Validation Study/Performance Check for Agilent GC 7890B ASAP Analytical IRD3 GC-FTIR (vapor phase)

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I. Introduction:

This validation study and performance check is designed to demonstrate the effectiveness of using GC-FTIR vapor phase in the analysis of legally significant substances. This analysis can effectively be used as a Category A test to determine structural information of an unknown compound and distinguish positional isomers that may be difficult to determine by GC-MS analysis. The ability for acquired spectra to differentiate and identify compounds with no ambiguity will be evaluated, except when compounds are thermally liable or have optical isomers (d and l ephedrine for example). The spectra obtained by the Agilent GC ASAP Analytical IRD3 (IRD3) will be compared to existing GC-FTIR libraries that have been built using Agilent/Thermo Scientific GC-FTIR instrumentation under similar operating conditions using the same type of column with previously validated methods (excluding new autosample, autosample splitless, and manual injection methods added for nitrites and lower molecular weight compounds) derived from literature and will be verified as fit for use. Based on the optical and mechanical parts of the IRD3, for optimal results it is recommended by ASAP Analytical that an auto calculate gain and resolution and co-add (scans) of 8-2 are used for analysis as well as nitrogen gas for purging and make-up gas. In comparison, the Agilent/Thermo Scientific GC-FTIR instrumentation parameters are set at gain of 1 or 2 and resolution and co-add (scans) of 8-4 and uses dried house air for purging and helium gas for the make-up gas. A study will be performed on the IRD3 to show that these recommended parameters are comparable to current validated instrumentation and provide even more data point information, resolved peaks, and less averaging within the peaks. Additionally, repeatability, limit of detection, and wavenumber accuracy will be determined with the IRD3 as outlined below. Once deemed fit for use, all analysts will be trained on the familiarization and use of the instrument.

Validation experiments/studies will include the following:

- Resolution and Co-Add (Scans)
- Repeatability
- Limit of Detection/Performance Check of Methods
- Wavenumber Accuracy

A. Resolution and Co-Add (Scans) – An Opioid standard mix will be analyzed at a resolution of 8 and co-add (scans) of 2 with a series of 3 auto sample injections. These results will be compared to the same Opioid standard mix analyzed with a series of 3 auto sample injections set at a resolution of 8 and co-add (scans) of 4. Chromatography will be evaluated along with the individual spectra to show comparable, if not improved, signal to noise, peak shape and separation with IRD3 parameters at the recommended manufacturer resolution and co-add (scans) of 8-2.

B. Repeatability – This experiment will involve preparing a 4-ethylethcathinone standard of known concentration on a specific method and running it multiple times across three days to show repeatable results.

C. Limit of Detection/Performance Check of Methods – A series of standards will be analyzed using the same column with previously validated autosample methods in order

to obtain their respective vapor phase IR spectra. In addition to the previously validated instrument methods, there will be an additional autosample, splitless autosample, and manual injection methods created for Butyl Nitrites and other low molecular weight compounds. In addition to analyzing standards by autosample, splitless autosample, and manual injection under multiple autosample instrument methods we will also use three different known concentrations (2 mg/mL, 1 mg/mL, and 0.5 mg/mL) to determine the limit of detection of each standard. It is important to note, that the limit of detection of each standard may vary due to each standard having distinctive interactions with infrared light.

D. Wavenumber Accuracy – Once spectra have been obtained using Limit of Detection experiments, their respective spectra will be evaluated to standard spectra within the existing GC-FTIR library for wavenumber accuracy. Their measured wavenumbers must be within an optical resolution of +/- 8 cm-1 when compared to the expected wavenumbers of the standard spectra within the existing GC-FTIR library.

II. Materials:

Column & Liner:

Agilent J&W GC HP-1 30 meter, 0.32 ID, 1.00 um column (P/N# 19091Z-213 S/N# USD717922H)

Restek Topaz Split/Splitless Straight with wool liner

Balance:

Mettler Toledo XS 225 DU
S/N BP03095311
Index 30

Pipette:

1000 uL Eppendorf Reference Pipette ID254432 Calibrated by Integrated Service Solutions on 07-22-19 Exp. 07-2020

For Resolution vs Co-add (Scans) Study

Solvent and Standards:

Methanol - Lot# 193741 Bottle #2 Exp. u/c LMN

THF4A Mix

Tramadol
SIGMA
Lot# BCBB9210
0.65 mg/mL → MeOH Exp. 01/29/21 GBE/LMN

4-ANPP
CAYMAN CHEMICAL
Lot# 0532667-4
0.61 mg/mL → MeOH Exp. 01/29/21 GBE/LMN

Heroin
Australian Government National Measurement Institute
Lot# 11-D-10
0.48 mg/mL → MeOH Exp. 01/29/21 GBE/LMN

Acetyl Fentanyl
CAYMAN CHEMICAL
Lot# 0497037-3
0.57 mg/mL → MeOH Exp. 01/29/21 GBE/LMN

Fentanyl Citrate

USP
Lot# 1275-F
0.78 mg/mL → MeOH Exp. 01/29/21 GBE/LMN

For Readability Study

Solvents and Standards:

0.5N NaOH – Lot# 185498 Exp. 08/01/2020 RH
CHCl3 – Lot# 193638 Bottle #1 Exp. July 2020 RH

4-Ethylethcathinone (4.11 mg)
CAYMAN CHEMICAL
Lot# 0433939-7
1 mg/mL → 0.5N NaOH → CHCl3 Exp. 02/18/21 IB/RH

For Limit of Detection Study/Performance Check of Methods

Solvents and Standards:

Methanol - Lot# 193741 Bottle #3 Exp. u/c 2-18-2020 IB
0.5N NaOH – Lot# 185498 Exp. 08/01/2020 RH
CHCl3 – Lot# 193638 Bottle #1 Exp. July 2020 RH

THF4A Mix

Tramadol (4.03 mg)
SIGMA
Lot# BCBB9210
2 mg/mL, 1 mg/mL, 0.5 mg/mL → MeOH Exp. 02/19/21 RH

4-ANPP (3.93 mg)
CAYMAN CHEMICAL
Lot# 0532667-4
2 mg/mL, 1 mg/mL, 0.5 mg/mL → MeOH Exp. 02/19/21 RH

Heroin (3.98 mg)
CAYMAN CHEMICAL
Lot# 0559235-2
2 mg/mL, 1 mg/mL, 0.5 mg/mL → MeOH Exp. 02/19/21 RH

Acetyl Fentanyl (4.15 mg)
CAYMAN CHEMICAL
Lot# 0497037-3
2 mg/mL, 1 mg/mL, 0.5 mg/mL → MeOH Exp. 02/19/21 RH

Fentanyl HCl (4.13 mg)

CAYMAN CHEMICAL
Lot# 0467883-34
2 mg/mL, 1 mg/mL, 0.5 mg/mL → MeOH Exp. 02/19/21 RH

Stanolone
STERALOIDS
Lot# B0350
2 mg/mL, 1 mg/mL, 0.5 mg/mL→ MeOH Exp. 02/21/21 RH

Amphetamine Mix

D-Amphetamine Sulfate (3.95 mg)
K AND K LABS
Lot# 83567
2 mg/mL, 1 mg/mL, 0.5 mg/mL → 0.5N NaOH → CHCl3 Exp. 02/18/21 IB/RH

Phentermine HCl (4.02 mg)
SIGMA
Lot# 47H0208
2 mg/mL, 1 mg/mL, 0.5 mg/mL → 0.5N NaOH → CHCl3 Exp. 02/18/21 IB/RH

A-(D-L) Methamphetamine (4.19 mg)
K AND K LABS
Lot# 732A
2 mg/mL, 1 mg/mL, 0.5 mg/mL → 0.5N NaOH → CHCl3 Exp. 02/18/21 IB/RH

Testosterone Propionate (4.17 mg)
SIGMA
Lot# 117F0618
2 mg/mL, 1 mg/mL, 0.5 mg/mL → MeOH Exp. 02/18/21 IB/RH

4-Ethylethcathinone (4.11 mg)
CAYMAN CHEMICAL
Lot# 0433939-7
2 mg/mL, 1 mg/mL, 0.5 mg/mL → 0.5N NaOH → CHCl3 Exp. 02/18/21 IB/RH

Diazepam (4.03 mg)
SIGMA
Lot# 105F0451
2 mg/mL, 1 mg/mL, 0.5 mg/mL → MeOH Exp. 02/18/21 IB/RH

GCMS Mix

Pethidine HCl (4.03 mg)
S. B. PENICK
Lot# 413NMN-027
2 mg/mL, 1 mg/mL, 0.5 mg/mL → MeOH Exp. 02/18/21 IB/RH

Cocaine Base (3.90 mg)
MERCK
Lot# U7472
2 mg/mL, 1 mg/mL, 0.5 mg/mL → MeOH Exp. 02/18/21 IB/RH

Hydrocodone Bitartrate (4.12 mg)
MERCK
Lot# V1526
2 mg/mL, 1 mg/mL, 0.5 mg/mL → MeOH Exp. 02/18/21 IB/RH

Butyl Nitrites Mix

Tert-Butyl Nitrite
SIGMA ALDRICH
Lot# BCCB6099
Exp. 02/19/21 RH

Butyl Nitrite
SIGMA ALDRICH
Lot# MKCL4353
Exp. 02/19/21 RH

Isobutyl Nitrite
SIGMA ALDRICH
Lot# MKCL3264
Exp. 02/19/21 RH

III. Methods/Procedures:

The following standards were run with corresponding methods on Hedwig (Agilent/ASAP Analytical IRD3 GC-FTIR):

The "IRD3" method on the ASAP Analytical IRD3 was used in conjunction with all Agilent GC methods. See Addendum for detailed method information.

For Resolution vs Co-add (Scans) Study

Autosample - (0.65, 0.61, 0.48, 0.57, 0.78 mg/mL)
120TO270 - THF4A Mix
Ran at a Resolution and Co-add (Scans) of 8-4 three times and a Resolution and Co-Add (Scans) of 8-2 three times.

For Repeatability Study

Autosample – 1mg/mL
120TO270 – 4-Ethylethcathinone
Ran ten times over a period of three days.

For Limit of Detection Study/Performance Check of Methods

Autosample – 2 mg/mL, 1 mg/mL, 0.5 mg/mL
60TO270 – Amphetamine Mix
120TO270 – 4-ethylethcathinone
120TO270(20MIN) – THF4A Mix
200TO270 – Pethidine, Cocaine, Hydrocodone
200TO270(20MIN) – Diazepam
ISO270(20MIN) – Testosterone Propionate
ISO270(30MIN) – Stanolone

Splitless Autosample – 1 mg/mL
60TO270 – Amphetamine Mix
120TO270 – 4-ethylethcathinone
120TO270(20MIN) – THF4A Mix
200TO270 – Pethidine, Cocaine, Hydrocodone
200TO270(20MIN) – Diazepam
ISO270(20MIN) – Testosterone Propionate
ISO270(30MIN) – Stanolone

Manual – 1 mg/mL
60TO270 – Butyl Nitrites Mix
120TO270 – 4-ethylethcathinone
120TO270(20MIN) – THF4A Mix
200TO270 – Pethidine, Cocaine, Hydrocodone
200TO270(20MIN) – Diazepam
ISO270(20MIN) – Testosterone Propionate
ISO270(30MIN) – Stanolone

IV. Experiments/Studies Performed:

This instrument consists of an Agilent Technologies 7890B Gas Chromatograph with Agilent Autosampler G4567A coupled to an ASAP Analytical Model 528A IRD3 GC-FTIR. It was installed and set up by ASAP Analytical service engineers. Existing Agilent/Thermo GC-FTIR's in our laboratory were previously validated and run at varying conditions to determine optimum values to obtain the best results. For this instrument, standards were analyzed using the same type of column with previously validated methods in order to obtain their respective vapor phase IR spectra. In addition to previously validated methods there was an additional autosample, splitless autosample, and manual injection methods created for Butyl Nitrites and other low molecular weight compounds. Any autosample methods created were set in Agilent to an injection volume of 1uL and manual injections were set at 1uL. As part of the validation study and performance check for this instrument, Resolution and Co-add (Scans), Repeatability, Limit of Detection and Performance Check of Methods, and Wavenumber Accuracy studies were performed.

A. Resolution and Co-Add (Scans) – Cerilliant, Cayman, USP, Sigma, and Australian Government NMI standards (Tramadol, 4-ANPP, Heroin, Acetyl Fentanyl, and Fentanyl (THF4A Mix) were analyzed at a resolution of 8 and co-add (scans) of 2 with a series of 3 auto sample injections. These results were compared to the same standards analyzed with a series of 3 auto sample injections set at a resolution of 8 and co-add (scans) of 4. Chromatography was evaluated along with signal to noise, peak shape and separation with each standard with IRD3 parameters set at the manufacturer recommended resolution and co-add (scans) of 8-2 and at 8-4.

B. Repeatability- This experiment involved preparing a 4-ethylethcathinone standard of 1 mg/mL using autosampler method 120TO270 and running it ten times with blanks running before each standard under the same method parameters of the standard across three days to show repeatable results.

C. Limit of Detection/Performance Check of Methods- Cerilliant, Cayman, USP, Sigma, Australian Government NMI, K & K Labs, Merck, S.B. Penick standards Amphetamine, Phentermine, and Methamphetamine (Amphetamine Mix), 4-ethylethcathinone, Tramadol, 4-ANPP, Heroin, Acetyl Fentanyl, and Fentanyl (THF4A Mix), Pethidine, Cocaine, and Hydrocodone (GCMS Mix), Diazepam, Testosterone Propionate, Stanolone, Isobutyl Nitrite, Butyl Nitrite, and Tert-Butyl (Butyl Nitrites Mix) were analyzed using the same type of column with previously validated methods in order to obtain their respective vapor phase IR spectra. Blanks were run before each standard under the same method parameters of the standard. In addition to the previously validated instrument methods, there was an additional auto sample, autosample spitless, and manual injection method created for the Butyl Nitrites Mix and other low molecular weight compounds. The Amphetamine Mix were analyzed using the autosample and splitless autosample methods and the Butyl Nitrites Mix were analyzed by headspace using the 60TO270 manual method. In addition to analyzing standards by autosample, splitless autosample, and manual injection under multiple instrument methods we also used three different known concentrations to determine the limit of detection of each standard for autosample methods (2 mg/mL, 1 mg/mL, and 0.5 mg/mL). Spectra obtained from each of the known concentrations will then be compared to known standards and to determine Limit of Detection. Limit of detection for compounds may vary slightly due to each compound having distinctive interactions with infrared light. Several standard mixes

were used to demonstrate optimal separation between different compounds across various methods using this particular type of column.

D. Wavenumber Accuracy – Once spectra was obtained using the 2 mg/ml standards that were used from the Limit of Detection experiment, their respective spectra was evaluated to standard spectra within the existing GC-FTIR library for wavenumber accuracy. Their measured wavenumbers must be within an optical resolution of +/- 8 cm-1 when compared to the expected wavenumbers of the standard spectra within the existing GC-FTIR library.

V. Results and Discussion:

For the Resolution and Co-Add (Scans) study all library matches of the Resolution and Co-Add (Scans) set at 8-2 and 8-4 (three runs each) were within +/- 2%, with a majority of spectra within a +/- 1% match. The concentrations of the different standards in the THF4A mix varied, however, and may have affected the quality match to the library. Well-defined peak shape and separation were noted for both, with 8-2 having narrower peak shape/width than 8-4. Signal to noise ratios were determined for both 8-2 and 8-4 and found to be comparable. In discussing with ASAP Analytical it was determined that there would be a greater significance in signal to noise using 8-2 vs 8-4 at lower detection limits due to less averaging of the baseline and would provide better visual representation of the peak. Therefore, it is recommended that a Resolution and Co-Add (Scans) of 8-2 be utilized in operating their IRD3 instrumentation. See attached data for results. The chart below outlines results obtained.

THF4a Mix	Signal to Noise	
	Resolution and Co-Add (Scans) 8-2	Resolution and Co-Add (Scans) 8-4
Injection 1		
Tramadol	141	142
4-ANPP	39	86
Heroin	26	50
Acetyl Fentanyl	22	66
Fentanyl	18	42
Injection 2		
Tramadol	159	137
4-ANPP	35	76
Heroin	50	42
Acetyl Fentanyl	31	39
Fentanyl	14	35
Injection 3		
Tramadol	80	132
4-ANPP	37	37
Heroin	37	62
Acetyl Fentanyl	28	34
4-ANPP	22	26

For Repeatability 4-ethylethcathinone 1mg/ml was injected ten times with blanks running before each standard injection across four days to show repeatable results. The first repeatability study was run on 2-26-20 and the instrument stopped in the middle of 4-Ethylethcathinone injection 9. The sequence was restarted on 2-28-20 to finish running blank and standard injections 9 and 10. Additionally, on 2-26-20 collection for blank injection 7 started to show a rise in baseline and 4-Ethylethcathinone abundance went down. The raised baseline and decreased abundance continued for 4-Ethylethcathinone injection 7 and for blank injection 8 and 4-Ethylethcathinone injection 8 and blank injection 9. This appears to be due to the fact that the instrument had last been filled with liquid nitrogen around 5AM that morning and blank injection 7 and the remaining injections, before the sequence stopped, ran by 6:34PM and later which was close and

then exceeded 14 hours of liquid nitrogen in the MCT. It is therefore not recommended to run samples without refilling liquid nitrogen in the MCT past 14 hours. The second repeatability study was run on 2-29-20 and the third repeatability study was run on 3-1-20. The average peak height for all three Repeatability studies was 35,494.5637 (excluding Injections 7 and 8 for Repeatability 1) with a standard deviation of 3454.2373. The peak height variance may be due to variations that might occur within the instrument or to the sample before injection, such as injection volume, how much liquid nitrogen is within the MCT, gas flow variations, oven temperature fluctuations, evaporation, etc. See attached data for results. The chart below outlines results obtained.

4-Ethylethcathinone	Repeatability 1 (Peak Height)	Repeatability 2 (Peak Height)	Repeatability 3 (Peak Height)
Injection 1	42933.1058	33887.8888	33089.8144
Injection 2	41707.7412	37283.7041	35273.2055
Injection 3	38594.7362	31019.3429	31252.7622
Injection 4	39924.8541	37199.8282	31492.6907
Injection 5	37227.6188	33711.5454	32266.6943
Injection 6	40710.4364	38579.2360	32823.2473
Injection 7	20419.5012	37340.4547	31750.9714
Injection 8	3589.7746	35038.4708	32918.3348
Injection 9	38343.0570	35479.7486	33469.1181
Injection 10	35712.0775	29842.9389	35154.1598

For Limit of Detection and Performance Check of Methods it was shown that detection levels at 2.0 mg/ml, 1.0 mg/ml and 0.5 mg/ml are distinguishable with well-defined peaks and spectra that can be compared to known standards in our GC-FTIR library. Several compounds such as the Testosterone Propionate and Stanolone showed a much lower instrument response for all concentrations, had less spectral definition at 0.5mg/ml, and may need to be more concentrated or run on different instrumentation for confirmation. Limit of detection appears to be at a value that is lower than 0.5 mg/mL and dependent on the type of compound. Therefore, some samples that fall below 0.5 mg/ml may not be suitable and fall below detection levels on the IRD3 requiring different instrumentation for confirmation. During the splitless autosample run the instrument stopped collecting in the middle of the blank for the 4-Ethylethcathinone due to a hardware issues and the IRD3 software not communicating with the IRD3. New boards were placed in the IRD3 on 2-25-20 by an ASAP Analytical service engineer and the splitless methods were rerun on 2-26-20. When the splitless methods were run on 2-26-20, the run stopped and was restarted at the blank for the GCMS Mix. Additionally, the splitless methods for the blank for the THF4A Mix and the THF4A Mix were reran on 2-28-20 due to the data collection stopping in the middle of the initial run for the THF4A Mix. Manual method runs were broken up between two days on the 2-28-20 and 3-2-20, stopping after injecting the GCMS Mix and starting on the blank for Diazepam. All standards and standard mixes within each autosample, splitless autosample, and manual methods eluted within the allotted time frame and temperature program, had resolved peaks, demonstrated optimal separation between mixtures of compounds, and were comparable to known standards in the GC-FTIR library. See attached data for results.

The Wavenumber Accuracy study was performed on the 2 mg/ml standards that were run on autosample in the Limit of Detection study. All wavenumbers of the 2 mg/ml

standards were within +/- 8 cm-1 of the known standards in our GC-FTIR library. The largest wavenumber difference was about +/- 6 cm-1 with the lowest wavenumber difference of about 1.5 cm-1. The individual wavenumbers that had larger wavenumber differences between the GC-FTIR library and the 2 mg/ml standards that ran, fell below 0.015 peak height. Although the exact concentrations of the known standards in our GC-FTIR library are not known, it was noted that the peak heights of the 2 mg/ml standards were less than the peak heights GC-FTIR library standards which may have slightly affected the wavenumber accuracy. See attached data for results.

Optical alignment values were monitored since the installation of the ASAP Analytical IRD3 Instrument and any necessary alignment and instrument inlet maintenance was performed prior to analyzing standards run. The chart below outlines results obtained.

Date	Optical Alignment Value
02/10/20	2.22
02/11/20	1.92
02/12/20	2.14
02/13/20	2.11
02/14/20	2.04
02/18/20	2.11
02/19/20	1.89
02/20/20	1.99
02/21/20	1.96
02/23/20	1.87
02/25/20	2.04 – Ferrule was tightened
02/26/20	2.00
02/27/20	1.81
02/28/20	1.92 – Performed optical alignment 2.14 – Reset Max
02/29/20	2.11 – Reset Max to 2.11
03/01/20	1.84 – Performed optical alignment 2.00
03/02/20	1.95
03/04/20	2.00

VI. Conclusions:

After performing studies on Resolution and Co-Add (Scans), Limit of Detection and Performance Check of Methods, Repeatability, and Wavenumber Accuracy, the ASAP Analytical IRD3 instrument showed comparable results to the existing GC-FTIR libraries and instrument responses. It is noted, that no limit of detection, repeatability, or wavenumber accuracy studies had been performed on existing GC-FTIR's for comparison, but appear to have consistent results with potentially greater sensitivity at lower detection limits. The instrument can now be verified as fit for use with analysts trained on the familiarization and use of instrument.

VII. References:

IRD3 ASIC Manual Rev 1-4.doc4 pages 1-33.

Essential FTIR – Operations Manual for GC IRD User Rev. 0-44 pages 1-36.

Forensic Drug Analysis by GC/FT-IR, Kempfert, Ken, Applied Spectroscopy, Volume 42, No. 5, 1988.

Instrumental Data for Drug Analysis Volume 6, Mills, Terry, III, et. Al., CRC Press, 1996.

VIII. Training:

Familiarization of the new Agilent GC and ASAP Analytical IRD3 software will be discussed and demonstrated with analysts. The demonstration will include preparing the instrument for use, setting up and running a sequence with a blank and standard mix in the IRD3-ASIC software, and how to review and print off the data in the Essential FTIR (eFTIR) software once it has been acquired. ASAP Analytical instructional manuals are available as well as operating procedures that have been created for this instrument. All analysts will review the validation and updated policies and procedures pertaining to this instrument prior to use. A roster will be kept of analysts who have successfully completed training including their initials and date.

IX. Competency Tests:

Prior knowledge and competency of existing GC-FTIR's are a part of the Forensic Chemistry training program and will not be required or necessary for the Validation Study/Performance Check of this instrument. Familiarization of the new Agilent GC and ASAP Analytical IRD3 instrumentation and software will be implemented as part of the Forensic Chemistry training program and GC-FTIR competency.