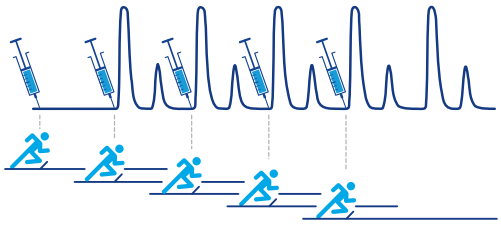
Science with Passion

KNAUER

Inject, collect, repeat - Stacked injection made easy



J. Menke, G. Greco, U. Krop, P. Slaby; menke@knauer.net

KNAUER Wissenschaftliche Geräte GmbH, Hegauer Weg 38, 14163 Berlin; www.knauer.net

SUMMARY

Stacked injections provide a straightforward means to increase productivity and improve efficiency in preparative chromatography. By nesting multiple injections within a single batch run, this approach maximizes throughput and minimizes downtime between peak collections. As a result, both time and solvent consumption are reduced. Furthermore, the automated fraction collection enhances ease of use and ensures high reliability. PurityChrom® 6 enables a simplified implementation of stacked injections and supports a variety of injection devices, such as autosamplers or sample pumps.

INTRODUCTION

For preparative LC applications solvent consumption is oftentimes high whereas obtaining a decent yield is rather challenging. A way to improve both are stacked injections. Using this method system downtime can be minimized thus leading to a more efficient overall purification process. The main aim of running stacked injections is to use the column bed more efficiently by reducing non elution times, whilst retaining peak purity. Stacked injections can be an alternative as well if a column overload with target compound is desired but not possible without coelution of impurities or matrix compounds. The sample is loaded in such volumes, that sufficient target compound resolution is still achieved. Usually the yield would be minimal, but via stacked injection multiples of the actual sample volume are repeatedly injected in one long run. For this method high robustness is needed, especially regarding retention time shifts. With a well-designed method, injection cycles can be repeated almost indefinitely which allows a semi-continuous process. Large batches of sample can be automatically processed without losing product purity. A typical use case for stacked injections are chiral separations of enantiomers. Here, expensive chiral stationary phase material can be saved using a smaller column dimension but sample throughput can be kept up using stacked injections.

Here we describe the efficient separation of caffeine and paracetamol using stacked injections with an autosampler or a sample pump. Manually programming stacked injection methods is oftentimes time-consuming and complicated. The PurityChrom® 6 software includes a stacked injection function which is described in detail in this application. It provides a tool for a dynamic method writing, making it easy to adjust cycle times and cycle numbers.

Inject, collect, repeat - Stacked injection made easy

SAMPLE PREPARATION

An exemplary sample mixture of 2 mg/mL caffeine and 12 mg/mL paracetamol (acetaminophen) was prepared in 20/80 ethanol/water (v/v) and filtered through a 0.45 µm RC-membrane (regenerated cellulose).

WORKFLOW FOR ESTABLISHING A STACKED INJECTION METHOD

A requirement for stacked injections is an isocratic method with all peaks eluting in proximity. Ideally the time frame between injection and target peak is free of impurities. Consequently, an impurity eluting with a long delay after the target peak makes stacked injections challenging if not impossible. Matrix compounds that elute without interacting with the stationary phase must be removed beforehand. Technical requirements for stacked injection are an automated sample injection system like a sample pump and an automated injection valve or an autosampler and a fractionation system to establish a stacked injection method 5 steps are necessary and descried in detail below:

Single Injection

First a single measurement with an existing isocratic method is performed **(Fig. 1)** Injection volume and sample concentration should match the parameters that are desired for the later stacked injection process. This is the base for the determination of the cycle time

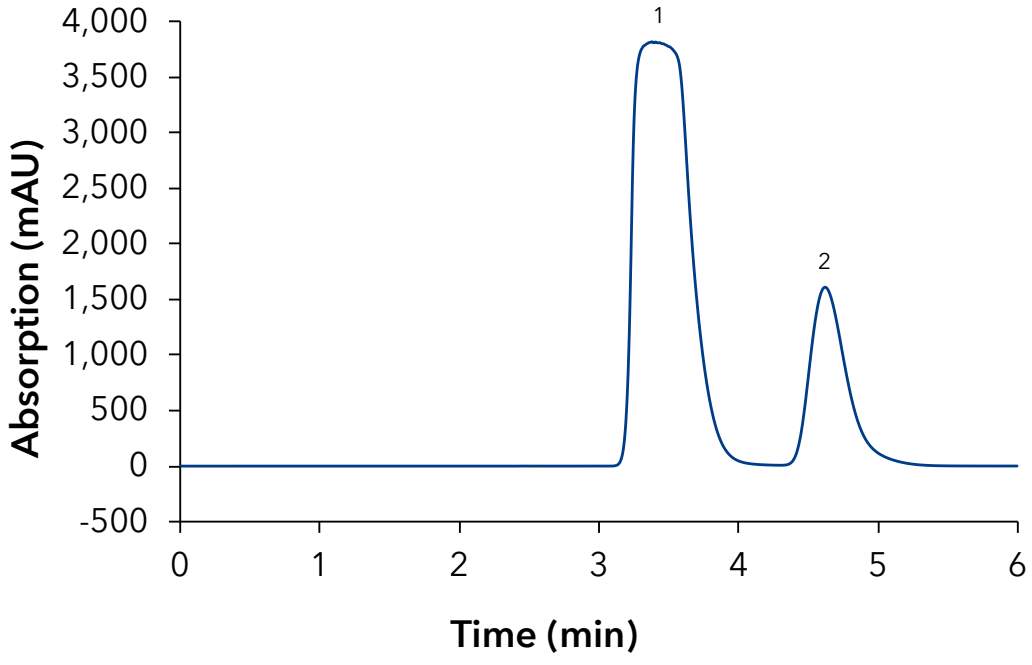


Fig. 1 Single injection of 500 µl sample; (1) paracetamol; (2) caffeine

Determination of cycle time and duration of injection

The cycle time defines the time between two injections. A baseline separation should be achieved (resolution > 1.7). To find a suitable cycle time, the single injection measurement chromatogram and a copy of the same chromatogram can be opened in compare overlay mode. The copy is then moved until the last peak of the original and the first peak of the copy are not overlapping anymore.

The time offset of the second chromatogram is the cycle time (see **Fig 2)**. The duration of the injection is shown in the measured chromatogram.

It is dependent of the chosen injection volume. To save time and solvent, an autosampler is set up to wash only between vials.

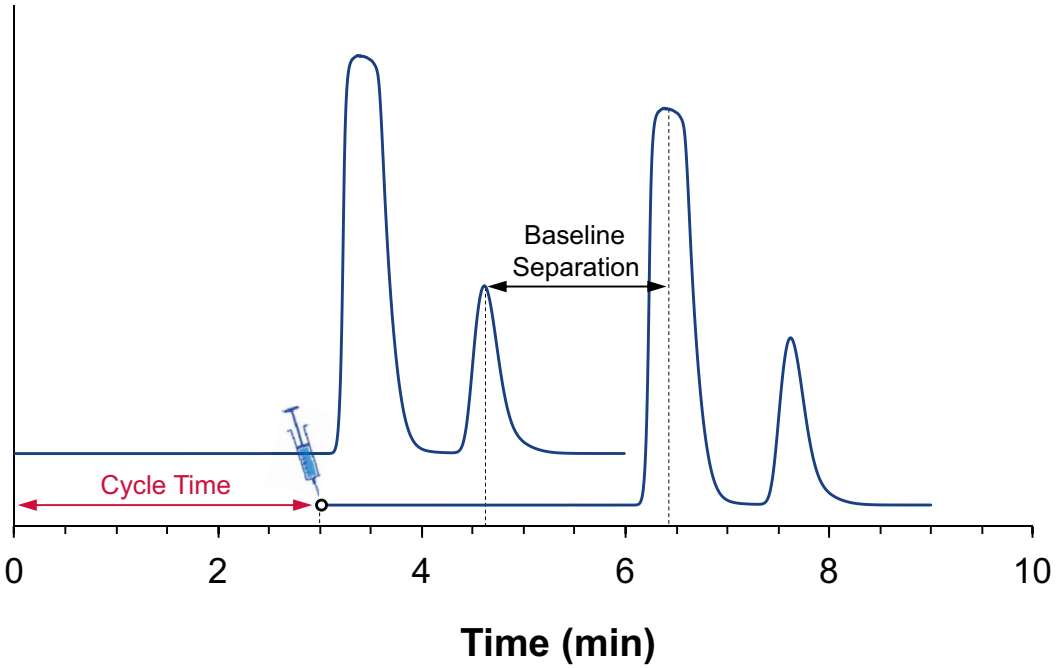


Fig. 2 Determination of cycle time

Preparing the stacked injection method

After running an initial single injection method, the software provides a dynamic approach to adjust for stacked injections. The single injection method needs to be split into 5 steps. To do so, more steps are added in the method procedure. These steps are conditioning, prepare injection, injection, separation and finish. When adjusted the user only needs to specify the desired number of injections and the method adjusts runtime and injection times automatically.

Inject, collect, repeat - Stacked injection made easy

Conditioning

The "conditioning" step is a command step, that prepares the system for the upcoming run. It is started as soon as the sequence is loaded. Typically, the system main pumps are activated, the solvent composition is set, the detector wavelength and data rate are set and an autozero is performed. The injection device is initialized. In case of an autosampler, the injection mode is chosen **(Fig. 3**A). In case of an automated valve, it is switched into the load position **(Fig. 3**B).

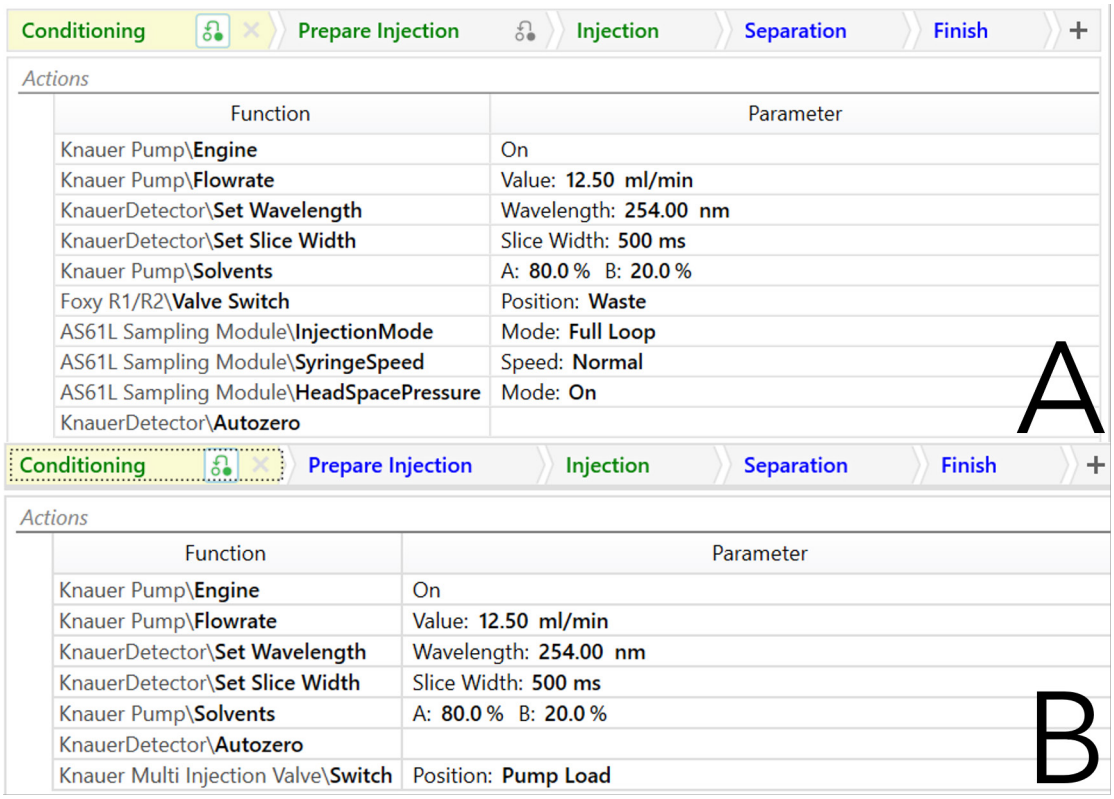


Fig. 3 Conditioning settings; A: autosampler; B: sample pump

Prepare Injection

The "Prepare Injection" step assures, that the sample is loaded, before the next injection is triggered. In case of an autosampler it is a command step with the command "Autosampler Sampling Module\Prepare Injection" together with a wait condition – "Autosampler Sampling Module\Status = ready". The autosampler will state ready as soon as the sample volume is aspirated and loaded into the loop and proceed to the next step **(Fig. 4**A).

In case of loading with a sample pump the "Prepare Injection" step needs to be a timetable instead of a command step. The injection valve is switched to the load position, the sample pump is started and run with suitable flow rate and duration to load the sample loop volume sufficiently. Thereafter, the sample pump is stopped **(Fig. 4**B)

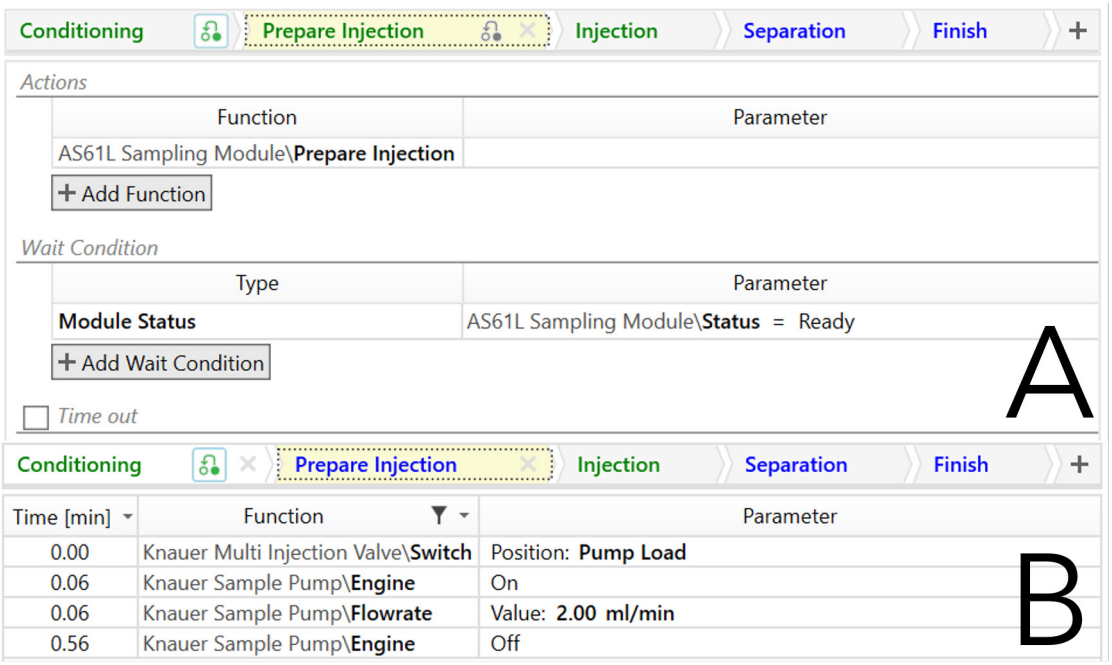


Fig. 4 Prepare injection settings; A: autosampler; B: sample pump

Inject, collect, repeat - Stacked injection made easy

Injection

In the "Injection" command step, the sample is introduced into the system. The autosampler valve or the injection valve are switched into the inject position (**Fig. 5**A).

In case of an autosampler a wait condition is added - "Autosampler Sampling Module\Status = Injected". This ensures that the full sample volume is introduced, before the separation continues (**Fig. 5**B).

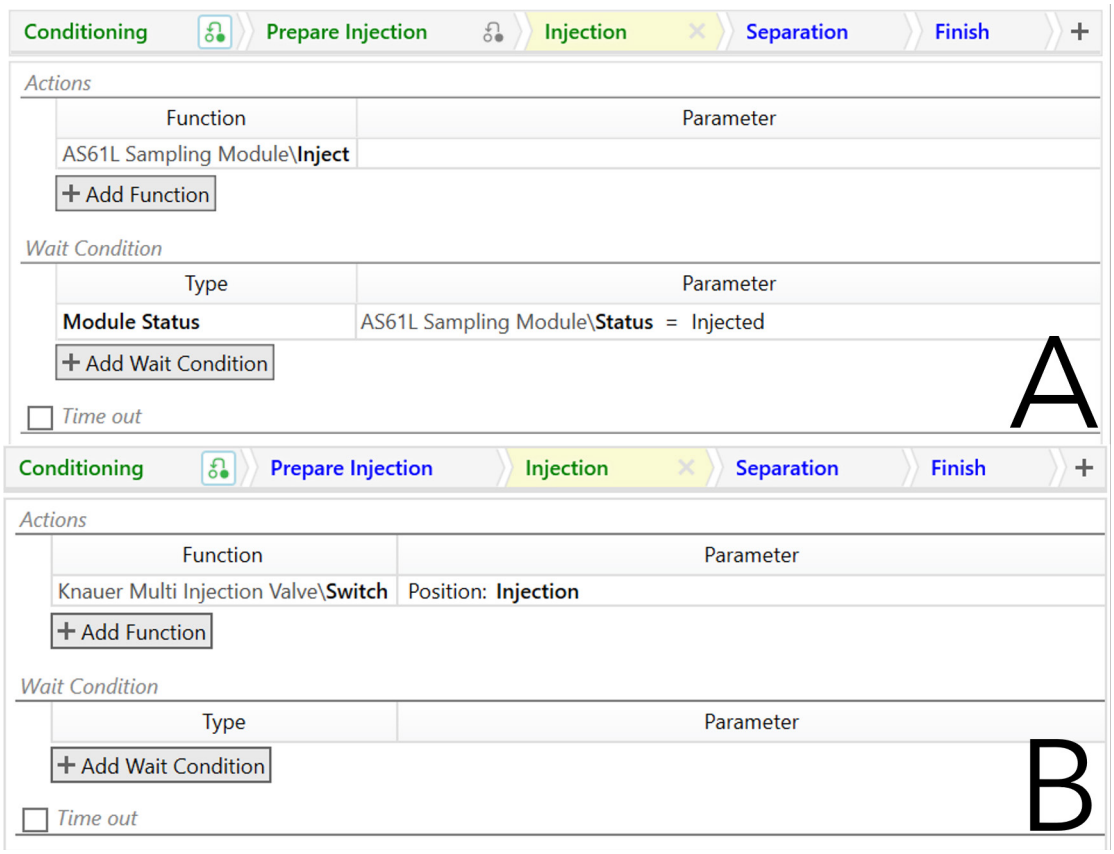


Fig. 5 Injection settings; A: autosampler; B: sample pump

Separation

The "Separation" step contains all the remaining functions originating from the single run method in a timetable. Here, the chromatogram is started and ended, the fractionation is programmed as well. For fractionation several options are possible. To fractionate each peak into its own container, a threshold on the UV-signal can be used. The threshold overstepping action switches the fraction collector valve to the fraction position. As understepping actions the fraction collector valve switches back to waste, and the collector is sent to the next position. If the target substances should be pooled immediately to the same vessel, the peak window function can be used. Every Peak gets its own peak window, where a collector position is stated. The "Separation" timetable is the step, that will be dynamically prolonged, depending on the number of stacked injections.

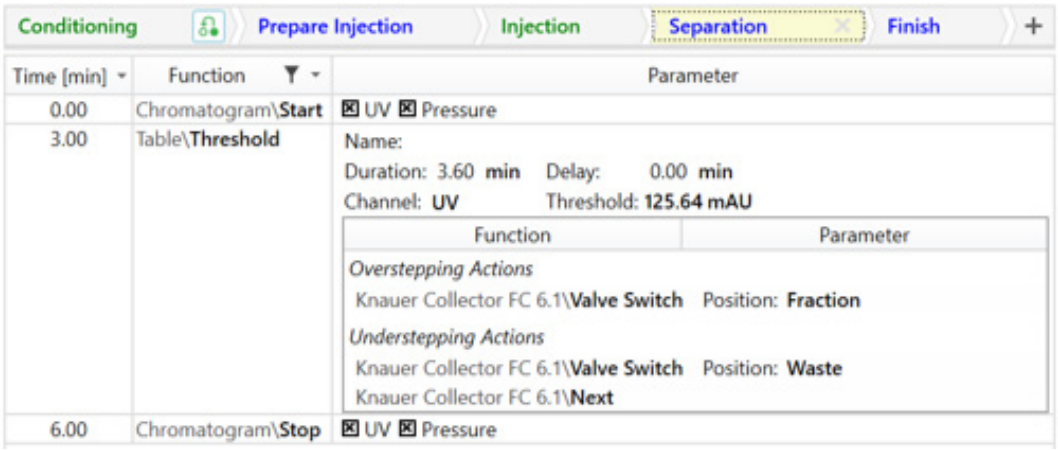


Fig. 6 Separation settings

Inject, collect, repeat - Stacked injection made easy

Finish
Last, the "Finish" step is used to end the run. It is a short timetable that typically stops all devices. It is run as soon as the last separation step has ended.

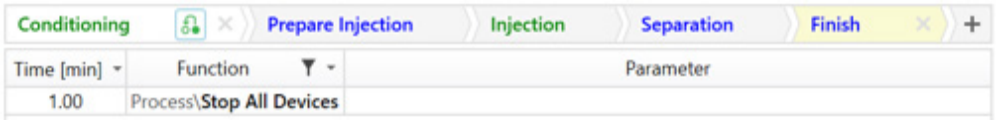


Fig. 7 Finish settings

Preparing a sequence for stacked injection
Once the stacked injection method is written, a new sequence file is created. In the sequence tool bar, "Stack Injections" is activated to get access to the stacked injection settings **(Fig. 8)**. A new line in the sequence table is created, that states the stacked injection method created beforehand. The desired number of injections are entered. The sample gets a Sample ID. In case of autosampler injection, the desired sample volume is entered as well.

In the stacked injection settings, the cycle time previously determined is entered. The "Load Before Inject" time is used to state when new sample volume should be loaded. It needs to be adjusted according to the time needed to load the desired sample volume with the injection device of choice.
The "Preparation Step", "Load Step", "Inject Step", "Cycle Step" and "Finish Step" are matched with the respective steps from method, using the dropdown menus.

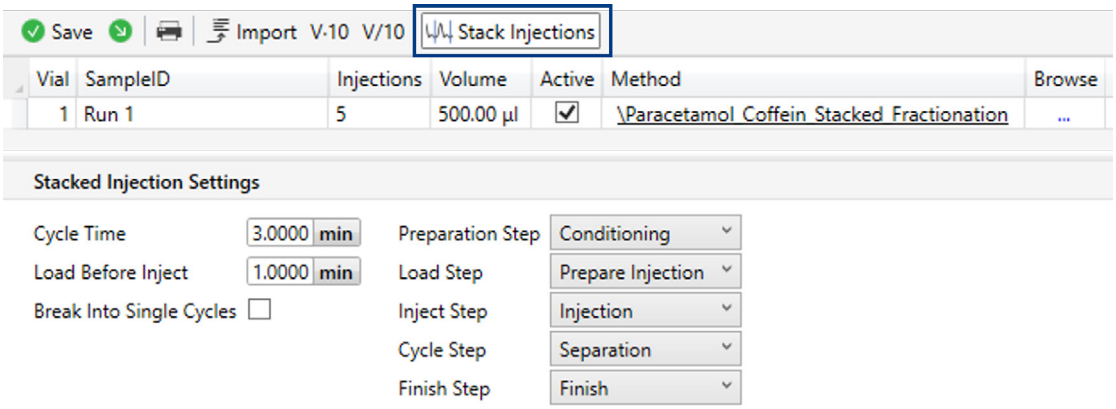


Fig. 8 Sequence settings

RUNNING STACKED INJECTIONS

At last, the sequence is started from the purification window. The conditioning step begins immediately. As soon as the start button is pressed, the stacked injection run will be performed automatically.

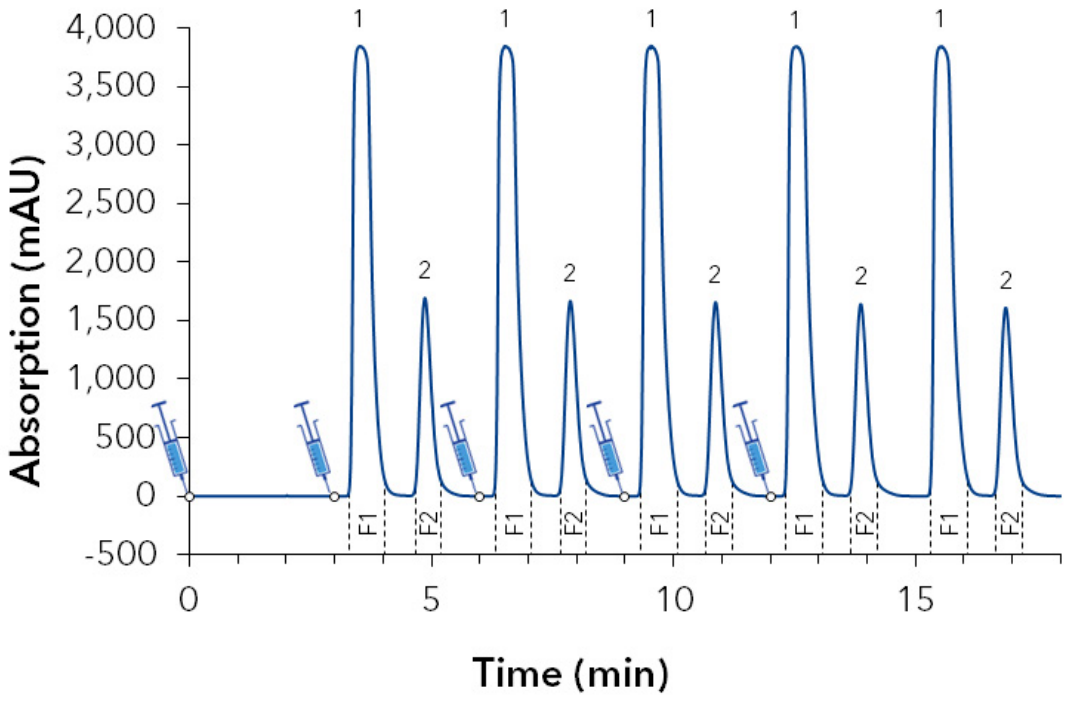


Fig. 9 5x stacked injections, (1) paracetamol, (2) caffeine

Inject, collect, repeat - Stacked injection made easy

RESULTS

The single injection showed good separation of paracetamol and caffeine. A cycle time of 3 minutes was determined. A sequence with 5 stacked injections was measured. All peaks still showed baseline separation. Comparing the necessary time for both single runs and stacked injections, the sample throughput was increased in this example by 40%, which consequently means a 40% reduction of eluent usage. In the time necessary for 3 single runs, 5 stacked injections were performed (see **Fig. 10**).

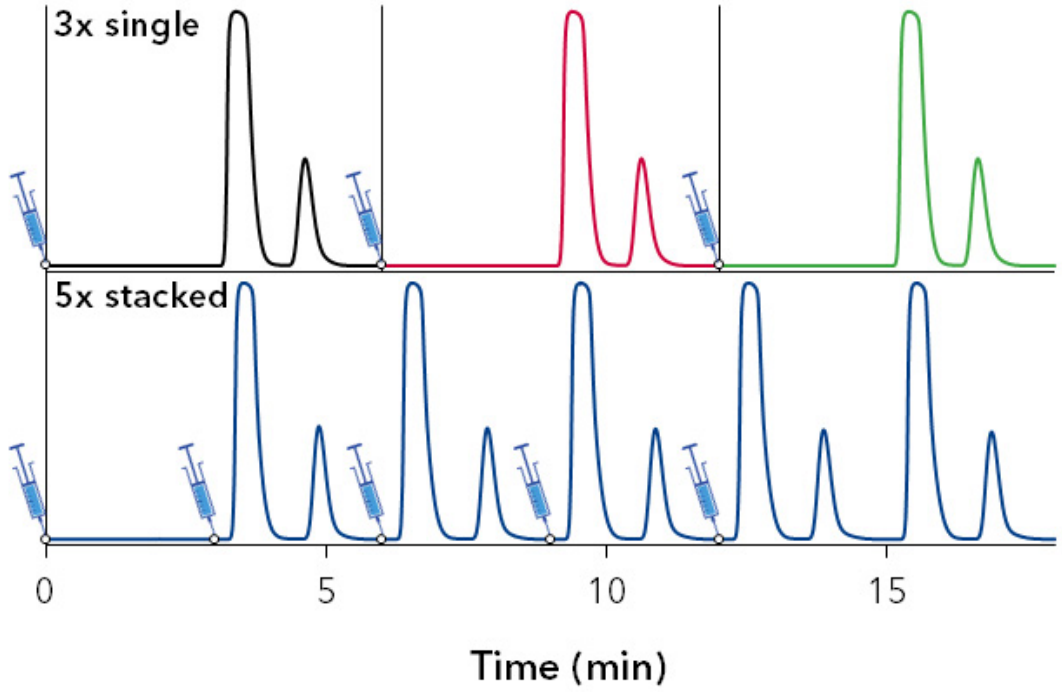
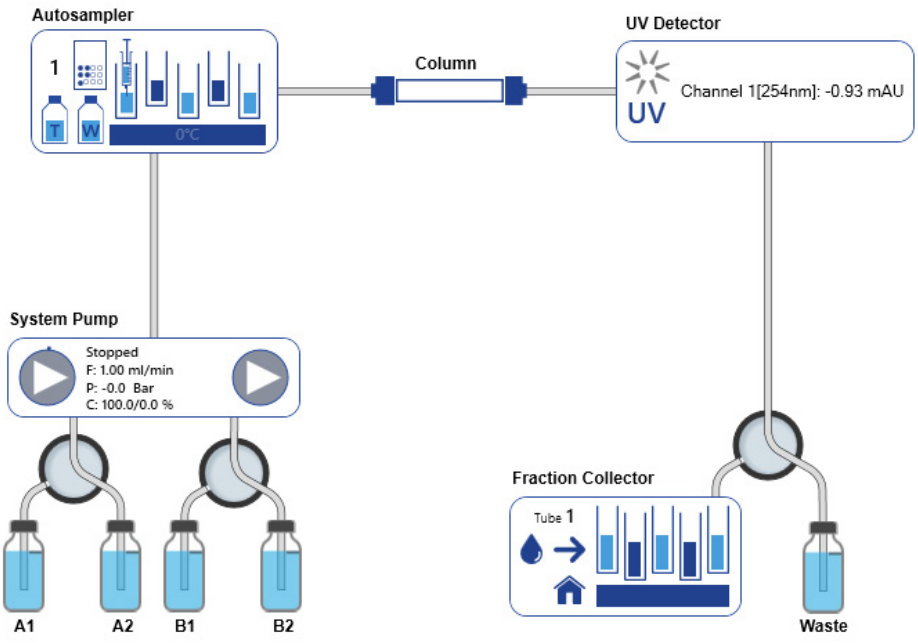


Fig. 10 Time comparison of single injection vs. stacked injection

SYSTEMS

Tab. 1 Semi-preparative system with autosampler

Instrument	Description	Article No.
LC system organisation	AZURA® Eluent tray E 2.1L	AZC00
Pump	AZURA® P 6.1L High Pressure Pump with 50 ml pump head, stainless steel, without Degasser	APH38FA
Autosampler	AZURA® Autosampler AS 6.1L (retrofitted with kit for large injection samples)	AAA50AA
Upgrade Kit	Kit for large injection samples	A50079
Detector	AZURA® UV/VIS Detector UVD 2.1L	ADA01XA
Flow Cell	Semi-preparative UV Flow Cell, 3 mm path length, 1/16", 2 µl volume, 300 bar, stainless steel	A4042
Fraction Collector	AZURA® FC 6.1 BIO Fraction Collector	AFA00
LC system organisation	Mounting bracket AZURA® L for columns with 25 - 29 mm AD	A9853-3
Column	Eurospher II 100-10 C18, Column 150x20 mm	15JE181E2N
Tubing	Classic capillary for HPLC (300 cm, 0.5 mm ID with 1/16" connections)	A0132



Inject, collect, repeat - Stacked injection made easy

Tab. 2 Semi-preparative system with sample pump injection

Instrument	Description	Article No.
LC system organisation	AZURA® Eluent tray E 2.1L	AZC00
Pump	AZURA® P 6.1L High Pressure Pump with 50 ml pump head, stainless steel, without Degasser	APH38FA
LC module docking station	AZURA® Assistant ASM 2.2L Left module: UV detector UVD 2.1S Middle module: Valve drive VU 4.1 Right module: Pump P 4.1S, 10 ml, stainless steel	AYASM EDA03XA AWA04 APG22EA
Flow Cell	Semi-preparative UV Flow Cell, 3 mm path length, 1/16", 2 µl volume, 300 bar, stainless steel	A4042
Valve	AZURA® V 4.1 Valve, Multi-injection valve, DLC stainless steel 1/16"	AVN96AE
Fraction Collector	AZURA® FC 6.1 BIO Fraction Collector	AFA00
LC system organisation	Mounting bracket AZURA® L for columns with 25 - 29 mm AD	A9853-3
Column	Eurospher II 100-10 C18, Column 150x20 mm	15JE181E2N
Tubing	Classic capillary for HPLC (300 cm, 0.5 mm ID with 1/16" connections)	A0132