Agilent

Trusted Answers

RapidFire for Automated High-Throughput LC/MS

Proven. Rapid. Complete.

Application Compendium

Table of Contents

Introduction

The Agilent RapidFire is the proven choice for high-throughput mass spectrometry, with over 15 years of development and publications. Strategies for automated method development and results in as little as 2 seconds per sample bring greater efficiency to your laboratory, while temperature-controlled sample storage and plate-handling robotics enable you to focus on outcomes instead of process. Whether you need the quantitative precision and sensitivity of triple quadrupole mass spectrometry or the investigative power of time-of-flight high resolution mass spectrometry, RapidFire delivers reliable results faster than ever before. Explore how RapidFire brings ease-of-use to high-throughput mass spectrometry and redefines what is possible.

Agilent RapidFire 400 with accessories

Application Note

Pharma & Biopharma

Agilent

Trusted Answers

High-Throughput (Sub-2.5 Second) Direct Injection Analysis by Mass Spectrometry

Using a modified Agilent RapidFire high-throughput MS system

Authors

Arrin Katz, William LaMarr, and Can Ozbal
PureHoney Technologies, Inc.
Billerica, MA, USA.

Peter Rye
Agilent Technologies, Inc.
Lexington, MA, USA

Abstract

Due to large numbers of samples and the associated analysis time for each, using mass spectrometry (MS) as a primary screening technique can be a long, tedious process. This Application Note presents a modified Agilent RapidFire high-throughput MS system (RapidFire) that bypasses SPE desalting and enables a sub-2.5 second sample throughput rate, making the analysis of 35,000 samples a day possible.

Introduction

The role of MS in early drug discovery, and especially in functional biochemical and binding assays, is well established. However, even fast techniques such as UHPLC or SPE/MS face challenges when primary screens of several hundreds of thousands of compounds need to be performed. With an eight seconds per sample throughput, a RapidFire can sample, desalt, and analyze 10,000 samples in 24 hours. However, a large screen of several hundreds of thousands of compounds still requires many weeks of effort.

We have modified a RapidFire to bypass the SPE desalting step, and inject samples directly into the MS at a throughput of less than 2.5 seconds per sample. This modified system enables the analysis of 35,000 samples, without cleanup, in 24 hours.

Experimental

Methods

The RapidFire was modified to achieve direct injection (Figure 1). The line between valves 1 and 2 was replaced with one that connected valve 1 directly to the MS. This resulted in pump 1 going to the MS, either around the sampling loop or through it depending on the position (load versus inject) of valve 1. Pumps 2 and 3 were plumbed to recycle water. The configuration files were altered to minimize unnecessary valve toggling. The modified system was connected to an Agilent 6470 triple quadrupole LC/MS. Samples, made to be representative of a high-throughput screen, were diluted and transferred to microplates. Plates were run using modified and conventional modes. AUC values were generated by Agilent RapidFire Integrator software, and analyzed using Microsoft Excel.

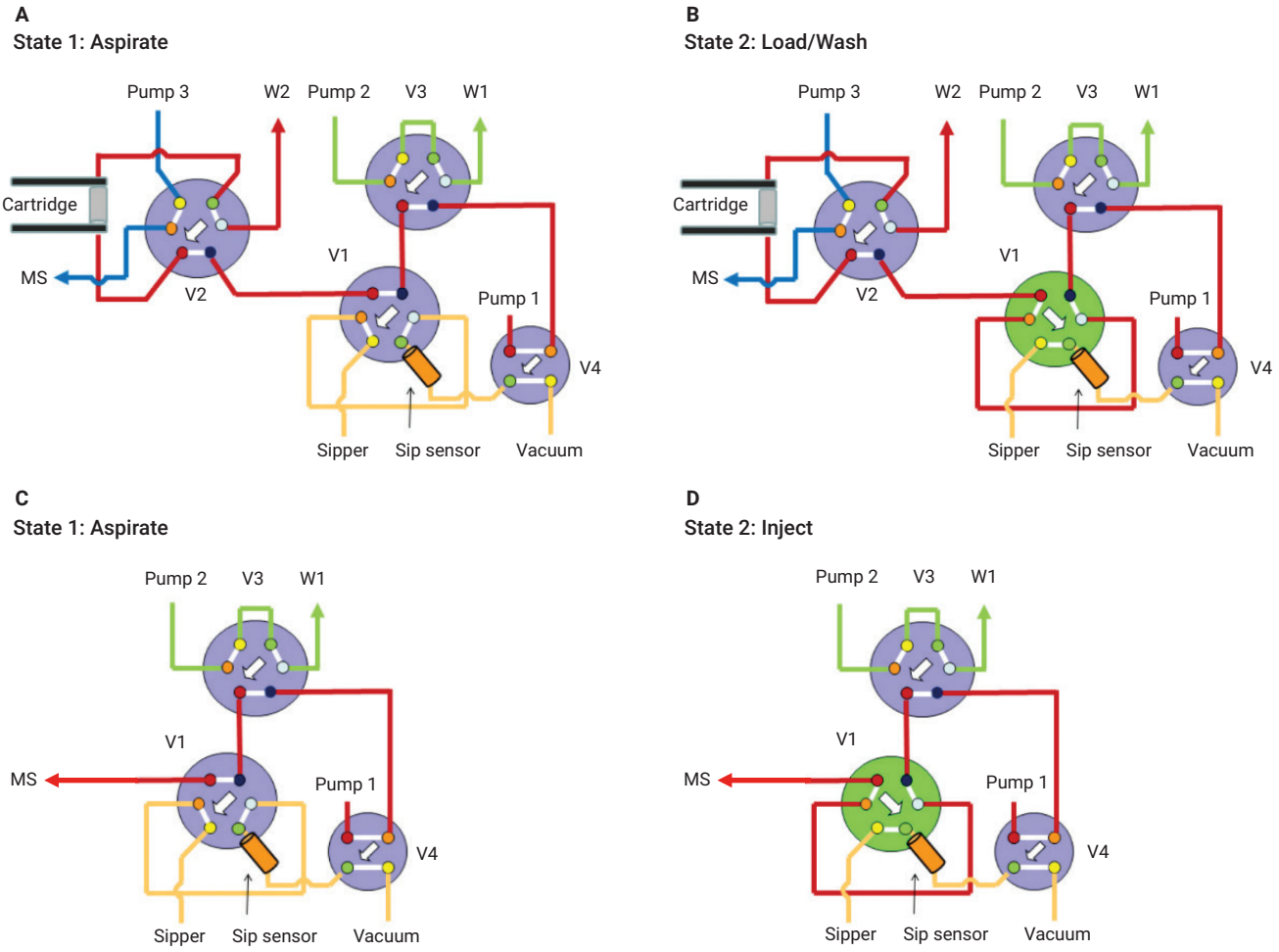


Figure 1. Flowpath diagrams for the RapidFire in standard and direct injection modes. In standard mode, the RapidFire aspirates sample from the plate into the sampling loop during state 1 (A) and loads/washes the sample onto the cartridge during state 2 (B). In subsequent states, not shown, the analytes are eluted into the MS. In direct injection mode, the RapidFire aspirates sample from the plate into the sampling loop during state 1 (C) and elutes that sample into the MS during state 2 (D).

Results and discussion

RapidFire in direct injection mode can sustain a throughput of just over two seconds per sample

To examine the speed of RapidFire in direct injection mode (Figure 2), replicate injections were made from a microplate containing a single dilution of S-adenosylhomocysteine (SAH). The flow rate of the elution pump and the elution time were optimized to balance throughput, peak-to-peak separation, and MS signal. Ultimately, a pump flow rate of 1.25 mL/min and an elution time of 500 msec was configured. The optimized method demonstrated that 60 injections could be measured in just over two minutes, sustaining a throughput of just over two seconds per sample.

Faster flow rates did result in greater peak-to-peak separation, which in turn allowed the method to be sped up further. However, faster flow rates also decreased the MS signal slightly. These results (data not shown) illustrate that the direct injection method can be tuned according to how different assay parameters are prioritized by the user.

RapidFire in direct injection mode provides a good concentration response

To demonstrate the concentration response of RapidFire in direct injection mode (Figure 3), 13 two-fold serial dilutions of SAH were made in water with 0.1 % formic acid starting from a concentration of 8,000 nM. Each of these 14 stock solutions (8,000, 4,000, 2,000, 1,000, 500, 250, 125, 62.50, 31.25, 15.62, 7.81, 3.91, 1.95, and 0.98 nM) was aliquoted into a microplate for analysis. One hundred replicate measurements were conducted of each concentration. These 1,400 injections were analyzed in 52 minutes. Data were integrated and exported in one minute using RapidFire Integrator. Results show a broad (spanning nearly four orders of magnitude) and linear (R^2 = 0.9997) concentration response.

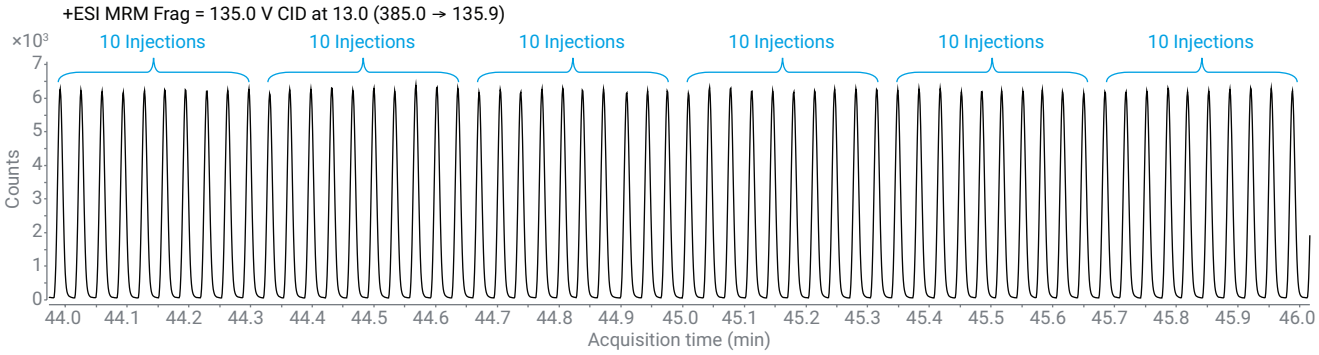


Figure 2. MRM chromatogram data for SAH analyzed by RapidFire-MS in direct injection mode, demonstrating the sampling and measurement of 60 injections in ~two minutes, or two seconds per sample

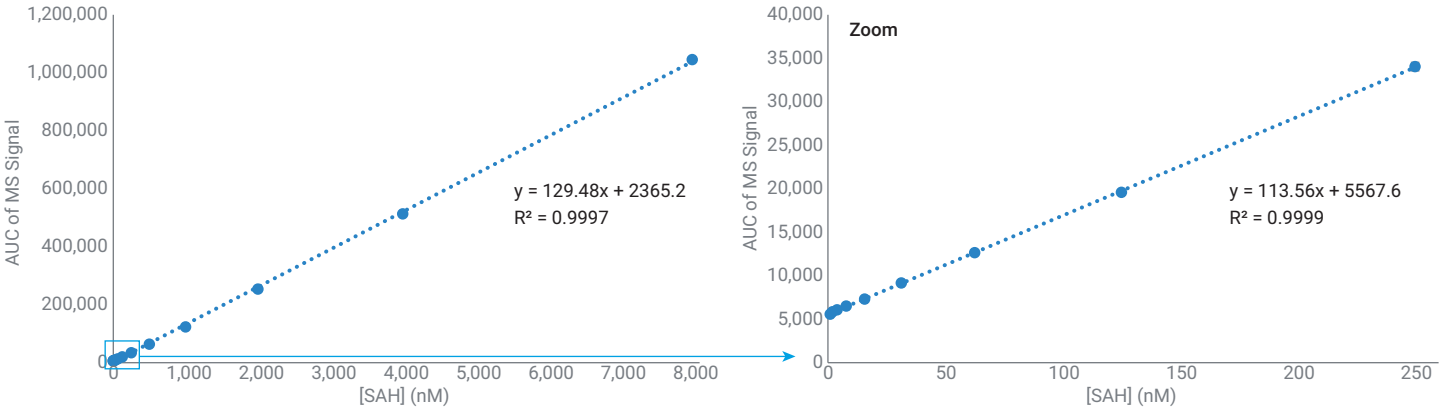
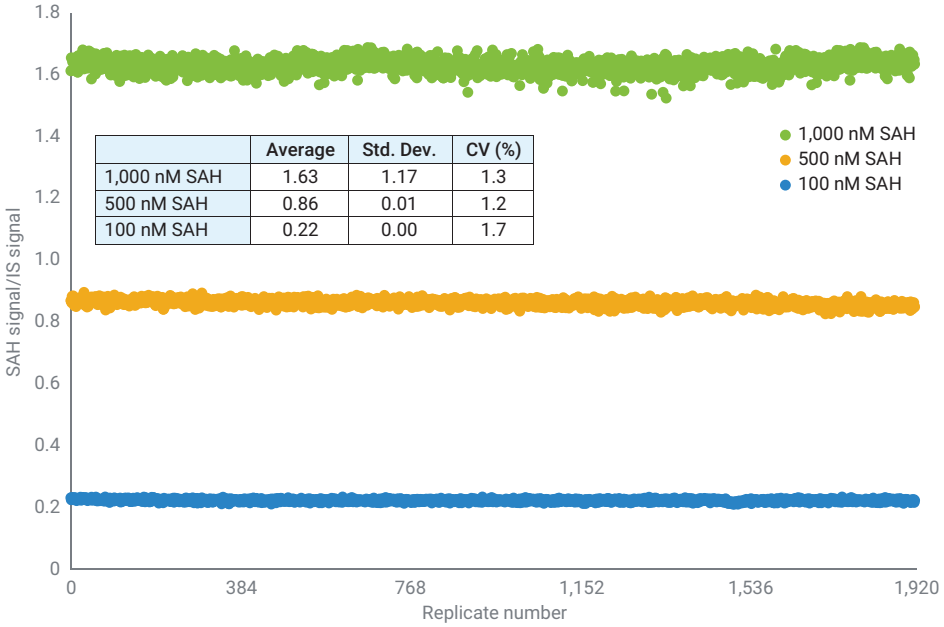


Figure 3. RapidFire-MS in direct injection mode provides a good concentration response. Error bars are shown to indicate the standard error of the mean. The plot of the entire concentration range is shown on the left. A zoom-in of the lower concentration data is shown on the right.

RapidFire in direct injection mode is reproducible

To test the reproducibility of the RapidFire in direct injection mode (Figure 4), bulk solutions of 100, 500, and 1,000 nM SAH were made, and each was supplemented with 500 nM S-adenosylmethionine (SAM) as an internal standard. Each solution was aliquoted into an individual microplate for analysis. For each of the three plates, 1,920 replicate injections were made in 72 minutes. In total, 5,760 injections were analyzed in ~3.5 hours. Data for each run were integrated and exported in one minute using RapidFire Integrator. Results showed excellent reproducibility. Coefficients of variation (CVs) were between 1 and 2 % for all concentrations tested.



	Average	Std. Dev.	CV (%)
1,000 nM SAH	1.63	1.17	1.3
500 nM SAH	0.86	0.01	1.2
100 nM SAH	0.22	0.00	1.7

Figure 4. RapidFire-MS in direct injection mode is reproducible. Replicate data for three concentrations of SAH were collected. The AUC for the SAH MS signal was divided by the AUC of the internal standard MS signal and plotted.

Data collected by RapidFire in direct injection mode correlate well with data from standard mode

To investigate the extent to which data collected by direct injection mode correlate with data from standard mode (Figure 5), four solutions of SAH were made (125, 250, 500, and 1,000 nM). Each was supplemented with 500 nM internal standard SAM, and 96 replicates of each solution were measured in both modes. The normalized data for the four samples, from each mode, were plotted against each other. In total, 768 measurements were used to generate the four-point unity plot, which illustrated good correlation between the data from the two RapidFire modes. The best fit line of the plot had an R^2 = 0.9983.

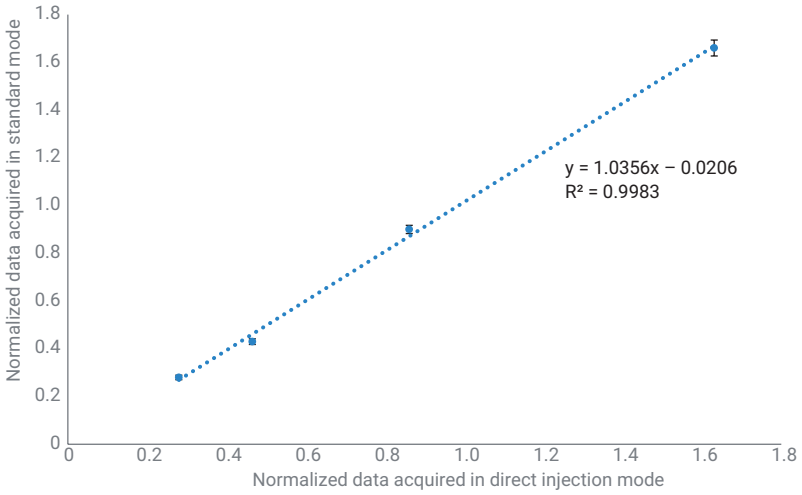


Figure 5. Data collected by RapidFire-MS in direct injection mode correlate well with data from standard mode.

Conclusion

The RapidFire was modified to bypass the SPE cartridge and perform direct injection of samples. This modification resulted in increased throughput, and could easily sustain a cycle time of less than 2.5 seconds per sample, representing a three to five-fold improvement over standard configuration analyses. The modified system also demonstrated a broad and linear concentration response, excellent reproducibility, and near perfect correlation with data acquired in standard mode. The RapidFire could be interconverted in less than 10 minutes, allowing users to balance the throughput and sensitivity requirements of their specific screens.

Though not described in detail here, the modified system can be beneficial in ways beyond increased throughput. As one example, because the sample composition is not subjected to SPE enrichment (which can affect the relative concentrations of analytes delivered to the MS), the modified system enables the analysis of diverse analyte panels in each sample. As another example, because the bind/elute process has been circumvented, the modified system allows the analysis of samples that are not amenable to SPE. That is, if a suitable SPE packing material cannot be identified, or if the sample matrix (high organic content, nonspecific binders, and so on) spoils the SPE binding process.

www.agilent.com/chem

DE44343.3690046296

This information is subject to change without notice.

© Agilent Technologies, Inc. 2019, 2021
Printed in the USA, June 22, 2021
5994-0781EN

Agilent
Trusted Answers

Application Note

Agilent
Trusted Answers

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

Authors

Lauren E. Frick and
William A. LaMarr
Agilent Technologies, Inc.

Abstract

Development of new analytical methods to monitor compounds of interest using SPE/MS/MS involves optimizing several parameters. The Agilent RapidFire 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 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.

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 to 15 seconds per sample, enabling various buffers and cartridges to be tested much more quickly. The RapidFire 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 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

Table 1. Cartridge selection guide.

Type	Packing	Typical Applications	Part Number
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 1: Make a constant concentration sample plate in a mock matrix

A 96-well plate was made containing 200 µL of 1 µM 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 the Agilent 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%.

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 trifluoracetic 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_2O + 0.1% formic acid	A 50% acetonitrile	A Acetonitrile + 0.1% formic acid
B ddH_2O + 0.1% trifluoroacetic acid	B ddH_2O	B Acetonitrile + 0.1% trifluoroacetic acid
C ddH_2O	C ddH_2O	C ddH_2O + 0.1% formic acid
D ddH_2O	D ddH_2O	D ddH_2O + 0.1% trifluoroacetic acid

Table 3. Agilent RapidFire high-throughput MS system methods to vary acid compositions.

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

Table 4. Sequences for acid and cartridge optimization experiment.

Sequence Number	Wells
1	A1 to A12
2	A1 to A12
3	A1 to A12
4	A1 to A12
5	B1 to B12
6	B1 to B12
7	B1 to B12
8	B1 to B12

Table 5. Batch for acid and cartridge optimization experiment.

Sequence Number	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). Accordingly, future experiments used 0.1% TFA in both the wash and the elution buffers.

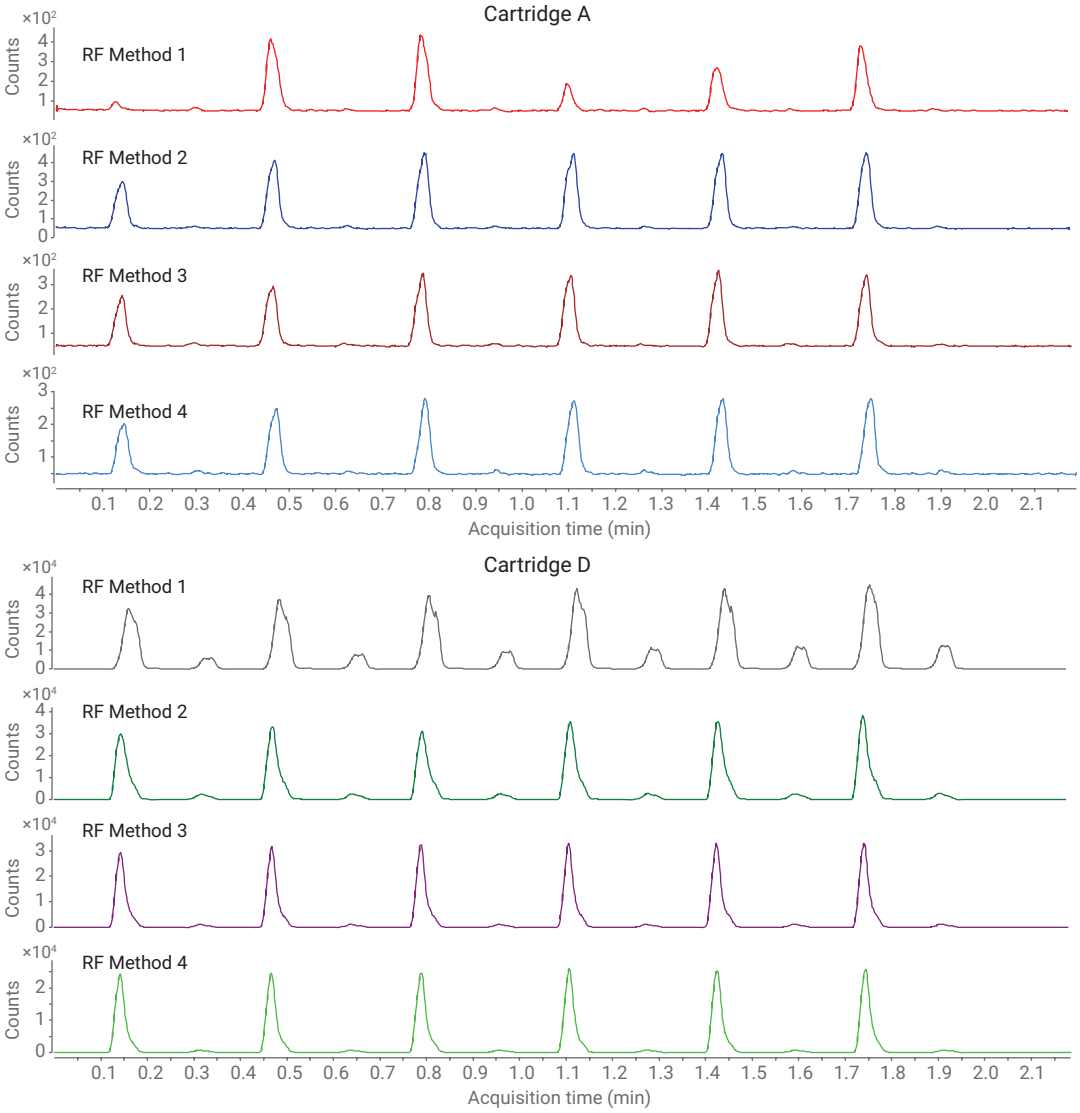


Figure 1. Example data acquired from the eight sequences run in experiment 1.

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).

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.

Table 6. Agilent RapidFire high-throughput MS system method setup for acetonitrile composition experiment.

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

Table 7. Sequence map for experiment 2.

Sequence Number	Wells
1	C1 to C12
2	C1 to C12
3	C1 to C12
4	D1 to D12
5	D1 to D12
6	D1 to D12

Table 8. Batch setup for experiment 2.

Sequence Number	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

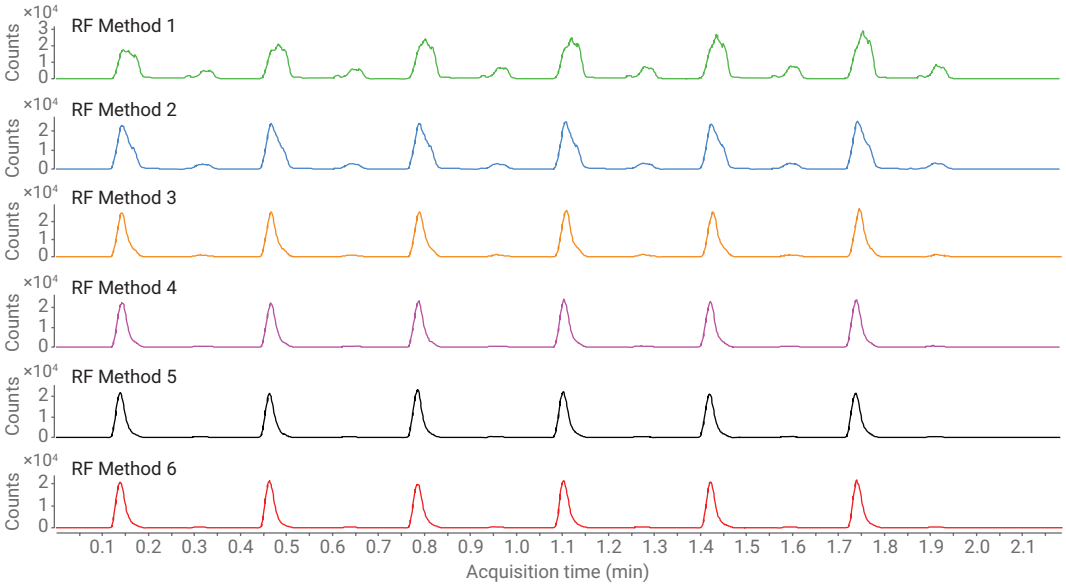


Figure 2. Example data acquired under acetonitrile concentrations ranging from 100% to 50%, showing dramatic alterations in peak shape and carryover.

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 ddH_2O + 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).

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

As shown 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.

Table 9. Agilent RapidFire 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 2: 2,500 3: 4,000 4: 500	1: 600 2: 3,000 3: 3,000 4: 500	1: 600 2: 3,000 3: 4,000 4: 500	1: 600 2: 3,000 3: 5,000 4: 500	1: 600 2: 3,000 3: 6,000 4: 500	1: 600 2: 3,000 3: 7,000 4: 500

Table 10. Sequence map for experiment 3.

Sequence Number	Wells
1	E1 to E12
2	E1 to E12
3	E1 to E12
4	F1 to F12
5	F1 to F12
6	F1 to F12

Table 11. Batch setup for experiment 3.

Sequence Number	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

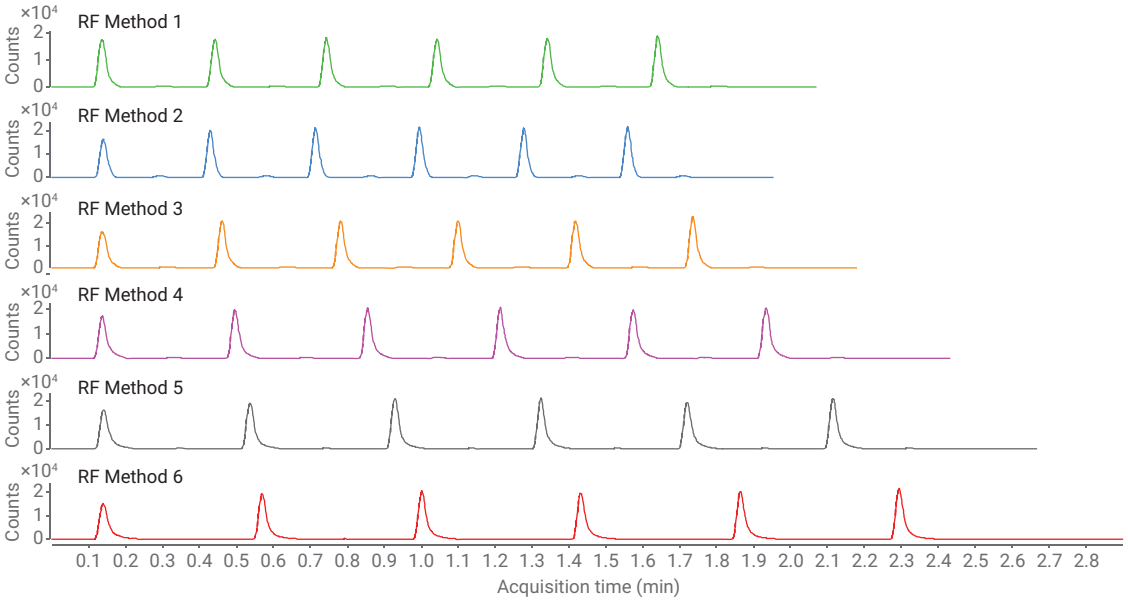


Figure 3. Example data showing the effects of various wash and elution state lengths.

Results and discussion

An Agilent RapidFire 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.

Conclusion

The speed of a RapidFire 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 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.

www.agilent.com/chem

DE44343.3796990741

This information is subject to change without notice.

© Agilent Technologies, Inc. 2014, 2021
Printed in the USA, July 8, 2021
5991-5222EN

Agilent
Trusted Answers

Technical Overview

Minimization of the Required Sample Volume for Agilent RapidFire High-Throughput Mass Spectrometry Systems

Author

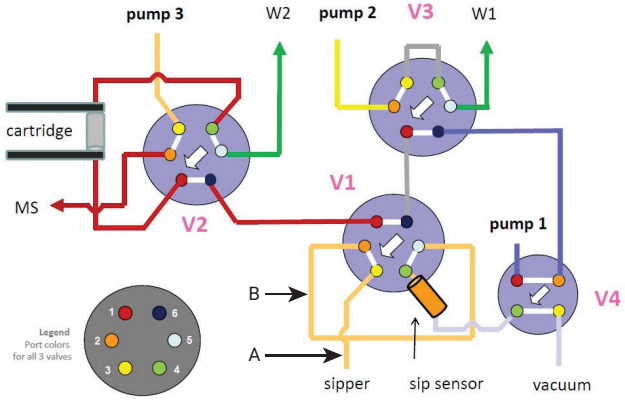
Peter Rye, Ph.D.
Agilent Technologies, Inc.

Abstract

This application note describes analysis with the Agilent RapidFire high-throughput MS system using tubing with a smaller inner diameter (id). This way, the necessary sample volume could be minimized. Two small id tubing configurations were successfully applied within this methodology, reducing the sample requirements to just 10 and 5 µL per well. The optimized configurations displayed excellent reproducibility across whole 96- and 384-well plates, producing data with coefficients of variation (CV) between 2.3 and 4.0%.

Introduction

The Agilent RapidFire high-throughput mass spectrometry (MS) system is designed to perform online SPE of samples with maximum speed. Consistent with these goals, the id of the tubing used to collect each sample is intentionally large, allowing the liquid from each well to be rapidly sipped by aspiration; sample collection from each well is regularly achieved in less than 200 ms. A consequence of using sample collection tubing (composed of a sipper tube and a sample loop, Figure 1) with a relatively large id is that the volume of liquid sampled from each well is relatively large. Typically, 10 µL of sample is used for each measurement, but dead volume before (in the sipper tube) and after (for the sip sensor) the sample loop results in a total consumption of ~35 µL per sample. This study evaluated the potential for decreasing RapidFire sample consumption, primarily using sample collection tubing with the same outer diameter (od) but a smaller id. A robust method requiring only 5 µL per well, for 384- and 96-well formats, was achieved, representing a seven-fold reduction in sample needs.



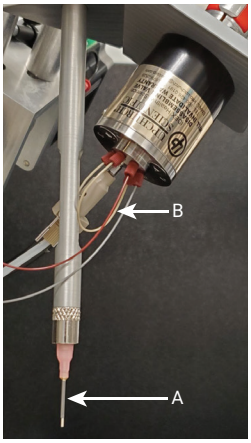


Figure 1. The Agilent RapidFire high-throughput MS comes standard with a beige sipper tube (A) and sample loop (B). The id of beige tubing is 0.015". This study replaced the sipper tube and sample loop with smaller id tubing (either gray with id 0.009" or red with id 0.005") to decrease the amount of sample required per well.

Experimental

Necessary parts and implementation

For 10 µL operation

Part Number	Quantity	Description	Notes
RF0052T	1	0.5 inch guide needle	Replaces 1.5 inch guide needle
RF0111T- 8	1	8 inch tubing, 0.009 inch id, gray	Cut to length (Table 1)
RF0094T	1 (10 pk)	Ferrule, 1/32 inch od, red	One per connection

For 5 µL operation

Part Number	Quantity	Description	Notes
RF0052T	1	0.5 inch guide needle	Replaces 1.5 inch guide needle
RF0112T-13	1	12 inch tubing, 0.005 inch id, red	Cut to length (Table 1)
RF0094T	1 (10 pk)	Ferrule, 1/32 inch od, red	One per connection

- Use the Agilent RapidFire sipper configuration wizard to teach the plate positions with the new needle configuration.
- Since vacuum levels can vary between labs, the optimal sip time for any volume must be empirically determined. Use the procedure described below to optimize the sip time for MS signal intensity and reproducibility.
- Best results were achieved using Greiner V-bottom 384-well plates (p/n 781280) and Greiner V-bottom 96-well plates (p/n 651201).

Modifications to RapidFire hardware

The RapidFire comes standard with a blunt ended 1.5" sipper guide needle, which allows the sipper to reach the bottom of deep-well plates, if necessary. For all the experiments, this needle was replaced with a 0.5" blunt ended needle (p/n RF0052T). The standard beige color (0.015" id) sipper tube and sample loop (Figure 1) were replaced with tubing that was either gray (0.009" id, p/n RF0111T-8) or red (0.005" id, p/n RF0112T-13). While mixing the id of the sipper tubing and the sample loop tubing could be useful, these remained matched in this study (both beige, gray, or red). Table 1 shows how the length and id of the sample collection tubing relates to the inner volume of the configuration.

Table 1. Relationship between tubing length, inner diameter (id), and inner volume.

	Beige Tubing (1/32" od × 0.015" id)	Gray Tubing (1/32" od × 0.009" id)	Red Tubing (1/32" od × 0.005" id)
Sipper Tube (4.5 inches)	~13.2 µL	~4.7 µL	~1.5 µL
Sample Loop (3.5 inches)	~10.0 µL	~3.6 µL	~1.1 µL
Total (8 inches)	~23.2 µL	~8.3 µL	~2.6 µL

Table 2. Evaluation of sipping behaviors using beige, gray, and red tubing configurations.

	Beige Tubing	Gray Tubing	Red Tubing
Time to Sip 1 mL Water	~23 seconds	~1 minute	>10 minutes
Time to Trigger Sip Sensor	~190 milliseconds	~180 milliseconds	~1,500 milliseconds

Results and discussion

Sipping behavior

It is possible for the efficiency of RapidFire sipping to be affected by clogs, insufficient vacuum, or loose/overtightened ferrules. In these cases, it is common to evaluate the RapidFire sipping behavior by timing how long it takes to aspirate 1 mL of water. In the standard configuration with beige tubing, ~23 seconds is typical and indicative of an unobstructed sample collection path. For comparison, the time required to sip 1 mL of water using beige, gray, and red tubing configurations was tested. Table 2 shows 1 mL sip times averaged from six replicates were ~23 seconds, ~1 minute, and >10 minutes, respectively. As liquids travel easier through less constrictive capillaries, this general trend was expected.

The sipping behavior of all three configurations was also characterized by averaging the sip time, as recorded by the sip sensor, across 16 sample replicates. The standard beige configuration showed an average sample sip time of ~190 ms, while the gray and the red sample sip times were ~180 and ~1,500 ms, respectively (Table 2). These results were expected, as the wide differences in sipping efficiencies were offset by the total sipping volumes. For example, even though the time to sip 1 mL using gray tubing was nearly threefold greater than the time for beige tubing, the corresponding sample volume sipped using the gray tubing was nearly threefold less. The net effect was a roughly equal sample sip time for both the beige and gray tubing configurations.

Optimization of sample volume and sip time

To optimize the sample volume and sip time, the RapidFire was plumbed for direct injection ("blaze") mode and samples containing S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), or both, were run. The plates used were either 384- (Greiner V-bottom, 781280) or 96-well (Greiner V-bottom, 651201) and centrifuged briefly prior to analysis to ensure that the liquid was at the well bottom. A sipper safe height of 1 mm was used. MS detection was conducted using the Agilent 6495C triple quadrupole MS.

Sample requirements can be reduced by a couple µL when the RapidFire sip sensor is disabled. In place of the sip sensor, the sip time for each configuration was optimized by measuring the MS signal as a function of RapidFire aspiration time and sample volume per well.

Using the optimization of the beige tubing configuration as an example, 30 µL of 500 nM SAM was added to each well, across multiple columns of a 384-well plate. Each column provided 16 replicates, and was analyzed using a different sip time (50, 75, 100, 125, 150, 175, and 200 ms). The average peak area for SAM was determined for each column and plotted. Once reproducible MS results for one or more sip times were confirmed, the experiment was repeated with a smaller volume per well (27.5, 25, or 22.5 µL). In this fashion, the optimization of sample volume and sip time was determined while monitoring the reproducibility of each condition.

The expectations in these experiments (Figure 2) were that sip times that were too short would result in less MS signal because the sample loop would not have time to fill completely. Likewise, sip times that were too long were also expected to result in less MS signal because some (or all) sample would have been aspirated through the sample loop to waste. The optimal sip time was therefore volume-dependent, where smaller sample volumes made the optimal sip time window narrower. Experimental results were consistent with these expectations.

Ultimately, each tubing configuration was optimized such that the required sample volume was just a couple µL more than the total tubing volume (Table 3). For the red tubing configuration, the optimized sample volume was just 5 µL per well, for both 384- and 96-well formats. These results represent a seven-fold reduction in sample requirements compared to when the RapidFire is used with the standard beige tubing and sip sensor enabled.

Reproducibility

Full 96- and 384-well plates were run to more thoroughly examine the robustness of each low sample volume method (Table 3). For each run, the plates were supplemented with a 2:1 mixture of SAM:SAH, and the area ratio was plotted for each injection. While the ratio of SAM to SAH was 2:1, the MS response factor for SAM was slightly greater than that for SAH, resulting in an average area ratio of ~2.3:1. Results showed excellent reproducibility for each configuration, with CVs between 2.3 and 4.0% (Figures 3, 4, and 5). No wells were missed during these analyses.

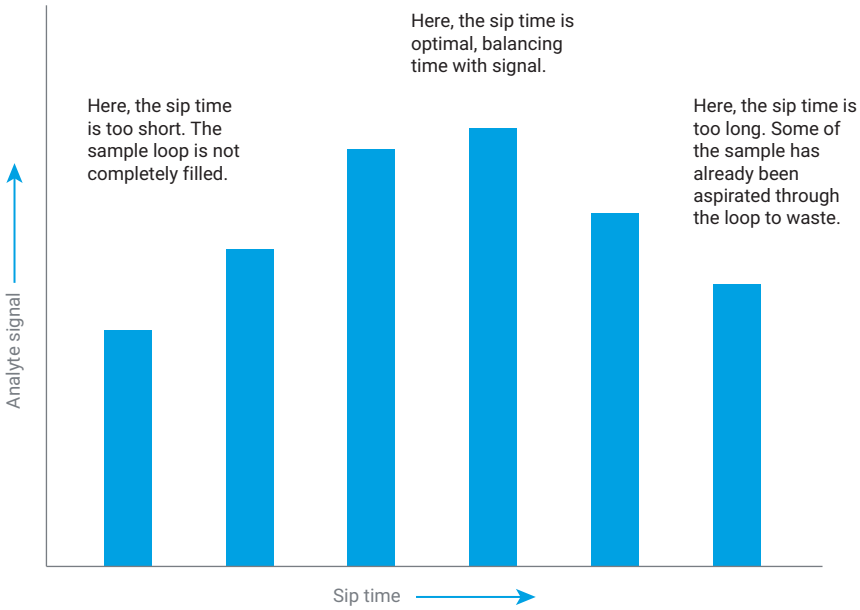


Figure 2. Analyte MS signal as a function of sip time. When the RapidFire sip sensor is off, which was done in this study to decrease material needs, the sip time must be optimized. If the sip time is too short, the sample loop will not be filled completely. If the sip time is too long, the liquid in the well will get aspirated all the way through the sample loop and leave it partially/completely empty. Determination of the sip time range that renders the sample loop full is critical for MS signal intensity and reproducibility.

Table 3. Optimized sample volume and sip time for the beige, gray, and red tubing configurations.

	Beige Tubing	Gray Tubing	Red Tubing
Optimized Sample Volume per Well	25 µL	10 µL	5 µL
Optimized Sip Time (Sip Sensor Off)	125 milliseconds	125 milliseconds	1,250 milliseconds

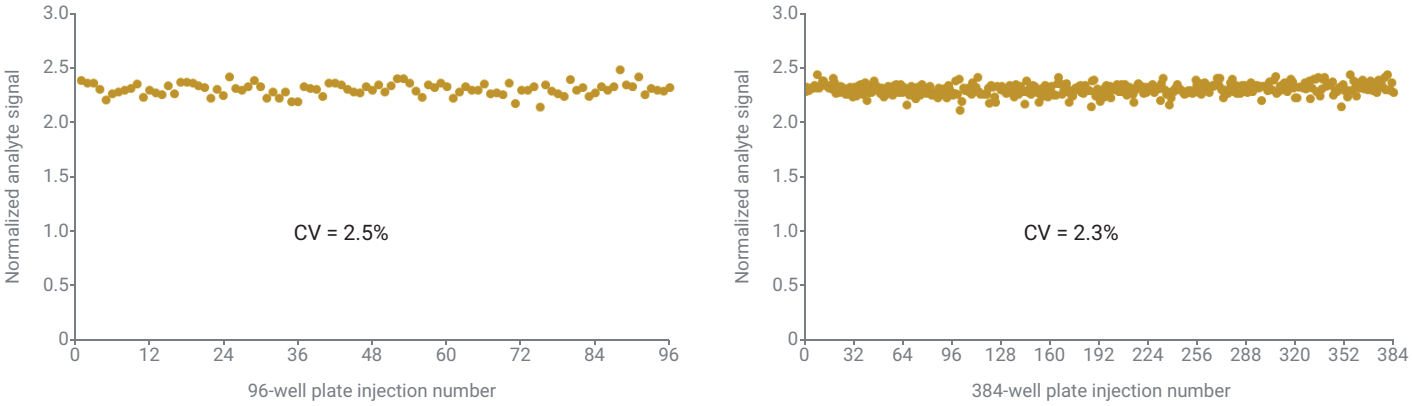


Figure 3. Reproducibility data from 96- (left) and 384-well (right) plates, for the optimized beige tubing configuration (25 µL per well).

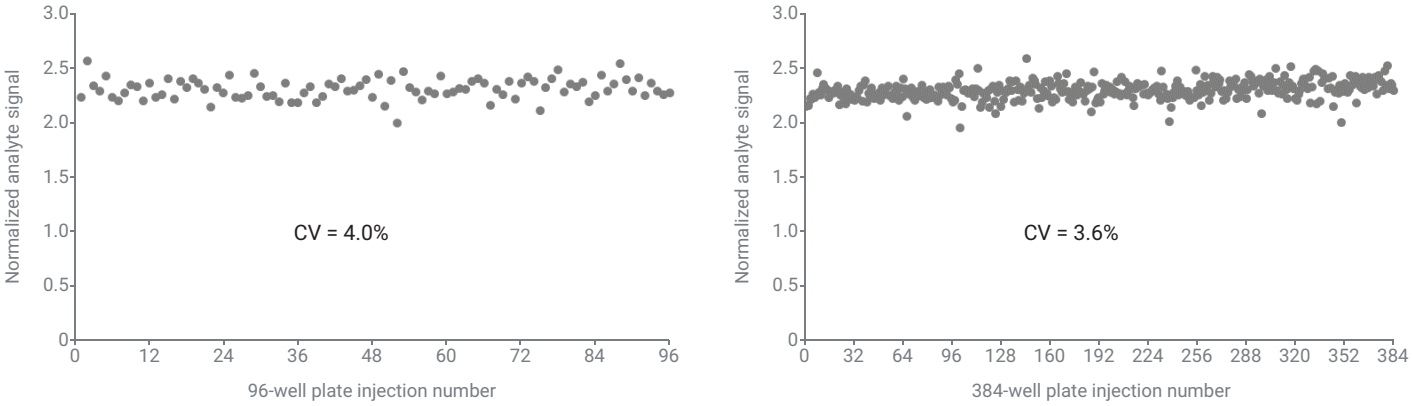


Figure 4. Reproducibility data from 96- (left) and 384-well (right) plates, for the optimized gray tubing configuration (10 µL per well).

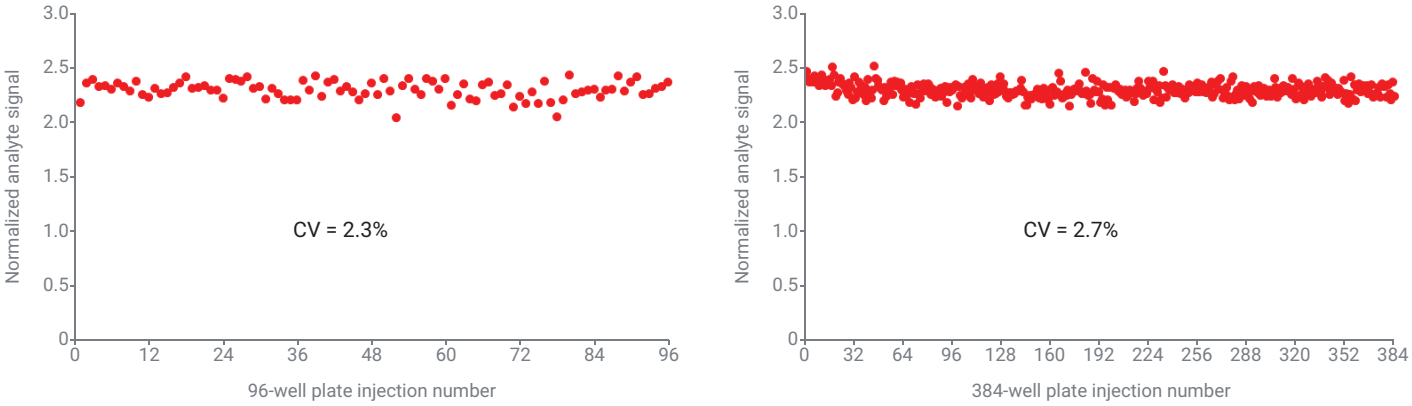


Figure 5. Reproducibility data from 96- (left) and 384-well (right) plates, for the optimized red tubing configuration (5 µL per well).

Conclusion

The goal of these studies was to use nonstandard sample collection tubing with a smaller id to decrease the material consumption of Agilent RapidFire MS analyses. Two smaller id tubing configurations were tested, and each was successfully optimized to decrease sample needs. In comparison to the standard beige tubing configuration, which requires 35 µL sample per well with the sip sensor is enabled, the gray and red tubing configurations decreased sample needs to 10 and 5 µL per well, respectively. These optimized methods performed reproducibly across entire plates and provided data with low CV.

www.agilent.com/chem

DE44243.1229398148

This information is subject to change without notice.

© Agilent Technologies, Inc. 2019, 2021
Printed in the USA, February 23, 2021
5994-1679EN

Agilent
Trusted Answers

Application Note

Agilent
Trusted Answers

High-throughput, Ion-Pairing-Free, HILIC Analysis of Oligonucleotides Using Agilent RapidFire Coupled to Quadrupole Time-of-Flight Mass Spectrometry

Author

Peter Rye, PhD
Agilent Technologies, Inc.

Abstract

This application note describes a high-throughput, ion-pairing-free method for oligonucleotide characterization using the Agilent RapidFire high-throughput MS system. The HILIC-based method achieves a 12-second cycle time, and demonstrates high robustness and reproducibility. Results include the identification of impurities less than 0.5% of the target, detection limits in the single-digit nanomolar range, and a linear concentration response over more than three decades. Analysis of nine unique oligonucleotides, comprising both unmodified and heavily modified components, illustrates that the method is highly versatile for samples with unique chemistries.

Introduction

LC/MS methods for the analysis of oligonucleotides (oligos) have traditionally been based on ion-pairing reverse-phase (IPRP) chromatography, because this approach generally delivers good separation and MS response in negative mode. However, considering that many ion-pairing reagents can present a memory effect which can diminish the performance of the system in positive mode, IPRP methods can be burdensome for mixed-use systems, pushing many laboratories to seek ion-pairing-free alternatives. In this work, a high-throughput, ion-pairing-free method for oligo characterization using an Agilent RapidFire 6545XT MS system is presented. This method leverages the Agilent HILIC-Z resin and MS-friendly, ammonium acetate-based mobile phases, which allow for subsequent positive mode use of the system without flushing or hardware changes. The method achieves a 12-second cycle time, along with the robustness, reproducibility, dynamic range, and sensitivity that are sought after for high-quality oligo characterization. Tests also demonstrate that the method is equally effective for unmodified and heavily modified oligos, including antisense (ASO) and aptamer samples.

Experimental

Analytical methods and samples

The RapidFire/Q-TOF instrument consists of an Agilent RapidFire 365 high-throughput MS system coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF equipped with an Agilent Jet Stream source. A HILIC cartridge (type H6, 4 µL bed volume, G9527) was used for online solid phase extraction. Data acquisition was performed with RapidFire Acquisition software, version 6.1, and MassHunter acquisition software for LC/MS systems, version 10.1. The RapidFire and MS methods used for this study are detailed in Table 1. LC/MS grade acetonitrile was sourced from Agilent. Water was sourced from a Milli-Q system. Mobile phase A (MPA) and mobile phase B (MPB) were prepared without any pH adjustments. All injection volumes in this study were 10 µL.

Following sampling by the RapidFire, samples were delivered to the cartridge and desalted using MPA at 1 mL/min for 5,000 ms (20 cartridge volumes of wash). The desalted oligo mixture was then eluted to the MS for measurement using MPB at 0.5 mL/min for 4,000 ms (eight cartridge volumes). The resulting chromatographic peaks were approximately 6 seconds wide and composed of 24 unique spectra.

The cartridge was then re-equilibrated with MPA at 1 mL/min for 500 ms (two cartridge volumes) before introduction of the next sample. There was insignificant benefit to longer load/wash, elute, or re-equilibration times (data not shown). The optimized method, including plate movements, sustained a 12-second cycle time.

Following acquisition, the MS data files were automatically parsed by the RapidFire software into individual injection files. Extracted ion chromatogram and Maximum Entropy deconvolution techniques were used in MassHunter BioConfirm software, version 10.0, for analysis.

Table 1. RapidFire and 6545XT MS methods used in this study.

RapidFire Conditions		
Cartridge	HILIC (PN G9527)	
Cartridge Temperature	Room temperature	
Injection Volume	10 µL	
Pump 1	MPA = 85% acetonitrile + 15 mM ammonium acetate	1.0 mL/min
Pump 2	MPB = 50% acetonitrile + 15 mM ammonium acetate	1.25 mL/min
Pump 3	MPB = 50% acetonitrile + 15 mM ammonium acetate	0.5 mL/min
State 1	Aspirate sample (sip sensor on)	600 ms
State 2	Load/wash (desalt)	5,000 ms
State 3	Extra wash	0 ms
State 4	Elute (inject)	4,000 ms
State 5	Reequilibrate	500 ms
6545XT Q-TOF Conditions		
Ion Polarity	Dual AJS Negative	
Data Storage	Both (Centroid and Profile)	
Gas Temperature	300 °C	
Drying Gas Flow	11 L/min	
Nebulizer Gas	35 psi	
Sheath Gas Temperature	350 °C	
Sheath Gas Flow	11 L/min	
Capillary Voltage	3,500 V	
Nozzle Voltage	2,000 V	
Fragmentor	175 V	
Skimmer	65 V	
Oct 1 RF Vpp	750 V	
Mass Range	100 to 3,200 *m/z*	
Acquisition Rate	4 spectra/sec	

Oligos used in this study (Table 2) were purchased from Integrated DNA Technologies (Coralville, Iowa) with standard desalting purification. Products were resuspended in water to make 1 mM stocks and diluted in MPA for analysis. See the individual experimental sections for the final concentrations used.

Results and discussion

Oligos of different sizes

To assess the applicability of the HILIC RapidFire/Q-TOF method to oligos of different sizes, an 18-mer (PR8), a 40-mer (PRL40), and a 60-mer (PRL60) were analyzed. A 10 µM sample (100 pmol on cartridge) of each oligo was analyzed, and results were compared to data previously collected using the IPRP technique.[1] Figure 1 shows ions for several expected charge states which were observed in the HILIC data (A) and the IPRP data (B) for each sample. Furthermore, several unique spectral qualities were observed in the RapidFire data.

First, the IPRP conditions resulted in a much wider charge state envelope for each oligo. In some cases, a bimodal distribution was observed; this is best exemplified by the 40-mer data in Figure 1B. These wide, bimodal distributions are thought to stem from portions of the oligo remaining in native conformation (leading to lower-charged species), while other portions are in a denatured conformation (facilitating formation of the higher-charged species). Spectra collected under HILIC conditions showed a much narrower charge state distribution shifted towards the less-charged species, suggesting that these oligos were maintained in their native state. This behavior would be consistent with observations from other mass spectrometry techniques, for example, analysis of native proteins in which ammonium acetate is commonly used.

Second, the IPRP conditions resulted in the larger oligos showing more charges than their smaller counterparts, resulting in a relatively consistent *m/z* range for spectral ions from oligos of different sizes. In fact, the most abundant charge state for the 18-mer (−4 at *m/z* ~1,375) had a higher *m/z* value than the most abundant charge state for the 60-mer (−19 at *m/z* ~970). In the case of the HILIC conditions, the *m/z* value for the most predominant charge state of each oligo trended higher as the oligo size increased. Again, this result is consistent with the HILIC conditions preserving a native folded state of the oligo, and charge-charge repulsion deterring the formation of higher-charged species. Strategies to mitigate this effect are required for the analysis of larger oligos on mass spectrometers with limiting *m/z* range. These studies are underway and will be described elsewhere.

Table 2. Oligonucleotides used in this study and their associated code notations. All sequences are written in the 5' to 3' orientation.

Name	Length	Approx. Molecular Weight	Sequence
PR1	20	6148	AGAGTTTGATCCTGGCTCAG
PR3	20	6007, 6031, 6047	TTTTTTTTTTTTTTTTTTTV
PR5	24	7289	CGCCAGGGTTTTCCCAGTCACGAC
PR7	21	6101	/5Phos/TTTTTTTTTTTTTTTTTTTT
PR8	18	5505	CTAGTTATTGCTCAGCGG
PRL40	40	12278	CTAGTTACTTGCTCAGCGGACTAGTTACTTGCTCAGCGGA
PRL60	60	18448	CTAGTTACTTGCTCAGCGGACTAGTTACTTGCTCAGCGGACTAGTTACTTGCTCA GCGGA
ASO	18	7127	/52MOErT/*/i2MOErC/*/i2MOErA/*/i2MOErC/*/i2MOErT/*/i2MOErT/* /i2MOErT/*/i2MOErC/*/i2MOErA/*/i2MOErT/*/i2MOErA/*/i2MOErA/* /i2MOErT/*/i2MOErG/*/i2MOErC/*/i2MOErT/*/i2MOErG/*/32MOErG/
Aptamer	28	9116	/52FC/mGmGrArA/i2FU//i2FC/mAmG/i2FU/mGmAmA/i2FU/mG/i2FC// i2FU//i2FU/mA/i2FU/mA/i2FC/mA/i2FU//i2FC//i2FC/mG/3InvdT/

Code	Description
*	Phosphorothioate bond
A	2'-deoxyribose adenine
C	2'-deoxyribose cytosine
G	2'-deoxyribose guanine
T	2'-deoxyribose thymine
mA	2'-O-methyl A
mG	2'-O-methyl G
rA	Ribose adenine
rG	Ribose guanosine
V	Mixed C, A, and G
/3InvdT/	3' inverted T

Code	Description
/32MOErG/	3' methoxyethoxy G
/5Phos/	5' phosphate
/52FC/	5' Fluoro C
/52MOErT/	5' 2-methoxyethoxy T
/i2FC/	Internal Fluoro C
/i2FU/	Internal Fluoro U
/i2MOErA/	Internal 2-methoxyethoxy A
/i2MOErC/	Internal 2-methoxyethoxy C
/i2MOErT/	Internal 2-methoxyethoxy T
/i2MOErG/	Internal 2-methoxyethoxy G

To compare the MS signal intensities for oligos of different sizes, scaling was removed by linking their Y-axes. Figure 2A shows that the HILIC conditions resulted in a significant drop in *m/z* ion intensities as the size of the oligo increased. More specifically, the height of the most predominant charge state for the 60-mer was approximately 25-fold less than that of the 18-mer. Similarly, comparison of the deconvolution peak heights (Figure 2B) shows an approximate 25-fold drop from the 18 to 60-mer. By comparison, the deconvolution peak heights for the 18-, 40-, and 60-mer, when run by IPRP, were within 2-fold of the lowest (data not shown).

These general observations have been made before. Specifically, Lobue *et al.* previously demonstrated that, in comparison to IPRP, HILIC analyses of oligos can result in (1) a narrower charge state distribution, (2) a most predominant charge state of lower charge, and (3) a right-shifting of the most predominant charge state as the oligo size increases.

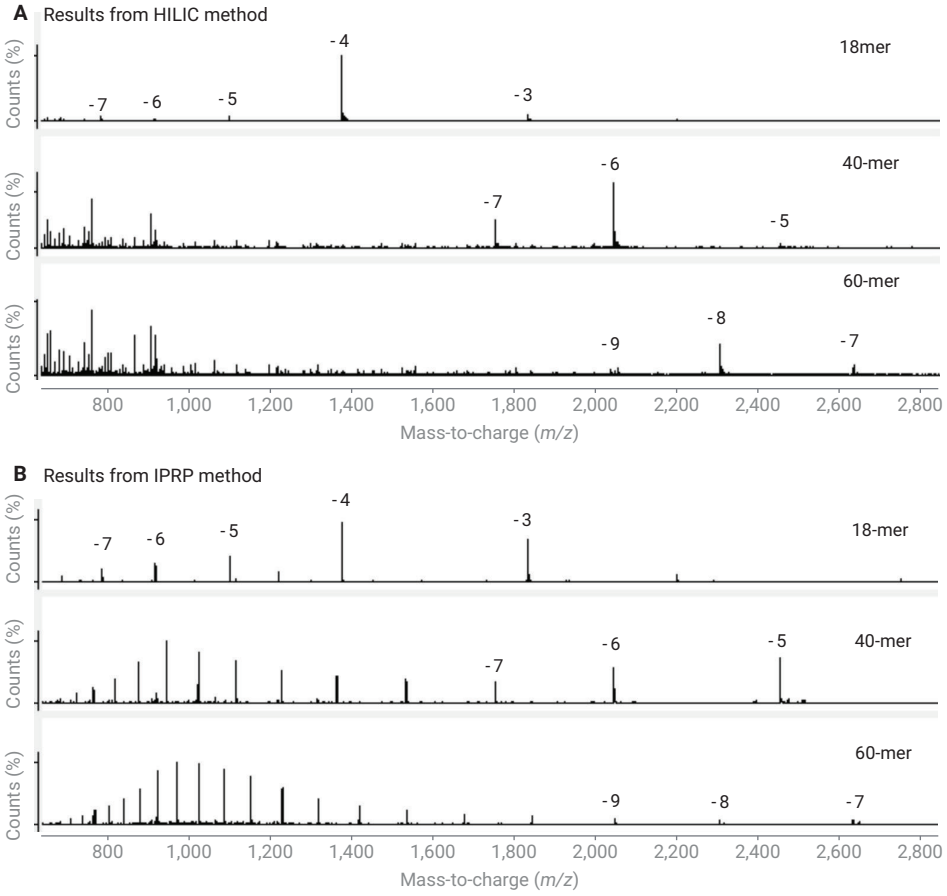


Figure 1. Raw *m/z* spectra for an 18-mer (top), a 40mer (middle), and a 60-mer (bottom) run by HILIC method (A) and, for comparison, an IPRP method (B). All spectra were each scaled to the largest peak within it. The predominant charge state clusters for HILIC method are labeled for each oligo.

Reproducibility

To test reproducibility of the HILIC RapidFire/Q-TOF method, 24 replicate injections of a poly-dT oligo with a 5' phosphate (PR7) were run and deconvoluted using an automated analysis method in BioConfirm. The resulting deconvolution spectra were then scaled to the largest peak in each spectrum and overlaid. The results demonstrate excellent reproducibility of the relative abundances within each sample, as the 24 spectra are superimposed near-perfectly (Figure 3). The total ion chromatograms for the replicates (Figure 3 insert) reveal consistent peak height and shape, illustrating that the absolute MS signals, in addition to the relative signals, are stable across many injections.

Determination of impurities

Oligo samples often contain a high number of low-abundance impurities, including truncated synthesis products, depyrimidations, and depurinations. It is therefore critical that analytical methods for oligo characterization demonstrate a wide dynamic range for impurity detection. This situation can be especially true for nonchromatographic methods, because the calculated purity can be overestimated if low abundance impurities are not detected in the presence of the highly abundant target oligo.

The 6545XT mass spectrometer used in this study was established to provide up to five orders of spectral dynamic range. Still, the dynamic range for this application was evaluated by comparing the relative deconvoluted peak heights of vastly different intensities for several samples.

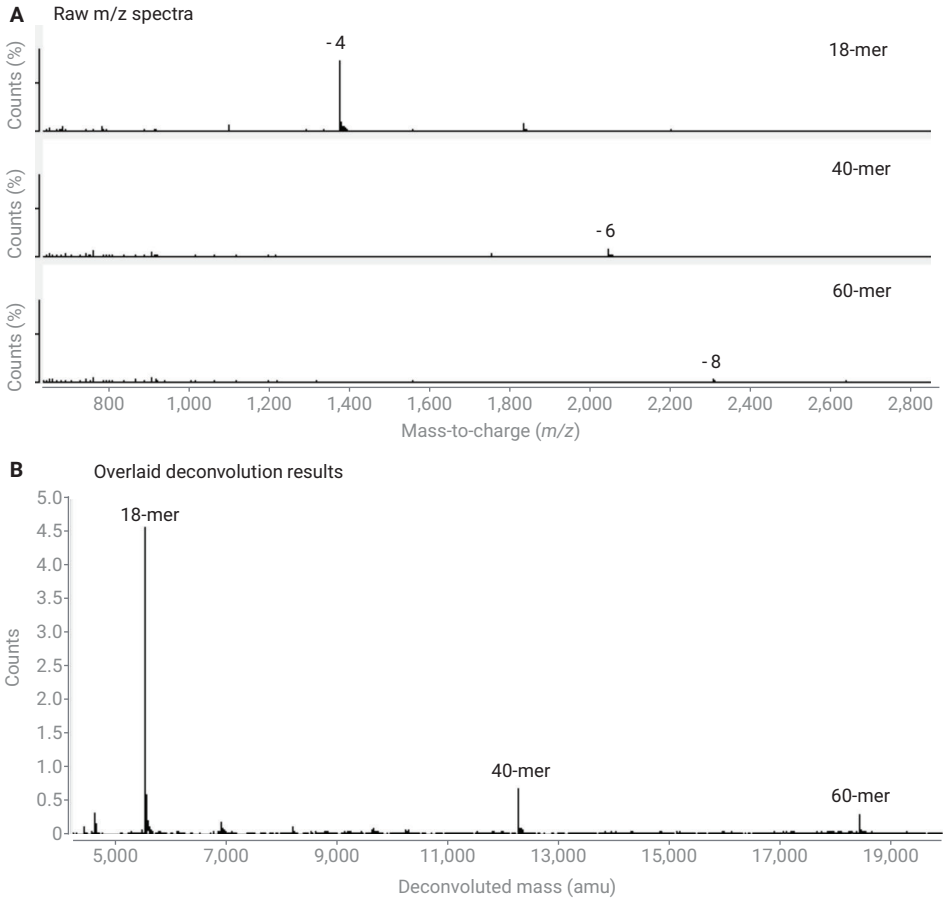


Figure 2. Comparison of MS signal intensities for an 18-mer, a 40-mer, and a 60-mer. Raw *m/z* spectra with linked Y-axis (A) and overlaid deconvolution results (B).

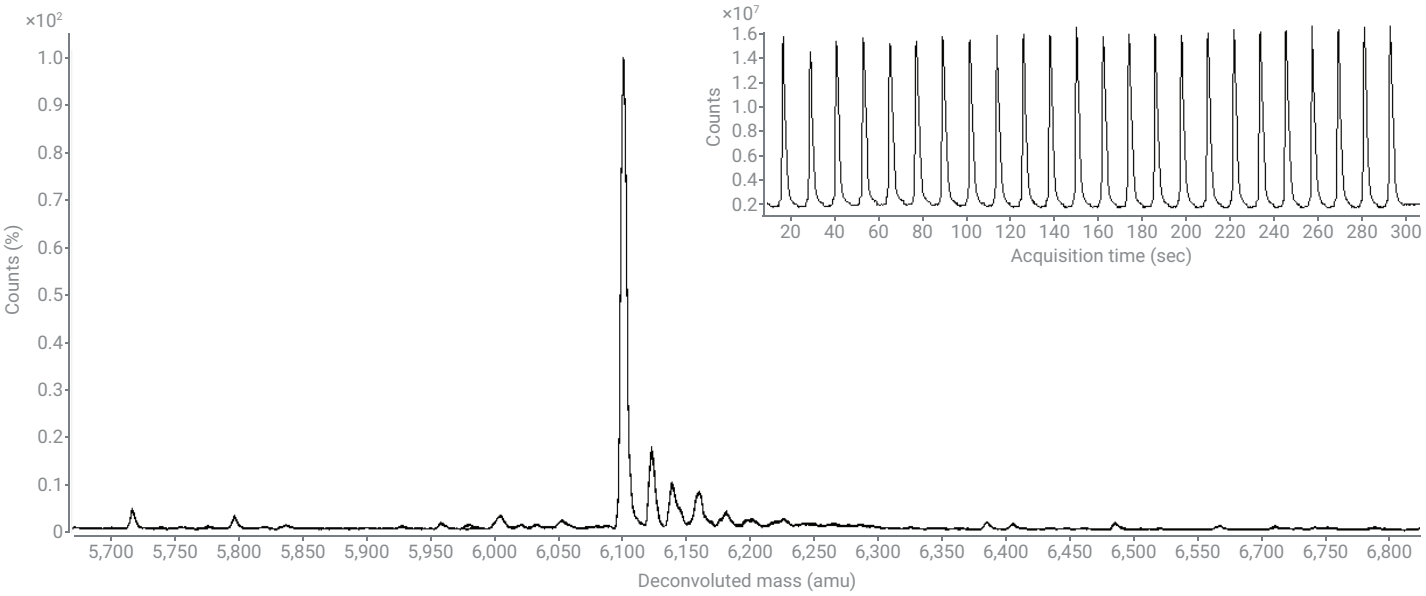


Figure 3. Reproducibility of total ion chromatogram (inset) and deconvolution results (main figure) for 24 replicate samples.

Figure 4 shows the deconvolution spectra from a 10 µM injection (100 pmol on cartridge) of a 20-mer DNA strand (PR1). The most abundant peak has a mass of 6,148 Da, consistent with the calculated mass of the target oligo. Several commonly observed metal adducts are present at masses larger than the target, and a large number of lower mass impurities are also observed. Close inspection of the mass ranges where commonly observed depurination (depur) and truncation (trunc) impurities were expected revealed several low-abundance peaks. Based on their mass differences from the target, 5' truncation of A, gas phase depurination of G, and hydrolytic depurination of G could all be assigned. The peak heights of the depurination impurities had a relative abundance of less than 1% of the target.

In some cases, it is necessary to analyze oligo mixtures containing individual components that are close in mass. To evaluate the ability of the HILIC RapidFire/Q-TOF method to mass resolve mixtures and their respective impurities of the components, 10 µM (100 pmol on cartridge) of a 20-mer poly-dT oligo containing a 3' variable base (C, A, or G) was injected (PR3). The *m/z* spectrum shown in Figure 5A illustrates multiple expected charge states, and the inset figure reveals good mass resolution of the isotopes for the –4 species. The deconvoluted result shown in Figure 5B clearly shows three predominant peaks that match the expected masses and relative abundances of the oligo with either C, A, or G on the 3' end. Moreover, for each of these species, the n-1 and n-2 impurities were observed. Loss of the 5'-T from the three species resulted in peaks of minus 304 Da, and loss of the 5'-TT from the three species resulted in peaks of minus 608 Da (304 + 304). For the oligo with a 3' G, comparison of the

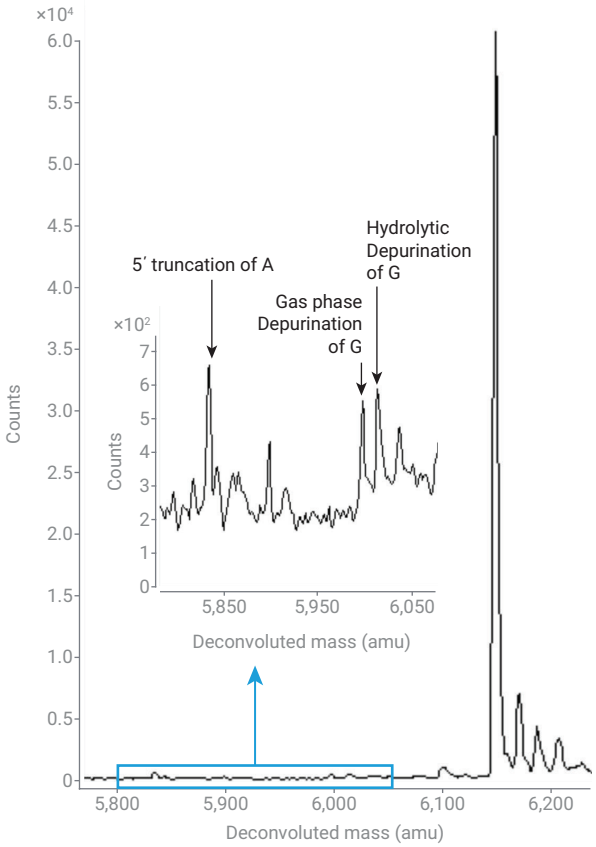


Figure 4. Identification of impurities in PR1.

peak heights corresponding to the target (2.5E5 counts) and loss of 5'-TT impurity (0.75E3 counts) demonstrates that 0.3% impurities are readily observed, despite the sample and spectral complexity.

Another noteworthy observation from this experiment was that the spectrum for the poly-dT oligo had a relatively wide distribution of charge states. Based on observations and discussion above, it appears poly-dT oligos do not readily adopt secondary structures that would otherwise reduce the charge states observed in the *m/z* spectrum.

Method sensitivity, linearity, and carryover

To evaluate the sensitivity and linearity of the HILIC RapidFire/Q-TOF method, triplicate injections for eight concentrations, plus a zero, of PR7 were analyzed. Two-fold serial dilutions starting at 1,250 nM were made down to 9.7 nM using MPA. A zero-concentration sample was injected between each replicate so that carryover could be studied at each concentration over the range. The resulting data for all 54 injections were analyzed two ways. First, for the targeted MS measurement, the extracted ion chromatogram for the –4 charge state (*m/z* ~1,524) was generated, smoothed, and integrated. The replicate areas for each concentration were averaged and plotted against their concentration. The standard deviations of the values were represented by error bars on that same plot, shown in Figure 6A and 6B. Second, for the untargeted deconvolution results shown in Figure 6C, the extracted ion chromatogram for the –4 charge state

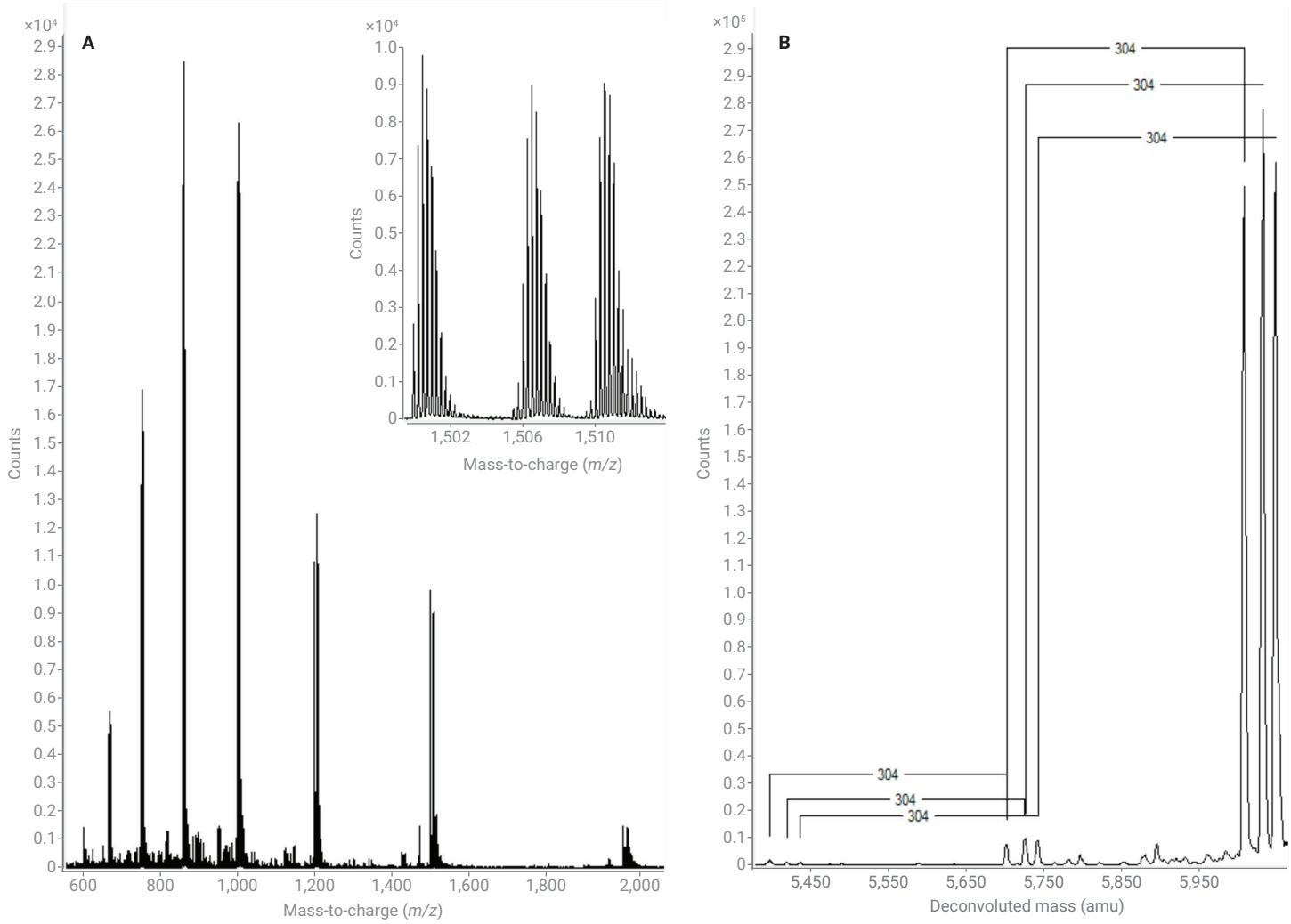


Figure 5. Identification of low abundance impurities in PR3 . Raw *m/z* spectra (A) and deconvolution results (B).

(*m/z* ~1,524) was generated, smoothed, and integrated. The average *m/z* spectra over the integrated peak was then extracted and deconvoluted.

Figure 6A shows that the oligo used in this study had a linear response over the nine concentrations studied, with an R^2 = 0.9988 for the best fit line. The blanks data shows a slope of ~2.8, versus ~192 for the samples, revealing less than 1.5% carryover across the concentration range. In subsequent experiments (data not shown), this value dropped to below 0.1% when the "blank injection in between each sample" feature of the RapidFire was selected.

However, because blanks between each sample double the cycle time, and the carryover without them satisfied the acceptance criteria, the additional blanks were deemed unnecessary. Focusing on the low end of the concentration data (Figure 6B), a clear difference can be seen in the AUC between the 0 and 9.7 nM concentrations. The signal-to-noise ratio was over 4 at 9.7 nM, almost 6 at 19.5 nM, and 28 at 39 nM. While the slope of the concentration response was much greater for IPRP conditions (2,898, data not shown) the signal-to-noise values were nearly identical to those from the HILIC conditions.

To test the limitations of measuring the target oligo in an untargeted fashion, the spectra for each concentration were deconvoluted. Representative results for the low concentration injections are shown in Figure 6C, and easily allow the determination of the target peak from low double-digit nM samples. These results indicate, as expected, that while targeted extraction provides more measurement sensitivity, untargeted deconvolution is still quite powerful for target identification from low concentration samples.

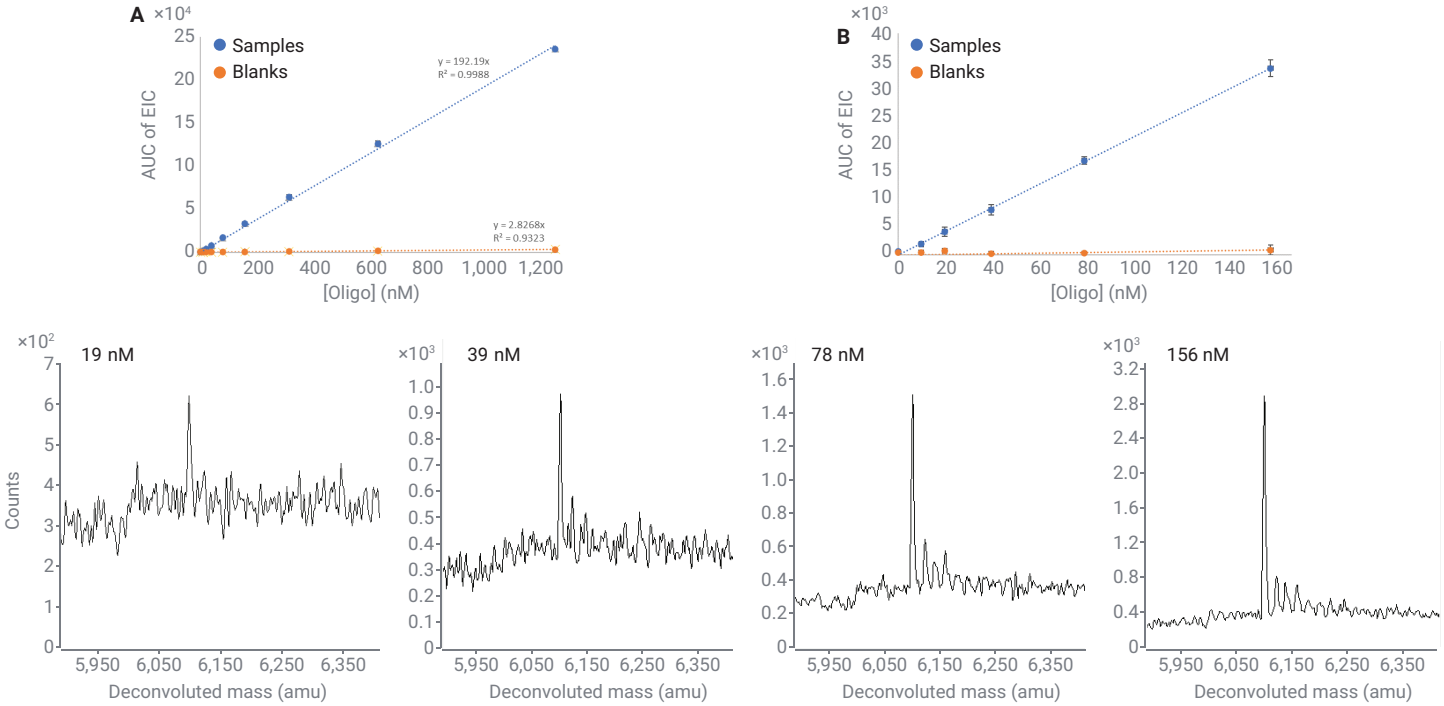


Figure 6. Concentration response of PR7 to evaluate method sensitivity, linearity, and carry over. The plot of signal against concentration (A), a zoom of the low concentration range (B) and deconvolution results (C).

Comparison of the sensitivity from HILIC versus IPRP methods, by others, has resulted in mixed reports. Lobue reported a greater MS signal response from HILIC conditions versus IPRP[2], attributing the gains to the higher organic content of the mobile phase under HILIC conditions, leading to more efficient desolvation. In other cases, less intense target peak heights under HILIC conditions versus IPRP have been blamed on increased levels of Na and K adduct ions. Further investigation is therefore required to compare the sensitivity of these techniques on a multitude of oligo sizes and chemistries, controlling for a wide host of acquisition and analysis parameters which can affect the result.

Method versatility

The chemistry of oligo samples can vary significantly. To evaluate the applicability of the HILIC RapidFire/Q-TOF method to oligos with different base compositions, linker types, and modifications, the data for a host of samples were acquired with the optimized method. These 10 µM samples included DNA strands (containing phosphodiester linkers and 5' phosphates), an ASO (containing phosphorothioate linkers and 2-methoxyethoxy building blocks), and an aptamer (containing inverted T, 2-methoxyethoxy groups, and fluorinated bases). The resulting deconvoluted spectra were each scaled to the largest peak and overlaid with each other. The results shown in Figure 7 reveal highly abundant target peaks, with excellent mass accuracy, for each sample. Common impurities could also be assigned for each sample (data not shown). These results illustrated that the HILIC RapidFire/Q-TOF method can provide high-quality data for a wide range of oligo types and chemistries in the 18- to 28-mer range.

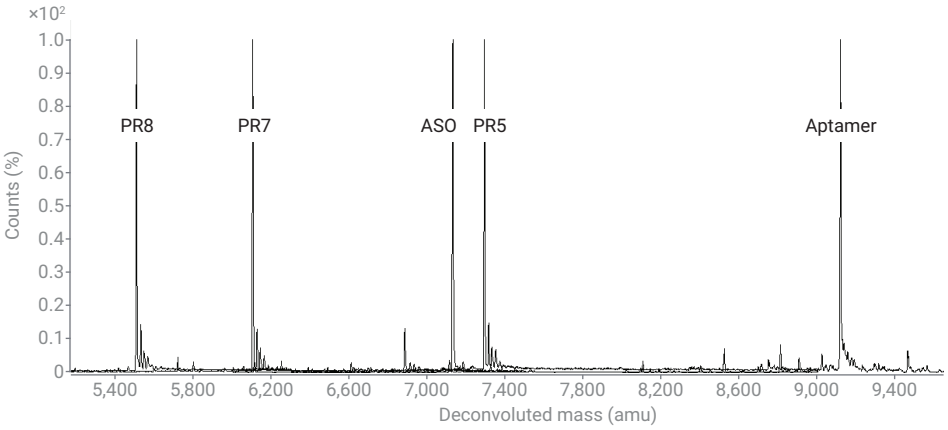


Figure 7. Overlaid deconvolution results for a wide variety of oligo chemistries run by RapidFire MS without ion pairing reagents.

Conclusion

The Agilent RapidFire high-throughput MS system, coupled to an Agilent 6545XT mass spectrometer, offers high-throughput oligo characterization by sustaining cycle times as fast as 12 seconds per sample during data acquisition. Acquisition methods include the previously described IPRP conditions[1], as well as the ion-pair-free HILIC conditions described here. The HILIC method was simple to set up and use, as it used standard Agilent products, and required no pH adjustments to the mobile phases.

The HILIC method displayed the robustness, reproducibility, dynamic range, and sensitivity that are sought after for high-quality oligo characterization. Tests on a variety of oligos illustrated high method performance on highly modified ASO and aptamer samples. Even though HILIC methods are commonly used for oligos approximately 25-mer in size, quality data on up to 60-mers were generated.

References

1. Rye, P. T.; Yang, Y. High-throughput Mass Spectrometry of Synthetic Oligonucleotides: A Comparison of Data from Fast LC and RapidFire Methods. *ASMS* **2020**. TP 434.
2. Lobue, P. A. *et al.* Oligonucleotide Analysis by Hydrophilic Interaction Liquid Chromatography-Mass Spectrometry in the Absence of Ion-Pair Reagents. *J. Chromatogr. A* **2019**, *1595*, 39–48.
3. Huang, M.; Xu, X.; Qiu, H.; Li, N. Analytical characterization of DNA and RNA oligonucleotides by hydrophilic interaction liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* **2021**, *1648*, 46–2184.

www.agilent.com

DE54527408

This information is subject to change without notice.

© Agilent Technologies, Inc. 2022

Printed in the USA, June 22, 2022
5994-4945EN

Agilent
Trusted Answers

Application Note

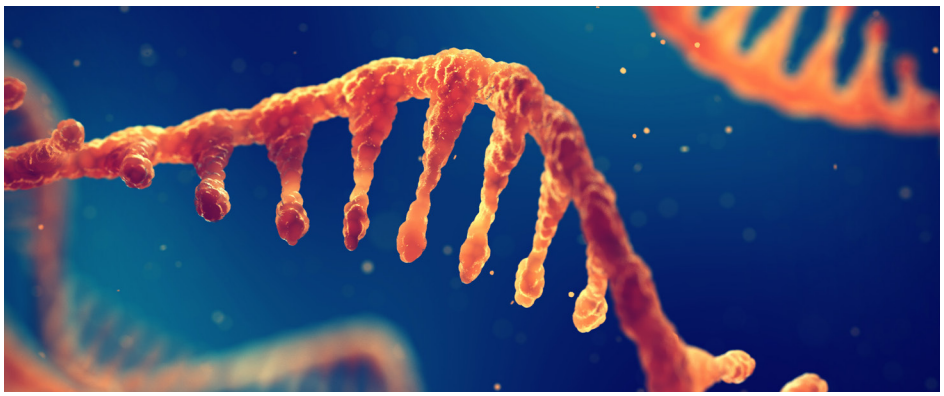
Agilent
Trusted Answers

High-throughput Mass Spectrometry Analysis of Synthetic Oligonucleotides

A comparison of data from Fast LC and RapidFire methods

Authors

Peter Rye, PhD and
Yanan Yang, PhD
Agilent Technologies, Inc.



Introduction

Liquid chromatography (LC) and mass spectrometry (MS) play a vital role in the characterization of synthetic oligonucleotides (oligos), and the appetite for higher throughput analytical methods has increased in the past years alongside the acceleration of oligo production and use. Traditional LC/MS of oligos, where separation is desired, can necessitate run times of many minutes. However, not all applications require chromatographic separation and desalting prior to MS measurement can be sufficient. This work describes and compares two methods, Fast LC and RapidFire, for the high-throughput sampling and desalting of oligos. Each method was optimized for speed on 18mers, then characterized for performance on a range of synthetic DNA and RNA, 18 to 100mer in length.

Experimental

For the Fast LC method, an Agilent 1290 Infinity II multisampler was equipped with dual injection needles that alternated between samples with smart overlap, providing analysis from one needle at the same time as sample draw from the other (Figure 1). The run time was further optimized by a fast gradient at high flow running through a guard column attached directly to the analytical nebulizer of the MS. The high flow rate for the Fast LC method was required to desalt the oligos quickly. In turn, the Fast LC acquisition rate was set to 10 spectra/sec to ensure at least 15 points across all chromatographic peaks (which were ~2 seconds wide, versus ~5 seconds for the RapidFire method). For the RapidFire method (Figure 2), the system performed a 6-second desalting (Pump 1, State 2) followed by a 6-second elute (Pump 3, State 4) on each sample. All resulting data were analyzed using MassHunter Bioconfirm B07.

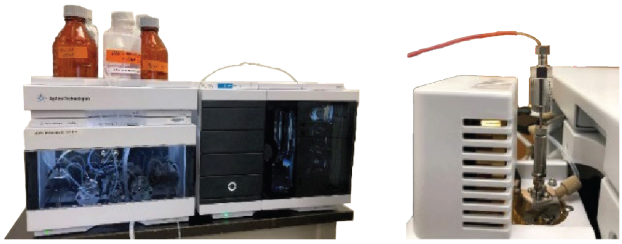


Figure 1. Fast LC method using an Agilent 1290 Infinity II multisampler equipped with dual injection needles.

Figure 2. Agilent RapidFire 400 high-throughput mass spectrometry system.

Fast LC conditions

Agilent 1290 Infinity II Binary Pump, Multisampler with Dual Needles	
Column	Agilent AdvanceBio Oligo UHPLC Guard column, 2.1 × 5 mm, 1.7 µm (p/n 821725-921)
Column Temperature	Room temperature
Injection Volume	10 µL
Smart Overlap	Enabled, alternating needle
Autosampler Temperature	5 °C
Needle Wash	Methanol/water 50/50
Mobile Phase	A) Water + 15 mM TEA + 400 mM HFIP B) Methanol
Flow Rate	1.75 mL/min
Gradient	Time (min) Time (sec) %B 0.00 0.00 20 0.03 1.80 20 0.24 14.4 50 0.25 15.0 100 0.30 18.0 100 0.31 18.6 20 0.59 35.0 20
Stop Time	0.60 min
Post Time	0.00 min

Agilent 6545 LC/Q-TOF	
Ion Polarity	Dual AJS Negative
Data Storage	Both (centroid and profile)
Gas Temperature	350 °C
Drying Gas Flow	13 L/min
Nebulizer Gas	60 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	2,000 V
Fragmentor	200 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Mass Range	400 to 3,200 *m/z*
Acquisition Rate	10 spectra/sec

RapidFire conditions

Agilent RapidFire 400		
Cartridge	Agilent PLRP-S, 30 µm, 1,000 Å, 4 µL bed volume	
Cartridge Temperature	Room temperature	
Injection Volume	10 µL	
Pump 1	Water + 7.5 mM TEA + 200 mM HFIP, 1.2 mL/min	
Pump 2	50% Methanol + 7.5 mM TEA + 200 mM HFIP, 0.6 mL/min	
Pump 3	50% Methanol + 7.5 mM TEA + 200 mM HFIP, 0.6 mL/min	
State 1	Aspirate sample (sip sensor on)	600 msec
State 2	Load/wash (desalt)	6,000 msec
State 3	Extra wash	0 msec
State 4	Elute (inject)	6,000 msec
State 5	Re-equilibrate	500 msec

Agilent 6545 LC/Q-TOF	
Ion Polarity	Dual AJS Negative
Data Storage	Both (centroid and profile)
Gas Temperature	275 °C
Drying Gas Flow	11 L/min
Nebulizer Gas	35 psi
Sheath Gas Temperature	325 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	2,000 V
Fragmentor	200 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Mass Range	400 to 3,200 *m/z*
Acquisition Rate	4 spectra/sec

Results and discussion

Throughput and reproducibility – RapidFire

The throughput of the RapidFire method is determined by the sum of the five states (~13 seconds, see Experimental) plus ~1.5 seconds for plate stage motion, and was just under 15 seconds per sample. For RapidFire MS, to circumvent the delay times associated with MS acquisition start/stop, a single data file is acquired per sample set and parsed post acquisition. Figure 3 shows the pressure for all three RapidFire pumps as one continuous file for a set of 24 replicate injections. For each pump, the pressure peaks and valleys were steady, and in the range between 0.5 and 10 MPa, consistent with a stable method.

Throughput and reproducibility – Fast LC

The throughput of the Fast LC method is determined by the gradient program (~35 seconds, optimized within the time of next sample draw) plus MS acquisition stop/start (~5 seconds), and was 40 seconds per sample. Figure 4 shows the overlaid pump pressure traces from 24 injections. The traces are superimposed, revealing good gradient reproducibility.

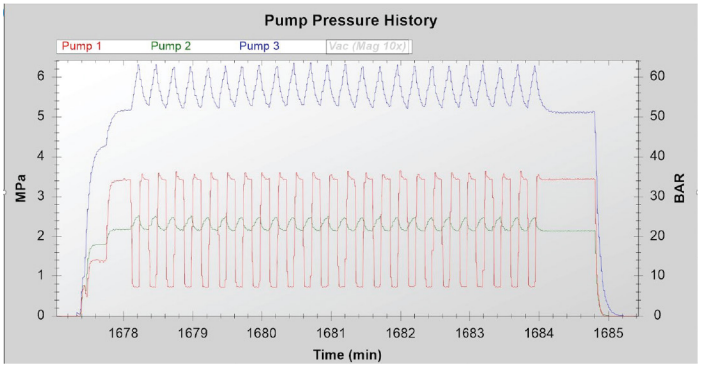


Figure 3. Overlay of three RapidFire pumps for 24 replicate injections.

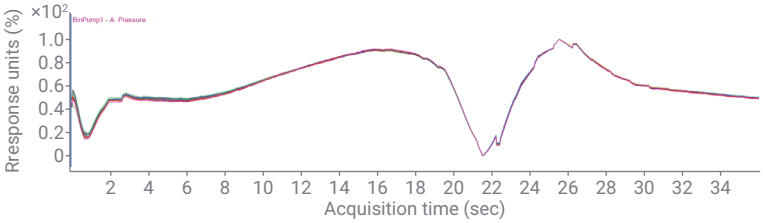


Figure 4. Pump pressure traces for 24 injections, revealing good reproducibility.

Desalting and signal intensity

Figure 5 shows the deconvoluted spectra from unpurified 18, 40, 60, 80, and 100mer oligos acquired using the RapidFire method (black) and the Fast LC method (red). Figure 5A represents the data scaled to the largest peak in each spectrum, and shows that the RapidFire method was more efficient than Fast LC at decreasing salt adducts, which appear as peaks +22 (Na) and +38 (K) Da. The relative percent of adducts, to the target peak, for each spectrum are indicated in blue. Very efficient desalting by the RapidFire method derives from the 6-second State 2 (see Experimental) on the 4 µL bed volume cartridge, which results in 15 cartridge volumes of wash. Figure 5B shows the same data as on top but with the Y-axis for each oligo size linked. Comparison of the absolute peak heights shows the Fast LC method provides less abundant target MS signals, which are indicated for each oligo in green. Despite the separative characteristics of Fast LC (see Figure 7), which can decrease ion suppression and thereby increase signal, the lower signals from Fast LC are the combined result from higher pump flow rate (1.75 versus 0.6 mL/min for RapidFire), faster acquisition rate (10 versus 4 spectra/sec for RapidFire), and less efficient desalting.

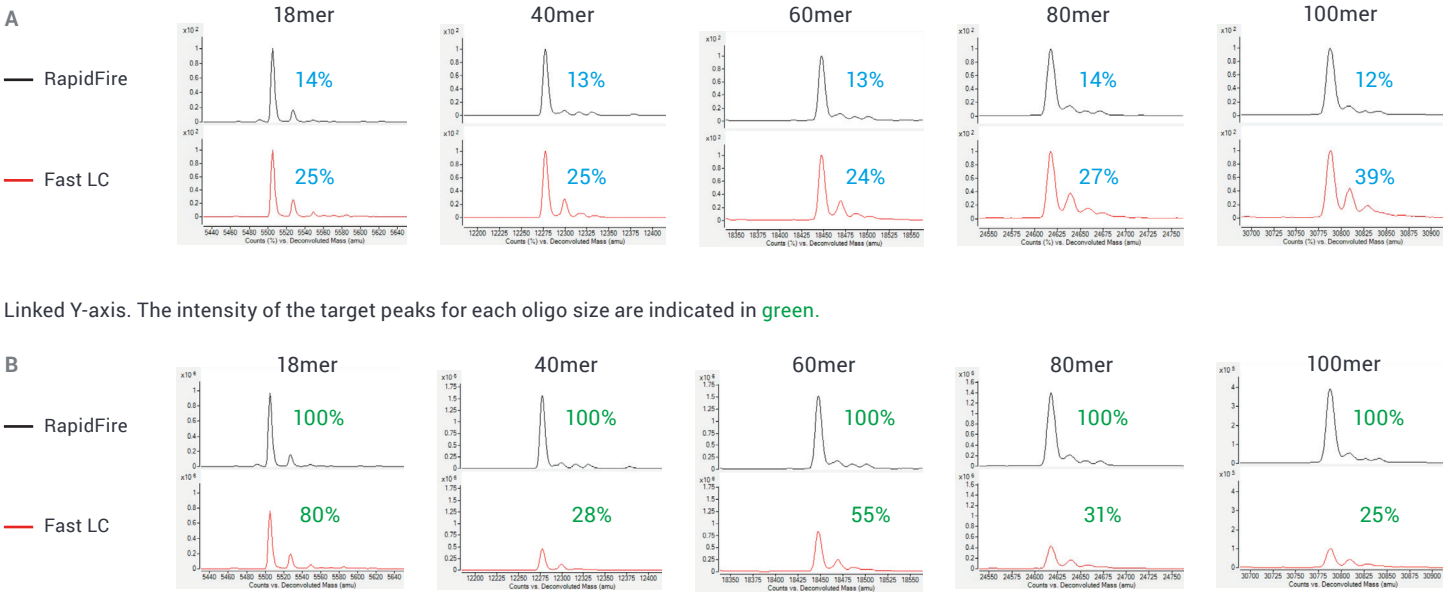


Figure 5. Deconvoluted spectra from unpurified oligos, acquired using the RapidFire and Fast LC methods.

Oligo retention – RapidFire

To evaluate oligo separation by the two methods, nineteen unique DNA and RNA samples ranging from 18 to 100mer in length were measured. In the RapidFire method, all of the oligos eluted from the cartridge at the same retention time. This result was expected as the RapidFire is specifically designed to prevent separation by switching from low to high organic conditions instantly (by valving) using cartridges with a small resin volume (4 µL), and eluting in the reverse direction to minimize analyte/cartridge interactions. Figure 6 shows the overlaid total ion chromatograms (TIC) for all 19 samples.

Oligo retention – Fast LC

In contrast to the RapidFire method, variable retention times were observed with the Fast LC method. Figure 7A shows the overlaid TIC for 19 unique DNA and RNA samples ranging from 18 to 100mer in length. For these samples, the retention times varied within a 7-second window. Figure 7B shows overlaid extracted ion chromatograms for a 20, 40, 60, 80, and 100mer that were injected as a single mixture, illustrating resolution of these products by a combination of chromatography and mass.

To evaluate the ability of the Fast LC method to separate and produce distinct deconvolution results for two oligos that were close in size, a 1:1 mixture of 18 and 20mer was run. Figure 7C shows the TIC, revealing the oligos produced peaks which the software integrated separately. Figure 7D shows the resulting deconvoluted spectra, revealing the two species, and their respective impurities. This separation could be easily improved by small changes to the gradient program (not shown).

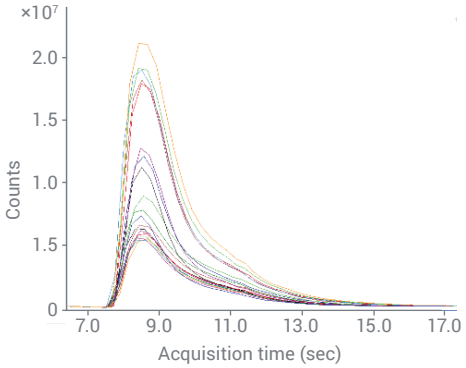


Figure 6. TIC for 19 samples. In the RapidFire method, all oligos had equivalent retention time.

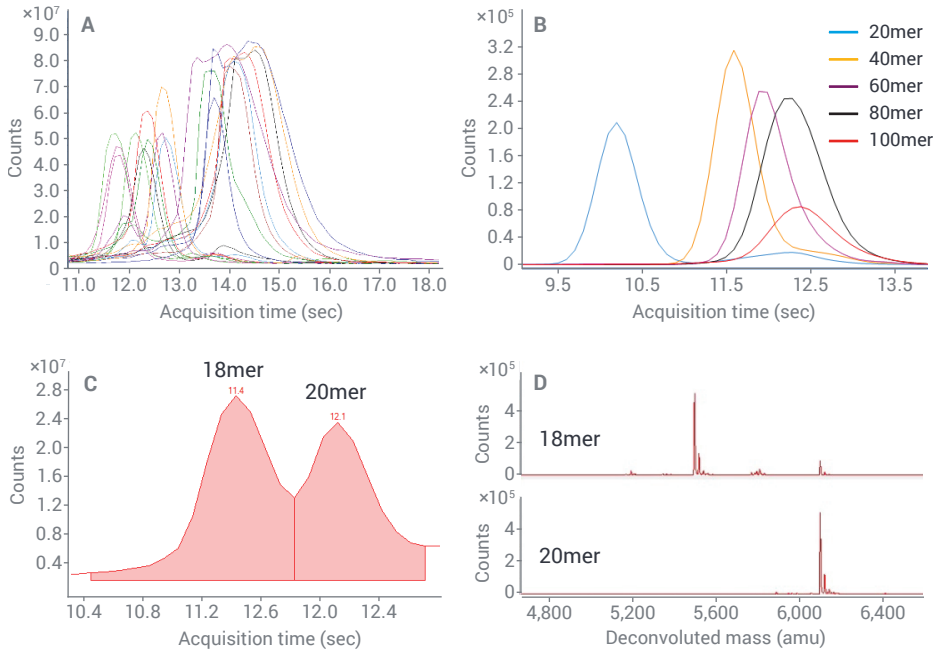


Figure 7. (A) Differential RT from the Fast LC method. (B) Overlaid EIC from an oligo mixture. (C) Separation of 18 and 20mer by Fast LC. (D) Deconvoluted spectra showing how oligo separation can simplify data interpretation.

Low-abundance impurity analysis

High-throughput purity assessment of oligos can be done by mass-resolving the products from a single chromatographic peak. Often, there are many low-abundance impurities coeluting with the highly abundant target, making MS measurement with a wide dynamic range, as well as software that can deconvolute complicated spectra, critical. To evaluate the detection of low-abundance impurities in the same chromatographic peak as the main product, the RapidFire method was used to analyze a 100mer guide RNA. Figure 8 shows that despite zero chromatographic separation, the deconvolution results reveal 100mer RNA as well as numerous impurities, many with a relative area as low as ~0.5%. As expected, this dynamic range was even better with separative/lower throughput methods (data not shown).

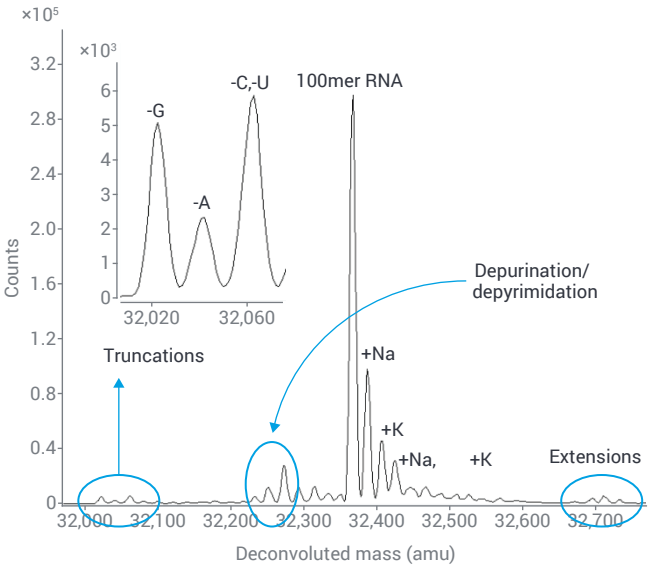


Figure 8. Deconvolution revealing low-abundance impurities.

Conclusion

- Both the RapidFire TOF and Fast LC TOF methods produced reproducible and high quality data for synthetic oligos.
- The RapidFire method sustained a throughput of 15 seconds per sample (240 samples an hour, 5,760 a day) while the Fast LC method sustained a throughput of 40 seconds per sample (90 samples an hour, 2,160 a day).
- The RapidFire method desalted oligos more efficiently than Fast LC, approximately 2- to 3-fold as oligo size increased.
- The Fast LC method produced less intense target signal than RapidFire, from 80 to 25% as oligo size increased.
- Small changes to the Fast LC method, with some compromise to throughput, further improved its performance.
- The Fast LC method afforded some separation of oligo species, a characteristic that could simplify the interpretation of data from mixtures and could also be adjusted to balance the throughput and separation needs of the application.
- In spite their speed over separation approach, both high-throughput systems provided excellent oligo data by mass resolving large numbers of low abundance impurities.

www.agilent.com/chem/6545-lc-q-tof

DE83824012

This information is subject to change without notice.

© Agilent Technologies, Inc. 2022
Printed in the USA, November 15, 2022
5994-3753EN

Agilent
Trusted Answers

Application Note

Agilent
Trusted Answers

Fragment-Based Drug Discovery: Comparing Labeled and Label-Free Screening

Screening of β-amyloid secretase (BACE-1) using fluorescence spectroscopy and ultrafast SPE/MS/MS

Authors

Lauren E. Frick and
William A. LaMarr
Agilent Technologies, Inc.

Introduction

Fragment-based screening offers advantages over traditional high-throughput screening by allowing more comprehensive coverage of chemical space, but the typical low potency of fragments leads to the frequent use of physical methods that detect binding. The few existing activity-based biochemical assays tend to use optical methods, such as fluorescence spectroscopy (FS), which can be subject to confounding factors due to the high concentrations of compound needed to detect activity. This application note screens β-amyloid secretase (BACE-1) against a fragment library using two substrates, a labeled and an unlabeled peptide, which were detected either by FS or ultrafast SPE/MS/MS using the Agilent RapidFire High-throughput Mass Spectrometry (MS) System. Different kinetic parameters, hit rates, and hit sets were obtained depending on the substrate and detection method, suggesting that using fluorescent labels and optical detection methods can lead to follow-up of compounds that are inactive against the unlabeled, more biologically relevant substrate. RapidFire-MS, which allows the direct study of native molecules, eliminates these potential pitfalls.

Experimental

Chemicals and reagents

Fluorescently-labeled or unlabeled BACE-1 substrate and product peptide standards were of the sequences Mca-SEVNLDAEFR-K(Dnp)-RR, Mca-SEVNL, DAEFR-K(Dnp)-RR, SEVNLDAEFR, SEVNL, and DAEFR. The unlabeled substrate peptide was purchased from Sigma-Aldrich (St. Louis, MO). The labeled substrate peptide and BACE-1 enzyme were purchased from R&D Systems, Inc., Minneapolis, MN. Standard peptides representing the cleavage products of both peptides were synthesized by American Peptide Company, Sunnyvale, CA. The reference inhibitor was purchased from EMD Biosciences, Inc., San Diego, CA. The fragment library was a 1,000-compound diversity subset of the Maybridge Ro3 Fragment Library, purchased from Thermo Fisher Scientific, Waltham, MA.

Sample preparation

BACE-1 reactions in a 50 µL volume were run using the following final conditions: 50 mM NaOAc pH 4.5, 50 mM NaCl, 0.03% BSA, 0.0025% Genapol, and 20 nM BACE-1 enzyme. Screening reactions were run with the following additions: 1 mM test compound, 2% DMSO vehicle as an uninhibited control, and 1 µM β-secretase Inhibitor IV in 2% DMSO as a fully-inhibited control. Labeled reactions contained 10 µM substrate and were run at room temperature for 120 minutes. Unlabeled reactions contained 2 µM substrate and were run at room temperature for 180 minutes. Both types of reactions were quenched with 10 µL 10% formic acid containing 2 µM of the opposite product standard as an internal standard (that is, labeled product standard in the unlabeled substrate reaction and vice versa).

Post quench, 45 µL ddH_2O was added to fluorescent reactions to increase the reaction volume height, allowing for sensitive and consistent data collection in the fluorescence spectrophotometer.

RapidFire triple quadrupole conditions

A RapidFire 360 High-throughput MS System and RapidFire integrator software were used for the analysis. Samples were analyzed at a rate of approximately 10 seconds per sample using the conditions shown in Table 1.

Fluorescence spectroscopy parameters

Samples were analyzed at a rate of approximately 2 seconds per sample using the conditions shown in Table 2. Wavelengths were optimized and data were collected for the Mca-SEVNL product peptide.

Data analysis

Cary Eclipse Advanced Reads software was used to acquire fluorescence data. RapidFire Integrator v3.6 software was used for MS peak integration. Microsoft Excel 2007 and GraphPad Prism 5 were used for data analysis and calculation of kinetic parameters. Hits were defined as fragments that produced normalized product signal less than three standard deviations below the average of the values obtained for the eight DMSO-only control wells on each plate. Similarly, autofluorescence was defined as unnormalized product signal greater than three standard deviations above the uninhibited average for each plate.

Table 1. RapidFire LC/MS conditions.

RapidFire Conditions		
Buffer A	Water with 0.1% formic acid; 1.5 mL/min flow rate	
Buffer B	100% acetonitrile with 0.09% formic acid and 0.01% trifluoroacetic acid; 1.25 mL/min flow rate	
Injection Volume	10 µL	
SPE Cartridge	Agilent RapidFire cartridge A (reversed-phase C4 chemistry, G9203A)	
RF State 1	Sip sensor	
RF State 2	3,500 ms	
RF State 3	5,000 ms	
RF State 4	500 ms	
MRM Transitions	Q1	Q3
Labeled Substrate	668.0	101.8
Labeled Product	777.3	532.1
Unlabeled Substrate	590.5	216.8
Unlabeled Product	561.3	217.0

Table 2. Fluorescence spectrometry conditions.

Cary Eclipse Fluorescence Spectrophotometer Conditions	
Data Mode	Fluorescence
Excitation Wavelength	394 nm
Emission Wavelength	326 nm
Excitation Slit	5 nm
Emission Slit	5 nm
Average Time	0.1 s

Results and discussion

Assay development

Functional biochemical BACE-1 assays were optimized around each substrate, with full characterization of buffer requirements, enzyme linearity, binding kinetics, DMSO tolerance, and inhibition by a reference compound (β-secretase Inhibitor IV). While the assays displayed similar linearity at room temperature, the BACE-1 enzyme exhibited very different affinities for the two different substrates (Figure 1, left panels). A standard K_m curve could be generated for the unlabeled peptide (calculated K_m of 22.4 µM), but curves could not be constructed for the labeled peptide, presumably due to poor substrate solubility at the higher concentrations required. These data suggest that the labeled peptide is a significantly less efficient substrate for the enzyme, which could alter the assay results.

Reference inhibition curves with Inhibitor IV, however, produced similar values of 16.2 nM for the unlabeled substrate and 24.6 nM and 24.5 nM for the labeled substrate by FS and MS, respectively (Figure 1, right panels). These values agreed quite well, both with each other and with the given literature value of 15 nM.[1] Z' values comparing DMSO-only wells with wells containing 1 µM inhibitor IV were between 0.61 and 0.71 for all assays, with n = 12 to 24.

Fragment library screening

After robust assays were developed, each substrate was used in a screen of BACE-1 against a 1,000-compound diversity subset of the Maybridge Ro3 Fragment Library. Compounds were screened in 96-well plate format at a final concentration of 1 mM. Initial screening of a fragment library generated different hits and hit rates among the various assay formats (Figure 2). Compounds of interest (primarily those registering as hits in certain assays but not others)

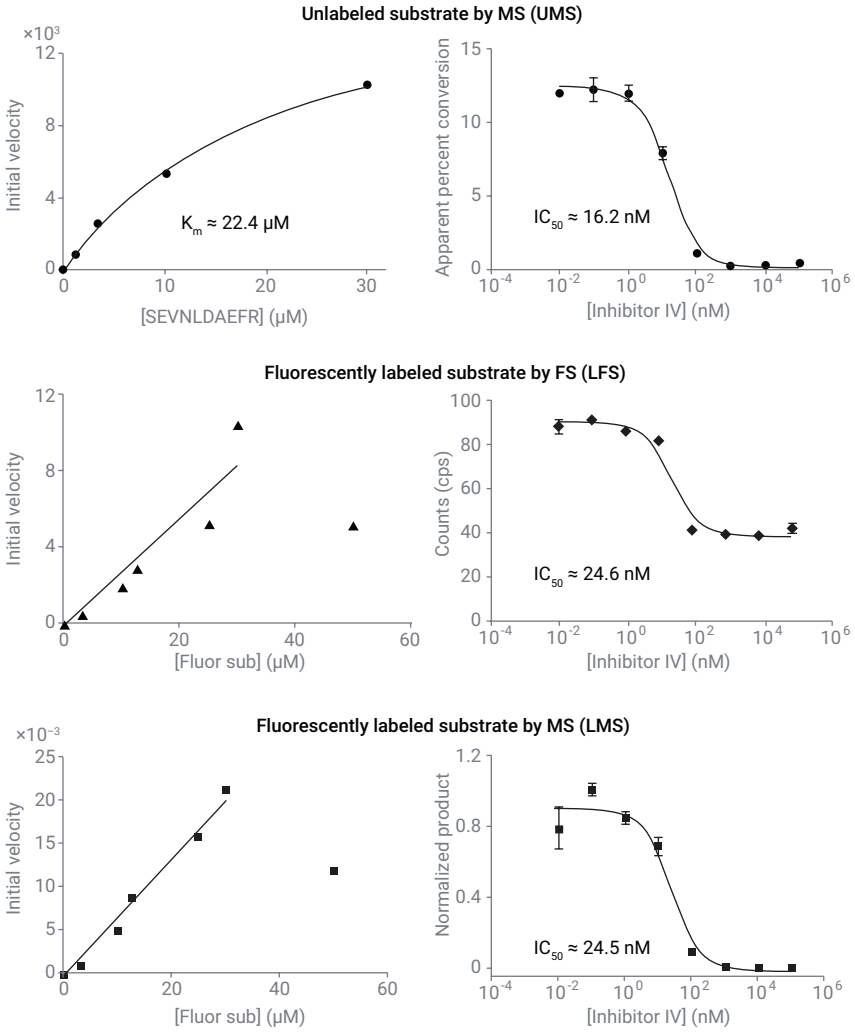


Figure 1. Kinetic parameters of different substrates by mass spectrometry (MS) and fluorescent spectroscopy (FS): unlabeled substrate by MS (UMS), fluorescently labeled substrate by FS (LFS), and fluorescently-labeled substrate by MS (LMS).

	UMS	LMS	LFS	All 3
UMS	211	14	41	–
LMS	14	32	22	–
LFS	41	22	122	–
All 3	–	–	–	8

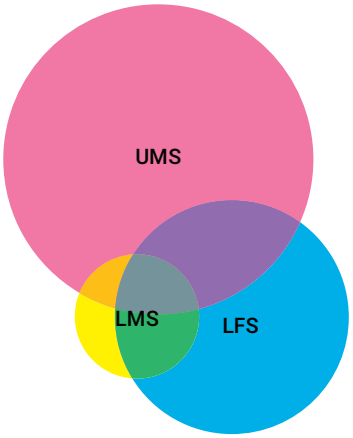


Figure 2. Table and Venn diagram of initial screening results displaying different hit rates and hit sets by assay format: unlabeled substrate by MS (UMS), labeled substrate by MS (LMS), and fluorescently labeled substrate by FS (LFS).

were chosen for confirmation screening. Follow-up studies of these selected hits revealed the presence of several classes of compounds with differing inhibitory characteristics towards the BACE-1 reaction.

Hits observed by MS only

Follow-up of selected hits confirmed that compound autofluorescence (AF) obscured several hits in the FS data, including the most potent analyte. Titration of that compound revealed a concentration-dependent increase in signal in the FS assay, suggesting AF, while the MS data were consistent with a traditional inhibition curve (Figure 3).

Hits observed with the unlabeled peptide only

A second class of inhibitors was detected in the unlabeled assay (UMS) whose members were not found with the fluorescent peptide (LFS or LMS). Because MS eliminates the need for unnatural modification of substrates, it allows the study of more biologically relevant molecules. These more realistic substrates could reveal activities that are lost with modified peptides, possibly due to altered binding, as in this case was clearly revealed by the K_m experiments.

Hits observed with the labeled peptide only

Yet another set of compounds was uncovered consisting of those molecules that appear as hits when the labeled peptide is used (as in the LFS and LMS assays), but do not show significant inhibition when the more native substrate is used (UMS, Figure 4). These results suggest that compounds may exist that interfere with the enzyme's ability to bind the peptide carrying the bulky label but not with the tighter binding exhibited by the enzyme for the unlabeled substrate, raising the possibility of misleading data being produced when modified substrates are used.

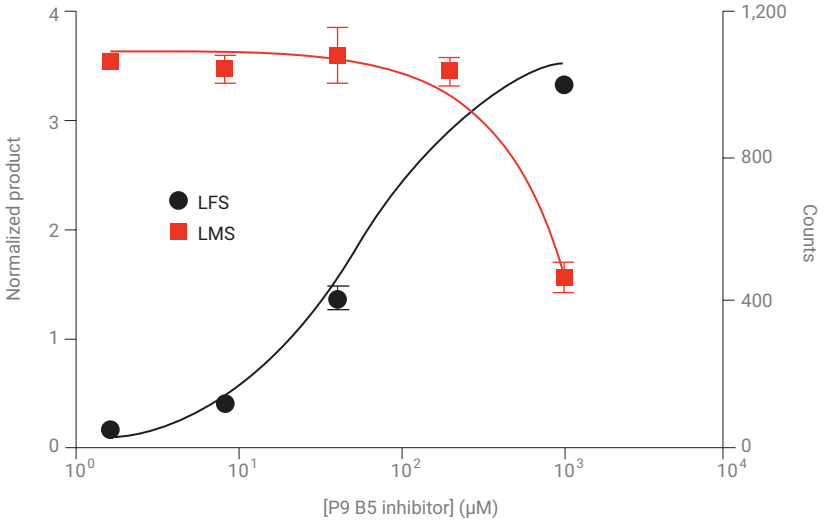


Figure 3. Inhibition observed by MS appears as concentration-dependent increase in signal by LFS.

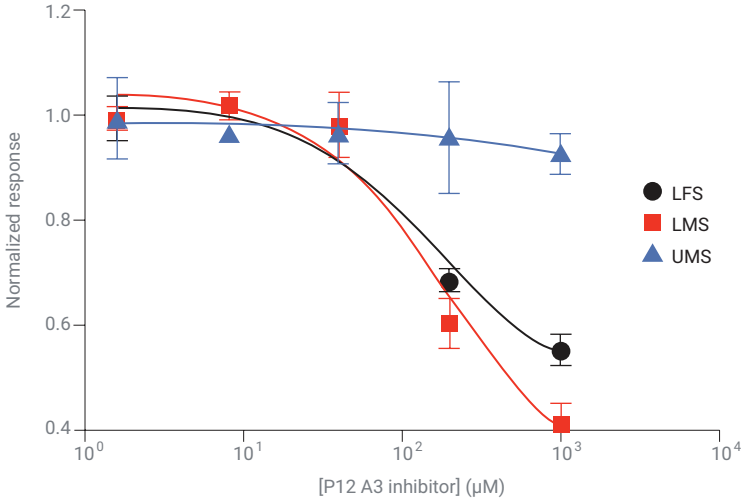


Figure 4. Inhibition observed with the labeled peptide is not seen with the unlabeled peptide.

Conclusion

Robust functional biochemical assays were developed for both a labeled and an unlabeled substrate of the BACE-1 enzyme, with data collection by both MS and FS. Using these assays to screen a fragment library against the labeled and unlabeled substrates using both detection methods produced three disparate hit sets and hit rates. Follow-up of selected compounds demonstrated the existence of different hit classes among the assays. Interestingly, FS and MS produced different hit sets when used as complementary detection methods on the same samples. While some MS hits (including the most potent) were obscured by autofluorescence in the FS assay, this phenomenon alone did not fully account for the discrepancy between techniques. MS also generated different hit sets for the labeled and the unlabeled peptide, finding both hits that were active against the labeled peptide but not the unlabeled, and vice versa. The existence of these two populations of compounds underscores the importance of substrate selection when setting up a new screen.

Pairing the RapidFire high-throughput system with MS solves the time bottleneck associated with MS detection, allowing an analysis rate of approximately 10 seconds per sample, and thus approaching the speeds of fluorescent plate readers. Label-free screening by high-throughput MS has proven to be a valid method for conducting activity-based screens of fragment libraries that enables the study of more native molecules and is less susceptible to confounding factors, such as autofluorescence.

Reference

1. http://www.emdmillipore.com/life-science-research/beta-secretase-inhibitor-iv/EMD_BIO-565788/p_moKb. s1OGx8AAAEjBopJNLpP, accessed 08/21/2012.

www.agilent.com/chem/rapidfire

This information is subject to change without notice.

© Agilent Technologies, Inc. 2013, 2022

Printed in the USA, April 14, 2022
5991-1338EN

Agilent
Trusted Answers

Application Note

Agilent
Trusted Answers

High-Throughput Lead Discovery with Agilent RapidFire/MS Systems

Analysis of stearoyl-coenzyme A desaturase (SCD)

Authors

William Lamarr, Lauren Frick, and Peter Rye
Agilent Technologies, Inc.

Introduction

The RapidFire high-throughput mass spectrometry system provides drug discovery researchers with mass spectrometry-based, high-throughput screening solutions for targets that have proven challenging to screen using conventional approaches. These intractable targets have substrates and products that are either too small to label or undergo modifications that are difficult to detect. RapidFire technology provides the most relevant data, with label-free native analyte detection that eliminates the need for cumbersome and costly labeling methods. RapidFire technology enables traditionally low-throughput, intractable assays to be converted into high-throughput assays processed at speeds approaching plate-based optical methods. In this application note, a stearoyl-coenzyme A desaturase (SCD) assay is used to illustrate the power of Agilent RapidFire/MS systems for screening intractable targets.

Using RapidFire high-throughput mass spectrometry to analyze SCD assay samples

The enzyme SCD catalyzes the conversion of stearoyl-coenzyme A (SCoA) to oleoyl-coenzyme A (OCoA) as shown in Figure 1. This enzyme plays a critical role in the desaturation of fatty acids and is an important therapeutic target for a range of disease states.

However, the reaction results in only the desaturation of a single carbon-carbon bond. This conversion is an extremely subtle change, which presents a number of significant challenges during the screening process. In addition, the use of a radiometric assay for these challenging lipophilic analytes is typically a barrier to efficient high-throughput screening of targets such as SCD. In the case of SCD, the radiometric assay is a tritiated water release assay that has been used for the determination of enzyme activity.

This application note presents an example of a RapidFire high-throughput mass spectrometry assay developed for SCD that overcomes the need for radioactive labeling, making this target class a candidate for a high-throughput screening approach.

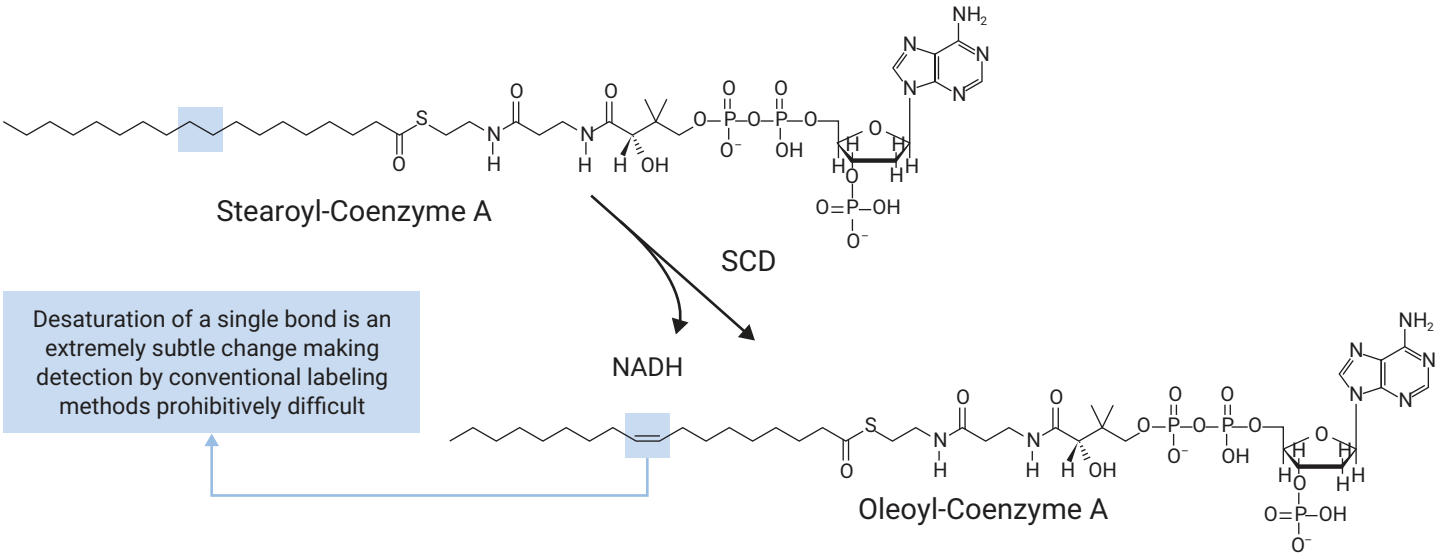


Figure 1. SCD assay reaction scheme.

Mass spectrometry is a highly sensitive method for detecting the small changes in mass and is well suited for detecting the single desaturation that occurs with SCD conversion. Both SCD substrate and product can be directly and accurately measured by mass spectrometry at sub-micromolar concentrations. The RapidFire method uses a solid phase extraction (SPE) sample cleanup step directly coupled to MS detection. Figure 2 shows standard measures of assay quality – linearity with respect to enzyme concentration for the indicated reaction time and initial reaction velocity within the tested range.

Furthermore, the RapidFire system yields highly repeatable results. Figure 3 demonstrates that the assay was reproducible for a set of 518 plates with an average Z' score of 0.597 and a median Z' score of 0.60.

The RapidFire screen yielded a number of potent and specific inhibitors, with 346 confirmed as active inhibitors of SCD activity. The RapidFire SCD assay effectively differentiates IC_{50} potencies during hit to lead expansion (Figure 4).[2]

The SCD example illustrates that RapidFire/MS delivers a high-throughput alternative, with integrated sample preparation and sensitive mass spectrometry detection that streamlines the drug discovery process for even the most difficult assays.

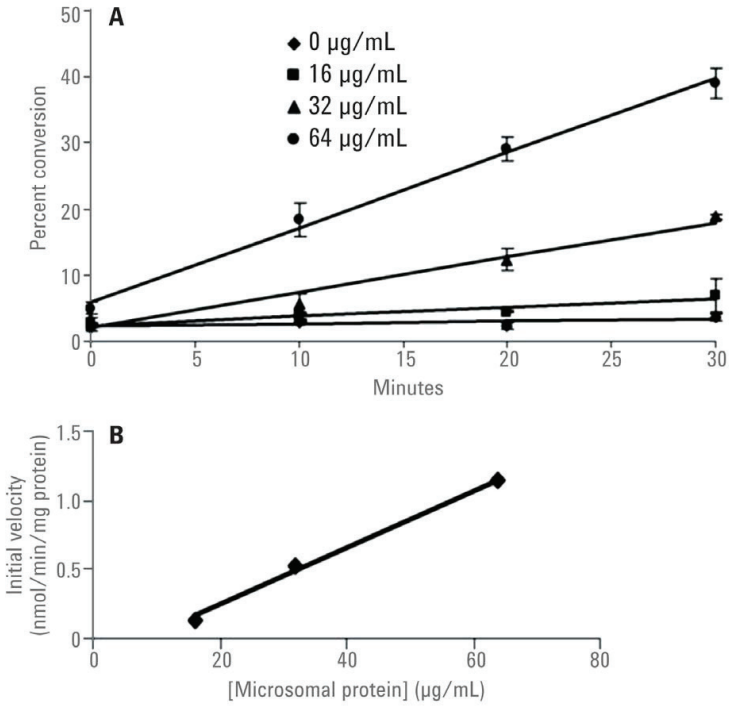


Figure 2. SCD1 assay linearity with respect to time (A) and microsomal protein concentration (B).

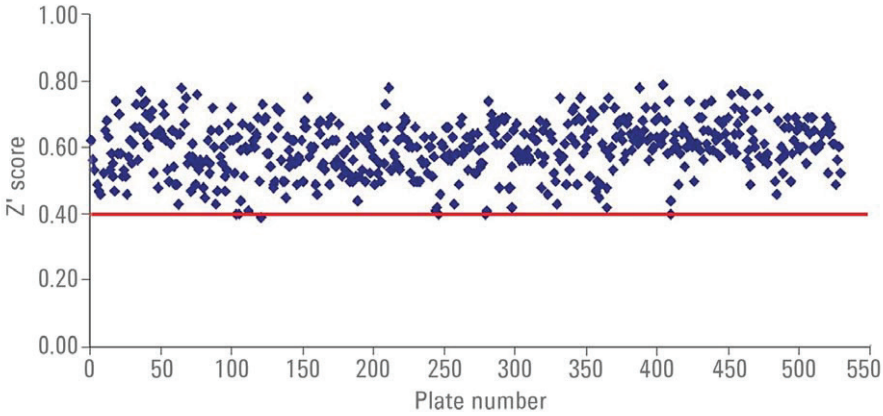
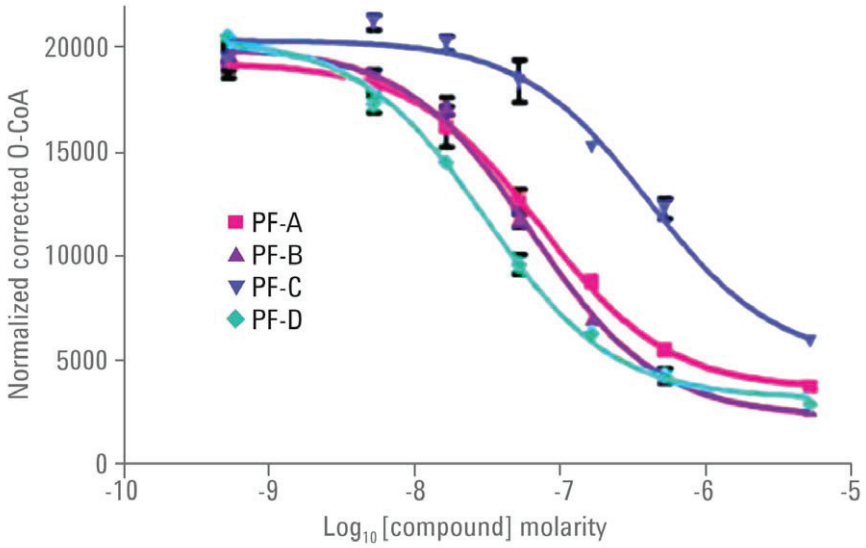


Figure 3. SCD1 assay quality as determined by Z' values for 518 plates (384-well).

Conclusion

The Agilent RapidFire high-throughput mass spectrometry system demonstrated a number of key benefits for the high-throughput screening of stearoyl-coenzyme A desaturase, an intractable target traditionally requiring extremely laborious labeling methods. RapidFire provides sample processing speeds of 6 to 10 seconds, increasing throughput over conventional methods by more than 10-fold.

RapidFire/MS enables sensitive and reliable analysis of challenging drug target classes with label-free, native molecule detection. RapidFire/MS can be used to efficiently screen chemical libraries with results comparable to optical methods. As a result, incorporation of RapidFire/MS systems into the lead discovery phase of the drug discovery process delivers efficiency and productivity advances unrivaled by other technologies.



	EC_{50}
PF-A	7.030e-008
PF-B	6.080e-008
PF-C	3.884e-007
PF-D	2.964e-008

Figure 4. Demonstration of secondary characterization HTS screen for SCD inhibitors.

References

1. Soulard, P. *et al.* Development of a High-Throughput Screening Assay for Stearoyl-CoA Desaturase Using Rat Liver Microsomes, Deuterium Labeled Stearoyl-CoA and Mass Spectrometry. *Anal. Chim. Acta.* **2008**, *627(1)*, 105–11.
2. Schilling, R. *et al.* The Use of High-Throughput Mass Spectrometry(HTMS) in Drug Discovery. Presented at the SBS 14th Annual Conference, **2008**, St. Louis, USA.

www.agilent.com/chem/rapidfire

DE.478275463

This information is subject to change without notice.

© Agilent Technologies, Inc. 2011, 2020, 2022

Printed in the USA, April 14, 2022
5990-9357EN

Agilent
Trusted Answers

Application Note

Pharmaceutical

Agilent

Trusted Answers

Ultrafast Analysis of *In Vitro* Microsomal Metabolic Stability using RapidFire Coupled to the Ultivo Triple Quadrupole Mass Spectrometer

Authors

Na-Young Choi
Agilent Technologies, Inc.

Nary Ha, Hee-jeong Ahn, and Hye-won Seo
Yuhan R&D Institute

Abstract

Metabolic stability studies are important steps in the initial drug discovery process. During the investigation of phase one drug metabolism, large quantities of samples are analyzed, creating the need for fast and reliable analytical methods. The Agilent RapidFire is an ultrafast, integrated mass spectrometry autosampler capable of automated solid phase extraction (SPE) sample cleanup. With cycle times ranging from 2 to 15 seconds, RapidFire dramatically reduces analysis times compared to traditional LC/MS without compromising data quality. This study compared the results of *in vitro* microsomal metabolic stability (MMS) assays analyzed by RapidFire/TQ and LC/TQ. Agilent MassHunter Optimizer software was used to automatically determine multiple reaction monitoring (MRM) transitions for 72 compounds. The results for the two systems correlated well (R^2 = 0.94), and RapidFire required only 10 seconds per sample, providing 10-times faster throughput than LC/TQ.

Introduction

Pharmacokinetic analysis is an important early process of drug discovery that aims to quantify absorption, distribution, metabolism, and excretion (ADME) of compounds over time. These initial analyses include a large set of samples, so high-throughput analytical methodology is desirable. The Agilent RapidFire harnesses the power of traditional liquid chromatography mass spectrometry (LC/MS) analysis but allows for a 10-times increase in throughput by replacing chromatography with on-line solid phase extraction (SPE). Also, with a large sample capacity of over 130,000 samples and integrated automated sample-handling robotics, RapidFire allows longer unattended operation than LC/MS, further improving productivity.

An *in vitro* microsomal metabolic stability (MMS) assay is one type of ADME experiment used to evaluate compounds of interest. It is widely used in early drug discovery studies because it is an *in vivo* stability indicator. When considering the pharmacokinetic properties of a drug candidate, the stability of a compound ultimately affects its efficacy as a drug.

In this study, MMS assays were performed on various drug candidates using the RapidFire 400 coupled to an Agilent Ultivo Triple Quadrupole Mass Spectrometer (TQ). This RapidFire/TQ system can produce analytical results that are equivalent to traditional liquid chromatography triple quadrupole mass spectrometry (LC/TQ) in just 10 seconds per sample. The findings in this study demonstrate that RapidFire/TQ is a suitable replacement for LC/TQ in these types of ADME assays.

Experimental

Sample preparation

Standard stock solutions for 72 compounds of interest were dissolved in acetonitrile. To assess the linearity and reproducibility of the method, a serial dilution of the stock solution was prepared using water containing 0.1% formic acid.

MMS assays were carried out in 96-well plates where target compounds were incubated with human liver microsomes (HLM; Corning). After incubation, the samples were transferred to a new plate where the reaction was quenched with an acetonitrile solution containing tolterodine as the internal standard (ISTD). Samples were centrifuged at 3,000 rpm at 4 °C for 10 minutes. The supernatant was transferred to a new plate, then diluted 1:2 with water containing 0.1% formic acid, before being injected for analysis.

Instrumentation

The RapidFire/TQ system consisted of a RapidFire 400 coupled to an Ultivo TQ. An Agilent RapidFire C4 (Type A) cartridge was used for SPE.

The LC/TQ system consisted of an Agilent 1290 Infinity II LC coupled to an Agilent 6470 TQ. Chromatography was performed using an Agilent ZORBAX Eclipse Plus C18, 2.1 x 50 mm, 1.8 µm column.

Data was acquired with Agilent MassHunter Acquisition (version 10.1) and analyzed with MassHunter Qualitative Analysis (version 10.1) and MassHunter Quantitative Analysis (version 10.1) software.

Instrument operating conditions are given in Tables 1 to 4.

Table 1. Agilent RapidFire parameters.

Parameter	Value
Pump 1	Water with 0.1% formic acid 1.5 mL/min flow rate
Pump 2	Acetonitrile 1 mL/min flow rate
Pump 3	60% acetonitrile with 0.1% formic acid 1 mL/min flow rate
Injection Volume	5 µL
SPE Cartridge	C4 (Type A; part number G9203A)
Aspiration	600 ms
Load/Wash	3,000 ms
Extra Wash	0
Elute	3,000 ms
Re-equilibrium	500 ms

Table 2. Agilent Ultivo TQ parameters.

Parameter	Value
Ion Source	ESI with Agilent Jet Stream
Acquisition Mode	MRM
Gas Temperature	350 °C
Gas Flow	12 L/min
Nebulizer	30 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
Capillary	(+)3000, (-)4,000 V
Nozzle voltage	(+)0, (-)1,500 V
Polarity	Positive/Negative

Table 3. Agilent 1290 Infinity II LC parameters.

Parameter	Value
Column	ZORBAX Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm
Column Flow	0.4 mL/min
Injection Volume	5 µL
Mobile Phase	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile
Gradient (%B)	60% isocratic for 2 min

Table 4. Agilent 6470 LC/TQ parameters.

Parameter	Value
Ion Source	ESI with Agilent Jet Stream
Acquisition Mode	MRM
Gas Temperature	350 °C
Gas Flow	12 L/min
Nebulizer	30 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
Capillary	(+)3,000, (-)4,000 V
Nozzle Voltage	(+)0, (-)1,500 V
Polarity	Positive/Negative

Results and discussion

To assess the accuracy and reliability of the RapidFire/TQ method, a complete MMS study of 72 compounds of interest was analyzed by RapidFire/TQ and LC/TQ. Tolterodine was used as an ISTD for all analyses. Warfarin was used as a target compound to verify linearity and reproducibility data on each system; it was also used as a reference compound in the MMS assay.

To ensure a direct comparison of RapidFire and traditional LC analysis, the same Ultivo TQ instrument was used for the linearity and reproducibility studies.

Correlation data was collected using the RapidFire/Ultivo and LC/6470.

A comparison of linearity

Warfarin was used to create a 7-point calibration curve ranging from 0.5 to 50 ng/mL. The results for RF/TQ (Figure 1A) and LC/TQ (Figure 1B) were equivalent, both showing excellent linearity (R^2 ≥0.999) and accuracy (between 90 and 110%).

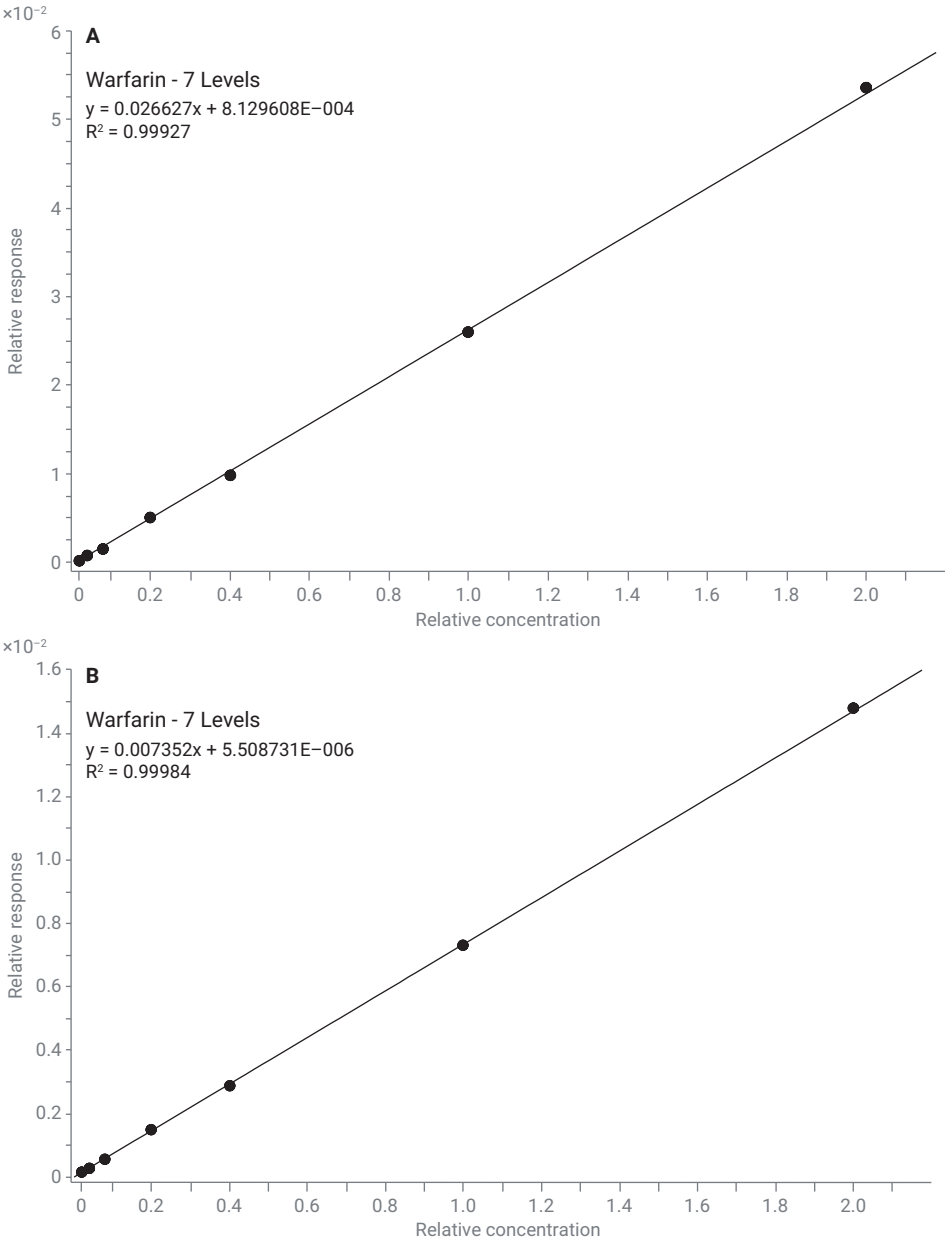


Figure 1. Calibration curves for warfarin obtained using (A) RapidFire/TQ and (B) LC/TQ.

A comparison of reproducibility

A 1 ng/mL warfarin standard was measured five times to assess the precision of each system. The relative standard deviation (%RSD) was calculated for the ratio of warfarin to tolterodine (ISTD). As shown in Table 5, both RapidFire/TQ and LC/TQ achieved highly reproducible results, with %RSDs of 4.87 and 2.06%, respectively.

Table 5. Reproducibility of measurement of 1 ng/mL warfarin standard using RapidFire/TQ and LC/TQ (n = 5).

Sample	Target/ISTD Ratio	
	RF/TQ	LC/TQ
1	0.00140	0.00030
2	0.00132	0.00030
3	0.00127	0.00031
4	0.00126	0.00031
5	0.00125	0.00030
Average	0.00130	0.00030
SD	0.00006	0.00001
%RSD	4.87	2.06

A comparison of correlation

To assess whether RapidFire/TQ can produce results equivalent to LC/TQ, identical MMS assays were analyzed by each system. The studies determined the amount of each compound remaining after the MMS assay and reported results as a percentage. A plot comparing RapidFire/TQ results to LC/TQ results (Figure 2) shows excellent correlation (R^2 = 0.9376), a slope of 1.0203, and a small y-intercept. The correlation data indicates that the systems produced equivalent MMS assay results, however, the RapidFire/TQ results were acquired 10 times faster than the LC/TQ data.

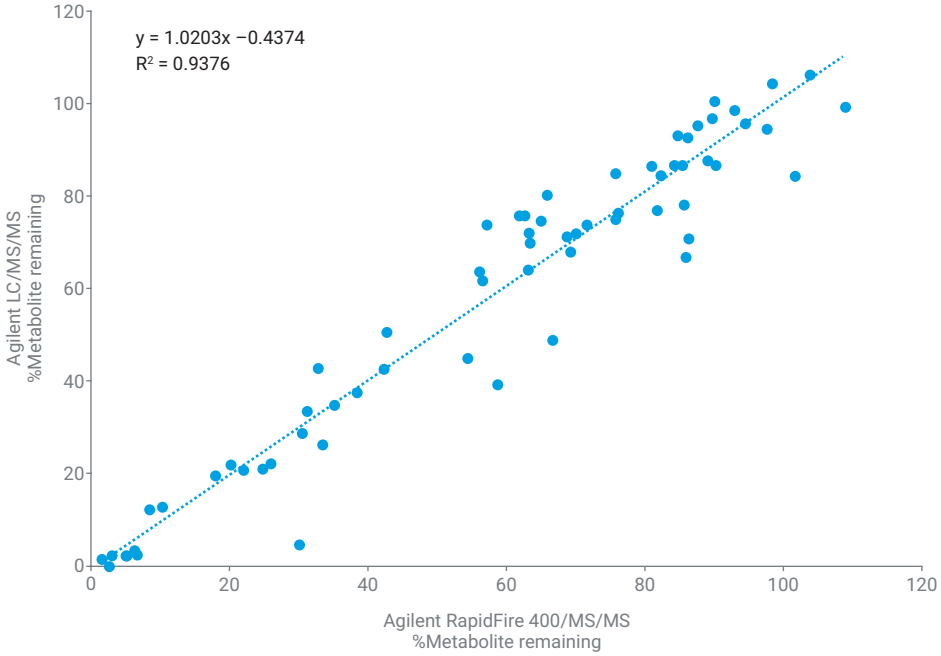


Figure 2. Correlation of RapidFire/TQ and LC/TQ results for percent of 72 compounds remaining at the end of MMS analysis.

Conclusion

A metabolic stability assessment of 72 different compounds was performed using an Agilent RapidFire coupled to an Agilent Ultivo triple quadrupole mass spectrometer (RapidFire/TQ). To determine if the RapidFire/TQ could produce equivalent results to traditional methods, the same samples were analyzed by liquid chromatography triple quadrupole mass spectrometry (LC/TQ). The comparison results showed that both sets of results were equivalent, but the RapidFire/TQ method, which uses solid phase extraction (SPE) rather than chromatography, was 10 times faster than LC/TQ. A comparison of the microsome metabolic stability (MMS) assay results obtained by RapidFire/TQ and LC/TQ showed excellent correlation between the methods.

The study has shown that RapidFire/TQ can improve the sample throughput, productivity, and efficiency of MMS assays and is potentially useful for other, similar *in vitro* ADME assays.

References

1. Ultrafast Analysis of Metabolic Stability Assays Using Agilent RapidFire High-Resolution MS, *Agilent Technologies Technical Overview*, 5990-8344EN, **2019**
2. High-Throughput *in vitro* ADME Analysis with Agilent RapidFire/MS System: Permeability Assays, *Agilent Technologies Technical Overview*, 5990-9081EN, **2021**
3. Di, Li *et al.* Application of High Throughput Microsomal Stability Assay in Drug Discovery. *Combinatorial Chemistry & High Throughput Screening*, *11(6)*, 469–476, **2008**
4. Di, Li *et al.* Optimization of a Higher Throughput Microsomal Stability Screening Assay for Profiling Drug Discovery Candidates. *Journal of Biomolecular Screening*, *8(4)*, 453-62, **2003**

www.agilent.com

For Research Use Only. Not for use in diagnostic procedures.

RA44585.6442824074-RA

This information is subject to change without notice.

© Agilent Technologies, Inc. 2022

Printed in the USA, February 17, 2022
5994-4532EN

Agilent

Trusted Answers

Application Note

Clinical Research

Agilent

Trusted Answers

Ultrafast Analysis of Levetiracetam in Serum

Using the Agilent RapidFire high-throughput mass spectrometry system

Authors

Michelle V. Romm and
Vaughn P. Miller
Agilent Technologies, Inc.

Abstract

Mass spectrometry-based analyses have emerged as a viable analytical method due to their sensitivity, specificity, and robustness. This application note evaluates the ability of an ultrafast SPE/MS/MS system (Agilent RapidFire High-throughput Mass Spectrometry system) which is capable of analysis times of <10 seconds per sample to analyze levetiracetam in human serum. The Agilent RapidFire/MS System had comparable accuracy, precision, linearity, and sensitivity to LC/MS/MS, but with a 10-fold faster sample analysis cycle time.

Experimental

The RapidFire/MS/MS system consisted of the following modules: an Agilent RapidFire 360, an Agilent 6460 Triple Quadrupole LC/MS system, Agilent MassHunter Triple Quadrupole Acquisition software B.04.01 with Qualitative Analysis B.04.00, and RapidFire Integrator software.

RapidFire-triple quadrupole conditions

Samples were analyzed at a rate of 9.5 seconds per sample using the conditions shown in Table 1. Levetiracetam and the internal standard were monitored simultaneously in all experiments (Table 1).

Chemicals and reagents

The analyte levetiracetam and its stable-labeled isotope internal standard levetiracetam-[D3] were purchased from Cerilliant Round Rock, TX. Quality control samples were purchased from UTAK Laboratories, Inc. Valencia, CA.

Sample preparation

Calibration standards were prepared by spiking human serum with levetiracetam to final concentrations ranging from 1 to 100 µg/mL. Commercially available quality control standards made in human serum were also analyzed. The serum samples were precipitated with acetonitrile containing internal standard. The precipitated samples were centrifuged, and the supernatant was removed and transferred to a 96-well plate for analysis.

Data analysis

RapidFire Integrator software was used for peak integration. The quantifier ion AUC of levetiracetam was normalized by the AUC of the internal standard. The data was subjected to linear regression with 1/x weighting.

Results and discussion

Prepared calibration standards and commercially available quality controls were analyzed using a RapidFire/MS system in triplicate over a series of days to establish both intra- and interday precision and accuracy. Levetiracetam (both the quantifier and qualifier ions) had intra- and interday accuracies within 15% and coefficient of variation values less than 10% for all concentrations within the linear range (Table 2). This method had excellent linearity within the measured range of 1 to 100 µg/mL with an R^2 value greater than 0.995 (Figure 1). Carryover was assessed by analyzing the AUC of a blank injection immediately following the highest standard curve concentration and calculated as a % of the mean peak area of the 1 µg/mL standard. No significant carryover (<1%) was seen using this method. Signal-to-noise ratios were calculated looking at peak-to-peak height and found to be greater than 20:1 at 1 µg/mL.

Table 1. RapidFire/MS/MS conditions.

RapidFire Conditions						
Buffer A	Water with 10 mM ammonium acetate, 0.1 % formic acid; 1.5 mL/min flow rate					
Buffer B	Methanol with 0.1 % formic acid; 1.25 mL/min flow rate					
Injection Volume	10 µL					
SPE Cartridge	Agilent RapidFire cartridge C (reversed-phase C18 chemistry, p/n G9203E)					
RF State 1	Sip sensor					
RF State 2	3,500 ms					
RF State 3	3,000 ms					
RF State 4	500 ms					
Triple Quadrupole Conditions						
Gas Temperature	350 °C					
Gas Flow	8 L/min					
Nebulizer	45 psi					
Sheath Gas Temperature	400 °C					
Sheath Gas Flow	9 L/min					
Nozzle Voltage	500 V					
Capillary Voltage	3,000 V					
	Q1	**Q3**	**Dwell**	**Fragmentor**	**CE**	**CAV**
IS	174.01	129.1	50	70	9	2
Quantifier	171.01	126.1	50	70	9	2
Qualifier	171.01	69.1	50	70	15	2

Table 2. Intraday and interday precision and accuracy for RapidFire/MS/MS analysis of leviteracetam in serum.

Leviteracetam (ng/mL)	Intraday % Accuracy (n = 3)	Intraday % Precision (n = 3)	Interday % Accuracy (n = 4)	Interday % Precision (n = 4)
1	104.3	2.5	105.9	2.9
5	93.5	0.5	91.8	2.4
25	100.9	2.3	100.9	2.9
50	102.4	1.5	102.6	2.3
100	98.8	1.3	98.8	1.6
UTAK1 (15.5)	95.9	1.4	95.2	4.3
UTAK2 (39.7)	16.2	0.5	15.5	3.1
UTAK3 (73.7)	104.3	0.6	105.1	2.9

Levetiracetam was spiked into bovine serum, processed, and run immediately at the Mayo Clinic, while identical samples were frozen and shipped to Agilent Technologies, Inc. The values determined at Agilent using RapidFire/MS were then compared to the values obtained by LC/MS/MS at the Mayo Clinic. The correlation between the two analytical methodologies was very good, R^2 value greater than 0.99 and slope within 1.0 ±0.1 (Figure 2).

Blinded human samples were processed and run immediately at the Mayo Clinic using LC/MS/MS, while identical samples were frozen and shipped to Agilent for RapidFire/MS analysis. The two methods had a very good correlation with an R^2 value greater than 0.995 and a slope within 1.0 ±0.1 (Figure 3).[1]

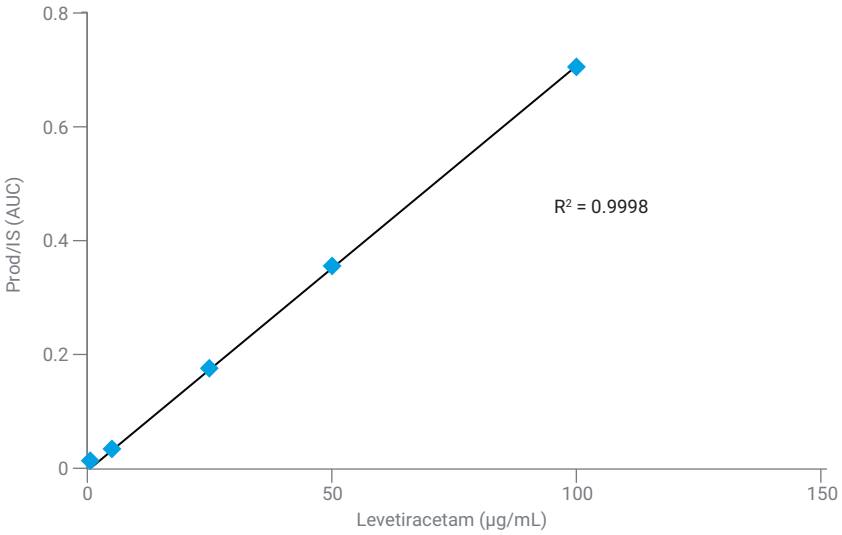


Figure 1. Representative standard curve for levetiracetam spiked into serum.

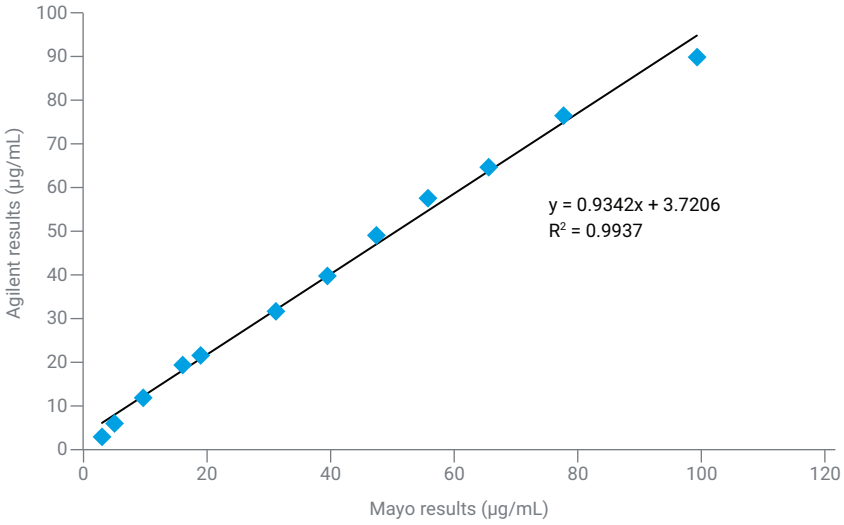


Figure 2. Correlation between RapidFire/MS/MS and LC/MS/MS for spiked levetiracetam samples.

Conclusion

Based on these results, levetiracetam can be accurately and precisely measured in human serum using the Agilent RapidFire/MS system at rates of 9.5 seconds per sample. While the analytical results of human samples were comparable to LC/MS/MS, the analysis time was approximately 10 times faster. RapidFire/MS may be useful for the fast and efficient analysis of similar targets of clinical research.

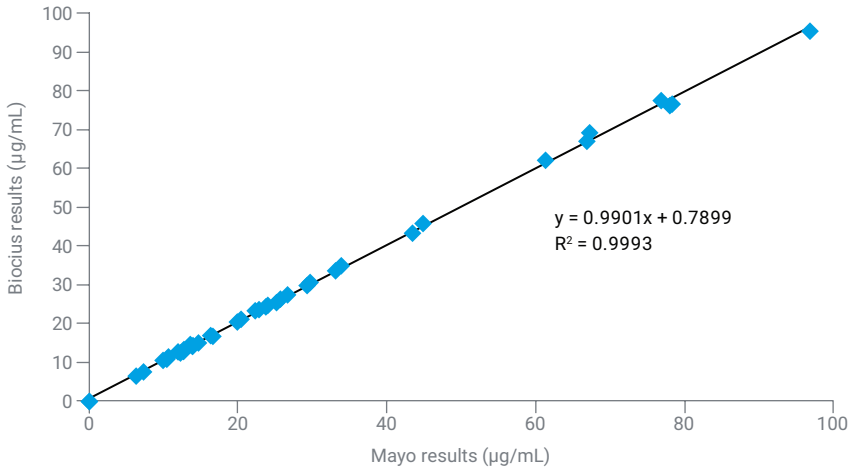


Figure 3. Correlation between RapidFire/MS/MS and LC/MS/MS for blinded human samples.

Acknowledgements

The authors gratefully acknowledge Frank Crow, Eric Korman, Christine Snozek, and Loralie Langman at the Mayo Clinic, Rochester, Minnesota for proving technical assistance and the blinded human samples used in this study.

Reference

1. Romm, M. V. *et al.* High-Throughput Analysis of Levetiracetam in Serum Using Ultrafast SPE/MS/MS. Poster #161 presented at the *59th ASMS Conference on Mass Spectrometry and Allied Topics*, June 7th **2011**, Denver, CO.

www.agilent.com/chem/rapidfire

For Research Use Only. Not for use in diagnostic procedures.

This information is subject to change without notice.

© Agilent Technologies, Inc. 2014, 2022
Printed in the USA, April 14, 2022
5990-9995EN

Agilent
Trusted Answers

Application Note

Clinical Research

Agilent

Trusted Answers

Feasibility of the Agilent RapidFire High-Throughput MS System for Ultrafast Screening of Drug Targets by Q-TOF

Authors

Brandon Nelson and
Frederick Strathmann
NMS Labs
Willow Grove, PA USA

Jennifer Hitchcock,
Julie Cichelli, Andy Gieschen,
and Kevin McCann
Agilent Technologies, Inc.

Abstract

The Agilent RapidFire high-throughput MS System and Agilent 6545 LC/Q-TOF (RF/Q-TOF) have been used to develop an efficient, qualitative method for the simultaneous analysis of a subset of analytes currently screened by ELISA. This method uses a supported liquid extraction (SLE) before RF injection and Q-TOF auto-MS/MS data acquisition. A personal compound database library (PCDL) provides scoring criteria to confirm the presence or absence of analytes of interest by comparing acquired spectra to known high-quality spectra at various collision energies (CEs). This methodology was compared to a 263-analyte postmortem blood screen currently used in our laboratory, which uses a liquid-liquid extraction (LLE) followed by a 10 minute LC/TOF analysis. RF/Q-TOF data acquisition averages 10 seconds per injection, illustrating the potential to improve the current screening time by 60x. Using the Q-TOF to acquire high-resolution accurate mass data that can be matched to a spectral database also gives a greater degree of confidence in positivity over the TOF's accurate mass and retention time data alone.

Introduction

Current methodologies in our laboratory for postmortem blood screening involve the use of a liquid-liquid extraction followed by LC/TOF analysis. One of the drawbacks to this type of analysis is that chromatography takes time to separate matrix components and resolve isobaric analytes of interest. Also, isobaric interferences found in whole human postmortem blood can cause false positives, which can lead to additional wasted time and instrument capacity when confirmation testing is performed. Furthermore, validating new analytes of interest as potential screening targets can be tedious as the method is very sensitive to modifications.

The Agilent RapidFire delivers ultrafast, simultaneous analysis of analytes with average injection times less than 15 seconds. When used in conjunction with the 6545 Q-TOF, it is possible to rapidly generate spectral data that can be matched to a library. Scoring criteria can be established to screen for drugs of interest in extracted samples, making it easy to distinguish true positive samples from false positives.

A subset of analytes currently screened by ELISA (Table 1) were used to prove the concept of using RF/Q-TOF analysis for high-throughput screening in blood. SLE was used for extraction of these analytes before injection onto the RF/Q-TOF. The average total injection time from sample to sample was 10 seconds. When compared to the current 10 minute chromatographic LC/TOF method, the RF/Q-TOF improved this time frame by 60 times. The RF settings used (Table 2) comprised a short load time to allow the very hydrophilic morphine to remain on the cartridge.

Table 1. ELISA screened analytes.

Initial Analytes Of Interest for RF/Q-TOF Analysis				
Analyte	**Precursor Mass**	**Targeted Concentration (ng/mL)**	**Collision Energy (V)**	**Sample**
Amphetamine	136.1121	100	10	5
Methamphetamine	150.1277	400	10	5
MDA	180.1019	100	10, 20, 40	6
MDMA	194.1176	100	10, 20, 40	6
Meprobamate	219.1339	200	10, 20	7
PCP	244.206	50	10, 20, 40	6
Carisoprodol	261.1809	200	10, 20	7
Tramadol	264.1958	100	10, 20, 40	7
EDDP	278.1903	100	10, 20, 40	6
Diazepam	285.0789	250	10, 20, 40	2
Morphine	286.1438	250	40	1
Hydromorphone	286.1438	100	40	3
Benzoylecgonine	290.1387	100	10, 20, 40	5
Codeine	300.1594	50	10, 20	1
Hydrocodone	300.1594	250	20	3
Oxymorphone	302.1387	100	10, 20	4
Cocaine	304.1543	100	10, 20, 40	7
Zolpidem	308.1757	100	10, 20, 40	6
Alprazolam	309.0902	100	10, 20	8
Methadone	310.2165	100	10, 20, 40	6
Clonazepam	316.0484	100	10, 20	8
Oxycodone	316.1543	250	10, 20	4
Lorazepam	321.0192	100	10, 20	8
6-Acetyl morphine	328.1543	25	10, 20	1
Fentanyl	337.2274	10	10, 20, 40	8
Buprenorphine	468.3108	20	10, 20, 40	8

Table 2. Agilent RapidFire conditions.

Agilent RapidFire Conditions	
Buffer A (Pump 1)	0.1% formic acid in HPLC grade water; 1.5 mL/min flow rate
Buffer B (Pump 2)	0.1% formic acid in 90% HPLC grade methanol:10% HPLC grade water; 1.25 mL/min flow rate
Buffer C (Pump 3)	0.1% formic acid in 90% HPLC grade methanol:10% HPLC grade water; 0.6 mL/min flow rate
Aqueous Wash	HPLC grade water
Organic Wash	LC/MS grade acetonitrile
Injection Volume	10 µL
SPE Cartridge	Agilent RapidFire cartridge C (reversed-phase C18, p/n G9205A)
RF State 1	600 ms
RF State 2	1,500 ms
RF State 3	0 ms
RF State 4	6,200 ms
RF State 5	500 ms

Longer elution times (RF state 4) with a lower flow (0.6 mL/min) for pump 3 resulted in a wider peak to give a greater area to use auto-MS/MS across. Tables 3 to 5 present Q-TOF source, tuning, and auto-MS/MS data acquisition settings.

Table 3. Agilent 6545 LC/Q-TOF source and tuning conditions.

Agilent 6545 LC/Q-TOF source conditions	
Ion Mode	Positive
Source	Agilent Dual AJS ESI
Capillary Voltage	3,500 V
Dry Gas Temperature	300 °C
Dry Gas Flow	12 L/min
Nebulizer Pressure	45 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
Nozzle Voltage	500 V
Fragmentor	125 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Mass Range	Low (1,700 *m/z*)
Fast Polarity Switching	Disabled
Slicer Mode	High resolution
Instrument Mode	Extended dynamic range (2 GHz)
Reference Mass	121.050873 and 922.009798

Table 4. Agilent 6545 LC/Q-TOF acquisition and reference mass conditions.

Agilent 6545 LC/Q-TOF auto MS/MS conditions	
MS Range	50 to 1,000 *m/z*
MS Acquisition Rate	20 spectra/sec
MS/MS Range	50 to 500 *m/z*
MS/MS Acquisition Rate	5 spectra/sec
Isolation Width	Medium (~4 *m/z*)
Collision Energy	10, 20, and 40 V
Max Precursor Per Cycle	10
Absolute Threshold	1,000 counts
Relative Threshold (%)	0.01%
Active Exclusion	Enabled
Excluded After	1 spectra
Released After	0.1 minutes
Use PC for MS/MS decisions	Disabled (if enabled will override collision energy tabs)
Isotope Model	Common organic molecules
Active Precursor Charge-State Selection and Preference	1, unkown
Sort Precursors by Abundance Only	Enabled
Scan Speed Varied Based on Precursor Abundance	Enabled
Target	25,000 counts/spectrum
Use MS/MS Accumulation Time Limit	Enabled
Reject Precursors That Cannot Reach Target TIC Within the Time Limit	Disabled
Purity Stringency	0%
Purity Cutoff	0%

Table 5. Agilent 6545 LC/Q-TOF preferred/exclude conditions.

Agilent 6545 LC/Q-TOF auto-MS/MS Preferred/Exclude Tab Example							
On	Prec. *m/z*	Delta *m/z* (ppm)	Z	Prec. type	RT (min)	Delta RT (min)	Iso. Width
Active	121.050873	100	1	Exclude	0	1	Medium (~4 *m/z*)
Active	922.009798	100	1	Exclude	0	1	Medium (~4 *m/z*)
Active	136.1121	100	1	Preferred	1	5	Medium (~4 *m/z*)
Active	150.1277	100	1	Preferred	1	5	Medium (~4 *m/z*)
Use preferred ion list only			Enabled				

Experimental

RapidFire/Q-TOF conditions

The Agilent RF/Q-TOF system consisted of the following modules: Agilent RapidFire 365, Agilent 6545 Quadrupole Time of Flight LC/MS using Agilent MassHunter Acquisition Software (B.09.00) with Qualitative Analysis Navigator (B.08.00), Qualitative Analysis Workflows (B.08.00), PCDL Manager (B.08.00) and RapidFire Acquisition Software (5.0.0.18130). Samples were analyzed at a rate of 10 seconds per sample. Preferred precursor masses were detected and fragmented using auto-MS/MS acquisition. Agilent Qualitative Analysis Workflows provided database and library search scores by referencing a PCDL created by Agilent.

Chemicals and reagents

All of the analytes were purchased from Cerilliant, Round Rock, Texas. HPLC grade water and methanol were from Honeywell, Mexico City, Mexico. LC/MS grade acetonitrile and isopropyl alcohol were from Honeywell. HPLC grade methylene chloride was from Fisher Scientific, Waltham, Massachusetts. Concentrated hydrochloric acid and ammonium hydroxide were from Fisher Scientific. HPLC grade methyl *tert*-butyl ether was from MilliporeSigma, Burlington, Massachusetts. High purity formic acid was from ProteoChem, Hurricane, Utah. Human whole blood was from BioIVT, Westbury, New York.

Sample preparation

Multiple samples were fortified with the drugs of interest at the targeted concentrations in Table 1 and extracted using the following procedure:

1. First, 500 µL of human whole blood was aliquoted to 12 × 75 mm glass tubes and buffered with 500 µL of 0.1% ammonium hydroxide (aqueous).
2. After vortex mixing for 10 seconds, the samples were loaded onto 1 mL SLE+ cartridges from Biotage (part number 820-0140-C) using a pipette with plastic tips to transfer. Positive pressure was applied through a System 48 CEREX Pressure Processor manifold at five psi for five seconds, and samples were allowed to bind for five minutes at ambient pressure.
3. Methylene chloride:isopropyl alcohol (95:5, v/v, 1 × 2.5 mL) was used to elute the analytes of interest by gravity into glass 13 × 100 mm tubes for five minutes, followed by positive pressure at five psi for five seconds. Methyl *tert*-butyl ether (2 × 2.5 mL) was used for further elution by gravity for five minutes after each aliquot, followed by positive pressure at five psi for five seconds.
4. A final pulse of positive pressure at 15 psi over 20 seconds yielded the final aliquots for evaporation.
5. Extracts were evaporated at 40 °C in the presence of 100 µL of hydrochloric acid (0.05%, methanol) using a Biotage Turbo Vap LV under the following gradient:
   - One minute (1.0 L/min)
   - Three minutes (1.6 L/min)
   - Eight minutes (3.0 L/min)
6. Once completely dry, the samples were reconstituted with 500 µL of HPLC grade water:methanol (90:10, v/v) to yield somewhat cloudy extracts. The samples were transferred to Agilent 0.5 mL polypropylene 96-well plates (part number 5042-1386) for RF/Q-TOF data acquisition.

Data analysis

System control and data acquisition were performed by MassHunter Acquisition Software in conjunction with RF Acquisition Software. Data analysis was completed using Qualitative Analysis Workflows in conjunction with PCDL Manager.

A compound discovery workflow was constructed using the Find By Auto MS/MS compound mining algorithm with library/database forward and reverse scores set to 0 to capture everything. Database search settings used mass only as values to match with a tolerance of 10 ppm. Since the RapidFire system does not provide chromatographic separation, retention time matching was not necessary. Only precursors resulting from +H charge carriers were looked at in this study. The overall score contribution for the database scoring was set to 100 for the mass score, and 5 for isotope spacing.

Library scores were calculated based on an average reverse score resulting from the fragmentation of the precursor masses of interest at 10, 20, or 40 V. Fragmentation data were compared to a PCDL containing unique spectra of the analytes of interest. The overall final score was weighted 50/50, composed of the database and library scores.

Results and discussion

Database scores indicated how close the precursor mass of the acquired spectra matched that of known spectra. Library scores indicated how close the fragmentation pattern of the acquired spectra matched that of known spectra. Initial runs used CEs at 10, 20, and 40 V for every preferred precursor mass. These scores were then compared to extracted blank blood samples to determine optimal CEs that gave unique fragmentation patterns for fortified samples to tease out isobaric interferences (Table 6). Amphetamine, methamphetamine, hydromorphone, and morphine appeared in blank blood with unusually high scores, which would make determining a real hit difficult, and lead to a large number of false positives. By excluding CEs at 20/40 V for amphetamine and methamphetamine as well as 10/20 V for morphine/hydromorphone, true hits can be distinguished from false positives. Library scores for drugs of interest are relatively high in fortified whole human blood when compared to blank blood (Table 6). Fortified morphine had the lowest score (at 67.11) using the optimized CEs, but this is highly distinguishable from a score of 11.14 for a blank blood sample.

Table 6. CE comparison of ELISA screened analytes.

Analyte	Database Score	Library Scores Using All CEs (10, 20, 40 V)		Library Scores Using Optimal CEs (see Table 1)	
		"Blank" Blood	Fortified Blood	"Blank" Blood	Fortified Blood
Amphetamine	97.19	14.64, 76.35, 62.06	98.52, 99.98, 97.27	2.26	97.17
Methamphetamine	97.54	17.19, 91.01, 78.23	99.99, 100, 99.99	17.19	99.93
MDA	96.91	0, 32.21, 32.39	98.06, 96.68, 93.14	5.65, 0, 4.06	96.68, 93.66, 91.9
MDMA	99.75		99.21, 98.28, 94.57		98.36, 92.45, 96.49
Meprobamate	96.2	0, 0, 85.16	86.9, 90.07, 86.4	0, 32.64	70.03, 79.59
PCP	99.97		91.59, 91.35, 91.3		93.3, 89.29, 89.36
Carisoprodol	95.1	4.11, 31.9, 55.24	96.63, 98.45, 99.74		96.43, 95.39
Tramadol	94.97		99.39, 100, 100		98.08, 100, 100
EDDP	99.92		100, 99.78, 96.64		98.55, 99.5, 88.66
Diazepam	99.75		99.87, 90.61, 84.06		99.89, 88.87, 83.8
Morphine	98.93		100, 98.27, 70.22	11.14	67.11
Hydromorphone	99.34	98.62, 78.44, 5.94	99.79, 93.14, 54.94		72.12
Benzoylecgonine	96.76		98.88, 95.8, 89.33		96.9, 96.95, 82.48
Codeine	94.97		100, 94.42, 27.29		100, 94.61
Hydrocodone	89.67		99.99, 98.75, 83.53		93.14
Oxymorphone	99.2		93.31, 78.29, 40.01		92.68, 85.98
Cocaine	99.79		97.19, 96.33, 89.49		98.1, 97.84, 87.78
Zolpidem	99.92		96.13, 97.68, 90.91		99.53, 98.53, 98.11
Alprazolam	97.25		99.53, 95.27, 84.08		96.82, 95.32
Methadone	96.93		91.46, 94.69, 94.93		98.75, 98.7, 98.34
Clonazepam	99.35		99.79, 86.38, 38.28		99.6, 97.4
Oxycodone	96.62		97.79, 94.4, 62.71		97.39, 97.74
Lorazepam	99.79		74.71, 64.23, 20.58		95.17, 93.95
6-Acetyl morphine	99.85		100, 96.19, 58.35		100, 96.17
Fentanyl	96.27		98.88, 78.31, 92.94		98.47, 86.74, 93.24
Buprenorphine	99.61		100, 100, 82.14		100, 100, 85.84

Using high-resolution accurate mass spectral matched fragmentation data gives more than enough confidence to distinguish real hits from false positives when using RF/Q-TOF in the absence of chromatographic separations typical of LC/Q-TOF.

The next step in testing RF/Q-TOF feasibility for rapid accurate drug screening in blood was to compare this method to the current postmortem blood screen used in our laboratory with a target scope of 263 analytes. Figure 1 demonstrates the time comparison between the currently used LC/TOF method (top) to our RF/Q-TOF method (bottom). Each injection required 10 minutes to analyze with LC/TOF, while it only takes 10 seconds to analyze an injection by RF/Q-TOF. This results in a 60x increase in sample throughput.

Table 7 summarizes a direct comparison of results from the LC/TOF and RF/Q-TOF methods. Twenty-six samples were prepared using the existing LLE method and analyzed by LC/TOF. Leftover extracts for each sample were then analyzed by RF/Q-TOF with no further modification. In 26 samples, 121 analytes were listed as positive hits using the LC/TOF and RF/Q-TOF methods. The RF/Q-TOF reported a total of 10 analytes as false positives, but this is based on unoptimized CEs. Manual investigation of the data showed that all 10 false positives were resolved using the optimized CEs. Finally, 132 analytes in-scope were not found using either method.

Table 7. Positivity comparison between LC/TOF and RF/Q-TOF. Manual investigation of the data shows that false positives are eliminated when using only optimal CEs.

N = 26	LC-TOF Positive	LC-TOF Negative
RF/Q-TOF Positive	121	10*
RF/Q-TOF Negative	0	132**

* CE of 40 is not optimal

** 132 Negative compounds not found in either

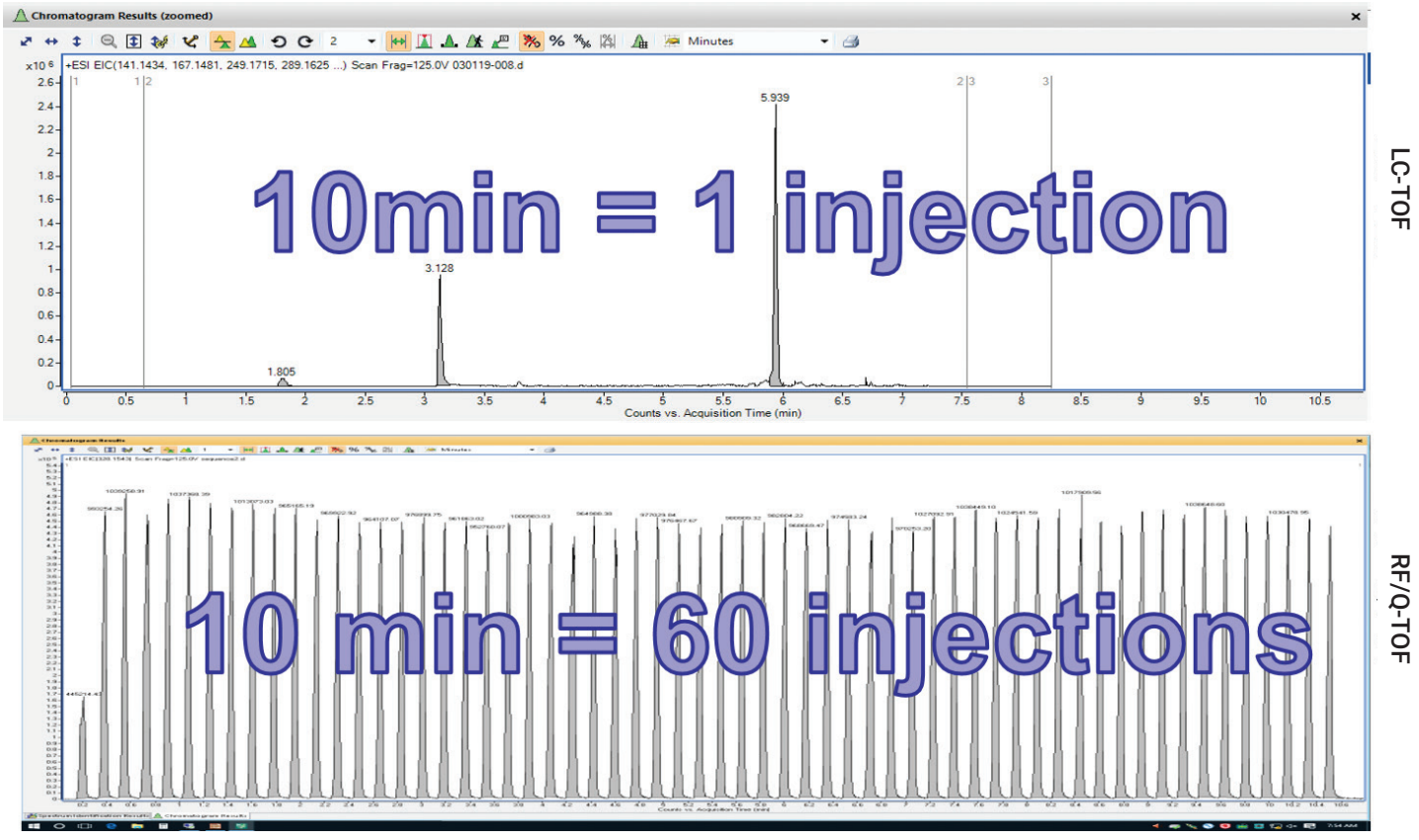


Figure 1. Injection comparison between LC/TOF and RF/Q-TOF.

Conclusion

A subset of ELISA screened analytes were studied to prove the utility of the Agilent RF/Q-TOF as a platform for high-speed drug screening in human whole blood. Auto-MS/MS, in conjunction with a PCDL, were used to accurately distinguish between a true positive sample and higher quantities of isobaric interferences. The RF/Q-TOF methodology provided results comparable to the current LC/TOF screen used in the lab, while increasing sample throughput by a factor of 60. Further development of this methodology could prove extremely beneficial to the forensic drug community when analyzing postmortem samples for the presence of a wide range of drug classes.

Learn more:
www.agilent.com/chem/rapidfire

Buy online:
www.agilent.com/chem/store

Get answers to your technical questions and access resources in the Agilent Community:
community.agilent.com

Find a local Agilent customer center in your country:
www.agilent.com/chem/contactus

U.S. and Canada
1-800-227-9770
agilent_inquiries@agilent.com

Europe
info_agilent@agilent.com

Asia Pacific
inquiry_lsca@agilent.com

RA44343.6601851852

For Research Use Only. Not for diagnostic procedures
This information is subject to change without notice.

© Agilent Technologies, Inc. 2023
Published in the USA, November 9, 2023
5994-6839EN

Agilent
Trusted Answers