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UHPLC Optimization Study for Improved LC-MS Performance and Throughput

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ABSTRACT

To efficiently use the qualitative and quantitative performance, most mass spectrometry (MS) based applications depend on automated sample introduction and chromatographic compound separation by liquid chromatography (LC). The ever-growing demand for LC-MS systems and the wide range of LC-MS applications in different markets ask for profound examinations which LC-MS combinations provide an optimum holistic system performance. Here, we evaluate the impact of three different ultra high performance UHPLC systems on retention time (RT) reproducibility, peak properties, and sample throughput to ensure optimal MS performance and capacity utilization. We used binary high pressure gradient (HPG) and quaternary low pressure gradient (LPG) UHPLC systems with different pressure specifications, gradient slopes, columns at different flow rates, and varied gradient delay volumes (GDVs) with varying mixer volumes.

INTRODUCTION

LC-MS technology-driven markets are embedded most commonly in a regulative environment that demands methodologies with high resolution, selectivity, sensitivity, robustness and number of molecules to be discovered, identified and quantified in one single run. One example is the environmental and food safety market where analysts need to meet strict regulatory requirements while providing high throughput capabilities at the same time. For consumer protection they must feel confident of the results obtained even from unknown matrices with unknown residues. Recent advances in HRAM and MRM MS analysis underline the capabilities of these highly accurate, precise and sensitive instrumentations. However, to efficiently extract the desired or "complete" information from diverse sample matrices, most MS applications depend on automated sample injections followed by LC separation prior to MS analysis. For this purpose, the hyphenation of UHPLC systems with MS is the state-of-the-art technology. Therefore, we test in our study three UHPLC systems (Figure 1), varying mixer volumes, LC gradient slopes and columns to evaluate their impacts on

(1) **Peak Properties**
(2) **Retention Time**
(3) **Throughput Consideration**
(4) **Throughput Maximization**

and to highlight the UHPLC performances for a variety of LC-MS application purposes. Here, we focus on the LC-MS analysis of pesticides.

MATERIALS AND METHODS

Standard Preparation

All pesticides were purchased from Sigma-Aldrich at high purity grade (PESTANAL®, analytical standard). Stock solutions of each pesticide were stored at -20˚C at a concentration of 10 mg/ml. Working solutions were freshly prepared (1:10000 dilution) prior to LC-MS analysis. Pesticides used: mepiquat chloride, methamidophos, carbendazim, fenuron, carbofuran, fluometuron, metazachlor, metalaxyl, boscalid, uniconazole, quinoxyfen.

LC-MS Analysis

To separate 1 µL injection volume of the pesticide mixture chromatographically, the three Thermo Scientific™ UHPLC systems: Thermo Scientific™ Vanquish™ UHPLC system, Thermo Scientific™ Vanquish™ Flex Binary UHPLC system and Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system, with the Thermo Scientific™ Hypersil GOLD™ Vanquish™ columns (100 x 2.1 mm, 1.9 µm and 50 x 2.1 mm, 1.9 µm) and the Thermo Scientific™ Accucore™ Vanquish™ C18+ (100 x 2.1 mm, 1.5 µm) column were used at 40 ˚C. Five gradient lengths were applied and compared at a flow rate of 0.5 mL/min: 2, 5, 10, 20 and 100 min for the gradient ramping from 5-95% solvent B (Figure 2). Solvent A: Water, 5 mM ammonium formate, 0.1% formic acid. Solvent B: Methanol, 5 mM ammonium formate, 0.1% formic acid. The Thermo Scientific™ Velos Pro™ ion trap mass spectrometer equipped with a HESI-II probe was utilized for mass spectrometric detection acquiring data in the full MS^1 mode at positive ESI polarity.

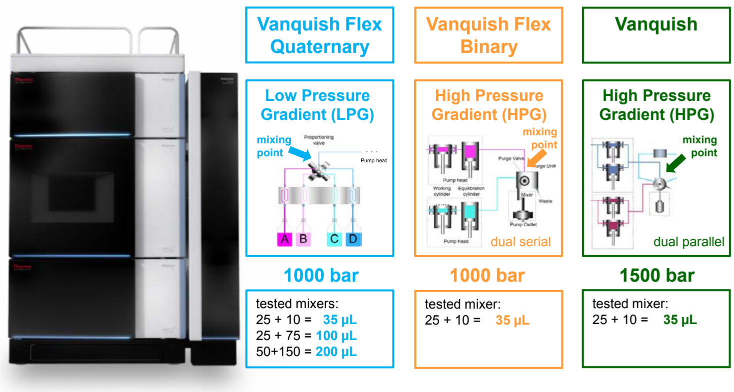

Figure 1. Vanquish Systems for MS analysis. Hardware configurations were chosen for best comparability between LC-MS systems.

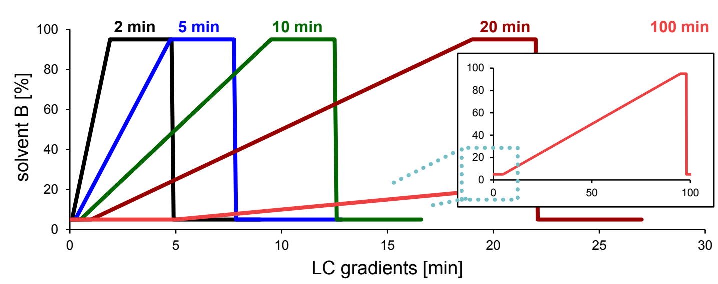

Figure 2. LC gradient studies. Application of five linear gradients in a range of 2-100 min with different gradient slopes, but same starting conditions of 5% solvent B and column wash and re-equilibration time (in total 8.0 min).

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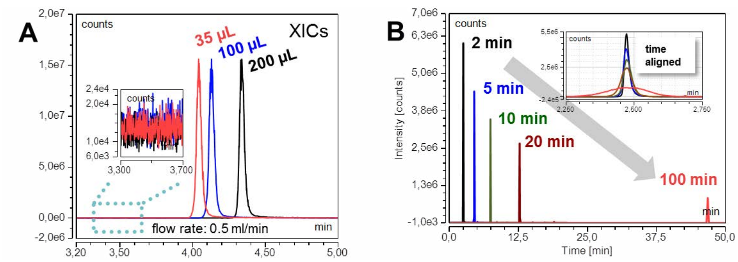
RESULTS AND DISCUSSION

(1) Peak Properties



(2) Retention Time

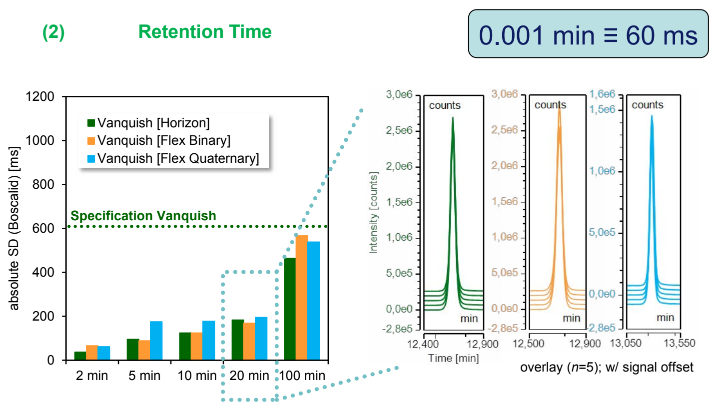


Figure 4. RT precision of LC gradients. RT precision of the three Vanquish systems and five gradients in quintuplicates (Figure 2), represented by absolute standard deviations (SDs) of boscalid. Overlaid XICs of boscalid from the 20 min gradient method.

Table 1. RT precision (*n*=199) of the two HPG systems [ms].

#	compound	Vanquish	Vanquish Flex Binary
2	Methamidophos	40	158
3	Carbendazim	100	97
4	Fenuron	71	62
5	Carbofuran	62	64
6	Fluometuron	48	63
7	Metazachlor	52	67
8	Metalaxyl	51	67
9	Boscalid	37	66
10	Uniconazole	35	71
11	Quinoxyfen	35	109
	Average	**53.1±20.4**	**82.3±31.1**

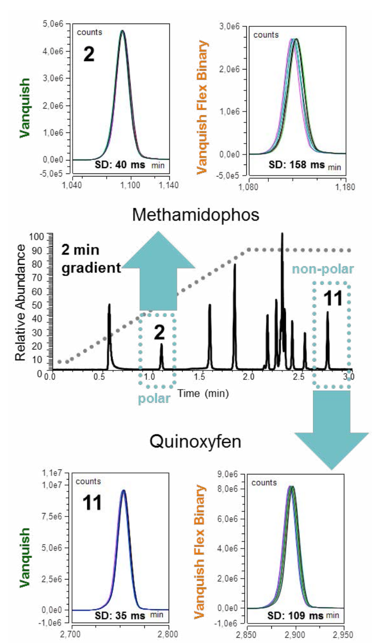


Figure 5. RT precision (*n*=199) of polar and non-polar pesticides. Representative chromatogram using the optimized 2 min gradient (*middle*). XICs of methamidophos *m/z* 142.0 (*top*) and of quinoxyfen *m/z* 308.3 (*below*). Normalized signal overlays (*n*=9; sample #10, #25, #50, #75, #100, #125, #150, #175, #199).

As quaternary LPG systems are often used with large mixer volumes for a low pump ripple, we tested several mixer volumes to determine the lowest possible mixer volume to provide an unaffected MS baseline ripple for the Vanquish Flex quaternary (Figure 3A). As a result, the 35 µL mixer could be used for all three Vanquish Systems for LC-MS analysis. To evaluate the Vanquish system performances we applied five different LC gradients (Figure 2). As expected, we observed gradient dependent RT shifts and peak broadening (Figure 3B). Direct comparison of RT precisions of the three Vanquish Systems revealed no significant differences across all gradients, exemplarily shown for boscalid and highlighted for the 20 min gradient (Figure 4). In line with chromatographic theory, the highest signal-to-noise ratio was obtained for the 2 min gradient, i.e. the steepest gradient (data not shown).

(3) Throughput Consideration

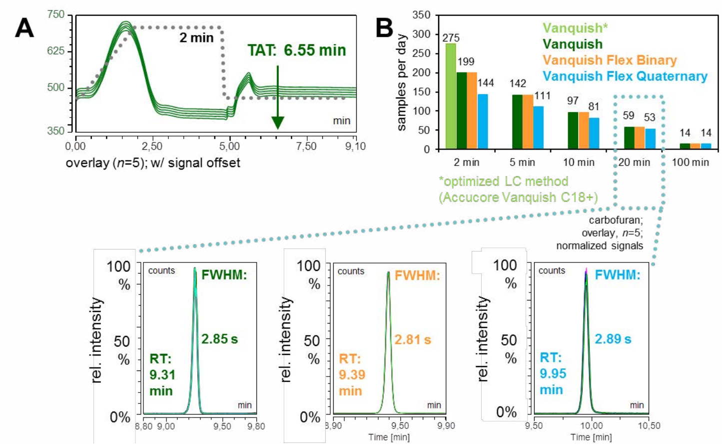


Figure 6. Total analysis time (TAT) of the Vanquish Systems in context of throughput per day. (A) Pump pressure profile of the 2 min gradient acquired on the Vanquish including TAT after method optimization. (B) Sample throughput and full width at half maximum (FWHM) between the Vanquish systems using the 20 min gradient.

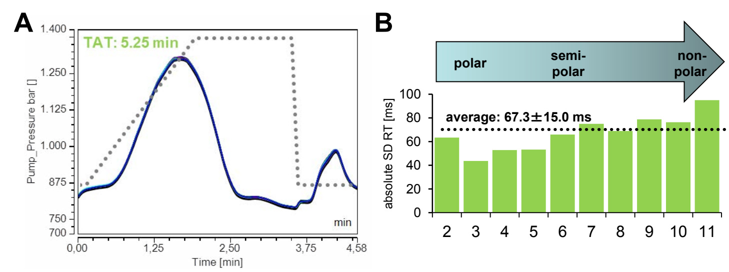


Figure 7. High throughput performance of the Vanquish (*n*=275). (A) 275 pump pressure overlays after LC optimization using the Accucore Vanquish C18+ column. (B) SD of absolute RT for 10 pesticides (2)-(11).

Besides the positive impact on signal sensitivity, short gradients also allow for high-throughput measurements and are of high interest for LC-MS applications. For this task, HPG systems are preferred due to their low GDV. To evaluate in this context the RT precision, we compared the Vanquish and Vanquish Flex binary systems. An average RT precision below 85 ms for 199 samples (Table 1) was obtained for all measured pesticides on both systems. Detailed analysis revealed that especially the polar pesticide methamidophos and the unpolar quinoxyfen showed reduced SD values for the Vanquish system compared to the Vanquish Flex binary system (Figure 5). For throughput evaluation of all Vanquish systems and used gradients, we modified the initially used gradient methods and calculated the achievable throughput per day considering a fix inter run cycle time. The initially kept constant column equilibration time was modified to account for GDV differences between HPG and LPG systems, e.g. the 2 min method with initially 9.1 min was reduced to 6.55 min for the HPG systems (Figure 6A). For the 2 min gradient a throughput of 199 samples on HPG systems compared to 144 samples per day on the LPG system could be achieved (Figure 6B). The throughput of 199 samples for HPG type pumps was verified experimentally (Figure 5).

(4) Throughput Maximization

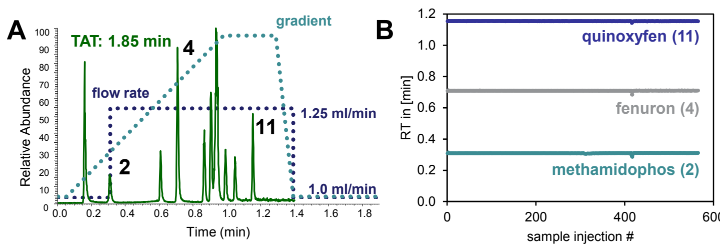


Figure 8. Proof of principle for ultrafast gradient application using the Vanquish (*n*=567). (A) XICs of pesticides after utilizing a 50 mm Hypersil GOLD Vanquish column and flow rate ramping from 1.0 ml/min to 1.25 ml/min. (B) RT stability of the three indicated pesticides obtained during 24 hrs measurements.

After shortening of all LC gradients, a point of similar throughput was reached for the 20 min gradient. Also, no remarkable differences in FWHM values between the three Vanquish systems were observed for the 20 min gradient (Figure 6B). Applying a 25 min gradient would result in a throughput difference below 10%. In a next step, we explored the high throughput possibilities with the 1500 bar Vanquish system. First, we optimized the 2 min LC gradient method using an Accucore Vanquish column (particle size: 1.5 µm). This resulted in a back pressures increase from 700 bar (Figure 6A) to 1300 bar (Figure 7A) but also in a sample throughput increase to 275 samples per day (Figure 6A). Overall, a RT precision below 70 ms on average was achieved under these conditions (Figure 7B). Finally, we developed an ultrafast gradient method within 1.85 minutes (Figure 8A) to measure 567 samples a day while still ensuring a similar peak resolution (Figure 8A) in a precise manner (Figure 8B). Under these conditions, FWHM values markedly below 1.0 s (data not shown) were obtained.

CONCLUSION

Here we highlight that the choice of the UHPLC system for LC-MS applications depends i.a. on the optimization possibilities, performance, and throughput considerations. Optimizations include e.g. the pump mixer volume change which can change the GDV dramatically. Based on the stable LC-MS signal baseline and peak shape, LC-MS applications are not prone to mixer volume changes. The Vanquish platform offers in addition an adjustable GDV contribution within the autosampler even between chromatographic runs. Regarding UHPLC system performance, there is a wide overlap within the range of fast to shallow gradient applications where all three Vanquish systems perform with high comparability. High throughput considerations lead us to recommend the Vanquish Flex quaternary system for gradients lengths exceeding 25 min with the used 35 µL mixer volume and provides in addition the flexibility to use four different solvents during a chromatographic run. Due to the low GDV, the two HPG systems provide clearly benefit for high throughput measurements and allow to perform steep gradients. In our example with the 2 min gradient, we achieved a high throughput increase of up to 38% per day compared to the LPG system (Figure 6B). Based on the larger scale throughput measurements (Figure 5), we recommend the Vanquish Flex binary system as a robust instrument for targeted and routine LC-MS analysis up to 1000 bar requirements. However, if a LC-MS analysis requires the maximum throughput or outstanding RT reproducibility and robustness under all LC conditions for all analytes shown in this study, the Vanquish system with its 1500 bar capability is the system of choice.