The Use of Microflow UHPLC in Veterinary Drug Residue Analysis

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INTRODUCTION

The levels and presence of veterinary drug residues in food of animal origin are legislated in the EU with limits often varying with the drug residue.[1] Traditionally in veterinary drug residue screening of food samples, samples are extracted and analyzed by LC/MS/MS usually at LC flow rates which are in excess of 500 µl/min and in combination with high pressures with smaller particle size HPLC columns to maintain sharp peaks and fast chromatography. These flow rates produce fast speeds and excellent peak shapes and results, but have a draw back in that they require higher volumes of organic solvent. The consumption of HPLC organic solvents, such as acetonitrile and methanol, is a growing cost of analysis and its disposal has an environmental impact. Therefore, ways to reduce solvent consumption in pesticide residue testing will be beneficial to the environment and reduce running costs of a testing lab.

Here we present new data using micro flow LC, running below 40 µL/min, in combination with a LC-MS/MS method developed on an AB SCIEX QTRAP® system utilizing the *Scheduled* MRM™ algorithm. Initially this approach has been applied to a screen of veterinary residues including sulfonamides to show its applicability in food analysis and data presented with compare Micro LC with traditional LC flow rates and show that low detection limits below legislated limits are easily possible by this approach.

MATERIALS AND METHODS

***Sample Preparation**:* The milk samples (2 g) was simply mixed with acetonitrile (8 ml) and roller mixed (20 minutes). After mixing the sample was centrifuged (5 minutes, 2500 rpm). The supernatant (4 ml) was evaporated to dryness (Eppendorf vacuum concentrator, 60 ºC) and then reconstituted into formic acid (2 ml). The reconstituted sample was centrifuged (13,000 rpm, 1 minute) and the top layer was decanted into plastic HPLC vials ready for injection.

For meat samples the sample preparation was exactly the same except the initial extraction solvent was 87.5% acetonitrile containing 12.5% water.

Figure 1. QTRAP® 5500 LC/MS/MS system

Figure 2. Eksigent ekspert™ microLC 200

***LC Separation**:* All micro LC method development and analysis was done using an Eksigent ekspert™ microLC 200 UHPLC system. Final extracted samples (2 µL) were separated over a 3.5 minute gradient (Table 2) where A = water and B = acetonitrile both containing 0.1 % formic acid. Apart form the initial development work which used the higher flows the drug residues were separated on a reversed-phase Triart C18 2.7 µm 50 x 0.5mm (YMC) column at 30 µL/min and at a temperature of 60ºC. For the high flow analysis a Shimadzu XR HPLC system was used at a flow rate of 600 µL/min and for this work the micro LC was run at 30 µL/min .

***MS/MS Detection**:* All analyses were performed on an AB SCIEX 5500 QTRAP® LC/MS/MS system using electrospray ionization (ESI). For Micro LC analysis the electrode was changed to a microLC hybrid electrode (50 µm ID) designed for MicroLC.[2]

Time (min)	A (%)	B (%)
0	98	2
2	98	2
7	40	60
7.2	5	95
8	5	95
8.1	98	2
10	98	2

Table 1. Gradient conditions used for the high flow vs. micro flow comparison

Time (min)	A (%)	B (%)
0	98	2
0.5	98	2
1.7	35	65
1.8	0	100
2.3	0	100
2.4	98	2
3.5	98	2

Table 2. Gradient conditions used for separation and sample analysis

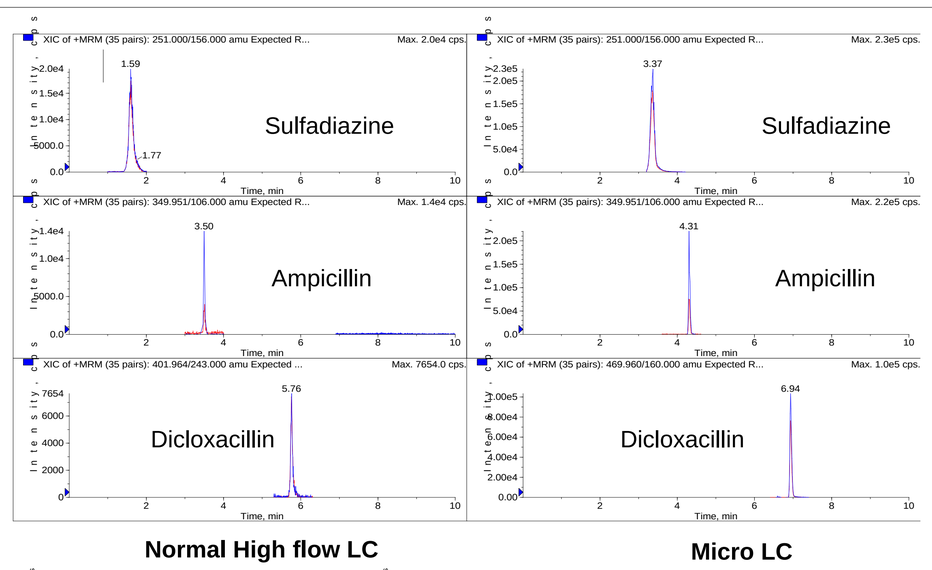
In the final method the Turbo V™ source conditions used were gas 1, gas 2 and the Curtain Gas™ interface was set to 30 psi, the temperature of the source was set at 350˚ C and the IS voltage was 5500 V. The peptides were analyzed using the *Scheduled* MRM™ algorithm with an MRM detection window of 40 s and a target scan time of 0.30 s. Q1 resolution and Q3 resolution was set to unit resolution. A total of 32 MRM transitions (Table 3) were evaluated for 15 veterinary drug residues over a 3.5 minute run time. Only a small set of residues were tested in this project but there is scope to add more residues to the same method.

Q1 Mass	Q1 Mass	Retention Time	Identity	MRM voltages (v)			
		(mins)		DP	EP	CE	CXP
349.951	106	1.3	Ampicillin 1	56	10	23	12
349.951	113.9	1.3	Ampicillin 2	56	10	41	12
435.996	277	1.9	Cloxacillin 1	51	10	19	26
435.996	160	1.9	Cloxacillin 2	51	10	17	20
469.96	160	2	Dicloxacillin 1	66	10	19	14
469.96	311	2	Dicloxacillin 2	66	10	21	12
415.032	199	1.9	Nafcillin 1	61	10	19	20
415.032	171	1.9	Nafcillin 2	61	10	47	16
401.964	243	1.8	Oxacillin 1	46	10	19	22
401.964	160	1.8	Oxacillin 2	46	10	17	18
351	160	1.8	Penicillin V 1	50	10	19	8
351	114	1.8	Penicillin V 2	50	10	45	8
335	160	1.7	Penicillin-G (benzylpenicillin) 1	50	10	15	14
335	176	1.7	Penicillin-G (benzylpenicillin) 2	50	10	19	14
215.04	155.9	1.2	Sulfacetamide 1	80	10	13	14
215.04	92	1.2	sulfacetamide 2	80	10	29	10
279.062	185.9	1.5	Sulfadimerazine 1	80	10	23	18
279.062	124	1.5	Sulfadimerazine 2	80	10	31	14
310.938	156	1.7	Sulfadimethoxine 1	71	10	29	18
310.938	92	1.7	Sulfadimethoxine 2	71	10	45	12
265.138	108	1.4	sulfamerazine 1	80	10	33	12
265.138	91.9	1.4	sulfamerazine 2	80	10	35	10
254.084	156	1.55	sulfamethaxazole 1	120	10	21	16
254.084	91.9	1.55	sulfamethaxazole 2	120	10	35	12
251	156	1.3	Sulfadiazine 1	66	10	26	8
251	108	1.3	Sulfadiazine 2	66	10	30	8
279.062	185.9	1.5	sulfamethazine 1	120	10	23	18
279.062	124	1.5	sulfamethazine 2	120	10	31	14
301	156	1.9	Sulfaquinoxaline 1	80	10	27	8
301	108	1.9	Sulfaquinoxaline 2	80	10	37	8

Table 3. MRM information for veterinary drug residues used in this evaluation

RESULTS

Before the micro LC was used for residue analysis the method was first compared against a higher flow method that had previously been developed for residue detection in meat and milk. A 1 part per billion (ppb) residue standard of a mixture of different veterinary residues was prepared and analyzed. For the high flow separation a 2.1 x 50 mm column Kinetex 2.6 µm XDB-C18 100Aº at a flow rate of 0.6 ml/min was used and a Triart C18 0.5 x 50 mm column was used for micro LC at 25 µ//min. The gradient conditions (Table 1) were kept the same as was the injection volume and column temperature and the results are shown in Figure 3. The results showed a typical sensitivity increase of factors greater than 8 fold for most of the veterinary residues when the analysis was moved from high flow to micro LC with none of the compounds showing a sensitivity loss. The gradient on the micro LC was then adjusted and the flow rate increased to 30 µ//min to shorten the run time down to 3.5 minutes. The results showed that for the late eluters there was some sensitivity loss due to peak broadening but again sensitivity gains were also observed for early eluters.



Normal High flow LC **Micro LC**

Figure 3. Comparison of a high flow injection with that of a Micro LC analysis using the same 1ppb standard.

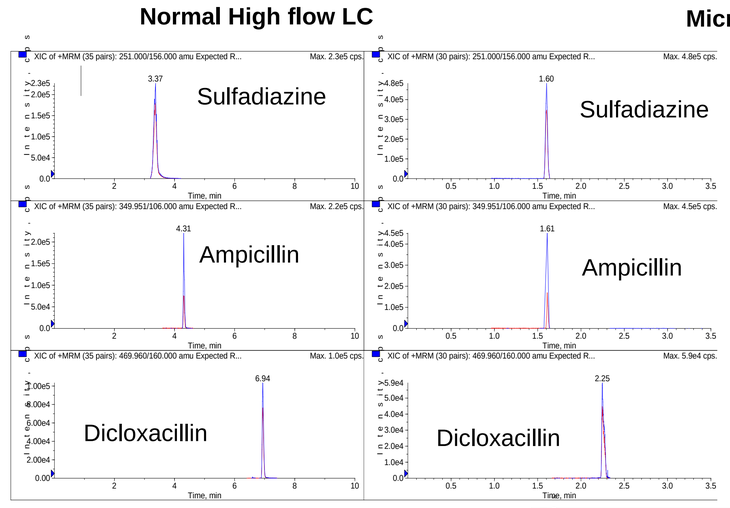
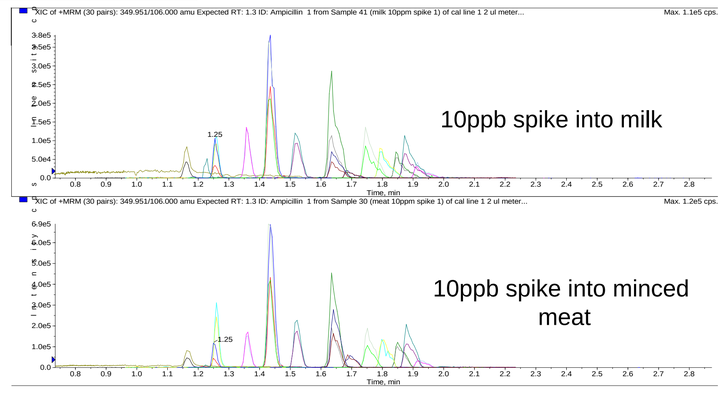


Figure 4.
This diagram shows the effect of speeding up the gradient elution with the analysis time moved from 10 minutes to 3.5 minutes. Generally in both methods peaks were seen to have widths at the peak base of 3s or less.

Figure 5. A comparison of a milk and meat sample which have been spiked at a 10 ppb level. The recoveries from meat were generally higher and it shows that recoveries are affected by the matrix and the method may require the use of internal standard(s) to get around some of these matrix affects or the use of matrix matched calibration lines or standard addition.



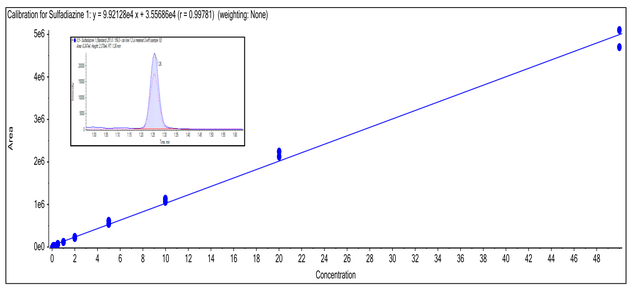
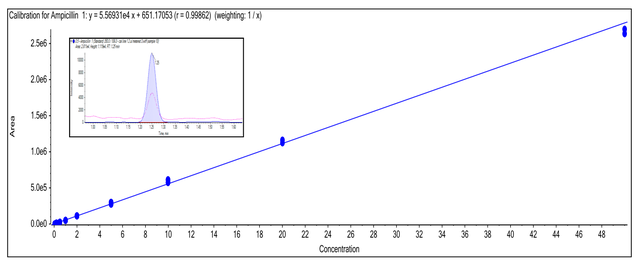


Figure 6. This is the calibration line from a Sulfadiazine from 0.05 – 50 ppb. The linearity is provided without the use of any internal standards and inset into the diagram is the chromatogram of a 0.5ppb standard.

Figure 7. This is the calibration line from a Ampicillin from 0.05 – 50 ppb. The linearity is provided without the use of any internal standards and inset into the diagram is the chromatogram of a 0.5ppb standard.



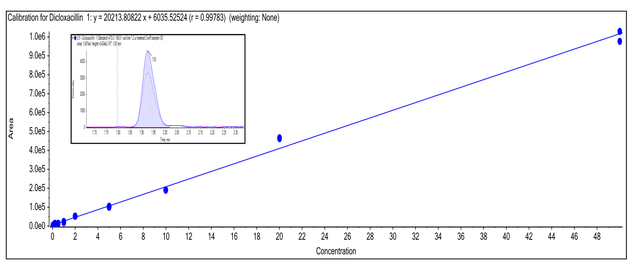


Figure 8. This is the calibration line from a Dicloxacillin from 0.05 – 50 ppb. The linearity is provided without the use of any internal standards and inset into the diagram is the chromatogram of a 0.5ppb standard.

CONCLUSIONS

This study has clearly demonstrated that using Micro LC is a valid approach in veterinary residue analysis. The method used was quick and sensitive and easily reached the requirements of current EU legislation. Micro LC offers the opportunity to cut the analysis time by over half without a loss in performance and in the majority of cases a gain in signal by over a factor of 8 for some residues. It also provides a huge cost saving to labs. With LC grade acetonitrile running at a cost of £100/L this 3 day study could have cost about £ 100 with convention chromatography (0.6 ml/min running for 24hrs a day) and < £10 with Micro LC. Over a year this amounts to savings of over £4000 (£90 x 50 weeks) in solvent consumption alone.

Although this method is still under development with plans to increase the number of compounds in this screen this work has shown the clear potential of Micro LC in this application area.

REFERENCES

1. COMMISSION REGULATION (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin.

2. Mriziq, K., Hobbs, S., Settineri, T and Neyer, D. (2011), Eksigent Technical Note entitled, 'Higher Sensitivity and Improved Resolution Microflow UHPLC with Small Diameter Turbo V™ Source Electrodes and Hardware for use with the Eksigent expressHT™Ultra System'.

TRADEMARKS/LICENSING