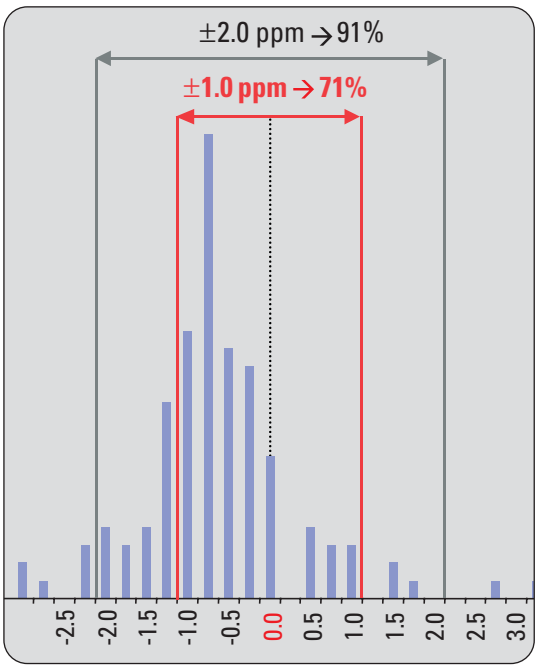
Agilent 1200 Series Rapid Resolution LC system and the Agilent 6210 TOF MS – Highest data content with highest throughput

Application Note



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Abstract

Fast and unambiguous determination of purity and identity of compounds derived from screening libraries is a common task for many analytical labs in the pharmaceutical industriy. The method of choice to determine the identity of compounds is mass spectrometry, preferably with accurate mass. As yet, data quality was usually compromised by gaining higher throughput. This Application Note demonstrates how a daily throughput of far more than 1000 samples can be achieved together with full spectral data acquisition and accurate mass information with close to FT-MS mass accuracy.

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Introduction

In the quest to achieve highest throughput in LC/MS analyses, the quality of the data is often compromised. There are certain approaches to increase the throughput of LC/MS systems. One approach is to do flow injection analysis. This probably delivers the highest possible throughput, however since no chromatographic separation occurs, the probability to loose compounds by the ion suppression effect during the ionization process is high. Orthogonal detection methods like UV detection do not succeed at all in flow injection analysis as all compound signals are overlaid. Approaches to achieve at least minimal chromatographic separation by using very short columns with 5 µm particles and ballistic gradients are an improvement in view of data quality, however, not state-of-the-art. Some manufactures have established parallel working instrumentation with a shared mass spectrometer and shared UV detector. Obviously, this also compromises data quality as the full acquisition rate of each instrument has to be shared on each LC channel[1].

With the introduction of an LC/MS system which facilitates the use of columns with sub two micron particles it is now possible to achieve short analyses times as well as high chromatographic resolution. Furthermore the system is able to acquire full UV spectral data and mass spectral data with accurate masses.

Experimental

The Agilent 1200 Series Rapid Resolution LC system is set up for alternating column regeneration (ACR)[2] using 2.1-mm id columns. The pumps are in the low delay volume configuration with an internal volume of only ca. 120 µL. All other modules are optimized for lowest delay volumes by using the low delay volume capillary kit (G1316-68744) and the alternating column regeneration kit (G1316-68721). Consequently, from the injection valve on only capillaries of 0.12 mm id are used. In the thermostatted column compartment the newly introduced low dispersion heat exchangers consisting of 1.6 µL internal volume have been used as well as the high pressure rated 2-position/10-port valve.

The instrument set-up is shown in figure 1:

- Two Agilent 1200 Series binary pumps SL with the new Agilent 1200 Series micro vacuum degasser placed between the two pumps eliminates the need for long tubing to the pumps.
- Agilent 1200 Series high performance autosampler SL.
- An Agilent 1200 Series thermostatted column compartment SL, equipped with a high pressure, 2-position/10-port valve, facilitating alternating column regeneration.
- An Agilent 1200 Series diode-array detector SL allowing a data acquisition rate of 80 Hz and equipped with a 500 nano liter flow cell with 0.12-mm id connecting capillaries.
- Agilent 6210 Time-of-Flight mass spectrometer allowing a maximum data acquisition rate of 40 Hz and equipped with a dual ESI source for parallel ionization of the analyte and a reference mixture.
- Two ZORBAX SB C18, 2.1 mm id x 50 mm, 1.8 µm columns
- As mobile phase gradient grade water with 0.1 % trifluoro acetic acid and acetonitrile with 0.08 % trifluoro acetic acid was used. No additional filtering of the solvents was made.

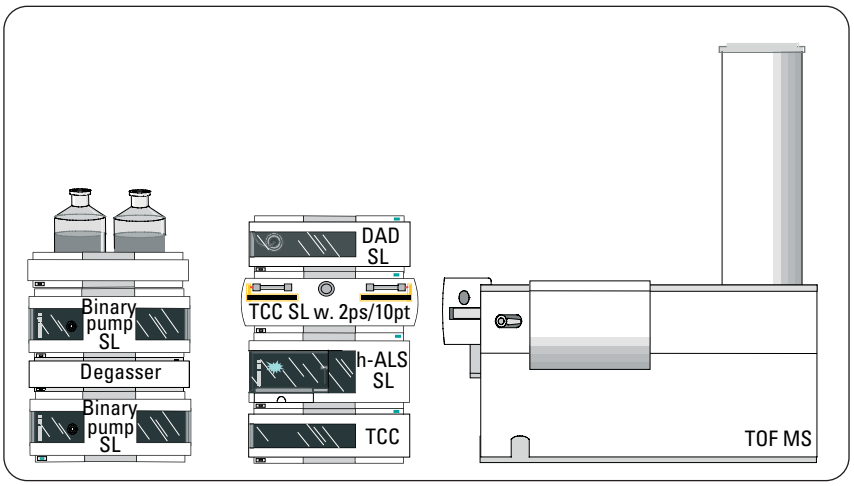

Figure 1
Agilent 1200 Series Rapid Resolution LC system with Agilent 6210 TOF-MS with low delay volume for high speed applications using 2.1-mm id columns with lengths ranging from 20 to 50 mm.

Instrument control and data acquisition was done by the Agilent TOF-software A02.01 running on a Hewlett-Packard xw 4300 workstation with an Intel dual core Pentium™ D840 CPU at 3.2 GHz.

Results and discussion

By applying elevated temperatures the viscosity of the solvent can be reduced which allows higher flow rates and therefore shorter gradient times. A maximum temperature of 80 °C was applied, which allowed a flow rate of 1.8 mL/min without hitting the pressure limit of the pump. This results in a linear velocity of approximately 11 mm/s for the 2.1 mm x 50 mm column (1.8 µm). With the help of the regeneration pump and the 2-position/10-port valve in the column compartment cycle times could be reduced significantly because one column is flushed with high organic content solvent and then re-equilibrated again with the starting composition of the gradient while on the second column the separation of a sample occurs. After this sequence the 10-port valve is switched and both columns are exchanged in the flow path. Details of alternating column regeneration and the correct setting of time points are described in another Application Note[2]. Despite the high flow rate (1.8 mL/min), the column effluent was not split prior to reaching the mass spectrometer. The standard ESI source specifies a maximum flow rate of up to 1 mL/min, however even these higher flows are tolerated if the drying gas temperature and flow rate are set to maximum and little condensation occurs. Condensation of water is practically eliminated when using ACR because equilibration is done on the column which

Data File	Sample Type	Inj Vol (µl)	Capillary	Fragmentor	Skimmer
opt_4000_215_60.wiff	Unknown	1	4000	210	60
opt_4000_215_60.wiff	Unknown	1	3000	210	60
opt_4000_215_60.wiff	Unknown	1	2000	210	60
opt_4000_215_60.wiff	Unknown	1	4000	180	60
opt_4000_215_60.wiff	Unknown	1	3000	180	60
opt_4000_215_60.wiff	Unknown	1	2000	180	60
opt_4000_215_60.wiff	Unknown	1	4000	150	60
opt_4000_215_60.wiff	Unknown	1	3000	150	60
opt_4000_215_60.wiff	Unknown	1	2000	150	60
opt_4000_215_60.wiff	Unknown	1	4000	210	40
opt_4000_215_60.wiff	Unknown	1	3000	210	40

Figure 2
Feature of the TOF software to modify the MS parameter from run to run.

Method:				
Solvent:	A = water (0.1% TFA), B = ACN (0.08% TFA)			
Temperature:	80 °C			
Flow:	1.8 mL/min			
Gradient:	0.00 min 5%B 0.50 min 90%B 0.51 min 5%B 0.65 min 5%B		**Regeneration:**	0.00 min 5%B 0.01 min 95%B 0.20 min 95%B 0.21 min 5%B 0.65 min 5%B
Stoptime:	0.65 min			no limit
Posttime:	off			off
DAD:	Wavelength:	210 nm (8), ref. off		
	Peak width:	>0.0025 min (0.05s responsetime), 80 Hz		
	Spectra:	no		
	Slit:	8 nm		
	Balance:	pre-run		
MS:	Scan range:	100-1000 m/z		
	Acquisition rate:	5, 20, 30 and 40 cycles/s		
	Data type:	profile data		
	Capillary voltage:	3000 V		
	Fragmentor:	180 V		
	Skimmer:	40V		
	Gas temperature:	350 °C		
	Gas flow:	13 L/min		
Injection volume:	1µL			
Injector:	Overlapped injection, Automatic delay volume reduction, Sample flush out factor = 10			
Valve position:	Next position			

Table 1
LC/MS method used for the data shown in figures 3-5. The method was also used to achieve the values in table 2.

is not connected to the detector. Generally the use of an Agilent multi mode source with a specified flow rate up to 2 mL/min even with pure water is recommended. The chromatographic conditions in table 1 were used to achieve gradient times of 0.5 min. Under these conditions, the peak capacity for the MS detection is in the range of >40 in 39 s. With the use of a 5-µm particle size column of the same dimension the peak capacity would only be half!

The detector of the Agilent 6210 TOF MS would be saturated if the compound concentrations used here to give also significant UV signals would be injected into the MS without special settings. Saturation of the MS detector would produce incorrect results in mass determination. The solution is to intentionally desensitize the TOF MS. This can be done quite easily by applying the functionality of the TOF software to alter the MS parameters from one run to the other,

simply by adding one or more "MS-parameter" columns to the worklist (figure 2). Select "add columns" from the worklist and then chose "MS-parameter" and the desired parameter. As the reference mixture is also affected by these settings, the concentration of the reference mixture was increased. Only the capillary voltage, the fragmentor voltage and the skimmer voltage were varied. The optimal conditions determined by this approach can be found in the method parameters in table 1.

In figure 3 the total ion chromatogram and the UV chromatogram achieved with conditions above (80 Hz DAD, 30 Hz TOF data acquisition rate) is shown for a five-component sample (58 ng/µL atenolol, 85 ng/µL primidon, 62 ng/µL metoprolol, 125 ng/µL verapamil and 75 ng/µL beclomethasone-dipropionat). The peaks of the total ion chromatogram are inherently broader than the peaks of the UV chromatogram because of additional extra column volume from the flow cell and also from connecting the capillary between the UV detector and ESI interface. But as can be seen in figure 3, the additional peak broadening of the MS peaks is only minor. The peak widths at half height of the MS peaks obtained under the highest data acquisition rate (40 Hz) are shown in figure 4 with values from as little as 0.34 to 0.42 s. The chromatograms shown in figure 5 were produced under the same chromatographic conditions, but with different data acquisition rates of the time-of-flight MS. The peak form and resolution are improved by having high data acquisition

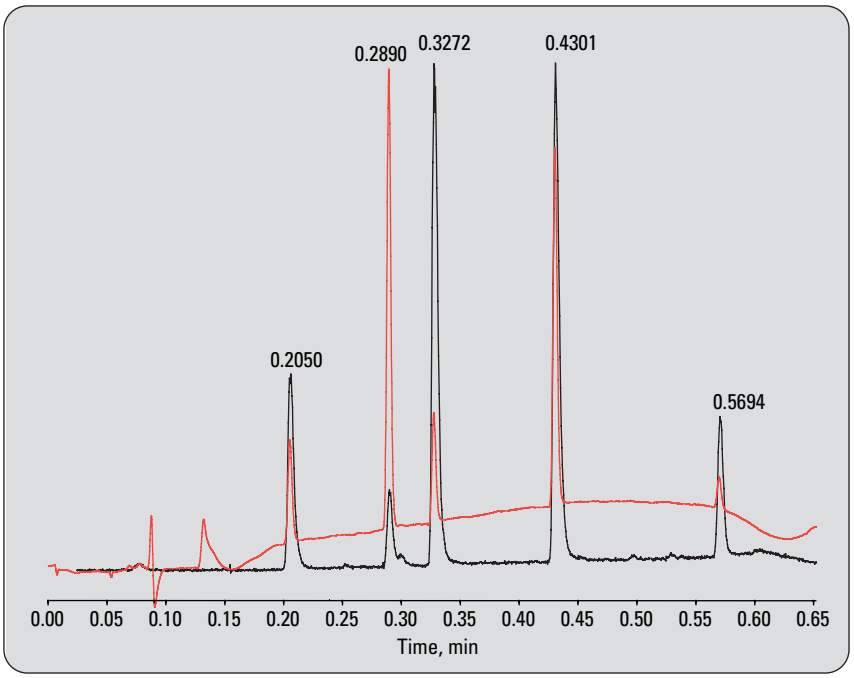

Figure 3
Comparison of corresponding peaks in the UV (red trace) and the MS detection (black trace).

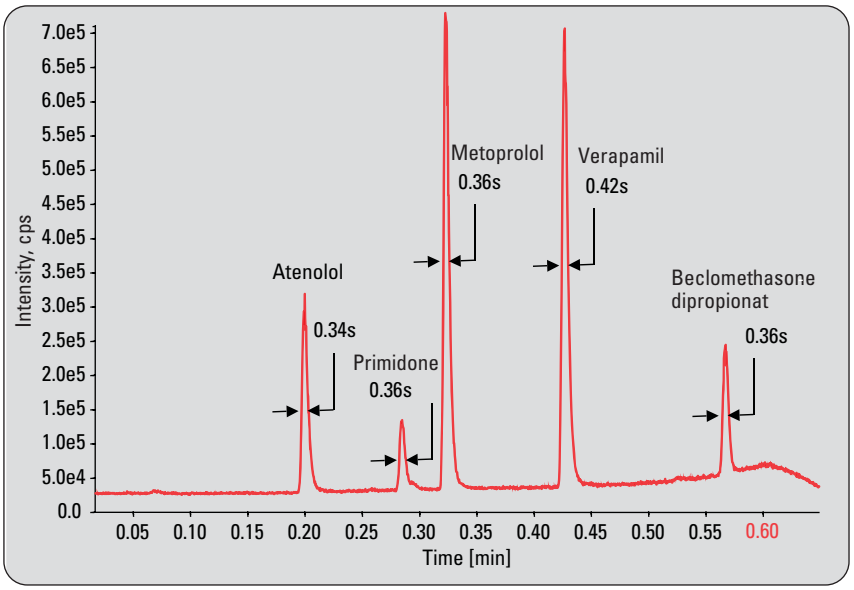

Figure 4
MS total ion chromatogram of highest speed LC-TOF-MS analysis (40 Hz TOF data acquisition rate).

rates in the MS which shows clearly in figure 5. The effect is nicely demonstrated on the little side peak next to the primidon peak – with 40-Hz data acquisition rate it is obvious that an additional compound shows up but with 5 Hz data acquisition rate this could not be differentiated from tailing of the primidon! The advantage, especially if MS quantization is necessary, is clear.

By applying the chromatographic conditions of table 1 and 80 Hz signal data acquisition of one wavelength and 30 Hz TOF centroid data, a cycle time of 49 s was achieved. The achievable cycle time is not only dependent on the used run time (that is the gradient time plus additional flush and re-equilibration times, or in Agilent terminology the stop time plus post time) but also very much dependent on the instrument overhead time. This is usually caused by communication between the data system and the individual LC/MS modules as well as the data system writing data to the hard disc and initiating certain processes. The overhead time caused by the data system can be significant if the computer's performance is not sufficient to handle the data amount or if other software programs or processes are consuming the power available. To decrease the cycle time it might be worth decreasing the amount of data acquired.

Table 2 shows the cycle times and the possible daily throughput depending on the DAD and MS settings. Since the MS data are constantly written to the hard disc during data acquisition, whereas the UV data are buffered and added to the data file after the stop time of

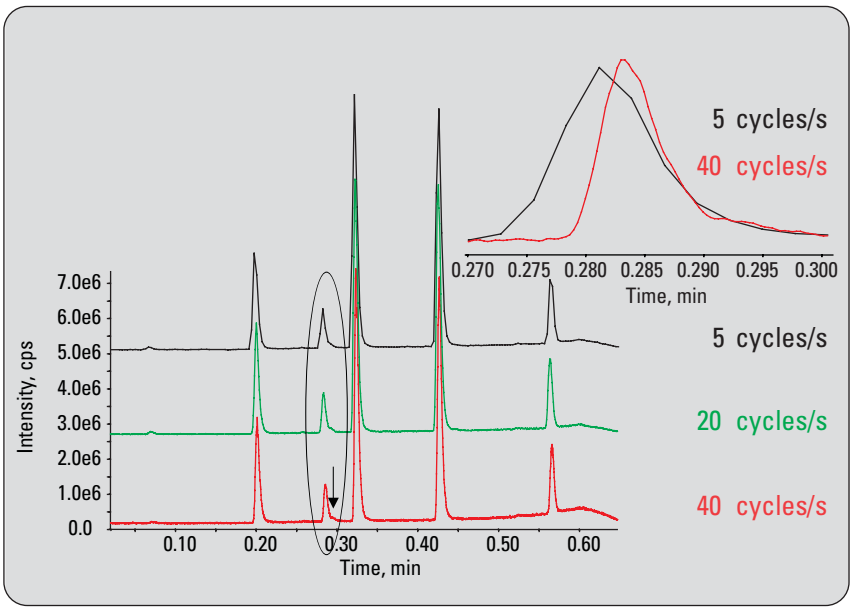

Figure 5
Total ion chromatograms recorded with varying data acquisition rates – dependence of the MS peak shape and resolution on the data acquisition rate.

DAD (80 Hz)		TOF (100 – 1000 Da)			Cycletime	Throughput
Type	Wavelength	Centroide	Profile	Data rate [Hz]	[s]	[Samples/day]
spectral	190-900 (1)		x	20	62	1394
spectral	190-900 (1)	x		20	62	1394
spectral	190-400 (2)		x	20	59	1464
spectral	190-400 (2)		x	40	59	1464
spectral	190-400 (2)		x	30	58	1490
signal	210/254		x	20	50	1728
signal	210	x		30	49	1763

Table 2
Dependence of the cycle time on the DAD and MS data acquisition settings, method stop-time was 0.65 min (39 s), pre-run balance was applied (ca. 2 s). The number in brackets for the DAD wavelength range stands for the scan width in nm.

the method, the cycle time depends more on the UV data amount than on the MS data amount. The cycle time was calculated from the time stamp each file gets assigned from the WindowsXP™ operating system after closing the file following data acquisition.

If using a TOF MS the attention is certainly focused on the accurate mass. The question may arise if the possibility to obtain low mass accuracy errors might suffer from these high speed conditions. Figure 6 shows the achieved mass accuracy errors of the analysis of 140 members of a chemical library used in a screening campaign by a pharmaceutical company. The shown error-values have been extracted from an automated empirical formula confirmation report and involved no manual interference. Sixteen of the compounds could not be ionized under positive ESI conditions and two compounds showed large mass errors of 11 and 15 ppm, probably caused by co-eluting isobaric impu-

rities. The cycle time was 90 s and was determined by a required injector program which allowed an on-line dilution of the samples directly prior to the analysis. Chromatographic conditions applied a 5-100 % water-acetonitrile (0.1 % TFA) gradient in 0.7 min at a flow rate of 1.5 mL/min and 60 °C column temperature. UV data acquisition to determine purity was done in the wavelength range of 210 to 500 nm with an acquisition rate of 80 Hz. The MS data acquisition rate was at 8 Hz to reduce the file size. The scan range was 120 – 1200 Da, capillary voltage 4000 V and the fragmentor voltage at 215 V. No ACR was applied and the flow to the MS was splitted in a 1:7.5 ratio.

More compelling is the histogram of the mass errors of these samples as shown in figure 7. More than 91 % of the ionizable compounds (outliers included) have a mass accuracy error in the range of ±2.0 ppm. Excluding the outliers even 93 % of the analyzed samples lie in-between the ±2.0 ppm range. In the ±1.0 ppm range which is FT-MS-like mass accuracy 71 % of the samples can be found (72 % excluding the outliers).

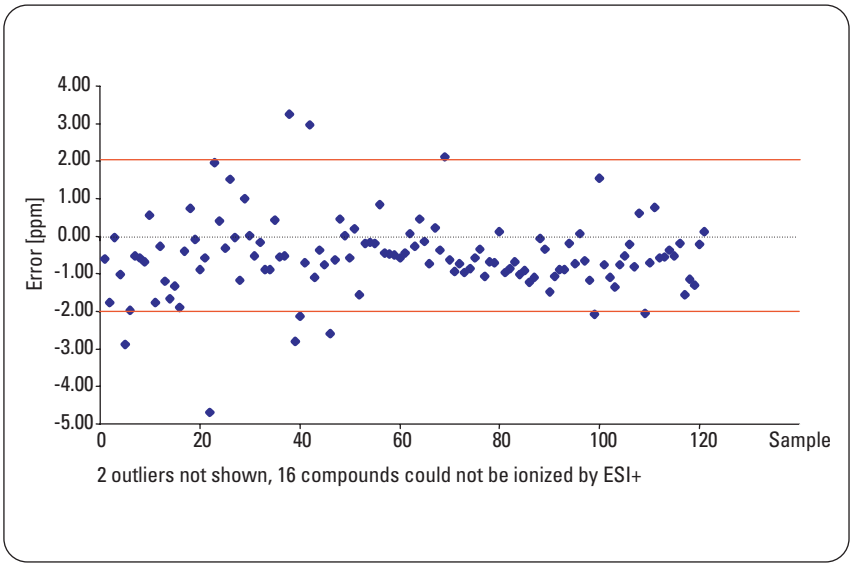

Figure 6
Mass accuracy errors of the analyses of a set of chemical library members under fast-LC conditions.

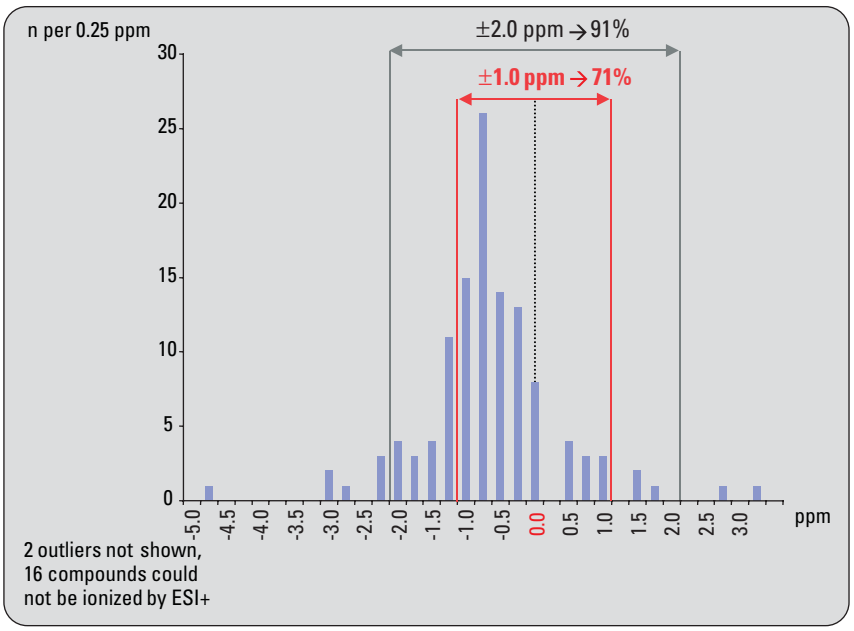

Figure 7
Histogram of the mass accuracy errors of the analyses of a set of chemical library members under fast LC conditions. The given populations of the ±1.0 ppm and ±2.0 ppm range include the outliers.

Conclusion

The Agilent 1200 Series Rapid Resolution LC system together with the Agilent 6210 Time-of-Flight mass spectrometer allows acquisition of a wealth of data to unambiguously determine the purity and identity of compounds in samples as they are typical for the high throughput analytical departments of pharmaceutical companies. In the time range of one minute high chromatographic resolution, full spectral diode-array data from 190-900 nm wavelength in a band width of 1 nm at an 80 Hz acquisition rate plus full MS spectral data from 100-1000 m/z with high acquisition rate and with an accurate mass with a mass error below ±2.0 ppm for more than 91 % of the samples could be acquired.

Using features like alternating column regeneration, overlapped injection, high temperatures, high flow rates together with highest data acquisition rates and most importantly stable and easy-to-use accurate mass, this system outperforms other high throughput LC/MS techniques used as yet in throughput and/or data quality. The linear velocities achieved were in the range of 11 mm/s and cycle times were as fast as 49 s for a run time of 41 s. Due to the columns with particle sizes of 1.8 µm, the UV peak capacities were still in the range of fifty and even the MS peak capacities were in the range of forty for a gradient time of 39 s.

References

1.

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2.

Udo Huber, „High throughput HPLC – Alternating column regeneration with the Agilent 1100 Series valve solutions" *Agilent Application Note, Publication number 5988-7831EN;* **2002.**

Most of the data herein was presented as a poster, titled "Non-multiplexed DAD-ToF Analysis of 1400 Samples/day" by Michael G. Frank, Edgar Naegele (Agilent Technologies, Waldbronn, Germany), Doug McIntyre (Agilent Technologies, Santa Clara, USA), Thilo A. Fligge, Stefan Buehler, Markus Christ (Boehringer-Ingelheim, Biberach, Germany), CO-1152, at the Pittcon conference 2006 in Orlando, Florida, USA.

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