A Review on Post-Column Derivatisation (PCD) using Active Flow Technology (AFT) in Reaction Flow (RF) Mode

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Post column derivatisation reactions coupled with HPLC is a powerful tool available to chemists. This technique is useful in the analysis of a wide range of compounds that do not react strongly to any of the detectors available for modern HPLC analysis. We present a discussion of common post column derivatisation (PCD) reactions along with the chemical and instrumental setups required for analysis as well as advantages and disadvantages of certain techniques where more than one PCD technique is available for the derivatisation of a given molecule. After this, we introduce a new advancement in HPLC-PCD analysis, namely the active flow technology (AFT) column operated in reaction flow (RF) mode. PCD applications of AFT-RF have so far been limited to a small range of derivatisation techniques, however, it has shown advantages over traditional techniques such as increased sensitivity, theoretical plate counts and resolution. These increases in performance are due to less peak broadening due to the ability to reduce post column dead volume. It is anticipated that these advantages will be duplicated in any PCD reaction where the AFT-RF column is utilised. An additional advantage of AFT-RF over conventional PCD techniques is the ability to monitor the central, underivatised, reaction stream as well as the derivatised stream coming from the outer radial region of the AFT column, enabling multiplexed detection which can be used to extract extra information about the target molecule(s). The use of the AFT-RF column is a new technique that has the ability to improve the observed separation efficiency in a range of traditional PCD techniques.

1. Introduction

Derivatisation reactions coupled with HPLC are a powerful tool available to chemists that are useful in the analysis of a large range of compounds. There are many compounds that can be separated by HPLC that do not yield strong signals to any of the detectors available for modern HPLC analysis [1]. To analyse these compounds by HPLC, derivatisation is necessary. Derivatisation techniques in HPLC can be split into two broad groups with the basic difference between the techniques being whether the derivatisation occurs before or after the separation. In pre-column derivatisation, the target molecule is derivatised prior to injection into the HPLC while in post column derivatisation, the target molecule is derivatised after separation, in between the column and the detector.

Compared to pre-column derivatisation, there are a number of advantages in the use of post-column derivatisation, namely, the technique is easier to automate and often requires less manual manipulation of the sample, peaks due to derivatisation reagents are not observed, less stable derivatives may be utilised as the derivatives need only to be stable until they are detected not for the entirety of the analytical run and management of the effluent flows allows for multiplexed detection of both derivatised and underivatised molecules. However, there are also several disadvantages of post column derivatisation techniques such as the need for extra hardware including reagent pumps and reaction loops, the additional post column dead volume of reaction loops and the resultant peak broadening and hence loss of separation power, the fact that larger quantities of reagent often need to be utilised, higher background signal caused by the mixing of effluent and reagent streams and the need for fast reaction kinetics for the derivatisation reaction.

There are a number of requirements for the successful application of a PCD system which have been described by Pickering [2] and need to be optimised for successful implementation of a PCD reagent. Pickering explained that the derivatisation reagent must be stable in solution for the course of the analytical run and the reaction should be reproducible as without this quantification is impossible. Furthermore, the reaction kinetics should be relatively fast allowing for the minimisation of reaction loop volume along with the associated peak broadening while maintaining a large signal. Additionally, the derivative should be stable enough so that it does not degrade prior to detection allowing for the maximisation of the analyte signal. Another requirement is for the underivatised reagent to show minimal detector response as a reagent with high detector response is a constant source of noise, possibly swamping the signal of the analyte peak(s). An additional requirement is that all components of the mixture must remain soluble in the solvent(s) used in the derivatisation system so the lines and detectors do not become blocked and potentially damaged. Finally, the

mobile phase and derivatisation reagent must be readily miscible with each other as improper mixing may cause refractive index discontinuities that can lead to increased detector noise.

In this article we highlight a new innovation in HPLC-PCD techniques, namely, active flow technology (AFT) columns in reaction flow (RF) mode. In this technique the derivatisation reagent(s) are pumped against the direction of mobile phase flow into the end fitting of the AFT column. Inside the end fitting of the column, the derivatisation reagent(s) are mixed with the effluent stream in a frit. This mixing process is more efficient than conventional mixing techniques, allowing for the minimisation of post column dead volumes and therefore improved observed separation efficiency. Three applications where RF-PCD techniques have been utilised [3-5] are presented as examples of the advantages of RF-PCD compared to conventional techniques. Finally, we also briefly discuss the detection of the underivatised central stream in RF-PCD techniques, which enables multiplexed detection using multiple destructive methods and/or without detection delay.

2. Post-column Derivatisation Techniques for Compounds

PCD techniques have been used for the analysis of a wide range of compounds. The choice of derivatisation regent and technique is mostly dependent on the availability of functional groups for reaction, however, other factors such as the mode of detection, matrix effects and the availability of hardware also play a role in the choice of derivatisation technique. A range of common PCD reactions are listed in Table 1 along with typical reaction conditions. Included in Table 1 are derivatisation regents for the analysis of a wide range of compounds such as amines, antioxidants, thiols, metals, antibiotics and toxins. Also included are a number of common derivatisation regents used for chemiluminescence detection. It should be noted that a number of the reactions included in Table 1 such as the derivatisation of amines using the ninhydrin reagent are not technically derivatisation reactions since it is not a tagged form of the target molecule that is detected, but an altered version of the derivatisation regent.

A second class of PCD reactions not included in Table 1 are reagentless PCD derivatisation methods, which use a physical process, such as, electromagnetic radiation or electrochemical processes to

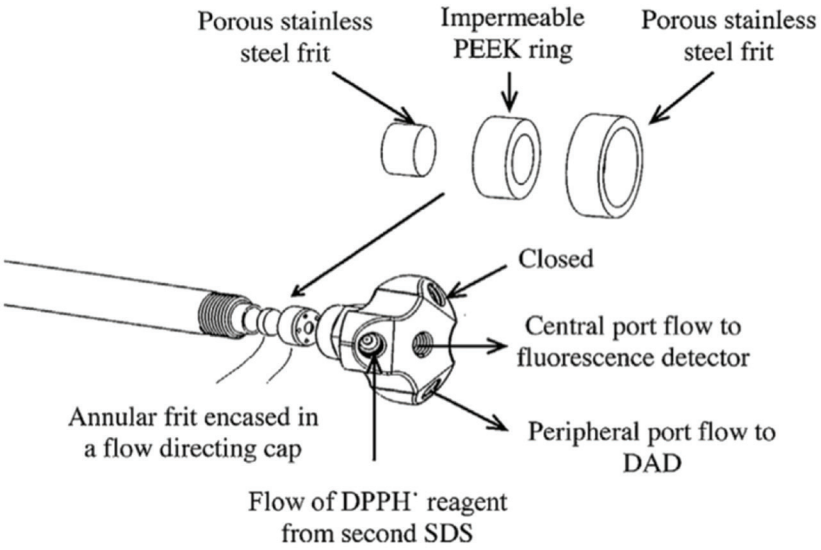

Figure 1: Illustration of AFT-PSF column end-fitting design consisting of flow separating frits and a multiport end-fitting. An example application of a single reagent AFT-RF system using DPPH•, multiplexed with fluorescence detector [3].

derivatise the analyte resulting in enhanced detection capabilities. Even though these methods are applicable to a relatively small range of compounds, their use is wide spread due to their simplicity and robustness. They have advantages over conventional PCD techniques as there is no use of PCD reagents so issues, such as,

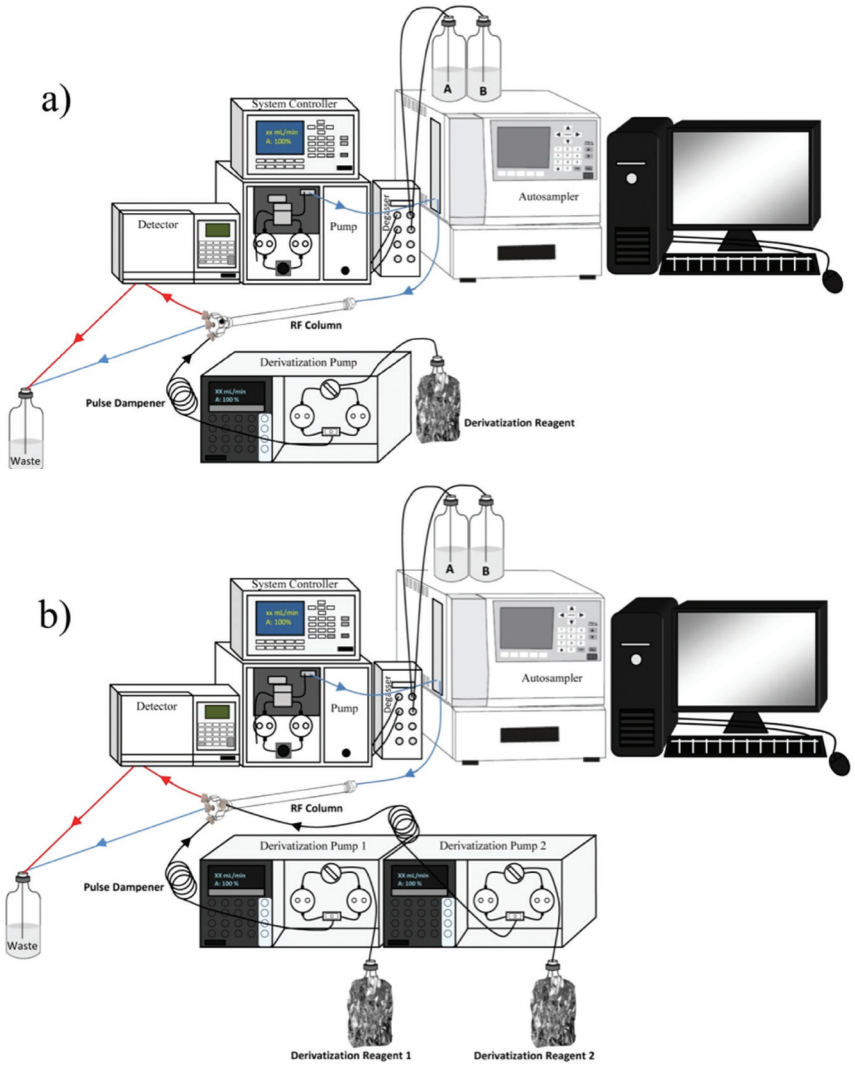

Figure 2: Illustration of typical instrumental setups in reaction flow chromatography using (a) a single reagent derivatisation setup and (b) a dual reagent derivatisation setup.

Table 1: Common post column derivatisation reactions along with typical reaction conditions.

Derivatisation Reagent	Target analyte	Detection Mode	Typical Detection Wavelength (nm)	Typical Reaction Loop Volume (µL)	Typical Reaction Temperature (°C)	Other Notes
Ortho-phthalaldehyde (OPA)	Primary amines or thiols	Fluorescence	340 excitation, 450 emission	200 - 1000	Room Temperature	Reaction requires the presence of both a primary amine and a nucleophilic agent. Secondary amines can be detected after chemical oxidation or UV decomposition
Fluorescamine	Primary amines	Fluorescence	390 excitation, 475m emission	< 200	Room Temperature	Secondary amines can be detected by UV absorption or after chemical oxidation
Ninhydrin	Primary and secondary amines	Visible absorbance	570 (primary amines), 440 (secondary amines)	> 500	85 - 130	
Benzofuran analogues	Amines, thiols, carboxylic acids, ketones and aldehydes	Fluorescence	460 excitation, 510 emission	> 1000	> 50	
Dimethylaminobenzaldehyde (DMAB)	Primary amines	Fluorescence	340 excitation, 390 emission	> 1000	Room Temperature	
1,2-Naphthoquinone-4-sulfonate (NQS)	Primary amines	Fluorescence	260 excitation, 435 emission	300 - 1000	60 - 80	
2,2-diphenyl-1-picrylhydrazyl radical (DPPH•)	Antioxidants	Drop in visible absorbance	520	20 - 1000	Room Temperature	
2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical (ABTS•+)	Antioxidants	Drop in visible absorbance	735	20 - 1000	Room Temperature	
Ferric reducing antioxidant potential (FRAP)	Antioxidants	Visible absorbance	593	1000	Room Temperature	
Copper reducing antioxidant potential (CuRAP)	Antioxidants	Visible absorbance	450	1000	Room Temperature	
Folin–Ciocalteu assay	Antioxidants	Visible absorbance	754	500	Room Temperature	
Alkyl esters or propionic acid	Thiols	UV absorbance	280 – 290	< 200	Room Temperature	2 step reaction process
Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB)	Thiols	Visible absorbance	412	> 1000	Room Temperature	
Iodide-Azide	Sulphur (II) contining Compounds	Drop in UV absorbance	350	20 - 1000	Room Temperature	Azide typically added to mobile phase, iodine added post column
Xylenol orange	Metals	Visible absorbance	618	< 100	Room Temperature	
Eriochrome black	Metals	Visible absorbance	512 or 640	< 100	Room Temperature	
Porphyrins	Metals	Visible absorbance	400 – 450	< 100	Room Temperature	
2-[(5-bromo-2-pyridil)-azo]-5-diethyl-aminophenol (5-Br-PADAP)	Metals	Visible absorbance	560 - 565	< 100	Room Temperature	

4-(2-pyridylazo) resorcinol (PAR)	Metals	Visible absorbance	520 - 530	< 100	Room Temperature	
1-(2-pyridylazo)-2-napthol (PAN)	Metals	Visible absorbance	510 – 530	< 100	Room Temperature	
Vanillin	Polyether antibiotics	Visible absorbance	520	1500 - 2000	100	
Iodine	Alfatoxins	Fluorescence	365 excitation, 440 emission	50 - 1000	60 - 90	
Bromine	Alfatoxins	Fluorescence	365 excitation, 440 emission	100	Room Temperature	Bromide prepared in situ using an oxidative current
Periodic acid	Paralytic shellfish toxins	Fluorescence	330 - 340 excitation, 390 - 410 emission	1000 - 2000	70 - 85	2 step reaction process
Acetoacetate	Trichothecene mycotoxins	Fluorescence	370 excitation, 460 emission	3000	115	2 step reaction process
2-Cyanoacetamide	Reducing carbohydrates and various others	Fluorescence	330 - 400 excitation, 370 - 450 emission	1500	60 - 100	2-Cyanoacetamide in mobile phase, post column addition of base to facilitate reaction
Potassium Iodide and Ammonium Molybdate	Bromate	UV absorbance	352	350 - 500	Room temperature up to 80°C	2 reagents combined prior to addition to the effluent stream
Luminol	Chemiluminescent derivatives	Chemiluminescence	425	< 100	Room temperature	
Tris(2,2'bipyridine) rythenium(III) (Ru(bpy)32+)	Chemiluminescent derivatives	Chemiluminescence	620	< 100	Room temperature	
Peroxylates	Chemiluminescent derivatives	Chemiluminescence	460 - 630	< 100	Room temperature	
Potassium permanganate	Chemiluminescent derivatives	Chemiluminescence	730 – 740	< 100	Room temperature	

derivatisation reagent degradation and mixing are avoided. Furthermore, these techniques can be combined with reagent based derivatisation techniques to provide an additional powerful tool that can be used to perform more complex multi-step processes to form a wider array of derivatised compounds. Post column dead volumes vary in reagentless derivatisation schemes and are dependent on the kinetics of the reaction in question, although they are broadly similar to those utilised in reagent based PCD schemes. Like reagent based PCD schemes, some reagentless derivatisation systems employ a heated reaction coil in order to enhance the speed of the derivatisation reaction.

3. Reaction Flow Chromatography

3.1 Active Flow Technology

Active Flow Technology (AFT) is a new column format designed to improve separation performance by overcoming the issues associated with column bed heterogeneity. AFT columns utilise a special purpose built outlet end-fitting composed of a multi-port end-fitting and a three component annular frit, this is known as Parallel Segmented Flow (PSF) mode [6, 7]. Figure 1 illustrates the frit design of an AFT-PSF column [3]. The inner frit is an impermeable annular ring that separates the central flow region from the wall flow region, preventing cross flow between regions. The central flow exits via the central port and the wall flow exits via the peripheral ports. The relative portion for these two streams can be adjusted to any ratio through pressure management for the optimisation of various functional aspects of column technology. There are numerous advantages of AFT columns, primarily: (1) central and peripheral flow regions are isolated [7], (2) analyte to mobile phase ratio is increased allowing increased sensitivity for light attenuating detectors [8], (3) multiplexing capabilities [9, 10] and (4) a platform for post-column derivatisations within the end-fitting known as reaction flow (RF) chromatography [3].

Figure 2 illustrates the instrumental setup and regent flows employed when using RF chromatography. Setups for both a single regent stream (figure 2a) and a dual reagent stream (figure 2b) have been shown.

3.2 AFT in RF mode

AFT in RF mode (AFT-RF) employs the same multi-port end fitting design as AFT-PSF. Reaction flow chromatography that is AFT-RF, is the introduction of derivatising reagents, using a reagent pump, into one or two of the outer peripheral port(s) of the AFT-RF column end-fitting against the direction of mobile phase flow. The column eluent is mixed with the derivatisation reagent(s) in the outer frit and is passed to the detector through a free outer port. Reaction flow can be used for either a single reagent derivatisation (1 port for the derivatisation reagent, 1 port to pass the

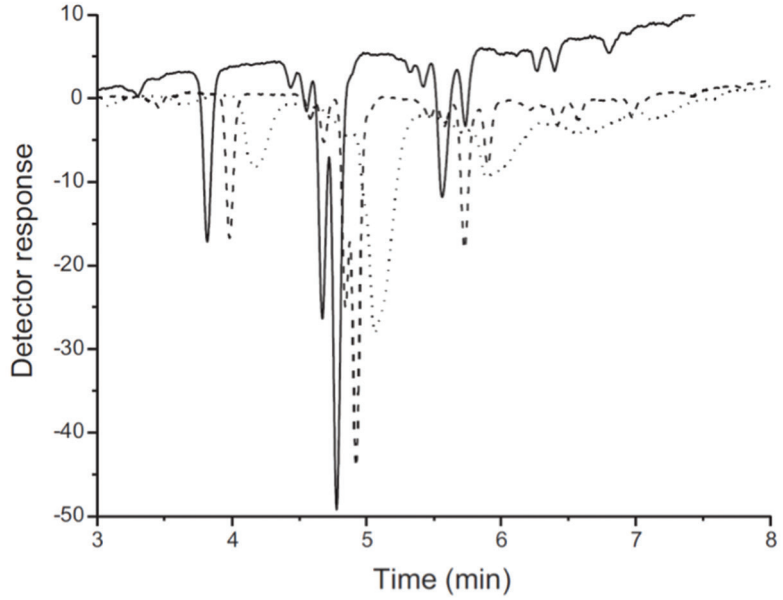

Figure 3: Chromatographic separation of antioxidants in coffee obtained using DPPH• with AFT-RF column with no reaction coil (solid trace), a standard conventional column with a 500 µL reaction coil (dotted trace) and a 50 µL reaction coil (dashed trace) [3].

column eluent to the detector and 1 port blocked) or a dual reagent system (2 ports for the derivatisation reagents and 1 port to pass the column eluent to the detector). The peripheral flow carries the derivatised analytes to the detector, whereas the central flow remains in its native form, underivatised. Camenzuli et al. have shown the effectiveness of the AFT end-fitting as a mixing device for the reaction/derivatisation to take place without the use of reaction coils [3].

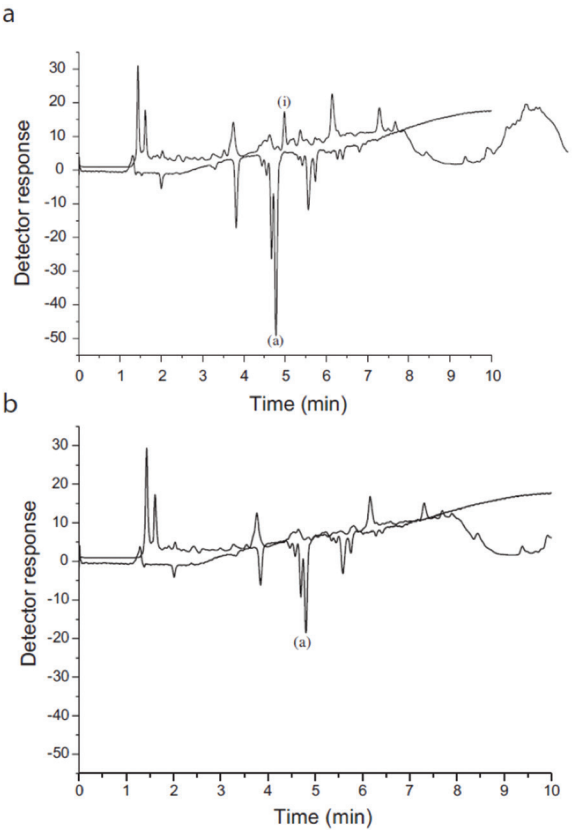

Figure 4: Chromatographic representation of AFT-RF with DPPH• detection on the peripheral flow and fluorescence detection on the native central flow for (a) Ristretto Coffee and (b) Decaffeinato Intenso coffee. Peak (i) is caffeine only present in the fluorescence response for Ristretto coffee and peak (a) is an antioxidant compound present in both coffee samples that closely elutes with caffeine [3].

An example of AFT-RF using a single reagent is the application of DPPH• for the detection of reactive oxygen species (ROS), i.e. antioxidants, in coffee [3]. In this study the DPPH• reagent was introduced into the peripheral port where the reaction between analyte and reagent takes place, the derivatised analytes exit through an available port to the detector (the third unused port is blocked), see Figure 1. Figure 3 represents the chromatographic separation of antioxidants using AFT-RF with no reaction coils compared to conventional DPPH• detection using a 500 µL reaction coil, typical of many setups described in the literature and also a setup using a 50 µL reaction coil. The efficient mixing in the frit of the AFT end-fitting eliminated the need to employ reaction coils and thus improved the separation performance in terms of sensitivity, efficiency and most importantly resolution of antioxidant compounds. Another application of AFT-RF involved the use of a two reagent component reaction/derivatisation using 4-aminoantipyrene and potassium ferricyanide for the detection of phenols. An interesting facet that occurred in this application was the detection of a non-phenolic compound under AFT-RF conditions [5].

3.3 Multiplexing AFT-RF

The underivatised flow emanating from the central outlet port can also be directed to another detector for monitoring the native analytes in the effluent. Through this procedure reaction flow chromatography with multiplexed detection can be achieved. In a well-designed detection system, a single analysis with multiplexed detection can provide substantial information in regards to the nature of the components within the sample. Importantly, destructive and non-destructive tests can be conducted at exactly the same time, without detection delay.

There are a limited number of reports on the use of AFT-RF in multiplexed detection. The study mentioned above, in the analysis of antioxidant compounds in coffee, the RF component was DPPH• for derivatisation and fluorescence detection (FLD) was used for monitoring the underivatised central effluent [3]. Figure 4 is the chromatographic representation of AFT-RF with DPPH• detection of coffee samples multiplexed with FLD. Another study involved the multiplexing of AFT-RF with fluorescamine reagent for analysis of primary amino acid using a UV-Vis and a second UV-Vis detector for analysing the native central flow [4].

3.4 Advantages of multiplexing AFT-RF

The advantages of multiplexed AFT-RF can be summarised to the following points:

- AFT-RF provides efficient mixing in the column end-fitting allowing the incorporation of post-column derivatisation techniques.
- The elimination of reaction coils increases the separation performance in terms of sensitivity, efficiency and resolution, by reducing the band broadening effects.
- The system set up of AFT-RF has been found to be simpler than the conventional methods of PCD.
- The impermeable frit inside the end-fitting prevents cross flow between flow regions, allowing derivatisation to take place in the outer region of the frit while the central region remains native, ideal for multiplexed detection.
- Multiplexing with AFT-RF allows for rapid sample screening or characterisation, allows

for easy peak matching between detection methods, reduces run time, and two destructive detection methods can be used simultaneously.

4. Conclusions

There have been a wide range of derivatisation reactions developed for use in HPLC-PCD applications, which can be utilised to detect an array of compounds that are otherwise not detectable using the suite of detectors currently available. These reactions are wide and varied in their chemistries and instrumental setups. The AFT column has been developed to improve separation efficiency. RF techniques have made use of the multiple exit port fittings of the outer region of the end fitting to allow more efficient mixing of the derivatisation reagent(s) and the effluent stream inside the outer frit, allowing for the reduction in post column dead volumes. Although relatively few applications of the technique have been published, it has shown a number of advantages over conventional PCD techniques such as increased sensitivity, theoretical plate counts and resolution. These advantages arise from a reduction in peak broadening as post column dead volume can be reduced. The large number of PCD applications in use today gives an insight into the number of analyses that may be improved due to the introduction of the AFT-RF technique. It is anticipated that the performance gains of the AFT-RF technique that have been noted in those reactions that have been currently tested will be applicable to any PCD technique that ATF-RF is used for. A final advantage of AFT-RF over conventional PCD techniques is the ability to monitor the central, underivatised, eluent stream as well as the derivatised stream coming from the outer radial region of the AFT column, enabling multiplexed detection which can be used to extract additional information about the target molecule(s). AFT-RF is a new development in the field of HPLC-PCD techniques that has so far only been trialled on a limited range of derivatisation reactions, but has shown great promise and advantages over conventional PCD techniques.

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