Determination of Pesticide Residues in Red Wine

Using a QuEChERS Sample Preparation Approach and LC-MS/MS Detection

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This application presents a fast, easy, and cost-effective method for the determination of 24 pesticide residues in red wine. Sample preparation involves the extraction of pesticides from red wine using the QuEChERS extraction method (AOAC version). The samples then undergo cleanup by dispersive solid-phase extraction (dSPE) using primary secondary amine (PSA) sorbent, which effectively retains organic acids, sugars, and phenolic pigments. A higher quantity of PSA than normally used in the dSPE step is required to sufficiently remove co-extracted phenolic compounds from red wine. The purified extract is subsequently separated using a solid core column prior to detection by a triple quadrupole mass spectrometer. The developed method was applied to commercially available red wine samples to test its applicability. Six out of the fourteen samples tested were found to contain pesticide residues at trace levels.

Red wine is one of the most commonly consumed alcoholic beverages in the world. It's also a rich source of phenolic antioxidants and is reported to reduce the risk of diabetes, cancer, Alzheimer's disease, and cardiovascular disease[1, 2]. To improve grape yields it is common practice in vineyards to use pesticides, such as fungicides and insecticides. However, if pesticide residues remain in the grapes prior to the winemaking process they can be transferred to the final product and, if present at significant levels, may be toxic to the consumer. Due to the health risk that pesticides pose to humans it is important to monitor for their presence in food and beverages. No maximum residue levels (MRLs) have been established for pesticide residues in red wine; however, MRLs set for the raw commodity (e.g. wine grapes) can be applied to the processed product (e.g. wine)[3], thus the pesticide residues detected in the red wines tested in this study will be compared to the MRLs in wine grapes set by European Union (EU)[4]. The analysis of pesticide residues in red wine is challenging due to the complexity of the matrix, which contains alcohol, organic acids, sugars, and polyphenols (e.g. anthocyanins, flavonols, and tannins). Traditional sample preparation methods for red wine include liquid-liquid extraction (LLE) with different organic solvents, solid-phase extraction (SPE) with reversed-phase C18 or polymeric sorbents, solid-phase microextraction (SPME), and stir bar sorptive extraction (SBSE). However, these traditional methods have their own limitations, such as being labor intensive, costly (e.g. need for expensive glassware and solvents), using large quantities of organic solvent (environmental impact and disposal costs), requiring extensive method development and optimization, and possibly suffering from a lack of reproducibility or accuracy.

The QuEChERS approach (acronym for Quick, Easy, Cheap, Effective, Rugged, and Safe) is a sample preparation technique that was first reported in 2003 by Anastassiades et al. for the analysis of pesticide residues in fruits and vegetables[5]. QuEChERS involves extracting pesticides (or other chemical residues) from a high aqueous sample into an organic solvent (most commonly acetonitrile) with the aid of salts, followed by dispersive solid-phase extraction (dSPE) to remove matrix co-extractives. This application note describes a modified QuEChERS extraction and dSPE cleanup method for the determination of pesticide residues in red wine. LC-MS/MS is used to accurately and quantitatively detect pesticides in red wine at low concentrations.

Thermo Scientific™ Accucore™ HPLC columns use Core Enhanced Technology™ to facilitate fast and high efficiency separations. The 2.6µm diameter particles have a solid core and a porous outer layer. The optimized phase bonding creates a series of high-coverage, robust phases. The tightly controlled 2.6µm diameter of Accucore particles results in much lower backpressures than typically seen with sub-2µm materials. Accucore aQ columns are compatible with with 100% aqueous mobile phases and offer special selectivity for polar analytes.

Consumables	Cat. No.
A 5mg/mL Triphenyl Phosphate Stock Solution in Methyl Tert-Butyl Ether was used as internal standard (IS).	–
Twenty-four neat pesticides (>96%) were obtained from a reputable supplier.	–
HPLC Grade Acetonitrile	**BDH83639.400**
HPLC Grade Methanol	**BDH20864.400**
Glacial Acetic Acid	**BDH3094-2.5LG**
Formic Acid (>95%) (ours is great than 90%?)	**97064-706**
Ammonium Formate (>99.995%)	**AA14517-30**
Ultra High Purity Water	**87003-236**

Sample Preparation Supplies	Cat. No.
50mL Polypropylene Centrifuge Tube, 50 mL	**89401-562**
Thermo Scientific™ Mylar® Pouch, contains 6g Magnesium Sulfate ($MgSO_4$) and 1.5g Sodium Acetate	**10047-124**
Thermo Scientific 2mL Centrifuge Tube containing 150mg $MgSO_4$ and 150mg PSA	**10841-610**
Thermo Scientific National™ Target™ 1mL All-Plastic Disposable Luer-Slip Syringes	**66064-752**
Thermo Scientific Target2™ 0.2µm, 17 Nylon Syringe Filters	**66030-861**
Screw Thread Glass Vials, Kit	**89239-026**
Thermo Scientific™ Finntip™ Pipet Tips, 0.50–250µL	**53516-150**

Preparation of Pesticide Stock Solutions: A 1mg/mL stock solution of each of the 24 pesticides was prepared by weighing 10mg of the neat standard into a 10mL volumetric flask and diluting to volume with acetonitrile.

Preparation of Pesticide Working Solutions:
(1) A 2 µg/mL pesticide working solution was prepared by mixing 100µL of each of the 1mg/mL stock solutions in a 50mL volumetric flask, and diluting to volume with acetonitrile. (2) A 0.2 µg/mL pesticide solution was prepared by mixing 1mL of the 2µg/mL pesticide working solution with acetonitrile in a 10mL volumetric flask, and diluting to volume with acetonitrile.

Preparation of Internal Standard Solution: A 30 µg/mL triphenyl phosphate working solution (IS) was made by mixing 60µL of the 5000µg/mL triphenyl phosphate solution with acetonitrile in a 10mL volumetric flask, and diluting to volume with acetonitrile.

Standard Storage: All stock standards and working solutions were transferred to amber glass vials with Teflon®-lined caps and stored at -20 °C until needed.

Sample Preparation

The AOAC acetate buffered procedure was selected for sample extractions as it provides higher recovery for pymetrozine compared to the EN15662 citrate buffered or original non-buffered procedure.

AOAC QuEChERS Extraction

1. Transfer 15mL red wine sample into a 50mL centrifuge tube.
2. Spike with 50µL of the 30µg/mL triphenyl phosphate solution (corresponding to 100 ng/mL).
3. Add 15mL of acetonitrile containing 1% acetic acid and vortex for 1 min.
4. Add contents of the Mylar pouch containing 6g $MgSO_4$ and 1.5g sodium acetate, and shake vigorously on a horizontal shaker or vortex for 1 min.
5. Centrifuge at ≥3,750 rcf for 5 min.
6. The supernatant is now ready for dSPE cleanup.

dSPE Cleanup

1. Transfer 1mL of the supernatant into a 2mL dSPE tube containing 150mg $MgSO_4$ and 150mg PSA and vortex for 30 s.
2. Centrifuge at ≥15,000 rcf for 5 min.
3. Transfer 0.3mL of the purified extract into an autosampler vial, add 0.3mL of reagent water, vortex, and filter with a 0.2µm syringe filter.
4. The sample extract is now ready for LC-MS/MS analysis.

Separation Conditions	
Instrumentation:	HPLC System
Column:	Thermo Scientific Accucore, 2.6 µm, 100 × 2.1 mm (Cat. No. **10039-148**)
Guard Column:	Thermo Scientific™ Accucore™ aQ Defender™, 2.6 µm, 10 × 2.1 mm (Cat. No. **10038-810**)
Run Time:	20 min. (including re-equilibration time)
Column Temperature:	40°C
Injection Volume:	10 µL
Autosampler Temperature:	10°C
Wash Solvent:	Methanol / Ultrapure Water (1:1, v/v)
Flow Rate:	200 µL/min.
Mobile Phase A:	0.3 % formic acid and 0.1 % ammonia formate in ultrapure water
Mobile Phase B:	0.1 % formic acid in methanol
Preparation of Mobile Phase:	A: Dissolve 3 mL formic acid and 1 g ammonium formate in 1 L ultrapure water, and sonicate for 30 min.
	B: Add 1 mL formic acid to 1 L methanol and sonicate for 30 min.

Mobile Phase Gradient:	Time (min)	B (%)
The mobile phase was diverted to waste from 0 to 0.5 min and 15 to 20 min to prevent ion source contamination.	0.0	1
	1.5	1
	3.5	80
	10.0	90
	12.0	100
	15.0	100
	15.2	1
	20.0	1

MS Conditions	
Instrumentation:	Mass Spectrometer
Ionization Mode:	ESI+
Spray Voltage:	4000 V
Vaporizer Temperature:	300 °C
Sheath Gas Pressure:	50 arbitrary units
Auxiliary Gas Pressure:	25 arbitrary units
Q1 and Q3 Peak Width:	0.2 and 0.7 Da
Collision Gas:	Argon at 1.5 mTorr
Cycle Time:	1 s
SRM Parameters:	See Table 1



SRM Transitions							
Pesticide	tR (min)	Precursor Ion	Product Ion 1	CE 1	Product Ion 2	CE 2	S-Lens (V)
Methamidophos	1.28	142.0	124.6	14	111.6	5	60
Pymetrozine	1.31	218.0	104.9	18	176.0	16	70
Carbendazim	6.39	192.1	132.1	29	160.1	17	81
Dicrotophos	6.47	238.0	126.6	17	108.6	33	73
Acetachlor	6.48	269.4	111.9	15	71.7	33	72
Thiabendazole	6.61	202.1	131.1	31	175.1	24	103
DIMP	7.30	181.3	96.6	13	78.6	32	44
Tebuthiuron	7.32	228.9	115.6	26	171.6	17	72
Simazine	7.34	201.4	67.7	33	103.6	24	85
Carbaryl	7.41	202.0	126.6	30	144.6	7	40
Atrazine	7.69	216.0	67.7	35	173.6	16	79
DEET	7.72	191.9	118.6	15	90.7	28	92
Pyrimethanil	8.10	200.1	107.1	23	183.1	22	66
Malathion	8.08	331.0	98.6	23	126.9	12	60
Bifenazate	8.21	300.9	169.8	15	197.6	5	51
Tebuconazole	8.71	308.0	69.7	29	124.6	35	97
Cyprodinil	8.78	226.1	77.0	40	93.1	33	88
Triphenyl phosphate (IS)	8.80	327.1	77.02	37	152.1	33	98
Diazinone	8.85	305.1	153.1	15	169.1	14	89
Zoxamide	8.85	335.8	186.5	20	158.5	38	102
Pyrazophos	8.95	374.1	194.1	20	222.1	20	104
Profenofos	9.56	372.3	302.4	19	143.5	35	104
Chlorpyrifos	10.18	350.0	96.9	32	197.9	17	69
Abamectin	11.13	890.5	304.4	18	306.7	15	102
Bifenthrin	12.67	440.0	165.2	39	180.4	11	66

Table 1: Compound Transition Details

Data Processing: Software packages available; contact your VWR Sales Representative.

Figure 1: Top: dSPE tubes with 150mg $MgSO_4$ and 150mg PSA before and after cleanup of 1mL red wine extract; Bottom: Red wine extract before and after dSPE cleanup.

Results

Visual Appearance: The use of a high amount of PSA (150 mg) in dSPE cleanup was necessary for the efficient removal of organic acids, sugars, and polyphenolic pigments in red wine samples. The purified sample (**Figure 1**) is a clear colorless extract that is ready for LC-MS/MS analysis (extract can be filtered if desired).

Linearity and Limit of Quantitation (LOQ)

Matrix-matched calibration curves were prepared at concentrations of 2, 10, 40, 100, 200, and 400ng/mL. An example of a calibration curve can be found in **Figure 2**. The responses were linear over the entire concentration range with correlation coefficient (R2) ≥ 0.9963 (**Table 2**) The signal-to-noise ratio (S/N) at the lowest calibration level (2 ng/mL) was found to be ≥10 for all 24 pesticides. Therefore, the LOQ was estimated to be ≤2 ng/mL in this study.

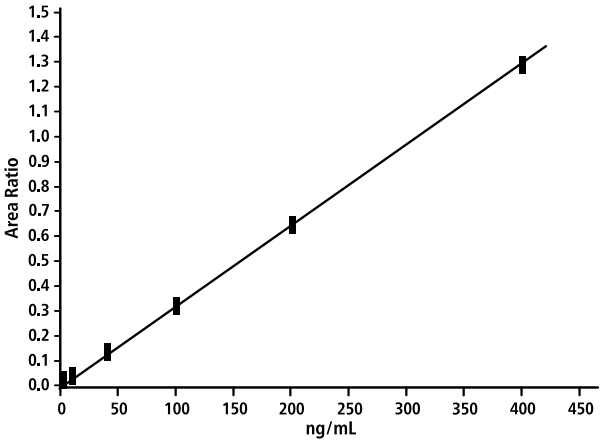


Figure 2: Simazine calibration curve

Pesticide	R^2
Methamidophos	0.9981
Pymetrozine	0.9979
Carbendazim	0.9989
Dicrotophos	0.9977
Acetachlor	0.9992
Thiabendazole	0.9966
DIMP	0.9998
Tebuthiuron	0.9996
Simazine	0.9998
Carbaryl	0.9986
Atrazine	0.9990
DEET	0.9996

Pesticide	R^2
Pyrimethanil	0.9983
Malathion	0.9997
Malathion	0.9997
Bifenazate	0.9987
Tebuconazole	0.9996
Cyprodinil	0.9995
Diazinone	0.9999
Zoxamide	0.9996
Pyrazophos	0.9997
Profenofos	0.9963
Chlorpyrifos	0.9965
Abamectin	0.9968
Bifenthrin	0.9991

Table 2: Linearity ranges and correlation coefficients (R^2)

Carryover: Blank acetonitrile was injected directly after the highest matrix-matched calibration standard (400 ng/mL) to check for sample carryover. No analyte carryover was observed.

Accuracy and Precision: Red wine made from organic grapes and determined to be free of pesticide residues was fortified with 10, 50, and 100ng/mL pesticides (n=6) and prepared according the experimental procedure described above. As outlined in **Table 3**, the majority of results (≥95%) were found to be within an acceptable recovery range of 70–120% and RSD values ≤20%, demonstrating that this method is suitable for pesticide residue analysis of red wine samples.

Pesticide	10 ng/mL (n=6)		50 ng/mL (n=6)		100 ng/mL (n=6)	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Methamidophos	78.5	6.1	84.2	2	91	11.4
Pymetrozine	64.5	5.5	61.9	2.4	63.3	12.1
Carbendazim	66.3	4.1	66.2	4.1	53.4	19.6
Dictrophos	82	2.4	80.2	1	81.4	13.6
Acetachlor	85.3	3.2	88.9	2.4	84.5	13.5
Thiabendazole	78.8	4.6	75.4	5.9	62.9	19.6
DIMP	95.8	2.9	94	4.3	91.4	13.2
Tebuthiruon	87.3	2.1	87.3	2.1	89.6	12
Simazine	97.7	2.5	99.3	2.5	92.2	11.4
Carbaryl	95.5	3.3	91.6	1.5	90	10.5
Atrazine	91	1.8	90.1	1.9	89.1	5.9
DEET	93.7	1.9	93.9	2.6	90.7	8.1
Pyrimethanil	94.2	3.1	91	2.1	82.7	13.7
Malathion	99	2.4	96.7	2.7	89.1	11.4
Bifenazate	103.3	3.4	97.5	3	84.5	11.3
Tebuconazole	95	3	94.1	3.1	93.6	8.4
Cyprodinil	98.7	2.3	96.6	2.3	90.4	5.2
Diazinone	98.5	2.5	100.1	3.5	80.2	17.6
Zoxamide	101.7	1.7	101.1	2.5	91.8	6.5
Pyrazophos	95.5	2.5	96.3	3.3	79.9	18.5
Profenofos	91.8	4.8	88.4	2.3	91.8	7.9
Chlorpyrifos	95.5	7.2	95.1	3.3	95.8	20.8
Abamectin	92.5	2.6	88.7	3.7	79.3	14.5
Bifenthrin	93.2	4.2	93.3	5.9	87.8	12.5
Overall Average	**90.6**	**3.3**	**89.7**	**2.9**	**83.2**	12.5

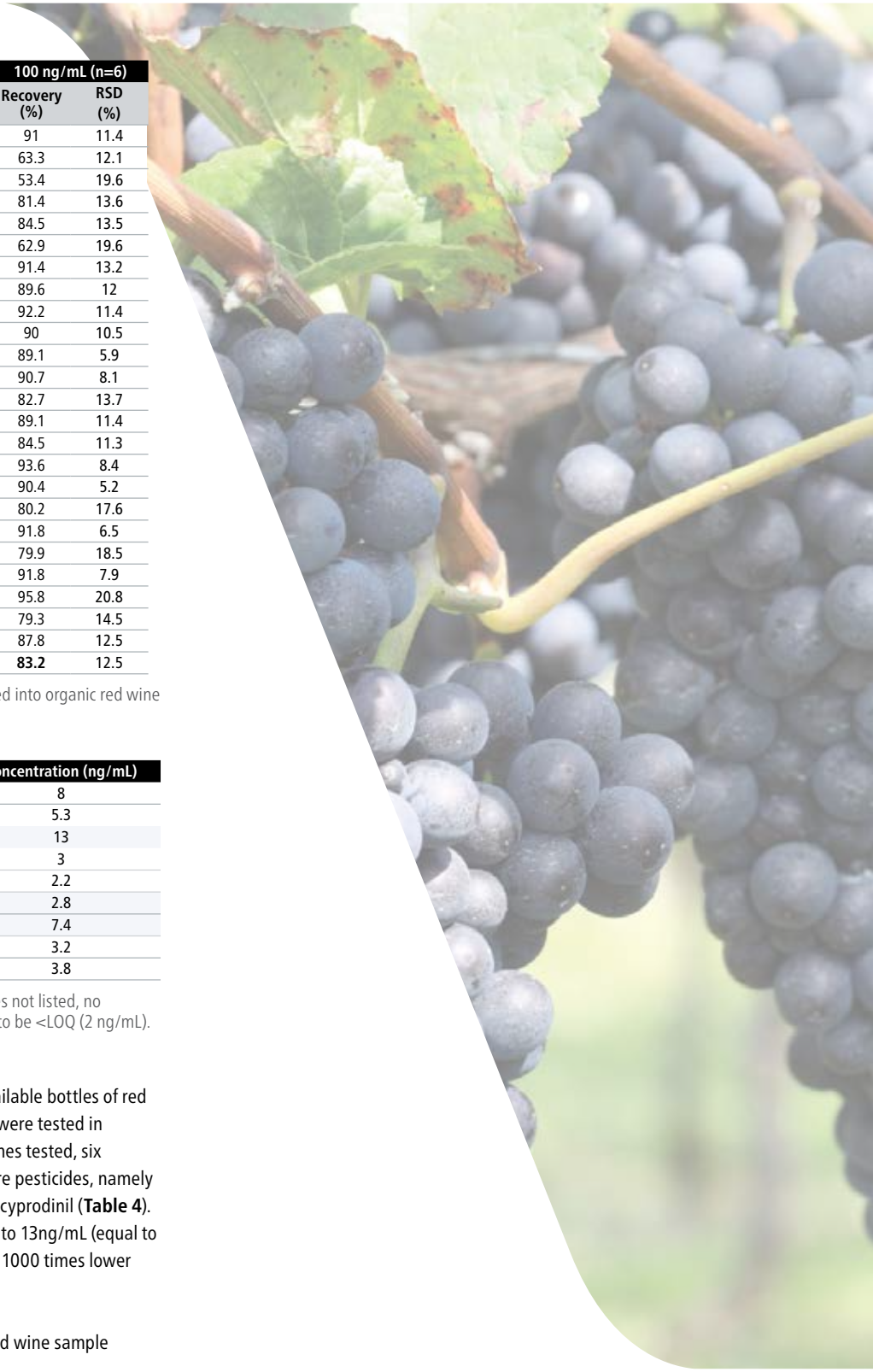
Table 3: Accuracy and precision data of the 24 pesticides fortified into organic red wine at three concentrations.

Pesticide Detected	Red Wine Sample	Concentration (ng/mL)
Carbendazim	#12	8
	#13	5.3
Pyrimethanil	#9	13
Bifenazate	#2	3
	#14	2.2
Tebuconzole	#11	2.8
	#14	7.4
Cyprodinil	#9	3.2
	#14	3.8

Table 4: Red wine samples and pesticides detected. For samples not listed, no pesticides were detected or the concentration was determined to be <LOQ (2 ng/mL).

Application to Real Samples: Fourteen commercially available bottles of red wine from various geographical regions around the world were tested in duplicate using the developed method. Of the fourteen wines tested, six samples (#2, #9, #11–14) were found to contain one or more pesticides, namely carbendazim, pyrimethanil, bifenazate, tebuconazole, and cyprodinil (**Table 4**). The concentrations of pesticides detected ranged from 2.2 to 13ng/mL (equal to 0.0022 to 0.013 mg/kg), which were approximately 100 to 1000 times lower than the MRLs set for wine grapes by the EU5.

Chromatograms: See **Figure 3** for chromatograms of a red wine sample fortified with pesticides at 50ng/mL.



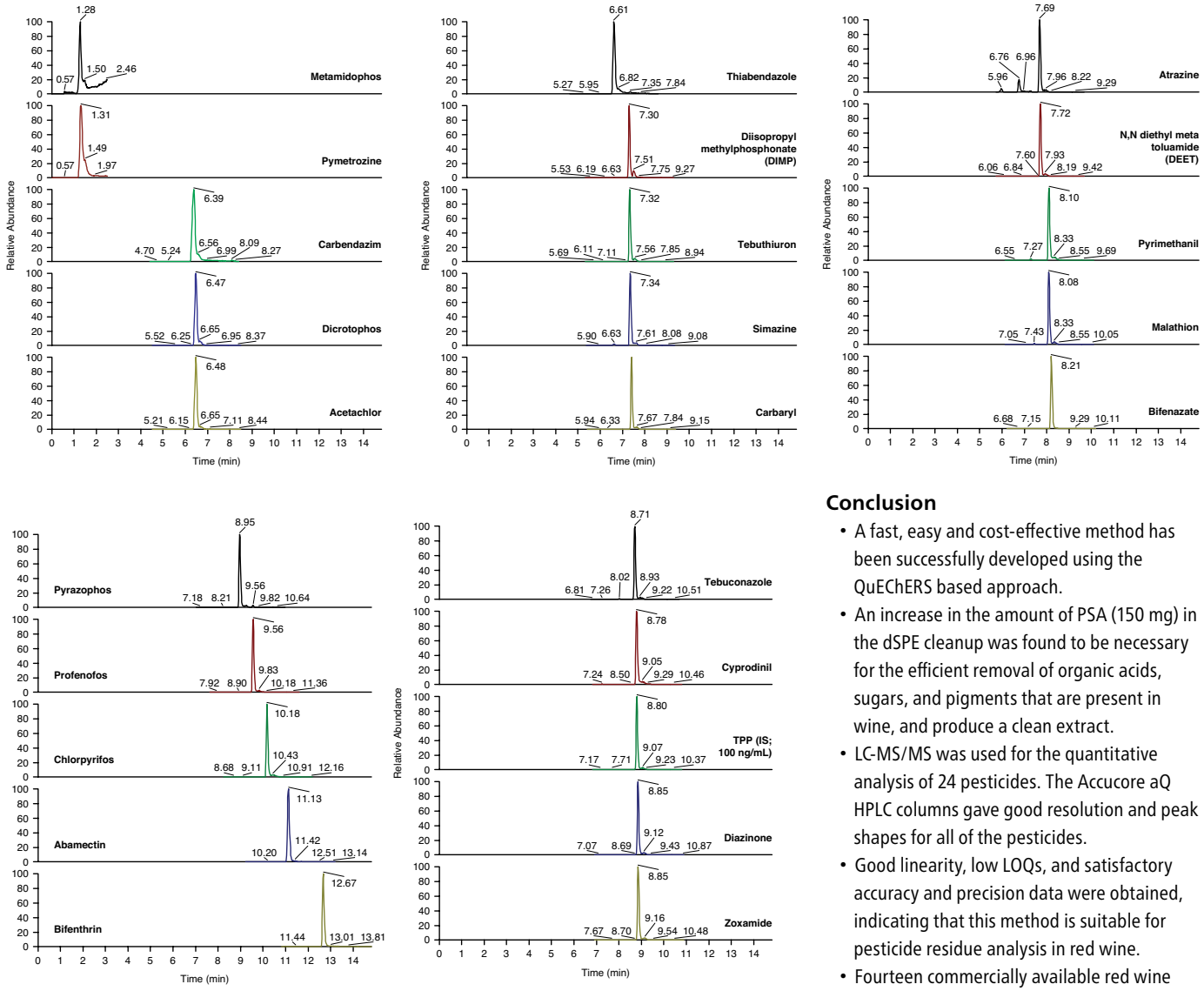


Figure 3: Chromatograms of a red wine sample spiked at 50ng/mL.

Conclusion

- A fast, easy and cost-effective method has been successfully developed using the QuEChERS based approach.
- An increase in the amount of PSA (150 mg) in the dSPE cleanup was found to be necessary for the efficient removal of organic acids, sugars, and pigments that are present in wine, and produce a clean extract.
- LC-MS/MS was used for the quantitative analysis of 24 pesticides. The Accucore aQ HPLC columns gave good resolution and peak shapes for all of the pesticides.
- Good linearity, low LOQs, and satisfactory accuracy and precision data were obtained, indicating that this method is suitable for pesticide residue analysis in red wine.
- Fourteen commercially available red wine samples were analyzed to test the applicability of the method. Six samples were found to contain one or more pesticides but at concentrations (0.0022–0.013 mg/kg) far below the MRLs in wine grapes set by EU.

References

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[5] M. Anastassiades, S.J. Lehotay, D. Stajnbaher and F.J. Schenck, J. AOAC Int. 2003, 86(2), 412-431.