Combining UHPLC with Advanced Chromatographic Techniques for Selected Food and Beverage Applications

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Introduction

Ultrahigh performance liquid chromatography (UHPLC) is a rapidly growing technique that takes advantage of HPLC columns with sub-3 µm particles. It offers ultrahigh resolution with maximum peak capacity, and can also significantly accelerate analyses through the use of higher linear velocities and shorter columns. This technology enables analysis of even medium-complex samples in the sub-minute range so that any further attempt to increase analysis speed approaches physical and technical limits. Further improvements in laboratory workflows beyond that level can only be achieved by increasing automation and total system utilization time.

This presentation describes technical solutions for increasing analytical throughput based on UHPLC in a dual system configuration, using the example of selected food and beverage applications. Improved productivity is achieved by combining UHPLC and advanced chromatographic techniques to enhance system utilization time compared to conventional LC. Significant enhancements in productivity are shown for the techniques of overlapping sample preparation, off-line column regeneration, and parallel LC. The poster discusses the advantages of running two different applications on such a dual UHPLC system, either simultaneously or sequentially. This is illustrated by examples of the simultaneous determination of water- and fat-soluble vitamins, and the automated subsequent analyses of water-soluble vitamins and soft drink additives.

Experimental

The experiments discussed on this poster were performed using four different UHPLC system configurations. All system configurations were controlled using the Thermo Scientific Dionex Chromeleon Chromatography Data System software.

System Setup for Conventional and UHPLC Experiments
The UltiMate 3000 RSLC system was used for demonstrating the productivity increase gained by transferring the water-soluble vitamin analysis from a conventional to a UHPLC column, and by implementation of overlapping sample preparation (Table 1).

Table 1. UltiMate™ 3000 RSLC System Configuration

Solvent Rack	Thermo Scientific Dionex SRD-3000 Solvent Rack
Pump	Thermo Scientific Dionex LPG-3400RS Rapid Separation Low-Pressure Gradient Pump
Autosampler	Thermo Scientific Dionex WPS-3000TRS Rapid Separation Thermostatted Autosampler
Column Compartment	Thermo Scientific Dionex TCC-3000RS Rapid Separation Thermostatted Column Compartment
Detector	Thermo Scientific Dionex DAD-3000RS Rapid Separation Diode Array Detector with 5 µL Semi-Analytical Flowcell, SST

System Setup for Advanced LC Techniques
All separations using advanced chromatographic techniques were performed on the UltiMate 3000 ×2 Dual RSLC system, configured for UHPLC tandem operation (off-line column regeneration), parallel operation, or automated application switching. Different valve configurations and Thermo Scientific Dionex Viper Capillary Kits were used to make all fluidic connections. An additional DAD-3000 Diode Array Detector was included in the ×2 Dual RSLC system for parallel operation configuration.

Table 2. UltiMate 3000 x2 Dual RSLC System Configurations

	Tandem (On-Line Column Registration)	Parallel	Automated Application Switching
Solvent Rack	Thermo Scientific Dionex SRD-3600 Solvent Rack with six degasser channels		
Pump	Thermo Scientific Dionex DGP-3600RS Rapid Separation Dual-Gradient Pump		
Autosampler	WPS-3000TRS Rapid Separation Thermostatted Autosampler		
Column Compartment	TCC-3000RS Rapid Separation Thermostatted Column Compartment		
Column Compartment Valves	2-position, 10-port valve	2-position, 6-port valve	2-position, 10-port valve, 2-position, 6-port valve
Detector	DAD-3000RS Rapid Separation Diode Array Detector with 5 µL Semi-Analytical Flowcell, SST (2× for parallel configuration)		
Capillaries	Viper™ Capillary Kit for Tandem Operation	Viper Capillary for Parallel Operation	Viper Capillary Kit for Automated Switching

HPLC
Water-Soluble Analyses
Five LC experiments were performed for the analysis of water-soluble vitamins: separation by conventional LC, by UHPLC without and with online sample preparation, UHPLC with off-line column regeneration, and parallel LC. Table 3 shows the method parameters for conventional LC and UHPLC with and without online sample preparation.

Table 3. Conventional to UHPLC method transfer for water-soluble vitamins analyses.

	Conventional LC			UHPLC		
Column	Thermo Scientific Acclaim PA2					
	5 µm, 250 × 4.6 min			2.2 µm, 100 × 2.1 min		
Mobile Phases	A) 30 mM Phosphate, pH 2.6 B) Acetonitrile					
Gradient	Time [min]	%A	%B	Time [min]	%A	%B
	0.0	0	0	0.0	0	0
	5.4	65	35	0.95	65	35
	7.8	65	35	1.37	65	35
	8.0	0	0	1.41	0	0
	22.7	0	0	4.00	0	0
Flow Rate	1.41 mL/min			0.66 mL/min		
Injection Volume	23 µL			2 µL		
Column Temperature	30 °C					
Detector Wavelength	210 nm, 244 nm, 262 nm, 264 mm, 292 nm					

The method parameters for the UHPLC experiments with off-line column regeneration and parallel operation are basically the same as shown for the UHPLC method in the table, only the gradients had to be adapted slightly and for the tandem LC experiment the Acclaim™ RSLC, PA2, 2.2 µm, 100 × 3 mm column was used at a flow rate of 1.35 mL/min.

Simultaneous Analyses of Water- and Fat-Soluble Vitamins
For the simultaneous analyses of water- and fat-soluble vitamins, the method parameters are shown in Table 4.

Table 4. Parallel analyses of water- and fat-soluble vitamins.

	Conventional LC			UHPLC				
Column	Acclaim RSLC, PA2, 2.2 µm (100 × 2.1 mm)			Acclaim RSLC, C18, 2.2 µm (100 × 2.1 mm)				
Mobile Phases	A) 30 mM Phosphate pH 2.6, B) Acetonitrile			A) Acetonitrile, B) Methanol, C) Water				
Gradient	Time [min]	%A	%B	Time [min]	Flow (mL/min)	%A	%B	%C
	-1.20	100	0	-2.80	0.995	85	5	10
	0.00	100	0	-1.60	0.995	85–95	5	10–0
	0.95	65	35	-1.30	0.600	95	5	0
	1.37	65	35	-1.15	0.995	95	5	0
	1.41	0	0	0.00	0.995	95	5	0
	3.00	0	0	3.80	0.955	95	5	0
	Time [min]	Exclusive Acess	ValveLet Position	Time [min]	Exclusive Acess		ValveLet Position	
	Initial	Acquire	1_2	Initial	Acquire		—	
	2.20	Release	—	-1.6	—		6_1	
Flow Rate	0.66 mL/min			See Gradient				
Injection Volume	2 µL			2 µL				
Temperature	30 °C			30 °C				
Detector Wavelength	210 nm, 244 nm, 262 nm, 269 nm, 264 nm,292 nm			210 nm, 247 nm, 266 nm, 326 nm				

Advanced Chromatographic Techniques

The main difference between an UltiMate 3000 Standard UHPLC and the ×2 Dual UHPLC system is that the pump actually houses two ternary gradient pumps that can operate independently. The column oven contains one or two valves to allow easy switching, a split-loop well plate autosampler, and a VWD or a DAD detector, which are fully controllable using the Chromeleon™ software.

Tandem UHPLC System Configuration
In the tandem UHPLC system configuration, two identical columns are switched between two flow paths—an analysis flow path and a regeneration flow path—to allow column washing and re-equilibration off-line.

While one column is equilibrated, the system injects the next sample on the other. This solution increases the sample throughput, without the need for new method development. Like parallel LC (see below), it takes away the need to revalidate methods and to revise corresponding documentation, as normally required when using other approaches to increase sample throughput.

Parallel UHPLC System Configuration
In the parallel UHPLC system configuration, one autosampler and one column compartment are shared between the two independent flow paths of the UltiMate 3000 ×2 Dual LC system with dual-gradient pump.

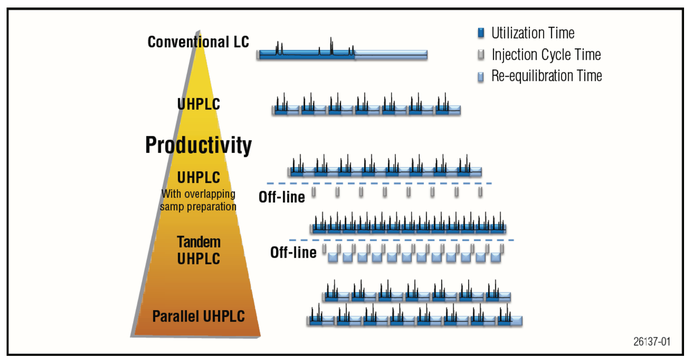
Automated Application Switching UHPLC System Configuration
The Automated Application Switching system configuration features the dual-gradient pump, delivering mobile phase to two different flow paths. The right pump and column 1 can be used to run one application and the left pump and column 2 can be used to run a second application.

The valve setup supports equilibration and flushing of one column while samples are analyzed on the other column. Initial instrument start-up and equilibration conditions, well defined shutdown procedures, and automated switch-over steps are defined, so that application switching becomes fully automated.

Results and Discussion

Definition of System Utilization Efficiency
In general, the run cycle time consists of three parts: the injection cycle time, the separation time, and the column re-equilibration time (Figure 1).

FIGURE 1. Comparison of utilization time, injection cycle time and re-equilibration time.



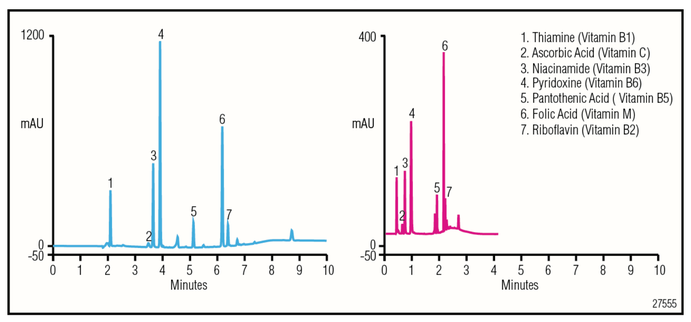
Useful data is only collected during separation time, which is the time between sample injection and the retention time of the last eluting peak of interest. This can also be defined as the utilization time. The injection cycle and the column re-equilibration time do not deliver useful data. System utilization efficiency is defined as the ratio of the utilization time vs. total run time including re-equilibration time and injection cycle time.

Means of Increasing Sample Throughput
In general, a higher sample throughput can be achieved by increasing the flow rate and keeping the gradient volume constant at the same time. In conventional LC, however, this cannot be realized without a significant loss in resolution. Therefore, conventional methods are frequently transferred to UHPLC conditions using columns with a particle diameter of 3 µm or lower. This allows for boosting the method even beyond van-Deemter minimum without sacrificing resolution. Sample throughput is increased by processing more samples in the same time while the system utilization efficiency is, in general, kept constant. A further gain in sample throughput can only be achieved by increasing the system utilization efficiency. This can be achieved by using advanced chromatographic techniques such as overlapping sample preparation, and Tandem or Parallel UHPLC based on a dual system configuration (Figure 1).

Analysis of Water-Soluble Vitamins—Increasing Productivity
We investigated the productivity increase for the analysis of seven water-soluble vitamins using the above mentioned approaches. As a first step we simply transferred the conventional HPLC method using an Acclaim PA2, 5 µm 250 × 4.6 mm column to UHPLC conditions using an Acclaim RSLC PA2, 2.2 µm 100 × 2.1 mm column (Figure 2).

By transferring the conventional HPLC method to the Parallel RSLC System, a productivity increase of up to a factor of ten could be achieved (Figure 2).

FIGURE 2. Analysis of seven water-soluble vitamins under A) conventional LC and B) UHPLC conditions on Acclaim PA2, 5 µm, 250 × 4.6 mm and Acclaim RSLC PA2, 2.2 µm, 100 × 2.1 mm columns, respectively.



The major gain in sample throughput was achieved by the method transfer to UHPLC itself (factor of 5). The Tandem LC approach further increased sample throughput by 67%, while with parallel LC the throughput was almost doubled.

Table 5. Overview of total run times and productivity for different approaches.

Productivity Approach	Total Run Time (min)	Productivity Increase Compared to HPLC Method	Productivity Increase Compared to UHPLC Method
Conventional HPLC Method	23.2/15.2	—	—
UHPLC Method with Overlapping Sample Preparation	4.3	× 5.4	× 1.08
Tandem UHPLC	2.8	× 8.4	× 1.67
Parallel UHPLC	4.8, two runs in parallel	× 9.8	× 1.96

Simultaneous Determination of Water- and Fat-Soluble Vitamins by Parallel UHPLC
In a next set of experiments, two distinct methods—the separation of water-soluble as well as the separation of fat soluble vitamins—were run on the Parallel RSLC system.

The gradient separation of the water-soluble vitamins was performed using an Acclaim RSLC PA2, 2.2 µm, 100 × 2.1 mm column. The mobile phase composition at gradient start time was 100% 30 mM phosphate buffer (pH 2.6). The isocratic separation of the fat-soluble vitamins was performed using an Acclaim RSLC C18, 2.2 µm, 100 × 2.1 mm column and used 95/5% acetonitrile/methanol as mobile phase (Figure 3).

FIGURE 3. Structural information from in-source decay pattern of glycerol trioleate (A) and glycerol stearate/oleate/linoleate (B).

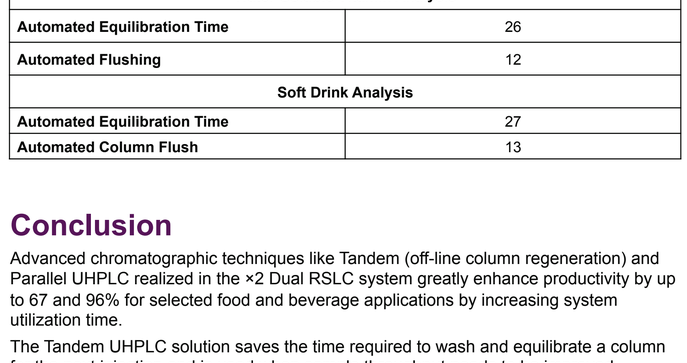
The advantage of this approach is that distinct chromatographic information from the same sample, injected on two different columns from the same vial at approximately the same time, is accessible to the analyst. This is especially important in the case of thermo-labile and light-sensitive analytes, such as water- and fat-soluble vitamins.

Even in such a complex setup the sample throughput is still increased by 20% compared to running both methods on a single instrument in a consecutive manner.

Automated Sequential Analysis of Water-Soluble Vitamins and Soft Drink Additives
If stable samples are to be analyzed with two different methods, the consecutive processing of both sequences is appropriate. Manual interference (e.g., change of mobile phase, check of column equilibration status) is usually required to prepare one single LC system for the first application and for switching between applications. In addition to increased labor time, this also leads to system idle times in the case that the sequence of the first application ends overnight or during the weekend.

In one set of experiments we tested the automated application switching approach with two different applications according to Figure 4.

FIGURE 4. Automated Application Switching workflow for the sequential analysis of water-soluble vitamins and soft drink analytes.



First, the dual-gradient pump of the ×2 Dual RSLC System was automatically purged with the mobile phase needed for both applications. The Acclaim RSLC PA2, 2.2 µm, 100 × 3.0 mm column was equilibrated for the separation of water-soluble vitamins (first application) using phosphate buffer and acetonitrile as mobile phase. Once the samples were analyzed, the column was washed and the sampler was flushed with a mixture of formate buffer and acetonitrile/formic acid for running the separation of soft drink analytes on an Acclaim RSLC C18, 2.2 µm, 100 × 3.0 mm column (second application). Again, the column was automatically equilibrated and the sequence was processed.

In the test scenario described here, the system operation time needed for automated pump purging, column equilibration and flushing was about 88 min (Table 6). During this time no manual interference and no check of the column equilibration status was needed. These tasks are processed by the ×2 Dual RSLC system in combination with the Chromeleon Chromatography Data System in a fully automated way.

Table 6. System operation times required for specified automated tasks.

System Operation Task	Time Saved (min)
Automated Pump Purging	23
Water-Soluble Vitamin Analysis	
Automated Equilibration Time	26
Automated Flushing	12
Soft Drink Analysis	
Automated Equilibration Time	27
Automated Column Flush	13

Conclusion

Advanced chromatographic techniques like Tandem (off-line column regeneration) and Parallel UHPLC realized in the ×2 Dual RSLC system greatly enhance productivity by up to 67 and 96% for selected food and beverage applications by increasing system utilization time.

The Tandem UHPLC solution saves the time required to wash and equilibrate a column for the next injection and is used when sample throughput needs to be improved significantly for a single LC method from different sample sets (Table 7).

FIGURE 7. Structural information from in-source decay pattern of glycerol trioleate (A) and glycerol stearate/oleate/linoleate (B).

Solution	Valves	Methods	Sample Throughput	Sample Sets	Sequences
Off-Line Column Regeneration (Tandem LC)	2p-10p	Same	++/+	Different	Single
Parallel LC	2p-6p	Same	++	Different	≥2, processed in parallel
		Similar	++/+	Same or Different	
		Different, But at Same Column Temperature	+	Same or Different	
Automated Application Switching	2p-6p/ 2p-10p	Different	0	Same or Different	Sequential

The Parallel UHPLC solution almost doubles sample throughput for the same LC application from different sample sets. In addition, this solution is also best suited if thermo-labile and/or light sensitive samples need to be analyzed with two different methods at the same time. In this case, however, the increase in sample throughput is lower compared to Parallel UHPLC of the same application from different sample sets, but valuable in-depth chromatographic data can be gathered from the same sample set (Table 7).

If, however, stable samples are to be analyzed with two different LC methods, using different mobile phases and different columns, the Automated Application Switching Solution is the ideal choice. It reduces manual interference to a minimum and increases system uptime significantly.

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