Analytical procedure for the determination of 3-nitrooxypropanol in cattle feed/TMR by HPLC-UV

Index

1 Foreword

This test method has been developed for the determination of 3-nitrooxypropanol in cattle feed/TMR.

2 Introduction

N/A

3 Title

Analytical procedure for the determination of 3-nitrooxypropanol in cattle feed/TMR by HPLC-UV

4 Warnings

Warning: Persons using this analytical method should be familiar with normal laboratory practice. This method does not purport to address all the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

Table 1: Safety aspects of chemicals

Compound	GHS word/Symbol	Hazard statements	Precautionary statements
Acetonitrile	Danger 	H225 Highly flammable liquid and vapor. H302 + H312 + H332 Harmful if swallowed, in contact with skin or if inhaled H319 Causes serious eye irritation.	P210 Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. P280 Wear protective gloves/ protective clothing. P305+P351+P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
3-Nitrooxypropanol	Danger 	H302 Harmful if swallowed H315 Causes skin irritation. H319 Causes serious eye irritation.	P264 Wash skin thoroughly after handling. P270 Do not eat, drink or smoke when using this product. P280: Wear protective gloves/protective clothing/eye protection/face protection P301 + P312 IF SWALLOWED: Call a POISON CENTER or doctor/ physician if you feel unwell. P337 + P313 If eye irritation persists: Get medical advice/ attention. P501 Dispose of contents/ container to an approved waste disposal plant.
Acetic Acid	Danger	H226 Flammable liquid and vapour.	P210 Keep away from heat, hot surfaces, sparks, open flames, and other ignition sources. No smoking.

		H314 Causes severe skin burns and eye damage.	

5 Scope

This described method is applicable for cattle feed / TMR samples which were dosed with the product containing ≥ 20 mg/kg and ≤ 1000 mg/kg 3-nitrooxypropanol as active principle adsorbed on a silicon dioxide carrier.
(Annotation: The exact range of the method depends on the sensitivity of the used UV-detector. In case a less sensitive detector is used, the range of the methods might need to be adapted to a higher concentration than 20 mg/kg 3-nitrooxypropanol).
Depending on the applied matrix, chromatographic interferences with the active substance may occur.

6 Normative reference

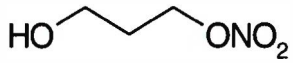
This analytical method was written according to the ISO 78-2 guideline.

7 Definitions

ISO	International Organization for Standardization
TMR	Total Mix Ration (TMR), cattle feed
3-NOP	3-(nitrooxy)propane-1-ol, 3-nitrooxypropanol, propanediol mononitrate, PDMN
MilliQ water	purified water
QC sample	Quality control sample

8 Principle

Cattle feed samples are extracted twice by liquid extraction with acetonitrile using shaking and sonication. Extracts are centrifuged before the supernatant is collected. Samples are diluted if necessary prior to analysis by LC-UV at 210 nm and 3-NOP is used as external standard.



Propanediol mononitrate $C_3H_7NO_4$

9 Reactions

n/a

10 Reagents and materials

10.1 General

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade, and distilled or demineralised water or water of equivalent purity.
The following materials were found to be suitable for use during the development / validation of this procedure. Alternative suppliers of these materials may be employed, provided adequate specificity, sensitivity and resolution are achieved.

10.2 Products used in their commercially available form

10.2.1 Acetonitrile (HPLC grade, e.g. Merck, Germany, product number 1.00030, CAS 75-05-8)

10.2.2 Acetic acid (ACS reagent ≥ 99.7 %, e.g. Sigma-Aldrich, Switzerland, product number 695092, CAS 64-19-7)

10.2.3 3-NOP (Analytical standard of known purity (see certificate of analysis), DSM Nutritional Products Ltd, CAS 100502-66-7)

10.3 Aqueous solutions

Different volumes may be employed when preparing the following solutions if the final concentrations are the same as stated in the analytical procedure.

10.3.1 Mobile phase A (water/acetonitrile/acetic acid (95/5/0.1, v/v/v))

Combine 950 mL of MilliQ water with 50 mL acetonitrile and 1 mL acetic acid. Mix well and degas before use. This solution is stable for at least 2 weeks at ambient temperature.

10.3.2 Mobile phase B (water/acetonitrile/acetic acid (80/20/0.1, v/v/v))

Combine 800 mL of MilliQ water with 200 mL acetonitrile and 1 mL acetic acid. Mix well and degas before use. This solution is stable for at least 2 weeks at ambient temperature.

10.4 Solutions of defined concentration

10.4.1 Standard reference solution

Different weights and volumes may be employed when preparing the following solutions if the analysed concentrations are within the linear range of the detector.

Accurately weigh approx. 20 mg of 3-NOP in a brown 20 mL volumetric flask, dissolve and make to volume with acetonitrile (final concentration of standard reference solution: 1000 µg/mL). To increase the accuracy of the calibration, prepare two individual standard reference solutions instead of one.
The standard reference solution is stable for at least 28 days at 2 - 8°C.
(The calculation of the concentration including purity correction is explained in chapter 14.1).

10.4.2 Standard solutions

Dilute the standard reference solution according to the following table (table 2) with acetonitrile. Analyse standard solutions C-H (* see also table 2) by LC-UV in order from the smallest to highest concentration.

Table 2: Standard solutions of 3-NOP

Standard ID*	Volume of Calibration solution taken [µL]	Stock used	Final volume [mL]	Final concentration of standard [µg/mL]
B	500	A	10	50
C	250	B	1	12.5
D	150	B	1	7.5
E	80	B	1	4.0
F	40	B	1	2.0
G	20	B	1	1.0
H	10	B	1	0.5

* Before analysing the standard solutions, see also chapter 13.4.

The standard solutions are stable for at least 28 days at 2 - 8°C.

10.4.3 Quality control solution

Accurately weigh approximately 100 mg of 3-NOP and make to volume with acetonitrile in a 25 mL volumetric flask. Mix well to give quality control stock solutions of 4000 µg/mL. Sonicate if required.

Depending on the 3-NOP target concentration of the samples, prepare QC samples according to table 3. At least a QC in duplicate of the concentration closest to the real samples needs to be prepared using 10 g blank cattle feed/TMR. Process the QC sample like a test sample (see 13.3).

Table 3: QC solutions of 3-NOP

QC ID	Volume of QC stock taken [mL]	Weight [g]*	Final concentration of QC sample [mg/kg]
Rec 1000	2.5	10	1000
Rec 400	1		400
Rec 200	0.5		200
Rec 120	0.3		120
Rec 40	0.1		40
Rec 20	0.05		20

* Weigh 10 g (± 450 mg) and calculate the concentration using a TMR sample weight of 10.0 g.

11 Apparatus

11.1 Equipment

11.1.1 Analytical balance (e.g. AT 261 Delta Range and PM 2000, Mettler-Toledo, Nänikon, Switzerland; accuracy of AT balance for reference standard: 0.01 mg and of PM 2000 balance for TMR: 0.01 g)

11.1.2 Glassware such as pipettes, volumetric flasks, funnels, test tubes, HPLC vials for the autosampler (or equivalent)

11.1.3 Grinder (e.g. Robot Coupe R8, Koller GmbH, Löchgau, Germany or an equivalent grinder)

11.1.4 Linear, side to side (horizontal) flatbed shaker (e.g. IKA HS 501, IKA Werke GmbH & CoKG, Staufen, Germany or Stuart SSL2, Cole-Parmer, Stone, Staffordshire, UK)

11.1.5 HPLC system: e.g. Agilent 1200, equipped with a (either binary or quaternary) pump capable of generating pressures of up to at least 200 bar, degasser, injector, column thermostat, DAD- or UV-detector, and integrator (Agilent software or comparable chromatography data system)

11.1.6 Ultrasonic bath (e.g. USC300D, 80 W, 45 kHz, heatable, VWR, Dietikon, Switzerland)

11.1.7 Centrifuge (e.g. Multifuge 1 Heraeus, Thermos Scientific, Langenselbold, Germany)

11.1.8 Centrifuge pot, 250 mL (e.g. Nalgene Centrifuge Bottels HDPE, Thermos Scientific, Langenselbold, Germany)

11.1.9 Accucore Polar premium, 2.6 µm, 150x4.6mm (Thermo, Product number 28026-054630) or equivalent

11.2 HPLC conditions

Column: Accucore Polar premium, 2.6 µm, 150x4.6mm (Thermo) or equivalent

Mobile phase: see 10.3.1 and 10.3.2

Gradient:

Time [min]	A [%]	B [%]	Flow rate [mL/min]
0	100	0	0.4
14	100	0	0.4
14.5	0	100	0.4
20	0	100	0.4
20.5	100	0	0.4
30	100	0	0.4

Pressure: approx. 100 bar (depending on instrument and age of column, pressure might differ. Therefore, check individually and monitor the pressure of the HPLC per sequence)

Column temperature: set to maintain 23 ± 0.8 °C

Injection volume: 5 µL

Autosampler temperature: ambient

Detection: 210 nm

Retention time: approx. 11 min (depending on instrument, age, and batch of column)

Run time: 30 min

11.3 Preparation of HPLC, system operation and trouble shooting

Equilibrate the HPLC column before starting each sequence. If a new HPLC column is being applied, flush the column with mobile phase A (gradient conditions at time point 0) for at least 4 hours. If the HPLC column has been used already, flush for 1 hour.
Then inject twice a blank (= pure acetonitrile) followed by a QC solution (10.4.3) or any other standard solution repeatedly, until the retention time stabilizes to ± 0.2 min. Only then start the sequence by injecting the standard solutions (or QC sample) followed by the sample solutions. After the last sample solution, inject another QC sample and at the very end a blank (= acetonitrile). If the HPLC column is being re-used for this method within 7 days, no additional cleaning of the HPLC column is necessary after the injection of acetonitrile. In any other case, flush the column with water/acetonitrile 50:50 solution for 2 h to get rid of the acetic acid before shutting down.

If the retention time does not stabilize, carry out some trouble shooting: e.g.

- test that there are no air bubbles in the system,
- check that gradient valve works properly,
- check for leaks,
- check pressure of system (if it continually goes up, test if blockages have occurred by cleaning the frits in the solvents reservoirs, flushing the column and injector thoroughly with mobile phase B; if this does not help,
- Replace and use a new HPLC column

Annotation:
If any chromatographic parameters described in this method need to be changed, carry out a system suitability test (10x injection of calibration solution F (2.0 µg/mL) to verify the stability of the change.

12 Sampling

A preparation of the laboratory sample is not necessary. A representative sample of approx. 200 g can be sent as such to the laboratory for analysis. Use aluminium foil as packaging material and seal the package air tightly. Apply storage conditions of -20°C (± 3°C).
If aluminium foil is not available, use a plastic container consisting of high-density polyethylene (HDPE) with seal and screw cap instead. Fill plastic container right till top to minimize headspace.

Use samples stored at room temperature or fridge directly, thaw frozen samples until at least 100 g can be further processed.
Before analysis, chop and homogenize the sample with an appropriate grinder (see also 11.1.3) until an optically homogeneous sub sample has been obtained before weighing an aliquot. If the sample is already ground, further homogenize the sample with a spatula thoroughly before weighing an aliquot.

After taking an aliquot, store the rest of the sample in a freezer.

13 Procedure

13.1 General

Determine each sample in duplicate.

13.2 Test portion

Into a 250 mL centrifuge pot (HDPE) accurately weigh the amount of ground test sample specified in the table below (table 4).

Table 4: Sample weights

Target concentration of 3-NOP [mg/kg]	Sample weight [g]
≥ 20 and ≤ 1000	10 ± 1

13.3 Determination - extraction and preparation of test solution

a. Add 100 mL of acetonitrile, cap and mix well.
b. Place sample on the flat bed shaker and shake for 15 ± 5 minutes (250 rpm/min).
c. Sonicate for 15 ± 5 minutes in a bath set to 50 ± 5°C.
d. Centrifuge at 4000 ± 500 rpm for 2 ± 1 minutes (ambient) to form a pellet. (Depending on the formula of TMR, it is possible that some floating ingredients might be left after centrifugation. They will be filtered off during the next step).
e. Filter supernatant through a paper filter into a 200 mL volumetric flask.
f. Repeat steps a-e
g. Combine the supernatants and make to 200 mL final volume with acetonitrile and mix well.
h. If necessary dilute the sample extract
i. Analyse by LC-UV

13.4 Calibration

Analyse the standard solutions H to C (see 10.4.2) using the HPLC system described in section 11.2. If two individual sets of standard solutions were prepared, inject these solutions once each. If only one set of standard solutions was prepared, inject each solution twice. Prepare the calibration curve by plotting the corresponding 3-NOP concentrations of the six standard solutions C to H in micrograms per millilitre and the areas of the integrated 3-NOP peaks (weighted 1/x, not forced through zero).

Annotation: Depending on the linearity of the detector, it might also be possible to inject the standard solution B (10.4.2) to extend the calibration curve if samples with higher concentrations need to be analysed.

Determination of LOQ:
If a detector with a low sensitivity is used, some of the lower calibration solutions might be below the LOQ of the method. Therefore, use the calibration solutions to determine the LOQ at this point. If the LOQ is higher than the lowest and or second lowest calibration solution, exclude them in the calculation of the calibration curve.

If the LOQ is higher than the third lowest calibration solution, the instrument is not suitable for this method and an HPLC with a more sensitive detector needs to be used.

Acceptance criteria for a valid calibration curve:

- Assay linearity will be acceptable if the calculated amount of at least 75 % of the calibration standards are within ± 15 % of the actual amount injected (± 20 % at the lower limit of linearity).
- No two consecutive calibration standards may be omitted and the correlation coefficient (R^2) should be ≥0.995.

Frequency of calibration:
Ideally, the calibration should be carried out before every sequence of analysis. Alternatively, it should be repeated at least after essential parts of the HPLC system were replaced (e.g. HPLC column, lamp, etc.). If the calibration is not carried out daily, it should be checked with a QC sample, e.g. a 3-NOP standard solution, daily.

14 Calculation and data acceptability

14.1 Calculation

Calculate the concentration of the relevant standard solutions 3-NOP in micrograms per millilitre:

$$c_{\text{standard solution 3-NOP}}[\mu g/mL] = \frac{m_{3\text{-NOP}} * 1000 * P}{20 * d * 100}$$

where

$m_{3\text{-NOP}}$ is the weight of 3-NOP in milligrams.
1000 is the conversion factor from milligrams to micrograms.
P is the purity of the reference standard of 3-NOP in percent (see certificate of analysis).
20 is the volume of the volumetric flask used to prepare the standard reference solution in millilitres.
d is the dilution factor used to prepare the standard solutions C-H.
100 is the conversion of percent purity.

Calculate the test sample concentration with the calibration equation in micrograms per millilitre according to the formula $y = m * c_{sample} + b$

$$c_{sample}\,[\mu g/mL] = \frac{y - b}{s}$$

where

y is the area of test sample peak in response units.
s is the slope of the equation.
b is the axis intercept.
c_{sample} is the unknown concentration of 3-NOP in test sample in micrograms per millilitre.

Calculate the concentration of 3-NOP in the test sample in milligram per kilogram according to the following formula (Annotation: the result is wet weight based!):

$$\text{3-NOP}\,[mg/kg] = \frac{c_{sample} * V * d}{w}$$

where

c_{sample} is the numerical value of the test sample concentration of 3-NOP in micrograms per millilitre.
V: is the volume of used extraction solvent in millilitre (200 mL).
d: is the dilution factor.
w: is the sample weight in gram.

Calculate the mean according to the following formula:

Mean:

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$$

where

x_i represents the test sample concentrations in milligram per kilogram.

n is the number of measurements.

Report the result with three significant figures.

If requested, convert the analysed concentration of 3-NOP from wet weight to dry weight basis according to the following formula:

3-NOP [mg/kg] DM = concentration 3-NOP WW * 100 / dry matter

where

DM:	is the abbreviation for dry weight basis
Concentration 3-NOP WW:	is the concentration of 3-NOP obtained by the described analytical method above on wet weight basis
100:	is the conversion to percent
Dry matter:	is the dry matter content of the sample in percent.