Perspective

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Generic Compound Isolation Using Solid-Phase Trapping as Part of the Chromatographic Purification Process. Part 1. Proof of Generic Trapping Concept

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In the past decade, there has been a movement toward the use of "generic" methods for many different types of analysis within the fields of analytical chemistry and biochemistry.[1,2] Developments in automation and the increased application of parallel chemistry formats have led to a dramatic rise in both the numbers and the relative speed of production of different types of compounds.[3−5] To maintain confidence in the ability to adequately check the quality of these compounds, adoption of high-throughput, fast, effective analysis methods have been crucial. Where possible, methods have been designed to be as universal as possible to avoid complications associated with too many options and also to reflect the fact that, early in a compound's history, for example, during the discovery phase, a general purity figure obtained from an analysis method will suffice. Reversed-phase gradient HPLC is generally used as the "universal" method of choice. These methods are also routinely scaleable, and further innovations, such as mass-directed fractionation, allow even more confidence in the ability to extract the specific target ion mass from chemically crude input materials, as well as to collect only one desired product from each crude target compound. These approaches form the basis of some very consistently used analysis and purification processes within pharmaceutical companies.

Fractions from reversed-phase HPLC are frequently required at various scales. These may include micro, semi-prep, and preparative and be loosely defined by reference to the diameter of the main column: for example, 2 mm, 4.6−10 mm, and 20 mm. Fraction collection, be it manual or automated, traditionally relies upon activation of a valve to temporarily divert HPLC eluent to a collection vessel. The collected solution may then be processed, typically by rotary evaporation or nitrogen blow-down, and solids can be recovered and weighed or redissolved in a suitable solvent to a target concentration. In most of the cases, the solids quantified by means of weighing are reconstituted in DMSO for library storage or immediate use in tests.

However, the rapid adoption of these types of processes has also led to common issues with sample recovery and isolation. The usual practice of using aqueous/solvent mobile phases, containing acid modifiers, and evaporation as a means of removing solvents, often means there are concerns that some amount of process residue is inevitably left with the sample after evaporation.[6] Depending on the process used, compound type and functionality, these residues can vary significantly in amounts present. There is a possibility, not just for "free" residue, such as water and acid, but also for "bound" entities, such as salts, to exist. If weighing is used as a post-purification method of quantification, there is an opportunity to introduce significant errors, which may be propagated into subsequent preparation of "known concentration" solutions. The type of acid used as a modifier also influences the type of salt form that will be produced, and this will affect the amount of mass error imparted to the weighing, based on the relative molecular weights of compound and salt form. Often, acids that are useful for

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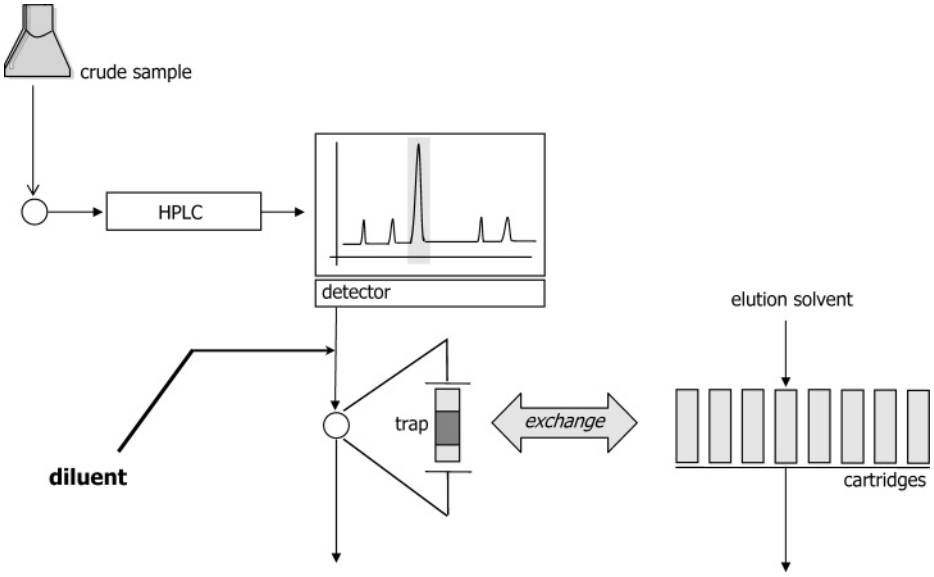

Figure 1. Schematic of solid-phase fraction trapping. A stationary-phase filled minicolumn (the trap) replaces traditional liquid fraction collection. Portable traps are exchanged in an automatic fashion to follow one pure, one-trap mode. Diluent solvent helps reduce the elutropic strength of the mobile phase to promote better immobilization.

chromatographic purposes, such as TFA, are not so desirable as the preferred salt form of an isolated, pure compound.

It was with these issues in mind, that the concept of on-line "trapping" of the purified compounds in the eluent stream from the HPLC system was investigated. The purpose of this initial work has been to study the feasibility of trapping (immobilizing) target compounds in selected fractions onto suitable sorbents that are configured in-line in a manner that enables subsequent washing, processing, storage, and, as required, elution. The overall objective was to isolate the compounds of interest as part of the purification process and present just the compound in a form that, when weighed, provided just the accurate amount of compound present. Due to the amount of work involved and the lack of purpose-designed equipment to facilitate automation, some aspects of this work, specifically, proof of salt form manipulation and fully optimized procedures for sample recovery, are not covered extensively in this paper but are intended to be published in greater detail in subsequent parts.

Concept Overview. The process can be envisaged as similar to that exploited in the common technique of online solid phase extraction (SPE) or column switching. In SPE, analytes generally present at low levels in aqueous solution are trapped on an activated and equilibrated (reversed phase) SPE sorbent prior to their elution in a more concentrated format (often onto an HPLC column) by a minimal amount of a solvent of high elutropic strength.[7] When used in "trapping mode", however, the highly retentive RP sorbent cartridge (the trap) is located downstream from the HPLC column and analyte delivered in a rather "concentrated" form as an HPLC band within the selected fraction (Figure 1). In this mode, then, we are not primarily concerned with the concentration effect's being our main objective because the inlet fractions are already concentrated, having been delivered from a preparative HPLC run. Our main intention is to exchange the background solvent and effectively remove any additives. There are, however, some very important differences between the approach described here and the well-known SPE-type applications. Ordinarily, SPE utilizes fairly cheap grade, large-particle, bonded silica, housed in low cost, disposable holders generally taking the form of a plastic syringe or, more recently, in 96-well microtiter plate format. This is consistent with their application limitations that require reasonable flow rates to be obtained at low operating pressures. The cost of achieving this is often poorly packed beds of large particles, which cause unnecessary dispersion of the analyte, flow channelling, and often necessitate significant excesses of solvent to guarantee reasonable analyte recovery. It is also widely reported that the reproducibility of this process can be variable.[8−10] Nevertheless, the concept is very appealing, and it was felt that if the components of this process could be improved such that improved speed and greater control of flow, sample dispersion, and sample recovery could be managed and used in a largely generic manner, then this would provide substantial benefits to the process.

The general concept of peak trapping has been proposed before and nowadays is gaining popularity, for example, in a quite specialized area of LC/NMR analyses for which sample focusing and solvent exchange are fundamental. Traps in the form of on-line SPE cartridges,[11,12] guard columns,[13] or even holding loops have been used.[14] LC fraction isolation prior to MS analysis with the use of short HPLC columns was also reported,[15] and a study of a more universal immobilization−retrieval process using LC/MS techniques has been performed.[16] Nevertheless, despite a growing number of applications, peak trapping remained a mere intermediary tool, often requiring customization to suit the needs of the specific analytical technique being used. It was conceived that there would be enormous utility in being able to generically trap the products of HPLC-based purification "on the fly" and without programming compound-specific conditions. It was also a key objective to decouple this compound isolation process from the recovery stage, which for many reasons was not required to be performed in-process and could be carried out in parallel, if required, as an independent stage.

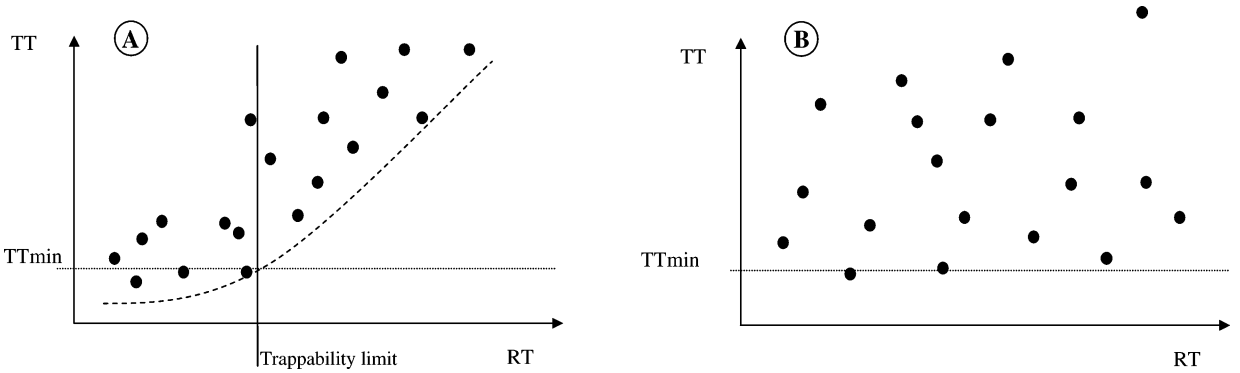

Figure 2. Desired behavior of trapping sorbent in terms of immobilization capability (TT) toward random analytes across the gradient envelope.

In the proposed method, the HPLC flow is diverted onto the trap in a fashion similar to classical liquid fraction collection by detector-based triggering. Because both the main (separation) column and the trap retain analytes by reversed-phase mechanisms it is necessary to weaken the fraction's mobile phase to promote on-trap immobilization by dilution with a solvent of low elutropic strength (e.g., water[17,18]). This dilution takes place only for the actual duration of trapping, that is, effectively, the volume of the target HPLC peak. Subsequently, the now off-line trap is washed with water to remove unwanted polar materials, such as TFA or buffers, and finally, the solvent is evacuated from the trap by passage of nitrogen. At this point, the trap is ready for elution with an arbitrary strong solvent suitable for further sample processing, or storage can be initiated by sealing the trap, which will help to protect the analyte from the impact of environmental factors, such as air, moisture, light, etc. Naturally, if the dry sample is needed, the evaporation process from 100% organic (and volatile) solvent is dramatically faster and cheaper. It may be that compound isolated in this manner will show favorable characteristics for ease of weighing, as compared to when isolated from evaporation of the HPLC mobile phase, for example, lower the incidence of gums, etc.

Trapping cartridges are conceived as compact, portable, identifiable, and trackable items easily movable between the trapping loop and their bulk source (e.g., the tray) in a fully automated manner. Likewise, cross-platform cartridge transfer, for example, for in-situ elution or postprocessing, can be easily arranged.

Experimental Section

Equipment and Materials. An Agilent HPLC 1100 series system equipped with diode array detection (DAD) (Agilent Technologies, Waldbronn, Germany) was used for chromatographic separations, and both data acquisition and processing were performed on Agilent ChemStation software. HPLC separations were performed on 100 × 2.1 mm Luna C18(2) 3-μm columns (Phenomenex, Torrance, CA) with the following linear gradient settings: solvent A, water/0.1% v/v formic acid; solvent B, acetonitrile/0.1% v/v formic acid; flow rate, 0.2 mL/min. Solvent B: 0−0.5 min at 0%; 0.5−14.5 min 0−100%; 14.5−16.5 min at 100%; 16.5−18.0 100−0%; 18.0−36.0 min equilibration at 0%. Water with either 0.1% v/v formic acid or 5.5 M ammonia was added to the HPLC eluent with the use of a second HPLC pump (also Agilent 1100 series). Mixing took place in a V-shaped passive mixing element, and a section of a number of short tubing pieces alternated with various unions to disturb the otherwise laminar flow.

The trapping section consisted of an on-line Prospect II SPE system (Spark Holland, The Netherlands) linked with the HPLC system via contact closure handling routines in Spark Holland's software. The post-cartridge signal was registered with another Agilent 1100 series DAD.

Spark-type, sorbent-filled SPE cartridges or other cartridges of the same dimensions as well as loose sorbents for testing were obtained from the following manufacturers: Phenomenex, Waters Corporation (Milford, CT), Spark Holland, Thermo Electron Corporation, (Runcorn, U.K.), and Polymer Labs.

Loose phases were packed by third parties (Spark Holland and Capital HPLC, Edinburgh, U.K.).

The model 8060-C CLND (Antek Instruments Inc., Houston, TX) instrument was controlled using Antek model 8060 Test Software version 1.06 and operated with a furnace temperature of 1050 °C, ozone and oxygen settings at 250 mL/min, and inlet and makeup helium flows at 50 mL/min.

Results and Discussion

How to Measure Effective Trapping. It is rather difficult to assess how well the analyte is immobilized on the trap while the trap is in-line during the trapping process. Therefore, in the course of the actual fraction isolation process, the trapping effectiveness is unknown. A prerequisite in trapping is to be highly confident the compound is being retained, rather than eluting it from the trap. Traditionally, it would be expected that measurement of "chromatographic" retention with varying organic mobile phase composition would be represented as a graphical plot of retention factor (or capacity factor k') against eluent composition. In these circumstances, however, with much smaller trap (column) volumes, variable mixing ratios of diluent, which may affect the mixing, and solute elution profiles that do not always give Gaussian-like distributions, it is difficult to be certain of these parameters. It was felt that for this work, a pragmatic definition of achievement of successful trapping could be applied as a simple limiting condition, for example, a minimum delay time through the trap that must be exceeded for trapping to be designated successful.

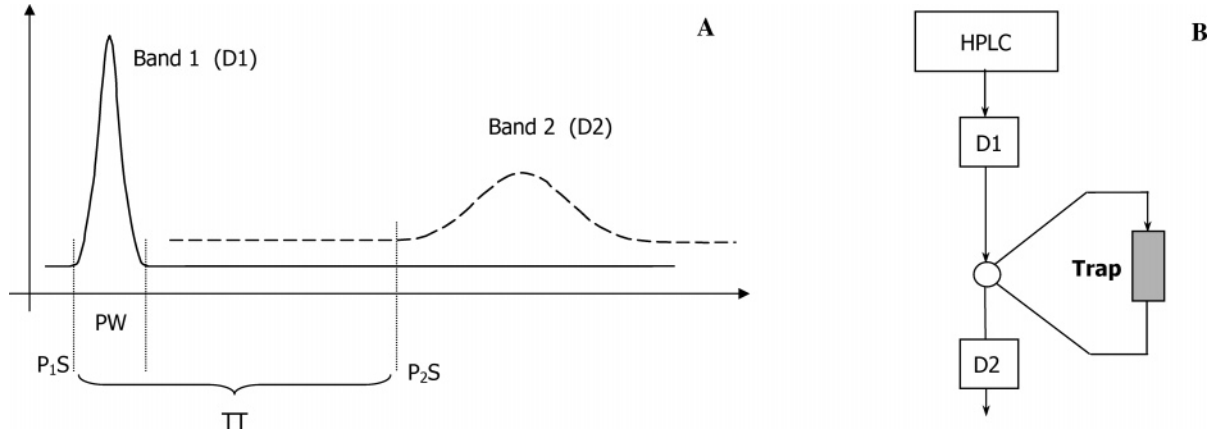

Figure 3. (A) Band delay on the trap and (B) equipment schematic, the trap between detectors D1 and D2.

Moreover, it is preferable that automated analyses should be based on generic protocols where possible, allowing for processing a wide range of diverse samples without specific method adjustment. To achieve this in solid-phase trapping (SPT), one has to develop a method which would quantifiably link the retentive capability of the given trap toward an analyte property known before the analysis, or obtained "on the fly" during the analysis, but before the actual trapping occurs. Additionally, this correlation between the trapping capability of a sorbent and any analyte's parameter has to be monotonic within some reasonable range of the parameter. On the basis of this monotonic trend, one could conceive a "minimum trappability" limit, in terms of the compounds' chemical nature, characteristic for the chromatographic conditions used. If this relationship holds, then a compound with a chromatographic retention corresponding to this limit can be chosen as a "limit marker", and it is reasonable to predict that anything with more chromatographic retention will trap at least as well.

As a natural choice, we selected the retention time of the analyte in the RP-HPLC gradient run as the most likely correlation parameter (in itself, this parameter is related to a molecule's hydrophobicity[19]). In this way, a critical requirement of the ideal trapping sorbent was not only to provide good immobilization for selected analytes, but also to comply with any monotonic restriction, as well (Figure 2A). In the event that no correlation could be found, it was felt that as long as the minimum delay required for effective "through-the-trap" compound passage exceeded the typical peak width of pure elution components (i.e., the peak is not eluting from the end while still entering the front of the trap column), then an arbitrary "trappability" threshold across the whole gradient, based on minimum trapping time (TT) in minutes, could be employed (Figure 2B).

Before a given sorbent is employed in a generic process, it is necessary to assess its trapping power, and a sorbent evaluation program was developed to achieve this. This program used a new parameter, trapping time, as a measure of the immobilization potential of a given trap toward a given analyte in specified chromatographic conditions.

TT is a measure of the delay experienced by an analyte band leaving the HPLC column (or effectively the detector D1) (Figure 3A, band 1) in the situation in which the trap is left in-line indefinitely so that the analyte can pass through it. To monitor the elution of the analyte from the trap (Figure 3A, band 2), a complementary detector (D2) is placed downstream from the trapping loop (Figure 3B).

TT is defined as the time delay between the fronts of band 2 (P_2S) and band 1 (P_1S) minus the hold-up time (DT) characteristic of the trap volume and plumbing (eq 1). The somewhat unorthodox employment of peak start times rather than apex retention times is a result of the often asymmetric band shape seen in detector 2. This is further evidence of the variable quality and lifetime of poorly prepared traps.

$$TT = P_2S - P_1S - DT \quad (1)$$

From the physicochemical point of view, TT can be assumed to be the shortest net time the analyte is delayed by the trap, and therefore, at the given chromatographic conditions, successful trapping will be achievable when TT is greater than the peak width (at the base) of the loading band (band 1). A healthy error margin was allowed to facilitate good method robustness.

TT is a derivative of chromatographic retention times, and its "behavior" is ruled by the familiar set of parameters. In a simplified model, it was decided to assume that four main variables will govern trapping of each analyte in a given trapping system. The trapping system is considered to be a trap of a specific geometry, containing a specific stationary phase and a specific two-component mobile phase pumped in the linear gradient mode. The variables are as follows:

- flow rate through the trap, FRT (mL/min),
- diluted elution point, DEP; composition of the mobile phase on the trap expressed in %B at the band front at the moment the band enters the trap,
- diluted gradient slope, DGS; steepness of the gradient after dilution (%B/min), and
- load, L, of the analyte.

FRT, DEP, DGS are direct derivatives of the HPLC parameters: flow rate through the column, FRC; original gradient slope, GS; and elution point, EP. For a mixing index of M, where $M = 0$ means undiluted eluent, $M = 1$ corresponds to 1:1 ratio with water, and so on, the following dependencies hold:

$$FRT = FRC \times (M + 1) \quad (2)$$

$$DEP = EP/(M + 1) \quad (3)$$

$$DGS = GS/(M + 1) \quad (4)$$

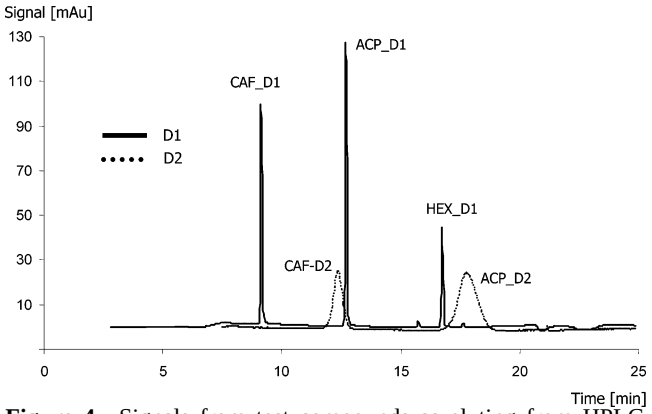

Figure 4. Signals from test compounds as eluting from HPLC column (D1) and after passing the trapping cartridge (D2). No hexanophenone signal indicates "permanent" trapping.

It is noteworthy that the elution point EP of any analyte is always strictly coupled with the flow rate and the gradient slope, the HPLC column being a coupling agent. In other words, any alterations to FR or GS will affect EP, and likewise, so will any change in the column characteristics (sorbent or geometry), even if the flow and gradient parameters stay the same. Any change in EP, in turn, could alter the overall trapping potential as the analyte is transported through the trap by a mobile phase of slightly different percentage of modifier. Due to these dependencies, care was taken to keep these parameters constant at all times while testing various sorbents for TT or when testing the same sorbent across different platforms and scales.

Stationary (Trapping) Phase Performance Comparisons. A key goal was to find a sorbent offering good retention characteristics and establish whether its retention capability, expressed as the TT, could be reasonably well correlated with the retention time of random analytes eluting across the gradient. For this purpose, a permanent HPLC system was set up for sorbent screening so that test results could be directly compared with one another, enabling exploration of parameter interdependencies. The chromatographic conditions employed are detailed in the Experimental Section. The trap size used in all tests was 2 × 10 mm (i.d. × *L*).

Three-Compound Test. A very simple test for quick phase screening was adopted, comprising three compounds normally eluting under early, middle, and late gradient conditions (Figure 4). The concentration of each component was maintained at ~0.5 mg/mL in water, and an injection volume of 10 μL was used to deliver a load of ~5 μg of each component. Tentatively, a TT of 1.5 min was arbitrarily selected as the threshold of reasonable retention above which trapping capabilities may be considered as being useful. At this stage of the experiment, the TT of 1.5 min was adopted to make allowance for the more typical peak widths (1−1.5 min) that would be obtained when using the separation column for real high-throughput preparative sample loads, rather than the relatively small peak widths associated with the analytical test rig.

Retention times measured at the peak start and corresponding EP (% MeCN) are given in Table 1. The time delays between the compounds' elution was sufficient to monitor trapping times up to 4 min, but the low compound loads employed minimized risk of interference, even when the compounds' overlap occurred on the trapping cartridge.

Table 1. Typical Peak Front Retention Times of Three Test Analytes in 14-min Gradient and Corresponding Elution Points

analyte	RT (min)	EP (% MeCN)[a]
caffeine	9.5	15.5
acetophenone	13.2	44.5
hexanophenone	17.5	75.5

[a] EP established by gradient sampling and MeCN content measured by CLND.

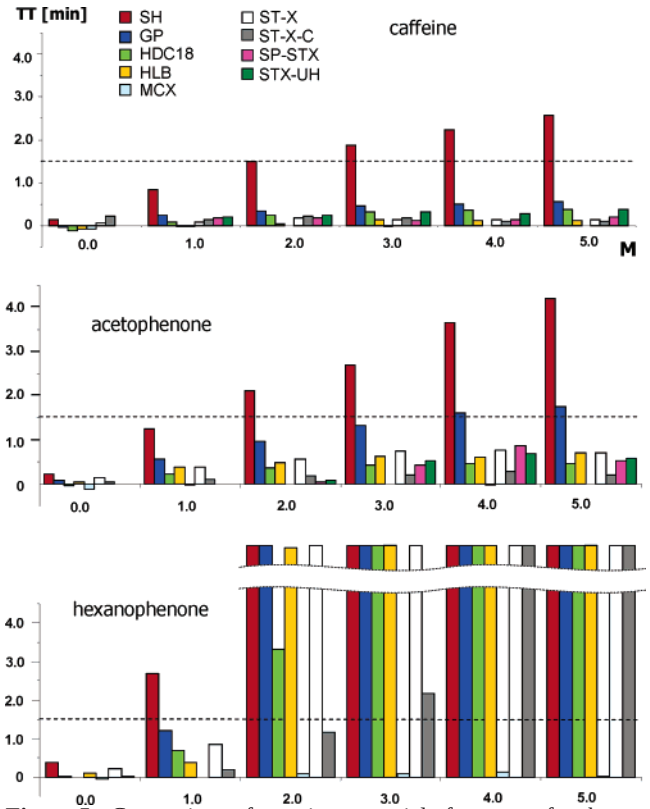

Figure 5. Comparison of trapping potential of a range of sorbents. Spark Holland sorbents: SH, strong hydrophobic; GP, general purpose; HDC18, high-density C18. Waters sorbents: HLB, Oasis hydrophilic−lipophilic balance; MCX, Oasis mixed-mode cation exchange. Phenomenex sorbents: ST-X, Strata-X; ST-X-C, Strata-X cation exchanger; SP-STX and STX-UH, noncommercial Strata-X derivatives. Analyte load, 5 μg; trap size, 2 × 10 mm (i.d. × *L*). HPLC as described in text.

Comparison tests with a first batch of available materials revealed the Spark Holland "strong hydrophobic" (SH) sorbent as being the most generic, offering good retention not only for hydrophobic analytes, but for polar compounds as well (Figure 5). This phase was promoted to the more complex retention tests, and from this point on, SH resin served as a reference for further evaluation of new sorbents for trapping. The other phases, especially Oasis HLB and Strata-X, performed poorly in this application. They were originally designed for extractions from mostly aqueous solvents and apparently do not cope well with the presence of significant amounts of acetonitrile in solution. Some cation-exchange sorbents (Oasis MCX and Strata X-C) were also included in the tests. The purpose of testing these resins was to probe their hydrophobic retention, should the need for mixed-mode action arise to be able to trap (polar) cations, such as protonated hydrophilic bases. Unfortunately, they showed

nearly no hydrophobic retention at all (MCX), or were able to retain only the most hydrophobic analytes (Strata X-C).

These initial tests proved that sorbents showing good retention toward all three compounds do exist (SH) and that it was likely that a stationary phase capable of trapping analytes across almost the whole gradient elution range could be found, even including compounds of significant polarity. To simplify the preliminary screenings of further sorbents, it was felt that the test could be tightened to test caffeine retention only, because clearly, if we could successfully trap caffeine, then the more hydrophobic compounds were also likely to work. Only stationary phases that retained caffeine well in gradient conditions would then undergo advanced inspection.

Trend Test. Candidate sorbents identified by the preliminary tests were then subjected to a multicomponent test involving chemicals of diverse properties (hydrophobicity, polarity, etc). The trend test for SH material performed on over 65 randomly chosen drug and druglike molecules (shown in appendix 1) covering the full gradient elution range gave very satisfactory results (Figure 6). Although not particularly tight at higher mixing multipliers, the trend is clearly visible, which allows for setting a trappability limit on the gradient scale, depending on the TT threshold adopted. For example, in these particular chromatographic conditions (HPLC S = 8.2%/min, equivalent to 14 min gradient, on 2 × 100 mm Luna C18 (2) column with FR = 0.2 mL/min, mobile phase water/MeCN/0.1% HCOOH, trap size 2 × 10 mm i.d. × *L*, analyte load of 10 μg) with an arbitrary TT threshold of 1.5 min and at the mixing ratio 1:5 ($M = 5$), the trapping limit would fall around 12−15% MeCN. This equates to all compounds eluting past this limit being trappable under these conditions. In a comparison of this elution point compared to a dataset of retention times (using the same method) of over 100k diverse structures that had been previously analyzed, it is predicted that >80% of these compounds would be effectively trapped using these conditions.

Dilution Ratio and Trap Size. The water mixing ratio is a critical issue in applying this trapping technique. Adding water promotes trapping by weakening the mobile phase elutropic strength prior to passage through the trap; but at the same time, retention kinetics may be compromised by increased flow rate through the cartridge. Isocratic measurements let us isolate the impact of one of the parameters (%B or FR) while keeping the other constant. The example of caffeine on the 2 × 10 SH trap shows the dramatic impact of the flow rate on retention (trapping time) within the trap, even at an eluent composition that is effective for trapping at lower flow rates (Figure 7). Combining these dependencies and allowing for the added influence of increasing %B in a gradient run (as the passage through the trap continues, the organic composition rises) results in a quasiasymptotic relation (Figure 8). Additionally, practical limits of flow rate have to be taken into account. As the dilution ratio increases, the backpressure across the trap becomes larger.

Having tested the impact of water mixing on trapping times for a range of compounds (also visible in Figure 6), we selected an arbitrary ratio of 1:5. This offers the most generic trapping conditions versus compound polarity, but it is also practically manageable in terms of flow rate demand and

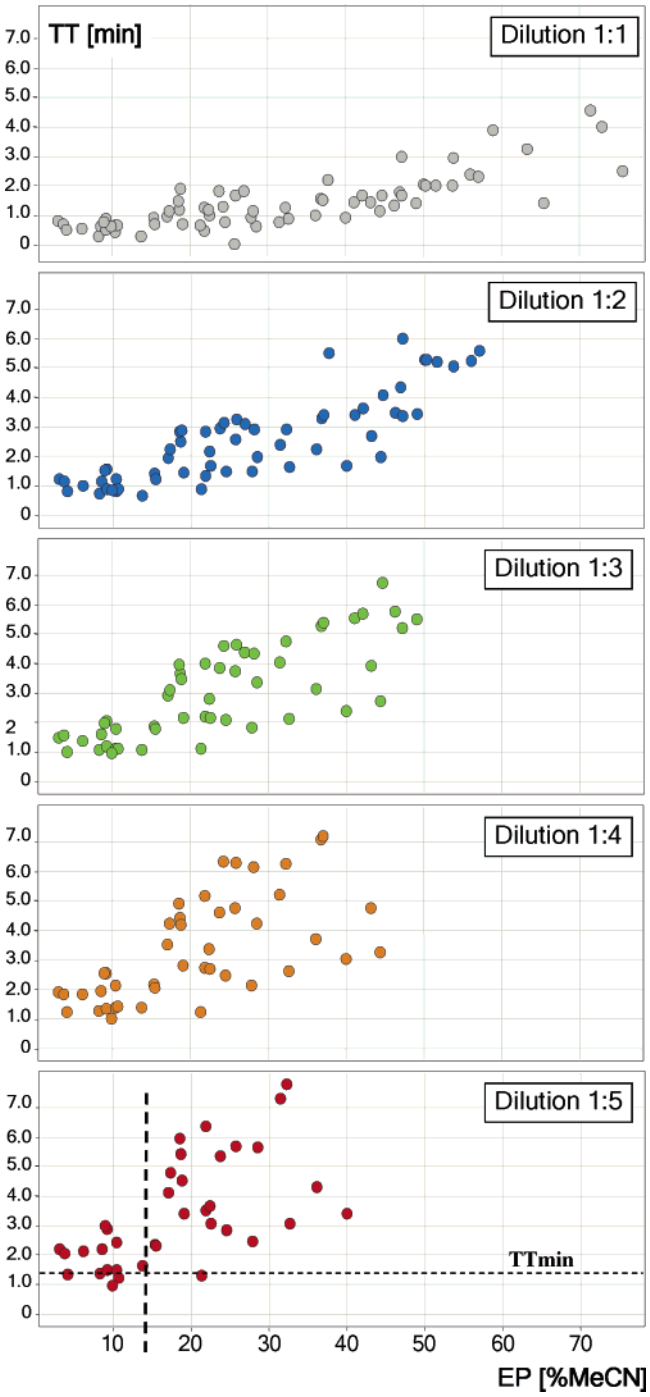

Figure 6. Good correlation between the TT and the EP at a range of dilution ratios in the test set of 65 random compounds of diverse properties "trapped" on the SH sorbent. HPLC conditions as previously. Trap size, 2 × 10 mm.

backpressure consequences. Increasing the dilution ratio beyond this point, for example, from $M = 5$ to $M = 12$, enhances the trapping time, but at the same time, the cumulative flow rate through the 2 × 10 mm cartridge reaches 2.6 mL/min, and there is a more than 70-bar pressure drop. These conditions would not be tolerable on larger scales of operation. Additionally, currently available SPE cartridges used as traps in this experiment proved to be rather poor in terms of packing quality, and the increased flow rates used could quite quickly lead to the analyte's leaking, which manifested itself by significant peak fronting, most likely due to instability and voiding of the packed bed.

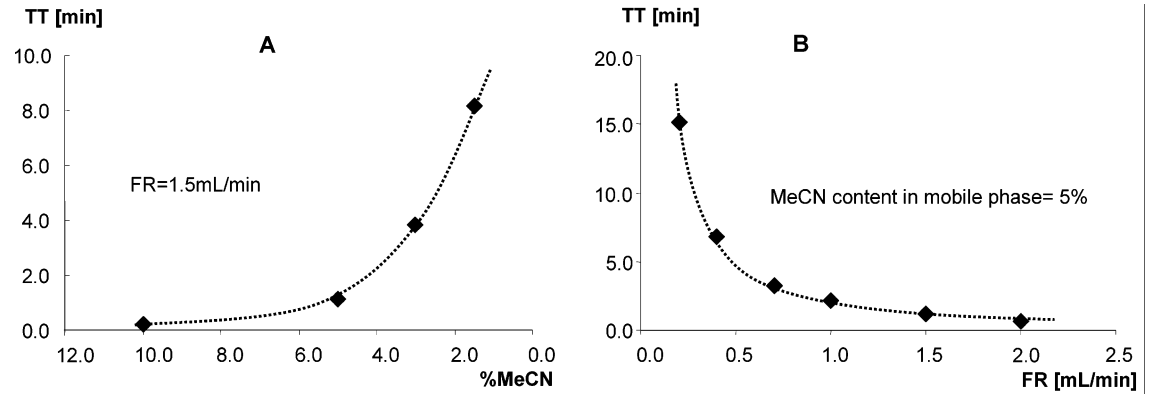

Figure 7. Trapping of caffeine (10 μg) in isocratic conditions, isolated impact of flow rate and percentage of organic modifier in the mobile phase on the retention. (A) Constant flow rate of 1.5 mL/min and variable percentage of acetonitrile, and (B) constant acetonitrile content of 5% and variable flow rate. Trap size, 2 × 10 mm; SH sorbent.

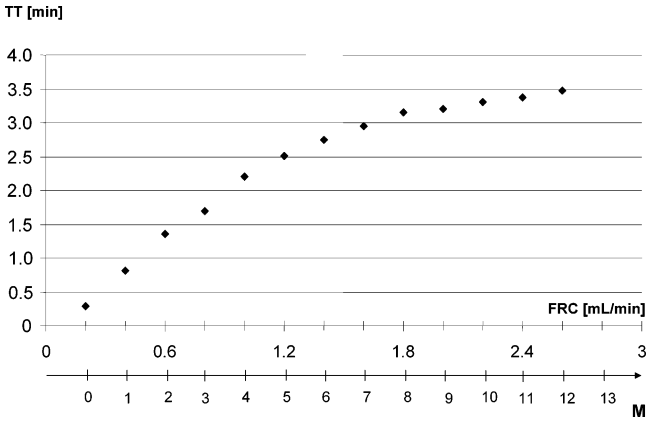

Figure 8. Trapping time of caffeine (10 μg) in gradient conditions as a function of flow rate through the trap; FRC corresponding to dilution ratio, *M*, for the good quality trap cartridge, well packed. HPLC conditions as described before. Trap size, 2 × 10 mm; SH sorbent.

Such reasoning leads to a general "rule of thumb" for the trap internal diameter. It seems that for fluidics reasons and to ensure reasonable solute−trap contact time, the most desirable trap internal diameter has to be roughly equal to that of the HPLC column used in the isolation setup. However, the precise trap geometry optimization has yet to be performed.

Trap Capacity, (SH Resin, 2 × 10-mm Trap Dimensions). The upper capacity limit of the trap was estimated by breakthrough measurements using purely aqueous solutions of relatively high solute concentration (2.08 mg/mL). A low flow rate of 0.1 mL/min was used to maximize the adsorption kinetics and to favor them over the longitudinal mass transfer. The breakthrough volume for caffeine as a solute, on the 2 × 10 mm trap (SH 5−25 μm, 18 mg of sorbent), measured between the curve breakdown point and first recognizable signal rise (breakthrough) was 19.98 min. This corresponds to 4.15 mg of caffeine deposited on the stationary phase. Full curve integration gave the result of ∼5 mg for equilibrium sorbent saturation using these conditions.

A mass of 4.15 mg (23% of the sorbent mass) is regarded as a sufficiently high capacity (and not unusual for polymeric resins) to be able to set the upper limit for further trapping tests. Note that this limit is unlikely to be obtained in real cases due to the presence of a certain amount of nonaqueous solvent and the different nature of the loading pattern (dynamic peak trapping rather then homogeneous saturation).

Loading tests at analyte loads ranging up to 2 mg performed "on-the-fly" in gradient conditions showed, as anticipated, some reduction of trapping times when compared with the TTs measured at minimum loads of 5−10 μg. Such a condition-specific "capacity", expressed as the maximum load of a certain analyte that can safely (beyond the arbitrary threshold) be immobilized on the cartridge in certain chromatographic conditions, was tentatively called the "dynamic capacity", and was estimated to be in the range of 0.5−1 mg per 2 × 10 mm SH cartridge for most of the compounds falling beyond the trappability limit. At this point, it was apparent that the major obstacle in successfully getting the analyte onto the cartridge was not the trap's dynamic capacity but more commonly, chromatographic column overload; in other words, the trap could accept more load than we could chromatograph on the test setup in a reproducible manner. Scaling up from a 2- to a 4.6-mm column was not a viable option, because it would produce too high a flow rate for the 2-mm-i.d. trap to cope with (∼6 mL/min at 1:5 mixing ratio and 1 mL/min HPLC flow rate). This outcome supports the earlier statement of the rule of thumb for trap dimension choice, but also has another interesting implication. Generally, a trap of diameter similar to that of the isolation column has ample capacity to trap the isolated compound, allowing isolation and trap diameters to be conserved in scale-up. Preliminary scale-up work performed on slightly longer (20-mm) traps up to 20-mm diameter has confirmed that compound amounts (even for compounds eluting near the trapping limit) of 150 mg are achievable, making this process largely scale-independent for most high-throughput purification needs.

Enhanced Trapping Option: High pH Trapping of Bases. By functionality, basic compounds make up probably the largest group of so-called active pharmaceutical ingredients, API. When chromatographed in the presence of typical pH-lowering and ion-pairing additives (buffers, HCOOH, TFA, etc.), this class of compounds will normally elute from an HPLC column in the ionized form, which is significantly more polar than its free base equivalent. Due to this tendency to be polar, hydrophilic, or both, elution is often in the early segment of a run when subjected to an organic gradient. In some cases, these compounds can be so hydrophilic as to be rendered ineffective for analysis by

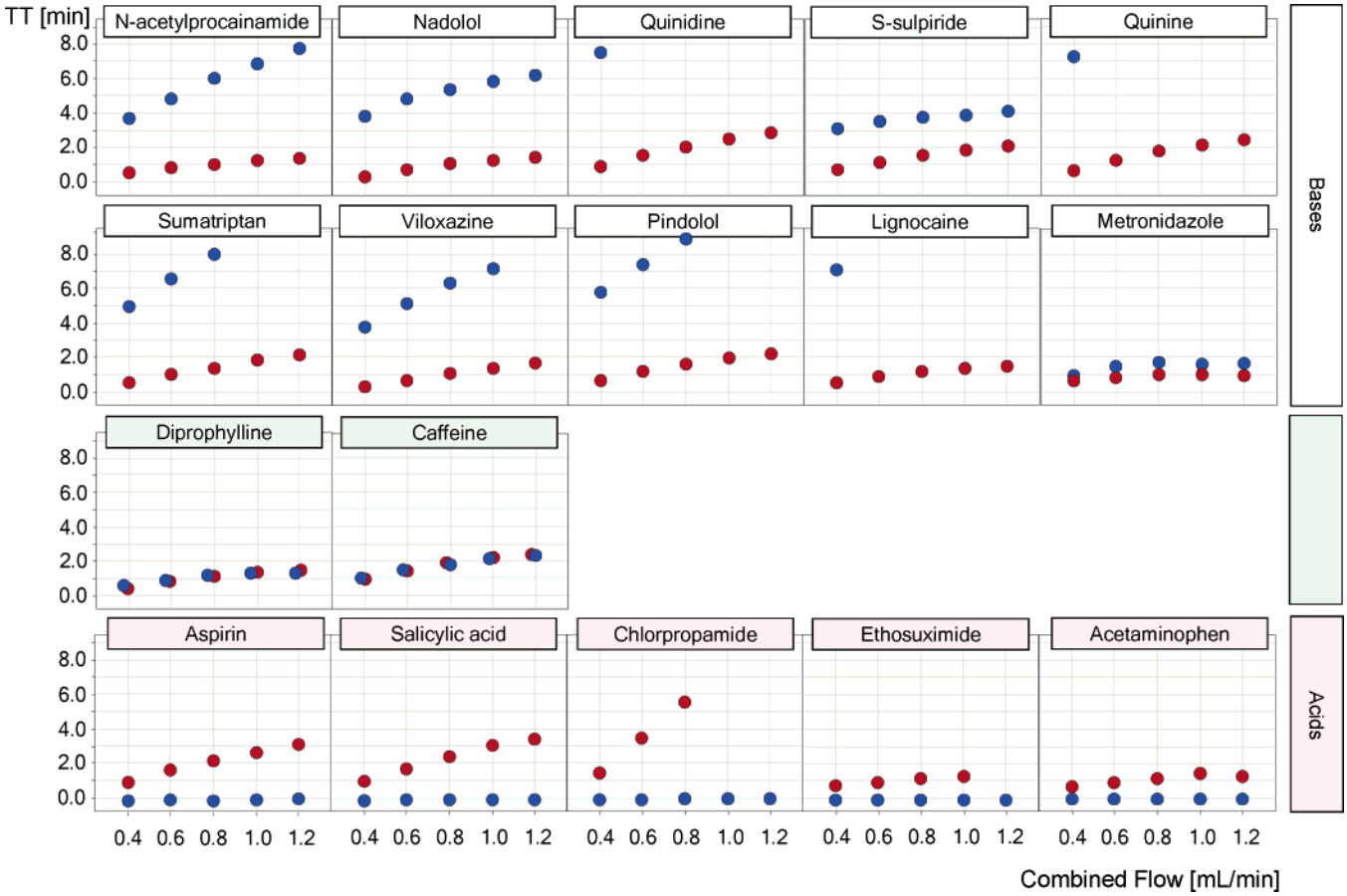

Figure 9. Trapping with the use of high pH diluent (5.5 M ammonia (blue dots), as compared with low pH trapping (red dots)) dilution water containing 0.1% HCOOH, pH 2.6 for compounds specified. Trap material, SH; 2 × 10 mm; mixing ratio, 1:5. HPLC conditions as before.

reversed-phase methodologies, due to insufficient retention. When attempting to trap compounds in this form, they pose a much bigger problem than other compounds from later parts of the elution gradient because the dilution effect is less effective. They do not have much more retention in an even higher aqueous mobile phase composition. The natural solution would appear to be to extend the trap length so that these weakly interacting compounds can get a better chance to be sufficiently delayed. Alternatively, it would be quite practical to simply switch the chemical form of these compounds from ionic to neutral before they reach the trap. The neutral form is always expected to be more hydrophobic. This could be achieved by using a diluent of suitable composition and pH so that, after combination with the original HPLC flow, the resulting solvent would maintain a pH higher than the pK_a of the (basic) analyte being trapped. Unfortunately, high pH mixing would also suppress trapping capacity toward acidic compounds. To avoid this effect, a prior knowledge of compound properties would allow a choice of diluent pH/composition. In this way, acidic and neutral compounds would be immobilized in neutral form (i.e., in the solvent of pH determined by the HPLC eluent) whereas bases would receive high pH mixing.

A preliminary test was performed to illustrate the influence of high pH mixing on trapping times of a range of test chemicals (Figure 9). The analytes were chromatographed and delayed on the trap as described in previous sections (0.1% HCOOH, which gives pH 2.6 as the aqueous component of the eluent) but now using a diluent of 5.5 M NH_3 (aq) (pH ~12). As expected, trapping capability with respect to the free base forms was significantly greater, whereas dissociated acids passed through the trap unretained. No retention change was observed for neutral or very weak bases. However, on the basis of the tests of the earlier 65 compounds, the "trapping limit" is seen to be extended for a significant number of compounds that would otherwise have been difficult to trap. This approach could substantially increase the number of compounds generically handled by this procedure.

Elution and Recovery. Having proven the feasibility of online trapping with the use of highly retentive polymeric material, the analyte recovery was briefly studied. The key demands put on the elution process are (1) complete recovery of the analyte; (2) recovery of a pure analyte; (3) analyte recovered in easily removable, purely organic solvent (no aqueous fraction); and (4) generic protocol allowing for recovery of all types of analytes from the specific trap type.

To profile the best elution conditions, two test compounds were loaded on a series of 2 × 10 mm SH cartridges by trapping HPLC peaks. The cartridges were dried with a gentle stream of nitrogen to remove water and subsequently were eluted with a range of solvents at different flow rates. Elution volumes were calculated on the basis of the widths of the elution peaks recorded on the UV−vis detector placed immediately downstream from the cartridge. Elution completion was, quite arbitrarily, visually assessed on the basis of the peak shape, assuming that all, or at least most, of the material is being released in the elution process. Deformed and noisy peaks were obtained as a rule, due to the presence

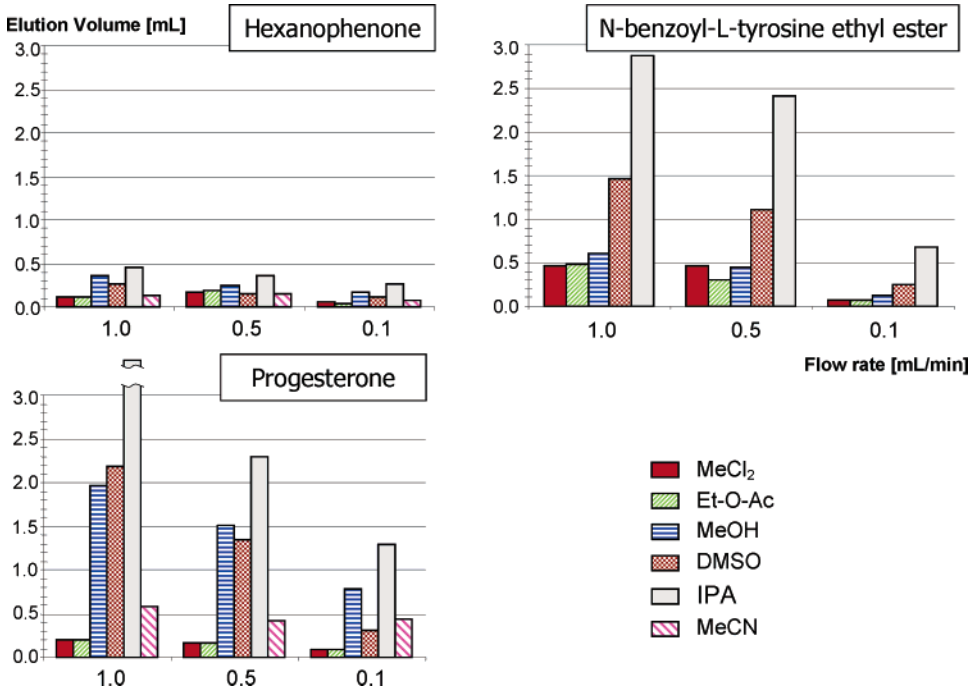

Figure 10. Elution volume as a function of solvent type and flow rate. Trap size, 2 × 10 mm; SH sorbent; analytes (0.5 mg) loaded by HPLC peak trapping at dilution ratio 1:5; and HPLC conditions as described before.

of air/N_2 in the trap, and therefore, any precise calculations based on the peak area were not attempted.

Typically, good solvents of high elutropic strength toward PS-DVB materials are (in order of strength[20]) $MeCl_2$ > EtOAc > MeCN > MeOH. Dimethyl sulfoxide, DMSO, and isopropyl alcohol, IPA, although good solvents, are usually quite weak mobile phases.[21] Data in Figure 10 seem to confirm this for the SH material. Additionally, it was observed that keeping the elution flow rate at a very low value (0.1 mL/min) in many instances allowed for significant reduction of the total elution volume.

The elution process may be considered as a combination of the two main orthogonal mass transfer processes: liberation of the analyte from the stationary phase and longitudinal mass transfer propelled by the flow of solvent. For liberation, one must consider the combination of processes that take place, depending on the form in which the analyte is deposited on the stationary phase: desorption with its concentration driven kinetics, diffusion into the bulk solvent, dissolving of aggregates/crystals of the solid analyte, etc. Apparently, for the overall case, a flow rate of 100 μL/min is more than enough to sustain efficient liberation. Increasing the flow does not speed up the process, but only increases the volume of solvent into which the analyte is displaced and, therefore, delivers a lower concentration fraction.

Preliminary work to check the feasibility of elution protocols was largely focused upon rather hydrophobic analytes that might be expected to be difficult to elute. The data suggests that, in general, it should be possible to recover 0.5−1.0-mg loads within 100−200 μL of solvent of suitable strength. For information purposes, although the "concentration" of trapped components was not the main objective, to some extent, this was achieved, particularly when we dealt with high (preparative) column loads of 1−2 mg of compound on the separation column (2-mm diameter). In this typical case, peak widths (volumes) of 1−1.5 min (200−300 μL), which upon dilution gave loading volumes of 1−1.5 mL, were comfortably eluted in as little as 100−200 μL of pure organic solvent. On this basis, it is not unreasonable to project that for larger-diameter traps of the same length (10 mm), we can safely isolate and efficiently recover up to 6 mg in <0.5 mL on a 5-mm trap and 80 mg in <8 mL on a 20-mm-diameter trap. It is expected that temperature-aided elution will prove very useful in further reducing elution volumes. These hypotheses, together with elution protocol optimization for a representative set of APIs of diverse physicochemical properties, will be tested as the subject of future work; however, at the time of submission, we have successfully trapped >100 mg of some compounds easily on 20 × 20 mm trap dimensions.

Following the findings to date with regard to best trapping and elution methods, a set of compounds was tested for "real life" trapping and subsequent elution with recovery confirmation. Analytes were loaded onto the SH polymer-filled traps by passing them (with significant overload) through the HPLC column (2 × 10 C18 at 0.2 mL/min, 14-min gradient) and trapping the target chromatographic bands onto the cartridges using a 1:5 water mixing ratio (as illustrated in Figure 11C). Equal amounts of each test compound were immobilized on three independent cartridges. Trapping was followed by a quick wash with pure water to remove remaining eluent background (this is covered in more detail in the following section), and the traps were subsequently dried by blowing nitrogen through at ~20 mL/min for 30 min using a well-tested protocol developed for identical traps for LC/NMR applications. In these NMR experiments, residual water is reduced to the levels associated with typical organic solvents. For reference purposes, liquid fractions were collected from HPLC runs using the same valve timing as that of on-cartridge trapping.

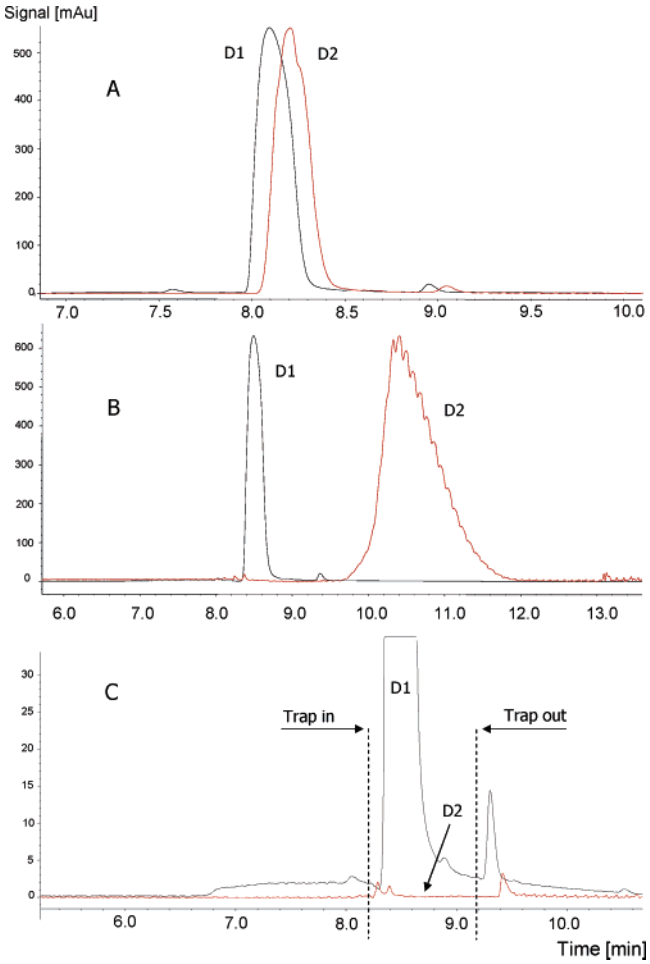

Figure 11. Illustration of trapping process for 2 mg of caffeine passed through 100 × 2 mm HPLC column. (A) 2 × 10 mm SH trap between detectors D1 and D2 left in-line, no mixing applied; (B) same trap with 1:5 mixing; (C) as above, but the trap switched in-line only for the duration of the loading peak. Absence of detector 2 peak indicates successful trapping. Units on signal axis correspond to D1, and D2 is normalized. In chromatograms A and B, the D2 signal was recorded at a wavelength different from that of D1.

Each trap was eluted with pure acetonitrile to ensure good sample compatibility with the reference liquid fractions. The faster flow used, 1 mL/min, imposed a slightly higher elution volume of typically 0.5−0.7 mL. Complete elution was estimated visually by the peak shape, and suitable margins allowed ensuring complete sample elution from the tubing postdetector. Sample recoveries were checked chromatographically by reinjecting both the trap-recovered and liquid fractions in identical isocratic conditions adjusted for each analyte.

The data suggest full recovery of all tested analytes, regardless of their hydrophobicity expressed as EP (Table 3). These results confirmed our expectations that the trapping materials also release the trapped solutes when eluted with certain organic solvents. The term "full recovery" indicates a satisfactory percentage recovery level from the sample load for our current purposes. Although UV−vis DAD has to date revealed no evidence of cross-contamination with cartridge reuse, it is recognized that some materials may fail to fully desorb during a generic elution protocol and so may require specific procedures to achieve full elution and avoid contamination of subsequent samples. This may entail use of larger volumes of elution solvent, thermally assisted elution, or use of specific wash procedures.

Removal of TFA Additive from Traps. The process of trapping acts as a means of reducing the background contamination of any liquid fraction simply by providing a limited volume (the interstitial trap volume, ∼50−60% of the total internal trap space available) for any background solvent to occupy. Therefore, any passage of a volume of aqueous TFA through a trap when this volume is larger than the trap volume can only leave behind one "trap full" of aqueous TFA, provided that there is no significant adsorption of TFA on the resin. In any event, however much TFA remains in the trap, we have the opportunity to wash out the background additives and effectively replace this volume with pure water. Water can then be removed by passage of a sufficient volume of warm nitrogen gas through the trap. To demonstrate the effectiveness of TFA removal, the following experiment was performed. Fractions of 1.3-min duration (∼1.3 mL mobile phase containing 0.1% w/v TFA + 5-fold water dilution, total 7.8 mL) were switched from the middle of the gradient in the standard HPLC gradient analysis (separation column, 4.6 × 15 mm Luna C18, flow rate 1 mL/min; A, water; B, MeCN; both 0.1% w/v TFA) and passed through the trap (4.6 × 30 mm dimensions). This trap was then subjected to various processes of either no wash with variable N_2 drying times or various washes of different volumes and compositions to determine the most effective way to remove the residual TFA. These were all compared to an untreated equivalent fraction volume of solution that acted as a reference sample for the amount of TFA initially present. The results are presented in Table 4, and the trends are shown graphically in Figure 12. It can be seen that although passage of the drying gas apparently gives a good reduction in TFA content, this is likely to be mainly due to expulsion of the aqueous TFA solution remaining in the trap. Better overall performance is achieved by washing with pure water for a reasonable number of trap volumes. At this stage of the work, this experiment was performed on fractions containing no actual solute components. Future work will concentrate more on investigating the effect of bound TFA or manipulation of the TFA salt form while it is on the trap resin. TFA residues were measured using ^{19}F NMR on solutions of TFA eluted in a standard 3-mL volume of acetonitrile and internally standardized (bendroflumethiazide).

Summary of the Proof of Concept. It has been shown that controlled trapping of analytes from HPLC fractions onto compact cartridges is possible. The process can be largely generic, provided a highly retentive sorbent and an adequate water mixing ratio are used. Recovery of the trapped material by elution with a suitable solvent shows extremely good recoveries for a small test compound set of druglike molecules that elute across the generic gradient range. Using existing hardware on the 2-mm chromatographic scale demonstrated in this paper, the technique could be immediately applicable for trapping of typically 1 mg of any compound eluting past the 15% MeCN point in typical HPLC "generic gradient" conditions. It is predicted and early measurements confirm that simple scaling of the column, trap diameters, and flow rates will allow this technique to

Table 2. Parameters of Selected Sorbents Used in Tests

name	code	manufacturer	type	surface area (m^2/g)	particle i.d. (μm)	pore diameter (Å)
Oasis HLB	HLB	Waters	*N*-vinylpyrolidone/divinylbenzene copolymer	810	15	80
Strata-X	Strata-X	Phenomenex	polar functionalized polystyrene/divinylbenzene	800	25	85
strong hydrophobic	SH	Spark Holland	modified polystyrene divinylbenzene (probably FC-hyper-cross-linked)	1222	5−25	
strong hydrophobic (version 2)	SH v2	Spark Holland	modified polystyrene divinylbenzene (probably FC-hyper-cross-linked)	1468	30	
general purpose	GP	Spark Holland	modified polydivinylbenzene		5−15	100
HyperCarb	HC	Thermo	porous graphitic carbon	120	5, 40	250
Amberchrome CG-161	CG-161	RohmHaas	macroporous polystyrene/divinylbenzene	900	35	150
Isolute ENV+	ENV+	Argonaut	hyper-cross-linked hydroxylated polystyrene/divinylbenzene	1000	90	
unspecified resin	PL50	Polymer Labs	macroporous polystyrene/divinylbenzene		50	

Table 3. Recovery from 2 × 10 mm SH Cartridges by MeCN Elution at 1 mL/min

analyte	EP[a] (% MeCN)	load (mg)	av recovery[b] (%)
caffeine	15	2.0	103
dibucaine	27	1.0	95
acetophenone	44	2.0	98
N-benzoyl-l-tyrosine ethyl ester	47	2.0	101
N-benzyl-4-chloro-5-sulfamoylanthranilic acid	50	0.5	96
N-[(R)-1-(10-naphthyl)ethyl]phthalamic acid	50	0.5	101
2-(4-chloro-3-nitrobenzoyl)benzoic acid	57	0.5	97
hexanophenone	75	1.0	102

[a] EP for 14-min gradient of water/MeCN/0.1% HCOOH, Luna 100 × 2 mm, 0.2 mL/min. [b] Average for three independent cartridge traps.

Table 4. Comparison of the Relative Effectiveness of Different TFA Removal Procedures

mobile phase sample			vol collected (mL)	wash (mL)	TFA in collected vol (μg/mL)	total TFA load (μg)	TFA reduction (% of original removed) (%)	Potential weighing error of TFA in typical 5-mg sample (%)
liquid fraction (untreated)		as is	1.275		1113.25	1419.39		28.39
trapped		no drying	2.985	0.0	27.82	83.05	94.15	1.66
	no wash	30-min drying	3.000	0.0	7.95	23.85	98.32	0.48
		5-h drying	3.000	0.0	3.97	11.92	99.16	0.24
			3.010	1.0	18.22	54.83	96.14	1.10
	water wash		3.025	2.0	12.92	39.08	97.25	0.78
	(no drying)		3.000	3.5	5.30	15.90	98.88	0.32
			3.040	5.0	0.66	2.01	99.86	0.04
			3.050	1.0	14.90	45.46	96.80	0.91
	5% MeCN wash		3.010	2.0	7.95	23.93	98.31	0.48
	(no drying)		3.040	3.0	3.97	12.08	99.15	0.24

be used easily at higher scales, with 80−100 mg of compound being easily possible on 20-mm-diameter traps.

Our data suggest that it should be possible to trap all analytes eluting at or above 15% B in generic reversed-phase water/MeCN gradients. This value of 15%, tentatively proposed as the trapping limit, should allow the majority of APIs of interest to the pharmaceutical industry to be trapped. Figure 12 depicts the obtainable trapping times on a 2 × 10 SH cartridge at a 1:5 mixing ratio projected against a typical distribution of many tens of thousands of structurally diverse compounds (internal source) according to their retention time in an in-house generic gradient. The elution scale [%EP] for TT measurements in our 14-min test gradient and the RT scale for compound distribution have been superimposed by running a number of compounds from the original distribution using the trapping methods. Both methods use the same HPLC column type (i.e., Luna C18), and this allows us to cross-calibrate the trapping scale and predict trapping conditions on the basis of the compound's RP retention time.

However, although already quite satisfactory, it is expected that the trapping capacity of the sorbent currently used can still be significantly improved by the trap geometry optimization. The trap length especially plays a significant role, allowing for longer contact times. On the other hand, the chromatographic conditions used here, although broadly generic, are just a very specific set of the infinite set of combinations of flow rates, column sizes, and gradient profiles. The

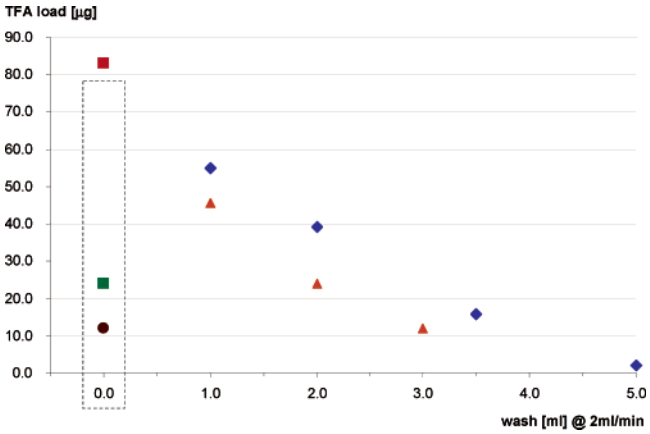

Figure 12. Trap of 4.6 × 30 mm dimensions containing residual TFA from passage of 1.3 mL of the mobile phase fraction. Trap: (red square) unprocessed, (blue diamond) washed with water, (gold triangle) washed with 5% MeCN in water, (green square) dried with N_2 for 30 min, and (purple circle) dried with N_2 for 5 h. All data points collected for the same trap.

truly generic trap and trapping protocol must work reliably and predictably under all possible circumstances.

Preliminary observations with regard to generic trap usability lead us to believe that the trap geometry can be optimized in such a way that will allow for safe immobilization of most of the compounds that are typically chromatographed in the RP mode regardless of their properties and chromatographic conditions used for separation.

Technique Benefits, Further Applications, and Developments. The proposed technique of on-cartridge trapping and fraction processing can be automated to a greater extent than in the case of liquid fraction handling, especially if the cartridges are to serve as a storage/transfer platform. Elimination of water from liquid fractions will make the process easier and less time-consuming. Moreover, apart from the cost reduction and increase in the labor efficiency of the separation/purification process, employment of the solid-phase trapping technique at the same time opens a wide variety of novel opportunities of on-line and off-line sample processing:

(1) On-Demand Elution with the Solvent of Choice. Elution can be performed online or offline at any convenient moment on a dedicated elution setup or any elution-capable LC equipment using the solvent appropriate for further sample processing. The cartridge may serve as a very efficient medium for cross-platform sample transfer.

(2) Solvent Exchange Option, for Example, to NMR-Compatible Solvents. An analyte immobilized in aqueous conditions may be dried on-cartridge and eluted directly with an NMR-compatible solvent or any other specialist solvent, avoiding the evaporation/reconstitution and sample-handling stage.

(3) Safe Storage in an Inert Environment of a Compact Cartridge. Cartridges may be used either as a tool, merely facilitating the fraction collection process by eliminating water from the fraction before the evaporation stage, as a cross-platform sample-transfer medium, or even as the ultimate long-term sample storage device. DMSO is often used as a solvent for long-term frozen storage but cannot be regarded as a fully inert medium. Unlike DMSO solutions, dry analyte deposited on the inert sorbent within a dark cartridge will be very effectively protected against decomposition or other transformations caused by UV/vis radiation or water contact. If saturated with inert gas and sealed airtight, it will also provide an anaerobic environment and, hence, better protect against sample oxidation.

(4) In-Cartridge Salt Exchange (TFA, Buffer Removal). Due to the hydrophobic interactions involved, the ionized base is held trapped in place on the stationary phase as long as the solvent surrounding it is weak, that is, aqueous. Thanks to this immobilization, ionized bases will be able to undergo an on-cartridge neutralization, salt exchange step, or both,

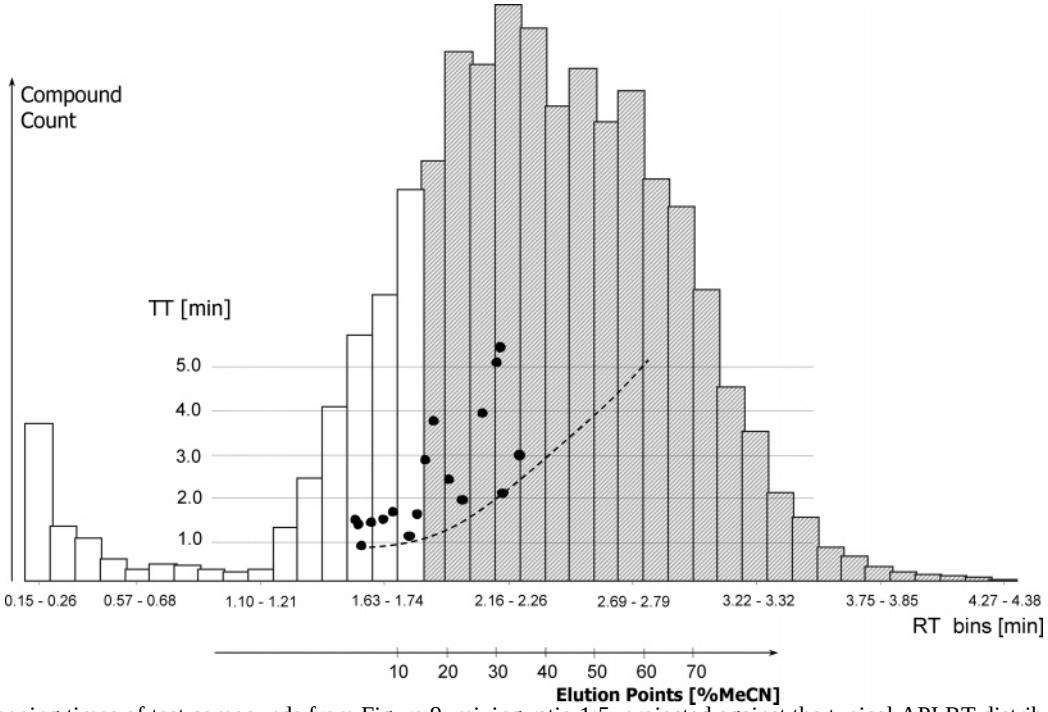

Figure 13. Trapping times of test compounds from Figure 9, mixing ratio 1:5, projected against the typical API RT distribution in generic in-house gradient. Shaded area represents portion of the compounds expected to trap well on the 2 × 10 mm SH trapping cartridge.

as a result of which a free base or a salt form other than TFA will be recovered from the trap with the use of the appropriate solvents.

(5) Optimization of Sample Recovery. Although a small amount of work is described here, sample recovery has not been fully explored. It is an area worthy of further investigation, and we can imagine that it could be performed in parallel microtiter plate format if required. There is also no practical reason recovery needs to be part of the on-line process. We intend to isolate (trap) our samples as part of the purification process and transfer them on-trap to another part of our process. This may be either recovery of a solid sample or submission to further analytical measurements, such as NMR or FT-MS, without further handling and the associated risks of sample losses. This is particularly relevant to small sample amounts, such as impurity isolation/identification.

Technical Hurdles/Problems with SPT Technique Overall, the work demonstrated here offers a more effective means of on-line capture of solutes, within an eluent or flowstream, as part of the real-time process; however, due to the generic application of *M* times dilution (often more dilution than is necessary to effectively trap more hydrophobic compounds) using a weaker elution solvent, such as water or dilute base, there is an opportunity for eluting samples to precipitate. This was rarely observed during this work, but for some compounds, it did occur, and in extreme cases, it could lead to system blockage. With our existing equipment setup, the best precaution that could be adopted was to carry out the dilution just prior to the trapping cartridge. This minimized these precipitation effects and allowed the vast majority of compounds to be effectively trapped without problems. We noted some cases in which in-cartridge precipitation seemed to occur, but this did not cause a system problem because the issue was confined to within the cartridge. In principle, this is a beneficial effect, as compared to system blockage, and in these cases, the cartridge acted as a part adsorbent/part filter-based trap. Recovery of the material from these traps did not present a problem; complete recovery was achieved in a small volume of a strong organic solvent. Nevertheless, we think that optimization of the in-line mixing with regard to composition, position, and fluid dynamics could prevent this problem from impacting the effectiveness of the process as a whole.

Conclusions

This work demonstrates the design and use of an on-line method to trap the products of chromatographic purification during the process using a generic approach. This approach is significantly different from previously reported low-pressure SPE-type approaches and offers considerable advantages. On-line SPT represents a significant move forward toward improved methods for obtaining pure, quantified compounds from high-throughput liquid chromatographic purification. Careful choice of trapping phase and adoption of generic mobile phase dilution conditions allow any compounds eluting in RP-LC beyond the 15% organic composition limit to be effectively trapped. Removal from the trap may be easily effected by passage of small volumes of pure, volatile organic solvent to leave residue-free, pure compound for further quantitation or recovery. These benefits combine to produce a much less labor-intensive process, which produces compounds with fewer opportunities for errors in quantification, particularly if gravimetric methods are used. Further opportunities exist to decouple compound recovery from the purification process, because storage on-trap is unlikely to promote compound decomposition. It is perceived that purposeful design of a trapping module in place of a traditional fraction collector device would enable seamless integration with existing instrumentation, therefore allowing an upgrade path to be able to harness the benefits of the new approach.

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Appendix

List of 65 drug and druglike molecules used in trapping tests, together with their retention times, RT, in 14-min linear gradient and corresponding elution points, EP

name	RT (min)	EP[a](% MeCN)	name	RT (min)	EP[a](% MeCN)
ranitidine	8.1	3.1	dibucaine	11.2	28.6
S-sulpiride	8.1	3.7	chloroprothixene	11.5	31.5
N-acetylprocainamide	8.2	4.2	colchicine	11.6	32.3
sumatriptan	8.4	6.2	aspirin	11.7	32.7
nadolol	8.7	8.3	phenobarbital	12.1	36.1
pindolol	8.7	8.5	hydrocortisone	12.2	36.9
trimethoprim	8.8	9.0	prednisone	12.2	37.0
lignocaine	8.8	9.3	flunarizine	12.3	37.8
quinidine	8.8	9.3	saliycylic acid	12.6	39.9
metronidazole	8.9	10.0	metyhylprednisolone	12.7	41.1
diprophylline	8.9	10.4	carbamazepine	12.8	42.1
quinine	9.0	10.5	benzocaine	13.0	43.1
acetaminophen	9.0	10.7	acetophenone	13.1	44.4
viloxazine	9.4	13.8	furosemide	13.1	44.7
caffeine	9.6	15.4	lorazepam	13.3	46.3
proxyphylline	9.6	15.6	*N*-benzoyl-L-tyrosine ethyl ester	13.4	46.9
zolpidem	9.8	17.1	nordazepam	13.5	47.2
papaverine	10.0	18.7	piroxicam	13.5	47.2
sulfapyridine	10.0	18.8	chlorpropamide	13.7	49.1
pyrimethamine	10.0	19.1	*N*-benzyl-4-chloro-5-sulfamoylanthranilic acid	13.8	49.9
ethosuximide	10.3	21.4	*N*-[(*R*)−1-(10-naphthyl)-ethyl]phthalamic acid	13.8	50.3
propranolol	10.3	21.8	griseofluvin	14.0	51.5
droperidol	10.4	21.9	ketoprofen	14.3	53.7
tinidazole	10.4	22.4	testosterone	14.3	53.8
antipyrine	10.4	22.6	diazepam	14.5	55.9
sulfamethazine	10.6	23.8	2-(4-chloro-3-nitro-benzoyl)-benzoic acid	14.7	57.0
dipyridamole	10.7	24.4	spironolactone	14.9	58.8
primidone	10.7	24.5	nabumetone	15.4	63.2
pentoxifylline	10.8	25.7	ibuprofen	15.7	65.4
haloperidol	10.8	25.8	progesterone	16.4	71.4
methoxysulfadiazine	11.0	26.9	tolfenamic acid	16.6	72.8
acetanilide	11.1	27.9	hexanophenone	16.9	75.5
perphenazine	11.1	28.1			

[a] EP established by gradient sampling, and MeCN content measured by CLND.

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