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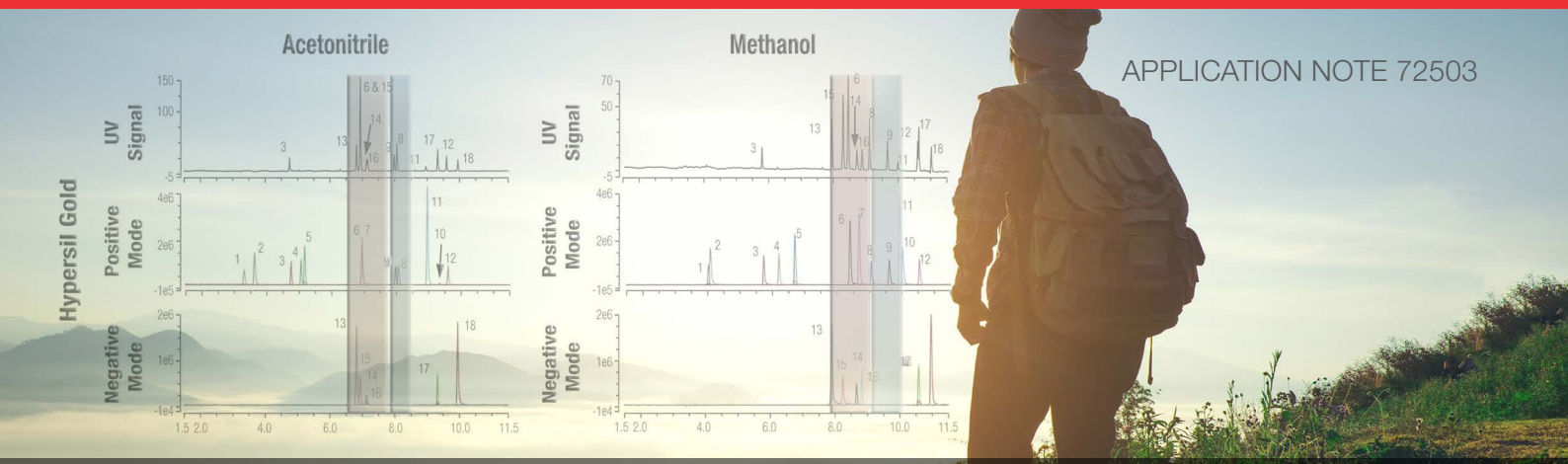

APPLICATION NOTE 72503

Accelerate method scouting with the Thermo Scientific ISQ EC single quadrupole mass spectrometer and the Thermo Scientific Vanquish Flex Quaternary UHPLC

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Keywords
Single quadrupole mass spectrometer, UHPLC, Method scouting, Method development, Custom variables

Goal

Demonstrate that mass spectrometric detection based method scouting reduces method development time compared to UV detection based approaches.

Introduction

LC method development is needed to provide an optimal method with sufficient peak separation and a short run time. Developing a separation method is often time-consuming and thus limits productivity. After defining method requirements, method scouting is usually the first step in the development process of an HPLC method. For method scouting, different column chemistries, solvents, mobile phase additives, temperatures, pH values, or gradients are evaluated to determine which set of parameters results in a method meeting the given requirements. Previously, we have demonstrated how this process can be simplified through hardware and software improvements.[1-3] Currently, the most common approach relies on UV detection for peak assignment during the method development

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process. The analytes are identified based on their UV spectra. Therefore, confident identification relies on several prerequisites, i.e. the analyte spectra need to be known and need to be sufficiently distinctive to ensure identification even during co-elution. If these prerequisites are not met, analyte standards must be measured separately, which makes method scouting even more time consuming.

This challenge can be overcome by adding mass spectrometric detection to the workflow. In addition to the analyte separation provided by the LC, mass spectrometry adds unique information for analyte identification. For small molecules, each analyte can be identified by its unique molecular mass, isotopic pattern, and – if needed – fragmentation pattern. Consequently, all analytes can be simultaneously analyzed and selectively visualized even during co-elutions. Since every peak is tracked, optimal method parameters can easily be determined. Thus, the additional selectivity provided by mass spectrometry significantly reduces the number of runs required for successful method scouting.

A reason for the limited usage of mass spectrometric detection for method scouting is that it is considered to be complex and difficult to use. The Thermo Scientific™ ISQ™ EC single quadrupole mass spectrometer (ISQ EC MS) was developed for operation by chromatographers. Its full integration into the Thermo Scientific™ Chromeleon™ 7.2 chromatography data system (CDS) and the Thermo Scientific™ AutoSpray™ smart method setup make LC-MS operation and data analysis straightforward and intuitive. The ISQ EC MS can operate in Full Scan and Single Ion Monitoring (SIM) mode, to either scan a mass range for detectable analytes or to be selective for specific compounds. It can run at scan rates suitable for fast UHPLC applications while delivering picogram detection limits. The new orthogonal source design provides high levels of instrument robustness, even with challenging matrices.

In the current work, the benefits of using the ISQ EC MS for method scouting are presented. A sample consisting of 18 pesticides was used to showcase that an MS-based workflow can be implemented in a straightforward manner. Four different column chemistries and two different organic eluents were tested. UV detection was included into the setup because combining MS and UV detection facilitates subsequent method transfer to UV-only based setups.

Experimental

Thermo Scientific™ QC pesticide standard mix (Table 1, 100 µg/mL for each pesticide) was used in the analysis.

Chromatographic separation was performed using LC-MS grade solvents (Table 2) on a Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system (Table 3). The system was set up using the Automated Method Scouting Kit for Vanquish (P/N 6036.2807) with one column compartment and two 6-position 7-port column switching valves. The fluidic setup is described in a previous publication.[3] A 75 cm long MP35N capillary with 100 µm inner diameter (P/N 6042.2390) was used for connecting the UHPLC system to the ISQ EC MS. In the presented work, a mix of 18 pesticides was analyzed with four different column chemistries (Table 4) and two different organic eluents.

The ISQ EC MS is fully integrated into Chromeleon 7.2 CDS, which was used for system operation and subsequent data analysis. This allowed easy sequence setup using custom variables within the sequence to select the different columns.

Chromatographic conditions and MS acquisition settings are listed in Tables 5 and 6.

Table 1. Overview of analytes ordered according to QC pesticide standard mix specification sheet.

#	Analyte	CAS	Chemical Formula	Molecular Weight	Monoisotopic Mass [M]	Detected *m/z* Green: $[M+H]^+$ Red: $[M-H]^-$
1	Omethoate	001113-02-6	$C_5H_{12}NO_4PS$	213.19	213.02	214.03
2	Propamocarb	024579-73-5	$C_9H_{20}N_2O_2$	188.27	188.15	189.16
3	Dicrotophos	000141-66-2	$C_8H_{16}NO_5P$	237.19	237.08	238.09
4	Vamidothion	002275-23-2	$C_8H_{18}NO_4PS_2$	287.34	287.04	288.05
5	Schradan	000152-16-9	$C_8H_{24}N_4O_3P_2$	286.25	286.13	287.14
6	Atrazine	001912-24-9	$C_8H_{14}ClN_5$	215.69	215.09	216.10
7	Cycluron	002163-69-1	$C_{11}H_{22}N_2O$	198.31	198.17	199.18
8	Azoxystrobin	131860-33-8	$C_{22}H_{17}N_3O_5$	403.39	403.12	404.13
9	Spirotetramat	203313-25-1	$C_{21}H_{27}NO_5$	373.45	373.19	374.20
10	Sulfotep	003689-24-5	$C_8H_{20}O_5P_2S_2$	322.32	322.02	323.03
11	Pirimiphos-methyl	029232-93-7	$C_{11}H_{20}N_3O_3PS$	305.33	305.10	306.11
12	Trifloxystrobin	141517-21-7	$C_{20}H_{19}F_3N_2O_4$	408.37	408.13	409.14
13	Bentazone	025057-89-0	$C_{10}H_{12}N_2O_3S$	240.28	240.06	239.05
14	2,4-D	000094-75-7	$C_8H_6Cl_2O_3$	221.04	219.97	218.96
15	Bromoxynil	001689-84-5	$C_7H_3Br_2NO$	276.92	274.86	273.85
16	MCPA	000094-74-6	$C_9H_9ClO_3$	200.62	200.02	199.01
17	Hexaflumuron	086479-06-3	$C_{16}H_8Cl_2F_6N_2O_3$	461.14	459.98	458.97
18	Fluazinam	079622-59-6	$C_{13}H_4Cl_2F_6N_4O_4$	465.09	463.95	462.94

Table 2. Solvents and additives.

Reagent	Grade	Supplier	Part Number
Acetonitrile	Optima™ LC-MS	Fisher Chemical™	A955-212
Formic acid	Optima™ LC-MS	Fisher Chemical™	A117-50
Methanol	Optima™ LC-MS	Fisher Chemical™	A456-212
Water	Ultra-Pure, 18.2 MΩ at 25°C	Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System	

Table 3. Vanquish Flex Quaternary UHPLC system modules.

Module	Part Number
Vanquish System Base F	VF-S01-A
Vanquish Quaternary Pump F (with 200 µL mixer)	VF-P20-A (6044.5110 and 6044.5026)
Vanquish Split Sampler FT	VF-A10-A
Vanquish Column Compartment H (6-position 7-port Column Switching Valves)	VH-C10-A (6036.1570)
Vanquish Variable Wavelength Detector F (2.5 µL SST flow cell)	VF-D40-A (6074.0360)

Table 4. Columns used for method scouting.

Stationary Phase	Particle Size	Dimension	Part Number
Thermo Scientific™ Accucore™ aQ	2.6 µm	2.1 × 100 mm	17326-102130
Thermo Scientific™ Hypersil GOLD™	1.9 µm	2.1 × 100 mm	25002-102130
Thermo Scientific™ Accucore™ RP-MS	2.6 µm	2.1 × 100 mm	17626-102130
Thermo Scientific™ Accucore™ Phenyl Hexyl	2.6 µm	2.1 × 100 mm	17326-102130

Table 5. UHPLC conditions.

Parameter	Value
Mobile Phase	A: Water with 0.1% formic acid B: Acetonitrile with 0.1% formic acid C: Methanol with 0.1% formic acid
Gradient	0–0.5 min: 2% B or C 0.5–10 min: 2–98% B or C 10–15 min: 2% B or C
Flow Rate	0.4 mL/min
Column Temperature	40°C, forced air mode Passive pre-heater, 40°C
Injection Volume	0.1 µL
UV detection	220 nm, 100 Hz

Table 6. MS conditions.

Parameter	Value
Source Settings	Default (easy mode)
Full Scan Positive Mode	
Time	0–15 min
Mass range	170–500 *m/z*
Dwell Time	0.2 s
Source CID voltage	20 V
Full Scan Negative Mode	
Time	0–15 min
Mass Range	190–500 *m/z*
Dwell Time	0.2 s
Source CID Voltage	20 V

Results and discussion

Utilizing MS detection in a method scouting workflow will result in more information about the sample. Analytes usually have a preferred ionization state, mostly either as protonated cations $[M+H]^+$ or as deprotonated anions $[M-H]^-$. Since a generically applicable method scouting workflow was desired, MS acquisition was performed in Full Scan mode with positive and negative polarity switching during the run. Polarity switching times were only 25 ms. UV data (220 nm wavelength) was acquired in addition to the MS data. Peak traces for the individual analytes were generated by extracting the respective mass-to-charge ratios (*m/z*) listed in Table 1 out of the Full Scan MS data. These extracted ion chromatograms (EICs) were subsequently used for calculating chromatographic parameters, such as retention time, peak width or resolution.

Using MS detection, all pesticides could be unambiguously detected using default settings. Twelve of them were detected in positive mode and six of them in negative mode (Figure 1). The observed masses deviated from the theoretical masses by 0.1 Da or less for all analytes (data not shown). UV detection revealed only ten peaks indicating co-elution or UV transparency at 220 nm wavelength for several compounds (Figure 1). By aligning the UV chromatogram with the EICs, the peaks in the UV chromatogram could be assigned. This revealed that six pesticides (omethoate, propamocarb, vamidothion, schradan, cycluron, and sulfotep) were not detectable at 220 nm in UV. The other twelve pesticides were detected with three of them co-eluting (atrazine, 2,4-D, and bromoxynil). The observation highlighted the clear benefit of MS data over UV data. The added selectivity of the mass information resulted in straightforward identification and full resolution of all analytes, even co-eluting ones. While UV data provides less information than MS data, combining UV and MS detection during method scouting is beneficial for the consecutive steps in the method development process. If an analyst wants to implement a simpler method using only UV-detection, the feasibility and the testing parameter will already be known based on the results of the method scouting.

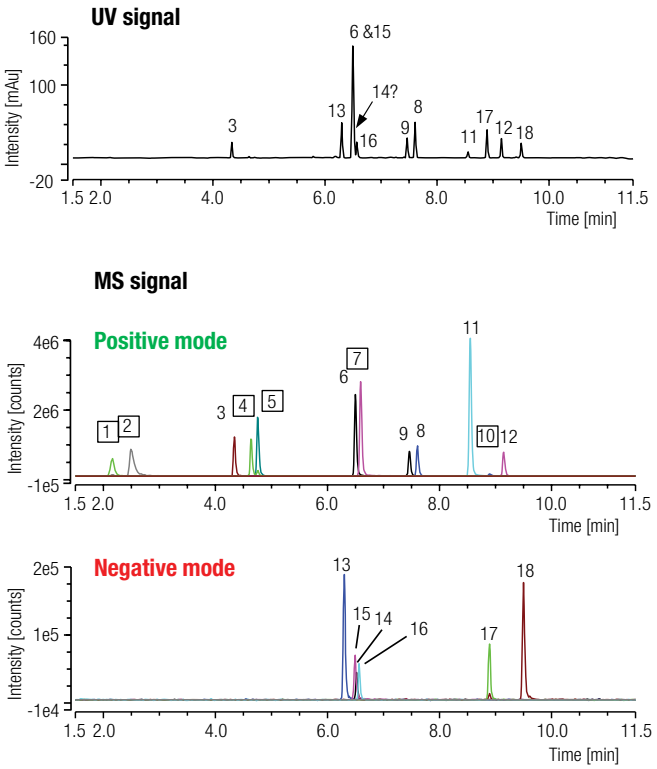

Figure 1. UV chromatogram and MS chromatograms of 18 pesticide analysis. The UV chromatogram is presented at the top. The MS chromatograms in positive mode and negative mode below. For both modes, EICs are shown. The analytes are numbered according to Table 1. Pesticides not detected by UV are labeled with framed numbers. For the presented data, an Accucore aQ column and acetonitrile as mobile phase were used.

So far, only the *m/z* ratio derived from the monoisotopic mass was used for analyte confirmation. More confidence in analyte identification can be achieved by comparing the measured isotopic pattern to the theoretical isotopic pattern. With Chromeleon 7.2 CDS, mass spectra can be generated automatically for the peak apex, front, or tail of the EICs, allowing a fast review of the isotopic pattern of the analyte. Several pesticides contained elements with very distinctive isotopic patterns (Table 7). For all pesticides, the theoretical patterns could be well

Table 7. Isotope distribution of selected, exemplary elements.

Element	Atomic Weight of Isotopes [u]	Relative Abundance [%]
^{12}C	12.00000	98.93
^{13}C	13.00335	1.07
^{35}Cl	34.96885	75.78
^{37}Cl	36.96590	24.22
^{79}Br	78.91834	50.69
^{81}Br	80.91629	49.31

matched to the measured ones, which confirmed their correct identification (data not shown). Two co-eluting analytes containing either two (2,4-D) chlorine atoms or two (bromoxylin) bromine atoms are shown (Figure 2). Based on the measured isotopic patterns, these two co-eluting analytes could be discriminated and each of them confidently identified.

For method scouting, we tested four column chemistries and two different organic eluents. Looking at the chromatograms, the differences in selectivity between the different columns and the effect of the organic eluents on the selectivity were clearly visible (Figure 3). This is in accordance with the reported chemical properties of these stationary phases.[4] Methanol generally resulted in better peak resolution than acetonitrile for all column chemistries, and elution patterns reversed when changing between organic eluents for some analytes, e.g. for azoxystrobin (#8) and spirotetramat (#9) (Figure 3, blue boxes). Several peaks were closely eluting and some of them overlapped, for example atrazine (#6), cycluron (#7), bentazone (#13), 2,4-D (#14), bromoxynil (#15), and MCPA (#16) (Figure 3, red boxes). These analytes could not have been separated by using only UV detection. With MS detection, all analytes were detected and the resolution between each of them could be accurately calculated (Table 8).

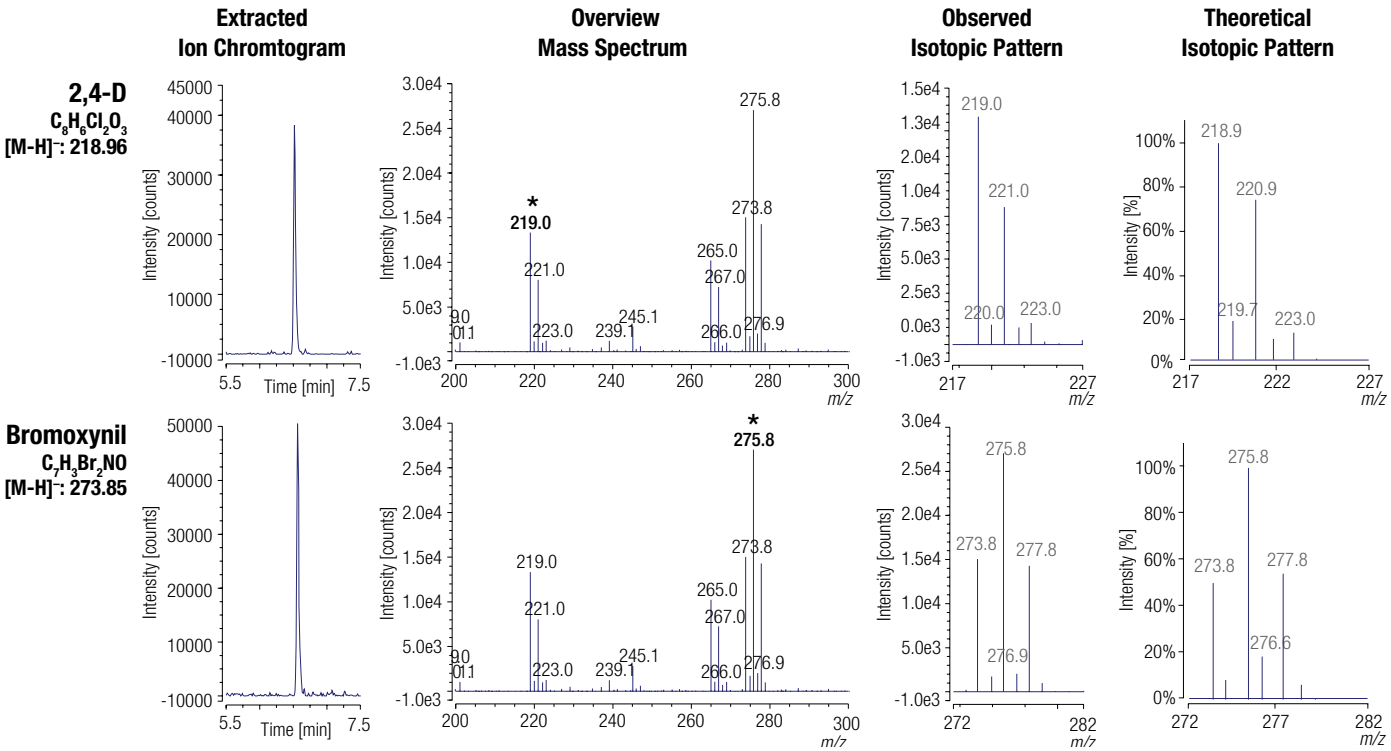

Figure 2. Confident analyte identification based on the isotopic patterns of the co-eluting analytes 2,4-D and bromoxylin. The EIC is depicted on the left, followed by the mass spectra, the zoom-in to the measured isotopic pattern of the analyte, and the theoretical isotopic distribution on the right.

In a real-life method scouting experiment, chromatographic requirements would have been defined beforehand. For instance, if atrazine (#6) and 2,4-D (#14) should be separated with a resolution of at least 1.5, then Hypersil GOLD and Accucore Phenyl Hexyl stationary phases in combination with acetonitrile, or Hypersil GOLD and Accucore RP-MS stationary phases in combination with methanol as organic solvent would be suitable (Table 8). If 2,4-D (#14) and MCPA (#16) need to be resolved with a resolution of at least 1.5, all tested columns in combination with methanol would be suitable (Table 8). Please note that parameters affecting stationary phase selectivity, such as column temperature or pH, were not tested to keep the application example concise.

Taken together, MS-based method scouting allows for fast and straightforward evaluation of separation parameters, e.g. resolution, since it utilizes the additional selectivity of mass spectrometric detection to confidently identify analytes and fully resolve them even when they are co-eluting. This simplifies and accelerates the scouting process. Single analyte samples are no longer required but standards containing all analytes can be used.

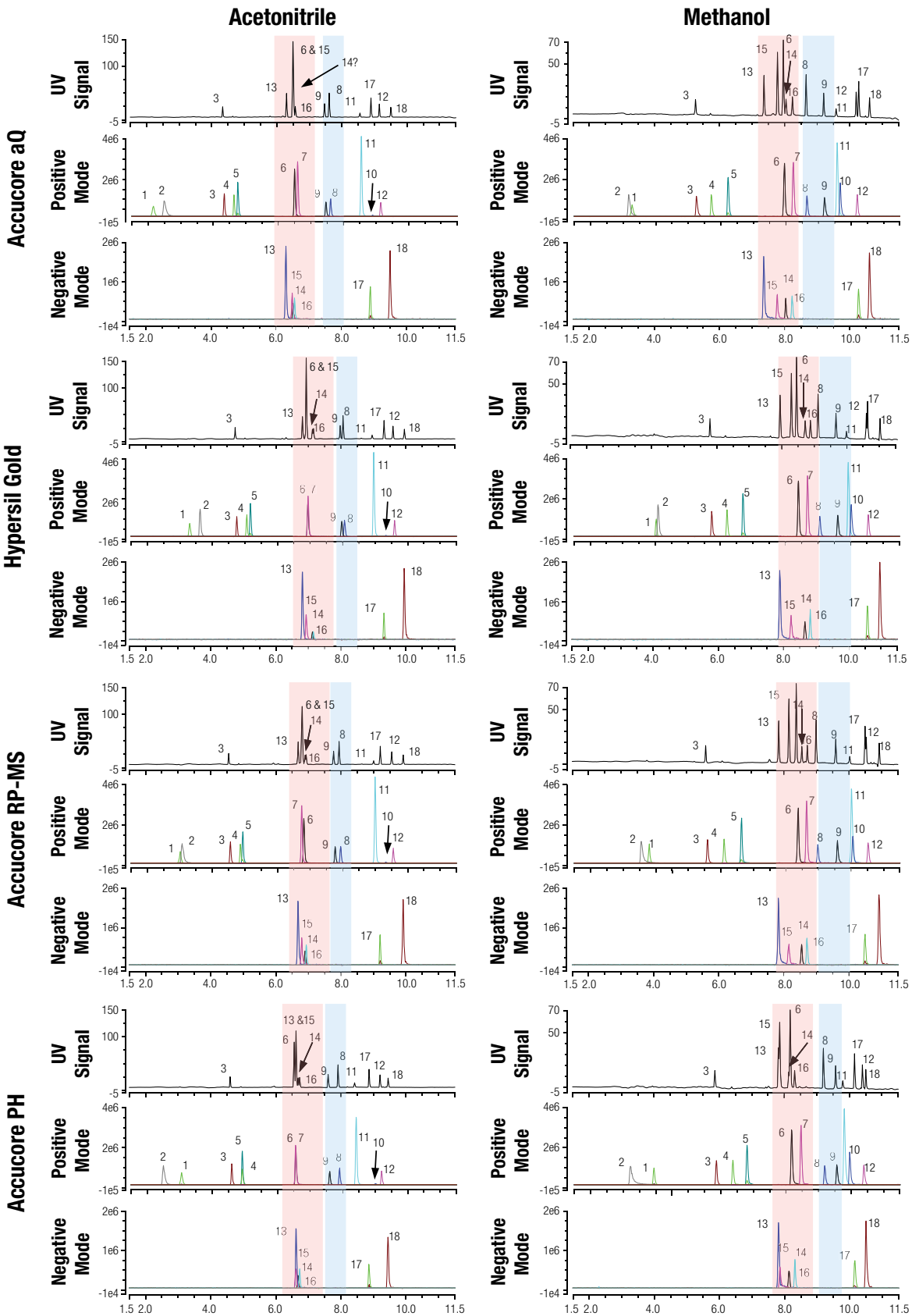

Figure 3. Overview of selectivity between different columns and organic eluents. Top to bottom: UV chromatograms, positive and negative mode EICs for four different column chemistries. Left: Acetonitrile as organic eluent. Right: Methanol as organic eluent. Peaks are numbered indicating the respective pesticides (according to Table 1). X-axis: Retention time in minutes. Y-axis: Signal intensity in mAU (UV) or counts (MS). Compounds exemplifying the changing of elution order depending on organic solvent (#8, 9) are highlighted in blue. Compounds exemplifying co-elution (#6, 7, 13-16) are highlighted in red.

Table 8. Peak resolution of atrazine, cycluron, bentazone, 2,4-D, bromoxynil, and MCPA under tested conditions. Peak resolution is calculated according to European Pharmacopeia (EP). Negative values indicate that analytes eluted in inverted order to their numbering in Table 1. Resolutions above 1.5 are highlighted. Resolutions used for example comparisons are in bold.

Column Chemistry	Analyte	Acetonitrile					Methanol				
		cycluron	bentazone	2,4-D	bromoxynil	MCPA	cycluron	bentazone	2,4-D	bromoxynil	MCPA
Accucore aQ	Atrazine	-1.28	2.87	**-0.32**	0.06	-1.05	-3.09	7.21	**-1.01**	2.40	-3.30
	Cycluron		3.99	1.13	1.43	0.41		10.12	2.61	5.75	0.05
	Bentazone			-3.54	-3.02	-4.21			-9.45	-5.62	-10.90
	2,4-D				0.41	**-0.85**				4.00	**-2.80**
	Bromoxynil					-1.21					-6.22
	MCPA										
Hypersil Gold	Atrazine	-0.20	1.44	**-2.84**	-0.15	-3.29	-3.18	5.90	**-3.27**	1.77	-5.64
	Cycluron		1.67	-2.63	0.05	-3.10		9.00	0.14	4.80	-1.94
	Bentazone			-4.72	-1.64	-5.16			-9.53	-3.90	-12.35
	2,4-D				2.74	**-0.61**				5.01	**-2.27**
	Bromoxynil					-3.21					-7.38
	MCPA										
Accucore RP-MS	Atrazine	0.82	1.66	**-1.21**	0.16	-1.81	-2.94	6.73	**-2.06**	2.37	-4.08
	Cycluron		0.87	-2.08	-0.71	-2.73		9.55	1.06	4.96	-0.88
	Bentazone			-2.93	-1.60	-3.60			-9.16	-3.61	-11.27
	2,4-D				1.45	**-0.57**				4.31	**-2.10**
	Bromoxynil					-2.10					-6.12
	MCPA										
Accucore PH	Atrazine	0.10	-0.80	**-1.66**	-0.72	-2.47	-3.38	4.23	**0.49**	3.90	-1.83
	Cycluron		-0.90	-1.75	-0.81	-2.56		7.85	3.97	7.64	1.98
	Bentazone			-0.89	0.00	-1.72			-3.82	-0.49	-6.75
	2,4-D				0.80	**-0.81**				3.47	**-2.44**
	Bromoxynil					-1.54					-6.47
	MCPA										

Conclusion

Mass spectrometry based method scouting with the ISQ EC single quadrupole mass spectrometer and the Vanquish Flex Quaternary UHPLC provides several benefits over traditional UV based approaches:

- Straightforward analyte identification and tracking reduces the risk of overlooking co-eluting analytes or incorrectly assigning peaks between different scouting runs.
- Analyzing all analytes in every scouting run drastically reduces method development times.
- UV and MS detection alignment facilitates transfer to UV-only methods.

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