METHOD OF ANALYSIS

N–methyl–2-pyrrolidone

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This method of detection for residues of *N*–methyl–2-pyrrolidone (NMP) in edible tissues of cattle was developed as required by 512(d)(1)(I) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 360b(d)(1)(I)) and is codified in 21 CFR part 500, subpart F through incorporation by reference.

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1. Method of analysis - *N*–methyl–2-pyrrolidone.

A method for the analysis of *N*-methyl-2-pyrrolidone (also known as *N*-methyl 2-pyrrolidinone, or NMP) in bovine liver over the range of 8 µg/g (ppm) to 20 ppm is described. After addition of the internal standard (deuterated NMP, d_9-NMP), NMP is extracted from bovine liver with methanol and then acetonitrile. After dilution, the NMP is separated by hydrophobic high performance liquid chromatography (HPLC) and detected by positive electro-spray tandem mass spectrometry (MS/MS). Quantitation is by external standard calibration of the ratio of the peak areas of NMP/d_9-NMP [mass/charge (m/z) 100→58 for NMP and m/z 109→62 for d_9-NMP] versus concentration. The quantitatively determined peak is confirmed to be NMP by monitoring four precursor/product ion transitions (m/z 100→82, m/z 100→69, m/z 100→58, and m/z 100→41) in Multiple Reaction Monitoring (MRM) mode. Peak areas of three transitions are compared to the fourth transition (quantitation ion) which is used as the reference transition to establish ion ratios. The profile of the ion ratios, above a minimum signal-to-noise (s/n), and retention times of each monitored transition in the samples are compared to those of standard injections for confirmation. In addition, the ion transition of m/z 100→100 must be present, but the ion ratio comparison is not determined.

2. Reagents (HPLC grade)

2.1. Acetonitrile (ACN)

2.2. Methanol (MeOH)

2.3. Water (H_2O)

2.4. Formic acid (FA)

2.5. HPLC mobile phase: ACN:H_2O (1:1, v:v), 0.1% FA. Measure 1 liter (L) ACN and 1 L H_2O separately using a graduated cylinder. Pour them into a 2-L glass bottle. Add 2.0 milliliters (mL) FA using a class A volumetric pipette and mix well. The HPLC mobile phase (MP) is stored at room temperature in a closed container and stable for three months.

3. Standards

3.1. NMP: Aldrich # 78769-5ML-F, 99.9% or equivalent

3.2. d_9-NMP: Aldrich #615854-1G, 99.3% or equivalent

4. General apparatus

4.1. Large Blender: Robot Coupe®, or equivalent

4.2. Small Blender: WARING® Commercial, laboratory blender, or equivalent.

4.3. Plastic Cups: VWR 8oz Cat#: 15706-603 or other suitable containers.

4.4. Balance: analytical, capable of weighing 1 mg accurately to the nearest 0.1 mg.

4.5. Balance: capable of weighing 2 g accurately (to the precision of at least ± 0.001 g).

4.6. Centrifuge: capable of ~2056 X g (3000 rpm).

4.7. Weighing Boat: suitable size weighing boat or small beaker.

4.8. Transfer Pipette: glass or plastic transfer pipette.

4.9. Volumetric flasks: 100-, 50-, 10-mL and other sizes as needed.

4.10. Transfer funnels: glass or plastic, suitable to transfer liquid into volumetric flasks.

4.11. Graduated cylinders: 1000-, 500-, 250-mL and other sizes as needed.

4.12. Glass solvent bottles with screw cap: 2-L, 1-L, 500-mL and other sizes as needed.

4.13. 50-mL polypropylene centrifuge tubes with caps.

4.14. Glass Volumetric Pipettes: Type A, 20-, 10-, 5-, 4-, 3-, 2-, 1-, ½-mL.

4.15. Pipettes: electronic and/or manual pipettes with disposable tips, 20-1,000 µL.

4.16. Vortex Mixer

4.17. Freezer: ≤ -10 °C, ≤ -20 °C for tissue storage.

4.18. HPLC vials: auto-sampler vial, 2-mL.

5. HPLC Equipment and Conditions

5.1. HPLC system: Waters Alliance 2795, or Agilent 1100, or Shimadzu 10A, or other equivalent HPLC system.

5.2. HPLC column and guard column

5.2.1. Preferred HPLC column and guard

5.2.1.1 Waters Atlantis HILIC-Silica, 3µm, 4.6x150mm, Part#: 186002029.

5.2.1.2 Waters Atlantis HILIC-Silica Guard, 3µ, 4.6x20mm, Waters Part#: 186002023.

5.2.1.3. Guard Holder: Waters Sentry Guard Holder WAT046910.

5.2.2. Alternate HPLC column and guard

5.2.2.1. Phenomenex Luna HILIC, 3µm, 4.6x150 mm, Part# 00F-4449-E0.

5.2.2.2. Phenomenex Luna HILIC SecurityGuard Cartridges, 3µm, 4x3.0mm, Part# AJ0-8329.

5.3. Column clean-up: Wash analytical and guard columns with ACN:H_2O (50:50, v:v) for 30 minutes (min) after use.

5.4. HPLC conditions:

5.4.1. flow rate: 0.4 mL/min

5.4.2. injection volume: 20 µL, based on instrument sensitivity

5.4.3. auto-sampler temperature (temp): ambient (18-21 °C)

5.4.4. column temp: ambient

5.4.5. retention time: 5-8 min

6. MS Equipment and Conditions

6.1. MS system: Micromass Quattro *micro*™, or Quattro *Ultima*™, or Applied Biosystems MDS SCIEX 3200 Q TRAP®, or other equivalent mass spectrometers with appropriate data acquisition/processing software for MS system used.

6.2. MS conditions: The MS should be tuned by direct infusion of an NMP standard solution which contains NMP and d_9-NMP with suitable concentrations, for example ~1 µg/mL. The MS conditions are optimized for maximum detection of ions m/z 58, 69, 82, 41 and 100 for NMP and 62 for d_9-NMP in MS/MS mode. The s/n ratio must be ≥ 10 for all of the transition ions. The resulting tune file should be used for all analyses, although the operator may vary conditions for optimum sensitivity. Table 6.2.1 lists suggested conditions for analysis. Table 6.2.2 lists the ion transitions for monitoring and their respective suggested instrument parameters.

The confirmatory method will use the transition ion of m/z 100→58 as the reference ion, which must be the base peak (most abundant ion) relative to any of the other three ions (m/z 100→69, m/z 100→82 or m/z 100→41). The abundance of ion m/z 100→100 can be higher than that of m/z 100→58, but the m/z 100→100 must only be present and is not used for ion ratio comparisons. Therefore, the collision energy level needs to be carefully selected to ensure that the maximum response is obtained for the transition ion of m/z 100→58 relative to the other three ions of m/z 100→69, m/z 100→82 and m/z 100→41. Generally, the optimized tuning of instrument conditions and ion transition monitoring parameters selected during the tuning process will result in the transition of m/z 100→58 being the most abundant. If this is not the case, the collision energy used for the transition of m/z 100→69 should be increased, so that the final response from the transition of m/z 100→69 would be sufficiently lower than the transition of m/z 100→58 for NMP to ensure that the transition of m/z 100→69 is never the base peak. The responses from ions of m/z 100→41 and m/z 100→82 are generally much smaller, thus the collision energy levels for these ions should be optimized to maximize their sensitivities. Overall, the MS tuning conditions should be optimized for the transition of m/z 100→58 to guarantee that the response of this ion transition is the base peak at all times.

Table 6.2.1. Micromass Quattro *Micro*™ instrument parameters for NMP analysis*

Source Parameters		Analyzer Settings	
Ionization mode	ESI+	LM1 resolution	13.0
Capillary	3.0 kV	HM1 resolution	13.0
Extractor	4.00 V	Ion energy 1	1.0
RF lens	0.0 V	Entrance slit	-7
Source temperature	120 °C	Exit slit	1
Cone	35 V	LM2 resolution	13.0
Desolvation temperature	350 °C	HM2 resolution	13.0
Cone gas flow	50 L/hr	Ion energy 2	2.0
Desolvation gas flow	650 L/hr	Multiplier	650

*These are suggested values only and may vary with instrument, operator, and/or time.

Table 6.2.2. Ion transitions and respective instrument parameters for sample analysis*

Required Transitions to Monitor for Confirmation				
Transition (m/z)	Usage	Dwell time (sec)	Cone voltage (V)	Collision Energy (V)
100→100	Confirmatory**	0.20	35.0	20.0
100→82	Confirmatory	0.20	35.0	20.0
100→69	Confirmatory	0.20	35.0	20.0
100→58	Quantitative and Confirmatory	0.20	35.0	25.0
100→41	Confirmatory	0.20	35.0	25.0
109→62	Internal standard for Quantitative	0.20	35.0	25.0

*These are suggested values only. Instrument parameters and m/z ratios may vary with instrument, operator and/or time.

**The ion transition m/z 100→100 is only used to check s/n. It is not used for ion ratio comparisons.

7. Standard Preparation

All standard solutions are stored in a freezer ≤ -10 °C and should be used within 8 weeks. Standard concentrations should be corrected for purity.

7.1. Preparation of NMP Stock Solution (~2000 µg/mL): Accurately weigh (± 0.1 mg) 100 to 120 mg of the NMP reference standard and transfer to a 50-mL volumetric flask. Record the precise weight and use this in subsequent calculations incorporating the purity. Bring to volume with ACN and mix well, ensuring that the compound dissolves completely.

7.2. Preparation of d_9-NMP Stock Solution (~2000 µg/mL): Accurately weigh (± 0.1 mg) 100 to 120 mg of the d_9-NMP reference standard and transfer to a 50-mL volumetric flask. Bring to volume with ACN and mix well, ensuring that the compound dissolves completely.

7.3. Preparation of Diluted NMP Stock Solution (1000 µg/mL): Transfer an appropriate volume (*e.g.*, ≤ 25 mL) of the NMP stock solution (~2000 µg/mL) into a 50-mL volumetric flask using glass volumetric pipettes and electronic (or manual) pipettes (accuracy ≤ 3%, precision ≤ 2%). Dilute to volume with ACN. The exact volume of the stock solution taken would depend on the concentration of the stock solution. The goal is to achieve a concentration as close as possible to 1000 µg/mL. The exact concentration should be calculated and reported.

7.4. Preparation of Diluted d_9-NMP Internal Standard Stock Solution (1000 µg/mL): Transfer an appropriate volume (*e.g.*, ≤ 25 mL) of the d_9-NMP stock solution (~2000 µg/mL) into a 50-mL volumetric flask using glass volumetric pipettes and electronic (or manual) pipettes (accuracy ≤ 3%, precision ≤ 2%). Dilute to volume with ACN. The exact volume of the stock solution taken would depend on the concentration of the stock solution. The goal is to achieve a concentration as close as possible to 1000 µg/mL. The exact concentration should be calculated and reported.

7.5. Preparation of NMP Working Standard Solutions: Transfer the appropriate volume of the diluted stock or working standard (WSTD) solution into a 100 mL volumetric flask using a glass volumetric pipette and dilute to volume with ACN. The exact concentration should be reported and used throughout all the calculations.

Table 7.5: Preparation of NMP Working Standard Solutions.

Working Standard Solution (WSTD)	Concentration of WSTD (µg/mL) Prepared	Use the stock or WSTD solution of	Concentration of the solution used (µg/mL)	Volumetric Pipette (mL)	Final Volume (mL)
WSTD_100	100	Diluted NMP stock *	1000	10	100
WSTD_4	4.0	WSTD_100	100	4	100
WSTD_3	3.0	WSTD_100	100	3	100
WSTD_2	2.0	WSTD_100	100	2	100
WSTD_1.5	1.5	WSTD_100	100	1.5	100
WSTD_1	1.0	WSTD_100	100	1	100
WSTD_0.5	0.5	WSTD_100	100	0.5	100

*prepared in step 7.3.

7.6. Preparation of d_9-NMP Working Internal Standard Solutions: Transfer the appropriate volume of the d_9-NMP diluted stock or working internal standard solution into a 100-mL volumetric flask using a glass volumetric pipette and dilute to volume with ACN. The exact concentration should be reported and used throughout all the calculations.

Table 7.6. Preparation of d_9-NMP Working Internal Standard Solutions

Working Internal Standard WINSTD solution	Concentration of WINSTD (µg/mL)	Concentration of diluted INS stock or WINSTD (µg/mL)	Volumetric Pipette used (mL)	Volume of the flask (mL)
WINSTD_100	100	1000*	10	100
WINSTD_10	10	100	10	100
WINSTD_1	1.0	10	10	100

*diluted d_9-NMP stock solution prepared in step 7.4.

7.7. Preparation of NMP Standard Curve Solutions: Six calibration points are used for the standard curve to quantitate NMP. Standard curve solutions are prepared by diluting a working standard solution to a known volume in order to achieve the required concentrations. When preparing a standard curve solution, transfer appropriate volumes of a working standard solution and internal standard solution into a volumetric flask, dilute to the volume with ACN and mix well. All standard curve solutions will contain a final concentration of 0.1 µg/mL d_9-NMP as the internal standard (IS).

Table 7.7. Preparation of NMP Standard Curve Solutions

Standard curve solutions	From NMP WSTD solutions	Concentration of WSTD solutions (µg/mL)	Volumetric Pipette used to take NMP WSTD (mL)	Volumetric Pipette used to take WINSTD_1 (mL)	Volumetric flask (mL)	Final Injection Concentration (µg/mL) NMP/d9-NMP	Equivalent NMP Concentration in Liver Samples (ppm)
std_0.4	WSTD_4	4.0	5	5	50	0.40/0.10	40
std_0.3	WSTD_3	3.0	5	5	50	0.30/0.10	30
std_0.2	WSTD_2	2.0	5	5	50	0.20/0.10	20
std_0.15	WSTD_1.5	1.5	5	5	50	0.15/0.10	15
std_0.1	WSTD_1	1.0	5	5	50	0.10/0.10	10
std_0.05	WSTD_0.5	0.5	5	5	50	0.05/0.10	5

Note: In order to reduce matrix effects, refer to Table 14.3.1. for preparation of diluted NMP standard curve solutions (see section 14, Notes to Analyst).

8. Sample Handling and Sampling

8.1. Processing Large Bovine Liver Sample Amounts Using a Large Blender - Fresh or partially thawed frozen livers are chopped into pieces (~1 inch cube) and transferred into the container of the food processor (see 4.1). The process is performed cryogenically. An equivalent bovine liver volume of dry ice (powdered or rice-pellet) is distributed on the bottom surface of the container and then the semi thawed chopped tissue is laid over the dry ice layer. Then a second layer of dry ice is placed on top of the liver pieces. The entire mixture is ground initially at a low speed or pulse speed until the desired consistency is reached (*i.e.,* ~60-90 seconds until the tissue is uniformly ground). The tissue is then ground at a higher speed until it becomes a uniform powder (~60-90 seconds). The powdered tissue (containing dry ice) is aliquoted into plastic cups (or bags) and capped left with a slight opening to allow the carbon dioxide to sublime, while stored at ≤ -10 °C. After the dry ice is completely sublimed (~12-16 hrs at ≤ -10 °C), the cups (or bags) are fully sealed for long-term storage. NMP is stable for up to 1 yr at ≤ -20 °C.

8.2. Processing Small Bovine Liver Sample Amounts Using a Small Blender - The frozen or semi thawed liver tissues should be chopped first into small pieces. The chopped tissue samples are weighed then processed with an equivalent amount of dry ice using a laboratory blender for about 1 minute. The sample is aliquoted and stored as above.

9. Sample Preparation Procedure

9.1. Accurately weigh (see 4.5.) 1 ± 0.1 g of processed control blank or incurred liver sample into a 50-mL polypropylene tube.

9.2. Add appropriate volumes (Table 9.2) of NMP working standard solution into QC samples. The NMP working standard solution should be added by dropping it on to the ground liver material. The IS is required for the quantitative assay. For each sample set, at least two control samples (one with and one without IS) and two samples fortified at Rm should be prepared (see section 11 for QC sample requirement).

Table 9.2. Preparation of QC Samples

Fortified QC samples	Fortified volume of NMP WSTD100 (µL)	Fortified volume of d_9-NMP WINSTD100 (µL)	Fortified concentration (µg/g or ppm) std/instd	Final injection concentration (µg/mL)
LQC_8	80	100	8.0/10.0	0.08/0.1
MQC_10	100	100	10.0/10.0	0.1/0.1
HQC_20	200	100	20.0/10.0	0.2/0.1

9.3. Except for the double blank control (no analyte or IS) samples, add 100 µL of IS solution (winstd_100) to all samples including blank control samples (blank with IS), QC samples, and incurred liver samples. The IS solution should be added by dropping it on to the ground liver material.

Note: If the tissue samples are analyzed for confirmation only, the IS does not need to be added. However, adding the IS is not a method deviation so that the samples can be used for both quantitation and confirmation.

9.4. After fortification, briefly vortex the samples and allow the samples to equilibrate at room temperature for at least 10 minutes, permitting the fortification solvent to evaporate.

9.5. Add 20 mL methanol into the sample tube, cap tight and vortex for ~1 min.

9.6. Centrifuge the sample tube for 10 min at ~3000 rpm (see 4.6).

9.7. Transfer the supernatant into a clean 50-mL volumetric flask using a funnel to simplify the transfer and rinsing the funnel with the dilution solvent from subsequent steps to achieve a quantitative transfer.

9.8. Repeat step 9.5. using 20 mL ACN, then repeat 9.6. and 9.7., combine the two extracted supernatants and mix well.

9.9. Rinse the funnel and take to volume with ACN and mix well. Transfer the final extract into a 50-mL centrifuge tube and centrifuge the extract for 10 minutes at ~1000 rpm (~250 x g) to ensure that the final extract solution is clear and free of floating particulate matters.

9.10. Dilute the above extract solution 1:1 by volume with ACN for analysis by mixing 0.5 mL extract solution and 0.5 mL ACN in an HPLC vial.

Note: In order to reduce matrix effects, refer to Sections 14.3.1 and 14.3.2 for preparation of further diluted sample extracts and diluted NMP standard curve solutions (see section 14, Notes to Analyst).

9.11. Prepare the NMP standard curve solutions as described in section 7.7 and transfer these solutions into HPLC vials for injection.

Note: At the beginning of each set, one or more injections of the lowest NMP standard solution and 10 or more injections of blank control bovine liver sample for conditioning of the LC column and LC-MS/MS system (see section 14, Notes to Analyst for further details) should be analyzed.

If the samples will not be injected immediately after preparation and/or if they need to be re-injected later, the sample solutions should be stored in a freezer at ≤ -10°C. The stored sample solutions should be warmed to room temperature and thoroughly mixed before injection. The samples should be analyzed within 7 weeks of their preparation. The extract samples and standard curve solutions can also be stored in a refrigerator at ~4 °C, but must be analyzed within 2 days.

10. Calculation and Expression of Results

10.1. Calculation and Expression of Results for Determination of NMP Residue

10.1.1. Calculation Method

The sample concentration is calculated by performing a non-weighted linear regression on the calibration, concentrations of the standards to first obtain a standard curve. The standard curve plots the concentration of the analyte against the response by the following calculation (this response is used as the y-variable subsequently):

$$\text{Response} = \text{NMP peak area} \bullet \frac{\text{IS concentration}}{\text{IS peak area}} \qquad (10.1.1\text{-}1)$$

All standards are run before and after (i.e., bracketing) bovine samples including one non-fortified control sample and one double blank control sample. The standard curve is not forced through the origin (zero) point. Non-weighted linear regression is applied to the standard curve. The regression equation (described in 10.1.1-2) is then used to calculate the concentration of NMP in the samples. The r^2 must be ≥ 0.99. If the

regression does not meet acceptance criteria, the set is deemed not acceptable and must be repeated by re-injecting the standards and samples or by preparing new standards and re-injecting the sample extracts with the standards, or by preparing new standards and samples.

The linear regression equation of the standard curve of the bracketing standards will be used to determine the concentration of the sample solutions injected using the following equation:

$$y = mx+b \qquad (10.1.1\text{-}2)$$

The concentration of each sample is calculated by using the formula

$$x = \frac{y - b}{m} \qquad (10.1.1\text{-}3)$$

Where:

y = MS detector calculated response using the IS ratio (see 10.1.1-1)
x = sample concentration
m = slope of the regression equation
b = y-intercept

10.1.2. Calculation of Unknown Concentrations from Incurred-residue Liver Samples and Fortified Samples

The following equation will calculate the concentration in µg/g (ppm):

$$C_T = \frac{(C_x \times V_f)}{S_w} \qquad (10.1.2\text{-}1)$$

Where:

C_T = concentration of NMP in µg/g (ppm) in the bovine liver tissue sample.
C_x = concentration of NMP in µg/mL in the final injection sample solution, which is equivalent to x as determined in the equation 10.1.1-3.
V_f = reconstitution volume of the sample residue multiplied by the dilution factor, for example 50 mL x 2.
S_W = weight in g of the initial sample (nominal weight of 1 g is used for fortified samples and exact weight is used for incurred and blank control samples).

An example of concentration calculation is given below:

For an incurred liver sample,

C_x = 0.15 µg/mL
Dilution factor = 2
V_f = 50 mL x 2 = 100 mL
S_W = 1.068 g

$$C_T = \frac{[0.15 \times (50 \text{ x } 2)]}{1.068} = 14.0 \text{ ppm}$$

10.1.3 Calculation of Recoveries from Fortified Samples

Recoveries are calculated from fortified samples using the equation:

$$\% \text{ Recovery} = \left(\frac{C_T}{C_F}\right) \times 100 \qquad (10.1.3\text{-}1)$$

Where:

C_T = concentration of NMP in µg/g (ppm)in the bovine liver tissue sample, determined using equation (10.1.2-1).
C_F = tissue fortification concentration in µg/g (ppm).

An example of fortified recovery calculation is given below:

A fortified bovine liver sample,

C_T=10.12 ppm*
C_F=10.24 ppm*

$$\% \text{ Recovery} = \left(\frac{10.12}{10.24}\right) \times 100 = 98.8\%$$

*Note: Rounding to three significant figures should be limited to the final result.

10.1.4. NMP Residue Determination Criteria

The linearity of the calibration standard curve, as indicated by the coefficient of determination, r^2, should be ≥ 0.99. The QC recovery should be 80 to 110%. Precision is expressed by the coefficient of variation (CV) and should be $\leq 10\%$.

10.2 Calculation and Expression of Results for NMP Confirmation

10.2.1 Calculation of Ion Ratios

The ion ratio (expressed as relative abundance) of the transition of interest compared to the reference transition is calculated for each sample according to the following equation:

$$\text{Ion Ratio} = \frac{\text{peak area of transition of interest}}{\text{peak area of reference transition}} \text{ x } 100\% \qquad (10.2.1\text{-}1)$$

The ion transitions are given in Table 6.2.2 (section 6.2) with the reference transition of m/z 100→58 for confirmation. Standards are injected contemporaneously with samples. Standards may be injected as a group prior and subsequent to samples or interspersed among the samples. The retention times and the ion ratios used for confirmation are averaged for all standards in that set. For each tissue sample, the retention time of NMP and ion ratios of the confirmation transitions are compared against the appropriate average of the standards for confirmation.

10.2.2 Confirmation Criteria

Analytical data must meet all of the following criteria in order to confirm the presence of NMP in a bovine tissue sample. Four ion transitions are monitored in MRM mode for ion ratio comparisons in order to confirm the presence of NMP in bovine liver, with one transition designated as the reference transition. The four monitored ion transitions are m/z 100→82, m/z 100→69, m/z 100→58 and m/z 100→41 with m/z 100→58 designated as the reference transition. The transition m/z 100→58 must be the base peak. If not, refer to Section 6.2 to modify the MS tuning conditions and ensure that the response of this ion transition is the base peak. The retention time and sensitivity of the analyte within the sample must meet the performance criteria. In addition, a fifth ion transition of m/z 100→100 must also be present (s/n $\geq$10:1) in order to confirm the presence of NMP in the bovine tissue sample.

10.2.2.1 Ion Ratios

The ion ratio (relative abundance) of m/z 100→69 to the reference transition m/z 100→58 must fall within ±20% (relative) of the average ion ratio of the standards. For example, if the average ion ratio for standards is 80%, the acceptable ion ratio range of sample would span 64% – 96%. The ion ratio values of m/z 100→41 and m/z 100→82 to the reference ion must fall within ±40% (relative) of the average ion ratio of the standards. For example, if an average ion ratio for standard is 30%, the acceptable ion ratio range of sample would span 18% - 42%. Because ion ratio values of m/z 100→41 and m/z 100→82 to the reference ion are generally below 30%, the criteria of ±40% of relative difference would be equivalent to less than 12% of absolute difference (*i.e.,* 30% ± 12%).

Table 10.2.2.1. Typical Ion Ratios (Relative Abundances) Obtained Using a Micromass *micro* Instrument

m/z Transitions	m/z 100→58 (Reference)	m/z 100→69	m/z 100→82	m/z 100→41
Typical Ion Ratios (Relative Abundances)	100%	~49.4%	~10.4%	~7.9%
Acceptable Range for Ion Ratios (Relative Abundances)	N/A	~39.5% to 59.3%	~6.2% to 14.6%	~4.7% to 11.1%
Acceptance Criteria (Relative % Differences)	N/A	±20%	±40%	±40%
Expressed as Absolute % Differences	N/A	±9.9%	±4.2%	±3.2%

10.2.2.2. Sensitivity

The s/n of each transition peak for m/z 100→58, m/z 100→69, m/z 100→82, m/z 100→41 and m/z 100→100 must be ≤ 10:1.

10.2.2.3. Retention Time

The retention time of NMP in an individual sample must fall within ± 2% (relative) of the average retention time of NMP in the standards.

11. Quality Control Samples

All analyses should include at least one blank control sample with IS and one double blank control sample (no analyte or IS). At least two non-fortified control blanks (one blank and one double blank) and two QC samples at each of two concentration levels (including Rm concentration) are used for a regular incurred sample assay set which analyzes four or more incurred samples. All QC samples should meet all of the confirmation criteria. The control blank and double blank samples should fail the criteria. The standard calibration curve should have at least 5 concentrations including the lowest concentration. All QC samples must be reported and meet the 80–110% recovery requirement for the set to be valid for quantitation.

If a sample is analyzed and it was found that quality control criteria were not met during that analysis, the sample may be re-injected or re-assayed after taking steps to improve system performance and re-establish quality control. If the QC samples still do not meet the acceptability criteria after MS optimization and re-injection, the whole sample set is invalid and must be re-extracted and re-analyzed.

12. System Suitability

Before analysis of unknown samples for the determination and confirmation of NMP, system suitability should be assessed. This system suitability assessment includes instrument tuning and test sample injections.

The instrument tuning is performed with infusion of a tuning solution, for example a standard solution which contains around 1 µg/mL NMP and d_9-NMP. All product ions for NMP confirmation and determination should be observed in MS/MS mode.

In addition, a short system suitability test will be run, which includes but is not limited to a test standard curve, a solvent blank, three injections of a blank control bovine liver extract, and three injections of an 8 ppm QC sample. The system suitability tests will not only check if the instrument functions properly, but also check sensitivity, retention time, carry over, injection reproducibility, matrix effects on ion suppression, QC sample recoveries, and so on. At the beginning of this short run, the instrument should be conditioned with one or more injections of the lowest NMP standard solution and 10 or more injections of blank control bovine liver sample.

The system suitability test passes for confirmation if the following criteria are met:

- The transition ion m/z 100→58 is the most abundant (base peak and reference ion);
- All injections of the 8 ppm QC sample must pass all the confirmation criteria (s/n, retention time, and ion ratios);
- All injections of blank solvent and blank control liver sample fail at least one of the confirmation criteria (s/n, retention time, or ion ratios).

The system suitability test passes for quantitation if the following criteria are met:

- Standard curve linearity, $r^2 \geq 0.99$;
- All injections of 8 ppm QC sample pass the recovery criteria of 80-110% and precision criteria (CV%) of $\leq$10%;
- The average peak area of the 8 ppm QC samples is > 25% of the peak area* calculated for an NMP standard concentration equivalent to 0.08 µg/mL ACN.

*Note: This calculation is to estimate the matrix effect and ion suppression for quantitative analyses of NMP. The peak area of 0.08 µg/mL NMP standard solution in ACN is calculated from the linear regression equation.

If the 8 ppm QC sample fails these criteria, the system suitability for quantitation analyses should be tested with a 5-fold further sample dilution (see section 14.3 below) to reduce the matrix effects and ion suppression. Quantitative analyses with 5-fold further sample dilutions may be used as an optional approach at all times as long as the sensitivity of instrument is not compromised (***e.g.,*** all system suitability criteria pass).

The system suitability assessment need only be performed once per analyst and per instrument before sample analyses in a series of runs. The system suitability assessment may be performed on one or more days prior to assay of unknown samples. If an instrument was serviced or not used for a period of time, or if a different mass spectrometer is to be used for this method, or at any other time that it is deemed needed, the system suitability test should be performed or repeated as described above.

13. Stability

NMP is stable for 8 weeks in stock solutions and standard curve solutions, and for 7 weeks in sample extracts when they are stored in freezers ≤ -10 °C. NMP stock solutions are stable at room temperature for 8 hours. NMP standard curve solutions and bovine liver extracts are stable at ~4 °C for 2 days. NMP is stable for ~12 months in fortified bovine liver samples when stored in freezer at ≤ -20 °C.

14. Notes to the Analyst

This method is designed to determine the NMP residue concentration and confirm the presence of NMP in bovine liver samples. Therefore, the same sample extracts may be analyzed for both NMP residue concentration determination and confirmation, or for NMP confirmation only.

The guard column and/or analytical column may need to be replaced if increased column back pressure, increased baseline noise, or bad peak shape are observed.

14.1. Instrument Conditioning

At the beginning of each set, inject one or more of the low concentration NMP standard to condition the LC column. The analyst should visually check the first one or more chromatograms to ensure that all the monitored transition ions are detected, the peak shape is good and the retention time of NMP is as expected. In addition, ten or more injections of the blank control QC sample are needed to condition the LC-MS/MS system for the sample matrix effects. These conditioning test injections are not used as raw data and not processed with the analytical set results.

14.2. Typical Test Results

The following are some typical test results obtained when performing the NMP analyses with this method.

Table 14.2.1. Typical Test Results Obtained for NMP Quantitation Analyses Using a Micromass *micro* Instrument

Nominal Fortification Concentrations (ppm)	Analyst I (6 replicates at each concentration per day over 4 days)		Analyst II (6 replicates at each concentration per day over 3 days)	
	Mean % Recovery Range	% CV Range	Mean % Recovery Range	% CV Range
8 (LOQ_8)	94.0 to 103	1.9 to 4.3	102 to 107	1.1 to 2.5
10 (MQC_10)	94.5 to 103	1.5 to 6.1	102 to 105	1.3 to 3.3
20 (HQC_20)	94.5 to 100	2.9 to 5.0	98.9 to 102	1.7 to 3.4

Table 14.2.2. Typical Test Results Obtained for NMP Confirmation Analyses Using a Micromass *micro* Instrument

m/z Transitions	m/z 100→58 (Reference)	m/z 100→69	m/z 100→82	m/z 100→41
Typical Ion Ratios (Relative Abundances)	100%	~49.4%	~10.4%	~7.9%
s/n Ratios	>530	>403	>42	>21
RetentionTimes (min.)	~7.03	~7.03	~7.03	~7.03

14.3. Quantitation Analyses in Order to Reduce Matrix Effects

For certain LC-MS/MS instruments, the matrix effects on ion suppression may be severe and the QC sample recoveries sometimes fail the 80-110% criteria. In this occurrence, the quantitation analyses should include a 5-fold further sample dilutions (see sections 14.3.1-14.3.2 below) to reduce the matrix effects and ion suppression. Accordingly, the NMP standard curve solutions are also diluted.

14.3.1. Preparation of Diluted NMP Standard Curve Solutions

Six calibration points are used for the diluted standard curve to quantitate NMP. This diluted standard curve ranges from 0.01 µg/mL to 0.08 µg/mL, which covers the equivalent NMP residue concentrations in bovine liver from 5 ppm to 40 ppm. Neat diluted standard curve solutions are prepared by diluting a working standard solution to a known volume in order to get the required concentrations. When preparing a diluted standard curve solution, transfer appropriate volumes of a working standard solution and internal standard solution into a volumetric flask, dilute to the marked volume with

ACN and mix well. All diluted standard curve solutions will contain a final concentration of 0.02 µg/mL d_9-NMP as the internal standard.

Table 14.3.1. Preparation of Diluted NMP Standard Curve Solutions

Standard curve solutions	From NMP WSTD solutions	Concentration of WSTD solutions (µg/mL)	Volumetric Pipette used to take NMP WSTD (mL)	Volumetric Pipette used to take WINSTD_1 (mL)	Volumetric flask (mL)	Final Injection Concentration (µg/mL) * NMP/d_9-NMP	Equivalent NMP Concentration in Liver Samples (ppm)
std_0.08	WSTD_4	4.0	1	1	50	0.08/0.02	40
std_0.06	WSTD_3	3.0	1	1	50	0.06/0.02	30
std_0.04	WSTD_2	2.0	1	1	50	0.04/0.02	20
std_0.03	WSTD_1.5	1.5	1	1	50	0.03/0.02	15
std_0.02	WSTD_1	1.0	1	1	50	0.02/0.02	10
std_0.01	WSTD_0.5	0.5	1	1	50	0.01/0.02	5

*The concentration of the solution placed in the sample vial on the HPLC-MS/MS.

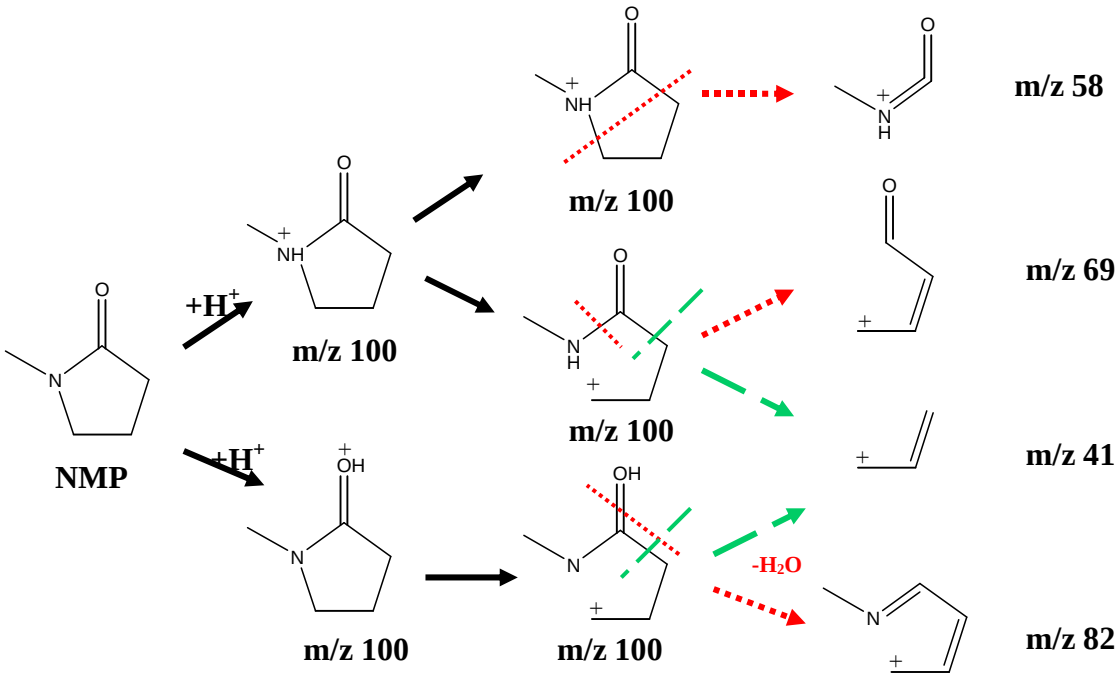
14.3.2 Sample Preparation Procedure in Order to Reduce Matrix Effects

14.3.2.1. Follow the same sample preparation procedure as described in sections 9.1 to 9.9.

14.3.2.2. Dilute the above extract solution 1:9 by volume with ACN for analysis by mixing 0.1 mL extract solution and 0.9 mL ACN in an HPLC vial.

14.3.2.3. Prepare the diluted NMP standard curve solutions as described in section 14.3.1 and transfer these solutions into HPLC vials for injection.

15. Proposed Fragmentation of *N*–Methyl–2-pyrrolidone (NMP).



Note: For NMP ion transition of m/z 100→58, there are two corresponding d_9-NMP transitions, m/z 109→61 and m/z 109→62. The transition of m/z 109→62 was selected for the quantitation.