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Long-term robustness of thiopental Ph. Eur. method performance on modern HPLC instrumentation

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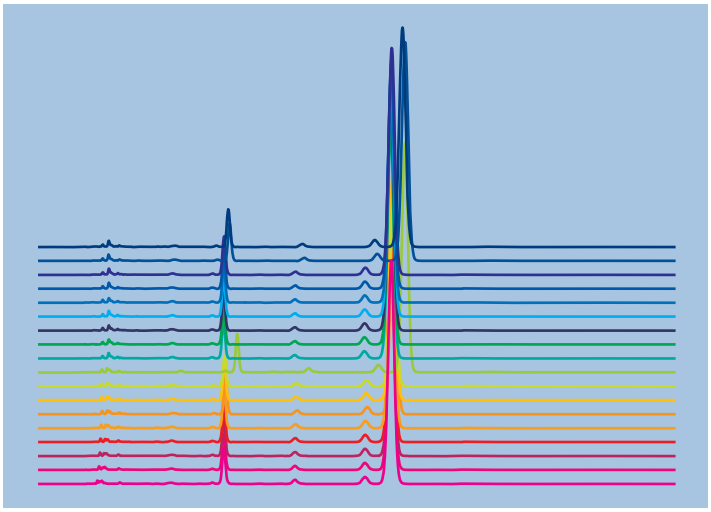

Demonstrated benefits

The results of a long-term robustness study run on a Thermo Scientific™ Vanquish™ Core Quaternary HPLC system show excellent intra-sequence reproducibility and merely minor variations of retention times and peak area over a timespan of more than 3 months. The biggest impact factors are the mobile phase preparation and shelf life, while the Vanquish Core system robustness excels all regulatory requirements with a retention time precision of 0.07% over 170 injections when the mobile phase is prepared by blending the pure solvents via the low-pressure gradient pump.

Goals

- Evaluate intra-system variability of retention times and peak areas on a larger time scale of several months
- Demonstrate the superiority of the pump proportioning performance compared to the original Ph. Eur. method by a 3-day sequence of 170 sequential injections

Introduction

A high reproducibility of key chromatographic parameters like retention time and peak area is crucial for a continuous use of high performance liquid chromatography (HPLC) systems, no matter if used in cutting-edge research or in routine analysis, such as in quality control and batch testing release laboratories. Method validations typically test for short-term precision data by addressing a limited number of injections (10, for example). However, for many users the long-term stability of key parameters is also of interest, for instance to avoid time-consuming adjustments on pre-defined peak integration settings in a processing method. High reproducibility among systems, which is needed for method transfer between laboratories, has already

been discussed for the Vanquish Core HPLC systems in a separate document.[1] In the present technical note, we investigate the long-term precision of an established routine analysis for thiopental and its by-products as described by the European Pharmacopoeia (EP)[2]. For that purpose, multiple sequences of thiopental analyses were repeated by one fixed operator with one fixed assigned Vanquish Core HPLC system and a total time window ranging over 3.5 months. In addition to this multi-sequence approach, we also looked at the robustness of this method when performed over a large number of injections covering a total run time of more than three consecutive days. In general, the reproducibility of the Vanquish Core systems within closed datasets was found to be excellent. Variability was mainly introduced by the sensitivity of the separation method against mobile phase composition.

Experimental details

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Acetonitrile, Fisher Chemical™ Optima™ LC/MS grade (P/N A955-212)
- Orthophosphoric acid, 85%, HPLC for electrochemical detection, certified, Fisher Chemical™ (P/N O/0515/PB08)
- EP Certified Reference Standard Thiopental for System Suitability CRS,[3] containing impurities A, B, C, and D (Catalogue code Y0001478)

Sample handling

- Fisherbrand™ Isotemp™ Stirring Hotplate (P/N S14365))
- Fisherbrand™ Mini Vortex Mixer (P/N 14-955-152)
- Vials (amber, 2 mL), Fisherbrand™ (P/N 03-391-6)
- Cap with Septum (Silicone/PTFE), Thermo Scientific™ (P/N 3-622-292)

Instrumentation

- Vanquish Core Quaternary HPLC system consisting of:
  - System Base Vanquish Core (P/N VC-S01-A-02)
  - Vanquish Quaternary Pump C (P/N VC-P10-A)
  - Vanquish Split Sampler CT (P/N VC-A12-A)
  - Vanquish Column Compartment C (P/N VC-C10-A-03)
  - Vanquish Diode Array Detector CG (P/N VC-D11-A)
  - Flow cell, analytical, 11 µL, VF-D11 (P/N 6083.0520)

Sample preparation

The thiopental standard sample was prepared as 1 mg/mL thiopental for system suitability CRS, containing the impurities A, B, C, and D, in premixed mobile phase. A 2 mg portion of the EP reference standard for system suitability was weighed in a 2 mL volumetric flask. The flask was then filled to 2 mL with pre-mixed mobile phase. The standard dissolved upon vortexing for about 1 minute.

Mobile phase preparation

Pre-mixed according to Ph. Eur.

The mobile phase was prepared by adding 1 g phosphoric acid (85%) to 900 mL of water in a 1000 mL volumetric flask and filling to volume with water. A 700 mL portion of acetonitrile was added to 1300 mL of the phosphoric acid solution in a 2 L eluent bottle, mixed by intensive stirring of the bottle content by a magnetic stirrer until a clear solution became visible, and degassed by placement for 5 minutes in an ultrasonic bath.

Dial-a-mix

Solvent A was prepared by adding 1 g phosphoric acid (85%) to 900 mL of water in a 1000 mL volumetric flask and filling to volume with water. Solvent B (acetonitrile) was used “as-is”. Mobile phase preparation was achieved in situ by the pump proportioning 65% A:35% B.

Chromatographic conditions

Table 1. Chromatographic conditions

Parameters	Value
Column	Thermo Scientific™ Hypersil GOLD™, 150 x 4.6 mm, 5 µm (P/N 25005-154630)
Mobile phase	Pre-mixed: 65:35 (v:v) 1 g/L phosphoric acid (85%) in water:ACN Dial-a-mix: Solvent A: 1 g/L phosphoric acid (85%) in water Solvent B: ACN A:B = 65:35
Run time	20 min
Flow rate	1 mL/min
Mixer volume	350 µL + 50 µL
Column temp.	25 °C with passive eluent pre-heater
Autosampler temp.	4 °C
UV wavelength	225 nm
UV data collection rate	10 Hz
UV response time	0.5 s
Injection volume	10 µL

Chromatography Data System

The Thermo Scientific™ Chromeleon™ Chromatography Data Systems (CDS), version 7.3 was used for data acquisition and analysis.

Results and discussion

Repeatability of a thiopental separation over multiple months

To define the baseline for this long-term study with a set series of experiments, one operator consistently using one Vanquish Core Quaternary HPLC generated the initial precision data for retention time and peak areas of the main constituents of a thiopental Ph. Eur. reference standard. The operator exactly followed the protocol for the HPLC analysis as outlined in the European Pharmacopoeia monograph, which includes preparation of the mobile phase by pre-mixing the two solvent portions and letting the HPLC pump deliver the mobile phase in isocratic mode with 100% flow rate on one solvent channel (A). Figure 1 shows a representative overlay of 10 replicate injections on the evaluated Vanquish Core system, showing excellent precision data of 0.02% retention time relative standard deviation (RSD) and peak area RSD values between 0.03 and 0.4%. The peak area precision of 0.83% RSD for impurity D is considered as an outlier due to the low concentration level of the compound only hardly meeting the limit of quantitation for this analyte.

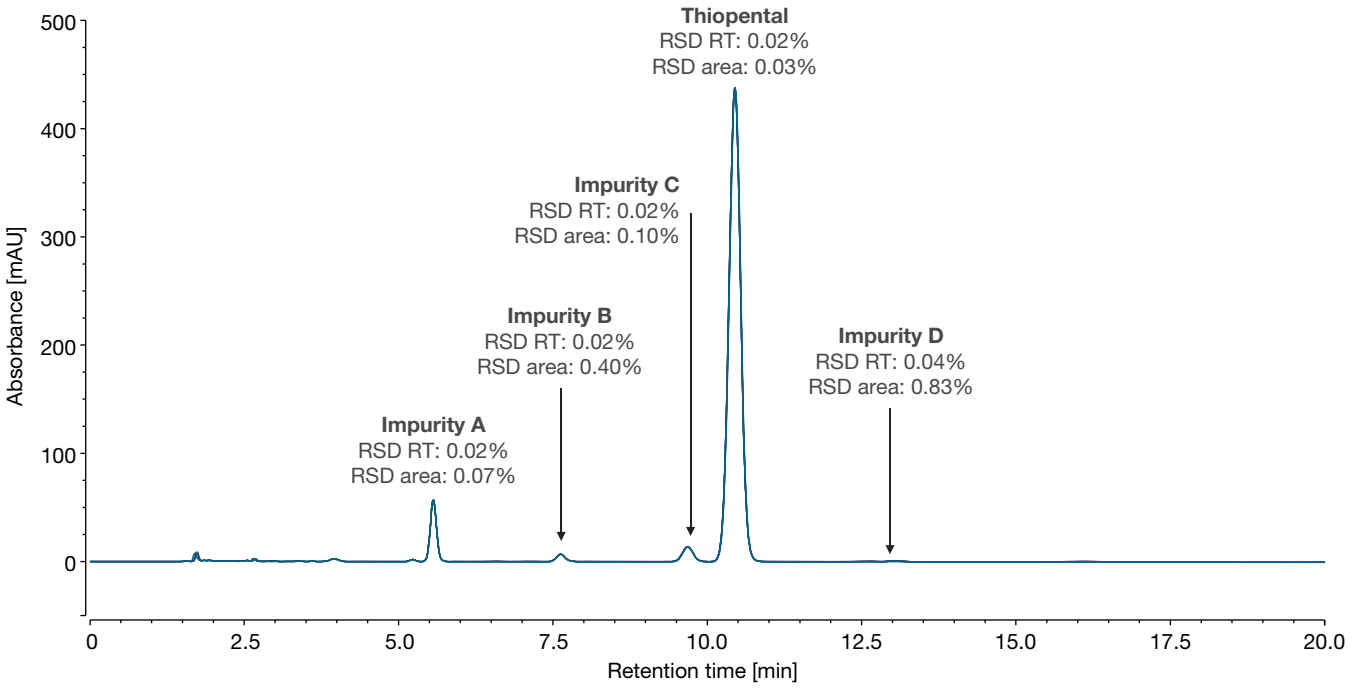

Figure 1. Overlay of 10 replicate injections of thiopental EP reference standard according to EP method protocol on a Vanquish Core Quaternary HPLC System using pre-mixed mobile phase

In a first approach to the long-term study, this analysis was performed three times over a period of 3.5 months, thus including not only continuous hardware stress by ongoing instrument use but also varying environmental conditions. Outer ambient conditions ranged from hot and low humidity summer with temperatures up to 35 °C to chilly and high humidity late fall. Despite an appropriate climate control of the laboratory environment by a powerful air conditioning system, such wide changes of atmospheric parameters cannot always completely be compensated, at least in the warm summer period. Table 2 lists the primary chromatographic parameters for the main components of the Thiopental Ph. Eur. reference standard.

The intra-sequence RSD values for retention times and peak areas are consistently excellent, with 0.02% or less for the active pharmaceutical ingredient (API) thiopental over all three sequences. This illustrates nicely the robustness of the Vanquish Core system, which shows no notable wear over the test period that would affect the precision, although the system has been in continuous use.

Table 2. Retention time and peak area precision for the main components for 10 replicates each. Three sequences run by one operator on one instrument were recorded over 3.5 months.

Time of experiment	Thiopental				Impurity A				Impurity C			
	Retention time [min]	RSD (RT)	Peak area [mAU.min]	RSD (Peak area)	Retention time [min]	RSD (RT)	Peak area [mAU.min]	RSD (Peak area)	Retention time [min]	RSD (RT)	Peak area [mAU.min]	RSD (Peak area)
Week 32 / 2019	11.206	0.02%	100.272	0.33%	5.932	0.01%	6.867	0.41%	10.379	0.02%	2.922	0.54%
Week 33 / 2019	11.299	0.02%	101.094	0.25%	5.966	0.04%	5.903	0.13%	10.462	0.02%	2.942	0.39%
Week 46 / 2019	11.082	0.00%	106.153	0.42%	5.829	0.01%	8.950	0.10%	10.256	0.01%	3.096	0.53%
Average	11.20		102.5		5.91		7.2		10.37		3.0	
RSD	1.0%		3.1%		1.2%		21.5%		1.0%		3.2%	

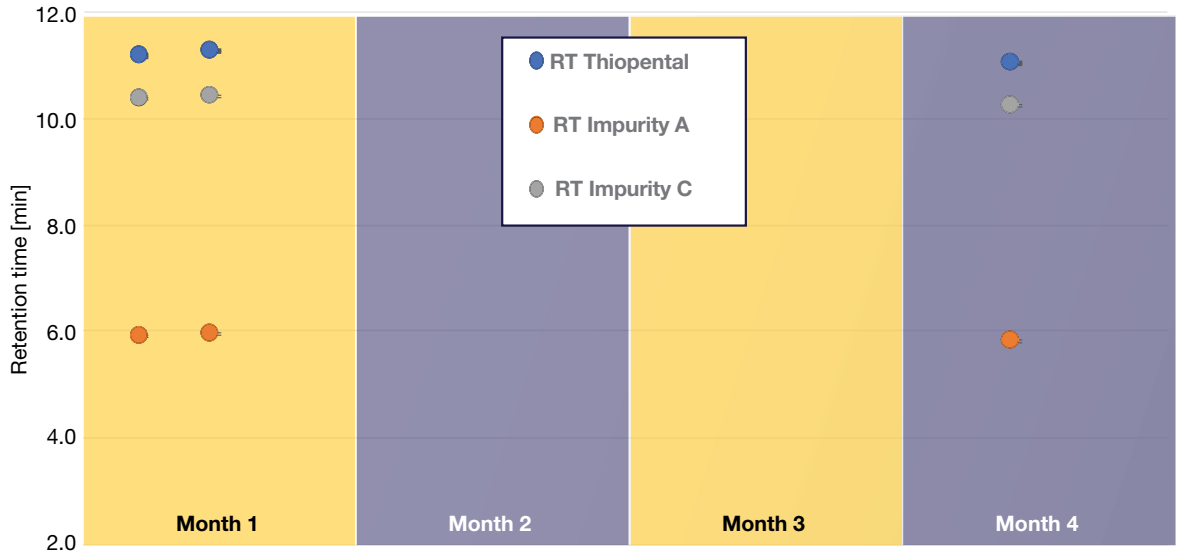

Figure 2. Long-term robustness data of thiopental sequences run on the same Vanquish Core Quaternary system between July and November. Intra-sequence precision (N=10) is excellent with RT RSD values of less than 0.02%.

What could already be shown elsewhere[1] is that the separation of thiopental reacts very sensitively to small variations in the mobile phase preparation. It must be noted that the lot-to-lot variability of the Thiopental Ph. Eur. reference standard is fairly high, which explains the poor peak area RSDs for the thiopental by-products when averaged over multiple sequences (Table 2). As each time a fresh standard lot must be used for sample preparation, the relative amounts of the impurities to the API varied substantially so that only the main component thiopental shows a good peak area precision over nearly 4 months.

Repeatability of a thiopental separation in a long-running sequence over multiple days

In addition to the long-term robustness of an HPLC separation utilizing a Vanquish Core system repeated over months, the retention time stability within one long-running sequence was also investigated. The main goal here was to verify if the HPLC system hardware suffered from constant wear on a small level that would eventually translate into trending chromatographic parameters. As an example, a drift of retention times rather than statistical scattering would, for instance, indicate minor shortcomings in flow delivery or thermostatting, potentially caused by seal wear or micro-leakages in the pump or limited capability of the column compartment to manage changes in the ambient laboratory atmosphere. Therefore, a sequence of 200 injections was started on the Vanquish Core system, applying the pre-mixed mobile phase as requested by the Ph. Eur. monograph method description for Thiopental. For the total sequence time of more than 3 days, a total amount of 5 L of pre-mixed mobile phase was prepared upfront as one batch to exclude inter-day changes by mobile phase preparation. The liquid was then aliquoted in 2 L portions, which were stored in a dark, dry place at ambient temperature in tight-closed eluent bottles. As one portion of 2 L lasted for a maximum of 33 hours, it was decided to replace a used bottle of mobile phase every 24 hours.

Figure 3 (A) illustrates the result of this 170-injection sequence experiment. For clarity, only every 10th injection is displayed in the overlay. The plot at the bottom shows the retention time values obtained in this experiment with a mobile phase prepared as outlined in the Ph. Eur. (orange data points).

It can be clearly seen that the retention times steadily drift to higher values; once a freshly opened bottle of mobile phase of the same batch is attached to the system, the retention time is set back to the original value, sometimes only after some time needed for re-equilibration, as indicated by the red arrows. This drift led to a retention time RSD of 0.8% for thiopental, a value which typically is not accepted by many test instructions and which also hampers correct peak assignment and quantitation in the chromatographic data system. Therefore, the experiment was stopped after 170 injections. Rising retention times in an isocratic separation can usually only go back to a decrease in the pump flow rate, to a reduction of the solvent strength, or to a temperature drift towards a cooler column over time. With the reset by using fresh mobile phase, both a temperature effect and a flow inconsistency could be excluded. Due to the pre-mixing of the mobile phase, the proportioning function of the pump has been excluded as a potential cause for this result. Further investigations revealed that a selective evaporation of acetonitrile while the mobile phase was standing on the HPLC solvent rack was the most likely root cause for this symptom.

To prove this assumption and to demonstrate the reliability of the HPLC hardware, this sequence was repeated with another set of 170 injections, keeping all parameters the same except the mobile phase preparation: now the pure aqueous and organic solvent were filled in channels A and B of the quaternary low-pressure mixing gradient pump to let the pump blend the mobile phase by running a method that mixes 65% A with 35% B. The result is depicted in Figure 3 (A and C, blue). When this dial-a-mix approach is applied to this separation, the Vanquish Core system allows for an excellent retention time precision of 0.07% over 170 injections and 3 days of uninterrupted operation.

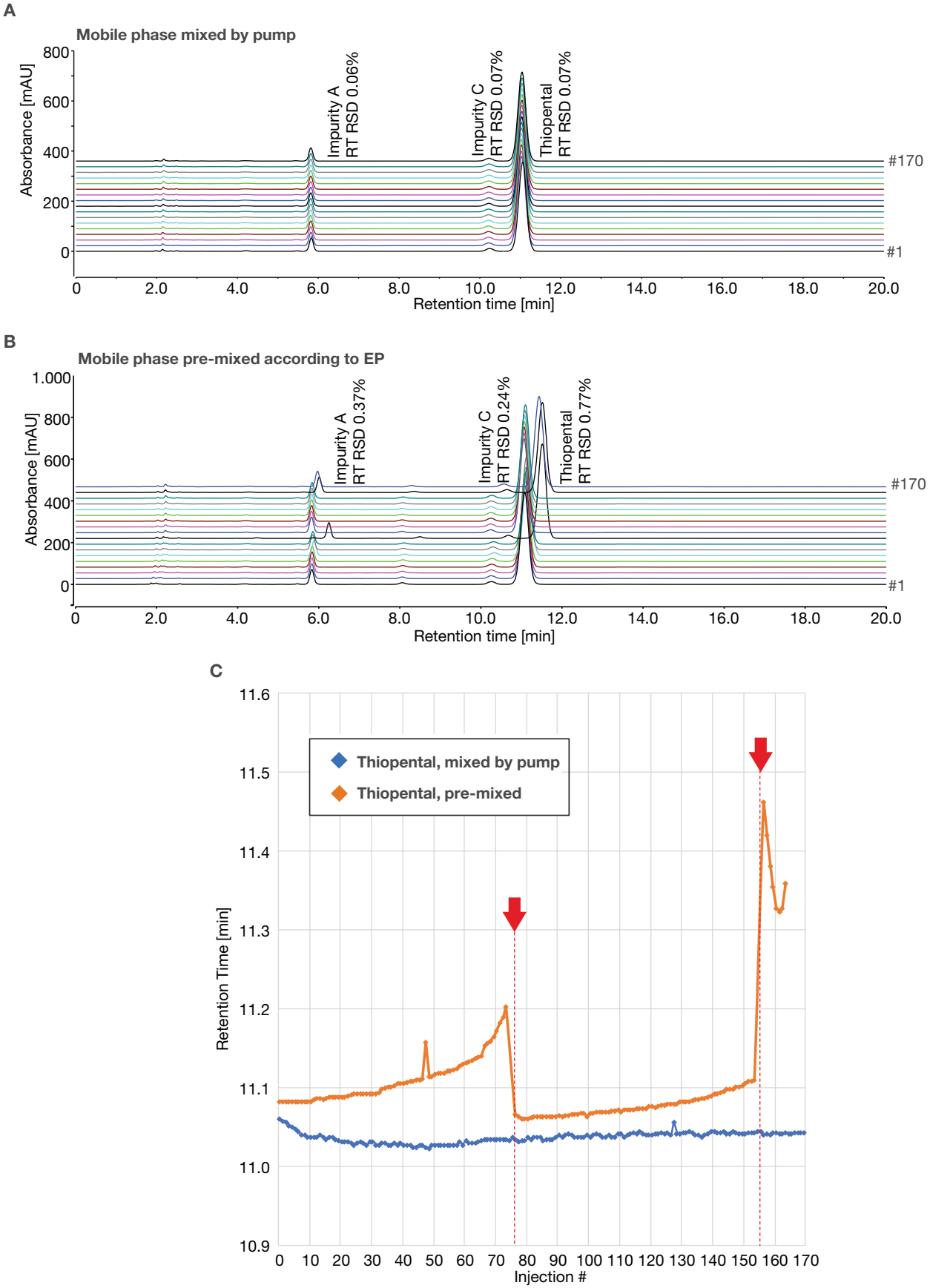

Figure 3. Comparison between sequences of 170 sequential injections in an uninterrupted system operation over 3 days, once with mobile phase preparation by the Vanquish Core Quaternary Pump C (mixed by pump, A) and once by pre-mixing the mobile phase according to EP (B), including trend charts of retention times (C). Red arrows indicate the replenishment of mobile phase from a freshly opened bottle of the same preparation batch for the sequence with pre-mixed mobile phase, orange plot).

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Conclusion

- The Vanquish Core HPLC systems offer excellent reproducibility for retention time and peak area over 3.5 months, with average retention time RSDs of less than 0.02% and peak area RSDs between 0.1 and 0.4%.
- Retention time reproducibility is impacted by eluent preparation; the shelf life of the pre-mixed mobile phase in multi-day sequences results in drifting retention times, which are reset once a freshly opened mobile phase of the same batch is used for replenishment.
- The Vanquish Core Quaternary HPLC systems allow for a variation of ~1% of retention times for sequences repeated over more than 3 months, including strong environmental condition changes from hot summer to chilly fall.
- It could be demonstrated that the Vanquish Core Quaternary HPLC system even provides a higher robustness (by dial-a-mix) than the manual preparation of the mobile phase according to EP allows for, with a total retention time RSD of 0.06% for the main compound over 170 injections in a row in 3 days.

References

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