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Right-First-Time Isocratic Preparative Liquid Chromatography-Mass Spectrometry Purification

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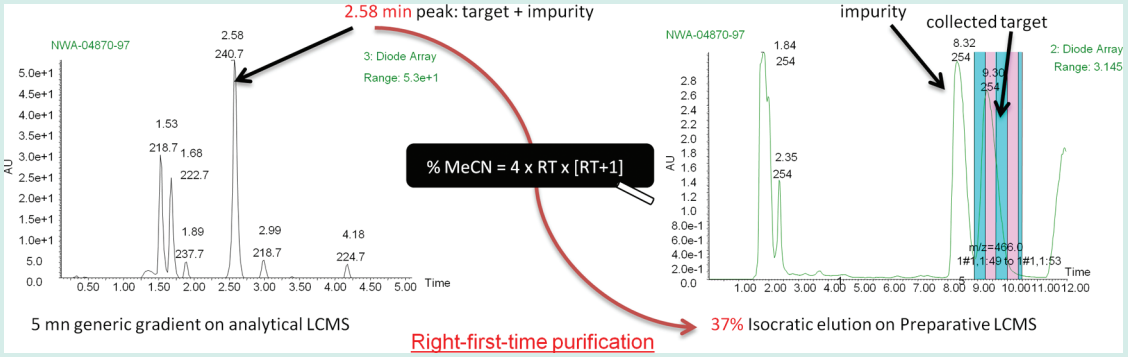

ABSTRACT: Preparative RP-LCMS is widely used to purify compounds from crude reaction mixtures in drug discovery today. Method development for preparative Liquid Chromatography-Mass Spectrometry (LCMS) consists of finding the right balance between speed and quality, often leading to the use of fast generic gradients (5−95% organic mobile phase) to purify compounds of various structures and polarity, which can be sometimes detrimental to the purity of the isolated compound. Isocratic elution is the best method to obtain high purity, but it usually requires a long trial and error method development to find the right eluent composition. Therefore it is mostly used to purify large quantities of product using for instance stack injections, but not for routine purification of structurally diverse compounds. Herein, we describe an empirical method that allows "right-first-time" purifications with isocratic elution on preparative RP-LCMS, permitting isolation of compounds in high purity and yield.

KEYWORDS: *mass-triggered preparative RP-LCMS, isocratic elution, purification, method development*

INTRODUCTION

The need for increased productivity in pharmaceutical industry R&D has led to significant improvements in multiparallel library production over the past few years. This has resulted in a major challenge for compound purification technology and its output speed: what can be done to align the rate of purification with the rapid delivery of the multiparallel syntheses? No matter what the speed of synthesis, it remains of paramount importance for compound libraries to be of high purity when performing lead discovery screening as any contaminant in samples may lead to false positive/negative biological results.

While it is important, from an analytical HPLC perspective, to obtain a good resolution for an entire gradient run, only the target compound requires a good resolution for preparative HPLC. Fast generic gradients are mainly used to purify compounds having a very large diversity in terms of structure and polarity, but the resulting resolution may not be sufficient to purify complex reaction mixtures. To improve resolution, several factors can be adjusted: the percentage and the type of the organic mobile phase B, the temperature, the column type, the pH of the mobile phase, and so forth. For routine purification of a diverse set of compounds, the easiest factor to vary is of course the percentage of solvent B.

Theoretical studies of quantitative structure-retention relationship (QSRR) have been published, with the aim of predicting a set of reversed-phase HPLC gradient conditions using descriptors like clogP.[1,2]

Many groups follow a practical approach in which the retention time of the product from a fast analytical run with a generic gradient is used to assign an optimized focused preparative gradient.[2−4,6,7] Recently, several vendors have launched analytical-to-preparative Liquid Chromatography-Mass Spectrometry (LCMS) softwares that electronically access the analytical LCMS data, extract the retention time of the product of interest, and then calculate a customized gradient for each sample of the library.[1,8] However, none of these methods apply to isocratic elution.

Snyder et al. published a formula to estimate the ideal conditions for isocratic elution in analytical HPLC based on an initial gradient run, in which the percentage of acetonitrile for isocratic elution is a linear function of the average retention time for the first and last peak.[9,10]

$$(\text{isocratic\%B}) = 6.33[(\text{RT})\text{avg} - t_D] - 0.02 \quad (1)$$

where t_D is the dwell volume, the value 6.33 being a 95% gradient over 15 min, and the value 0.02 resulting from the

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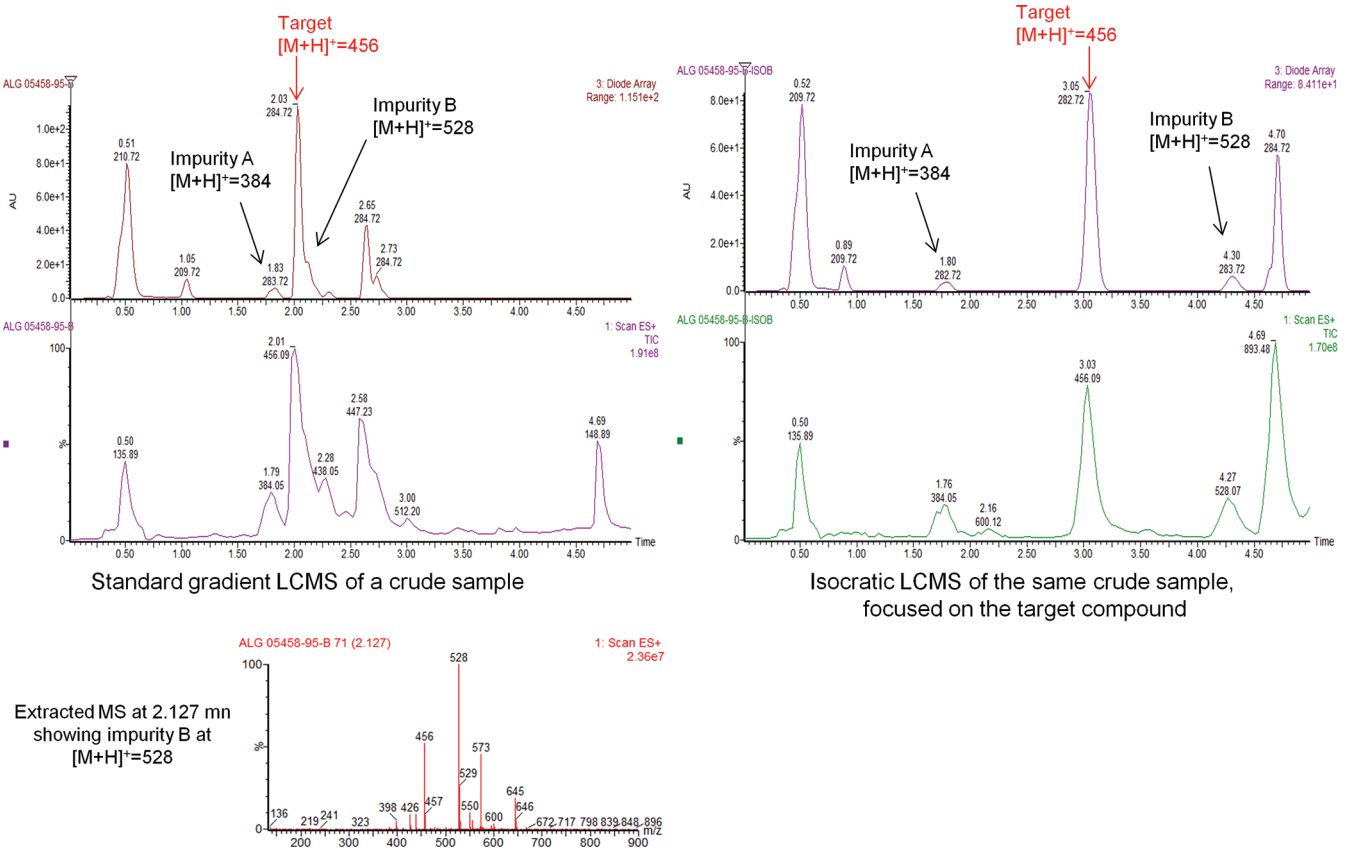

Figure 1. Isocratic elution focused on the target compound vs gradient elution. (Standard gradient used 5% to 95% of MeCN, isocratic conditions set to 22% of MeCN).

starting gradient value, retention factor, and a constant. The calculation is based on an average retention time, and is not focused on a target component, so it is not optimized enough for preparative chromatography. Moreover, in preparative chromatography, the fact that the columns are often overloaded has consequences on the elution, that is, the migration of one component in the column affects in a complex manner the migration of the others,[11] thus rendering the formula invalid for many cases of preparative chromatography.

Several groups have compared isocratic and gradient performances on analytical systems.[9,12,13] Gradient elution is often seen as a slower technique[3] because of longer re-equilibration time and is less reliable because of ghosts peaks and other disturbances. Isocratic elution also provides for easier method transfer, fewer experimental problems or demands on the operator, and the use of simpler instrumentation.[12,14] However, a purification run can be longer with an isocratic method if the method is not focused on the target compound.

We consider isocratic elution as the most effective method to obtain good resolution from close impurities when focused on the target compound (Figure 1). Our goal was to develop an isocratic method with the same short run time as a gradient method so that we would gain in quality but without compromising speed.

RESULTS AND DISCUSSION

Snyder et al. published in their latest book a preparative LC method development based on touching peak separation (T-P).[14] This method requires four steps, the first three being done on the analytical system: (1) an initial separation after adjusting %B; (2) maximizing the separation factor for the product peak; (3) maximizing by trial and error the sample size such that the broadened product peak touches just one of the two surrounding peaks; (4) scale-up by well-known formulas in which flow rate, sample volume, and loading are increased in proportion to the column's cross sectional area.

In our daily work it was highly desirable to find a more general method that could be applied to every crude reaction mixture and necessitate as little optimization as possible, given the large structural diversity of our molecules.

The general philosophy was to find a correlation between our analytical and our preparative LCMS systems. To that matter, we believed it was important to use the same packing material and chromatographic conditions (solvents and modifier) for both analytical and preparative systems.

Preliminary work enabled us to determine the maximum average loading of our column. Good results were obtained with 0.3 mmol of product in 1.5 mL of solvent per injection, which is roughly our reaction scale for final products synthesis. The optimized flow rate was set to 60 mL/min, following the vendor's recommendations for X-Bridge 5 μm 30 × 150 mm columns. We then wished to establish a general formula for the %MeCN in an preparative isocratic elution to isolate the target compound without the risk of either losing the sample in the rinsing phase or eluting it too close to the void volume.

To accomplish this, we analyzed a series of compounds of varying polarity using a standard gradient condition (5% to 95% MeCN on a 5 min run time). We then performed isocratic preparative elution on these samples, establishing by trial-and-error the amount of MeCN to use to bring the desired peak off

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the column in the second half of the chromatogram (approximately 6 min in a 10 min run), to achieve the best possible separation. We found this isocratic % of acetonitrile to be proportional to the analytical retention time in the gradient analysis, designating the conversion coefficient as Ω:

$$\%\mathrm{MeCN} = \Omega \times \mathrm{RT} \quad (2)$$

We also observed that Ω varies whether the sample is hydrophilic or lipophilic. For example, four samples of different polarities were injected on our analytical system using standard gradient from 5% to 95% MeCN on a 5 min run time. The observed retention times for compound 1 and compound 2 were respectively RT_1 = 3 min and RT_2 = 1.8 min (Table 1).

Table 1. Proportionality between the Analytical Retention Time and the % of Acetonitrile Used to Obtain a Good Purification in Isocratic Preparative LCMS for 4 Different Compounds

RT (min) in analytical gradient run	1.8	2.2	2,6	3
% MeCN in preparative isocratic run	25	35	47	60
Ω	14	16	18	20

On the preparative system, the right percentage of acetonitrile was adjusted by approach for each sample to obtain a good isocratic elution in the second half of the chromatogram, that is, around 6 min on our 10 min run time method. We experimentally found $\%\mathrm{MeCN}_1$ = 60% and $\%\mathrm{MeCN}_2$ = 25% for these samples, which leads to the corresponding values of Ω using formula 2: Ω_1 = 20 and Ω_2 = 14 (Table 1).

As Table 1 shows, our second observation was that Ω is a linear function of the retention time (RT):

$$\Omega = A \times \mathrm{RT} + K \quad (3)$$

Solving the following equations using the values RT and Ω previously found, gives A = 5 and K = 5.

$$\Omega_1 = A \times \mathrm{RT}_1 + K \quad \text{and} \quad \Omega_2 = A \times \mathrm{RT}_2 + K$$

Combining eqs 2 and 3, we have

$$\%\mathrm{MeCN} = \Omega \times \mathrm{RT} = (A \times \mathrm{RT} + K) \times \mathrm{RT}$$

With our values of A and K, we deduce

$$\%\mathrm{MeCN} = (5 \times \mathrm{RT} + 5) \times \mathrm{RT} = 5 \times \mathrm{RT}(\mathrm{RT} + 1)$$

To ensure that the target compounds really elute in the second half of the chromatogram we realized that we had to further refine the above equation and add a correction factor of 0.88, which was found to hold experimentally over numerous tests using samples with different polarities. Hence, the final formula for translation between gradient and isocratic conditions for our system is

$$\%\mathrm{MeCN} = 5 \times \mathrm{RT}(\mathrm{RT} + 1) \times 0.88 \quad (4)$$

It must be pointed out that this formula is only valid for our systems:

- analytical LCMS using a 4.6 × 50 mm, 3.5 μm X-Bridge column at 2.5 mL/mn at 50 °C on a 5 min run time
- preparative LCMS using a 30 × 150 mm 5 μm X-Bridge column at 60 mL/mn at 20 °C on a 10 min run time.

The correction factor compensates for differences in tubing, dead volume, and other factors between the two different instruments. In other words, changing the temperature or the flow rate would require adjustment of the correction factor so that the target compound still elutes in the second half of the chromatogram.

While this formula applies only to the system on which it was created, the method can be used for any paired analytical/preparative LCMS system. Any user can set up a formula that would suit his instruments using the full procedure above. Interestingly, the correction factor seems to be independent of column within the same type, since we found that no modification of formula 4 was necessary for a C18 column from a different vendor tested on our analytical and prep systems (Phenomenex's Gemini NX 4.6 × 50 mm, 5 μm for analytical LCMS and Gemini NX, 30 × 150 mm, 5 μm for prep LCMS). Moreover, while most of our work were performed with ammonium carbonate as a basic modifier, we observed that we could change to an acidic one (trifluoroacetic acid 0.1%) and still use the formula without changing the correction factor.

To improve the purification of some more complex mixtures that could not be perfectly purified on a 10 min method, we set up a new method on a 12 min method. Extension of the isocratic run to 12 min was easily accomplished using a lower correction factor of 0.8 to keep the target compounds eluting in the second half of the chromatogram. This longer method leaves more time for close peaks to separate. Even longer run times are not advisible as peaks tend to broaden in isocratic elution, moreover, short run times are needed to maintain high productivity when purifying libraries of compounds.

The method can also be applied to the analytical LCMS, allowing for an easy and effective correlation between our generic gradient on the analytical LCMS and an isocratic elution on the same analytical LCMS. In this case, a correction factor of 0.72 was used to delay elution to the second half of the run. This is very useful in many cases, such as when we suspect the presence of isomers, secondary products, or impurities hidden below the main product peak in the generic gradient run. Analytical LCMS of the same sample under the predicted isocratic conditions often resolves such hidden peaks in a single attempt.

The different correlations for our systems are summarized in Figure 2. We have found the formulas to be valid for our systems using C18 stationary phases, but it would be interesting to test another type of bonded phase to check if a similar formula can be determined.

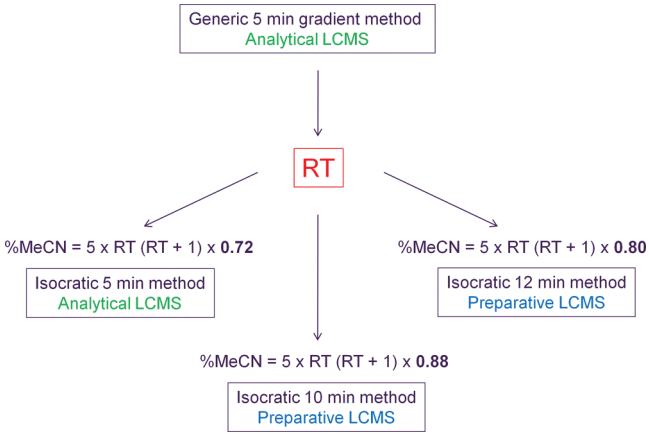

Figure 2. Adjusting the correction factor for the different LCMS systems.

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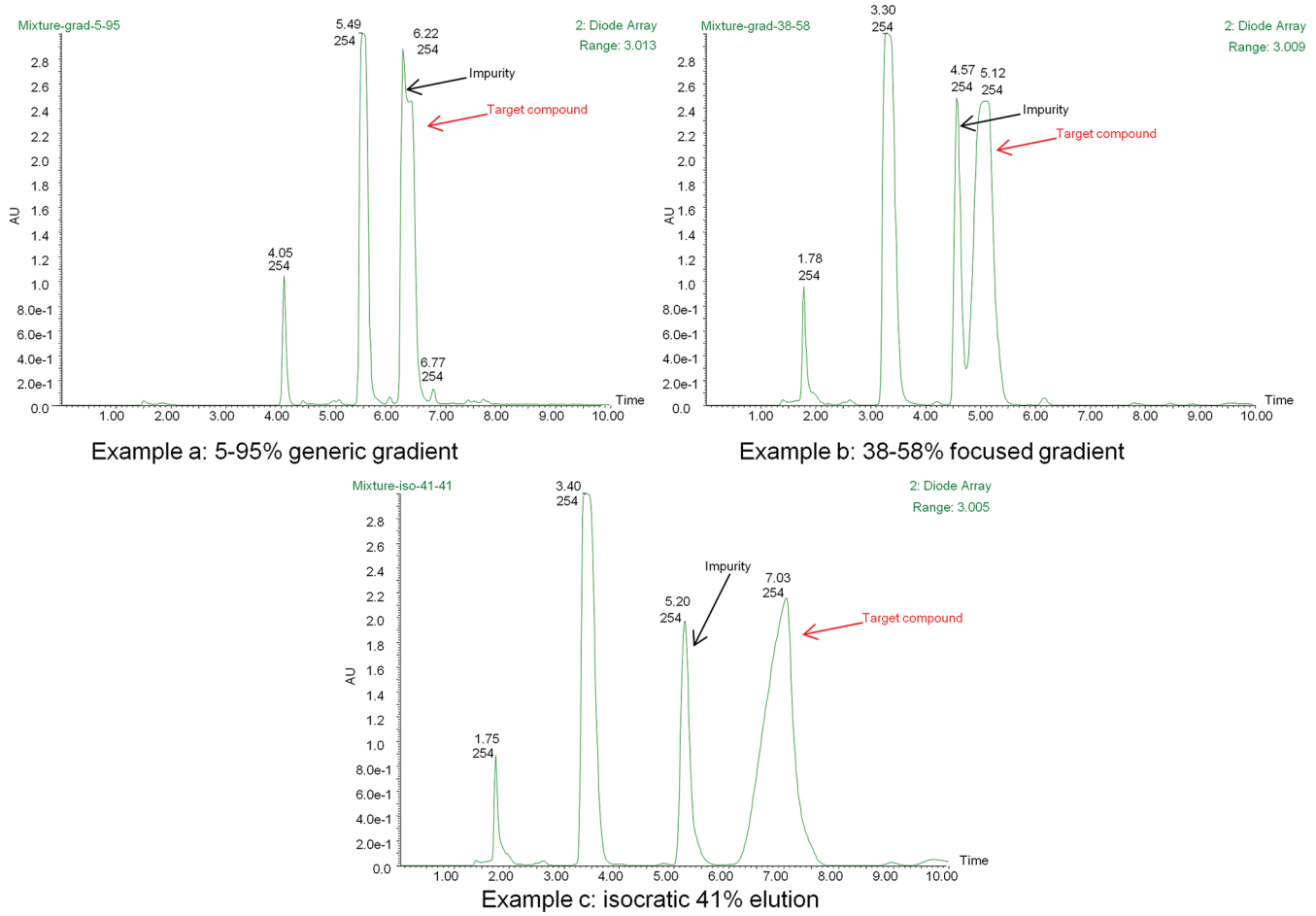

Figure 3. Comparison of (a) a generic gradient, (b) a focused gradient, and (c) an isocratic elution.

APPLICATIONS

While compound-focused gradients are described in the literature as more efficient than generic gradients,[2,3,5−7] isocratic elution proved to be even more powerful. The example in Figure 3 shows preparative LCMS purifications of a mixture of four components. Three methods were tested on the same run time of 10 min. First, a generic 5−95% gradient (Figure 3a) showed the desired compound as the most lipophilic peak at 6.22 min, but, as can be seen from the shape of the peak, coelution with an impurity was evident. An isocratic gradient was calculated using formula 4 based on the analytical retention time of the target (Figure 3c) and a focused gradient (Figure 3b) was also created around that value. The focused gradient enabled separation of the coeluting compounds that were not separated using the generic 5−95% gradient, but the resolution was not good enough to obtain the compound with an acceptable recovery and purity. In contrast, isocratic elution dramatically increased the resolution and allowed an efficient purification of the target compound.

Figure 4 shows another example in which the crude reaction mixture looked fine by analytical LCMS: the peak of the expected product at 2.58 min was not particularly large (Figure 4a). However, the mass spectrum indicated two masses associated with that peak, the expected product, and another one of lower mass, that was thought to be either a fragment or an impurity. To address that issue, the sample was analyzed by isocratic analytical LCMS (%MeCN = 5 × RT (RT + 1) × 0.72 = 33%) (Figure 4b), clearly revealing the previously hidden impurity peak. The crude reaction mixture was purified by a 12-min preparative LCMS run using the corresponding formula % MeCN = 5 × RT (RT + 1) × 0.80 = 37% (Figure 4c).

Formula 4 proved to be efficient regardless of the chemical diversity of our compounds, covering a large range of lipophilicity. Some examples of such chemical structures are given in Figure 5.[15−19] As described by Degorce et al., the closely related compounds **5**, **6**, and **7** were nicely separated in one shot from one single reaction mixture.[15]

Figure 6 gives an overall picture of the chemical space accommodated by our isocratic HPLC method in terms of hydrogen bond acceptors, donors, molecular weight, calculated logP, and molecule ion class. Thus, the technique covers much of the range of properties of molecules that need to be purified in contemporary pharmaceutical R&D. The purity average calculated over 1208 crude reaction mixtures purified meets our standards for compounds to be sent for primary in vitro evaluation such as biological, DMPK, and physicochemical assays. As shown in Figure 6, 87% of the samples were obtained in greater than 95% purity using a single preparative LC run set up according to eq 4 from a standard preliminary gradient analysis.

CONCLUSION

To meet the challenge of high throughput purification in drug discovery, we have recently optimized our preparative LCMS method, improving both speed and quality. An empirical formula has been set up that allows a right-first-time

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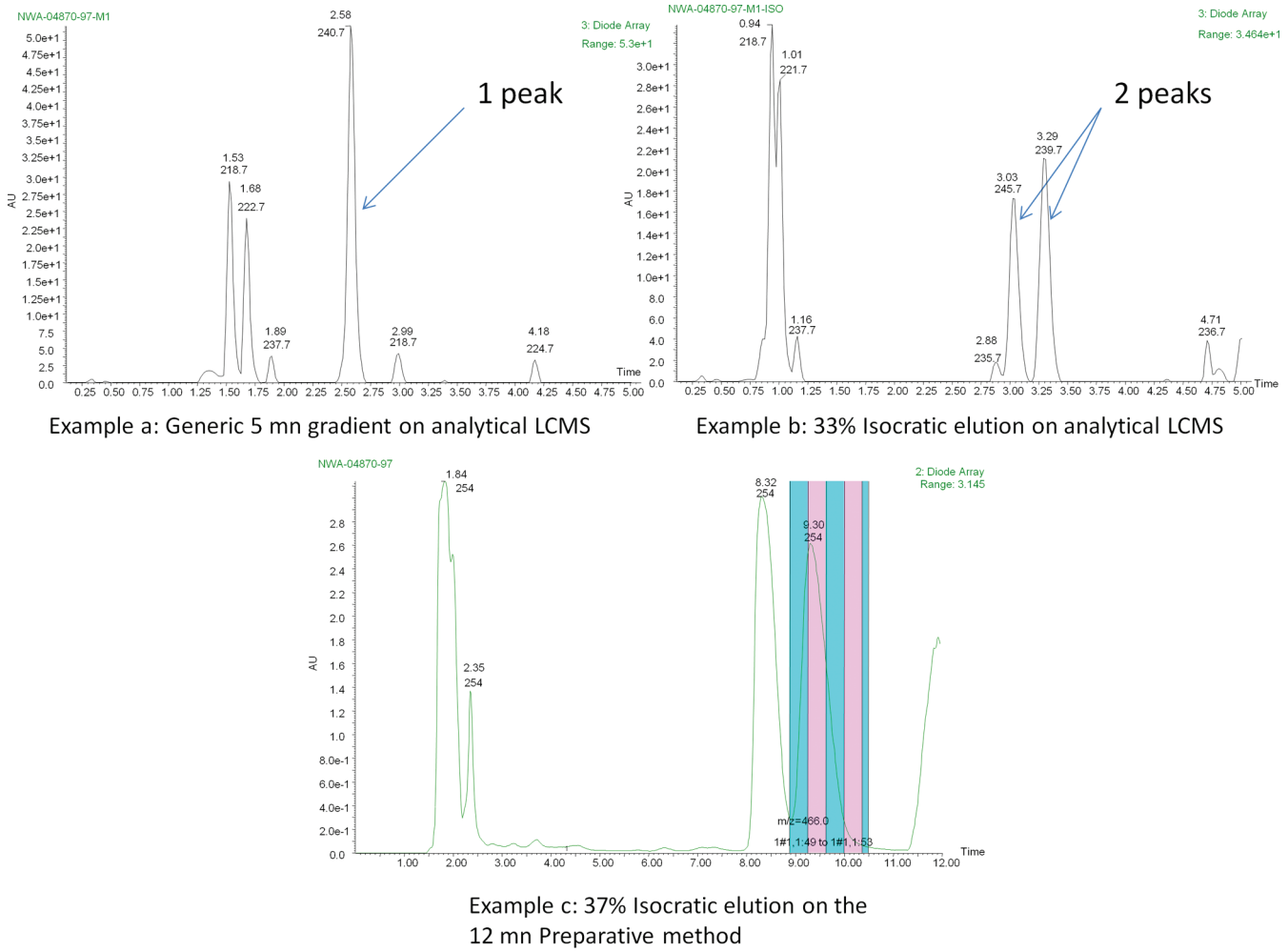

Figure 4. Example of a hidden peak revealed by isocratic analytical LCMS.

purification using isocratic elution, which is, to our knowledge, the first isocratic transfer method from analytical to preparative LCMS. This high performing purification system originally designed to improve library purifications led us to develop another activity within the lab: a purification service dedicated to lead optimization chemists, which is now purifying 74% of the chemists' final products. With a turnover of less that 24 h including solvent evaporation, a recovery ratio of 90% and an attrition of 0.2% (2 samples lost over 1000), this efficient method is now used every day and has enabled us to increase the purification productivity by 150% with an average of 2600 purifications per year, without loss in quality. The purification service saves time for the project chemists, so that they can focus on high value adding activities such as designing new routes and making compounds.

EXPERIMENTAL PROCEDURES

General Methods. Preparative LCMS was carried out using Binary Waters 2525 Pumps linked to an injector/fraction collector Waters 2767, and a Waters Active flow splitter for Waters ZQ 2000 mass spectrometer and a UV detector 2487. The mass spectrometer, LC and fraction collection are controlled via Masslynx and FractionLynx Waters software. Samples are routinely filtered and injected at concentrations of reaction scale, that is, around 0.3 mmol in 1.5 mL of DMF on a Waters Xbridge Column, C18, 5 μm, 30 × 150 mm, with ammonium carbonate 2 g/L (solvent A) as the aqueous modifier (pH = 8.9) and acetonitrile (solvent B) using a 10 mn preparative method (Waters Autopurif), 60 mL/min. Samples injected are crude reaction mixtures. No preliminary workup was done even if metals were used for the reaction, as we found our column's lifetime acceptable (∼ 2000 injections). We preferred not to use column prefilters to avoid elution problems or loss of compound through precipitation.

The 10 min preparative method is set as follows with X the percentage of acetonitrile calculated with formula 4 for isocratic elution: t = 0 min, %A/B = 95/5 25 mL/min; t = 0.5 min, %A/B = (100 − X)/X, 60 mL/min, t = 8.5 min, %A/B = (100 − X)/X, t = 8.51 min, %A/B = 0/100, t = 9.5 min, %A/B = 0/100, t = 10 min, %A/B = 95/5. The 12 min preparative method is set as follows: t = 0 min, %A/B = 95/5 25 mL/min; t = 0.5 min, %A/B=(100 − X)/X, 60 mL/min, t = 10.5 min, %A/B = (100 − X)/X, t = 10.51 min, %A/B = 0/100, t = 11.5 min, %A/B = 0/100, t = 11.51 min, %A/B = 95/5, t = 12 min, %A/B = 95/5.

The flow rate ramps up at the start of the method and then goes back to the initial conditions at the end of the method, ie. %A/B = 95/5 at 25 mL/min, so that the instrument is ready for the next injection. This lower flow rate allows for an efficient and safe injection of the product on the column. The preparative methods start at 95% A and ramp to the calculated % of organic eluent in 0.5 min so that the pumps does not change too abruptly in solvent delivery. One minute of rinsing

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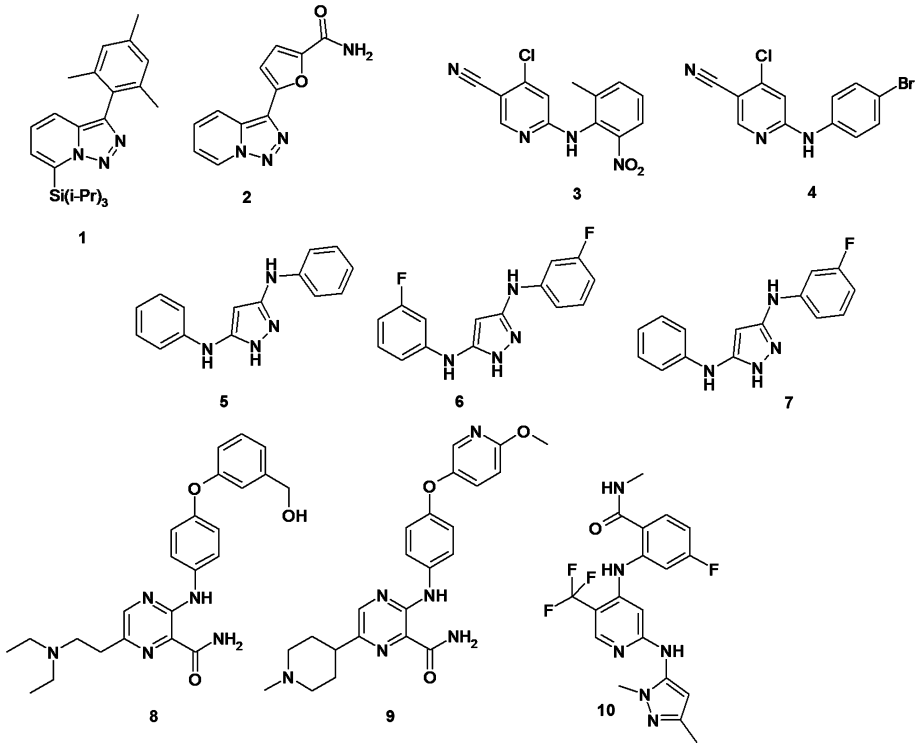

Figure 5. Heterocyclic compounds purified from crude reaction mixtures using our method.

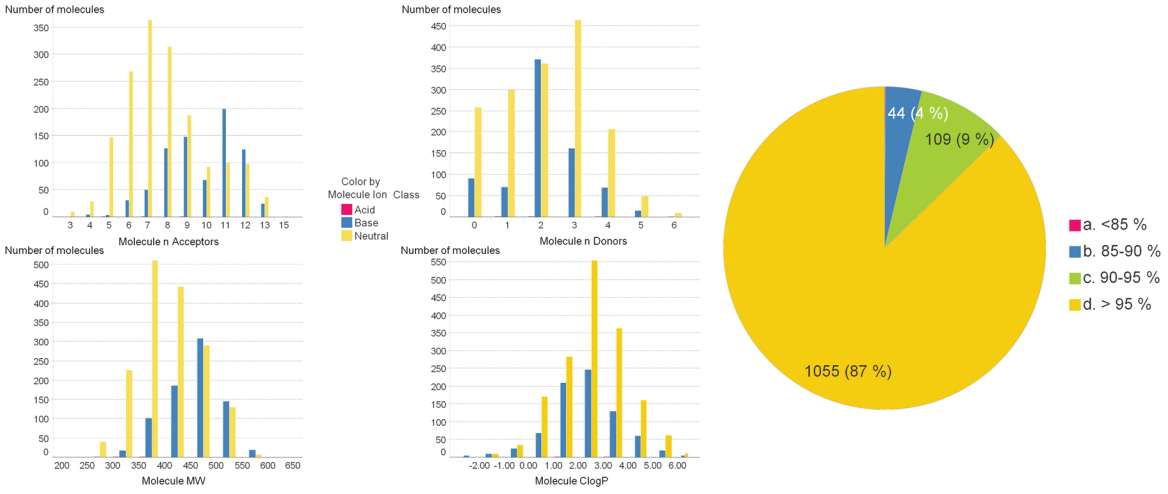

Figure 6. (Left) Chemical space covered by the compounds purified with our method. (Right) Purities for 1208 products purified by isocratic HPLC according to eq 4.

at 100% of B at the end of the run is necessary to elute the compounds that are still on the column and make sure the column is clean for the next injection. We observed that a total of 0.5 min plus the time between injections is sufficient to re-equilibrate to 95% A.

Analytical LCMS was carried out using Waters Alliance 2695 system linked to a Waters ZMD mass spectrometer and a 2996 PDA detector. Samples are routinely filtered and injected on a Waters Xbridge Column, C18, 3.5 μm, 4.6 × 50 mm, with ammonium carbonate 2 g/L as the aqueous modifier (pH = 8.9), using a standard gradient from 5% to 95% MeCN on a 5 min run time, 2.5 mL/min, 50 °C. The analytical method is set as follow: t = 0 min, %A/B = 95/5; t = 4 min, %A/B = 5/95, t = 4.75 min, %A/B = 5/95, t = 4.76 min, %A/B = 95/5, t = 5 min, %A/B = 95/5. The column oven is heated to 50 °C to allow for a higher flow rate and good peak resolution. A modification of the temperature would change the retention time and consequently the correction factor of the formula would need to be re-evaluated. The preparative column is kept at 20 °C for practical reasons.

Solvents are evaporated during a night time program using the Genevac HT-4 and HT-4X apparatus.

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Notes

The authors declare no competing financial interest.

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