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# Semi-preparative high-resolution recycling liquid chromatography

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ABSTRACT

A semi-preparative high-resolution system based on twin column recycling liquid chromatography was built. The integrated system includes a binary pump mixer, a sample manager, a two-column oven compartment, two low-dispersion detection cells, and a fraction manager (analytical). It addresses challenges in drug/impurity purification, which involve several constraints simultaneously: (1) small selectivity factors ($\alpha < 1.2$, poor resolution), (2) mismatch of elution strength between the sample diluent and the eluent causing severe band fronting or tailing, (3) diluent-to-eluent mismatch of viscosity causing viscous fingering and unpredictable band deformation, (4) low abundance of the impurity relative to the active pharmaceutical ingredient (API) (<1/100), and (5) yield and purity levels to be larger than 99% and 90%, respectively.

The prototype system was tested for the preparation of a trace impurity present in a concentrated solution of an API, estradiol. The ultimate goal was to collect ~1 mg of impurity (>90% purity) for unambiguous structure elucidation by liquid state nuclear magnetic resonance (NMR 600 MHz and above). First, the particle size (3.5 μm) used to pack the 4.6 mm × 150 mm long twin columns is selected so that the speed-resolution of the recycling process is maximized at 4000 psi pressure drop. Next, the production rate of the process is also maximized by determining the optimum number (7) of cycles and the corresponding largest sample volume (160 μL) to be injected. Finally, the process is fully automated by programming the time events related to (1) sample cleaning, (2) transfer of the targeted impurity from one to the second twin column, and (3) impurity collection. The process was tested without interruption during one week for the collection of a trace impurity ($\alpha = 1.166$, strong acetonitrile-methanol sample diluent, concentration ~2 mg/L) from a concentrated (10 g/L) stock solution (60 mL total) of estradiol. The process enriches the impurity content relative to the API by about a factor ~5000. For the lack of a sufficient collected amount (~120 μg only) of the pure impurity (purity 50% only), NMR experiments could not provide reliable results. Instead, the combination of LC–MS (single ion monitoring) and UV absorption spectra ($\lambda_{max}$ shift) revealed that the targeted impurity was likely the low-abundant enol tautomeric form of the ketone estrone, a possible intermediate or by-product of the synthesis reaction of estradiol.



## 1. Introduction

Combining ultra-high resolution and semi-preparative liquid chromatography in a single separation/purification process remains a serious challenge because these two system characteristics are by essence antagonist. On one hand, preparative chromatography handles large sample volumes and high concentrations [1] while, on the other hand, ultra-high performance chromatography relies on infinitely small sample loads and diluted concentrations (linear chromatography) [2]. For that all, high-resolution semi-preparative chromatographic systems are highly demanded by the pharmaceutical industry, which has to comply with strict regulations before a synthesized drug can be released on the market. For instance, isolating and preparing a sufficient amount of an unknown, targeted, and low-abundant impurity, which nearly co-elutes with the highly concentrated API, remains unsolved with current technologies (simulated moving bed and steady state recycling). The unambiguous structure elucidation of such a critical impurity is usually achieved by liquid state NMR which requires as much as 1 mg of pure material. Not only the purity

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but also the recovery level (or yield) of the separation/purification process should be larger than 90% because the impurity concentration is usually much smaller (by 2–4 orders of magnitude) than that of the API in the stock solution.

On one hand, ultra-high resolution can be routinely performed by gas chromatography [3], very high pressure liquid chromatography (vHPLC) [4–9] using sub-2 μm particles and pressures as large as 1 kbar, supercritical fluid chromatography (SFC) using tandem columns and low-viscosity eluents [10], heart cutting two-dimensional liquid chromatography (2D-LC) [11], and various hyphenated techniques [12]. However, the amount of pure material that can be prepared by these analytical techniques is insufficient for NMR-based structural investigations. On the other hand, batch preparative chromatography is based on shorter and wider inner diameter (i.d.) columns, which have lower resolution power relative to analytical GC, SFC, or vHPLC columns [1]. It is well suited for the separation and collection of a few targeted and abundant compounds present in a simple feed mixture and easily separated from the other (large selectivity factors). The production rate and the overall performance of batch preparative chromatography can be significantly increased by either continuous or semi-continuous purification systems. These processes are based on simulated moving bed chromatography [13–15], two-column open-loop simulated moving-bed chromatograph [16], steady state recycling [17,18], or multi-column countercurrent solvent gradient processes [19]. However, for the lack of resolution power, these preparative systems are not well adapted to extreme separation/purification problems such as the collection of a trace compound that nearly co-elutes with the most abundant compound. A typical example is a stock solution containing the main API, several minor compounds, and many trace impurities. The most serious problem arises when one of the trace impurities nearly co-elute with the main API peak. Most often, these critical targeted impurities are undesirable intermediates or by-products which have structures very similar to that of the API. Alternative techniques that will cope with their isolation and collection are then in high-demand from the pharmaceutical industry.

Discontinuous twin column recycling separation process (TCRSP) was efficiently used in the past to fractionate polymer mixtures by gel permeation chromatography [20–25], to prepare pure isotopes and isomers by gas chromatography [26,27], and to collect optically active compounds by chiral liquid chromatography [28–31,17]. This recycling technique was recently extended to modern HPLC and vHPLC adsorption and size-exclusion columns in order to provide unprecedented resolutions of shape isomers (polycyclic aromatic hydrocarbons) [32], enantiomers [33,34], isotopes [33,34], polymers [33], and monoclonal antibodies from their aggregated forms [33]. Despite the production rate of the discontinuous TCRSP being intrinsically lower than that of continuous or semi-continuous processes, it has several advantages: the experimental set-up is easily and rapidly assembled by the non-expert [33], it can cope with extremely challenging separation problems (selectivity factor <1.2 and abundance ratio <1/100 [33,34]), and its long-time automation is straightforward by controlling and maintaining steady the eluent and column temperature. As a result, the retention times of all the compounds are nearly perfectly reproducible and hundreds of successive injections can be repeated in order to collect the desired amount of the pure targeted impurity.

In this work, the potential of the discontinuous TCRSP is investigated from both a fundamental and practical viewpoint. The preparation of a low-abundant (impurity-to-API concentration ratio ~1/5000) and closely eluting impurity (selectivity factor $\alpha = 1.166$) present in an API (estradiol) stock solution (60 mL, 10 g/L) is carried out by coupling the TCRSP with a fraction manager (analytical). First, the production rate of the TCRSP is calculated and optimized under two different case scenario: (1) the elution strength of the sample diluent matches exactly that of the mobile phase and (2) the elution strength of the sample diluent is much stronger than that of the mobile phase (as it is observed) because the largest possible amount of API (and impurity) has to be initially dissolved in a strong diluent for maximum productivity. In each scenario, the optimum pair of the cycle number and largest injection volume to be applied in the TCRSP run is unambiguously determined. Secondly, the process is automated to prepare over a long-time period (days) about 1 mg of the unknown critical impurity. Besides sample diluent effects, the separation also involves viscous fingering effects, which causes additional band deformation. Viscous fingering results from large injection volumes (100 μL) and from the significant mismatch between the viscosity of the sample diluent and that of the mobile phase. The capabilities of the developed TCRSP as an ultra-high resolution and semi-preparative system is reported and discussed regarding the preparation of 1 mg of pure impurity for NMR-based structure elucidation. Its limits are also pointed out and it is shown how single ion monitoring mass spectrometry and UV absorption spectra (photo diode array detectors) can propose impurity structures when NMR investigations are not possible for the lack of a sufficient amount of pure material.

## 2. Theory

In the next two sections, the production rate of the semi-preparative TCRSP is optimized from a theoretical viewpoint. In a first step, the resolution power of the TCRSP used to separate the targeted impurity from the API is optimized from the construction of speed-resolution plots at the imposed maximum pressure drop along the two twin columns ($\Delta P = 4000$ psi), each of them having a fixed length $L = 15$ cm. The best commercially available particle diameter $d_{p,opt}$ is then determined for the purification process. In a second step, imposing a yield and a purity level of at least 99.7% and 99.0%, respectively, the production rate is maximized by finding the largest injection volume ($V_{p,opt}(n_{opt})$) at the optimum cycle number ($n_{opt}$) using two 4.6 mm inner diameter (i.d.) × 15 cm length columns. It is assumed in the calculations that the adsorption isotherms of both the impurity and API are linear. In practice, the user could simply perform mock experiments with two arbitrary columns of fixed length $L$ and find the minimum number of cycles required to isolate the impurity for given injection volume, yield, and purity. However, this does not provide the optimum particle size $d_p$ for performance optimization of the TCRSP and the optimum pair [$V_{p,opt}$;$n_{opt}$] that will maximize the production rate of the process. The following sections provide the fundamental rationale for the selection of these three relevant experimental parameters.

### 2.1. Ultra-high resolution chromatography: performance optimization of the recycling system

The TCRSP is based on recycling multiple times a targeted separation zone (the entire band of the trace impurity and a fraction of API band) from one to the other twin column. Based on the observed pressure drop, $\Delta P$, along the two twin columns and the fixed length, $L$, of each column, the optimum particle sizes, $d_{p,opt}$, that maximize either resolution power or speed-resolution performance can be unambiguously determined. All the necessary details for the selection of $d_{p,opt}$ are given in [34]. The main results are summarized below.

The method is based on the construction of speed-resolution plots for the TCRSP. Under ideal conditions (no loss in column effi-

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