Reproducibility Testing of the HP CE Instrument

The final chemical test developed for the HP CE instrument implicitly checks various instrument functions by determining the reproducibility of migration time and peak area measurements for well-defined chemical samples. The injection type was selected by testing four different types in a series of reproducibility tests. The final test can be used in production, at a customer site, or for teaching CE basics.

by Ulrike Jegle

Instrument tests are indispensable for quality assurance of highly integrated systems. Only tests of the whole system guarantee a quick setup of the instrument at the customer site and a minimum failure rate in daily use. Although the components of the HP CE instrument are tested separately for functionality, the integrated system has to be tested for successful coexecution of all built-in elements. Besides functionality tests, the procedure for the HP CE instrument includes measurements of detector properties (noise detection) and reliability determination within a reasonable test time.

For the final test of the HP CE instrument, a test procedure was implemented that exercises the instrument under real-life conditions. A chemical test procedure had to be developed for this. Besides testing the instrument functions, other important aspects had to be considered for this chemical test. The final test had to be fast and easy to execute and use commercially available and nontoxic chemicals. In addition, it was desirable that the test be usable as a customer-site test. Teaching aspects were also considered in choosing the test separation procedure, so that the test could be used to show the basics of the relatively new capillary electrophoresis technique. The advantages of dual use of the final test are cost reduction and, more important, the possibility of obtaining comparable results in final test and customer tests for validation reasons.

Test Description

For the chemical final test, bare fused silica capillaries are used to separate a set of three vitamins—thiamine (positively charged), nicotinamide (no charge), and nicotinic acid (negatively charged)—using a 20-mM sodium phosphate buffer of pH 7 as run buffer. The vitamins come in a lyophilized mixture that has to be dissolved in water before use.

To complete a high number of runs within a reasonable test time, short preconditioning and separation intervals are required. Short run times can be achieved by high electro-osmotic flow and high analysis temperature.

Table I
Conditions for Chemical Final Test

Capillary:	Bare fused silica Total length: 48.5 cm Effective length: 40 cm Inner diameter: 50 μm
Conditioning: (pretreatment before first use)	Flush: 10 min 1M NaOH Flush: 2 min 0.1M NaOH Flush: 3 min run buffer All steps carried out at 60°C
Run Buffer:	20 mM sodium phosphate pH 7
Solute:	1 mM each thiamine, nicotinamide, nicotinic acid dissolved in water
Preconditioning: (before every run)	Flush: 2 min 0.1M NaOH Flush: 3 min run buffer
Separation:	Voltage: 25 kV Polarity: positive Temperature: 40°C
Detection:	Wavelength: 215 nm Bandwidth: 16 nm Reference: off
Data Analysis:	Initial threshold: −2

To guarantee a high electroosmotic flow, the capillary is pretreated before its first use with 1M sodium hydroxide at 60°C. Treatment of the fused silica wall with 1M sodium hydroxide solution at an elevated temperature results in a maximum of charged silanol groups on the surface, which is the basic prerequisite for high electroosmotic flow. A second rinsing step consisting of a 2-minute flush with 0.1M sodium hydroxide and a 3-minute flush with phosphate buffer is added to be as consistent as possible with the preconditioning step used before every run. The capillary conditioning with 1M NaOH is carried out once before the first use of the capillary for the final test and is not repeated

Table II
Factors Influencing Migration Time Reproducibility

Instrument Parameters

Constant Cassette Temperature
- Peltier Element
- Fans

Constant Separation Voltage
- Power Supply
- Connections

Constant and Reproducible Liquid Level in Replenished Vials
- Pressure System
- Valves
- Tube Connections

Chemical and Physical Parameters

Electroosmotic Flow (Zeta Potential†)
- Fused Silica Material
  - Bulk
  - Surface
- Buffer
  - pH
  - Ionic Strength
  - Type
  - Viscosity (strongly temperature dependent)
- Equilibrium State
  - Preconditioning
  - Buffer Type

† Zeta potential is the potential difference between the capillary wall surface and the bulk solution.

during the test. The preconditioning is a flush step carried out before every single run. Here weaker conditions are used: 0.1M NaOH and run buffer.

In addition to creating a high electroosmotic flow, the run time of the vitamin separation is optimized by using a run temperature of 40°C even though this impairs the separation efficiency. Increasing the temperature results in a decrease of buffer viscosity of 2% per degree. This causes higher flow velocity of the run buffer. The preconditioning and separation conditions are listed in Table I.

It has been shown (see "Final Test and Results" below) that the test mixture of thiamine, nicotinamide, and nicotinic acid can be separated with high reproducibility of migration time and peak area under the conditions listed in Table I. Typical relative standard deviation values calculated for six runs were below 1% in migration time and below 3% in peak area.

Test Parameters

The metrics collected in the final test are the reproducibilities (relative standard deviations) of the measured migration times and peak areas of the well-defined vitamin samples. The instrument parameters implicitly tested by determining time reproducibility are the stability of the cassette temperature and the accuracy of the replenishment system. The entire injection and detection system is tested by the determination of area reproducibility. Of course, the basic instrument functions like tray and lift movements need to work correctly during the final test for any results to be obtained.

Time and area reproducibility depend not only on instrument parameters, but also strongly on chemical and physical influences. For testing instrument functions, the test tool, which is the vitamin analysis in this case, should have constant parameters, ideally with no failure possibilities. However, variations and failures of chemical systems cannot be excluded. Therefore, failures and their impacts have to be well-known to distinguish between chemical and instrumental failures.

Tables II and III show instrumental and chemical/physical parameters having direct influences on migration time and peak area.

Time Reproducibility

The most important parameters influencing the migration time reproducibility of separations carried out in bare fused silica capillaries are variations in the electroosmotic flow and the degree of equilibration of the silica surface with respect to the buffer.

The Smoluchowski equation describes the parameters determining the electroosmotic mobility:[1]

$$\nu_{EOF} = -\frac{\varepsilon_0 \varepsilon_r \zeta}{\eta} = \mu_{EOF} E,$$

where ν_{EOF} is the electroosmotic velocity, ε_0 is the permittivity of vacuum, ε_r is the permittivity of the electrolyte solution, ζ is the zeta potential, η is the viscosity of the electrolyte solution, μ_{EOF} is the electroosmotic mobility, and E is the electrical field strength. According to the Smoluchowski equation, the electroosmotic flow is influenced by the zeta potential, which is affected by the properties of the fused silica material. The electroosmotic flow is also affected by the composition and pH of the buffer and the preconditioning solution.

Table III
Factors Influencing Peak Area Reproducibility

Instrument Parameters

Injection System
- Pressure
- Valves

Detection System

Chemical and Physical Parameters

Expulsion of Sample
- Single-Step Injection
- Injection + Postinjection
- Voltage Ramp

Reproducibility of Injection Plug
- Injection Volume
- Zero Injection Effect

Time Reproducibility
- $\int t \cdot \text{absorbance}$

pH and Concentration of Conditioning and Preconditioning Solutions. Fused silica capillaries are drawn under highly dehydrating conditions of high temperatures (around 1600°C) and dry inert gas (argon). When a capillary is treated with aqueous conditioning solutions the first time before use, silanol groups are created, a gel layer at the silica/liquid interface is developed, and the electrical double layer is built up (in the case of untreated fused silica, this consists of negative charges on the capillary surface and positive charges in the liquid near the surface, as explained in the article on page 6).

In general, NaOH flushes at elevated temperatures are used for conditioning, resulting in a maximum surface density of charged silanol groups. NaOH is known to dissolve fused silica strongly and is therefore expected to have the best cleaning effect on the surface. On the other hand, the use of NaOH is a very severe method that is expected to leave a rough surface which could create electroosmotic flow variations and local eddies. This can decrease the separation efficiency.[2] The impact of NaOH is not entirely clear at this time and the subject is still under ongoing research.

Acidic solutions like phosphoric acid are also used for conditioning, especially when the subsequent separation is carried out in acidic buffer media.

Before every run, capillaries are normally preconditioned for the subsequent separation for cleaning and equilibration purposes. This step can consist just of one buffer flush but often includes several steps with different flush solutions. Preconditioning solutions that differ from the run buffer influence the status of the surface. For example, when NaOH flushes followed by run buffer flushes are used for the vitamin analysis, the resulting electroosmotic flow and surface condition are different than if only run buffer is used for preconditioning. In general, the electroosmotic flow is higher using NaOH flushes. In such cases, the surface is in a quasistable state and not in an equilibrium state.

Buffer pH, Concentration, and Type. The separation buffer affects the density of charged groups and the electrical double layer at the surface as well as the thickness and composition of the gel layer.

The type of buffer determines the equilibration behavior of the surface and the buffer. Phosphate buffers especially are known to have a slow equilibration behavior because phosphate migrates into and out of the bulk silica, continuously changing the zeta potential of the wall as long as migration takes place.[3,4] Using phosphate buffer at low pH after initial NaOH cleaning, surface equilibrium adjustment times over 20 days can be observed. For citrate or borate buffers, the migration mechanisms as described for phosphate are not known. Equilibration seems to occur faster with those buffers.

Fused Silica. The manufacturing of capillary material and the process of capillary production are expected to have an impact on the zeta potential of the surface. The type and magnitude of the influence of these parameters have not been clarified yet and are still under ongoing research.

Area Reproducibility

Besides the reproducibility of the injection pressure and voltage, which are instrument parameters, the type of injection procedure is one of the important factors that determine area reproducibility. The zero injection effect (explained later) has a similar influence on area reproducibility.

Type of Injection. The most common injection procedure is just to inject a sample plug of a certain length either by voltage or pressure. For better reproducibility, the sample introduction can be combined with an additional buffer plug injection (postinjection) with or without a voltage ramp to start the separation.

Theoretically, the following considerations apply. When a separation voltage is applied to a capillary after injection, a heat pulse is created. This results in the volume expansion of the buffer and sample in the capillary. Volume expansion causes liquid to be pushed out of the capillary. If the sample plug is located directly at the end of the capillary, some sample is expelled. This process is a cause of low reproducibility. Under the conditions in which the vitamin analysis is carried out, this effect has been calculated to have a minor impact.[5,6] Experiments (described later) have shown that the geometry of the capillary end is much more important. Even if the capillary has an accurately cut rectangular edge and the polyimide is burned off at the ends, the microscopic edge properties differ from capillary to capillary. In addition, physical interactions between the surface tension of the liquid and the properties of the inner and outer surfaces of the capillary contribute to the inconsistency of injected and expelled sample amounts. These deficiencies of the capillary ends can be overcome by the introduction of a buffer plug after the sample plug, as confirmed in the experiments.

Zero Injection Effect. All injection procedures are subject to the *zero injection effect*. Zero injection is caused by capillary forces when a capillary is immersed in liquid. Reproducibility is mainly affected by the interaction of the surface tension of the liquid and the capillary inner and outer surfaces, and by the inner diameter and length of the capillary.

For a capillary with an inner diameter of 50 μm and a total length of 48.5 cm, the amount of zero injection plug can be expected to be around 0.2 mm. This amount is added linearly to each injection plug. The influence of the zero injection effect decreases with increasing injection plug length, but increasing the injection plug length strongly decreases the efficiency of every peak. Efficiency and zero injection are opposite effects. A general rule says that the injection plug length should not exceed 1% to 2% of the total capillary length.[1]

Reproducibility Tests

To examine the exact influence of zero injection and injection type, a test suite was set up consisting of four different three-vitamin analyses. The conditions, except those for injection, were held constant and were the same as used in the final test procedure. The exact injection conditions are described in Table IV. Fig. 1 shows the uncorrected absolute areas of all three vitamin peaks (thiamine, nicotinamide, and

Table IV
Injection Conditions for Four Three-Vitamin Analyses

Method	Injection Time (min)	Injection Pressure (mbar)	Postinjection Time (min)	Postinjection Pressure (mbar)	Voltage Ramp at Separation	Sample Plug Length (mm)
1	4.6	40	4	40	no	2.9
2	2	40	no	no	2.4 s/20 kV	1.3
3	2	40	no	no	no	1.3
4	zero	zero	4	40	no	zero injection plug: 0.2

nicotinic acid). Fig. 2 shows the relative standard deviations of the peak areas shown in Fig. 1. The method numbers in Fig. 2 correspond to those in Table IV. Six repetitions of each method (injection type) were carried out for the relative standard deviation calculation. In addition, the test set of the four injection types was repeated four times to show the reproducibility of each.

As expected, the highest relative standard deviation was calculated for zero injection. Typical relative standard deviation values were around 13% to 20%. Comparing the test conditions, the best reproducibility, with relative standard deviation values smaller than 3% in all repetitions, was realized using the postinjection procedure (method 1). As expected, postinjection was the best means of overcoming the disadvantageous impact of the many different forces at the capillary ends. Using no postinjection but a linear voltage ramp of 20 kV in 2.4 seconds led to a failure range of around 5% relative standard deviation. As expected from theory,[5,6] the voltage ramp showed minor impact in that case.

Final Test and Results

The final test procedure was chosen on the basis of the above tests and considerations to minimize the effects of the factors that degrade migration time and peak area reproducibility.

At the beginning of the test procedure the capillary is flushed with 1M and 0.1M NaOH and run buffer (capillary conditioning), independent of whether the capillary is being used for the first time or has been stored in buffer for a longer period of time. Afterwards, 25 vitamin separations are carried out for each test. The first 18 runs are for equilibration of the capillary to an acceptable degree. The nineteenth run is used as a calibration run for the reproducibility report. From the following six runs, calculations for time and area reproducibility are done. Injection is carried out in a two-step procedure consisting of a sample injection by pressure followed by a postinjection of run buffer, also by pressure. From a statistical point of view the total number of test runs (25) gives a high enough probability that any instrument failure will be discovered.

Time Reproducibility. Why new capillaries have to be conditioned before first use can be directly inferred from the theoretical aspects described above. Cleaning and the creation of high electroosmotic flow are the main reasons. But the capillary conditioning with 1M NaOH also has to be carried out for capillaries stored in buffer. Theory leads to the expectation that storage in buffer over several days should result in an equilibrium state of the surface. However, if the capillary is used directly after storage, a bigger and longer electroosmotic flow drift during test separations is observed compared to the drift obtained after the conditioning method-with 1M NaOH. Probably, the preconditioning with 0.1M NaOH before every run destroys the equilibrium reached during the contact with buffer only. But elimination of the 0.1M NaOH flushes before every run leads to a worse peak shape

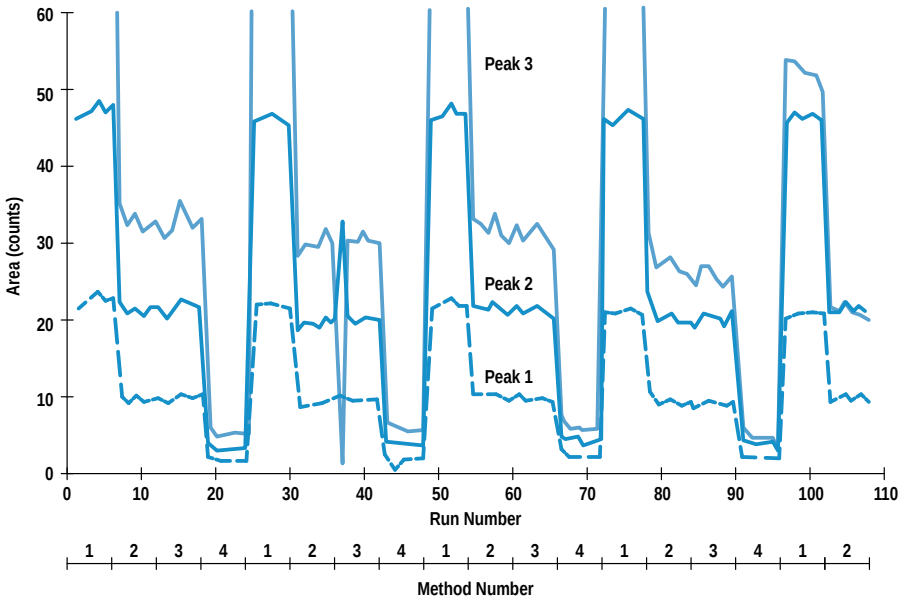

Fig. 1. Peak areas for thiamine (peak 1), nicotinamide (peak 2), and nicotinic acid (peak 3) using the four methods (injection types) described in Table IV. Six runs were done using each method and the entire sequence was repeated four times.

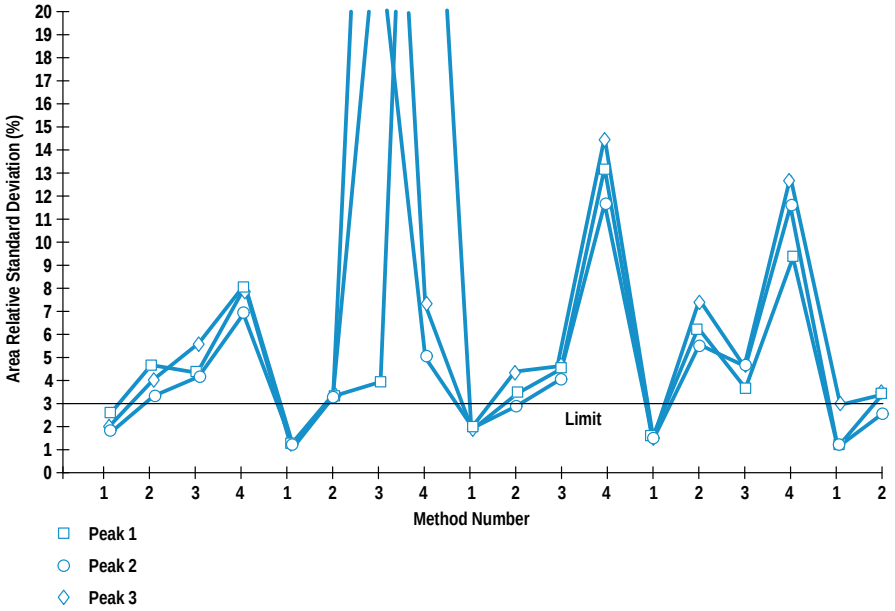

Fig. 2. Relative standard deviations of peak areas for thiamine (peak 1), nicotinamide (peak 2), and nicotinic acid (peak 3) using the four methods (injection types) described in Table IV. Six runs were used for the relative standard deviation calculation for each method and the entire sequence was repeated four times. The final test procedure for the HP CE instrument uses method 1.

for thiamine after a certain number of runs. In our tests, the best stability was reached by carrying out the capillary conditioning method once before the start of every test, followed by analysis procedures consisting of preconditioning with 0.1M NaOH and then run buffer, and finally separation.

Even when this procedure is used, the capillary needs 15 to 20 runs until stable enough conditions are established to ensure highly reproducible migration times. The procedure was verified in a series of five test sequences carried out using different capillaries. Figs. 3 to 5 show the migration times of the three vitamins thiamine, nicotinamide, and nicotinic acid plotted against the run number. Nicotinamide, the non-charged molecule, is only transported by electroosmotic flow. Therefore, this molecule can be used for the determination of electroosmotic flow. Figs. 3 to 5 clearly show variations within the first 15 to 20 runs which led to the deviations in migration times of all three test molecules. The impact of electroosmotic flow on negatively charged ions is much greater than on positively charged ions because negative ions migrate against the electroosmotic flow, so the influence of a fluctuating electroosmotic flow is much larger. This effect can be also deduced from Figs. 3 to 5.

Figs. 3 to 5 also show a comparison of the migration times of different sequences run with different capillaries. Differences between absolute values of electroosmotic flow for different capillaries are obvious. Electroosmotic flow variations up to 7% from capillary to capillary and up to 20% from batch to batch of bare fused silica could be observed. This is a well-known phenomenon described in the literature.[7,8]

Up to 1000 vitamin analysis runs can be carried out with a bare fused silica capillary treated as described above. In

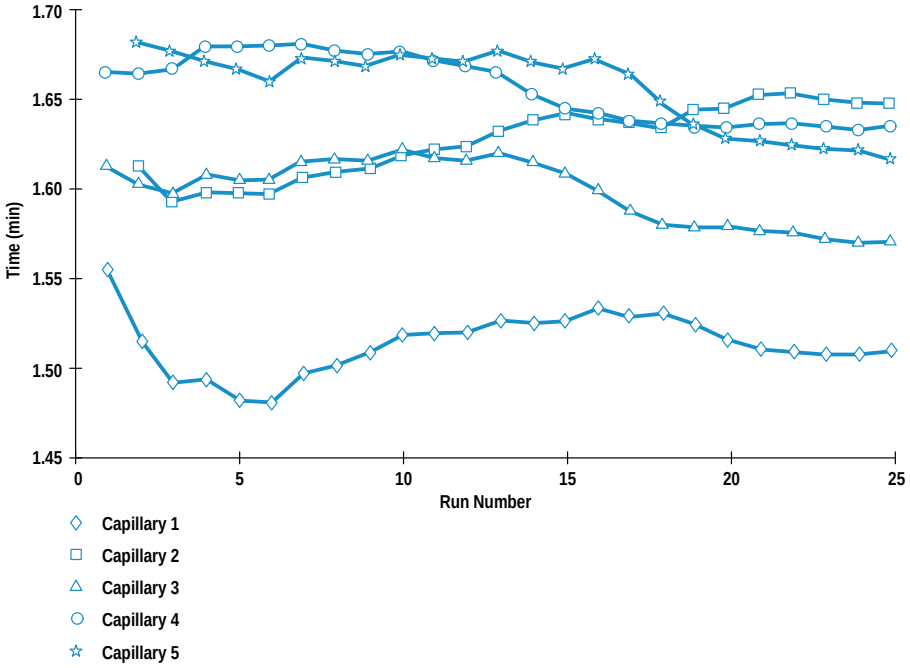

Fig. 3. Migration times for thiamine during the final test procedure for the HP CE instrument. The five plots are for five different capillaries.

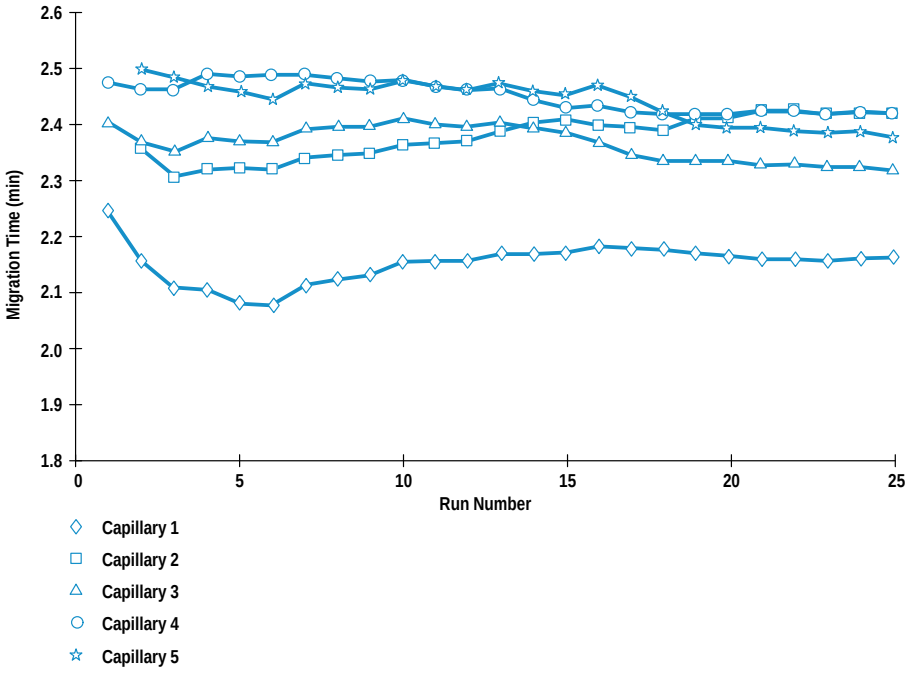

Fig. 4. Migration times for nicotinamide during the final test procedure for the HP CE instrument. The five plots are for five different capillaries.

general, a long-term drift to higher electroosmotic flow has been observed in long sequences.

Area Reproducibility. The injection procedure in the final test consists of two steps: the sample injection using the pressure mode is followed by a postinjection of run buffer by pressure as well. Both the sample plug and the buffer plug have a length of 3 mm. This plug length is a compromise between minimizing the zero injection effect and maximizing separation efficiency.

Another very important aspect for area reproducibility is the choice of the outlet vial during the injection procedure. The outlet home vial, which is the run buffer vial for the separation, has to be in position at the start of sample injection and must not be changed after sample injection. If the capillary outlet end is taken out of one vial and immersed into another, physical forces like surface tension of the aqueous liquid, inside and outer surface properties of the capillary, and pressure differences can cause the contents of the capillary to move. This can lead to either introduction of air or expulsion of liquid (sample) at the inlet side, the other end of the capillary. Like zero injection, this effect is hard to control and results in very high (failing) relative standard deviations. This problem is avoided by positioning the outlet home vial for all separations before injection.

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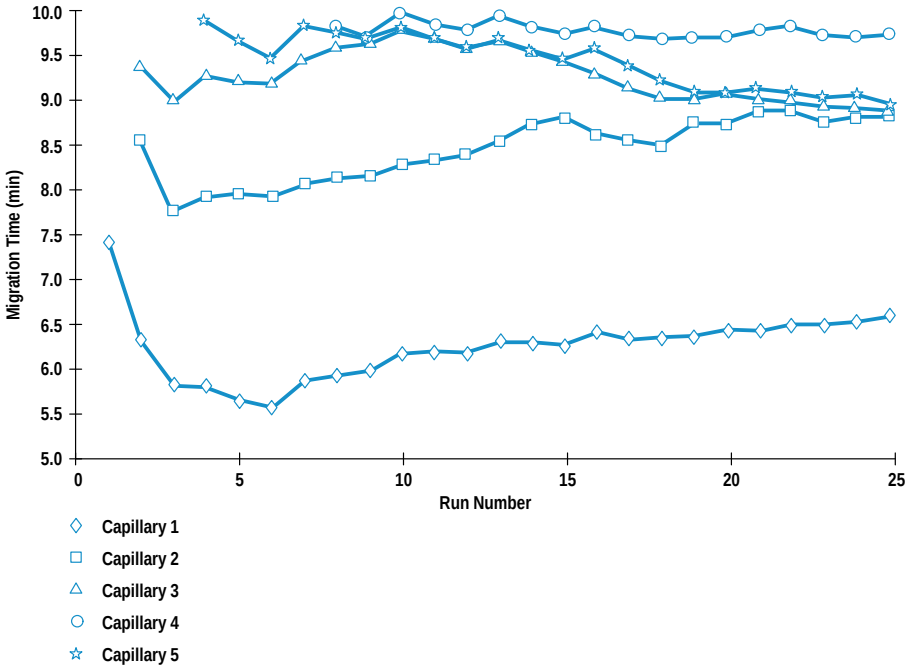

Fig. 5. Migration times for nicotinic acid during the final test procedure for the HP CE instrument. The five plots are for five different capillaries.

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