Application Note

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B Vitamin Analysis in Dietary Supplements on the Agilent 1290 Infinity III LC System

Using ion-pairing reagent and supported by time-saving automation of routine tasks using the Agilent InfinityLab Assist

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Abstract

B vitamins are present in a large variety of vitamin supplements on the market. As quality control of vitamin supplements is an important step, analysis of B vitamins such as B1, B2, B5, B6, B7, B9, and B12 commonly used in supplements is necessary to ensure compliance with the stated nutritional values.

In this application note, an analytical method capable of detecting seven B vitamins using ion-pairing reversed-phase LC and UV detection is presented. Furthermore, the benefits of using the Agilent InfinityLab Assist "Make Ready" task to ensure reproducible and fast system preparation are demonstrated. The schedulable "Make Ready" and "Standby" tasks can clearly reduce errors during daily laboratory work, which can also help to reduce unnecessarily long instrument idle states or solvent consumption.

Introduction

There is a huge variety of (multi)vitamin supplements on the market, with different vitamin combinations and different concentrations available. To ensure that the claimed vitamin contents are met, analysis of those supplements is necessary.

To ensure constant and good analytical performance, preparation of the analytical system is fundamental. There are different approaches to preparing the system for analysis. Purging the solvent lines from bottle to pump removes possible air bubbles, especially after longer breaks between instrument usage or changing eluent to the ion-pairing and buffer-containing eluent after the weekend. Equilibration can either be done by a calculated time (10–20 column volumes)[1], set as a fixed experimentally determined value, or manually monitored. Using a fixed time for equilibration can lead to unnecessary high solvent consumption, as equilibration may already be established and no monitoring was previously done. In contrast, manual monitoring of equilibration can be time-consuming and error-prone. For example, if there are no written rules regarding key points for equilibration of the column, its determination can be subjective (constant pressure and baseline of e.g. UV absorption).

As vitamins are grouped according to their water solubility and not their chemical structure, separation behavior can greatly differ as they have different charges at physiological pH. In such cases, ion-pairing reagents such as hexane sulfonic acid can enable the separation of those vitamins while using a reversed phase column. As ion-pairing LC depends on good equilibration to ensure the presence of ion-pairing reagent within the system, the process of preparing the instrument is an inevitable step. This is essential for good and reproducible analytical results.

With the Agilent InfinityLab Assist, making your instrument ready is no longer a time-consuming and unpleasant procedure.[1] This application note demonstrates the separation of multiple B vitamins in multivitamin tablets and effervescent tablets using an Agilent 1290 Infinity III LC System. Furthermore, it is shown how the InfinityLab Assist can help users ensure good chromatographic results, save time, and reduce errors by replacing manual tasks with InfinityLab Assist tasks.

Experimental

Equipment

- Agilent 1290 Infinity III High-Speed Pump (G7120A)
- Agilent 1290 Infinity III Multisampler (G7167B) with Agilent InfinityLab Sample Thermostat (G4761A)
- Agilent 1290 Infinity III Multicolumn Thermostat (G7116B) with Agilent InfinityLab Quick Connect Heat Exchanger (G7116-60015)
- Agilent InfinityLab Level Sensing (G7175A)
- Agilent InfinityLab Assist (G7180A) with Agilent InfinityLab Assist Control Software, version 2.0
- Agilent 1290 Infinity III Diode Array Detector (G7117B) with Agilent InfinityLab Max-Light Cartridge Cell (10 mm, 1.0 µL, G4212-60008)

Software

Agilent OpenLab CDS, version 2.8, update 7 (or later versions)

Column

Agilent InfinityLab Poroshell 120 EC-C18, 2.1 mm × 100 mm, 2.7 µm column (p/n 695775-902).

Table 1. Chromatographic conditions.

Parameter	Value
Mobile Phases	A) 50 mM NaH_2PO_4 + 5 mM hexane sulfonate, filtrated using a 0.2 µm membrane filter (p/n 5191-4340), pH 3.07 with phosphoric acid B) Methanol
Flow Rate	0.400 mL/min
Gradient	Time (min) %A %B 0.00 97.00 3.00 1.00 97.00 3.00 3.85 80.00 15.00 13.25 45.00 55.00 13.50 97.00 3.00 Stop time: 13.50 min Post time: 3 min
Multicolumn Thermostat (MCT) Temperature	40 °C
Diode Array Detector	Wavelengths (10 nm width): – 210 nm for vitamin B5, B6, B7, B9, and B12 – 246 nm for vitamin B1 – 268 nm for vitamin B2 Reference wavelength: 533 nm (20 nm width) Peak width: > 0.013 min (0.25 s response time) (20 Hz)
Injection	Injection volume: 5 µL Needle wash: 50:50 (v/v) water:acetonitrile Thermostat temperature: 10 °C

Analytes

Table 2. List of B vitamins used, their chemical name, and the supplier.

Vitamin	Chemical Name	Supplier
B1	Thiamine hydrochloride	Supelco
B2	Riboflavin	Sigma-Aldrich
B5	Calcium-d-pantothenate	Supelco
B6	Pyridoxal hydrochloride	Sigma-Aldrich
B7	Biotin	Supelco
B9	Folic acid	Supelco
B12	Cyanocobalamin	Sigma-Aldrich

Solvents and modifier

- Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak)
- Methanol (Agilent InfinityLab Methanol for LC/MS (gradient grade), part number 5191-5110*)
- Orthophosphoric acid (Supelco LiChropur, 85%)
- Sodium hexane sulfonate (Sigma-Aldrich, ≥ 98%)
- Sodium phosphate monobasic (Sigma-Aldrich)

* Only available in select countries

Samples

Vitamin standards were each dissolved in ultrapure water and then mixed to a standard solution at a concentration of 5 µg/mL. Multivitamin tablets and multivitamin effervescent tablets were ground separately with a mortar and pestle and dissolved in ultrapure water. Before injecting, aliquots of the samples were filtered with a regenerated cellulose syringe filter (Agilent Captiva Premium Syringe Filter, p/n 5190-5111). The injected amount of vitamin for each sample can be found in Table 3.

Table 3. Supplements investigated and the injected amounts on column of vitamins calculated according to the vitamin content stated on the packaging.

Sample	B Vitamin Amount on Column (ng)						
	B1	B2	B5	B6	B7	B9	B12
Supplement 1 (Effervescent Tablet)	34	39	146	49	–	–	0.06
Supplement 2 (Tablet)	56.9	72.41	310.34	72.41	2.59	10.34	0.13
Supplement 3 (Tablet)	117.45	134.23	402.68	167.79	10.07	15.1	0.08
Supplement 4 (Mini-Tablet)	–	–	1,723.40	–	–	–	47.87

InfinityLab Assist Settings for "Make Ready" and "Standby" Task

Table 4. InfinityLab Assist settings for automatic equilibration and standby.

Make Ready	Setting	Setpoint
Purge System	Purge mode	Automatic
	Channels to purge	A1 and B1
Preflush	Flush volume	5 mL (estimated preflush time 2 min)
	Flow	2.5 mL/min
	Solvent selection valve	A1 and B1
	Composition	97% A and 3% B
	Pressure limit	Max. 600 bar
	Flow path	Mainpass – Needle to waste
Equilibrate	Equilibration mode	Automatic
	Equilibration monitoring sensitivity	Low/Standard/Strict
	Solvent Delivery	
	Flow	0.400 mL/min
	Solvent selection valve	A1 and B1
	Composition	97% A and 3% B
	Pressure limit	Max. 600 bar
	Sample Delivery	
	Thermostat status	On*
	Temperature	10 °C
	Separation	
	Thermostat temperature left	40 °C
	Thermostat temperature right	Combined
	Detection	
	Switch on UV lamp	Yes
	Equilibration monitoring	Yes
Standby	**Setting**	**Setpoint**
Flush System	Flush volume	3.0 mL (estimated flush time 5 min)
	Solvent Delivery	
	Flow	0.600 mL/min
	Solvent selection valve	A1 and B1
	Composition	90% A and 10% B
	Pressure limit	Max. 600 bar
Standby	Standby mode	User-defined
	Solvent delivery flow	0.000 mL/min
	Energy Saving Options	
	Switch off column thermostat	Yes
	Switch off detector	Yes
	Switch off sample thermostat	No*

* As samples were stored in the sampler overnight, the sampler thermostat was not switched off after measurements and was already cooled down before starting the Make Ready Task.

Conditions for Manual Instrument Make Ready

Table 5. Settings for Manual Make Ready of the instrument.

Manual Make Ready		Setpoint
Modules	**Separation**	
	Thermostat temperature left	40 °C
	Thermostat temperature right	Combined
	Sample Delivery	
	Thermostat status	On*
	Temperature	10 °C
	Detection	
	Switch on UV lamp	On
Purge System	Purge channels A1 and B1 for 3 min at 5 mL/min each	
Equilibrate	**Solvent Delivery**	
	Flow	0.400 mL/min
	Solvent selection valve	A1 and B1
	Composition	97% A and 3% B
	Pressure limit	Max. 600 bar

* As samples were stored in the sampler overnight, the sampler thermostat was not switched off after measurements and was already cooled down before starting the Make Ready task.

Results and discussion

Separation of vitamin B1, B2, B5, B9, and B12

To analyze vitamin B1, B2, B5, B6, B7, B9, and B12 in vitamin supplements, different columns, mobile phases, and gradients were evaluated. The selected chromatographic conditions (Table 1) showed the best separation from peak to peak and additional matrix peaks. Hexane sulfonate was needed as an ion-pairing reagent to ensure good peak shapes, as all vitamins investigated, except vitamin B12, are expected to be charged based on their pKa values.[3] The method detects the B vitamins investigated in vitamin tablets and effervescent tablets as demonstrated in Figure 1, showing chromatograms of aqueous standards and real samples. As some vitamins are present at higher doses, such as the 12 mg tablet (vitamin B5) compared to the 2.5 µg tablet (B12) in supplement 3 (Figure 1), parallel analysis is rather difficult, and not all vitamins present were detectable in each supplement.

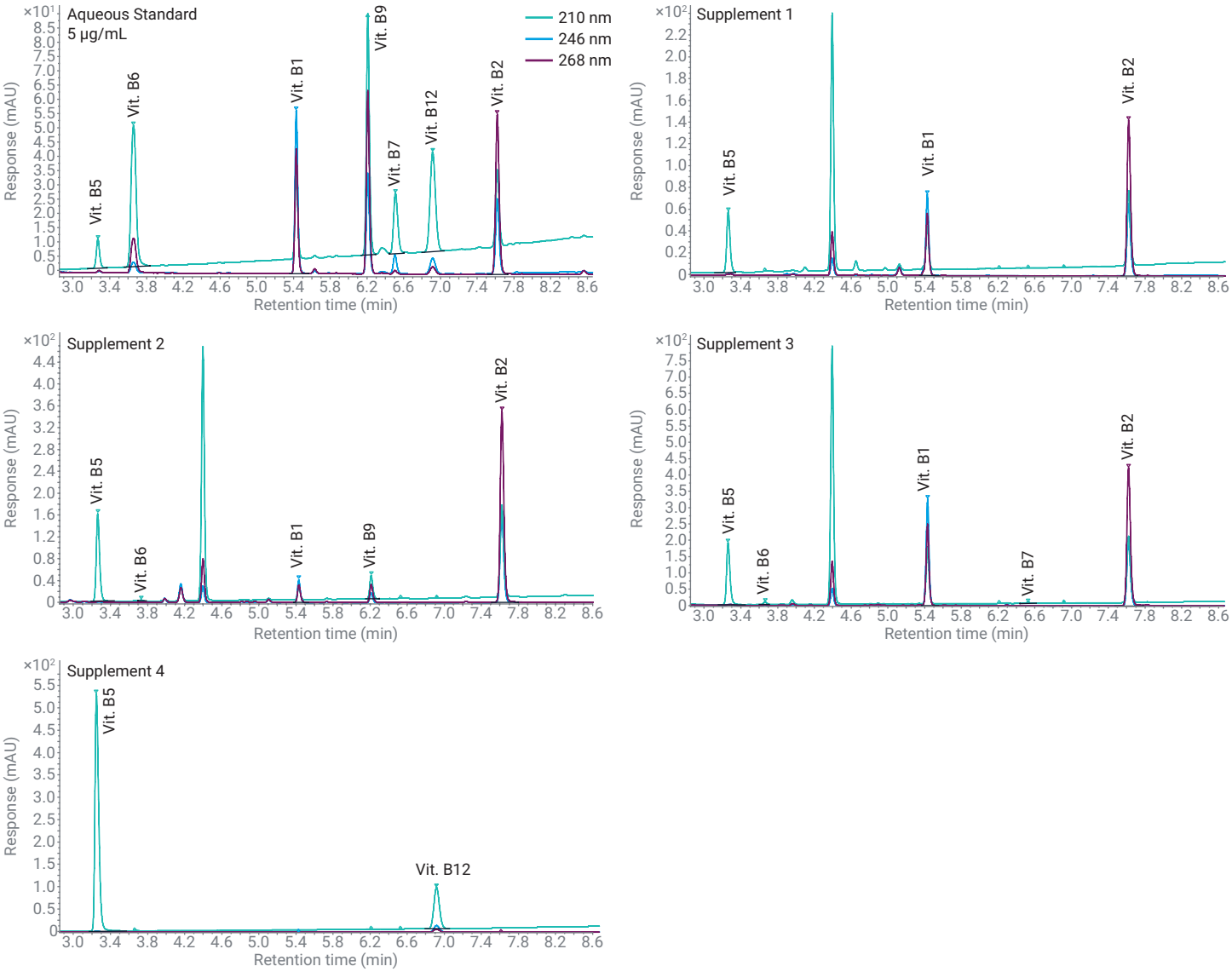

Figure 1. Chromatograms of aqueous vitamin standards and multivitamin supplement extracts under the chromatographic conditions described in Table 1.

System preparation for measurements and instrument standby

In Figure 2, equilibration times of the system are shown depending on the technique used. Each equilibration consists of purging solvents, switching the multicolumn thermostat (MCT) and diode array detector on, and flushing the column under the chromatographic starting conditions described in Table 1. For the automatic "Make Ready" task implemented in InfinityLab Assist (for configuration, see Table 4), an additional step to preflush the system (flowpath needle to waste) was used. Conditions for manual "Make Ready" can be found in Table 5. Furthermore, different modes of automatic equilibration monitoring sensitivity were tested (see Figure 2). In this case, sensitivity describes the acceptable variation of pump and detector signals until conditions are regarded as stable. With relative standard deviations of the equilibration time < 0.11% for low and standard mode, and 0.67% for strict equilibration mode, automatic monitored equilibration times were reproducible between test runs.

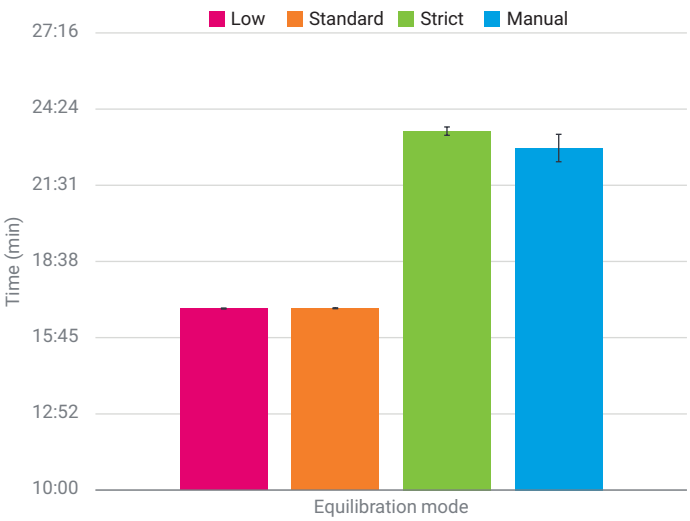

Figure 2. Time needed for system preparation using the automated "Make Ready" task or manual equilibration.

As expected, strict equilibration mode took longer (approximately 6.5 minutes) than low and standard mode. Time needed for manual equilibration is comparable with strict mode, but with a higher standard deviation (2.26%) of the total system preparation time, even though experiments were conducted by the same person.

This is explained by the manual determination of successful equilibration and time needed to conduct different steps of system preparation, which can vary day-by-day. In a busy laboratory environment, tasks such as sample preparation and system setup are often carried out in parallel, which can significantly extend the overall system preparation time. In contrast, no monitoring of the "Make Ready" task is necessary when using InfinityLab Assist. By scheduling this task, the instrument can be ready directly at the beginning of the workday. Alternatively, the premade task can be started anytime by the operator who can now fully focus on other tasks such as sample preparation or sequence writing. Differences between individual operators can be reduced, as the perception of an equilibrated system may differ among operators. For traceability reasons, signals during the "Make Ready" task are documented, can be reviewed (Figure 3), and exported to a PDF file in InfinityLab Assist.

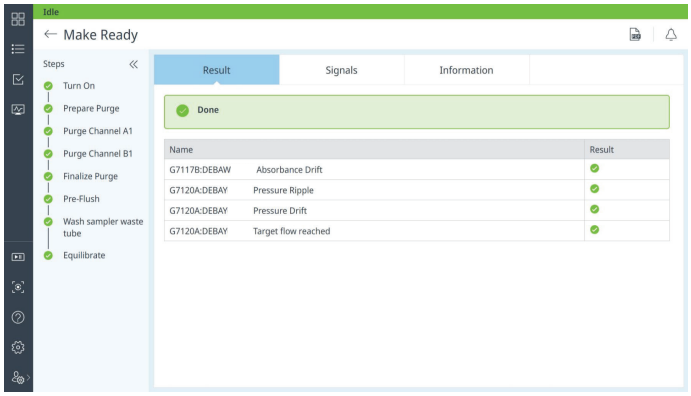

Figure 3. Report following successful automatic equilibration of an LC system.

Additionally, putting the instrument into standby is a substantial process in the daily business of a laboratory. Especially when using third-party software, submitting a shutdown method after sample runs may be not possible or can be a difficult task, which can lead to unnecessarily long idle time of the instrument. Using the possibility to schedule the InfinityLab Assist "Standby" task, this manual step can easily be omitted.[2] Furthermore, adjustment of the task is possible.[2] For example, to ensure safe sample storage during the conducted experiments, the sampler thermostat was held at a constant temperature of 10 °C. For this reason, the "Standby" task was modified to switch off the MCT, detector, and set no pump flow, but the sample thermostat was left running (see Figure 4). This can also reduce energy consumption depending on the time between "Standby" and "Make Ready". Additionally, if the system is not used for a longer time, the system should not be stored with buffer such as K_2HPO_4 in the flowpath to prevent salt precipitation.

This can be easily achieved by including a flushing step with ultrapure water in the automatic standby task. This option saves time by reducing the effort required for the operator to start and monitor such a task, as well as the risk of forgetting such a task.

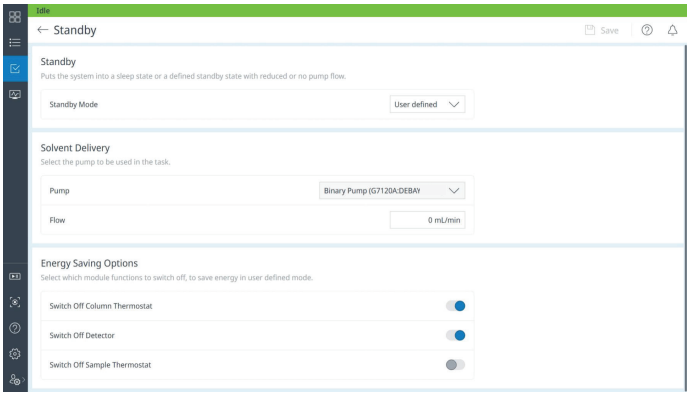

Figure 4. Modified settings of the InfinityLab Assist "Standby" task used during the experiments.

The total process of analyzing B vitamins in supplements is clearly eased by the automatic tasks of the InfinityLab Assist. Using the automatic "Make Ready" task with medium sensitivity, reproducible equilibration of the system especially with the buffer and ion-pairing reagent, is assured without manual monitoring.

Conclusion

Using the Agilent 1290 Infinity III LC System setup detection of vitamins B1, B2, B5, B6, B7, B9, and B12 in effervescent tablets and multivitamin supplement matrix, good separation was achieved. Using the Agilent InfinityLab Assist "Make Ready" task, the system was prepared for B vitamin analysis using only one automated task and no manual monitoring. Running this task automatically by scheduling saved more than 20 minutes of working time. Additionally, the time required for medium equilibration monitoring sensitivity was reduced by approximately six minutes, compared to manual equilibration. Furthermore, using the schedulable InfinityLab Assist "Standby" task, the risk of errors regarding instrument standby can be drastically reduced. User-defined settings enable adjustment for different purposes, such as switching of all modules except the sampler thermostat.[2] The ability to remove the used buffer and ion-pairing reagent from the flow path for longer storage by scheduling a standby task also greatly helps to prevent salt precipitation in the system without additional effort. Also, the InfinityLab Assist can clearly help to accelerate checking the instrument status while preparing the instrument, as well as during running samples.[2]

References

1. Best Practices for Using an Agilent LC System. Agilent Technologies technical note, publication number SD-29000194, 2016–**2022**.
2. Agilent InfinityLab Assist: A Local User Interface to Control and Automate Your HPLC System. *Agilent Technologies white paper*, publication number 5994-7572EN, **2024**.
3. Dhale, M.; Singh, R.; Sharma, R.; Arora, S. Quantification of All B Vitamins in a Single Run Using Ion-Pair Modified Liquid Chromatography with UV Detection. *J. Food Compos. Anal.* **2023**, *123*, 105602. https://doi.org/10.1016/j.jfca.2023.105602

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