# Impurity Profiling with the Agilent 1200 Series LC System

## Part 3: Rapid Condition Scouting for Method Development

## Application Note

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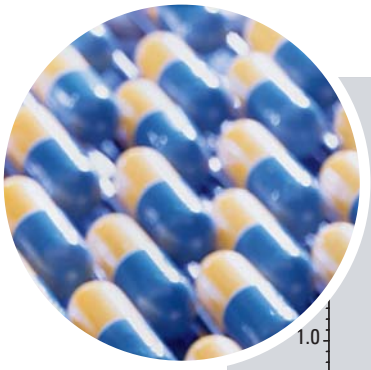

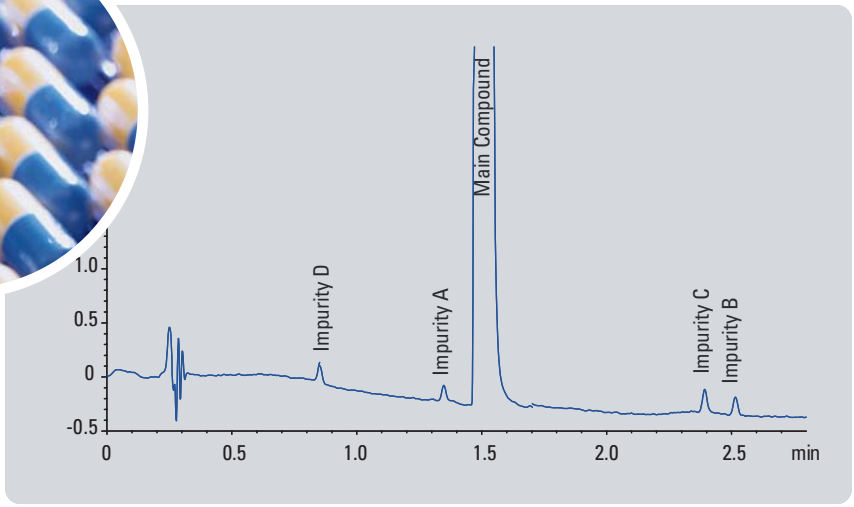



### Abstract

Method development is one of the most time-consuming tasks in today's pharmaceutical analytical laboratories.[1] Usually, condition scouting is the first step followed by fine-tuning of the best set of parameters found. A means of speeding up the entire process by utilizing Agilent Rapid Resolution HT columns in combination with the Agilent 1200 Series Rapid Resolution LC (RRLC) system is described. How separation condition scouting and method fine-tuning resulted in a robust method for the quantization of diasteromeric and regioisomeric impurities of an active pharmaceutical ingredient within one and a half days is demonstrated. The final method not only is much faster than comparable conventional methods but also saves a significant amount of solvent.


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## Introduction

Developing and validating new analytical methods is costly and time consuming. So, any means to reduce the run-time of each individual analysis is beneficial for the overall process, but no compromise in resolution, sensitivity, and especially robustness must be accepted. The availability of columns packed with sub-2 µm particles and the high quality of LC instruments required for more than 1000-fold applied methods, nowadays allows a significant acceleration in method development. At the outset often a broad scouting of separation conditions like stationary phases, mobile phase parameters (% B, buffer, pH, solvent) and operation parameters, such as gradient slope, temperature etc. are used to find the optimum starting point for fine tuning. In this Application Note we demonstrate the use of the Agilent 1200 Series Rapid Resolution LC (RRLC) system together with a variety of Agilent ZORBAX RRHT columns to search for separation conditions with run times below four minutes per analysis and yet the efficiency to get diastereomeric compounds and regioisomers separated.

## Experimental

### Instrumentation

- Agilent 1200 Series binary pump SL with an Agilent 1200 Series micro vacuum degasser.
- Agilent 1200 Series high performance autosampler SL with a thermostat.
- Agilent 1200 Series thermostatted column compartment SL.
- Agilent 1200 Series diode-array detector SL. In this experiment 5 µL/6 mm and 13 µL/10 mm cells were used.
- ZORBAX RRHT 1.8-µm particle columns with various stationary phases and dimensions.

Gradient grade water from a Millipore device with different modifiers and different pH ranges was used as the mobile phase. Gradient grade acetonitrile and methanol from Merck, Darmstadt were used as the strong solvents. No additional filtering of the solvents was required. Instrument control and data acquisition were performed by the Agilent ChemStation B02.01 SR1 software.

## Results and discussion

The objective of this method development was to provide a quick and robust method for quantization of production impurities of a pharmaceutical compound, which is a basic salt with a pKa of 9.4 and easily soluble in water, especially under acidic conditions. From previous experiments[2], it was known that the reactants (3-bromanisole and impurity E, see figure 1) could easily be detected by means other than HPLC, e.g. GC or TLC. Focus was placed on the separation of the diasteromeric impurity A and the regioisomeric dehydroxylation products B and C as well as on the demethylation product D. Structures have been elucidated by means of ion trap MS analysis, Time-of-Flight-MS analysis[2], and after preparative purification by NMR techniques[2].

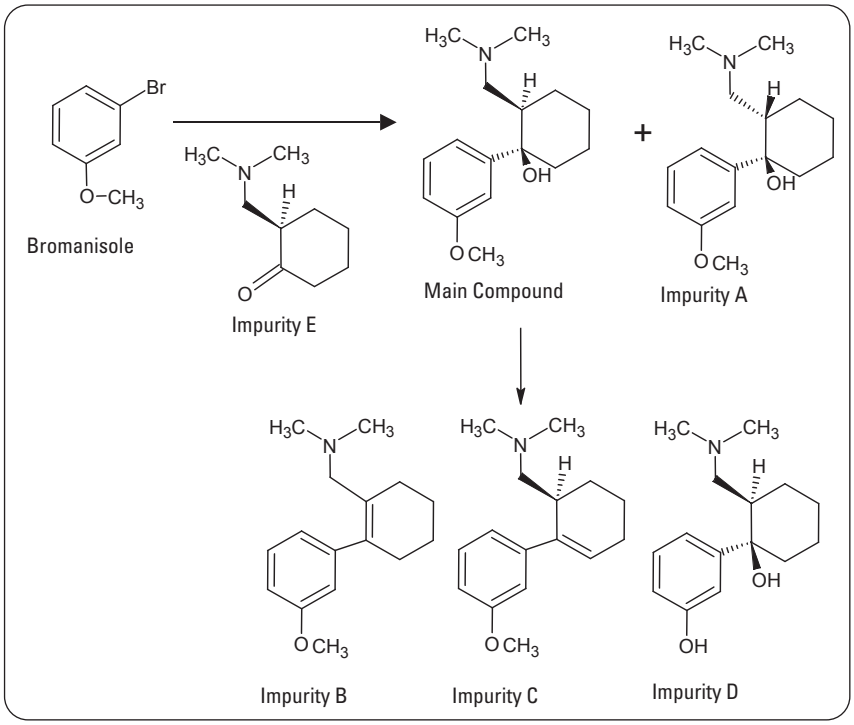


**Figure 1**
**Structures of the by-products of the active pharmaceutical ingredient.**

Comparison of the chromatograms at different wavelengths acquired by the diode-array detector led to the decision to choose 270 nm as the monitoring wavelength, because all compounds of interest had a strong absorption band at this wavelength (figure 2) and the baseline showed the fewest gradient shifts. As the main compound, as well as all impurities, had an excellent solubility in pure water, it was chosen as the solvent for dissolving the sample. Sample concentration was 0.7 mg/mL. At substantially higher concentrations, a severe tailing of the main compound could be seen due to overloading effects. During the initial condition scouting, an impurity enriched sample was used and a broad gradient of 5-95 % B was applied with a different set of stationary and mobile phases. All columns used were Agilent ZORBAX columns with a dimension of 50 mm x 3.0 mm ID and with a 1.8-µm particle size. The pump was operated in the low delay volume configuration and the detector cell was a 5 µL/6 mm cell. Column temperature was set to 40 °C. The stationary and mobile phases used are listed in table 1 and the chro-

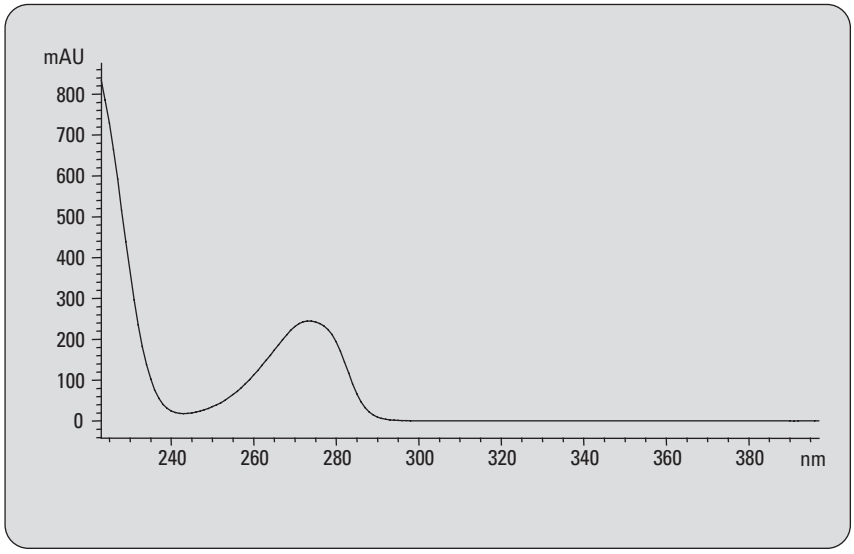


**Figure 2**
**UV spectrum of the main compound.**

| Exp. Number | Stationary phase | pH | Mobile phase | Modifier | Comment |
|---|---|---|---|---|---|
| 1 | SB CN | 1.92 | $H_2O$/ACN | TFA | 5-95 %B gradient, 40°C |
| 2 | Extend C18 | 1.92 | $H_2O$/ACN | TFA | 5-95 %B gradient, 40°C |
| 3 | XDB C18 | 1.92 | $H_2O$/ACN | TFA | 5-95 %B gradient, 40°C |
| 4 | XDB C8 | 1.92 | $H_2O$/ACN | TFA | 5-95 %B gradient, 40°C |
| 5 | SB C18 | 1.92 | $H_2O$/ACN | TFA | 5-95 %B gradient, 40°C |
| 6 | XDB C8 | 6.01 | $H_2O$/ACN | Phosphate-Buffer | 5-95 %B gradient, 40°C |
| 7 | XDB C18 | 6.01 | $H_2O$/ACN | Phosphate-Buffer | 5-95 %B gradient, 40°C |
| 8 | Extend C18 | 6.01 | $H_2O$/ACN | Phosphate-Buffer | 5-95 %B gradient, 40°C |
| 9 | SB CN | 1.92 | $H_2O$/MeOH | TFA | 5-95 %B gradient, 40°C |
| 10 | XDB C18 | 1.92 | $H_2O$/MeOH | TFA | 5-95 %B gradient, 40°C |
| 11 | Extend C18 | 1.92 | $H_2O$/MeOH | TFA | 5-95 %B gradient, 40°C |
| 12 | SB C18 | 1.92 | $H_2O$/MeOH | TFA | 5-95 %B gradient, 40°C |
| 13 | XDB C8 | 1.92 | $H_2O$/MeOH | TFA | 5-95 %B gradient, 40°C |
| 14 | SB CN | 6.01 | $H_2O$/ACN | Phosphate-Buffer | 5-95 %B gradient, 40°C |
| 15 | SB C18 | 6.01 | $H_2O$/ACN | Phosphate-Buffer | 5-95 %B gradient, 40°C |
| 16 | Extend C18 | 11.0 | $H_2O$/ACN | $NH_4OH$ | 5-95 %B gradient, 40°C |
| 17 | SB C18 | 1.92 | $H_2O$/ACN | TFA | Transfer of Exp. 5 to 4.6mmID column |
| 18 | SB C18 | 1.92 | $H_2O$/ACN | TFA | 5-50 %B gradient, 40°C |
| 19 | SB C18 | 1.92 | $H_2O$/ACN | TFA | 15-50 %B gradient, 40°C |
| 20 | SB C18 | 1.92 | $H_2O$/ACN | TFA | 15-50 %B gradient, 20°C |
| 21 | SB C18 | 1.92 | $H_2O$/ACN | TFA | 15-50 %B gradient, 60°C |
| 22 | SB C18 | 1.92 | $H_2O$/ACN | TFA | 17-45%B gradient, 30°C, final method |

**Table 1**
**Stationary and mobile phases used for the initial condition scouting and fine tuning (lower part).**

matographic results (retention times and resolution of the critical pairs) are shown in figures 3 and 4. As expected, a change of selectivity by varying the mobile and/or the stationary phase had a dramatic effect on the chromatographic result. Choosing the wrong set of conditions could lead to the false impression that a rather simple mixture with just three components – one of them being the main compound – is present (for example phosphate buffer combined with the Extend C18 column). By changing the conditions, the regioisomers B and C start to separate (e.g. phosphate buffer using the ZORBAX XDB C18 column). Eventually the diasteromeric impurity A and the main component also split up to reveal the actual five compounds present in this sample.

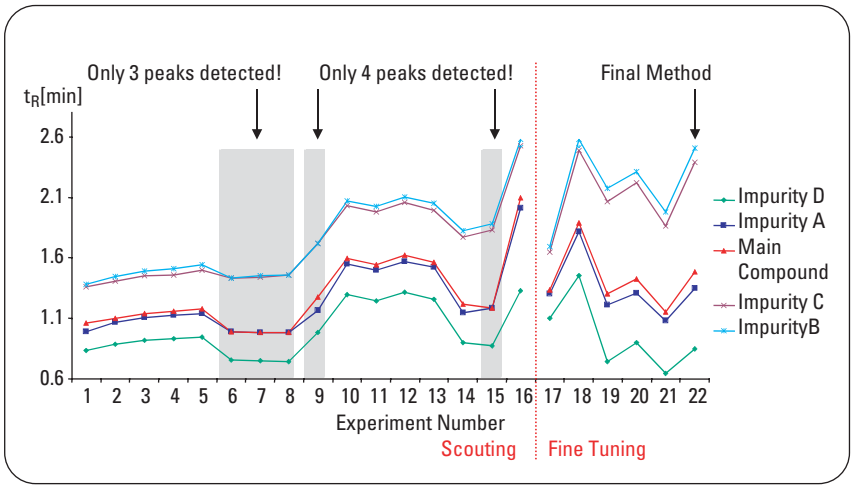


**Figure 3**
**Retention times of the initial condition scouting and the fine tuning.**

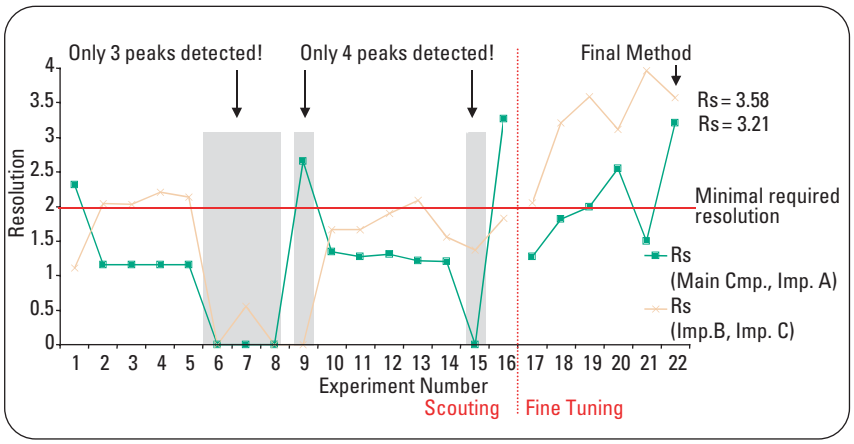


**Figure 4**
**Resolution of the critical pairs of the initial condition scouting and the fine tuning.**

| | | |
|---|---|---|
| **Scouting method:** | | |
| Solvent: | (see table 1) | |
| Temperature: | 40 °C | |
| Flow: | 1.2 mL/min | |
| Gradient: | 0.00 min | 5 %B |
| | 3.00 min | 95 %B |
| | 3.50 min | 95 %B |
| Stop time: | 3.50 min | |
| Post time: | 1.00 min | |
| DAD: | Spectra | 190-500 nm (bandwidth 1nm), all spectra |
| | Signal A: | 270 nm (10 nm), ref. 500 nm (100 nm) |
| | Peak width: | >0.03 min (0.2 s response time) |
| | Slit: | 8 nm |
| | Balance: | pre-run |
| Injection volume: | 5 µL | |
| Injector: | Automatic delay volume reduction, Sample flush out factor = 20 Needle wash 10 s (methanol) | |

In figures 5 and 6 some examples are given to demonstrate the effect of changing the selectivity. Evaluation of the achieved retention times and resolutions from the different scouting runs demonstrates that, in general, the poorest results were obtained with weak acidic conditions (phosphate buffer). All retention times were shifted to higher values when using methanol as the strong solvent, due to its higher viscosity. In the search for the best starting point for the optimization, only conditions which did not have more than one critical pair with a resolution below 2 and none with a resolution close to zero were taken into account. Only the experiments 1-5, 13 and 16 remained. Experiment 16 yielded the best initial resolution, but also the highest retention times, and was therefore deferred (also because of the less favorable basic conditions). Experiment 13 exhibited higher retention times at comparable resolutions. Ultimately, one of a number of very similar experiments, 1 through 5, had to be selected for further optimization and fine tuning. Testing the Cyano-phase with a narrower gradient failed to significantly increase the resolution of the two regioisomeric impurities B and C. Since the differently bonded C18 stationary phases as well as the C8 stationary phase exhibited only minor differences, the StableBond C18 column was chosen for further fine tuning. Initially, the conditions of experiment 5 were transferred to a 4.6-mm ID column, which is the preferred column ID in a manufacturing QA/QC envi-

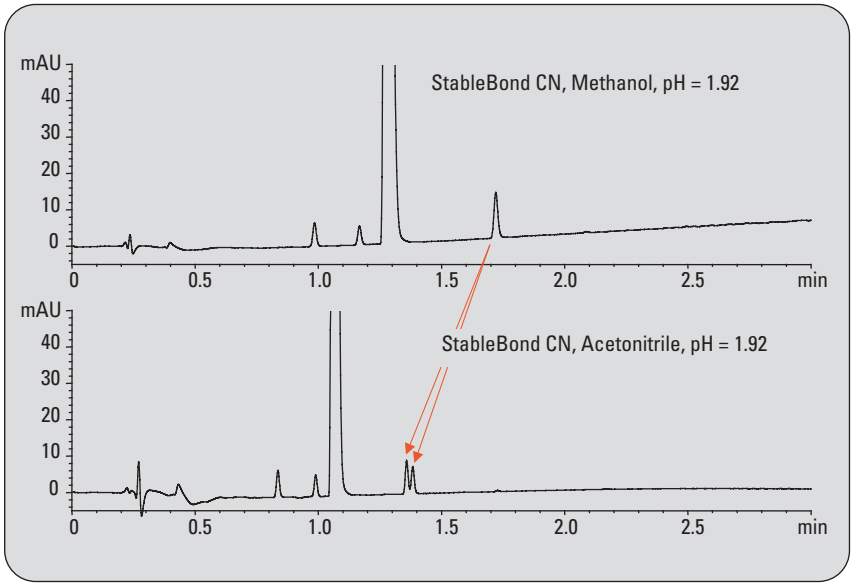


**Figure 5**
**The regioisomers B and C appear as a single, perfect Gaussian-shaped peak using methanol as the strong solvent together with a StableBond CN column (upper trace). Just by switching to acetonitrile as the strong solvent, the two compounds split up.**

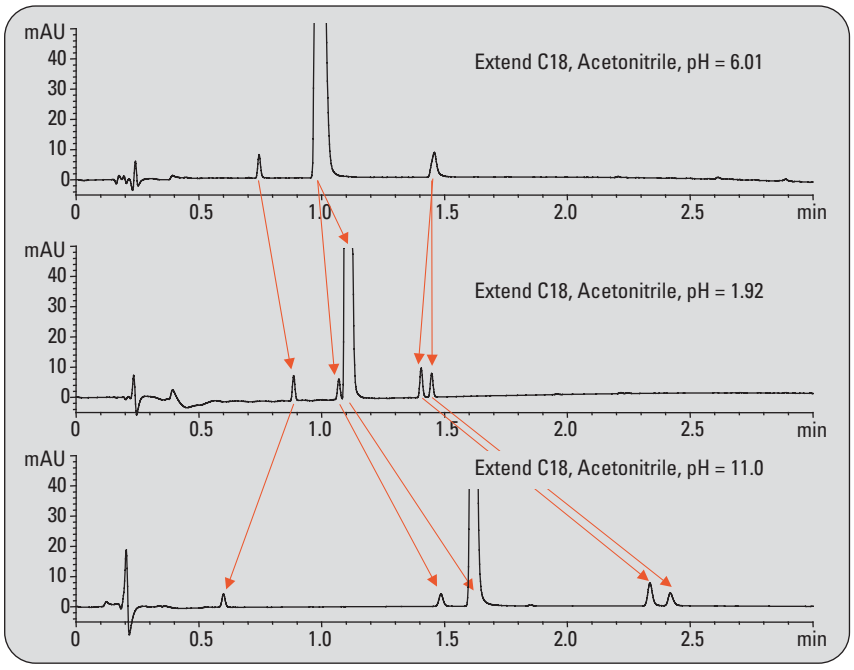


**Figure 6**
**The effect of changing the pH is illustrated here using the ZORBAX Extend C18 column with acetonitrile as the strong solvent and phosphate buffer at pH = 6.0 in the upper trace, 0.2% TFA as acidic modifier in the middle trace (pH = 1.9) or 0.2% ammonia as the basic modifier in the bottom trace (pH = 11).**

ronment. The transfer was made by geometrical scaling of the flow rate. In addition, the pump was operated in the standard delay volume configuration, the standard heat exchangers with 3 µL internal volume were used, and a 13 µL/10 mm cell was used in the diode-array detector. The resolutions remained practically constant, whereas a slight increase of the retention times could be detected due to the additional delay volume of the mixer and damper. The gradient range was narrowed and also the effect of different temperatures was explored to fine-tune the method (figures 3 and 4). Unfortunately, varying the temperature achieved an opposite effect on the two critical pairs. When lowering the temperature, the resolution between impurity A and the main compound increased, however it decreased between the two regioisomers B and C. Increasing the temperature produced a better resolution for the regioisomers, but a worse resolution between impurity A and the main compound. It was decided to use 30 °C as a compromise. A resolution of more than three could be achieved for all compounds with the final method by applying a 17- 45 % acetonitrile gradient in water at pH = 1.92 (0.2 % TFA) in 2.8 min with a hold of 0.2 min at 45 % B, using a ZORBAX StableBond C18 column (50 mm x 4.6 mm ID, 1.8 µm), at a temperature of 30 °C (figure 7). Table 2 lists some characteristic values for the separation of a sample containing impurities at the lower reporting level (0.05 % of main compound). This method was then validated and checked for its robustness[4] before being made available for analyzing samples in the manufacturing QA/QC environment[5].

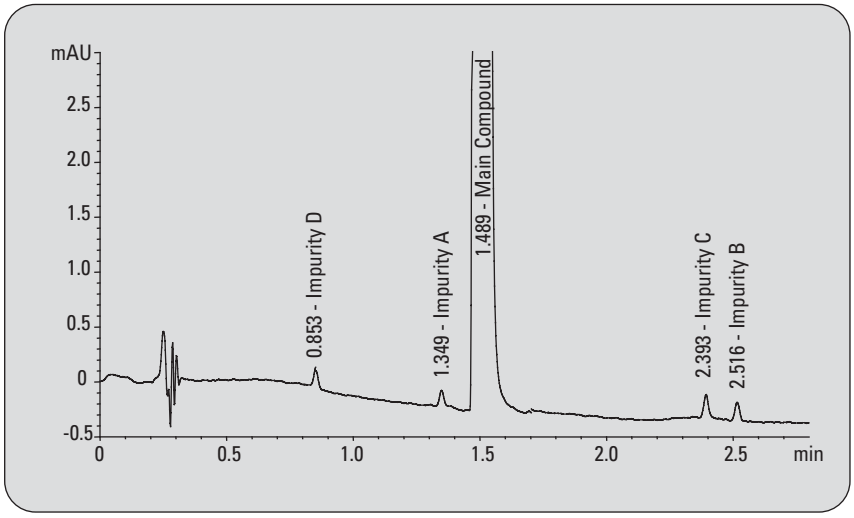


**Figure 7**
**Applying the final method conditions to a sample containing impurities at the lower reporting level (0.05 % of main compound).**

| **Final method:** | | |
|---|---|---|
| Solvent: | A = Water (0.2 volume.-% TFA), B = acetonitrile (0.16 volume-% TFA) | |
| Temperature: | 30 °C | |
| | Flow: | 2.2 mL/min |
| Gradient: | 0.00 min | 17 %B |
| | 2.80 min | 45 %B |
| | 3.00 min | 45 %B |
| Stop time: | 3.00 min | |
| Post time: | 1.00 min | |
| DAD: | Spectra | 190-500 nm (bandwidth 1nm), all spectra |
| | Signal A: | 270 nm (8 nm), ref. 500 nm (100 nm) |
| | Peak width: | >0.03 min (0.2 s response time) |
| | Slit: | 8 nm |
| | Balance: | pre-run |
| Injection volume: | 5 µL | |
| Injector: | no automatic delay volume reduction, Needle wash 10 s (methanol) | |

| Compound | Time [min] | Resolution | Area [mAU · s] | Height [mAU] | Width [s] | Area [%] | Mass [ng] |
|---|---|---|---|---|---|---|---|
| Impurity D | 0.853 | --- | 0.25 | 0.180 | 1.30 | 0.050% | 1.76 |
| Impurity A | 1.349 | 15.96 | 0.23 | 0.150 | 1.38 | 0.046% | 1.62 |
| Main Compound | 1.489 | 3.21 | 497.10 | 222.200 | 2.11 | 99.791% | 3492.69 |
| Impurity C | 2.393 | 19.83 | 0.33 | 0.220 | 1.36 | 0.066% | 2.32 |
| Impurity B | 2.516 | 3.58 | 0.23 | 0.170 | 1.27 | 0.046% | 1.62 |

**Table 2**
**Characteristic values of the separation of a sample containing impurities of the main compound at the lower reporting level.**

## Conclusion

The method development time for the demanding separation of diastereomeric and regioisomeric impurities of an active pharmaceutical ingredient could be dramatically reduced by using sub-2 µm particle columns for condition scouting and method fine-tuning. Since rather short scouting runs of 4.5 min cycle time were required, a large set of conditions could be tested merely within a day (including replicates and blanks). Additional fine-tuning lasted another half day, so after just one and a half days a method could be provided for subsequent method validation, which was also dramatically reduced in time[4] due to the short individual run time of 4.0 min per analysis with the final method. Also keep in mind the numerous later analyses in the manufacturing QA/QC lab. During the many years of compound-production analysis time is also reduced to a fraction of old-fashioned methods, resulting in a constantly shorter release time of the product, saved storage costs of intermediates, making reactors earlier available for other production campaigns and saved solvent costs per analysis because just 8.8 mL mobile phase are consumed per analysis.

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