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LC–MS solvent composition monitoring and chromatography alignment using mobile phase tracer molecules

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Abstract

In the field of proteomics, reproducible liquid chromatographic description of analytes is often a key element for the differentiation or identification of proteins or peptides for clinical or biological research projects. However, analyte identification by retention time can be problematic in proteomics where lack of standardization can result in significantly different chromatography for the same analytes analyzed on different machines. Here we present a novel method of monitoring the mobile phase gradient of LC–MS/MS analyses by monitoring the ion current signal intensities of tracer molecules dissolved in the mobile phase solvents. The tracers' ion current signal intensities chronicled gradient fluctuations, did not adversely affect the number or quality of CID-based sequence identifications, and had lower run-to-run variance when compared to retention time.

Keywords: Liquid chromatography; Electrospray mass spectrometry; Proteomics; Peptide; Tracer molecules

1. Introduction

The liquid chromatographic (LC) retention time of an analyte is often used to determine the analyte's identity and/or differentiate it from other analytes. For example, pharmaceutical and environmental compounds are commonly identified and characterized by their retention times relative to reference standards using HPLC analysis coupled with UV detection [1,2]. In the field of proteomics where samples are complex polypeptide mixtures, LC retention time is often paired with additional peptide and protein information to find differences in the patterns obtained from different samples. For example, retention time, the accurate mass, and MS peak signal intensity from LC–MS analyses have previously been used to create polypeptide maps for compositional comparison between normal and malignant breast epithelial cell lysates [3,4]. These maps were compared against each other to screen for differential protein patterns between the cell lines [3], and for differential protein patterns between cells treated with estrogen and control cells [4]. The additional peptide characteristic information gained from retention time has also been used to aide peptide mass fingerprinting identifications by providing an additional peptide identifying factor [5]. In addition, LC retention time coupled with accurate peptide mass has also been used to characterize peptides and proteins via global cataloging (a.k.a. tagging) values for subsequent identification of these sequences by their peptide tags [6,7].

Though chromatographic peak positions are often described by the peaks' retention times, there are several drawbacks to using retention time as a chromatography descriptor. First, retention time values are LC system specific. Identical analytes analyzed on different chromatographic columns, pumping systems or mobile phase gradients can have significantly different retention time values. In the pharmaceutical and environmental fields, this variability is minimized by standardized chromatography columns and automated HPLC systems. However, this standardization is not common in the field of proteomics where experiments between laboratories are often analyzed on columns of different dimensions and packing materials, and on systems with different gradient delay times. Thus, chromatographic retention times acquired in one proteomic laboratory can be difficult to replicate in other proteomic laboratories.

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Another drawback is that retention time can be affected by external factors that are not sample dependent such as changes in the ambient or column temperature, poor solvent mixing, small gas bubbles in the chromatographic systems, and other random events. Some of these factors are unpredictable and difficult to control and mathematically model.

LC retention time shifting is especially a problem when aligning multiple chromatography runs to each other. Methods to correct for these fluctuations and to align multiple chromatography runs have included calculating relative LC peak retention times with respect to reference peaks [1,2,8], and employing computer algorithms to shift peaks and warp chromatographic runs to fit target chromatographic patterns [9,10]. One problem with these methods is that they typically do not employ enough reference peaks to allow for continuous monitoring of the gradient. Thus, alignment in the region around a reference peak can be inaccurate if the reference peak is not detected. Even if all the reference peaks are identified, some chromatography alignment algorithms assume linear or simple polynomial relationships between reference peaks that do not account for unpredicted chromatography deviations that can cause alignment errors. Other methods that use direct chromatography mapping by regressive optimization can also fail if peaks are ill-defined and poorly aligned between runs.

Another way of describing peak positions in LC is to describe the chromatography using the mobile phase solvent composition to define the chromatographic positions. One advantage of directly monitoring the solvent composition is that we can detect gradient fluctuations and systemwide events as they occur. Monitoring the gradient can also simplify run-to-run chromatography alignment by removing the need for reference analyte peaks and mathematical models to describe the chromatography.

In this paper, we present a novel method of describing LC gradients of LC–MS and LC–MS/MS experiments using tracer molecules dissolved in the mobile phase solvents. We followed the signal intensities of the ion current from the MS analysis of two tracer molecules, one dissolved in solvent A and one dissolved in solvent B, and calculated the ratio of the signal intensities to describe the mobile phase solvent composition relative to each other throughout a chromatographic run. Using this method, we simultaneously monitored the LC gradient in real-time and identified peptide sequences by MS/MS. In addition, we show run-to-run chromatographic gradient alignment of peptide samples analyzed using different LC gradients and on different ion trap mass spectrometer systems by describing peak positions using the tracer molecules' signal intensity ratios to describe the chromatographic gradient position. From these results we demonstrate that the solvent composition, as determined from the ratio of tracer molecules is a better parameter for pattern alignment than peak retention time.

2. Experimental

2.1. Liquid chromatography mobile phase solvents

Buffer A, the aqueous phase solvent, consisted of 0.4% (v/v) acetic acid (Fisher Scientific, Fairlawn, NJ, USA) and 0.005% heptafluorobutyric acid (HFBA) (Fluka, Sigma–Aldrich, St. Louis, MO, USA) in HPLC quality water with 0.01 mg/ml maltitol (Fluka, Sigma–Aldrich, St. Louis, MO, USA) as the aqueous phase tracer molecule. Buffer B, the predominantly organic phase solvent, consisted of 0.4% acetic acid and 0.005% HFBA in 80% acetonitrile (Fisher Sci, Fairlawn, NJ, USA) and 20% water with 0.01 mg/ml lactose (Sigma, St. Louis, MO, USA) as the organic phase tracer molecule.

2.2. LC–MS/MS system

Two different LC–MS/MS systems were used for the study presented here. Both systems contained Agilent 1100 binary HPLC pumps. These pumps were fed into a reverse phase capillary column using the pre-column flow-splitting set-up previously described [11]. The LC reverse phase capillary column (Polymicron Technologies, Phoenix, AZ) was packed to a length of 12 cm with Magic C18 resin (Michrom BioResources, Auburn, CA). The pump flow rate was set to 0.12 ml/min. This flow was split to achieve an elution flow rate of 200 nl/min off the reverse phase column. Unless otherwise specified, the gradient was as follows (gradient number 1): 0 min, 5% buffer B; 5 min, 15% B; 65 min, 35% buffer B; 80 min, 100% buffer B; 87 min, 100% buffer B; 95 min, 5% buffer B. For the peak alignment experiment, gradient number 2 was used: 0 min, 4% buffer B; 5 min, 10% B; 60 min, 40% buffer B; 70 min, 100% buffer B; 85 min, 100% buffer B; 85 min, 5% buffer B. A 15 min equilibration time was used between analyses.

The two mass spectrometers used to analyze the chromatography eluent were a ThermoFinnigan LCQ Classic and a Finnigan LCQ-Deca XP ion trap tandem mass spectrometer. The peptides were ionized using inline ESI as previously described [11]. The instruments were set to acquire masses from 400 to 2000 Da in positive ion mode and the dynamic exclusion was set for 2 min. The characteristic masses from lactose and maltitol were put on the dynamic exclusion list so they would not be selected for CID analysis throughout the chromatographic run. The exclusion list window was set to ± 2 Da.

MS/MS sequence identifications were obtained using the SEQUEST algorithm [12] and the quality of these identifications were assessed by computing the probability that the SEQUEST derived scores are typical of a correct identification using the Peptide Prophet and Protein Prophet algorithms [13,14]. Those sequences with Peptide Prophet scores greater than 0.9 were considered high-scoring identifications. This corresponded to a false positive rate of 0.7%.

2.3. Tracer molecule pre-testing for selection

Solubility of the potential tracer molecules was assessed by first dissolving 0.001, 0.01, 0.1, and 1 mg of the tracer compounds in 1 ml of 0.4% (v/v) acetic acid in water. Those compounds that resulted in a clear solution at all solubility concentrations were considered soluble. The soluble compounds were further tested for solubility in acetonitrile by dissolving 1 mg in 20 ml 0.4% (v/v) acetic acid in water and bringing the volume up to 100 ml with acetonitrile resulting in a solution

of 0.01 mg/ml tracer in 80% acetonitrile. Solubility was once again determined by the clarity of the solution after incubation in 80% acetonitrile after approximately 5 min. Approximately 2 μg of the compounds that were soluble in both solvents were then loaded onto a C18 LC capillary column for analysis by MS through the course of a full gradient from 5 to 80% acetonitrile. Those compounds that eluted as a single slug at the beginning of the gradient and had no detectable masses trailing throughout the gradient were considered to have low retention on the C18 chromatography packing. The compounds that also produced a single predominant mass in the MS spectra were considered viable tracer candidates. The tracer candidates were further tested by dissolving 1 mg of tracer in 100 ml of LC solvent (buffers A or B depending on the molecule) and running a blank LC–MS analysis containing no analyte through a full gradient program using the solvent with the tracer molecule. If the extracted ion chromatogram of the tracer followed the course of the gradient without signs of degradation or instrument contamination, it was considered a viable tracer molecule. Several molecules were tested as potential tracers, of which lactose and maltitol combined the highest number of desirable features. Molecules tested but not selected as suitable tracer molecules were lactulose, palatinose, melezitose, lacitol, saccharin, lactobionic acid, raffinose, maltulose, lacitol, sucrose, melebiose, and L-saccharopine. The results from this testing are provided in the supplementary information.

2.4. Peptide samples

Extracts of yeast proteins derived from wild type strain *S288C* were proteolyzed using previously described methods [15,16]. Trypsinized yeast peptides were purified and separated by ion exchange chromatography [17], and desalted over a Waters SepPak C18 cartridge (Milford, MA, USA) using two column volumes of 0.4% acetic acid as the washing solvent, and two column volumes of acetonitrile as the column elution solvent. The peptide samples were lyophilized to dryness on a Speedvac centrifugal vacuum system (Thermo Savant, Holbrook, NY) and reconstituted in 30 μl of 0.4% acetic acid. The eluted peptides were subsequently analyzed by LC-mass spectrometry. Approximately 20 μg of peptides were loaded onto the LC column for MS analysis.

A mixture of four commercially purchased peptide standards was also analyzed by LC–MS (New England Biolabs, Beverly, MA, USA). This mixture consisted of angiotensin I, neurotensin, ACTH (1–17), and ACTH (18–39) mixed in equal volumes. One microliter corresponding to 12 ng of each peptide was loaded onto a C18 reverse phase capillary LC column for mass spectrometric analysis.

2.5. Data processing

The tracer-based gradient chromatograms were generated from extracted ion chromatograms of the masses corresponding to maltitol (689–690) and lactose (685–686) created using the Xcalibur software provided with the ThermoFinnigan mass spectrometers (San Jose, CA). The signal intensity ratios and moving window average signal intensity values were generated by first converting the LCQ mass spectrometer .dta files to the mzXML format [18], and then collating the retention time and corresponding signal intensities of peaks with m/z between 689 and 690 (maltitol) and 685 and 686 (lactose) into a Microsoft Excel (Redmond, WA, USA) spreadsheet. The moving window average values for the signal intensities were calculated in 1 min increments (approximately 25 scans) to smooth the signal over time. The average variability in retention time of individual peaks in our system was found to be ±2 min (data not shown) and a 1 min time window of approximately 25 scans was selected to smooth the noise without generating a significant loss in signal peak intensity due to the averaging.

The retention time was derived from the highest signal intensity point of the peak corresponding to each peptide. The calculated gradient composition was calculated from the gradient value programmed in the LC pump corresponding to the peptide retention time. The tracer signal intensity ratio was calculated by first normalizing the extracted ion current signal intensity of each tracer to unity when the corresponding buffer concentration was equal to 100%.

To compare the values of the different peak position metrics, the corresponding metric values for each of the analyzed peptides was taken as the average from three runs on the same sample. The chromatographic deviation between different instruments and gradients was calculated by taking the differences between the averages, and the percentage deviation was calculated by dividing this difference by the average of the two values in question.

3. Results and discussion

The goal of this report is to present a novel method of describing LC separations and monitoring of the mobile phase gradient during LC–MS and LC–MS/MS analyses. To monitor the gradient, we first selected for suitable solvent composition tracer molecules. These tracers were dissolved in the mobile phase solvents, and the extent to which the tracer molecules' signal strengths described the solvent composition was assessed. The ratio of the tracer signals was subsequently assessed as a potential gradient descriptor for run-to-run gradient and chromatography alignment.

3.1. Selection and analysis of tracer molecules

Gradient tracer molecules must possess the following properties: good solubility and stability in the mobile phase solvents, detectable masses as measured by the mass spectrometer, signal intensities corresponding to the amount of molecule analyzed, and the tracer molecule must not stick to the LC column, contaminate the LC system, and/or hinder the chromatographic separation. These properties are noted in Table 1 for easy reference. We found sugar molecules to be ideal tracer candidates for our system because they generally have low retention on C18 resins, are detectable by mass spectrometry, and are soluble in aqueous solutions. Thus, several sugar molecules were pretested as possible tracers. Details of the tracer molecule testing

Table 1
Factors for tracer molecule selection

Desired properties	Properties to avoid
Solubility and stability in mobile phase solvents	Insolubility in one or both mobile phase solvents
Elutes off column with solvent front	High retention on chromatography column
Characteristically identifiable mass by mass spectrometer	Multiple masses due to polymerization or fragmentation
Does not contaminate the LC system or adversely affect the operation of the LC–MS system	Tracer molecule instability in chromatography buffers
Signal intensity corresponds to amount of molecule analyzed	Tracer contaminates the LC–MS system
Does not hinder chromatographic separations	Tracer molecule adversely affects LC–MS analyses

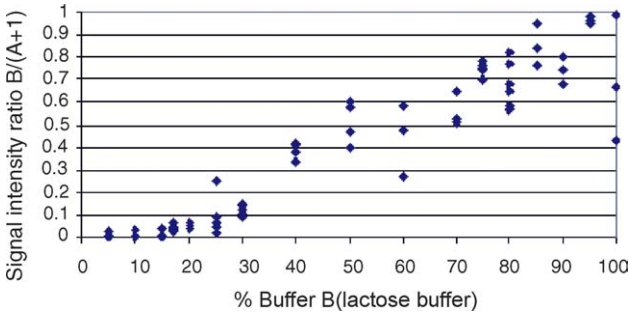

Fig. 2. Signal intensity ratio. The signal intensity ratio of lactose:maltitol increases with increasing proportion of buffer B. The ratio is calculated from the value of $B/(A+1)$, where B is the set of values corresponding to the lactose ion current and A is the set of values corresponding to the maltitol ion current at each time point. This plot demonstrates measured values of the signal intensity ratio of the two tracers at each buffer concentration.

and selection are given in the experimental methods. In general, the pre-testing involved determining the molecule's solubility in the organic and aqueous phase LC solvents, analyzing for characteristic molecular masses on the mass spectrometer, and ensuring the molecule was not retained on the LC-column and/or did not contaminate our LC–MS system. Sucrose, melezitose, lacitol, palatinose, melebiose, saccharin, lactobionic acid, raffinose, L-saccharopine, maltulose, and lactulose were eliminated from the list of potential tracers because they were either not soluble in the mobile phase solvents, did not present distinctive or quality mass spectra, or contaminated our LC system. Lactose and maltitol possessed the desired tracer molecule properties and were selected for further gradient tracer testing.

To determine the predominant tracer masses to monitor, we dissolved lactose and maltitol in their respective solvents and analyzed them on the mass spectrometer. The most intense ion peak observed for lactose was 685.5 and for maltitol was 689.4 (Fig. 1A and B). Since lactose and maltitol have molecular masses of 342 and 344 respectively, these masses are likely the (2M + H) dimers as has been previously noted [19].

To follow changes in the HPLC solvent composition throughout a chromatographic run, the signal intensity strength of the tracer molecules must accurately reflect the amount of tracer molecule present. We found that the extracted ion current signal intensities of the individual lactose and maltitol masses increased as the corresponding concentrations of these molecules increased. More importantly for the purposes of gradient monitoring, the ratio of the signal intensities of the tracer molecules reflected the amount of tracer molecule present (Fig. 2). When the signal intensities of the tracer molecules were combined as a ratio, the solvent concentrations were described relative to each other, giving a description of the mobile phase composition. To calculate this ratio, we first normalized for differences in the ion current signal intensities of the two tracer molecules caused by the differences in the stability and ionization of lactose versus maltitol. This was done by dividing the ion current signal intensities by the respective values when the concentration was 100%. Thus, at 100% concentration, the ion current signal intensities of each tracer are equal to unity and the values between the two tracers are comparable. The ratio of the extracted ion current signal intensities (SI-ratio) was calculated from the following equation:

$$\text{SI-ratio} = \frac{B}{A+1}$$

where B is the normalized ion current signal intensity of lactose and A is the normalized ion current signal intensity of maltitol. Using this relationship, the SI-ratio is theoretically equal to unity when B = 100% lactose buffer. When B = 0% lactose buffer, the SI-ratio is theoretically equal to zero. To note, since buffer B contains 80% acetonitrile, the concentration of 100% lactose buffer is actually equivalent to an 80% organic 20% aqueous solution.

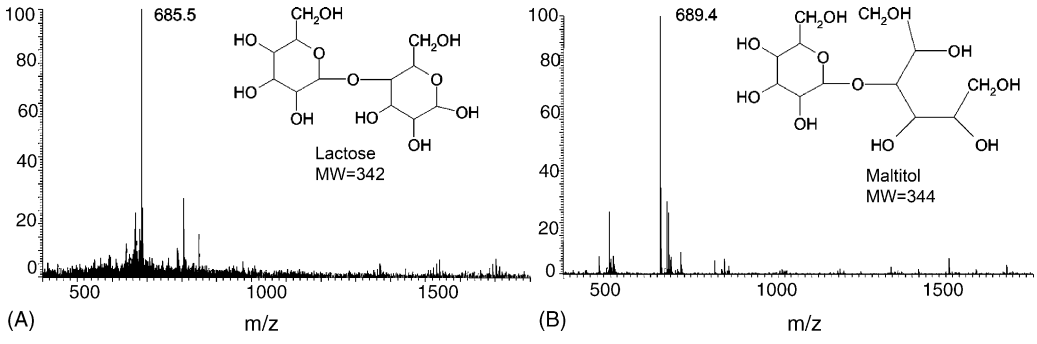

Fig. 1. Gradient tracer molecules. The characteristic mass spectrum for the gradient tracer molecules (A) lactose and (B) maltitol.

In practice, the signal intensities of the tracer molecules exhibit some fluctuation in values due to the random nature of electrospray ionization. Likewise, the SI-ratio also exhibits fluctuations as seen in Fig. 2. As the amount of buffer B increased, we saw a greater variability in the signal intensity. This was likely due to the limitations of the HPLC pump to provide stable flow at extremely low concentrations of either of the mobile phase buffers. Also, we noticed the values of the SI-ratio tended to be lower than the theoretically predicted values. This could partially be attributed to the differences in the relationships between the observed signal strength for a given tracer concentration. We normalized the individual signals prior to calculating the SI-ratio and differences in these relationships may be responsible for some of the observed deviation from the theoretically predicted values after calculating the SI-ratio.

3.2. Gradient monitoring

In the previous section we described a calibration curve that showed that the signal intensity ratio of the extracted ion current of the tracer masses (SI-ratio) changes accordingly with increasing amounts of buffer B (containing lactose). To determine whether the tracer signal intensities and SI-ratio reflect changes in the mobile phase gradient during a chromatographic run, we monitored the extracted ion current signal intensities of the lactose and maltitol masses throughout a LC–MS analysis of a mixture of four standard peptides. The resulting chromatograms show that the tracer molecules' ion current signal intensities increase and decrease with respect to increases and decreases in the programmed gradient (Fig. 3A–D). And, the resulting SI-ratio based chromatogram generally reflected the changes of the programmed gradient. As noted for Fig. 2, when buffer B was equal to 100% in the gradient, we achieved a SI-ratio less than 1 for the run depicted in Fig. 3A. This could be due to a shift in the SI-ratio values due to, or after signal normalization of the individual tracer signal intensities. It could also be due to HPLC pump inaccuracy at low buffer concentrations (i.e. low %A or %B). SI-ratios less than 1 at 100% B were observed repeatedly for the batch of runs from which the sample chromatogram was derived.

Also shown in Fig. 3A, we observed a deviation between the SI-ratio monitored gradient and the programmed gradient in the form of a time delay between the programmed gradient and the monitored gradient that was caused by the time necessary for the solvent to move from the mixing chamber of the pump, through the tubing and reverse phase column, to the mass spectrometer.

In these experiments, we noted that the tracer molecule ion current signal intensities were above the background noise of approximately 10^4 (counts) and below the typical peptide ion current signal intensity of between 10^6 and 10^8. Our tracer signal intensity range was between 10^5 and 10^6 for the lactose and maltitol concentrations tested (0.01 mg/ml). Thus, the signal intensity values of the background noise, tracer molecules, and peptides were differentiable by a factor of 10 or more. Further evidence of the signal discrimination between the background noise, tracer molecules, and peptides was provided by the detection of the four added peptides independently of the

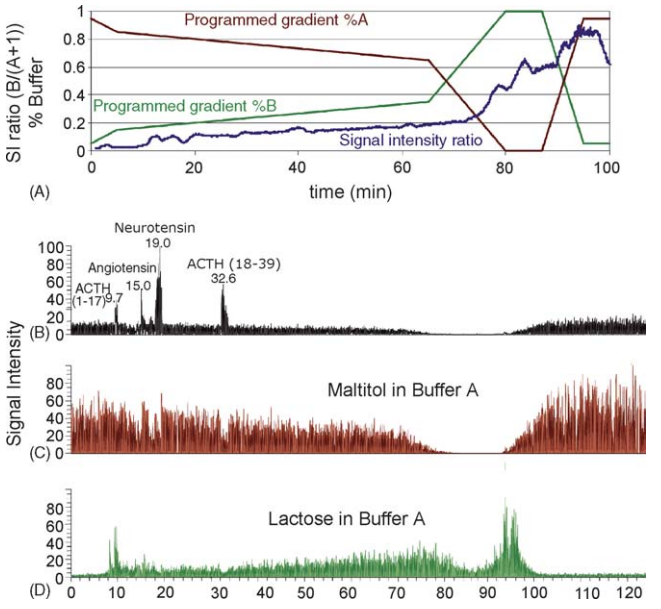

Fig. 3. Tracer molecule monitoring of the reverse phase gradient. (A) The gradient programmed into the binary HPLC pump and the resulting signal intensity ratio in comparison to the gradient (gradient 1 detailed in the Experimental methods). (B) The base peak chromatogram of the four peptide standard analyzed with tracer molecules in the mobile phase solvents. (C) The extracted ion current signal intensity chromatogram of maltitol tracer mass range (689–690) in buffer A. (D) The extracted ion current signal intensity chromatogram of lactose tracer mass range (685–686) in buffer B.

tracer molecules (Fig. 3B), and the maltitol and lactose masses independently of the background noise (Fig. 3C and D).

One use for the presented gradient monitoring method is to simultaneously monitor the reverse phase gradient in real-time and acquire MS/MS spectra for the purpose of peptide sequence identifications from complex biological samples. An obvious concern with the presented strategy was that the presence of these tracer molecules could change the solvent composition and as a result changes the ability to obtain MS/MS sequence identifications. To address this concern, yeast peptide fractions were analyzed by LC–MS/MS without (Fig. 4B) and with (Fig. 4C) lactose and maltitol tracers in the mobile phase solvents. Using the programmed gradient, we found that we could simultaneously describe the gradient using the SI-ratio and identify yeast peptide sequences using MS/MS (Fig. 4A). This experiment was repeated with three other ion exchange fractions from yeast and in our analyses, we consistently identified a higher number of sequences in the presence of tracer molecules than in their absence when the data were filtered against a constant false positive error rate [14]. Thus, the presence of the tracer molecules did not hinder the number or quality of identifications we obtained. However, it did increase the number of sequences identified in every run. This consistently larger number of identifications may be due to a slight spreading of the chromatography in the presence of the tracer molecules. In all instances this spreading caused a larger number of peptides to be identified, most likely because the expanded chromatography increases the possible number of MS/MS scans that may be acquired from a given peptide.

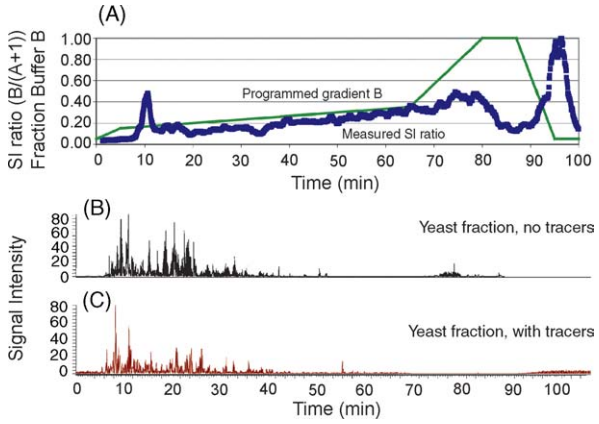

Fig. 4. Simultaneous LC-gradient monitoring and MS/MS based peptide sequence identification from yeast. Using LC gradient 1 as described (A) we identified 300 high-scoring peptide sequences in the yeast fraction without tracers (B) and 320 high-scoring peptide sequences in the yeast fraction with tracers (C). We were also able to follow the gradient using the SI-ratio while acquiring CID-spectra of these yeast peptides (A).

To summarize the results from the tracer molecule selection and testing for gradient monitoring, we found that the ion current signal intensities corresponding to the maltitol and lactose tracers reflected the changes in the programmed gradient, and the corresponding signal intensity ratio was proportional to the mobile phase solvent composition. We also noted the ability to detect gradient shifts, and the ability to simultaneously monitor the gradient and acquire MS/MS based sequence identifications from yeast in the presence of these tracer molecules.

3.3. Run-to-run chromatography comparisons and alignment

Traditionally, multiple chromatographic runs have been aligned by matching reference peaks common between the runs and adjusting the chromatography accordingly. These peaks are typically defined by their retention times—values that can vary with different LC systems and analysis conditions. Small gradient shifts can complicate chromatography alignment when using the retention time of reference peaks to match chromatographic positions between runs. Monitoring the solvent concentrations with tracers helps identify these unpredicted gradient shifts that can make traditional alignment methods difficult. It also provides a method of peak position description where the values are somewhat independent of the LC system used.

The run-to-run reproducibility of the SI-ratio was measured as the degree of scatter in the SI-ratio values between multiple analyses. For blank analyses where the gradient is run with no peptide samples loaded on the column, the average standard deviation in the SI-ratio from 15 repeated sampled runs at various chromatographic retention times was calculated to be ±0.3%. This run-to-run reproducibility of the gradient is demonstrated in Fig. 5 which shows three runs acquired using the same instrumental conditions. The figure shows the full runs (Fig. 5A), the gradient in the MS/MS acquisition zone prior to signal smoothing (Fig. 5B), and after signal smoothing (Fig. 5C). For repeated

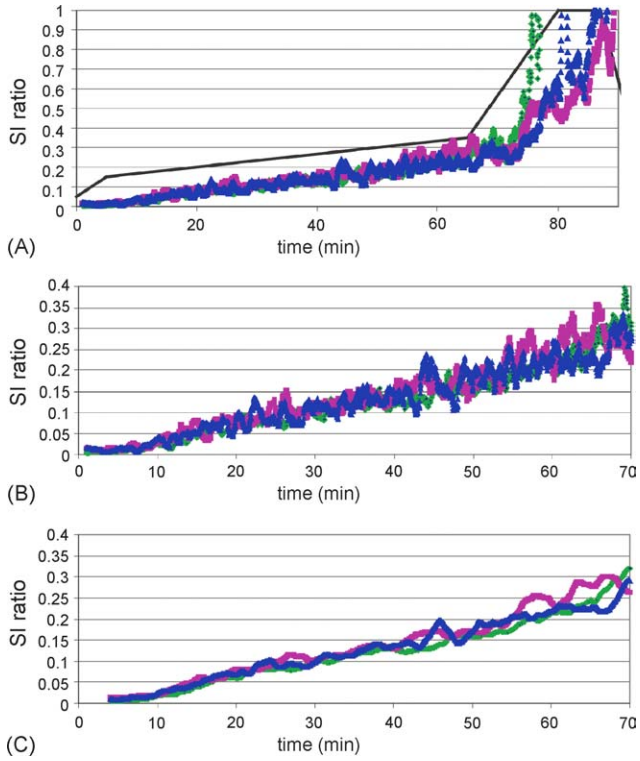

Fig. 5. Run-to-run reproducibility of SI-ratio for three runs using gradient 1. (A) Chromatogram of entire 90 min run. (B) SI-ratio during the typical peptide elution range. (C) SI-ratio with signal-to-noise smoothing.

analyses of the same peptide mixture, the average difference in the SI-ratio for individual peptides between runs was found to be ±1% at a given chromatography retention time. This difference in the reproducibility (0.3% versus 1%) is likely due to the variability in the eluted gradient composition versus the variability in the composition at which a peptide elutes.

To determine whether the SI-ratio translates into an accurate chromatographic peak position descriptor, we analyzed a mixture of four peptides using two different LC gradients, determined the average position descriptor values for each of the peptides, and calculated the percentage difference in the descriptor values between the two gradients (Table 2). The descriptors calculated were the peak retention time, calculated solvent composition, and SI-ratio based on real time solvent composition monitoring. The SI-ratio had the lowest average percent difference between the gradients with a value of 5%, whereas retention time had a difference of 20% and the calculated gradient had a difference of 12%. These results were calculated as averages from multiple analyses and indicate that the SI-ratio based solvent composition is potentially a more accurate peptide peak descriptor than the retention time. Further detail on how these values were calculated is provided in Section 2.

To determine whether the SI-ratio could be used to align the gradients from analyses acquired on different LC–MS/MS instruments, we analyzed the four peptide mixture using the same LC gradient (gradient 1) acquired on different ion trap mass spectrometers (ThermoFinnigan LCQ Classic and LCQ Deca XP) equipped with separate LC chromatography systems and columns. Once again, the SI-ratio based solvent composition

Table 2
Average values and deviations of peak position descriptors of four standard peptides analyzed with two different gradients

	Retention time (min)				Programmed gradient (%)				Signal intensity ratio			
	Grad1	Grad2	Δ[a]	% Δ[b]	Grad1	Grad2	Δ[a]	% Δ[b]	Grad1	Grad2	Δ[a]	% Δ[b]
ACTH (1–17)	12.4	9.8	2.6	24	11.2	12.6	1.4	12	12.0	12.0	0	0
Angiotensin	21.5	15.5	6.0	32	15.2	15.7	0.5	3	12.3	12.7	0.4	3
Neurotensin	22.4	19.1	3.3	16	15.6	18.0	2.4	14	12.3	13.1	0.8	6
ACTH (18–39)	35.3	32.6	2.7	8	21.2	25.1	3.9	17	13.0	14.5	1.5	11
Overall average				20				12				5

Grad 1: 0 min, 5% buffer B; 5 min, 15% B; 65 min, 35% buffer B; 80 min, 100% buffer B; 87 min, 100% buffer B; 95 min, 5% buffer B.
Grad 2: 0 min, 4% buffer B; 5 min, 10% B; 60 min, 40% buffer B; 70 min, 100% buffer B; 80 min, 100% buffer B; 85 min, 5% buffer B.
[a] These columns represent the difference between the values acquired between the two gradients.
[b] These columns represent the percentage difference between the values acquired between the two gradients.

was the most accurate and retention time was the least accurate peak position descriptor (Table 3).

These results showed that in our testing, peak positions were more accurately described by the solvent composition than by retention time or the programmed gradient. We believe the signal intensity ratio had the lowest variance because it directly describes the solvent composition and defines peak positions in terms of a value that is somewhat LC system independent. In contrast, retention time is LC system dependent and can vary significantly with differing gradients and chromatography conditions. The significance of these results is that the signal intensity ratio can potentially be used to align chromatographic gradients and compare chromatographic separations acquired on different machines allowing one to potentially share LC information between laboratories.

3.4. Additional comments

The gradient monitoring method described here can be used to describe LC–MS or LC–MS/MS operations in real-time. Events such as delays in the system plumbing and solvent composition can be detected using the gradient tracers. This gradient monitoring method also has the advantage that it can potentially detect run-to-run changes in the pump, column, or mass spectrometry system that may occur over time. Unlike online UV or fluorescence detection methods which monitor the chromatography separately from the mass spectrometer, chromatography gradient tracers generate gradient information that is included with each mass spectral scan. This can simplify peak position definition because it does not require additional LC event-to-MS scan synchronization. Also, since the tracer signal intensities are present in each scan, they have the potential to be used as internal quantitation standards with which peptide peaks can be normalized against.

The efficacy of this gradient monitoring method can be limited by the tracer molecules selected for use in the mobile phase solvents. Lactose and maltitol, the molecules used as tracers in this study, are suitable for use with C18 chromatography and ESI mass spectrometers but may not be suitable for analyses conducted with different column types and on mass spectrometers with different ionization methods. Also, those peptides with masses that overlap the mass ranges of lactose and maltitol are not sequenced because we do not acquire CID spectra within $\pm$2 Da of the tracer molecule masses. Though this is typically not a problem if other peptides from the same protein source are present, it may be of concern when monitoring a specific peptide isobaric in mass to the tracer molecules. In these cases, other well-chosen tracer molecules could be substituted without changing the principle of the method. For example, isotopic molecules with similar ionization energies could be used as tracers, resulting in fewer differences in concentration-to-signal intensity dependence on the mass spectrometer. These other tracer molecules may or may not be sugars. Identification of other tracer molecules will most likely require some trial and error and adherence to the recommendations presented in Table 1.

The effectiveness of the tracer method for run-to-run chromatography alignment can also be compromised in cases where

Table 3
Average values and deviations of peak position descriptors of four standard peptides analyzed on two different mass spectrometers

	Retention time (min)				Programmed gradient (%)				Signal intensity ratio			
	#1	#2	Δ[a]	% Δ[b]	#1	#2	Δ[a]	% Δ[b]	#1	#2	Δ[a]	% Δ[b]
ACTH (1–17)	11.9	11.4	0.5	4	13.8	13.6	0.2	2	0.14	0.13	0.008	6
Angiotensin	22.0	16.1	5.8	31	16.5	14.9	1.6	10	0.16	0.15	0.008	5
Neurotensin	26.1	16.7	9.4	44	17.6	15.1	2.5	15	0.17	0.15	0.014	9
ACTH (18–39)	44.7	36.9	7.8	19	22.5	20.4	2.1	10	0.21	0.24	0.035	15
Overall average				24				9.2				8.8

#1, ThermoFinnigan LCQ Classic with Agilent 1100 HPLC pump; #2, ThermoFinnigan LCQ Deca XP with Agilent 1100 HPLC pump.
[a] These columns represent the difference between the LCQ Classic and the LCQ XP values.
[b] These columns represent the percentage difference between the LCQ Classic and the LCQ XP values.

significant chromatography system changes cause solutes to elute at different eluent compositions. In these cases, other chromatography alignment methods using algorithm based chromatography warping [10] or other methods of standardizing the elution profile [20] may be used for alignment. Regardless, the gradient tracer method could still be useful in describing changes or deviations in the chromatography that would otherwise remain undetected.

The method presented here provides another measure for describing when a peptide will elute. Thus, factors that affect the solvent conditions such as temperature and problems with the pump will affect this chromatographic peak position descriptor just as it would affect chromatographic retention time. However, in applying the gradient tracer SI-ratio to describe chromatographic position, we found that it was a more accurate descriptor of peak position than retention time. When applied to the analysis of a complex protein mixture from yeast, these tracers were shown to describe the chromatographic gradient while not adversely affecting the number of CID based peptide identifications. Thus, gradient description by tracer molecules can potentially be applied for simultaneous chromatographic gradient description and the analysis of complex peptide mixtures.

The method described here monitors the LC gradient independently of the sample analyzed, system delay time, column dimensions, or column packing material. Ideally, one would like to ensure that the separation conditions are identical when running duplicate analyses. However, this is not always accomplished and we demonstrated an improved ability to align peptide peaks analyzed using different LC gradients, as well as, on different LC–MS ion trap instruments within our laboratory in comparison to chromatographic retention time. Though not demonstrated here, this method could potentially be used to align chromatographic gradients from analyses acquired in different laboratories. Thus, it can potentially be used as a chromatography standard for a reference database of peptides for use amongst multiple laboratories. In this scenario, multiple laboratories could submit their peptide identification information (including the LC based signal intensity ratio) to a central database which could be used by other laboratories as a reference for subsequent high-throughput identifications.

4. Conclusions

We have described a method of LC gradient monitoring during LC–MS and LC–MS/MS analyses in real-time by following the ion current signal strength over time of tracer molecules dissolved in the mobile phase solvents. Lactose and maltitol were selected as the solvent tracers, and by using the ratio of the signal intensity of these tracer molecules to indicate the gradient position, we were able to describe chromatographic positions of peaks from LC–MS analyses conducted with different gradients and on different systems more accurately than when described by retention time. Also, since this method monitors the gradient in real-time, it can potentially detect chromatography deviations that would otherwise be undetected. The presence of these tracer molecules in the mobile phase solvents did not hinder tandem mass spectrometric analysis—rather, the number of sequences increased due to the chromatographic spread caused by the presence of these molecules.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2005.09.039.

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