WHITE PAPER

Migration of Nutraceutical Methods from HPLC to UHPLC

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PerkinElmer

UHPLC SYSTEMS CAN DECREASE ANALYSIS TIMES UP TO TEN FOLD

Introduction

Recently, there has been much interest in the nutraceutical sector to speed up the analytical liquid chromatographic process to increase productivity as well as decrease consumption of mobile phase solvent to reduce cost and waste. This interest has been sparked by the development of ultra high pressure liquid chromatography (UHPLC) systems, such as the PerkinElmer Flexar FX-10 and FX-15, and new columns packed with porous sub-2 µm stationary phases. These UHPLC systems can decrease analysis times by up to ten fold and reduce mobile phase consumption by as much as 90% due to shorter analysis times and lower flow rates. Many laboratories wish to convert some of their traditional HPLC methods to fast UHPLC analysis but are hesitant due to their lack of experience and the cost of new instrumentation. This article will demonstrate the ease of method migration from HPLC to UHPLC and the cost savings realized in higher productivity and reduction in mobile phase consumption and cost of waste disposal.

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Determining Migration Candidates

One must first carefully consider which current chromatographic separations can and should be migrated to UHPLC in order to fully realize the advantages. The first factor to consider is the type of stationary phase used in the current HPLC analysis. For ease of migration it is best to choose a UHPLC stationary phase type that is identical or very similar to the one currently used so retention characteristics are similar. Currently only reversed phase columns are available in sub-2 µm particle sizes. Many manufacturers offer an array of sub-2 µm columns including PerkinElmer, which offers 1.9 µm C18, Aqueous C18, PFP Propyl, Biphenyl, and IBD stationary phases in 2.1 mm I.D. x 30 mm, 50 mm, or 100 mm lengths. When choosing a column length that will maintain the efficiency of the current HPLC separation, a good rule of thumb is if a 100 mm HPLC column is currently used, try a 30 mm UHPLC column, for 150 mm HPLC column go to a 50 mm UHPLC column, and for a 250 mm HPLC column go to a 100 mm UHPLC column. This is just a rule of thumb and there are other factors to consider when migrating to UHPLC. There are many in-depth references available on UHPLC migration including a white paper available on the PerkinElmer web site entitled "Guidelines for the use of UHPLC Instruments" by D. Guillarme and J. Veuthey.

To simplify method translation, an easy-to-use UHPLC method translator tool is available from PerkinElmer (Figure 1). This tool allows you to input your current HPLC method, and provides recommended UHPLC method conditions based on the equations used subsequently in this paper. The tool also provides valuable information about how much time and mobile phase solvent are saved by using UHPLC.

Time of analysis should also be considered when migrating to UHPLC. Longer HPLC analyses (>20 min.) are the best candidates for migration to UHPLC in order to truly take advantage of the speed of the system. In this paper the analysis of ginsenosides in ginseng will be used as an example to demonstrate the ease of migration to UHPLC.

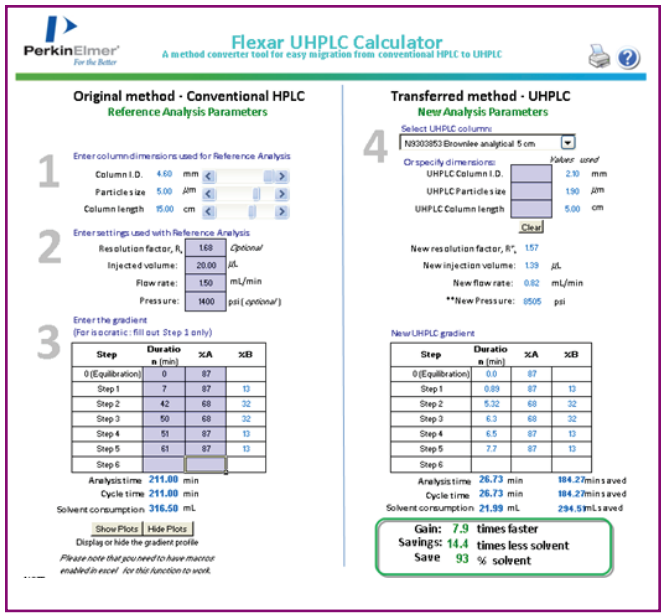

Figure 1. Flexar UHPLC Calculator: A method converter tool for easy migration from conventional HPLC to UHPLC.

Ginsenosides from ginseng are commonly separated by traditional HPLC using a C18 column and an acetonitrile/water gradient. In this study, the HPLC separation was performed on a PerkinElmer Brownlee Analytical C18, 150 mm x 4.6 mm, 5 µm column (P/N N9303513). The total analysis time was 61 minutes at a flow rate of 1.5 mL/min, a UV detection wavelength of 203 nm, column temperature of 30°C and injection volume of 20 µL. The chromatograph (Figure 2) consists of seven components with retention times ranging from 23.45 min to 45.84 min. The two most critical components to separate are Rg1 and Re which exhibit near baseline separation at a resolution (R_S) of 1.680.

The column selected for the UHPLC analysis was a PerkinElmer Brownlee Analytical C18, 50 mm x 2.1 mm, 1.9 µm (P/N N9303853). The main parameters that must be adjusted when converting an HPLC separation to UHPLC are flow rate, injection volume, time of isocratic steps, and time and slope of gradient steps. There are simple equations described in the aforementioned PerkinElmer white paper and elsewhere in the literature that can be utilized to predict all of these parameters for a UHPLC analysis. These calculations give a good approximation of the conditions for UHPLC separation but fine tuning is usually required to arrive at the final conditions for the analysis. Yet, the time required for fine tuning the method is significantly reduced compared to HPLC method development due to the faster analysis time and lower column volume which reduces column re-equilibration time.

The flow rate (F) for UHPLC should be adjusted so the mobile phase linear velocity (u) is similar to that used in the HPLC column. The linear velocity within a column is directly proportional to the column diameter (d_c) but also depends on the particle size (d_p) of the stationary phase. Therefore, a constant u^*d_p product must be maintained to account for changes in column diameter and particle size. The UHPLC flow rate (F_2) can be predicted using equation 1. In this equation (and all subsequent equations) subscripts 1 and 2 are related to HPLC and UHPLC, respectively. The flow rate calculated for the 50 mm x 2.1 mm, 1.9 mm column was 0.82 mL/min which is a 1.8 fold decrease in flow rate (originally 1.5 mL/min).

$$F_2 = F_1 \cdot \frac{d_{c_2}^{\,2}}{d_{c_1}^{\,2}} \cdot \frac{d_{p_1}}{d_{p_2}} \qquad \text{(Eq. 1)}$$

The injection volume (V_{inj}) must be adjusted to avoid column overload as well as to maintain sensitivity and reduce extra-column band broadening. As a rule, injected volumes should not exceed 1-5% of the column volume. The column volume is a function of the column length (L) and internal diameter (d_c) but

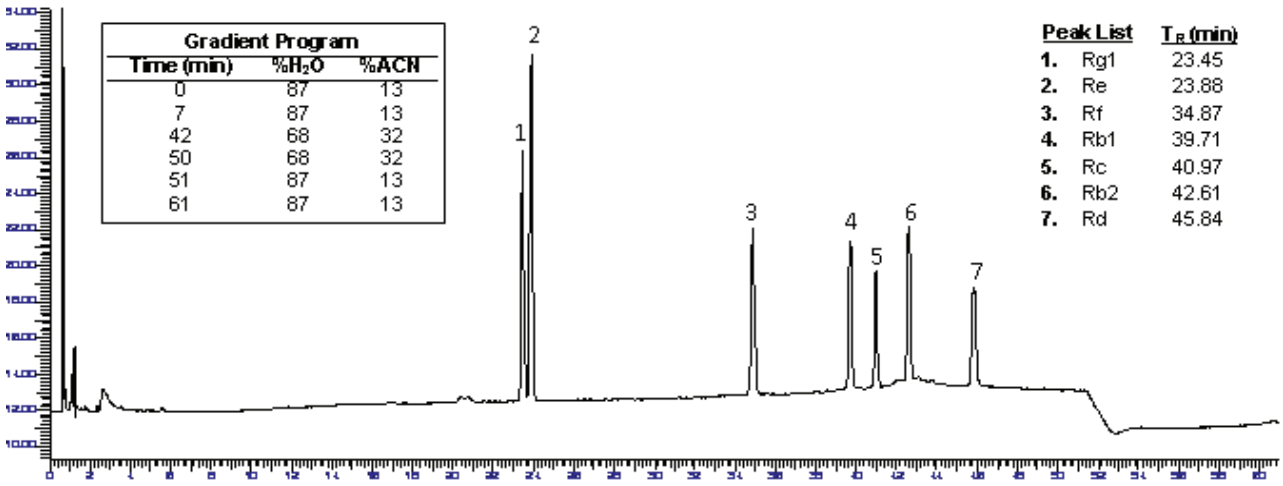

Figure 2. Chromatogram of ginsenosides from ginseng obtained on PerkinElmer Series 200 HPLC using a PE Brownlee Analytical C_{18}, 150 mm x 4.6 mm, 5 µm column.

is independent of stationary phase particle size. Therefore, the new injection volume (V_{inj_2}) was calculated with equation 2, which maintains the ratio of injection volumes and column dead volumes between HPLC and UHPLC. The new injected volume calculated was 1.4 µL which was a 14-fold decrease in volume.

$$V_{inj_2} = V_{inj_1} \bullet \frac{d_{c_2}^{\ 2}}{d_{c_1}^{\ 2}} \bullet \frac{L_2}{L_1} \qquad \text{(Eq. 2)}$$

The ratio between the isocratic step time (t_{iso}) and the column dead time must be maintained between HPLC and UHPLC conditions. The column dead time depends on the flow rate, column diameter and length. The isocratic step times were calculated using equation 3.

$$t_{iso_2} = t_{iso_1} \bullet \frac{F_1}{F_2} \bullet \frac{d_{c_2}^{\ 2}}{d_{c_1}^{\ 2}} \bullet \frac{L_2}{L_1} \qquad \text{(Eq. 3)}$$

The initial and final compositions in any gradient step should initially be maintained in the UHPLC method. The slope and time of a gradient step in the UHPLC method must be adjusted so the product of the gradient slope and dead time remain constant between the traditional HPLC method and the UHPLC method. Equation 4 and 5 were used to calculate the new slopes ($slope_2$) and subsequently the new gradient times (t_{grad_2}) for the UHPLC method.

$$slope_2 = slope_1 \bullet \frac{d_{c_1}^{\ 2}}{d_{c_2}^{\ 2}} \bullet \frac{L_1}{L_2} \bullet \frac{F_2}{F_1} \qquad \text{(Eq. 4)}$$

$$t_{grad_2} = \frac{(\%B_{final_1} - \%B_{initial_1})}{slope_2} \qquad \text{(Eq. 5)}$$

Table 1 shows a comparison of the traditional HPLC gradient program used to acquire the chromatogram shown in Figure 2 and the new gradient program calculated for the UHPLC method

Table 1. Comparison of HPLC gradient program and the calculated UHPLC gradient program.

Step	HPLC Time, min.	UHPLC Time, min.	%H2O	%ACN
1	0	0	87	13
2	7	0.9	87	13
3	42	5.3	68	32
4	50	6.3	68	32
5	51	6.4	87	13
6	61	7.7	87	13

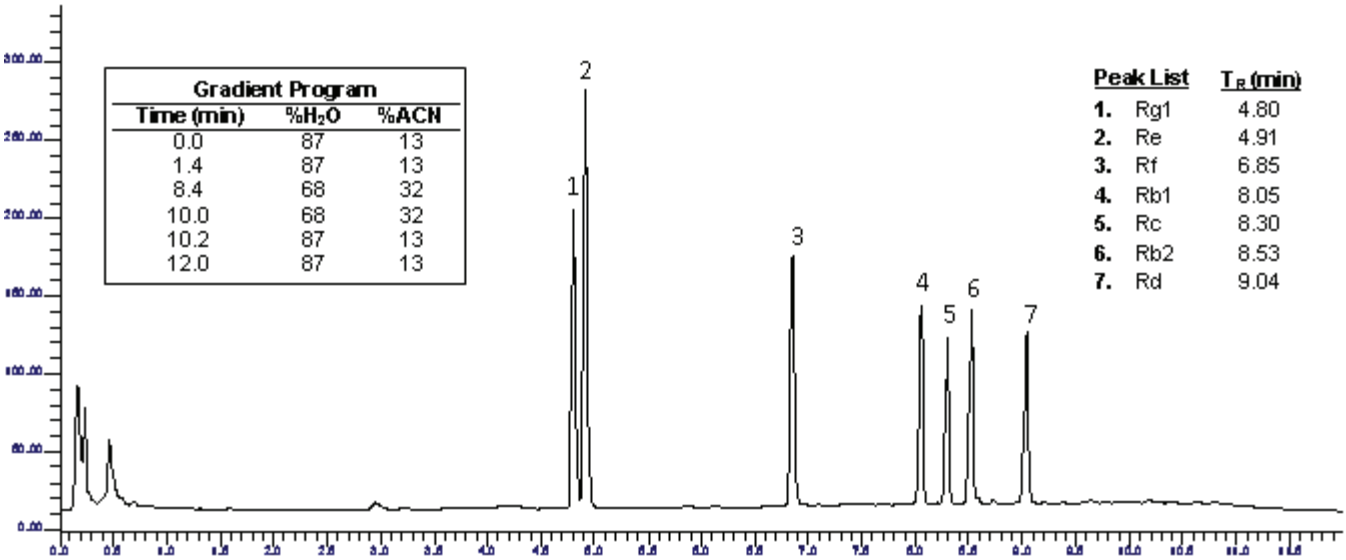

Figure 3. Chromatogram of ginsenosides from ginseng obtained on PerkinElmer Series 275 HRes LC using a PE Brownlee Analytical C18, 50 mm x 2.1 mm, 1.9 µm column.

using equations 3 & 4. There was an 8-fold decrease in analysis time and a 93% solvent savings predicted by the calculated UHPLC gradient program. This program was initially used in the UHPLC separation of the ginsenosides. The critical pair of components (Rg1 and Re) coeluted under these conditions so it was necessary to decrease the gradient slope at step 3 of the gradient program. The gradient time was systematically increased until adequate resolution of Rg1 and Re was obtained. The time of this step increased from 4.4 minutes to 7.0 minutes. The other step times were also fine tuned to minimize runtime while maintaining retention time repeatability. The optimized UHPLC conditions and chromatogram are shown in Figure 3.

Adjustments were also systematically made in the flow rate, injection volume, and column temperature to optimize the UHPLC conditions. The calculated flow rate was 0.82 mL/min, but after optimization a flow rate of 0.7 mL/min was found to be sufficient for separation of all components. This constitutes a 2.1-fold decrease in flow rate as compared to the HPLC conditions. The injection volume was increased from the calculated value of 1.4 µL to 5 µL to maximize sensitivity. This constitutes a 4-fold decrease in sample volume as compared to the 20 µL injection used in the HPLC analysis. Over time this could result in a significant savings in standards and samples especially critical in cases where samples are precious and standards are expensive. Finally the column temperature was increased to 35°C to lower the viscosity of the mobile phase and lower the overall pressure. The maximum pressure during the HPLC analysis was ~1400 psi while the maximum pressure during the UHPLC analysis was ~8100 psi. Column temperatures are commonly elevated on a UHPLC system to control the column backpressure but one must be careful not to compromise efficiency and peak resolution when elevating the temperature.

The time required to convert this analysis to UHPLC was less than one working day. As one can see, using the tools described here, method migration can be relatively easy. The result is a ~5-fold increase in productivity due to the reduction in runtime and a 91% savings in mobile phase consumption due to the reduction in flow rate. Additionally sensitivity is increased while consumption of samples and standards is reduced. Given the savings realized in increased productivity and decreases in the cost of mobile phase and waste disposal a UHPLC system can pay for itself in less than one year. This should be ample motivation to obtain a UHPLC system and start converting those long traditional HPLC separations to fast UHPLC analysis.

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