Quantification of tramadol and its metabolites in molasses using automated EVOLUTE® EXPRESS CX SPE

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Introduction

Molasses is commonly used in equine feed manufacturing as a palatability enhancer or feed binder. However, it may occasionally be contaminated with pharmacologically active substances. Tramadol, a synthetic opioid analgesic, along with its primary metabolites, pose a risk of anti-doping rule violations if inadvertently introduced via feed.

An accurate and precise quantification method for the analysis of tramadol and its metabolites in molasses is vital to ensure fair play within horse racing. This poster will demonstrate a robust and automated solid-phase extraction (SPE) procedure using Biotage® EVOLUTE® Express CX (30mg) plates. Analysis was performed using LC-MS/MS. The method is tailored for doping control and regulatory compliance in the equine industry.

Experimental

Reagents

Analytical standards (of tramadol and its metabolites), sulfuric acid 95-98% and ammonia solution 25% were purchased from Sigma-Aldrich. Methanol and ethyl acetate were LCMS grade and purchased from Rathburn Chemicals Ltd (Scotland, UK). Water used was ultrapure (18.2 MΩhm-cm) and drawn daily from a Milli-Q® system (Merck Millipore, Germany). Formic acid (Honeywell Fluka™) was mass spectrometry grade and was purchased from Fisher Scientific.

Sample Preparation

Molasses samples were processed to reduce matrix complexity and viscosity. Each sample was subjected to acidic aqueous dilution and centrifugation before SPE.

Sample pre-treatment

3g ± 0.05g of molasses were weighed into a 50mL polypropylene centrifuge tube. 20mL of a 50mM H_2SO_4 (aq) solution was added to the molasses sample. The tube was vortexed to mix thoroughly and placed in an ultrasonic bath at room temperature for 15 minutes. The sample was then centrifuged at 4000 rpm for 20 minutes at room temperature. 1.7mL of supernatant was transferred to a 96-well plate ready for automated SPE.

***Figure 1**. Biotage® Extrahera™ Classic*

Solid Phase Extraction:
Pre-treated molasses was extracted with the Biotage® EVOLUTE® Express CX 30mg 96-well plates (601-0030-PX01), processed using the Biotage® Extrahera™ Classic (414001) automated sample preparation system, shown in ***Figure 1.*** Final extraction protocol is demonstrated in ***Table 1.*** No conditioning or equilibration step was necessary for this method due to the water wettable surface of the polymeric sorbent.

***Table 1**. SPE Procedure.*

Step	Solvent	Volume
Conditioning	N/A	
Equilibration	N/A	
Load	Pre-treated molasses	1.5 mL
Wash 1	50mM H_2SO_4 (aq)	2 x 1 mL
Dry	10 minutes at full flow	
Wash 2	MeOH	2 x 1 mL
Dry	5 minutes at full flow	
Elute	EtOAc/NH_3 (97:3 v/v)	2 x 900 µL

A full automated sample preparation method using the Biotage® Extrahera™ Classic is available upon request.

Post extraction

Evaporation was performed on the TurboVap® 96 Dual evaporator. Both the gas (N_2) and the plate temperatures were maintained at 30°C; flow rate was set to 45 L min^{-1}; and the plate height set to 55mm. The eluates were evaporated for 30 minutes, until dryness.

The residues were reconstituted using 200 µL of mobile phase (0.1% Formic Acid, 10% MeOH in Water). The plates were subsequently shaken for 5 minutes, transferred to an autosampler and stored at 4°C until analysis.

UPLC Conditions

Instrument: Thermo Scientific Dionex UltiMate 3000 UHPLC+.
Column: Supelco® Ascentis Express C18 (50mm x 2.1mm, 2.7µm; P/N: 53822-U) with an Ascentis Express C18 guard cartridge (5mm x 2.1mm, 2.7µm; P/N: 53501-U).
Mobile phase:
A: 0.1% Formic Acid in Water.
B: 0.1% Formic Acid in Methanol.
Flow rate: 0.5 mL min^{-1}.
Column temp: 40 °C.
Injection volume: 10 µL.
Gradient: See ***Table 2***.

***Table 2**. UHPLC Gradient Conditions*

Step	Time (min)	Flow Rate (mL min^{-1})	A (%)	B (%)
1	0.00	0.5	95	5
2	1.00	0.5	95	5
3	2.00	0.5	5	95
4	3.00	0.5	5	95
5	3.50	0.5	95	5
6	6.00	0.5	95	5

MS Conditions

Instrument: All experiments were conducted using a Thermo Scientific TSQ Quantum Access MAX triple quadrupole mass spectrometer. Detection was facilitated via electrospray ionisation (ESI) in positive ion mode, operating under multiple reaction monitoring (MRM) conditions.

Spray Voltage: 5000kV.
Vaporiser Temperature: 300°C.
Capillary Temperature: 320°C.
CID gas (argon) flow: 135kPa.

***Table 3**. Mass Spectrometry Parameters*

Analyte	MRM Transition	Collision (eV)
TRM	264.11 > 58.42	15
O-TRM	250.09 > 58.39	15
N-TRM	250.09 > 44.49	15
N,O-TRM	236.04 > 44.46	15
1,6-TRM	246.09 > 58.37	15

Results

Evaporation

Evaporation was carried out using a TurboVap® 96 Dual evaporation system shown in ***Figure 2.*** Gas flow, temperature, plate temperature and plate height were optimised for an efficient evaporation.

***Figure 2**. TurboVap® 96 Dual nitrogen evaporator*

Initial evaporation and reconstitution tests were run to ensure maximum recovery of tramadol and its metabolites after evaporation to dryness. Full removal of ethyl acetate was required and reconstitution in a LC-MS/MS compatible solvent. Reconstitution recoveries for all analytes were in the range of 81-98%, showing minimal evaporation losses, shown in ***Figure 3.***

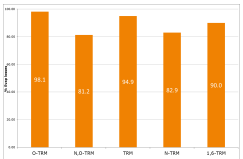


***Figure 3**. Evaporation and reconstitution recoveries*

Selectivity/Specificity

Selectivity of the assay was checked by injecting a blank matrix sample and a spiked matrix sample. Tramadol and the metabolites thereof were quantified accurately without interference. Exemplar chromatograms of extracted calibration standards are presented in ***Figure 4.***

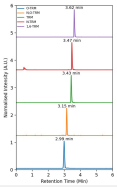
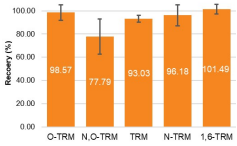


***Figure 4**. Separation of tramadol and its metabolites. Chromatograms are normalised and offset for clarity.*

Accuracy and Precision

Accuracy was checked via the analysis of blank samples spiked with 3 ng mL^{-1} tramadol and the metabolites thereof. Recovery, expressed as a percentage, was calculated for each injection. Analyte recoveries were acceptable for the concentrations used and were in the 78—101 % range. Precision is expressed as percentage relative standard deviation (%RSD; also reported as coefficient of variance, %CV). Samples spiked with 3 ng mL^{-1} tramadol and its metabolites were injected seven times and the RSD values calculated (2.99—15.00 %) were appropriate for the concentrations used.
Average recoveries and %RSD values (indicated by error bars) are presented in ***Figure 5.***

***Figure 5**. Average recoveries of tramadol and its metabolites. %RSDs are shown as error bars.*



Matrix factors determined moderate ion suppression and good removal of matrix interferences, shown in ***Figure 6.***

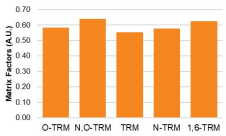
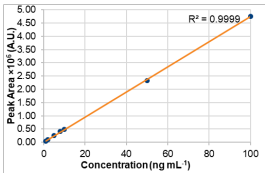


***Figure 6**. Matrix factors*

Range and Linearity

The method had a good linearity across 1 – 100 ng mL^{-1} concentration range. Linearity was assessed via linear regression analysis of 7 concentrations of standard: 1, 2, 5, 8, 10, 50 and 100 ng mL^{-1}, extracted and injected in duplicate. All calibration curves had R^2 values greater than 0.99. An exemplar calibration curve is presented in ***Figure 7.***

***Figure 7**. Extracted calibration of O-Desmethyltramadol using 7 concentrations*



Limits of Detection and Quantification

Sensitivity of the assay is expressed as the limit of detection (LOD) and the limit of quantification (LOQ), with signal-to-noise ratios of 3 and 10, respectively. The LODs and LOQs for each analyte are presented in ***Table 4.***

***Table 4**. LOD and LOQ of Tramadol and its metabolites*

Analyte	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
TRM	0.05	0.20
O-TRM	0.05	0.20
N-TRM	0.20	0.50
N,O-TRM	0.50	1.00
1,6-TRM	0.10	0.50

Automation

Transfer of a manual method onto the Biotage® Extrahera™ Classic, ***Figure 1,*** helped to improve the method efficiency. By fully automating the sample preparation method, it allowed the processing of 96 samples in 75 minutes.

Automating the full process supported the laboratory efficiency by freeing up analysts for other important tasks, such as data processing and system set ups. It also removed manual labor tasks providing accurate, reproducible results.

Conclusion

- This poster demonstrates a robust and sensitive method for the quantification of Tramadol and its primary metabolites in molasses.
- High reproducible recoveries for tramadol and its metabolites were observed when analysing on the Biotage® Extrahera™ Classic.
- The automated sample preparation method is efficient, processing up to 96 samples in 75 minutes.