

Optimal Approach for Multiplexed NanoLC-MS

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Overview

The growing use of LC-MS in drug discovery, biomarkers detection and proteomics research has made increasing sample throughput without compromising sensitivity extremely desirable. Gradient methods preclude Hadamard Transform (HT) multiplexing techniques that have been developed successfully for GC¹ and CE² applications since these techniques require that the separation methods do not have time-variant elements. Multiplexing independent LC's running different (or the same) gradient or isocratic methods for a single MS will be demonstrated. Methods for multiplexing may range from the sequential input (one-on-at-a-time, or 1/N duty cycle, N=number of LC's), to intermediate duty cycle, e.g., Hadamard Transform (HT), which has a ~50% duty cycle, to one-off-at-a-time, i.e., (N-1)/N duty cycle. The sequential method is the most straightforward, but sensitivity and resolution of MS detection suffers because of diminishing duty cycle as the number of LC's increase. This report evaluates the various approaches multiplexing 3 and 7 nanoLC- for signal detection, and the methods of deconvoluting the data.

The three approaches for LC-MS multiplexing

All the multiplexing approaches are based on the use of discrete "time bins" T_n of a fixed time duration. Since the chromatographic peak widths were from 15 s to 30 s, the time bin was chosen to be 1 to 2 s long so that each peak would contain sufficient time-bins to give adequate time resolution for the data deconvolution. The number of nanoLC's to be multiplexed is 3 for the experimental section, and up to 7 for theoretical consideration. The method for starting and stopping sprays is by turning the spray voltage on and off. No mechanical device was employed to block the spray into the mass spectrometer.

I. The sequential approach

Waters' MUX™ platform that sequentially samples 4 or 8 LC inputs for a single mass spectrometer is the state-of-the-art commercially available technology. Using similar sequential spray techniques, the eluates from two different columns have been sprayed into a mass spectrometer for detection by means of a robotic arm that positioned the spray tips alternately in front of the mass spectrometer.³

In this experiment, the spray voltages for the three spray emitters were sequentially turned on for a time bin duration of 2 s each. "Deconvolution" of the data simply involves assigning the MS signal collected during T₁ to spray emitter 1, T₂ to spray emitter 2, etc. The signal counts in the discrete time bins after spray emitter assignments are then interpolated to smooth the LC peak features. We limit the number of nanoLC's to 3 for the sequential approach because a higher number of nanoLC's will make the resolution and sensitivity of any chromatographic peak features too low to be usable since the duty cycle is reduced is 1/N.

II. The Hadamard-based Multiplexing Approach

The Hadamard-based multiplexing scheme is based on the mathematics of Hadamard matrices and uses cyclic N by N Hadamard simplex matrices (consisting of 1's and 0's instead of the 1's and -1's in the regular Hadamard matrices) to define the on-off patterns that are sequentially and regularly applied to the nanospray spray tips. These 0's and 1's represent the "off" or "on" states of the spraying of the spray tips: each column of the matrix gives the on-off patterns of the N spray tips at any given time interval or "time bin". Cyclic simplex matrices exist only for certain N (see Ref. 4). The lowest values are N = 3, 7, 11, 15, 19, 23, 31, 35 Each column has exactly (N+1)/2 ones, so the duty cycle for each source is about 50%.

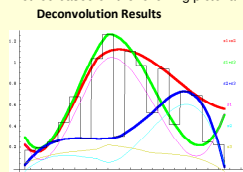
The deconvolution method of the Hadamard-based technique is depicted in the following set of diagram.

The Hadamard-based Multiplexing of LC Sources

For the case of N=3, the shaded portions of the top graphs indicate intervals during which the spray of the nanospray source is turned off. Reading vertically for time bin=1, the Hadamard sequence representing the on/off pattern of the nanospray sources is 110. For time bin=2, the Hadamard sequence is 101, etc. The data represented by the MS output are the sum of the three sources, only two of which are on for a given time bin. Data points are generated by averaging in each time bin. A transform of dimension N is then carried out for each data point, utilizing N neighboring data points of which that data point is the central one. The result is approximate and relies on the time variation of the neighboring points being small. We can correct exactly for errors caused by non-vanishing slopes and curvatures. Numerical simulations have shown that the deconvolution works well if the sharpest Gaussian peaks of the sources are at least N bins wide. In this diagram, the deconvoluted signal is represented by the shaded rectangular bars at each time bin. The signal from each source spraying separately and continuously is superimposed onto the deconvoluted signal to show the degree of approximation. It is clear from this diagram that the higher the number of time bins that fit into a peak, the better the approximation. The maximum number of columns or sprayers supported by this method is limited by the peak width, the scan speed of the mass spectrometer, the switching time of the high voltage for inducing the spray, and also the physical space available for placing the spray devices at the MS inlet.

The Interpolation method of deconvolution

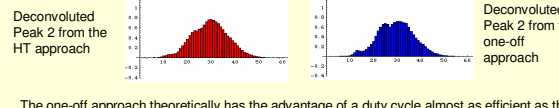
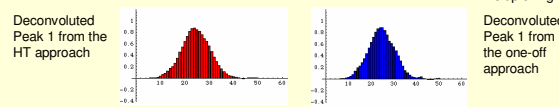
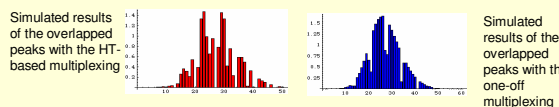
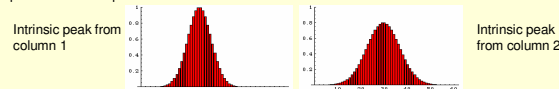
Since N is a small number in our multiplexing scheme, an alternative deconvolution method based on the following pictorial concept offers a far simpler approach.



The binned data from a paired-spray experiment are indicated in black. The thick curves interpolate every third bin and provide a continuous estimate of each pair intensity, passing through the points at which its value is known exactly. The thin curves show for the intensities of the individual sources, e.g. $s1 = ((s1 + s2) + (s1 + s3) - (s2 + s3))/2$

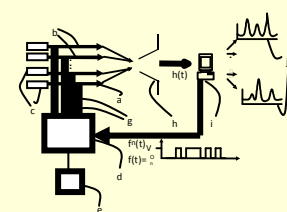
III. The One-off Approach

For N < 15, it is possible to have unambiguous assignment of the sprays to the spray emitters by having just one spray emitter turned off during any time bin. The theoretical duty cycle of the signal detection can thus be increased to (N-1)/N. The method and its deconvolution is illustrated below. The deconvolution method used here is interpolation. The peak from column 1 has Gaussian peak of width 7 bins, height= 1; the peak from column 2 has a peak offset by 5 bins (the two peaks overlap substantially), width= 10 bins, height=0.8. The other five columns produce have no peak features.



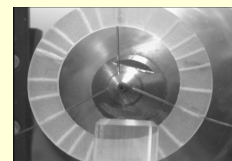
The one-off approach theoretically has the advantage of a duty cycle almost as efficient as that of individual uninterrupted LC runs. However, the example shown above indicates that the deconvoluted results of the one-off approach do not appear to be superior than those of the Hadamard-based (~50% duty cycle) approach. Moreover, we discover that the "ghost" features, artifacts that resemble LC peaks resulting from the deconvolution process become more severe as the duty cycle increases. The experiments described here will provide feedback for refinements to our theoretical findings.

Experimental Design



A schematic drawing of the multiplexing techniques for columns running different methods including gradient elution. a: spray devices; b: separation columns (or separation lanes of a multidimensional devices); c: LC pump devices; d: high speed high voltage switch box for supplying the spray voltages in the form of repetitive sequences; e: high voltage power supply; f: the chosen sequence applied to the spray devices; h: the mass spectrometer inlet; i: the computer control for generating the spray sequence and the deconvolution; j: the deconvoluted mass chromatograms of the individual separations. This technique is patent-pending.

Experimental Method Multiplexing 3 nanoLC-MS separations



The spray devices used were the tapered fritted ends of C18, 5 μm columns. The non-clogging spray tips sprayed eluates directly into the mass spectrometer without dead volume. They were arranged in a circle so that the spray from each device was equivalent in position and orthogonal to the MS (LCQ Advantage) inlet. An electronic circuit and the accompanying software switched the high voltage required for nanospray (2.4 to 4 kV) on (HV) ms time scale every 2 s (2 s time bin duration). For the experiments in this report, the HV used was 3.8 kV. The column holder can accommodate up to 23 columns.

Three separations with two distinct peptide mixtures were used in these experiments.

The peptide standards (2pmole/mL)		Neuropeptide standards (2 μg/mL)	
Name	[MH+2]2+	Name	[MH] ⁺ [MH+2]2+
γ-Endorphin	930.0	Angiotensin II	524
Bradykinin1-9	531	Val-Tyr-Val	380
Bradykinin2-9	453	Leu-enkephalin	556
		Met-enkephalin	574

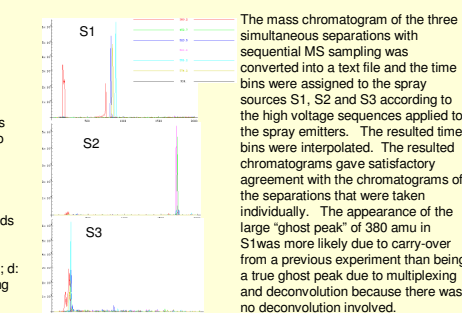
The run buffers were A: 98% H₂O + 0.1% formic acid
B: 98 % acetonitrile+ 0.1% formic acid.

The methods and samples for the three sprayers are:

S1	S2	S3
75 μm i.d., 5 μm C18 capillary column	75 μm i.d., 5 μm C18 capillary column	75 μm i.d., of 5 μm, C18 capillary column
Neuropeptide sample	Peptide standard mix	Neuropeptide sample
30 minutes of equilibration at 10% B	Same as S1	Isocratic elution 70%A/30%B
0-3 minutes 5% B		
3-4 minutes 5-20%B		
4-36 minutes 20-60% B		
37-38 minutes 60-98%B		
39-45 minutes 98% B		
40-50 minutes 90%B		
500 nL/min splitless pump	500 nL/min splitless pump	~500 nL/min split from 100 μL/min

Results

I. Sequential



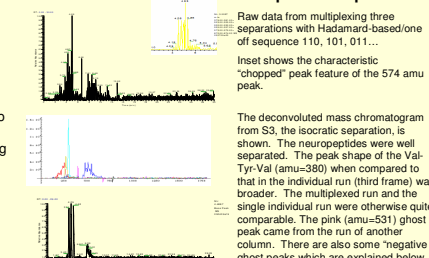
Acknowledgements: This work was partially funded by an NIH SBIR grant.

References:

- 1) Trapp, O., Angew. Chem. 2007, 46, 5609-5613.
- 2) McKeynolds, J.A., Leyi, G., Barber-Singh J., Shippy, S.A., J. Separation Sci. 2005, 28, 128-126.
- 3) Bonnell E., Tessier C., Carrier A., Thibault P., Electrophoresis 2005 26, 4675-89.
- 4) Harwit, M., Sliane, N.J.A., Hadamard Transform Optics, Academic Press, New York, 1979.

Results

II. Hadamard-based/one-off Multiplexed Separations



Inset shows the characteristic "chopped" peak feature of the 574 amu peak.

The deconvoluted mass chromatogram from S3, the isocratic separation, is shown. The neuropeptides were well separated. The peak shape of the Val-Tyr-Val (amu=380) when compared to that in the individual run (third frame) was broader. The multiplexed run and the single individual run were otherwise quite comparable. The pink (amu=531) ghost peak came from the run of another column. There are also some "negative ghost peaks" which are explained below.

Discussion

For pairwise injections, we have deconvoluted the spectra by assuming, for example, that intensity s12 from simultaneous injection of sources 1 and 2 equals the sum s1 + s2 of the intensities of these sources if injected separately. The pairwise intensity for each of the three pairs is obtained for a particular time bin by interpolating the measured numbers occurring at every third bin. The calculated value for s3 would then be (s23 + s13 - s12)/2. But, if, for as yet unconfirmed reasons such as unpredictable fluctuations in the spray intensities, s12 is instead given by the linear combination c12 s1 + c21 s2, the calculation will give the incorrect result which reduces to s3 only if all the c_{ij}'s equal 1. Expressions for the other calculated sources are obtained by cyclic permutation of the indices. These expressions show that if a large peak is present in only one of the 3 sources, (say s1 and s2 are negligible compared to s3), then a large negative value can appear in one of the other sources, and a large positive value of exactly the same magnitude will be calculated for the remaining source. In the example, this magnitude will be +/- s3 (c31 - c32)/2. We can use this property to identify peaks as ghost peaks if a negative peak of about the same magnitude appears in the calculated value for another source. In the future, we hope to be able to determine the constants c_{ij} experimentally, and we will then be able to calculate the correct source spectra without anomalous peaks.

Summary

Three different approaches for multiplexing nanoLC-MS have been presented and demonstrated to be feasible for increasing nanoLC sample throughput, even for gradient elution.

The sequential method of multiplexing 3 LC-MS appears to be adequate for obtaining mass chromatograms that are comparable to those obtained individually. This method is applicable to separations of reasonably concentrated analytes in samples. For N>3, the sequential method will have a duty cycle too diminished to be useful.

The Hadamard-based approach has the potential of improving the signal detection duty cycle to ~50% for N>3, provide an adequate signal level for data deconvolution. The disadvantage of the Hadamard approach is the more frequent appearances of "ghost peaks" which requires refinement of the deconvolution techniques.

However, for N>3, the Hadamard-based approach appears to be optimal compared to the one-off approach which provides a better duty cycle of (N-1)/N for signal detection, but does not appear in our preliminary results to gain better deconvoluted features. Moreover, it suffers more severe ghost peaks.