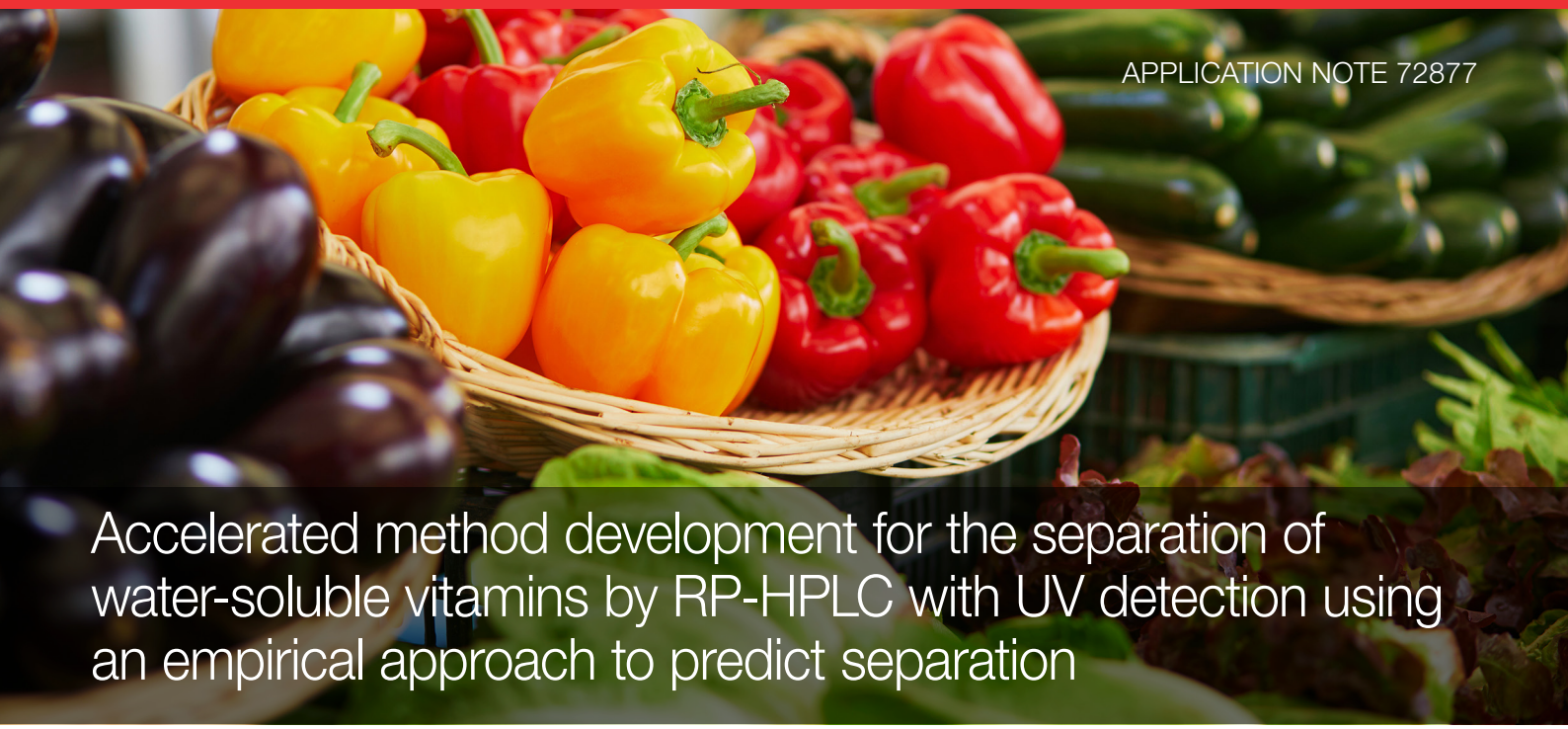
thermo scientific



APPLICATION NOTE 72877

Accelerated method development for the separation of water-soluble vitamins by RP-HPLC with UV detection using an empirical approach to predict separation

Authors

Sylvia Grosse, Soo Hyun Park, Mauro De Pra, Frank Steiner

Thermo Fisher Scientific, Germering, Germany

Keywords

Water soluble vitamins, reversed-phase chromatography, empirical approach, retention time prediction, Vanquish Horizon, UHPLC, ultraviolet detection, UV

Application benefits

- Fast method development using an empirical approach to predict separation
- First all-in-one method to achieve baseline separation of all known water-soluble vitamins on a reversed-phase (RP) column
- Excellent retention time prediction accuracy and method performance

Goal

To demonstrate the application of a software-based method development approach for the advanced separation of all known water-soluble vitamins in reversed-phase mode with ultraviolet detection. To achieve transferability of the predicted method with excellent agreement between predicted and experimental retention times.

Introduction

Vitamins are important constituents of our daily diet. Either vitamin deficiency or overdose can lead to many problems and diseases.[1] Comprehensive analysis of the vitamin composition of various foods, drinks, and dietary supplements is therefore of great importance. In particular, the group of water-soluble vitamins (WSVs) poses analytical challenges, since they are

ThermoFisher SCIENTIFIC

structurally diverse and some are highly polar. The separation capabilities of the most common technique, reversed-phase (RP) chromatography, may be insufficient, and two or more components may co-elute. A mass spectrometer could provide the extra selectivity in this case. However, the coupling of high liquid performance chromatography (HPLC) to mass spectrometry (MS) requires a substantial financial investment, as well as more specialized skills and knowledge of the analyst, which cannot be provided by all laboratories.

The poor retention of polar compounds in conventional reversed-phase columns, can be mitigated by using highly aqueous mobile phase conditions and polar embedded or polar end-capped columns. In the current study, a software-based method development approach was chosen to solve the difficult separation problem with the help of predictive models. Ultraviolet detection (UV) was used as the detection technique. An empirical approach, namely making a few initial isocratic runs and entering the experimental retention data of each analyte into ChromSword Offline software, was successfully tested and implemented to predict gradient profiles and retention times. The final optimized method was transferred to a Thermo Scientific™ Vanquish™ Horizon UHPLC system to measure method performance.

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Optima™ Acetonitrile LC/MS grade (P/N A955-212)
- Fisher Scientific™ Optima™ Methanol LC/MS grade (P/N A456-212)
- Fisher Scientific™ Formic acid LC/MS grade (P/N A117-50)
- Fisher Scientific™ Acetic acid LC/MS grade (P/N A113-50)
- Fisher Scientific™ Ammonium acetate LC/MS grade (P/N A114-50)
- Fisher Scientific™ Ammonium formate LC/MS grade (P/N A115-50)
- Fisher Scientific™ Potassium hydroxide, >85% (P/N 60-012-29)
- Fisher Scientific™ Potassium hydrogen carbonate (P/N AC446301000)

The vitamins ascorbic acid, biotin, cyanocobalamin, folic acid, nicotinamide, nicotinic acid, pantothenic acid, pyridoxal, pyridoxine, thiamine, and riboflavin (all analytical grade) were purchased from a reputable vendor.

Equipment

- Vials (amber, 2 mL), Fisher Scientific (P/N 11545884)
- Snap Cap with Septum (silicone/PTFE), Fisher Scientific (P/N 10547445)

Column and mobile phase screening

The ChromSwordAuto™ Developer module was used for column and mobile phase screening of four different stationary phases, five different aqueous eluents, and two organic eluents (Table 1). Afterwards, the best conditions were selected and used for further method development.

Table 1. Columns and eluents used for the ChromSwordAuto Developer-rapid optimization task

Columns
Thermo Scientific™ Hypersil GOLD™ aQ C18 (100 × 2.1 mm, 1.9 µm)
Thermo Scientific™ Acclaim™ VANQUISH™ Polar Advantage II (150 x 2.1 mm, 2.2 µm)
Thermo Scientific™ Acclaim™ Polar Advantage (100 × 2.1 mm, 2.2 µm)
Thermo Scientific™ Accucore™ aQ C18 (100 × 2.1 mm, 2.6 µm)
Aqueous Eluents
10 mM ammonium acetate, pH 3.8
10 mM ammonium formate, pH 3.8
50 mM ammonium formate, pH 3.8
50 mM ammonium formate, pH 6.5
0.015% formic acid in water, pH 3.1
Organic Eluents
Acetonitrile
Methanol

Preparation of standards

Stock solutions of ascorbic acid, biotin, cyanocobalamin, folic acid, nicotinamide, nicotinic acid, pantothenic acid, pyridoxal, pyridoxine, thiamine, and riboflavin were prepared at a concentration of 1 mg/mL in water, except for riboflavin and biotin in 5 mM KOH, and folic acid in 20 mM $KHCO_3$ due to limited solubility in water.

Standards for method development process

A mixture of biotin, folic acid, nicotinamide, nicotinic acid, pantothenic acid, pyridoxal, pyridoxine, thiamine, and riboflavin, as well as single solutions for peak assignment, were prepared with a concentration of 100 µg/mL, except for biotin and pantothenic acid (500 µg/mL) in water.

Standards for method performance data

For method performance experiments, ascorbic acid and cyanocobalamin were added to the compound list of the method development process to complete the set of WSV. Calibration samples were prepared according to the concentrations listed in Table 2 and injected in triplicate. For each vitamin, the optimal UV wavelength was selected for data evaluation.

The limit of detection (LOD) and the limit of quantitation (LOQ) values were estimated by extrapolation from the signal-to-noise (S/N) ratio of the 0.5 µg/mL calibration point, with the exception of biotin and pantothenic acid, for which the 100 µg/mL standard was applied as a reference. To assess the relative standard deviation (RSD) of retention time (RT) and peak area, the 100 µg/mL calibration standard, and for biotin and pantothenic acid the 750 µg/mL standard, was injected ten times.

Instrumentation

Thermo Scientific™ Vanquish™ Horizon system consisting of:

- System Base Vanquish Horizon (P/N VH-S01-A)
- Thermo Scientific™ Vanquish™ Binary Pump H (P/N VH-P10-A)
- Thermo Scientific™ Vanquish™ Split Sampler HT (P/N VH-A10-A)
- Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A)
- Thermo Scientific™ Vanquish™ Diode Array Detector (P/N VH-D10-A) with Thermo Scientific™ LightPipe™ Standard flow cell, 10 mm (P/N 6083.0100)

Table 2. Calibration concentration range and UV wavelength used for method performance assessment

Common name	Chemical name	Concentration [µg/mL]	UV [nm]
Vitamin C	Ascorbic acid	0.1–50	245
Vitamin B7	Biotin	100–750	245
Vitamin B12	Cyanocobalamin	1–100	280
Vitamin B9	Folic acid	0.1–100	280
Vitamin B3 derivative	Nicotinamide	0.5–100	245
Vitamin B3	Nicotinic acid	0.1–100	270
Vitamin B5	Pantothenic acid	100–750	210
Vitamin B6 derivative	Pyridoxal	0.1–100	280
Vitamin B6	Pyridoxine	0.1–100	280
Vitamin B1	Thiamine	0.1–100	245
Vitamin B2	Riboflavin	0.1–100	270

Table 3. LC conditions of final method used for method performance data

Column:	Hypersil GOLD aQ C18, 100 × 2.1 mm, 1.9 µm (P/N 25302-102130)	
Mobile phase:	A: 50 mM ammonium formate, pH 3.8 B: methanol	
Flow rate:	0.3 mL/min	
Gradient:	*Time [min]*	*% B*
	0	1
	0.2	2
	0.5	2
	0.7	4
	0.9	4
	1.3	6
	1.4	21
	12.0	21
	12.1	1
	22.0	1
Mixer volume:	10 + 25 µL	
Column temp.:	25 °C (passive pre-heater and Still Air mode)	
Injection volume:	1 µL	
Autosampler temp.:	5 °C	
UV wavelength 1:	210 nm	
UV wavelength 2:	245 nm	
UV wavelength 3:	270 nm	
UV wavelength 4:	280 nm	
3D scan:	190–360 nm	
UV data collection rate:	10 Hz	
UV response time:	0.5 s	

Data processing and software

ChromSword Auto 5 Software 5.0.437.633 with the module of ChromSwordAuto Developer for column and mobile phase screening was used. The ReportViewer module of ChromSwordAuto was used for data analysis and evaluation during the screening process.

ChromSword Offline Software 4.30.10.13 was used during the method development process to predict gradient profiles and retention times of the analytes by using the empirical approach.

Thermo Scientific™ Chromeleon™ 7.2.8 Chromatography Data System (CDS) was used for retention data acquisition and processing, to experimentally verify (or confirm) the predicted retention data by ChromSword offline modelling.

Results and discussion

Method development

As a first method development step, a column and mobile phase screening (Table 1) was performed using ChromSwordAuto Developer. ChromSword acquired a series of chromatograms, which were visually inspected. The Accucore aQ column and the Acclaim Polar Advantage column delivered poor resolution for the highly polar vitamins, while the Hypersil GOLD aQ column and Acclaim VANQUISH Polar Advantage II (PA2) column resulted in baseline separation for these critical compounds. However, the run time with the Acclaim Vanquish PA2 column was long compared to the one with the Hypersil GOLD aQ column. Therefore, the Hypersil GOLD aQ C18 column was selected as the most suitable column for further development. The Hypersil GOLD aQ column provided the best resolution when used with 50 mM ammonium acetate pH 3.8 and methanol, which was selected as solvent pair for method development.

A software-based approach was applied using ChromSword Offline software to speed up the development process. The broad hydrophobicity range of the molecules complicates baseline separation on a RP column of all the compounds in one method and in a reasonable analysis time. The empirical approach of the ChromSword Offline software allows the optimization of gradient profiles and prediction of retention times after few initial experiments.

Firstly, three initial runs were performed using an isocratic method with 10%, 15%, and 20% methanol. The retention times obtained were entered into the ChromSword Offline software. The software then suggested a suitable gradient profile for the separation of the analytes using predictive models and provided expected retention times for all analytes. The simulated chromatogram after optimization is shown in Figure 1.

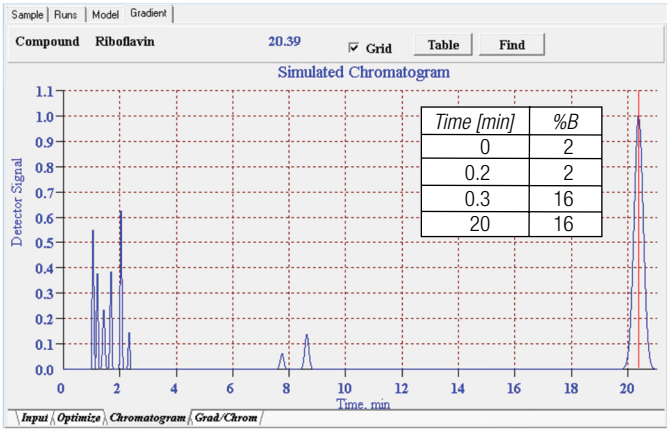


Figure 1. Simulated chromatogram with suggested gradient profile for nine WSVs. Experimental data used for prediction were obtained at 10%, 15%, and 20% methanol.

As the chromatogram shows, the separation of the polar vitamins (first six peaks) is not yet optimal, showing resolution <2.0. Furthermore, there are larger retention time gaps in the chromatogram between the polar and the non-polar WSVs (last three eluting peaks), which is not ideal, since the analysis time is unnecessary long.

The software delivered long run times because of the high diversity of analyte polarity. To circumvent the problem, the compounds were divided into two groups: a polar group with nicotinic acid, nicotinamide, pantothenic acid, pyridoxal, pyridoxine, and thiamine, and a non-polar group consisting of folic acid, biotin, and riboflavin. In this way, we intended to obtain two separate methods, one for the highly polar and one for the less polar, that we could try to combine in a single method afterwards. For the new approach, additional isocratic experiments were performed for the polar group with 5% methanol and for the non-polar group with 30%, 40%, and 50% methanol to extend the analysis window.

Upon entering into the program the four experimentally obtained retention datasets of 5%, 10%, 15%, and 20% methanol for the polar group, a method with 5 min analysis time was suggested by the software. Figure 2A shows the simulated chromatogram with baseline separation of all six polar WSVs.

After performing four initial runs of 20%, 30%, 40%, 50% methanol for the non-polar WSVs and entering the retention data into the software, the simulated chromatogram in Figure 2B was achieved, showing separation of all three analytes within 24 min. Again, the run time was unnecessarily long.

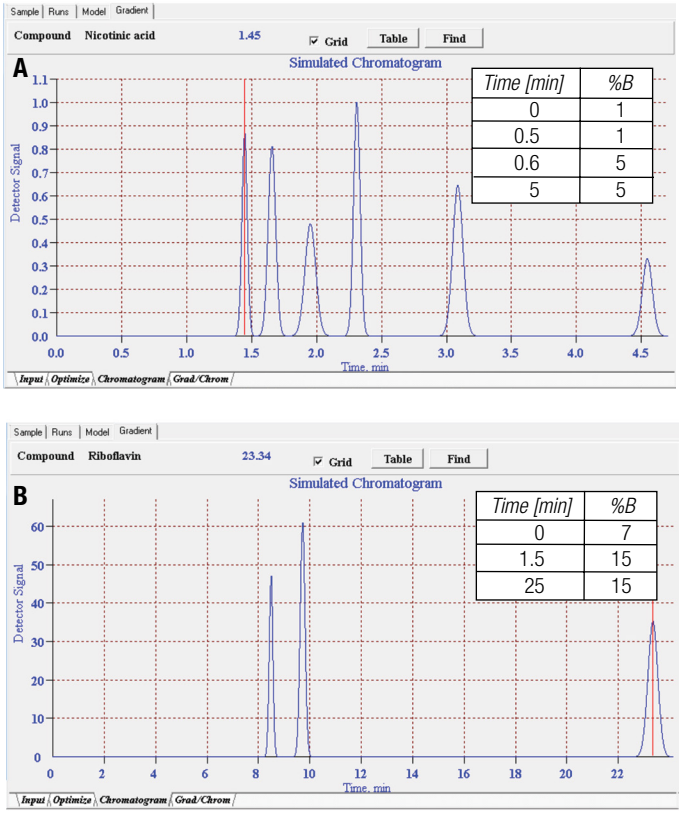


Figure 2. Simulated chromatograms A) for polar group (six polar vitamins) and B) for non-polar group (three non-polar vitamins), each with the suggested gradient profile after optimization. Experimental data used for prediction were obtained at 5%, 10%, 15%, and 20% methanol of the polar group (A) and 20%, 30%, 40%, 50% methanol of the non-polar group (B).

The final gradient condition (%B) of the polar group was suggested with 5% methanol, while the starting condition of the non-polar group was achieved with 7% methanol. If these methods were combined, there would be a slight shift in the retention conditions. Therefore, we tried to get the same organic condition (%B), namely 5% methanol for the polar and non-polar method, by adding the last eluting polar compound, pantothenic acid, to the set of non-polar analytes. After this change, the software suggested an initial gradient condition of 5% methanol and furthermore a much shorter method, with riboflavin being the last eluting compound at 12 min (Figure 3).

The obtained methods (gradient profile of Figure 2A and gradient profile of Figure 3) were run to compare the predicted retention times with the experimentally determined ones. The achieved results are summarized in Table 4. Excellent agreement between predicted and experimental retention times could be observed, with less than 0.28 min difference for all analytes, independent of their hydrophobicity.

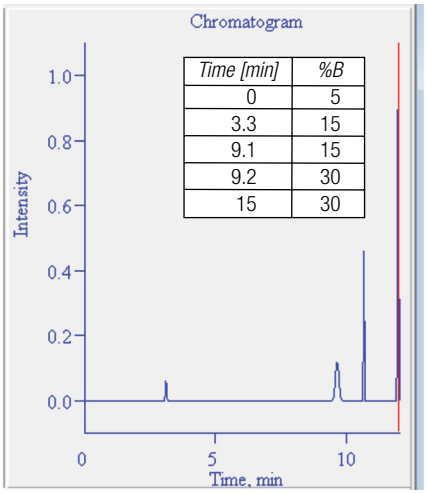


Figure 3. Predicted gradient profile and simulated chromatogram for the extended non-polar group of four WSVs. Experimental data used for prediction of the extended non-polar group were obtained at 20%, 30%, 40%, and 50% methanol.

The goal of the study was to develop a method that separates all nine vitamins. Therefore, the two separated methods needed to be combined. The fact that 5% methanol was suggested by the software as both the initial gradient composition of the extended non-polar group and the final gradient composition for the polar group made this step particularly easy, and no additional optimization was required other than simply extending the polar WSVs gradient with the non-polar WSVs gradient. Figure 4 shows the chromatogram obtained for the combined method. Single standards of each analyte were injected for peak assignments. Baseline separation was achieved within 17 min.

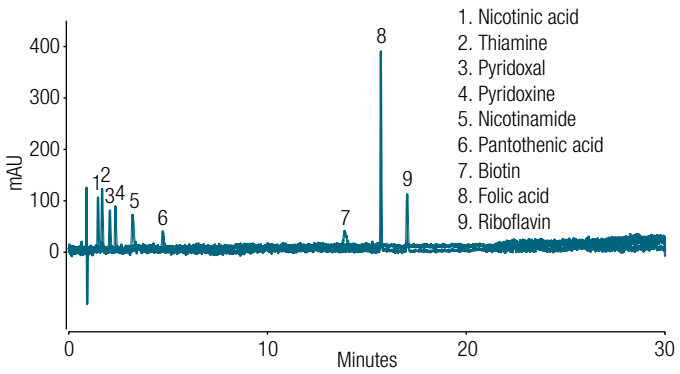


Figure 4. Chromatogram at 210 nm obtained after combining polar and extended non-polar gradient profiles into one method

Another promising strategy was the use of all the retention data for nine vitamins at 5%, 10%, 15%, and 20% methanol. Since the retention times of the three most retained vitamins (biotin, folic acid, and riboflavin) could not be experimentally determined at 5% methanol because the mobile phase was too weak (retention factor k>20), the retention times of these vitamins at 5% methanol were predicted by using Equation 1, which was derived using the retention datasets at 20%, 30%, 40%, and 50% methanol.

$$\ln k' = \ln k_0' + a_1c^1 + a_2c^2 + a_3c^3$$

Equation 1. Used for prediction of retention times for biotin, folic acid, and riboflavin at 5% methanol

where k' is retention factor and c the concentration of organic solvent (i.e., methanol (%)). The values are of retention coefficients $\ln k_0'$, a_1, a_2, and a_3 and are listed in Table 5.

Table 4. Comparison of predicted and experimentally determined retention times for the polar and extended non-polar group of WSVs

Polar group			
Vitamin	**RT predicted [min]**	**RT experimental [min]**	**Δ RT (exp.-pred.) [min]**
Nicotinic acid	1.45	1.48	+0.03
Thiamine	1.66	1.71	+0.05
Pyridoxal	1.95	2.08	+0.13
Pyridoxine	2.31	2.36	+0.05
Nicotinamide	3.09	3.23	+0.14
Pantothenic acid	4.55	4.72	+0.17
Extended non-polar group (including pantothenic acid)			
Vitamin	**RT predicted [min]**	**RT experimental [min]**	**Δ RT (exp.-pred.) [min]**
Pantothenic acid	3.10	3.31	+0.21
Biotin	9.66	9.94	+0.28
Folic acid	10.68	10.72	+0.04
Riboflavin	12.00	12.05	+0.05

Table 5. Values of ln k_0', a_1, a_2, a_3 used in Equation 1

Compound	ln k_0'	a_1	a_2	a_3
Riboflavin	6.929	-0.288	0.0037	0
Folic acid	5.903	-0.285	0.0035	0
Biotin	4.305	-0.160	0.0014	0

After entering all the RT data (combining the predicted RT data for the three vitamins with all the other experimental data) into the software, it was possible to perform the method optimization and prediction for all nine vitamins with a broader analysis window. This approach led to a rapid method in which all nine vitamins could be baseline separated within 11 min (Figure 5). After experimental use of this method, the retention time accuracies (Δ RT (exp.-pred.)) obtained are excellent, being less than ±0.10 min (Table 6) for six out of nine WSVs. Slightly lower accuracies could be observed for folic acid (+0.51 min), biotin (+0.25 min) and riboflavin (+0.82 min). The deviation may be due to the poor accuracy of the applied model used for these three analytes since these compounds could not be experimentally determined with 5% methanol. The use of predicted instead of experimental data in the model could have led to worse RT accuracy. Nevertheless, the values are still in a fully acceptable range <1 min.

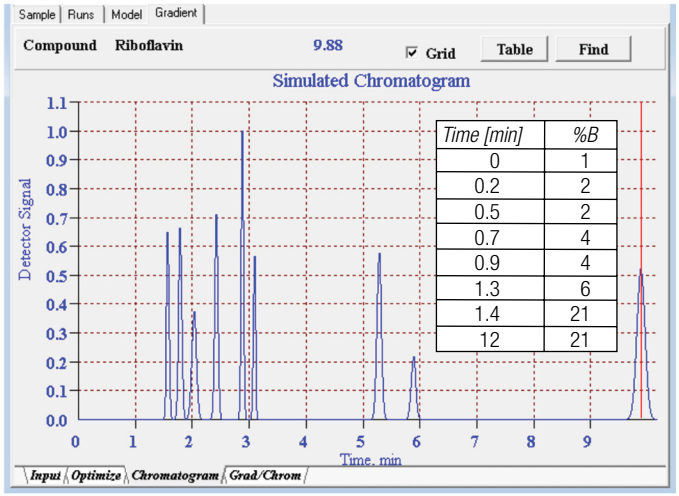


Figure 5. Simulated chromatogram and gradient profile obtained for nine WSVs. Combined experimental (six vitamins) and predicted (three vitamins at 5% methanol) datasets of 5%, 10%, 15%, and 20% methanol were used for prediction.

This approach, compared to the approach of combining the separately modelled methods for the polar and extended non-polar groups, provided the best gradient profile in terms of separation and analysis time and was further selected for method performance evaluation. With only a few experimental runs, the method for the WSVs was developed in less than two days.

Table 6. Comparison of predicted and experimentally determined retention times for the method of Figure 5

Vitamin	RT predicted [min]	RT experimental [min]	Δ RT (exp.-pred.) [min]
Nicotinic acid	1.57	1.48	-0.09
Thiamine	1.79	1.69	-0.10
Pyridoxal	2.03	2.05	+0.02
Pyridoxine	2.42	2.40	-0.02
Nicotinamide	2.88	2.89	+0.01
Pantothenic acid	3.09	3.11	+0.02
Folic acid	5.28	5.79	+0.51
Biotin	5.89	6.14	+0.25
Riboflavin	9.88	10.70	+0.82

Method performance

To obtain method performance data of RT, resolution, RSD of RT, and peak area for a complete set of all known water-soluble vitamins, ascorbic acid and cyanocobalamin were included. As ascorbic acid is known to be very unstable, it was removed from the method development process to avoid problems with degradation and the generation of degradation peaks.

A standard mixture of 100 µg/mL WSVs, including ascorbic acid, nicotinic acid, thiamine, pyridoxal, pyridoxine, nicotinamide, folic acid, cyanocobalamin, and riboflavin, as well as 750 µg/mL individual standard solutions of pantothenic acid and biotin were injected ten times. All eleven WSVs, including ascorbic acid and cyanocobalamin show baseline separation (Figure 6). Peak 1, ascorbic acid, shows low retention, which may be critical for some applications, as matrix peaks could co-elute with the analyte.
On the other hand, the results presented in Table 7 show a minimum resolution of 2.3 for the most critical peak pair folic acid (peak 8) and biotin (peak 9), which is above the required minimum resolution of 2.0 needed for reliable quantification. Good method reproducibility for retention time and peak area was achieved for all vitamins with <0.16% for RT and <1% for peak area. Only pantothenic acid and biotin show slightly higher, but still acceptable, RSD values for peak area with 3.13 and 3.11, respectively.

The linearity of the calibration for the WSVs was examined by making three injections of at least four calibration points in the calibration range listed in Table 8. Excellent correlation coefficients (R^2) could be achieved for all

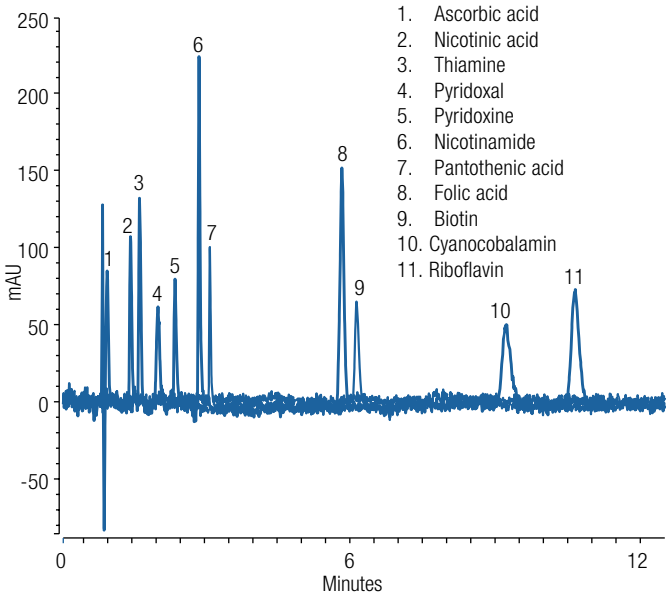


Figure 6. Overlaid chromatograms obtained for a 100 µg/mL WSVs mixture and individual solutions of 750 µg/mL of biotin and pantothenic acid injected to the final method of Figure 5 with 12 min gradient profile; UV trace shown at 210 nm

vitamins. The LOD and LOQ values were estimated by using the 0.5 µg/mL calibration point, or 100 µg/mL in case of pantothenic acid and biotin, as a reference and extrapolating the concentration for LOD to S/N 3 and LOQ to S/N 10. The lowest LOD value was found for folic acid with 0.030 µg/mL (±0.005 µg/mL) and the highest value for pantothenic acid with 75.96 µg/mL (±9.55 µg/mL). For each vitamin, the optimal wavelength was chosen (Table 2). Biotin and pantothenic acid show no specific UV absorption above 210 nm, which decreases the detection sensitivity significantly, since most of the solvents used for HPLC absorb strongly themselves at this wavelength.[2]

Table 7. Results of RT, resolution, and RSD of RT and Area for the complete set of WSVs (n=10)

Peak	Vitamin	RT [min]	Resolution	RSD (RT)	RSD (Area)
1	Ascorbic acid	0.99	6.1	0.09	0.52
2	Nicotinic acid	1.48	2.6	0.12	0.17
3	Thiamine	1.69	3.3	0.16	0.17
4	Pyridoxal	2.05	3.2	0.07	0.28
5	Pyridoxine	2.40	7.0	0.04	0.32
6	Nicotinamide	2.89	3.7	0.05	0.55
7	Pantothenic acid	3.11	25.5	0.08	3.13
8	Folic acid	5.79	2.3	0.14	0.21
9	Biotin	6.14	12.7	0.11	3.11
10	Cyanocobalamin	9.26	4.5	0.14	0.95
11	Riboflavin	10.70	-	0.08	0.17

thermo scientific

Table 8. Calibration results with linearity and LOD, LOQ estimations with standard deviation (± S.D.) (n=10)

Vitamin	Calibration range [µg/mL]	R^2	LOD ± S.D. [µg/mL]	LOQ ± S.D. [µg/mL]
Ascorbic acid	0.1–50	0.9998	0.142 ± 0.039	0.473 ± 0.131
Nicotinic acid	0.1–100	0.9999	0.043 ± 0.008	0.145 ± 0.026
Thiamine	0.1–100	0.9999	0.052 ± 0.014	0.172 ± 0.045
Pyridoxal	0.1–100	0.9999	0.072 ± 0.012	0.239 ± 0.039
Pyridoxine	0.1–100	0.9999	0.054 ± 0.009	0.179 ± 0.029
Nicotinamide	0.5–100	0.9996	0.098 ± 0.025	0.325 ± 0.083
Pantothenic acid	100–750	0.9965	75.96 ± 9.55	253.19 ± 31.84
Folic acid	0.1–100	0.9999	0.030 ± 0.005	0.102 ± 0.017
Biotin	100–750	0.9990	34.87 ± 7.79	116.22 ± 25.97
Cyanocobalamin	1–100	0.9997	0.399 ± 0.063	1.330 ± 0.211
Riboflavin	0.1–100	0.9999	0.044 ± 0.008	0.145 ± 0.026

To our best knowledge this is the first presentation of a RP-HPLC-UV method of all known WSVs in one method achieving full baseline separation with resolution above 2.0 for all analytes. In addition, the analysis time is very short at 11 min. The developed method can be considered as a fast and efficient method for routine analysis and reliable quantification of WSVs.

Conclusion

- ChromSword Offline software offered the possibility to predict gradient profiles for the baseline separation of WSVs.
- Excellent retention time accuracy was achieved between predicted and experimentally determined retention times.
- A fast and efficient method of all known WSVs with resolution >2.0 was developed.
- Good method performance and reproducibility was proven.

References

1. Kamangar, F. and Emadi, A., Vitamin and Mineral Supplements: Do We Really Need Them?, Int. *J. Prev. Med.* **2012,** *3*(3), 221–226.
2. Walash, M. I., Sheribah, Z. A., Salim, M. M., Kinetic Spectrophotometric Determination of Biotin in Pharmaceutical Preparations, *Int. J. Biomed. Sci.* **2008,** *4*(3), 238–24.

Find out more at **thermofisher.com/vanquish**

ThermoFisher SCIENTIFIC