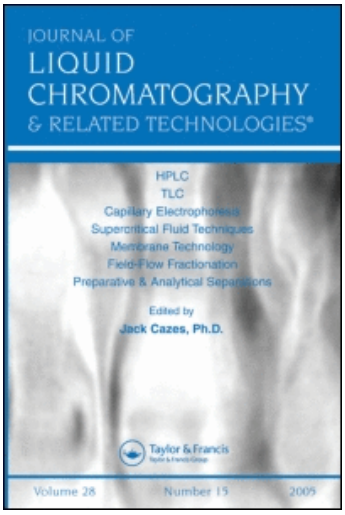

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Optimization and Standardization of Liquid Chromatography-Mass Spectrometry Systems for the Analysis of Drug Discovery Compounds

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Abstract: An approach for improving the speed and effectiveness of orthogonal, low and high pH, LC/MS-based methods for routine applications is presented. Considering HPLC column performance as an integral part of an LC/MS system, advantages and disadvantages of three modern column technologies are thoroughly discussed as alternatives to conventional silica-based packing materials. Instrument performance assessment strategies, using a standard mix composed of four drug-like compounds, are discussed where parameters such as peak capacity and retention are used as key performance indicators. These procedures have been standardized and evaluated across two different sites within Lilly Research Laboratories. The value of alternating orthogonal low and high pH methods, in a high throughput mode, on a single system is demonstrated. The development of software for simulating the LC/MS open access sample queues is also presented.

Keywords: Liquid chromatography-mass spectrometry, Drug discovery compounds, Orthogonality, Low pH and high pH methods, Peak capacity, Standard mix, System suitability test, Simulation software

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INTRODUCTION

The implementation of new technologies in drug discovery, such as automated parallel chemistry, targeted libraries, and screening technologies, demands the creation of new and faster separation strategies for quantitative and qualitative analysis.[1–4] In the past few years, efforts in pharmaceutical laboratories have been devoted to define, in a high throughput manner, the most appropriate analytical techniques to support the great diversity of chemical structures. Today, liquid chromatography-mass spectrometry (LC/MS) is widely acknowledged as the most versatile and robust separation and detection analytical technique for purity assessment and identification of drug discovery compounds.[5] Due to it's robustness, LC/MS has been adopted as a user friendly, open access service, within medicinal chemistry, biological and ADME/Tox laboratories.[1,6]

Although LC/MS has come of age in the 21st century, its application to the analysis of pharmaceutical compounds, mostly bases, is still a very ambitious and challenging goal. Many compounds with different functionalities and physicochemical properties need to be analyzed with diverse LC/MS methods that allow greater sensitivity and accuracy.[7] We have recently discussed the benefits of orthogonal reversed-phase LC/MS-based methods, to accurately determine the identities and purities of drug discovery compounds.[5] Practical advantages of suitable column chemistry architecture, mobile phase pH, and gradient time were highlighted. It is clear that LC/MS methods, with a high degree of orthogonality, provide higher information content.[8,9]

In the present study, we have examined an approach for improving the speed and effectiveness of orthogonal methods for routine purity assessment and characterisation. Starting with existing LC/MS methods, efficient procedures for the development of fast and high resolution separation and detection methods are proposed. Considering HPLC column performance as an integral part of the LC/MS system, advantages and disadvantages of three modern column technologies, Gemini®, XTerra® and XBridge® are thoroughly discussed as alternatives to conventional silica-based packing materials. Whereas the general influence of mobile phase pH has been previously reviewed,[10,11] the impact on peak capacity, resolution, and ionization will be summarized.

Based on the concept that the LC/MS hardware, electronics, operation, configuration, and analyte constitute an integrated system that can be evaluated as a whole, system suitability tests have been developed to monitor LC/MS system performance in greater detail.[12] These tests have been used to verify that the resolution and reproducibility of the system deliver the expected data quality, qualified by specific criteria measured in advance of core hour analysis by utilization of overnight timed events. Tang et al. introduced a test mix composed of four components for the evaluation and validation of a LC/MS method.[13] More recently,

Goetzinger et al. described the use of a test mix composed of eight components for similar purposes.[14] The analysis of a cross suitability sample (standard test mix) allows us to define specific HPLC column, UV, and MS parameters that will be used as predefined parameters to validate the transfer of methods from one laboratory to another. These procedures have been standardized and evaluated in two different Lilly Research Laboratories as described below.

Acknowledging LC/MS as a critical 'walk-up' tool to the medicinal chemist for rapid purity assessment of intermediates and characterization of products, it is important to maximize throughput, and minimize queue or lead time (defined as the length of time from sample submission to a user receiving his or her results), for each LC/MS analysis. The lead time is a function of many factors. The number of samples in the queue plays a part. Another key factor is the cycle time, which is the time it takes an instrument from starting one sample to starting the next sample. The cycle time includes data collection ("value added time"), equilibration, data processing, and sample introduction such as moving of auto-sampler robot arms and manipulation of vials ("non-value added time"). In trying to optimize the lead time, it is clearly more desirable to reduce or remove non-value adding elements than value adding ones. Thus, strategies for alternating orthogonal low and high pH methods, in a high throughput mode, on a single system, as well as the development of software for simulating the LC/MS open access sample queues are keys to successful implementation.

EXPERIMENTAL

Instrumentation

Experiments were performed with an Agilent 1200 Series Rapid Resolution LC/MSD SL system or Agilent 1100 Series LC/MSD equipped with a solvent degasser, binary pump, auto sampler, column compartment and a diode array detector (Agilent Technologies, Waldbronn, Germany). The UV wavelength was set at 300 nm, band width 200 nm. Electrospray mass spectrometry measurements were performed on a MSD quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) interfaced to the HP1200 or HP1100 HPLC system. MS measurements were acquired simultaneously in both positive and negative ionization modes (fragmentor 80 V, threshold spectral abundance 80, MS peak width 0.3 minutes) or in positive ionization mode (fragmentor 120 V, threshold spectral abundance 150, MS peak width 0.04 minutes) over the mass range of 100–800. The following ion source parameters were used: drying gas flow, 5.0 L/min; nebulizer pressure, 60 psig; drying gas temperature, 350°C; capillary voltage, 1000 V for the 1200 Rapid Resolution system and drying gas flow, 15.0 L/min; nebulizer pressure, 60 psig; drying gas temperature,

350°C; capillary voltage, 4000 V for the 1100 system. Data acquisition and integration for LC/UV and MS detection were collected by means of the Chemstation software (Agilent Technologies). HPLC 1100 instruments were optimized with the Agilent Rapid Resolution HT1100 Series LC modification Kit (part number 5188-5328) or a low volume mixer to reduce as much as possible system dead volume and gradient delay. Connections between the injector, the column, and detectors were made using 0.17 mm i.d. stainless steel or PEEK tubing.

Reagents and Columns

Water, acetonitrile (ACN), and methanol (MeOH) were HPLC grade from Lab Scan (Dublin, Ireland). Formic acid (FA), trifluoroacetic acid (TFA), water with 0.1% FA and 0.01% TFA, acetonitrile with 0.1% FA and 0.01% TFA, ammonium hydroxide and ammonium hydrogencarbonate were from Sigma-Aldrich (Steinheim, Germany).

The drug-like compounds were obtained from Sigma-Aldrich. 5 mg of each compound was weighed and dissolved firstly with 30 mL of acetonitrile and diluted with water in a 100 mL volumetric flask. The resulting per component concentration equals 50 μg/mL. The injection volume was 2 μL.

The HPLC columns were Gemini C_{18} 50 × 2.0 mm, 3 μm (Phenomenex, Torrance, CA), XTerra MS C_{18} and XBridge C_{18} 50 × 2.1 mm, 3.5 μm (Waters, Milford, MA) columns. The characteristics of the columns given by the manufactures are listed in Table 1. The acidic mobile phases were water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid (FA) or 0.05% trifluoroacetic acid (TFA) or mixtures of 0.1% FA and 0.01% TFA. Meanwhile, the alkaline mobile phases were water (solvent A) with 10 mM ammonium hydrogencarbonate (NH_4HCO_3) adjusted to pH 9 with ammonium hydroxide (NH_4OH) or 0.1% ammonium hydroxide and acetonitrile (solvent B). The gradient programs employed are described in each chromatogram. The flow rate prior to the mass spectrometer was 1 mL/min, which was split at a ratio of 3:1 in order to deliver 250 μL/min into the electrospray interface and 750 μL/min to the waste reservoir.

Table 1. Physico-chemical properties of the columns used in this study. 1; Two-in-one technology. 2; HPT: hybrid particle technology. 3; BEH: bridged ethyl hybrid technology. 4; data not available

Column technology	Pore size (Å)	Surface area (m^2/g)	Coverage ($\mu mol/m^2$)	Carbon load (%)
Gemini C18 Twin[1]	110	375	[4]	14
XTerra MS C18 HPT[2]	125	175	2.2	15.5
XBridge C18 BEH[3]	135	185	3.2	18

RESULTS AND DISCUSSION

Test Mix

The drug-like compounds (three amines and one acid) used in this study were selected based on their variety of physicochemical properties and chemical functionalities and represent drug molecules encountered in pharmaceutical laboratories. Because of their variable chromatographic behavior (i.e., mainly hydrophobic and electrostatic interactions) and ionization response, as a function of the mobile phase pH, the test mix was found to be well-suited for RP-LC and ESI-MS studies. It is well known that over 80% of commercialized drugs are bases or possess basic functionalities.[15] Basic drug discovery compounds are usually prepared as free bases and/or salts for biological/toxicological assays. Thus, propranolol HCl (pKa: 9.2, logP: 3.1), verapamil HCl (pKa: 9.0, logP: 3.9), terfenadine (pKa: 9.5 and 13.3, logP: 6.5) and niflumic acid (pKa: 1.7 and 4.7, logP: 4.9)[16] were the components of the test mix for the studies carried out with low pH mobile phases. Diltiazem HCl (pKa: 8.9, logP: 3.6) was used instead of propranolol for the mixture employed for high pH analyses.

Column Architecture/Chemistry and Mobile Phase pH for Orthogonal Separations

Conventional silica C_{18} stationary phases were the packings used in our primary method for the analysis of final products with low pH mobile phases (mobile phase A: H_2O 0.1% FA and mobile phase B: ACN 0.1% FA). A 10 minute gradient time from 5% to 99% B on a 100 × 3 mm and 5 μm column operated at a flow rate of 1 mL/min with column temperature of 50°C was optimized for maximum sample resolution (total run time was 14 minutes). In fact, the average peak capacity value for such a method was found to be 90. We observed that under conditions of high throughput, within an open access environment, the column lifetime was not acceptable. Thus XTerra, the first commercialized hybrid technology phase, was the target of intensive research due to its high chemical and thermal stability.[10] Satisfactory results were obtained with this packing operated with acidic and alkaline mobile phases. Peak capacity was found to be 75 for our high pH method (mobile phase A: H_2O 10 mM NH_4HCO_3 pH 9 and mobile phase B: ACN) with a 10 minute gradient elution from 20% to 99% B on XTerra MS C18 100 × 3 mm and 5 μm column operated with the same chromatographic conditions developed for the low pH method.

Even though those results were quite satisfactory, new column technologies like Gemini C_{18} and XBridge C_{18} columns were tested as potential alternatives to classical silica C_{18} and XTerra MS C_{18} packing materials. Due to the need for faster analysis and greater separation power, our

methods were further improved by replacing the above column architecture with smaller dimension columns packed with smaller particle size. 100×3 mm column dimension packed with 5 μm particles were replaced by a 50×2.1 mm column containing 3–3.5 μm particles. According to Eq. (1), the flow rate for the 2.1 mm i.d column should be 0.5 mL/min.

$$\text{Flow}_{\text{col 2}} = \text{Flow}_{\text{col.1}} \times (\text{Diameter}_{\text{col 2}}/\text{Diameter}_{\text{col 1}})^2 \quad (1)$$

Compared with 5 μm columns, the 3 and 3.5 μm columns have a higher optimum linear velocity.[17,18] However, the use of small particles results in higher operating conditions, as column back pressure is proportional to the square of the particle size. In this context, our goal was to use this new column size to achieve improved efficiency and shorter analysis time. To compensate for the high column back pressure, elevated column temperature was applied. The optimum flow rate at which 3–3.5 μm columns were more efficient, under the practical conditions used by the authors, was 1.0 mL/min. Thus critical parameters that influence column peak capacity like column dead volume (the region where there is no separation possible) and peak width were substantially reduced. Employing the conditions described in Table 2, switching from the 100 mm to 50 mm column length permitted a reduction of gradient time and solvent usage per analysis by 1.4 and 1.6 fold respectively.

Mobile phase pH and the identity of the column were simultaneously modified to explore differences in selectivity, retentiveness and separation

Table 2. HPLC conditions for the methods with low pH mobile phases. Gradient elution: from 5 to 100% B in 7 min, stays at 100% B for 1 min, and then 0.5 min to initial conditions

Mobile Phase (A)	H_2O (0.1% FA pH 2.9)	H_2O (0.05% TFA pH 2.5)	H_2O (0.1% FA + 0.01% TFA pH 2.7)
Mobile Phase (B)	ACN (0.1% FA)	ACN (0.05% TFA)	ACN (0.1% FA + 0.01% TFA)
HPLC columns	Gemini C18 batch 1 XTerra MS C18 batch 1	Gemini C18 batch 2 XTerra MS C18 batch 2	Gemini C18 batch 1 and 2 XTerra MS C18 batch 1 and 2
Flow rate	1 mL/min	1 mL/min	1 mL/min
Temperature	50°C	50°C	50°C
Maximum column back pressure	Gemini C18: 194 Bar XTerra MS C18: 204 Bar	Gemini C18: 202 Bar XTerra MS C18: 194 Bar	Gemini C18: 202 Bar XTerra MS C18: 194 Bar
Injections n°/ column batch	100	100	100

power. Gemini and XTerra columns possessing different surface properties were selected for the development of the new low pH method with three acidic MS compatible additives; FA, TFA, and a synergy of both FA/TFA (see Table 2). The latter, was used to balance the benefits and limitations of FA and TFA on chromatographic resolution and MS sensitivity. For practical purposes and to confirm the high column chemical and thermal stability reported by the manufactures, two column batches of each packing material were used. The chromatographic behavior of the four test compounds is depicted in Figure 1. Clearly, marked difference in selectivity and resolution as a function of both column chemistry and mobile phase modifier was observed. High chromatographic resolution is ideal for the challenging task of accurate purity assessment and/or impurity profiling. As was expected, peak resolution was superior on the Gemini column as a consequence of the smaller particle size. Interestingly, the largest peak resolution on the Gemini column was observed for the mobile phases containing FA. Resolution (Rs) was calculated according to the tangent method (United State Pharmacopoeia) Eq. (2),

$$\mathrm{Rs} = 2(\mathrm{T2} - \mathrm{T1})/(\mathrm{W2} + \mathrm{W1}) \quad (2)$$

T1 and T2 are the retention times of peaks 1 and 2, and W1 and W2 are the widths of the peaks at baseline. Thus, the Rs values for the critical peaks 3 and 4 (terfenadine and niflumic acid) on the Gemini column were 19.0, 1.7, and 9.9 respectively for different mobile phases (see chromatograms of Figure 1 A–C). Rs values for the same pair on the XTerra column were 5.8, 4.2, and 3.1, respectively (see chromatograms of Figure 1 D–F). The reversal of elution order observed for these two peaks on XTerra and Gemini columns with the mobile phase containing TFA alone was an indication of the high degree of separation orthogonality between these low pH methods (see chromatograms of Figures 1B and 1E). The higher retention time for terfenadine is due to the ion-pairing capability of TFA (pKa $<$ 1) with protonated bases.

The resolving power of the low pH methods studied is summarized in Figure 2. Herein, PC was calculated according to the equation described by the authors in reference [5]. The average PC in all methods was superior to 110. The results on the Gemini column revealed that FA yielded the higher PC value (i.e. 139 for peak 2) in comparison with the other acidic additives. Although not as dramatic, the average PC with FA using the XTerra column was also slightly better than with the mobile phases with TFA. It is worth noting that similar and/or lower column back pressure (i.e., 10 bar) was observed for Gemini columns packed with smaller particles. This intriguing data could be related to the influence of the surface chemistry of the packing materials (differences of the carbon content and surface coverage) on the column back pressure as was recently postulated by Guiochon et al.[19] In terms of column lifetime and stability the XTerra column

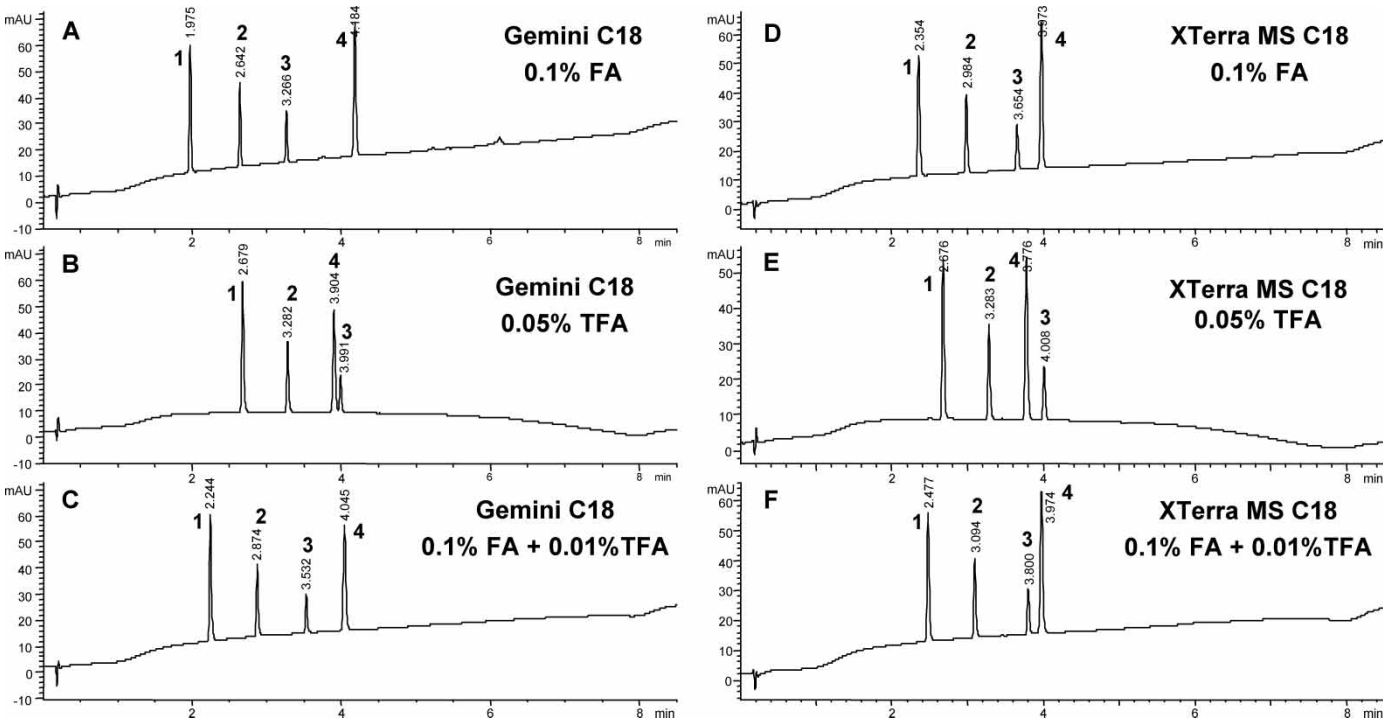


Figure 1. UV chromatograms of the standard mixture. Peaks: (1) propranolol; (2) verapamil; (3) terfernadine; (4) Niflumic acid. Resolution between peaks: RsA_{1-2}: 15.6; RsA_{2-3}: 15.2; RsA_{3-4}: 19.0; RsB_{1-2}: 12.9; RsB_{2-4}: 12.1; RsB_{4-3}: 1.7; RsC_{1-2}: 13.8; RsC_{2-3}: 14.5; RsC_{3-4}: 9.9; RsD_{1-2}: 12.1; RsD_{2-3}: 13.2; RsD_{3-4}: 5.8; RsE_{1-2}: 12.0; RsE_{2-4}: 9.0; RsE_{4-3}: 4.2; RsF_{1-2}: 12.3; RsF_{2-3}: 13.9; RsF_{3-4}: 3.1.

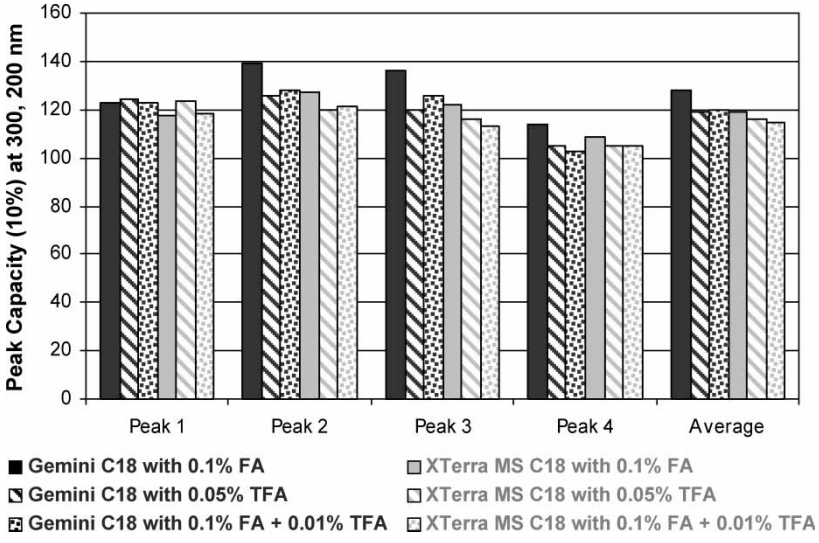


Figure 2. Average peak capacity values of the six low pH methods used in this work. Peaks: (1) propranolol; (2) verapamil; (3) terfernadine; (4) niflumic acid.

exhibited more consistent PC values over the testing period. In contrast, the Gemini packing material was a concern. Earlier studies with older column batches revealed that PC dropped 20% after 400 injections when columns were used in an open access environment. According to the manufacturer this shortcoming was associated with a void at the head of the column. Today's new column batches should not be as susceptible to voids.[20] Data described in section 4 confirm this assumption. Regarding MS sensitivity, FA was the additive that provided higher MS signal and sensitivity. All additives gave a good ionization response in positive mode, although TFA and the blended FA/TFA showed suppression of the signal in negative mode. The co-elution of peaks 3 and 4 in the XBridge column influenced the average PC across all four peaks. Thus, no data is presented for the XBridge column with low pH mobile phases.

The conditions for the development of the orthogonal method with high pH mobile phases are described in Table 3. Most basic drug-like compounds are not ionized at high pH. As a result, higher retention in the analysis of structurally related compounds is observed, given that hydrophobic interactions between the analyte and the stationary phase of the column are favored. Allowance was made with a 5% increase of the initial eluent strength to avoid retentions outside the gradient time. The other conditions were maintained as those described for the low pH methods. Two series of three column chemistries were used for these experiments.

Chromatograms illustrated in Figure 3 highlight the retention of the four standards at high pH. The separations at high pH look nearly identical regardless

Table 3. HPLC conditions for the methods with high pH mobile phases. Gradient elution: from 10 to 100% B in 7 min, stays at 100% B for 1 min, and then 0.5 min to initial conditions

Mobile Phase (A)	H_2O (10 mM NH_4HCO_3 pH 9)	H_2O (0.1% NH_4OH pH 10.5)
Mobile Phase (B)	ACN	ACN
HPLC columns	Gemini C18 batch 3 XTerra MS C18 batch 3 XBridge C18 batch 1	Gemini C18 batch 4 XTerra MS C18 batch 4 XBridge C18 batch 2
Flow rate	1 mL/min	1 mL/min
Temperature	50°C	50°C
Maximum column back pressure	Gemini C18: 210 Bar XTerra MS C18: 232 Bar XBridge C18: 230 Bar	Gemini C18: 210 Bar XTerra MS C18: 248 Bar XBridge C18: 216 Bar
Injections n°/column batch	100	100

of column or modifier. The only difference is the early elution of niflumic acid compared to the low pH conditions; it is obvious since the acidic functionality is negatively charged. Although excellent chromatographic resolution was obtained in all the separations at high pH, Gemini and XBridge columns operated with ammonium hydrogencarbonate buffer showed higher PC values (see Figure 4). As observed with the development of low pH methods, column back pressure was slightly lower on Gemini columns. In the context of column lifetime and stability, all columns exhibited consistent PC values over the testing period with ammonium hydrogencarbonate buffer. This behavior was later confirmed with the analysis of real samples (>500 injections) in an open access operation (data not shown). On the contrary, the XBridge column technology exhibited greater column lifetime with mobile phases containing 0.1% ammonium hydroxide at the end of the testing period. It is important to remark, that better MS sensitivity was obtained with the mobile phases with ammonium bicarbonate buffer.

UV and MS Hardware Configuration

In addition to chromatographic parameters such as column chemistry and mobile phase pH, other parameters related to the performance of the LC/MS hardware configuration had to be considered during method optimization. For instance, because PC and Rs are dependent on peak width, LC/MS integrity and the subsequent interpretation of data from lab to lab are directly tied to hardware configuration performance. In his context, the main points in the LC/MS configuration are the DAD and MS parameters.

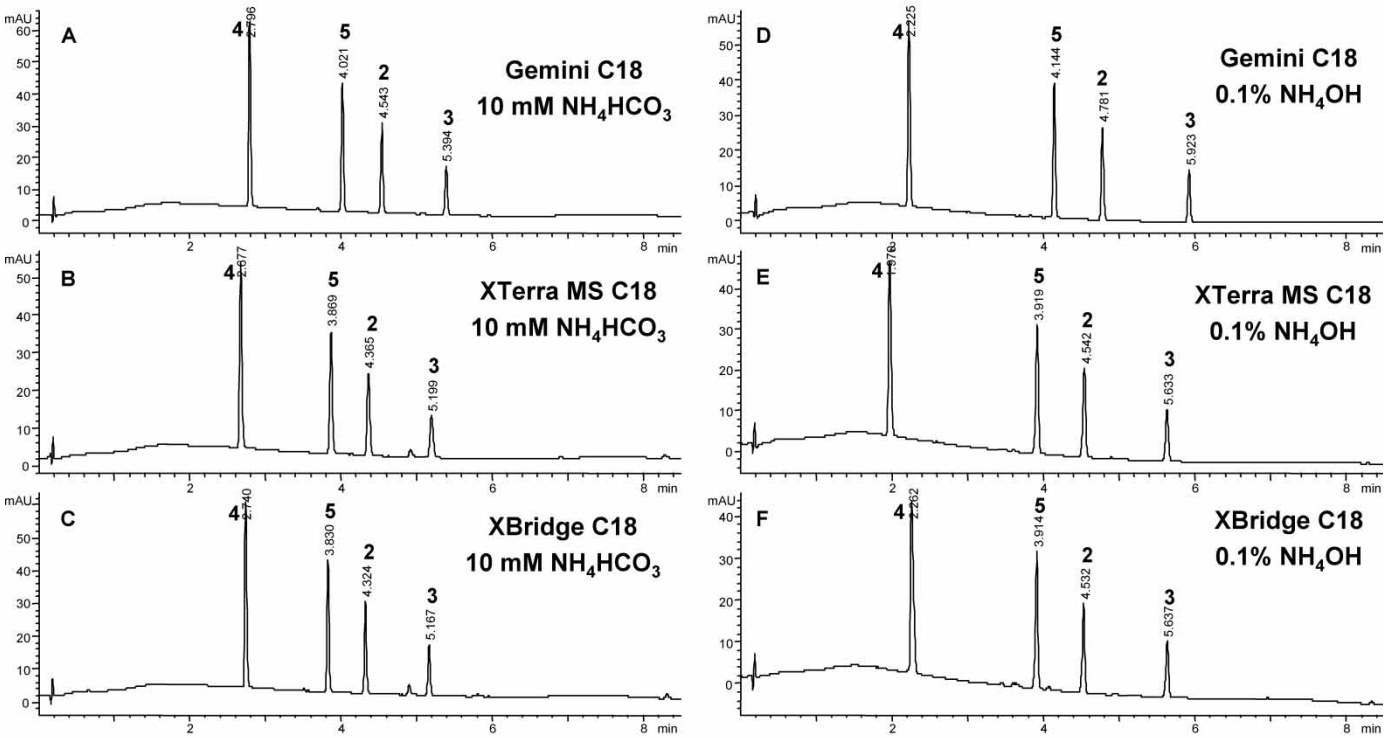


Figure 3. UV chromatograms of the standard mixture. Peaks: (4) niflumic acid; (5) diltiazem; (2) verapamil; (3) Terfernadine. Resolution between peaks: RsA_{4-5}: 24.9; RsA_{5-2}: 10.2; RsA_{2-3}: 15.8; RsB_{4-5}: 20.2; RsB_{5-2}: 7.7; RsB_{2-3}: 11.7; RsC_{4-5}: 22.0; RsC_{5-2}: 9.7; RsC_{2-3}: 15.5; RsD_{4-5}: 34.9; RsD_{5-2}: 11.8; RsD_{2-3}: 19.9; RsE_{4-5}: 30.2; RsE_{5-2}: 9.7; RsE_{2-3}: 16.1; RsF_{4-5}: 26.3; RsF_{5-2}: 10.9; RsF_{2-3}: 18.8.

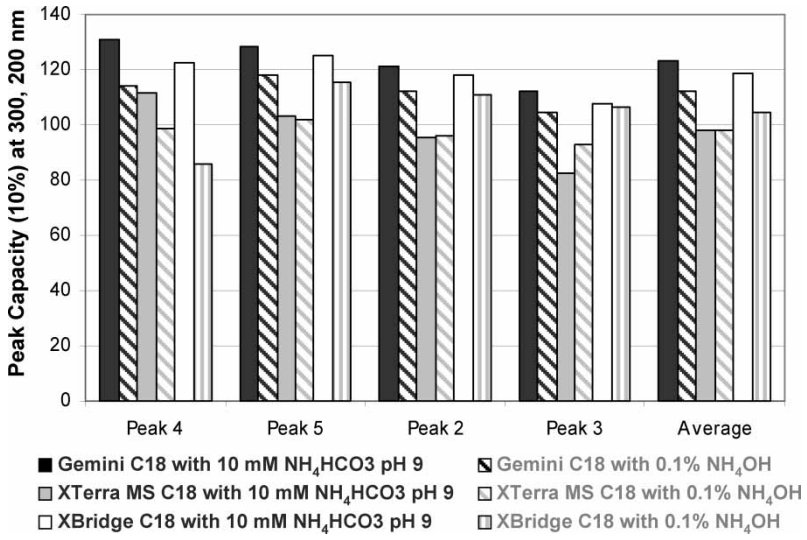


Figure 4. Average peak capacity values of the six high pH methods used in this work. Peaks: (4) niflumic acid; (5) diltiazem; (2) verapamil; (3) terfernadine.

For the DAD parameters, the peak width sets the optimum response time for the DAD. It is defined as the width of a peak, in minutes, at half the peak height.[21] It has a significant impact on the measured peak width and, consequently, on the chromatographic resolution and peak capacity. During the LC/MS standardization study, in two different Lilly research laboratories, this parameter was cautiously studied in combination with the slit width in order to assess their influence on peak capacity. The slit width allows selection of the optical bandwidth of the detector[21] and, therefore, it was modified between 2 and 4 nm in this study. Table 4 shows the corresponding response time that is set automatically and the appropriate data rate that is selected when the different peak widths are set.

Figure 5 illustrates the importance that these parameters can have on the peak capacity of the system and the effect of these parameters on the file size.

Table 4. Corresponding response time and the appropriate data rate for signal and spectra acquisition

Peak width (min)	Response time (sec)	Signal data rate
>0.03	0.5	10 Hz
>0.05	1.0	5 Hz
>0.10	2.0	2.5 Hz

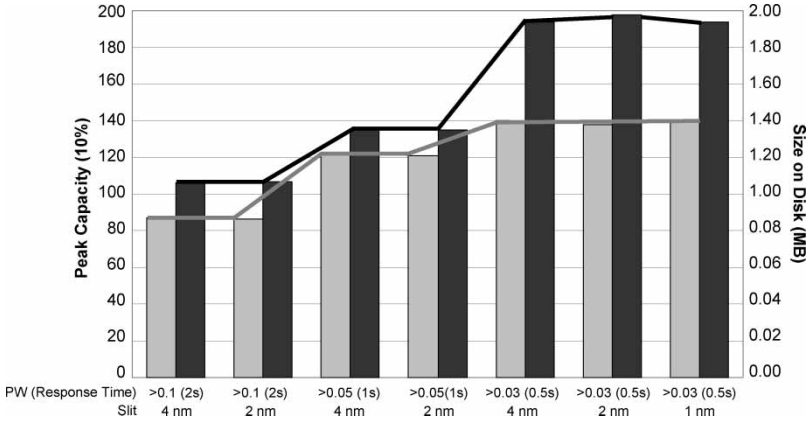


Figure 5. Influence of peak width (response time) and slit on UV peak capacity (10%) and on size on disk.

When the peak width is set to 0.5 s, the peak capacity is approximately 140. When the data rate is decreased from 10 to 2.5 Hz, the increased response time causes an increased peak width and a drop of peak capacity by 40%. On the other hand, when the response time decreases, the peak capacity increases but adversely the size of the files on the disk increases. A compromise between peak capacity and file size was therefore adopted, selecting a standard peak width of 1s for all the analysis. Figure 5 also illustrates that the slit does not influence the peak capacity or the file size.

Another important point relating to UV collection is the optimal wavelength to carry out the purity determination. The use of a diode array detector allows us to collect a combined wavelength trace from 200–400 nm, minimizing selectivity issues that can occur at a single wavelength due to different extinction coefficients. The collection of diode array data also opens up the possibility of Peak Purity Determination and the use of UV spectra for characterization or identification purposes. However, the collection of diode array data and representation of the results as a combined wavelength trace can lead to issues with quantitation, sensitivity and resolution. To compliment the combined wavelength trace we also report data at a single wavelength 214 $(+/-1)$.

Electrospray mass spectrometry (ESI-MS) was the ionization mode of choice because it enables the detection of most drug discovery compounds. Due to the chemical diversity, both positive and negative ion modes are used to cover the chemical space and to detect analytes in a high throughput environment. Since most of the compounds in drug discovery present basic characteristics, it is important to optimize MS parameters that permit an increase in the sensitivity when only positive acquisition mode is used. Basic compounds usually form protonated molecules $[M+H]^+$ which can be analyzed in positive ion mode to give a peak at mass $M+1$. Figure 6

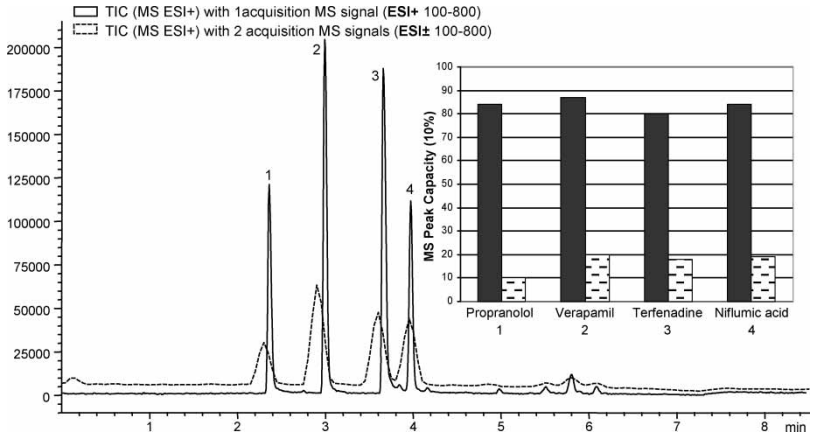


Figure 6. TIC of the standard mix acquired in the positive ionization mode with a MS peak width of 0.04 minutes (solid line) and in both positive/negative ionization mode with a MS peak width of 0.3 minutes (dashed line). Analysis carried out on XTerra MS C_{18} column with 0.1% of FA. Gradient elution as described in Table 2.

shows the differences in sensitivity and peak capacity of the standard mix when the MS detector was set up for acquiring positive/negative modes simultaneously and in positive mode only. Selecting only positive acquisition and modifying parameters such as the fragmentor, the threshold spectral abundance and the MS peak width, it was possible to get an increase in the MS sensitivity and peak capacity by 2.5 and 5 fold respectively.

Standardization and System Suitability Test

The goal of the standardization process is to acquire and publish consistent LC/MS data for new chemical entities (NCE's), independent of research site. To be sure that data obtained in different laboratories was comparable a cross site validation study was performed and standard test protocols developed. This was assessed by means of an inter-laboratory trial and was considered for the standardization of an analytical procedure.[12] For this, 10 consecutive injections of the standard mix were run using the same procedure at two different Lilly Research Laboratories. Parameters such as PC and the RSD of retention times were compared between these laboratories. A summary of these results is shown in Table 5. It was found that the average PC values were comparable and higher than 130. The RSD values obtained for these experiments were appropriate to consider that results from these laboratories were equivalent.

The data obtained from reproducibility experiments was also used to develop a system suitability test to monitor the performance of the LC/MS

Table 5. PC and RSD of retention times obtained at two different labs. Analysis carried out on HPLC 1100 systems with the rapid resolution kit (laboratory 1) and a low volume mixer (laboratory 2). Separations were performed on Gemini C18 columns with 0.1% of FA. Gradient elution: from 5 to 100% B in 7 min, stays at 100% B for 1 min, and then 0.5 min to initial conditions. UV detection: 300, 200 nm. Temperature: 50°C. Flow rate: 1.0 mL/min

n = 10 Injections		Laboratory 1			Laboratory 2		
	Name	Ret Time (min)	RSD (%)	PC (10%)	Ret Time (min)	RSD (%)	PC (10%)
1	Propranolol	1.58	0.241	141	1.47	0.566	151
2	Verapamil	2.17	0.171	148	2.13	0.354	176
3	Terfenadine	2.79	0.145	148	2.78	0.304	175
4	Niflumic acid	3.57	0.032	110	3.58	0.076	111
	Average		0.147	137		0.325	153

systems. The above discussed parameters as well as UV and MS signal intensities can be monitored and compared from injection to injection. The daily collection of such data was found to be a helpful tool for trouble-shooting and to detect problems and failures associated with any of the components of a LC/MS system.

Strategies for Alternating Low and High pH LC/MS Methods on a Single System

Traditionally, we have set up instruments with a specific low or high pH LC/MS configuration, but with increasing sample numbers, there was a need to adopt a multi functional design where both the low and high pH methods were released on the same LC/MS instrument. One of the options was to use external valves that permit the combination of more solvents and columns in the same instrument. However, new instruments with low dead volumes allow the possibility of using the same solvent tubing for low and high pH mobile phases without interference. In addition, the 6 port valve on the HP1100 column compartment allows alternating two columns and automation of the method selection process. This delivers improved productivity for the LC/MS equipment. A number of experiments were carried out to ensure both methods could be run on the same system without compromising data integrity and with no impact on system reliability that would increase downtime due to increased maintenance. The analysis of the generated data revealed that both methods could be performed on the same equipment without increased downtime.

A minimum equilibration time (1 min at a flow rate of 1.2 mL/min) was employed for alternating pH methods to be run without affecting retention time and peak shape. Figure 7 shows 5 overlaid chromatograms at low and high pH respectively that correspond to 10 consecutive injections in the same instrument. The low RSDs (<0.6%) obtained from the 5 low and 5 high pH alternating injections corroborate that this configuration permits alternating pH methods with good repeatability.

Simulation Software

To improve the lead time for LC/MS or, indeed, any other analysis, it is necessary to increase the exit rate (i.e., the number of samples the LC/MS set up can complete in a given period of time) from the analysis. This could be achieved by reducing the cycle time or by increasing the number of instruments on which the desired method is available. However, it is less clear how much of an effect any particular change would have. To implement a change takes effort, and to gather statistically meaningful metrics on impact takes time as patterns of sample submission show variation. At the end of this testing period, the results may not be what were anticipated. For example, while it is clear a reduction in cycle time cannot be detrimental, it is not obvious how great an effect it will have and whether that will repay the effort or risk involved in the change. Furthermore, changing instrument

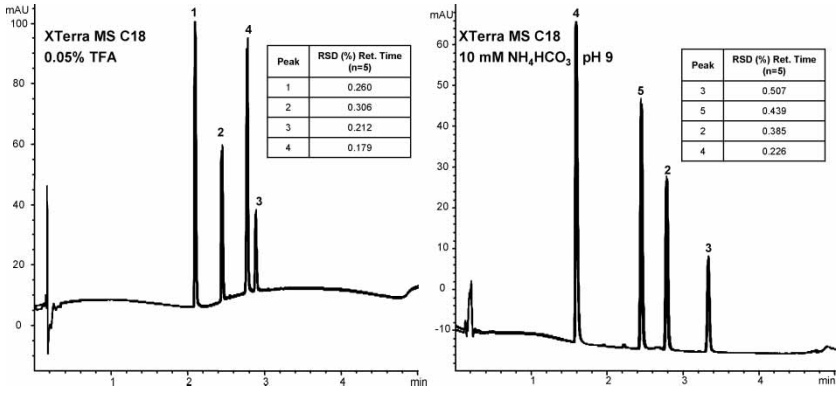


Figure 7. Five overlaid UV chromatograms at low (left) and high pH (right) with switching conditions between each injection. Peaks: (1) propranolol; (2) verapamil; (3) terfernadine; (4) niflumic acid; (5) diltiazem. Analysis carried out on XTerra MS C_{18} columns with 0.05% of TFA for low pH method and 10 mM ammonium bicarbonate at pH 9. Gradient elution for low pH: stays at 5% B for 0.5 min, from 5 to 100% B in 4 min, stays at 100% B for 0.5 min. Gradient elution for high pH: stays at 20% B for 0.5 min, from 20 to 100% B in 4 min, stays at 100% B for 0.5 min. Temperature: 50ºC. Flow rate: 1.2 mL/min.

configuration, such as which methods are available on which machines, might actually make queues worse. Thus although there may be many potential solutions to a problem, it is far from clear which is the best, and it is very difficult to test out many possibilities.

To solve this dilemma, we developed software for simulating the LC/MS queues. The simulation is able to predict sample lead time for any number of instrument configurations; many months worth of data can be simulated in a few seconds, allowing the testing of many potential solutions and thus the discovery of the optimum conditions. The algorithm employed for the simulation is summarized in Figure 8. A key feature of our model is the use of historical sample submissions made by users. These are fed into the program and their predicted finish time in the modeled environment is calculated. All of our submissions are logged into a central Oracle database, with details of the time, date and method requested. Thus, this information could be retrieved by a simple query of the database. The algorithm operates on each sample from this list in turn. First, the requested method is mapped to currently available methods in the simulation, and the need to run the sample or not is determined. These steps are necessary as we might want to test the time savings made by combining methods. For example, we might choose to replace separate positive and negative ion methods with pos/neg switching: in this case some injections where both were requested for a single sample would become unnecessary. The next step is to determine which instrument

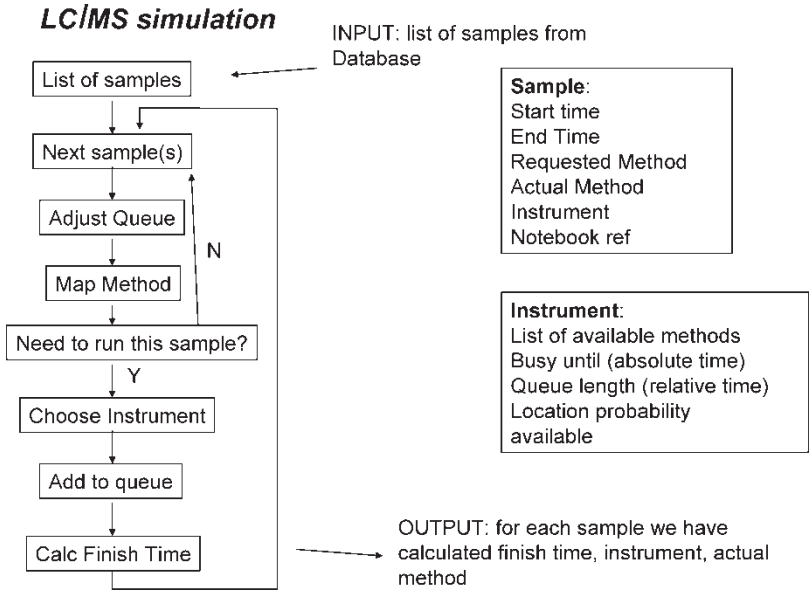


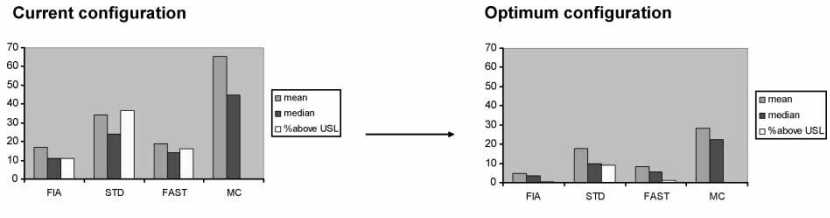
Figure 8. Schematic of the algorithm used in our LC/MS simulation.

should 'run' the sample. This decision considers a number of factors such as on which instruments the required method is available, and the current queue on those instruments. The algorithm assumes that all things equal, a user will choose the instrument with the shortest queue. However, as our previous analyses of patterns of instrument use had concluded that conveniently located instruments were far more likely to be chosen even if they had longer queues, we also built in a probability factor influencing instrument choice to model this behavior. Once an instrument is chosen, the algorithm determines how long this sample will take and adds it to the queue of the appropriate instrument. The next sample is then read, the queues adjusted according to the time of this request, and the process repeated until all samples in the simulation have had their lead times calculated.

When considering the results of the simulation, a number of statistics on the predicted lead times can be calculated. The median is perhaps the most useful measure of central tendency in this context as the average is influenced by outliers. Another useful statistic is the percentage 'out of specification'. The specification was determined in surveys of users as the time they would be willing to wait for an LC/MS result. 30 minutes was the most common answer here. Reducing the number of out of specification lead times is a clear goal of changing the system.

Many configurations were considered using the simulation, and their relative merits were ranked using the statistics described above. The simulation showed that some of the options we were considering were not worth pursuing. For example, we were concerned with the extra equilibration time triggered when initial solvent conditions changed. The simulation showed that this had a negligible effect on lead times, as it was actually triggered very rarely. Other changes were shown by the simulation to be more beneficial than our expectations would have predicted. For example, we discovered that we could reduce the post time in all our runs by taking into account two things: First, our users never made more than one injection per vial, and second, the time taken for the auto-sampler to change vials was close to one minute. We thus shortened our post time by one minute, effectively carrying out these activities in parallel rather than in series. Clearly doing this would have to be beneficial to the queue, but the increase in exit rate from the process achieved by this change could actually prevent queues from building up. The simulation clearly showed this in the resulting statistics. One is rather reminded of traffic at a busy junction: a relatively small obstruction slowing the traffic can lead to significant delays.

The simulation also allowed us to study the effect of the availability of methods on instruments. One might imagine many scenarios, but a fundamental question for us was whether to make machines as general as possible or whether to have machines devoted to specific methods. In particular an 'express' machine with only a fast method might seem attractive. However, the simulation clearly showed the optimum solution was to make instruments as similar as possible. The average time for 'fast' methods was the same in



	mean	median	%above USL
FIA	16.85	11	11.08
STD	34.36	24	36.74
FAST	18.96	14	15.94
MC	65.34	45	0.11

	mean	median	%above USL
FIA	4.9	3.5	0.26
STD	17.74	10	9.14
FAST	8.39	5.5	1.03
MC	28.31	22.5	0

Figure 9. Comparison of simulation results for pre and post optimization scenarios. Results are divided into general method categories of FIA (loop injection), standard (4 minute gradient), fast (2.5 minute gradient) and MC (final product characterization, 7 minute gradient). Lead times are in minutes.

both scenarios, but the average time for longer methods was dramatically reduced by having both available on several instruments.

The simulation told us (see Figure 9) that the optimum set up should give us significant improvements over the current configuration, including a 50% reduction in the median lead time. The changes modeled in the simulation included moving all instruments to a central communal area; reducing non value added cycle time by carrying out equilibration in parallel with other activities such as sample manipulation and reporting; standardizing methods across multiple LC/MS systems thereby reducing the impact of maintenance through redundancy; and optimizing specific chromatographic methods. The overall strategy to reduce lead time by implementing these changes offers significant financial saving compared to the option of investing in additional LC/MS capacity. We implemented the set up as determined by the simulation, and were pleased to see that the improvements predicted by the simulation were indeed borne out in practice (see Table 6).

Table 6. Statistics for comparison of predicted improvements (from simulation) and actual improvements. Before and after are the actual lead times (in minutes) before and after the changes were implemented. The % reduction shows the actual change between these two numbers, predicted shows the predicted % reduction. The reduction achieved compares very well to that predicted

	Before	After	%Reduction	Predicted
Mean	38.3	12.0	68.7	52.0
Median	20.0	7.3	63.3	59.0
% OS	32.3	5.6	82.6	84.0

CONCLUSIONS

Orthogonal low and high pH LC/MS-based methods with new column technologies have been developed using a standard mix composed of four drug-like compounds. Methods were optimized for selectivity, separation power (peak capacity), MS sensitivity and stability. Our standard 14 min separation time using generic gradient methods have been reduced by 1.6 fold when switching from 3.0 × 10 mm, 5 μm to 2.1 × 50 mm, 3−3.5 μm columns. Although all tested columns exhibited good separation performance, higher average PC values are observed on the Gemini column with formic acid and the XBridge column with ammonium bicarbonate buffer. A standardized process to record standard and consistent LC/MS data for new chemical entities, independent of research site has been proposed. The standardized process has been evaluated by means of a reproducibility study, using a system suitability test that has been incorporated in all the laboratories to monitor the LC/MS systems performance. The strong influence of DAD parameters, such as the response time on the peak capacity, or the influence of MS parameters on MS sensitivity has been shown. The value of strategies for alternating orthogonal low and high pH methods, in a high throughput mode, on a single system has been evaluated without compromising data quality. The development and implementation of software for simulating the LC/MS open access sample queues has been described. A significant financial saving compared to the option of investing in additional LC/MS capacity is noted.

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