



Automated Method Development Using the Agilent RapidFire 365 High-Throughput Mass Spectrometry System

Application Note

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Abstract

Development of new analytical methods to monitor compounds of interest using SPE/MS/MS involves optimizing several parameters. The Agilent RapidFire 365 High-throughput MS System's expanded capabilities allow the user to automate much of this optimization. This Application Note demonstrates a procedure for optimizing a method for an small example molecule, cyclic AMP. The final method has a CV of < 3 %, greatly improved peak shape, and 20-fold reduced carryover as compared to the generic starting method. The optimization required 12 minutes of hands-on time, and 74 minutes of walk-away run time. The rapid throughput and the ability of the RapidFire 365 to switch solvents and cartridges automatically allow the acquisition of finely detailed data, enabling the head-to-head comparison of slightly different conditions, and resulting in greater confidence in the final optimized method.



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Introduction

New method development for mass spectrometry-based assays can be time-consuming. Multiple packing materials and buffer systems must often be explored before a suitable combination is found. LC methods require several minutes each, and trying different options can quickly add up to a significant time investment. The Agilent RapidFire High-throughput MS System addresses this bottleneck by allowing sample analysis in 8–15 seconds per sample, enabling various buffers and cartridges to be tested much more quickly. The RapidFire 365 takes this improvement one step further by offering the ability to switch solvents and cartridges in an automated fashion. Method development can then be set up to run automatically, allowing the user to attend to other tasks. This Application Note follows an example protocol to optimize a RapidFire method for a representative small molecule, cyclic adenosine monophosphate (cAMP), for which mass spectrometric conditions have already been determined (resources for MS optimization, such as how to use Optimizer, are available on the Agilent web site). The following protocol is not intended to be comprehensive, nor to apply to all potential analytes of interest, but rather to serve as a general guideline for how a new optimization could be approached, and to illustrate the ability of the RapidFire 365 to assist in method development.

Experimental

Planning and instrument setup

Commonly, a RapidFire method is optimized from a generic starting point chosen based on the size and polarity of the molecule. Method components that are frequently explored include cartridge packing material, wash and elution buffer identity and additives, RapidFire state timings, and possibly RapidFire flow rates. The example method development detailed here involves three rounds of optimization:

- Cartridge and acid composition of wash and elution buffers
- Percentage of acetonitrile in the elution buffer
- RapidFire timing

Step 1: Make a constant concentration sample plate in a mock matrix

A 96-well plate was made containing 200 μ L of 1 μ M cAMP in 50 mM Tris pH 7.5 containing 0.1 % formic acid in odd-numbered columns, and 50 mM Tris pH 7.5 + 0.1 % formic acid in even-numbered columns to assess carryover from sample wells into buffer wells.

Step 2: Choose cartridge(s) to be tested based on the general cartridge selection guide (Table 1)

Following our general cartridge selection chart, a C4 (A) and a graphitic carbon (D) cartridge were chosen as the most likely packing materials to work well with this small hydrophilic molecule.

Step 3: Choose buffers and additives to be tested

Based on the MS transition optimization, cAMP is ionized most efficiently in positive mode. Therefore, buffers containing formic acid and/or trifluoroacetic acid were used. A common starting point for reverse phase applications is water with or without acids as buffer A, and some percentage of acetonitrile with or without acids as buffer B. Because acids and pairing agents can impact the success of a method severely, they were optimized first using a generic acetonitrile concentration of 80 %.

Table 1. Cartridge selection guide.

Type	Packing	Typical applications	Part no.
A	C4	Small molecules, peptides, oligos	G9203A
B	Cyano	Hydrophobic compounds	G9204A
C	C18	Proteins, small molecules	G9205A
D	Graphitic carbon	Hydrophilic compounds, small molecules	G9206A
E	C8	Proteins, peptides, small molecules	G9207A
F	Phenyl	Aromatic compounds	G9208A
H	HILIC	Hydrophilic compounds, small molecules	G9209A
Z	Custom	Custom applications	G9210A

Step 4: Set up buffer bottles on RapidFire

Set up buffer bottles on RapidFire pumps to cover the range of buffers and additives to be explored. Purge the pumps, assigning 25 % of flow to each of the four channels, and elevating the flow rate to 10 mL/min for at least 1 minute.

To allow the exploration of acid combinations from 0 to 0.1 % formic or trifluoroacetic acid and acetonitrile concentrations up to 100 %, the pumps were set up as in Table 2.

Experimental setup

Step 5: Create RapidFire method files to run the cartridge/buffer combinations of interest

Four RapidFire methods were created (Table 3) to vary the acid composition of both the wash and the elution buffers. All methods used a sip height of 1, a pump 2 composition of 100 % channel A, generic state timings of (1) 600, (2) 3,000, (3) 4,000, and (4) 500 ms, and flow rates of 1.5 mL/min for pump 1 and 1.25 mL/min for pumps 2 and 3.

Step 6: Create a plate map containing one sequence for each condition to be tested

Because there are eight conditions to be tested in the first experiment (four acid combinations × two cartridge types), a plate map containing eight sequences, each corresponding to one row, was created (Table 4).

Step 7: Set up a batch

Set up a batch to pair the RapidFire methods with the cartridges to be tested. Assign a mass spec method to each sequence, if using synchronization.

A batch was created (Table 5) to run the eight sequences under four different elution solvents and on two cartridges. All eight experiments used the same MS method.

Table 2. RapidFire pump setup.

Pump 1		Pump 2		Pump 3	
A	ddH ₂ O + 0.1 % formic acid	A	50 % acetonitrile	A	Acetonitrile + 0.1 % formic acid
B	ddH ₂ O + 0.1 % trifluoroacetic acid	B	ddH ₂ O	B	Acetonitrile + 0.1 % trifluoroacetic acid
C	ddH ₂ O	C	ddH ₂ O	C	ddH ₂ O + 0.1 % formic acid
D	ddH ₂ O	D	ddH ₂ O	D	ddH ₂ O + 0.1 % trifluoroacetic acid

Table 3. Agilent RapidFire 365 High-throughput MS System methods to vary acid compositions.

	Method 1		Method 2		Method 3		Method 4	
	0.1 % FA		0.09 % FA 0.01 % TFA		0.05 % FA 0.05 % TFA		0.1 % TFA	
Pump 1 composition	A	100	A	90	A	50	A	0
	B	0	B	10	B	50	B	100
	C	0	C	0	C	0	C	0
	D	0	D	0	D	0	D	0
Pump 3 composition	A	80	A	72	A	40	A	0
	B	0	B	8	B	40	B	80
	C	20	C	18	C	10	C	0
	D	0	D	2	D	10	D	20

Table 4. Sequences for acid and cartridge optimization experiment.

Sequence no.	Wells
1	A1 – A12
2	A1 – A12
3	A1 – A12
4	A1 – A12
5	B1 – B12
6	B1 – B12
7	B1 – B12
8	B1 – B12

Table 5. Batch for acid and cartridge optimization experiment.

Sequence no.	RapidFire method	Cartridge	MS method
1	1	A	cAMP.m
2	1	D	cAMP.m
3	2	A	cAMP.m
4	2	D	cAMP.m
5	3	A	cAMP.m
6	3	D	cAMP.m
7	4	A	cAMP.m
8	4	D	cAMP.m

Step 8: Load the batch, press play

The system will automatically calculate the number of mix injections required to clear the dead volume of the instrument with the mix of solvents specified in the RapidFire method assigned to sequence 1. Following these mix injections, MS acquisition will begin, if chosen, and the sample injections will follow. Upon completion of sequence 1, the mixing injections will occur again if the RapidFire method assigned to sequence 2 calls for a different buffer composition. If the three compositions are all the same, mixing injections will be skipped, and sequence 2 will begin immediately.

Step 9: Analyze the acquired data

Upon run completion, analyze the acquired data (Figure 1). Choose final cartridge and buffers for the method, or use the data to inform the next round of optimization.

Criteria for an optimal method includes proper peak shape, baseline peak separation, maximum peak height, and minimum carryover from sample wells into buffer wells. Here, large improvements in analyte retention and peak reproducibility are seen on the graphitic carbon cartridge

(D, peak heights of approximately $4E4$) as compared to the C4 cartridge (A, peak heights of approximately $4E2$). Additionally, a substantial reduction in carryover is associated with increasing concentrations of TFA (from 0 % TFA in RF method 1 to 0.1 % TFA in RF method 4).

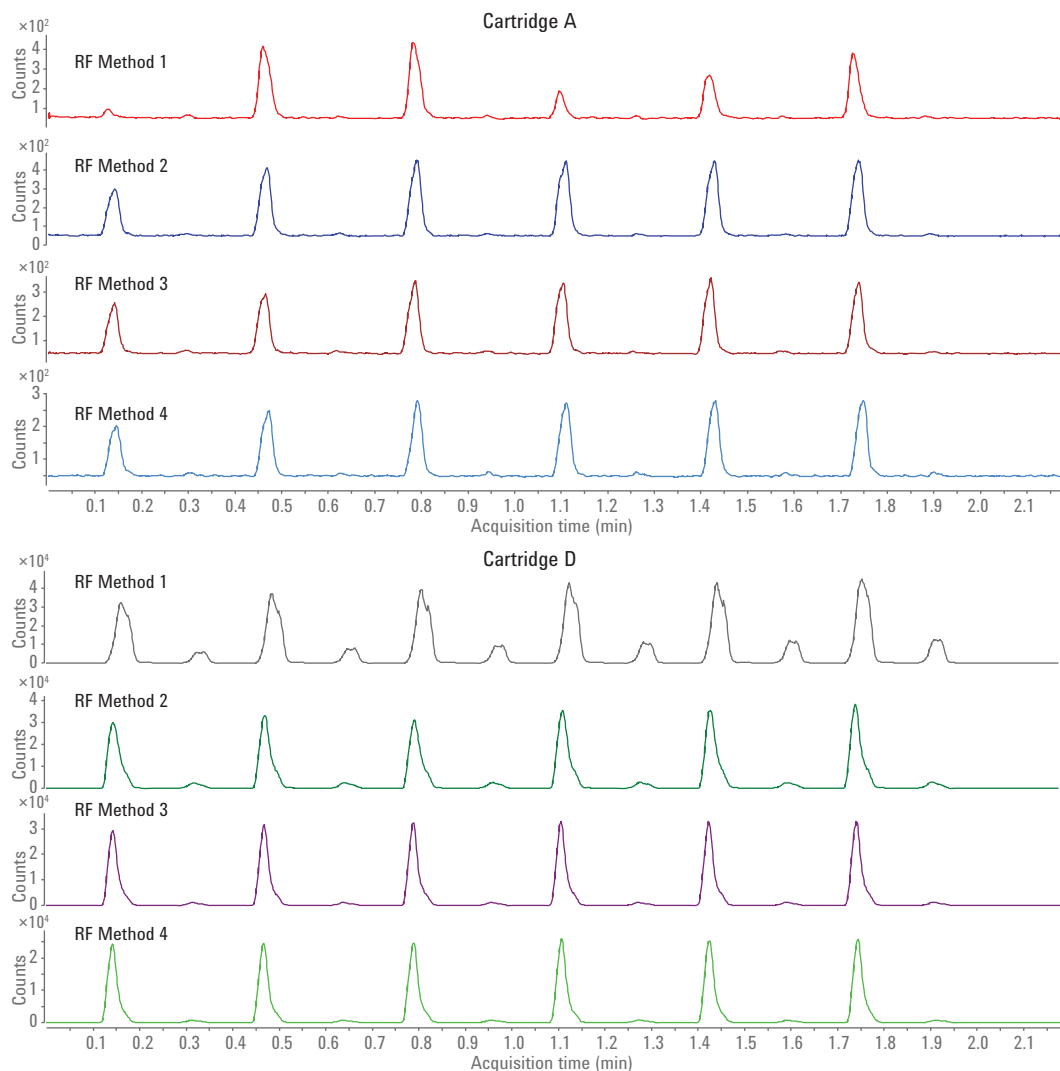


Figure 1. Example data acquired from the eight sequences run in experiment 1.

Step 10: Determine whether further method optimization is required

Decide if further buffer, additive, or RapidFire method optimization is necessary. If it is, repeat steps 5 through 9 until sufficient information is gathered to generate an optimized final method.

The best conditions from the first experiment are cartridge D and 0.1 % TFA. As percentages of acetonitrile other than the default of 80 % have not yet been explored, a six-sequence batch was set up to optimize acetonitrile concentration.

Six RapidFire methods were created, as in Table 6, to vary the acetonitrile composition of the elution buffer. All methods used a TFA concentration of 0.1 %, a sip height of 1, a pump 2 composition of 100 % channel A, state timings of (1) 600, (2) 3,000, (3) 4,000, and (4) 500 ms, and flow rates of 1.5 mL/min for pump 1 and 1.25 mL/min for pumps 2 and 3.

A plate map was created containing six sequences (Table 7).

A batch was created to run each sequence under a different RF method (Table 8).

Table 6. Agilent RapidFire 365 High-throughput MS System method setup for acetonitrile composition experiment.

	1 100 % ACN		2 90 % ACN		3 80 % ACN		4 70 % ACN		5 60 % ACN		6 50 % ACN	
Pump 1 composition	A	0	A	0	A	0	A	0	A	0	A	0
	B	100	B	100	B	100	B	100	B	100	B	100
	C	0	C	0	C	0	C	0	C	0	C	0
	D	0	D	0	D	0	D	0	D	0	D	0
Pump 3 composition	A	0	A	0	A	0	A	0	A	0	A	0
	B	100	B	90	B	80	B	70	B	60	B	50
	C	0	C	0	C	0	C	0	C	0	C	0
	D	0	D	10	D	20	D	30	D	40	D	50

Table 7. Sequence map for experiment 2.

Sequence no.	Wells
1	C1 – C12
2	C1 – C12
3	C1 – C12
4	D1 – D12
5	D1 – D12
6	D1 – D12

Table 8. Batch setup for experiment 2.

Sequence no.	RapidFire method	Cartridge	MS method
1	1	D	cAMP.m
2	2	D	cAMP.m
3	3	D	cAMP.m
4	4	D	cAMP.m
5	5	D	cAMP.m
6	6	D	cAMP.m

The batch was run and the results were analyzed (Figure 2).

Here, a large improvement in peak shape is observed as the percentage of acetonitrile is reduced from 100 %. An enormous reduction in carryover is also observed. Analyte signal is comparable under all conditions tested, so a concentration of 60 % acetonitrile + 0.1 % TFA was selected as the final elution solvent.

Finally, to further reduce carryover, a six-sequence batch was created to optimize RapidFire state timings using six RapidFire methods (Table 9). All methods used ddH₂O + 0.1 % TFA as buffer A, 60 % acetonitrile + 0.1 % TFA as buffer B, a sip height of 1, a pump 2 composition of 100 % channel A, and flow rates of 1.5 mL/min for pump 1 and 1.25 mL/min for pumps 2 and 3.

A plate map was created containing six sequences (Table 10).

A batch was created to run each sequence under a different RF method (Table 11).

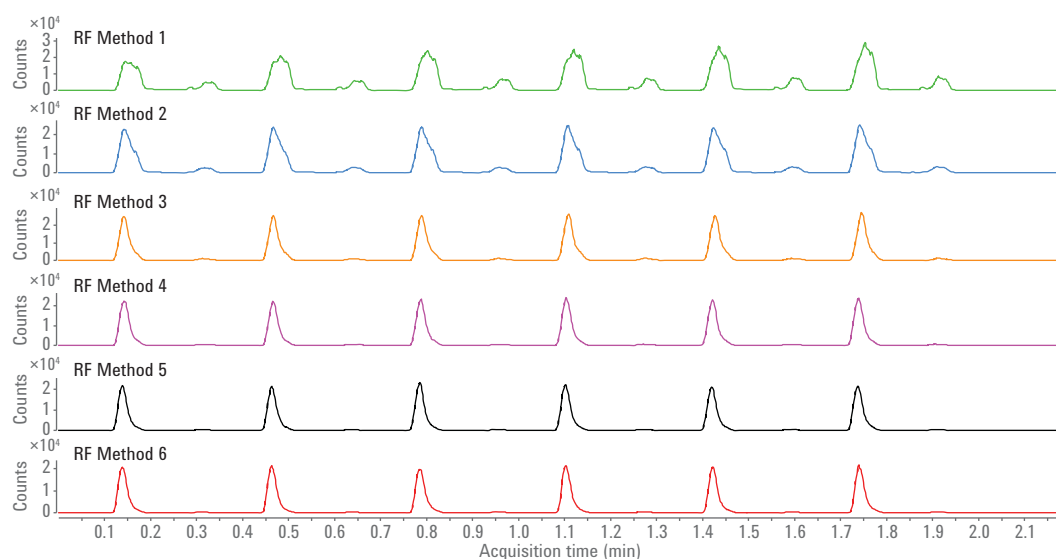


Figure 2. Example data acquired under acetonitrile concentrations ranging from 100 % to 50 %, showing dramatic alterations in peak shape and carryover.

Table 9. Agilent RapidFire 365 High-throughput MS System method setup for state timing experiment.

	Method 1		Method 2		Method 3		Method 4		Method 5		Method 6	
State timings (ms)	1	600	1	600	1	600	1	600	1	600	1	600
	2	2,500	2	3,000	2	3,000	2	3,000	2	3,000	2	3,000
	3	4,000	3	3,000	3	4,000	3	5,000	3	6,000	3	7,000
	4	500	4	500	4	500	4	500	4	500	4	500

Table 10. Sequence map for experiment 3.

Sequence no.	Wells
1	E1–E12
2	E1–E12
3	E1–E12
4	F1–F12
5	F1–F12
6	F1–F12

Table 11. Batch setup for experiment 3.

Sequence no.	RapidFire method	Cartridge	MS method
1	1	D	cAMP.m
2	2	D	cAMP.m
3	3	D	cAMP.m
4	4	D	cAMP.m
5	5	D	cAMP.m
6	6	D	cAMP.m

The batch was run and the results were analyzed (Figure 3).

As seen here, a longer wash can assist in desalting and result in higher signal, and a longer elution can reduce carryover. State timings for this method were set to 600, 3,000, 6,000, and 500 ms to minimize carryover but keep the cycle time as short as possible.

Results and Discussion

An Agilent RapidFire 365 High-throughput MS System method for a representative small molecule was developed in an automated fashion from a generic starting point through three rounds of optimization to determine the cartridge, buffer composition, buffer additives, and RapidFire timings that gave the best peak shape, baseline separation, and peak height, as well as an absence of carryover. The CV for six sample injections under the final optimized method was 2.9 %. Peak shape was greatly improved and carryover was reduced 20-fold from the generic starting point method. This optimization required about 12 hands-on minutes spent setting up the instrument and the required batches. Total hands-off optimization time, including solvent mixing, was 74 minutes.

Conclusions

The speed of a RapidFire 365 High-throughput MS System eliminates guesswork from new method development, as many conditions can simply be tried very quickly and the real results observed. The rapid throughput and the ability of the RapidFire 365 to switch solvents and cartridges automatically allow the acquisition of finely detailed data, enabling the head-to-head comparison of slightly different conditions, and resulting in greater confidence in the final optimized method. The ability to develop robust, reliable new methods with minimal hands-on time frees up researchers to attend to other tasks while maintaining or even improving the quality of the work accomplished.

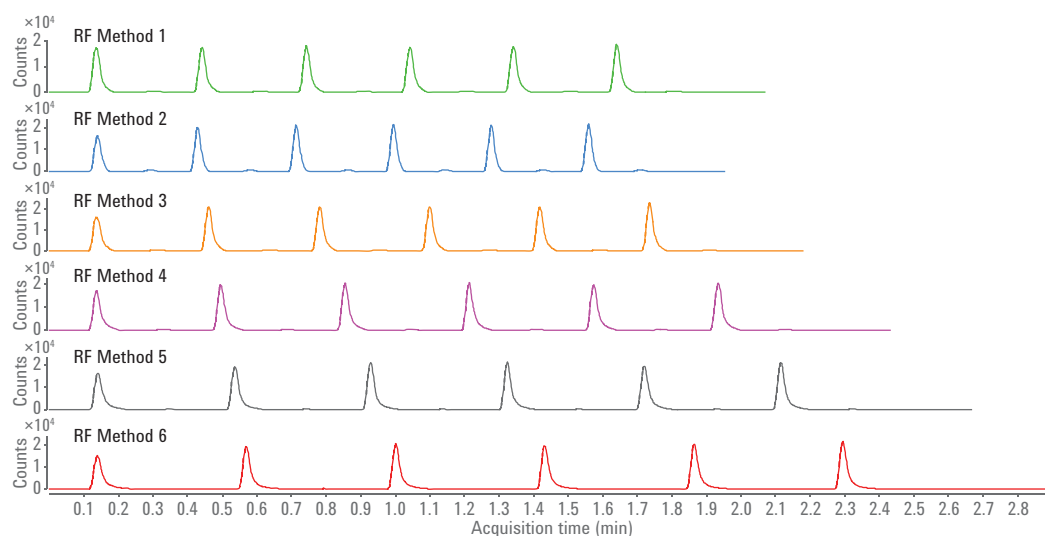


Figure 3. Example data showing the effects of various wash and elution state lengths.

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Published in the USA, September 30, 2014
5991-5222EN



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