HOW DO YOU DEVELOP, TROUBLESHOOT AND VALIDATE AN ANALYTICAL METHOD?

APPLICATION NOTE

Modification of an Analytical Method for the Analysis of Clopyralid in Animal Tissues and the Pitfalls Encountered

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ABSTRACT

Purpose: The objective of the study was to convert a method utilizing analyte derivatization with GC/MS detection to a direct analysis by LC/MS-MS.

Methods: The original analytical method consisting of a basic hydrolysis/extraction technique followed by HLB solid-phase extraction (SPE) column (Waters, 200 mg/6 mL) cleanup, propylation, and detection by capillary gas chromatography with negative-ion chemical ionization mass spectrometry (GC-NCI-MS) was modified for direct analysis by LC/MS-MS. The original extraction procedure and a modification of the HLB SPE clean-up procedure were utilized to provide successful analysis by LC/MS-MS without matrix enhancement/suppression or interferences. Conversion of a method from one utilizing derivatization followed by GC/MS analysis to that of a direct detection by LC/MS-MS can pose unique analytical challenges to obtain comparable sensitivity and specificity. This paper will give an overview of the unique recovery, interference and sensitivity issues overcome during the process.

For the final method, residues of clopyralid are extracted from animal tissue samples with 2.5N NaOH with heating at approximately 105 °C for a minimum of 2 hours. Optional cleanup for poultry liver is affected by partitioning the basic extract with dichloromethane (DCM). An aliquot of the extract is acidified with HCl and submitted to a polymeric reversed-phase solid phase extraction column (Waters, HLB SPE) cleanup and elution with DCM. After removal of the DCM using nitrogen blow down, the sample is reconstituted in 10:90, acetonitrile:0.1% formic acid. The final extract is filtered through a 0.2 m PTFE syringe filter and then analyzed by liquid chromatography coupled with negative-ion electrospray ionization tandem mass spectrometry (ESI LC/MS-MS).

CHALLENGES

LC/MS-MS Parameters: The first step in developing a new method was to set up the instrument detection system. Since the original method was capillary gas chromatography with negative-ion chemical ionization mass spectrometry, the LC/MS-MS parameters had to be developed. The clopyralid dissolved in methanol was infused directly into the triple quad detector and a mass spectrum was generated in the negative-ion mode using

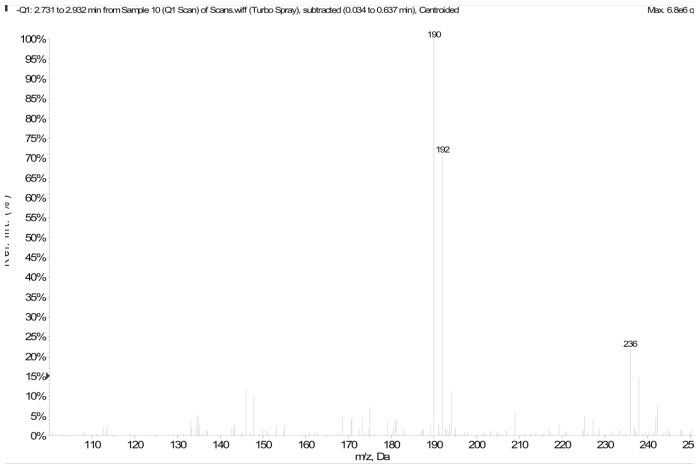

Figure 1: Negative-Ion electrospray mass spectrum for Clopyralid

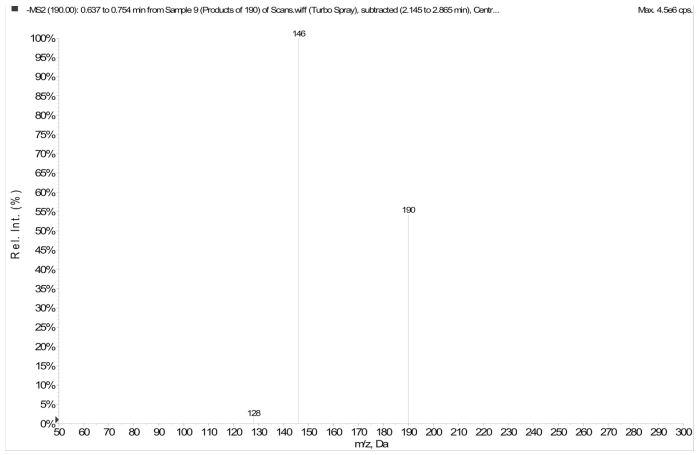

Figure 2: Product-ion mass spectrum of m/z 190 showing a major transition ion at m/z 146

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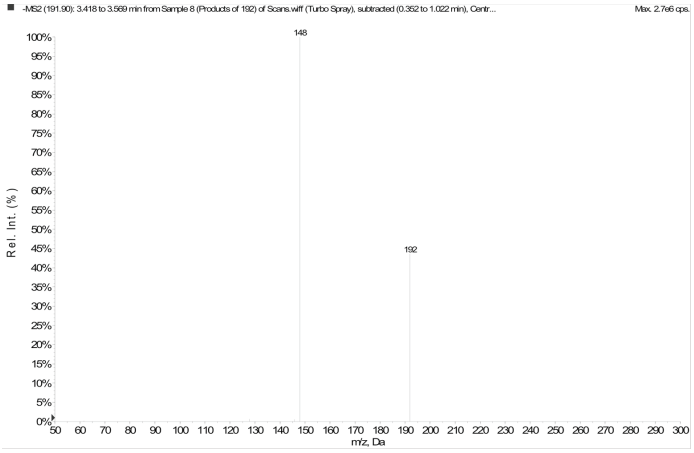

Figure 3: Product-ion mass spectrum of m/z 192 showing a major transition ion at m/z 148

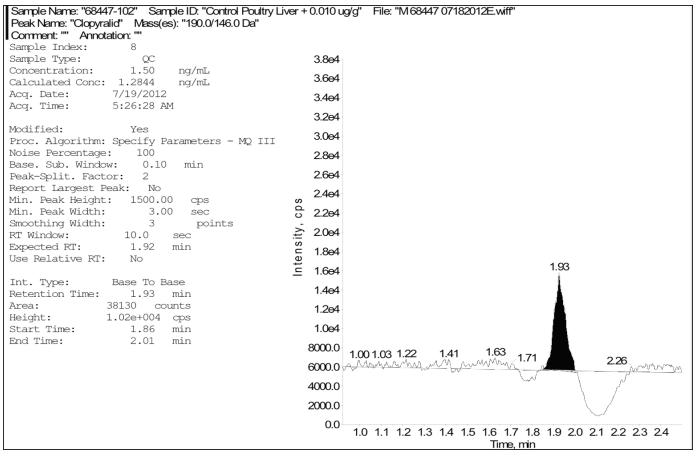

Figure 4: Poultry liver sample fortified with Clopyralid at 0.010 mg/kg (LOQ) (68447 102) without DCM cleanup.
Mobile Phase A: 0.1% Formic acid in water,
Mobile Phase B: 0.1% Formic acid in MeOH, 35 µL injection

electrospray. The clopyralid structure is presented in Table 1 and the spectrum in Figure 1. The most intense molecular ion (M-H)- 190 m/z with the corresponding product ion of 146 m/z was used for quantitation (Figure 2). Both EPA and European guidelines require that a confirmation transition be monitored to confirm the identity of the molecule being detected. A second product ion generated from the same molecular ion is preferred. However, no other product ions were generated in sufficient quantities to be a satisfactory confirmation transition. Since the molecule contained chlorine, it was decided to use the +2 molecular ion [(M-H)- +2] 192 m/z and its corresponding product ion 148 m/z (Figure 3).

Once the quantitation and confirmation transitions were selected, the HPLC parameters needed to be established to obtain adequate sensitivity and separation from sample matrix. HPLC parameters utilizing a methanol/water system with 0.1% formic acid a modifier was developed. This system seemed to be adequate until issues were encountered with the poultry liver matrix which contained

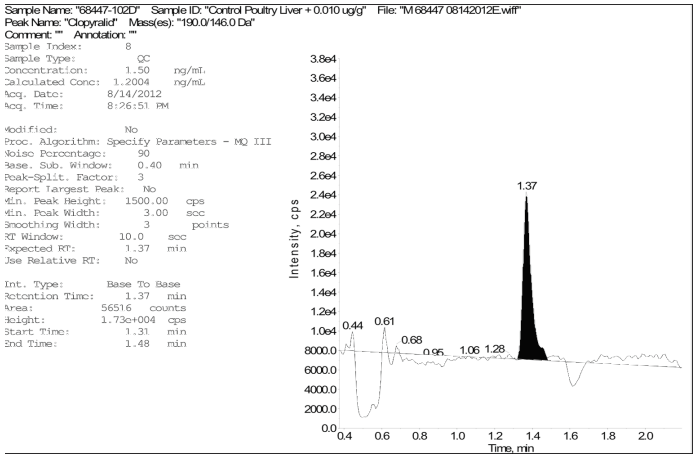

Figure 5: Poultry liver sample fortied with Clopyralid at 0.010 mg/kg (LOQ) (68447 102D) with DCM cleanup.
Mobile Phase A: 0.01% Formic acid in water
Mobile Phase B: 0.01% Formic acid in 60:40, MeOH:ACN, 15 µL injection

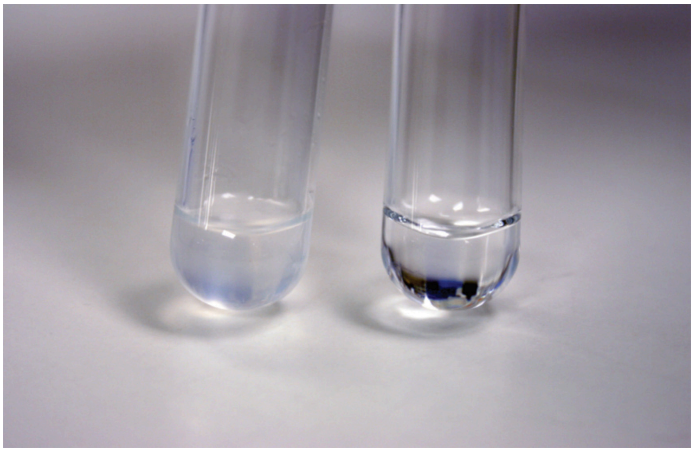

Figure 6: Example of proper dissolution of the final sample

negative peaks on both sides of the clopyralid peak (Figure 4). To further clean up the sample, a dichloromethane partitioning step on the basic extract was added which removed the negative peak eluting before the clopyralid peak (Figure 5).

To further affect separation from the negative peak eluting after of the clopyralid, the organic phase was modified to 60:40, methanol:acetonitrile. To increase the sensitivity and reduce the baseline noise, the modifier was reduced to 0.01% formic acid. As can be seen in Figure 5 the sensitive was increase by 48% even though the injection volume was reduced from 35 uL to 15 uL.

Recovery Issues: During the development of the cleanup method, tests were conducted to determine if any analyte losses would be incurred during the blow down step. No losses were seen when reagent spikes were blown down under a stream of nitrogen at a water bath temperature of 40°C even after continuing the blow down for 15 minutes after all dichloromethane had been removed. However, low recoveries were seen during the blow down step in the presence of matrix with post SPE spikes. The

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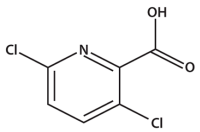
Common Name of Compound	Structural Formula and Chemical Name
Clopyralid Molecular Formula: $C_6H_3Cl_2NO_2$ Formula Weight: 192.00 Nominal Mass: 191 CAS Number 1702-17-6	 3,6-Dichloropicolinic Acid

Table 1: Identity and structure of Clopyralid

Sample Number	Matrix	Analysis Date[a]	Added	Quantitation Transition (Q1/Q3 m/z 190/146)		Confirmation Transition (Q1/Q3 m/z 192/148)		Confirmation Ratio Difference[b]
				Found	Recovery	Found	Recovery	
			(mg/kg)	(mg/kg)	(Percent)	(mg/kg)	(Percent)	(Percent)
68447-093D	Liver	17-Jul-2012	NA[c]	ND[d]	NA[c]	ND[d]	NA[c]	NA[c]
68447-100D	Liver	18-Jul-2012	NA[c]	ND[d]	NA[c]	ND[d]	NA[c]	NA[c]
68447-101D	Liver	18-Jul-2012	0.0030	0.0021	NA[c]	0.0014	NA[c]	-30.21
68447-094D	Liver	17-Jul-2012	0.010	0.0075	75	0.0065	65	-16.19
68447-095D	Liver	17-Jul-2012	0.010	0.0080	80	0.0080	80	-0.09
68447-102D	Liver	18-Jul-2012	0.010	0.0080	80	0.0067	67	-17.07
68447-103D	Liver	18-Jul-2012	0.010	0.0075	75	0.0069	69	-7.09
68447-104D	Liver	18-Jul-2012	0.010	0.0077	77	0.0067	67	-11.81
Mean 0.01 (n=5)				0.0077	77	0.0069	69	
SD 0.01 (n=5)				0.00023	2.3	0.00060	6.0	
RSD (%) 0.01 (n=5)				2.9	2.9	8.6	8.6	

[a] The 'Date of Analysis' indicates the date that the samples were extracted.

[b] The 'Percent Difference' is relative to the average confirmation ratio found for the standards.

[c] NA — not applicable.

[d] ND — not detected. The residue was below the 0.003-mg/kg limit of detection.

Table 2a: Recovery of Clopyralid from poultry liver

use of vortexing and sonication to dissolve the residues yielded variable recoveries. When multiple samples were analyzed in one set they were placed in a test tube rack during sonication. The samples remained clear (Figure 6, right test tube) even after prolonged sonication and vortexing and recoveries were low. While troubling shooting this step a single sample was handheld and placed in the sonicator and the sample became cloudy.

As the sample was rotated and tilted to assure the solvent came in contact with the entire inner surface of the test tube during sonication the sample became more cloudy (Figure 6, left test tube). Further investigation determined that eluting the SPE with dichloromethane was actually dissolving a portion of the plastic that contained the HLB column bed. As the dichloromethane was evaporating under nitrogen blow down, the plastic was trapping

Sample Number	Matrix	Analysis Date[a]	Added	Quantitation Transition (Q1/Q3 m/z 190/146)		Confirmation Transition (Q1/Q3 m/z 192/148)		Confirmation Ratio Difference[b]
				Found	Recovery	Found	Recovery	
			(mg/kg)	(mg/kg)	(Percent)	(mg/kg)	(Percent)	(Percent)
68447-096D	Liver	17-Jul-2012	1.00	0.8928	89	0.8970	90	2.38
68447-097D	Liver	17-Jul-2012	1.00	0.8599	86	0.8659	87	2.59
68447-098D	Liver	17-Jul-2012	1.00	0.8801	88	0.8857	89	2.53
68447-105D	Liver	18-Jul-2012	1.00	0.8678	87	0.8995	90	1.69
68447-106D	Liver	18-Jul-2012	1.00	0.8339	83	0.8147	81	-4.29
Mean 1.0 (n=5)				0.8669	87	0.8726	87	
SD 1.0 (n=5)				0.0223	2.2	0.0349	3.5	
RSD (%) 1.0 (n=5)				2.6	2.6	4.0	4.0	
Mean Total (n=10)				NA[c]	82	NA[c]	78	
SD Total (n=10)				NA[c]	5.3	NA[c]	10.5	
RSD (%) Total (n=10)				NA[c]	6.5	NA[c]	13.3	

[a] The 'Date of Analysis' indicates the date that the samples were extracted.

[b] The 'Percent Difference' is relative to the average confirmation ratio found for the standards.

[c] NA — not applicable.

[d] ND — not detected. The residue was below the 0.003-mg/kg limit of detection.

Table 2b: Recovery of Clopyralid from poultry liver

the clopyralid on the sides of the test tube. Since the plastic was not soluble in the 90% aqueous final diluent, simple vortexing or sonication in a vertical position was not sufficient to loosen the plastic residue and liberate the clopyralid. Thus, it was a necessity to sonicate each sample by hand, insuring the solvent came in contact with the entire inner surface of the test tube to physically remove the plastic from the tube surface allowing the clopyralid to dissolve in the solvent. This is the most critical step in the procedure and accounts for the majority of analyte losses. Having a cloudy final solution is an indication of proper dissolution of the sample. Filtration through a 0.2-m PTFE syringe filter removes all cloudiness.

Results: The method validation study was conducted to determine the recovery levels and the precision of the method for the determination of residues of clopyralid in animal matrices. The performance of the analytical method was determined with each set of samples by fortifying aliquots of the appropriate control matrix with a clopyralid solution and analyzing the set following the procedures described within this report. Samples were fortified at the limit of detection (LOD) of 0.003 mg/kg,

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the limit of quantitation (LOQ) of 0.010 mg/kg, and at 1.0 mg/kg. Samples fortified at the LOD were analyzed only to demonstrate observable peaks at the LOD level; the results were not included in average percent recovery calculations. An unfortified control matrix and reagent blank were also included in each set.

The results of the quantitation and confirmatory recoveries for poultry liver are listed in Table 2. For the quantitation results, the individual recoveries for all samples fell within the range of 70 to 110% and the average recoveries at each fortification level also fell within the range of 70 to 110%. The average recoveries for all fortification levels fell within the range of 70 to 110%. Relative standard deviations at each fortification level were all less than 20%.

The method is selective for the determination of clopyralid by virtue of the chromatographic separation and MS/MS detection system used. Using published guidelines (1), when detection is by tandem mass spectrometry methods, confirmation of the presence of the analyte should require the observation of a precursor ion plus one structurally significant product ion observed at the same retention time. By monitoring multiple MS/MS ion transitions for each analyte, the confirmation ratios were calculated for clopyralid in each sample set and compared to the average for the calibration standards. The results are listed in Table 2. The confirmation ratios in all samples (accept the LOD) were within ±20% of the average found for the standards, indicating that the method is selective for the determination of clopyralid in poultry liver.

Conclusions: The analytical method for the determination of clopyralid in animal matrices has been demonstrated to be satisfactory in terms of accuracy, precision, linearity, and selectivity. The method was validated over the concentration range of 0.003-1.0 mg/kg for poultry liver with a limit of quantitation of 0.010 mg/kg.

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