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Application News

Liquid Chromatograph Mass Spectrometer LCMS-8050

Methods for the determination of residual veterinary drugs in Raw Milk using LCMS-8050

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User Benefits

- Modified QuEChERS and SPE combined with Ultra-fast technologies of LCMS-8050 for quantification of veterinary drugs residues at trace levels.
- Method covering different drugs with different chemical properties
- Shorter analytical time can provide more high throughput analyses.

1. Introduction

The use of veterinary drugs in dairy farming is important for the prevention and treatment of diseases in dairy cows. However, veterinary drugs may remain in raw milk due to the misuse or withdrawing period of drugs, which can lead to health issues for consumers as milk is an important food in the diet, especially for infants and children. Hence to ensure the safety of milk products, the safety regulation for veterinary drug residues in food has been strengthened. To meet these stringent regulations a quantitative and highly sensitive analytical method is required. The aim of this study is to develop a sensitive analytical method covering this wide range of different veterinary drugs.

Based on these requirements, Shimadzu has developed and validated 4 simple, sensitive and high throughput, multiresidue methods for the determination of available veterinary drugs (total 69) in milk using LCMS-8050. The extraction was performed with modified QuEChERS[1] method and SPE. Method I covers 44 compounds, Method II covers 15 compounds, Method III covers 4 compounds and Method IV covers 6 compounds. Number of veterinary drugs covered under different regulations are shown in Table 1.

Table 1 Coverage of veterinary drugs as each regulation

Compliance / Regulation	No. of drugs regulated	No. of drugs covered in this method
FSSAI	97	48
EU	82	30

2. Materials and Methods

The reference standards were procured from Sigma.
Milk sample was procured from local market to prepare matrix-matched calibration standards and spiked samples.

Fig. 1 Shimadzu LCMS-8050

The calibration standards were prepared in the range as given in Table 2.

Table 2 Calibration standard range

Method I	Method II	Method III	Method IV
1 to 50 ug/L	2 to 50 ug/L	1 to 50 ug/L	2 to 50 ug/L

Calibration curves were generated by external standard method and using weighted regression of $1/C^2$. Spiked samples were prepared in six replicates of each LOQ and 2 × LOQ. LOQ'S achieved were below MRL.(refer Table 4)

Shimadzu LCMS-8050 with Nexera X3 (Fig. 1, Shimadzu Corporation) was used in order to quantify veterinary drug residue in milk sample.

Shimadzu's LC-MS/MS Method Package for veterinary drugs Ver.2 and LC-MS/MS Method Package for Aminoglycoside Antibiotics enables quick instrumental method optimization for higher throughput. For most of the compounds, 1 target and 2 reference MRM transitions were included in the method.

Shimadzu's data processing software LabSolutions Insight™ was used for data processing, which helped in evaluating validation parameters with ease.

2.1. Sample preparation

This study uses 4 different extraction procedures in which modified QuEChERS method and SPE was adopted. Method I: Sample was deproteinised with acetonitrile. Anhydrous MgSO4 and NaCl was added for Separation and centrifuged.

dSPE clean-up was followed using C18. Aliquot was evaporated under nitrogen stream followed by reconstituting with the mobile phase.

Method II: Sample treated with Disodium Hydrogen Phosphate buffer and deproteinised with acetonitrile. Anhydrous NaSO4 and NaCl was added for separation and centrifuged.

dSPE clean-up was followed using C18. Evaporated the aliquot under nitrogen stream and reconstituted with mobile phase.

Method III: Sample deproteinised with trichloro acetic acid and centrifuged. Aliquot was passed through HLB cartridge, eluted with Methanol and diluted with mobile phase.

Method IV: Sample diluted with Ion pairing reagent and filtered before injection.

All samples were analyzed with each condition, respectively, shown in Table 3.

2.2. Analytical Conditions

Table 3 Instrument configuration and analytical conditions: LC-MS/MS

System Configuration	
LC-MS/MS	: LCMS-8050
Auto-sampler	: Nexera X3 with SIL -40C
Column	: Shim-pack™ Velox C18, P/N 227-32010-03 (3.0 mm i.d. × 100 mm , 2.7 µm)

Method I and II	
Flow rate	: 0.3 mL/min
Mobile phase A	: 0.1 % Formic acid in water
Mobile phase B	: Methanol
Gradient program	: 5 %B (0.0 mins to 1.0 mins) → 5-80 %B (1.0 min to 6.5 min) → 80-80 %B (6.5 min to 7.5 min) → 80-100 %B (7.5 min to 9.0 min) → 100-100 %B (9.0 min to 12.5 min) → 100-5 %B (12.5 min to 13.0 min)
Run time	: 17 min
Injection volume	: 10 µL (Co-injection with water)
Column oven temp	: 40 °C

Method III	
Flow rate	: 0.3 mL/min
Mobile phase A	: 0.01 M Oxalic acid in water
Mobile phase B	: Methanol
Gradient program	: 5 %B (0.0 mins to 1.0 mins) → 5-80 %B (1.0 min to 6.5 min) → 80-80 %B (6.5 min to 7.5 min) → 80-100 %B (7.5 min to 9.0 min) → 100-100 %B (9.0 min to 12.5 min) → 100-5 %B (12.5 min to 13.0 min)
Run time	: 17 min
Injection volume	: 10 µL (Co-injection with water)
Column oven temp	: 40 °C

Method IV	
Flow rate	: 0.3 mL/min
Mobile phase A	: 0.2 % HFBA in water
Mobile phase B	: Methanol
Gradient program	: 10-95 %B (0.0 mins to 6.0 mins) → 95-95 %B (6.0 min to 7.0 min) → 95-10 %B (7.0 min to 8.0 min) → 10-10 %B (8.0 min to 12.0 min)
Run time	: 17 min
Injection volume	: 10 µL
Column oven temp	: 40 °C

MS	
Ionization	: ESI
Nebulizing gas flow	: 3 L/min
Heating gas flow	: 8 L/min
Drying gas flow	: 8 L/min
Interface temp.	: 250 °C
DL temp.	: 200 °C
Heating block temp.	: 350 °C

3. Result and Discussion

Validation parameters like specificity, linearity, recovery and precision were studied as per SANTE guidelines[2]. Results obtained are shown in Table 4. FSSAI and EU regulated compounds are marked with # and * respectively.

3.1. System precision and specificity

System precision was evaluated by calculating variation of the peak area and retention time of six replicates of 10 µg/L mixture of veterinary drugs solvent standard.

The %RSD was found to be less than 20 for peak area and retention times were within tolerance limit of ±0.1 min. Specificity of the method was determined by comparing the response of blank sample (reagent and matrix) against reporting level. Response in reagent/matrix blank sample was well within 30 % of the reporting limit and met the acceptance criteria.

3.2. Linearity study

For linearity study, matrix match calibration standards were used. All calibration standards were found within 80 to 120 % accuracy as per SANTE guidelines. The linearity graphs of some representative compounds are shown in Fig. 2.

3.3. Recovery study

Recovery was evaluated by analysing spiked samples at LOQ and 2 × LOQ (six replicates at each level) against matrix match calibration curve. Mean recoveries for most of the compounds were found within 70-120 %. As per SANTE guidelines, all the compounds were found to be reproducible at their LOQ levels.

3.4. Precision study

For precision, repeatability and within-laboratory reproducibility studies were carried out. Concentrations of spiked samples were back calculated against matrix matched linearity.

Repeatability (RSD_r):

Repeatability experiment was performed by injecting six replicates of veterinary drugs standard mix at LOQ and 2XLOQ concentration levels. The %RSD for repeatability of six injections at their respective LOQ levels were found to be ≤20%. (Refer to Table 4)

Reproducibility (RSD_R):

Reproducibility experiment for recoveries was performed on six different spiked samples at LOQ and 2 × LOQ concentration levels. The %RSD for recovery of seven spiked samples at their respective LOQ levels were found to be ≤ 20%. (Refer to Table 4)

Table 4 Summary of Results

Compound Name	Ret. Time (min)	Target MRM (m/z)	CE	Covered under Method	LOQ (mg/kg)	Recovery at LOQ (%)	EU MRL (mg/kg)	FSAAI MRL (mg/kg)	Precision	
									% RSD_R (n=6)	% RSD_r (n=6)
*Albendazole 2 Amino Sulfone	4.29	240.00>132.95	-27	I	0.002	82.8	0.1	NA	7.67	3.52
*Albendazole Sulfone	6.26	298.00>158.90	-37	I	0.002	88.56	0.1	NA	4.53	6.39
*Albendazole Sulfoxide	6.04	282.20>239.90	-14	I	0.002	87.19	0.1	NA	12.33	6.82
*#Ampicillin	5.14	350.10>106.00	-22	I	0.002	52.7	0.004	0.01	14.4	16.57
#Amprolium	1.57	242.95>150.00	-12	I	0.002	75.56	NA	0.01	10.68	8.63
*#Ceftiofur	6.39	524.00>241.00	-16	I	0.005	80.84	0.1	0.1	15.43	6
#Clopidol	4.69	192.10>100.90	-26	I	0.005	101.13	NA	0.01	10.59	11.73
*#Cloxacillin	6.01	435.50>98.90	-22	I	0.002	76.76	0.03	0.01	18.88	14.01
*Dicloxacillin	7.76	470.10>159.90	-16	I	0.002	88.56	0.03	NA	10.62	11.28
#Diminazene	6.04	282.20>239.85	-14	I	0.002	86.98	NA	0.15	7.13	6.76
*#Enrofloxacin	4.92	360.05>315.95	-19	I	0.005	41.37	0.1	0.01	19.1	5.39
*Erythromycin A	6.88	734.35>158.05	-30	I	0.002	70.27	0.04	NA	4.08	4.62
#Ethopabate	6.83	237.90>135.95	-26	I	0.002	83.8	NA	0.01	15.35	6.58
*#Febental	8.05	447.10>198.90	-20	I	0.002	80.01	0.01	0.1	10.47	4.97
*#Fenbendazole Sulfone	6.8	332.20>299.85	-22	I	0.002	78.51	0.01	0.1	7.21	6.7
*#Flunixin	6.27	297.60>158.90	-35	I	0.002	102.42	0.04	0.01	8.57	13.9
#Halofuginone	6.26	414.00>100.00	-22	I	0.002	62.54	NA	0.01	17	6.21
*#Lincomycin	4.51	407.10>126.00	-30	I	0.002	51.21	0.15	0.15	6.88	3.6
#Mepyramine	5.63	286.30>120.95	-22	I	0.002	109.33	NA	0.01	9.45	23.91
Metronidazole	3.8	172.30>127.90	-16	I	0.002	88.67	NA	NA	8.12	4.56
*Morantel	5.11	221.10>77.00	-41	I	0.002	82.73	0.05	NA	11.65	13.62
#Nimesulide	7.76	306.80>121.95	36	I	0.002	72.5	NA	0.01	12.2	8.52
*#Nitroxinil	7.23	288.70>162.15	22	I	0.002	91.48	0.02	0.01	9.84	15.21
*#Oxfendazole	6.67	316.10>158.90	-32	I	0.002	79.66	0.01	0.1	5.56	3.89
*#Oxyclozanid	4.87	398.10>380.85	-12	I	0.005	44.48	0.01	0.01	19.43	19.88
Phenyl Butazone	1.52	309.10>152.80	-21	I	0.002	58.31	NA	NA	3.6	2.39
#Praziquantel	8.11	313.10>203.00	-17	I	0.002	79.14	NA	0.01	8.81	7.25
#Promazine	6.76	285.10>85.95	-19	I	0.002	46.9	NA	0.01	18.99	6.21
*Spiramycin I	5.51	422.20>101.00	-18	I	0.002	75.75	0.2	NA	19.67	11.51
#Sulfadiazine	3.94	251.15>155.95	-15	I	0.002	96.04	NA	0.01	18.98	14.49
Sulfadimethoxine	4.97	279.00>185.90	-17	I	0.002	70.69	NA	NA	14.81	19.77
#Sulfdimidine	1.54	215.15>137.20	-10	I	0.002	94.22	NA	0.025	7.26	6.77
Sulfguanidine	4.5	265.00>155.90	-16	I	0.002	57.35	NA	0.01	1.89	0.65
Sulfmerazine	5.31	254.15>91.95	-26	I	0.005	75.03	NA	NA	12.81	18.56
Sulfamethoxazole	5.06	281.15>155.90	-17	I	0.002	72.35	NA	NA	16.13	19.87
#Sulfamethoxypyradizine	4.16	255.80>155.90	-14	I	0.002	79.99	NA	NA	11.57	14.44
#Sulfpyridine	6.18	311.20>155.95	-20	I	0.002	84.29	NA	NA	7.25	10.51
Sulphathiazole	4.3	250.15>107.95	-26	I	0.002	81.52	NA	0.01	19.91	16.78
#Tiamulin	6.72	494.25>192.00	-21	I	0.002	79.19	NA	0.01	5.95	1.82
*Tilmicosin (isomers)	6	869.40>173.95	-45	I	0.002	76.68	0.05	NA	6.4	9.09
Tinidazole	4.55	248.00>120.90	-16	I	0.002	93.27	NA	NA	19.42	9.21
*#Trimethoprim	4.51	290.95>230.00	-24	I	0.002	82.86	0.05	0.01	5.71	3.9
*Tylosin	6.82	916.50>174.00	-39	I	0.002	81.73	0.05	NA	12.67	11.62
#Xylazine	5.11	220.90>89.95	-22	I	0.002	83.69	NA	0.01	7.79	2.31
*#Chlortetracycline	5.95	479.10>443.85	-21	III	0.02	58.06	0.1	0.1	8.84	14.37
Doxicyclin	6.56	445.10>427.90	-19	III	0.02	56.67			9.26	15.74
*#Oxytetracycline	5.21	461.10>425.95	-20	III	0.02	95.17	0.1	0.1	8.6	16
*#Tetracycline	6.45	445.05>427.90	-20	III	0.02	51	0.1	0.1	6.38	18
*#Albendazole	7.67	266.20>233.90	-19	II	0.005	64.47	0.1	0.1	7.76	7.51

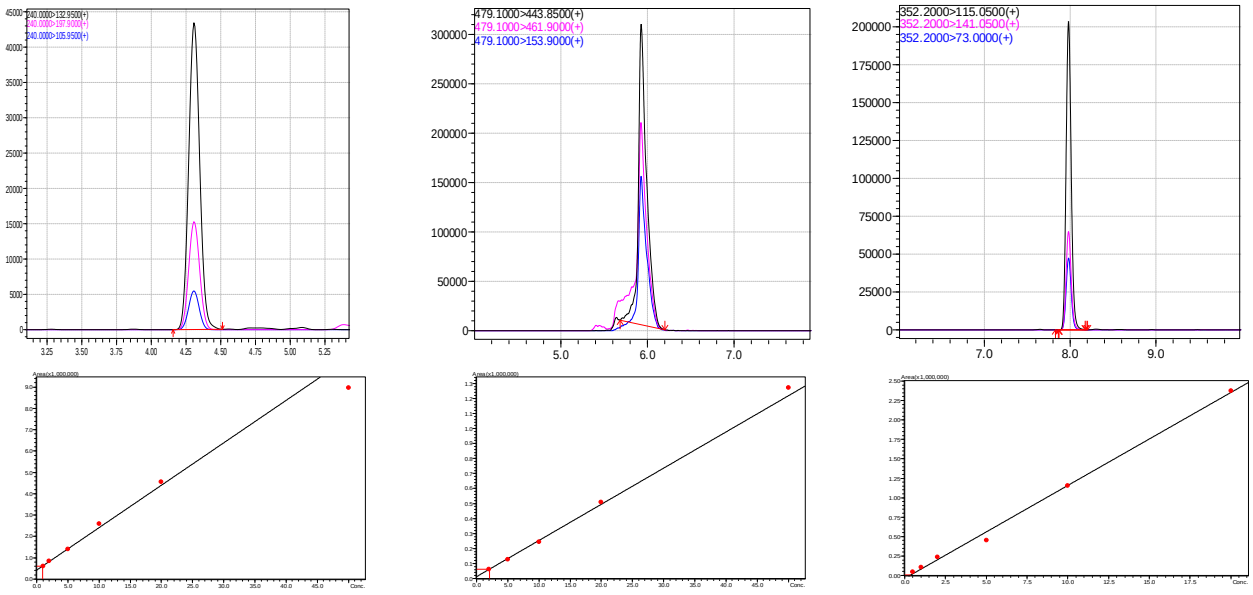
Table 4 Summary of results (Contd.)

Compound Name	Ret. Time (min)	Target MRM (m/z)	CE	Covered under method	LOQ mg/kg	Recovery at LOQ (%)	EU MRL (mg/kg)	FSAAI MRL (mg/kg)	Precision	
									% RSD_R (n=6)	% RSD_r (n=6)
#Buparvaqunone	11.21	325.00>186.00	33	II	0.01	37.63	NA	0.01	8.03	5.23
#Buserelin	6.51	620.50>592.20	-16	II	0.005	63.32	NA	0.01	18.30	10.03
#Carboprost Tromethamine	8.45	367.00>323.15	20	II	0.01	66.90	NA	0.01	18.72	17.00
#Cloprostenol sodium	8.25	422.90>126.90	27	II	0.01	71.02	NA	0.01	14.66	9.62
#Diethyl carbamazine	1.60	200.00>99.95	-16	II	0.01	99.04	NA	0.01	11.91	7.58
#Ivermectin	11.29	892.40>307.00	-22	II	0.005	101.80	NA	0.01	19.97	11.94
#Maduramicin	11.17	915.20>613.25	25	II	0.005	52.80	NA	0.01	19.81	17.36
*#Meloxicam	7.99	352.20>115.05	-22	II	0.01	71.11	0.015	0.01	6.26	8.19
#Niclosamide	10.31	324.70>171.00	26	II	0.005	58.84	NA	0.01	14.26	12.90
#Parbendazole	7.53	247.90>216.00	-21	II	0.005	62.11	NA	0.01	8.02	7.02
#Propofol	1.57	179.00>101.00	-10	II	0.01	43.35	NA	0.01	5.37	3.65
#Salinomycicin	11.30	773.30>431.05	-52	II	0.005	66.81	NA	0.01	14.54	16.03
#Sulfachlorpyridazine	5.41	284.80>155.95	-15	II	0.005	61.55	NA	0.01	7.18	10.79
#Sulfaquinoxaline	6.48	301.00>155.85	-16	II	0.005	69.10	NA	0.01	14.37	10.57
#Apramycin	4.10	540.00>217.00	-28	IV	0.01	107.27	NA	0.01	9.57	6.81
*#Dihydrostreptomycin	4.03	584.00>263.05	-32	IV	0.01	103.65	0.2	0.02	6.09	4.76
*Gentamicin	4.14	464.00>322.10	-16	IV	0.01	87.50	0.1	NA	2.79	11.65
*Kanamycin	4.06	485.00>163.00	-26	IV	0.01	105.21	0.15	NA	2.99	4.05
*#Neomycin	4.14	615.00>163.00	-36	IV	0.02	75.14	1.5	1.5	16.26	13.53
*#Spectinomycin	3.94	351.00>98.30	-20	IV	0.01	116.11	0.2	0.2	3.59	5.68

Note: * EU regulated and # FSSAI regulated

Out of total compounds analyzed, mean recoveries for 48 were found to be within 70-120 %, and for 20 within 35-70 %. As per SANTE guidelines, recoveries of all the compounds were found to be reproducible with ≤20 %RSD at their LOQ levels compounds (Refer to Table 4)

The method successfully achieved 2 µg/kg LOQ on LC-MS/MS for 40 compounds, 5 µg/kg LOQ for 14 compounds, 10 µg/kg LOQ for 11 compounds and 20 µg/kg LOQ for 5 compounds (Refer to Table 4). Representative chromatograms of few compounds at their LOQ levels are shown in Fig. 2.



R^2 = 0.9971

Albendazole-2-Amino Sulphone

R^2 = 0.9962

Chlortetracycline

R^2 = 0.9898

Meloxicam

Fig. 2 Representative chromatograms at LOQ level and linearity curves.

4. Conclusion

A simple, sensitive and rapid methods have been developed to quantify veterinary drugs by LC-MS/MS in milk sample.

The method developed on Shimadzu LC-MS/MS proved to be highly sensitive and reproducible as most of the compounds showed good RSD_r and RSD_R (as per SANTE guidelines REF2) at trace levels.

This highlights the reliability of the method and enables its use for milk samples in testing laboratories as per FSSAI regulations.

5. References

1. M. Anastassiades, S. J. Lehotay, D. Štajnbaher, F. J. Schenck, Fast and Easy Multiresidue Method Employing acetonitrile Extraction/Partitioning and "Dispersive Solid-Phase Extraction" for the Determination of Pesticide Residues in Produce, J. AOAC Int., 86; 412–431, 2003.
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