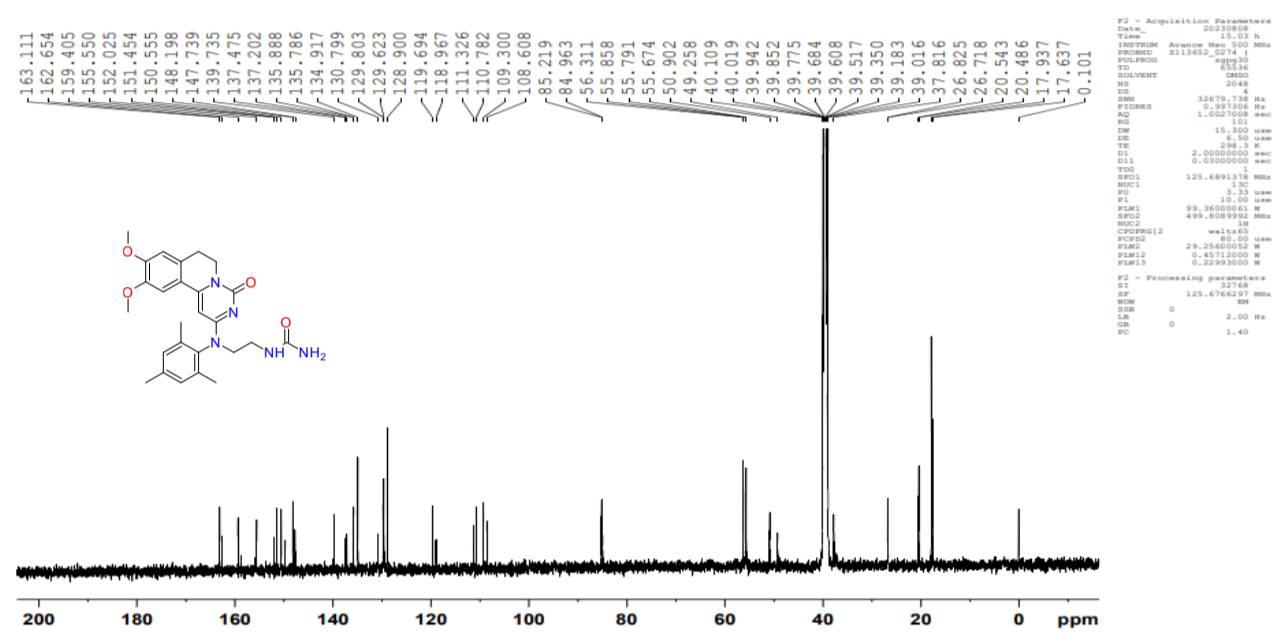
HPLC: ENF-N-alkyl impurity detected at retention time 38.476 min.

IR and UV spectra confirms the functional group involved in the impurity

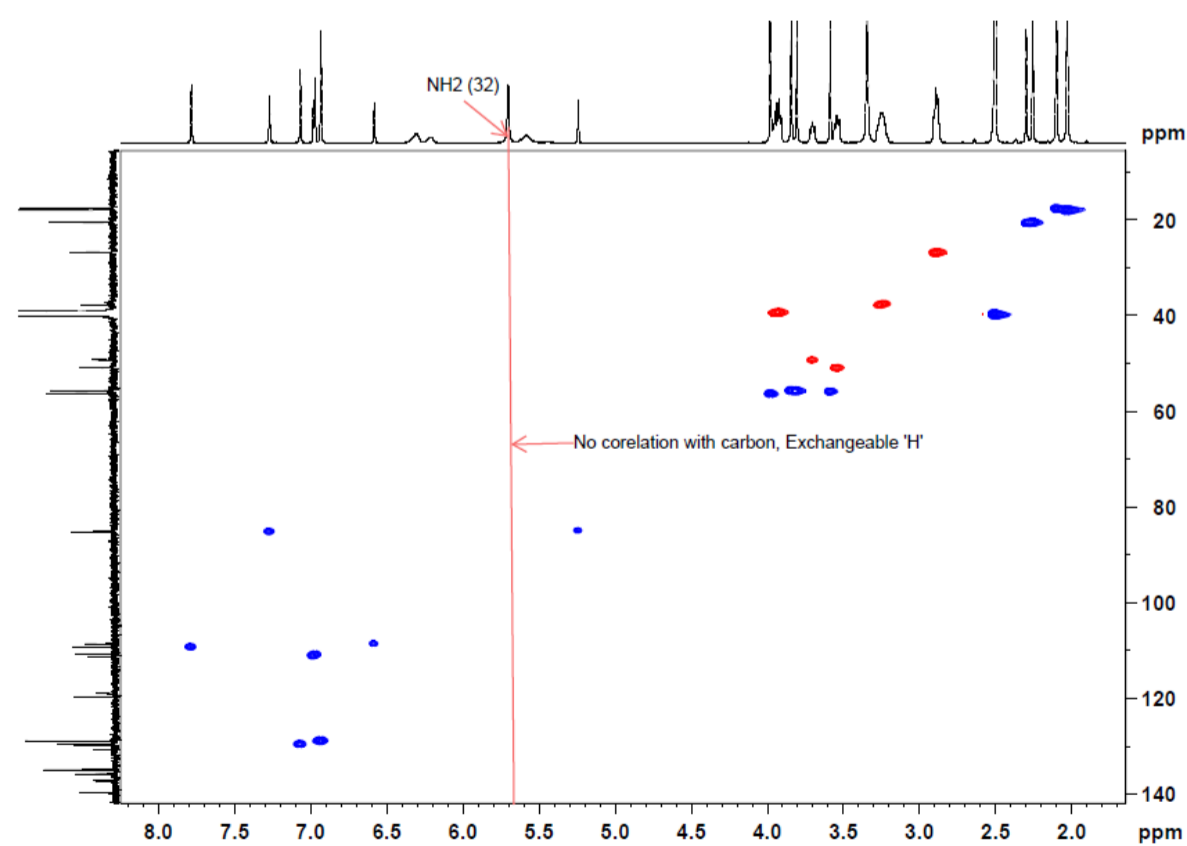
<sup>1</sup>H NMR of ENF N-alkyl Ensifentrine impurity (9a)



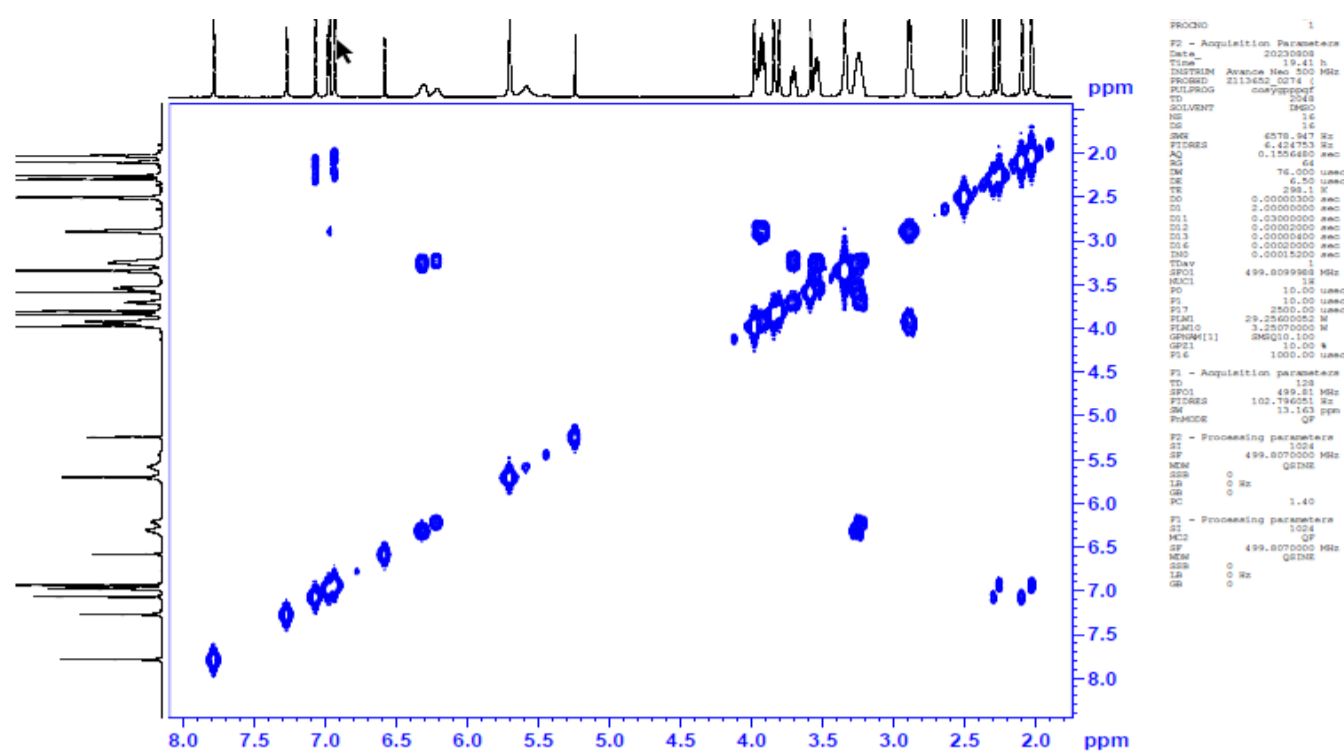
<sup>13</sup>C NMR of ENF N-alkyl Ensifentrine impurity (9a)



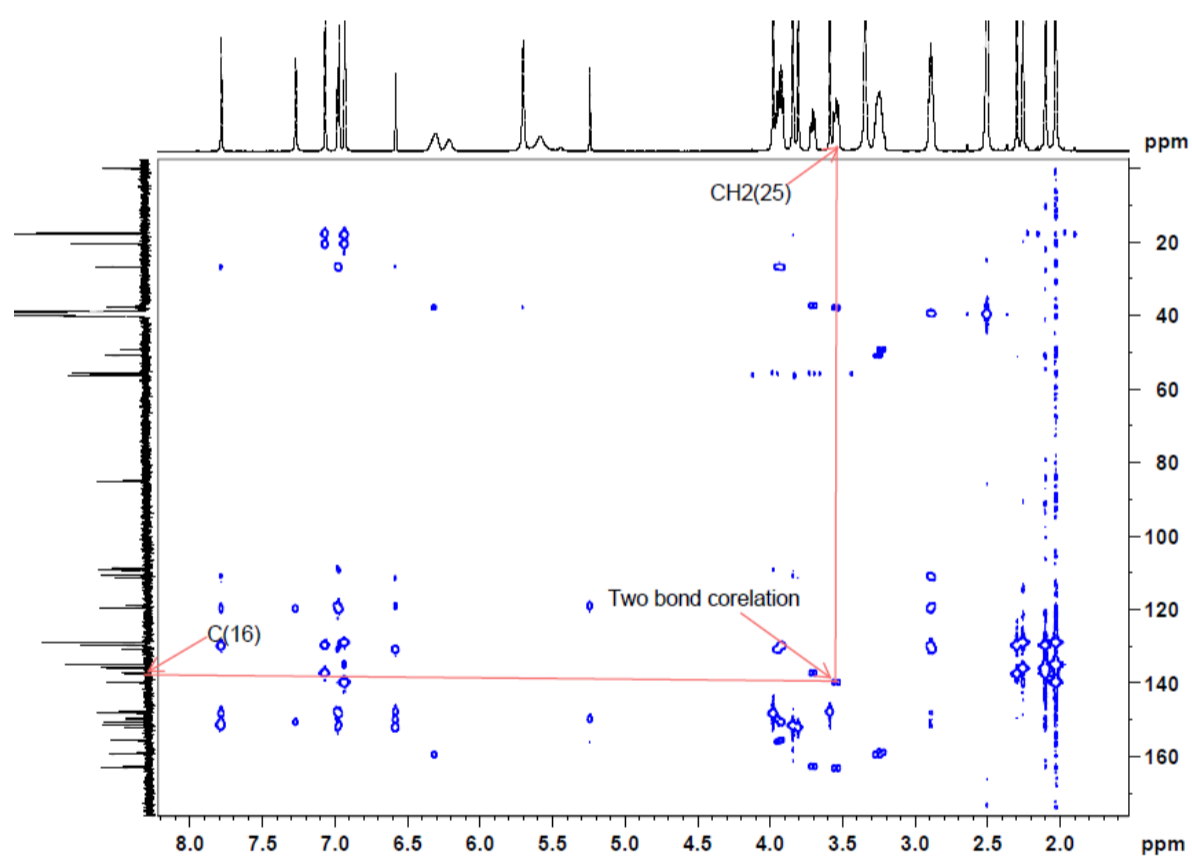
HSQC of ENF N-alkyl Ensifentrine impurity(9a)



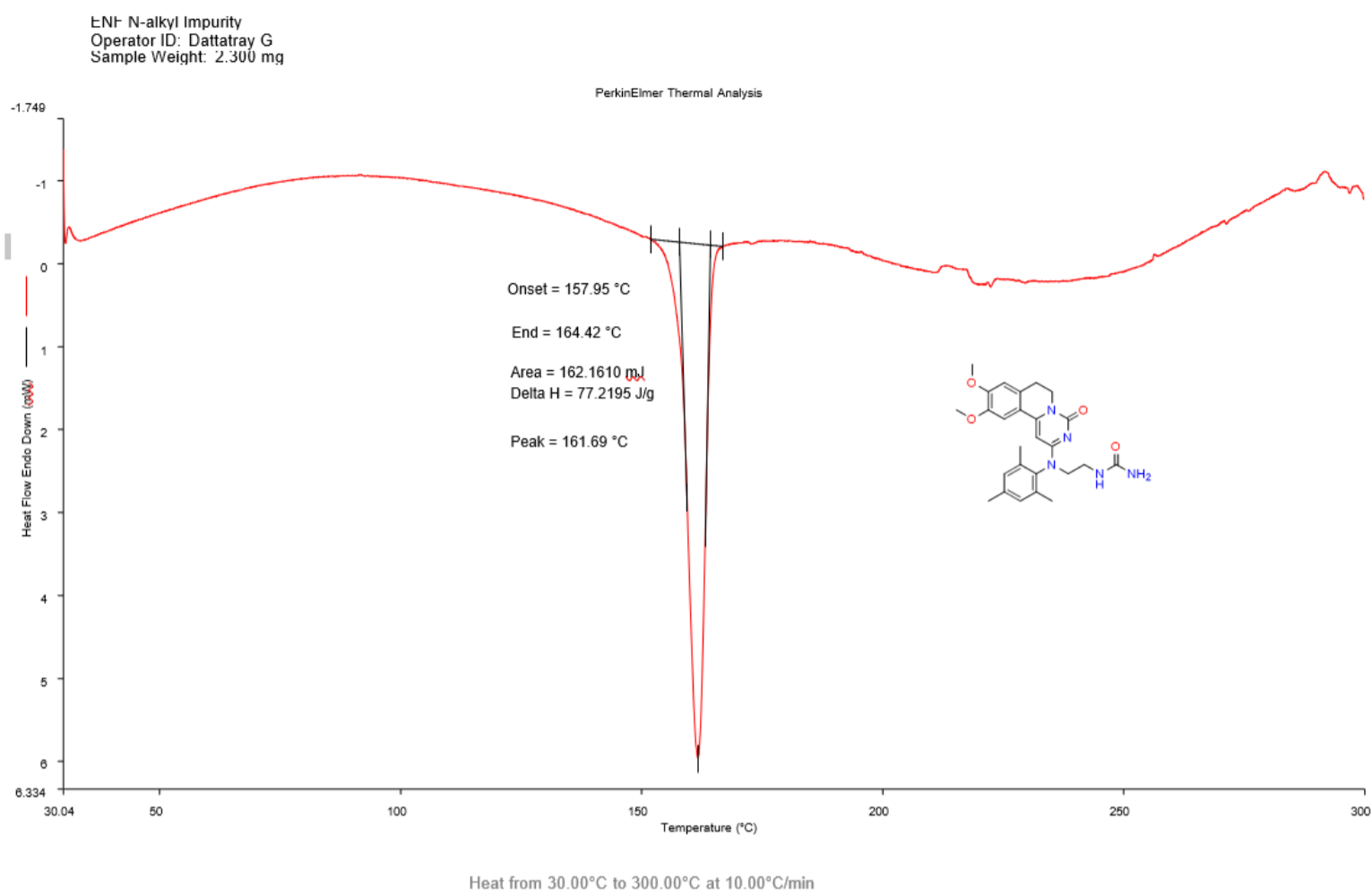
COSY of ENF N-alkyl Ensifentrine impurity(9a)



HMBC of ENF N-alkyl Ensifentrine impurity(9a)

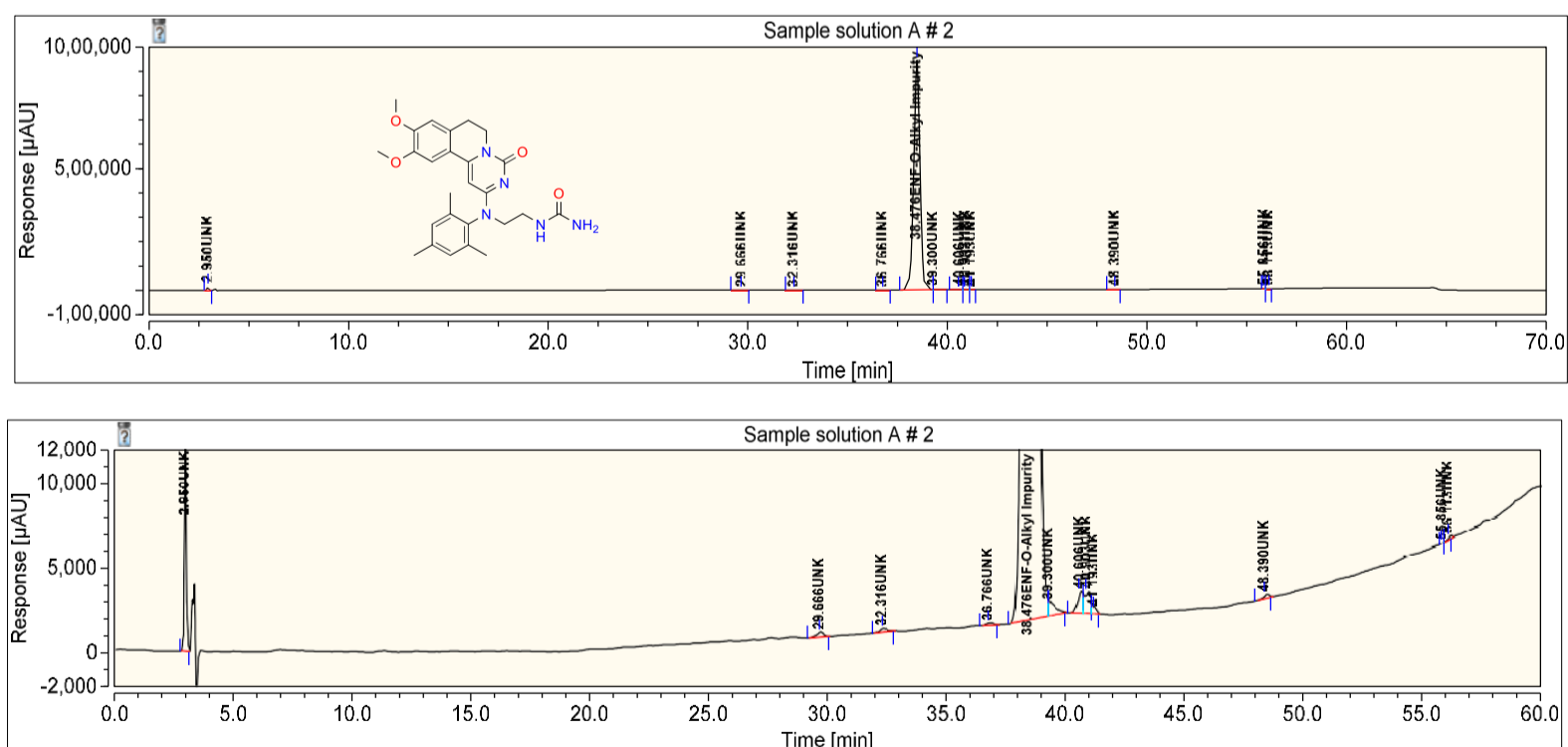


### DSC Analysis of ENF N-alkyl Ensifentrine impurity(9a)

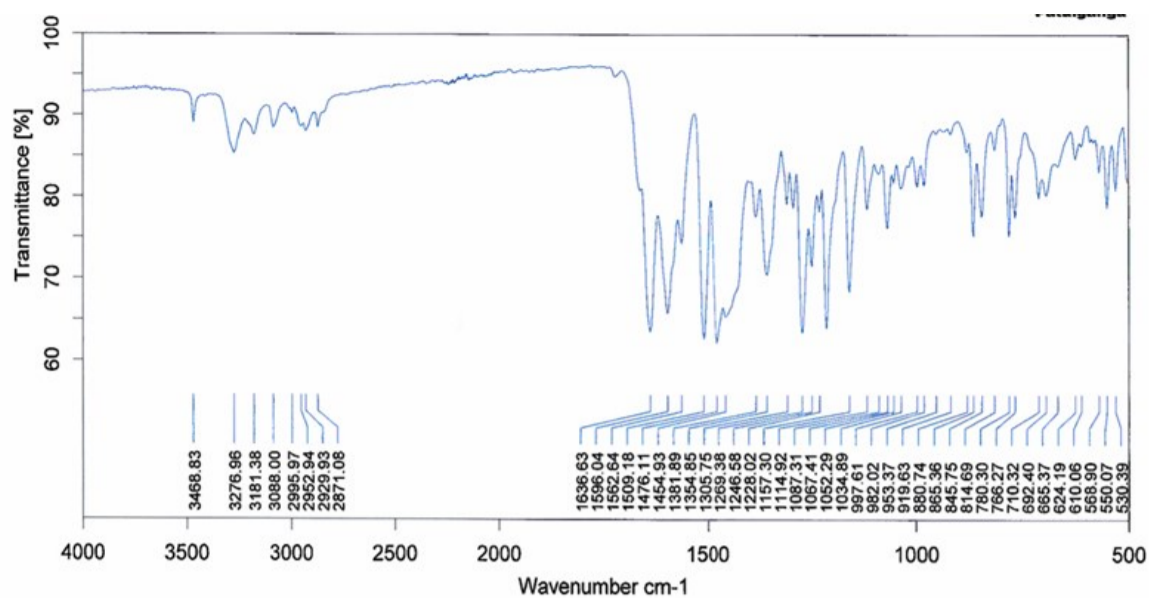


### HPLC Analysis of ENF N-alkyl Ensifentrine impurity(9a)

ENF N-alkyl Impurity  
Inst. Name & No: SHIMADZU\_LC2030C\_I-105  
Detector: VWD\_Signal 1  
Injection volume: 10.0 µL.  
Wavelength: 225 nm



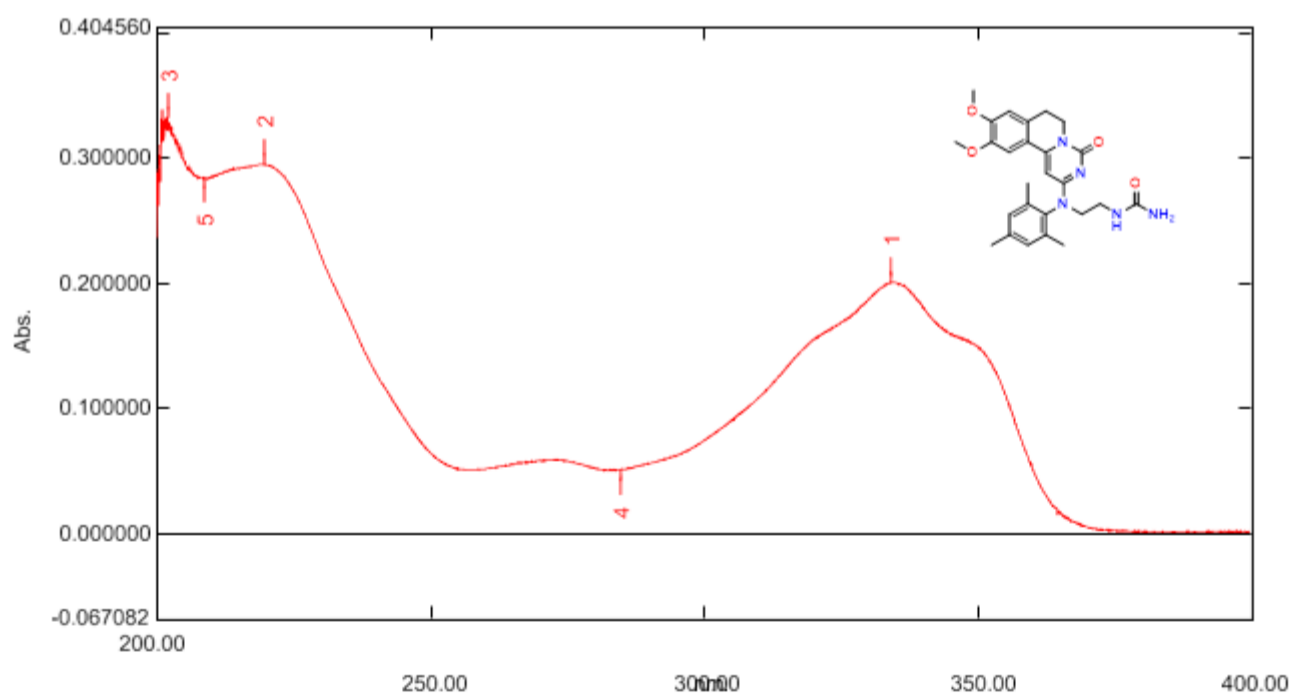
IR Spectra of ENF N-alkyl Ensifentrine impurity(9a)



UV Spectra of ENF N-alkyl Ensifentrine impurity(9a)

Spectrum Measurement Report

Data set: 0843872\_Identification\_SPL\_29 - RawData



Software Information  
 Software Name: UVProbe  
 Version: 2.62  
 Mode: LabSolutions

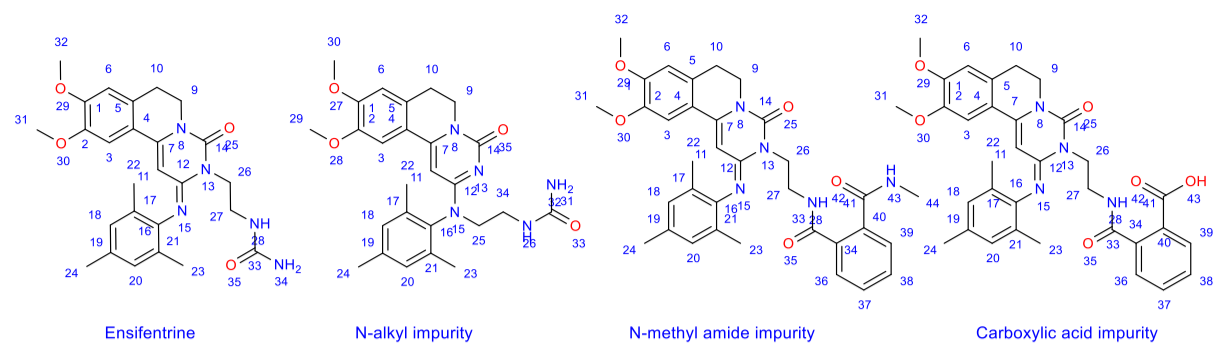
Data Information  
 Data is:  
 Method: I-003 - Identification By UV\_200\_

Instrument Information  
 Instrument Name: UV 1800  
 Instrument Type: UV-1800 Series  
 Model (S/N): UV Vis Spectrophotometer (A11454907900)

[Measurement Properties]  
 Wavelength Range (nm.): 200.00 to 400.00  
 Scan Speed: Medium  
 Sampling Interval: 0.05  
 Auto Sampling Interval: Disabled  
 Scan Mode: Single

[Instrument Properties]  
 Instrument Type: UV-1800 Series  
 Measuring Mode: Absorbance  
 Slit Width: 1.0 nm  
 Light Source Change Wavelength: 360.0 nm  
 S/R Exchange: Normal

No.	P/V	Wavelength	Abs.	Description
1	↑	334.60	0.199554	
2	↑	219.70	0.293839	
3	↑	202.05	0.331497	
4	↓	284.85	0.049988	
5	↓	208.95	0.282349	

**References:**

- (1) *US20250171436A1 - Tricyclic Fused Heterocyclic PDE3/4 Dual Inhibitor and Use Thereof - Google Patents.*  
<https://patents.google.com/patent/US20250171436A1/en> (accessed 2025-10-25).