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Abstract

In this work, an innovative method is described for multi-residue pesticide analysis by liquid chromatography coupled to targeted mass spectrometry. Called “Scout-MRM, this new acquisition mode lies in the monitoring of complex transitions groups triggered by either endogenous or spiked Scout compounds, hence fully releasing the monitoring of target molecules from time scheduling. As a proof of concept, a Scout-MRM method was built where 5 transitions groups tracking a total of 191 pesticides where successively triggered under the control of 5 spiked-in deuterated pesticides. As expected from its retention time independency, Scout-MRM demonstrates strong detection robustness towards modifications of gradient parameters, as well as easy method transfer between distinct analytical platforms with nearly 100 % recovery after a single run. Finally, Scout-MRM was used for the multi-residue screening and quantification of pesticides in real surface water samples, by applying an external calibration procedure and comparing it with classical scheduled reaction monitoring methods.

Keywords: MRM : Multiple Reaction Monitoring ; sMRM Scheduled Multiple Reaction Monitoring, scout-MRM, retention time independency, multiplexing capability

1. Introduction

In the framework of European environmental legislation, various protocols have been developed for assessing the environmental risk of chemicals. For example, pesticides and biocides undergo rigorous assessment in compliance with Directives 91/414/EEC and 98/8/EC. There are more than 1,000 pesticides currently available for use while the number of regulations aimed at protecting human health is increasing. Pesticide testing has become a prerequisite. With hundreds or even thousands of samples to be screened within tight deadlines and according to strict legislation, an efficient solution is required to detect and identify pesticide compounds across multiple classes. Thus, environmental analysis laboratories develop analytical multiplexed methods, known as "multi-residue methods", that allow the simultaneous identification and quantification of many organic contaminants. Due to its sensitivity and specificity, liquid chromatography coupled to mass spectrometry is an appropriate method for detecting organic contaminants and has been widely used for the analysis of pesticide residues [1,2]. Among the different mass spectrometry techniques available, targeted approaches with triple quadrupole analysers in selected reaction monitoring (MRM or MRM) mode are widely used. For each pesticide, 2 or 3 MRM transitions are monitored to reduce the risk of false positives [3] (a transition corresponds to a precursor/fragment ion couple): one transition for quantification and two transitions for qualification. Thus, for example, the analysis of 300 compounds requires monitoring from 600 [4] to 900 transitions. As the number of transitions in a method increases, it is necessary to either reduce the dwell times or to increase the cycle time for each scan. It should be remembered that the longer the mass spectrometer spends following a transition, the higher the signal-to-noise ratio. Slow scanning of the triple quadrupole limits the development of highly multiplexed methods. Indeed, to guarantee enough data points per peak (e.g., 15), it is not possible to increase the overall cycle time which is the sum of each dwell time. Thus, instead of programming the recording of a transition throughout the duration of the chromatographic separation, it is monitored only within a time-limited window. This time segmentation can be relatively large and allows monitoring the transitions of several different molecules. However, the strategy most used is to record the specific transitions for a compound only within a window around its retention time. The manufacturers of mass spectrometers have developed their own processes, bearing different names: "Scheduled Multiple Reaction Monitoring" for Sciex (sMRM); [1] "Timed MRM" for Thermo [5]; "Dynamic MRM" for Agilent [6,7]. Whatever the time segment methods used dynamically or not, a retention time must be documented for each pesticide in the method. The major drawback of this procedure is that any unattended shift of retention time may partially or, even worse, totally exclude a compound of interest from its monitoring window, resulting in either a truncated or non-detected peak. [8]. Truncated chromatographic peaks make integration more difficult and the data obtained are of low-quality. Thus, during large analytical campaigns or the transfer of analytical method, the smallest variations in analytical conditions, such as (i) the use of different LC configurations with different solvent delays (e.g., of chromatographic chains), and (ii) differences in applied flow rates, imply readjustments of retention times.

As a consequence, a new mode of multiplexed targeted analysis, called “Scout-MRM”, in the context of multi pesticide residue monitoring has been reported. This method, which

does not require retention time setting, solves the limitation affecting large multi pesticide assays regarding chromatography reproducibility and offers plug and play implementation between laboratories.

2. Materials and Methods

2.1 Chemicals and reagents

All the reagents were of analytical grade. Water, methanol and acetonitrile (LC-Ms grade) were purchased from Fisher Scientific (Strasbourg, France). Acetone, formic acid (FA) and formate ammonium (LC–MS grade) was obtained from Sigma–Aldrich (St Quentin-Fallavier, France).

Over 191 pesticides of different classes were used to develop the Scout-MRM method. All the substances were certified for internal calibration or as external calibration reference materials and were provided by Sciex (Toronto, Canada), in its iDQuant™ Standards Kit for Pesticide Analysis. As scout standard, five deuteurated pesticides ([D_4]-Cyromazine, [D_6]-Fenitrotion, [D_6]-Linuron [D_5]-Simazine and [D_9]-Tebuconazole) were purchased from Dr. Ehrenstorfer (Augsburg, Germany) and Sigma Aldrich (St Quentin-Fallavier, France). These pesticides were then formulated by Anaquant (Lyon-France) and coated on READYBEADS™. This technology is used to obtain the fast and reproducible preparation of standard solutions and, in particular, it avoids storage, weighing and stability issues.

To perform the comparison between Scout-MRM and sMRM reference method, a mixture of 17 pesticides in acetone was used to establish the calibration curves (acetochlore, atrazine, azoxystrobin, chlorpyriphos methyl, chlortoluron, 3.4-dichloroaniline, 3-(3,4-dichlorophenyl)-1-methylurea (DCPMU), diflufenicanil, diuron, isoproturon, linuron, metolachlore, norflurazon, norflurazon desmethyl, procymidone, simazine, tebuconazole). Depending on the pesticide, concentrations of stock solutions ranged from 200 to 8000 ng/mL. Nine calibration standard mixtures were prepared following initial 1/100 dilution in water containing 10% acetonitrile and serial dilutions in water (See supplementary table 3) External calibration was used. The Low Limit of Quantitation (LLOQ) ranged from 0.002 to 0.8 ng/mL. A coated READYBEADS™ was placed in each standard vial during the dilution process and the final volume of the solvent was 1 mL.

2.2 Sample extraction

Ten water samples (named real sample) were collected from rivers (3 up or downstream massif Central site, Beaujolais Veneyard downstream), stored at 4°C and filtered within 24 hours with 0.7 µm GF/F glass fibre membranes (Millipore). Pesticide concentrations in water samples were determined after solid phase extraction (SPE) followed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The SPE extraction was carried out with 6 mL/200 mg Oasis HLB cartridges (Waters) conditioned with 3 mL of methanol, 3 mL of acetonitrile and 3 mL of ultrapure water. 250 mL of filtered water was extracted at a flowrate of 10mL/min. Elution was performed with 6 mL of acetonitrile at a flowrate of 1 mL/min. Organic extract was gently evaporated under nitrogen and finally diluted in 250 µL of water/acetonitrile (80/20, v/v) containing deutered diuron D_6, used as

the injection internal standard (IS). The concentration factor was 1000. Each sample was split into two vials. The first vial was used for pesticide determination with the sMRM reference method and the second was diluted 10-fold with ultra-pure water and a one scouts standard READYBEADS™ was added in each vial before analysis with Scout-MRM method.

2.3 LC-MS/MS analysis

2.3.1 LC-MS/MS in University of Lyon

The Scout-MRM method was performed on a 1290 HPLC system (Agilent Technologies) coupled with a QTRAP 6500® from SCIEX (Concord, Canada) equipped with a Turbo V™ source (SCIEX). Instrument control, data acquisition and processing were performed using modified Analyst 1.6.2 software®. Implementing the "Scout-MRM" tool in the Analyst® 1.6.2 software required computer developments by David Cox at the Sciex R&D laboratory. Chromatographic separations were performed on a 3.5µm BEH C_{18}, 100 x2.1mm (Waters, Milford, MA, USA), at ambient temperature. The flow rate was to set at 300 µl/min and an elution gradient was used. The mobile phase consisted of (A) water containing 0.05 % formic acid and 2 mM ammonium formate and methanol containing 0.05% formic acid, 2mM formate ammonium. The gradient started with 10% of B. This was held for the first 2 min. Then, the amount of solvent B was increased to 70% within 15 min. Afterwards, solvent B was increased again to 100% within 0.1 min. This was held for 3 min. Afterwards solvent B was decreased to 100% within 0.1 min and held for 5 min. before the next injection. The sample injection volume was 1 µL and the run time of the assay was 25 min. MS analysis was carried out in positive ionization mode using an ion spray voltage of 5500 V. The curtain gas (nitrogen) and the nebulizer (nitrogen) flows were set at 50 and 70 psi respectively. The Turbo V™ ion source was set at 550 ◦C with the auxiliary gas flow (nitrogen) set at 60 psi.

2.3.2. LC-MS/MS in Toronto (Sciex)

The Scout-MRM method was performed on an HPLC Model- LC 20 AT Prominence, (Shimadzu, Japan) coupled with a QTRAP 5500® from SCIEX (Concord, Canada) equipped with a Turbo V™ source (SCIEX). Instrument control, data acquisition and processing were performed using modified Analyst 1.6.2 software®. Implementing the "Scout-MRM" tool in the Analyst® 1.6.2 software required computer developments by David Cox at the Sciex R&D laboratory. Chromatographic separations were performed on 3 µm X Bridge C_{18}, 100 x 2.1mm (Waters, Milford, MA, USA), operated at 50°C. The flow rate was to set at 600 µl/min. An elution gradient was used. The mobile phase consisted of (A) water containing 0.05 % formic acid and 2 mM ammonium formate and methanol containing 0.05% formic acid, 2mM formate ammonium. The gradient started with 5% of B. This was held for the first min. Then, the amount of solvent B was increased to 95% within 25 min. This was held for 5 min. Afterwards solvent B was decreased to 100% within 1 min and held for 5 min. before the next injection. The sample injection volume was 10 µL and the run time of the assay was 36 min. MS analysis was carried out in positive ionization mode using an ion spray voltage of 5500 V. The curtain gas (nitrogen) and the nebulizer (nitrogen) flows were

set at 25 and 30 psi, respectively. The Turbo V™ ion source was set at 550°C with the auxiliary gas flow (nitrogen) set at 70 psi.

2.3.3. LC-MS/MS sMRM method in INRAE (ex IRSTEA)

Liquid chromatography was performed on a Shimadzu series Nexera X2 UHPLC system (Marne-la-Vallée, France). Pesticides were separated on an Acquity HSS T3 1.8 µm, 2.1 × 100 mm Waters column (Milford, MA, USA). The column temperature was set at 30°C +/- 0.5°C. The injection volume was 20 µL. The chromatographic separation of analytes was carried out with acetonitrile and ultrapure water, both with formic acid (0.1%) in an analytical gradient (from 0 to 90% acetonitrile) of 15 min at 0.6 mL/min. The UHPLC system was coupled to a triple-quadrupole mass spectrometer (MS/MS) API 4000 from Sciex (Concord, Canada). The electrospray ion source (Turbo-Ionspray from AB Sciex) was set to positive mode at 600°C. The ion spray voltage was +5500 V. Nitrogen was used as curtain and collision gas, while air was used as nebulizer and drying gas. The pesticides analysed were identified and confirmed by their specific retention times, two characteristic precursor-product ion transitions (quantifier and qualifier), and specific ratios of the intensities of the product ions, in compliance with European Commission Decision 2002/657/EC (European_Commission, 2002). Quantification was performed with deuterated diuron d6. Within-laboratory validation was performed to evaluate the analytical performances of the SPE-UHPLC-MS/MS protocol according to the following criteria: linearity, limit of quantification (LOQ), precision and trueness, inspired from reference standards (AFNOR, 2009; SANCO/12495/2011, 2012). Quantification limits were between 5 and 50 ng/L depending on the pesticide and recoveries ranged from 67 % to 107 %. Analytical uncertainties were determined using the within-laboratory reproducibility relative standard deviation. An expanded coverage factor of k=2 was used to calculate the expanded uncertainty with a confidence interval of 95% (AFNOR, 2003; International_Organization_for_Standardization, 2012). Uncertainties were evaluated between 30% and 60% on the whole range of the method's application.

3. Results and discussion

3.1 Principle of Scout-MRM

Scout MRM is a new targeted Multiple Reaction Monitoring mode enabling the deployment of large multiplexed assay. The major breakthrough relies on the fact that it releases the acquisition from scheduling retention windows. As already described in the context of relative peptide quantification [9,10], scout molecules are compounds dispatched along the chromatogram , which individually controls the triggering of a transition group. Hence, each transition group is framed by two scouts. Once the signal intensity of a given scout exceeds a user-defined threshold, it triggers the monitoring of a transition group while the previous one is stopped and so on (Figure 1). Two major advantages arise from this innovative process: i) the easiness of building a multiplexed assay by simply positioning the target molecules between two scouts; ii) the release of the acquisition from the retention time dimension which makes any unattended retention time

shift with no consequence An advantage of not using time segmentation is the possibility of using the real multiplexing capabilities of MRM methods to their maximum.

To illustrate the benefits of the approach, a proof of concept has been carried where a scout-MRM assay was developed targeting 191 pesticides. Five labelled pesticides were used as spiked-in scouts compounds and judiciously selected in order to dispatch the 191 pesticides targets into groups of approximately the same number of transitions. In their order of use, these scouts were: Cyromazine-D4; Simazine-D5; Linuron-D6; Fénitrothion-D6; and Tebuconazole-D9. At the beginning of the run, the mass spectrometer was scheduled to follow the most intense transition of Cyromazine-D4 (Scout 1).When the two most intense transitions of Cyromazine-D4 were detected above a user-predefined threshold, then the monitoring of all the transitions of group A was triggered (Group A, list of pesticides in supplementary Table 1). Group A is thus defined as the set of transitions tracking 42 pesticides eluted between Cyromazine-D4 (Scout 1) and Simazine-D5 (Scout 2). Once the transition signals of Simazine-D5 reach triggering defined threshold, group A monitoring is stopped while the one of Group B transitions is initiated with Linuron-D6 as third scout, and so on. This triggering process continued throughout the chromatographic elution of the pesticides with Scout 4 (Fénitrothion-D6) and 5 (Tebuconazole-D9). Since "Scout-MRM" acquisition is a succession of MRM experiments triggered only by signals of the scout whose thresholds are user-defined, pesticides transitions can be arranged in a completely random manner within a group.

The only constraint to consider during a Scout-MRM based assay is the upper limit of concurrent transitions positioned within the same group in such a way that the duty cycle compulsorily ensures the required minimum of data points per peak. If additional pesticides were to be included in the Scout-MRM test, then it would be sufficient simply to add new deuterated scout pesticides.

3.2 Performance of Scout-MRM under different chromatographic conditions

As described above, Scout-MRM approach developed here, differs from conventional methods in which a scheduled time windows or retention time are fixed. In conventional methods this implies not only detailed knowledge requirements of the expected retention time for all the pesticides monitored but also stable and reproducible liquid chromatography conditions. This is no longer an issue with this approach, as shown in Figure 2. In this case, no retention time is programmed. This presents an advantage because we no longer have to take into account any changes in retention time for pesticide detection. The isocratic step of the LC method was deliberately extended by 5 minutes or the gradient slope was decreased (Figure 2 A and B). In this case as shown, all the pesticides were detected (100%). The detection repeatability of Scout-MRM was however compared to Scheduled-MRM on the same instrument by calculating the coefficient of variation (CV) of triplicate injections. Identical ranges of CVs were obtained for both modes of acquisition (supplementary figure 1).

To assess Scout-MRM robustness, the portability of the assays between two distinct sites and experimental set-up was evaluated. Indeed, the comparison of the same analysis (set of samples) at two different laboratories has been performed. For this purpose, one Excel file was sent containing the MRM transitions and their Scout-MRM group affiliation was

sent to Sciex Research Laboratory in Toronto. While the chemistry of the C_{18} stationary phase was identical, the particle sizes, flow rate, elution gradient and temperature were different. Despite these substantial modifications, 92% of the pesticides were detected as illustrated in figure 2A. The remaining 8% corresponded to group changes probably due to a modification of chromatographic selectivity. Indeed, if the order of elution of the compounds varies to cause a peak inversion between a "Scout" and a compound of interest, the latter will not be followed at the right time and cannot be detected. A second analysis to identify undetected pesticides by positioning them in adjacent groups ensures that they are detected correctly. Peak intensities were different since electrospray ionisation [11] depending on mobile phase composition and / or comparisons were performed on different generations of mass spectrometers (API 6500 QTRAP *vs* API5500 QTRAP).

This result unequivocally demonstrate the capacity of Scout-MRM to facilitate the sharing and implementation of ready-to-use-assay following a plug-and-play philosophy.

3.3 Applications and comparison of Scout-MRM vs MRM

Having preliminarily demonstrated the detection repeatability and robustness towards radical change of chromatographic conditions of Scout-MRM mode, Scout-MRM was ultimately compared to conventional MRM and scheduled MRM towards pesticides evaluation in river water samples. The goal was here not to deliver accurate evaluation of surface water contamination but to evaluate whether different instruments and distinct modes of acquisition might provide reliable quantification results. Again, distinct experimental set-up were used during the study, in particular an older generation instrument at the INRAE lab (API 4000 triple quadrupole instrument) on which measurements were carried out routinely in MRM mode. Although only 16 pesticides frequently detected in surface waters were subjected to analysis in the INRAE study (sMRM reference method on an API4000), the method containing all pesticides was applied.

In the INRAE study only 9 out of the 17 targeted pesticides were detected and quantified. The Scout-MRM method was used for the acquisition but only the concentrations of the 9 pesticides measured by INRAE were measured precisely and reported in table 1. With our more sensitive API 6500 QTRAP, more pesticides were detected but not quantified (more than 65, see Supplementary Table 2). As shown in Table 1, the results are similar except for atrazine, whose detection was about between -28% and -55% lower. This result can be explained by the matrix effect, which was different due to sample dilution. Indeed, as API 6500 QTRAP is more sensitive, the water samples must be diluted by 10 before analysis. This was supported by the fact that the comparison between Scout-MRM and sMRM on API 6500 QTRAP gave approximatively the same concentrations for atrazine and all the other pesticides.

4. Conclusion

The present proof-of-concept study was intended to illustrate how Scout-MRM may advantageously replace conventional time-scheduled acquisition mode for implementing multiresidue pesticide assay. Scout-MRM mode has been originally conceived to fully release a highly multiplexed assay from the absolute retention time of the respective targeted compounds. Thus, 100% recovery were obtained for a 191-plex pesticide panel despite radical modifications of the chromatographic parameters between runs. Even more impressive than expected, 92% recovery of the 191-plex pesticide panel was obtained across a first run analysis after a simple mail transfer of Scout-MRM method to a distinct lab and, again, despite substantial differences in the chromatographic set-up. This supports the idea that Scout-MRM is the fit-for-purpose tool for the dissemination and implementation of ready-to-use methods, especially between different laboratories. Finally, the fact that any unattended retention shift does not translate into a false negative detection strengthens the confidence level of pesticide assays and the resulting results. It should also be noted that routine laboratories running high-throughput pesticide assay are used to introduce few internal standards in each run. This function can be here advantageously fulfilled by the deuterated scout pesticides, hence without any radical change in the procedure. The Scout-MRM provisional software patch is available for academic research on request from SCIEX (please contact yves.leblanc@sciex.com) and will be integrated automatically in the MS controlling software instruments.

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Figure caption

Figure 1 : Principe of scout-MRM acquisition mode

Figure 2: Example of 3 chromatograms in different LC conditions. Extraction of 5 pesticides of Scout-MRM group D (Flufenacet, Fenbuconazole, Picoxystrobin, Dimosystrobin and Benalaxyl). (A) LC-MS/MS analysis in Toronto with different LC conditions (different LC and MS systems; different gradient), (B) and (C) same LC-MS/MS systems with different isocratic steps. Pesticides concentration was 1 ng/mL.

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Fig. 1

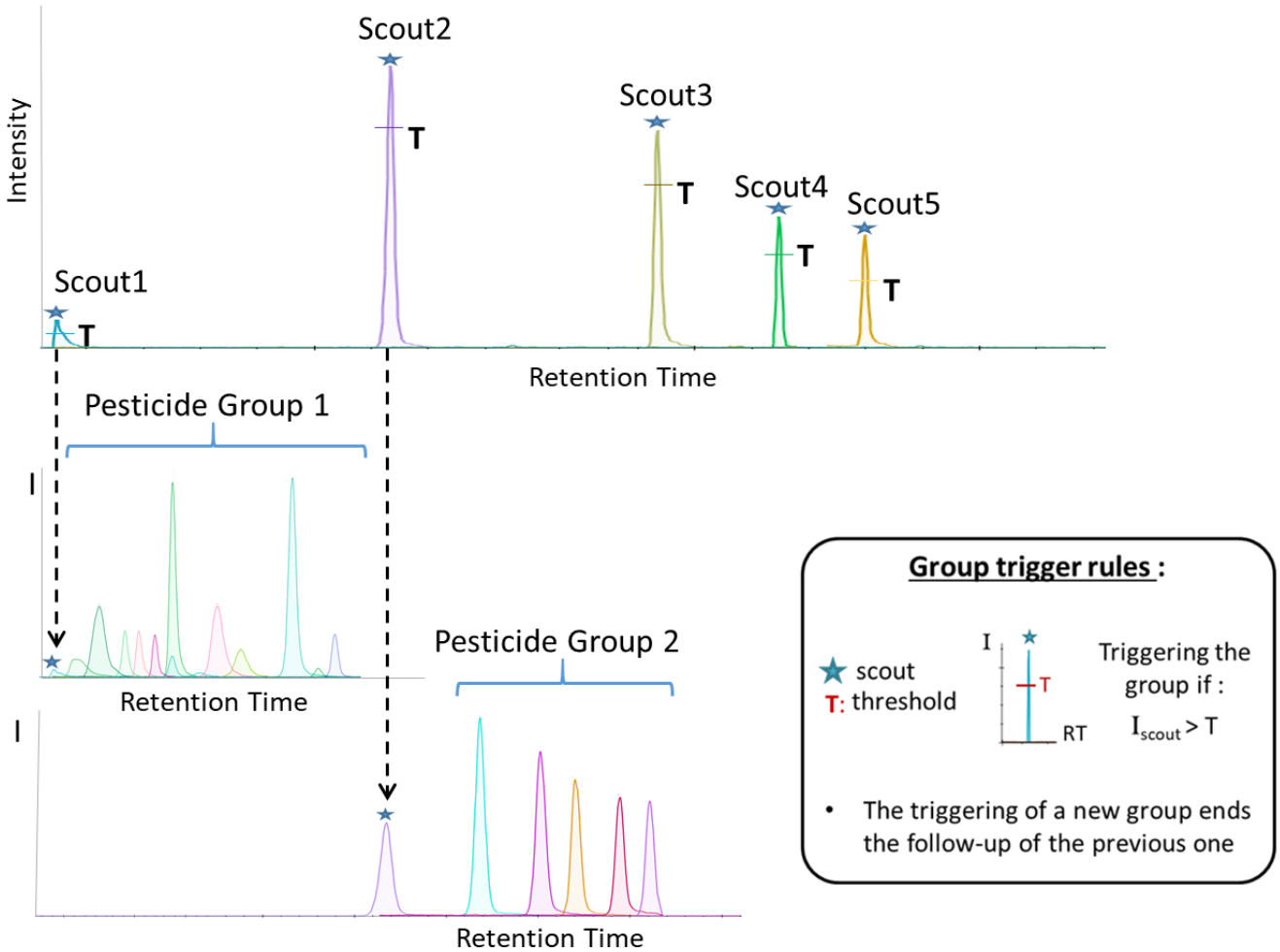
Intensity
Scout1
Scout2
Scout3
Scout4
Scout5
T
Retention Time
Pesticide Group 1
Pesticide Group 2
I
Retention Time
Group trigger rules :
scout
T: threshold
I
T
RT
Triggering the group if :
$I_{scout} > T$
The triggering of a new group ends the follow-up of the previous one

Fig. 2

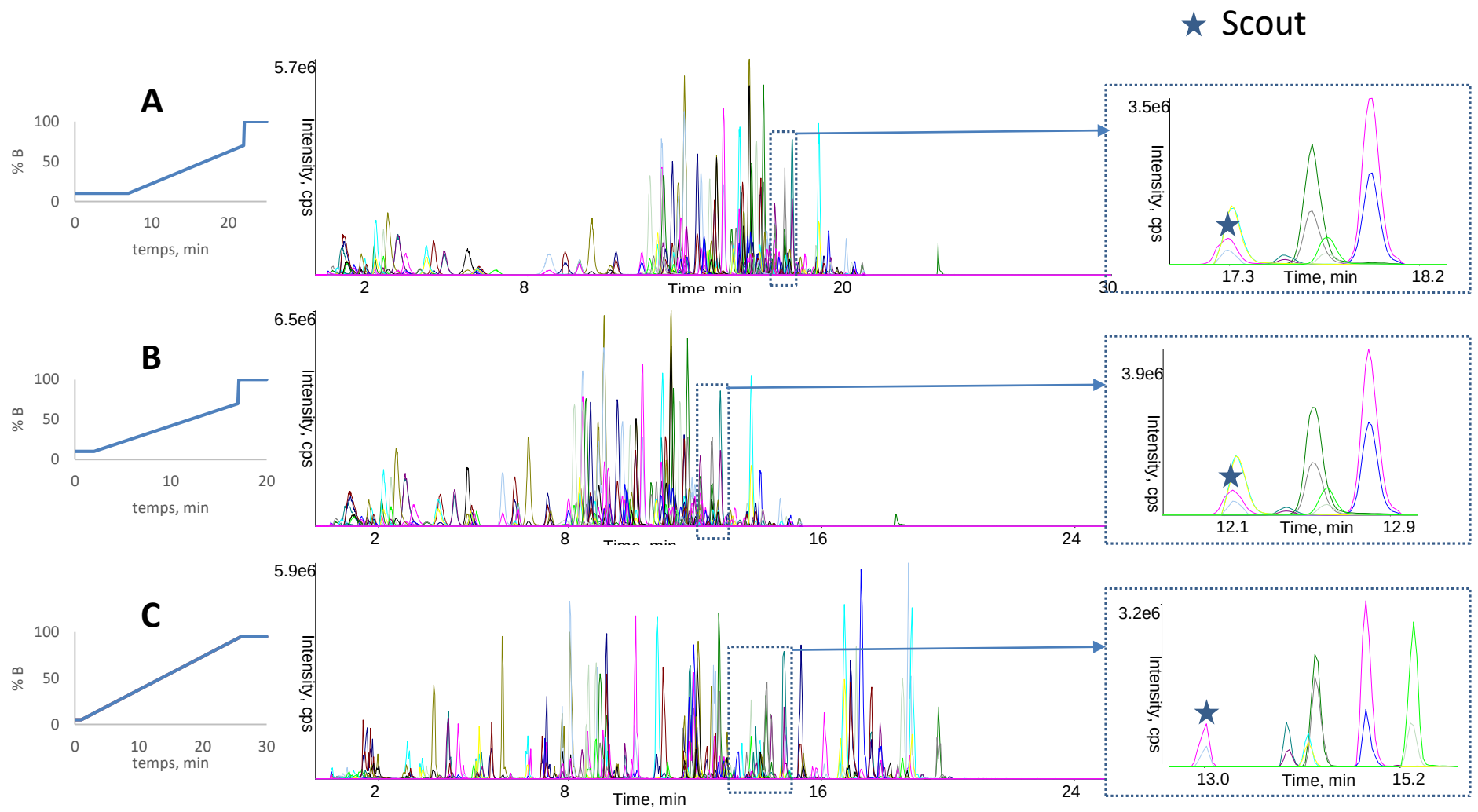
★ Scout
A
B
C
% B
100
50
0
0
10
20
30
temps, min
5.7e6
6.5e6
5.9e6
Intensity, cps
2
8
16
20
24
30
Time, min
3.5e6
3.9e6
3.2e6
17.3
18.2
12.1
12.9
13.0
15.2

Table 1: Limit of Quantification and pesticide measurements in river water samples using a reference method (A) on a API4000 triple quadrupole mass spectrometer (Irstea Lab), and (B) Scout-MRM and (C) sMRM approach on an hybrid quadrupole ion trap API6500 mass spectrometer (ISA lab). () in brackets error calculation between method A and B; in square brackets error calculation between method B and C.

		LOQ	56_01	56_02	56_03	56_04	56_05	56_06	56_09	56_11	56_13	56_14
Irstea – MRM (Method A)	Atrazine	0.20	2.4	2.3	1.1	1.0	3.0	2.7	2.0	2.3	2.0	2.0
	Azoxystrobin	0.50	2.3			5.6	6.3	6.1				
	Chlortoluron	1.00					25.3					
	3-(3,4-dichlorophenyl diuron	1.00								4.9		
		0.50					8.2	5.3	11.3	8.7	6.3	8.0
	isoproturon	1.00				9.2						
	metolachlore	1.00	17.2	6.6	25.5	5.6			6.1			
	norflurazon	0.50							9.3	9.8	9.2	8.6
	simazine	0.20	1.2				2.7	1.6	4.7	6.3	4.6	4.8
	tebuconazole	0.50					5.7	9.6	20.4	15.9		16.2
		LOQ	56_01	56_02	56_03	56_04	56_05	56_06	56_09	56_11	56_13	56_14
ISA - Scout (Method B)	Atrazine	0.04	1.7 (-29)	1.7 (-28)	0.5 (-55)	0.6 (-39)	1.7 (-44)	1.7 (-35)	1.2 (-43)	1.6 (-33)	1.2 (-40)	1.2(-39)
	Azoxystrobin	0.005	2.1 (-9)			4.9 (-12)	5.0 (-21)	5.2 (-14)				
	Chlortoluron	0.05					23.5 (-7)					
	3-(3,4-dichlorophenyl diuron	0.01								4.8 (-1.5)		
		0.10					7.0 (-14)	4.6 (-13)	9.4 (-17)	8.6(-1)	6.4(1)	7. 8(-3)
	isoproturon	0.01				8.4 (-8)						
	metolachlore	0.05	14.8 (-14)	6.8 (-2)	19.1 (-25)	5.9 (5)			6.0 (-2)			
	norflurazon	0.05				0.3			8.5 (-9)	10.7 (9)	10.4 (13)	9.6 (12)
	simazine	0.02	1.3 (2)				2.5(-7)	1.7 (3)	4.4 (-6)	6.7 (8)	4.8 (5)	5.0 (6)
	tebuconazole	0.05					4.8 (-15)	8.6 (-10)	17.7 (-13)	16.8 (6)		19.3 (20)
		LOQ	56_01	56_02	56_03	56_04	56_05	56_06	56_09	56_11	56_13	56_14
ISA – sMRM (Method C)	Atrazine	0.04	1.7 [3]	1.6 [6]	0.5 [0]	0.6 [-4]	1.6 [7]	1.6 [11]	1.2 [0]	1.6 [-1]	1.3 [10]	1.1 [7]
	Azoxystrobin	0.005	2.1 [2]			3.8 [28]	5.2 [-3]	5.3 [-3]				
	Chlortoluron	0.05					22.6 [4]					
	3-(3,4-dichlorophenyl diuron	0.01								5.1 [-5]		
		0.10					7.2 [-2]	4.8 [-4]	9.5[-1]	8.9 [-3]	6.7[-5]	8.2 [-5]
	isoproturon	0.01				7.9 [7]						
	metolachlore	0.05	14.5 [2]	6.7 [1]	18.2 [5]	5.8 [1]			6.1 [-2]			
	norflurazon	0.05							8.8 [-4]	10.7 [-1]	11.0 [-5]	9.7 [-1]
	simazine	0.02	1.2 [7]				2.3 [10]	1.4 [20]	4.2 [5]	6.4 [5]	4.5 [6]	4.7 [7]
	tebuconazole	0.05					5.1 [-6]	8.9 [-3]	19.0 [-7]	18.7 [-10]		20.6 [-6]