Chapter

Turbulent Flow Chromatography: A Unique Two-Dimensional Liquid Chromatography

Francesca Di Gaudio, Annamaria Cucina and Sergio Indelicato

Abstract

Among 2D-LC techniques, a particular approach is commercialized by Thermo Fisher Scientific that may enable the direct introduction of biological samples into an online automated extraction system without any additional pre-treatment: the TurboFlow technology. It combines chemical and size exclusion capability of chromatography columns packed with porous particles in which a turbulent solvent flow is able to separate smaller molecules from larger ones (e.g. proteins). Once extracted, the small molecules can also be transferred to an analytical column for improving separation prior to detection. This is done through a unique plumbing and customized valve-switching arrangement that allows the focusing of molecules onto the second column. This enables a very efficient chromatographic separation. The use of the TurboFlow not only eliminates extensive sample preparation, thus reducing inter-operator variability and matrix effects, but also increases the capacity for high-throughput analyses due to a unique multiplexing technology, in which multiple LC channels are connected to a single detector.

Keywords: 2D-LC, turbulent flow chromatography, TurboFlow, multiplexing, extraction, purification

1. Introduction

High-performance liquid chromatography (HPLC) is a powerful technique for the separation of compounds. However, one-dimensional liquid chromatography (1D-LC) cannot easily handle complex matrices and complex mixtures of analytes. The matrix effect is relevant when dealing with biological matrices, such as urine, saliva, serum, and whole blood in liquid chromatography coupled with mass spectrometry (LC–MS). This effect is mainly due to co-elution of endogenous compounds, such as proteins, lipids, sugars, or salts [1, 2]. In fact, early approaches in LC–MS, tending to simplify sample preparation methods, such as "dilute and shoot" and quick chromatographic analysis time, soon revealed that by not removing the matrix components, these could interfere with the ionization process in an unpredictable and inconsistent way (e.g. ion suppression).

For these reasons, a thorough and robust sample preparation is crucial for quantitative analysis. Among others, protein precipitation (PP) methods are widely used. In

these procedures, a small volume of sample is mixed with a certain volume of protein precipitation reagent. The interaction between proteins and this reagent determines the alteration of the protein conformation and the consequent precipitation. Once the precipitate is separated, the analytes of interest, remaining in solution, are ready for analysis. Although PP protocols are inexpensive and simple, they are time-consuming and unable to purify the samples enough. More recently, modified PP approaches, such as membrane-based PP filter plates, have been proposed to overcome these limitations [3, 4].

Liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are alternative sample preparation methods. The first one consists in extracting the analytes from one liquid phase to another immiscible liquid phase. Once shaken or vortex-mixed, the phases are separated, and the molecules collected for analysis. LLE presents several limitations, such as the need for large sample volume, variable recovery, and difficulty on the extraction of compounds with varying lipophilicities. A more efficient upgrade of this technique is represented by salting-out assisted LLE (SALLE), in which appropriate salts are added to the solvent to enhance the extraction [5].

SPE technique allows extraction and enrichment of analytes of interest by loading a liquid sample onto a column/cartridge/plate packed with a sorbent material. While the analytes are retained, the interferents are either eliminated during the loading phase or washed away during the washing steps. The analytes are then eluted and collected for LC–MS analysis [6]. Even if SPE has many advantages, it has limited selectivity and sensitivity because matrix constituents can be adsorbed together with the analytes, determining matrix effects. Furthermore, SPE cartridges are usually single use. More recently, several approaches have been developed to improve SPE technique, such as dispersive solid-phase extraction, solid-phase micro-extraction, and stir bar sorptive extraction [7]. Furthermore, SPE has been automated in online solid phase extraction, coupled with HPLC [8]. This technique can thus be considered as an online two-dimensional liquid chromatography (2D-LC).

The online 2-D approaches are faster and more reproducible, but they require specific interfaces and more complex operative modes.

There are two main approaches to the online 2D-LC: heart-cutting and comprehensive [9]. The first one enables the re-injection of a definite number of multicomponent effluent fractions from a primary to a secondary column, while the second one determines the separation of the entire sample in both dimensions [10]. When this technique is employed, a combination of different chromatographic separation methods is usually utilized. Frequent combinations are size exclusion chromatography (SEC) with reverse phase liquid chromatography (RPLC), RPLC with hydrophilic interaction liquid chromatography (HILIC), or normal-phase liquid chromatography with RPLC [11].

Regardless of the operative mode, 2D-LC presents some disadvantages. In fact, the separation typically takes longer than in 1D-LC, and the detection sensitivity may decrease because of the dilution or loss of the sample during the two separations. In addition, considering that the 1D effluent is the injection solvent of the second dimension, incompatibility issues may be raised. Incompatibility could be determined by partial or complete immiscibility of the two mobile phases, difference in solvent strength, but also by the excessive difference in viscosity, that could result in peak deformation or splitting. Besides, an increased instrumentation and conceptual complexity must be considered. Active-modulation techniques, such as active solvent modulation (ASM) and stationary-phase assisted modulation (SPAM), could surmount these limitations. However, a constraint to the ASM approach is the time

needed to displace the fractions of effluent from the sampling loop to the second column. The required extra-time can be significant for short 2D cycles [12]. SPAM separation methods of analytes with completely different chemical properties could lead to incomplete recovery and discrimination effects, due to a partial trapping of all analytes with the chosen dilution solvent. Moreover, the trapping columns of SPAM could reduce the robustness of the system [13].

Turbulent flow chromatography (TFC) overcomes the limitations of 2D-LC by removing potential interferences in a fast and efficient manner. In addition, the application of staggered, parallel methods ("multiplexing") maximizes the use of detector time, saving time and solvents. Furthermore, the extraction columns are re-usable for hundred injections [14].

In this chapter, theory and hardware aspects of the TFC, with particular reference to the TurboFlow system, will be discussed. Moreover, method development and applications will be brought up.

2. Theory of turbulent flow chromatography

In the ideal chromatographic process, the analytes form narrow bands while moving along the column. The band or peak narrowness is a measure of the efficiency of the separation procedure that can be quantified by the number of theoretical plates, *N*. These latter are hypothetical zones or stages in which two phases, the stationary phase, and the liquid mobile phase in the case of HPLC, establish an equilibrium with each other. If the peak shape is Gaussian, the theoretical plate number *N* can be approximated according to Eq. (1).

$$N = 5.54 * \left(\frac{t(r)}{W(1/2)}\right)^2 \tag{1}$$

where *t(r)* is the retention time of a compound, and *W(1/2)* is the peak width measured at half of the peak height. As described in the Eq. (2), the theoretical plate number *N* depends on the ratio between the column length and the height equivalent of a theoretical plate, *h*.

$$N = \frac{L}{h * d(p)} \tag{2}$$

where *L* is the column length, *N* is the number of theoretical plates, and *d(p) is* the particle diameter. The smallest value of *h* corresponds to the highest efficiency. Thus, *h* is a more effective way to compare efficiency of the chromatographic processes.

Column efficiency, linked to band broadening processes, in traditional HPLC was first described by van Deemter and colleagues [15]. Van Deemter described these mechanisms with the Eq. (3).

$$h = A + \frac{B}{v} + Cv \tag{3}$$

where *A*, *B,* and *C* are constants, and υ is the average linear velocity (cm/s). The optimum value of *h* will be obtained when

$$\frac{dh}{dv} = 0$$

so that

$$v = \sqrt{\frac{B}{C}}$$

The term A is defined as the tortuosity factor, and it is affected by the size and the distribution of the stationary phase particles. The term B depends on the diffusion of the molecules in the longitudinal direction, linked to the motion of solute molecules in the mobile phase. This coefficient is significant at low flowrates. The term C refers to the resistance to the mass transfer of the analytes through the two phases and their diffusion on the surface. **Figure 1** presents a simplified van Deemter plot, which allows us to identify the optimum flowrate, corresponding to the minimum value of h.

In 1966, Pretorius and Smuts firstly observed that, using open tubular columns, it is possible to improve speed and efficiency of mass transfer with turbulent rather than laminar flow [16]. Turbulent flow in fact, dominated by rotational motion, reduces band-broadening, presenting a profile flatter than laminar flow (**Figure 2**).

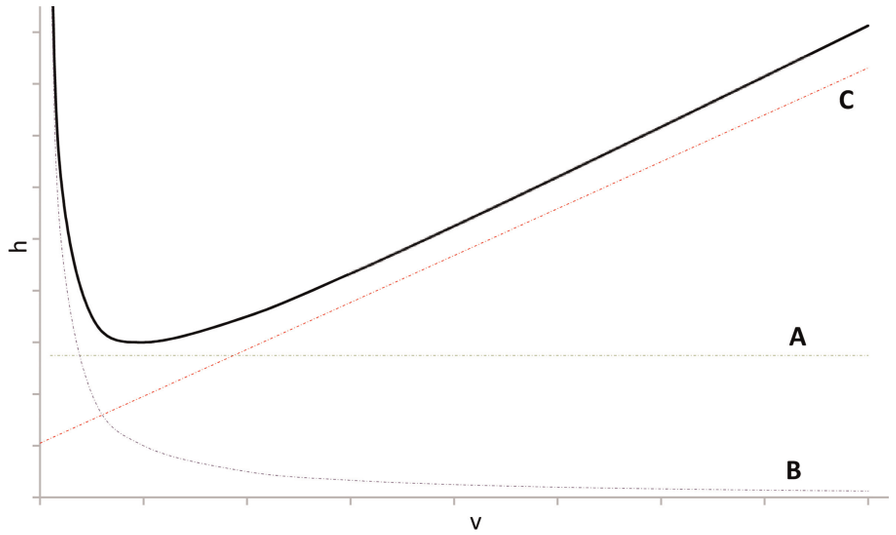

Figure 1.
Simplified van Deemeter plot with A, B and C components.

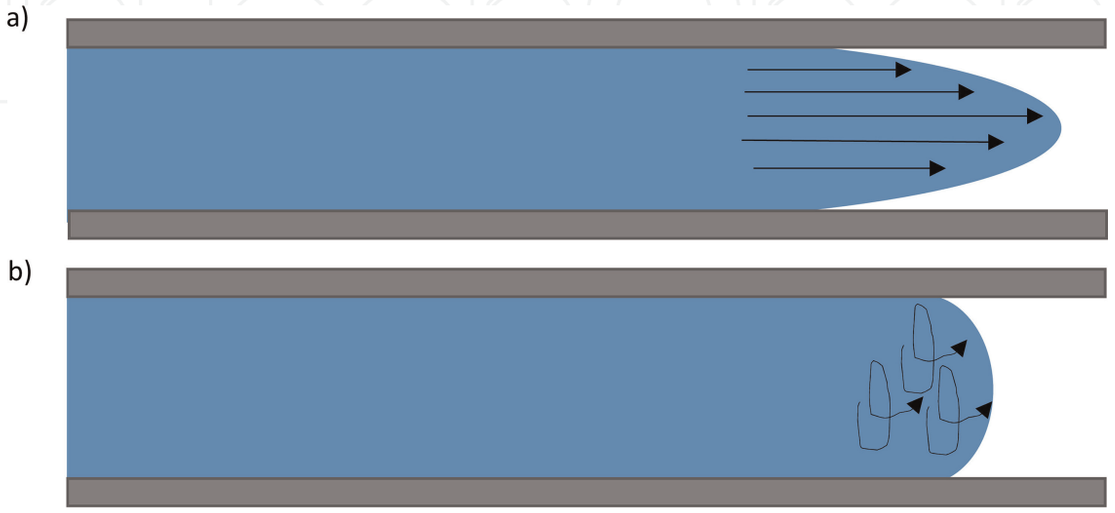

Figure 2.
a) Laminar flow; b) turbulent flow.

Physicist Osborne Reynolds found that the flow of a fluid through a straight and smooth tube transitions from laminar to turbulent as the momentum of the fluid becomes around two thousand times greater than its resistance to flow. The momentum, which is a certain amount of mass moving at a certain velocity, takes into account the fluid density and the diameter of the tube. It is possible to increase the fluid momentum by increasing its velocity, or by increasing the diameter of the tube, or both. The resistance to flow is expressed as the absolute or dynamic viscosity of the fluid [17].

The Reynolds number, Re, is a dimensionless parameter, defined as the ratio of the inertial to viscous forces present in a fluid system (Eq. 4).

$$\Re = \frac{\mu 0 * I}{\eta} \tag{4}$$

where μ_0 is the mean linear velocity of the fluid, and η is the dynamic viscosity of the mobile phase. In a packed bed such as an HPLC system, the characteristic length scale I is related to particle diameter and external porosity.

The transition from laminar to turbulent flow occurs as Re overcomes some reference values. For a very straight and smooth cylinder the transition from laminar to turbulent flow occurs at a Reynolds number of 2400, while for a packed bed of uniform spheres, turbulence occurs between 1 and 10 Re [18]. Even if turbulent flow presents the advantage of better mass transfer, the backpressures required to obtain this flow would be excessively high, particularly in terms of pumping systems and particle architecture. In the 1990s, it was observed that using large irregularly shaped particles (50–100 μm), the backpressures were sufficiently low to generate turbulent flow and to improve column efficiency at increasing flowrate thanks to large interstitial spaces between particles (around 100 Å). In addition, it was observed that larger molecules in solution diffuse more slowly than smaller ones. Thus, larger molecules do not interact with the stationary phase, while smaller molecules have time to diffuse in and out of the pores and to interact with the stationary phase depending on their affinity. In 1997, these packed columns (now called TurboFlow™ columns) were patented (US patent no. 5,919,368) [19]. Although the mechanism is not fully understood, TurboFlow has demonstrated to be a very efficient method to separate large molecules, such as matrix components (e.g. proteins and lipids), from small molecules [14].

2.1 Column chemistries

TurboFlow™ columns are available in a wide variety of packing materials to enable different applications. The selection of the appropriate column must be based on the polarity of the analytes and the mobile phases needed to solubilize them. Initially, these columns presented an internal diameter of 1 mm and required a flowrate of 4–5 mL/min. Recent columns have an internal diameter of 0.5 mm to reduce solvent consumption and require a flowrate around 1.5–2 mL/min. It is possible to group TurboFlow™ columns in silica-based and polymer-based. Among silica-based columns, suitable for pH ranging from 2 to 9, packing includes Cyclone C18, Cyclone C8, and Cyclone C2 modified columns. Furthermore, Cyclone Fluro and Cyclone C18-P columns are also available. Styrene/divinylbenzene copolymers-based columns, suitable for pH ranging from 1 to 13, are also available in different chemistries: Cyclone, Cyclone-P, Cyclone mixed-mode with anion—(Cyclone MAX), cation—(Cyclone MCX), and mixed anion–cation-exchange phases (Cyclone MCX-2). Details about column applications are described in **Table 1** [20].

Column	Application
Silica-based columns	
Cyclone C18	For non-polar and moderately polar solutes (f.e. pharmaceuticals, fatty acids)
Cyclone C18-P	For polar and non-polar solutes (f.e. polar pharmaceuticals, metabolites)
Cyclone C8	For solutes excessively retained by C18 (f.e. pharmaceuticals, metabolites)
Cyclone Fluro	For highly-lipophilic solutes and perfluorinated compounds
Cyclone C2	For extremely non-polar solutes (f.e. extremely non-polar multi-functional pharmaceuticals)
Polymer-based columns	
Cyclone	For non-polar analytes in complex matrices and silanophilic compounds
Cyclone-P	For non-polar and moderately polar compounds (f.e. steroids)
Cyclone MAX	For polar solutes and weakly acidic compounds (f.e. polar pharmaceuticals, metabolites, antibiotics)
Cyclone MCX	For polar solutes and weakly basic compounds (f.e. polar pharmaceuticals, metabolites, drugs of abuse)
Cyclone MCX-2	For polar solutes and weakly basic compounds (f.e. polar pharmaceuticals, metabolites, metanephrines, melamine)

Table 1.
Silica-based and polymer-based column applications.

3. Hardware

The turbulent flow chromatography, whose main peculiarity lies in the particular characteristics of its columns (described above), requires a specific hardware platform capable of exploiting the potential that these can express. The only instruments on the market designed to take full advantage of TurboFlow columns are marketed by Thermo Fisher Scientific as Transcend TLX systems.

These systems can be used as traditional HPLC, injecting the sample directly onto the analytical column, or for 2D-LC. In order to handle a turbulent flow chromatography and eventually to couple it with a second analytical column, a complex and unique hardware, which presents at least two pump systems and a valve interface module, is required. In addition, the systems can include multi-channel technologies. Depending on its configuration, each system can include one, two, or four pairs of pumps that enable the system to work either in single or 2D-LC mode over one, two, or four separate channels. When a sample is extracted/purified, the system uses one pump, named loading pump, to carry the sample into the TurboFlow column, and a second pump, the eluting pump, to resolve the extracts over a traditional analytical column. The overall plumbing of the system is intricate since it can include up to four pairs of valves each lodging a different couple of columns (the TurboFlow column in valve A and the analytical one in valve B) and, eventually, a special extraction loop, together with other valves for channel selection and for flow diversion to the detector or to the waste (**Figure 3**). Moreover, the system controls up to four couples of injection ports (for the online extraction and for the traditional elution) using one or two autosampler arms. Some interesting accessories can complete and further complexify the configuration, as for example a multicolumn compartment module (MCM) that allows to install simultaneously up to six extraction and six analytical

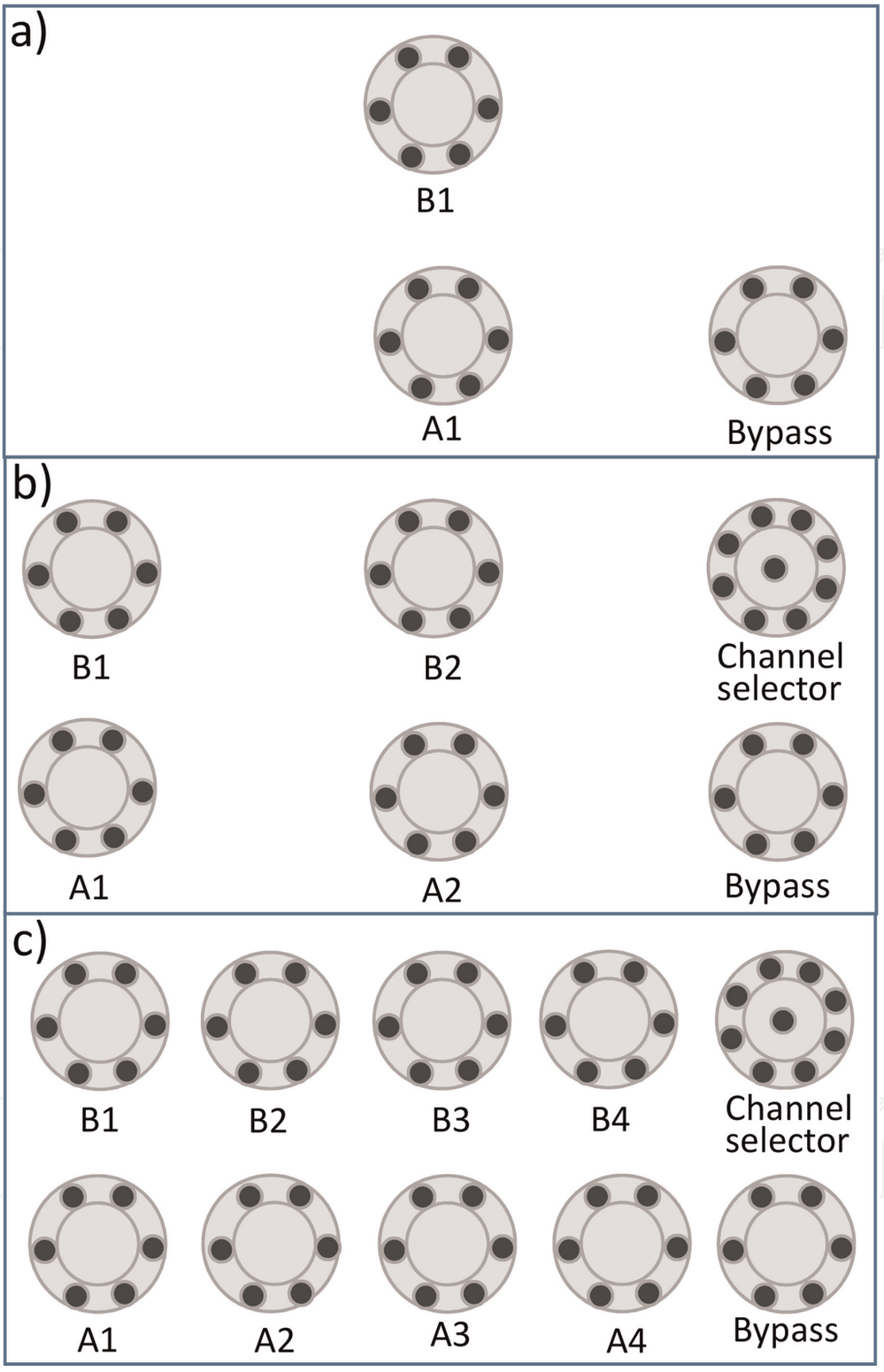

Figure 3.
Valve interface module of a) single channel, b) double channel, c) four channel systems. The "bypass" valve diverts the flow to the waste or to the detector.

columns (or eleven columns of the same type) and to select the column to be used via software, without the need to plug and unplug them. Considering the complexity of Transcend systems, a dedicated software (Aria MX™) simplifies their use by controlling and coordinating the valve switching processes and the multiplexing capabilities.

Using Transcend systems for online extraction, two modes of operation are possible: Quick Elute Mode and Focus Mode (**Figures 4** and **5**). These differ in complexity, field of application and plumbing, being the Quick Elute mode simpler and faster.

Quick Elute is generally used for single compound quantification and/or when speed is the priority. In this case, in which only a TurboFlow column is needed, chromatographic separation is performed, while compounds are eluted from the column. This operating mode presents the advantages of being fast and simple to be set even when analysing complex matrices. Furthermore, it requires minimal method development. However, this separation presents a limited resolution, because of the large irregularly shaped particles of TurboFlow columns, so possible isobaric interferences should be taken into account. Nonetheless, it is possible to add an analytical column between the valve B and the detector for a rapid 2-D separation, similar to an online SPE, with the advantages that a turbulent flow gives to the extraction phase.

The Quick Elute Mode consists of the following steps: loading, eluting, column cleaning, and reconditioning. The sample is firstly injected into the loading pump mobile phase flow. The analytes are brought to and retained by the column, installed on the A valve, while the matrix macromolecules, not retained by the column, are washed to the waste connected in the B valve. If the analytes are not retained, they will be washed away in this step, if they are too retained, they will be washed out during the column cleaning process. After the loading step and before the elution, the TurboFlow column can be rinsed with a stronger mobile phase to purify the sample.

By switching the B valve, the mobile phase from the eluting pump flows through the TurboFlow column, the analytes are eluted to the detector or, if the case, to an analytical column. It is possible to elute the analytes off the TurboFlow column using

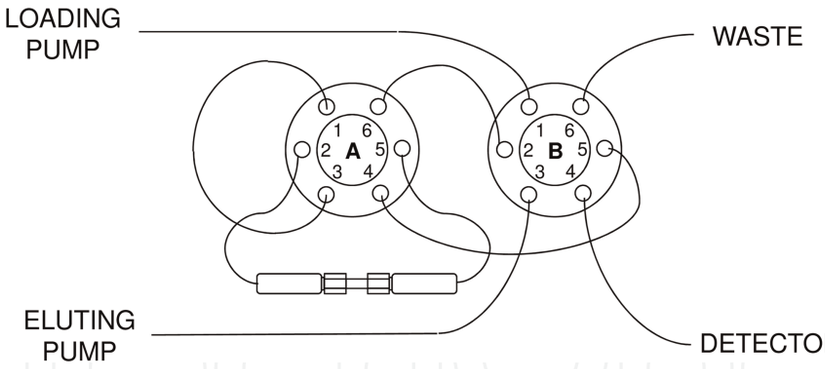

Figure 4.
Quick elute valve arrangement without analytical column.

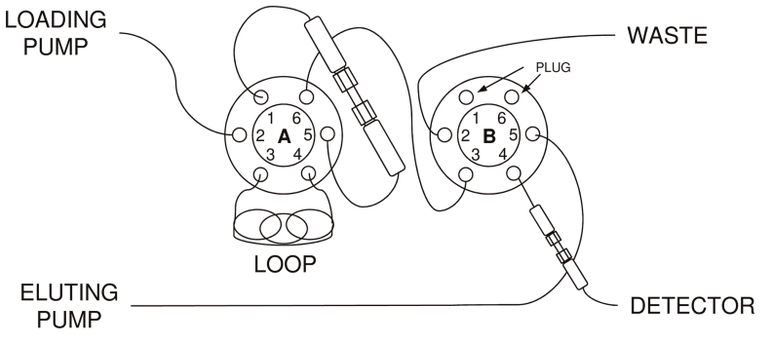

Figure 5.
Focus mode valve arrangement.

the same flow direction as the loading step (forward flush), or in the opposite direction (back flush), by switching the A valve. After the elution step, the extraction column is washed by the loading pump flow with an appropriate organic solvent. The re-conditioning presents the same parameters of the loading step, but for a longer time to better equilibrate the system.

When the quantification of multiple compounds is requested, Focus Mode is indispensable. It involves a TurboFlow and an analytical column and requires more extensive method development. In addition to the Quick Elute mode setup, the Focus Mode requires an elution loop in the A valve for the transfer step and a customized tee-piece rotor seal in the B valve (**Figure 6**).

The Focus Mode consists of six steps: loading, sample washing, transfer, system washing, loop-filling, and reconditioning (**Figure 7**) [20]. In the loading step, samples are injected into the TurboFlow column at high flow rates so that macromolecules of the matrix, salts and ionic compounds, not retained by the column, flow to waste (**Figure 7a**). Once loaded, the sample is cleaned from eventual contaminants using an appropriate mobile phase (**Figure 7b**). During these two steps, the mobile phase of the eluting component flushes to the analytical column connected to the detector.

During the transfer step, the valves A and B are switched, the optimized loop content is pushed by the mobile phase into the TurboFlow column, and the analytes are eluted off and transferred to the analytical column (**Figure 7c**). In this step, the transfer flow is diluted, via the tee-piece rotor seal, in the B valve, with the mobile phase coming from the eluting pump, in order to focus the molecules of interest to the head of the analytical column. These first three steps typically take around 2 minutes.

When the transfer is completed, the B valve is switched, the loading and eluting flows are again separated and, while the analytes are resolved on the analytical column and eluted to the detector, the TurboFlow column and the extraction components are washed to avoid carryover (**Figure 7d**). At this moment, the loop is filled with the proper transfer solution to be used in the following injection (**Figure 7e**). In the final step, the elution loop is isolated, and the columns are re-equilibrated to the conditions of the loading step (**Figure 7f**).

3.1 Operations in multichannel systems

In a traditional LC run, analytes of interest are usually eluted in a small portion of the entire chromatogram. It means that, during the pre-injection phase (needle and injector wash, sample withdrawn, etc.), but also during the first and last minutes of a run, the detector acquires useless data or the flow is diverted to waste. TurboFlow is multichannel systems, in which each "channel" is an independent HPLC equipment with its own pump(s) and injector port(s). These channels are connected to one single detector, and the systems are able to maximize the productivity, reducing the idle

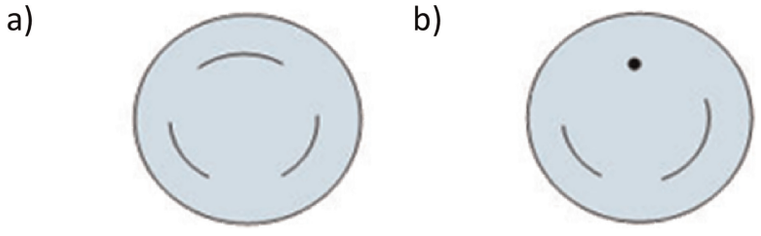

Figure 6.
Rotor seals in a) quick elute mode, b) focus mode.

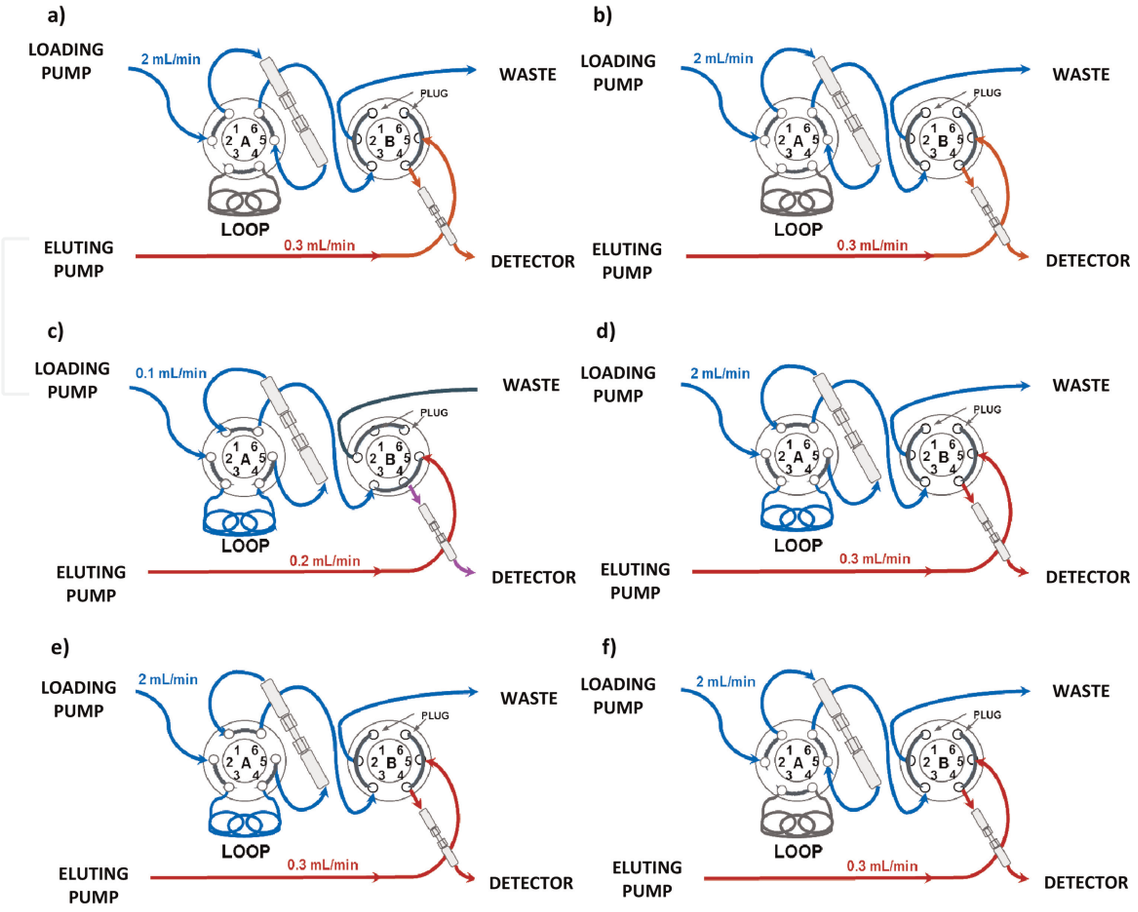

Figure 7.
a) Loading; b) sample washing; c) transfer; d) system washing; e) loop filling; f) reconditioning.

time of the detector. Thanks to the multiplexing, in fact, TurboFlow staggers injections, by overlapping the channels to optimize the time of the acquisition window. In particular, two- or four-channel systems are currently available (**Figure 8**). Aria MX™ software is able to calculate automatically the timing of injections based on the relative duration of acquisition windows in comparison with the duration of the entire chromatogram and the pre- and post-injection operations of the auto-sampler. On each of

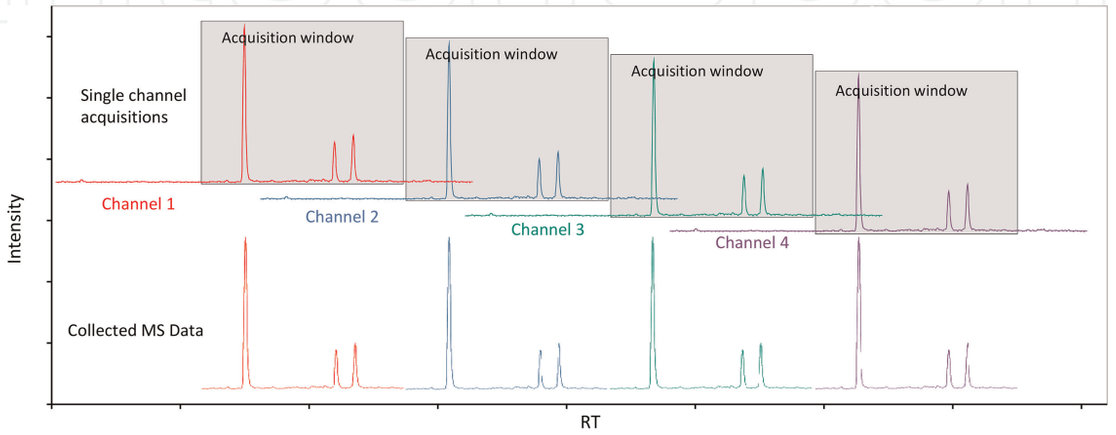

Figure 8.
Representation of staggered injections in a multiplexing system.

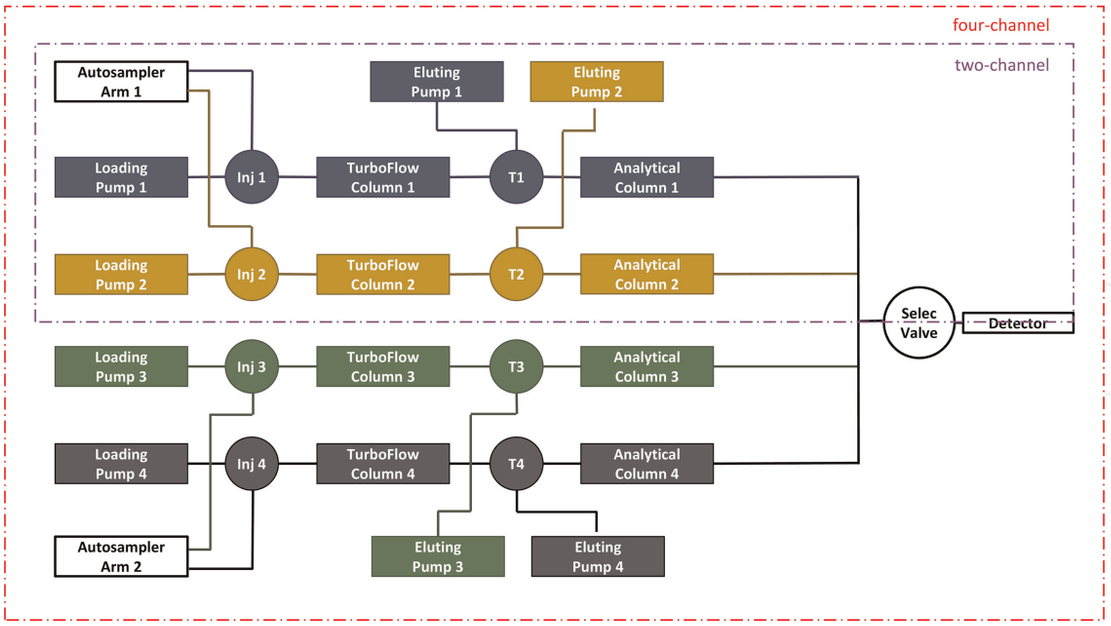

Figure 9.
Diagram showing the setup of a two- (squared in purple) and a four-channel (squared in red) multiplexing.

the different channels, the system is able to handle runs with the same or different instrumental method, maximizing the productivity up to four times when using a four-channel system. However, the hardware complexity is also significantly increased (**Figure 9**).

4. Development of a focus mode method

The two approaches described above require different method development. However, considering the complexity of the Focus Mode, in the present paragraph only its method development will be explained.

A Focus Mode involves at least two columns, a TurboFlow and an analytical column. Two mobile phases (appropriately chosen) for the analytical gradient elution and at least two mobile phases for the extraction component for loading, transfer and washing steps are also requested. It is generally suggested to start with the choice of the ideal analytical column and elution conditions, able to chromatographically resolve the analytes. Then, considering the type of chromatography for the extraction process (i.e. reverse phase, ionic exchange, etc.) intended to perform, a TurboFlow column has to be chosen, compatible with the chemical characteristics of the analytes, the mobile phases, and the column selected for the analytical separation. Considering the possible combinations of couples of columns, a multiple column module (MCM) can help in this evaluation. Once the separation conditions are set, loading, transfer and washing conditions have to be optimized. This process that can be facilitated by the Aria software, consists of studying the analytes chromatographic behaviour when mobile phase composition, duration, and flowrate are changed during the different steps. In fact, one of the software useful features allows one to test different conditions for a specific method parameter, defining it as "variable" and programming its values in the acquisition sequence instead of writing one method per condition that has to be tested. When optimizing these parameters, the analytical column has to be

removed. A T-union, which splits the flow of the eluting mobile phase between detector and waste, is connected to detect the analytes elution at all steps in the method (**Figures** 7 and **10**).

During the optimization of the loading and washing step conditions in a classic TurboFlow method, in which reverse phase chromatography for analytes extraction is used, different percentages of organic solvent have to be tested to establish the highest % of organic phase able to wash the sample without eluting analytes to the waste. In the wash step all interferences more polar than the molecules of interest are ideally washed out. In the example reported in **Figure 11**, tetrahydrocannabinol (THC) was loaded in a Cyclone-P column in 100% aqueous phase and an increasing percentage of organic phase was tested for the washing step. The ideal condition of the washing step

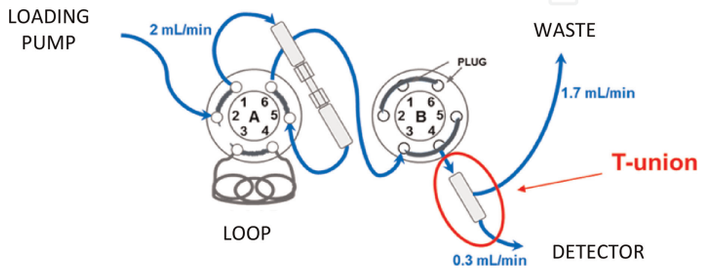

Figure 10.
Method development setup: The analytical column is replaced by a T-union diverting only the proper flow from the TX column to the MS detector.

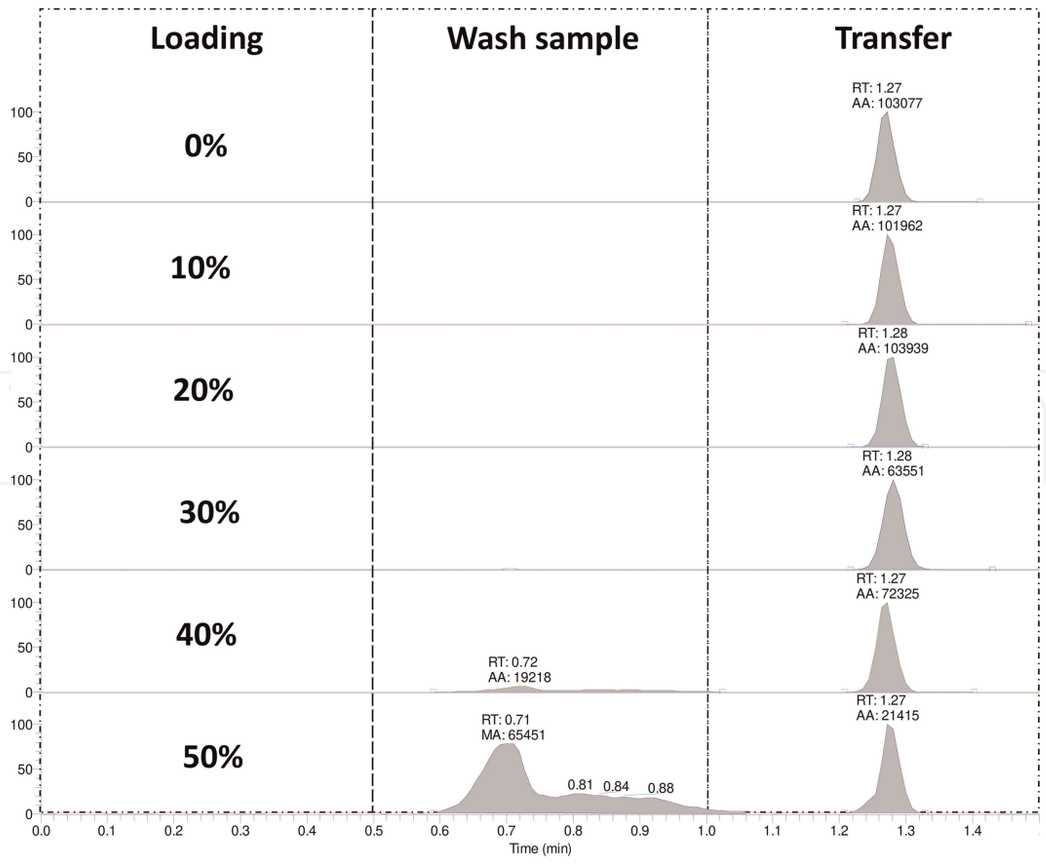

Figure 11.
Optimization of loading conditions for the analysis of THC in a cyclone-P column. The percentages refer to the organic phase.

was at 30% of B (**Figure 11**). Results with a higher organic content (40% and 50% in **Figure 11**) showed peaks appearing during the wash step, therefore demonstrating that the analytes are no longer retained on the TurboFlow column. Some loss during the loading/washing steps might be accepted as long as this is not impacting the required limits of quantification, since this will make the sample cleaning more efficient.

In the Transfer step, the analytes elute from the TurboFlow column and are transferred to the analytical column by the contents of the transfer loop. The solvent strength of the loop content is diluted before entering the analytical column by the eluting pump flow. The goal in this step is to be able to quantitatively elute the analytes from the TurboFlow column and to focus them on the head of the analytical column while reducing as much as possible the hydrophobic interferences from the sample matrix (matrix effect). So, three development stages are required: optimization of the transfer loop content, review of the transfer time using different transfer flowrates, and finally optimization of the transfer step dilution ratio.

Regarding the transfer loop content, the goal at this stage is to determine the minimum amount of organic solvent that quantitatively transfers all the target analytes to the analytical column, without eluting more hydrophobic compounds. The lower the content of organic solvent in the loop, the higher the retention of late eluting components (e.g. phospholipids) in the TurboFlow column (then washed to waste during the wash step), reducing matrix effect during the chromatographic elution.

If the solvent strength of the loop is too low, the analytes remain retained in the TurboFlow column and will be washed to waste during the washing step. On the other hand, if the solvent strength is too high, focusing of the analytes on top of the analytical column might be affected, leading to chromatographic peaks distortion or lack of retention. Moreover, the purification process would be less efficient. During the method development, the optimization of the loop content is performed without the analytical column.

In the example of the method development for the analysis of THC in the Cyclone-P column, decreasing percentages of organic phase were tested, and the ideal one was identified as 50% (**Figure 12**). Indeed, for lower content of organic solvent in the loop, part of the analyte was not transferred and was washed out in the washing step.

It is advisable to evaluate the transfer step time for different loading pump flowrates in preparation for the next step of the method development, the dilution ratio. If the flowrate of the loading pump during the transfer step is high, the method will be faster. However, the dilution from the eluting pump will be lower (taking into account that the sum of loading plus eluting flow cannot exceed the flowrate suitable for the analytical column), and hence, it will be more difficult to focus the target analytes on the head of the analytical column. This evaluation step consists in recording the time it takes for the analytes to reach the detector as the eluting flow varies in the transfer step. For the transfer time evaluation, there are no right or wrong values, but the information about the transfer that will be used in the final method, once the dilution ratio will be established, is just collected.

After the installation of the analytical column, the dilution ratio can be assessed, keeping the goal to focus the extracted analytes on the analytical column. If the organic concentration of the mobile phase in the transfer step is too high when it enters the analytical column, the analytes move through the analytical column rather than focus on it. In order to reduce this effect, the eluting pump dilutes the organic mobile phase from the loop with aqueous mobile phase reducing the solvent strength of the combined flow into the analytical column. The final solvent strength is

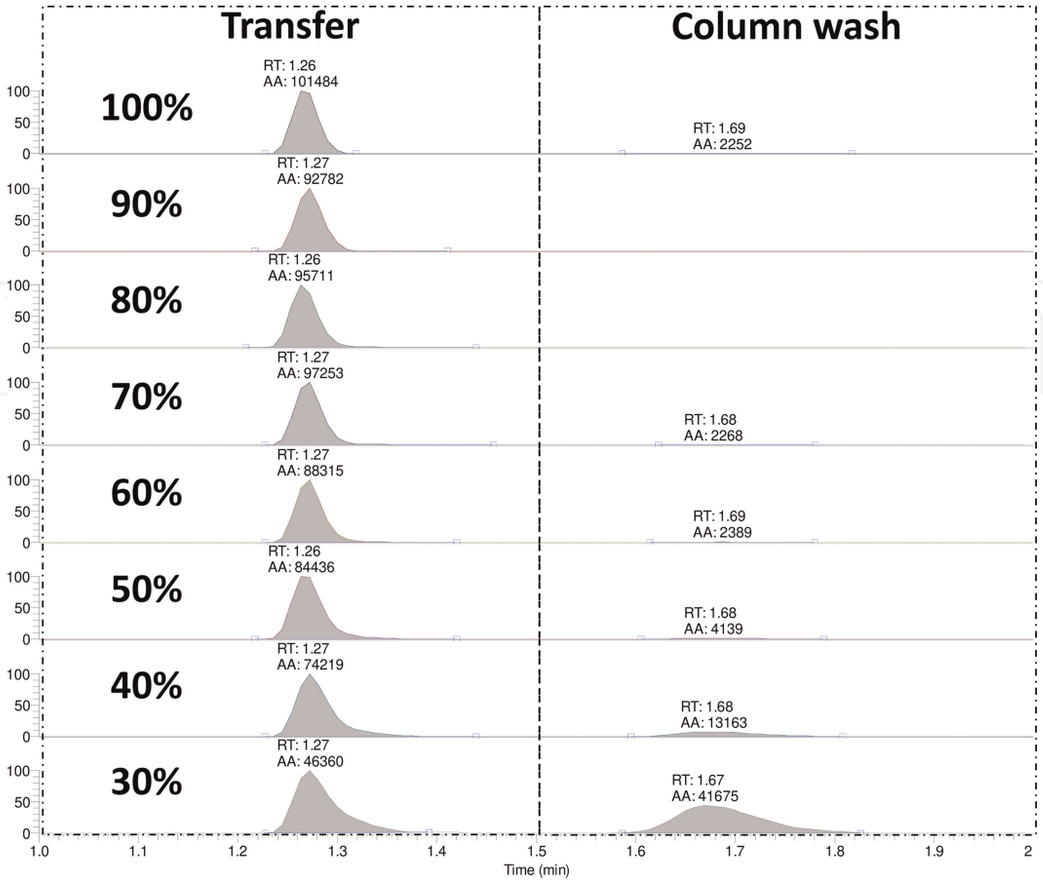

Figure 12.
Optimization of loop fill content for the analysis of THC in a cyclone-P column. The percentages refer to the organic phase.

influenced by the organic concentration of the loop contents and of the eluting pump flow (already optimized in the previous steps) and by the relative ratio between the eluting and loading pump flowrates (Eq. 5).

$$final\%organicsolvent = \left(\frac{LP}{T}\right) * OL + \left(\frac{EP}{T}\right) * OEP \tag{5}$$

where T is the total flowrate, the combined flowrate of the loading and eluting pumps during the Transfer step; LP is the loading pump flowrate during the transfer step; EP is the eluting pump flowrate during the transfer step; OL is the organic content percentage in the loop, and OEP is the organic percentage in the eluting pump flow.

Thus, the transfer dilution ratio has to be optimized by testing the effect of different combinations of loading and eluting flowrates on chromatography, taking into consideration that the total flowrate should match the flowrate used for the analytical column.

Peak fronting, poor resolution, and breakthrough in the final chromatography could be observed if solvent strength is too high during transfer and should be fixed trying to reduce the overall organic content during the transfer step.

Once the method is optimized, the maximum injection volume, which follows the same rules of a regular liquid chromatography method, has to be evaluated. The injectable sample volume will be higher when its composition is more similar to the

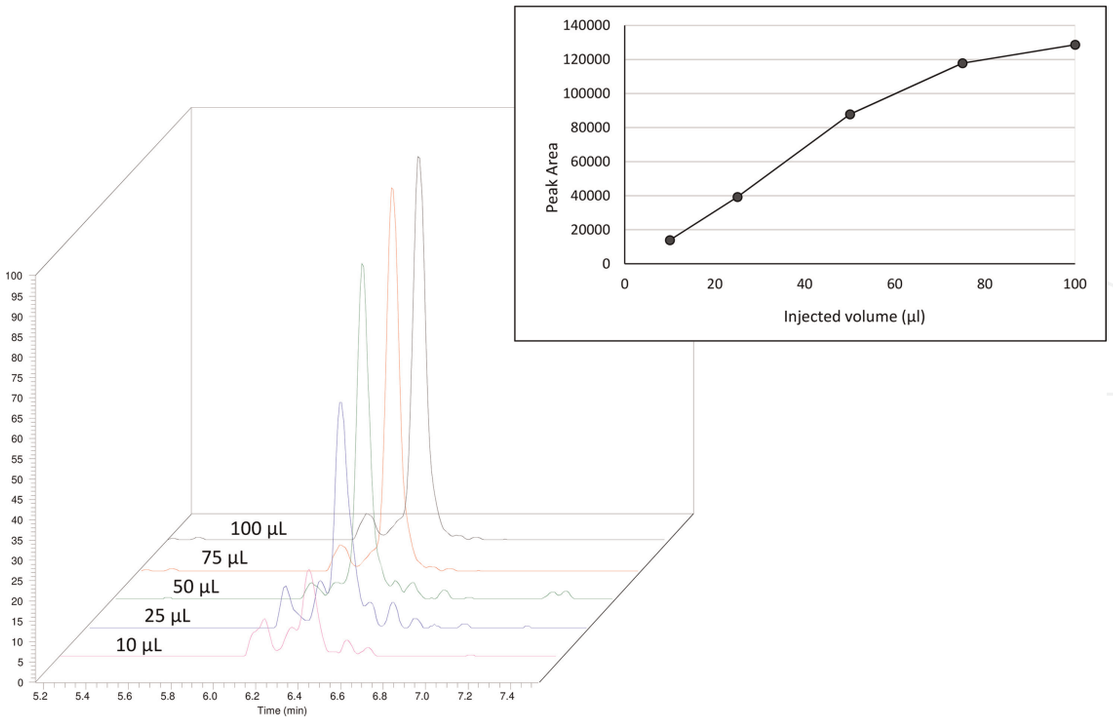

Figure 13.
Maximum injection volume identification.

initial mobile phase composition. However, it has to be considered that turbulent flow, compared to laminar flow, allows the use of a higher percentage of organic solvent. Increasing injection volumes of an extracted matrix sample, correlated to proportional increase of peak area, should be tested. The maximum injection volume corresponds to the volume that causes a flattened out peak area. The example in **Figure 13** shows that the maximum injection volume is 75 µL. Nonetheless, thanks to high carbon load values, the columns capabilities usually can handle volumes of 100 µL, significantly improving the overall method sensitivity, compared with injection volumes of conventional methods (5–10 µL).

5. Applications

Successful applications of the turbulent flow technique have been reported in different fields, such as therapeutic drug monitoring and environmental analysis, applied to various matrices, such as urine, plasma, but also food materials and water (**Table 2**).

Most of the published papers are focused on clinic applications. In this field, sample pretreatment, separation, and detection need to be more integrated in order to make mass spectrometry routinary even for not specialized laboratories. Online multidimensional chromatography, like TurboFlow methods, combining sample preparation and analysis, facilitate sample introduction, ease-of-use, and speed, even when dealing with complex matrices. Turbulent flow chromatography allows a simplified analysis of plasma, serum, or whole blood [21–26]. Hervious and colleagues reported the development, validation, and application of LC–MS/MS coupled with TurboFlow for the quantification of irinotecan, a cytotoxic agent used for metastatic

Reference	Matrix	TurboFlow column	Analytes	Application
Michopoulos [21]	Blood plasma	Cyclone	metabonomic analysis	Clinic
Chassaing [22]	Blood plasma	Cyclone	fluconazole, UK-112,166, dofetilide, candoxatril, UK-250,300, UK-141,495, doxazosin, CP-122,288, Compound I	Clinic
Gous [23]	Blood plasma	Cyclone C18-P-XL	anticoagulants: apixaban, dabigatran, edoxaban, and rivaroxaban	Clinic
Lindner [24]	Blood serum	Cyclone	amitriptyline, atomoxetine, citalopram, clomipramine, clozapine, descitalopram, desfluoxetine, desipramine, desmirtazapine, doxepine, dulozetine, fluoxetine, fluvoxamine, imipramine, maprotiline, mianserin, mirtazapine, norclomipramine, norclozapine, nordoxepine, nortryptiline, nortrimipramine, norvenlafaxine, paroxetine, protryptiline, reboxetine, sertraline, trazodone, trimipramine, venlafaxine	Clinic
Peake [25]	Blood	Cyclone-P	cyclosporine A, tacrolimus, and sirolimus	Clinic
Couchman [26]	Blood plasma or serum	C18	antifungal drugs: fluconazole, voriconazole, posaconazole, ketoconazole, itraconazole, hydroxyitraconazole, R051012	Clinic
Hervious [27]	Blood plasma	Cyclone P	irinotecan	Clinic
Helfer [28]	Blood plasma	Cyclone and Phenyl-	mixture A: morphine, levetiracetam, amphetamine, codeine, risperidone, diphenhydramine, quetiapine, promethazine, amitriptyline, sertraline, thiopental, diazepam, diclofenac, flufenamic acid; mixture B: moxonidine, hydrochlorothiazide, molsidomine, triamterene, minoxidil. Torasemide-M (HOOC-), bisoprolol, ivabradine, torasemide, ramipril-M (deethyl-), doxazosin, verapamil, aliskiren, amlodipine, ramipril, losartan, losartan-m (HOOC-), bezafibrate, spironolactone-M (carnenone), phenprocoumon, amiodarone-m (deethyl-), amiodarone	Clinic
Couchman [26]	Blood plasma or serum	Cyclone	TKIs	Clinic
Kasper [29]	Dried bloodspots	Cyclone P	enzymes for high-throughput screening of lysosomal storage disorders	Clinic
Frederiksen [30]	Urine	Cyclone P	biphenol A and other phenols	Clinic
Lopez-Garcia [31]	Urine	Cyclone P	neonicotinoids: imidacloprid, acetamiprid, clothianidin, dinotefuran, nitenpyram, thiacloprid, thiamethoxam, acetamiprid-N-desmethyl	Toxicology

Reference	Matrix	TurboFlow column	Analytes	Application
Lopez-Garcia [32]	Urine	Cyclone P	pesticides: organophosphates, pyrethroid insecticides	Toxicology
Helfer [33]	Urine	Cyclone and Phenyl-	toxins	Toxicology
Schaefer [34]	Urine	Cyclone MAX	opiates, cocaine, amphetamines, methadone, benzodiazepines	Forensic
Ates [35]	Wheat, maize, animal feed	Cyclone MCX-2	fusarium mycotoxins	Food quality
Ates [36]	Wheat, maize, animal feed	Cyclone MCX-2	plant and fungal metabolites	Food quality
Roach [37]	Infant formula	Cyclone MCX-2	melamine	Food quality
Mottier [38]	Honey	Cyclone	fluoroquinolones	Food quality
Lopez-Gutierrez [39]	Royal belly	Cyclone P	polyphenols	Food quality
Presta [40]	Wine	C18	flavonoids	Food quality
Fan [41]	Chinese cabbage and cucumber	C18	pesticides: imidacloprid, 3-hydroxycarbofuran, acetamiprid, pirimicarb, aldicaarb, propoxur, carbofuran, carbanyl, isoprocarb, metalaxyl, carbendazim, isazofos, diflubenzuron, chlorbenzuron, phoxim	Food quality
Hollosi [42]	Wheat flour and carrot-based puree	Cyclone MCX-2	48 polar, mid- and non-polar pesticides	Food quality
Bousova [43]	Chicken meat	Cyclone P	antibiotics	Food quality
Nathanail [44]	Cereal grains	Cyclone MCX-2	deoxynivalenol and its derivative	Food quality
Asperger [45]	Water and surface	C18, Phenyl, Cyclone (tested also SPE with Oasis HLB and porous graphitized carbon–Hypercarb)	pesticides: isoproturon, diuron, chlortoluron, atrazine, simazine, terbutylazine	Environment
Koal [46]	Water and surface	C18, Phenyl, Cyclone (tested also SPE with Oasis HLB and porous	28 pesticides (triazines, phenylureas, organophosphorous species and others)	Environment

Reference	Matrix	TurboFlow column	Analytes	Application
		graphitized carbon–Hypercarb)		
Lopez-Serna [47]	Water	Cyclone, Cyclone P, Cyclone MCX, C18, C18-P XL and Cyclone MAX	58 pharmaceuticals and 19 metabolites/ transformation products	Environment

Table 2.
Some applications of turbulent flow chromatography reported in literature.

colorectal cancer treatment, and its active and inactive metabolites, SN38 and SN38-G, respectively, in plasma after protein precipitation [27]. The same approach was applied for human plasma screening of various drugs that could be identified also below the therapeutic concentration [28]. Therapeutic drug monitoring of tyrosine kinase inhibitors (TKIs) is crucial for various cancers treatment. Early analytical methods involved HPLC-UV. However, not all TKIs present a good UV absorbance. TurboFlow LC–MS/MS was successfully used for the quantification of these compounds in a single analysis. In this protocol development, a precipitation step before online extraction was proven to maximize column life-time and minimize risk of autosampler blockage [14]. Multiplexed, multi-dimensional uHPLC–MS/MS was applied not only to the analysis of human plasma, but also to the high-throughput screening of lysosomal storage disorders in newborn dried bloodspots to obtain an online sample clean-up [29]. Determination of contaminants in urine, such as Bisphenol A and other environmental phenols [30], pesticides and metabolites [31, 32] or toxins [33] have been reported. Turbulent flow chromatography finds its application also in forensic toxicology. Mueller and colleagues developed a fully automated toxicological screening system for online urine extraction and analysis [48]. In the context of driving ability diagnostic, TurboFlow methods for the quantification of opiates, cocaine, amphetamines, methadone, and benzodiazepines have been validated in urine matrices [34].

Although the applications in drug monitoring are predominant, the use of TurboFlow is also reported in food and environmental quality. In fact, food products contain analytes in low levels, and other constituents, potentially interferents, such as sugars, proteins, and pigments in significantly higher concentrations, determining the necessity of sample clean-up and/or pre-concentration. The applicability of this technique in food analysis has already been proved in the determination of Fusarium mycotoxins [35] and of plant and fungal metabolites in wheat, maize, and animal feed [36], but also in the determination of melamine in infant formula [37]. A successful method has been developed and applied by Mottier and colleagues for the determination of 16 fluoroquinolones in honey, used to treat bee's bacterial diseases [38]. This technique allowed the reduction of extraction time and elimination of interferences in complex samples such as royal jelly and the determination of polyphenols [39]. A TurboFlow method was validated for the quantification of flavonoids and resveratrol in different types of wine. The authors suggested applying the method for the quantification of flavonoids, which could be correlated to the type of pesticides and of grapes

used to produce wine [40]. Online purification based on turbulent flow chromatography for the simultaneous quantification of multiple pesticides residues is reported in different matrices, such as Chinese cabbage and cucumber samples [41], grape, wheat flour, and carrot-based puree baby food [42]. Veterinary drug residues of 36 antibiotics from seven different chemical classes were identified and quantified in chicken meat, bringing the benefits of automation and cost-effectiveness [43]. The versatility in the analysis of different matrices has been confirmed in a study comparing conventional and online sample clean-up system for the determination of deoxynivalenol and its conjugated derivative, deoxynivalenol-3-glucoside, in cereal grains [44].

Recently, laboratories have begun to move towards alternative and greener methods for environmental analysis, in response to the growing awareness of more sustainable and environmentally friendly techniques. Online sample pretreatment responds to this trend, providing the development of effective approaches with low or no solvent and chemical consumption for the analysis of trace contaminants, such as pesticides. A TurboFlow method, for example, was successfully applied to the analysis of a wide spectrum of trace level pesticides, in drinking and surface water samples, taken from several sampling sites. Different TurboFlow columns were tested, showing that polymer-based columns offered the best performance [45, 46].

Pharmaceuticals are other relevant environmental trace contaminants. Their ecotoxicology is well known, while less is known about their metabolites and transformation products effects. Thus, multi-residues analytical methods are crucial to assess the risk of their presence in the environment. Furthermore, due to their low concentration, a pre-concentration procedure is often mandatory. Lopez-Serna and coworkers presented the development of an efficacious method for 58 pharmaceuticals and 19 metabolites/transformation products with an online pre-treatment based on turbo-flow chromatography in environmental aqueous samples. The combination of three TurboFlow columns in sequence showed the highest sensitivity [47].

6. Conclusion

Turbulent flow chromatography is a useful technique, able to remove potential interferences and to reduce preparation steps in an efficient way. In addition, the multiplex system allows to obtain faster results, by switching between different methods with minimum manpower, enhancing laboratory high-throughput productivity. Even if potentially heavy-matrix samples, as biological ones, could be directly injected into the TurboFlow column without any pre-purification, when performing quantitative analysis, in which the use of an internal standard is advisable, a simple addition of an organic solution of the internal standard to the samples could be performed, partially precipitating the protein content of the solution to be injected. In this way, the lifetime of the columns would be maximized. The resistance of the columns, granting hundreds of injections, and the possibility to integrate the TurboFlow to a pre-existent mass spectrometer, whose sensitivity is improved, guarantees also cost-effectiveness. Nonetheless, a high hardware and method development complexity have to be considered. This approach has only lately been increasing its applications, probably due to the complexity of the analytical methods development and optimization. Clinic and toxicology, where mass spectrometry must be handled even by not specialized laboratories, are the fields in which TurboFlow finds major application. However, TFC started to find use in food and environmental quality control, and it is intended to expand in these fields in the future.

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Author details

Francesca Di Gaudio[1], Annamaria Cucina[2*] and Sergio Indelicato[2]

1 PROMISE-Promotion of Health, Maternal-Childhood, Internal and Specialized Medicine of Excellence "G. D'Alessandro", Palermo, Italy

2 Chromatography and Mass Spectrometry Section, Quality Control and Chemical Risk (CQRC), Ospedali Riuniti Villa Sofia – Cervello, Palermo, Italy

*Address all correspondence to: annamariacucina1@gmail.com

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