Application Note

Food Testing & Agriculture

Analysis of Formamidine Pesticides and Metabolites in Pork and Porcine Liver Using Agilent Captiva EMR—Lipid and LC/MS/MS

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Abstract

Formamidine pesticides are widely used in pest control for crops and livestock, creating a potential risk in animal-origin food safety. This Application Note presents a reliable and robust method using QuEChERS extraction followed by Agilent Captiva EMR—Lipid cleanup in the residue analysis of amitraz, chlordimeform, and their metabolites in pork and porcine liver. The method delivers good accuracy (>60.0 %) and precision (RSD<12 %) for the four analytes at all levels, providing a fast and effective analysis in high-fat samples.

Introduction

The formamidines are a group of acaricides, unique both in chemical structural and biological activities[1]. They are used in the control of mites, cattle ticks, and certain orders of insects that have become resistant to conventional acaricides and insecticides[2]. Chlordimeform (CMD) and amitraz (AMZ) are two formamidine acaricides that are applied widely as repellents against insects or pests by spraying onto farm livestock. As a result, these compounds can be absorbed by the animal. CMD has been banned, but AMZ is still widely used worldwide. Maximum residue levels (MRLs) exist for AMZ and its metabolites in its use on a wild range of farm animals in EN commission regulation 2017/623[3].

Agilent Enhanced Matrix Removal—Lipid (EMR—Lipid) provides high efficiency lipid removal with a limited impact on target analytes in many applications[4,5]. The selective and efficient lipid removal by EMR—Lipid cleanup is due to the unique combination of both size exclusion and hydrophobic interactions. Only the long unbranched aliphatic chains on lipid-like molecules are trapped by the EMR—Lipid sorbent. EMR's unique pass-through functionality simplifies the sample preparation workflow. In addition, the reduced water amount needed to achieve desired lipid removal also improves hydrophobic analyte recovery.

This study investigates the use of Captiva EMR—Lipid cartridge cleanup in the analysis of AMZ, CMD, and their metabolites in pork and porcine liver. Table 1 shows chemical information for the studied pesticides.

Experimental

Regent and chemicals

All reagents and solvents were HPLC or analytical grade. Acetonitrile (ACN) was from Honeywell (Muskegon, MI, USA). Formic acid (FA) and ammonium hydroxide (NH_4OH) were from J&K Scientific Ltd. (Beijing, China). The pesticides and metabolite standards were purchased from Alta (Tianjin, China).

Solution and standards

Individual standard stock solutions were made in ACN at 10 mg/mL in amber glass vials, and stored at –20 °C. The combined standard spiking solution (1 mg/mL) was prepared with ACN just before use. The extraction solvent ACN was stored at –20 °C for cold extraction.

Equipment and material

Separation was carried out using an Agilent 1290 Infinity LC coupled with an Agilent 6495B triple quadrupole LC/MS system with an Agilent Jet Stream electrospray ionization source. Agilent MassHunter Workstation software was used for data acquisition and analysis.

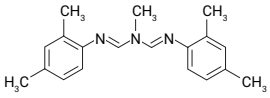
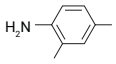
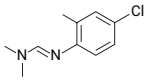
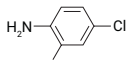
Other equipment and material used for sample preparation include:

- SPEX SamplePrep 2010 Geno/Grinder (Metuchen, NJ, USA)
- Eppendorf Centrifuge 5810R (Hamburg, Germany)
- Agilent Captiva EMR—Lipid cartridge, 6 mL, 600 mg (p/n 5190–1004)
- Agilent Vac Elut 20 Manifold (p/n 12234101)
- Agilent QuEChERS extraction kit for veterinary drugs (p/n 5982–0032)

Instrument conditions

Figure 1 shows typical chromatograms of four neat standard analytes, compared to pork and porcine liver matrix blanks using a developed sample preparation method. As shown, all the analytes can be well quantitated at the level of 0.1 ng/g or lower in pork or porcine liver matrices. An exception is 2,4-DMA, which is set at 1 ng/g. Interferences from complex matrices can impact the response of a small molecule such as 2,4-DMA (MW 121 Da). Therefore, more caution was taken in the selection of the MRM quantitative ion pair. The third highest pair (*m/z* 121.1 → 105) was selected for quantitation as it demonstrated the lowest interference from the matrices.

Table 1. Target analytes, chemical structures, molecular weight, log P, pKa, and MRL (amitraz and its metabolites are expressed in total) in EN regulation 2017/623.

Analyte	Chemical structure	MW (Da)	logP	pKa	MRL (ng/g)
Amitraz (AMZ)		293.41	5.5	4.2	400 (pork) 200 (porcine liver)
2,4-Xylidine (2,4-DMA)		121.18	1.68	4.89	
Chlordimeform (CMD)		196.68	2.89	6.8	Not applicable
4-Chloro-*o*-toluidine (DCMD)		141.6	2.27	3.85	

HPLC conditions

Parameter	Value
Column	Agilent ZORBAX Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm (p/n 959757-902)
Column temperature	35 °C
Autosampler temperature	10 °C
Injection volume	10 µL
Mobile phase	A) 0.1 % FA in water B) 0.1 % FA in ACN
Gradient	Time (min) %B Flow rate (mL/min) 0 5 0.3 0.1 5 0.3 3 45 0.3 5 95 0.3 7 95 0.3 7.1 5 0.3
Stop time	10 minutes

MS conditions

Parameter	Value
Positive/negative mode	Positive
Gas temperature	210 °C
Gas flow	13 L/min
Nebulizer	35 psi
Sheath gas heater	400 °C
Sheath gas flow	12 L/min
Capillary	3,500 V
Delta EMV (+)	400 V

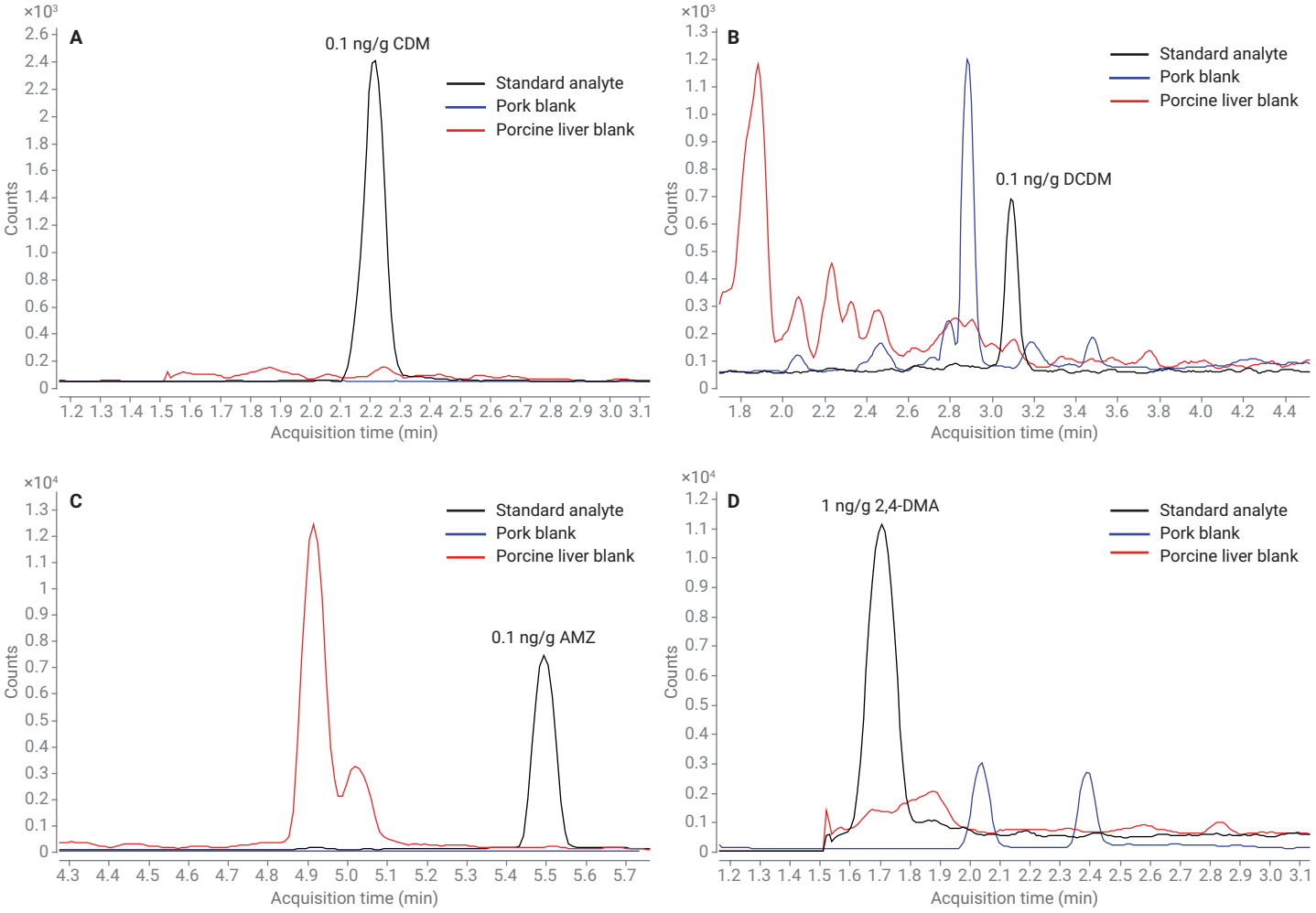


Figure 1. LC/MS/MS MRM chromatograms for four neat standard analytes (black trace), and pork blank (blue trace)/porcine liver blank (red trace) under quantitative MRM ion pair monitoring.

MRM parameters

Compound	Retention time (min)	Precursor ion (*m/z*)	Quantifier ion (*m/z*) (CE, V)	Qualifier ion (*m/z*) (CE, V)	Cell accelerator voltage (V)
AMZ	5.5	294.2	163.1 (9)	122.0 (32)	3
2,4-DMA	1.7	122.1	105 (17)	107 (21) 77 (33) 103 (25)	3
CMD	2.2	197.1	46.1 (21)	116.9 (29)	3
DCMD	3.1	142.0	107 (21)	125 (21)	3

Sample preparation

Figure 2 shows the sample preparation procedure. Pork is generally dry and needs 1 mL of water to help homogenization; porcine liver is much easier to homogenize without adding extra water. However, porcine liver is more complex, which appeared to induce AMZ precipitation after dilution with water at a ratio of 1:2 before injection. Therefore, 2 µL of the porcine liver samples were injected without any dilution after EMR—Lipid cleanup.

Calibration standards and quality control (QC) samples

Matrix-matched calibration standards and post spiked QC samples were prepared by spiking appropriate standard solutions into the matrix blank after EMR—Lipid cartridge cleanup. The spiking concentrations for calibration standards were 0.1, 0.5, 1, 5, 10, 50, 100, and 200 ng/g in pork or porcine liver.

Prespiked QC samples were fortified by spiking the appropriate standard working solution into the homogenized pork or porcine liver sample with six replicates of low (0.1 and 1 ng/g), mid (5 ng/g), and high levels (50 ng/g).

Results and Discussion

Extraction optimization

For animal-origin sample matrix, solvent extraction is a commonly used method to remove proteins. We investigated the effect of solvent extraction using acidic, neutral, and basic ACN on the responses of the four analytes, as shown in Figure 3. AMZ shows degradation issues with acidic extraction, while ACN extraction without any pH adjustment delivered the best responses.

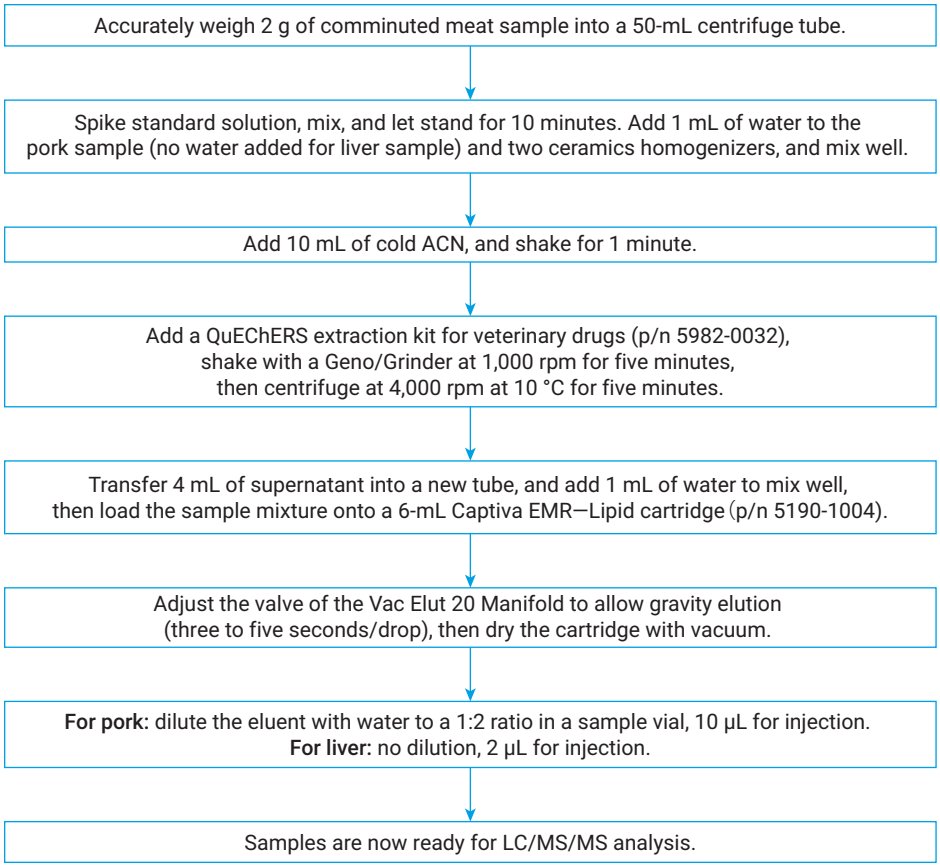


Figure 2. Pork sample extraction and following cleanup procedure using an Agilent Captiva EMR—Lipid 6-mL cartridge.

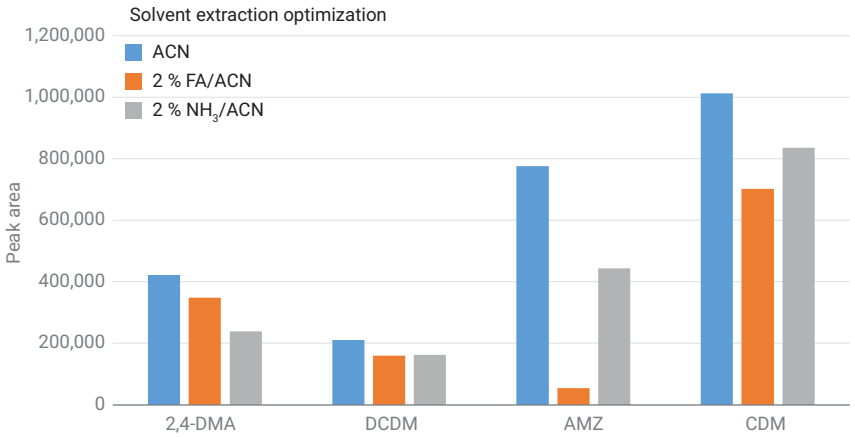


Figure 3. The effect of extraction solvent on the responses of four analytes.

QuEChERS and solvent extraction were compared for sample extraction. As a recommendation, 20 % water is required to mix with the organic sample extraction when loading sample onto the Captiva EMR—Lipid cartridge, to achieve satisfactory lipid removal. This can be achieved using a 20:80 water/ACN solvent extraction. However, it creates some problems for accurate calculation without the use of internal standard correction. Also, it affects the extraction efficiency of the hydrophobic analytes. We compared two extraction procedures, a QuEChERS extraction kit for veterinary drugs by Agilent, and a solvent extraction with ACN, with the results shown in Figure 4. From the results, QuEChERS extraction improved the extraction of DCDM, CDM, and AMZ, and was selected for this application.

Linearity

The data were processed with MassHunter quantification software. A calibration curve gave R^2 values >0.990 for all pesticides and metabolites. Table 2 lists the data.

Recovery and precision results

The optimized extraction and cleanup method was validated by running spiked samples with three or four QC levels. The recovery was calculated by comparing the peak area of prespiked and post spiked QCs. Table 2 lists the quantitative results in detail. Acceptable recoveries were achieved for all analytes at all levels in both pork and porcine liver. The RSD values for six replicates of these four analytes were below 11.1 %, with the typical RSD being 5.2 %.

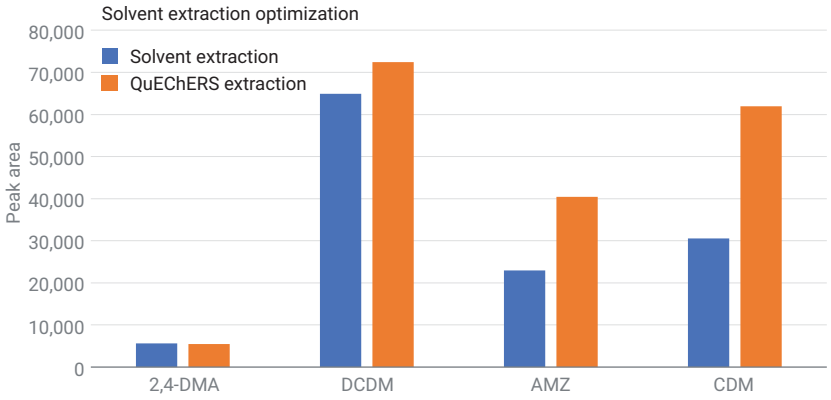


Figure 4. The effect of extraction type on the responses of four analytes.

Table 2. Method quantitation results for four analytes in pork and porcine liver.

Analyte	Matrix	R^2	Linear range (ng/g)	0.1 ng/g QCs (n = 6)		1 ng/g QCs (n = 6)		5 ng/g QCs (n = 6)		50 ng/g QCs (n = 6)	
				Rec%	RSD	Rec%	RSD	Rec%	RSD	Rec%	RSD
2,4-DMA	Pork	0.995	1~200	–	–	87.3	5.6	82.0	10.2	87.1	5.6
	Porcine liver	0.999	1~200	–	–	90.1	7.3	98.4	2.3	91.9	0.7
AMZ	Pork	0.993	1~100	–	–	127.4	1.9	118.7	4.7	79.1	5.4
	Porcine liver	0.991	1~200	–	–	60.9	2.7	69.2	6.7	74.8	11.1
DCMD	Pork	0.999	0.1~100	76.6	10.4	87.1	6.8	89.8	5.3	95.2	4.6
	Porcine liver	0.999	0.1~100	86.5	7.9	97.2	7.3	97.3	1.3	91.7	1.9
CDM	Pork	0.998	0.1~100	73.9	1.5	66.7	3.8	68.3	10.1	66.1	7.3
	Porcine liver	0.997	0.1~200	105.6	8.5	60.4	3.6	64.4	0.8	60.2	1.6

Conclusion

An easy and reliable sample preparation method using QuEChERS extraction followed by Captiva EMR—Lipid cleanup was established and validated for the quantitative determination of formamidine pesticides and metabolites in pork and porcine liver. The method delivers excellent results with higher than 60 % recoveries and less than 12 % RSD in both pork and porcine liver matrices. The limit of quantitation (LOQ) of the banned pesticide CDM and its metabolite DCDM were below 0.1 ng/g. The LOQ of the regulated pesticide AMZ and its metabolite 2,4-DMA were far below the MRL specified by the European Union.

References

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