1.0 Purpose

The purpose of this procedure is to define the method for the determination of water soluble vitamins in raw materials and finished products by LC-MS.

2.0 Scope

This procedure applies to the determination of vitamin B1 (thiamin hydrochloride, thiamin mononitrate), vitamin B2 (riboflavin), vitamin B3 (niacinamide, nicotinic acid), vitamin B5 (pantothenic acid, calcium pantothenate), vitamin B6 (pyridoxine, pyridoxine hydrochloride), vitamin B7 (biotin), and vitamin B9 (folic acid) in the QC laboratory at Ion Labs.

3.0 Responsibility

3.1 It is the responsibility of QC Chemists to follow this procedure.

3.2 It is the responsibility of QC Laboratory Management to ensure that this procedure is being followed.

3.3 It is the responsibility of QC Laboratory Management and/or Analytical Development to keep this procedure aligned with current practices.

4.0 Definitions

4.1 **LC-MS** – Liquid chromatography – mass spectrometry

4.2 **QC** – Quality control

4.3 **ACN** – Acetonitrile

4.4 **DMSO** – Dimethylsulfoxide

4.5 **$Na_2EDTA \cdot 2H_2O$ –** Ethylenediaminetetraacetic acid disodium salt dihydrate

4.6 **H_2O** – Deionized water (>18MΩ·cm)

5.0 References

5.1 PRTCL-23-0088, Protocol, Validation of a Method for the Determination of Water Soluble Vitamins by LC-MS

5.2 PRTCL-24-0031, Protocol, Validation of a Method for the Determination of Water Soluble Vitamins by LC-MS

5.3 D-903, SOP, Conversion Factors Used in Analytical Determinations and New Product Formulations

6.0 Supplies

6.1 Chemicals

6.1.1 Reference standards (traceable to USP or other pharmacopeia preferred)

6.1.1.1 Thiamin hydrochloride

6.1.1.2 Pyridoxine hydrochloride

6.1.1.3 Niacinamide

6.1.1.4 Nicotinic acid

6.1.1.5 Calcium-D-pantothenate

6.1.1.6 Folic acid

6.1.1.7 Biotin

6.1.1.8 Riboflavin

6.1.2 Isotopically labelled internal standards (100µg/mL solution or neat solid)

6.1.2.1 Thiamin-$^{13}C_3$

6.1.2.2 Pyridoxine-$^{13}C_3$

6.1.2.3 Niacinamide-$^{13}C_6$

6.1.2.4 Nicotinic acid-$^{13}C_6$

6.1.2.5 Pantothenic acid-$^{13}C_3$,^{15}N

6.1.2.6 Folic acid-$^{13}C_5$,^{15}N

6.1.2.7 Biotin-D_4

6.1.2.8 Riboflavin-$^{13}C_4$,$^{15}N_2$

6.1.3 ACN (HPLC or LC-MS grade as specified in Section 7)

6.1.4 Ammonium formate (HPLC or LC-MS grade as specified in Section 7)

6.1.5 Ammonium acetate (HPLC grade)

6.1.6 Citric acid (ACS reagent grade)

6.1.7 $Na_2EDTA{\cdot}2H_2O$ (ACS reagent grade)

6.1.8 Formic acid (LC-MS grade)

6.2 Glassware and Disposables

6.2.1 Class A volumetric glassware as required for standard and sample preparations

6.2.2 HPLC vials, 2mL with screw-cap enclosures and septa

6.2.3 Tips for adjustable pipettes

6.2.4 0.22 or 0.45 µm nylon or PVDF syringe filters

6.2.5 10-mL plastic syringes with luer lock fitting

6.3 Equipment

6.3.1 Suitable gradient HPLC system consisting of a pump, autosampler, and column oven

6.3.2 Agilent Ultivo mass spectrometer using MassHunter software

6.3.3 Analytical balance

6.3.4 Micro balance

6.3.5 Sonicator

6.3.6 Wrist action shaker

6.3.7 Adjustable pipettes

7.0 Preparation of Mobile Phase, Extraction Solvent, Standards, Internal Standards, and Samples

7.1 Mobile Phase A (20 mM ammonium formate + 0.05 % formic acid in H_2O)

7.1.1 Transfer 1000 mL of H_2O to a suitable container.

7.1.2 Add 1.26 g of ammonium formate (LC-MS grade).

7.1.3 Add a stir bar, and adjust to pH 3.80 ± 0.05 using formic acid.

7.2 Mobile Phase B (ACN)

7.2.1 Use 100% ACN (LC-MS grade).

7.3 Extraction Solvent (70% buffer containing 100 mM ammonium acetate, 25 mM citric acid, and 5 mM $Na_2EDTA{\cdot}2H_2O$ / 30% ACN)

Note: The Extraction Solvent for extraction of nicotinic acid from samples is 100% DMSO. For all other analytes and for preparation of standard and internal standard nicotinic acid solutions, prepare as outlined below.

7.3.1 Transfer 5.4 g of ammonium acetate to a 1000-mL bottle.

7.3.2 Add 3.36 g of citric acid.

7.3.3 Add 1.3 g of $Na_2EDTA{\cdot}2H_2O$.

7.3.4 Add 700 mL H_2O.

7.3.5 Add 300 mL ACN (HPLC grade).

7.3.6 Stir until completely dissolved, and equilibrate to room temperature.

7.4 Diluent (20 mM ammonium formate + 0.005% formic acid)

7.4.1 Transfer 1000 mL of H_2O to a suitable container.

7.4.2 Add 1.26 g of ammonium formate (HPLC grade).

7.4.3 Add 50 µL of formic acid.

7.4.4 Mix until completely dissolved.

7.5 Internal Standard Stock Solution (2.5 µg/mL)

7.5.1 The Internal Standard Stock Solution is prepared from individual 100 µg/mL isotopically labelled standards which are commercially available. Alternatively, 100 µg/mL solutions may be prepared from solid materials by dissolving 1 mg of isotopically labelled standard in 10 mL of Extraction Solvent.

7.5.2 Transfer 1.0 mL of each 100 µg/mL isotopically labelled internal standard solution into a 50-mL low-actinic (red) volumetric flask.

7.5.3 Add 34 mL of Extraction Solvent, and mix well.

7.5.4 Purge with argon, and store at -20°C. The solution has an expiry of one year.

7.6 Internal Standard Working Solution (100 ng/mL)

7.6.1 Transfer 1.0 mL of Internal Standard Stock Solution to a 25-mL low-actinic (red) volumetric flask.

7.6.2 Dilute to volume using Diluent.

7.6.3 Store at 4°C. The solution has an expiry of one month.

7.7 Stock Standard (~10 mcg/mL)

7.7.1 Accurately weigh and transfer about 5 mg of each reference standard into a 500-mL low-actinic (red) volumetric flask.

7.7.2 Dilute to volume using Extraction Solvent.

7.7.3 Add a stir bar, and stir for 1 hour or until **completely dissolved**.

7.7.4 Store at 4°C. The solution has an expiry of 10 days.

7.8 Working Standard (~0.3 mcg/mL)

7.8.1 The Working Standard is prepared fresh on the day of use.

7.8.2 Remove the Stock Standard from refrigeration, and stir for at least one hour.

7.8.3 Transfer 3.0 mL of Stock Standard to a 100-mL low-actinic (red) volumetric flask.

7.8.4 Dilute to volume with Diluent.

7.8.5 Combine 0.5 mL of the Working Standard with 0.5 mL of the Working Internal Standard in an HPLC vial, and **vortex to mix**.

7.9 Stock Sample Preparation

7.9.1 Specific sample testing details are provided in each products profile. If a specific testing details section is not available, then follow preparation procedure as described below, maintaining concentration within the linear range of this method.

7.9.2 The Stock Sample is prepared fresh on the day of use.

7.9.3 The default sample weight is 1.0 g.

7.9.3.1 Finished products with large dosage units and/or very small amount of target analyte may require larger sample size.

7.9.3.2 For raw materials or finished products with a very large amount of target analyte, the sample size may be decreased with a minimum recommended weight of 50 mg.

7.9.4 Based on the label claim or potency, ensure that the analyte concentration in the Stock Sample does not exceed the solubility listed below:

7.9.4.1	Vitamin B1 (thiamin)	5 mg/mL
7.9.4.2	Vitamin B2 (riboflavin)	0.4 mg/mL
7.9.4.3	Vitamin B3 (niacinamide)	5 mg/mL
7.9.4.4	Vitamin B3 (nicotinic acid)	5 mg/mL
7.9.4.5	Vitamin B5 (pantothenic acid)	5 mg/mL

7.9.4.6 Vitamin B6 (pyridoxine) 5 mg/mL

7.9.4.7 Vitamin B7 (biotin) 0.5 mg/mL

7.9.4.8 Vitamin B9 (folate) 0.1 mg/mL

7.9.5 Ensure that the sample is homogeneous prior to weighing.

7.9.5.1 For capsules, combine the fill material from at least 10 dosage units and homogenize in a mortar and pestle if necessary.

7.9.5.2 For tablets, combine at least 10 dosage units and homogenize in a mortar and pestle.

7.9.5.3 For chewable gels (gummies), homogenize at least 10 dosage units as outlined in D-793.

7.9.6 Transfer 1.0 g of sample to a 100-mL low-actinic (red) volumetric flask.

7.9.7 Add about 65 mL of Extraction Solvent (use 100% DMSO as extraction solvent for nicotinic acid).

7.9.8 Shake on a wrist-action shaker for 30 min.

7.9.9 Dilute to volume with Extraction Solvent (or DMSO for nicotinic acid), and shake vigorously.

7.9.10 If the sample is laden with particulate matter, filtration through a 0.22 or 0.45 µm membrane (discarding 2 – 3 mL before collecting the sample for further dilution) may be necessary prior to further dilution of the sample.

7.10 Working Sample Preparation

7.10.1 The linear range of the method for each analyte is listed below. The working sample preparation must be within the linear range of the method.

7.10.1.1 Vitamin B1 (thiamin) 14 – 700 ng/mL

7.10.1.2 Vitamin B2 (riboflavin) 15 – 773 ng/mL

7.10.1.3 Vitamin B3 (niacinamide) 82 – 824 ng/mL

7.10.1.4 Vitamin B3 (nicotinic acid) 13 – 1600 ng/mL

7.10.1.5 Vitamin B5 (pantothenic acid) 16 – 793 ng/mL

7.10.1.6 Vitamin B6 (pyridoxine) 31 – 781 ng/mL

7.10.1.7 Vitamin B7 (biotin) 13 – 1600 ng/mL

7.10.1.8 Vitamin B9 (folate) 2 – 1228 ng/mL

7.10.2 The Working Sample is prepared fresh on the day of use.

7.10.3 Dilute the Stock Sample to the target concentration using Diluent. Multiple dilutions may be required.

7.10.4 Filter through a 0.22 or 0.45 µm membrane (discarding 2 – 3 mL before collecting the sample for analysis). If the stock sample was filtered, filtration of the working sample is not required.

7.10.5 Combine 0.5 mL of the Working Sample with 0.5 mL of the Working Internal Standard in an HPLC vial, and **vortex to mix**.

7.11 Instrument Method Parameters

7.11.1 Column: InfinityLab Poroshell 120 EC-C18, 2.7 µm, 2.1 mm x 150 mm

7.11.2 Sampler

7.11.2.1 Injection Volume: 5 µL

7.11.2.2 Enable Needle Wash: Selected

7.11.2.3 Mode: Flush Port

7.11.2.4 Time: 10 sec

7.11.2.5 Sample Flush-Out Factor: 5.0 times injection volume

7.11.2.6 Overlapped Injection Mode: Off

7.11.3 Binary Pump

7.11.3.1 Flow Rate: 0.2 mL/min

7.11.3.2 Gradient

Time (min)	%A	%B
0.0	95	5
9.0	60	40
9.1	95	5
12.0	95	5

7.11.4 Column Oven

7.11.4.1 Temperature: 35 °C

7.11.5 QQQ

7.11.5.1 Acquisition

7.11.5.1.1 Scan type: dMRM

7.11.5.1.2 Cycle Time: 600 ms

7.11.5.1.3 Polarity: Positive

7.11.5.1.4 Acquisition Parameters for Target Analytes

Note: Retention times and/or window may be adjusted if peaks shift outside of the listed retention time window.

Analyte	Int Std	MS1 Res	Precursor (m/z)	MS2 Res	Product (m/z)	RT (min)	Window (min)	Frag (V)	CE (V)
thiamin	thiamin-$^{13}C_3$	Unit	265	Unit	122	2.05	1.2	76	13
pyridoxine	pyridoxine-$^{13}C_3$	Unit	170	Unit	134	2.20	1.0	86	21
niacinamide	niacinamide-$^{13}C_6$	Unit	123	Unit	80	2.60	1.0	106	21
nicotinic acid	nicotinic acid-$^{13}C_6$	Unit	124	Unit	80	2.30	1.5	75	20
pantothenic acid	pantothenic acid-$^{13}C_3$	Unit	220	Unit	90	3.65	1.9	96	11
folic acid	folic acid-$^{13}C_5$,^{15}N	Unit	442	Unit	295	6.60	2.0	95	15
biotin	biotin-D_4	Unit	245	Unit	227	9.10	2.0	70	10
riboflavin	riboflavin-$^{13}C_4$,$^{15}N_2$	Unit	377	Unit	243	8.50	1.8	129	22

7.11.5.1.5 Acquisition Parameters for Internal Standards

Int Std	MS1 Res	Precursor (m/z)	MS2 Res	Product (m/z)	RT (min)	Window (min)	Frag (V)	CE (V)
thiamin-$^{13}C_3$	Unit	268	Unit	122	2.05	1.2	76	13
pyridoxine-$^{13}C_3$	Unit	173	Unit	137	2.20	1.0	86	21
niacinamide-$^{13}C_6$	Unit	129	Unit	85	2.60	1.0	106	21
nicotinic acid-$^{13}C_6$	Unit	130	Unit	85	2.30	1.5	75	20
pantothenic acid-$^{13}C_3$	Unit	224	Unit	94	3.65	1.9	96	11
folic acid-$^{13}C_5$,^{15}N	Unit	448	Unit	295	6.60	2.0	95	15
biotin-D_4	Unit	249	Unit	231	9.10	2.0	70	10
riboflavin-$^{13}C_4$,$^{15}N_2$	Unit	383	Unit	249	8.50	1.8	129	22

7.11.5.1.6 Source Parameters

Source	Gas Temp (°C)	Gas Flow (L/min)	Nebulizer Pressure (psi)	Sheath Temp (°C)	Sheath Flow (L/min)	Capillary Voltage (V)	Nozzle Voltage (V)
AJS ESI	350	7	25	400	12	2500	0

7.12 Recommended Sequence

7.12.1 Make at least 2 injections of Diluent.

7.12.2 Make 5 injections of the Working Standard.

7.12.3 Make a single injection of each Working Sample.

7.12.4 Make a single injection of the Working Standard after every six samples and at the end of the run.

7.13 System Suitability Requirements

7.13.1 The %RSD for five consecutive injections of Working Standard is NMT 2.0%.

7.13.2 The %RSD for all injections of Working Standard is NMT 3.0%.

7.13.3 If significant interference is present in the diluent injection (the relative response is greater than 0.5% of that in the sample injection), it must be subtracted from the sample result.

7.14 Column Cleaning

7.14.1 It is recommended to clean the column after every run.

7.14.2 Clean the column at 0.2 mL/min with Mobile Phase A / Mobile Phase B (20/80) for at least 30 min.

7.15 Column Storage

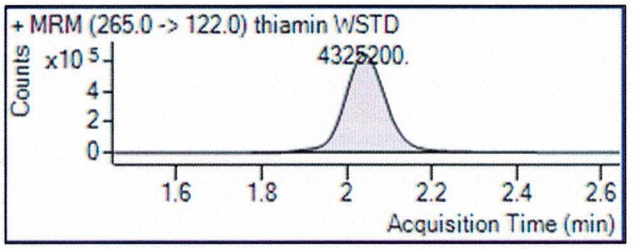
7.15.1 Store the column in Mobile Phase A / Mobile Phase B (20/80).

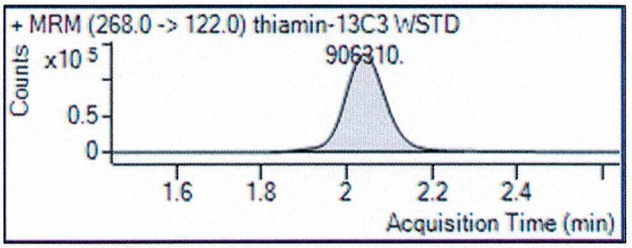
8.0 Example Calculation

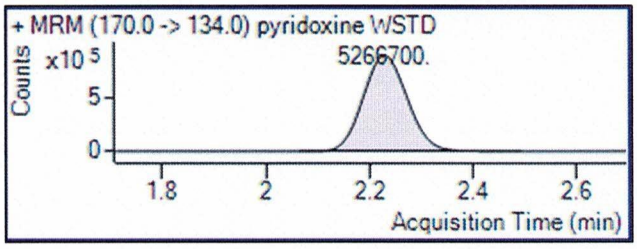
$$\%\ \text{assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{SS}{Spl_{wt}} \times \frac{V_{spl}}{LA} \times MULT \times 100$$

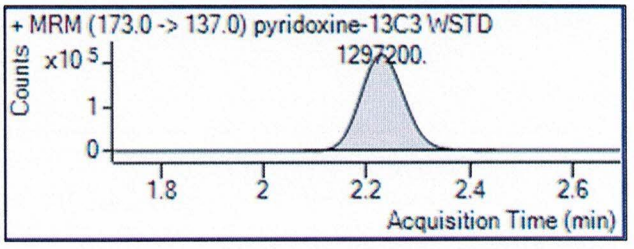
R_u	Sample relative response
R_s	Mean standard relative response
Wt_{std}	Weight of reference standard in mg
V_{std}	Volume of the standard preparation accounting for dilutions in mL
P	Purity of the reference standard in decimal format
SS	Serving size: Weight of a single dosage unit in mg or 1 for raw materials.
Spl_{wt}	Sample weight in mg
V_{spl}	Volume of the sample preparation accounting for dilutions in mL
LA	Label amount in mg per dose or 1 for raw materials
MULT	Multiplier (from D-903)

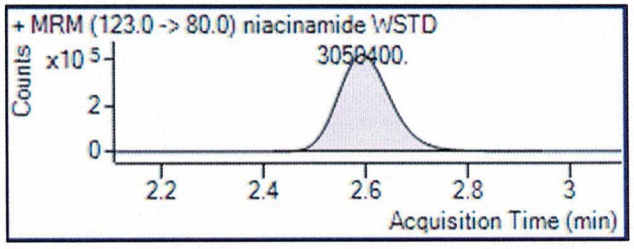
9.0 Example Chromatograms

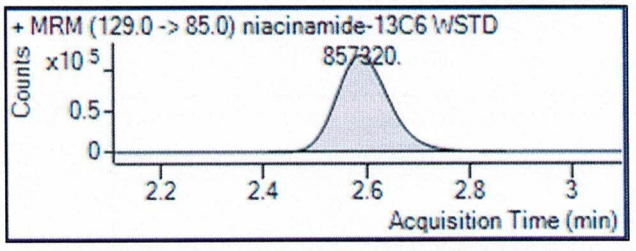


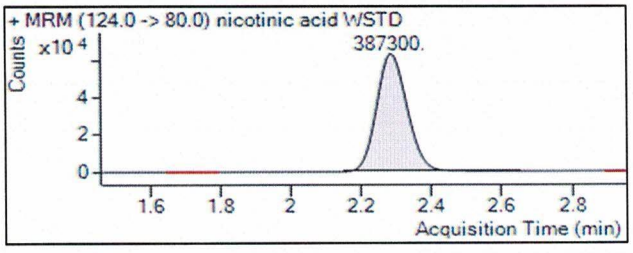


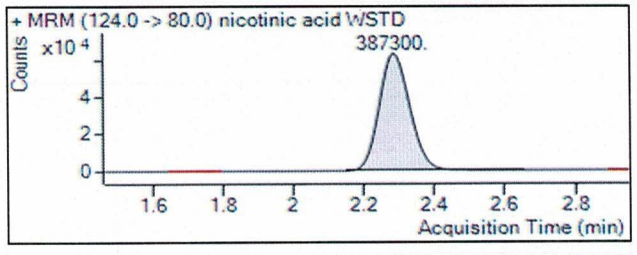


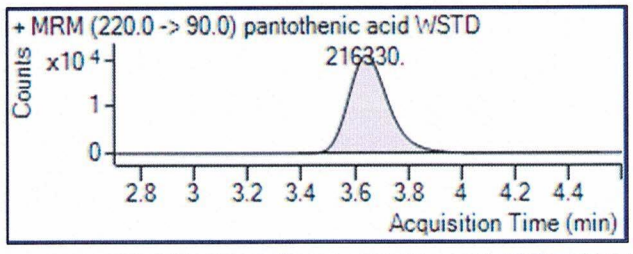


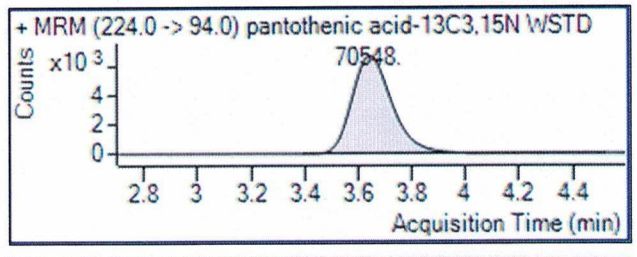


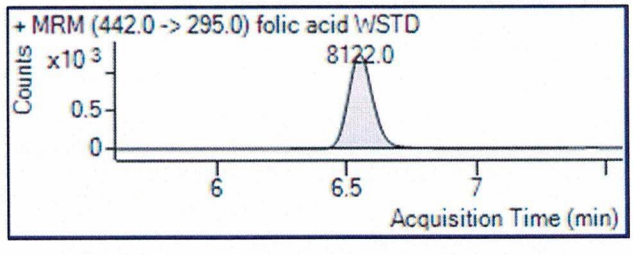


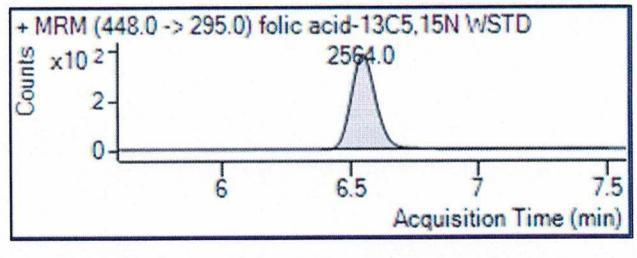


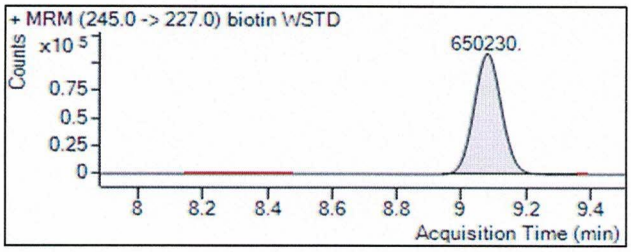


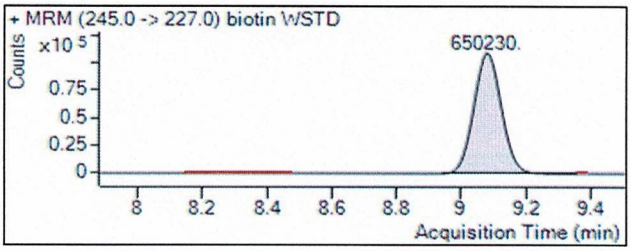


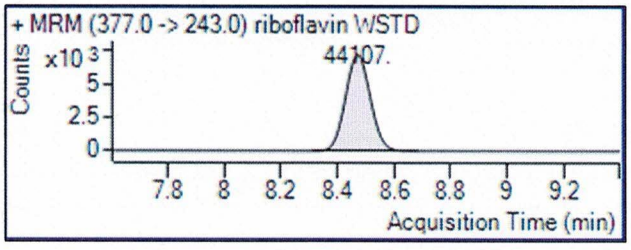


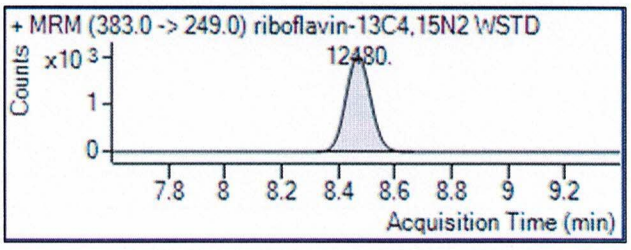












10.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	01/12/24	New procedure.	N/A	S. Sassman
1	06/06/24	Add biotin and nicotinic acid as analytes, change mobile phase prep for more careful control of pH, add preference for traceable standards, add grade requirements for chemicals.	CC-24-0257	S. Sassman